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Research Article

Untersuchung der Auswirkung von Injektionsanästhesie mit Propofol über die Hämatologische und Biochemische Parameter bei den Katzen

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Abstract

Ziel der vorliegenden Studie soll es sein, die Kombinationen von Ketamin/Diazepam/Propofol hinsichtlich ihrer Wirkungen und Nebenwirkungen bei der Katze zu untersuchen. Für das Versuchsvorhaben wurden insgesamt 12 Katzen eingesetzt. In der vorliegenden Arbeit wurde Ketaminhydrochlorid (10 mg/kg) und Diazepam (0.5 mg/kg) intramuskulär gespritzt, um die Praemedikation bei den Katzen zu schaffen. Zur Fortführung der Anästhesie wurde 3 mg/kg Propofol intravenös verabreicht. Vor- und während der Anästhesie wurde von allen Tieren Blutproben entnommen, um labordiagnostische und biochemische Untersuchungsparameter auszuwerten. Während der Anästhesie zeigten die Atemfrequenz und die Körpertemperatur ein signifikanten Abfall (P<0.01) im Vergleich zum Ausgangswert, der statistisch bedeutend war. Die Werte von Monozyten (P<0.01), Hämatokrit (P<0.05), Kreatinin (P<0.05), Neutrophile (P<0.01), Lymphozyten (P<0.01) und Gesamtprotein (P<0.05) zeigten eine signifikante Veränderungen während der Anästhesie im Vergleich vor der Anästhesie, aber diese Werte liegen im Referenzbereich. Auffälligste Nebenwirkung von Propofolinjektion war eine vorübergehende Apnoe. Diese Kombination scheint als, die für kurz dauernde chirurgische Eingriffe und zur Prämedikation vor der Inhalationsanästhesie geeignet sind.

Keywords: *Anästhesie, Diazepam, Ketamin, Propofol, Katze*

Kedilerde Propofol Enjeksiyon Anestezisinin Hematolojik ve Biyokimyasal Parametreler Üzerine Etkisinin Araştırılması

Özet

Bu çalışmanın amacı, kedilerde Ketamin/Diazepam/Propofol'le oluşturulan kombinasyon anestezisinin olası etkileri ve yan etkilerini araşırmaktır. Çalışma materyalini 12 kedi oluşturdu. Bu çalışmada, kedilerde 10 mg/kg Ketamin HCl ve 0.5 mg/kg Diazepam intramusküler yolla uygulanarak premedikasyon sağlanmıştır. Anestezinin devamı için 3 mg/kg Propofol intravenöz olarak verilmiştir. Anestezi öncesi ve anestezi sırasında hayvanlardan kan örnekleri alınarak klinik ve biyokimyasal parametreler incelendi. Solunum frekansı ve vücut ısısında anestezi sırasında anestezi öncesine göre azalma (P<0.01) istatistiki açıdan önemli bulunmuştur. Yine monosit (P<0.01), hematokrit (P<0.05), kreatinin (P<0.05) nötrofil (P<0.01), lenfosit (P<0.01) ve total protein (P<0.05) değerlerinde anestezi esnasında anestezi öncesine göre fizyolojik sınırlar içerisinde istatistiksel olarak anlamlı bir değişiklik gözlenmiştir. Bu kombinasyonun en önemli yan etkisi; geçici bir apnö oluşturmasıdır. Sonuç olarak bu kombinasyonun kedilerde kısa süreli cerrahi girişimlerin anestezisinde ve inhalasyon anestezisinden önce preanestezik olarak kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Anestezi, Diazepam, Ketamin, Propofol, Kedi*

EINLEITUNG

Die Anästhesie bei der Katze stellt andere Anforderungen an den Tierarzt als die Anästhesie des Hundes. Katzen sind wenig duldsam und durch Zwangsmaßnahmen nur schlecht ruhigzustellen. Viele Untersuchungen und Eingriffe, die beim Hund in Sedation oder mit einer Lokalanästhesie durchgeführt werden können, erfordern bei der Katze eine Allgemeinanästhesie. Weiterhin ist die Narkose bei der Katze mit einem höheren Risiko verbunden als beim Hund [1,2]. Clark und Hall [3] zeigten in einer Studie, daß bei Katzen auf 600 und bei Hunden auf 900 Narkosen ein tödlicher Zwischenfall zu beobachten ist.

Ketamin ist ein chemisch den Halluzinogenen nahestehendes Phencyclinderivat und erzeugt eine

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dissoziative Anästhesie, einen Zustand, in dem die Tiere von ihrer Umgebung abgekoppelt erscheinen, ohne daß ein normaler Schlafzustand eintritt. Diese geht mit einer ausgeprägten Analgesie einher, die gegenüber somatischen Schmerzen stärker als gegenüber viszeralen Schmerzen ausgeprägt ist. Ketamin verursacht als einiges Narkosemittel eine Stimulation des Herz-Kreislauf-Systems, wobei Herzfrequenz und mittlerer Blutdruck ansteigen. Eine dosisabhängige Atemdepression nach Ketamingabe ist zu verzeichnen, die mit charakteristischen Änderungen des Atemrhythmus einhergehen kann. Neben Phasen mit vertieften Atemzügen und Abnahme der Frequenz sowie kurzzeitigen apnoeischen Phasen zeigen sich auch Phasen mit seufzerartigen Inspirationen [4-9]. Ketamin bewirkt keine Muskelrelaxation, sondern führt im Gegenteil zu erhöhtem Muskeltonus und zu tonisch klonischen Krämpfen sowie unkontrollierten Spontanbewegungen, was die Kombination mit einer muskelrelaxierend und sedativ wirkenden Substanz erfordert. Dafür kommen a_2-Adrenozeptoragonisten, Benzodiazepine sowie Phenothiazinen in Frage [7,10-13].

Diazepam gehört zur Gruppe der Benzodiazepine und ist als Injektionslösung erhältlich. Diazepam wirkt nach Löscher und Mitarbeiter [8] dosisabhängig anxiolytisch, antikolvulsiv, antiaggressiv, sedierend, hypnotisch und zentral muskelrelaxierend. Die Patienten reagieren individuell verschieden auf Diazepam. Die gewünschte Sedierung kann bisweilen ausbleiben oder es zeigt sich sogar paradoxe Wirkung wie gesteigerte Nervosität, Aufregung oder Aggressivität. Diazepam wird sowohl beim Hund als auch bei der Katze zur Unterbrechung eines Status epilepticus eingesetzt. Bei anorektischen Katzen bewirkt Diazepam eine gesteigerte Futteraufnahme [12-15].

Propofol bewirkt eine Dämpfung des Zentralnervensystem (ZNS), indem es die hemmende Wirkung des Neurotransmitters Gamma-aminobutyric acid (GABA) steigert. Die Auswirkungen von Propofol auf das Kreislaufsystem sind gering. Durch Reduktion der Kontraktilität des Myokards sowie arterielle und venöse Vasodilatation kann es eine geringgradige, systemische Hypotension hervorrufen. Über Stimulation der Barorezeptoren wird die Herzfrequenz gesteigert. Nach der intravenösen Narkoseeinleitung mit Propofol wird bei einigen Patienten ein etwa ein minütiger Atemstillstand beobachtet [10,16-20]. Die Atemfrequenz sinkt nach Hyperventilation während der Narkose auf ca. 30 Atemzüge pro Minute ab. Fodor und Mitarbeiter [17] beobachten zeitweise Atemfrequenzen unter 10/min. Etwa zwei Drittel der Hunde zeigen nach intravenöser Gabe von 6 mg/kg Propofol eine zufriedenstellende Muskelrelaxation. Leber, Nierenfunktion und Kortisolsynthese werden nicht beeinflusst [10,11,20]. Morgan und Mitarbeiter [21] stellten in ihrer Arbeit bei 207 Katze fest, daß der durchschnittlichen Dosis von Propofol für die Einleitung der Anästhesie ohne Prämedikation 8.03 mg/kg war. Nach der Prämedikation mit einem Neuroleptikum reduzierte der durchschnittlichen Dosis von Propofol etwa 20% (5.97 mg/kg). Die Aufwachzeit dauerte 27 bis 38 Minute und die Aufwachphase verlief schnell und exzitationslos.

Propofol ist ein reines Hypnotikum, es hat keine klinisch relevanten analgetischen Eigenschaften. Ist eine Schmerzausschaltung notwendig, muss diese durch die zusätzliche Applikation eines Analgetikums (z.B. Ketamin, Medetomidin, Fentanyl) erfolgen [9,15,19,20,22].

Ziel der vorliegenden Studie soll es sein, die Kombinationen von Ketamin/Diazepam/Propofol hinsichtlich ihrer Wirkungen und Nebenwirkungen bei der Katze zu untersuchen.

MATERIAL und METHODEN

Die Untersuchung wurde an 12 klinisch gesunden Katzen (6 weiblich, 6 männlich) im Durchschnittsalter von 2 Jahren (6 Monate bis 4 Jahre) vorgenommen. Das durchschnittliche Körpergewicht betrug 2.1 kg (1.6 kg-4.8 kg). 12 Stunden vor der Anästhesie wurden die Tiere in Einzelboxen untergebracht und nüchtern gesetzt. Wasser steht *ad libitum* zur Verfügung. Am Tag der Anästhesie wurde den Tieren zur Prämedikation, 10 mg/kg Ketamin hydrochlorid (Ketalar®-50) und 0.5 mg/kg Diazepam (Diazepam-Ratiopharm®) intramuskulär eingespritzt. 5 Minuten nach der Prämedikation wurden Propofol 4 mg/kg zur Fortführung der Anästhesie über einen venösen Zugang in die *V. cephalica antebrachii* verabreicht. Eine halbe Stunde vor der Prämedikation und 15, 30, 45, 60 Minuten nach der Anästhesie wurde von allen Tieren Blut entnommen, um haematologische (Leukozyten, Neutrophilen, Lymphozyten, Monozyten, Eosinophilen, Hämoglobin und Hämatokrit)und biochemische Untersuchungsparameter (Gesamtprotein, Kreatinin, AST und ALT) auszuwerten. Die serologischen Untersuchungen wurden mit einem Analysegerät (Refletron Plus, Roche) durchgeführt.

Die Zeitspanne bis zum Verlust des Standvermögens wurde ermittelt. Während der Anästhesie wurden Herzfequenz, Atemfrequenz und die Körperinnentemperatur gemessen. Weitere Beobachtungsmerkmale waren das Vorhandsein von Lidschlagreflex, Zwischenzehenreflex und Analreflex. Nebenwirkungen wie Speichelfluß, Erbrechen, Zyanose und Muskelzittern wurden ebenfalls erfaßt. Von ausreichender Sedation, Analgesie und Muskelrelaxation und damit dem Erreichen eines chirurgischen Toleranzstadiums wurde ausgegangen, wenn der Zwischenzehenreflex nicht auslösbar war und der Fang ohne Widerstand maximal geöffnet werden konnte. Außerdem wurde bei allen Tieren geprüft, ob die Tiere die Intubation tolerieren konnten.

Die rechnerische Aufbereitung des Datenmaterials erfolgten mit dem Statistikprogrammpaket SPSS© (Statistical Package for the Social Science, Version 14.0). Für alle untersuchten Parameter wurden der arithmetische Mittelwert, der Medianwert und die Standardfehler

errechnet. Unterschiede der einzelnen Messparameter wurden mit Hilfe vom t-Test für abhängigen Stichproben errechnet. Das Tierversuchsvorhaben ist durch die Adnan Menderes Universität genehmigt worden.

ERGEBNISSE

Durchschnittlich 4 Minuten nach erfolgter i.m. Anwendung von Ketamin/Diazepam haben die Patienten ihr Standvermögen verloren. Bei allen Patienten waren Lidschlag- und Analreflex über die gesamte Narkosedauer nicht auslösbar. Der Zwischenzehenreflex war 5 Minuten nach Anflutung des Propofols nicht mehr auslösbar und trat im Durchschnitt 45 Minuten nach der Injektion wieder auf *(Tabelle 1)*. Erbrechen, verstärkter Speichelfluss oder Exzitationen konnten in der Einleitungs- und Aufwachphase sowie während der Anästhesie in keinem Fall beobachtet werden. 6 Minuten post injektionen konnte in allen Fällen eine ausreichende Analgesie und Muskelrelaxation erreicht werden. Das Stehvermögen war im Schnitt nach anderthalb Stunden wiedererlangt. Die Aufwachphase verlief in allen Fällen ruhig und exzitationslos. Schmerzreaktionen aufgrund der Injektion konnten in keinem Fall beobachtet werden. Bei allen Tieren war die Intubation spätestens 5 Minuten nach Injektion des Propofols möglich. Nach der Verabreichung von Propofol trat bei 5 Tieren eine transiente Apnoe auf, die 1 bis 3 Minuten dauerte, ein therapeutisches Eingreifen nicht notwendig machte.

Die Atemfrequenz und die Körperkerntemperatur zeigten während der Anästhesie ein signifikanten Abfall (P<0.01) zum Ausgangswert. Im Vergleich zum Vorwert veränderten sich die Werte von Neutrophilen (P<0.01), Lymphozyten (P<0.01), Monozyten (P<0.01), Hämatokrit (P<0.05), Kreatinin (P<0.05) und Gesamtprotein (P<0.05) während der Anästhesie lagen jedoch im Referenzbereich *(Tabelle 2)*.

DISKUSSION

Der Wirkungseintritt von Propofol bei Hund und Katze erfolgt sehr rasch und ohne Exzitationserscheinungen. Die Wirkdauer ist im Vergleich zum Hund bei der Katze etwas länger. Hunde sind 15-30 Minuten nach einer einzelnen intravenösen Gabe vollständig erwacht, während bei Katzen die Aufwachzeit etwa 30 Minuten beträgt. Diese verlängerte Wirkung ist auf den eingeschränkten Phenolmetabolismus der Katzen zurückzuführen. Die Aufwachphase ist exzitationslos und vollständig. Dieser Wirkungseintritt wird bereits von vielen Autoren [4,10,17-20] bestätigt.

Die Anästhesie ausschließlich mit Propofol ist nur bei wenig schmerzhaften Untersuchungen empfehlenswert,

Tabelle 1. *Einschlafphase und Aufwachzeiten bei Propofol Anästhesie (Minute) (x=Mittelwert, Sx=Standardfehler)*
Tablo 1. *Propofol anestezisinde anesteziye giriş ve anesteziden uyanma süreleri (dakika) (X=Ortalama değer , Sx = standart hata)*

Tier	Einschlafzeit (Minute Post Injectionen) X±Sx	Aufwachzeit (Minute Post Injectionen) X±Sx	Chirurgische Toleranz (Minute Post Injectionen) X±Sx
Katze (n=12)	2±0.5	85±35	40±10

Tabelle 2. *Tabellarische Darstellung der klinischen Parameter, des Blutbildes sowie der klinisch-chemischen Laborparameter im Blutserum vor der OP. und während der OP. sowie nach der OP. (x=Mittelwert, Sx=Standardfehler)*
Tablo 2. *Propofol anestezisinde anestezi öncesi, sırası ve sonrasında bazı klinik ve biyokimyasal parametreler (X=Ortalama değer , Sx = standart hata)*

Phase	0 (Vorwert) (X±Sx)	15. Minute (X±Sx)	30. Minute (X±Sx)	45. Minute (X±Sx)	60. Minute (X±Sx)
Herzfrequenz (min^{-1})	162.6±13.90	176.0±6.11	166.3±12.60	162.6±9.64	154.6±7.12
Atemfrequenz (min^{-1})	30.7±3.37	16.0±3.57**	16.±2.65**	21.1±3.62	20.66±3.12
Körperkerntemperatur (°C)	38.23±0.21	37.21±0.20*	36.48±0.48**	35.82±0.50**	34.86±0.77**
Leukozytenzahl ($10^3/mm^3$)	11.59±0.58	13.32±1.36	13.66±0.90	13.35±1.27	12.53±0.95
Neutrophile (%)	60.66±1.54	62.00±6.04	55.83±7.20	50.16±5.02	47.16±2.86**
Lymphozyten (%)	27.33±3.68	29.61±6.53	32.66±7.18	33.66±4.02	37.16±2.65**
Monozyten (%)	1.16±0.16	3.00±0.73**	1.66±0.33	2.5±0.67	1.50±1.11
Eosinophile (%)	4.33±0.55	7.0±1.82	6.66±1.30	7.50±1.62	7.66±1.64
Hämoglobin (g/dl)	10.67±0.48	11.59±0.37	10.29±0.24	9.88±0.36	9.42±0.17
Hämatokrit (%)	34.16±1.64	26.83±2.90	24.66±0.61*	23.66+0.42*	24.5±0.61*
Gesamtprotein (mg/dl)	6.46±0.80	6.40±0.89	6.38±0.27	5.31±0.20*	5.34±0.24*
Kreatinin (mg/dl)	1.31±0.12	1.46±0.02*	1.44±0.02	1.41±0.02	1.22±0.04
AST (U/L)	16.5±2.10	13.5±2.12	15.1±1.83	13.0±0.85	15.3±0.21
ALT (U/L)	43.16±1.86	42.0±1.60	35.6±5.95	40.5±4.29	52.16±4.97

** P<0.05 (Irrtumwahrscheinlichkeit <5%, signifikant) signifikanter Unterschied zum Vorwert; ** P<0.01 (Irrtumwahrscheinlichkeit <1%, hochsignifikant) signifikanter Unterschied zum Vorwert*

so können z.B. Röntgenaufnahmen angefertigt oder Zahnstein entfernt werden. Bei der Durchführung schmerzhafter Eingriffe ist zur Ergänzung mit einem analgetischen Medikamente notwendig. Eine sedativ-analgetische Prämedikation bewirkt eine notwendige Schmerzausschaltung und durch ihren sedativen Effekt senkt sie den Propofolbedarf. Unter diesem Anästhesieregime können schmerzhafte Verbandwechsel, Wundbehandlungen, Repositionen von Luxationen, Punktionen und ähnliches durchgeführt werden [4,10,17-20,23]. In vorliegender Arbeit wurde Propofol mit Ketamin und Diazepam kombiniert.

Die Atemfrequenz fiel während der Anästhesie signifikant gegenüber dem Ausgangsniveau herab. Die atemdepressive Wirkung des Ketamin und des Propofol ist bereits im Schrifttum hinreichend beschrieben [4,9-12,21].

Am Anfang der Anästhesie lag die Herzfrequenz über dem Ausgangsniveau. Die herzfrequenzsteigernde Wirkung des Propofol und Ketamin ist bereits im Schrifttum hinreichend beschrieben [4,9-12,21].

Der signifikante Abfall der Rektaltemperatur, der am Ende der Anästhesie deutlich ausgeprägt war, wird auch von anderen Untersuchern beschrieben [1,4,10,11,20].

Die Alanin-Amino-Transferase (ALT) kann bei Hund und Katze als leberspezifisch bezeichnet werden. Die ALT kommt nur im Zytoplasma vor. Sie ist also bereits bei Membrandesintegrationen erhöht, ohne daß es zu Leberzellnekrosen gekommen sein muss [24]. In der vorliegender Arbeit erhöht sich zwar die ALT am Ende der Anästhesie, aber sie liegt im physiologischen Referenzbereich.

Kreatinin ist ein Produkt des endogenen Muskelstoffwechsels und wird aus Kreatin und Phosphokreatin gebildet. Die Serumkonzentration steht in gewisser Beziehung zur Muskelmasse des Individuums. Kreatinin hat gegenüber dem Harnstoff den Vorteil, daß es nicht nahrungsabhängig ist und auch vom endogenen Proteinmetabolismus nicht beeinflusst wird [24]. Auch die Kreatininwerte steigen in unserer Arbeit signifikant. Aber die Werte liegen noch im oberen Referenzbereich.

Somit kann man sagen, dass die Funktion von Leber und Nieren durch die Anästhetika, die während dieser Arbeit verwendet wurden, bei therapeutischer Dosierung nicht beeinträchtigt wird.

Das Gesamtprotein und das Hämatokrit fiehl am Ende der Anästhesie signifikant herab. Es könnte an der Blutentnahme für die laborchemischen und klinisch-chemischen Untersuchungen liegen.

Smith und Mitarbeiter [20] untersuchte in ihrer Arbeit die Nebenwirkungen des Propofol mit verschiedenen Kombinationen (Diazepam/Propofol, Azepromazin/Propofol und Butarphanol/Propofol). Sie stellten dabei fest, daß die häufigste Nebenwirkungen Apnoe (bei 34 Hunden von 40 Hunden), Erbrechen (bei 6 Hunden von 40 Hunden), verstärkter Speichelfluss (bei 8 Hunden von 40 Hunden), Zyanose der Zungenschleimhaut (bei 3 Hunden von 40 Hunden), und Exzitationen (bei 4 Hunden von 40 Hunden) sind. Die Apnoe dauerte die Mehrheit der Hunden 1-5 Minuten. In eigener Untersuchung wurde Erbrechen, verstärkter Speichelfluss, Zyanose der Zungenschleimhaut oder Exzitationen in der Einleitungs- und Aufwachphase sowie während der Anästhesie nicht beobachtet. Hall und Mitarbeiter [1] stellten in ihrer Arbeit bei der Katze fest, daß die Zyanose die häufigste Nebenwirkung waren. Nach der Verabreichung von Propofol trat bei 5 Tieren eine transiente Apnoe auf, die 2 bis 5 Minuten dauerte, ein therapeutisches Eingreifen nicht notwendig machte. Dieses Ergebnis stimmt mit denen anderer klinischer Studien überein [25,26].

Mit der Kombination Ketamin/Diazepam/Propofol wurde bei allen Tieren ein schneller exzitationsloser Eintritt der Anästhesie erreicht. Als auffälligste Nebenwirkung zeigte sich eine vorübergehende Apnoe. Diese Kombination scheint als Anästhesie für kurze Eingriffe und als Prämedikation vor einer Inhalationsanästhesie geeignet.

LITERATURVERZEICHNIS

1. Hall TL, Duke T, Townsed HG, Caulkett NA, Cantwell SL: The effect of opioid and acepromazine premedication on the anesthetic induction dose of propofol in cats. *Can Vet J*, 40, 867-870, 1999.

2. Hosgood G, Scholl DT: Evaluation of the age and American Society of Anesthesiologists (ASA) physical status as risc factors for perianesthetic morbidity and mortality in the cat. *J Vet Emerg Crit Care*, 12, 9-16, 2002. DOI: 10.1046/j.1534-6935.2002.00002.x

3. Clarke KW, Hall LW: A survey of anesthesia in small animal practice: AVA/BSAVA report. *J Vet Anesth*, 17, 4-10, 1990. DOI: 10.1111/j.1467-2995.1990.tb00380.x

4. Alef M, Schmidt-Oechterning GU: Injektionsanaesthesie für kurze Eingriffe. **In,** Schmidt-Oechterning GU, Alef M (Hrsg): Neue Aspekte der Veterinaeranaestesie und Intensivtherapie. 34-45, Paul Parey Verlag, Berlin, 1993.

5. Büch HP, Büch U: Narkotika. **In,** Forth W, Henschler D, Rummel W (Hrsg): Allgemeine und Spezielle Pharmakologie und Toxikologie. 232-253. B.I. Wissenschafsverlag, Mannheim, 1992.

6. Coper H, Helmchen H: Psychopharmaka. **In,** Forth W, Henschler D, Rummel W (Hrsg): Allgemeine und Spezielle Pharmakologie und Toxikologie. 281-303, B.I. Wissenschafsverlag, Mannheim, 1993.

7. Larsen, R: Anaesthesie. 3. Aufl, Urban und Schwarzenberg, München, 1990.

8. Löscher W, Ungemach FR, Kroker R: Grundlagen der Pharmakotherapie bei Haus- und Nutztieren. Paul Parey, München, 1994.

9. Moens T, Forgetton X: A comparative study of medetomidine/ketamin and xylazin/ketamin anaesthesie in dogs. *Vet Rec*, 127, 567-571, 1990.

10. Jones RS: Propofol anaesthesia in the dog and cat. *Vet Ann*, 30, 200-202, 1990.

11. Haskins SC, Farver TB, Patz JD: Ketamin in dogs. *Am J Vet Res*, 46, 1855-1860, 1985.

12. Haskins SC, Farver TB, Patz JD: Cardiovascular changes in dogs given diazepam and diazepam-ketamine. *Am J Vet Res*, 47, 795-798, 1986.

13. Muir WW, Hubbel JAE: Cardiopulmonary and anesthetic effects of ketamine and its enantiomers in dogs. *Am J Vet Res*, 49, 530-534,

1988.

14. Balderssani RJ: Benzodiazepines. **In,** Goodman GA, Goodman GS (Eds): The Pharmacological Basis of Therapeutics. 433-437, Macmillan Publishing Company, New York, 1985.

15. Hunkeler W, Möhler H, Piere L, Polc P, Bonetti EP, Cumin R: Selective antagonist of benzodiazepines. *Nature*, 290, 514-516, 1981. DOI: 10.1038/290514a0

16. Branson KR, Gross ME: Propofol in veterinary medicine. *J Am Vet Med Assoc*, 204, 1888-1890, 1994.

17. Fodor G, Kaser-Hotz B, Kuhn D: Erfahrungen mit Propofol als Injektionsnarkotikum bei der Strahlentherapie von Hund und Katze. *Tierärztl Prax*, 24, 62-67, 1992. DOI: 10.1046/j.1439-0442.2001.00357.x

18. Galatos AD, Savas I, Prassinos NN, Raptopoulos D: Gastro-oesophageal reflux during thiopentone or propofol anesthesia in the cat. *J Vet Med A*, 48, 287-294, 2001. DOI: 10.1046/j.1439-0442.2001.00357.x

19. Paddleford RR, Erhardt W: Anaesthesie bei Kleintieren. Schattauer, Stuttgart, 1992.

20. Smith JA, Gaynor JS, Bednarski RM, Muir WW: Adverse effects of administration of propofol with various preanesthetic regimens in dogs. *JAVMA*, 202, 1111-1115, 1993.

21. Morgan, DW, Legge K: Clinical evaluation of propofol as an intravenous anesthetic agent in cats and dogs. *Vet Rec,* 124, 31-33, 1989. DOI: 10.1136/vr.124.2.31

22. Kramer S, Engelka A, Nolte I: Motorische Krampfanfälle unter Propofol Anästhesie beim Hund. *Kleintierpraxis*, 40, 181-192, 1995.

23. Weaver BMQ, Raptopoulos D: Induction of the anesthesia in dogs and cats with propofol. *Vet Rec*, 126, 617-620, 1990.

24. Kraft W, Dürr UM: Klinische Labordiagnostik für die Tiermedizin. **In,** Kraft W (Hrsg): 3. Aufl. Schattauer, Stuttgart, 1995.

25. Kılıç N, Paşa S, Seyrek K: Kedide ketamin/diazepam/propofol'le kombinasyon anestezisi. *VI. Ulusal Veteriner İç Hastalıkları Kongresi,* 113, 4-7 Temmuz, Kars, 2005.

26. Özaydın İ, Atalan G, Uzun M, Kılıç E, Çenesiz M: Köpeklerde medetomidin, propofol ve ketamin kombinasyonunun anestezik özellikleri ile klinik, kardiyovasküler ve respiratorik etkilerinin değerlendirilmesi. *Kafkas Univ Vet Fak Derg,* 7, 71-76, 2001.

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Research Article

Immunohistochemical and Bacteriological Investigations of *Mannheimia haemolytica* in Sheep Bronchopneumonia

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Abstract

Mannheimia haemolytica infection is one of the most common etiologic agents of sheep pneumonia almost all over the world. Ovine pneumonic Mannheimiosis is characterized by severe fibrinous pleuropneumonia. Subacute to chronic cases progress to purulent bronchopneumonia and its squeals include abscessation and fibrous pleural adhesions. In the present study, lungs of 8986 sheep were inspected grossly in the Ahvaz abattoir and totally 65 lungs with visible signs of bronchopneumonia were selected for pathological and bacteriological examinations. *Mannheimia haemolytica* antigens were detected in 63.07% of immunoperoxidase stained tissue sections while 52.30% of the lungs were positive in bacteriological culture. Suppurative, necrotic and fibrinous types of bronchopneumonia were the most abundant lesions and right cranial lobes, specifically their cranial portions, were the most affected areas. McNemar test showed a significant difference between the diagnostic power of immunohistochemistry (IHC) and bacterial culture in detection of *M. haemolytica* (κ=0.66). Considering IHC as a golden test, sensitivity and specificity of bacterial culture were estimated as 78.05 and 91.67%, respectively. Chi- squared test showed significant correlations between the distribution of the lesions and bacterial isolation (P=0.04), types of lesions and IHC results (P=0.01), and also types of bronchopneumonia and mixed/pure isolation (P=0.008). This study showed the significant role of *Mannheimia haemolytica* in causing pneumonic lesions of studied sheep.

Keywords: *Immunohistochemistry, Mannheimia haemolytica, Sheep, Lung, Bacteriology*

Koyun Bronkopnömonilerinde *Mannheimia haemolytica*'nın İmmunohistokimyasal ve Bakteriyolojik İncelenmesi

Özet

Mannheimia haemolytica enfeksiyonu tüm dünyada koyun pnömonilerinin en yaygın etiyolojik etkenidir. Ovine pnömonik mannheimiozis fibrinli pleuroplnömoni ile karakterizedir. Subakut ve kronik vakalarda purulent bronkopnömoni gelişir ve takibinde abseleşme ve fibrinli plöral yapışma şekillenir. Bu çalışmada, 8986 koyuna ait akciğer Ahvaz kesimevinde makroskopik olarak incelendi ve görsel olarak bronkopnömoni tespit edilen toplam 65 akciğer patolojik ve bakteriyolojik inceleme için seçildi. *Mannheimia haemolytica* antijenleri immunperoksidaz boyalı doku kesitlerinde %63.07 oranında belirlenirken örneklerin %52.30'u bakteriyolojik kültür ile pozitif olarak tespit edildi. Suppuratifi nekrotik ve fibrinli tipteki bronkopnömoniler en yaygın belirlenen lezyonlar iken sağ kranial loblar ve özellikle de onların kranial lopları en çok etkilenen bölgeler olarak belirlendi. McNemar testi immunohistokimya (İHK) ile bakteriyolojik kültür yöntemlerinin *M. haemolytica* (κ=0.66) etkenini tespit etme güçleri arasında anlamlı bir fark olduğunu gösterdi. İHK yöntemi altın test olarak düşünüldüğünde bakteriyolojik kültür yönteminin özgüllüğü ve özgünlüğü sırası ile %78.05 ve %91.67 olarak belirlendi. Ki-kare testi lezyonların yayılımı ile bakteriyal izolasyon arasında (P=0.04), lezyon tipi ile IHK sonuçları arasında (P=0.01) ve bronkopnömoni tipi ile miks/saf izolasyon arasında (P=0.008) ilişki olduğunu gösterdi. Bu çalışma incelenen koyunlarda pnömonik lezyonların şekillenmesinde *Mannheimia haemolytica*'nın önemli rol oynadığını göstermektedir.

Anahtar sözcükler: *İmmunohistokimya, Mannheimia haemolytica, Koyun, Akciğer, Bakteriyoloji*

INTRODUCTION

Bronchopneumonia is the most common type of pneumonia in domestic animals [1], causing great economic losses in lambs [2]. With few exceptions, it is characterized by the cranioventral consolidation of the lungs [1]. Several infectious agents such as ovine respiratory syncytial virus, *Mannheimia haemolytica* and *Pasteurella multocida* have

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been isolated from bronchopneumonia in small ruminants. Generally bronchopneumonia is usually caused by two or more infectious agents working together, but some agents can also cause a significant disease alone [2]. As a rule, the causing agents arrive in the lungs via inhaled air, either from infected aerosols or from the nasal flora [1].

Mannheimia haemolytica is one of the most important bacterial agents of sheep pneumonia [3,4] and is involved in ovine pneumonic Mannheimiosis, septicemia in young lambs, ovine enzootic pneumonia, and sporadic severe gangrenous mastitis in ewes. In pneumonic form, lesions are characterized by severe fibrinous pleuropneumonia. Subacute to chronic cases progress to purulent broncho-pneumonia and its sequels may include abscessation and fibrous pleural adhesions. In contrast to ovine pneumonic Mannheimiosis, chronic enzootic pneumonia, causes only a mild to moderate pneumonia and is rarely fatal [1].

Immunoperoxidase technique (IPT), by clear visualization of antigens, is a preferred method for determining a correlation between histopathological findings, causative organisms, and their location in tissues. Since the identification of *M. haemolytica* with bacterial culture is often difficult in some situations (antibiotic therapy, frozen and autolytic samples, fixed tissue, etc.), immunohistochemical analyses for detecting *M. haemolytica* antigens can be employed to overcome many problems associated with this method [5].

This study was performed to investigate the diagnostic capabilities of IPT and bacterial isolation for the detection of *M. haemolytica* in bronchopneumonic lungs of slaughtered sheep in Ahvaz, southwest of Iran.

MATERIAL and METHODS

Sample Collection and Pathology

Lungs of 8986 sheep were inspected grossly for 4 months (from February to May 2011) in the Ahvaz abattoir and 65 lungs with visible signs of bronchopneumonia were collected for pathologic, bacteriologic, and immuno-histochemical studies. Tissue samples were taken from the affected areas and were fixed in 10% neutral buffered formalin. After routine tissue processing and paraffin embedding, 5 µm thick sections were routinely cut and stained with haematoxylin and eosin for histopathological investigation.

Bacterial Culture and Identification

According to the conventional method, sterile swab samples were taken aseptically from the deep areas of the lesions and cultured on blood and McConkey agar plates. Plates were incubated aerobically at 37°C and examined for growth of bacteria. Characterization of suspected bacterial isolates to *Pasteurella, Mannheimia* and *Bibersteinia* was carried out using classic methods based on bacterial morphology, biochemical tests, and reference tables [6-8]. *M. haemolytica* (local isolate) was used as a positive control for identification procedures.

Preparation of anti-M. haemolytica Polyclonal Antibody

Polyclonal antibody against *M. haemolytica* was prepared with immunization of 2 rabbits by intramuscular administration of 2 ml *M. haemolytica* inoculum prepared with Freund's adjuvant. Immunization was done six times in two weeks intervals [9]. Blood samples were taken two weeks following the last injection. The collected sera samples were evaluated for antibody titer against *M. haemolytica* (local isolate) and cross-reaction with antigenically closest to Pasteurellaceae *(Pasteurella multocida, Bibersteinia trehalosi)* and *E. coli* (representative of gram negative bacteria) by microagglutination, Dot-ELISA and indirect IPT [10,11]. In order to eliminate cross-reactivity of the polyclonal anti-body, it was diluted and adsorb with the above bacteria (whole cell antigens) for 1 h. The mixture was then centrifuged at 4000 rpm for 20 min and the supernatant was evaluated again for probable cross-reactivity.

Indirect Immunoperoxidase Tests

Tissue sections, 3 µm thick, were deparaffinized and rehydrated and antigen demasking was carried out using commercial solution (Target retrieval solution, S1699, DAKO, USA) at 97°C for 20 min [12]. Sections were rinsed in Tris buffer (pH 7.6) before endogenous peroxidase activity was blocked using 3% hydrogen peroxide in absolute methanol for 30 min. A further rinse in tap water for 10 min was followed by application of commercial blocking solution (Protein block, serum free, X0909, DAKO, USA) for 10 min at room temperature. Treatment for 1 h with the primary antibody (rabbit polyclonal anti *M. haemolytica)*, at room temperature and at a 1/200 dilution followed. Sections were then washed three times (5 min each) in Tris buffer before incubation at room temperature for 1 h with secondary antibody (Goat anti-rabbit IgG, HRPO-Conjugated, A9169, Sigma- Aldrich, USA) at 1/800 dilution. Further rinsing for three times (5 min each) in Tris buffer was followed by peroxidase development using DAB (D5905, Sigma-Aldrich, USA) in 1 h and sections were counterstained with Mayer's haematoxylin [10].

As a negative control, a normal sheep lung tissue (based on negative bacteriological and histological findings) was used. For serum control, replicate sections of the pneumonic lungs were processed, substituting the primary antibody with the rabbit pre-immune serum. This serum was free of antibodies against *M. haemolytica, P. multocida, B. trehalosi* and *E. coli* based on the results of Dot ELISA.

Statistical Analysis

McNemar test and kappa coefficient were used to compare the diagnostic power and the agreement of the two diagnostic methods, respectively. Chi square or Fisher

exact tests were also used to evaluate the data distribution. Results are shown using *P*-values with 5% level of significance. All analysis was performed using SAS software (SAS, 9.1).

RESULTS

Pathological Findings

Grossly, the common feature of the selected lungs was the various degrees of consolidation affecting the cranioventral lobes. In 21 cases, consolidation was seen exclusively in the right lung. The lesion in one case was restricted to the left lung and in 42 cases it included both the left and the right lungs. In one case, consolidation was observed in the whole lung. Among the lobes, affected regions were frequently observed in the right cranial lobe, especially in its cranial portion (57 cases). In the cut surfaces, at least one of the following manifestations was noted: multiple irregular pale necrotic foci with 2-7 mm diameter *(Fig. 1)*, grey nodules 1-4 mm in diameter, gelatinous thickening of interlobular septa and suppurative or mucous exudate in the bronchi.

Histologically, suppurative bronchopneumonia was observed in 44 cases, followed by necrotic bronchopneumonia (9 cases), fibrinous bronchopneumonia (8 cases), ovine pulmonary adenocarcinoma (6 cases), bronchointerstitial pneumonia (1 case), and pyogranulomatous pneumonia (1 case).

Suppurative bronchopneumonia was categorized into 3 subtypes; acute, subacute and chronic. Filling of the airspaces (bronchi, bronchioles and alveoli) by infiltrated leukocytes, mainly neutrophils, was a common feature of 3 subtypes, especially the acute ones (10 cases). The microscopic features of OPA (papillary hyperplasia of pneumocytes type II and the Clara cells) was detected in 2 of acute suppurative bronchopneumonic lungs. In 12 subacute suppurative pneumonic cases, there was epithelial hyperplasia of some bronchioles and increasing number of infiltrated mononuclear leukocytes such as macrophages within the airspaces. Chronic suppurative bronchopneumonia (22 cases) was characterized by peri-airways lymphoid hyperplasia, bronchial squamous metaplasia and bronchiolar goblet cell metaplasia.

Necrotic bronchopneumonia was characterized by irregular areas of coagulative necrosis surrounded by a dense zone of necrotic leukocytes, specially neutrophils. Heavy infiltration of neutrophils and/or deposition of fibrin were also seen within the adjacent bronchioles and alveoli. This type of pneumonia was observed in 1 specimen along with OPA.

In fibrinous bronchopneumonia, fibrin was the predominant component of the filling exudates of the airspaces. Hyperemia, dilation of lymphatic vessel, infiltration of neutrophils, and fibrin deposition were common findings in the interlobular septa and the pleura. In one of the affected lungs, the localized chronic suppurative bronchopneumonia was also seen.

Bacteriological Findings

The culture result of the 65 specimens showed bacterial isolation in 62 specimens (95.38%). Because of mixed infections in some specimens, the total number of the isolate increased to 92. The most isolated bacteria were *M. haemolytica* (34; 52.30%) and then *P. multocida* and *B. trehalosi* (12; 18.46%). *M. haemolytica,* that was isolated from 34 lungs, grew in 20 lungs as pure and mixed with other bacteria in 14 specimens *(Table 1)*. *Table 1* shows other bacteria isolated with a low frequency in the lungs.

Immunohistochemical Findings

The presence of *M. haemolytica* or its antigens were detected in 41 (63.07%) of 65 lung specimens. IHC positive

Fig 1. Cut surface of left cranial lobe of an affected lung showing sharply demarcated irregular shaped foci of necrosis *(asterisks)* within consolidated region

Şekil 1. Enfekte bir akciğere ait sol kranial lobun kesit yüzeyinde konsalide bölge içerisinde yer alan keskin kenarlı düzensiz şekilli nekroz odakları *(yıldızlar)*

Table 1. *Bacteriological, histopathological and immunohistochemical findings in 65 suspected bronchopneumonic lungs of sheep*
Tablo 1. *Bronkopnömoni şüpheli 65 koyun akciğerine ait bakteriyolojik, histopatolojik ve immunohistokimyasal bulgular*

Lesion	Count	Culture positive		IHC positive		Isolated bacteria of IHC negative lungs										
		Pure	Mixed	Mild	Severe	B. t	P. m	P sp A	M. h	C. s	St. a	St. d	St.	Pr. v	En. a	N. G.
Chronic suppurative bronchopneumonia	22	9	4	15	0	3	1	1	1	0	1	1	0	0	1	1
Subacute suppurative bronchopneumonia	12	6	2	9	0	0	1	2	0	0	0	0	0	0	0	0
Acute suppurative bronchopneumonia	10	4	2	5	0	1	1	1	1	2	0	0	1	0	0	1
Necrotic bronchopneumonia	9	0	4	0	8	1	0	0	0	0	0	0	0	0	0	0
Fibrinous bronchopneumonia	8	0	2	3	0	1	2	0	0	0	0	0	0	1	0	1
Bronchointerstitial pneumonia	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Total	62	20	14	33	8	6	5	4	2	2	1	1	1	1	1	3

P. m: *Pasteurella multocida*, ***B.t:*** *Bibersteinia trehalosi*, ***St.:*** *Streptococcus spp.*, ***P. sp A:*** Pasteurella species A, ***St.a:*** *Streptococcus agalactiae*, ***C.s:*** *Corynebacterium pseudotuberculosis*, ***Pr.v:*** *Proteus vulgaris*, ***St.d:*** *Streptococcus dysgalactiae*, ***En.a:*** *Enterobacter aerogenes*, **N.G.:** no growth bacteria

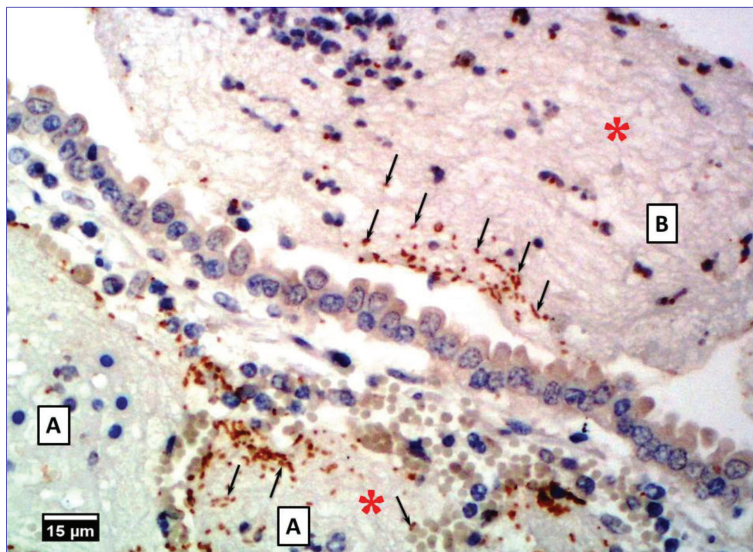


Fig 2. Positive immunostaining of *M. haemolytica (large arrows)* within fibrinous exudate *(asterisks)* in a bronchiole (B) and adjacent alveoli (A), Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 2. Bronşiol (B) ve bitişiğindeki alveollerde (A) fibrinli eksudat içerisinde *(yıldızlar) M. haemolytica (büyük oklar)* pozitif immunboyanma, İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

cases were divided into 2 groups based on the intensity of immunoreactivity.

Group 1 which contained 8 specimens (19.51%) was identified by a strong reaction against the antigen in necrotic foci and surrounding areas. The reaction was restricted to fibrinous exudate *(Fig. 2)*, around necrotic cells *(Fig. 3)* and inside surrounding neutrophils and macrophages. In other areas, the presence of the bacteria and antigens were similar to Group 2.

Group 2 (33 specimens; 80.48%) was typified by weak diffuse reaction against the antigen, especially in the exudate or inflammatory cells within the airways or alveoli. The same reaction was seen within or on the luminal surface of the airway's epithelial cells *(Fig. 4)*.

In both groups, the bacterial antigens were seen in the cytoplasm of affected cells in mild, moderate, and severe patterns. In the mild pattern, the antigens were observed in the cytoplasm as multiple large and small inclusions especially in large macrophages *(Fig. 5)*. In the moderate pattern, the cytoplasm had been occupied mainly by the antigens in which nucleus and only the rim of the cytoplasm were observable. In the severe pattern, the cells were completely filled with the antigens *(Fig. 6)*.

Comparison of Bacteriological and IHC Results

McNemar test showed a significant difference between diagnostic power of IHC and bacterial culture in the detection of *M. haemolytica* (The Kappa correlation coefficient was 0.66). *Table 2* demonstrates a correlation between frequency distribution of samples detected by both methods. Considering IHC as a golden test, sensitivity

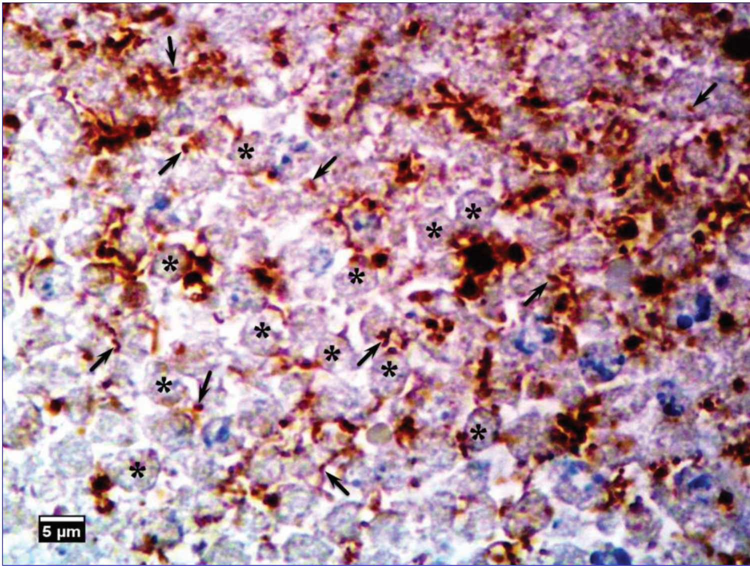
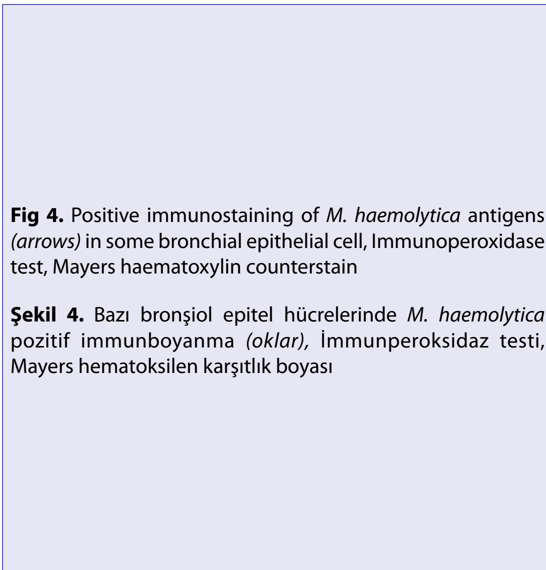


Fig 3. Immunopositive staining of *M. haemolytica (arrows)* around necrotic cells *(asterisks)*. Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 3. Nekrotik hücrelerin *(yıldızlar)* çevrelerinde *M. haemolytica* immunpozitif boyanma *(oklar)*. İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası



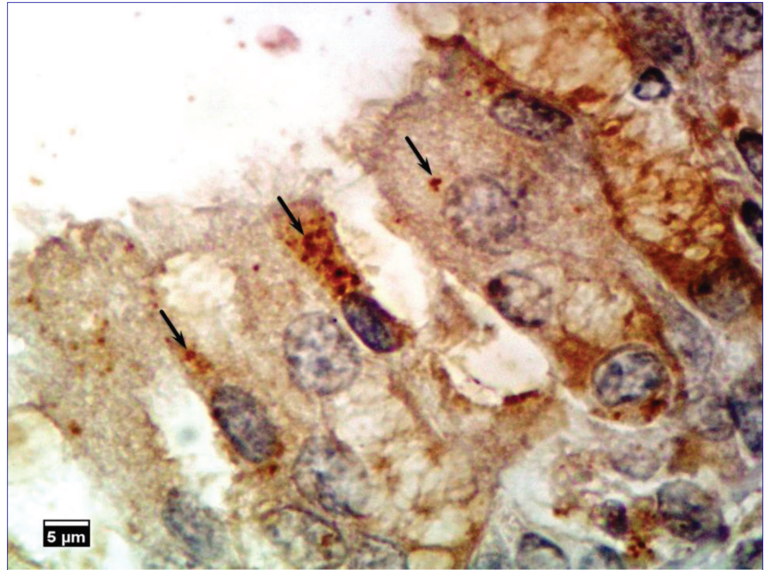


Fig 4. Positive immunostaining of *M. haemolytica* antigens *(arrows)* in some bronchial epithelial cell, Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 4. Bazı bronşiol epitel hücrelerinde *M. haemolytica* pozitif immunboyanma *(oklar),* İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

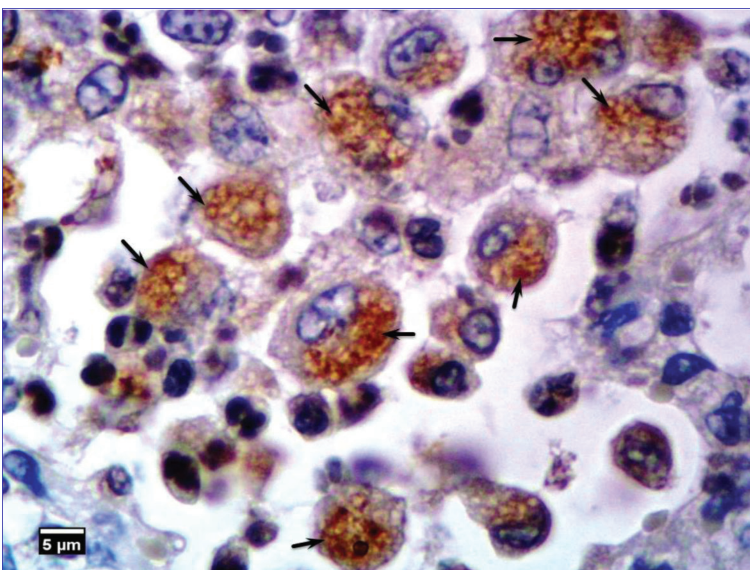


Fig 5. Mild pattern of immunostaining of *M. haemolytica* antigens *(arrows)* in large macrophages within an alveolus, Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 5. Bir alveol içerisinde büyük makrofajlarda *M. haemolytica* antijenlerinin orta dereceli immunboyanması *(oklar),* İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

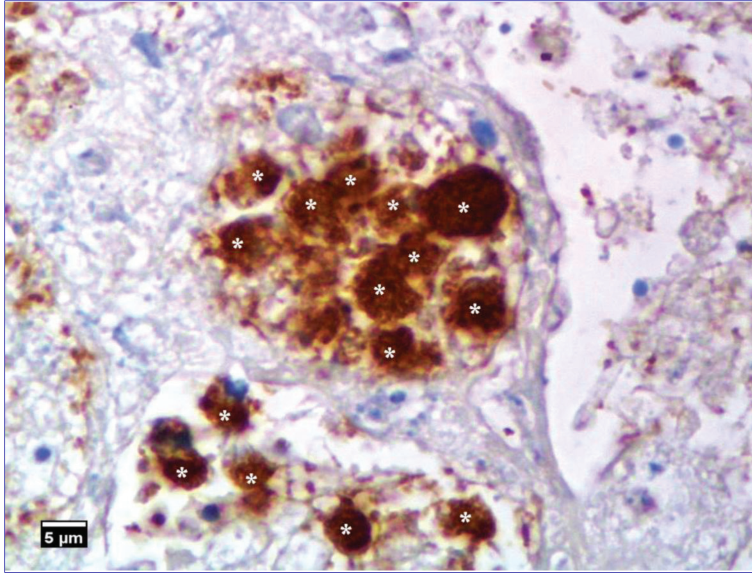


Fig 6. Intense pattern of immunostaining of *M. haemolytica* antigens and filling of macrophages *(asterisks)* within alveoli, Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 6. Alveol içerisinde makrofajlarda *M. haemolytica* antijenlerinin yoğun dereceli immunboyanması *(yıldızlar)*, İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

Table 2. *Frequency (percentage) of positive and negative samples in IHC or culture methods*

Tablo 2. *İHK veya kültür metodlarında pozitif ve negative örneklerin sıklığı (yüzde olarak)*

Culture	IHC		
	Positive n (%)	Negative n (%)	Total n (%)
Positive	32 (49.23)	2 (3.08)	34 (52.31)
Negative	9 (13.85)	22 (33.85)	31 (47.69)
Total	41 (63.08)	24 (36.92)	65 (100)

Table 3. *The frequency (percentage) of pulmonary lesions and IHC detection of M. haemolytica*

Tablo 3. *Pulmoner lezyonların ve İHK ile M. haemolytica tespit edilebilme sıklıkları (yüzde olarak)*

Lesion	IHC Results		
	Positive n (%)	Negative n (%)	Total n (%)
Suppurative bronchopneumonia	29 (46.03)	14 (22.22)	43 (68.25)
Necrotic bronchopneumonia	8 (12.70)	1 (1.59)	9 (14.29)
Fibrinous bronchopneumonia	3 (4.76)	5 (7.94)	8 (12.70)
OPA	0	3 (4.76)	3 (4.76)
Total	40 (63.49)	23 (36.51)	63 (100.00)

and specificity of bacterial culture were estimated as 78.05 and 91.67%, respectively.

In the Chi-square test, significant correlations were observed between the distribution of the lesions and the bacterial isolation (P=0.04), types of the lesions and IHC results (P=0.01) as well as the types of bronchopneumonia and mixed/pure isolation (P=0.008) *(Table 3, Table 4)*. In the statistical comparison, the dominant form of bronchopneumonia was compared. The concomitant and low frequent lesions have not been included.

DISCUSSION

In this study, *M. haemolytica* was isolated from 52.3% of samples, while its antigens were detected in 63.7% of lungs by IHC. These results demonstrate a significant difference between bacteriological and immunohistochemical detection of *M. haemolytica*. Such results have been reported in other studies that confirmed preference of immuno-histochemistry compared to bacterial culture [5,13,14]. Also, a significant difference was observed between the diagnostic power of IHC and culture in detection of *M. haemolytica* (P=0.03) and an acceptable coefficient of agreement was seen between the results κ) = 0.66), which has not been implied in earlier studies.

Table 4. *The frequency (percentage) of pulmonary lesions and isolation of M. haemolytica*

Tablo 3. *Pulmoner lezyonların ve M. haemolytica izolasyonlarının sıklıkları (yüzde olarak)*

Lesion	Kind of Isolation			
	Negative n (%)	Pure n (%)	Mixed n (%)	Total n (%)
Suppurative bronchopneumonia	17 (27.87)	19 (31.15)	8 (13.11)	44 (72.13)
Necrotic bronchopneumonia	5 (8.20)	0	4 (6.56)	9 (14.75)
Fibrinous bronchopneumonia	6 (6.84)	0	2 (3.28)	8 (13.11)
Total	28 (45.90)	19 (31.15)	14 (22.95)	61 (100.00)

M. haemolytica was not isolated from 9 IHC positive lungs in which 4 samples had necrotic bronchopneumonia (with strong positive reaction) and the others (with weak positive reaction) had chronic suppurative (3 cases), subacute suppurative and fibrinous bronchopneumonia (1 case each). The identification of *B. trehalosi* from 2 lungs with necrotic lesions as a specific sign of Mannheimiosis [4,15], and not the isolation of *M. haemolytica*, may be explainable with the results of Dassanayake et al.[16]. They proved *B. trehalosi*, due to higher growth rate, inhibits the growth of *M. haemolytica in vitro* and concluded that if these patterns occur *in vivo,* they may cause failure to routinely isolate *M. haemolytica* from pneumonic lungs. Negative culture results in the weak IHC lungs may be due to the low concentration of bacteria or dead bacteria in the tissues examined [5]. The negative IHC results of two bacteriologically positive lungs may be explained by different sampling location for both methods as well as the focal accumulation of bacteria in the lungs [5,17].

Bacterial culture of one IHC negative lung with necrotic lesions was negative for *M. haemolytica* and positive for *B. trehalosi*. It was proved that necrotic lesions in pneumonic sheep can be induced by other bacteria such as *Histophilus somni*, *Trueperella pyogenes* [18] or *B. trehalosi* [4]. Although *B. trehalosi* is a known cause of septicemia in lambs over 5 months [1], it has been isolated from pneumonic lungs [19]. Sasani et al.[20] isolated the bacterium from fibrinous pneumonic lung of a dead lamb with coagulative necrosis (without signs of septicemia). Also in an experimental study by Onderka and Wishart [21], *B. trehalosi (Pasteurella haemolytica* biotype T) has been isolated from the big horn sheep lungs with necrotizing fibrinopurulent broncho-pneumonia.

In the present study, 0.68% of all examined lungs had bronchopneumonia in which suppurative, necrotic and fibrinous types were recognized in 67.69, 13.85 and 12.31% of the selected lungs, respectively. In a retrospective study by Oruc [3], *M. haemolytica* was isolated from 56.14% of the lungs and the most frequent isolation was reported from fibrinous pneumonia (42.19%) and catarrhal-purulent bronchopneumonia (15.63%). In our study, the broncho-pneumonic IHC positive lungs were mostly suppurative (46.03%) and the necrotic and fibrinous bronchopneumonia were in the next ranking (P=0.01). In addition, most chronic and some subacute suppurative bronchopneumonic lungs had known features of chronic enzootic pneumonia: bronchiolar epithelial metaplasia, bronchial epithelial hyper-plasia and peri-bronchiolar lymphoid hyperplasia [19,22,23]. The etiology of the chronic enzootic pneumonia is complex. The disease is often subclinical [19] and has devastating effects on animal growth rate and food conversion ratio [24]. *Mycoplasma ovipneumoniae*, *M. haemolytica* and some viruses are known to cause the disease [19]. Sheehan et al.[25], identified the sign of the chronic enzootic pneumonia in 60% of suspected pneumonic lambs but in 90% of them, isolation of *Mycoplasma ovipneumoniae* or IHC detection of its antigen was reported. In this study, *M. haemolytica* was isolated from 30% of lungs, especially with purulent lesions. Due to observation of the largely intact infiltrated leukocytes and the lobular pattern of lesions, the authors concluded that the role of *M. haemolytica* in the pathogenesis of chronic enzootic pneumonia appears significantly different from acute pneumonic pasteurellosis. Therefore, because of having no following *Mycoplasma* and the pneumonic viruses in our study, we cannot ignore the probability occurrence of chronic enzootic pneumonia in some animals. In other words, isolation of the bacteria from chronic and/ or subacute purulent bronchopneumonic lungs with weak immunoreaction may support a secondary role for *M. haemolytica* in this type of pneumonia. Some authors believe that although mycoplasmas can interfere with host ciliary activity and therefore provide the invasion of other pathogens such as *M. haemolytica* [23], they may also modulate growth or toxin production of *M. haemolytica* [19].

In this study, a significant association was observed between *M. haemolytica* isolation (pure or mixed) and the types of bronchopneumonia. In acute bronchopneumonia, particularly fibrinous and necrotic, *M. haemolytica* was isolated mixed, and in more chronic lesions, it was isolated pure. *M. haemolytica* is a single cause of ovine pneumonic Mannheimiosis [1] and infection with the pneumonic viruses can increase the severity of the disease. However, the role of other ubiquitous opportunistic bacteria in ruminants' population [2] in reducing the resistance of animals to the *M. haemolytica* challenges and facilitating rapid proliferation and descent of *M. haemolytica* into the lower respiratory tract and induction of fatal bronchopneumonia should not be ignored [26,27].

In summary, in the present study, *M. haemolytica* or its antigens were identified by either bacterial culture or IHC in 66.15% of the samples. The sheep lung has anatomical features (minor collateral ventilation and extensive inter-lobular septa) which limits its ability to resolve pneumonic episodes and the capacity to expel alveolar exudate [28]. Therefore, occurrence of chronic pneumonic lesions in ruminant is more frequent in sheep than other animals [4]. However, due to the high storage capacity of the lung, focal pneumonic lesions often remain clinically silent but the presence of inflammatory exudate (even mild) has disruptive effects on respiratory performance. Thus detection of 55.55% and 59.25% of *M. haemolytica* antigens in subacute or chronic lesions (suspected chronic enzootic pneumonia) and acute lesions (suspected respiratory Mannheimiosis), respectively showed the significant role of the bacteria in causing pneumonic lesions in the studied sheep.

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REFERENCES

1. Lopez A: Respiratory system, mediastinum and pleurae. **In,** Zachary JF, McGavin MD (Eds): Pathologic Basis of Veterinary Disease. 5th edn., 459-464, 509, 516, Mosby Elsevier, Missouri, 2012.

2. Woolums AR, Ames TR, Baker JC: The bronchopneumonias (Respiratory disease complex of cattle, sheep and goats). **In,** Smith BP (Ed): Large Animal Internal Medicine. 4th edn., 602, Mosby Elsevier, St. Louis, 2009.

3. Oruc E: The pathologic and bacteriologic comparison of pneumonia in lambs. *Turk J Vet Anim Sci,* 30, 593-599, 2006.

4. Caswell JL, Williams KJ: Respiratory System. **In,** Maxie MG (Ed): Jubb, Kennedy and Palmer's Pathology of Domestic Animals. Vol. 2, 5th edn., 525, 528-530, 561-564, 601-604, Saunders Elsevier, Edinburgh, 2007.

5. Haziroglu R, Diker KS, Turkarslan J, Gulbahar MY: Detection of *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* antigens by an immunoperoxidase technique in pneumonic ovine lungs. *Vet Pathol,* 33, 74-76, 1996. DOI: 10.1177/030098589603300108

6. Markey BK, Leonard FC, Archambault M, Culinane A, Maguire D: Clinical Veterinary Microbiology. 2nd edn., 307- 316, Mosby Elsevier, London, 2013.

7. Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S, Hartigan PJ: Veterinary Microbiology and Microbial Disease. 2nd edn., 207-212, 263-284, 300-308, Wiley-Blackwell, Chichester, 2011.

8. Winn Jr WC, Allen SD, Janda WM, Koneman EW, Procop GW, Schreckenberger PC, Woods GL: Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th edn,. 223-235, 458-467, 709-715, Lippincott Williams & Wilkins. Philadelphia, 2006.

9. Diker KS, Akan M, Kaya O: Evaluation of immunogenicity of Pasteurella hemolytica serotypes in experimental models. *Turk J Vet Anim Sci*, 24, 139-143, 2000.

10. Jones EL, Gregory J: Immunoperoxidase methods. **In,** Catty D (Ed): Antibodies: A Practical Approach. Vol. 2, 156-177, IRL Press, Washington DC, 1988.

11. Tuchinda K, Naknam B, Sunthornandh P, Kittikul C, Lawhavinit O: Detection of *Salmonella* in food samples by dot-ELISA using polyclonal antibody. *Kasetsart J (Nat Sci)*, 45, 444-450, 2011.

12. Kumar GL, Rudbeck L: Damasking of Antigens. **In,** Kumar GL, Rudbeck L (Eds): Education Guide, Immunohistochemical (IHC) Staining Methods. 5th edn., 52, Dako North America, Carpinteria, 2009.

13. Haziroglu R, Diker KS, Gulbahar MY, Kul O: Immunoperoxidase examination of pneumonic bovine lungs naturally infected with *Pasteurella haemolytica. Isr J Vet Med,* 56, 59-61, 2001.

14. Yener Z, Ilhan F, Ilhan Z, Saglam YS: Immunohistochemical detection of *Mannheimia* (*Pasteurella*) *haemolytica* antigens in goats with natural pneumonia. *Vet Res Commun*, 33, 305-313, 2009. DOI: 10.1007/s11259-008-9178-z

15. Haritani M, Nakazawa M, Oohashi S, Yamada Y, Haziroglu R, Narita M: Immunoperoxidase evaluation of pneumonic lesions induced by *Pasteurella haemolytica* in calves. *Am J Vet Res*, 48 (9): 1358-1362, 1987.

16. Dassanayake RP, Call DR, Sawant AA, Casavant NC, Weiser GC, Knowles DP, Srikumaran S: *Bibersteinia trehalosi* inhibits the growth of *Mannheimia haemolytica* by a proximity-dependent mechanism. *Appl Environ Microbiol*, 76, 1008-1013, 2010. DOI: 10.1128/AEM.02086-09

17. Haritani M, Ishino S, Oka M, Nakazawa M, Kobayashi M, Narita M, Takizawa T: Immunoperoxidase evaluation of pneumonic lesions in calves naturally infected with *Pasteurella haemolytica. Jpn J Vet Sci,* 51 (6): 1137-1141, 1989.

18. Haritani M, Nakazawa M, Hashimoto K, Narita M, Tagawa Y, Nakagawa M: Immunoperoxidase evaluation of the relationship between necrotic lesions and causative bacteria in lungs of calves with naturally acquired pneumonia. *Am J Vet Res,* 51 (12): 1975-1979, 1990.

19. Gilmour NJL, Gilmour JS: Pasteurellosis of sheep. **In,** Adlam C, Rutter JM (Eds): Pasteurella and Pasteurellosis. 223-260, Academic Press Limited, London, 1989.

20. Sasani F, Atyabi N, Raei Dehaghi M: Pneumonic pasteurellosis (Fibrinous bronchopneumonia) in lamb due to *Pasteurella hemolytica* (Biotype T). *J Vet Res,* 57 (3): 73-74, 2002.

21. Onderka DK, Wishart WD: Experimental contact transmission of *Pasteurella haemolytica* from clinically normal domestic sheep causing pneumonia in rocky mountain bighorn sheep. *J Wildlife Dis*, 24 (4): 663-667, 1988. DOI: 10.7589/0090-3558-24.4.663

22. Gilmour JS, Jones GE, Rae AG: Experimental studies of chronic pneumonia of sheep. *Comp Immunol, Microbiol Infect Dis*, 1, 285-293, 1979. DOI: 10.1016/0147-9571(79)90030-4

23. Ayling RD, Nicholas RAJ: Mycoplasma respiratory infections. **In,** Aitken ID (Ed): Diseases of Sheep. 4th edn., 231- 235, Blackwell Publishing, Oxford, 2007.

24. Jones GE, Field AC, Gilmour JS, Rae AG, Nettleton PF, McLauchlan M: Effects of experimental chronic pneumonia on bodyweight, feed intake and carcass composition of lambs. *Vet Rec*, 110, 168-173, 1982. DOI: 10.1136/vr.110.8.168

25. Sheehan M, Cassidy JP, Brady J, Ball H, Doherty ML, Quinn PJ, Nicholas RAJ, Markey BK: An aetiopathological study of chronic bronchopneumonia in lambs in Ireland. *Vet J*, 173, 630-637, 2007. DOI: 10.1016/j.tvjl.2006.01.013

26. Dassanayake RP, Shanthalingam S, Herndon CN, Subramaniam R, Lawrence PK, Bavananthasivam J, Cassirer EF, Haldorson GJ, Foreyt WJ, Rurangirwa FR, Knowles DP, Besser TE, Srikumaran S: *Mycoplasma ovipneumoniae* can predispose bighorn sheep to fatal *Mannheimia haemolytica* pneumonia. *Vet Microbiol*, 145, 354-359, 2010. DOI: 10.1016/j.vetmic.2010.04.011

27. Porter JF, Connor K, Krueger N, Hodgson JC, Donachie W: Predisposition by an ovine isolate of *Bordetella parapertussis* to subsequent infection with *Pasteurella haemolytica* A2 in specific pathogenfree lambs. *J Comp Pathol*, 112, 381-389, 1995.

28. Ackermann MR, Brogden KA: Response of the ruminant respiratory tract to *Mannheimia (Pasteurella) haemolytica*. *Microbes Infect*, 2, 1079-1088, 2000. 10.1016/S1286-4579(00)01262-4

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Research Article

A New ^{99m}Tc Labeled Peptide: ^{99m}Tc β-Casomorphin 6, Biodistribution and Imaging Studies on Rats [1]

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Abstract

Peptide radiopharmaceuticals have an increasing significance in nuclear medicine practice. β-casomorphin is a digestive peptide with 6 amino acids (Tyr-Pro-Phe-Pro-Gly-Pro). N terminal amino acid chain mainly tyr-pro-phe-pro structured exogen opioid peptid type beta cosomorphin are μ-receptor agonistic activity with priority. Animal studies show that β-casomorphins can act as opioid receptor agonists. The aim of this study was to label β-casomorphin with ^{99m} Tc and to examine its usefulness as an opioid receptor binding radiopharmaceutical in *Albino Wistar rats* and cancer cells. β-casomorphin was labeled with ^{99m} Tc radionuclide using bifunctional chelating agent. Quality control studies were done by Instant Thin layer chromatography (ITLC) and High performance liquid chromatography (HPLC) methods. Binding efficiency of the compound was more than 99%. It was observed as stable for at least 3 h at room temperature. Lipophilicity was also performed for labeled molecule. Imaging studies for ^{99m} Tc labeled molecule was done in rats by using gamma camera. For biodistribution studies; ^{99m} Tc labeled molecule was injected to the rats from tail vein and radioactivity per gr weight of each organ was measured as count per second (cps). Receptor specificity was evaluated in imaging and biodistribution studies in experimental animals. Cell labeling studies were also performed on *breast* and ovarien cancer cells. In terms of evaluating the biodistribution of ^{99m} Tc-β-casomorphin molecule in rats, *uterus* and *ovary* displayed high involvement. It was also confirmed by cell labeling studies. If the radiopharmaceutical is radiolabeled with therapeutic radionuclides it would be useful for therapy for *uterus, ovary* and *breast* tumors.

Keywords: *β-casomorphin, Cancer cell , Peptide, ^{99m}Tc, receptor*

^{99m}Tc Bağlı Yeni Bir Peptid: Ratlarda ^{99m} Tc β-Kazomorfin 6 Molekülünün Biyodağılım ve Görüntüleme Çalışması

Özet

Peptit radyofarmasötikleri nükleer tıp pratiğinde giderek artan bir öneme sahiptir. β- kazomorfin 6 amino asit (Tyr-Pro-Phe-Pro-Gly-Pro) ile bir sindirim peptitidir. Tyr-Pro-Phe-Pro aminoasit zinciri ile başlayan ekzojen opioid peptid türevi β-kazomorfinler öncelikle μ-resptör agonistik aktiviteye sahiptir. Hayvan çalışmaları β-kazomorfin türevlerinin bir opioid reseptör agonisti olduğunu göstermiştir. Bu çalışmanın amacı, β-kazomorfin'i ^{99m} Tc ile işaretleyerek opioid reseptörler radyofarmasötiği olarak kullanabilirliğini Albino Wistar ratlarda ve kanser hücrelerinde incelemektir. β-kazomorphin bifonksiyonel molekül kullanılarak ^{99m} Tc radyonüklidi ile işaretlendi. Kalite kontrol çalışmaları, ince tabaka kromatografisi (ITLC) ve yüksek performanslı sıvı kromatografisi (HPLC) yöntemleri ile yapıldı. Bileşiğin işaretlenme verimi %99'dan fazla idi. Oda sıcaklığında, en az 3 saat boyunca stabil kaldığı tespit edildi. İşaretli molekülün lipofilite çalışması da gerçekleştirildi. ^{99m}Tc ile işaretli molekülün görüntüleme çalışmaları sıçanlarda gamma kamera kullanılarak gerçekleştirildi. Biyolojik dağılım çalışmaları için ^{99m}Tc işaretli molekül kuyruk veninden sıçanlara enjekte edildi ve her organın gr ağırlığı başına radyoaktivitesi sayım/saniye olarak ölçüldü. Molekülün reseptöre bağlanma özelliği deney hayvanlarında görüntüleme ve biyolojik dağılım çalışmaları ile değerlendirildi. Hücre işaretleme çalışmaları meme ve yumurtalık kanser hücreleri için yapıldı. ^{99m} Tc β-kazomorfin molekülünün biyolojik dağılımı sıçanlarda değerlendirildiğinde, rahim ve yumurtalıkta yüksek tutulum gösterdiği görüldü. Molekülün reseptör etkinliği hücre işaretleme çalışmalarıyla da doğrulandı. Molekül tedavi radyonüklitleri ile işaretlendiğinde ise rahim ve yumurtalık tümörleri tedavisinde yararlı olabilecektir.

Anahtar sözcükler: *β-kazomorfin, Kanser hücresi , Peptid, ^{99m}Tc, reseptör*

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INTRODUCTION

Peptides are receptor specific molecules. They have an important role not only in diagnosis but also in treatment modalities. Peptide radiopharmaceuticals are developed for this purpose became important in nuclear medicine practice. Use of receptor-specific peptide compounds has an increasing significance not only in imaging, but also in treatment modalities [1-6].

Imaging of receptors on tumor cell surface using radiolabeled regulatory peptides becoming ingradually important strategy in oncology research [7]. High affinity of radiolabeled peptides for receptors indicates molecular targets for diagnosis and treatment of many cancer types.

Several clinically relevant radionuclides have been used for labeling bioactive peptides either for diagnostic imaging (^{99m}Tc, ^{111}In, $^{68/66}$Ga, ^{18}F, ^{123}I, ^{64}Cu) or for therapy (^{111}In, $^{64/67}$Cu, ^{90}Y, ^{177}Lu, ^{213}Bi) [2-4,6].

Small-sized peptides (compounds having short chain with less than 30 amino acids) manifest rapid pharmacokinetics. They easily penetrate into extravasculary tissue space, are excreted rapidly and their clearance from the blood is fast [7-10].

Rapid pharmacokinetics are ideal for labeling peptides with a radioisotope that has a short half-life, such as ^{99m}Tc. Among all the radioisotopes used in nuclear medicine ^{99m}Tc is still the most widely applied for diagnostic purpose, mainly because of the ready availability, low cost, excellent imaging properties, favorable dosimetry and high specific activity [11,12]. ^{99m}Tc-labeled peptides, particularly those of a lipophilic nature, are often excreted through the hepatobiliary system, and the subsequent accumulation in the intestine may obscure receptor-mediated uptake in tumor sites in the pelvis [13,14]. In our previous studies CCK8 (Asp-Tyr(SO3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) was labeled with ^{99m}Tc using bifunctional chelates [Glucoheptonate (GH) and Diethylene-Triamine-Pentaacetate (DTPA)] and it was shown that this could have a high potential for practice in tissues containing CCK receptors [15]. We also studied on ^{99m}Tc exorphin C peptide molecule for biodistribution and imaging on tumor bearing rats [16].

β-casomorphin is an exogenous bioactive opioid peptide derivative from enzymatic digests of bovine casein [17]. β-casomorphin is a β-casein derive peptide sequence present in the milk protein. β-casomorphin release from parent protein molecule during *gastrointestinal* digestion. It was reported that *uterus* and *ovary* express opioid receptors [17,18].

Animal studies show that β-casomorphins can act as opioid receptor agonists are derived-casein.

Animal studies clearly show that β-casomorphins derived from β-casein are opioid-like peptides [19,20].

N terminal amino acid chain mainly tyr-pro-phe- pro structured exogen opioid peptid type beta casomorphin are receptör agonistic activity with priority [21,22].

In this study β-casomorphin has 6 aminoacid (Tyr-Pro-Phe-Pro-Gly-Pro) sequenced peptide molecule was labeled with ^{99m}Tc and investigated its radiopharmaceutical potential by imaging and biodistribution studies in rats.

MATERIAL and METHODS

All animals were treated in accordance with protocols approved by the Animal Care and Use Committee of Ege University.

HPLC equipment: is an equipment which has high pressure-four gradient LCD display screen microprocessor controlled pump, variable wave length UV/VIS detector, sample automatic injection and control unit (Perkin Elmer Series, 200 USA). There is a fraction collector on the equipment to collect the carrier phase (Foxy 200).

Gamma camera: Philips Forte Gamma Camera (for scintigraphy).

In this study, linear peptide molecule was designed with 6 amino acid sequences (Tyr-Pro-Phe-Pro-Gly-Pro) and it was synthesized by PepMetric Technologies commercially. All other chemicals were supplied from Sigma-Aldrich Co.

UV absorption spectral analysis was performed by spectrophotometer. Purity of synthesized molecule was checked in HPLC equipment at 214 nm wave length by using C-18 RP as column and as eluents 0.1% TFA and water as solution. Purity of synthesized molecules was 97%.

Labeling and Quality Control Studies with ^{99m}Tc

- Preparation of Solutions

Preparation of peptide solution: Peptide was dissolved in pure water. Peptide solutions were fractioned and kept in tapped tubes at -20°C to be used 1 tube (250 µg/mL) at each time. Out of 250 µg/mL peptide solution, 0.1 mL was used for labeling process.

Labeling of peptides with ^{99m}Tc: In labeling procedures, firstly direct labeling method was used, but since it could not reveal sufficient labeling, desired result was obtained similar with our previous study [15,16,23].

GH molecule was used as bifunctional agent in indirect labeling method. Ten mg of GH (Sigma Chemical Co.) was dissolved in 1 ml distilled water, 300 µg (in 0.5 ml water) stannous chloride and 259 MBq of ^{99m}Tc (in 0.5 mL), 25 µg peptide (in 0.1 mL), were added to the solution and then the mixture was heated for 10 min at 90°C. Firstly reduction

of technetium was (^{99m}Tc) was accomplished by tin and then reduced ^{99m}Tc was bound to peptide molecules using bifunctional agent GH at pH value of 5 *(Fig. 1)*.

After cooling to room temperature, quality control assessments of ^{99m}Tc-β-casomorphin, was done by ITLC and HPLC methods. Rf values of labeled compounds for 0.9% NaCl and acetone were determined using ITLC-SG (1x10) strips *(Table 1, Fig. 2, Fig. 3)*.

Conditions for HPLC: The wavelength of labeled compound was measured as 195 nanometers in the spectrophotometer. Solutions of 0.1% TFA and acetonitrile at ratio of 20/80 with a flow velocity of 1 mL/min was set up, wavelength was set up 195 nanometer for UV detector *(Fig. 4)*.

- Determination of Lipophilicity

Lipophilicity values are important in understanding biologic transport characteristics of the peptide molecule. Distribution of a single molecule between two different phases can be defined as distribution factor.

Table 1. *Rf values of relevant complexes*
Tablo 1. *İlgili komplekslerin Rf değerleri*

Compound	Physiological Serum (*Rf*)	Acetone (*Rf*)
Na^{99m}TcO4	1	1
^{99m}Tc O2	0	0
^{99m}Tc GH	0.4	0
^{99m}Tc-β-casomorphin	1	0

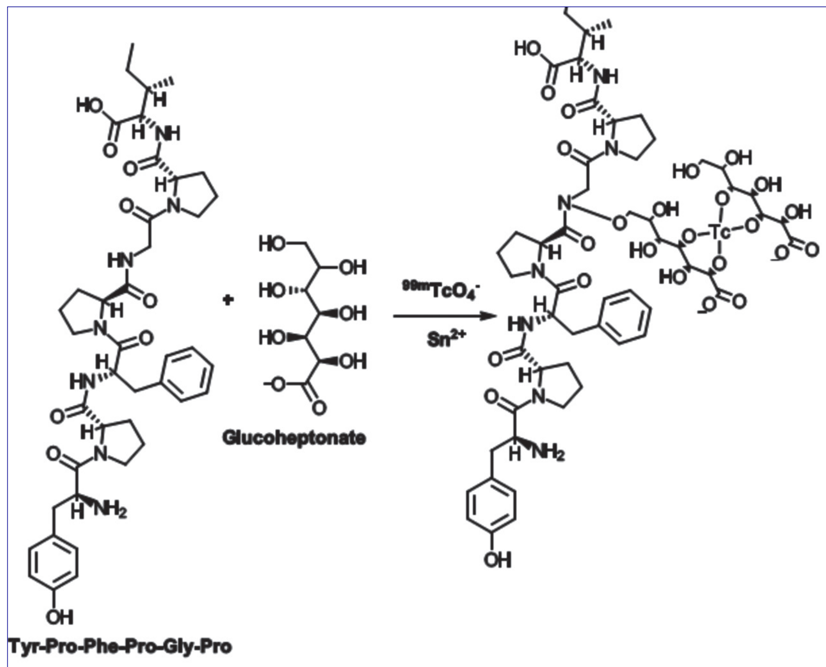

Fig 1. β-casomorphin + GH + ^{99m}Tc O4

Şekil 1. β-kasomorphin + GH + ^{99m}Tc O4

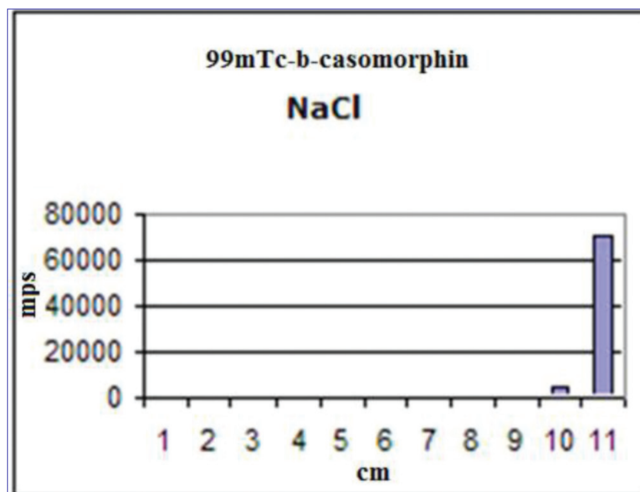

Fig 2. ITLC-SG diagram of ^{99m}Tc-β-casomorphin in NaCl 0.9%
Şekil 2. %0.9 NaCl'de ^{99m}Tc-β-kazomorphin'in ITLC-SG diyagramı

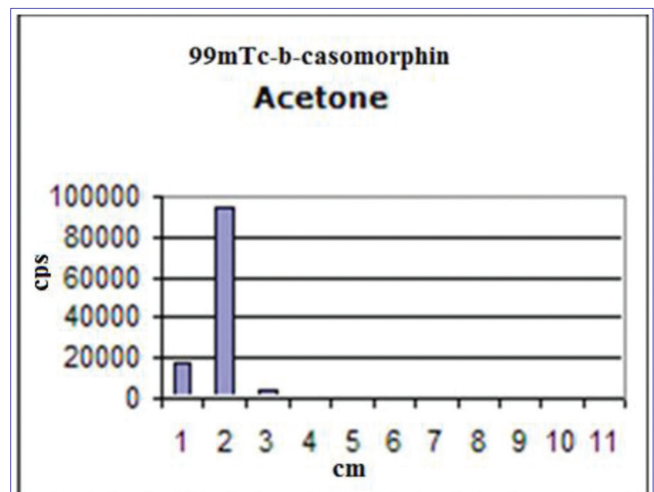

Fig 3. ^{99m}Tc-β-casomorphin ITLC-SG in acetone
Şekil 3. Aseton'da ^{99m}Tc-β-kazomorphin ITLC-SG diyagramı

Experimental lipophilicity values of labeled compounds were determined by extraction method carried out in octanol for two different solutions 0.9% NaCl and H_2O. For lipophilicity, activities in octanol and water phase and those in octanol and 0.9% NaCl phase were counted in counter equipment as cps. Lipophilicity values were obtained by rationing the counts in both phases and calculating their logarithm. After mixing 500 μL octanol, 500 μL 0.9% NaCl or H_2O and 10 microcurie ^{99m}Tc labeled peptide in a vortex mixer for 1 min, they were centrifuged at 4.000 rpm. Low phase and upper phase radioactivity were counted in dose calibrator and phases were rationed and logarithm was calculated [22,24,25]. The lipophilicity was calculated using the formula

logP = A0/Aw

Besides, theoretical lipophilicity values were compared with experimental values using ACD chemsketch computer program *(Table 2)*.

Experimental Animal Studies

Albino wistar female rats weighing 150-200 g were used for animal experiments. Animals were housed under constant conditions of temperature (20-22°C) and lighting (12 h light-12 h dark). All animals had access to regular rat chow and water *ad libitum* except when scheduled for testing and for sacrifice.

- Scintigraphic Studies

Labeling of peptide molecule with ^{99m}Tc was performed and after reaching a sufficient binding level, scintigraphy and biodistribution experiments were done on experimental animals.

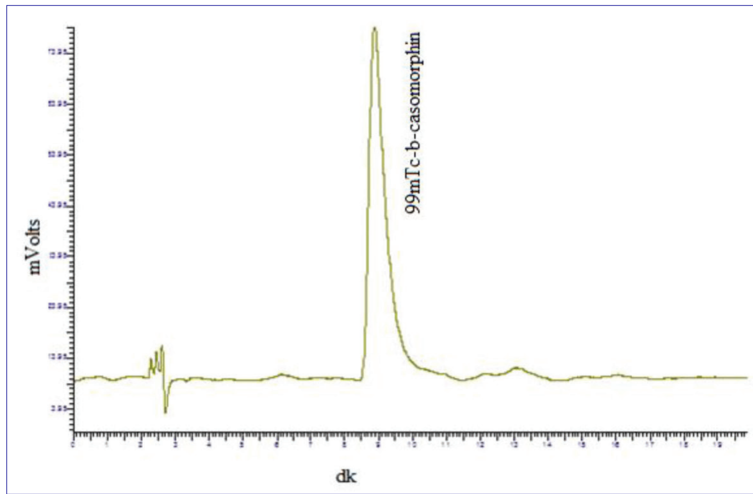

Fig 4. HPLC diagram of ^{99m}Tc-β-casomorphin

Şekil 4. ^{99m}Tc-β-kazomorfin'in HPLC diyagramı

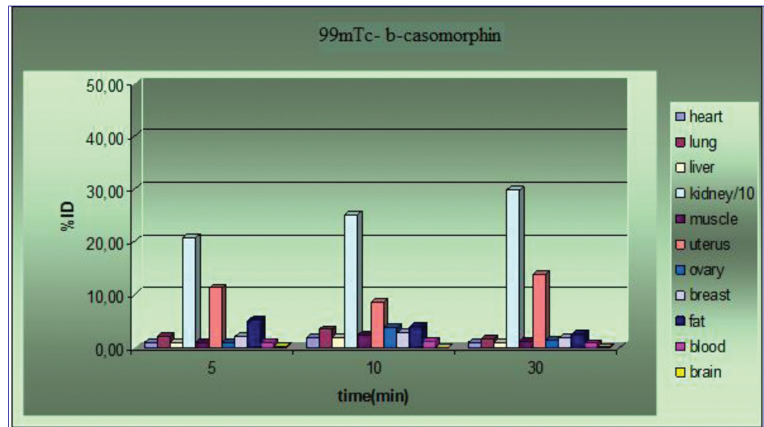

Fig 5. ^{99m}Tc-β-casomorphin biodistribution studies on rats

Şekil 5. Ratlarda ^{99m}Tc-β-kazomorfin biyodağılım çalışması

Table 2. *Therotical lypophilicity value of unlabeled β-casomorphin was similar with ^{99m}Tc-β-casomorphin*

Tablo 2. *Bağlanmamış β-kazomorfin'in kuramsal lipofilitesi ^{99m}Tc-β-kazomorphin ile benzerdir*

Peptide Molecule	Lypophilicity Values (logP)		
	Water	%NaCl	Theoretical
^{99m}Tc-β-casomorphin	1.8±0.1	1.27±0	1.42±0.95

Quality control studies demonstrated that labeled compounds can be administered to living beings. Experimental animals were used as three rats per group. Rats were given general anesthesia, 100 microcurie ^{99m}Tc-β-casomorphin was injected from tail vein and scintigraphy was performed using angle gamma camera. Distribution in the body was followed up by obtaining static images in (256x256) matrix at 5, 10, 15, 20, 30,

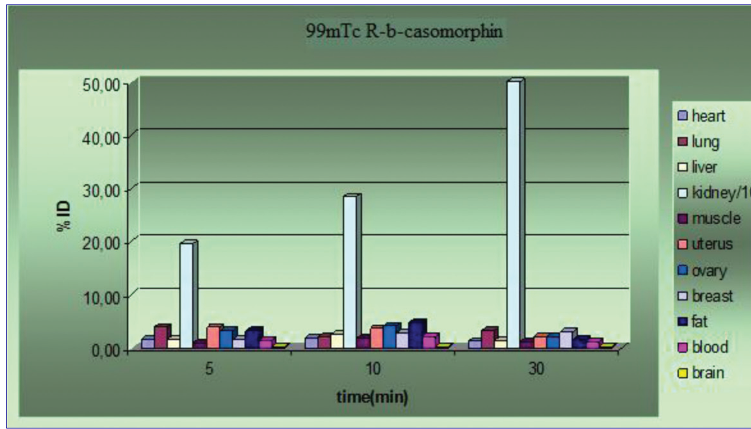

Fig 6. ^{99m}Tc-β-casomorphin reseptor saturated biodistribution studies on rats

Şekil 6. Ratlarda satüre ^{99m}Tc-β-kazomorfin reseptör biyo-dağılım çalışması

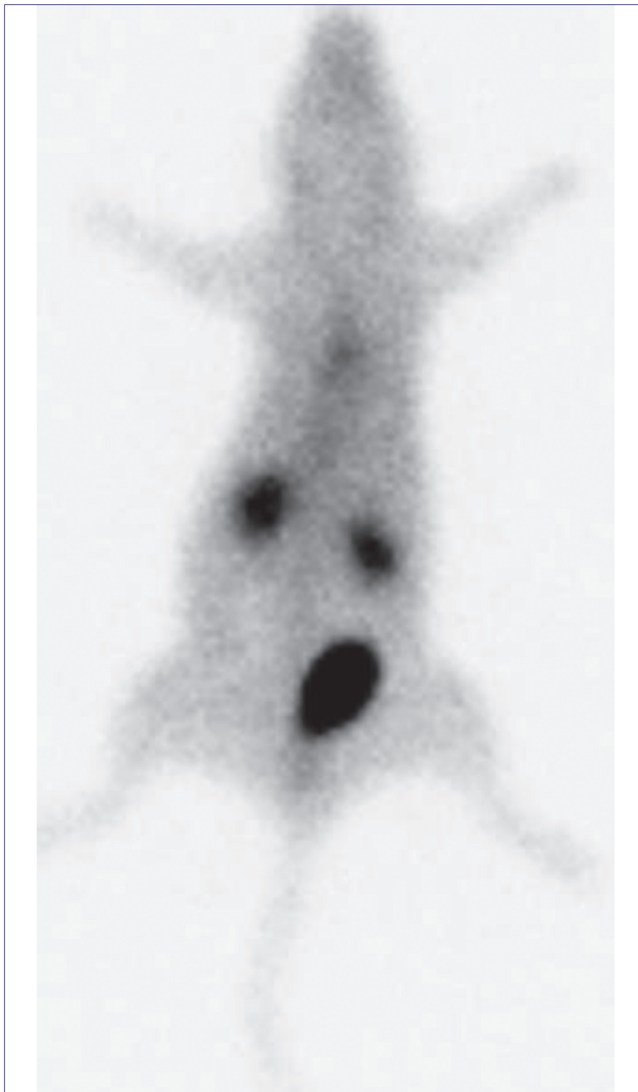

Fig 7. ^{99m}Tc-β-casomorphin scintigraphic imaging on rats

Şekil 7. Ratlarda ^{99m}Tc-β-kazomorfin sintigrafik görüntüleme

60, 120 min and 24 h. For receptor saturation studies, pure β-casomorphin was given prior to radioactive substance and scintigraphic imaging was done under the same conditions *(Fig. 5)*.

***Table 3.** Uptake rate of peptide molecule for target organs*

***Tablo 3.** Hedef organlarda peptid molekülünün tutulum oranı*

99mTc-β-Casomorphin (Organs)	Unsaturated Receptor		Saturated Receptor	
	%ID/g	Organ/Bg	%ID/g	Organ/Bg
uterus	7.02	7.97	0.69	1.23
ovary	5.61	6.7	0.41	0.70
breast	5.3	6.02	0.72	0.48
muscle=bg	0.88	1	0.88	1

***Table 4.** The labeling efficiency of peptide molecule in accordance with cell types*

***Tablo 4.** Hücre tiplerinde peptide molekülünün bağlanma etkinliği*

Tc-99m Labeled Molecule	Cell Cultures		
	MCF7	MDAH2774	WL38(control)
Tc-99m-β-Casomorphin	90.71±9%	80.32±10%	31±8%

For each organ, a graphic was drawn corresponding with time intervals using the counts obtained from images cps.

- Biodistribution Studies

Biodistribution studies were also done on rats. Rats given 100 microcurie labeled molecule was sacrificed at 5th, 10th and 30th min, being three rats from each group. Each organ was extracted and activity was measured in gamma counter. Percentage of activity per gram corresponding cps was calculated. Receptor saturation studies were carried out under the same conditions. Just before radioactive substance injections at 5th, 10th and 30th min, rats were injected 10 μg/0.5 mL pure peptide molecule for receptor saturation. Biodistribution graphic was created with percentage of injected dose amount (ID/g%) for counts per grams corresponding time interval *(Fig. 6, Fig. 7, Table 3)*.

Cell Culture Studies

The radiopharmaceutical potentials of peptide radio-pharmaceuticals in cancerous cell and normal cell were examined in the cell culture studies of peptide radio-

pharmaceutical. The cell culture process took place at the Department of Medical Biology of Faculty of Medicine in Ege University. All cell lines were supplied from American Type Culture Collection (ATCC, UK). BIOAMF-1 (Biological Industries, Israel), BIOAMF-1 supplement for the reproduction of WL-38 (Lung Fibroblast) cell lines in culture medium and RPMI 1640 (Biological Industries, Israel) for the reproduction of MDAH-2774 (Ovarian endometriotid adenocarcinoma) and MCF-7 (breast cancer) cell lines were used by adding 2 mM L-glutamin, 1% Penicillin Streptomycin to the medium. The cells were incubated in the incubator (Thermo, US) maintaining at 95% relative humidity and with 5% CO_2 until the adequate reproduction was obtained from the cells. The proliferation, passaging (by Trypsinization method) and follow-up of cells were monitored through the inverted microscope (Olympus, Japan). Production and passaging processes were continued until the number of cells in each cell line reached 107 per mL.

Cell Labeling Studies

The cells were examined under the microscope at 10x, 40x and 100x magnifications. It was observed that the cells were healthy and enough in number. The cell numbers were studied to be 10^7 cell/mL. The cells were put into falcon tubes and 2 mL medium (RPMI 1640) was added. They were centrifuged at 2.500 rpm for 5 min. The supernatant was removed. 5 mCi/mL Tc-99m-β-casomorphin was added on the cells (MCF7, MDAH2774 and WL38) After the incubation at room temperature for 10 min, 2 mL RPMI-1640 was added. They were centrifuged at 2.500 rpm for 5 min. The upper layer was taken and the labeling efficiency of cells was calculated through the measurement of activities in the lower layer and upper layer *(Table 4)*.

RESULTS

In this study, peptide molecule was labeled with ^{99m}Tc and its usability as opioid receptor pharmaceutical was analyzed. Peptides were labeled with ^{99m}Tc using GH as bifunctional agent. Binding efficiency of the labeled compound was more than 99%. It was observed to be stable for 3 h at room temperature.

Imaging and biodistribution studies on rats were also evaluated for crucial organs. Since *heart* and *lungs* are crucial organs, long term involvement of radiopharmaceutical in these organs is not desired. Our studies on imaging and biodistribution favor these results. It was observed that the radiopharmaceutical was excreted by the *kidneys*. ^{99m}Tc-β-casomorphin molecule, which is slightly lipophilic, was also excreted through *hepatobiliary* route beside *kidneys*.

Uterus, ovaries and *breast* are considered as target organs in studies about biodistribution of ^{99m}Tc-β-casomorphin molecules on rats. Especially in receptor saturation studies, activity decreased during first minutes in *uterus ovaries* and *breast* of the rats while it increased in *stomach* and *small intestines*. This suggests that *uterus ovaries* and *breast* of the female rats constitute targets. Radioactivity in all other tissues was unremarkable. Each of the six amino acids in the sequence Tyr-Pro-Phe-Pro-Gly-Pro appears to be integral to the site recognized by cancer cells through the cell-surface receptors. This conclusion is based on the complete lack of activity that was observed with synthetic peptides in which substitutions were made by using the most closely related amino acid. Since these conservative substitutions give inactive peptides, it seems reasonable to conclude that other, less closely related amino acids [21].

The partition coefficient is defined for dilute solutions as the molar concentration ratio between octanol and water phases at equilibrium: P=A0/Aw [21]. The lipophilicity for ^{99m}Tc- β-casomorphin was given as -2.21±1.44 while 1.42±0.95 for β-casomorphin and -4.07±0.90 for GH by the ACD programmer and found to be 1.8±0.1 for Tc-99m β-casomorphin, experimentally (n=5). Theoretical lipophilicities of ^{99m}Tc complexes cannot be predicted by ACD. It seems that ^{99m}Tc complexing is increasing the lipophilicity. On the other hand, lipophilicity of β-casomorphin was reduced by conjugation with GH, since β-casomorphin has more lipophilicity than GH. Similar lipophilicity value for another ^{99m}Tc labeled seven amino acid sequenced ^{99m}Tc-YGGSLAK (Tyr-Gly-Gly-Ser-Leu-Ala-Lys) lipophilicity value was 1.46±0.13 while theoretical lipophilicity was 1.73. They reported extraction way as hepatobiliary however the target organs were not *ovaries, uterus* or *breast* [13]. In receptor saturation studies, target organs activity decreased with blocking opioid receptors while excretion rate increased. This means that opioid receptor expressing organs uptake labeled molecule, in receptor blocking situation, molecule can not be uptaken by receptor and it is excreted by *kidney*.

In the cell labeling studies, MCF7, MDAH2774 (breast and ovarien cancer) and WL 38 (normal tissue) cells were labeled in vitro with Tc-99m-labeled peptides. It was observed that Tc-99m-β-casomorphin bound to MCF7 and MDAH2774 cancer cell with a high efficiency such as 90.71±9 %80.32±10% respectively.

DISCUSSION

Renal excretion was also observed as expected from small peptides [9]. Moreover, the radiopeptide is rapidly cleared via the *urinary system*, showing much lower *liver* uptake. After saturating receptors, there was increased *renal* and *gastrointestinal* excretion. Increased activity was seen in *intestine, kidney* and *bladder* after receptor saturation study.

Animal studies clearly show that β-casomorphins

opioid-like peptides are derived from β-casein [19-26]. β-Casomorphins are exogenous opioid peptides derivatives containing the common N-terminal amino acid sequence Tyr-Pro-Phe-Pro and have preferential μ-receptor agonistic activity [25].

In terms of evaluating the biodistribution of ^{99m}Tc-β-casomorphin molecule in rats, *uterus* , ovary and breast displayed high involvement at 5th and 10th min in normal biodistribution, it decreased at 30th min. On the contrary, if the receptors were saturated, low involvement was observed in first minutes and increased activity occurred at 30th min. A reverse receptor-peptide reaction is suggested by binding of the peptide molecule at first and then decreasing of the binding as time passes. After saturation of the receptors, this gradual increase in activity that was low at first shows that the radiopharmaceutical is specific to *uterus, ovaries* and *breast* receptors.

In ^{99m}Tc-β-casomorphin molecule, however when the receptor is compared to saturated biodistribution, increase of activity in *kidneys* and *urinary bladder* indicate that labeled molecule is excreted from the body more rapidly. When the radiopharmaceutical is evaluated in terms of *uterus, ovaries* and *breast,* the molecule seems to be more specific for these organs.

In biodistribution studies on rats, the facts that the compound had a low *hepatobiliary* excretion, higher *renal excretion* and rapid pharmacokinetics in the organs correspond to expected characteristics. In cell labeling studies, when breast, ovarien and uterus cancer are compared with control cells results show that labeled molecule can be used for both diagnosing and treatment these type of cancer cells.

Analysis of variance (ANOVA) was applied to the results and data were analyzed statistically. It is suggested that the radiopharmaceutical can be used in diagnosis of especially *uterus ovaries* and *breast* cancer and when labeled with therapeutic radionucleotide, treatment radio-pharmaceutical potential would be attributed to it. For *uterus, ovaries* and *breast* and for the tumors related to these organs, it is demonstrated that a convenient radio-pharmaceutical can exist.

Radionuclide labeled molecule ^{99m}Tc-β-casomorphin is useful for *uterus ovaries* and *breast* cancer cells. The peptide molecule must have appropriate chemical design to the receptor in order to be able to have affinity for tumor tissue at cellular level. The affinity towards cancer cells can be increased through causing change in the amino acid sequence of peptide molecule. The fact that radiolabeled peptides have high affinity towards cancer cell receptor shows the molecular targets for the diagnosis and treatment for *uterus ovaries* and *breast* cancer. The use of peptide for peptide receptor radionuclide therapy when it is labeled with the therapy radionuclides such as ^{177}Lu, whose amino acid sequence is Tyr-Pro-Phe-Pro-Gly-Pro can be convenient in conformity with *in vivo* applications.

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REFERENCES

1. Fischman AJ, Babich JW, Strauss WH: A ticket to ride: Peptide radiopharmaceuticals. *J Nucl Med*, 34, 2253-2263, 1993.

2. Fani M, Maecke HR: Radiopharmaceutical development of radiolabelled peptides. *Eur J Nucl Med Mol Imaging,* 39, 11-30, 2012. DOI: 10.1007/s00259-011-2001-z

3. Dong ZL, Wang F: Peptide-based radiopharmaceuticals for targeted tumor therapy. *Curt Med Chem*, 21, 139-152, 2014.

4. Giblin MF, Veerendra B, Smith CJ: Radiometallation of receptor-specific peptides for diagnosis and treatment of human cancer. *In Vivo,* 19, 9-29, 2005.

5. Ruzza P, Calderan A: Radiolabeled peptide-receptor ligands in tumor imaging. *Expert Opin Med Diagn*, 5, 411-424, 2011. DOI: 10.1517/17530059.2011.592829

6. Okarvi SM: Anti-tumour treatment, peptide-based radiopharmaceuticals and cytotoxic conjugates: Potential tools against cancer. *Cancer Treat Rev*, 34, 13-26, 2008. DOI: 10.1016/j.ctrv.2007.07.017

7. Behr T, Gotthardt M, Barth A, Behe M: Imaging tumors with peptide-based radioligands. *Q J Nucl Med Mol Im,* 45, 189-200, 2001.

8. Lee S, Xie J, Chen X: Peptide-based probes for targeted molecular imaging. *Biochemistry* 23, 1364-1376, 2010. DOI: 10.1021/bi901135x

9. Thakur ML, Pallela VR, Consigny PM, Rao PS, Vessileva-Belnikolovska D, Shi R: Imaging vascular thrombosis with Tc-99m labeled fibrin α-chain peptide. *J Nucl Med*, 41, 161-168, 2000.

10. Lister-James J, Moyer BR, Dean TV: Small peptides radiolabeled with ^{99m}Tc. *Q J Nucl Med Mol Im,* 40, 221-233, 1996.

11. Dijkraaf I, Wester HJ: Peptides, multimers and polymers, **In**, Semmler W, Schwaiger M (Eds): Moleculer Imaging II, Handb Exp Pharmacol. 61-92, Springer-Verlag Berlin Heidelberg, Germany, 2008.

12. Fichna J, Janecka A: Synthesis of target-specific radiolabeled peptides for diagnostic imaging. *Bioconjugate Chem,* 14, 3-17 2003. DOI: 10.1021/bc025542f

13. Trejtnar F, Laznicek M, Laznickova A, Mather SJ: Pharmacokinetics and renal handling of 99mTc-labeled peptides. *J Nucl Med,* 41, 177-182, 2000.

14. Ertay T, Unak P, Tascı C, Biber FZ., Zihnioglu F, Durak H: Tc-99-exorphin C: A new peptide radiopharmaceutical for tumor imaging. *JRNC,* 265, 473- 479, 2005. DOI: 10.1007/s10967-005-0851-1

15. Kakali D, Susmita C, Santanu G, Bhart S, Mridula M: Synthesis and radiobiological evaluation of a new ^{99m}Tc-labeled small peptide: ^{99m}Tc-YGGSLAK as imaging agent. *J Label Compd Radiopharm,* 54, 374-381, 2011. DOI: 10.1002/jlcr.1883

16. Ertay T, Unak P, Bekis R, Yurt F, Biber FZ, Durak H: New radiolabeled CCK-8 analogues [Tc-99m-GH-CCK-8 and Tc-99m-DTPA-CCK-8]: preparation and biodistribution studies in rats and rabbits. *Nucl Med Biol* 28, 667-678, 2001. DOI: 10.1016/S0969-8051(01)00196-2

17. Zhu Y, Pintar JE: Expression of opioid receptors and ligands in pregnant mouse uterus and placenta. *Biol Reprod,* 159, 925-932, 1998. DOI: 10.1095/biolreprod59.4.925

18. Kornyei JL, Vertes Z, Oszter A, Kovacs KA, Rao CV, Vertes M: Opioid peptides inhibit the action of oestradiol on human myometrial cells in culture. *Mol Hum Reprod,* 5, 565-572, 1999. DOI: 10.1093/MOLEHR/5.6.565

19. Volterra A, Restani P, Brunello N, Galli CL, Racagni G: Interaction of beta-casomorphins with multiple opioid receptors: *İn vitro* and *in vivo* studies in the newborn rat brain. *Brain Res,* 395, 25-30, 1986. DOI: 10.1016/0165-3806(86)90126-4

20. Brantl V, Teschemacher H, Henschen A, Lottspeich F: Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein peptone. *Hoppe Seyler Z Physiol Chem*, 360, 1211-1216, 1979.

21. Noni, ID, Cattaneo S: Occurrence of b-casomorphins 5 and 7 in commercial dairy products and in their digests following *in vitro* simulated gastro-intestinal digestion, *Food Chemistry,* 119, 560-566, 2010. DOI: 10.1016/j.foodchem.2009.06.058

22. Nguyen DD, Johnson SK, Busetti F, Solah VA : Formation and degradation of beta-casomorphins in dairy processing. *Crit Rev Food Sci Nutr*, 55, 1955-1967, 2015. DOI: 10.1080/10408398.2012.740102

23. Ertay, T, Unak P, Tascı C: Scintigraphic imaging with Tc-99m exorphin C in rabbits. *Appl Radiat Isot*, 62, 883-888, 2005. DOI: 10.1016/j.apradiso.2004.10.016

24. Meisel H: Chemical characterization and opioid activity of an exorphin isolated from *in vivo* digests of casein. *FEBS Lett,* 196, 3363, 1986. DOI: 10.1016/0014-5793(86)80251-4

25. Suzuki S, Oldberg A, Hayman EG, Pierschbacher MD, Ruoslahti E: Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO J,* 4, 2519-24, 1985.

26. Buchwald P, Bodor N: Octanol-water partition of nonzwitterionic peptides: Predictive power of a molecular size-based model. *Proteins*, 30, 86-99, 1998. DOI: 10.1002/(SICI)1097-0134(19980101)30:1<86::AID-PROT8>3.0.CO;2-I

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Research Article

Vakum Paketli Şavak Tulum Peynirlerinde Potasyum Sorbatın Kullanımı [1]

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Özet

Bu çalışma potasyum sorbat ilaveli vakum paketli Şavak tulum peynirlerinin raf ömrünün belirlenmesi için yapıldı. Bunun için koyun sütü K (kontrol) grubu, A grubu (%0.05 potasyum sorbat), B grubu (%0.1 potasyum sorbat) ve C grubu (%0.2 potasyum sorbat) olmak üzere 4 gruba ayrıldı. Örnekler buzdolabında (4°C) muhafaza edildi. Muhafazanın 0., 15., 30., 60., 90., 120., 150., 180., 210., 240. günlerinde mikrobiyolojik (toplam mezofilik aerob, LLP, lactic streptococlar, koliformlar, lipolitikler, proteolitikler, küfler, mayalar, *E. coli, Staph. aureus* ve *Cl. perfringens*), kimyasal (pH, asitlik, aw, yağ, tuz, kuru madde, kül ve sorbik asit) ve duyusal (ambalaj, görünüm, yapı, koku, tat ve toplam) olarak analizleri yapıldı. Muhafazanın 120. gününde kontrol grubu, 150. gününde A grubu, 180. gününde B grubu ve 270. gününde ise C grubu duyusal olarak bozuldu. *Cl. perfringens* bakterisine hiçbir grupta rastlanılmadı. *E.coli* bakterisi kontrol grubunda 90., A ve B grubunda 15. ve C grubunda ise 0. günden itibaren üreme göstermedi. *Staph. aureus* ise kontrol grubunda 30., A ve B grubunda 15. ve C grubunda ise 0. günden itibaren tespit edilmedi. Sorbik asit miktarı muhafazanın 120. gününde A grubunda 0.002 ppm, 150. gününde B grubunda 0.006 ppm ve 240. gününde ise C grubunda 0.11 ppm olarak saptandı. Duyusal olarak ise en çok beğenilen C grubu oldu. Sonuç olarak hem tulum peynirlerinin vakum paketlenerek küçük porsiyonlar halinde kullanılabileceği hem de potasyum sorbatların *E. coli* ve *Staph. aureus* üzerinde inhibe edici etkisinin olduğu görüldü.

Anahtar sözcükler: *Vakum paket, Tulum peyniri, Potasyum sorbat*

The Use of Potassium Sorbate in Vacuum Packaged Şavak Tulum Cheese

Abstract

This study was carried out to determine the shelf life of vacuum packaged Şavak tulum cheese supplemented with potassium sorbate. For this purpose, ewe milk was divided to four groups as K (control group), A (added with 0.05% potassium sorbate), B (added with 0.1% potassium sorbate), and C (added with 0.2% potassium sorbate). The groups were stored in refrigerator (4°C) and analyzed for microbiological (Total mesophilic aerobic bacteria, LLP, lactic streptococcus, coliforms, lipolytic bacteria, proteolytic bacteria, yeast and mold, *E. coli, Staph. aureus,* and *C. perfringens*), chemical (pH, acidity, aw, fat, salt, dry matter, ash, and residue sorbic acid) and sensorial (package, appearance, structure, odor, taste and general acceptability) attributes on days 0, 15, 30, 60, 90, 120, 150, 180, 210.and 240. of storage. The control group, A, B and C groups were deteriorated as regards sensorial on days 120, 150, 180. and 270. of storage, respectively. *C. perfringens* was found in none of the groups. *E. coli* was found to be below detection limit after 90 days in control group, 15 days in A and B groups, and on day 0 in C group. *Staph. aureus* wasfound to be below detection limit after 30 days in control group, 15 days in A and B groups, and on day 0 in C group.Residue sorbic acid level was detected as 0.002 ppm in group A on day 120, 0.006 ppm in group B on day 150, and 0.11 ppm in group Con day 240. The most popular group by panelists was group C. Consequently, it was seen that savak tulum cheese can be vacuum packaged and consumed in small porsions by consumers, and the use of potassium sorbate in vacuum packaged Savak tuulum cheese has an inhibitory effect on *E. coli* and *Staph.aureus.*

Keywords: *Vacuum package, Tulum cheese, Potassium sorbate*

GİRİŞ

Ülkemizde en çok üretilen peynirler arasında bulunan tulum peyniri hammaddenin peynir mayası kullanılarak pıhtılaştırılması ile elde edilen telemenin fermantasyonunu takiben ufalanıp tuzlanması, daha sonra gıdaya temasa uygun bir ambalaj malzemesine veya deri tulumlara sıkıca basılarak üretilen ve olgunlaştırıldıktan sonra piyasaya

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arz edilen çeşidine özgü karakteristik özellikler gösteren peynirdir [1]. Bölgesel ürün çeşidi olarak bilinen Şavak tulum peyniri ise genellikle Elazığ ve Tunceli iline bağlı köylerde yaşayan Şavak aşireti mensupları tarafından yapılmaktadır [2,3]. Şavak tulum peyniri geçimini hayvancılıkla sağlayan yöre halkına hem gelir temini hem de beslenmelerine önemli bir kaynak olmaktadır. Genellikle çiğ sütten üretilen tulum peynirlerinin kalitesini ve raf ömrünü etkileyen birçok faktör bulunmaktadır. Bunlardan biri de ambalaj materyalinin yapısıdır. Önceleri hijyenik olmayan hayvansal kökenli tulumlara basılan peynirler daha sonraları plastik bidonlara basılmaya başlanmıştır. Bu konuda yapılan çalışmalar oldukça sınırlıdır [3,4]. Ancak literatür taramalarında tulum peynirlerinin vakum paketlenip küçük porsiyonlar halinde üretilmesi ile ilgili her hangi bir bilgiye ulaşılamamıştır. Gıda maddelerinin raf ömrünü artırmada etkili olan faktörlerden biri de ambalajlama şekli ve ambalajlamada kullanılan yöntemlerdir. Vakum paketleme yöntemi de gıda maddelerinde özellikle süt ve süt ürünlerinde geniş bir kullanım alanına sahiptir. Bu yöntemle kap içerisindeki oksijeni çıkartarak, uçucu bileşiklerin buharlaşmasını engellemek, gıdanın rengini ve tadını korumak, gıdayı çevresel faktörlerden korumak ve daha esnek kalıplarla daha az yer kaplayarak tüketicinin albenisini artırmak hedeflenmektedir. Vakum paketlemenin dezavantajı ise oluşabilecek migrasyon durumlarında güvenilir ve halk sağlığı açısından önem arz eden plastiklerin seçiminin çok dikkatli yapılmaması ve ucuz ambalaj materyalinin kullanılma ihtimalinin olmasıdır [5].

Gıdaları muhafaza etmede yaygın olarak kullanılan maddelerden biri de sorbik asit ve tuzlarıdır. Sorbik asit doğada *Sorbus aucuparia* L. olarak bilinen üvez ağacı ve meyvelerinde fazla miktarlarda bulunmaktadır [6,7]. Genel olarak sorbatlar şeklinde nitelendirilen tuzlarının ve bunlar içinde özellikle potasyum tuzunun suda oldukça yüksek çözünürlük oranına sahip olması nedeniyle gıda maddelerine uygulamada potasyum sorbat tercih edilmektedir [6,8]. Sorbik asit ve potasyum tuzu antimikrobiyal bir koruyucu olarak oldukça yaygın bir şekilde gıdalarda kullanılmaktadır. Genel olarak gıdalarda %0.01-0.2 mg/kg arasında değişen oranlarda kullanılmaktadır [9]. Çeşitli gıdalarda kullanılan sorbik asit ve tuzlarının insan vücudunda doğal olarak yağ asitlerine benzer bir şeklide metabolize olduğu bilinmektedir [7]. Gıda koruyucusu olarak kullanılan sorbatların uygulama alanları içerisinde peynir ve peynir ürünleri, yoğurt, ekşi krema gibi süt ürünleri, kek ve kek karışımları, pastalar, şekerli çörek, yumuşak şekerlemeler, şekerli krema, fırıncılıkta kullanılan soslar gibi birçok gıda maddeleri bulunmaktadır [6,10]. Sorbik asit ve tuzlarının en çok kullanım alanının peynir endüstrisi olduğu bilinmektedir. Yaklaşık olarak 51 çeşit peynirde ve peynirden yapılan ürünlerde kullanıldığı ifade edilmektedir [11]. İspanyol tipi yumuşak peynirlerde *Salmonella* türlerinin gelişimi ve kontrolünün yapıldığı bir çalışmada %0.3 oranında potasyum sorbat ilavesinin 6-30°C'de muhafaza edilen peynirlerde bu bakterinin gelişimini önemli ölçüde etkilediği ifade edilmektedir [12]. Yapılan başka bir çalışmada [13]; yumuşak taze beyaz peynir örneklerinden izole edilen *E.coli* ve *Staph. aureus*'un toksik türlerini %0.02 sodyum sorbat ilave edilerek hazırlanan skim milk vasatında sorbik asit türlerinin bu mikroorganizmalar üzerine bakteriyostatik etki gösterdiği bulunmuştur. Diğer bir araştırma grubu [6] ise sorbata direnç gösterebilen bazı küf türlerinin %0.3 oranında sorbat ihtiva eden peynirlerde mikotoksin üretimi yapamadığını belirlerken, bir diğer araştırma grubu [14] aynı ortamda sorbat oranını sub-inhibitör seviyelere düşürmüşler ve mikotoksin üretiminin stimüle edildiğini bulmuşlardır. Nizamlıoğlu ve ark.[15] yapmış oldukları bir çalışmada deneysel olarak yaptıkları kaşar peynirlerini %1, %2, %3 oranlarında potasyum sorbat içeren solüsyonlara daldırmışlar ve olgunlaşmanın 60. gününe kadar mikrobiyolojik ve kimyasal yönden analizler yapmışlar ve sonuç olarak bu maddenin *Staphylococcus-Micrococcus* ile maya ve küfler üzerine etkili olduğunu bulmuşlardır. Doğruer ve ark.[16] deneysel olarak beyaz peynir yapımında kullanılan süt ve salamura suyuna %0.015 ve %0.030 oranında potasyum sorbat ilave etmişler ve peynirleri 60 gün boyunca olgunlaşmaya almışlar. Mikrobiyolojik açıdan potasyum sorbatın koliform grubu, fekal streptokok mikroorganizmalar ile maya ve küfler üzerinde etkili olduğunu bulmuşlardır. Ancak, ülkemizde yaygın bir şekilde tüketimi yapılan tulum peynirlerinde sorbatların kullanılabilirliği ile ilgili herhangi bir veri bulunmamaktadır. Bu çalışmada, yöre halkı tarafından sevilerek tüketilen Şavak tulum peynirlerine nontoksik olarak kabul edilen potasyum sorbatın ilave edilmesi ve vakum paketlenerek raf ömrünün belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Materyal

Bu amaçla, Fırat Üniversitesi Tarım ve Hayvancılık Araştırma Merkezi'nden getirilen yaklaşık 70-80 L koyun sütü kullanıldı. Gelen sütler 4 eşit gruba ayrıldı. K (kontrol-potasyum sorbat katılmayan), A [%0.05 potasyum sorbat (Mediko Kimya, İstanbul-Türkiye) ilaveli], B (%0.1 potasyum sorbat ilaveli) ve C (%0.2 potasyum sorbat ilaveli) grubu. Çiğ sütten yapılan tulum peyniri örnekleri ortalama 100-110 gramlık olacak şekilde vakum paketlendi. Örnekler buzdolabı koşullarında (+4°C) muhafaza edildi. Muhafazanın 0., 15., 30., 60., 90., 120., 150., 180., 210. ve 240. günlerinde mikrobiyolojik (toplam mezofilik aerob, *Lactobacillus-Leuconostoc-Pediococcus*, lactic streptococlar, koliformlar, lipolitikler, proteolitikler, küfler, mayalar, *E. coli*, *Staph. aureus* ve *Cl. perfringens*), kimyasal (pH, asitlik, a_w, yağ, tuz, kuru madde, kül ve sorbik asit) ve duyusal (ambalaj, görünüm, yapı, koku, tat ve toplam) analizleri yapıldı. Çalışma bir ay arayla üç kez tekrar edildi.

Metot

Deneysel olarak yapılan tulum peyniri örneklerinin her birinden 10 g alınarak bir parçalayıcının (Bag Mixer

İnterscience 78860 St. France-Stochmaer) özel steril torbasına bırakıldı. Üzerine steril ¼ Ringer (Merck1.5525 - Darmstadt - Germany) çözeltisinden 90 mL ilave edilerek parçalayıcıda homojen hale getirildi. Böylece örneğin 10^{-1} (1/10)'lik dilüsyonu hazırlandı. Bu dilüsyondan aynı seyrelticiyi kullanmak suretiyle örneğin 10^{-9}'a kadar diğer dilüsyonları yapıldı. Örneklerin her bir seyreltisinden 1'er mL kullanılarak çift paralelli şekilde dökme plak metoduyla ve yayma yöntemiyle ekimleri yapıldı. İnkübasyon süresi sonunda 30-300 koloni içeren plaklar değerlendirilmeye alındı [17,18].

Örneklerdeki toplam mezofilik aerob mikroorganizmaların sayımı için Plate Count Agar (PCA) (LABM - LAB10) (35±1°C'de 48 saat) [19], *L.L.P* sayımında de Man Rogosa Sharpe Agar (LABM - LAB093) (37±1°C'de 48 saat) [20], laktik streptokoklar için M17 Agar (LABM– LAB092) (30±1°C'de 48-72 saat) [21,22], koliform grubu bakterilerin sayımı için Violet Red Bile Agar (VRB) (Acuamedia 7165-A) (30±1°C'de 24 saat) [21], lipolitik mikroorganizmalar için Tributyrin Agar (Merck 1.01957) (30±1°C'de 48 saat) [21] proteolitik mikroorganizmalar için Calcium Caseinat Agar (Merck 1.05409) (30±1°C'de 48 saat) [21] küfler için Saboauraud Agar (22-25°C'de 3 gün) [21], mayalar için Word Agar (22-25°C'de 5 gün) [21], *E. coli* sayımı için Tryptone Bile X-Glucuronide Medium (Oxoid-CM945) (30°C'de 4 saat, daha sonra 44°C'de 18 saat) [23] koagulaz pozitif *Staphylococcus aureus* sayımı için Egg Yolk Tellurite Emulsion (Oxoid SR54) ilave edilmiş Baird Parker Agar (Oxoid CM275) (36±1°C'de 30 saat) kullanıldı. Petrilerde üreyen spesifik koloniler Brain Heart Infusion Broth (BHI, CM0225, Oxoid) veya TSB Broth'a geçildi ve 37°C'de 24 saat inkübe edildi. Steril boş tüpler içerisine 0.1 mL Broth'larda üreyen kültürlerden ilave edildi. Bu tüplerin üzerine üzerindeki tarife göre hazırlanan Bactident Coagulase'dan (Merck, 1.13306-0001, Rabbit Plasma With EDTA, lyophilizied) 0.3 mL eklendi. Tüpler 37°C'de 4 saat inkübe edildi. Pıhtı veya jel oluşumuna göre karar verildi. Koagulaz test sonucu pozitif olan kolonilerin sayısı şüpheli kolonilerin sayısıyla çarpılıp, 5'e bölünerek koagulaz pozitif *Staphylococcus aureus*'un sayısı belirlendi [24,25]. Sülfit indirgeyen anaerobların sayımı için Sülfit Polymyxin Sulfadiazin (SPS) agar kullanılarak "rol tüp" tekniği ile 37°C'de 24 saat inkübasyondan sonra oluşan siyah koloniler sayılarak değerlendirildi [16]. *Cl. perfringens*'in sayısının tespiti için bu kolonilerden rastgele seçilen 5 tanesi %0.3 agarlı nitratlı peptonlu suya inoküle edilerek tüpler anaerobik koşullarda 37°C'de 24 saat inkübe edildi ve daha sonra pozitif tüpler değerlendirildi. *Cl. perfringens*'in sayısı pozitif tüplerin sayısının 5'e bölünmesinden elde edilen sayının, sülfiti indirgeyen mikroorganizmaların sayısı ile çarpılarak bulundu [26].

Örneklerin pH değerleri, pH metre (Selecta - pH 2001) ile saptandı [27]. Ayrıca, peynir örneklerinin a_w değerleri a_w metrede (Testo - 650) ölçüldü [28]. Yağ tayini için Gerber Metod'u [29] kullanıldı. Tuz miktarlarının tespiti için Mohr metodu [30], asitlik tayini (%l.a) için titrasyon yöntemi [31], kuru madde miktarlarının tayini için gravimetrik yöntem [29], kül miktarının tayini için TSE'nin önerdiği metot [31] ve sorbik asit tayini için ise AOAC'nin önermiş olduğu metot esas alındı [32]. Duyusal analizler için en az 5 kişilik panelist değerlendirme yaptı. Ambalaj 8, görünüm 28, yapı 12, koku 20 ve tat ise 32 olmak üzere toplamda 100 puan üzerinden sonuçlar hesaplandı [33].

İstatistiksel Analizler

Mikrobiyolojik, kimyasal ve duyusal analiz sonuçlarında varyans analizi yapıldı. Örnek alma günlerinde gruplar arasındaki değişimler modellendirildi. Modellemede lineer regresyon (GLM) kullanıldı. İstatistiksel analizler Statistical Analysis System (SAS) paket programı kullanılarak yapıldı [34]. İstatistiksel önem derecesi P<0.05 olarak kabul edildi.

BULGULAR

Bu çalışmada farklı oranlarda potasyum sorbat ilave edilen vakum paketli Şavak tulum peynirlerinin 4±1°C'de muhafazası esnasında mikrobiyolojik, kimyasal ve duyusal özelliklerinde meydana gelen değişimler incelendi. Deneysel tulum peyniri örneklerinin mikrobiyolojik analiz bulguları *Tablo 1* ve *Tablo 2*'de kimyasal analiz bulguları *Tablo 3*'te ve duyusal analiz bulguları ise *Tablo 4*'te gösterilmektedir.

TARTIŞMA ve SONUÇ

Toplam mezofilik aerob bakteri sayısı tüm gruplarda muhafazanın 0. gününden itibaren sürekli olarak artmaya başladı. Kontrol grubunda muhafazanın 90. günde 8.89 log_{10}kob; A grubunda 120. günde 9.55 log_{10}kob/g; B grubunda 150. günde 9.89 log_{10}kob/g ve C grubunda ise 240. günde 9.90 log_{10}kob/g en yüksek seviyeye çıktı. Kontrol grubu muhafazanın 120. gününden, A grubu 150. günden, B grubu 180. günden ve C grubu ise 270. günden itibaren duyusal olarak bozulmaya başladı. Uygulanan potasyum sorbat miktarı arttıkça ürünün raf ömrünün uzadığı görüldü *(Tablo 1)*. Potasyum sorbatın en fazla kullanıldığı gruptaki (C) toplam mezofilik aerob bakteri sayısının kontrol grubuna göre yaklaşık olarak 1 log_{10}kob/g kadar daha düşük seviyede olduğu belirlendi. Elde edilen bu sonuçlar olgunlaşma süresi boyunca toplam mezofilik aerob bakteri sayısının arttığını bildiren [35,36] sonuçlarıyla benzerlik arz etmektedir. İstatistiksel olarak gruplar arasındaki fark önemli bulunmadı (P>0.05).

Laktik asit bakteri grubunda yer alan ve ürünlerin kendine has lezzet, aroma ve dayanma süresi üzerine etki eden önemli bir bakteri grubu da *Lactobacillus-Leuconostoc-Pediococcus* mikroorganizmalarıdır [3]. Bu grup mikroorganizmalar olgunlaşma periyodu boyunca sürekli olarak bir artış gösterdi. *Lactobacillus* sayısındaki bu artış hem uygulanan ısıl işleme hem de potasyum sorbatın bakteriyel yükü azaltmasına bağlı olarak laktik asit bakterilerinin gelişimi için uygun bir ortam sağlamasından kaynaklanmış

Tablo 1. *Tulum peyniri örneklerinin mikrobiyolojik analiz sonuçları (log_{10} kob/g ± Standart sapma)*
Table 1. *The microbiological analysis results of tulum cheese samples (log_{10}cfu/g ± Standard deviation)*

Mikroroganizma	Gruplar	Gün							
		0	15	30	60	90	120	150	180
Toplam Mezofilik Aerob	Kontrol	8.05 ± 0.04^{b}	8.12 ± 0.11^{ab}	8.38 ± 0.20^{ab}	8.76 ± 0.20^{ab}	8.89 ± 0.04^{a}	-	-	-
	A	8.27 ± 0.21^{c}	8.41 ± 0.34^{bc}	8.52 ± 0.31^{bc}	8.93 ± 0.01^{abc}	9.18 ± 0.25^{ab}	9.55 ± 0.28^{a}	-	-
	B	8.34 ± 0.11^{d}	8.41 ± 0.45^{d}	8.72 ± 0.17^{cd}	9.05 ± 0.35^{bcd}	9.38 ± 0.2^{abc}	9.65 ± 0.14^{ab}	9.89 ± 0.03^{a}	-
	C	8.40 ± 0.31^{d}	8.50 ± 0.28^{d}	8.78 ± 0.17^{cd}	9.01 ± 0.31^{bcd}	9.40 ± 0.08^{abc}	9.70 ± 0.17^{ab}	9.80 ± 0.08^{ab}	9.85 ± 0.04^{ab}
Lactobacillus-Leuconostoc-Pediococcus	Kontrol	8.12 ± 0.11^{a}	8.20 ± 0.11^{a}	8.37 ± 0.21^{a}	8.54 ± 0.57^{a}	8.97 ± 0.01^{a}	-	-	-
	A	8.19 ± 0.31^{a}	8.34 ± 0.20^{a}	8.58 ± 0.31^{a}	8.75 ± 0.21^{a}	8.94 ± 0.03^{a}	9.17 ± 0.57^{a}	-	-
	B	8.20 ± 0.31^{b}	8.36 ± 0.17^{b}	8.60 ± 0.25^{ab}	8.80 ± 0.14^{ab}	8.98 ± 0.06^{ab}	9.22 ± 0.79^{ab}	9.50 ± 0.34^{a}	-
	C	8.20 ± 0.08^{c}	8.40 ± 0.06^{c}	8.65 ± 0.10^{bc}	8.88 ± 0.08^{abc}	8.95 ± 0.04^{abc}	9.26 ± 0.34^{abc}	9.55 ± 0.13^{ab}	9.50 ± 0.17^{ab}
Lactic Streptococlar	Kontrol	7.29 ± 0.30^{Yc}	7.49 ± 0.16^{Yc}	7.81 ± 0.07^{bc}	8.40 ± 0.21^{ab}	8.79 ± 0.18^{a}	-	-	-
	A	7.99 ± 0.23^{XYc}	8.22 ± 0.31^{XYbc}	8.46 ± 0.48^{bc}	8.89 ± 0.07^{ab}	9.05 ± 0.38^{ab}	9.47 ± 0.33^{a}	-	-
	B	8.32 ± 0.17^{Xc}	8.40 ± 0.31^{XYc}	8.68 ± 0.13^{bc}	8.94 ± 0.06^{abc}	9.38 ± 0.31^{ab}	9.50 ± 0.45^{ab}	9.74 ± 0.03^{a}	-
	C	8.30 ± 0.17^{Xd}	8.45 ± 0.10^{Xcd}	8.65 ± 0.07^{cd}	8.90 ± 0.03^{bcd}	9.30 ± 0.24^{abc}	9.60 ± 0.17^{ab}	9.70 ± 0.11^{ab}	9.81 ± 0.08^{a}
Koliform	Kontrol	4.34 ± 0.14^{X}	3.85 ± 0.06^{X}	3.70 ± 0.20^{X}	3.34 ± 0.11	3.20 ± 0.11	-	-	-
	A	3.90 ± 0.07^{X}	3.56 ± 0.31^{XY}	3.43 ± 0.66^{XY}	3.23 ± 0.86	3.12 ± 0.17	3.00 ± 0.01	-	-
	B	3.50 ± 0.17^{XY}	3.36 ± 0.08^{XY}	3.13 ± 0.24^{XY}	2.98 ± 0.11	2.85 ± 0.04	2.55 ± 0.07	2.4 ± 0.06	-
	C	2.60 ± 0.20^{Y}	2.53 ± 0.10^{Y}	2.48 ± 0.06^{Y}	2.30 ± 0.40	2.25 ± 0.20	2.20 ± 0.28	2.1 ± 0.68	2.05 ± 0.30
Lipolitik	Kontrol	7.12 ± 0.20^{Xb}	7.29 ± 0.17^{Xab}	7.44 ± 0.28^{Xab}	7.79 ± 0.20^{Xab}	8.06 ± 0.25^{Xa}	-	-	-
	A	7.02 ± 0.51^{Xc}	7.13 ± 0.13^{Xbc}	7.34 ± 0.37^{Xabc}	7.63 ± 0.38^{Xabc}	7.86 ± 0.08^{Xab}	8.0 ± 0.0^{Xa}	-	-
	B	6.90 ± 0.08^{Xc}	6.94 ± 0.06^{Xc}	7.12 ± 0.33^{Xbc}	7.48 ± 0.31^{Xabc}	7.70 ± 0.17^{Xabc}	7.85 ± 0.07^{Xab}	8.0 ± 0.0^{Xa}	-
	C	5.50 ± 0.31^{Yf}	5.80 ± 0.17^{Yef}	5.94 ± 0.01^{Ydef}	6.08 ± 0.17^{Ycdef}	6.24 ± 0.11^{Ybcdef}	6.50 ± 0.17^{Yabcde}	6.75 ± 0.10^{Yabcd}	6.88 ± 0.06^{abc}
Proteolitik	Kontrol	7.46 ± 0.07^{Xc}	7.71 ± 0.04^{Xbc}	7.96 ± 0.03^{Xabc}	8.43 ± 0.44^{Xab}	8.65 ± 0.24^{Xa}	-	-	-
	A	6.34 ± 0.68^{Yc}	6.78 ± 0.03^{Ybc}	7.08 ± 0.51^{Ybc}	7.20 ± 0.0^{Yab}	7.29 ± 0.28^{Yab}	7.95 ± 0.07^{Xa}	-	-
	B	6.24 ± 0.08^{Yc}	6.40 ± 0.14^{Yc}	6.71 ± 0.07^{Ybc}	6.90 ± 0.08^{Yabc}	7.0 ± 0.0^{Yabc}	7.40 ± 0.11^{Xab}	7.76 ± 0.11^{Xa}	-
	C	5.30 ± 0.17^{Ze}	5.48 ± 0.23^{Zde}	5.70 ± 0.08^{Zde}	5.88 ± 0.06^{Zcde}	6.10 ± 0.23^{Zbcde}	6.30 ± 0.17^{Yabcd}	6.58 ± 0.11^{Yabc}	6.78 ± 0.06^{ab}
Küf	Kontrol	3.60 ± 0.27^{Xc}	3.77 ± 0.24^{Xbc}	4.34 ± 0.23^{Xabc}	4.60 ± 0.20^{Xab}	5.18 ± 0.38^{Xa}	-	-	-
	A	3.31 ± 0.30^{XY}	3.59 ± 0.18^{XY}	3.71 ± 0.18^{XY}	3.65 ± 0.04^{X}	3.41 ± 0.55^{Y}	3.11 ± 0.57^{X}	-	-
	B	3.12 ± 0.17^{XYb}	3.45 ± 0.04^{XYab}	3.69 ± 0.08^{XYab}	3.52 ± 0.11^{XYab}	3.35 ± 0.07^{Yab}	$3.25.0\pm0.0^{Xa}$	3.18 ± 0.20^{a}	-
	C	2.70 ± 0.17^{Yc}	2.76 ± 0.06^{Ybc}	2.84 ± 0.08^{Yabc}	2.70 ± 0.08^{Yabc}	2.55 ± 0.0^{Zabc}	2.32 ± 0.20^{Yabc}	2.28 ± 0.20^{abc}	2.10 ± 0.20^{abc}
Maya	Kontrol	3.68 ± 0.31^{Xc}	3.97 ± 0.03^{Xbc}	4.49 ± 0.34^{Xbc}	4.73 ± 0.30^{Xab}	5.49 ± 0.30^{Xa}	-	-	-
	A	3.49 ± 0.20^{Xb}	3.72 ± 0.17^{Xab}	3.86 ± 0.08^{Xab}	3.60 ± 0.07^{XYab}	3.42 ± 0.57^{Yab}	3.25 ± 0.50^{Xa}	-	-
	B	3.26 ± 0.23^{Xb}	3.50 ± 0.11^{Xab}	3.67 ± 0.04^{Xab}	3.51 ± 0.08^{Yab}	3.38 ± 0.0^{Yab}	3.13 ± 0.24^{Xa}	3.09 ± 0.27^{a}	-
	C	2.20 ± 0.11^{Yc}	2.55 ± 0.07^{Ybc}	2.63 ± 0.10^{Ybc}	2.50 ± 0.08^{Zbc}	2.40 ± 0.08^{Zab}	2.24 ± 0.08^{Yab}	2.15 ± 0.14^{ab}	2.10 ± 0.06^{a}

XYZ: *Aynı sütunda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***a-e:*** *Aynı satırda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***K:*** *Kontrol,* ***A:*** *%0.05 potasyum sorbat,* ***B:*** *%0.1 potasyum sorbat,* ***C:*** *%0.2 potasyum sorbat*

olabilir. Kontrol grubuyla diğer gruplar arasında önemli bir fark görülmedi (P>0.05) *(Tablo 1)*. Elde edilen sonuçlar bu grup mikroorganizmalar üzerine potasyum sorbatın inhibe edici etkisinin olmadığını ifade eden bazı araştırmacıların sonuçlarıyla benzerlik arz etmektedir [6,15,35,37].

Laktik streptokoklar da starter kültür olarak süt ürünleri üretiminde yaygın olarak kullanılan bakterilerden biridir [3]. Bu grup mikroorganizmalar olgunlaşma süresince sürekli olarak artış gösterdi. Muhafazanın 90. gününde kontrol grubunda 8.79 log_{10}kob; A grubunda 9.05 log_{10}kob/g; B grubunda 9.38 log_{10}kob/g ve C grubunda ise 9.30 log_{10}kob/g olarak tespit edildi *(Tablo 1)*. İstatistiksel olarak gruplar arasındaki farklılığın önemli olmadığı görüldü (P>0.05). Elde edilen bu sonuçlar [15,36,37] bazı araştırmacıların sonuçlarıyla benzerlik arz etmektedir.

Koliform grubu bakterilerin gıda maddelerinde yüksek sayılarda bulunması sanitasyon işlemlerinin ve ürüne uygulanan ısı işlemlerinin yetersiz olduğunun yada işlem sonrası rekontaminasyonun mevcut olduğunun göstergesidir. Koliform grubu mikroorganizmalar muhafaza süresince

Tablo 2. *Tulum peyniri örneklerinin patojen bakteri analiz sonuçları (log_{10} kob/g±Standart Sapma)*

Table 2. *The pathogenic bacteria analysis results of tulum cheese samples (log_{10} cfu/g±Standard Deviation)*

Patojen Mikroorganizmalar	**Gruplar**	**Gün**									
		0	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**
E. coli	K	2.12 ± 0.51^{Xa}	2.01 ± 0.18^{a}	1.78 ± 0.23^{ab}	1.02 ± 0.11^{b}	-	-	-	-	-	-
S.aureus		1.47 ± 0.07^{Xa}	1.10 ± 0.14^{b}	-	-	-	-	-	-	-	-
Cl. perfringens		-	-	-	-	-	-	-	-	-	-
E. coli	A	1.0 ± 0.00^{Y}	-	-	-	-	-	-	-	-	-
S. aureus		1.0 ± 0.00^{Y}	-	-	-	-	-	-	-	-	-
Cl. perfringens		-	-	-	-	-	-	-	-	-	-
E. coli	B	1.0 ± 0.00^{Y}	-	-	-	-	-	-	-	-	-
S.aureus		1.0 ± 0.00^{Y}	-	-	-	-	-	-	-	-	-
Cl. perfringens		-	-	-	-	-	-	-	-	-	-
E. coli	C	-	-	-	-	-	-	-	-	-	-
S. aureus		-	-	-	-	-	-	-	-	-	-
Cl. perfringens		-	-	-	-	-	-	-	-	-	-

XYZ: *Aynı sütunda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***a-e:*** *Aynı satırda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***K:*** *Kontrol,* ***A:*** *%0.05 potasyum sorbat,* ***B:*** *%0.1 potasyum sorbat,* ***C:*** *%0.2 potasyum sorbat*

sürekli bir azalma gösterdi. B ve C grubundaki koliform sayıları ile kontrol grubundaki sayıları arasında yaklaşık olarak 1 log_{10}kob/g seviyesinde azalma olduğu belirlendi. Muhafazanın 90.gününde kontrol grubunda 3.20 log_{10}kob; A grubunda 3.12 log_{10}kob/g; B grubunda 2.85 log_{10}kob/g ve C grubunda ise 2.25 log_{10}kob/g olarak tespit edildi *(Tablo 1)*. Ancak istatistiksel olarak gruplar arasındaki farklılığın önemli olmadığı görüldü (P>0.05). Elde edilen bulguların bazı araştırmacıların [15,35-37] bulgularıyla benzerlik arz ettiği görülmektedir.

Lipolitik bakteri sayımı lipoliz olayında etkili olan ve lipolitik aktiviteye sahip mikroorganizmaların varlığını belirlemek amacıyla yapılmaktadır. Bu grup mikroorganizmaların muhafaza süresi boyunca sürekli olarak bir artış gösterdiği görüldü. Muhafazanın 90. gününde kontrol grubunda 8.06 log_{10}kob; A grubunda 7.86 log_{10}kob/g; B grubunda 7.70 log_{10}kob/g ve C grubunda ise 6.24 log_{10}kob/g olarak bulundu. Potasyum sorbat miktarının artmasına bağlı olarak lipolitik mikroorganizmaların sayısında bir azalma olduğu ve C grubundaki sayının kontrol grubuna göre yaklaşık 2 log_{10}kob/g daha az olduğu belirlendi *(Tablo 1)*. İstatistiksel olarak ise C grubu ile diğer gruplar arasındaki farkın önemli olduğu saptandı (P<0.05).

Proteolitik mikroorganizmalar özellikle süt ürünlerinde proteini parçalayarak istenmeyen tat ve kokulara neden olurlar. Bu grup mikroorganizmalar tüm gruplarda muhafaza esnası boyunca sürekli artış gösterdi. Uygulanan potasyum sorbat miktarının artmasına bağlı olarak bu grup mikroorganizmaların sayılarında bir azalma olduğu ve C grubu ile kontrol grubu arasında 2 log_{10}kob/g seviyesinde bir düşüş olduğu belirlendi. Muhafazanın 90. gününde kontrol grubunda 8.65 log_{10}kob; A grubunda 7.29 log_{10}kob/g; B grubunda 7.00 log_{10}kob/g ve C grubunda ise 6.10 log_{10}kob/g olarak bulundu *(Tablo 1)*. İstatistiksel olarak gruplar arasındaki farkın önemli olduğu görüldü (P<0.05).

Küf sayısı üzerinde potasyum sorbatın inhibe edici etkisinin olduğu bilinmektedir. Özellikle C grubundaki sayının olgunlaşmanın 0. gününden itibaren muhafazanın sonuna kadar diğer gruplardan yaklaşık 1 log_{10}kob/g daha az seviyelerde seyrettiği görüldü. Muhafazanın 90. gününde kontrol grubunda 5.18 log_{10}kob; A grubunda 3.41 log_{10}kob/g; B grubunda 3.35 log_{10}kob/g ve C grubunda ise 2.55 log_{10}kob/g olarak bulundu *(Tablo 1)*. İstatistiksel olarak gruplar arasındaki fark önemli bulundu (P<0.05).

Maya sayısı kontrol grubu hariç diğer gruplarda muhafazanın ilerlemesine bağlı olarak 60. günden itibaren azalamaya başladı. Olgunlaşmanın 90. gününde kontrol grubunda 5.49 log_{10}kob; A grubunda 3.42 log_{10}kob/g; B grubunda 3.38 log_{10}kob/g ve C grubunda ise 2.40 log_{10}kob/g olarak bulundu *(Tablo 1)*. İstatistiksel olarak gruplar arasındaki fark oldukça anlamlıydı (P<0.05). Potasyum sorbatın peynirlerin üretiminde maya ve küflerin üremesini engelleyen ve en çok kullanılan inhibitör maddelerden biri olduğunu birçok araştırmacı bildirmektedir [6,15,34-38].

Cl. perfringens bakterisine hiçbir grupta rastlanılmadı. *E. coli* bakterisi kontrol grubunda 90. günden, A ve B grubunda 15.günden ve C grubunda ise 0. günden itibaren üreme göstermedi. *Staph. aureus* ise kontrol grubunda 30. günden, A ve B grubunda 15. günden ve C grubunda ise 0. günden itibaren tespit edilmedi *(Tablo 2)*.

Tulum peyniri örneklerinde pH değerleri kontrol grubu hariç diğer gruplarda muhafazanın 30. gününe kadar sürekli bir artış gösterdikten sonra azalmaya başladı. En düşük pH değerleri kontrol grubunda 90. günde 4.96, A grubunda 120. günde 4.67, B grubunda 150. günde

Tablo 3. *Tulum peyniri örneklerinin kimyasal analiz sonuçları (Ortalama Değer ± Standart sapma)*
Table 3. *The chemical analysis results of tulum cheese samples (Mean value ± Standard deviation)*

Değer	Gruplar	Gün									
		0	15	30	60	90	120	150	180	210	240
pH	Kontrol	5.10±0.08^{X}	5.10±0.08^{X}	5.06±0.03^{Y}	4.98±0.00^{X}	4.96±0.00^{X}	-	-	-	-	-
	A	4.76±0.03^{Xa}	5.00±0.03^{Ya}	5.00±0.03^{Xb}	4.92±0.00^{Xb}	4.87±0.04^{Yd}	4.67±0.00^{Yc}	-	-	-	-
	B	4.73±0.04^{Yb}	4.91±0.03^{Xa}	4.94±0.00^{Ya}	4.90±0.00^{Xa}	4.83±0.00^{Xab}	4.63±0.03^{Xab}	4.58±0.02^{b}	-	-	-
	C	4.33±0.03^{Ze}	4.76±0.04^{XYc}	4.86±0.02^{Xa}	4.82±0.04^{Xb}	4.79±0.01^{Xb}	4.60±0.02^{Xc}	4.55±0.01^{c}	4.42±0.02^{c}	4.40±0.03^{d}	4.30±0.06^{e}
Asitlik (%)	Kontrol	0.18±0.03^{Yb}	0.25±0.03^{ab}	0.27±0.0^{ab}	0.30±0.00^{a}	0.35±0.004^{a}	-	-	-	-	-
	A	0.25±0.03^{Xab}	0.29±0.00^{ab}	0.30±0.01^{b}	0.33±0.06^{ab}	0.35±0.00^{a}	0.38±0.03^{a}	-	-	-	-
	B	0.27±0.04^{XYd}	0.30±0.03^{cd}	0.30±0.00^{bcd}	0.35±0.03^{bcd}	0.39±0.01^{abc}	0.42±0.00^{ab}	0.45±0.03^{Xa}	-	-	-
	C	0.29±0.04^{XYd}	0.32±0.03^{cd}	0.34±0.00^{cd}	0.38±0.03^{d}	0.40±0.00^{cd}	0.44±0.00^{bcd}	0.48±0.03^{Ybcd}	0.51±0.02^{abc}	0.55±0.03^{ab}	0.62±0.02^{a}
a_w	Kontrol	0.90±0.0^{Ya}	0.93±0.0^{Ya}	0.94±0.0^{Za}	0.91±0.01^{Ya}	0.90±0.0^{Zb}	-	-	-	-	-
	A	0.90±0.0^{Xb}	0.93±0.0^{Xab}	0.93±0.0^{Xa}	0.90±0.0^{Xb}	0.89±0.0^{Xb}	0.87±0.01^{Xc}	-	-	-	-
	B	0.92±0.0^{Xa}	0.92±0.0^{Xa}	0.91±0.0^{Ya}	0.89±0.0^{Yb}	0.88±0.0^{Yb}	0.86±0.0^{Yc}	0.85±0.0^{Xc}	-	-	-
	C	0.88±0.0^{Zb}	0.90±0.0^{Yb}	0.90±0.0^{Ya}	0.88±0.0^{Yb}	0.87±0.0^{Yb}	0.85±0.0^{Xb}	0.84±0.0^{Yc}	0.82±0.0^{d}	0.80±0.0^{e}	0.80±0.0^{e}
Yağ (%)	Kontrol	40.0±0.00	40.0±0.00	41.5±2.12	42.0±0.00	42.0±2.83	-	-	-	-	-
	A	40.0±0.00	40.0±0.00	42.5±0.85	42.5±0.71	43.0±0.00	44.0±2.83	-	-	-	-
	B	40.0±2.83	40.0±0.00	41.0±2.83	42.0±0.00	42.5±2.83	43.0±0.00	43.0±4.24	-	-	-
	C	40.0±0.00	40.0±2.83	41.0±1.41	42.0±2.83	42.5±1.41	43.0±2.83	44.0±2.83	44.0±2.12	45.0±3.54	45.5±4.95
Tuz (%)	Kontrol	4.45±0.07^{Za}	4.31±0.04^{Yab}	4.22±0.02^{Zb}	4.21±0.01^{Xb}	4.21±0.01^{Xb}	-	-	-	-	-
	A	4.91±0.04^{Xa}	3.98±0.00^{Xc}	4.68±0.03^{Yb}	3.74±0.00^{Yd}	3.98±0.00^{Yc}	3.74±0.0^{Yd}	-	-	-	-
	B	4.68±0.03^{Yc}	4.91±0.04^{Xb}	4.84±0.03^{Xa}	3.74±0.02^{Yf}	3.74±0.06^{Zf}	3.98±0.0^{Xe}	4.21±0.04^{d}	-	-	-
	C	4.45±0.07^{Za}	4.21±0.03^{Yb}	4.14±0.03^{Zb}	3.74±0.03^{Yc}	4.21±0.04^{Xb}	3.74±0.03^{Yc}	4.21±0.06^{b}	4.20±0.00^{b}	4.40±0.07^{a}	4.45±0.01^{a}
Kuru Madde (%)	Kontrol	59.10±0.03	59.64±0.06	60.22±0.06	61.42±0.08	63.60±0.06^{X}	-	-	-	-	-
	A	59.80±0.00	59.85±0.03	60.30±0.08	61.42±0.03	63.81±0.41^{XY}	64.08±0.31	-	-	-	
	B	59.85±0.24	60.60±0.17	63.45±1.77	65.21±1.71	67.29±1.17^{XY}	69.23±1.43	70.22±2.11	-	-	-
	C	59.96±1.75^{ab}	60.75±1.80^{b}	64.81±1.71^{b}	69.30±2.86^{b}	69.23±1.67^{Yb}	72.22±1.30^{ab}	73.50±2.40^{ab}	75.40±0.57^{a}	75.90±0.06^{a}	78.70±1.87^{a}
Kül (%)	Kontrol	4.57±0.78^{Yc}	6.43±0.24^{Xab}	5.81±0.16^{Yb}	6.46±0.20^{ab}	6.42±0.11^{a}	-	-	-	-	-
	A	6.54±0.03^{Xa}	6.74±0.03^{Xa}	5.79±0.04^{Yb}	6.50±0.03^{a}	6.48±0.00^{ab}	6.19±0.04^{ab}	-	-	-	-
	B	6.08±0.0^{Xa}	5.57±0.04^{Yb}	6.01±0.13^{Xa}	6.56±0.03^{a}	6.59±0.04^{a}	6.45±0.04^{a}	6.52±0.06^{a}	-	-	-
	C	6.72±0.04^{X}	6.82±0.03^{X}	6.81±0.03^{X}	6.85±0.00	6.81±0.00	6.80±0.03	6.84±0.03	6.84±0.00	6.86±0.03	6.92±0.03
Sorbik Asit (ppm)	Kontrol	-	-	-	-	-	-	-	-	-	-
	A	0.0018±0.0^{Y}	0.0018±0.0^{Y}	0.0019±0.0^{Y}	0.0019±0.0^{Y}	0.0020±0.0^{Y}	0.002±0.0^{Y}	-	-	-	-
	B	0.0030±0.0^{Y}	0.0040±0.0^{Y}	0.0040±0.0^{Y}	0.0050±0.0^{Y}	0.0052±0.0^{Y}	0.0055±0.0^{Y}	0.0060±0.0^{Y}	-	-	-
	C	0.070±0.0^{Xe}	0.075±0.004^{Xe}	0.082±0.0^{Xd}	0.085±0.0003^{Xd}	0.090±0.0^{Xc}	0.098±0.0^{Xb}	0.10±0.0^{Xb}	0.10±0.0^{b}	0.11±0.0^{a}	0.11±0.0^{a}

XYZ: *Aynı sütunda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***a-e:*** *Aynı satırda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***K:*** *Kontrol,* ***A:*** *%0.05 potasyum sorbat,* ***B:*** *%0.1 potasyum sorbat,* ***C:*** *%0.2 potasyum sorbat*

4.58 ve C grubunda ise 240. günde 4.30 olarak belirlendi. Sorbatların antimikrobiyal etkisi ayrışma sabitesi olan (pKa) 4.75'e yaklaştıkça artmaktadır. Buna göre uygulanan potasyum sorbat miktarı arttıkça pH değerinin arttığı fakat muhafazanın ilerlemesine bağlı ise sürekli bir azalma gösterdiği tespit edildi. Elde edilen bu sonuçlar bazı araştırmacıların bulgularıyla benzerlik arz etmektedir [37,38]. İstatistiksel olarak ise gruplar arasındaki farklılığın önemli olduğu görüldü (P<0.05) *(Tablo 3)*.

Asitlik değeri (% l.a olarak) tüm gruplarda muhafaza süresi boyunca sürekli bir artış gösterdi. En yüksek seviye kontrol grubunda 90. günde 0.35, A grubunda 120. günde 0.38, B grubunda 150. günde 0.45 ve C grubunda ise 240. günde 0.62 olarak saptandı. Potasyum sorbat miktarının artmasına bağlı olarak gruplardaki asitlik değeri de artış gösterdi *(Tablo 3)*. Bu sonuçların bazı araştırmacıların sonuçlarıyla benzerlik arz ettiği görüldü [37,38]. İstatistiksel olarak ise gruplar arasındaki fark önemli bulundu (P<0.05).

Gıdalara ilave edilen tuz ve şeker gibi maddelerin potasyum sorbatın antimikrobiyel etkinliğini arttığı bilinmektedir [5]. Bu nedenle potasyum sorbatın ilave edilmesiyle a_w (su aktivitesi) değerinin düştüğü ve ürünün raf ömrünün uzadığı görüldü. a_w değeri tüm gruplarda muhafazanın 30. gününe kadar artış gösterdikten sonra azalmaya başladı *(Tablo 3)*. Gruplar arasındaki farklılığın önemli olduğu saptandı (P<0.05).

Yağ miktarı kontrol grubu dahil diğer gruplarda da muhafazanın ilerlemesine bağlı olarak dalgalanmalar gösterdi. Kontrol grubunda 90. günde %42.0, A grubunda

Tablo 4. *Tulum peyniri örneklerinin duyusal analiz sonuçları (Ortalama değer ± standart sapma)*

Table 4. *The sensory analysis results of tulum cheese samples (Mean value ± Standard deviation)*

Özellik	Gruplar	Gün									
		0	15	30	60	90	120	150	180	210	240
Ambalaj	Kontrol	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	-	-	-	-	-
	A	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	-	-	-	-
	B	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	-	-	-
	C	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00	8.00±0.00
Görünüm	Kontrol	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	-	-	-	-	-
	A	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	-	-	-	-
	B	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	-	-	-
	C	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00	28.00±0.00
Yapı	Kontrol	12.00±2.83	12.00±2.83	10.00±0.00	10.00±0.00	8.00±0.00	-	-	-	-	-
	A	12.00±0.0^{a}	12.00±0.00^{ab}	12.00±0.00^{ab}	10.00±0.00^{ab}	10.00±0.00^{ab}	8.00±0.00^{b}	-	-	-	-
	B	12.00±0.0^{a}	12.00±0.00^{a}	12.00±0.00^{a}	10.00±2.83^{ab}	10.00±0.00^{ab}	8.00±0.00^{b}	8.00±0.00^{b}	-	-	-
	C	12.00±0.0^{a}	12.00±0.00^{a}	12.00±0.00^{a}	10.00±0.00^{ab}	10.00±0.00^{ab}	8.00±0.00^{bc}	8.00±0.00^{bc}	8.00±0.00^{bc}	8.00±0.00^{bc}	5.00±0.00^{c}
Koku	Kontrol	20.00±0.0^{a}	20.00±0.0^{a}	20.00±0.0^{a}	15.00±2.83^{b}	12.00±0.00^{Yb}	-	-	-	-	-
	A	20.00±0.0^{a}	20.00±0.0^{a}	20.00±0.0^{a}	18.00±2.83^{ab}	15.00±2.83^{XYbc}	12.00±2.83^{c}	-	-	-	-
	B	20.00±0.0^{a}	20.00±0.0^{a}	20.00±0.0^{a}	18.00±2.83^{ab}	15.00±2.83^{XYbc}	15.00±0.00^{bc}	12.00±2.83^{c}	-	-	-
	C	20.00±0.0^{a}	20.00±0.0^{a}	20.00±0.0^{a}	18.00±0.00^{a}	18.00±0.00^{Xa}	15.00±0.00^{ab}	15.00±0.00^{ab}	12.00±0.00^{bc}	10.00±0.00^{bc}	8.00±0.00^{c}
Tat	Kontrol	32.00±0.0^{a}	32.00±0.0^{a}	28.00±2.83^{a}	20.00±5.66^{Yb}	15.00±1.41^{Zb}	-	-	-	-	-
	A	32.00±0.0^{a}	32.00±0.0^{a}	30.00±2.83^{ab}	25.00±2.83^{XYbc}	20.00±0.00^{YZcd}	15.00±2.83^{Ycd}	-	-	-	-
	B	32.00±0.0^{a}	32.00±0.0^{a}	32.00±0.0^{a}	28.00±2.83^{Xab}	25.00±0.00^{XYbc}	20.00±0.00^{Ycd}	15.00±1.41^{Yd}	-	-	-
	C	32.00±0.0^{a}	32.00±0.0^{a}	32.00±0.0^{a}	30.00±0.0^{Xab}	28.00±0.00^{Xab}	28.00±0.00^{Xab}	25.00±0.0^{Xbc}	20.00±0.00^{cd}	20.00±0.00^{cd}	15.00±0.0^{d}
Toplam	Kontrol	100.0±0.0^{a}	100.0±0.0^{a}	94.00±2.83^{a}	81.00±8.49^{Yb}	71.00±1.41^{Zb}	-	-	-	-	-
	A	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	89.00±2.83^{XYb}	81.00±2.83^{YZbc}	71.00±2.82^{Yc}	-	-	-	-
	B	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	92.00±8.49^{XYab}	86.00±5.66^{XYb}	79.00±0.00^{Ybc}	71.0±4.24^{Ybc}	-	-	-
	C	100.0±0.0a	100.0±0.0^{a}	100.0±0.0^{a}	94.00±0.00^{Xab}	92.00±0.00^{Xab}	87.00±0.00^{Xbc}	84.0±0.0^{Xbcd}	76.00±0.00^{cd}	74.00±0.00^{de}	64.00±0.00^{e}

XYZ: *Aynı sütunda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***a-e:*** *Aynı satırda yer alan ortalamalardan farklı üst simgeyi taşıyanlar istatistiksel bakımdan önemlidir (P<0.05);* ***K:*** *Kontrol,* ***A:*** *%0.05 potasyum sorbat,* ***B:*** *%0.1 potasyum sorbat,* ***C:*** *%0.2 potasyum sorbat*

120. günde %44.0, B grubunda 150. günde %43.0 ve C grubunda ise 240. günde %45.5 olarak belirlendi *(Tablo 3)*. Gruplar arasındaki farklılığın önemli olmadığı görüldü (P>0.05).

Tuz miktarı tüm gruplarda olgunlaşmanın 0. gününden itibaren sürekli artma ve azalma şeklinde bir değişim gösterdi. Muhafazanın 90. gününde kontrol grubunda %4.21, A grubunda %3.98, B grubunda %3.74 ve C grubunda ise %4.21 olarak belirlendi. Bu günde gruplar arasındaki farklılığın önemli (P<0.05) olduğu saptandı *(Tablo 3)*.

Kuru madde miktarı kontrol grubu dahil tüm gruplarda muhafazanın 0. gününden itibaren sürekli olarak bir artış gösterdi. En yüksek değer kontrol grubunda 90. günde %63.60, A grubunda 120. günde %64.08, B grubunda 150. günde %70.22 ve C grubunda ise 240. günde %78.70 seviyesinde görüldü. İstatistiksel olarak gruplar arasındaki farklılıkların oldukça önemli (P<0.05) olduğu belirlendi *(Tablo 3)*.

Kül miktarı tüm gruplarda muhafazanın ilk gününden son gününe kadar azalma ve artmalar şeklinde dalgalanmalar gösterdi. Tulum peyniri örneklerini 0. gün ile 240. günler arasında değerlendirmeye alacak olursak kül miktarı en düşük olarak %4.57 ve en yüksek olarak ise %6.92 değerlerinde bulundu *(Tablo 3)*. Gruplar arasındaki farklılıkların ise istatistiksel olarak önemli olmadığı görüldü (P>0.05).

Gıdalara ilave edilen koruyucu katkı maddelerinin miktarını doğru olarak tespit etmek bazen zor olmaktadır. Çünkü bu duruma etki eden birçok faktör bulunmaktadır. Örn; gıdanın tipi, başlangıçtaki mikrobiyel yükü, ürünün işleme ve depolama şartları kullanılan koruyucu maddenin konsantrasyonunu önemli derecede etkilemektedir. Sorbik asit miktarı (ppm) A,B ve C gruplarında muhafazanın ilerlemesine bağlı olarak sürekli bir artış gösterdi. A grubunda 120. günde 0.002 ppm, B grubunda 150. günde 0.006 ppm ve C grubunda ise 0.11 ppm düzeyinde bulundu *(Tablo 3)*. Kullanılan sorbat miktarının artmasına bağlı olarak muhafaza süresinin uzadığı ve gruplar arasındaki farklılıkların oldukça önemli olduğu görüldü (P<0.05).

Tulum peyniri örnekleri duyusal değerlendirme sonucuna göre [33] kontrol grubu dahil tüm gruplarda muhafaza süresi boyunca ambalaj [8] ve görünüm [28] yönünden tam puan aldılar. Yapı bakımından ise gruplar arasında farklılıklar olduğu ancak bu farklılıkların istatistiksel olarak önemli olmadığı görüldü (P>0.05). Koku, tat ve toplam puan

üzerinden değerlendirme yapıldığı zaman ise muhafazanın 60. gününden itibaren gruplar arasında önemli farklılıklar olduğu belirlendi (P<0.05). Kontrol grubu 120. günden, A grubu 150. günden, B grubu 180. günden ve C grubu ise 270. günden itibaren değerlendirmeye alınmadılar *(Tablo 4)*. Duyusal olarak en çok beğenilen C grubu oldu.

Genellikle çiğ sütten yapılan Şavak tulum peyniri örneklerinde potasyum sorbatların kullanılması ve bu örneklerin vakum paketlenerek muhafazaya alınması ile ilgili literatür taramalarında herhangi bir bilgi bulunmadı. Sonuç olarak hem tulum peynirlerinin vakum paketlenerek kahvaltı sofralarında küçük porsiyonlar halinde kullanılabileceği hem de potasyum sorbatların önemli sayılan bazı patojen bakteriler özellikle *E.coli* ve *Staph. aureus* üzerinde inhibe edici etkisinin olduğu görüldü. Bu çalışmanın ileriki dönemlerde yapılacak olan başka çalışmalara ışık tutabileceği düşünülmektedir.

KAYNAKLAR

1. Peynir Tebliği: Türk Gıda Kodeksi Peynir Tebliği (Tebliğ No: 2015/6). *Resmi Gazete,* Sayı: 29261, 8 Şubat 2015.

2. Kurt A, Öztek L: Studies on the production technique of Savak Tulum cheese. *J Agric Faculty*, 153, 65-67, 1984.

3. Ateş G, Patır B: Starter kültürlü tulum peynirinin olgunlaşması sırasında duyusal, kimyasal ve mikrobiyolojik niteliklerinde meydana gelen değişimler üzerine araştırmalar. *FÜ Sağ Bil Derg*, 15, 45-56, 2001.

4. Güven M, Konar A: Physical, chemical and sensory properties of tulum cheese made from cow's milk and ripened in different materials. *J Food Protect*, 19, 287-293, 1994. DOI: 10.1111/j.1471-0307.1976.tb00440.x

5. Üçüncü M: Gıda Ambalajlama Teknolojisi. Ambalaj Sanayicileri Derneği İktisadi İşletmesi Derneği Matbaacılık, İstanbul, 2011.

6. Liewen MB, Marth EH: Growth and inhibition of microorganisms in the presence of sorbic acid. A rewiew. *J Food Protect*, 48, 364-375, 1985.

7. Sofos JN, Busta FF: Antimicrobial activity of sorbate. *J Food Protect*, 44, 614-622, 1981.

8. Çakmakçı S, Çelik İ: Gıda Katkı Maddeleri. Atatürk Üniv Ziraat Fak Ders Notu, Erzurum, 1995.

9. Türk Gıda Kodeksi: Gıda Katkı Maddeleri Yönetmeliği. Sayı: 28693, Ankara, 2013.

10. Marin S, Abellena M, Rubinat M, Sanchis V, Ramos AJ: Efficacy of sorbates on the control of the growth of *Eurotium* species in bakery products with near neutral pH. *Int J Food Microbiol*, 87, 251-258, 2003. DOI: 10.1016/S0168-1605(03)00068-0

11. Dziezek JD: Antimicrobial agents. *J Food Technol*, 40, 104-111, 1986.

12. Kasrazadeh M, Genigeorgis C: Potential growth and control of *Salmonella* in Hispanic type soft cheese. *Int J Food Microbiol*, 22, 127-140, 1994. DOI: 10.1016/0168-1605(94)90137-6

13. El-Zayat AT: Toxic bacteria in soft cheese. *Egyptian J Food Sci*, 16, 127-134, 1988.

14. Yousef AE, Marth EH: Incorporation of (^{14}C) acetate by Aspergillus parasiticus in presence of antifungal agents. *Eur J Appl Mic Biotech*, 18, 103-108, 1983. DOI: 10.1007/BF00500832

15. Nizamlıoğlu M, Gürbüz Ü, Doğruer Y: Potasyum sorbatın kaşar peynirin kimyasal ve mikrobiyolojik kalitesine etkisi. *Selçuk Univ Vet Bil Derg*, 12, 23-29, 1996.

16. Doğruer Y, Gürbüz Ü, Nizamlıoğlu M: Potasyum sorbatın beyaz peynirin kimyasal ve mikrobiyolojik kalitesine etkisi. *Selçuk Univ Vet Bil Derg*, 12, 109-116, 1996.

17. Harrigan WF: Laboratory Methods in Food Microbiology. 3rd edn., Academic Press, London, 1998.

18. ICMSF: International Commission on Microbiological Specifications for Foods. Microorganism in Foods 1. Their Significance and Methods of Enumeration, London: Univto Toronto Press, 1982.

19. Maturin LJ, Peeler JT: Aerobic plate count. **In**, Bacteriological Analytical Manual, chapter 3, 2001. http:// www. cfsan.fda.gov/ebam/bam-3htm, *Accessed:* 26.01.2011.

20. American Public Health Association: Standarts Methods for the Examination of Dairy Products. 15th edn., American Public Health Association, New York, 1995.

21. Halkman AK: Merck Gıda Mikrobiyolojisi Uygulamaları. Başak Matbaacılık, Ankara, 2005.

22. Terzaghi BE, Sandine WE: Improve medium for lactic streptococci and their bacteriophages. *Appl Microbiol*, 29, 807-813, 1975.

23. ISO 16649-2: Microbiology of food and animal feding stuffs-Horizontal method for the detection and enumeration of β-glucuronidase-positive *Escherichia coli*. Part 2, Colony-count technique a 44°C using 5-brome-4-chloro-3-indoly-beta-D-glucuronide, Geneve, Switzerland, 2001.

24. ISO 6888 – 1: 1999 / AMD 1: 2003. Cogulase (+) *Staphylococcus aureus* Identification. http://www.iso.org/iso/iso_catalogue/. *Accessed:* 07.04.2015.

25. Lancette GA, Bennett RW: *Staphylococcus aureus* and staphylococcal enterotoxins. **In**, Downes FP, Ito K (Eds): Microbiological Examination of Foods. 4th edn., 387-404, American Public Health Association, Washington DC, 2001.

26. Anonim: http://www.fda.gov/Food/ScienceResearch LaboratoryMethods/ Bacteriological Analytical Manual BAM/UCM071400, 19.04.2011.

27. Case RA, Bradley RL, Williams RR: Chemical and physical methods. Chapter 18, **In**, Richardson GH (Ed): Standart Methods for the Examination of Dairy Products. 15th edn., American Public Health Association, Washington DC, 1985.

28. Lang KW, Sternberg MP: Calculation of moisture content of a formulated food system to any given water activity. *J Food Sci*, 45, 1228-1230, 1998. DOI: 10.1111/j.1365-2621.1980.tb06527.x

29. Association of Official Analytical Chemists: Official Methods of Analysis. 14th ed., Association of Analytical Chemists, Washington DC, 1984.

30. Demirci M, Gündüz HH: Süt Teknoloğunun El Kitabı. Hasat Yayıncılık, İstanbul, 1994.

31. Türk Standardları Enstitüsü: Tulum Peynir Standardı. TS 3001. Türk Standartları Enstitüsü, Ankara, 1995.

32. Association of Official Analytical Chemists: Official Methods of Analysis. 15th edn., Association Official Analytical Chemists (AOAC), Washington DC, 1990.

33. Uysal H, Kınık Ö, Kavas G: Süt ve Süt Ürünlerinde Uygulanan Duyusal Test Teknikleri (Yardımcı Ders Kitabı). Ege Üniv Ziraat Fak Yay No: 560, İzmir, 2004.

34. Statistical Analysis System (SAS): Version 8.0. SAS Institute. Cary, North Caroline, USA, 1999.

35. Öksüztepe G, İlhak Oİ, Dikici A, Çalıcıoğlu M, Patır B: Effect of potassium sorbate on some microbiological properties of cokelek stored at different temperatures. *Kafkas Univ Vet Fak Derg*, 16 (Suppl-A): S99-S105, 2010. DOI: 10.9775/kvfd.2010.1840

36. Şeker P, Patır B: Kısa ve uzun ömürlü ayranlarda potasyum sorbat uygulamasının mikrobiyolojik kaliteye etkisi. *FÜ Sağ Bil Vet Derg*, 25, 23-30, 2011.

37. Özdemir C, Demirci M: Selected microbiological properties of kashar cheese samples preserved with potassium sorbate. *Int J Food Prop*, 9, 515-521, 2006. DOI: 10.1080/10942910600596191

38. Şeker P: Kısa ve Uzun Ömürlü Ayranlarda Potasyum Sorbat Uygulamasının Kaliteye Etkisi. *Doktora Tezi*. Fırat Üniv. Sağlık Bil. Enst., 2008.

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Research Article

Effects of Local Sodium Bentonite as Aflatoxin Binder and Its Effects on Production Performance of Laying Hens

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Abstract

The study was designed to examine the effect of sodium bentonite (SB) as mycotoxin binder in poultry feed and its effects on productions performance of laying hens. A total of forty-five production hens white leghorn aged 34 weeks were caged in a naturally aired laying house into five different groups. Group A was kept as control group, Group B was fed with higher level 170 ppb aflatoxin without binders, and Group C feed contained 170 ppb aflatoxin with 1.5% SB binder, Group D contained 170 ppb aflatoxin with 2% SB and Group E feed contained 170 ppb aflatoxin with 2.5% SB. Aflatoxin contamination adversely affected the egg production and feed consumption. Addition of 2% and 2.5% SB increased ($P<0.05$) in feed intake and egg productions. SB appears to be useful as toxin binding additive and counteracting the deleterious effects of aflatoxin. However there were no obvious effects observed among groups in respect to egg yolk and albumin content. Significant effect was observed among the groups for feed consumption, egg production, weight, shell weight and thickness and for FCR value. The addition of 2% SB showed better feed and protein utilizations leading an increased egg production and reduced eggs defects in layer hens.

Keywords: *Mycotoxins binder, Sodium bentonite, Poultry feed, Laying hen, Layer*

Aflatoksin-Bağlayıcı Lokal Sodyum Bentonitin'in Yumurtacı Tavukların Üretim Performansı Üzerine Etkileri

Özet

Çalışma, kanatlı yemlerinde sodyum bentonit (SB)'in mikotoksin bağlayıcı olarak etkisi ve yumurta tavuklarında üretim performansı üzerine etkilerini incelemek için tasarlandı. Toplam kırk beş adet 34-haftalık beyaz leghorn üretim tavuğu beş farklı grup tarzında doğal havalandırmalı kafesler içine alındı. Grup A: Kontrol grubu olarak tutuldu; Grup B: Bağlayıcılı üst düzey 170 ppb aflatoksin ile beslendi; Grup C: Yem %1.5'lik SB bağlayıcılı 170 ppb aflatoksin içerdi; Grup D: %2'lik SB bağlayıcılı 170 ppb aflatoksin içerdi; Grup E: Yemler %2.5'lik SB bağlayıcılı 170 ppb aflatoksin içerdi. Aflatoksin kontaminasyonu yumurta üretimi ve yem tüketimini olumsuz yönde etkiledi. SB'nin %2 ve %2.5'lik ilavesi yem tüketimi ve yumurta üretimlerini artırdı ($P<0.05$). SB'nin toksin-bağlayıcı katkı özelliği sayesinde aflatoksinin zararlı etkilerine karşı faydalı olduğu gözükmektedir. Ancak, gruplar arasında yumurta sarısı ve albümin içeriği yönünden gözlenen belirgin bir etkisi yoktu. Gruplar arasında yem tüketimi, yumurta verimi, ağırlığı, kabuk ağırlığı ve kalınlığı ile FCR değeri için ise önemli etki gözlendi. SB'nin %2'lik ilavesinin, yumurta tavuklarında daha iyi yem ve protein kullanımı sayesinde yumurta üretiminde artışa ve yumurta kusurlarında düşmeye yol açtığı gözlendi.

Anahtar sözcükler: *Mikotoksin bağlayıcı, Sodyum bentonit, Kanatlı yemi, Yumurtacı tavuk, Yumurtacı*

INTRODUCTION

The demand in egg and broiler has increased, which put tremendous pressure and compression on apposite feeding of poultry. In order to sustain and uphold poultry industry a good management and proper feeding is indispensable. So far the notable problem in poultry feed is contamination of different toxin that not only reduces income but also impairing farm operations. Mycotoxin is odorless and invisible compound that can not be detected by

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smell or taste. The main types of mycotoxins are aflatoxins, trichothecene, zearalenone and fumonisin. Even a small quantity of these mycotoxins is extremely dangerous and their presence in poultry feeds lead to a poor performance of different poultry products [1]. Aflatoxins as major mycotoxins are produced by fungi such as *Aspergillus* and *Penicillium* species. These filamentous fungi contaminate cereals and grains during harvesting, storage, transportation and processing. Aflatoxicosis, severely affected the development of broiler as well egg production in layers and produced symptoms like anemia, enlargement of hemorrhagic liver, fat accumulation, kidney paleness and reduced feed uptake [2,3]. Another important mycotoxin in poultry diet is Ochratoxin A (OTA), which is produced by *Aspergillus and Penicillium* species of fungi, and have carcinogenic effects especially on kidney and liver [4-6]. Low-level exposure to OTA leads to reduced feed consumption and increase susceptibility to diseases [7]. These naturally occurring toxin vary through different climatic and geographical positions; however their occurrence and exposure in poultry feed is worldwide. The well-known carcinogenic mycotoxins are aflatoxins which includes AF B1, B2, G1, G2 and M1 but aflatoxin B1 is considered the major hepato-carcinogenic, which effects differently with age, species, sex and nutritional conditions in animals.

Poultry are considered sensitive to aflatoxins that severely reduce growth, feed intake and cause kidney and liver damage and eventually leads to death. Their feed consists of several types of agro industrial byproducts and cereal grains i.e., corn grain which can be easily contaminated by fungi under favorable temperature and humidity [8]. In this way, fungus contaminates feed and produces mycotoxin affecting animal health and nourishment and ultimately influence human health [9].

Different strategies have been used for detoxification of aflatoxin-contaminated feedstuff such as thermal inactivation, physical separation, microbial degradation, irradiation and chemicals treatment. Ammonia treatment is being used to decontaminate aflatoxin in feeds [10]. Chemical detoxification is considered the best method for aflatoxin containing poultry diet and reported more effective than physical and biological methods [11]. These chemicals are used as feed additives and also mixed in animal feed. These compounds have no side effects and are clay minerals that can immediately bind to aflatoxin because of opposite charge. Different aflatoxin binders like zeolites, calcium and sodium alumino-silicate have been tested for binding capability. In turkey and chicken feeds, phyliosilicate clay and hydrated sodium-calcium silicates have been reported to efficiently absorb the aflatoxin [1].

Bentonite is a hydrophilic compound, colloidal alumino-hydrosilicate in nature and has high swelling ability and act as enterosorbant in animal diet and can bind and reduce aflatoxin absorption [12-14]. It has capability to form complex with aflatoxin and prevents absorption through intestinal epithelium. It is also used as lubricating; binding agents improve food uptake efficiency and growth rate in broilers. Apart from this it also improves protein utilization and prolonged the foodstuff passage time. Nowadays, there are different commercial adsorbents available on the market and can be used as feed additives to control or neutralize the toxic effects. The present strategy was to bind the aflatoxins with sodium bentonite (SB) and inhibit absorption in digestive tract of layers and excrete the complex in feces. SB is a three-layer structure compound of aluminum silicate that can absorb a variety of compounds selected for aflatoxins binding in broiler feed. This study was planned to evaluate the ability of indigenous SB as aflatoxins binder, to observe its effects on production performance of layer and also to determine the most appropriate level of SB. To the best of author's knowledge the only well known naturally occurring deposit SB in Northwestern part of Pakistan.

MATERIAL and METHODS

Birds were handled with proper care and kept in cages at the poultry unit of the University poultry Farm, the University of Agriculture Peshawar-Pakistan from August to September 2014 inclusive. The bird care protocol used in this study was reviewed and approved by the Ethics Committee headed by the Chairman Department Live Management & Animal Breeding and Genetics with three members each from Department of Poultry Science, Department of Animal Nutrition and Department of Animal Health, Faculty of Animal Husbandry and Veterinary Sciences, the University of Agriculture Peshawar-Pakistan. Daily requirement including light, water, air and feed was given according to the bird's standard protocols during the experiment period including 1st week of adaptation period. Daily observation of bird's health was regularly monitored and problems were recorded. The feed testing for aflatoxins was performed at the Veterinary Research Institute (VRI) Peshawar-Pakistan.

Experimental Design

A total of forty-five hens of white leghorn breed (RIR and Fyoumi) aged 34 weeks and divided into five different treatment groups from the day 1st, while each group consisted of 9 birds. Group A was kept as control group and was given feed (F-1) with least level 15 ppb of aflatoxins without binders, Group B was given feed (F-2) with higher level 170 ppb aflatoxins without binders, and Group C feed (F-3) contained 170 ppb aflatoxins with 1.5% SB binder, Group D feed (F-4) contained 170 ppb aflatoxins with 2.0% SB and Group E feed (F-5) contained 170 ppb aflatoxins with 2.5% SB.

Selection Feed and Chemical Analysis

Feed samples were randomly collected from different feed sellers in the capital city of Khyber Pakhtonkhwa

province of North-western part of Pakistan and tested for aflatoxins load (Feed-1: aflatoxins B1 (AFB1)=35 ppb, AFB2=10 ppb; Feed-2: AFB1=15 ppb; Feed-3: AFB1=40 ppb, AFB2=10 ppb; Feed-4: AFB1=95 ppb, AFB2=05 ppb, AFG1, 5.5 ppb; Feed-5: AFB1=150 ppb, AFB2=11.11 ppb, AFG1, 8.9 ppb). The well-known carcinogenic mycotoxins are aflatoxins including AF B1, B2, G1, G2 and M1 but aflatoxin B1 is considered the major hepatocarcinogen which effects differently with age, species, sex, and nutritional conditions in animals.

Feed samples from different feed dealers from the local market were randomly collected and tested for mycotoxins load using Thin Layer Chromatography (TLC) and feed containing the highest load of total mycotoxins was selected. TLC procedure carried according the standard protocol described briefly below:

A 25 g dry feed sample was mixed with 100 mL of acetone: Distilled water (81 mL: 19 mL) solution and was shaken for 40 min. The feed sample was filtered and mixed with 85 mL (0.2 N NaOH), 15 mL (6.6% $FeCl_3$), 2.5 g cellite powder and 1.5 g $CuCO_3$. The mixture was shaken for 20 min and then filtered. The filtrate was then mixed with 75mL of 0.03% of H_2SO_4 and 10 mL of 35% of chloroform and then shaked vigorously for 5 min. The solution was then suspended in a separating funnel for 30 min and the lower layer (precipitate) in flask was collected and 50 mL of KOH + KCl solution in the ratio of 1:10 was added and was suspended for 30 min again. Then again the lower layer in china dish was collected and it was dried and dissolved in 1 mL chloroform and spotted on TLC plates along with standard spots. The plates were then developed in developing tank having chloroform + acetone as 90:10 solution and air dried and observed under ultra violet lamp.

Feed Preparation

Sodium bentonite clay samples were collected from different parts of the Northwestern part of Pakistan. SB samples were crushed and grinded and submitted to the Pakistan Council of Scientific and Industrial Research (PCSIR) lab Peshawar-Pakistan for chemical analysis (Silica, 52.61; Iron, 13.30; Aluminum, 10.78; Calcium, 3.07; Magnesium, 0.43; Sodium, 3.56; Potassium, 2.50, Moisture, 5.69; loss on ignition, 8.00). After aflatoxins quantification, three rations were prepared i.e., 1.5%, 2% and 2.5% of SB and mix with commercial layers ration obtained from the local market.

Egg Production and Feed Intake

Egg were collected at 2 pm daily form each replicate of all groups to calculate egg production. Egg were collected and counted daily. Hen day egg production for all the groups was determined using the following formula.

Average Hen Day Egg production (%) = No of egg produced on 1 day/No of live birds in that group at that day) multiply 100.

Similarly feed intake was calculated on daily bases. It was measured by subtracting the feed rejected from the total feed given,

Feed consumption = Feed Offered-Feed Refused

Feed Conversion Ratio

Feed conversion ratio (FCR) was calculated on weekly basis. FCR was calculated for each replicate by the following formula;

$$\text{FCR per dozen of egg} = \frac{\text{Weekly feed consumption per replicate}}{\text{Weekly number of egg produced per replicate}} \times \frac{1}{12}$$

Egg Quality and Quantity Parameters

Two eggs from each group were taken and weighed individually, and then their mean was calculated. Weight eggs contents (*i.e.,* yolk, albumen, shell) were recorded on weekly basis. Two eggs per group were broken individually. The yolk was separated with help of egg yolk separator (Kitchen Craft, UK).

Yolk weight = Egg weight-(Albumin weight + Shell weight)

Albumen weight was calculated by subtracting yolk weight and shell weight from total egg weight. Similarly eggshell weight was determined after removing eggshell membranes. Eggshell thickness was determined with help of digital micrometer screw gauge. Shell thickness was determined without inner and outer membranes at three different regions to get the average value.

Statistical Analysis

Statistical analysis was performed with a commercially available software program SPSS version 18, SPSS Inc., Chicago, IL, USA. The data were analyzed using one-way analysis of a variance (ANOVA) between treatments. Least Significant Difference (LSD) test was applied when significant differences were found. The value of P<0.05 was considered to be significant.

RESULTS

Feed Intake

Laying birds that were fed with 2% and 2.5% SB level consumed higher (P<0.05) feed than other treatments groups *Table 1*. Feed consumption was significantly (P<0.05) affected by aflatoxins in treatment B. It was also cleared that lower feed intake was shown by group B, while high feed intake were observed by group D, and E as recorded in our experiment. It is also clear that SB increases efficiently uptake of feed in layers. Although group C, do not show

any effects (P>0.05) as compared to group D and E. From these results we observed that SB neutralized the effect of aflatoxins in layer bird efficiently.

Egg Productions

The average number of eggs production in group D and E showed uniformity while in group B there was low eggs production recorded. It is clear that aflatoxins adversely affected egg production in layers. The addition of 2% & 2.5% SB level effectively increased (P<0.05) production of eggs *Table 2*. Because SB neutralized the major effects of aflatoxins in groups D, and E when compared to group B.

Feed Conversion Ratio

In 1st week our results showed that in groups D and E have significantly higher FCR when compared to other experimental groups. However in week 2nd, high FCR was recorded in group C which is followed by group B, and groups D and E, while groups B and C showed similarity results. In 3rd week, B and C showed significant (P<0.05) increase FCR followed by D and E. In 4th week again groups B and C had highest FCR when compared to groups A, D and E. From these results the best FCR was noticed in group A, followed by groups D and E during our observation period *Table 3*.

Egg Quality and Metric Parameters

Our data showed that egg weight was significantly (P<0.05) heavier in group D and E than B, C and in groups A *Table 4*. Eggs weight in group A and C showed uniformity while in group B were lighter in weight, however eggs in group D was significantly heavier.

Eggshell weight was significantly affected by SB level. Eggshell weight was higher (P<0.05) recorded in group D while lowest was found in group B. However 2% SB level do not only increase egg weight but also increase shell thickness. Our results also indicated that the addition of SB could not increase (P>0.05) in weight of yolk and albumin level. Egg yolk weight was not significantly (P>0.05) affected by different level of SB. So it is clear that SB couldn't affect egg yolk weight and there is no interaction between yolk, Albumen and SB concentration. However, eggshell

Table 1. *Total feed intake (Kg) by five groups of laying hen with different diet (Mean ± SE)*
Tablo 1. *Farklı rasyon verilen beş farklı gruptaki yumurta tavuklarının toplam yem tüketimi (Kg) (Ortalama ± SE)*

Groups	Week 1st	Week 2nd	Week 3rd	Week 4th	Total Feed Intake
A	0.61±0.03^{bc}	0.71±0.04^{bc}	0.75±0.02^{bc}	0.80±0.04^{bc}	2.87±0.04^{bc}
B	0.53±0.03^{c}	0.62±0.04^{c}	0.66±0.02^{c}	0.72±0.06^{c}	2.53±0.03^{c}
C	0.62±0.02^{b}	0.73±0.02^{b}	0.80±0.02^{b}	0.82±0.05^{b}	2.97±0.04^{b}
D	0.68±0.01^{a}	0.83±0.01^{a}	0.88±0.05^{a}	0.88±0.06^{a}	3.27±0.04^{a}
E	0.69±0.01^{a}	0.84±0.01^{a}	0.88±0.02^{a}	0.89±0.07^{a}	3.30±0.05^{a}

Mean values within the same column with the different superscripts are significantly different at (P<0.05). Where capital alphabet A refer to control group, B, 170 ppb aflatoxin added, C, 170 ppb aflatoxin plus 1.5% SB added, D, 170 ppb aflatoxin plus 2% SB added, and E,170 ppb aflatoxin plus 2.5% SB added. The letters in the following tables have the same meanings

Table 2. *Weekly egg production by laying hens used in the experiment*
Tablo 2. *Çalışmada kullanılan yumurta tavuklarındaki haftΩalık yumurta üretimi*

Groups	Week 1st (Mean ± SE)	Week 2nd (Mean ± SE)	Week 3rd (Mean ± SE)	Week 4th (Mean ± SE)	Total Egg in % (Mean ± SE)
A	2.28±0.01^{b}	2.32±0.01^{bc}	2.35±0.02^{bc}	2.36±0.01^{bc}	2.32±0.01^{bc}
B	2.20±0.02^{c}	2.27±0.01^{c}	2.28±0.02^{c}	2.30±0.02^{c}	2.26±0.02^{c}
C	2.24±0.01^{bc}	2.30±0.01^{bc}	2.37±0.02^{bc}	2.37±0.02^{bc}	2.34±0.02^{bc}
D	2.30±0.00^{a}	2.35±0.01^{a}	2.42±0.01^{a}	2.44±0.01^{a}	2.40±0.02^{a}
E	2.31±0.02^{a}	2.35±0.02^{a}	2.41±0.02^{a}	2.41±0.02^{a}	2.37±0.02^{a}

Mean values within the same column with the different superscripts are significantly different at (P<0.05)

Table 3. *Weekly FCR (Ratio) in laying hens fed on different diets (Mean ± SE)*
Tablo 3. *Farklı rasyonlarla beslenen tavuklarda haftalık FCR (Oran) (Ortalama ± SE)*

Groups	Week 1st	Week 2nd	Week 3rd	Week 4th	Total FCR
A	2.60±0.02	2.75±0.03	2.75±0.03	2.80±0.05	2.72±0.04
B	2.58±0.02	2.85±0.05	2.97±0.05	2.99±0.06	2.85±0.09
C	2.61±0.02	2.88±0.02	2.90±0.04	2.92±0.04	2.82±0.07
D	2.67±0.04	2.75±0.04	2.76±0.05	2.82±0.06	2.76±0.03
E	2.68±0.04	2.73±0.04	2.79±0.05	2.80±0.06	2.75±0.02

Table 4. *Effects of SB supplementation on production performances of laying hens fed on aflatoxins contaminated diets (Mean ± SE)*
Tablo 4. *SB ilavesinin aflatoksinlerle kontamine rasyonlarla beslenen tavukların üretim performanslarına etkileri (Ortalama ± SE)*

Groups	Egg Production %	Egg Weight (g)	Albumin (g)	Yolk (g)	Shell (g)	Shell Thickness (mm)
A	80.64±0.03^{bc}	59.10±0.01^{bc}	27.95±0.08	14.99±0.07	5.81±0.08^{b}	0.376±0.0023^{b}
B	76.72±0.04^{c}	58.10±0.03^{c}	27.66±0.19	14.91±0.12	5.34±0.05^{c}	0.368±0.0007^{c}
C	80.92±0.03^{bc}	59.10±0.01^{bc}	27.47±0.20	14.82±0.10	5.72±0.10^{bc}	0.375±0.0021^{bc}
D	84.28±0.03^{a}	59.50±0.01^{a}	27.67±0.19	14.87±0.13	6.07±0.14^{a}	0.381±0.0024^{a}
E	82.60±0.02^{a}	59.25±0.01^{a}	27.47±0.23	14.86±0.14	5.85±0.09^{b}	0.377±0.0027^{b}
P value	0.032	0.002	0.63	0.87	0.002	0.024

Mean values within the same column with the different superscripts are significantly different at (P<0.05)

Table 5. *Economic cost (US $) of different groups and their egg production performances*
Tablo 5. *Farklı grupların ekonomik maliyeti ve yumurta üretim performansı (Amerikan Doları)*

Items	Groups				
	A	B	C	D	E
Feed consumption in kg	28	28	28	28	28
Total feed cost	10.66	10.66	10.66	10.66	10.66
Medication cost	1.9	1.9	1.9	1.9	1.9
Sodium bentonite cost @ one-half of a US dollar/kg	0	0	0.20	0.26	0.33
Total cost	12.57	12.57	12.57	12.57	12.57
Total egg (For Selling)	1.52	1.26	1.47	1.61	1.59
Per egg price	0.07	0.09	0.08	0.07	0.07
Cost per dozen eggs	0.94	1.14	0.98	0.9	0.91

thickness was significantly affected by SB levels. Highest value (P<0.05) was measured in group D, while group A and E show uniformity and Group B has the lowest value followed by group C and A.

Economic Coast

The cost of feeding was calculated according to the rates of feed, eggs, medication, additives and selling prevailed during the experiment. From economic point of view, only group D & E were profitable and cheap in price while group A and C were satisfactory, but in group B showed high price per dozen of egg and no increase in production parameters in contrast to others experimental groups *Table 5*.

DISCUSSION

Sodium bentonite supplemented diets had highest amount of feed intake when compared to aflatoxin containing negative control group. This may be disturbance in the normal metabolism that results in decreased appetite and hepatic degeneration [15-17]. Adsorbent like SB mixed in feed has high economical competence value for ducks by adding 0.5 to 1% SB in feed, which enhance (P<0.05) feed consumption [18]. From the results of our study, we assumed that SB clay possibly has absorptive and selective character that improves digestion. Pervious study also considers SB, as strong colloidal material [19]. In another report, it was described that SB as good additive in feed intake of laying hens [20-22]. In studies, SB, as feed additives 1% and 1.5% found significant increase in feed intake [19,22]. However aflatoxin contaminated feed treatment showed significant decrease in feed efficiency when compared to positive control group [23]. From our results, feeding efficiency was enhanced by adding either 2% or 2.5% SB as compared to positive and negative control diets. These results indicate that 2% SB supplementation is productive in layers to maximize feed intake. Our results are in agreement with another study that ascertains 1.5% SB as effective for higher feed consumption in laying hens [24]. The reason behind the increase in feed consumption could be possibly prolonged passage time of feed in the intestine and energy and protein utilization in the presence of SB [25-27].

The present findings were different from the reports of other studies, who reported a non-significant on overall performance of white leghorn on aflatoxin level and sodium bentonite, which slightly decreased in feed intake after addition of 5% SB to the diet [26]. The difference with their results may be due to the lower level of aflatoxins i.e., 50 ppb and SB composition as well as it concentration. SB containing diets work well in egg production and maintaining good health condition when compared to negative control group. Egg production per layer hen

during experimental study was significantly improved and the average increase of total egg production was 3.9% for 2% SB and 3% for 2.5% SB as compared to negative control group. Another study found that hen day egg production and egg mass boost when feed mix with sodium bentonite containing 10 g/kg [28,29]. The enhancement in egg production can be attributed to SB clay content possibly containing some minerals, vitamins, salts and other beneficial additives that helps eggs production and aflatoxins neutralization. Our results are also in agreement with other reports, indicating that a significant increase in egg number with addition of 1 to 2% of SB contents [18,21].

Dietary level of SB has showed better FCR as compared to toxin containing diets. From our results, highest FCR was shown by B group that consist 170 ppb aflatoxins only. The feeds containing SB 2.0% and 2.5% have good FCR. The results of FCR are substantiated by the findings of others [24]. They concluded that 1.5-3.0% of SB as supportive in FCR. Moreover, another suggested that feed with 2-8% SB showed significantly improved FCR [20]. Study also reported 1% SB containing feed as supportive for FCR [18]. Literature reports higher FCR for aflatoxin containing diets while overall best FCR for 2% SB containing feed in laying hens [23]. In our results, overall FCR was better in groups A and D than B and C. SB groups significantly ($P<0.05$) reduced the toxicity of aflatoxin on FCR in groups E and D when we compared to B although 1.5% SB was not as effective as 2% and 2.5% SB. The reason could be better digestibility of nutrients that is achieved at 2% and 2.5% SB level. Our results were also in agreement with the reports of others, who found improvement in FCR upon supplementation of toxin binders in poultry feeds [30-33].

In our study egg weight was significantly ($P<0.05$) increased during the whole experimental period. These results were also in accordance with the study that found increase in egg weight by use of dietary SB. According to this observation, SB significance on increasing in egg weight. These findings could be attributed to SB composition that can efficiently increase nutrient digestibility [26]. Our results were also in agreement with another study, who reporting increase in egg weight, when fed SB feed as compared to the toxin supplemented feed diet [20].

The most striking results in our egg traits were increase in eggshell weight and thickness. Maximum egg shell weight and thickness were observed in treatments D followed by E while lowest was observed in group B. Overall the result of treatments groups were significant ($P<0.05$) when compared to control treatment groups. The eggshell thickness of group three was almost comparable to group one that is without aflatoxin. Study has reported significant increase in relative ducks egg shell weight and thickness but to date no reports are available about shell thickness increase in layer [18]. Another group of observers reported that significances of sodium bentonite may be due to some minerals of bentonite and its natural values as well as the bentonite can be a natural anti-contamination of poultry feed used [25]. Egg trait parameters such as yolk and albumin weights were not significant ($P>0.05$) even within SB supplementation groups, although this level increase most of egg traits significantly. Yolk weight was higher in a group followed by group B, D and E. The lowest weight was shown by C in comparison to treatments groups with 1.5% SB but was not significant i.e. $P>0.05$. Our results are in agreement with a study, has reported that weight of yolk and albumen were not significantly affected [18]. Another study, suggested that egg quality parameters were not affected by adding sodium bentonite at any level to the different diet [19]. From different egg quality traits, egg weight, shell weight and thickness were increased ($P<0.05$) the entire experimental period.

The maximum egg production, feed conversion, feed consumption and minimum cracked and shell less egg were observed in treatments D and E. The highly affected group with minimum egg production and feed intake was treatment B that has not only produced less numbers of eggs but also showed reduced egg weight. Thus we conclude that the reason of significance can be the SB neutralizing effect and also aflatoxin binding abilities and has high mineral and vitamins content. The most important content in SB clay is calcium and ion exchange capability that helps in improvements of various traits [34]. In our study we observed significant difference among groups for egg production, feed consumption, egg weight, eggshell weight and thickness and FCR. Overall performance including egg production, feed intake and FCR were prominently affected in the birds feed on aflatoxin containing feed. These birds also showed better feed and protein utilizations having effect on increasing egg weight and reduced most of the soft, cracked and abnormal egg. These disastrous effects were efficiently diminished by the addition of SB at levels of 1.5, 2 and 2.5% in treated groups.

REFERENCES

1. Huff WE, Harvey RB, Kubena LF, Rottinghaus GE: Toxic synergism between aflatoxin and T-2 toxin in broiler chickens. *Poult Sci,* 67, 1418-1423, 1988. DOI: 10.3382/ps.0671418

2. Ortatatli M, Oğuz H: Ameliorative effects of dietary clinoptilolite on pathological changes in broiler chickens during aflatoxicosis. *Res Vet Sci,* 71, 59-66, 2001. DOI: 10.1053/Rvsc.2001.0487

3. Solmaz A, Bahman P, Mahmood R, Behrooz A: Aflatoxin M1 contamination in pasteuriezed milk in Shiraz (south of Iran). *Food cont,* 17,582-584, 2006. DOI: 10.1016/J.Foodcont.2005.03.009

4. Krogh P, Elling F, Friis C, Hald B, Larsen AE, Lilleh EB, Ravnskov U: Porcine nephropathy induced by long-term ingestion of ochratoxin A. *Vet Path Online J,* 16, 466-475, 1979. DOI: 10.1177/030098587901600410

5. Pfohl-Leszkowicz, A, R.A. Manderville: Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Moli Nutr Food Res,* 51, 61-99. Review Erratum, 51, 1192, 2007. DOI: 10.1002/mnfr.200600137

6. Marquardt RR, Frohlich AA: A review of recent advances in understanding ochratoxicosis. *J Anim Sci,* 70, 3968-3988, 1992. DOI: 10.2527/1992.70123968x

7. Stoev SD, Anguelov G, Ivanov I, Pavlov D: Influence of ochratoxin A and an extract of artichoke on the vaccinal immunity and health in

broiler chicks. *Exp Toxico Path,* 52, 43-55, 2000. DOI: 10.1016/S0940-2993(00)80014-7

8. Sun X, Zhao X, Tang J Gu, Zhou X, Chu FS: Development of an immuno-chromatographic assay for detection of aflatoxin B1 in food. *Food Cont*, 17, 256-262, 2006. DOI: 10.1016/J.Foodcont.2004.10.007

9. Bintvihok A, Ponpornpisit A, Tangtrongpiros J, Panichkriangkrai W, Rattanapanee R, Kumagai S: Aflatoxin contamination in shrimp feed and effects of aflatoxin addition to feed on shrimp production. *J Food Prot,* 66, 882-885, 2003.

10. Ali S, Ejaz N, Shah FH: Detoxification of aflatoxin in poultry feed. *Pak J Indi Res*, 30, 401-407, 2000.

11. Huwig A, Freimund S, Käppeli O, Dutler H: Mycotoxin detoxication of animal feed by different adsorbents. *Toxiclett,* 122, 179-188, 2001. DOI: 10.1016/S0378-4274(01)00360-5

12. Temur S, Bentonit Yatakları Endüstriyel Hammaddeler, Su. Mühendislik-Mimarlik Fak., Konya, 1994.

13. Demire H, Karapinaran, Akca K: Bentonite ve diğer killerin absorbant olarak kullanımı. *Endüstriyel Hammaddeler Sempozyumu*. 21-22 Nisan, İzmir, Türkiye, 1995.

14. Piyarat C, Johanna FG: Evaluation of the aflatoxin B1 adsorbent capacity of bentonite using an in vitro method mimicking monogastric gastro-intestinal tract conditions. *Thai J Vet Med,* 34, 13-19, 2004.

15. Reddy AR, Rao PV, Reddy VR, Yadgiri B: Effects of experimentally induced aflatoxicosis on performance of commercial broiler chicks. *Indi Anim Sci,* 52, 405-410, 1982.

16. Shaline KSB, Howrth JB, Wyatt RD: Effect of dietary aflatoxin in reproductive performance on mature white leghorn. *Poult Sci,* 59, 1311-1315, 1980. DOI: 10.3382/ps.0591311

17. Nandkumar RD, Rao PV, Reddy VR, Yadgiri B: Effect of selected levels of dietary aflatoxin on performance of broiler chicken. *Ind J Anim Sci,* 54, 68-73, 1984.

18. Fatouh MHA, Awad AL, Ghonim AIA: Effect of dietary sodium bentonite supplementation on laying performance of domyati and kampell ducks. *Egypt Poult Sci,* 32, 497-514, 2012.

19. Hashemipour H, Kermanshahi H, Pilevar M: Intercative effect of sodium bentonite with pigment on performance and egg quality of laying hens. *J Anim Vet Adv*, 9, 2179-2184, 2010.

20. Olver MD: Sodium bentonite as a component in layer diets. *Brit Poult Sci,* 30, 841-846, 1989. DOI: 10.1080/00071668908417210

21. Yenice E, Mizrak C, Ceylan N, Yildiz T, Gültekin M, Atik Z: Effects of dietary sodium bentonite and mannan oligosaccharide supplementation on performance, egg quality, blood and digestion characteristics of laying hens fed aflatoxin contaminated diet. *Kafkas Univ Vet Fak Derg,* 2, 211-218. 2015. DOI: 10.9775/kvfd.2014.12035

22. Nasir AT, Zafar IC, Zaheen AF: Effect of different levels of HIMAX BS-7(Sodium bentonite) on the performance of commercial layers. *Pak Vet J,* 20, 105-106, 2000.

23. Manafi M, Murthy HNN, Mohan K, Narayana SHD: Evaluation of different mycotoxin binders on broiler breeders induced with aflatoxin B: Effects on visceral organ weight and organ lesions parameters. *American-Eurasian J Agri Envir Sci,* 12, 574-578, 2012.

24. Nasir AT, Chughtai, ZI, Farooqi ZA: Effect of different levels of himah bs-7 (sodium bentonite) on the performance of commercial layers. *Pak Vet J,* 20,105-106, 2000

25. Khan AD, Ijaz N, Shahzad K: Role of bentonites in poultry feed. *Agro Vet News,* 2-3, 2001.

26. Pasha TN, Farooq MU, Khattak FM, Jabbar MA, Khan AD: Effectiveness of sodium bentonite and two commercial products as aflatoxin absorbents in diets for broiler chickens. *Anim Feed Sci Tech,* 132, 103-110, 2007. DOI: 10.1016/J.Anifeedsci.2006.03.014

27. Saçakli P, Calik Ali, Bayraktaroğlu AG, Ergün A, Şahan Ö, Özaydin S: Effect of clinoptilolite and/or phytase on broiler growth performance, carcass characteristics, intestinal histomorphology and tibia calcium and phosphorus levels. *Kafkas Univ Vet Fak Derg,* 21, 729-737, 2015. DOI: 10.9775/kvfd.2015.13283

28. Saeed MK, Ahmad I, Sakhawat A, Ashraf M, Khurram S, Haq IU, Shaikh MA: Comparative evaluation of sorbatox and bentonite for detoxification of aflatoxin contaminated layer feed. *Pak J Food Sci,* 19, 27-31, 2009.

29. Gilani A, Kermanshahi H, Tahmasbi AM: Impact of sodium bentonite addition to the diets containing cottonseed meal on productive traits of HY-line W-36 hens. *J Anim Plant Sci,* 23, 411-415, 2013.

30. Denli M, Blandon JC, Guynot ME, Salado S, Perez JF: Effects of dietary Afla-Detox on performance, serum biochemistry, histopathological changes, and aflatoxin residues in broilers exposed to aflatoxin B1. *Poult Sci,* 88, 1444-1451, 2009. DOI: 10.3382/ps.2008-00341

31. Haq A, Rasool S, Shah TH, Anjum I: Effect of sodium bentonite as aflatoxin binder in broiler feeds containing fungal infected grains. *Pak J Agri Sci,* 37, 163-165, 2000.

32. Indresh H, Devegowda G, Ruban SW, Shivakumar M: Effects of high grade bentonite on performance, organ weights and serum biochemistry during aflatoxicosis in broilers. *Vet World,* 6, 313-317. 2013. DOI: 10.5455/vetworld.2013.313-317

33. Khanedar F, Vakili R, Zakizadeh S: Effects of two kinds of bentonite on performance, blood biochemical parameters, carcass characteristics and tibia ash of broiler chickens. *Irani J Appli Anim Sci,* 3, 577-581, 2013.

34. Grim RE: Applied Clay Mineralogy. Geologiska Föreningen Stockholm Förhandlingar, 84 (4), 1962.

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Research Article

Effects of Photoperiod Length and Light Intensity on Performance, Carcass Characteristics and Heterophil to Lymphocyte Ratio in Broilers [1]

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Abstract

The aim of this study was to investigate the effects of photoperiod length and light intensity on performance, carcass characteristics and heterophil to lymphocyte ratio in broilers. A total of 272 1 day-old male broiler chicks (Ross 308) were randomly assigned to four treatment groups based on the photoperiod length (23L:1D or increasing duration of light) and light intensity (20 lux vs. a dim, reducing intensity) with four replicates. At 42 d of age, effects of photoperiod length and light intensity on performance traits were not significant. The heterophil/lymphocyte ratio in 20 lux and dim, reducing light intensity groups were 0.30 and 0.15 (P<0.001), respectively. On the other hand, the effect of light intensity has no influence on heterophil/lymphocyte ratio. Cold and hot carcass weights and whole breast meat and wing weights were found lower in the dim, reducing light intensity group than 20 lux light intensity group. The effects of photoperiod length and light intensity on carcass characteristics were not significant, statistically. In conclusion, it can be said that body weight, feed consumption, feed convertion ratio, whole breast meat and wing weights were increased by providing the increasing photoperiod used with a 20 lux light intensity in broiler breeding.

Keywords: *Broiler, Carcass, Heterophil/lymphocyte ratio, Light intensity, Performance, Photoperiod*

Etlik piliçlerde Fotoperiyot Uzunluğu ve Işık Şiddetinin Performans, Karkas Özellikleri ve Heterofil/Lenfosit Oranı Üzerine Etkileri

Özet

Bu çalışmanın amacı fotoperiyot uzunluğu ve ışık şiddetinin etlik piliçlerde performans, karkas özellikleri ve heterofil/lenfosit oranı üzerine etkilerinin araştırılmasıdır. Bir günlük yaşta toplam 272 adet (Ross 308) erkek civcivler fotoperiyot uzunluğu (23A:1K veya giderek artan aydınlık süre) ve ışık şiddeti (20 lüks veya giderek azalan ışık şiddeti) faktörlerine göre dört gruba, dört tekrarlı olacak şekilde rastgele olarak dağıtılmıştır. Kırkiki günlük yaşta, performans özellikleri üzerine, fotoperiyot uzunluğu ve ışık şiddetinin etkileri önemsiz bulunmuştur. Yirmi lüks ve giderek azalan ışık şiddeti gruplarında, heterofil/lenfosit oranı sırasıyla 0.30 ve 0.15 olarak bulunmuş olup, gruplar arası farklar istatistik bakımdan önemli (P<0.001) çıkmıştır. Diğer taraftan, ışık şiddetinin heterofil/lenfosit oranı üzerine önemli bir etkisinin olmadığı saptanmıştır. Sıcak ve soğuk karkas ağırlık ortalaması, bütün göğüs eti ve kanat ağırlık ortalaması değerleri giderek azalan ışık şiddeti grubunda, 20 lüks ışık şiddeti grubuna göre daha düşük olarak belirlenmiştir. Tüm karkas özellikleri üzerine fotoperiyot uzunluğu ve ışık şiddetinin etkisi istatistiksel olarak önemsiz çıkmıştır. Sonuç olarak, etlik piliç yetiştiriciliğinde, 20 lüks ışık şiddeti altında, giderek artan aydınlık süre kullanımının canlı ağırlık, yem tüketimi, yemden yararlanma oranı, bütün göğüs eti ve kanat ağırlıklarını olumlu yönde etkilediği söylenebilir.

Anahtar sözcükler: *Etlik piliç, Karkas, Heterofil/lenfosit oranı, Işık şiddeti, Performans, Fotoperiyot*

INTRODUCTION

Light is an important factor in the regulation and control of production, reproduction and health of poultry. Growth rate and welfare of the broiler is influenced to a great degree by at least three components of light: photoperiod, intensity and color or wavelength of the light [1-3]. Broiler chickens have usually been reared under continuous

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(24L:0D) or near-continuous (23L:1D) photoperiods to maximize feed consumption (FC) and growth rate. It has been reported that broilers exposed to continuous or near-continuous lighting programs to provide constant visual access to feed and water, resulting in maximum FC, increased live weight gain and growth rate [4,5]. However, several studies indicated that, using continuous lighting programs might result in inadequate sleep and as a result of sleep deprivation physiological stress responses were increased [6,7]. Amid these conflicting results EU [8] have established guidelines on behalf of poultry welfare on light intensities, and amounts and durations of darkness that must be provided to broilers daily. On this contex, the use of photoperiods longer than 20 h and intensities less than 21.52 lux were restricted. Therefore, recent studies have focused on limited lighting programs (such as increasing photoperiod), as an alternative to the continuous lighting program, to improve the productivity of broilers, Rahimi et al.[9] reported that physical activity and energy consumption were low during darkness period.

Although there is a lot of studies on photoperiod, the effect of light intensity on production is less studied in broilers. The effect of light intensity (ranging from 1 to 150 lx) on body weight (BW), FC, feed conversion ratio (FCR) and mortality in broiler chickens was reported as statistically nonsignificant by some studies [10-12]. Processed fillet weights were reported to be higher in 1.08 lux light intensity (dim light) than those kept in 161.4 lux light intensity (bright light) [13]. Deep et al.[1] observed that carcass, thighs and drums yields decreased linearly with increasing light intensity from 1 to 40 lx in broiler chickens.

The objective of this study was to evaluate the effects of photoperiod and light intensity on performance traits (body weight, feed consumption, feed convertion ratio and mortality), carcass characteristics (cold and hot carcass weights and parts weights) and physiological stress response (heterophil to lymphocyte (H/L) ratio) in broilers.

MATERIALS and METHODS

Animals and Diets

A total of 272 1-d old male broilers (Ross 308) obtained from a commercial hatchery were used in the study. From the first day, chicks were housed on deep litter of wood shavings in an experimental barn with controlled heating and hygienic and feeding patterns according to standard management requirements for broilers. Heat was provided by an electric forced draft heater in each treatment room. Birds were fed with a starter diet from 1 to 21 d of age (3060 kcal ME/kg, 23% crude protein) and a grower diet from 22 to 42 d of age (3200 kcal ME/kg, 21.5% crude protein). Feed and water were available *ad libitum* during the experiment. Two 40 W incandescent bulbs, which were controlled by a rheostat and automatic timer, used for lighting. The lights were attached 1.90 m above the floor. Light intensity was monitored at chick head level using a digital illuminometer (Datalogging light meter, Extech HD 450, Extech Instruments, USA) thrice weekly. Walls and ceilings in the rooms were painted white to ensure light intensity was consistent. The ambient barn temperature was gradually decreased from 32±1°C on d 1 to 23±1°C on the last day of fattening (d 42). The relative humidity was varied 50 to 60%.

Experimental Treatments

All the procedures used in this study were approved by Adnan Menderes University Animal Experiments Local Ethics Committee (No: 64583101/2013/088). A 2 x 2 factorial design was used with two levels of photoperiod length and light intensity treatment groups for which have four photoperiod and light intensity subgroups. Photoperiod lengths were either near-continuous (CPL) (23L:1D from 1 to 42 d) or increasing photoperiod (IPL) (23L:1D from 1 to 8 d, 14L:10D from 9 to 15 d, 16L:8D from 16 to 22 d, 18L:6D from 23 to 29 d, 20L:4D from 30 to 36 d, followed by 23L:1D from 37 to 42 d). It should be noted that 23L was applied for the last 6 d before slaughter in the increasing photoperiod group because of recent EU guidelines [8]. Light intensities were either bright (BLI) or dim, reducing (DRLI). Broilers in the BLI group were exposed to 20 lux from d 1 to 42 d while those in dim, reducing DRLI group were exposed to 5 lux from d 1 to 8, 2.5 lux from d 9 to 15 and 1.25 lux from d 16 to 42.

Traits Measured

Individual BW and FC were recorded on d 8, 15, 22, 29, 36 and 42. According to collected data, FCR was also calculated. Mortality from which cumulative mortality ratio was calculated (0-42 d) recorded at daily basis. On d 41, blood samples from a total of 160 birds that were randomly selected (40 birds (10 birds for each replication) per group were used for heterophil to lymphocyte (H/L). Blood samples were taken from the vena basilica of broilers in each photoperiod and light intensity group. Following the blood film preparation, films were painted with May-Grünwald and Giemsa dyes [14]. After 100 leucocytes were counted in light microscope with (x100) magnification, H/L ratio was calculated by dividing heterophil count to lymphocyte count. At 42 d of age, eight broilers from each pen, a total of 128 broilers were randomly selected for processing. Feed was withdrawal 12 h prior to slaughter. Slaughtering is conducted by cutting the jugular veins and carotid arteries. Broilers were then scalded for 150 s at 53°C, before mechanically plucking (35 s) and eviscerated. Whole carcasses (without neck, giblets) were weighed and recorded as hot carcass weight. Cold carcass weights were recorded after the carcasses were stored at +4°C for 24 h. Skinless, boneless breast fillets *(pectoralis major muscles)*, breast tenders *(pectoralis minor muscles)*, total breast meat, wings, whole legs (thigh and drum) and abdominal

fat pads were removed from each carcass and weighed to determine carcass parts weight. Breast skin was removed and then weighted.

Statistical Analyses

Statistical analyses were performed by using Statistical Package for the Social Sciences for Windows (SPSS) 22.0 [15]. The data was subjected to ANOVA using the GLM procedure with photoperiod length and light intensity as the main effects along with their interactions included in the following model: $x_{ijk} = \mu + M_i + D_j + (MD)_{ij} + e_{ijk}$, where: x_{ijk} = Analyzed measurement, μ = Overall mean, M_i = Effect of photoperiod length (23L:1D and increasing photoperiod), D_j = Effect of light intensity (bright and dim, reducing), $(MD)_{ij}$ = Effect of interaction, ε_{ijk} = Residual random error. In analysis, GLM was designed to reveal the effects of photoperiod length and light intensity on performance, carcass characteristics and H/L ratios. The partial effects of photoperiod length and light intensity for each factor were analyzed with Least Squares Means Test and multiple comparisons were performed with a Duncan test [16]. Chi-square test was performed for mortality.

RESULTS

Least square means and standard errors of BW of broilers from 8 to 42 days of age were summarized in *Table 1*. Body weights of CPL group were higher than that of IPL group (P<0.01) at 15 days of age. At 42 d, there was not significant difference between CPL and IPL groups. The FC level was found as 699.19 and 683.26 g for CPL and IPL groups at 15 d (P<0.05) *(Table 2)*. The differences between light intensity groups for FC and FCR were not significant for d 0-42. The mortality rate was found as 0.74% for CPL group while there was no death in IPL group. And, there was no death in BLI group while one death (0.74%) recorded in DRLI group. It was also determined that photoperiod length and light intensity has no significant effects on mortality ratio. Least square means and standard errors of live weights, carcass characteristics and parts weights and H/L ratio of broilers were given in *Table 3*. The differences between light intensity groups for H/L ratio were found significant (P<0.001) statistically.

DISCUSSION

On d 15, average BW was 24.68 g (4.50%) less (P<0.01) in IPL group than CPL ones. This difference at BW's can be explained by the suppression of FC's for birds subjected to increasing light IPL group. It was determined that the increasing photoperiod treatment had caused a decrease in FC, which resulted in reduced BW at d 0-15. On d 42, average BW was 30.64 g less in CPL group than IPL ones. There was no significant difference between

Table 1. *Influences of photoperiod length and light intensity on body weights of broilers* [1]
Tablo 1. *Fotoperiyot uzunluğu ve ışık yoğunluğunun etlik piliçlerde canlı ağırlık üzerine etkileri* [1]

Treatment Main Effects	Body Weight (g)											
	n	d 8	n	d 15	n	d 22	n	d 29	n	d 36	n	d 42
Photoperiod length												
Near Continuous (CPL)	136	194.57	135	548.98^a	135	1020.22	135	1685.79	135	2315.23	135	2916.72
Increasing (IPL)	136	196.87	136	524.30^b	136	998.55	136	1681.42	136	2329.11	136	2947.36
Light intensity												
Bright (BLI)	136	198.70^a	136	543.45^a	136	1004.71^b	136	1688.43	136	2318.32	136	2944.70
Dim, reducing (DRLI)	136	192.74^b	135	529.83^b	135	1014.05^a	135	1678.78	135	2326.03	135	2919.38
SEM^2		0.91		2.47		4.62		8.78		13.75		17.24
Photoperiod length x light intensity												
CPL + BLI		199.82		560.97		1027.94		1714.71		2340.06		2980.63
IPL + BLI		189.31		525.93		981.49		1662.15		2296.54		2908.77
CPL + DRLI		197.58		537.00		1012.49		1656.88		2290.41		2852.80
IPL + DRLI		196.16		522.67		1015.60		1700.69		2361.64		2985.96
SEM^3		1.83		4.92		9.25		17.57		27.51		34.47
Significance of main effects	P value											
Photoperiod length		0.209		0.006		0.314		0.583		0.779		0.463
Light intensity		0.001		0.001		0.020		0.803		0.614		0.375
Photoperiod length x light intensity		0.013		0.037		0.008		0.006		0.051		0.003

[1] *Data presented as the least square means,* ^{a,b} *Means with different superscript letters in the same row differ (P<0.05),* [2] *Pooled SEM for main effects,* [3] *Pooled SEM for interaction effects*

Table 2. *The least square means for cumulative feed consumption and feed conversion between days 8 and 42*
Tablo 2. *Sekizinci-42. günler arasında kümülatif yem tüketimi ve yemden yararlanma oranlarına ait en küçük kareler ortalamaları*

Treatment Main Effects	Cumulative Feed Consumption (g/bird)							Cumulative Feed Conversion (g of feed/g of gain)					
	n	d 0-8	d 0-15	d 0-22	d 0-29	d 0-36	d 0-42	d 0-8	d 0-15	d 0-22	d 0-29	d 0-36	d 0-42
Photoperiod length													
Near- Continuous (CPL)	8	163.55	699.19[a]	1363.47	2374.00	3544.81	4732.49	1.09	1.39[b]	1.40	1.45	1.56	1.65
Increasing (IPL)	8	165.81	683.26[b]	1350.10	2379.28	3582.66	4809.98	1.09	1.43[a]	1.42	1.44	1.56	1.64
Light intensity													
Bright (BLI)	8	169.14[a]	688.01	1356.83	2398.85	3583.19	4797.75	1.09	1.38[b]	1.41	1.46	1.58	1.65
Dim, reducing (DRLI)	8	160.22[b]	694.43	1356.74	2354.44	3544.27	4744.71	1.08	1.44[a]	1.40	1.43	1.54	1.64
SEM[1]		1.40	3.32	5.88	13.72	23.85	33.97	0.01	0.01	0.00	0.01	0.01	0.01
Photoperiod length x light intensity													
CPL + BLI		169.00	698.54	1379.30	2437.63	3629.93	4856.48	1.09	1.36	1.41	1.46	1.58	1.66
IPL + BLI		169.28	677.49	1334.35	1360.36	3536.46	4739.02	1.09	1.41	1.42	1.46	1.57	1.65
CPL + DRLI		158.09	699.84	1347.63	2310.67	3459.69	4608.50	1.08	1.42	1.39	1.44	1.54	1.64
IPL + DRLI		162.34	689.03	1365.85	2398.21	3628.85	4880.92	1.09	1.45	1.41	1.43	1.55	1.64
SEM[2]		2.79	6.63	11.75	27.45	47.69	67.94	0.01	0.01	0.01	0.01	0.02	0.02
Significance of main effects		P value						P value					
Photoperiod length		0.434	0.033	0.278	0.851	0.443	0.276	0.854	0.011	0.055	0.722	0.837	0.809
Light intensity		0.008	0.352	0.995	0.132	0.430	0.450	0.582	0.001	0.155	0.068	0.082	0.472
Photoperiod length x light intensity		0.491	0.455	0.020	0.011	0.017	0.014	1.000	0.339	1.000	0.859	0.632	0.903

[a,b] Means with different superscript letters in the same row differ ($P<0.05$), [1] Pooled SEM for main effects, [2] Pooled SEM for interacion effects

photoperiod groups in terms of final BW, FC and FCR. This finding was found to be consistent with other studies [17-19]. Similary, Downs et al.[4] reported that BW and FC in continuous photoperiod was higher than increasing photoperiod group at early ages. But, at the market age (d 56), photoperiod treatment has no significant effects on BW and FC. Similarly, in other studies the effect of photoperiod on FCR was found to be statistically not significant [4,11,13,17,20]. There was photoperiod length x light intensity interaction on BW of broilers at different periods of growth, except for 36 d. As Downs et al.[4], Lien et al.[11], Çoban et al.[21] reported that the photoperiod length has no statistically significant effects on mortality. It might be arised from the genetic selection of metabolic and skeletal disorders. However, Schwean-Lardner et al.[22] indicated that when photoperiod increased linearly from 14 to 23 h, mortality would gradually increase. It also has been noted that rapid growth rates in the early stages of rearing along with increasing lighting programmes resulted in increased mortality [5,23]. It was determined that the increasing photoperiod length had led to an increase in hot and cold carcass weight, whole leg and abdominal fat pad weights and a decrease in whole breast weights, but this has not reached statistically significance. These findings were in consistent with other studies reporting that decreases in breast meat and increases in wing and leg weights were caused from increasing photoperiod programs [4,17]. Similarly, as reported in some studies that the extension of the light period from 18 h to 23 h [11] and from 14 h to 23 h [22] resulted in heavier whole breast. Lewis et al.[24] also indicated that continuous lighting increased the weight of breast meat. However, a reduction (0.2%) in breast yield during an increasing photoperiod program was reported by Newcombe et al.[25]. The diversity of carcass parts might be explained by some growth retardation of legs and wings by light limitation at early ages. On the other hand, photoperiod has no effect on H/L ratio. Similar results were reported in some studies carried out in broilers in which the effect of photoperiod on H/L ratio were statistically non-significant [11]. However, Coban et al.[21] had recorded lower H/L ratio in 16L:8D photoperiod group than counterparts subjected to continuous lighting ($P<0.001$).

At d 42, light intensity was not determined to have significant effect on BW. Similarly, Kristensen et al.[10], Blatchford et al.[12], Deep et al.[1], Ahmad et al.[2] reported that light intensity has no significant effects on BW at market age. Newberry et al.[26] also found no influence between light intensity groups (180 and 6 lux) on BW. However, Charles et al.[27] found improved BW and FCR with low light intensities (5.4 lux) compared to birds given more light (150 lux). BW differences can be attributed to increased activity of broilers exposed to high bright light. The FC level was found as 169.14 and 160.22 g for BLI and

Table 3. *The least square means for carcass parameters, carcass part weights and heterophil/lymphocyte (H/L) ratios in treatment groups*
Tablo 3. *Deneme gruplarındaki etlik piliçlerin karkas parametreleri, karkas parça ağırlıkları ve heterofil/lenfosit oranlarına ait en küçük kareler ortalamaları*

Treatment Main Effects	Live Weight (g)	Hot Carcass Weight (g)	Cold Carcass Weight (g)	Whole Breast Weight (g)	Breast Skin Weight (g)	Fillets Weight (g)	Tenders Weight (g)	Whole Leg Weight (g)	Thighs Weight (g)	Drums Weight (g)	Wing Weight (g)	Abdominal Fat Pad Weight (g)	H/L Ratio
Photoperiod length													
Near- Continuous (CPL)	2930.00	2271.91	2241.20	711.37	59.68	595.85	115.53	840.09	583.70	256.39	182.58	42.26	0.21
Increasing (IPL)	2955.61	2280.16	2249.29	704.20	61.44	587.92	116.28	851.05	589.50	261.55	178.91	44.99	0.24
Light intensity													
Bright (BLI)	2947.14	2293.16	2258.66	720.83	60.20	602.42	118.41	840.88	583.22	257.66	182.58	44.10	0.30[a]
Dim, reducing (DRLI)	2938.47	2258.91	2231.83	694.74	60.92	581.35	113.39	850.25	589.97	260.28	178.92	43.14	0.15[b]
SEM[1]	25.17	20.44	20.02	7.41	1.13	6.46	1.37	8.84	6.20	2.99	1.66	1.21	0.02
Photoperiod length x light intensity													
CPL + BLI	2966.28	2327.94	2294.81	743.51	61.47	624.49	119.03	839.10	581.73	257.37	186.09	41.75	0.32
IPL + BLI	2928.00	2258.39	2222.52	698.15	57.90	580.35	117.80	842.67	585.67	255.42	179.07	46.46	0.28
CPL + DRLI	2893.72	2215.88	2187.59	679.23	58.94	567.21	112.03	841.08	584.72	257.95	179.07	42.77	0.09
IPL + DRLI	2983.22	2301.94	2276.06	710.25	63.95	595.49	114.76	859.42	594.28	265.14	178.76	43.52	0.20
SEM[2]	50.13	40.71	39.88	14.75	2.25	12.86	2.73	17.61	12.54	5.94	3.31	2.40	0.03
Significance of main effects							**P value**						
Photoperiod length	0.612	0.840	0.840	0.629	0.437	0.540	0.783	0.537	0.641	0.389	0.272	0.260	0.334
Light intensity	0.863	0.404	0.504	0.081	0.752	0.105	0.069	0.597	0.587	0.661	0.273	0.691	<0.001
Photoperiod length x light intensity	0.207	0.059	0.051	0.011	0.061	0.006	0.470	0.677	0.821	0.445	0.314	0.415	0.026

[a,b] *Means with different superscript letters in the same row differ (P<0.05),* ***Whole breast*** *= combined weight of right and left pectoralis major and minor.* ***Fillet*** *= combined weight of right and left pectoralis major;* ***Tender*** *= combined weight of right and left pectoralis minor,* [1] *Pooled SEM for main effects,* [2] *Pooled SEM for interacion effects*

DRLI groups at 8 d (P<0.01). In the following weeks, there was no significant effect of light intensity on FC. Similarly, Downs et al.[4], Lien et al.[11], Charles et al.[27] reported that there was no effect of light intensity on FC. Kristensen et al.[10] also reported no effect of intensities varying from 53.80 lux to 64.56 lux as in contrast to 107.6 lux to 124.82 lux was observed on FC. Whereas, Lien et al.[13] found that FC increased gradually by providing 1.75 vs. 162 lux of light intensity. Inconsistencies between studies are most probably related to the amount of light intensity. Also, it can be concluded that light intensity varying from 1.25 to 20 lux have no significant effect on FC. Similarly, light intensity did not have any effect on FCR. Similar results were reported by various authors about light intensity in different growth periods [4,11,13,27]. Buyse et al.[28] reported that increasing light intensity from 5 to 51 lux has no significant effect on FCR. According to, Deep et al.[1], Ahmad et al.[2], Kristensen et al.[10], Lien et al.[11], and Lien et al.[13] light intensity has no significant effect on mortality. However, Newberry et al.[26] observed an increase in mortality due to light intensity ranging from 6.45 to 194 lux. The differences between studies regarding the effect of light intensity on mortality may be arised from timing, severity and duration of light intensity and combined effect of light intensity with other management factors. The cold carcass weight of broilers reared at BLI group was higher (2258.66 g) than DRLI group (2231.83 g). Similary, Lien et al.[11] reported that higher cold carcass weight has been reported in broilers reared under 10.76 lux compared to 1.08 lux (P<0.01). Parallel to this result, several authors reported that there were no significant differences in abdominal fat pad weight among light intensity groups [1,4,13]. In another study in which Deaton [29] used two levels of light intensity (2 or 52 lux) found that the proportion of abdominal fat pad was unaffected by light intensity. In contrast, Charles et al.[27] reported that carcasses of broilers exposed to 150 lux had a lower percentage of fat than those exposed to 5 lux. Moreover, it was determined that light intensity has no effects on the most valuable part of the carcass in breast meat [1,4,11,13]. This study revealed that whole leg, thigh and drum weights were not affected by light intensity. Likewise, Downs et al.[4] reported an 1.35% improvement in 56-d whole leg weight when female broilers were exposed to 2.69 lux (777.7 g), as in contrast to 21.52 lux (767.2 g), with no influence on whole leg weight. Although wing weight was not influenced by light intensity, an increase of wing weight in broilers exposed to low light intensity has been indicated by other studies [1,4,13]. Likewise Deep et al.[1], light intensity has no significant effect on the weight of breast skin. The genotype and gender of broilers, severity of light intensity and light intensity in combination with some environmental factors can be responsible for the differences in some studies regarding to the effect of light intensity on carcass part weights. Interaction effect of photoperiod length and light intensity on many carcass parts weights were not significant. The H/L ratio is a sensitive indicator of stress, and 0.2, 0.5 and 0.8 characterize low, optimum and high levels of stress, respectively [30]. In this study, the highest H/L ratio (0.30) was obtained for broilers in BLI group, whereas the broilers in DRLI group had the lowest H/L ratio (0.15). This result suggests that broilers in light intensity indicated a low level of stress. However, Lien et al.[11] reported no effect of light intensity on the H/L of 40-days old female broilers.

These results indicated that increasing photoperiods have negative effects on FC in d 0-15. However, in later periods, it was determined that broilers exposed to increasing photoperiods has reached similar BW's. As to light intensity, the birds exposed to a dim, reducing light intensity showed a reduced BW in d 8, 15 and 22. On the other hand, BWs rebounded by d 42 to weights similar to those for birds on bright light intensity. The low levels of H/L ratios indicated that light intensity was a non-stressful event by broilers. Increasing photoperiod and 20 lux light intensity would appear to produce the best BW benefits for the commercial broiler producers, as well as the 20 lux light intensity did promote heavier whole breast meat and wing weights. It's believed that further studies should be designed to understand the physiological pathways and welfare status of broilers exposed to different photoperiods and light intensities.

REFERENCES

1. Deep A, Schwean-Lardner K, Crowe TG, Fancher BI, Classen HL: Effect of light intensity on broiler production, processing characteristics and welfare. *Poult Sci*, 89, 2326-2333, 2010. DOI: 10.3382/ps.2010-00964

2. Ahmad F, Haq AU, Ashraf M, Abbas G, Siddiqui MZ: Effect of different light intensities on the production performance of broiler chickens. *Pak Vet J*, 31, 203-206, 2011.

3. Olanrewaju HA, Miller WW, Maslin WR, Collier SD, Purswell JL, Branton SL: Influence of photoperiod, light intensity and their interaction on health indices of modern broilers grown to heavy weights. *Int J Poult Sci*, 14, 183-190, 2015. DOI: 10.3923/ijps.2015.183.190

4. Downs KM, Lien RJ, Hess JB, Bilgili SF, Dozier WA: The effects of photoperiod length, light intensity and feed energy on growth responses and meat yield of broilers. *J Appl Poult Res*, 15, 406-416, 2006. DOI: 10.1093/japr/15.3.406

5. Schwean-Lardner K, Fancher BI, Classen HL: Impact of day length on the productivity of two commercial broiler strains. *Br Poult Sci,* 53, 7-18, 2012. DOI: 10.1080/00071668.2012.659652

6. Campo JL, Davila SG: Effect of photoperiod on heterophil to lymphocyte ratio and tonic immobility duration of chickens. *Poult Sci*, 81, 1637-1639, 2002. DOI: 10.1093/ps/81.11.1637

7. Kliger CA, Gehad AE, Hulet RM, Roush WB, Lillehoj HS, Mashaly MM: Effect of photoperiod and melatonin on lymphocyte activities in male broiler chickens. *Poult Sci*, 79, 18-25, 2000. DOI: 10.1093/ps/79.1.18

8. EU: Laying down minimum rules for the protection of chickens kept for meat production. EUCD 2007/43/EC 28 June. *O J L*, 182, 19-28, 2007.

9. Rahimi G, Rezaei M, Hafezian H, Saiyahzadeh H: The effect of intermittent lighting schedule on broiler performance. *Int J Poult Sci*, 4, 396-398, 2005.

10. Kristensen HH, Perry GC, Prescott NB, Ladewig J, Ersboll AK, Wathes CW: Leg health and performance of broiler chickens reared in different light environments. *Br Poult Sci*, 47, 257-263, 2006. DOI: 10.1080/00071660600753557

11. Lien RJ, Hess JB, McKee SR, Bilgili SF, Townsend JC: Impact of light intensity and photoperiod on live performance, heterophil to lymphocyte

ratio and processing yields of broilers. *Poult Sci*, 86, 1287-1293, 2007. DOI: 10.1093/ps/86.7.1287

12. Blatchford RA, Klasing KC, Shivaprasad HL, Wakenell PS, Archerand GS, Mench JA: The effect of light intensity on the behavior, eye and leg health and immune function of broiler chickens. *Poult Sci*, 88, 20-28, 2009. DOI: 10.3382/ps.2008-00177

13. Lien RJ, Hess JB, McKee SR, Bilgili SF: Effect of light intensity on live performance and processing characteristics of broilers. *Poult Sci*, 87, 853-857, 2008. DOI: 10.3382/ps.2007-00277

14. Gross WB, Siegel HS: Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis*, 27, 972-979, 1983. DOI: 10.2307/1590198

15. Statistical Packages for the Social Sciences. SPSS base 22.0 user's guide, IBM United States Software Announcement, 213-309, August 13, 2013.

16. Duncan DB: Multiple range and multiple F tests. *Biometrics,* 11, 1-42, 1955. DOI: 10.2307/3001478

17. Renden JA, Bilgili SF, Kincaid SA: Research Note: Comparison of restricted and increasing light programs for male broiler performance and carcass yield. *Poult Sci* 72, 378-382, 1993. DOI: 10.3382/ps.0720378

18. Lott BD, Branton SL, May JD: The effect of photoperiod and nutrition on ascites incidence in broilers. *Avian Dis,* 40, 788-791, 1996. DOI: 10.2307/1592299

19. Lien RJ, Hooie LB, Hess JB: Influence of long- bright and ıncreasing-dim photoperiods on live and processing performance of two broiler strains. *Poult Sci*, 88, 896-903, 2009. DOI: 10.3382/ps.2008-00309

20. Renden JA, Moran Jr ET, Kincaid SA: Lighting programs for broilers that reduce leg problems without loss of performance or yield. *Poult Sci,* 75, 1345-1350, 1996. DOI: 10.3382/ps.0751345

21. Çoban O, Lacin E, Genç M: The effect of photoperiod length on performance parameters, carcass characteristics and heterophil/lymphocyte-ratio in broilers. *Kafkas Univ Vet Fak Derg,* 20, 863-870, 2014. DOI: 10.9775/kvfd.2014.11186

22. Schwean-Lardner K, Classen HL, Fancher BI: Daylength effects on production traits of modern broilers. *Poult Sci,* 85 (Suppl. 1): 169, 2006.

23. Schwean-Lardner K, Fancher BI, Gomis S, Van Kessel A, Dalal S, Classen HL: Effect of day length on cause of mortality, leg health and ocular health in broilers. *Poult Sci,* 92, 1-11, 2013. DOI: 10.3382/ps.2011-01967

24. Lewis PD, Danisman R, Gous RM: Photoperiodic responses of broilers. I. Growth, feeding behaviour, breast meat yield and testicular growth. *Br Poult Sci,* 50, 657-666, 2009. DOI: 10.1080/00071660903338452

25. Newcombe M, Cartwright AL, Harter-Dennis JM: The effect of increasing photoperiod and food restriction in sexed broiler-type birds. I. Growth and abdominal fat cellularity. *Br Poult Sci,* 33, 415-425, 1992. DOI: 10.1080/00071669208417479

26. Newberry RC, Hunt JR, Gardiner EE: Influence of light-intensity on behavior and performance of broiler chickens. *Poult Sci,* 67, 1020-1025, 1988. DOI: 10.3382/ps.0671020

27. Charles RG, Robinson FE, Hardin RT, Yu MW, Feddes J, Classen HL: Growth, body composition and plasma androgen concentration of male broiler chickens subjected to different regimens of photoperiod and light intensity. *Poult Sci*, 71, 1595-1605, 1992. DOI: 10.3382/ps.0711595

28. Buyse J, Simons PCM, Boshouwers FMG, Decuypere E: Effect of intermittent lighting, light intensity and source on the performance and welfare of broilers. *World's Poult Sci J*, 52, 121-130, 1996. DOI: 10.1079/WPS19960012

29. Deaton JW, Lott BD, Branton SL, Simmons JD: Effect of differing light intensities on abdominal fat deposition in broilers. *Poult Sci*, 67, 1239-1242, 1988. DOI: 10.3382/ps.0671239

30. Siegel PB, Gross WB: General principles of stress and well-being. **In,** Grandin T (Ed): Livestock Handling and Transport. 27-41, CABI, Wallingford, UK, 2000

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Research Article

Can *Taraxacum officinale* (Dandelion) Extract be an Alternative of Paracetamol in Inflammatory and Painful Cases? An Evaluation with Regard to Biochemical and Reproductive Parameters [1]

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Abstract

The aim of this study was to investigate the usage of *Taraxacum officinale* extract (TOE) in inflammatory and painful cases as an alternative to paracetamol (PRC) through the assessment of biochemical and reproductive parameters. Totally, 30 male Sprague Dawley rats aged eight weeks old, were used in this study. The animals were obtained from Atatürk University Experimental Research and Application Centre and kept under standard laboratory conditions. Commercial pellet chow and fresh drinking water were available *ad libitum*. Rats were divided into five groups: Group I (n= 6); referred as control. Group II (n=6); referred as TOE150 (150 mg/kg). Group III (n=6); referred as TOE200 (200 mg/kg). Group IV (n=6); referred as TOE250 (250 mg/kg). Group V (n=6); referred as Paracetamol (PRC) (2 g/kg). The treatment was performed for consecutive 8 days. The animals were tranquilized and sacrificed on 9th day of study. Blood samples, cauda epididymal semen samples and testes tissues were collected. Routine semen examinations were performed and oxidative stress levels of testicular tissues were assayed. Reproductive organ weights [total testes weight (TTW) and total cauda epididymal weights (TCEW)] were recorded. Motility in TOE250 group was significantly higher when compared to the other groups (P<0.05). Velocity of sperm cells in PRC group was significantly lower when compared to the other groups (P<0.05). Dead sperm rate in control group was significantly higher when compared to the other groups (P<0.001). On the other hand, the lowest TCEW was in TOE150 group (P<0.05). There were no differences in terms of TTW among all groups. Malondialdehyde (MDA) level of PRC group was significantly higher than the treatment groups (P<0.05). Besides, glutathione peroxidase (GPx) levels of PRC group were lower than the other groups (P<0.001). Superoxide dismutase (SOD) level of PRC group was significantly lower than the treatment groups (P<0.001). The lowest catalase (CAT) level was in PRC group and the highest glutathione (GSH) level was in T200 group (P<0.001). In conclusion, it was observed that TOE could use as alternative of PRC and hence can be avoided from negative effects of PRC on biochemical and reproductive parameters.

Keywords: *Taraxacum officinale, Paracetamol, Reproduction, Rat, Biochemical parameters*

Karahindiba Ekstresi Yangılı ve Ağrılı Durumlarda Parasetamolün Alternatifi Olabilir mi? Biyokimyasal ve Reprodüktif Parametreler Yönünden Bir Değerlendirme

Özet

Bu çalışmanın amacı, biyokimyasal ve reprodüktif parametreleri değerlendirmek suretiyle karahindiba ekstresinin (*Taraxacum officinale extract, TOE*) parasetamole bir alternatif olarak yangılı ve ağrılı durumlarda kullanımını araştırmaktı. Çalışmada sekiz haftalık yaşta toplam 24 adet erkek Sprague Dawley rat kullanıldı. Hayvanlar Atatürk Üniversitesi Deneysel Araştırma ve Uygulama Merkezi'nden temin edildi ve standart laboratuvar koşullarında tutuldu. Ticari pelet yemi ve taze içme suyu *ad libitum* olarak verildi. Ratlar beş gruba bölündü: Grup I (n= 6) kontrol grubu olarak adlandırıldı. Group II (n=6) TOE150 (150 mg/kg) olarak adlandırıldı. Grup III (n=6) TOE200 (200 mg/kg) olarak adlandırıldı. Grup IV (n=6) TOE250 (250 mg/kg) olarak adlandırıldı. Grup V (n=6) parasetamol (PRC) (2 g/kg) olarak adlandırıldı. Tedavi ardışık 8 gün boyunca uygulandı. Çalışmanın 9. gününde hayvanlar trankilize edilerek kesildi. Kan örnekleri, kauda epididimal sperm örnekleri ve testis dokuları toplandı. Rutin sperma muayeneleri yapıldı ve testis dokularının oksidatif stres düzeyleri ölçüldü. Reprodüktif organ ağırlıkları [toplam testis ağırlığı (*TTW*) ve toplam kauda epididimis ağırlıkları (*TCEW*)] kaydedildi. Diğer gruplarla karşılaştırıldığında motilite TOE250 grubunda anlamlı olarak yüksekti (P<0.05). Diğer gruplarla karşılaştırıldığında velosite PRC grubunda anlamlı olarak düşüktü (P<0.05). Diğer gruplarla karşılaştırıldığında ölü sperm oranı kontrol grubunda anlamlı olarak yüksekti (P<0.001). Diğer taraftan en düşük total kauda epididimal ağırlık (*TCEW*), TOE150 grubundaydı (P<0.05). Bütün gruplar arasında total testis ağırlığı (*TTW*) açısından fark yoktu. Malondialdehit (MDA) düzeyi PRC grubunda tedavi gruplardan anlamlı olarak yüksekti (P<0.05). Bunun yanında, glutatyon peroksidaz (GPx) düzeyi PRC grubunda diğer gruplardan düşüktü (P<0.001). Superoksit dismutaz düzeyi (SOD) PRC grubunda tedavi gruplarına göre anlamlı olarak düşüktü (P<0.001). En düşük katalaz (CAT) düzeyi PRC grubunda ve en yüksek glutatyon (GSH) düzeyi T200 grubundaydı (P<0.001). Sonuç olarak, TOE'nin PRC'nin alternatifi olarak kullanılabileceği ve bu nedenle PRC'nin biyokimyasal ve reprodüktif parametreler üzerine olumsuz etkilerinden kaçınılabileceği gözlenmiştir.

Anahtar sözcükler: *Karahindiba, Parasetamol, Reprodüksiyon, Rat, Biyokimyasal parametreler*

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INTRODUCTION

In recent years, it is observed that the increase of comparative experimental studies in laboratory animals [1].

Paracetamol (acetaminophen or N-acetyl-p-amino phenol, PRC) is a mild analgesic, antipyretic agent and also a non-steroidal anti-inflammatory drug [2]. PRC is moderate effective drug when taken in appropriate doses and used against headache, fever, migraine, toothache, influenza infections, muscle and joint pain, middle ear pain, sinusitis, injuries and variety pains for long years [3].

Taraxacum officinale (dandelion) plant has phyto-chemical properties when analyzed as a whole in view of roots, leaves and flowers [4]. There are available studies showing TOE is a type of rich plant in view of vitamin and minerals [5]. It is an important plant in terms of A, B, C, D, vitamin E, as well as choline, inositol, lecithin, minerals and oligoelements (calcium, sodium, magnesium, iron, copper, phosphorus, zinc, manganese). In addition, dandelion, has the nutritional value, is one of the best natural sources of potassium, since it contains high levels of potassium [6,7]. TOE has been widely used as a folkloric medicine for its anti-inflammatory, anti-rheumatic, diuretic, hypolipidemic, anti-carcinogenic, antiulcer, antioxidant, antiviral, anti-allergic, anti-coagulant, anti-hyperglycemic, analgesic, prebiotic and chloretic properties. Furthermore, TOE has inhibiting effect on reactive oxygen species [8,9].

Male reproductive development is mainly regulated by testosterone, which stimulates the development of testes and reproductive tract at puberty and supports spermatogenesis and fertility in adulthood [10].

Motility is a characteristic function of mature sperm. Sperm motility is under the control of many biochemical factors such as mitochondrial function and enzyme activity [11]. Velocity expressing the position change depend on time and motion, is the primary determinant of sperm competition success [12]. The non-viable sperm term, is primarily related to the loss of integrity of the sperm plasma membrane, leading them to an irreversibly loss of homeostasis, immotility and death [13].

Free radicals are short-lived reactive atoms or molecules contain unpaired electrons in the outer orbitals. The most common radicals are hydrogen (H^+), superoxide (O_2^-), hydroxyl (^-OH), nitrogen oxide (NO) and nitrogen dioxide (NO_2). Free radicals can lead to gene mutation, aging and tissue-cell destruction caused by the molecular changes such as generated by normal metabolism. On the other hand, MDA acts on the ion-exchange through the cell membranes, leads to crosslinking of the compounds in the membrane and provokes the adverse consequences such as alteration of the ion permeability and the enzyme activity [14]. Besides, antioxidants try to prevent the free radicals and reactions [15]. There are various endogenous defense mechanisms against free radicals, such as the enzymes SOD, GSH, GPx and CAT, whose activities eliminate superoxide, hydrogen peroxide and hydroxyl radicals [16].

The aim of this study was to investigate the usage of TOE in inflammatory and painful cases as an alternative to PRC through the assessment of biochemical and reproductive parameters.

MATERIAL and METHODS

The approval of Committee for Institutional Animal Care and Use was provided from Ataturk University Local Board of Ethics (The approval number: 2013/132) before the study had been planned.

Plant Material

T. officinale L. samples were collected in September 2013 from Erzurum (Turkey) and identified by Saban KORDALI (Atatürk University, Faculty of Agriculture, Department of Plant Protection, Erzurum). A voucher specimen has been deposited in the Herbarium of Ataturk University, Erzurum (Turkey).

Preparation of the Samples

Plant materials were dried under shade and powdered coarsely before extraction. The dried *T. officinale* L samples were powdered in a blender and then 100 g of sample was extracted individually with 500 mL ethanol at room temperature. The extract was filtered and evaporated to dryness in a vacuum at 40°C with a rotary evaporator after 48 h. Filtration, the organic solvents were evaporated under reduced pressure and temperature. The dried extracts were stored at 4°C until used. The extract was dissolved in 0.5% aqueous carboxymethylcellulose (CMC) suspension in distilled water prior to intraperitonally administration to animals by using needle eight days [17].

PRC Material

2 g/kg of PRC was dissolved in 1% carboxymethyl cellulose (CMC), 1x Phosphate buffered saline (PBS) buffer [18].

Preparation of PBS Buffer

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 was weighed, pH adjusted to 7.4 in 980 mL and the solution was completed to 1 L.

Animals and Experimental Procedure

In the study, 24 male Sprague Dawley rats aged eight weeks old and weighted 250-300 gr, were used. The animals were obtained from Atatürk University Experimental and Application Research Centre and housed in standard laboratory conditions. Commercial pellet chow and fresh drinking water were available *ad libitum*. Rats were divided into five groups: Group I (n= 6); referred as control, physiolo-

gical saline was administered via intraperitonally (IP) route. Group II (n=6); referred as *Taraxacum officinale* extract (TOE) 150, 150 mg/kg TOE, was given IP. Group III (n=6); referred as TOE200, 200 mg/kg TOE was injected IP. Group IV (n=6); referred as TOE250, 250 mg/kg TOE was administered IP. Group V (n=6); referred as Paracetamol (PRC), paracetamol administered animals were fasted 24 h and 2 g/kg per os (p.o.). The treatment was performed for consecutive 8 days. The animals were tranquilized and sacrificed on 9th day of study.

Preparation of Plasma

The blood samples was transferred to the vacuum tubes with coagulant and anticoagulated. Plasma and serum were separated at 3000 rpm, +4°C during 10 min by centrifugation. Samples were stored at deepfreeze, -20°C until biochemical analyzes would made.

Collection of Samples

Following decapitation procedure, the testes and cauda epididymidis of the rats were removed from the corpse and cleaned from connective tissues such adipose or connective tissues with anatomical scissors and tweezers. Cauda epididymal semen samples and testes tissues were collected. Routine semen examinations were performed and oxidative stress levels of testicular tissues were assayed. Reproductive organ weights [total testes weight (TTW) and total cauda epididymides weights (TCEW)] were recorded.

Semen Evaluation

One of cauda epididymidis was used to obtain semen sample for each animal. For this purpose, randomly selected cauda epididymidis was minced in Petri dish including 5 mL of physiological saline. To provide the migrations of spermatozoa from cauda epididymidis to fluid, 5 min incubation period was obtained on warmed stage (at 35°C). Following the incubation period, cauda epididymidis residue was removed by using anatomical tweezers from the Petri dish. The fluid remaining in the Petri dish was used as semen sample. Evaluation of semen was conducted using routine spermatological parameters including motility, velocity and dead sperm rate.

To evaluate the percentage of sperm motility and velocity, light microscope (Primo Star; Carl Zeiss, Oberkochen, Germany) equipped with the heated stage was used. Briefly, a slide was placed on a heated stage warmed up to 35°C placed on a conventional light microscope. Approximately 20 µL of semen sample was dropped on the slide. The percentage of sperm motility was detected by visual investigation of the sample. To estimate the sperm motility, randomly selected three different fields from each sample were interpreted. The average of three field estimations was calculated as the final motility score of the sample [19,20]. To determine the sperm velocity, the distance of five head positions were measured and were divided into time spent for moving such distance. Velocity of approximately 50 to 60 motile sperm was measured for each individual [21].

Sperm viability was evaluated with light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan) after eosin nigrosin staining [22]. The smear was prepared for counting. A total of 200 cells were counted and the results are presented as percentages.

Biochemical Evaluations of Testicular Tissues

For assaying MDA, CAT, SOD levels and GSH activity homogenates were centrifuged for 15 min at 1000 g at +4°C while to assay the GPx activity of testicular homogenates were centrifuged for 20 min at 9000 g at +4°C. Following the centrifuge process, the obtained supernatant was subjected to enzyme assays as soon as possible. The homogenisation of testicular tissues was carried out in Teflon-glass homogenizer with a buffer containing 1.15% KCl to obtain1:10 (w/v) whole homogenate.

The malondialdehyde (MDA) level of testicular tissues was measured by the thiobarbituric acid reaction method of Placer et al.[23]. The values of MDA were expressed as nmol/g tissue. The CAT activity of testicular tissue was determined according to the method of Goth [24]. The values of CAT were expressed as kU/g protein. The SOD activity of testes was measured as the level of decrease in the absorbance at 560 nm and SOD values of testicular homogenates were expressed as EU/mg protein. To assay superoxide dismutase (SOD) activity of testicular tissues, the method of Sun et al.[25] was used. The GPx activity of testes was determined using the method of Matkovics et al.[26] method. The GPx activity of testicular homogenates was expressed as U/mg protein. The GSH content of testicular homogenates was determined at 412 nm according to the method; described by Ball [27], Fernandez and Videla [28]. GSH levels were expressed as mmol/g tissue. The protein content of the testicular tissues was measured according to the method described by Lowry et al.[29].

Statistical Analysis

Statistical comparisons of data were analysed using General Linear Model/Repeated Measures (SPSS, Version IBM 20.0 Microsoft, Chicago, IL, USA) in-group comparisons. Data were expressed as mean ± standard error of the mean (SEM). Differences were considered significant when $P<0.05$.

RESULTS

The results of the study are presented in two tables as reproductive and biochemical parameters. As it is seen in *Table 1*, motility value was significantly higher in TOE250 group when compared to the other groups ($P<0.05$).

Velocity of sperm cells in PRC group was significantly lower when compared to the other groups (P<0.05). Dead sperm rate in control group was significantly higher when compared to the other groups (P<0.001). On the other hand, the lowest TCEW was in TOE150 group (P<0.05). There were no differences in terms of TTW among all groups. According to our findings, it was found that the T250 group was more positive effective than the other groups in terms of reproductive parameters. In *Table 2*, malondialdehyde (MDA) level of PRC group was significantly higher than the other groups (P<0.05). Besides, glutathione peroxidase (GPx) and superoxide dismutase (SOD) levels of PRC group were significantly lower than the other groups (P<0.001). The lowest catalase (CAT) level was in PRC group and the highest glutathione (GSH) level was in T200 group (P<0.001). We observed that the T200 group was more favourable than the other groups in terms of biochemical parameters. Besides, it was determined that PRC has negative impact particularly in terms of biochemical parameters.

DISCUSSION

It is tried to determine the usage of *Taraxacum officinale* extract (TOE) in inflammatory and painful cases as an alternative to paracetamol (PRC) through the assessment of biochemical and reproductive parameters. We observed that the administration of TOE at doses of 250 mg/kg had beneficial effects upon the reproductive parameters and 200 mg/kg of TOE had positive effects upon the biochemical parameters in present study.

Reduction of antioxidant defense causes the ROS (reactive oxygen species) production and the formation of oxidative stress [30]. The increasing of ROS production stems from very intensely oxidative modification of enzymatic proteins [31].

There are limited literature data demonstrating the efficacy of *Taraxacum officinale* extract and PRC on reproductive parameters in rats. Due to restricted literature data, our findings generally were interpreted by comparison with biochemical parameters.

PRC is widely used anti-inflammatory, analgesic and in the group of non-prescription drug [32]. Whereas in overdose, PRC can be detrimental to various tissues [20,33-34]. High dose PRC transform into NAPQI (N-Acetyl-p-benzoquinone imine) - toxic metabolite by cytochrome p450 enzymes in the biological systems. This form leads to the oxidative stress, glutathione consumption and increased lipid peroxidation taking hydrogen from polyunsaturated fatty acids [35,36]. These reactive metabolites initiates cell stress through various mechanisms such as glutathione consumption or these metabolites can be connected to enzymes, lipids, nucleic acids and other cellular structures [37]. The cause of lipid peroxidation is a great aldehyde generating polyunsaturated fatty acids in the cell membrane and

Table 1. *The values (Mean ±SEM) of reproductive parameters in male rats*
Tablo 1. *Erkek ratların reprodüktif parametre değerleri (Ortalama± SEM)*

Groups	Motility	Velocity (0 -5 Intervals)	Dead Sperm Rate	Total Testis Weight - TTW (g)	Total Cauda Epididymal Weight -TCEW (g)
Control	45.22±0.83^{a}	3.46±0.18^{b}	42.30±0.53^{c}	2860.00±52.61	408.77±9.60^{b}
TOE150	47.90±1.58^{ab}	3.20±0.20^{b}	36.94±1.94^{ab}	2631.00±47.49	345.00±25.72^{a}
TOE2OO	45.58±1.42^{a}	3.00±0.00^{b}	39.40±1.33^{bc}	2768.40±64.01	408.20±16.30^{b}
TOE250	50.80±0.00^{b}	3.00±0.00^{b}	34.98±0.74^{a}	2814.88±52.73	417.75±12.32^{b}
PRC	46.16±2.17^{a}	2.20±0.20^{a}	37.64±2.99^{ab}	2894.60±66.98	408.00±14.86^{b}
P Value	*	*	***	--	*

*$^{a-c}$ The values represented by different letters within the same row are significantly different from each other; * P<0.05, *** P<0.001*

Table 2*. The values (Mean ±SEM) of biochemical parameters in male rats*
Tablo 2. *Erkek ratların biyokimyasal parametre değerleri (Ortalama± SEM)*

Groups	MDA (µmol/g)	GSH µmol/g	CAT (kU/g)	GPx U/mg	SOD (EU/mg)
CONTROL	38.10±2.65^{b}	0.79±0.02^{bc}	294.64 ±6.27^{bc}	0.05±0.01^{b}	9.01±0.127^{a}
TOE150	31.58±2.03^{a}	0.79±0.03^{bc}	273.89±17.42^{b}	0.07±0.01^{bc}	9.61±1.15^{b}
TOE2OO	29.66±0.80^{a}	0.83±0.01^{c}	316.71±1.63^{c}	0.07±0.01^{c}	10.63±0.07^{c}
TOE250	28.90±1.84^{a}	0.76±0.02^{b}	301.23±5.92^{c}	0.07±0.01^{bc}	10.52±1.14^{c}
PRC	49.75±1.50^{a}	0.56±0.04^{a}	215.29±20.63^{a}	0.04±0.01^{a}	8.70±1.14^{a}
P Value	*	***	***	***	***

*$^{a-c}$ The values represented by different letters within the same row are significantly different from each other; * P<0.05, *** P<0.001;* ***nmol/g****= Nanomole per gram testis tissue;* ***µmol/g****= mikromol/gram tissue;* ***kU/g****= Katalunite/gram tissue;* ***/mg****= Unite /miligram tissue;* ***EU/mg****= Enzymeunite/miligram*

results in the increasing of MDA level [38]. In PRC induced hepatotoxicity studies when toxication group in comparison with control group, it was observed significant increase in MDA level ($P<0.05$) in rats [39,40]. Aksu et al.[20] determined that the decreasing of GSH-px and SOD activity of testicular tissue in the PRC group when compared to the control group in rats. In present study, it is thought that the increasing in MDA level, the decreasing in GSH level, CAT, SOD and GPx activities arise from the harmful effect of PRC via NAPQI pathway.

PRC generated a reduction on sperm motility and count significantly but did not cause any pathological lesion in testis in a study in rats [41]. Aksu et al.[20] showed that PRC toxicity decreased the sperm motility. Similarly, we observed statistically significant reduction in terms of motility in the PRC group in comparison with TOE250 group in the present study. It is thought that the similar results stem from the mode of administration and toxic dose.

It might need to use anti-inflammatory and analgesic substances due to these harmful effects as alternative of PRC.

Natural antioxidants not only protect food lipids from oxidation but also provide a positive contribution on health precluding damage caused by biological degeneration [42]. Some studies clearly showed the antioxidant activity, anti-inflammatory effect and liver protection of *Taraxacum officinale* [43,44]. The antioxidant effects of TOE were determined to reduce the lipid peroxidation [45].

Astafieva et al.[46] specifed the characterization of ToHyp1 and ToHyp2 peptides from *Taraxacum officinale* flowers and exhibited antimicrobial activity of ToHyp2 against Gram-positive and Gram-negative bacteria.

There are numerous studies showing the negative effects of lipid peroxidation and bacterial contamination on reproductive parameters in biological systems [47-50].

It is thought that the mentioned physiological features of TOE can play an active role on the reproductive and biochemical parameters in present study.

Taraxacum officinale increased the antioxidant levels (glutathione and β-carotene) and the activity of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase) and significantly decreased the lipid peroxidation in mice [51]. It was observed the development in endogenous antioxidant profile via implementation of the roots and leaves of *Taraxacum officinale* in rats [52,53]. These findings support our results.

It can be emphasized that the antioxidant characteristic of TOE has an effect on the oxidative stress and the antioxidant parameters via suppression reactive oxygen species and prevention lipid oxidation [54].

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly known as a marker of oxidative stress and the antioxidant status in cancerous patients [55]. Sumanth and Rana [56] determined that alcohol extract of the *Taraxacum officinale* roots in dose of 100 mg/kg orally, significantly decreased the level of MDA compared to toxicity group ($P<0.01$). Furthermore it was observed that when toxicity group compared to the control group, the level of MDA significantly elevated ($P<0.001$). Similarly, we observed that the level of MDA significantly decreased in the groups of TOE administered (150, 200 and 250 mg/kg TOE). Besides, the level of MDA significantly increased in the group of PRC ($P<0.05$).

Glutathione (γ-glutamyl-ʟ-cysteinylglycine, GSH) is an acidic molecule characterized by a cysteine residue and a γ-linked amino acid which provides protection against hydrolysis by cellular proteases [57]. Parmar et al.[39] determined that the decrease in GSH level of the group of PRC toxicity in comparison with the control group in rats. In other study, Park et al.[58] showed that the significantly decrease ($P<0.05$) in GSH level of toxicity group compared to the control group and significantly increase ($P<0.05$) in GSH level of the water extract of *Taraxacum officinale* leaves compared to the toxicity group in rats. In addition, Sumanth and Rana [56] administered the alcohol extract of the *Taraxacum officinale* roots in two divided doses as 50 and 100 mg/kg orally,100 mg/kg dose of TOE significantly increased the level of GSH, compared to toxicity group ($P<0.001$), besides it was observed that any significance is not detected in 50 mg/kg dose. According to our results, the lower GSH level was in PRC group and the higher GSH level was in TOE200 group ($P<0.001$). In current study, it is thought that the administration of toxic dosage of PRC led to the oxidative stress, glutathione consumption and increased lipid peroxidation by NAPQI. TOE200 group has provided improving effect in GSH level thank to the antioxidant properties.

Glutathione peroxidase (GPx), an enzyme dependent on the micronutrient selenium (Se), plays a critical role in the reduction of lipid and hydrogen peroxides [59]. Sumanth and Rana [56] observed that 100 mg/kg dose of alcohol extract from *Taraxacum officinale* roots significantly increase the GPx level compared to the toxicity group ($P<0.001$). Besides, they determined the significantly decrease in GPx level of toxicity group in comparison with the control group ($P<0.001$). There is another study evidentally the increasing level of GPx by the using of hepatoprotective plants [60]. In other study, Cho et al.[61] determined that the significant increasing in the level of glutathione peroxidase of *Taraxacum officinale* group (aqueous extract of *Taraxacum officinale* leaves) in comparison with control group. Especially TOE200 group showed the higher level compared to toxicity (PRC) group in the level of GPx in our study

(P<0.001). This result implied that the TOE200 dosage reduce the lipid peroxidation.

Catalase (CAT) is an important antioxidant enzyme in organisms which could catalyze H_2O_2 to H_2O and O_2 to maintain the redox balance [62]. Sumanth and Rana [56] determined that 50 and 100 mg/kg dose of alcohol extract from *Taraxacum officinale* roots significantly increase the CAT level compared to the toxicity group (P<0.01, P<0.001; respectively). Cho et al.[61] applicated the water extract of *Taraxacum officinale* leaves to the rats, and observed the increasing of CAT level in TOE groups. In our study, although TOE150 group is lower compared to the control group in terms of CAT, TOE200 and TOE250 groups showed the improver effect in CAT level.

Superoxide dismutases catalyze the dismutation reaction of O^{2-} and transforming it into hydrogen peroxide and oxygen for defending against ROS [63,64]. Alcohol extract of the *Taraxacum officinale* roots in dose of 50 and 100 mg/kg, significantly increased the level of liver SOD compared to toxicity group in rats (P<0.01, P<0.001; respectively) [56]. The groups of T200 and T250 showed high protective effect in terms of SOD in the present study and demonstrated conformity to the above study despite the differences of tissues.

Tahtamouni et al.[65] carried out the aqueous extract of *Taraxacum officinale* orally to adult male rats at doses of 1065 and 2130 mg/kg body weight and recorded the motility values 33.9±7.8% and 30.7±7.8%, respectively. The motility value was 85.1±6.2%. There was significantly difference between treatment groups and control group (P≤0.0001). We observed that the motility value of TOE250 group was higher in comparison with the other groups except TOE150 group (P<0.05). In terms of velocity, we determined that the lower value in PRC group when compared to the other groups (P<0.05). Besides, TOE250 group has provided protection upon sperm cells by reducing the rate of dead sperm in the present study. The administration of the aqueous extract of *Taraxacum officinale* resulted in a significant decrease in testis weight in the two experimental groups (aqueous extract of *Taraxacum officinale* at doses of 1065 and 2130 mg/kg body weight, orally) in comparison to the control group in rats [65]. In the current study, although there is no significant difference, it was observed a decrease in the total testis weight in the TOE groups. Furthermore, TOE150 group showed significantly lower value in terms of total cauda epididymal weight in comparison with the other groups (P<0.05) in the present study.

Consequently, it was observed that the especially administration of TOE at doses of 250 mg/kg had beneficial effects upon the reproductive parameters and 200 mg/kg of TOE had positive effects upon the biochemical parameters studied. When considering the effects on the reproductive and biochemical parameters, in case of the inflammation and pain, it was concluded that TOE could use as alternative of PRC and therefore can be avoided negative effects of PRC on biochemical and reproductive parameters. It is thought that the determination of the effects of obtained extract upon the reproductive parameters will contribute to the literature.

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REFERENCES

1. Yarsan E, Durgut R: Farmakoloji ve toksikolojide rat modelleri. **In,** Yücel O (Ed): Küçük Deney Hayvanlarından Rat. 97-105. Matris Tanıtım Baskı Hizmetleri, Ankara - Türkiye, 2012.

2. Roberts LJ, Morrow JD, Hardman JG, Limbird LE, Gilman AG: Goodman & Gilman's the Pharmacological Basis of Therapeutics, 10th edn., McGraw-Hill, New York, 2001.

3. Süzer Ö: Farmakoloji Ders Notları. www.onersuzer.com, *Erişim tarihi:* 23.03.2016.

4. Schütz K, Carle R, Schieber A: Taraxacum - A review on its phytochemical and pharmacological profile. *J Ethnopharmacol*, 107, 313-323, 2006. DOI: 10.1016/j.jep.2006.07.021

5. Sigstedt SC, Hooten CJ, Callewaert MC, Jenkins AR, Romero AE, Pullin MJ, Kornienko A, Lowrey TK, Slambrouck SV, Steelant WF: Evaluation of aqueous extracts of *Taraxacum officinale* on growth and invasion of breast and prostate cancer cells international journal of oncology. *Int J Oncol,* 32, 1085-1090, 2008. DOI: 10.3892/ijo.32.5.1085

6. Gallaher RN, Gallaher K, Marshall AJ: Mineral analysis of ten types of commercially available tea. *J Food Compost Anal,* 19, 53-57, 2006. DOI: 10.1016/j.jfca.2006.02.006

7. Hu C, Kitts DD: Antioxidant, prooxidant, and cytotoxic activities of solvent-fractionated dandelion *(Taraxacum officinale)* flower extracts *in vitro. J Agric Food Chem*, 51, 301-310, 2003. DOI: 10.1021/jf0258858

8. Omur AD, Apaydın Yıldırım B, Kandemir FM, Akman O, Aktas Senocak E, Aksu EH: Investigation of efficacy of *Taraxacum officinale* extract on reproductive and biochemical parameters in male rats. *IInd International VETistanbul Group Congress,* 7-9 April 2015, Saint-Petersburg, Russia, 2015.

9. Bajaj YPS: Medicinal and Aromatic Plants, *Taraxacum officinale* Weber (Dandelion): *In Vitro* Culture, Micropropagation and Production of Volatile Metabolites. 356-369, 6th edn., Springer-Verlag, Berlin, Heidelberg, 1994.

10. Hong J, Chen F, Wang X, Bai Y, Zhou R, Li Y, Chen L: Exposure of preimplantation embryos to low-dose bisphenol A impairs testes development and suppresses histone acetylation of StAR promoter to reduce production of testosterone in mice. *Mol Cell Endocrinol*, 427, 101-111, 2016. DOI: 10.1016/j.mce.2016.03.009

11. Zhu X, Shi D, Li X, Gong W, Wu F, Guo X, Xiao H, Liu L, Zhou H: TLR signalling affects sperm mitochondrial function and motility via phosphatidylinositol 3-kinase and glycogen synthase kinase-3α. *Cell Signal,* 28, 148-156, 2016. DOI: 10.1016/j.cellsig.2015.12.002

12. Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA: Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. *Curr Biol,* 14, 44-47, 2004. DOI: 10.1016/j.cub.2003.12.028

13. Roca J, Parrilla I, Gil MA, Cuello C, Martinez EA, Rodriguez-Martinez H: Non-viable sperm in the ejaculate: Lethal escorts for contemporary viable sperm. *Anim Reprod Sci,* DOI: 10.1016/j.anireprosci.2016.02.028

14. Kalender S, Kalender Y, Öğütçü A, Uzunhisarcıklı M, Durak D, Açıkgöz F: Endosulfan-induced cardiotoxicity and free radical metabolism

in rats: The protective effect of vitamin E. *Toxicology*, 202, 227-235, 2002. DOI: 10.1016/j.tox.2004.05.010

15. Dündar Y, Aslan R: Hekimlikte Oksidatif Stres ve Antioksidanlar. 29-95, Afyon Kocatepe Üniv. Yayınları, Afyon, 2000.

16. Soto C, Recoba R, Barron H, Alvarez C, Favari L: Silymarin increases antioxidant enzymes in alloxan-induced diabetes in rat pancreas. *Comp Biochem Physiol C Toxicol Pharmacol,* 136, 205-212, 2003. DOI: 10.1016/S1532-0456(03)00214-X

17. Kesdek M, Kordali S, Coban K, Usanmaz A, Ercisli S: Larvicidal effect of some plant extracts on the pine processionary moth, *Thaumetopoea pityocampa* (Denis & Schiffermuller) in laboratory conditions. *Acta Sci Pol Hortorum Cultus*, 13, 145-162, 2014.

18. Albayrak A: Serotonin-7 reseptörlerinin parasetamol ile indüklenen deneysel karaciğer toksisistesindeki yeri ve önemi. Türk Farmakoloji Derneği Farmakoloji Eğitiminde Kuşaklararası Bilimsel Etkileşme Seminerleri Programı, Bursa, 2013.

19. Turk G, Atessahin A, Sonmez M, Ceribasi AO, Yuce A: Improvement of cisplatin - induced injuries to sperm quality, the oxidant–antioxidant system, and the histologic structure of the rat testis by ellagic acid. *Fertil Steril,* 89, 1474-81, 2008. DOI: 10.1016/j.fertnstert.2007.04.059

20. Aksu EH, Ozkaraca M, Kandemir FM, Omur AD, Eldutar E, Kucukler S, Comaklı S: Mitigation of paracetamol-induced reproductive damage by chrysin in male rats via reducing oxidative stress. *Andrologia*, 2016 (In press). DOI: 10.1111/and.12553

21. Alavi SMH, Drozd B, Hatef A, Flajšhans M: Sperm morphology, motility, and velocity in naturally occurring polyploid European weatherfish *(Misgurnus fossilis* L.) *Theriogenology,* 80, 153-160, 2013. DOI: 10.1016/j.theriogenology.2013.04.009

22. Akyol N, Azkur AK, Ekici H: Comparison of flow cytometric analysis and eosin-nigrosin staining methods for determining some morphological characteristics of bull epididymal spermatozoa. *Kafkas Univ Vet Fak Derg,* 21, 659-664, 2015. DOI: 10.9775/kvfd.2015.13008

23. Placer ZA, Cushman LL, Johnson BC: Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Anal Biochem,* 16, 359-64, 1966. DOI: 10.1016/0003-2697(66)90167-9

24. Goth L: A simple method for determenation of serum catalase activity and revision of reference range. *Clin Chim Acta,* 196, 143-152, 1991. DOI: 10.1016/0009-8981(91)90067-M

25. Sun Y, Oberley LW, Li Y: A simple method for clinical assay of superoxide dismutase. *Clin Chem*, 34, 497-500, 1988.

26. Matkovics B, Szabó L, Sz Varga I: Determination of enzyme activities in lipid peroxidation and glutathione pathways. *Laboratóriumi Diagnosztika*, 15, 248-250, 1988 (in Hungarian).

27. Ball CR: Estimation and identification of thiols in rat spleen after cysteine or glutathione treatment: Relevance to protection against nitrojen mustards. *Biochem Pharmac,* 15, 809-816, 1966. DOI: 10.1016/0006-2952(66)90157-2

28. Fernandez V, Videla LA: Effect of acute and chronic ethanol ingestion on the content of reduced glutathione of varios tissues of the rat. *Experientia,* 37, 392-394, 1981. DOI: 10.1007/BF01959881

29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with folin reagent. *J Biol Chem,* 193, 265-275, 1951.

30. Parola M, Robino G: Oxidative stress-related molecules and liver fibrosis. *J Hepatol*, 35, 297-306, 2001. DOI: 10.1016/S0168-8278(01)00142-8

31. Gonzalez-Castejon M, Visioli F, Rodriguez-Casado A: Diverse biological activities of dandelion. *Nutr Rev,* 70, 534-547,2012. DOI: 10.1111/j.1753-4887.2012.00509.x

32. Anderson BJ, Holford NHG, Woollard GA, Chan PLS: Paracetamol plasma and cerebrospinal fluid pharmacokinetics in children. *Br J Clin Pharmacol*, 46, 237-243, 1998. DOI: 10.1046/j.1365-2125.1998.00780.x

33. El-Shafey MM, Abd-Allah GM, Mohamadin AM, Harisa GI, Mariee AD: Quercetin protects against acetaminophen-induced hepatorenal toxicity by reducing reactive oxygen and nitrogen species. *Pathophysiology,* 22, 49-55, 2015. DOI: 10.1016/j.pathophys.2014.12.002

34. Ratnasooriya WD, Jayakody JR: Long-term administration of large doses of paracetamol impairs the reproductive competence of male rats. *Asian J Androl,* 2, 247-255, 2000.

35. Vermeulen NPE, Bessems JGM, Vandestreat R: Molecular aspects of paracetamol induced hepatotoxicity and it mechanism based prevention. *Drug Metab Rev*, 24, 367-407, 1992. DOI: 10.3109/03602539208996298

36. Grypioti AD: Liver oxidant stress induced by paracetamol overdose. *Int J Pharmacol,* 4, 7, 2006.

37. Pauli-Magnus C, Stieger B, Meier Y, Kullak-Ublick GA, Meier PJ: Enterohepatic transport of bile salts and genetics of cholestasis. *J Hepatol,* 43, 342-357, 2005. DOI: 10.1016/j.jhep.2005.03.017

38. Slater RF: Free radicals and tissue injury: Fact and fiction. *Br J Cancer Suppl,* 8, 5-10, 1987.

39. Parmar SR, Vashrambhai PH, Kalia K: Hepatoprotective activity of some plants extract against paracetamol induced hepatotoxicity in rats. *J Herb Med Toxicol,* 4, 101-106, 2010.

40. Sundaria K, Karthik D, Ilavenil S, Kaleeswaran B, Srigopalramd S, Ravikumar S: Hepatoprotective and proteomic mechanism of Sphaeranthus indicus in paracetamol induced hepatotoxicity in wistar rats. *Food Bioscience*, 1, 57-65, 2013. DOI: 10.1016/j.fbio.2013.03.004

41. Oyedeji KO, Bolarinwa AF, Ojeniran SS: Effect of paracetamol (acetaminophen) on haematological and reproductive parameters in male albino rats. *IOSR-JPBS,* 4, 65-70, 2013.

42. Davies KJA: Oxidative Stress: The Paradox of Aerobic Life. **In,** Rice-Evans C, Halliwell B, Lunt GG (Eds): Free Radical and Oxidative Stress: Environments, Drugs and Food Additives. 1-31, Portland Press, Great Britain, 1995.

43. Hudec J, Burdová M, Kobida L, Komora L, Macho V, Kogan G, Turianica I, Kochanová R, Lozek O, Habán M, Chlebo P: Antioxidant capacity changes and phenolic profile of *Echinacea purpurea,* nettle *(Urtica dioica* L.), and dandelion *(Taraxacum officinale)* after application of polyamine and phenolic biosynthesis regulators. *J Agric Food Chem,* 55, 5689-5696, 2007. DOI: 10.1021/jf070777c

44. Fallah HH, Zaree MA, Naghdi BH, Alavian SM, Mohammadi SR, Mehdizadeh M: The protective effect of medicinal herbs extracts including *Cynara scolymus* L., *Cichorium intybus* L., *Taraxacum officinale* L. and *Berberis vulgaris* L. in single and in combination form in CCl_4 induced rat liver toxicity. *J Med Plants,* 41, 78-85, 2012.

45. Popovic M, Kaurinovic B, Mimica-Dukic N, Vojinovic-Miloradov, Dordevic M: Combined effects of plant extracts and xenobioticson liposomal lipid peroxidation. Part 3. Dandelion extract-CCl_4/fullnerol. *Oxid Commun,* 24, 335-343, 2001.

46. Astafieva AA, Enyenihi AA, Rogozhin EA, Kozlov SA, Grishin EV, Odintsova TI, Zubarev, RA, Egorov TA: Novel proline-hydroxyproline glycopeptides from the dandelion *(Taraxacum officinale* Wigg.) flowers: De novo sequencing and biological activity. *Plant Science*, 238, 323-329, 2015. DOI: 10.1016/j.plantsci.2015.07.002

47. Molina I, Mari M, Martínez JV, Novella-Maestre E, Pellicer N, Pemán J: Bacterial and fungal contamination risks in human oocyte and embryo cryopreservation: Open versus closed vitrification systems. *Fertil Steril*, 2016 (In Press). DOI: 10.1016/j.fertnstert.2016.03.024

48. Mohamed HM, Mohamed MA: Effect of different doses of nandrolone decanoate on lipid peroxidation, DNA fragmentation, sperm abnormality and histopathology of testes of male Wister rats. *Exp Toxicol Pathol,* 67, 1-11, 2015. DOI: 10.1016/j.etp.2014.09.003

49. Sangeeta S, Arangasamy A, Kulkarni S, Selvaraju S: Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim Reprod Sci,* 161, 82-88, 2015. DOI: 10.1016/j.anireprosci.2015.08.008

50. Boonthai T, Khaopong W, Sangsong J, Sooksawat T, Nimrat S, Vuthiphandchai V: Semen collection methods affect the bacterial composition of post-thawed semen of silver barb *(Barbodes gonionotus). Anim Reprod Sci,* 166, 90-98, 2016. DOI: 10.1016/j.anireprosci.2016.01.007

51. Kim MY, Cheong SH, Kim MH, Son C, Yook HS, Sok DE, Cho JHK, ChunH, Kim MR: Leafy vegetable mix supplementation improves lipid profiles and antioxidant status in C57BL/6J mice fed a high fat and high cholesterol diet. *J Med Food*, 12, 877-884, 2009. DOI: 10.1089/

jmf.2008.1125

52. Champe PC, Harvey RA, Ferrier DR: Lippincott's Illustrated Reviews: Biochemistry. Çeviri: Ulukaya E, Biyokimya. 3rd edn., Nobel, İstanbul, 2007.

53. Kuntz E, Kuntz HD: Hepatology Textbook and Atlas. 3rd edn., Springer Press, Germany, 2008.

54. Hu C, Kitts DD: Dandelion *(Taraxacum officinale)* flower extract suppresses both reactive oxygen species and nitric oxide and prevents lipid oxidation *in vitro*. *Phytomedicine,* 12, 588-597, 2005. DOI: 10.1016/j.phymed.2003.12.012

55. Gaweł S, Wardas M, Niedworok E, Wardas P: Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiad Lek,* 57, 453-455, 2004.

56. Sumanth M, Rana AC: *In vivo* antioxidant activity of hydroalcoholic extract of *Taraxacum officinale* roots in rats. *Indian J Pharmacol,* 38, 54-55, 2006.

57. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND: Glutathione metabolism and its implications for health. *J Nutr,* 134, 489-492, 2004.

58. Park CM, Cha YS, Youn HJ, Cho CW, Song YS: Amelioration of oxidative stress by dandelion extract through CYP2E1 suppression against acute liver injury induced by carbon tetrachloride in sprague-dawley rats. *Phytother Res,* 24, 1347-1353, 2010. DOI: 10.1002/ptr.3121

59. Arthur JR: The glutathione peroxidases. *Cell Mol Life Sci,* 57, 1825-1835, 2000. DOI: 10.1007/PL00000664

60. Hassan HA, Yousef MI: Ameliorating effect of chicory *(Cichorium intybus* L.) -supplemented diet against nitrosamine precursors-induced liver injury and oxidative stress in male rats. *Food Chem Toxicol*, 48, 2163-2169, 2010.

61. Cho SY, Oh YJ, Park JY: Effect of dandelion *(Taraxacum officinale)* leaf extracts on hepatic antioxidative system in rats fed high cholesterol diet. *J Korean Soc Food Sci Nutr*, 2, 458-463, 2003.

62. Lushchak VI: Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol*, 101, 13-30, 2011. DOI: 10.1016/j.aquatox.2010.10.006

63. Zelko IN, Mariani TJ, Folz RJ: Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med*, 33, 337-349, 2002.

64. He JY, Chi CF, Liu HH: Identification and analysis of an intracellular Cu/Zn superoxide dismutase from *Sepiella maindroni* under stress of *Vibrio harveyi* and Cd^{2+}. *Dev Comp Immunol*, 47, 1-5, 2014. DOI: 10.1016/j.dci.2014.06.010

65. Tahtamouni LH, Alqurna NM, Al-Hudhuda MY, Al-Hajj HA: Dandelion *(Taraxacum officinale)* decreases male rat fertility *in vivo*. *J Ethnopharmacol,* 135, 102-109, 2011. DOI: 10.1016/j.jep.2011.02.027

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Research Article

Total Sialic Acid, Oxidative Stress and Histopathological Changes in Rainbow Trout Saprolegniasis *(Oncorhynchus mykiss)*

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Abstract

Saprolegniasis is known as one of the most important fungal diseases of salmonids along with high mortality and economic problems. One hundred and seven fish suffering from cutaneous *Saprolegnia* infections and the same number of healthy fish were selected and blood parameters along with histopathology assay were performed in all ones. The results indicated a significant increase (P≤0.01) in total sialic acid, malondialdehyde, urea, creatinine, aspartate aminotransferase, total protein in plasma and a decrease in glucose, catalase, glutathione peroxidase and paraoxonase. Meanwhile, no significant alterations of alanine aminotransferase and superoxide dismutase were revealed in infected fish. Also, the histopathological findings observed in liver, especially glycogen storage and fatty inclusion and melanomacrophage centres in spleen. Tubular vascular degeneration along with cystic formation was identified in kidney. The results suggest that saprolegniasis develops substantial histopathological and blood profile changes in rainbow trout and recommend to pay more attention on some biochemical profiles such as MDA and TSA, due to cell health and defence against fungus on the skin respectively, along with hepatocyte function index (aspartate aminotransferase) and nitrogen metabolism (creatinine and urea) during disease management.

Keywords: *Saprolegniasis, Blood parameters, Histopathology, Rainbow trout*

Saprolegniyazis`li Gökkuşağı Alabalıklarında *(Oncorhynchus mykiss)*, Toplam Siyalik Asit, Oksidatif Stres ve Histopatolojik Değişiklikler

Özet

Saprolegniya enfeksiyonu salmonid balıklarda yüksek mortalite ve ekonomik sorunlarla birlikte en önemli mantar hastalıklardan biri olarak bilinir. Yüz yedi adet Saprolegniya enfekte ve aynı sayıda sağlıklı balıklar seçilerek, histopatolojik analizler`ile birlikte bazı kan parametreleri incelendi. Plazma toplam siyalik asit, malondialdehit, üre, kreatinin, aspartat aminotransferaz ve toplam protein düzeylerinde artış (P≤0.01) görülürken, glukoz miktarı miktarı`ile, katalaz, glutatiyon peroksidaz ve paraoksonaz aktivitesinde ise azalma (P≤0.01) görüldü. Alanin aminotransferaz ve süperoksit dismutaz aktivitesinde anlamlı bir değişiklik görülmedi. Ayrıca, karaciğerde gözlenen histopatolojik bulgularda, glikojen depolanması ve yağ içerme ve dalakta melanomakrofaj merkezleri görüldü. Böbrek`de kistik oluşum`ile birlikte tubular vacuolar dejenerasyonu tespit edildi. Sonuçlar gökkuşağı alabalığında önemli histopatolojik ve kan profil değişikliklerini sebep olduğunu gösteriyor ki bu arada MDA ve TSA tertible hücre sağlığı ve deride oluşan mantara karşı savunmada özel önem taşıyorlar. Ayrıca hepatosit fonksiyon indeksi (aspartat aminotransferaz) ve azot metabolizması (kreatinin ve üre) gibi bazı biyokimyasal parametrelerin hastalık yönetiminde önemli rolları olabileceği düşünüldü.

Anahtar sözcükler: *Saprolegniasis, Kan parametreleri, Histopatoloji, Gökkuşağı alabalık*

INTRODUCTION

Fungal diseases involve severe management problems in aquaculture farms and saprolegniasis is known as one of the main types salmonids diseases [1,2]. Saprolegniasis in addition to salmonid fish, has been also reported in crayfish *(Astacus leptodactylus)* [3]. *Saprolegnia* as major genus of water molds of Oomycete class possesses opportunistic, saprotrophic and necrotrophic traits. Naturally, it is found in fresh water ecosystems and environmental factors such as low whirlpool, low soluble oxygen, stress and over-crowding facilitate saprolegniasis occurrence in aquaculture

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farms along with mortality in fresh water fish [4]. Acetylated derivative of neuraminic acid is sialic acid (SA) that is extensively dispensed in entirely vertebrate tissues, body fluids and in higher invertebrate [5,6]. It is worth mentioning that SA indwells the terminal location on macromolecules, cell membranes and it is known as an inflammatory marker [7-9]. Therefore, the determination of SA may be a valuable indicator for diagnosis and prognosis of inflammatory diseases. Also, evidences reveal the changes of serum SA in different diseases [5,10,11]. However, assay has not been observed about SA levels in fungal fish diseases such as saprolegniasis.

Free Radicals (FRs) such as superoxide anion, hydroxyl radical, as well as non-radical molecules like hydrogen peroxide are generated during normal metabolic processes into the cells and oxidative stress is acquired due to either excess production of FRs or shortage in antioxidant enzymes such as SOD, CAT, and GSH-Px [12-14]. Free radicals devastate polyunsaturated fatty acids of cell membrane lipids and causes lipid peroxidation which is used as determinant of oxidative stress and cellular injury indicator [15,16]. Malondialdehyde (MDA), one of the lipid peroxidation by-products, is considered the most abundant and reliable biomarker either in assessment of lipid peroxidation or indirect detection of FRs levels [17-19]. MDA is merged with cell membrane and leads to intracellular damage, cross-linking with phospholipid and enzymes [20,21]. Consequently, overwhelming of antioxidant defence is believed to be the trigger of broad difference processes on various organs which are conducive to diseases and tissue damage [22,23]. Determination of antioxidant enzymes in fish diseases has been performed in various studies [23-25] and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px have been determined in most fish species [23,26]. Recently, one of the other antioxidant enzymes which has been paid more attention, is PON. It synthesized in liver and liberated into blood and belongs to esteric hydrolases that hydrolysis xenobiotics such as organophosphates. Furthermore, PON alleviate oxidative stress in tissues and cells and its activity has been reported in fish [27,28].

To our knowledge, this is first study to evaluate alterations of some blood parameters such as sialic acid (as inflammatory marker), oxidative stress indices and other parameters along with histopathology in *Oncorhynchus mykiss* saprolegniasis and likely some of them be effective in the management of saprolegniasis in Rainbow trout.

MATERIAL and METHODS

Examination of Saprolegniasis

This study was conducted in Urmia city during 2014-2015, Iran. Fish with lack of any internal and external parasites along with lack of bacterial infection which examined with clinical signs were selected for the study. Water quality consists of normal conditions such as water temperature 11°C heat low C, pH 7.2, oxygen concentration 6.8 mg/L. Three hundred and fifty-eight rainbow trout were assessed from eight aquaculture farms of Urmia and among them, one hundred and seven saprolegniasis rainbow trout without UDN (ulcerative dermal necrosis) disease symptoms were detected by white or gray patches on the head and fin with cotton-like appearance and were diagnosed by GYPS agar plates (Glucose-Yeast-Peptone agar) which is specific for *Saprolegnia* culture [29]. Also, the same number fish that were examined for lack of any saprolegniasis and other diseases (parasitic, viral and bacterial) signs were selected as the healthy group. This study was confirmed with the ethical approval (No: 0552762948126) in the Ethics Committee of Urmia Islamic Azad University.

Preparation of Blood Samples

Anesthesia was not performed on the fish because it can induce adverse effects on blood parameters [30]. Blood samples were collected from all fish via caudal vein into EDTA-contained tubes for hematologic tests and plasma preparation. Thereafter, all tubes were centrifuged with 4000 RPM for 10 min at room temperature.

Hematology and Plasma Chemistry

Standard micro-hematocrit method was carried out for packed cell volume (PCV) determination. Hemoglobin (Hb) measurement was determined according to cyano-methemoglobin method with spectrophotometer at 540 nm absorbance. White blood cells (WBC) and red blood cells (RBC) count were carried out with Dacie`s solution as a diluting fluid [31]. Determination of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) as the erythrocyte indexes were detected by Haney et al.[32] method. Moreover, blood smears were stained with Giemsa solution 5% for differential count of leukocyte and blood parasites examination and specification of each cell percentage were counted in one hundred cells. TSA was determined by Sydow [33] method (spectrophotometric, Spekol-1500). SOD, CAT, GPx activities were assessed in erythrocyte hemolysis on the basis of manual methods, Abei [34] for CAT and commercial kits (Randox laboratories Ltd. G.B) for SOD, GSH-Px and total antioxidant capacity (TAC), (Auto analyzer, Alcyon-300, USA) as well as, GL, Urea, CREA, TPP, AST, ALT detected by commercial kits (Pars azmoon., Chemical co., Tehran, Iran) with Auto analyzer, Hitachi- 917, Japan. Finally, MDA levels and PON activity was detected by Satoh [35] and Furlong [36] method respectively (spectrophotometer, model Cecil, Italy).

Histopathological Examination

Tissue specimens of healthy and infected fish from liver, spleen and kidney were taken and rapidly fixed in neutral buffered formalin 10%. Thereafter, conventional paraffin wax embedding technique was performed in

fixed specimens. Then, the sections were cut 5 microns in thickness and were stained by Hematoxyline and Eosin (H&E) and Periodic Acid Schiff (PAS) staining methods.

Statistical Analysis

Statistical analysis was accomplished in all analyses. Mean ± SD and determination of variation between the data results were carried out with Student's *t*-test with SAS version 9.1 and significance level was specified at (P<0.01).

RESULTS

Saprolegniasis causes significant alterations in the majority of plasma biochemical profiles of infected fish compared to healthy ones (P≤0.01), which was documented in *Table 1*. Moreover, hematological values assessment was revealed in *Table 2*. According to the hematological results, HCT, Hb amount and RBC count decreased (P≤0.01). Neutrophilia, lymphopenia, monocytosis and eosinophilia occurred in saprolegniasis group than healthy ones. In respect of nitrogen metabolism, an increase in Urea and CREA and TPP was observed. In comparison with healthy ones, marked decrease of GL was exhibited in diseased fish. ALT and AST concentration as the liver function indices were different, there was significant elevation in AST with no remarkable alterations in ALT. High levels of TSA and MDA were determined in infected ones than healthy group and concentration of antioxidant enzymes considerably reduced. In the case of SOD, there are no significant alterations were detected.

Histopathologic results of this present study show that cutaneous saprolegniasis in Rainbow trout causes three lesions in different tissues. These are as follows: Liver: focal necrosis, edema, the increase of melanomacrophage centers, fatty change and glycogen storage state *(Fig. 1, Fig. 2)*. Spleen: the increase of melanomacrophage centers *(Fig. 2)*. Kidney: Tubular vacuolar degeneration, glomerular atrophy and partly increase of melanomacrophage centers *(Fig. 3)*.

Table 1. *The following table reveals all parameter alterations in patient compared with control group*

Tablo 1. *Aşağıdaki tablo, tüm parametre değişikliklerini hasta grubun kontrol grubu ile karşılaştırıldığında ortaya çıkarır*

Parameters	Control Group	Patient Group
TSA (mg/dl)	39.26±4.03	57.63±3.21†
MDA (nmol/ml)	6.49±1.417	8.84±0.922†
PON (U/L)	79.72±22.585	42.64±10.164†
SOD (U/gHb)	1569.66±214.719	1789.31±769.025
CAT (k/gHb)	102.70±13.547	61.85±8.102†
GSH-Px (U/mgHb)	98.35±11.086	53.48±9.294†
TAC (mmol/L)	0.86±0.7	0.47±0.11†
GL (mg/dl)	78.24±5.861	56.92±5.943†
CREA (mg/dl)	1.172±0.328	3.01±0.296†
UREA (mg/dl)	6.32±1.573	12.6±2.236†
TPP (g/dl)	5.03±0.367	5.85±0.506†
AST (U/l)	179.64±55.3164	247.32±21.7595†
ALT (U/l)	56.92±19.7081	68.56±18.6303

Data are expressed as mean ± standard deviation; † Significantly different from the control group (P<0.01)

Table 2. *The following table denotes hematological alterations in saprolegniasis group compared with healthy ones*

Tablo 2. *Aşağıdaki tablo, hematolojik değişiklikleri Saprolegniasis grubunda sağlıklı olanlarla karşılaştırıldığında gösterir*

Parameters	Control Group	Patient Group
PCV	42.68±3.14	30.8±2.34†
Hb	9.24±0.73	6.25±0.84†
RBC	3.09±0.46	1.26±0.15†
MCV	289.99±41.15	277.04±17.79
MCH	66.76±7.65	67.12±3.86
NEU	18.48±2.16	30.80±2.04†
LYM	69.68±1.84	55.44±5.23†
MONO	2.60±1.41	6.36±1.91†
EOS	5.48±1.29	10.92± 4.15†

Data are expressed as mean ± standard deviation; † Significantly different from the control group (P<0.01)

DISCUSSION

Sialic acid (SA) is known as a valuable marker for diagnosis and prognosis of inflammatory diseases. Motoi et al.[37]. In this study, SA levels increased in infected group than non-infected ones. SA concentrations have been specified high during the course of many diseases. Citil et al.[11]. We could not find any published studies about SA levels in rainbow trout saprolegniasis. In these circumstances, rise in TSA may be attributed to acute phase proteins elevation, because acute phase proteins, such as α1-acid glycoprotein, are sialated glycoproteins [37]. The another probable reason can be ascribed to inter-relationship between SA and innate immune system of skin mucous. Fish skin mucous is involved as first line of defence against pathogens [38]. The multiple components of innate immune system are aggregated in fish skin mucous, such as glycoproteins, lysozyme, immunoglobulin, anti-microbial peptides, lectins, C-reactive proteins [39]. Besides, skin mucous secretions contain sialic acid in various fish species and sialated glycoproteins constitute in skin mucous ingredients [40]. They involve in restrain and protection against bacterial break down and viral invasion [41]. Since SA is firmly bounded to bacterial macromolecules and bacteria, thereby impede the adherence of pathogen agents to epithelial cells [42]. It is likely that sialic acid of skin mucous interacts with *saprolegnia spp* for hindering its extension. Therefore, SA increases in blood for compensation of SA consumption in skin mucous.

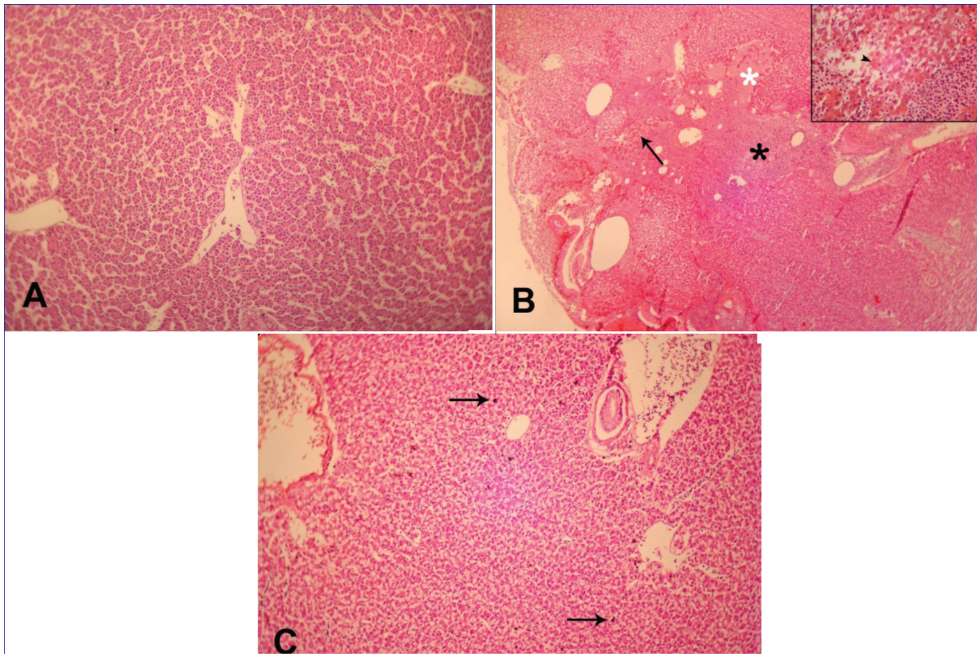

Fig 1. A. Control group: Hepatic tissue is normal and clear vacuoles in hepatocytes are very little, (H&E), X200; **B.** Cutaneous saprolegniasis group: (Main figure): Focal edema with serosal fluids *(white asterisk)* in liver, hemorrhage and focal infiltration of lymphocytic cells *(black asterisk)*, local hepatic necrosis *(arrow)*, (H&E), X80. (Inner figure): Necrotic hepatocytes containing eosinophilic cytoplasm and destructed nuclei *(arrowhead)*, (H&E), X800; **C.** Cutaneous saprolegniasis group: Increase of clear vacuoles in most of hepatic cells is seen. Dispersion of Melanomacrophage centers has increased in hepatic tissue *(arrow)*, (H&E), X200

Şekil 1. A. Kontrol grup: Karaciğer dokusu normaldir ve hepatositlerde net vakuoller çok az vardır, (H&E), X200; **B.** Deri Saprolegniasis grubu: (Ana Şekil): Karaciğerde serozal sıvıları ile fokal edema *(beyaz yıldız)*, kanama ve lenfositik hücre odak infiltrasyonu *(siyah yıldız)*, lokal hepatik nekroz *(ok)*, (H&E), X80. (iç şekil): Nekrotik hepatositler ki içinde eozinofilik sitoplazma ve tahrip olmuş çekirdek var *(okbaşı)*, (H&E), X800; **C.** Deri Saprolegniasis grubu: Karaciğer hücrelerinin çoğunda açık vakuoller artışı görünür. Melanomacrophage merkezlerinin dispersiyonu karaciğer dokusunda artmıştır *(ok)*, (H&E), X200

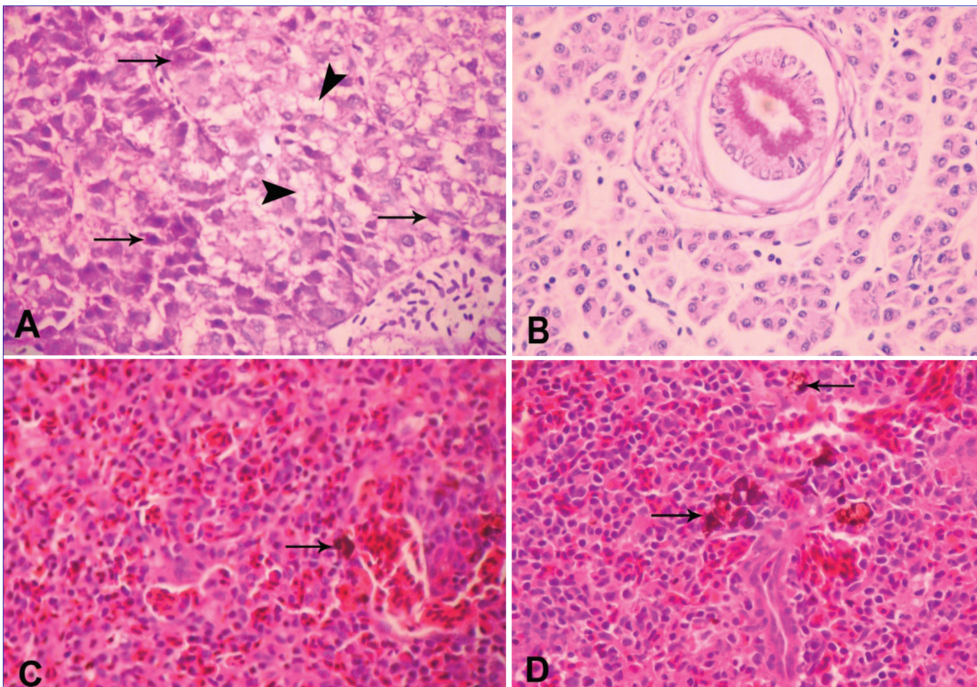

Fig 2. A. Cutaneous saprolegniasis group: Focal fatty change *(arrowhead)* and cytoplasmic aggregations of glycogen in hepatocytes *(arrow)*, (PAS), X960; **B.** Control group: Normal appearance of hepatic tissue, (PAS), X960; **C.** Control group: Normal splenic tissue containing few melanomacrophege centers *(arrow)*, (H&E), X960; **D.** Cutaneous saprolegniasis group: Hyper activation and increase of melanomacrophage centers *(arrow)* in spleen, (H&E), X960

Şekil 2. A. Deri Saprolegniasis grubu: Fokal yağlı değişim ve hepatositlerin sitoplazmasında glikojen toplamaları *(ok)*, (PAS), X960; **B.** Kontrol grubu: Karaciğer dokusunun normal görünümü, (PAS), X960; **C.** Kontrol grubu: Normal dalak dokusu içeren birkaç melanomakrofaj merkezleri *(ok)*, (H&E), X960; **D.** Deri Saprolegniasis grubu: Hiperaktivasyon ve dalakta melanomacrophage merkezlerinin artışı *(ok)*, (H&E), X960

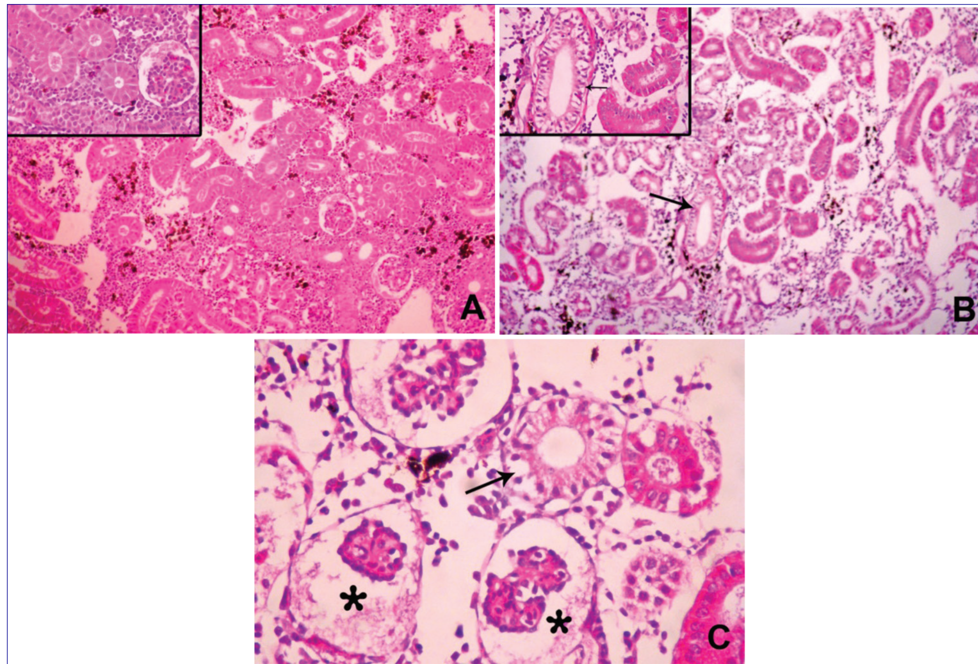

Fig 3. A. control group: (Main figure): Normal appearance of renal glomeruli and tubules, (H&E), X240. (Inner figure): Higher magnification of normal kidney, (H&E), X960; **B.** Cutaneous saprolegniasis group: Vacuolar degeneration in some of renal tubules *(arrow)*, (Main figure): (H&E), X240. (Inner figure): (H&E), X960. **C.** Cutaneous saprolegniasis group: Glomerular atrophy and cystic formation *(asterisk)* and tubular vacuolar degeneration *(arrow)*. (H&E), X960

Şekil 3. A. Kontrol grubu: (Ana şekil): Böbrek glomerül ve tübülün normal görünümü, (H&E), X240. (iç şekil): Normal böbrek üstü büyütme. (H&E), X960, **B.** Deri Saprolegniasis grubu: Vakuolar dejenerasyon bazı böbrek tübüllerinde *(ok)*, (Ana şekil): (H&E), X240. (iç şekil): (H&E), X960; **C.** Deri Saprolegniasis grubu: Glomerüler atrofi ve kistik oluşumu *(yıldız)* ve tübülar vakuolar dejenerasyonu *(ok)*, (H&E), X960

Enhancement of Urea and Crea in *oncorhynchus mykiss* have been revealed in viral haemorrhagic septicemia (VHS) [43] and after seven-day post-infection of *Tilapia nilotica* with *saprolegnia parasitica* [4] which may be attributed to kidney insufficiency due to the infestation of *Saprolegnia* hyphae. Versus, Rehulka [43] reported Crea decrease in Atlantic salmon vibriosis. In the present study, high nitrogen metabolism values (Urea and Crea) in infected ones can be attributed to saprolegniasis induced-kidney insufficiency. Infected fish exhibited an increase in TPP concentration compared to healthy ones. TPP is considered as health index in fish medicine and some factors induce hyperproteinemia, such as metabolism severity, nutrition, health status and high stocking density [44,45]. This might have been the case in our study as high TPP is attributed to fluid volume disorders, overcrowded stockings and/or elevation of acute phase proteins due to saprolegniasis. In the present study, AST increase was accompanied by no significant alterations of ALT, which may suggest effects of fungus toxins or presence of *Saprolegnia spp* hyphae in liver. Also, histopathological results confirm liver damage. High activity of ALT was exhibited in Atlantic Salmon Vibriosis, Rehulka [43] but Zaki et al.[4] reported AST increase in *Tilapia* nilotica affected *Saprolegnia parasitica*. Moreover, bacterial infection of Rainbow trout such as *Aeromonas salmonocida* contributes in significant elevation of trans-aminases [43]. Plasma MDA as an indicator of lipid per-oxidation and oxidative stress index, Elia et al.[24] also significantly increased in present study. We did not find any study about plasma MDA changes in fish fungal diseases. It suggests the production of free radicals and lipid peroxidation. Arabi and Alaeddini [26] and Vutukuru et al.[20] revealed MDA increase in *Oncorhynchus mykiss* gill hemogenate and visceral tissue of *Esumus danricus*, respectively. However, some studies have reported low levels of MDA in rainbow trout and Teleost [25,46] which is not in accordance with our study. In recent study, antioxidant enzymes (CAT, GSH-Px and TAC) were determined to be low. It postulated that their impression on oxidative stress lead to alleviation and/or is ascribed to low nutrition conditions. Mathew et al.[47] indicated significant reduction of CAT, GSH-Px and TAC in infected *Penaeus monodon* with white spot syndrome virus (WSSV) and Castex et al.[18] reported decrease of CAT and GSH-Px activities in affected *Litopenaeus stylirostris* with *Vibrio nigripulchritudo* infection. There was no significant change in SOD activity which agrees with unchanged liver SOD activity in affected rainbow trout with copper rich foods. Knox et al.[48]. However, SOD alterations have been investigated in some studies [25,49]. The GL concentration as the main analyte of carbohydrate metabolism accompanied with significant decrease. This may have been the case in our study as saprolegniasis causes anorexia and/or induction of glycogenesis (glycogen accumulation) in liver, which is confirmed with histopathological findings (PAS staining). We could not find any information regarding possible link

between plasma GL with glycogen storage in rainbow trout saprolegniasis. Pescador et al.[50] revealed relevance of hypoxia with glycogen accumulation and indicated that hypoxia-inducible factor causes glycogen synthase promotion in human muscle as well as hypoxia induce steatohepatitis in mice [51]. Since, based on Brauner and Wang study [52], hypoxia arises during anaemia. Therefore, it is possible that anaemia-induced hypoxia leads to glycogen accumulation along with fatty changes (steatohepatitis) in liver of saprolegniasis group. Furthermore, bacterial infections and VHS diminish GL concentration in rainbow trout while some of diseases such as overcrowding stress, heavy metals, herbicides and pesticides cause GL enhancement [43,45,53,54]. PON plays a vital role in Xenobiotic biotransformation and protects against lipid peroxidation [55,56]. Many studies reported PON activity in human [57], but, no comprehensive studies have been done regarding PON in veterinary medicine. In this study, PON concentration was found to be low. Most probably PON involves as inhibitory role on lipid peroxidation reaction. In this study, saprolegniasis caused marked alterations in hematology parameters than healthy ones including low values of PCV, RBCs count and Hb without significant changes in MCV, MCH, which indicated anaemia. Low count of peripheral RBC may associate with lose of hemapoietic tissue. Erslev [58] reported concurrent incidence of chronic renal failure along with uremia which is regularly accompanied by anaemia and its intensity is correlated with uremia severity. It is worth noting that many published studies have reported induction of anaemia in salmonids with bacterial and fungal infection [43,59]. It is postulated that kidney insufficiency-mediated uremia is caused anaemia in saprolegniasis group. Zaki [4] also pointed out correlation between plasma cortisol level with PCV, Hb and RBC reduction in *Tilapia nilotica*. It is more likely that occurrence of high cortisol level through disease induced- stress plays another cause in decrease of PCV, Hb and RBC with above mentioned mechanism in saprolegniasis. The significant increase of WBCs such as neutrophil, eosinophil and monocyte with lymphocyte decrease were noticed in the present study, which denotes cellular-immunity system interaction with fungal infection, Jamalzadeh [59]. Also, lymphopenia is attributed to three different mechanisms. Firstly, stress induced lymphopenia with following mechanisms, lymphocyte re-dispensation to lymphoid organs, cell destruction or decrease in blood circulation due to high levels of cortisol. Secondly, hypoxia-induced lymphopenia and thirdly, lymphocyte infiltration to tissues which it has been documented in *Fig. 1-B* [60-62].

Histopathological process carried out in liver, kidney and spleen. The results suggest that saprolegniasis-induced hypoxia may involve in glycogen accumulation and fatty changes into hepatocyts, second, the observed liver and renal damage could attribute to invasion of *saprolegnia* spp hyphae. In spleen, the increase of melanomacrophage centers usually occurs through antigens confrontation. According to the comparison of histopathologic results of control group with saprolgniasis ones, it indicated that the increase of melanomacrophage centers in spleen may ascribe to contamination by *Saprolegnia* spp.

In conclusion, these results showed the saprolegniasis impresses on blood parameters of rainbow trout with histopathological changes in different organs. These findings may persuade attempts to expand the importance of biochemistry and clinical hematology in the health screening programs of the rainbow trout in intensive aquaculture.

REFERENCES

1. Hossein MMA, Hatai K: *Saprolegnia salmonis* sp. nov isolated from sockeye salmon, *Onchrhynchus nerka*. *Mycoscience,* 40, 387-391, 1999. DOI: 10.1007/BF02464392

2. Espeland S: Prevention of *Saprolegnia* on Rainbow Trout Eggs. *BSc Thesis,* University of the Faroe Islands, 2004.

3. Avsever ML, Polat SH, Turk N, Metin DY: *Saprolegnia sp.* and *Aeromonas hydrophila* Isolation from Freshwater-Crayfish *(Astacus leptodactylus)*. *Kafkas Univ Vet Fak Derg,* 17, 873-875, 2011. DOI: 10.9775/kvfd.2011.4714

4. Zaki SM, Fawzi OM, Jackey EJ: Pathological and biochemical studies in tilapia nilotica infected with *Saprolegnia parasitica* and treated with potassium permanganate. *Am Eurasian J Agric & Environ Sci,* 3, 677-680, 2008. DOI: 10.1016/j.ijvsm.2013.04.001

5. Guzel M, Askar TK, Kaya G, Atakisi E, Erbil Avci G: Serum sialic acids, total antioxidant capacity, and adenosine deaminase activity in cattle with theileriosis and anaplasmosis. *Bull Vet Ins Pulawy,* 52, 227-230, 2008.

6. Lacomba R, Salcedo J, Alegia AM, Lagarda J, Barber R, Matencio E: Determination of sialic acid and gangliosides in biological samples and dairy products: A review. *J Pharm Biomed Analysis*, 51, 346-357, 2010. *DOI*: 10.1016/j.jpba.2009.04.023

7. Haq M, Haq S, Tutt P, Crook M: Serum total sialic acid and lipid-associated sialic acid in normal individuals and patients with myocardial infarction and their relationship to acute phase proteins. *Annals Clin Biochem,* 30, 383-386, 1993. DOI: 10.1177/000456329303000406

8. Thougaard AV, Hellmen E, Jensen AL: Total serum sialic acid is a general disease marker rather than a specific tumour marker in dogs. *J Vet Med Series A*, 45, 471-479, 1998. DOI: 10.1111/j.1439-0442.1998.tb00850.x

9. Nazifi S, Haghkhah M, Asadi Z, Ansari-Lari M, Tabandeh M, Esmailnezhad Z, Aghamir M: Evaluation of sialic acid and acute phase proteins (haptoglobin and serum amyloid a) in clinical and subclinical bovine mastitis. *Pakistan Vet J*, 31, 55-59, 2011.

10. Chrostek L, Cylwik B, Panasiuk A, Adamusiak DB, Gruszewska E: Lipid-bound sialic acid (LSA) in liver diseases of different etiologies. *Annals Hepatol,* 10, 150-154, 2011.

11. Citil M, Gunes V, Karapehlivan M, Atalan G, Marasli S: Evaluation of serum sialic acid as an inflammation marker in cattle with traumatic reticulo peritonitis. *Revue Méd Vét*, 155, 389-392, 2004.

12. Abdelmajeed NA: Oxidative tissue damage induced by citalopram in rat different organs. *Res J Med and Med Sci,* 4, 580-586, 2009

13. Atmaca M, Tezcan E, Kuloglu M, Ustundag B, Tunckol H: Antioxidant enzyme and malondialdehyde values in social phobia before and after citalopram treatment. *European Arch Psych Clin,* 254, 231-235, 2004. DOI: 10.1007/s00406-004-0484-3

14. Dimri U, Sharma MC, Yamdagni A, Ranjan R, Zama MMS: Psoroptic mange infestation increases oxidative stress and decreases antioxidant status in sheep. *Vet Parasitol,* 168, 318-322, 2010. DOI: 10.1016/j.vetpar.2009.11.013

15. Magni F, Panduri G, Paolocci N: Hyprothermia triggers iron-

dependent lipoperoxidative damage in the isolated rat heart. *Free Radical Biol Med*, 16, 465-476, 1994. DOI: 10.1016/0891-5849(94)90124-4

16. Halliwell B, Chirico S: Lipid peroxidation: Its mechanism, measurement, and significance. *Am J Clin Nut,* 57, 715-725, 1993.

17. Crnogaj M, Petlevski R, Mrljak V, Kis I, Torti M, Kucer N, Matijatko V, Sacer I, Stokovic I: Malondialdehyde levels in serum of dogs infected with *Babesia canis*. *Vet Med*, 55, 163-171, 2010.

18. Castex M, Lemaire P, Wabete N, Chim L: Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. *Fish Shellfish Immunol,* 4, 622-631, 2010. DOI: 10.1016/j.fsi.2009.12.024

19. Deger S, Deger Y, Bicek K, Ozdal N, Gul A: Status of lipid peroxidation, antioxidants, and oxidation products of nitric oxide in equine babesiosis: Status of antioxidant and oxidant in equine babesiosis. *J Equine Vet Sci,* 29, 743-747, 2009. DOI: 10.1016/j.jevs.2009.07.014

20. Vutukuru SS, Chintada S, Madhavi KR, Rao JV, Anjaneyulu Y: Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *somus danricus*. *Fish Physiol Biochem*, 32, 221-229, 2006. DOI: 10.1007/s10695-006-9004-x

21. Rice-Evans C, Baurdon R: Free radical-lipid interactions and their pathological consequences. *Prog in Lipid Res*, 32, 71-110, 1993. DOI: 10.1016/0163-7827(93)90006-I

22. Fontagné S1, Lataillade E, Brèque J, Kaushik S: Lipid peroxidative stress and antioxidant defense status during ontogeny of rainbow trout *(Oncorhynchus mykiss). British J Nut,* 100, 102-111, 2008. DOI: 10.1017/S0007114507876215

23. Trenzado C, Hidalgo MC, García-Gallego M, Morales AE, Furné M, Domezain A, Domezain J, Sanz A: Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture*, 254, 758-767, 2006. DOI: 10.1016/j.aquaculture.2005.11.020

24. Elia AC, Galarini R, Taticchi M.I, Dorr AJ, Mantilacci L: Antioxidant responses and bioaccumulation in *Icttalurus melas* under mercury exposure. *Ecotoxicol and Environ Safety,* 55, 162-167, 2003. DOI: 10.1016/S0147-6513(02)00123-9

25. Ates B, Orun I, Talas ZS: Effects of sodium selenite on some biochemical and hematological parameters of rainbow trout *(Oncorhynchus mykiss* Walbaum, 1792) exposed to Pb^{2+} and Cu^{2+}. *Fish Physiol and Biochem,* 34, 53-59, 2007. DOI: 10.1007/s10695-007-9146-5

26. Arabi M, Alaeddini MA: Metal-ion-mediated oxidative stress in the gill homogenate of rainbowtrout *(Oncorhynchus mykiss)* antioxidant potential of manganese, selenium and albumin. *Biol Trace Element Res,* 108, 155-168, 2005. DOI: 10.1385/BTER:108:1-3:155

27. Karatas T, Kocaman EM: Comparison of Paraoxonase activity, malondialdehyde and high-density lipoprotein levels in cultuvated normal and albino rainbow trout reared in the same conditions. *Kafkas Univ Vet Fak Derg,* 18, 87-90, 2012. DOI: 10.9775/kvfd.2011.4971

28. Bastos C, Folly E, Rossini A, Ceccarelli PS, Senhorini JA, Bastos JC: Paraoxonase activity in liver of Paco, *Piaractus mesopotamicus holmberg* (Characidae). *Rev Bras Zool,* 15, 677-685, 1998. DOI: 10.1590/S0101-81751998000300012

29. Willoughby LG, Pickering AD: Viable *saprolegniaceae* spores on the epidermis of the salmonid fish *Salmo trutta* and *Salvelinus alpinus*. *Trans Br Mycol So,* 68, 91-95, 1977. DOI: 10.1016/S0007-1536(77)80157-5

30. McKnight IMA: Hematological study on the mountain whitefish *Popium willasemi*. *J Fisheries Res Board Canada,* 23, 45-64, 1996.

31. Blaxhall PC, Daisley KW: Routine haematological methods for use fish with blood. *J Fish Biol,* 5, 771-781, 1973. DOI: 10.1111/j.1095-8649.1973.tb04510.x

32. Haney DC, Hursh DA, Mix MC, Winton JR: Physiological and hematological changes in chum salmon artificially infected with erythrocytic necrosis virus. *J Aquatic Anim Health,* 4, 48-57, 1992. DOI: 10.1577/1548-8667(1992)004<0048:PAHCIC>2.3.CO;2

33. Sydow G: A simpllifield quick method for determination of sialic acid in serum. *Biomed Biochim Acta*, 44, 1721-1723, 1985.

34. Aebi H: Catalase *in vitro*. *Methods Enzymol*, 105, 121-126, 1984. DOI: 10.1016/S0076-6879(84)05016-3

35. Satoh K: Serum lipid peroxide in cerebrovascular disorders determined by new colorimetric method. *Clinica Chimica Acta*, 90, 37-43, 1978. DOI: 10.1016/0009-8981(78)90081-5

36. Furlong CE, Richter RJ, Seidel SL, Motulsky AG: Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the metabolites chlorpyrifos oxon and paraoxon. *Am J Human Genetics,* 43, 230-238, 1988.

37. Motoi Y, Kimura Y, Wakamatsu H, Shimbayashi K: Determination and clinical evaluation of sialic acid and mucoprotein in bovine blood. *National Ins Anim Health,* 37, 643-649, 1984. DOI: 10.12935/jvma1951.37.643

38. Ellis AE: Innate host defence mechanisms of fish against viruses and bacteria. *Devel Comp Immunol,* 25, 827-839, 2001. DOI: 10.1016/S0145-305X(01)00038-6

39. Shephard KL: Functions for fish mucus. *Rev Fish Biol and Fisheries*, 4, 401-429, 1994. DOI: 10.1007/BF00042888

40. Sumi T, Hama Y, Maruyama D, Asakawa M, Nakagawa H: Isolation and properties of a sialoglycoprotein from the skin mucus of the stingray *Dasyatis akajei*. *Fish Sci*, 63, 453-458, 1997. DOI: 10.2331/fishsci.63.453

41. Nigam AK, Kumari U, Mittal S, Mittal AK: Comparative analysis of innate immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches. *Fish Physiol and Biochem*, 38, 1245-1256, 2012. DOI: 10.1007/s10695-012-9613-5

42. Traving C, Schauer R: Structure, function and metabolism of sialic acids. *Cellular and Mol Life Sci*, 54, 1330-1349, 1998. DOI: 10.1007/s000180050258

43. Rehulka J: Haematological analyses in rainbow trout Oncorhynchus mykiss affected by viral haemorrhagic septicaemia (VHS). *Dis Aquat Organ,* 56, 185-193, 2003. DOI: 10.3354/dao056185

44. Svoboda M, Kouril J, Hamackova J, Kalab P, Savina L, Svobodova Z, Vykusova B: Biochemical profile of blood plasma of Tench *(Tinca tinca L.*) during pre- and post spawing period. *Acta Vet Brno,* 70, 259-268, 2001. DOI: 10.2754/avb200170030259

45. Joseph JP: Alteration of certain blood parameters of freshwater teleost *Mystus vittatus* after chronic exposure to Metasystox and Sevin. *Fish Physiol and Biochem,* 33, 15-20, 2006. DOI: 10.1007/s10695-006-9112-7

46. Varghese S, Oommen OV: Long-term feeding of dietary oils alters lipid metabolism, lipid peroxidation, and antioxidant enzyme activities in a Teleost *(Anabas testudineus* Bloch). *Lipids*, 35, 757-762, 2000. DOI: 10.1007/s11745-000-0582-2

47. Mathew S, Kumar KA, Anandan R, Viswanathan Nair PG, Devadasan K: Changes in tissue defense system in white spot syndrome virus (WSSV) infected *Penaeus monodon*, comparative biochemistry and physiology - Part C. *Toxicol & Pharmacol,* 145, 315-320, 2007. DOI: 10.1016/j.cbpc.2007.01.001

48. Knox D, Cowey CB, Adron JW: Effects of dietary zinc intake upon copper metabolism in rainbow trout *(Salmo gairdneri). Aquaculture,* 40, 199-207, 1984. DOI: 10.1016/0044-8486(84)90187-X

49. Zikic RV, Stajn AS, Pavlovic SZ, Ognjanovic BI, Saicic ZS: Activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of goldfish *(Carassius auratus gibelio* Bloch) exposed to cadmium. *Physiol Res,* 50, 105-111, 2001.

50. Pescador N, Villar D, Cifuentes D, Garcia-Rocha M, Ortiz-Barahona A,Vazquez S, Ordonez A, Cuevas Y, Morales DS, Garcia- Bermejo ML, Landazuri MO, Guinovart J, Del Peso L: Hypoxia promotes glycogen accumulation through hypoxia inducible factor (hif)-mediated induction of glycogen synthase, *PLoS ONE*, 5(3): e9644, 2010. DOI: 10.1371/journal.pone.0009644

51. Piguet AC, Stroka D, Zimmermann A, Dufour JF: Hypoxia aggravates non-alcoholic steatohepatitis in mice lacking hepatocellular PTEN. *Clin Sci,* 118, 401-410, 2010. DOI: 10.1042/CS20090313

52. Brauner C.J, Wang T: The optimal oxygen equilibrium curve: A comparison between environmental hypoxia and anemia. *Am Zool,* 37, 101-108, 1997. DOI: 10.1093/icb/37.1.101

53. Montero D, Izquierdo MS, Tort L, Robaina L, Vergara JM: High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiol and Biochem,* 20, 53-60, 1999. DOI: 10.1023/A:1007719928905

54. Velisek J, Svobodova Z, Piackova V, Sudova E: Effects of acute exposure to metribuzin on some hematological, biochemical and histopathological parameters of common Carp *(Cyprinus carpio L.*). *Bull Environ Cont and Toxicol,* 82, 492-495, 2009. DOI: 10.1007/s00128-009-9648-1

55. James RW: A long and winding road: defining the biological role and clinical importance of paraoxonases. *Clin Chem and Lab Med,* 44, 1052-1059, 2006. DOI: 10.1515/CCLM.2006.207

56. Aviram M, Rosenblat M: Paraoxonase and cardiovascular diseases: Pharmacological and nutritional influences. *Curr Op Lipidol,* 16, 393-399, 2005.

57. Turk R, Juretic D, Geres D, Turk N, Rekic B, Simeon-Rudolf V, Svetina A: Serum paraoxonase activity and lipid parameters in the early postpartum period of dairy cows. *Res Vet Sci*, 76, 57-61, 2004. DOI: 10.1016/j.rvsc.2003.08.001

58. Erslev AJ, Besarab A: Erythropoietin in the pathogenesis and treatment of the anemia of chronic renal failure. *Kidney Int,* 51, 622-630, 1997. DOI: 10.1038/ki.1997.91

59. Jamalzadeh HR, Keyvan A, Ghomi MR, Gerardi F: Comparison of blood indices in healthy and fungal infected Caspian salmon *(Salmo trutta caspius). Afr J Biotechnol,* 8, 319-322, 2009.

60. Choi K: Modulation of immune function parameters in fish caused by sudden environmental changes dissertation. University of North Carolina, 2004.

61. Dolin R, Reichman RC, Fauci AS: Lymphocyte populations in acute viral gastroenteritis. *Infect Immun,* 14, 422-428, 1976.

62. Ishikawa NM, Ranzni-Paiva MJT, Lombardi JV, Ferreira CM: Hematological parameters in Nile *Tilápia, Oreochromis niloticus* Exposed to sub-lethal concentrations of mercury. *Braz Arch Biol Tech,* 4, 619-626, 2007. DOI: 10.1590/S1516-89132007000400007

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Research Article

Prevalence and Characterization of ESBL- and AmpC-producing *Escherichia coli* from Cattle [1]

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Abstract

In this study, it was aimed to determine the prevalence of extended spectrum β-lactamase (ESBL) and/or AmpC type β-lactamase (AmpC) producing *Escherichia coli* from cattle in Hatay. For this purpose, 312 rectal swabs samples were collected from apparently healthy cattle. ESBL production was phenotypically investigated by disc combination method and double disc synergism test and β-lactamase genes (bla_{CTX-M}, bla_{CMY-2}, bla_{SHV}, bla_{OXA}, *and* bla_{TEM}) and plasmid mediated quinolone resistance (PMQR) genes (*qnr*A, *qnr*B, *qnr*S and *aac*(6')-Ib) were screened by polymerase chain reaction (PCR) and subsequent sequence analysis. Antimicrobial susceptibility of the isolates were determined using disc diffusion method and their phylogenetic groups were also searched by PCR. Twenty six (8.3%) isolates were found to be ESBL producer by phenotypic tests. The following ESBL/AmpC genes were detected: $bla_{CTX-M-15}$ (n= 12), $bla_{CTX-M-1}$ (n=11), $bla_{CTX-M-3}$ (n=2), and bla_{CMY-2} (n=1). PMQR genes were detected in 11 (42.3%) ESBL producing *E. coli* isolates and these isolates were only positive for *aac(6')-Ib-cr* and *qnr*S1 genes. Twenty two (84.6%) of the isolates exhibited multidrug resistance (MDR) phenotype. ESBL/AmpC producing *E. coli* isolates were observed to be belonged to B1 (50%), A (34.6%) and D (15.4%) phylogroups. This study was the first to describe the presence of CTX-M-15, CTX-M-3, CTX-M-1 and CMY-2 producing *E. coli* in cattle in Turkey and the co-existence of *aac(6')-Ib-cr* and *qnr*S1 genes in some isolates.

Keywords: *Escherichia coli, Cattle, ESBL, AmpC*

Sığırlarda GSBL ve AmpC Sentezleyen *Escherichia coli*'nin Prevalansı ve Karakterizasyonu

Özet

Bu çalışmada, Hatay ilinde sığırlarda genişlemiş spektrumlu β-laktamaz (GSBL) ve/veya plazmid aracılı AmpC tip β-laktamaz (AmpC) sentezleyen *E. coli*'nin prevalansının belirlenmesi hedeflendi. Bu amaçla, sağlıklı görünüşlü 312 sığırdan rektal sıvab örneği toplandı. Fenotipik GSBL sentezi disk kombinasyon metodu ve çift disk sinerji testi ile β-laktamaz (bla_{CTX-M}, bla_{CMY-2}, bla_{SHV}, bla_{OXA} ve bla_{TEM}) ve plazmid aracılı kinolon direnç (PAKD) genleri (*qnr*A, *qnr*B, *qnr*S ve *aac*(6')-Ib) polimeraz zincir reaksiyonu (PZR) ve daha sonra sekans analizi ile araştırıldı. Ayrıca, GSBL ve/veya AmpC sentezleyen *E. coli* izolatlarının antimikrobiyal duyarlılıkları disk difüzyon metodu ile filogenetik gruplarının belirlenmesi ise PZR ile incelendi. Yirmialtı izolat (%8.3) GSBL üretimi yönünden fenotipik testlerle pozitif bulundu. Bu izolatlarda $bla_{CTX-M-15}$ (n= 12), $bla_{CTX-M-1}$ (n= 11), $bla_{CTX-M-3}$ (n= 2) ve bla_{CMY-2} (n=1) genleri belirlendi. PAKD genleri 11 (%42.3) ESBL sentezleyen *E. coli* izolatında saptandı ve bu izolatlar sadece *aac(6')-Ib-cr* ve *qnr*S1 genleri yönünden pozitif bulundu. İzolatlarının 22'si (%84.6) çoğul direnç fenotipi gösterdi. GSBL/AmpC sentezleyen *E. coli* izolatlarının B1 (%50), A (%34.6) ve D (%15.4) filogruplarına ait olduğu görüldü. Bu çalışma ile, ilk kez Türkiye'de sığırlarda CTX-M-15, CTX-M-3, CTX-M-1 ve CMY-2 tip β-laktamaz sentezleyen *E. coli* varlığı ve bu izolatların bazılarında *aac(6')-Ib-cr* ve *qnr*S1 genlerinin birlikte bulunduğu gösterilmiştir.

Anahtar sözcükler: *Escherichia coli, Sığır, GSBL, AmpC*

INTRODUCTION

Emergence and dissemination of extended spectrum β-lactamase (ESBL) and/or AmpC type β-lactamase (AmpC) producing *Escherichia coli* are public health concern worldwide in the intestinal microbiota of food-producing animals [1]. Since ESBL and/or AmpC producing *E. coli* isolates frequently contain resistance genes to other classes

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of antimicrobial (e.g. fluoroquinolones, aminoglycosides and tetracyclines), therapeutic options are very limited [2]. Another public concern is the transmission of these bacteria to humans through the food chain [1]. Even though different ESBL types have been reported in *E. coli* isolates from food-producing animals, CTX-M have widely been encountered not only in humans but also in animals [3].

AmpC type β-lactamases are either chromosomally mediated or plasmid-mediated in the *Enterobactericeae*. In contrast to ESBLs, they hydrolyze cephamycins (cefoxitin and cefotetan) and are not inhibited by β-lactamase inhibitors. Therefore, cefoxitin insusceptibility has been used for screening of AmpC producers. Despite the fact that AmpC type β-lactamases are of numerous types, CMY-2 is the most encountered in Enterobactericeae members from different host [4]. So far, the presence of AmpC producing *E. coli* has not yet been shown in food producing animals in Turkey.

Data on the occurrence and dissemination of ESBL/AmpC producing *E. coli* in cattle are very scarce in Turkey [5]. Therefore, the objectives of this study were to determine the prevalence of ESBL and/or AmpC producing *E. coli* from cattle. Antimicrobial resistance profiles and the phylogenetic groups of the isolates were also studied.

MATERIAL and METHODS

Bacterial Isolates

Rectal swabs (n=312), of which 172 were taken from dairy cattle and 140 from beef cattle, were collected from different cattle farms located in Hatay, Turkey, from March 2012 to June 2013. The study was approved by the Animal Ethical Committee of Mustafa Kemal University (2012/95). The rectal swabs were streaked onto Eosin Methylen Blue (EMB) agar supplemented with cefotaxime (2 µg/mL) and incubated at 35°C for 24 h. One typical colony for *E.coli* per plate was selected and identified by conventional methods and confirmed by polymerase chain reaction (PCR) [6].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates for confirmed ESBL producer *E. coli* isolates were determined by disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines (CLSI) [7]. The following antimicrobial disks (Bioanalyse, Turkey) were used: ampicillin (10 µg), amoxicillin/clavulanic acid (20 µg/10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), imipenem (10 µg), gentamicin (10 µg), streptomycin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), sulfamethoxazole-trimethoprim (1.25 µg/23.75 µg), tetracycline (30 µg) and chloramphenicol (30 µg). The isolates resistant to third generation cephalosporins were confirmed as ESBL producer by double disk synergy [8] and disk combination method according to guidelines of CLSI [7]. Cefoxitin resistance were considered positive for AmpC production [7]. All phenotypically positive isolates were examined for ESBL/AmpC genes. *E. coli* standard strain ATCC 25922 were used for quality control.

Phylogenetic Grouping

The ESBL/AmpC producing *E. coli* isolates were phylogenetically grouped into A, B1, B2 or D using the triplex PCR reaction as previously reported by Clermont et al.[9].

Detection of ESBL, AmpC and PMQR Genes

ESBL genes (*bla*$_{CTX\text{-}M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$) [10], *bla*$_{CMY\text{-}2}$ [11], and plasmid-mediated quinolone resistance (PMQR) genes (*qnr*A, *qnr*B, *qnr*S and *aac(6')-Ib*) [12,13] were investigated as previously reported.

Statistical Analysis

Statistical differences between beef and dairy cattle were investigated by use of IBM SPSS Statistics package Version 23 (IBM Corp., Armonk, NY, USA). Differences were considered significant at P<0.05.

RESULTS

Out of 312 rectal swab samples, 26 (8.3%) *E. coli* isolates were ESBL/AmpC phenotype, comprising 10 (5.8%) from dairy cows and 16 (11.4%) from beef cattle (P=0.075). Following PCR amplification and sequencing of ESBL/AmpC producing *E. coli* strains, 12 (46.2%) harbored *bla*$_{CTX\text{-}M\text{-}15}$, 11 (42.3%) *bla*$_{CTX\text{-}M\text{-}1}$, two (7.7%) *bla*$_{CTX\text{-}M\text{-}3}$ and one (3.8%) *bla*$_{CMY\text{-}2}$. In addition to ESBL/AmpC genes, other β-lactamase genes were detected in 22 isolates (84.6%), of which 21 isolates harbored *bla*$_{TEM\text{-}1b}$ and one isolate harbored *bla*$_{OXA\text{-}1}$ in combination with ESBL/AmpC genes *(Table 1)*.

Most of ESBL/AmpC producing *E. coli* isolates belonged to group B1 (50%) and group A (34.6%), to lesser extent to group D (15.4%). However, group B2 was not found in the isolates.

All isolates were resistant to ampicillin (100%) but susceptible to imipenem. Isolates were also found to be resistant to streptomycin (80.8%), sulfamethoxazole-trimethoprim (76.9%), tetracycline (73.1%), chloramphenicol (53.8%), kanamycin (50%), amoxicillin/clavulanic acid (46.2%), nalidixic acid (46.2%), ciprofloxacin (23.1%) and cefoxitin (3.8%). Multiple resistance phenotype (resistance to three or more antimicrobials) were found in 22 (84.6%) isolates *(Table 1)*. PMQR genes were only detected in 11 ESBL producing *E. coli* isolates. Among the 11 PMQR positive isolates, eight carried *aac(6')-Ib-cr*, two *qnr*S1 and one both *qnr*S1 and *aac(6')-Ib-cr*. All of the isolates tested were negative for the *qnr*A, *qnr*C, *qnr*D and *qep*A *(Table 1)*.

Table 1. *Characteristics of ESBL/AmpC producing E. coli isolates from cattle*
Tablo 1. *Sığırlardan izole edilen ESBL/AmpC sentezleyen E. coli özellikleri*

Isolate ID	Phylogenetic Group	ESBL Type	Other Beta-lactamase Genes	AmpC Type	PMQR Gene	Antimicrobial Resisitance Phenotype*
22	A	CTX-M-15	TEM-1b	-	-	AMP, S, CN, TE, SXT, C
37	A	-	TEM-1b	CMY-2	*aac(6')-Ib-cr*	AMP, AMC, FOX, S, TE, C
38	D	CTX-M-15	TEM-1b	-	*qnr*S1	AMP, S, TE, SXT, C
40	D	CTX-M-15	TEM-1b	-	-	AMP, S, K, TE, SXT
44	A	CTX-M-15	TEM-1b, OXA-1	-	*aac(6')-Ib-cr*	AMP, AMC, NA, CIP, S, CN, K, TE, SXT
57	B1	CTX-M-15	-	-	*qnr*S1	AMP
60	D	CTX-M-15	TEM-1b	-	-	AMP, NA, S, K, TE, SXT
76	B1	CTX-M-3	-	-	*aac(6')-Ib-cr*	AMP
78	A	CTX-M-15	TEM-1b	-	-	AMP, AMC, S, SXT
80	B1	CTX-M-3	-	-	-	AMP
88	D	CTX-M-15	-	-	-	AMP
192	B1	CTX-M-1	TEM-1b	-	-	AMP, S, TE, SXT
197	B1	CTX-M-1	TEM-1b	-	*aac(6')-Ib-cr*, *qnr*S1	AMP, S, TE, SXT
221	B1	CTX-M-15	-	-	-	AMP, S, CN, TE, SXT, C
224	A	CTX-M-15	TEM-1b	-	-	AMP, S, TE, SXT, C
242	A	CTX-M-15	TEM-1b	-	*aac(6')-Ib-cr*	AMP, AMC, NA, CIP, S, SXT, C
246	A	CTX-M-1	TEM-1b	-	*aac(6')-Ib-cr*	AMP, AMC, NA, CIP, S, K, TE, SXT, C
257	B1	CTX-M-15	TEM-1b	-	*aac(6')-Ib-cr*	AMP, S, K, TE, SXT
276	A	CTX-M-1	TEM-1b	-	-	AMP, AMC, NA, S, K, TE, SXT, C
277	B1	CTX-M-1	TEM-1b	-	-	AMP, NA, S, K, TE, SXT, C
279	A	CTX-M-1	TEM-1b	-	-	AMP, AMC, NA, S, K, TE, SXT, C
280	B1	CTX-M-1	TEM-1b	-	-	AMP, AMC, NA, S, K, TE, SXT, C
282	B1	CTX-M-1	TEM-1b	-	-	AMP, AMC, NA, S, K, TE, SXT, C
283	B1	CTX-M-1	TEM-1b	-	-	AMP, AMC, NA, CIP, S, K, TE, SXT, C
286	B1	CTX-M-1	TEM-1b	-	*aac(6')-Ib-cr*	AMP, AMC, NA, CIP, K
287	B1	CTX-M-1	TEM-1b	-	*aac(6')-Ib-cr*	AMP, AMC, NA, CIP, S, K, TE, SXT, C

**AMP: ampicillin, AMC: amoxicillin/clavulanic acid, FOX: cefoxitin, CN: gentamicin, S: streptomycin, K: kanamycin, NA: nalidixic acid, CIP: ciprofloxacin, SXT: sulfamethoxazole-trimethoprim, TE: tetracycline; C: chloramphenicol*

DISCUSSION

ESBL and/or AmpC producing Enterobacteriaceae have been a growing problem throughout the world [1], and it has increasingly been reported in food producing animals (EFSA) [14]. The prevalence rate of ESBL/AmpC producing *E. coli* in cattle (8.3%) was higher than those previously reported in France (5.8%) [15] and Hong Kong (3.1%) [16], but lower than those reported in Switzerland (16%) [17] and Japan (31.3%) [18]. However, in a study conducted in Poland, no ESBL/AmpC producing *E. coli* were observed [19].

In the current study, beef cattle (11.4%) showed higher prevalence rate than dairy cattle (5.8%) (P=0.075). Ohnishi et al.[20] reported a prevalence rate of 2.6% in dairy farms in Japan, whereas Schmid et al.[21] reported higher prevalence in dairy cattle (41.1%) and in beef cattle (18.9%) in Germany.

Up until now, very little data have been present in literature on the occurence and molecular characterization of ESBL/AmpC producing *E. coli* among food producing animal in Turkey. Küçükbasmacı et al.[5], the first to report ESBL producing *Enterobactericeae* from food producing animals in Turkey, reported a prevalence of 2.1% (5/277) among *Enterobactericeae* from cattle, of which only three isolates were *E. coli*. In their study, the authors detected only OXA-10 and SHV-5 as ESBL enzymes together with TEM-1. Recently, Önen et al.[22] reported higher contamination rate of chicken meat with ESBL producing *E. coli*, but low contamination rate for beef meat (7%) in Turkey. In contrast, Başaran Kahraman et al.[23], who investigated the presence of ESBL and AmpC β-lactamase producing *E. coli* from faecal samples of broiler and egg-type healthy chicken belonging to 43 flocks, reported prevalence of ESBL and AmpC producers as 7.8% and 3.6%, respectively.

Considerable differences in ESBL/AmpC β-lactamase types have been detected in *E. coli* from food producing animals in the world [1]. In this study, CTX-M-15 and CTX-M-1 were the most frequently detected ESBL enzymes, which are also commonly detected in human clinical *E. coli* isolates in Turkey [24,25]. CTX-M-1 was detected as predominant ESBL enzyme in France [15] and Switzerland [17], whereas CTX-M-2 in Japan [20] and CTX-M-14 in China [26] were reported as predominant ESBL enzyme. Moreover, detection of CMY-2 type β-lactamase in one *E. coli* isolate was an important finding that have never been repoted in Turkey so far. The results suggest that there have been a drastic change in the epidemology of ESBL producing *E. coli* in cattle in Turkey.

E. coli strains found on microbioata of farm animals were reported to belong largely B1 and A phylogenetic groups, and to lesser extent, to phylogenetic groups B2 and D [27]. In this study, phylogenetic analysis of *E. coli* strains revealed that ESBL/AmpC producing strains were mainly belonged to group B1 and showed absence of phylogenetic group B2.

In the present study, vast majority of the isolates (84.6%) were also resistant to non-β-lactam antibiotics, and showed multi drug resistance. A similar finding was also reported by Valentin et al.[28], who found that ESBL/AmpC producing *E. coli* strains were also resistant to other class of antimicrobials agents. This could partly be explained by the fact that the plasmids harboring ESBL/AmpC genes frequently carry other resistance genes that are responsible for other class antimicrobials, such as fluoroquinolones, aminoglycosides and trimethoprim-sulphamethoxazole [28].

For the first time, PMQR genes were found together with ESBL/AmpC genes from cattle in Turkey. The most prevalent PMQR gene was a*ac(6')-Ib-cr* (42.3%) whereas *qnr*S1 (11.5%) was the only *qnr* gene detected. This higher co-existence could be explained by overuse and misuse of fluoroquinolones in animals. In accordance with our results, this finding has been previously documented in *E. coli* isolates from animals of different origin [29,30].

In conclusion, the results indicate that cattle are potential reservoir of ESBL/AmpC producing *E. coli* in Turkey. Thus, ESBL/AmpC producing bacteria should be monitored in both microbiota of healthy animals and clinical materials regularly, and prudent use of antimicrobial agents is necessary to prevent spread of these bacteria.

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REFERENCES

1. Trott D: β-lactam resistance in gram-negative pathogens isolated from animals. *Curr Pharm Des*, 19, 239-249, 2013.

2. Pitout JD: Infections with extended-spectrum beta-lactamase-producing enterobacteriaceae: Changing epidemiology and drug treatment choices. *Drugs*, 70, 313-333, 2010. DOI: 10.2165/11533040-000000000-00000

3. Seiffert SN, Hilty M, Perreten V, Endimiani A: Extended-spectrum cephalosporin-resistant gram-negative organisms in livestock: An emerging problem for human health? *Drug Resist Update*, 16, 22-45, 2013. DOI: 10.1016/j.drup.2012.12.001

4. Philippon A, Arlet G, Jacoby GA: Plasmid-determined AmpC-type-lactamases. *Antimicrob Agents Chemother*, 46, 1-11, 2002. DOI: 10.1128/AAC.46.1.1-11.2002

5. Kucukbasmaci O, Ciftcioglu G, Midilli K, Issa G: Detection of extended spectrum β-lactamase producing Enterobacteriaceae from food animals In Turkey. *Revue Méd Vét,* 159, 586-592, 2008.

6. Wang G, Clark CG, Rodgers FG: Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR. *J Clin Microbiol*, 40, 3613-3619, 2002. DOI: 10.1128/JCM.40.10.3613-3619.2002

7. CLSI: Clinical and Laboratory Standart Institute. Performance standarts for antimicrobial susceptibility testing; Twenty-Second informational supplement; M100-S20. Wayne PA USA, 2012.

8. Jarlier V, Nicolas MH, Fournier G, Philippon A: Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: Hospital prevalence and susceptibility patterns. *Rev Infect Dis*, 10, 867-878, 1988.

9. Clermont O, Bonacorsi S, Bingen E: Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*, 66, 4555-4558, 2000. DOI: 10.1128/AEM.66.10.4555-4558.2000

10. Ahmed AM, Motoi Y, Sato M, Maruyama A, Watanabe H, Fukumoto Y, Shimamoto T: Zoo animals as a reservoir of Gram negative bacteria harboring integrons and antimicrobial resistance genes. *Appl Environ Microbiol*, 73, 6686-6690, 2007. DOI: 10.1128/AEM.01054-07

11. Zhao S, White DG, Mc Dermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, Walker RD: Identification and expression of cephamycinase bla_{CMY} genes in *Escherichia coli* and Salmonella isolates from food animals and ground meat. *Antimcrob Agents Chemother*, 45, 3647-3650, 2001. DOI: 10.1128/AAC.45.12.3647-3650.2001

12. Kim HB, Park CH, Jacoby GA, Kim CJ, Hooper DC, Kim EC: Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrob Agents Chemother*, 53, 639-645, 2009. DOI: 10.1128/AAC.01051-08

13. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC: Prevalence in the United States of *aac(6)-Ib-cr* encoding a ciprofloxacin modifying enzyme. *Antimicrob Agents Chemother,* 50, 3953-3955, 2006. DOI: 10.1128/AAC.00915-06

14. European Food Safety Authority (EFSA): Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. *EFSA Journal,* 9, 2322, 2011. DOI: 10.2903/j.efsa.2011.2322

15. Hartmann A, Locatelli A, Amoureux L, Depret G, Jolivet C, Gueneau E, Neuwirth C: Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France (Burgundy Region). *Front Microbiol,* 3, 83, 2012. DOI: 10.3389/fmicb.2012.00083

16. Duan RS, Sit TH, Wong SS, Wong RC, Chow KH, Mak GC, Yam WC, Ng LT, Yuen KY, Ho PL: *Escherichia coli* producing CTX-M beta-lactamases in food animals in Hong Kong. *Microb Drug Resist,* 12, 145-148, 2006. DOI:

10.1089/mdr.2006.12.145

17. Geser N, Stephan R, Hächler H: Occurrence and characteristics of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. *BMC Vet Res,* 8, 21, 2012. DOI: 10.1186/1746-6148-8-21

18. Hiroi M, Yamazaki F, Harada T, Takahashi N, Iida N, Noda Y, Yagi M, Nishio T, Kanda T, Kawamori F: Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in food-producing animals. *J Vet Med Sci,* 74, 189-195, 2012. DOI: 10.1292/jvms.11-0372

19. Wasy ID, Hasman H, Cavaco LM, Aarestrup FM: Prevalence and characterization of cephalosporin resistance in nonpathogenic *Escherichia coli* from food-producing animals slaughtered in Poland. *Microb Drug Resist,* 18, 79-82, 2011. DOI: 10.1089/mdr.2011.0033

20. Ohnishi M, Okatani AT, Esaki H, Harada K, Sawada T, Murakami M, Marumo K, Kato Y, Sato R, Shimura K: Herd prevalence of Enterobacteriaceae producing CTX-M-type and CMY-2 β-lactamases among Japanese dairy farms. *J Appl Microbiol,* 115, 282-289, 2013. DOI: 10.1111/jam.12211

21. Schmid A, Hörmansdorfer S, Messelhäusser U, Käsbohrer A, Sauter-Louis C, Mansfeld R: Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* on bavarian dairy and beef cattle farms. *Appl Environ Microbiol*, 79, 3027-3032, 2013. DOI: 10.1128/AEM.00204-13

22. Pehlivanlar Önen S, Aslantaş Ö, Yılmaz Şebnem E, Kürekci C: Prevalence of β-lactamase producing *Escherichia coli* from retail meat in Turkey. *J Food Sci*, 80, 2023-2029, 2015. DOI: 10.1111/1750-3841.12984

23. Başaran Kahraman B, Diren Sığırcı B, Çelik B, Gümüş B, Metiner K, Adıgüzel MC, Bağcıgil AF, İkiz S, Özgür NY: Detection of extended-spectrum β-lactamase and AmpC β-lactamase producing *Escherichia coli* Isolates from chickens. *Kafkas Univ Vet Fak Derg*, 22, 591-596, 2016. DOI: 10.9775/kvfd.2016.15121

24. Copur Cicek A, Saral A, Ozad Duzgun A, Yasar E, Cizmeci Z, Ozlem Balci P, Sari F, Firat M, Altintop YA, Ak S, Caliskan A, Yildiz N, Sancaktar M, Buda EE, Erturk A, Ozgumus DB, Sandalli C: Nationwide study of *Escherichia coli* producing extended-spectrum β-lactamases TEM, SHV and CTX-M in Turkey. *J Antibiot Tokyo*, 66, 647-650, 2013. DOI: 10.1038/ja.2013.72

25. Gür D, Gülay Z, Akan OA, Aktaş Z, Kayacan CB, Cakici O, Eraç B, Gültekin M, Oğünç D, Söyletir G, Ünal N, Uysal S: Resistance to newer beta-lactams and related ESBL types in gram-negative nosocomial isolates in Turkish hospitals: Results of the multicentre HITIT study. *Mikrobiyol Bul,* 42, 537-544, 2008.

26. Zheng H, Zeng Z, ChenS, Liu Y, Yao Q, Deng Y, Chen X, Lv L, Zhuo C, Chen Z, Liu JH: Prevalence and characterisation of CTX-M β-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. *Int J Antimicrob Agents,* 39, 305-310, 2012. DOI: 10.1016/j.ijantimicag.2011.12.001

27. Escobar-Paramo P, Clermont O, Blanc-Potard AB, Bui H, Le Bouguenec C, Denamur E: A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. *Mol Biol Evol,* 21, 1085-1094, 2004. DOI: 10.1093/molbev/msh118

28. Valentin L, Sharp H, Hille K, Seibt U, FischerJ, Pfeifer Y, Michael GB, Nickel S, Schmiedel J, Falgenhauer L: Subgrouping of ESBL-producing *Escherichia coli* from animal and human sources: an approach to quantify the distribution of ESBL types between different reservoirs. *Int J Med Microbiol,* 304, 805-816, 2014. DOI: 10.1016/j.ijmm.2014.07.015

29. Li L, Wang B, Feng S, Li J, Wu C, Wang Y, Ruan X, Zeng M: Prevalence and characteristics of extended-spectrum ß-lactamase and plasmid-mediated fluoroquinolone resistance genes in *Escherichia coli* isolated from chickens in Anhui Province, China. *PLoS One,* 9(8), e104356, 2014. DOI: 10.1371/journal.pone.0104356

30. Wang Y, He T, Han J, Wang J, Foley SL, Yang G, Wan S, Shen J, Wu C: Prevalence of ESBLs and PMQR genes in fecal *Escherichia coli* isolated from the non-human primates in six zoos in China. *Vet Microbiol*, 159, 53-59, 2012. DOI: 10.1016/j.vetmic.2012.03.009

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Research Article

Influence of Body Condition Score and Ultrasound-Determined Thickness of Body Fat Deposit in Holstein-Friesian Cows on the Risk of Lameness Developing

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Abstract

The aim of this study was to examine the correlations between ultrasound measurement of thickness of fat over the tuber ischiadicum (TFT), body condition scoring (BCS) and the risk of lameness developing in Holstein-Friesian dairy cows. The 100 cows were enrolled from a population of dry cows on one farm. TFT was measured with ultrasound, and BCS and locomotion score were determined during lactation. Of the 100 cows, 31% developed lameness during lactation. The highest proportion of lame cows was in cows with BCS≥4.25 (66.7%). The risk of lameness developing was higher in cows with BCS≥4.25 (OR=7) and ≤3.25 (OR=2) than in cows with optimal BCS=3.75. Cows in the lower TFT quartile had a higher proportion of lameness, but not those in the upper quartile. TFT may have some value as a predictor of lameness in thin cows. The best prediction of lameness in both fat and thin cows (ROCAUC=0.8725, P<0.01) occurred when both BCS and TFT values were used together. The risk of developing lameness was positively correlated with BCS, negatively correlated with TFT and negatively correlated with their interaction. For fat cows, BCS assessment is a suitably strong predictor of lameness. In normal or thin cows, lameness prediction required the combination of both BCS and TFT measurements.

Keywords: *Dairy cow, Lameness, Body condition score*

Siyah-Alaca İneklerde Vücut Kondisyon Skoru ve Ultrasonla Belirlenmiş Vücut Yağ Katmanı Kalınlığının Topallık Gelişme Riski Üzerine Etkisi

Özet

Bu çalışmanın amacı, Siyah-Alaca süt ineklerinin tuber ischiadicum üzerindeki yağ kalınlığının (TFT) ultrasonla ölçümü, vücut kondisyonu skorlama (BCS) ve topallık gelişme riski arasındaki korelasyonu incelemekti. Bir çiftlikteki kurudaki inek popülasyonundan 100 baş inek kaydedildi. TFT ultrason ile ölçülürken, BCS ve hareket puanı emzirme döneminde belirlendi. Yüz baş ineğin %31'inde laktasyon döneminde topallık gelişti. En yüksek topal inek oranı BCS≥4.25 (%66.7) ineklerde oldu. Topallık gelişme riski, BCS=3.75 optimum olan ineklere kıyasla BCS ≥4.25 (OR=7) ve ≤3.25 (OR=) olan ineklerde daha yüksekti. Daha düşük TFT çeyrekli inekler, daha yüksek çeyreğe göre daha yüksek bir topallık oranına sahipti. Zayıf ineklerdeki TFT topallığın göstergesi olarak belli bir değere sahip olabilir. Hem yağlı hem de zayıf ineklerdeki topallığın en iyi tahmini (ROCAUC=0.8725, P<0.01), BCS ve TFT değerleri her ikisi birlikte kullanıldığı zaman oluştu. Topallığa yakalanma riski BCS ile pozitif, TFT ile negatif ve bunların etkileşimi ile de negatif korelasyonlu idi. Yağlı inekler için, BCS tayini topallığın uygun şekilde güçlü bir göstergesidir. Normal veya zayıf ineklerde ise, topallık tahmini hem BCS hem de TFT ölçümlerinin kombinasyonunu gerektirdi.

Anahtar sözcükler: *Sütçü inek, Topallık, Vücut kondisyon skoru*

INTRODUCTION

Lameness is one of the most important endemic diseases of cattle, particularly in the dairy sector. It has a significant impact on health and welfare and leads to a range of production losses [1]. Furthermore, it reduces longevity [2] causes pain [3], influences milk production [4,5] and reproductive performance [6,7], and consequently, has

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a great economic effect [8]. Lameness in cattle is not a single condition, but rather is a symptom of a wide range of different diseases. The etiology and pathogenesis of many of these diseases remain relatively poorly understood. Claw horn disruption (CHD) is a common underlying cause of lameness in dairy cattle and leads to compromised animal welfare and production losses [9]. A greater risk of lameness and claw horn disruption lesions developing in cows with lower body condition score (BCS) and lower digital cushion thickness (DCT) has been described in cross sectional study [10]. Cows with low BCS (≤2 on a scale 0 to 5) are more likely to be treated for lameness in the four months following such a score [11]. This supports the hypothesis that low BCS are correlated with reduced digital cushion thickness, which can be associated with claw horn disruption lesions [10-14]. BCS is the most common method to evaluate the subcutaneous adipose tissue depot of the cow [12]. This is a widely accepted management tool to estimate the amount of adipose tissue laid down as energy storage at parturition, but which can be lost after parturition. This data can be used to predict the lactation performance, reproduction, and general health of the cow. As a part of dairy herd management, BCS can be used as an attempt to assess the magnitude of the energy deficit [13]. In a former study [14], we found that ultrasound determination of digital cushion thickness can be used for predicting CHD lesion development. However, ultrasound examination of the cattle acropodium has significant obstacles. First of all, there is a hard horn with moisture content lower than skin and loose layers filled with air. In a preliminary study [15], it was reported that an absolute requirement for ultrasound examination is claw trimming with removal of the air-filled layer of hoof horn and to make a flat surface. This procedure involves restraining the animal in a crush, which is very stressful. Then, BCS is influenced by the experience of the observer and many inter-observer differences can occur.

The purpose of this study was to examine the correlations between ultrasound measurement of thickness of fat over the *tuber ischiadicum* (TFT), BCS scores, and risk of lameness developing.

MATERIAL and METHODS

Animals and Study Design

Altogether, 100 Holstein-Friesian cows were enrolled. The cows were selected from population of dry cows, and were housed on a dairy farm with a cubicle housing system. They were fed a total mixed ration based on alfalfa hay, sugar beet pulp, corn silage, and concentrate. The average milk yield at the previous lactation was 7794±1210 kg/305 days. All cows were under the competent and permanent supervision of an employed veterinarian, with daily veterinary examination.

Ultrasound Measurement

The thickness of fat over the *tuber ischiadicum* (TFT) was measured with ultrasound using a linear probe (8 MHz) and ultrasound device Falco vet (Esaote Pie Medical). The measurement was taken at the point of *tuber ischiadicum* *(Fig. 1; Fig 2)* during the dry period, 4 to 6 weeks prior to calving.

Body Condition Score

A BCS system 1-5 [16] which incorporates a numerical scale, with thin animals receiving lower scores and fat animals receiving higher scores, was employed. All cows were scored in the dry period, 4 to 6 weeks prior to calving.

Lameness Diagnosis

All cows were scored for locomotion in the dry period

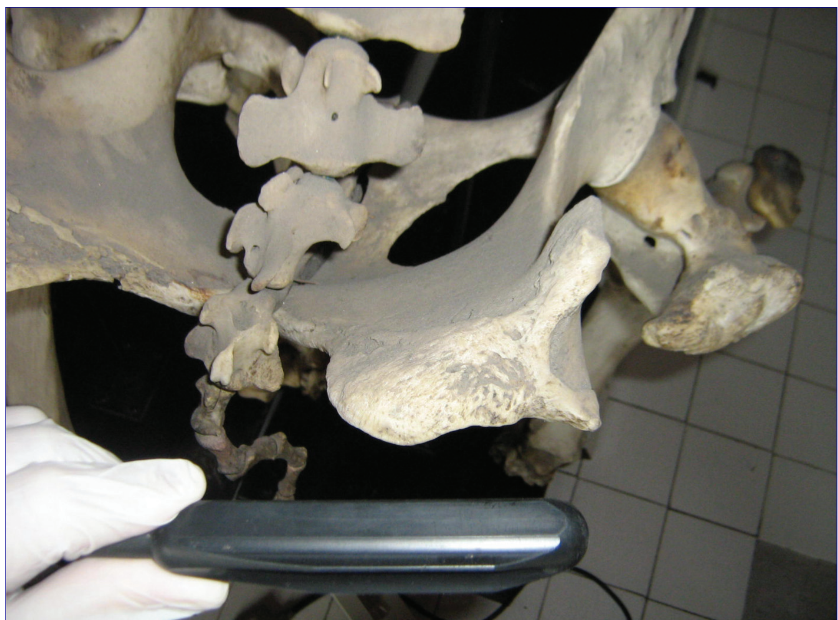

Fig 1. The point of *tuber ischiadicum* measurement

Şekil 1. *Tuber ischiadicum* ölçüm noktası

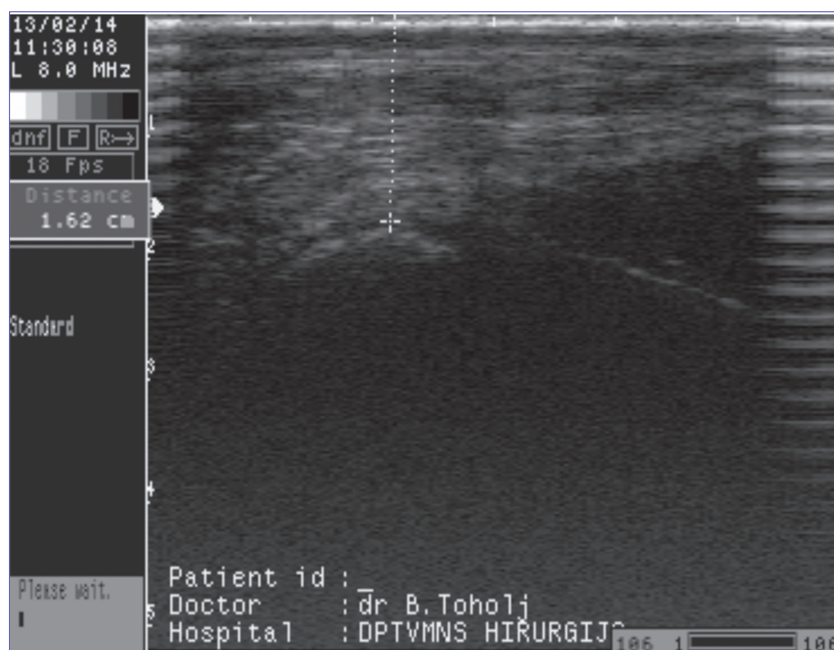

Fig 2. Ultrasound image of a typical *tuber ischiadicum* viewed among the 100 dairy cows

Şekil 2. *Tuber ischiadicum*'un yüz inek arasında gözlemlenen tipik ölçüm noktası

and six month after calving. The locomotion scoring system as developed by [17] using a 1 to 5 score, was employed. A score of 1 and 2 denotes sound locomotion, whereas scores of 3 or higher describe clinical lameness.

Statistical Analyses

The correlation between BCS and TFT was determined using Spearman's coefficient of correlation.

The influence of BCS and TFT on lameness developing was determined using a logistic regression model, separately for BCS, TFT and BCS×TFT interaction.

Risk of lameness development in cows with high or low BCS and TFT, in comparison with the optimal values, was analyzed by calculation of odds ratio using a 2×2 table. Tables contained the proportions of cows with lameness when they were classified into different classes of BCS (BCS≤3.25; BCS 3.5; BCS 3.75; BCS 4; BCS≥4.25) and TFT (lower quartile ≤1.27; lower medial quartile 1.28-1.40; upper medial quartile 1.41-1.54; higher quartile ≥1.54). Differences in proportions were determined using a t-test for proportion.

Prediction capacity of BCS, TFT and BCS×TFT interaction for lameness development was analysed by ROC curve and the area under the ROC curve (AUC ROC). ROC curve is plot that illustrated the relationship between X - true positive cow and Y - false positive cow in logarithmic regression model: $Y= a \times \ln(x)+b$. AUC ROC gives information about correct detection of lameness in cows with different BCS and TFT values, so that correct detection of cows will be estimated as: fail (AUC ROC=0.5-0.6), poor (0.6-0.7), fair (0.7-0.8), good (0.8-0.9) or excellent (0.9-1). We constructed the following ROC curve: 1) true positive cows (lame cows with BCS≥4.25 or ≤3.25) and Y - false positive cows (lame cows with optimal BCS 3.5-4); 2) true positive cows (lame cows with TFT in the lower or upper quartiles) and Y - false positive cows (lame cows with TFT in the optimal-median quartile); 3) true positive cows (lame fat cows BCS≥4.25, TFT ≥1.54; lame thin cows ≤3.25, TFT≤1.27) and Y - false positive cows (lame cows with optimal BCS and optimal TFT).

For this investigation we used a statistical software Statgraphic centurion and Microsoft Office Excel.

RESULTS

BCS and TFT Correlation

Result showed a strong positive correlation between values of TFT and BCS. In 28% of cases, the TFT variation depended on the BCS variation *(Fig. 3)*.

Relationships Between BCS, TFT and Lameness Development

Relationships between BCS and TFT in the dry period with lameness development during lactation were not statistically significant. However, lameness was positively regressed with BCS, but negatively regressed with TFT. Importantly, though, lameness was statistically significantly correlated to both BCS and TFT (BCS×TFT). Models and tests of regression parameters (variables) and intercept are presented in *Table 1*.

Different Level of BCS and TFT and Risk of Lameness Development

Altogether, 31% of cows develop lameness in lactation. The highest proportion of lame cows was in the group of cows with BCS≥4.25 (66.7%), and lowest proportion was found in group with optimal BCS=3.75 (20%) *(Table 2)*. The risk of lameness developing in cows with BCS≤3.25 was

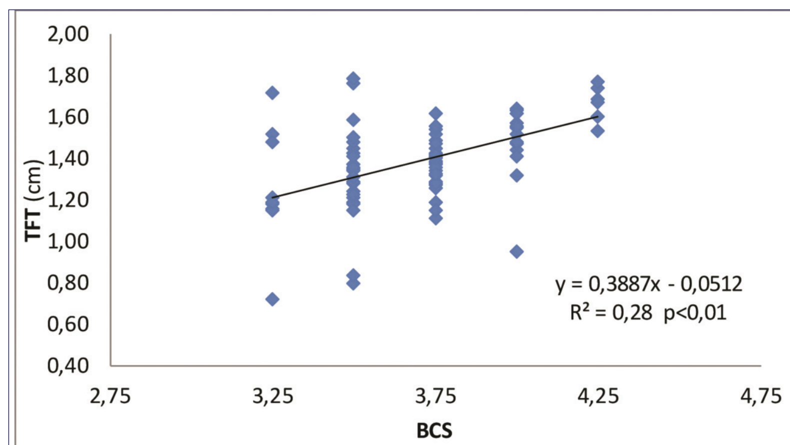

Fig 3. Correlation between TFT and BCS value in dairy cows

Şekil 3. Sütçü ineklerde TFT ve BCS arasındaki korelasyon

Table 1. *Testing of logistic regression model parameters for lameness developing as a function of BCS, TFT and BCS×TFT*
Tablo 1. *BCS, TFT ve BCSxTFT'nin bir işlevi olarak lojistik regresyon modeli parametre testi*

Independet Variable	Regression Formula						
BCS	**Lameness occurrence=1/(1+exp (-(-3.75+0.79×BCS))) Chi Square=0.99; df=1; p=0.31ns**						
	Variable	**Coeff.**	**SE**	**P**	**O.R.**	**Low**	**High**
	BCS	0.79	0.80	0.32	2.22	0.46	10.71
	Intercept	-3.75	3.00	0.21			
TFT	**Lameness occurrence =1/(1+exp (-(1.12-1.38×TFT))) Chi Square=1.67; df=1; p= 0.19ns**						
	Variable	**Coeff.**	**SE**	**P**	**O.R.**	**Low**	**High**
	TFT	-1.38	1.07	0.19	0.25	0.03	2.06
	Intercept	1.12	1.49	0.45			
BCS×TFT	**Lameness occurrence =1/(1+exp (-(-4.55+2.14×BCS-3.05×TFT))) Chi Square= 5.99; df=2; p=0.04**						
	Variable	**Coeff.**	**SE**	**P**	**O.R.**	**Low**	**High**
	BCS	2.14	1.08	0.04	8.53	1.01	72.94
	TFT	-3.05	1.44	0.03	0.047	0.028	0.8
	Intercept	-4.55	3.18	0.15			

ns Non-significant model P>0.05

two times higher (O.R.=2) then in the group of cows with BCS=3.75 *(Table 3)*. The cows with BCS≥4.25 were seven times more likely to develop lameness compared with cows with BCS=3.75. The risk of lameness development was not statistically significant from 1 between other groups of BCS.

The highest proportion of lame cows were in the first TFT quartile (42.3%) and the lowest proportion of lame cows were in third quartile (20.8%) *(Table 4)*. The risk of lameness developing in cows in the lower TFT quartile was higher then in cows in the other quartiles. Cows in higher TFT quartiles did not show a statistically significant increased risk of developing lameness *(Table 5)*.

Prognostic Value of BCS, TFT and BCS×TFT in Lameness Development

High BCS≥4.25 in the dry period was a good indicator for lameness development during lactation (AUCROC=0.7556; P<0.05; *Fig. 4*). Prediction of lameness in thin cows (BCS<3.25) was not statistically significant (AUCROC=0.62; P>0.05). The possibility of lameness prediction using TFT was most relevant in the lowest quartiles of TFT values compared with upper quartiles. However, it was impossible to strictly determine a numeric risk (in terms of TFT) for lameness developing (AUCROC 0.57; P>0.05). The best model for predicting lameness in cows was obtained when both BCS and TFT were used in the prediction model for both fat and thin cows (ROCAUC=0.8725, P<0.01; *Fig. 5*).

DISCUSSION

The mean prevalence of lameness in dairy herds is approximately 20% [18,19]. In our investigation, 31% of enrolled cows develop lameness in lactation. The prevalence of lameness in Europe has been estimated at 1.2% in 34 zero-grazing herds in The Netherlands [20], 5% on 101 farms in Sweden [21], and 22% on 53 farms in England [22]. The

Table 2. *Number or proportion of cows with lameness within each BCS category*

Tablo 2. *Her bir BCS kategorisindeki topal inek sayısı veya oranı*

Parameter	BCS				
	≤3.25	3.5	3.75	4	≥4.25
Lame (n)	4	8	7	8	4
Sound (n)	7	17	28	15	2
Proportion (%)	36[a]	32[a]	20[a]	34.7[a]	66.7[b]

[a,b] *Numbers with different superscripts in a row are significantly different; P<0.01*

Table 3. *Risk for lameness occurrence (odds ratio; OR) in cows with low BCS≤3.25 or high BCS≥4.25 in comparison with optimal BCS=3.5-4.0*

Tablo 3. *Optimal BCS=3.5-4.0 olanlara kıyasla, düşük BCS≤3.25 veya yüksek BCS≥4.25 ineklerdeki topallık oluşum (odds ratio; OR) riski*

BCS Risk	OR	Low	High
3.25 to 3.50	1.07	0.90	2.20
3.25 to 3.75	2.00*	1.50	3.60
3.25 to 4.00	1.07	0.90	2.20
3.25 to 4.25	0.29	0.11	0.42
4.25 to 3.25	3.50*	2.50	4.20
4.25 to 3.50	3.75*	2.80	4.40
4.25 to 3.75	7.00*	4.90	9.20
4.25 to 4.00	3.75*	2.80	5.10

** Risks that are statistically greater than; **1** = Real, increased risk*

Table 4. *Number or proportion (%) of cows with lameness within each TFT quartile grouping*

Tablo 4. *Her bir TFT çeyrek gruplandırması içindeki topal inek sayısı veya oranı (%)*

Parameter	TFT Quartiles			
	≤1.27	1.28-1.4	1.41-1.54	≥1.54
Lame (n)	11	7	5	8
Sound (n)	15	18	19	17
Proportion (%)	42.3[a]	28[b]	20.8[b]	32[b]

[a, b] *Numbers with different superscripts in a row are significantly different; P<0.01*

Table 5. *Risk of lameness occurrence (odds ratio; OR) in cows with TFT scores in the lower and upper quartiles in comparison with median quartiles*

Tablo 5. *Median çeyrekli olanlara kıyasla, daha düşük ve daha yüksek çeyrekli TFT skorlu ineklerde topallık oluşum (odds ratio; OR) riski*

TFT Risk	OR	Low	High
0.10-1.27 to 1.28-1.4	1.88	0.90	2.60
0.10-1.27 to 1.41-1.54	3.23*	1.80	4.30
0.10-1.27 to >1.54	1.55	0.74	2.42
>1.54 to 1.28-1.4	1.11	0.60	2.10
>1.54 to 1.41-1.54	1.25	0.55	2.20

** Risks that are statistically greater than; **1** = Real, increased risk*

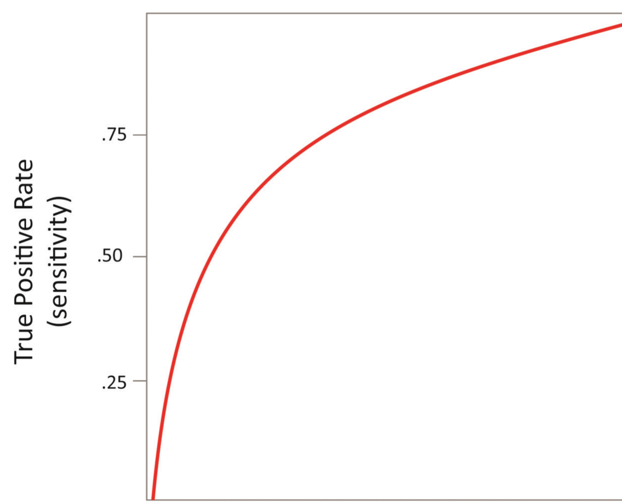

Fig 4. ROC curve and area under curve in prognosis of lameness in cows with high BCS

Şekil 4. Yüksek BCS'li ineklerde topallık prognozunda ROC eğrisi ve eğri-altı alanı

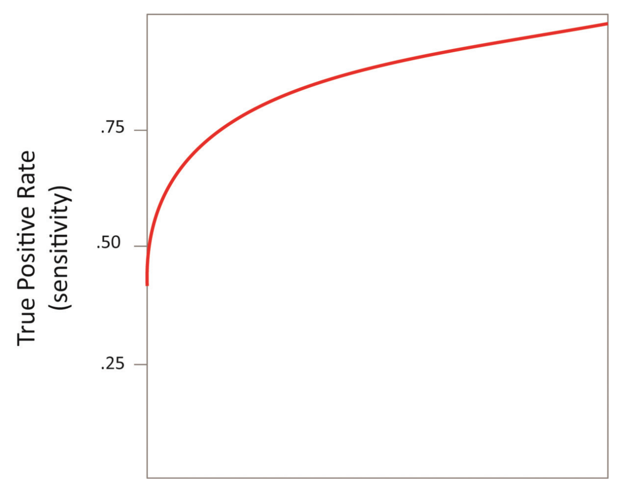

Fig 5. ROC curve and area under curve in prognosis of lameness in cows with BCS×TFT interaction

Şekil 5. BCS × TFT etkileşimli ineklerde topallık prognozunda ROC eğrisi ve eğri-altı alanı

prevalence of dairy cow lameness in our study was even higher, but this could have been a result of us enrolling a lower number of animals from one herd only. This was done because the purpose of our study was to find if there was any possible correlation of BCS and TFT with lameness.

An investigation into the incidence [23] of lameness in the United Kingdom revealed that approximately 50 cases/100 cows were stricken with lameness annually. The poor correlation between lameness incidence rates and records of treatments for lameness on-farm has been highlighted in some research [24], which suggests that the true incidence of lameness is likely to be higher than the rates cited above.

BCS and TFT Correlation

The ideal body condition during each stage of lactation is that which optimizes milk production, minimizes reproductive and health disorders, and maximizes economic returns [25]. A precise assessment of body energy stores is needed to increase the efficiency of milk production. In our investigation, an ultrasound device was used to measure the thickness of fat over the *tuber ischiadicum* (TFT). The image is generated by the sound waves being reflected from boundaries between different tissue densities [26]. In this particular case, the boundaries existed between adipose tissue and bone surface. Our results show a strong positive correlation between TFT values and BCS. This can be easily explained because there is a lot of fat surrounding the cow tail structure and BCS mostly depends on the amount of fat deposits. Ultrasound measurement of back fat thickness has been described earlier as a valuable method for assessing the body fat deposits in cows. The most common place for measuring of back fat thickness was at an imaginary line between the hooks and pins at the sacral examination site [13]. Our method seems to be more easily learned, because there are not a lot of tissue structures at the site where our measurement is taken, and we only measure the distance between skin and bone. If fat thickness in the gluteal region is measured, the examiner needs to distinguish the fat deposits among many other structures, such as superficial and deep gluteal fascia, gluteal muscles, etc.

BCS and Risk for Lameness Developing

Several cow-level factors have been associated with an increased incidence of lameness. BCS has been reported as being a suitable indicator of risk for lameness in several studies [11,27]. In our investigation, the highest proportions of lame cows were in the groups of cows with BCS≤3.25 (64.3%) and BCS≥4.25 (66.7%). Therefore, it seems that BCS, which are not optimal could be associated with an increased risk of lameness developing. One study has shown that cows with low BCS around parturition had 3 to 9 times higher odds of developing lameness compared with cows with higher BCS [29]. Cows with low BCS≤2 (on a scale 0 to 5) are more likely to be treated for lameness in the 2 or >2 to 4 months following such a score [11]. This can be explained by reduced capability of the digital cushion (fat tissue) to serve as shock absorbers during walking, which results in increased pressure on corium and consequently, the development of claw horn disruption lesions. The impaired shock-absorbing properties of the digital cushion seem to be a crucial factor leading to concussions of the corium and developing a claw horn disruption lesions [29]. The digital cushion is a complex structure (composed mostly of adipose tissue) located underneath the distal phalanx and plays an important function of dampening compression of the corium tissue beneath the cushion. The biomechanical importance of the digital cushion in alleviating compression under the *tuberculum flexorum* of the distal phalanx is well known [29]. This finding supports the hypothesis that low BCS is associated with claw horn lesions, possibly due to reduced digital cushion thickness, which has been correlated with low BCS [10]. At the opposite end of the scale, a group of cows with high BCS and an increased proportion of lameness were also noticed. We hypothesize that increased BCS could also mean increased body weight, and therefore, the increased proportion of lame cows in this group may be due to increased body weight pressure on the claws.

TFT and Risk for Lameness Developing

Regardless of the fact that BCS was strongly positively correlated with our ultrasound-determined TFT, this value has limited importance in predicting a lameness event. The highest proportion of lame cows was in the first TFT quartile (42.3%) and the lowest proportion of lame cows was in third quartile (18.5%). The possibility of lameness prediction using TFT would be most relevant in the lowest quartiles. To date, we are not aware of any comparable data from other investigations, which studied the relationship between ultrasound-determined TFT and risk of lameness developing.

In conclusion, the results showed a strong positive correlation between TFT values and BCS. The cows with BCS≥4.25 or below 3.25 were much more likely to develop lameness in comparison with cows with normal BCS. The risk of lameness developing positively correlates with BCS, but negatively correlates with TFT and with their interaction (BCS×TFT). For fat cows, BCS assessment is a suitable predictor of future lameness. In normal or thin cows, lameness prediction requires a combination of BCS and TFT. In further research, the influence of TFT on prediction of lameness in normal and thin cows should be investigated.

REFERENCES

1. Huxley JN: Lameness in cattle: An ongoing concern. *Vet J,* 193, 610-611, 2012. DOI: 10.1016/j.tvjl.2012.06.039

2. Booth CJ, Warnick LD, Grohn YT, Maizon DO, Guard CL, Janssen D: Effect of lameness on culling in dairy cows. *J Dairy Sci,* 87, 4115-4122, 2004. DOI: 10.3168/jds.S0022-0302(04)73554-7

3. Rushen J, de Passille AM: Effects of roughness and compressibility of flooring on cow locomotion. *J Dairy Sci,* 89, 2965-2972, 2006. DOI: 10.3168/jds.S0022-0302(06)72568-1

4. Warnick LD, Janssen D, Guard CL, Grohn YT: The effect of lameness on milk production in dairy cows. *J Dairy Sci,* 84, 1988-1997, 2001. DOI:

10.3168/jds.S0022-0302(01)74642-5

5. Green LE, Hedges VJ, Schukken YH, Blowey RW, Packington AJ: The impact of clinical lameness on the milk yield of dairy cows. *J Dairy Sci,* 85, 2250-2256, 2002. DOI: 10.3168/jds.S0022-0302(02)74304-X

6. Hernandez J, Shearer JK, Webb DW: Effect of lameness on the calving-to-conception interval in dairy cows. *J Am Vet Med Assoc,* 218, 1611-1614, 2001. DOI: 10.2460/javma.2001.218.1611

7. Garbarino EJ, Hernandez JA, Shearer JK, Risco CA, Thatcher WW: Effect of lameness on ovarian activity in postpartum Holstein cows. *J Dairy Sci,* 87, 4123-4131, 2004. DOI: 10.3168/jds.S0022-0302(04)73555-9

8. Ettema JF, Ostergaard S: Economic decision making on prevention and control of clinical lameness in Danish dairy herds. *Livest Sci,* 102, 92-106, 2006. DOI: 10.1016/j.livprodsci.2005.11.021

9. Cook NB, Nordlund KV, Oetzel GR: Environmental influences on claw horn lesions associated with laminitis and subacute ruminal acidosis in dairy cows. *J Dairy Sci,* 87, E36-E46, 2004. DOI: 10.3168/jds.S0022-0302(04)70059-4

10. Bichalo R, Machado VS, Caixeta LS: Lameness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. *J Dairy Sci,* 92, 3175-3184, 2009. DOI: 10.3168/jds.2008-1827

11. Green LE, Huxley JN, Banks C, Green MJ: Temporal associations between low body condition, lameness and milk yield in a UK dairy herd. *Prev Vet Med,* 113, 63-71, 2014. DOI: 10.1016/j.prevetmed.2013.10.009

12. Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G: A body condition scoring chart for Holstein dairy-cows. *J Dairy Sci,* 72, 68-78, 1989. DOI: 10.3168/jds.S0022-0302(89)79081-0

13. Schroder UJ, Staufenbiel R: Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of back fat thickness. *J Dairy Sci,* 89, 1-14, 2006. DOI: 10.3168/jds.S0022-0302(06)72064-1

14. Toholj B, Cincović M, Stevančević M, Spasojevic J, Ivetic V, Potkonjak A: Evaluation of ultrasonography for measuring solar soft tissue thickness as a predictor of sole ulcer formation in Holstein-Friesian dairy cows. *Vet J,* 199, 290-294. 2014. DOI: 10.1016/j.tvjl.2013.11.005

15. Kofler J, Kübber P, Henninger W: Ultrasonographic imaging and thickness measurement of the sole horn and the underlying soft tissue layer in bovine claws. *Vet J,* 157, 322-331, 1999. DOI: 10.1053/tvjl.1998.0315

16. Ferguson JD, Galligan DT, Thomsen N: Principal descriptors of body condition score in Holstein cows. *J Dairy Sci,* 77, 1695-1703, 1994. DOI: 10.3168/jds.S0022-0302(94)77212-X

17. Sprecher DJ, Hosteler DE, Kaneene JB: A lameness scoring system that uses posture and gait to predict dairy cattle reproductive performance. *Theriogenology,* 47, 1179-1187, 1997. DOI: 10.1016/S0093-691X(97)00098-8

18. Cook NB: Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface. *J Am Vet Med Assoc,* 223, 1324-1328, 2003. DOI: 10.2460/javma.2003.223.1324

19. Espejo LA, Endres MI, Salfer JA: Prevalence of lameness in high-producing Holstein cows housed in freestall barns in Minnesota. *J Dairy Sci,* 89, 3052-3058, 2006. DOI: 10.3168/jds.S0022-0302(06)72579-6

20. Smits MC, Frankena JK, Metz JM, Noordhuizen J: Prevalence of digital disorders in zero-grazing dairy cows. *Livest Prod Sci,* 32, 231-244, 1992. DOI: 10.1016/S0301-6226(12)80004-2

21. Manske T: Hoof lesions and lameness in Swedish dairy cattle. *PhD. Diss.* Acta Univ Agric Sueciae Vet, Skara, Sweden, 2002.

22. Whay HR: Locomotion scoring and lameness detection in dairy cattle. *In Pract,* 24, 444-449, 2002. DOI: 10.1136/inpract.24.8.444

23. Why HR, Main CJ, Green LE, Webster AJF: Assessment of the welfare of dairy cattle using animal-based measurements: Direct observations and investigation of farm records. *Vet Rec,* 153, 197-102, 2003. DOI: 10.1136/vr.153.7.197

24. Gearhart MA, Curtis CR, Erb HN, Smith RD. Sniffen CJ, Chase LE, Cooper MD: Relationship of changes in condition score to cow health in Holsteins. *J Dairy Sci,* 73, 3132-3140, 1990. DOI: 10.3168/jds.S0022-0302(90)79002-9

25. Houghton PL, Turlington LM: Application of ultrasound for feeding and finishing animals: A review. *J Anim Sci,* 70, 930-941, 1992. DOI: 10.2527/1992.703930x

26. Hoedemaker M, Prange D, Gundelach Y: Body condition change ante- and postpartum, health and reproductive performance in German Holstein cows. *Reprod Domest Anim*, 44, 167-173, 2009. DOI: 10.1111/j.1439-0531.2007.00992.x

27. Lim PY, Huxley JN, Willshire JA, Green MJ, Othman AR, Kaler J: Unravelling the association between lameness and body condition score in dairy cattle using a multistate modelling approach. *Prev Vet Med*, 118, 370-377, 2015. DOI: 10.1016/j.prevetmed.2014.12.015

28. Raber M, Lischer C, Geyer H., Ossent P: The bovine digital cushion, a descriptive anatomical study. *Vet J,* 167, 258-264, 2004. DOI: 10.1016/S1090-0233(03)00053-4

29. Logue DN, Offer JE, McGovern RD: The bovine digital cushion-how crucial is it to contusions on the bearing surface of the claw of the cow? *Vet J,* 167, 220-221, 2004. DOI: 10.1016/j.tvjl.2004.02.004

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Research Article

Ameliorative Effect of Omega-3 in Carbon Tetrachloride Toxicity

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Abstract

Omega-3 is a polyunsaturated fatty acid known to have immunomodulatory functions. In the present study, ameliorative potential of omega-3 in experimental carbon tetrachloride (CCl_4) toxicity was investigated. Total of 40 adult male Wistar albino rats were allocated into five groups and were subcutaneously given once every two days for 6 weeks the followings: Group 1 (Control): 0.5 mL/kg serum physiologic, Group 2 (Omega): 0.5 g/kg omega-3, Group 3 (Vehicle): 0.5 ml/kg pure olive oil, Group 4 (CCl_4): 0.5 mL/kg CCl_4, Group 5 (CCl_4 + Omega): 0.5 mL/kg CCl_4 plus 0.5 g/kg omega-3. At the end of the treatments, blood samples were collected and necropsy was performed for collection of liver tissues. Serum AST, ALT, GGT, TAC, TOC, triglyceride, and visfatin levels were detected. Liver morphology and immunoreactivities against TGF-α, TGF-β, PPAR-α, and PPAR-γ were assessed. Serum AST, ALT, GGT, and TOC levels significantly increased while TAC level decreased in CCl_4 given animals as compared to the control group. No significant changes were observed in triglyceride and visfatin levels. Immunohistochemical staining revealed increased TGF-α and TGF-β expressions and decreased PPAR-α and PPAR-γ expressions in liver of CCl_4 given animals. Omega-3 supplementation has prominent effects in correcting the biochemical and immunohistochemical parameters studied as well as the tissue morphology. The results of the investigation indicated that omega-3 has ameliorative effects on the oxidative tissue degeneration and inflammatory processes induced by CCl_4 treatment in rats.

Keywords: *CCl_4, Omega-3, TGF-α, TGF-β, PPAR-α, PPAR-γ*

Karbon Tetraklorür Toksikasyonunda Omega-3'ün Koruyucu Etkisi

Özet

Omega-3 bağışıklığı düzenleyici fonksiyonları olduğu bilinen çoklu doymamış bir yağ asididir. Bu çalışmada, deneysel karbon tetraklorür (CCl_4) toksikasyonunda omega-3'ün koruyucu potansiyel etkisi araştırılmıştır. Toplam 40 adet ergin erkek Wistar albino rat eşit beş gruba ayrıldı ve 6 hafta süresince her iki günde bir olmak üzere subkutan yolla deneklere tarif edilen uygulamalar yapıldı; Grup 1 (kontrol): 0.5 mL/kg serum fizyolojik, Grup 2 (Omega): 0.5 g/kg omega-3, Grup 3 (Taşıt): 0.5 mL/kg saf zeytin yağı, Grup 4 (CCl_4): 0.5 mL/kg CCl_4, Grup 5 (CCl_4 + Omega): 0.5 ml/kg CCl_4 artı 0.5 g/kg omega-3. Araştırma süresinin sonunda, kan örnekleri alınan deneklere karaciğer doku örneklerinin toplanması amacıyla nekropsi uygulandı. Serum AST, ALT, GGT, TAC, TOC, trigliserid ve visfatin seviyeleri belirlendi. Karaciğer morfolojisi incelendi ve TGF-α, TGF-β, PPAR-α ve PPAR-γ immunreaktiviteleri tespit edildi. Kontrol grubu ile karşılaştırıldığında CCl_4 verilen hayvanlarda serum AST, ALT, GGT ve TOC seviyelerinin anlamlı derecede arttığı, TAC seviyesinin ise azaldığı gözlemlendi. Trigliserid ve visfatin seviyelerinde anlamlı bir değişikliğin olmadığı belirlendi. İmmunohistokimyasal boyamalarda CCl_4 verilen hayvanlarda TGF-α ve TGF-β immunreaktiviteleri artarken PPAR-α ve PPAR-γ immunreaktivitelerinde azalma dikkati çekti. Omega-3 takviyesinin incelenen biyokimyasal ve immunohistokimyasal parametreler üzerine olumlu etki gösterdiği belirlendi. Çalışma sonuçları omega-3'ün CCl_4 uygulanan ratlarda oluşan oksidatif doku hasarı ve yangısal süreci azaltmada etkili olduğunu göstermektedir.

Anahtar sözcükler: *CCl_4, Omega-3, TGF-α, TGF-β, PPAR-α, PPAR- γ*

INTRODUCTION

Liver is the primary organ in various metabolic activities. However, countless physiological and biochemical functions as well as its anatomic localization subject the organ in development of infectious and toxic degenerations [1,2]. Liver may recover after acute degeneration, if not chronic inflammation and/or death may develop. The worst result

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of the chronic inflammation is cirrhosis. Liver cirrhosis may cause portal hypertension, hepatic encephalopathy, and organ failure that may result in death of the patient [3,4].

Carbon tetrachloride (CCl_4) is a chemical compound frequently used in experimental studies to induce liver cirrhosis in laboratory animals as a model for human. It is a transparent, non-flammable, easily vaporizable, and colorless liquid [5,6]. In mammalian body, CCl_4 is first metabolized to trichlormethyl (CCl_3) by the enzymatic reduction of cytochrome p-450 and then converted to trichlormethylperoxy radical ($OOCCl_3$) in the presence of oxygen [5]. These reactive free radical products of CCl_4 may react with polyunsaturated fatty acids and cause the production of reactive oxygen derivatives, which triggers lipid peroxidation in biological membranes [7-9]. Oxygen radicals can induce a cascade of events that result in cellular degeneration, increased Kupffer cell activation in liver, and increased expression of some pro-fibrogenic and pro-inflammatory factors such as Tumor necrosis factor alpha (TNF-α) and Transforming growth factor beta 1 (TGF-β1) [10]. TGF-β1 functions in conversion of quiescent hepatic stellate cells into myofibroblast-like cells and suppresses degradation and stimulates production of extracellular matrix proteins [10], thereby is important in the development of hepatic fibrosis [11].

Omega-3 fatty acids are unsaturated fatty acids that have double bounds at the third carbon atom from the end of the carbon chain. Omega-3 fatty acids, which include dokozaheksanoic asit (DHA), eikozapentaenoic acid (EPA) and α-linolenic acid (ALA), are the part of cellular membranes and required for normal biological functioning. DHA and EPA are members of long chained polyunsaturated fatty acids and found in fish oil in large amounts [12,13]. Studies on omega-3 fatty acids showed that they have anti-oxidant, anti-inflammatory, anti-hypertensive, and anti-apoptotic effects [14,15].

Free radicals are known to involve in degeneration of cellular molecules and hence play role in aging, cancer, arteriosclerosis, and cirrhosis. Omega-3 fatty acids were shown to partially protect against these conditions by inhibiting development of ischemia, inflammation and production of free radicals [2,16-18].

Peroxisome proliferated activated receptors (PPARs) are nuclear receptors and found in 3 isoforms, namely PPAR-α, -β, and -γ [19,20]. PPAR-α is most frequently expressed in organs rich in fat tissue such as liver, heart, skeletal muscle, brown adipose tissue and kidney. Monocytes, macrophages, lymphocytes, vascular endothelial and smooth muscle cells show expression of PPAR-α the most. While PPAR-β is expressed mostly in fat tissue, skin and brain, PPAR-γ is seen in fat tissue, large intestine, heart, kidney, pancreas and spleen [21-23]. PPARs are activated by fatty acids and their derivatives. PPAR-α is activated by leukotriene B4 while PPAR-β with prostaglandin J2 [24,25].

Visfatin is an adipokin having insulin like functions and excreted from adipose tissue. Its expression is affected by TNF-α and interleukin 6 [26]. It was shown that visfatin play roles in lipid metabolism and inflammation [27]. It was shown that PPAR-γ ligands could increase the gene expression of visfatin in macrophages [28].

In this study, the roles of TGF-α, TGF-β, PPARs and recently discovered molecule, visfatin, were investigated in an experimental hepatic fibrosis model in rats induced by CCl_4 and also the potential protective effect of omega-3. Oxidant and antioxidant capacities as well as enzymatic changes in liver tissue in relation to the tissue degeneration were investigated by immune-histopathological and biochemical means.

MATERIAL and METHODS

Animal and Treatments

The ethical approval for the research was confirmed by Kafkas University Animal Care and Use Committee (Registration Number: 2012-71). All procedures were conducted in accordance with the 'Guide for Care and Use of Laboratory Animals' published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

Forty male Wistar-Albino rats weighing 270-300 g at 3 months of age were used in the study. The rats were housed at 20±2°C and 12 h/12 h light/dark cycle through the study. Standard pellet diet and tap water were provided *ad libitum*. The animals were divided into five groups each containing 8 animals and treated once every two days during a 6-week period with subcutaneous injections of the followings: Group 1 (Control): 0.5 ml/kg serum physiologic, Group 2 (Omega): 0.5 g/kg omega-3 (18% eicosapentaenoic acid and 12% docosahexaenoic acid), Group 3 (Vehicle): 0.5 ml/kg pure olive oil, Group 4 (CCl_4): 0.5 ml/kg CCl_4 mixed 1:1 volume with olive oil, and Group 5 (CCl_4 + Omega): 0.5 ml/kg CCl_4 mixed 1:1 volume with olive oil plus 0.5 g/kg omega-3.

Biochemical Analysis

At the end of the 6-week treatment period, intracardiac blood samples were collected into the serum tubes under ether anesthesia. Serum was separated by centrifugation of the blood samples at 3000 rpm for 10 minutes. The samples were maintained at -20°C until further analyses. Serum aspartate aminotransferase (AST) (ERBA DDS), alanine aminotransferase (ALT) (ERBA DDS), gamma glutamyl transferase (GGT) (TML), triglyceride (IBL), TAC and TOC (REL Assay Diagnostics, Gaziantep-Turkey) were measured colorimetrically using a spectrophotometer (Eon Biotex, USA), serum visfatin (SUN RED CAT NO: 201-11-0472) levels were determined using an ELISA kit.

Histopathology

Animals were sacrificed by decapitation under ether anesthesia. At necropsy, liver samples were collected and fixed in 10% buffered formaldehyde solution. After routine procedures, paraffin blocks were prepared and cut at 5 µ thickness for hematoxylin and eosin staining. The liver sections were viewed under light microscope for evaluation of pathological changes.

Immunohistochemistry

Avidin biotin peroxidase method with diaminobenzidine substrate color development was used for immunohistochemical staining for PPAR-α, PPAR-γ, TGF-α, and TGF-β in liver tissue sections. Antigen retrieval was accomplished by 0.01% trypsin treatment at 37°C for 30 min. Antibody dilutions were as follow: PPAR-α (abcam ab8934) 2 µg/mL, PPAR-γ (abcam ab19481) 4 µg/mL, TGF-α (abcam ab112030) 5 µg/mL, and TGF-β (abcam ab66043) 5 µg/mL. All antibody incubations were done at room temperature for 1 h. Liver sections were finally counterstained with Mayer's hematoxylin and examined under light microscope. Immunoreactivity was evaluated based on the density of the immunostain as weak, moderate, and strong.

in situ TUNEL Method for Apoptosis

Apoptotic cell death in liver was studied by DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA). Tissue sections cut at 4 µ thickness were processed through xylene and alcohol series. After several rinses in phosphate buffered saline (PBS), the sections were treated with Proteinase K solution for 30 min. Then, the sections were placed in an equilibration buffer and incubated with the reaction buffer composed of biotinylated nucleotide mix and terminal deoxynucleotidyl transferase at 37°C for 1 h. After incubation with sodium citrate solutions, endogenous peroxidase activity was blocked by 3% H_2O_2. The sections were incubated with streptavidin horseradish peroxidase solution, and color development was accomplished by 3,3-diaminobenzidine/H_2O_2. The sections were rinsed in distilled H_2O, counterstained with 0.1% methyl green, rinsed in distilled H_2O_2, dehydrated in butanol and xylene and finally coverslipped with Permount.

Statistical Analysis

Statistical analysis were performed by the statistical package SPSS, version 10.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis of data was carried out using one-way analysis of variance (ANOVA) followed by Duncan test. Results were expressed as mean ± standard error (mean ± SE). *P* values less than 0.05 were considered significant.

RESULTS

Biochemical Findings

The findings of biochemical investigations are summarized in *Table 1*. There were significant changes at activities of serum ALT, AST, GGT, TAC, TOC and triglycerides in the only CCl_4-treated group as compared to the control group, indicating that CCl_4 caused liver damage ($P<0.05$). No significant changes in visfatin were recorded in any of the groups studied.

Histopathological Findings

Normal liver histomorphology was observed in groups of control *(Fig. 1a)*, omega, and vehicle (Figures not shown). In only CCl_4 given group, hydropic degeneration, occasional coagulation necrosis, and sinusoidal dilatation were observed. Formation of fibrosis starting from the portal regions through the parenchyma with presence of occasional necrotic hepatocytes and mononuclear cellular infiltration was evident. Interlobular areas were widened due to development of fibrosis. Pseudolobule formations were also recognizable in severely affected regions *(Fig. 1b)*. The severity of the histopathological changes decreased in CCl_4 + Omega given group as compared to the only CCl_4 given group. The most recognizable findings in CCl_4 + Omega group were the fatty and hydropic degeneration of hepatocytes located close to the portal region. No fibrotic

Table 1. *Serum levels of AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma glutamyl transferase, TAC: Total antioxidant capacity, TOC: Total oxidant capacity, triglycerides, and visfatin in groups*

Tablo 1. *AST: Aspartat aminotranferaz, ALT: Alanin aminotransferaz, GGT: Gama glutamil transferaz, TAC: Total antioksidan kapasite, TOC: Total oksidan kapasite, trigliserit ve visfatin serum seviyeleri*

Parameter	Control	Omega	Vehicle	CCl_4	CCl_4 + Omega	*P* value
ALT (U/L)	$21.32±2.31^{bc}$	$15.60±1.97^{c}$	$25.88±4.28^{bc}$	$47.81±1.02^{a}$	$32.80±3.75^{b}$	0.000
AST (U/L)	$77.87±3.55^{c}$	$80.76±6.66^{c}$	$84.02±7.6^{bc}$	$118.36±6.96^{a}$	$99.50±4.18^{b}$	0.000
GGT (U/L)	$1.93±0.154^{bc}$	$1.98±0.296^{bc}$	$0.80±0.475^{c}$	$6.23±1.199^{a}$	$3.30±0.419^{b}$	0.001
TAC (µmol Trolox Eq/L)	$0.96±0.03^{a}$	$1.005±0.02^{a}$	$1.002±0.05^{a}$	$0.66±0.04^{c}$	$0.822±0.04^{b}$	0.000
TOC (µmol H_2O_2 Eq/L)	$11.52±1.66^{b}$	$12.71±3.06^{b}$	$18.80±3.02^{b}$	$20.80±1.60^{a}$	$16.73±2.06^{ab}$	0.037
Triglycerides (mg/dL)	$104.33±6.115^{b}$	$107.62±5.47^{b}$	$132.49±12.67^{a}$	$103.47±3.55^{b}$	$109.42±6.20^{b}$	0.05
Visfatin (ng/mL)	80.64±5.72	67.72±1.08	72.46±2.86	68.74±1.80	70.34±3.88	Ns

Values in a row with different superscripts differ from each other significantly (P<0.05)

changes were observed in any of the animals in this group. Hepatocytes located in midzonal and central areas were normal in histomorphology *(Fig. 1c)*.

Immunohistochemistry

- ***TGF-α:*** In the control group, mainly weak immunoreactivity against TGF-α antibody was seen throughout the hepatic regions *(Fig. 2a)*. Compared to the control, increased immunoreactivity was noted in liver of rats given CCl_4 only *(Fig. 2b)*. The degree of immunoreactivity against TGF-α antibody in CCl_4 + Omega group was near to *(Fig. 2c) that* observed in the control group. Immunoreactivity in omega and vehicle groups was also similar to that of the control group (Figures not shown).

- ***TGF-β:*** Very weak immunoreactivity against TGF-β was observed in the control group *(Fig. 3a)*. In CCl_4 only given group, cytoplasmic immunoreactivity was observed in the hepatocytes throughout the liver sections *(Fig. 3b)*. This immunoreactivity was stronger in hepatocytes located close to the portal regions. TGF-β immunoreactivity in CCl_4 +

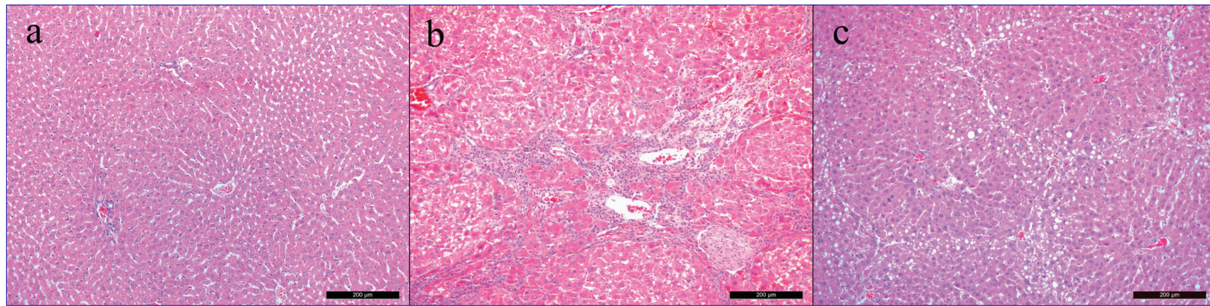

Fig 1. a) Control: Normal liver histomorphology, H&E, **b)** CCl_4: Hepatic fibrosis developing from the portal region through the hepatic lobules. Degenerated and occasional necrotic hepatocytes, accumulation of mononuclear cells, and some attempt for ill-formation of hepatic lobules, H&E, **c)** CCl_4 + Omega: Periportal fatty and hydropic degeneration, no fibrotic changes, H&E

Şekil 1. a) Kontrol: Karaciğerin normal histomorfolojisi, H&E, **b)** CCl_4: Portal bölgeden hepatik lobüllere doğru gelişen hepatik fibrozis. Dejenere ve nekrotik hepatositler, mononüklear hücrelerin toplanması ve bazı hatalı hepatik lobüllerin oluşma çabası, H&E, **c)** CCl_4 + Omega: Periportal alanda yağ ve hidropik dejenerasyonu, fibrotik değişiklikler bulunmamaktadır, H&E

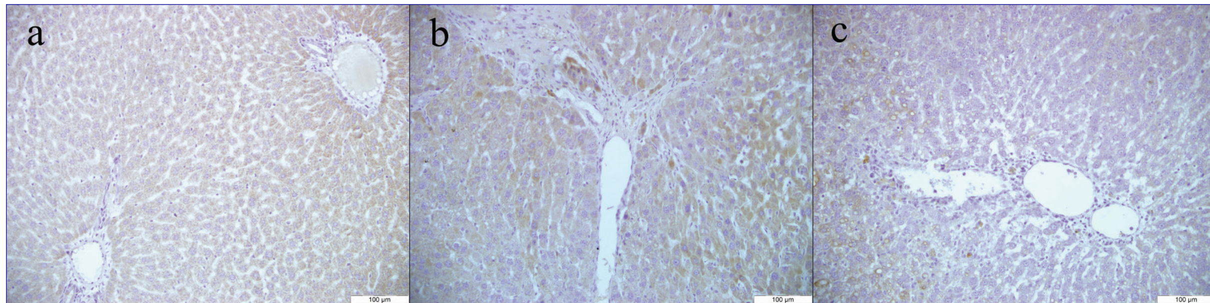

Fig 2. TGF-α immunohistochemistry; **a)** Control: Weak immunoreactivity, **b)** CCl_4: Moderate to strong immunoreactivity, **c)** CCl_4 + Omega: Weak immunoreactivity

Şekil 2. TGF-α immunohistokimyası; **a)** Kontrol: Zayıf immunoreaktivite, **b)** CCl_4: Ortadan güçlüye değişen derecede immunoreaktivite, **c)** CCl_4 + Omega: Zayıf immunoreaktivite

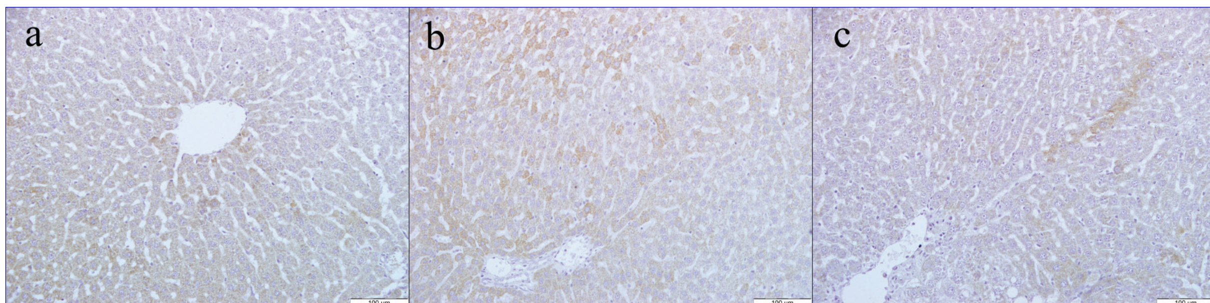

Fig 3. TGF-β immunohistochemistry; **a)** Control: Weak immunoreactivity, **b)** CCl_4: Moderate to strong immunoreactivity, **c)** CCl_4 + Omega: Weak immunoreactivity

Şekil 3. TGF-β immunohistokimyası; **a)** Kontrol: Zayıf immunoreaktivite, **b)** CCl_4: Ortadan güçlüye değişen derecede immunoreaktivite, **c)** CCl_4 + Omega: Zayıf immunoreaktivite

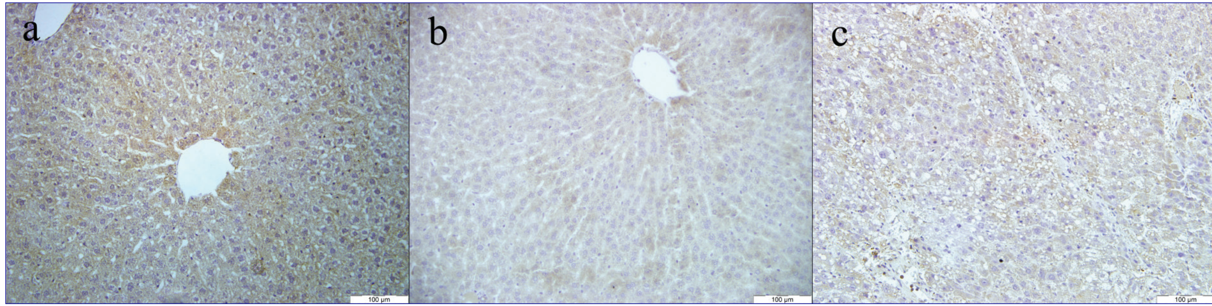

Fig 4. PPAR-α immunohistochemistry; **a)** Control: Strong immunoreactivity, **b)** CCl_4: Very weak immunoreactivity, **c)** CCl_4 + Omega: Weak to moderate immunoreactivity

Şekil 4. PPAR-α immunohistokimyası; **a)** Kontrol: Güçlü immunoreaktivite, **b)** CCl_4: Oldukça zayıf immunoreaktivite, **c)** CCl_4 + Omega: Zayıftan ortaya değişen derecede immunoreaktivite

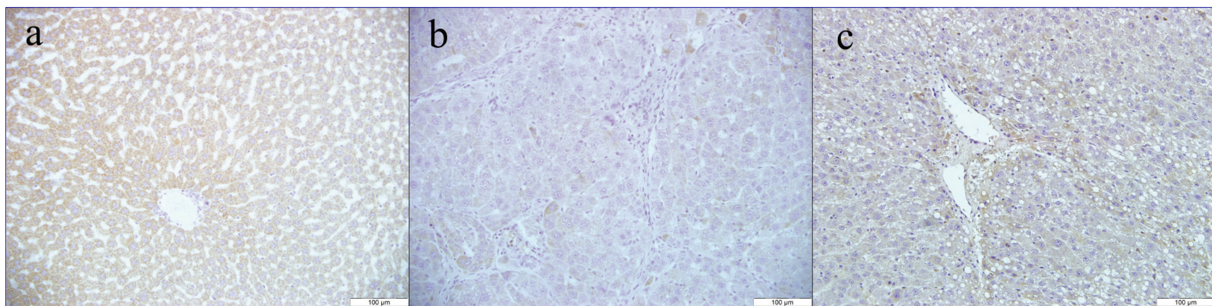

Fig 5. PPAR-γ immunohistochemistry; **a)** Control: Moderate to strong immunoreactivity, **b)** CCl_4: Very weak immunoreactivity, **c)** CCl_4 + Omega: Moderate immunoreactivity

Şekil 5. PPAR-γ immunohistokimyası; **a)** Kontrol: Oratadan güçlüye değişen derecede immunoreaktivite, **b)** CCl_4: Oldukça zayıf immunoreaktivite, **c)** CCl_4 + Omega: Orta derecede immunoreaktivite

Omega group was prominently reduced compared to the CCl_4 only given group *(Fig. 3c)*. The pattern and density for TGF-β immunoreactivity in group 2 and 3 were similar to that of the control group (Figures not shown).

- ***PPAR-α:*** Strong immunoreactivity against PPAR-α was observed in the control group *(Fig. 4a)*. Immunoreactivity was stronger in the periportal region as compared to the central region. In CCl_4 only given group, mostly no immunoreactivity was seen except in few scattered cells *(Fig. 4b)*. Weak to moderate immunoreactivity against PPAR-α was observed in CCl_4 + Omega group *(Fig. 4c)*. PPAR-α immunoreactivity in group 2 and 3 were similar to that of the control group (Figures not shown).

- ***PPAR-γ:*** Moderate to strong immunoreactivity against PPAR-γ was noted in the control group *(Fig. 5a)*. In CCl_4 only given group, weak immunoreactivity was observed. Immunoreactivity in this group was mostly observed in the Kupffer cells though hepatocytes also showed weak immunoreactivity *(Fig. 5b)*. Moderate immunoreaction was recognized in CCl_4 + Omega group *(Fig. 5c)*. PPAR-γ immunoreactivity in group 2 and 3 were similar to that of the control group (Figures not shown).

Apoptosis

No apoptotic cell death was detected by *in situ* TUNEL method in any of the groups including CCl_4 (Figures not shown).

DISCUSSION

Liver acts as the center of many important biological activities such as synthesis and/or storage of substances in the body. Hence, it might be considered that it is the most important organ in many metabolic activities. During the metabolic activation and/or detoxification of substances degenerative changes may take place resulting acute or chronic toxicity. Fatty accumulation and cirrhosis can also develop as a result of long lasting activities [1,29,30]. Despite the efforts of medical treatments, the medical management of the cases with liver degenerations might be insufficient and require other regimens [31]. The use of herbal substances and/or other non-classical medical treatments might be considered as a supplemental application [32,33]. In this study, the effect of omega-3 supplementation was assessed in the CCl_4-mediated experimental liver degeneration model in rats.

CCl_4-induced liver injury is a well-known animal model in hepatic toxicity studies [6,15,33,34]. The mode of toxicity induced by CCl_4 is that it is metabolically converted to CCl_3 by cytochrome P450, and CCl_3, under molecular oxygen rich environment, reacts with cellular proteins and poly-

unsaturated fatty acids to form more toxic trichlormethyl peroxy radicals and other oxygen radicals such as O_2^-, OH, and H_2O_2. Adduct formation with membrane phospholipids are primarily important in cellular degeneration through development of lipid peroxidation [5]. Disrupted membrane integrity is the main cause of cellular degeneration and resulting necrosis observed microscopically. CCl_4-induced liver degeneration and necrosis of hepatocytes were previously described by others [5,6,35-37]. In the present study, severe necrotic changes were as well observed due to CCl_4 treatment. Direct damage of lipid membranes as a result of free radicals is responsible for cellular degeneration. Increased TOC and decreased TAC levels support the notation that oxidative cellular degeneration is the cause of liver degeneration and necrosis.

Mononuclear cell infiltration and fibrosis was described in experimental CCl_4 toxicity studies [5,7,15,35,36]. Same findings were also noted in the present study. Additionally, fatty degeneration observed in many hepatocytes in CCl_4 given animals in the current study can be explained by interruption of triglyceride passage to blood and as a result their accumulation inside the cells resulting cellular vacuolation. Some studies also described apoptotic cellular death in experimental CCl_4 toxicities [38,39]. Such a cellular death was not observed in the present study. Severe cellular degeneration probably did not allow the development of apoptotic pathways as necrotic changes was the dominant cellular change in the CCl_4 given rats.

In evaluation of liver degeneration, liver function tests are commonly applied. Biochemical markers such as ALT, AST, GGT, albumin etc, can therefore provide priceless clinical clues about the development and degree of liver injury [40,41]. Upon hepatocellular degeneration release of these substances into the circulatory system increases as a result of the loss of membrane integrity. CCl_4 has been previously shown to cause increased serum levels of AST and ALT [1,32,36,42,43]. In the present study, serum ALT and AST levels were significantly higher in CCl_4 given rats as compared to the control. ALT is normally found higher in liver and kidney tissues whereas AST is higher in heart and skeletal muscles. Therefore, significant increase in serum ALT level correlates with liver injury. It has also been reported that AST level might increase in the early phase of liver degeneration and then the level could decrease with time [40,41]. In the present study increased levels of both ALT and AST clearly shows the presence of liver injury.

GGT is expressed in liver hepatocytes located close to the bile ducts, as well as renal proximal tubules, pancreas and small intestines. Serum GGT level mostly reflects the liver GGT level, and hence increased serum GGT level is usually an indicator of liver injury [40,41]. In the present study, increased serum GGT level may indicate the presence of degeneration in those hepatocytes located close to the portal regions. In histopathological observations, fibrotic changes and periportal degenerations in hepatocytes correlates the findings of biochemical analysis. Therefore, increased serum ALT, AST, and GGT levels as well as histopathological observations indicate the presence of liver injury in CCl_4 given rats.

In estimating the presence of cellular degeneration in general, detection of reactive oxygen species is used commonly [15,33,36,43]. However, detection of individual oxidant and/or antioxidant products can be very difficult as well as time and money consuming. Besides, analyzing the individual results from different oxidant and antioxidant products can be very complicated. Therefore, measuring total oxidant and antioxidant capacities and making comparisons are much in use and practical today. Even TAC and TOC can be used in monitoring the treatment in use for a given disease or experimental study [44,45]. In the present study, TOC increased while TAC decreased in CCl_4 given animals as compared to the control. The role of oxidative stress in CCl_4-induced hepatic toxicity has been previously shown as well [1,46,47]. On the other hand, in a CCl_4 toxicity study, unchanged TAC level has also been recorded though presence of increased TOC [48]. Omega-3 supplementation in the current investigation significantly increased the level of TAC as compared to the CCl_4 only given group. In addition, TOC level reduced, though not significantly, in the omega-3 supplemented group. These findings, together with the results of serum ALT, AST, and GGT levels, clearly indicated that omega-3 supplementation reduced the oxidative degenerative effects that are induced by CCl_4.

Liver is a key organ in lipid metabolism and plays important roles in fatty acid uptake, conversion, oxidation, and synthesis. It has been shown that CCl_4 administration increases cholesterol and triglycerides levels [1,49-51]. In the current investigation, an increase in triglyceride level was observed only in the vehicle group in that olive oil was given to the rats. However, no significant changes were observed among the other groups. Therefore, the result of this investigation contradicts the previous studies. It has been suggested that the increase in hepatic triglyceride content in olive oil given mice might be due to the disrupted mitochondrial fatty acid oxidation [52]. This might be the case in the present study.

In regulation of lipid metabolism, adipocytokines, which are released from the adipose tissue, may be critical. They have been also shown to be responsible for differentiation of stem cells to myofibroblast-like cells, which produces large amount of extracellular matrix substances [4,31,53]. Therefore, some of the adipocytokines have been suggested to be responsible in development of hepatic fibrosis [53,54]. Visfatin, which is a newly discovered adipocytokine found in adipocytes, hepatocytes, lymphocytes, monocytes and neutrophils, was studied to investigate its relationship with liver injury in the present study. The role visfatin in acute and chronic liver degenerations and fibrosis has not been well studied before and there are some controversial results. While it has been shown that visfatin concentration

is lower in cirrhotic patients [55], in other studies no correlation was found between fibrosis and serum visfatin level [56,57]. We also did not detect any significant changes in serum visfatin level on any of the groups studied indicating that visfatin has no apparent role on the omega-3 supplementation or the liver injury induced by CCl_4.

TGF-α and TGF-β expression in liver were investigated immunohistochemically to estimate the roles of these cytokines in liver injury [15,43]. TGF-α is known to be present in healthy liver as well as many other organs. It is a strong mitogen and thought to play role in hepatocyte regeneration after hepatic injury. Harada et al.[58] stated that TGF- α expression is closely related to severity of liver dysfunction. TGF-α is able to activate hepatic stellate cells, and that triggers the development of hepatic fibrosis [59]. It has been shown that following CCl_4 treatment expression of TGF-α rapidly increases and after few days prominently decline indicating that TGF-α play role early in the liver degeneration [38]. Similar finding and continously increased expression of TGF-β1 was also described by Tian et al.[60]. We have detected increased TGF-α immunoreactivity in rats given only CCl_4, and the immunoreactivity was near to control group in omega-3 supplemented animals indicating that omega-3 has beneficial effects in inhibiting the degenerative effects of CCl_4.

TGF-β has been described as an important cytokine in development of hepatic fibrosis. Sources of TGF-β in liver has been described primarily to be Kupffer cells and stellate cells [61]. TGF-β is known to be the most profibrotic cytokine and its expression significantly increases in hepatic cirrhosis [57]. It has been shown in a rat model of CCl_4 toxicity that TGF-β mRNA expression significantly increases as the Kupffer cell number increase indicating that these cells are primarily responsibly in development of hepatic fibrosis [14]. In the present investigation, TGF-β immunoreactivity increased in CCl_4 given animals, and omega-3 supplementation lowered the immunoreactivity to the level that was observed in the control group.

In the present investigated, we have studied the expression of PPARs in liver tissue. All three isoforms of PPAR, -α, -β, and -γ, are known to have important regulatory roles in not only inflammatory processes but also lipid biosynthesis, glucose metabolism, cellular proliferation and differentiation [19,62]. It has been suggested that PPAR-α plays a role in hepatic steatosis, and ciprofibrate, which is a potent ligand for PPAR-α, has a potential to prevent the negative effects, indicating that PPAR-α overexpression or activation is important in amelioration of degenerative changes in liver [63]. PPAR-γ overexpression in liver has also been suggested to be important and inhibits the fibrotic changes in liver [34,64]. The number of PPAR-γ expressing cells has also been shown to decrease by CCl_4 treatment [34,42]. In the current investigation, immunohistochemical reactivity of both PPAR-α and PPAR-γ, increased in omega-3 supplemented rats compared to the only CCl_4 injected animals. Therefore, our results correlate the findings of previous studies [42,65,66] indicating CCl_4 treatment prominently reduce the expression of PPAR-γ, and that omega-3 supplementation has beneficial effects in reducing the development of degenerative changes induced by CCl_4.

In conclusion, we have observed that intraperitoneal injection of CCl_4 at a dose of 0.5 mg/kg in rats causes fatty degeneration and necrosis as well as fibrosis especially in the periportal region in liver. Biochemical analysis for AST, ALT and GGT confirmed the hepatic injury. Decreased TAC and increased TOC levels indicated that the degenerative changes in liver were mediated by oxidative pathways. Visfatin, a recently discovered adipocytokine known to play role in fat metabolism, was shown to have no significant effect in hepatic fibrosis in the given experiment. On the other hand, increased TGF-β and decreased PPAR-α and PPAR-γ expressions were recognized to be associated with development of hepatic fibrosis in CCl_4-treated animals. Omega-3 supplementation, on the other hand was shown to normalize the above-mentioned parameters studied, indicating clearly that it has beneficial effect in amelioration of the degenerative changes induced by CCl_4. It seems that the protective effect of omega-3 is mediated primarily by inhibiting the lipid peroxidation.

REFERENCES

1. Kober H,Tatsch E, Torbitz VD, Cargnin LP, Sangoi MB, Bochi GV, da Silva AR, Barbisan F, Ribeiro EE, da Cruz IB, Moresco RN: Genoprotective and hepatoprotective effects of Guarana *(Paullinia cupana* Mart. var. sorbilis) on CCl_4-induced liver damage in rats. *Drug Chem Toxicol*, 39, 48-52, 2016. DOI: 10.3109/01480545.2015.1020546

2. Lee S, Kim S, Le HD, Meisel J, Strijbosch RAM, Nose V, Puder M: Reduction of hepatocellular injury after common bile duct ligation using omega-3 fatty acids. *J Pediatr Surg*, 43, 2010-2015, 2008. DOI: 10.1016/j.jpedsurg.2008.05.030

3. Berardis SI, Dawisthi Sattwika P, Najimi M, Sokal EM: Use of mesenchymal stem cells to treat liver fibrosis: Current situation and future prospects. *World J Gastroenterol,* 21, 742-758, 2015.

4. Raafat N, Abdel Aal SM, Abdo FK, El Ghonaimy NM: Mesenchymal stem cells: *In vivo* therapeutic application ameliorates carbon tetrachloride induced liver fibrosis in rats. *Int J Biochem Cell Biol*, 68, 109-118, 2015. DOI: 10.1016/j.biocel.2015.09.003

5. Manibusan MK, Odin M, Eastmond DA: Postulated carbon tetrachloride mode of action: A review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev,* 25, 185-209, 2007. DOI: 10.1080/10590500701569398

6. Hong RT, Xu JM, Mei Q: Melatonin ameliorates experimental hepatic fibrosis induced by carbon tetrachloride in rats. *World J Gastroenterol,* 15, 1452-1458, 2009.

7. Muriel P, Alba N, Pérez-Alvarez VM, Shibayama M, Tsutsumi VK: Kupffer cells inhibition prevents hepatic lipid peroxidation and damage induced by carbon tetrachloride. *Comp Biochem Physiol C Toxicol Pharmacol*, 130, 219-226, 2001. DOI: 10.1016/S1532-0456(01)00237-X

8. Sheweita SA, Abd El-Gabar M, Bastawy M: Carbon tetrachloride-induced changes in the activity of phase II drug-metabolizing enzyme in the liver of male rats: Role of antioxidants. *Toxicology,* 165, 217-224, 2001. DOI: 10.1016/S0300-483X(01)00429-2

9. Basu S: Carbon tetrachloride-induced lipid peroxidation: Eicosanoid formation and their regulation by antioxidant nutrients. *Toxicology,* 189, 113-127, 2003. DOI: 10.1016/S0300-483X(03)00157-4

10. Nan YM, Kong LB, Ren WG, Wang RQ, Du JH, Li WC, Zhao SX,

Zhang YG, Wu WJ, Di HL, Li Y, Yu J: Activation of peroxisome proliferator activated receptor alpha ameliorates ethanol mediated liver fibrosis in mice. *Lipids Health Dis*, 12, 1-10, 2013. DOI: 10.1186/1476-511X-12-11

11. Cheng K, Yang N, Mahato RI: TGF-beta1 gene silencing for treating liver fibrosis. *Mol Pharm*, 6, 772-779, 2009. DOI: 10.1021/mp9000469

12. Simopoulos AP: The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, 56, 365-279, 2002. 10.1016/S0753-3322(02)00253-6

13. de Lorgeril M, Salen P: New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. *BMC Med*, 10, 1-5, 2012, DOI: 10.1186/1741-7015-10-50

14. Pastor-Clerigues A,Marti-Bonmati E, Milara J, Almudever P, Cortijo J: Anti-inflammatory and anti-fibrotic profile of fish oil emulsions used in parenteral nutrition-associated liver disease. *PLoS One*, 9, 1-25, 2014. DOI: 10.1371/journal.pone.0115404

15. Shaaban AA, Shaker ME, Zalata KR, El-kashef HA, Ibrahim TM: Modulation of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis by olmesartan and omega-3. *Chem Biol Interact*, 25, 81-91, 2014. DOI: 10.1016/j.cbi.2013.10.008

16. Aguilera CM, Ramirez-Tortosa CL, Quiles JL, Yago MD, Martínez-Burgos MA, Martínez-Victoria E, Gil A, Ramirez-Tortosa MC: Monounsaturated and omega-3 but not omega-6 polyunsaturated fatty acids improve hepatic fibrosis in hypercholesterolemic rabbits. *Nutrition*, 21, 63-71, 2005. DOI: 10.1016/j.nut.2004.06.029

17. Calder PC: Polyunsaturated fatty acids and inflammation. *Prostaglandins Leukot Essent Fatty Acids*, 75, 197-202, 2006. DOI: 10.1016 /j.plefa.2006.05.012

18. Raptis DA, Limani P, Jang JH, Ungethüm U, Tschuor C, Graf R, Humar B, Clavien PA: GRP120 on Kupffer celss mediates hepato-protective effects of ω-3 fatty acids. *J Hepatol*, 60, 625-632, 2014. DOI: 10.1016/j.jhep.2013.11.006

19. Issemann I, Green S: Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, 347, 645-650, 1990. DOI: 10.1038/347645a0

20. Pawlak M, Lefebvre P, Staels B: Molecular mechamism of PPAR-α action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J Hepatol*, 62, 720-733, 2015. DOI: 10.1016/j.jhep.2014.10.039

21. Wolfrum C, Borrmann CM, Borchers T, Spener F: Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors alpha - and gamma-mediated gene expression via liver fatty acid binding protein: A signaling path to the nucleus. *Proc Natl Acad*, 98, 2323-2328, 2001. DOI: 10.1073/pnas.051619898

22. Akbiyik F, Ray DM, Bozkaya H, Demirpence E: Ligand- and species-dependent activation of PPAR alpha. *Cell Physiol Biochem*, 14, 269-276, 2004. DOI: 10.1159/000080336

23. Tunca R, Devrim AK, Sözmen M, Dağ S, Güngör O, İpek E: The effects of gemfibrozil and ovariectomy on the peroxisome proliferator activated receptors (PPARs) in mice with experimentally induced obesity. *Kafkas Univ Vet Fak Derg*, 19, 783-792, 2013. DOI: 10.9775/kvfd.2013.8788

24. Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W: The PPAR alpha-leukotriene B4 pathway to inflammation control. *Nature*, 384, 39-43, 1996. DOI: 10.1038/384039a0

25. Xin X, Yang S, Kowalski J, Gerritsen ME: Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and *in vivo*. *J Biol Chem*, 74, 9116-9121, 1999. DOI: 10.1074/jbc.274.13.9116

26. Hutcheson J: Adipokines influence the inflammatory balance in autoimmunity. *Cytokine*, 75, 272-279, 2015. DOI: 10.1016/j.cyto.2015.04.004

27. Al-Suhaimi EA, Shehzad A: Leptin, resistin and visfatin: The missing link between endocrine metabolic disorders and immunity. *Eur J Med Res*, 18, 1-13, 2013. DOI: 10.1186/2047-783X-18-12

28. Mayi TH, Rigamonti E, Pattou F, Staels B, Chinetti-Gbaguidi G: Liver x Receptor (LXR) activation negatively regulates visfatin expression in macrophages. *Biochem Biophys Res Commun*, 404, 458-462, 2011. DOI: 10.1016/j.bbrc.2010.12.002

29. Lee SH, Heo SI, Li L, Lee MJ, Wang MH: Antioxidant and hepatoprotective activities of *Cirsium setidens* NAKAI against CCl_4 -induced liver damage. *Am J Chin Med*, 36,107-114, 2008. DOI: 10.1142/S0192415X0800562X

30. Kopec AK, Joshi N, Luyendyk JP: Role of hemostatic factors in hepatic injury and disease: Animal models de-liver. *J Thromb Haemost*, 14,1337-1349, 2016. DOI: 10.1111/jth.13327

31. Thompson AJ, Patel K: Antifibrotic therapies: Will we ever get there? *Curr Gastroenterol Rep*, 12, 23-29, 2010. DOI: 10.1007/s11894-009-0080-9

32. Chang BY, Lee DS, Lee JK, Kim YC, Cho HK, Kim SY: Protective activity of kudzu *(Pueraria thunbergiana)* vine on chemically-induced hepatotoxicity: *in vitro* and *in vivo* studies. *BMC Complement Altern Med*, 16, 1-8, 2016. DOI: 10.1186/s12906-016-1023-2

33. Lu Y, Hu D, Ma S, Zhao X, Wang S, Wei G, Wang X, Wen A, Wang J: Protective effect of wedelolactone against CCl_4-induced acute liver injury in mice. *Int Immunopharmacol*, 34, 44-52, 2016. DOI: 10.1016/j.intimp.2016.02.003

34. Wang Z, Xu JP, Zheng YC, Chen W, Sun YW, Wu ZY, Luo M: Peroxisome proliferator-activated receptor gamma inhibits hepatic fibrosis in rats. *Hepatobiliary Pancreat Dis Int*, 10, 64-71, 2011. DOI: 10.1016/S1499-3872(11)60009-X

35. Jadhav VB, Thakare VN, Suralkar AA, Deshpande AD, Naik SR: Hepatoprotective activity of Luffa acutangula against CCl_4 and rifampicin induced liver toxicity in rats: A biochemical and histopathological evaluation. *Indian J Exp Biol*, 48, 822-829, 2010.

36. Suzek H, Celik I, Dogan A, Yildirim S: Protective effect and antioxidant role of sweetgum *(Liquidambar orientalis)* oil against carbon tetrachloride-induced hepatotoxicity and oxidative stress in rats. *Pharm Biol*. 54, 451-457, 2015. DOI: 10.3109/13880209.2015.1045086

37. Sen I, Turgut K, Ok M, Kıran MM, Güzelbekteş H, Ortatatlı M, Birdane FM, Altunok V: Effects of nutritional therapy or n-acetyl-cysteine (NAC) treatment on biochemical markers and liver histology in dogs with ccl4-induced hepatic necrosis. *Revue Med Vet*, 156, 483-490, 2005.

38. Liu J, Zhang QY, Yu LM, Liu B, Li MY, Zhu RZ: Phycocyanobilin accelerates liver regeneration and reduces mortality rate in carbon tetrachloride-induced liverinjury mice. *World J Gastroenterol*, 21, 5465-5472, 2015. DOI: 10.3748/wjg.v21.i18.5465

39. Özsoy ŞY: The protective effect of kefir on carbon tetrachloride-induced histopathological changes in the livers of rats. *Kafkas Univ Vet Fak Derg*, 22, 403-408, 2016. DOI: 10.9775/kvfd.2015.14825

40. Limdi JK, Hyde GM: Evaluation of abnormal liver function tests. *Postgrad Med J*, 9, 307-312, 2003. DOI: 10.1136/pmj.79.932.307

41. Giannini EG, Testa R, Savarino V: Liver enzyme alteration: A guide for clinicians. *CMAJ*, 172, 367-379, 2005. DOI: 10.1503/cmaj.1040752

42. Guo C, Xu L, He Q, Liang T, Duan X, Li R: Anti-fibrotic effects of puerarin on CCl_4-induced hepatic fibrosis in rats possibly through the regulation of PPAR-γ expression and inhibition of PI3K/Akt pathway. *Food Chem Toxicol*, 56, 436-442, 2013. DOI: 10.1016/j.fct.2013.02.051

43. Chu X, Wang H, Jiang YM, Zhang YY, Bao YF, Zhang X, Zhang JP, Guo H, Yang F, Luan YC, Dong YS: Ameliorative effects of tannic acid on carbon tetrachloride-induced liver fibrosis *in vivo* and *in vitro*. *J Pharmacol Sci*, 130, 15-23, 2016. DOI: 10.1016/j.jphs.2015.12.002

44. Aydin M, Oktar S, Yonden Z, Ozturk OH, Yilmaz B: Direct and indirect effects of kisspeptin on liver oxidant and antioxidant systems in young male rats. *Cell Biochem Funct*, 28, 293-299, 2010. DOI: 10.1002/cbf.1656

45. Sözen S, Kisakürek M, Yildiz F, Gönültaş M, Dinçel AS: The effects of glutamine on hepatic ischemia reperfusion injury in rats. *Hippokratia*, 15, 161-166, 2011.

46. Recknagel RO, Glende EAJ, Dolak JA, Waller RL: Mechanisms of carbon tetrachloride toxicity. *Pharmacol Ther*, 43,139-154, 1989. DOI: 10.1016/0163-7258(89)90050-8

47. Weber LW , Boll M, Stampfl A: Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model.

Crit Rev Toxicol, 33, 105-136, 2003. DOI: 10.1080/713611034

48. Demiroren K , Dogan Y, Kocamaz H , Ozercan IH , Ilhan S, Ustundag B, Bahcecioglu IH: Protective effects of L-carnitine, N-acetylcysteine and genistein in an experimental model of liver fibrosis. *Clin Res Hepatol Gastroenterol*, 38, 63-72, 2014. DOI: 10.1016/j.clinre.2013.08.014

49. Ince S, Keles H, Erdogan M, Hazman O, Kucukkurt I: Protective effect of boric acid against carbon tetrachloride-induced hepato-toxicity in mice. *Drug Chem Toxicol,* 35, 285-292, 2012. DOI: 10.3109/01480545.2011.607825

50. Li G, Wang XY, Suo YR, Wang HL: Protective effect of seed oil of Herpetospermum pedunculosum against carbon tetrachloride-induced liver injury in rats. *Saudi Med J*, 35,981-987, 2014.

51. Yuan C, Li Z, Yi M, Wang X, Peng F, Xiao F, Chen T, Wang C, Mushtaq G, Kamal MA: Effects of polysaccharides from selenium-enriched Pyracantha fortuneana on mice liver injury. *Med Chem,* 11, 780-788, 2015.

52. Ferramosca A, Savy V, Zara V: Olive oil increases the hepatic triacylglycerol content in mice by a distinct influence on the synthesis and oxidation of fatty acids. *Biosci Biotechnol Biochem*, 72, 62-69, 2008. DOI: 10.1271/bbb.70369

53. Anty R, Lemoine M: Liver fibrogenesis and metabolic factors. *Clin Res Hepatol Gastroenterol,*1, 10-20, 2011. DOI: 10.1016/S2210-7401(11)70003-1

54. Kukla M, Mazur W, Bułdak RJ, Zwirska-Korczala K: Potential role of leptin, adiponectin and three novel adipokines-visfatin, chemerin and vaspin-in chronic hepatitis. *Mol Med*, 17, 1397-1410, 2011. DOI: 10.2119/molmed.2010.00105

55. de Boer JF, Bahr MJ, Böker KH, Manns MP, Tietge UJ: Plasma levels of PBEF/Nampt/visfatin are decreased in patients with liver cirrhosis. *Am J Physiol Gastrointest Liver Physiol*, 296, G196-201, 2009. DOI: 10.1152/ajpgi.00029.2008

56. Baranova A,Jarrar MH, Stepanova M, Johnson A, Rafiq N, Gramlich T, Chandhoke V, Younossi ZM: Association of serum adipocytokines with hepatic steatosis and fibrosis in patients with chronic hepatitis C. *Digestion*, 83, 32-40, 2011.

57. Waluga M, Kukla M, Żorniak M, Kochel-Jankowska A, Kajor M, Krzemiński T, Kotulski R: Visfatin and TGF-β1 in primary biliary cirrhosis and two other common liver diseases. *Folia Med Cracov*, 55, 59-70, 2015.

58. Harada K, Shiota G, Kawasaki H. Transforming growth factor-alpha and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. *Liver*, 19, 318-325, 1999. DOI: 10.1111/j.1478-3231.1999.tb00056.x

59. Ohyama T, Yamazaki Y, Sato K, Horiguchi N, Ichikawa T, Kakizaki S, Takagi H, Mori M: Transforming growth factor-α attenuates hepatic fibrosis: Possible involvement of matrix metalloproteinase-1. *Liver Int*, 31, 572-584, 2011. DOI: 10.1111/j.1478-3231.2011.02475.x

60. Tian XF, Ji FJ, Zang HL, Cao H: Activation of the miR-34a/SIRT1/p53 signaling pathway contributes to the progress of liver fibrosis via inducing apoptosis in hepatocytes but not in HSCs. *PLoS One*, 11, 1-14, 2016. DOI: 10.1371/journal.pone.0158657

61. Seki E, Brenner DA: Recent advancement of molecular mechanisms of liver fibrosis. *J Hepatobiliary Pancreat Sci,* 22, 512-518, 2015. DOI: 10.1002/jhbp.245

62. Abbott BD: Review of the expression of peroxisome proliferator-activated receptors alpha (PPAR alpha), beta (PPAR beta), and gamma (PPAR gamma) in rodent and human development. *Reprod Toxicol,* 27, 246-257, 2009. DOI: 10.1016/j.reprotox.2008.10.001

63. Rao MS, Papreddy K, Musunuri S, Okonkwo A: Prevention/reversal of choline deficiency-induced steatohepatitis by a peroxisome proliferator-activated receptor alpha ligand in rats. *In Vivo,* 16, 145-152, 2002.

64. Yang L, Chan CC, Kwon OS, Liu S, McGhee J, Stimpson SA, Chen LZ, Harrington WW, Symonds WT, Rockey DC: Regulation of peroxisome proliferator-activated receptor-gamma in liver fibrosis. *Am J Physiol Gastrointest Liver Physiol*, 291, G902-G911, 2006. DOI: 10.1152/ajpgi.00124.2006

65. Liu Q, Wang CY, Liu Z, Ma XS, He YH, Chen SS, Bai XY: Hydroxysafflor yellow A suppresses liver fibrosis induced by carbon tetrachloride with high-fat diet by regulating PPAR-γ/p38 MAPK signaling. *Pharm Biol*, 52, 1085-1093, 2014. DOI: 10.3109/13880209.2013.877491

66. Orfila C, Lepert JC, Alric L, Carrera G, Béraud M, Pipy B: Immunohistochemical distribution of activated nuclear factor kappa B and peroxisome proliferator-activated receptors in carbon tetrachloride-induced chronic liver injury in rats. *Histochem Cell Biol,* 123, 585-593, 205. DOI: 10.1007/s00418-005-0785-2

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Research Article

Multiple Hypothesis Testing in a Genome Wide Association Study of Bovine Tuberculosis [1]

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[1] *Part of this study was presented as poster at XIVth Congress of Medical Biology and Genetics at Muğla/Turkey (27-30 October 2015) and published in congress abstract book*

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Abstract

Genome-wide association studies (GWAS) have been used to detect single nucleotide polymorphisms (SNPs) related to various animal traits. The outcome of GWAS is based on quality of the both phenotypic and genotypic datasets. False positive (or negative) associations can be obtained due to multiple hypothesis testing procedures, quality control measures, or an undetected population structure. The objectives of this study were to 1) investigate different multiple hypothesis testing procedures with different quality measures and 2) to detect and correct ancestral stratification using different single SNPs models of the bovine tuberculosis GWA data set. Based on a regression model, SNPs from chromosomes 2, 7, 8 and 13 were detected at a significance level of P<0.001 without correction for multiple hypothesis testing. However, after Bonferroni correction, Hochberg's method and permutation test for multiple hypothesis correction genomic signals, it became non-significant. Only a false discovery rate approach detected weak signals (at level of 0.54) from chromosomes 2, 8, and 13. We used a model that took into account the effect of linkage disequilibrium to the multiple hypothesis testing procedures by combining adjacent SNPs test statistics with windows sizes of 2, 4 and 6. We detected strong genomic signals from chromosomes 13, 8, 6 and 2 at windows size 6. The results of this study showed that multiple hypothesis testing procedures are related to false positive genomic signals. It is difficult to suggest universally acceptable multiple hypothesis testing and QC measures and their thresholds due to sources of variations between species and within populations. However, additional analytical approaches and studies are needed to evaluate the effects of linkage disequilibrium on the multiple hypothesis testing procedures and QC measures (especially for minor allele frequencies) to GWAS under various scenarios including, but not limited to, level of heritability, linkage disequilibrium, population structure, and population size.

Keywords: *Genome wide association analyses, Multiple hypothesis testing, Quality control procedures*

Sığır Tüberkülozu İçin Çoklu Hipotez Düzeltmesi İle Genom Tabanlı İlişki Analizi

Özet

Genom tabanlı ilişki çalışmaları (GTİÇ) kullanılarak çiftlik hayvanlarının verimleri ile ilişkili tekil nükleotid polymorfizmler (TNP) belirlenebilmektedir. GTİÇ'den elde edilecek sonuçlar hem fenotip hem de genotip veri setlerinin kalitesine bağlı olacaktır. Populasyon tabakası, çoklu hipotez düzeltim yöntemleri ve kalite kontol süreçleri yanlış pozitif (veya negatif) ilişki sonuçlarına yol açabilir. Bu çalışmanın amaçları: bir sığır tüberküloz GTİÇ veri setine 1) değişik kalite ölçütleri ve çoklu hipotez düzeltme yöntemlerinin 2) bazı TNP regresyon yöntemleri ile atasal tabakaların belirlenmesi ve düzeltilmesinin etkilerinin incelenmesidir. Çoklu hipotez düzeltmesi olmadan TNP regresyon modeli ile 2, 7, 8 ve 13. kromozomdan önemli TNP'ler (P<0.001) için belirlendi. Ama çoklu hipotez düzeltmesi Bonferroni düzeltmesi, Hochberg yöntemi ve permutasyon ile gerçekleştirildiğinde genomik sinyallerin önemsiz çıktığı gözlemlendi. Sadece yanlış keşif oranı yöntemi 0.54 seviyesinde zayıf genomik sinyalleri 2, 8 ve 13. kromozomdan belirledi. Çoklu hipotez testlerinde dengesiz bağıntıyı, 2, 4 ve 6 TNP pencere büyüklüğü için modele tanıttık. Pencere büyüklüğü 6 olunca 2, 6, 8 ve 13. kromozomlardan güçlü genomik sinyaller tespit ettik. Bu çalışmanın sonuçları çoklu hipotez test yöntemlerinin yanlış genomik sinyallerin keşfedilmesinde önemli olduğunu ortaya koydu ve doğruladı. Hem türler arası hem de populasyonlar içi varyasyon kaynakları nedeniyle evrensel kalite kontrol ölçütleri önermek oldukça zordur. Bununla birlikte kalıtım derecesi seviyeleri, dengesiz bağıntı, populasyon yapısı ve populasyon büyüklüğü dahil farklı senaryoların varlığında dengesiz bağıntının çoklu hipotez test yöntemlerine etkileri farklı kalite kontrol ölçütleri kullanılarak (özellikle farklı minör alel sıklığı seviyelerinde) GTİÇ için analitik olarak incelenmelidir.

Anahtar sözcükler: *Genom tabanlı ilişki analizi, Çoklu hipotez test yöntemleri, Kalite kontrol yöntemleri*

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INTRODUCTION

P values have been heavily used in frequentist statistics (and all other branch of sciences) to evaluate whether null hypothesis (stating there is no treatment effects) true or not. Optimal use of hypothesis testing is an active research area: recently the American Statistical Association published how to correctly use and interpret the p values [1]. P values could be used over time or space. For example Karacaören [2] employed longitudinal p values for modeling time effect in genomic studies. Zaykın et al.[3] used the combined p values over neighboring chromosomal locations (or space) in genome wide association studies (GWAS).

GWAS have been used to detect single nucleotide polymorphisms (SNPs) related to various animal traits. GWAS compare the allele frequencies of cases and controls to determine significant SNPs. Assumptions regarding the genetic architecture of the trait facilitate different statistical models in GWAS. If a trait is assumed to be controlled by many rare variants, a large amount of hypothesis testing must be conducted in piecemeal manner to detect the association [4]. Due to the SNPs depending on chromosomal locations, the naive use of multiple hypothesis testing procedures might lead to a loss of power [5,6]. Therefore, the outcome of GWAS is dependent on the model of multiple hypothesis testing and the quality of both the phenotypic and genotypic datasets [7,8]. False positive (or negative) associations may be obtained due to multiple hypothesis testing process, quality control measures and undetected population structure.

For example, current practices of animal breeding employ assortative mating to obtain higher selection responses in animal production. A GWAS model should take this relatedness structure into account using pedigree and/or genomic information. Several approaches have been proposed for detecting and correcting the effects due to common ancestral clusters using single SNP approaches. Although principal component-based approaches are commonly used in the literature, mixed model-based approaches have also gained popularity recently [9]. Price et al.[10] used principal component analysis to take into account of ancestral population stratifications. Aulchenko et al.[11] suggested conducting genome-wide rapid association using mixed model and regression (GRAMMAR) for phenotypes based on polygenic effects of pedigree structure. Amin et al.[12] extended the GRAMMAR approach by using genomic information instead of pedigree information. Svishcheva et al.[13] extended the original GRAMMAR approach by introducing a gamma factor to adjust the inflation factor. Karacaören [14] defined the original GRAMMAR approach in a Bayesian framework.

Bermingham et al.[15] used GRAMMAR and regional heritability mapping approaches to detect variants using a bovine tuberculosis (BT) dataset. BT is an infectious disease with annual economic costs estimated as €2 billion [16]. However availability (and therefore investigation) of public genomic livestock datasets especially for BT is uncommon. Main reasons are associated with economical and strategical values of the datasets [17]. Since BT dataset included high (617885 SNPs) number of explanatory variables: usage of different multiple hypothesis testing procedures may lead to interesting results. The objectives of this study were to 1) investigate different multiple hypothesis testing procedures with different quality measures and 2) to detect and correct ancestral stratification using different single SNP models for simulated [18] and the BT GWA dataset.

MATERIAL and METHODS

QTL-MAS Simulated DataSet

The quantitative trait locus marker assisted selection (QTL-MAS) simulated data set [18] included 2326 individuals from 10031 biallelic SNPs over 5 chromosomes. The pedigree was simulated according to the half sib family structure. A quantitative trait simulated in association with 37 QTLs. Major QTLs were located on chromomes 1 and 3. We considered mapped QTLs as if the predicted SNPs were located within 5Mb distance from true QTL position. We also investigated number of single mapped QTLs using the same criteria. More details about the dataset could be found at [18].

Phenotypes and Genotypes of the BT Dataset

The BT dataset was obtained from 1151 Holstein -Friesian cows in Northern Ireland [15]. The cases (n=592) and controls (n=559) were defined by single intradermal comparative tuberculin test and abattoir inspection. Individuals were genotyped using BovineHD Illumina Bead 617885 SNPChip. More details about the dataset could be found at [15].

Methods

We applied various quality control measures to the real BT genotypic data set. Due to selection over generations we are not expecting Hardy Weinberg equilibrium in the population. However minor allele frequencies (MAF), individual and genotypic call rates might be important for the outcome of GWAS [19]. Genomic inflation factors could be used to asses if the results of the GWA results are biased using distribution of the test statistics. Multidimensional scaling plots using genomic kinship matrix could be used to investigate genetic outliers in the populations.

Association mapping to detect quantitative trait locus controlling tuberclosis was implemented in R using the various GRAMMAR functions in the GenABEL package [20]. GRAMMAR (raw, genomic control or gamma versions) function uses a two step linear model. In the first step of the GRAMMAR analysis, we estimated the errors for the

phenotype using an animal model as was implemented in GenABEL;

$$y = \mathbf{X}b + \mathbf{Z}a + e \qquad \text{(i)}$$

where y contains the observations, b is the fixed effects of age, breed, season of year of and reason of tuberculosis, a is the additive genetic effect, matrices **X** and **Z** are incidence matrices, and **e** is a vector containing residuals.

$$Var\begin{pmatrix} a \\ e \end{pmatrix} \sim N\left[\mathbf{0}; \begin{pmatrix} \mathbf{A}\sigma_a^2 & \mathbf{0} \\ \mathbf{0} & \mathbf{I}\sigma_e^2 \end{pmatrix}\right]$$

For the random effects, it is assumed that A is the additive genomic relationship matrixfor the animals; I is an identity matrix, σ_a^2 is the additive genetic variance and σ_e^2 is the residual variance. In the second step, assuming a single SNP model for the quantitative trait, we could detect the most significant SNPs using the following model:

$$\mathbf{y} = \mathbf{X}\mathrm{f} + \boldsymbol{\eta} + \mathbf{e} \qquad \text{(ii)}$$

where y represents vector of n observations (residuals from (i)), $\boldsymbol{\eta}$ is intercept, $\mathbf{X}$ is a design matrix relating observations with f regression coefficients vector to be estimated, e is a vector of residuals assumed to be normally distributed. Svishcheva et al.[13] extended GRAMMAR approach by introducing gamma factor for adjusting inflation factor. Genetic stratification could also be detected by principal components [10]. Phenotypes of (i) were corrected by principal components for ancestral stratification as was implemented in GenABEL.

We used genomic relationship matrix [21] to take into account of pedigree structure: $A = WW'/c$ where c is a normalizing constant and $W_{ik} = X_{ik} + 1 - 2p_k$, p_k is the frequency of the 1 allele at marker k. We used the GWA function for association analysis was implemented in R by rrBLUP package [21]. Similar to the GRAMMAR; the GWA function use a mixed linear model to take into account of genetic stratification by genomic relationship matrix. Here the variance of random effect is assumed to be $2\mathbf{A}V_g$ where A is the genomic kinship matrix and V_g is the maximum likelihood estimate of polygenic variance.

Assumptions regarding underling genetic architecture in GWAS is crucial for choosing optimal statistical models for detection of causal variants. Since we assumed that many rare variants are in effect with bovine tuberculosis; we employed a single SNP regression in (ii). However evaluation of null hypothesis for huge number of SNPs may lead to false positive findings. In order to avoid type 1 errors (rejecting of a true null hypothesis); Bonferroni correction, Hochberg's method or false discovery rate approach could be used.

Since huge number of hypothesis testing needs to be done to detect the genomic association; type 1 errors should be controlled as such at α=0.05 level. If we have *m* number of SNPs to test: Bonferroni correction tests each SNPs at the significance level of 0.05/*m*. Different from Bonferroni correction: Hochberg's method compares each *p* value with different significance levels: 0.05/(*m*+1-*k*) using ordering values of *k*. Original false discovery rate approach use critical threshold value of *i**0.05/*m* with ordering values of *i*. Full details of this models could be found in [22,23]. However failing to taken into account of linkage disequilibrium in multiple hypothesis correction by adjacent SNPs may lead to loose of power. Zaykin et al.[3] suggested to use truncated product method for combining p values by chromosomal location of adjacent SNPs in various windows sizes.

RESULTS

QTL-MAS Dataset

QTL-MAS dataset were used for validation of multiple hypothesis testing procedures (Bonferroni, Hochberg and false discovery rate methods) and Zaykin et al.[3] model for QTL mapping. QTLs were mapped by different success rates: both Bonferroni and Hochbergs methods resulted as 0.75 mapping success. However both model did not able to detect 29 (total number of QTLs were 37) single QTLs. Although false discovery rate approach mapping success found to be lower as 0.67: It detected much higher number of single QTLs (20 true QTLs). We combined p values over chromosomal locations using Zaykin et al.[3] approach. Both windows sizes of 4 and 6 gave the same mapping success as 0.74. However Zaykins model predicted highest number of true single QTLs (33 true single QTLs) using both 4 and 6 windows sizes by Bonferroni correction. We noted that Zaykins model detected false positive QTLs from chromosome 5, where there was no QTL on this chromosome, for windows size of 0.

BT Dataset

The Manhattan plot of the transformed (-log) p values using GWA option of rrBLUP [21] revealed strong genomic signals from chromosomes 8 and 13 without any quality control *(Fig. 1)*. We fixed the minor allele frequencies at 0.01 (Quality Control 1, QC1) and 0.05 (Quality Control 2, QC2) and accordingly created two genotypic datasets.

We excluded 10 SNPs with a minor allele frequency <1% and 44,783 SNPs with a minor allele frequency <5%. There was a 0.90 call rate for both animals and SNPs, leaving 617,875 SNPs and 573,102 SNPs in the analyses with 0.01 and 0.05 minor allele frequencies, respectively. We excluded one animal due to a sex chromosome error,

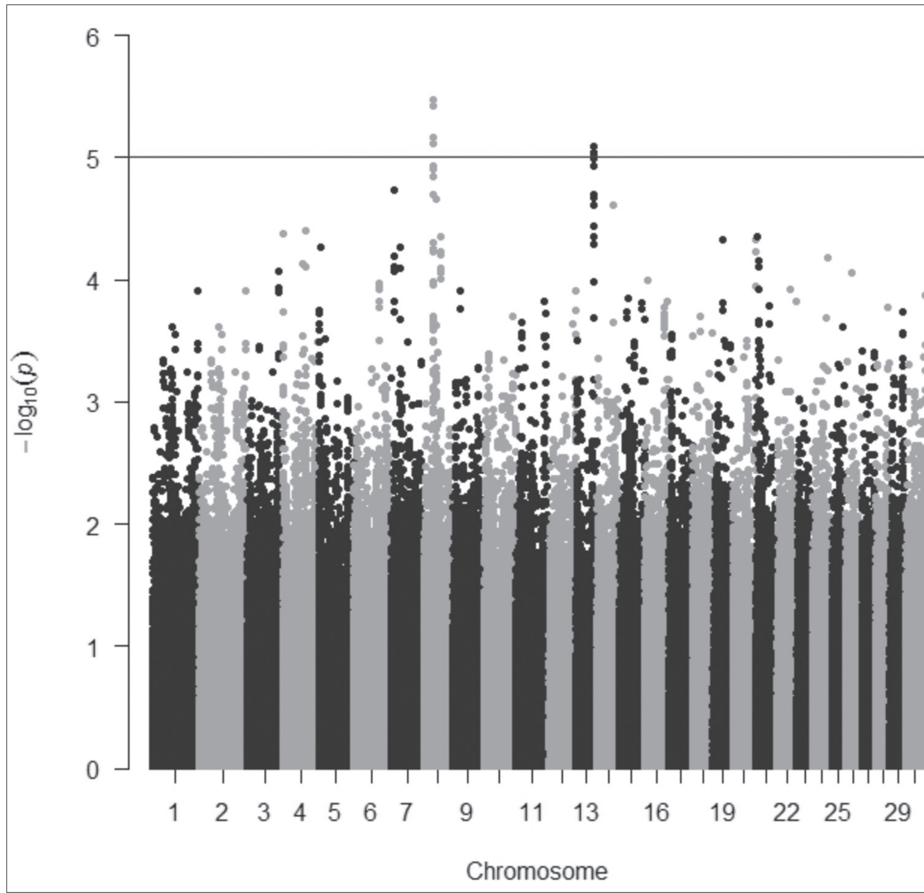

Fig 1. Manhattan plots of -log 10 (P values) using all SNPs (without quality control filters)

Şekil 1. Kalite kontrol süzgeçleri olmadan elde edilen bütün TNP›lere ait -log 10 (p değerleri) için Manhattan şekli

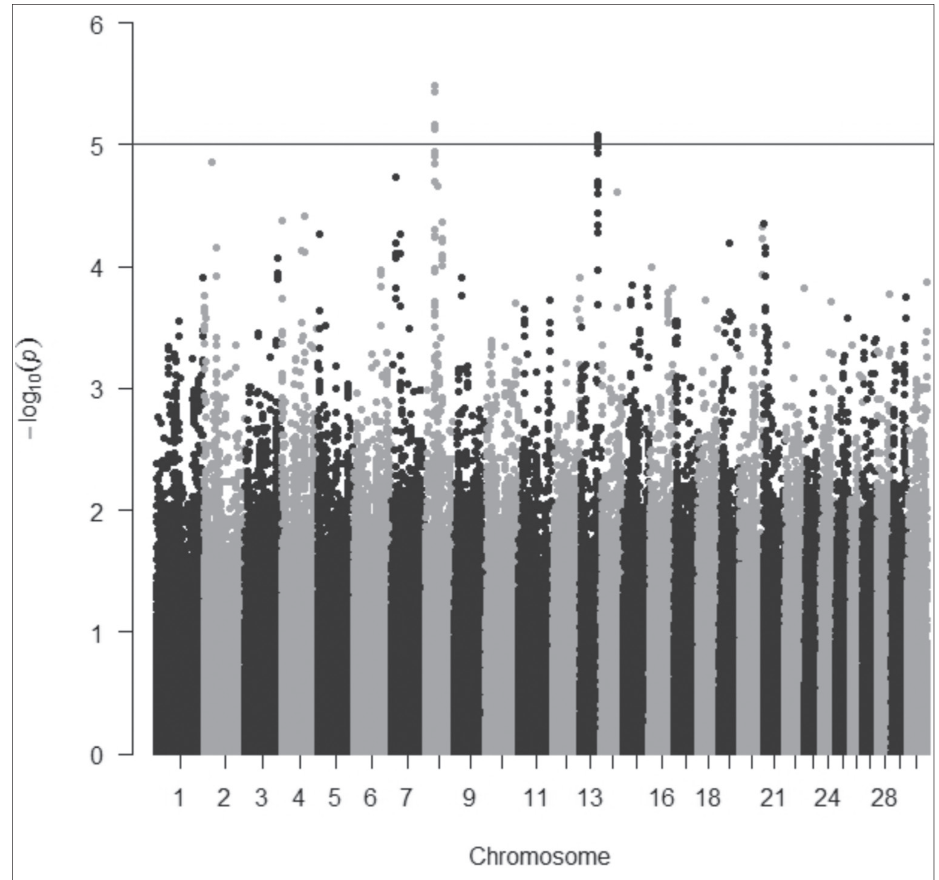

Fig 2. Manhattan plots of -log 10 (P values) using all SNPs with minor allele frequency 0.05, calling rate for animals 0.10, calling rate for genotypes 0.10

Şekil 2. 0.05 minör alel sıklıklı, 0.10 genotip ve hayvan başına çağırım oranlı bütün TNP›lere ait -log 10 (p değerleri) için Manhattan şekli

Table 1. *Genome wide association results of QTLMAS dataset using GRAMMAR and different windows sizes (0, 2, 4 and 6)*
Tablo 1. *Farklı pencere büyüklüklü (0, 2, 4 ve 6) GRAMMAR genom tabanlı ilişki sonuçları*

SNP	P-VAL	SIZE_0	SIZE_2	SIZE_4	SIZE_6
5143	1.313E-16	1.23E-12	3.66E-25	6.52E-48	8.97E-71
5144	0.103	1	1.56E-20	9.39E-43	3.04E-70
5145	7.439E-05	0.696142	2.11E-24	5.28E-43	3.32E-70
906	0.3508	1	4.57E-24	8.7E-42	1.5E-68
905	3.501E-12	3.28E-08	1.15E-20	4.98E-41	2.26E-67
904	0.1411	1	6.08E-10	3.02E-44	3.25E-67
5146	0.5324	1	8.34E-10	5.27E-47	4.71E-65
5153	1.196E-06	0.011192	5.99E-15	1.44E-42	1.29E-64
5141	1.172E-05	0.109676	6.31E-36	5.15E-53	2.08E-64
5147	2.487E-14	2.33E-10	4.49E-19	9.51E-47	2.5E-61

Table 2. *Genome wide association results with 0.05 minor allele frequencies of GRAMMAR using genomic control*
Tablo 2. *Genomik kontrol, 0.05 minör alel sıklığı ile GRAMMAR kullanılarak elde edilen genom tabanlı ilişki sonuçları*

Single Nucleotide Polymophism	Chromosome	Location	No of Individuals	Chi Square	P Val	P Val-Permuted
BovineHD0200007460	2	25899036	1057	18.76	1.48E-05	0.93
BovineHD4100005792	7	17622873	1150	18.41	1.78E-05	0.95
BovineHD1300020589	13	71788784	1149	18.30	1.89E-05	0.96
BovineHD1300020586	13	71784332	1146	18.17	2.02E-05	0.96
BovineHD1300020584	13	71782488	1150	18.06	2.14E-05	0.97
BovineHD1300020585	13	71783216	1150	18.06	2.14E-05	0.97
BovineHD1300020590	13	71789620	1150	18.06	2.14E-05	0.97
BovineHD1300020591	13	71791844	1150	17.91	2.32E-05	0.97
BovineHD0800010042	8	33645693	1150	17.76	2.51E-05	0.98
BovineHD4100010384	13	71781867	1139	17.49	2.89E-05	0.99

Table 3. *Principal components corrected genome wide association results with 0.05 minor allele frequencies*
Tablo 3. *0.05 minör alel sıklığı ve temel bileşenler analizi ile düzeltilerek elde edilmiş genom tabanlı ilişki sonuçları*

Single Nucleotide Polymophism	Chromosome	Location	No of Individuals	Chi Square	P Val	P Val-Permuted
BovineHD0800010042	8	33645693	1150	24.62	6.99E-07	0.16
BovineHD1300020589	13	71788784	1149	24.28	8.32E-07	0.18
BovineHD1300020586	13	71784332	1146	24.16	8.86E-07	0.19
BovineHD1300020584	13	71782488	1150	24.04	9.44E-07	0.19
BovineHD1300020585	13	71783216	1150	24.04	9.44E-07	0.19
BovineHD1300020590	13	71789620	1150	24.04	9.44E-07	0.19
BovineHD0800010045	8	33655169	1150	23.70	1.12E-06	0.22
BovineHD1300020591	13	71791844	1150	23.67	1.15E-06	0.23
BovineHD4100010384	13	71781867	1139	23.13	1.51E-06	0.29
BovineHD1300020582	13	71776870	1149	22.63	1.97E-06	0.35

retaining 1150 animals in the analyses. The mean identity by state was found to be 0.72 (0.009) 0.69 (0.008) using QC1 and QC2, respectively. The mean heterozygosis was found to be 0.36 (0.01) and 0.38 (0.01) using QC1 and QC2, respectively. We estimated heritability to be 0.23 and 0.22 using genomic kinship matrix and QC1 and QC2, respectively. Although a multi-dimensional scaling method detected two slightly separated clusters in the population (data not shown), as suggested by Birmingham et al.[15], we retained all of the animals in the dataset for further analyses. Since the results of both the GWA function of rrBLUP *(Fig. 2)* and GenABEL were similar, we opted to discuss only those results obtained from GenABEL.

Table 4. *Genome wide association results with 0.01 minor allele frequencies of GRAMMAR using genomic control*
Tablo 4. *Genomik kontrol, 0.01 minör alel sıklığı ile GRAMMAR kullanılarak elde edilen genom tabanlı ilişki sonuçları*

Single Nucleotide Polymophism	Chromosome	Location	No of Individuals	Chi Square	P Val	P Val-Permuted
BovineHD0200007460	2	25899036	1057	18.71	3.69E-06	0.95
BovineHD1300020589	13	71788784	1149	18.36	4.57E-06	0.97
BovineHD4100005792	7	17622873	1150	18.31	4.69E-06	0.97
BovineHD1300020586	13	71784332	1146	18.22	4.95E-06	0.98
BovineHD1300020584	13	71782488	1150	18.11	5.29E-06	0.98
BovineHD1300020585	13	71783216	1150	18.11	5.29E-06	0.98
BovineHD1300020590	13	71789620	1150	18.11	5.29E-06	0.98
BovineHD1300020591	13	71791844	1150	17.97	5.76E-06	0.99
BovineHD0800010042	8	33645693	1150	17.63	7.06E-06	0.99
BovineHD4100010384	13	71781867	1139	17.56	7.37E-06	0.99

Table 5. *Principal components corrected genome wide association results with 0.01 minor allele frequencies*
Tablo 5. *0.01 minör alel sıklığı ve temel bileşenler analizi ile düzeltilerek elde edilmiş genom tabanlı ilişki sonuçları*

Single Nucleotide Polymophism	Chromosome	Location	No of Individuals	Chi Square	P Val	P Val-Permuted
BovineHD0800010042	8	33645693	1150	24.59	7.10E-07	0.17
BovineHD1300020589	13	71788784	1149	24.26	8.44E-07	0.19
BovineHD1300020586	13	71784332	1146	24.13	9.00E-07	0.20
BovineHD1300020584	13	71782488	1150	24.01	9.58E-07	0.22
BovineHD1300020585	13	71783216	1150	24.01	9.58E-07	0.22
BovineHD1300020590	13	71789620	1150	24.01	9.58E-07	0.22
BovineHD0800010045	8	33655169	1150	23.66	1.15E-06	0.26
BovineHD1300020591	13	71791844	1150	23.63	1.17E-06	0.26
BovineHD4100010384	13	71781867	1139	23.11	1.09E-06	0.32
BovineHD1300020582	13	71776870	1149	22.59	1.01E-06	0.40

The genomic control inflation factors of QC1 for the GRAMMAR genomic control, principal components corrected association and GRAMMAR gamma were 0.87 (7.85×10^{-6}), 1.14 (1.23×10^{-5}) and 0.99 (8.98×10^{-6}), respectively. The genomic control inflation factors of QC2 for GRAMMAR genomic control, the principal components corrected for association and GRAMMAR gamma were 0.87 (9.18×10^{-6}), 1.14 (9.19×10^{-6}) and 0.99 (1.05×10^{-5}), respectively.

Results of the QC1 and QC2 association analyses using, GRAMMAR with genomic control and principal components approaches including permutation tests are reported in *Table 2*, *Table 3*, *Table 4,* and *Table 5*.

DISCUSSION

QTL-MAS Dataset

Investigation of QTL-MAS simulated dataset showed that Bonferroni and Hochberg methods gives conservative results (only 8 QTLS were detected out of 37) as was also concluded by Johnson et al.[24]. Fu et al.[6] showed advantaged of using linkage disequilibrium information in multiple hypothesis testing. As was also showed in *Table 1*: taking into account of linkage disequilibrium structure leads to higher p values proportional to the windows sizes. On the basis of these observations we found that: the Zaykins model built from different windows sizes found the highest number of true QTLs (33 out of 37). Similarly Hu et al.[25] showed by simulation that grouping of p values leads to higher statistical power in the genetic association studies.

BT Dataset

We found that the genomic control inflation factors were similar for each association model using QC1 and QC2. The GRAMMAR gamma inflation factors were approximately 1, which indicates that this method is unbiased compared with other correction methods. Bermingham et al.[15] also noted a minimal increase in the lambda values, similar to the results of GRAMMAR with gamma inflation factors.

Since Bermingham et al.[15] possibly used GRAMMAR with gamma inflation factors, we did not reproduce the same results in our study. The results of the QC1 and QC2 association analyses using GRAMMAR with genomic

control and principal component approaches including permutation tests are reported in *Tables* 2-5. We detected SNPs from chromosomes 2, 7, 8 and 13 at a significance level of P<0.001 without correcting for multiple hypothesis testing. However, after the Bonferroni correction, Hochberg's method and the permutation test for multiple hypothesis correction *(Tables 2-5)*, the genomic signals became non-significant. Only the false discovery rate approach detected weak signals (at a level of 0.54) from chromosomes 2, 8 and 13.

Type 1 errors would increase without correcting for multiple hypothesis testing. However, traditional correction models do not take into account dependency among hypotheses. In GWAS, one of the sources for the correlated hypothesis may be linkage disequilibrium. In addition, failing to take into account linkage disequilibrium among adjacent SNPs may lead to a reduction of power. We used the Zaykin et al.[3] model to take into account the effect of linkage disequilibrium on the multiple hypothesis testing procedures by combining adjacent SNP test statistics with window sizes of 2, 4 and 6. We detected strong genomic signals from chromosome 13 (-log(p)=90.33), chromosome 8 (-log(p)=51.34), chromosome 16 (-log(p)=49.47) and chromosome 2 (-log(p)=44.77) at a window size of 6. However, the test statistics will increase as the window size increases using the approach of Zaykin et al.[3]. Although top SNPs have been found to be the same irrespective of the windows size, larger windows sizes may lead to anti-conservative results [26].

The results of this study have shown that multiple hypothesis testing procedures are related to false positive genomic signals. Although the level of minor allele frequencies did not make a difference in terms of genomic signals *(Tables 2-5)*, these results cannot be generalized [7]. It is difficult to suggest universally acceptable multiple hypothesis testing [24] and quality control measures and their thresholds due to sources of variations between species and within populations. However, additional analytical approaches and studies are necessary to evaluate the effects of linkage disequilibrium on the multiple hypothesis testing procedures and QC measures (especially for minor allele frequencies) to GWAS under various scenarios including, but not limited to, the level of heritability, linkage disequilibrium, population structure and population size.

REFERENCES

1. Wasserstein RL, Lazar NA: The ASA's statement on p-values: Context, process, and purpose. *Am Stat*, 70, 129-133, 2016. DOI: 10.1080/00031305.2016.1154108

2. Karacaören B: A bayesian random walk approach for mapping dynamic quantitative trait. *J App Non Dyn*, 5, 105-115, 2016. DOI: 10.5890/JAND.2016.03.008

3. Zaykin DV, Zhivotovsky LA, Westfall PH, Weir BS: Truncated product method for combining P-values. *Genet epid*, 22, 170-185, 2002. DOI: 10.1002/gepi.0042

4. Sham PC, Purcell SM: Statistical power and significance testing in large-scale genetic studies. *Nat Rev Genet*, 15, 335-346, 2014. DOI: 10.1038/nrg3706

5. Kang G, Ye K, Liu N, Allison DB, Gao G: Weighted multiple hypothesis testing procedures. *Stat Appl Genet Molec Biol*, 8, 1-22, 2009. DOI: 10.2202/1544-6115.1437

6. Fu G, Saunders G, Stevens J: Holm multiple correction for large-scale gene-shape association mapping. *BMC Genet*, 15 (Suppl. 1): S5, 2014. DOI: 10.1186/1471-2156-15-S1-S5

7. Tabangin ME, Woo JG, Martin LJ: The effect of minor allele frequency on likelihood of obtaining false positives. *BMC Proc*, 3 (Suppl. 7): S41, 2009. DOI: 10.1186/1753-6561-3-S7-S41

8. Chen G, Lee SH, Robinson MR, Trzaskowski M, Zhu Z, Winkler TW, Day FR, Croteau-Chonka DC, Wood AR, Locke AE, Kutalik Z, Loos RJF, Frayling TM, Hirschhorn JN, Yang J, Wray NR, The Genetic Investigation of Anthropometric Traits (GIANT) Consortium, Visscher PM: Across-cohort QC analyses of GWAS summary statistics from complex traits. *Eur J Hum Genet*, 2016 (In press). DOI: 10.1038/ejhg.2016.106

9. Eu-ahsunthornwattana J, Howey RA, Cordell HJ: Accounting for relatedness in family-based association studies: Application to genetic analysis workshop 18 data. *BMC Proc*, 8, (Suppl. 1): S79, 2014. DOI: 10.1186/1753-6561-8-S1-S79

10. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D: Principal components analysis corrects for stratification in genome-wide association studies. *Nat genet*, 38, 904-909, 2006. DOI: 10.1038/ng1847

11. Aulchenko YS, De Koning DJ, Haley C: Genomewide rapid association using mixed model and regression: A fast and simple method for genomewide pedigree-based quantitative trait loci association analysis. *Genetics*, 177, 577-585, 2007. DOI: 10.1534/genetics.107.075614

12. Amin N, Van Duijn CM, Aulchenko YS: A genomic background based method for association analysis in related individuals. *PloS one*, 2, e1274, 2007. DOI: 10.1371/journal.pone.0001274

13. Svishcheva GR, Axenovich TI, Belonogova NM, van Duijn CM, Aulchenko YS: Rapid variance components-based method for whole-genome association analysis. *Nat Genet*, 44, 1166-1170, 2012. DOI: 10.1038/ng.2410

14. Karacaören B: Admixture mapping of growth related traits in F 2 mice dataset using ancestry informative markers. *J Bioinform Comput Biol*, 12, 2014. DOI: 10.1142/S0219720014410108

15. Bermingham ML, Bishop SC, Woolliams JA, Pong-Wong R, Allen AR, McBride SH, Ryder JJ, Wright DM, Skuce RA, McDowell SWJ, Glass EJ: Genome-wide association study identifies novel loci associated with resistance to bovine tuberculosis. *Heredity*, 112, 543551, 2014. DOI: 10.1038/hdy.2013.137

16. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsempe C, Simon S, Harris B, Atkin R, Doggett J, Mayes R, Keating L, Wheeler PR, Parkhill J, Barrell BG, Cole ST, Gordon SV, Hewinson RG: The complete genome sequence of *Mycobacterium bovis. Proc Natl Acad Sci USA*. 100, 78-77, 82, 2003. DOI: 10.1073/pnas.1130426100

17. Cleveland MA, Hickey JM, Forni S: A common dataset for genomic analysis of livestock populations. *G3: Genes Genom Genet*, 2, 429-435, 2012. DOI: 10.1534/g3.111.001453

18. Szydlowski M, Paczynska P: QTLMAS 2010: Simulated dataset. BMC Proc, 5 (Suppl 3): S3, 2011. DOI: 10.1186/1753-6561-5-S3-S3

19.Turner S, Loren LA, Yuki B, Christopher SC, Dana CC, Andrew T. C, Mariza de A, Kimberly FD, Jonathan LH, Geoffrey H, Gail J, Lan J, Iftikhar JK, Rongling L, Hua L, Teri AM, Martha M, Catherine AM, Andrew NM, Daniel BM, Justin EP, Elizabeth WP, Luke VR, Russell AW, Rebecca LZ, Marylyn DR : Quality control procedures for genome-wide association studies. *Curr prot hum genet*, 1-19, 2011. DOI: 10.1002/0471142905.hg0119s68

20. Aulchenko YS, Ripke S, Isaacs A, Van Duijn CM: GenABEL: An R library for genome-wide association analysis. *Bioinformatics*, 23, 1294-1296, 2007. DOI: 10.1093/bioinformatics/btm108

21. Endelman JB: Ridge regression and other kernels for genomic

selection with R package rrBLUP. *Plant Genome*, 4, 250-255, 2011. DOI: 10.3835/plantgenome2011.08.0024

22.Balding DJ: A tutorial on statistical methods for population association studies. *Nat Rev Genet*, 7, 781-791,2006. DOI: 10.1038/nrg1916

23. Goeman JJ, Solari A: Multiple hypothesis testing in genomics. *Stat Med*, 33, 1946-1978, 2014. DOI: 10.1002/sim.6082

24. Johnson RC, Nelson GW, Troyer JL, Lautenberger JA, Kessing BD, Winkler CA, O'Brien SJ: Accounting for multiple comparisons in a genome-wide association study (GWAS). *BMC genomics*, 11, 1, 2010. DOI: 10.1186/1471-2164-11-724

25. Hu X, Zhang W, Zhang S, Ma S, Li Q: Group-combined P-values with applications to genetic association studies. *Bioinformatics*, 2016 (In press). DOI: 10.1093/bioinformatics/btw314

26. Lin WY, Lou XY, Gao G, Liu N: Rare variant association testing by adaptive combination of P-values. *PloS one*, 9 (1): e85728, 2014. DOI: 10.1371/journal.pone.0085728

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Research Article

Effects of BCRP and P-gp Modulators on the Penetration of Aflatoxin B_1 into the Mouse Brain [1]

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Abstract

This study was conducted to determine whether the plasma and brain concentrations of AFB_1 are affected by the modulation of P-gp and BCRP using zosuquidar (ZQR) and prazosin (PRZ), respectively. In this study, a total of 40 healthy adult male BALB/c mice (32±3.7 g) were used. The animals were randomly divided into 5 groups, with 8 animals per group. Group 1 was used for method validation. Group 2 (AF) received intraperitoneal AFB_1 at a dose of 20 mg/kg of body weight. Groups 3 (AF+PRZ), 4 (AF+ZQR), and 5 (AF+PRZ+ZQR) received 20 mg/kg of AFB_1 intraperitoneally 30 min after the intraperitoneal administration of prazosin (0.3 mg/kg), zosuquidar (25 mg/kg), and prazosin+zosuquidar (0.3 mg/kg prazosin + 25 mg/kg zosuquidar), respectively. Six hours after the administration of AFB_1, blood and brain samples were collected from the animals in Groups 2 to 5. AFB_1 concentrations were determined using an HPLC system with fluorescence detection. Individual and simultaneous administration of prazosin and zosuquidar significantly reduced the brain concentrations of AFB_1 in comparison to a single administration of AFB_1 ($P<0.05$). The brain/plasma ratio of the AF group was higher than that of the other groups (AF+PRZ, AF+ZQR, and AF+PRZ+ZQR) ($P<0.05$). Inducers of transmembrane proteins, especially BCRP, can be life saving during acute AFB_1 poisoning.

Keywords: *Aflatoxin B_1, Brain, Drug transporter proteins, Modulation, Mice*

Aflatoksin B_1'in Fare Beynine Geçişi Üzerine BCRP ve P-gp Modülatörlerinin Etkisi

Özet

Çalışma, zosuquidar ve prazosin tarafından sırasıyla P-gp ve BCRP modülasyonunun AFB_1'in plazma ve beyin konsantrasyonlarının etkileyip etkilemediğini belirlemek için gerçekleştirildi. Bu çalışmada, 40 adet sağlıklı, erkek BALB/c ırkı fare (32±3.7 g) kullanıldı. Hayvanlar her grupta 8 fare olacak şekilde rastgele 5 gruba ayrıldı. Grup 1, metod validasyon çalışmalarında kullanıldı. Grup 2 (AF)'ye AFB_1 20 mg/kg dozda intraperitoneal yolla verildi. Grup 3 (AF+PRZ), 4 (AF+ZQR) ve 5 (AF+PRZ+ZQR)'e ise sırasıyla intraperitoneal yolla prazosin (0.3 mg/kg), zosuquidar (25 mg/kg) ve prazosin+zosuquidar (0.3 mg/kg prazosin + 25 mg/kg zosuquidar) uygulamalarından 30 dk. sonra AFB_1 20 mg/kg dozda intraperitoneal yolla uygulandı. Grup 2-5'de bulunan hayvanlardan AFB_1 uygulamasından sonraki 6. saatte kan ve beyin örnekleri alındı. AFB_1 düzeyleri fluoresans dedektör içeren HPLC sisteminde tayin edildi. Prazosin ve zosuquidarın tek ve eşzamanlı uygulanması AFB_1'in beyin konsantrasyonlarında sadece AFB_1 uygulamasına göre önemli oranda azalmaya neden oldu ($P<0.05$). AFB_1 grubunda AFB_1'in beyin/plazma oranı diğer gruplardan (AF+PRZ, AF+ZQR, and AF+PRZ+ZQR) önemli oranda daha yüksekti ($P<0.05$). Akut AFB_1 zehirlenmelerinde özellikle BCRP gibi transmembran proteinlerin indüklenmesi hayatta kalma oranını artırabilir.

Anahtar sözcükler: *Aflatoksin B_1, Beyin, İlaç taşıyıcı proteinler, Modülasyon, Fare*

INTRODUCTION

Aflatoxin B_1 (AFB_1), which is an environmental dietary carcinogen, is one of the most toxic mycotoxins and causes significant losses of livestock [1-3]. While the clinical signs of acute aflatoxicosis include epistaxis, blood stained faeces, and convulsions, sudden death is observed as the clinical sign of severe acute aflatoxicosis [4,5]. The elimination half-life of AFB_1 was determined to be 77 min after intraperitoneal administration in mice [6]. There is a

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high interaction potential to occur of AFB_1 with food/feed because AFB_1 is a common food/feed contaminant. Also, the ingredients found in the food/feed compositions can cause to change the efficiency and disposition of drug and toxic substances through enzyme-and transporter [7-9].

Aflatoxin B_1 is a substrate of BCRP, and BCRP function may affect systemic exposure to this mycotoxin [10,11]. Additionally, AFB_1 and its metabolite aflatoxin B_1-epoksit-glutathione are substrates of multidrug resistance protein 1 (MRP1), although the affinity of MRP1 for AFB_1 is low [12-14]. BCRP substrates tend to overlap with P-gp substrates [13,15], but no information is available concerning whether AFB_1 is a substrate of P-gp. Van Herwaarden et al.[10] noted that BCRP plays an important role in the renal excretion of AFB_1. If AFB_1 is a substrate of both BCRP and P-gp, the tissue penetration of AFB_1 may be changed by the modulation of these transmembrane proteins.

The blood-brain barrier (BBB) protects the brain from a variety of endogenous and exogenous substances. The BBB not only limits substance flow from the blood to the brain tissue via the paracellular and transcellular routes but also permits the efflux of substances via several trans-membrane proteins, such as P-glycoprotein (P-gp) and the breast cancer resistance protein (BCRP). P-gp (ABCB1) and BCRP (ABCG2) are members of the ATP-binding cassette transporter superfamily. Both P-gp and BCRP are expressed in mammalian capillary endothelial cells at BBB sites, and these transporters work in tandem to limit the accumulation of substances in tissues [11,16-19]. P-gp and BCRP are inducible and inhibitable *in vivo* and *in vitro* by various substances. The efflux activity of these transmembrane proteins has been described as saturable [19]. Because P-gp and BCRP have a broad substrate specificity and tissue distribution, the modulation of these transmembrane proteins may result in significant alterations in the pharmacokinetics, pharmacodynamics, and toxicity of their substrates. For example, Van Herwaarden et al.[10] found that the brain concentration of [^{14}C] 2-amino-3-methylimidazo[4,5-f] quinoline, which is a BCRP substrate, was higher in bcrp-/- mice than wild-type mice.

The aim of this study was 1) to determine the passage of AFB_1 into the brain and 2) to evaluate whether the plasma and brain concentrations of AFB_1 are affected by the modulation of P-gp and BCRP using prazosin and zosuquidar, respectively. This study is the first *in vivo* experimental study intended to evaluate the modulation of AFB_1 passage into the brain.

MATERIAL and METHODS

Chemicals and Reagents

All reagents were of recognised analytical grade. AFB_1 was obtained from Biopure Chemical Co. (Romer Labs Inc., 1301 Stylemaster Drive Union) and dissolved in corn oil for injection. Zosuquidar hydrochloride (99%) was obtained from MedKoo Biosciences (Canada, USA) and dissolved in a solution that included glycine (15 mg) and mannitol (200 mg) in 1 mL of distilled water for injection. Prazosin hydrochloride (≥99%) was purchased from Sigma Chemical Co. (Saint Louis, MO, USA) and dissolved in 1 mL of distilled water for injection. Immunoaffinity columns (Aflatest® WB) were purchased from Vicam (Watertown, MA, USA). Monobasic potassium phosphate (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium chloride (NaCl), potassium bromide (KBr), nitric acid (4 mol/L), and HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All water used in this study was deionised and distilled. For the high performance liquid chromatograpgy (HPLC), water was passed through a water purification system (aqua Max-Ultra System, Younglin Instrument Co. Ltd., South Korea). Phosphate buffer solution (PBS) was prepared by dissolving 0.2 g of KCl, 0.2 g of KH_2PO_4, 1.16 g of anhydrous Na_2HPO_4, and 8 g of NaCl in 1,000 mL of water, and the pH of the solution was adjusted to 7.4 with 0.1 N NaOH. A Tween-20 (Amresco, USA) solution was prepared by adding 15 mL of Tween to 85 mL of PBS and mixing.

Experimental Design

In this study, a total of 40 healthy adult male BALB/c mice (32±3.7 g) were used. Mice were supplied from Experimental Animal Production and Research Laboratory, Faculty of Veterinary Medicine, University of Mehmet Akif Ersoy, Burdur, Turkey. All experimental administrations on animals were carried out in here. The animals were housed individually in plastic cages with grated stainless steel floors in a controlled environment (temperature 25±1°C, relative humidity 60±10%, and artificial lighting sequenced to generate a 12-h light/dark cycle). The animals had *ad libitum* access to water and a commercial diet (Optima Feeds, Kırklareli, Turkey) that included the following: 89% dry matter, 21% crude protein, 2850 kcal/kg metabolic energy, 5% crude fibre, 0.75% methionine and cysteine, 1.0-2.0% calcium, 0.5-1.0% phosphorus, and 0.5% sodium.

All animal protocols in this study were approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Mehmet Akif Ersoy (No: 2014-71).

The animals were randomly divided into 5 groups of 8 animals each. Group 1 was used for method validation without any treatment of the animals. AFB_1 was administered intraperitoneally at a dose of 20 mg/kg of body weight to mice in Group 2-5. In the present study, the dose of AFB_1 was determined by taking into consideration concerns about AFB_1 analysis, the natural poisoning state and the dose previously reported Bastaki et al.[6] because no experimental research is available concerning the passage of AFB_1 into the brain. Group 2 (AF) received intraperitoneal (IP) AFB_1 at a dose of 20 mg/

kg of body weight 30 min after the IP administration of 1 mL of the glycine/mannitol solution. Groups 3 (AF+PRZ), 4 (AF+ZQR), and 5 (AF+PRZ+ZQR) were administered IP AFB_1 at a dose of 20 mg/kg of body weight 30 min after the IP administration of prazosin (0.3 mg/kg of body weight) plus the glycine/mannitol solution (1 mL), zosuquidar (25 mg/kg of body weight), and prazosin (0.3 mg/kg of body weight) plus zosuquidar (25 mg/kg of body weight), respectively. The animals were observed for general behavioural changes, signs of toxicity, and mortality continuously for 6 h following the administration of AFB_1. Six hours after the administration of AFB_1 in Groups 2 to 5, blood samples from the hearts of animals under ether anaesthesia were collected into tubes containing heparin. All animals were euthanized using the cervical dislocation method. Plasma and brain tissues were collected from the animals and carried to Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Selcuk, Konya, Turkey for HPLC analysis. All samples were stored at -70°C until the time of analysis.

HPLC and Chromatographic Conditions

HPLC analyses were performed using an Agilent 1100 series HPLC system, which consisted of a G1379A degasser, a G1310A isocratic pump, a G1313A autosampler, a G1316A column oven, and a fluorescence detector (model G1321A, Agilent Technologies, Palo Alto, California, USA). Data acquisition was performed using the Chemstation 3D software (Agilent Technologies, Palo Alto, California, USA). For HPLC analysis, a reverse-phase ACE C18 analytical column (5 mm particle size, 4.6 x 250 mm) was employed. The column temperature was maintained at 30°C. Postcolumn derivatisation resulting in enhanced fluorescence was achieved with electrochemically-generated bromine in a Cobra cell (Coring System Diagnostics GmbH, Gernsheim, Germany), using a reaction tube that consisted of a 0.5 mm id x 34 cm length of polyether ketone tubing. The LC mobile phase solvent used with electrochemically-generated bromine was water/methanol/acetonitrile (60:20:20, v/v/v). To each litre of mobile phase, 120 mg of potassium bromide and 350 mL of 4 M nitric acid were added. The flow rate was 1 mL/min. The fluorescence detector was set to excitation and emission wavelengths of 360 and 430 nm, respectively. The injection volume for the standards and samples was 100 µL.

Standard Preparation

A stock solution was prepared by dissolving 5 mg of AFB_1 in 5 mL of methanol and stored at -70°C. The stock solution was further diluted with water/methanol/acetonitrile (60:20:20, v/v/v) to generate a series of working standard solutions. Calibration standards of AFB_1 (0.5, 1, 5, 10, 25, 50, 100, 200 ng/mL) were prepared by adding 20 µL of the working standard solution to 180 µL of plasma or 180 mg of brain tissue. Quality control samples (QC) at low (5 ng/mL), medium (25 ng/mL) and high (200 ng/mL) concentrations were used for the method validation program. These calibration standards and QC samples were processed according to the described method of sample preparation prior to HPLC analysis.

Sample Preparation

Plasma and brain tissue samples were extracted and cleaned up according to the procedures described by Association of Official Analytical Chemist [20] and Chiavaro et al.[21], with some modifications. Briefly, 200 µL of plasma or 200 mg of tissue sample were blended with 200 mg of NaCl in 1.2 mL of methanol-water (80:20) using a tissue homogeniser (Heidolph Silent Crusher M, Germany) at 9.000 rpm for 1 min. The extract was filtered through Whatman No. 4 filter paper. A 1-mL filtrate sample was diluted to 7.2 mL with PBS containing 0.1% Tween-20. The final filtrate was passed through an immunoaffinity column at a flow rate of 1 drop/s. The column was then washed twice with 4 mL of ultrapure water. AFB_1 was eluted with 0.5 mL of methanol followed by 0.5 mL of water in a glass vial. Finally, 100 µL of the sample was injected into the HPLC system.

Method Validation

The specificity, linearity, sensitivity, recovery, precision, and accuracy of the employed method were determined to evaluate the performance of the analytical method. The specificity of the method was evaluated to identify interference from plasma and brain tissue. The calibration curve of AFB_1 was constructed using seven calibration standards over a calibration range of 0.5-200 ng/mL. The limits of detection (LOD) and quantification (LOQ) were determined via signal-to-noise ratio evaluations of samples spiked from 0.1-10 ng/mL. The LOD was defined at a signal-to-noise (S/N) ratio of 3:1. The LOQ was defined as the lowest quantifiable concentration of analyte with an accuracy within 20% and a precision <20%. The recovery of AFB_1 was evaluated at concentrations corresponding to the three QC values (5, 25, and 200 ng/mL), with six replicates. This parameter was determined by comparing the peak areas of the extracted QC samples with those of the working standards. The intra- and inter-day precision and accuracy were assessed by extracting and analysing five replicates of each QC sample. The intra- and inter-day precision and accuracy of the assay were determined based on the percent coefficient of variation (CV) and percent relative error (RE) values, respectively.

Statistical Analysis

All data were expressed as the mean ± SD. Data obtained from plasma and brain concentrations were analysed using one-way ANOVA, followed by the *Duncan* test. Statistical significance was assigned at P<0.05. The SPSS (Version 16.0 for Windows, Chicago, USA) statistical software was used for the statistical analyses.

RESULTS

No interfering peaks were observed in the blank plasma and brain tissue samples after the extraction. The assay was linear from 0.5 to 200 ng/mL, and the concentrations of the calibration standards were back-calculated using the peak area ratios of AFB_1. The data were analysed using linear regression analysis, and the correlation coefficients for the calibration curves prepared from plasma and brain tissue were ≥0.9996 and ≥0.9994, respectively. The LOD values were determined to be 0.2 and 0.5 ng/mL in plasma and brain tissue, respectively. The LOQ values were determined to be 0.5 and 1 ng/mL in plasma and brain tissue, respectively. The values obtained for the recovery of AFB_1 ranged from 89.3 to 97.9% for the plasma and brain samples and from 86.4 to 94.3% for the QC samples. In the plasma and brain samples, the intra-day precision that was calculated from three QC samples that were injected six times on the same day was low, with a precision ranging from 3.94% to 10.27% for the CV and an accuracy ranging from 94.3% to 107.6%. The inter-day precision and accuracy varied from 1.54% to 12.48% and from 92.1% to 109.71%, respectively.

***Table 1.** The plasma and brain concentrations of aflatoxin B_1 at six hours following the intraperitoneal administration of aflatoxin B_1 (20 mg/kg) in mice (n=8)*

***Tablo 1.** Farelerde aflatoksin B_1'in (20 mg/kg) intraperitoneal yolla uygulanmasını takiben 6. saatte alınan plazma ve beyin örneklerindeki AFB_1 konsantrasyonları (n=8)*

Group	Plasma (ng/mL)	Brain (ng/g)
AF	23.45±13.84^{ab}	181.23±89.40^{a}
AF+PRZ	15.53±10.10^{b}	6.69±2.81^{b}
AF+ZQR	31.96±14.28^{a}	24.07±19.64^{b}
AF+PRZ+ZQR	13.37±9.50^{b}	24.85±11.66^{b}

*^{a,b} Different letters in the same column indicate statistically significant differences (P<0.05); **AF:** aflatoxin B_1; **PRZ:** prazosin; **ZQR:** zosuquidar*

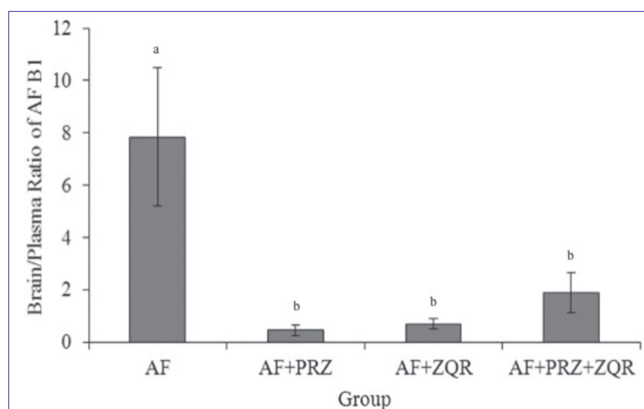

Fig 1. Ratios of brain concentrations to plasma concentrations of aflatoxin B_1. AF; aflatoxin B_1, PRZ; prazosin, ZQR; zosuquidar. ^{a,b} Different letters are statistically significant (P<0.05)

Şekil 1. AFB_1'in beyin/plazma konsantrasyon oranları. AF; aflatoksin B_1, PRZ; prazosin, ZQR; zosuquidar. ^{a,b} Farklı harfler istatistiksel açıdan önemlidir (P<0.05)

The effects of prazosin and zosuquidar on murine plasma and brain concentrations of AFB_1 after the IP administration of AFB_1 at a dose of 20 mg/kg of body weight are presented in *Table 1*. Single and simultaneous administrations of prazosin and zosuquidar significantly reduced brain concentrations of AFB_1 in comparison to a single administration of AFB_1 (P<0.05). The ratios of the brain concentrations of AFB_1 to the plasma concentrations of AFB_1 in the mice are shown in *Fig 1*. The brain/plasma ratio in the AF group was higher than those of the groups (AF+PRZ, AF+ZQR and AF+PRZ+ZQR) that received prazosin and zosuquidar (*Fig. 1*, P<0.05).

No mortality was observed in the AFB_1-treated groups during the study; however, signs of toxicity, including oedema of the lower extremities, lethargy, muscular weakness, and convulsions, were observed in the AF group.

DISCUSSION

Although AFB_1 was found at levels of 1-5 ppb in human brain biopsy samples, as reported by Hooper et al.[22] no experimental study investigated the ability of AFB_1 to cross the BBB. However, AFB_1 was reported to be unable to pass into the rat brain [23]. In this study, AFB_1 crossed the BBB after the administration of 20 mg/kg AFB_1, and the brain/plasma ratio of AFB_1 was 7.85 six hours after administration. Our results suggest that AFB_1 passed more intense to the brain and cannot be removed immediately. We concluded that AFB_1 accumulates in tissues, such as the brain, and cannot be removed immediately due to the greater concentration of AFB_1 in the brain than that in the plasma at the 6-hour blood collection time.

Contrary to expectation in this study, prazosin administration caused a statistically significant decline in the brain concentration of AFB_1 (P<0.05); however, the decline in the plasma concentration of AFB_1 was not statistically significant in the AF group (P>0.05). The brain/plasma ratio of AFB_1 in prazosin-treated mice was 0.45 *(Fig. 1)*. Our results indicate that prazosin is an inducer of BCRP, although some authors reported that prazosin is a BCRP inhibitor and substrate [24-27]. The reason for this contradiction may be differences in the materials used in the studies and in the employed doses of BCRP inhibitors and substrates. The drug affinity and the expression rate of BCRP differ among species, breeds, and organs [28,29]. Studies of inhibitors and inducers are typically conducted *in vitro*. The majority of *in vivo* studies concerning the passage of BCRP and P-gp substrates into the brain were conducted using transgenic experimental animals (bcrp/p-gp -/-), such as mice and rats. Our study was conducted *in vivo* using wild-type mice. The type of modulation (i.e., induction and/or inhibition) and the effectiveness of a substance on transporter proteins are reported to depend on the dose, the type of tissue, the substrate examined, and the time of administration [30,31]. Additionally, the

difficulties of predicting *in vivo* relationships based on *in vitro* transport assays were discussed by Enokizono et al.[32]. Alternatively, the causes of the decreased AFB_1 concentration in the brain that is caused by prazosin may be associated with the roles of AFB_1 as a P-gp substrate and prazosin as a P-gp inducer. The inhibition of BCRP by prazosin may cause the activation of P-gp in the brain capillaries of mice. A compensatory relationship has been reported to exist between P-gp and BCRP, and P-gp has also been reported to be more prominent and inducible than BCRP at the BBB [33,34] Prazosin has been indicated to be only an inducer of P-gp [23-27]. In addition, many BCRP and P-gp substrates and modulators overlap [13,27,35].

In the present study similar to the results of prazosin, zosuquidar, which is a potent and selective P-gp inhibitor *in vitro*, caused a non-statistically significant increase in the plasma concentration of AFB_1 but a statistically significant decrease in the brain concentration of AFB_1 ($P<0.05$). Cripe et al.[34] reported that the *in vivo* inhibition of P-gp by zosuquidar might lead to the activation of non-P-gp transmembrane proteins, such as BCRP. In addition, BCRP and P-gp presumably work in synergy or in a compensatory manner to restrict the passage of their concerted substrates into the CNS in mice, and P-gp can compensate for the deletion of BCRP, as suggested by Zhou et al.[33]. In addition, zosuquidar can be considered to be an inducer of P-gp or a dual P-gp/BCRP inducer in brain capillaries. Zosuquidar is described as a specific P-gp inhibitor [13,19,35], and a number of BCRP and P-gp substrates and modulators overlap [3,27,35].

Although the co-administration of prazosin and zosuquidar reduced the level of AFB_1 in the brain, this reduction was not statistically significant in comparison to the other two treatment groups. Thus, prazosin and zosuquidar do not show the combined effect to prevent the passage of AFB_1 into the brain. This may be associated with that prazosin or zosuquidar in combined use altere the response to the modulation of other transmembrane protein. Also, it has been reported that BCRP and P-gp work in synergy or in a compensatory manner for the efflux of their substrates and the modulation of a transmembrame protein by the inducer/inhibitor alteres the efflux effect of other transmembrane protein [33,34].

The administration of prazosin, zosuquidar and prazosin+ zosuquidar did not cause a statistically significant reduction of the plasma concentrations of AFB_1 in comparison to a single administration of AFB_1 (AF group). Similar to our findings, some previous studies reported that trans-membrane protein modulators caused no changes in the plasma concentrations or pharmacokinetics of the substrates of transmembrane proteins but did cause important changes in tissue concentrations [34,36,37]. Based on the results of studies in this field, we believe that this difference may occur because the transmembrane proteins in each tissue respond differently to inducers and because drugs/substances are transported by more than one transmembrane protein. Demeule et al.[38] found that dexamethasone, which is a P-gp inducer, increased P-gp expression in the liver and the lung but reduced the expression of this molecule in the kidney. Transmembrane proteins in each tissue respond differently to inducers, as stated by Drescher et al.[39].

AFB_1 is activated via the conversion into AFB_1 8-9 epoxide by cytochrome 450 enzymes, especially CYP1A2 and CYP3A4, and glutathione S-transferases are the most important enzymes for detoxifying AFB_1 in all species, including mice [40-42]. The plasma concentration of AFB_1 in the ZQR-AF group was found to be significantly higher than those of the PRZ-AF and PRZ+ZQR-AF groups. The causes of this difference may be the inhibition of AFB_1 metabolism by zosuquidar and/or the induction of BCRP in excretion organs by prazosin. Van Herwaarden et al.[10] posited that BCRP plays an important role in the renal excretion of AFB_1. P-gp inhibitors have also been reported to inhibit CYP3A activity, which plays an important role in AFB_1 metabolism.

In summary, both prazosin and zosuquidar significantly reduced the brain concentration of AFB_1 but not the plasma concentration of this molecule. Thus, prazosin is a better inducer than zosuquidar for both BCRP and P-gp in brain capillaries. In addition, AFB_1 may be a substrate of both BCRP and P-gp. Inducers of transmembrane proteins, such as prazosin, can be life-saving during acute poisoning with AFB_1, based on the overall health status and brain concentrations of AFB_1 in mice. The results of *in vitro* studies in this issue should be confirmed with *in vivo* studies of wild-type animals.

REFERENCES

1. Dalvi RR: An overview of aflatoxicosis of poultry its characteristics, prevention and reduction. *Vet Res Commun*, 10, 429-443, 1986. DOI: 10.1007/BF02214006

2. Oguz H: A review from experimental trials on detoxification of aflatoxin in poultry feed. *Eurasian J Vet Sci*, 27, 1-12, 2011.

3. Zain ME: Impact of mycotoxins on humans and animals. *J Saudi Chem Soc*, 15, 129-144, 2011. DOI: 10.1016/j.jscs.2010.06.006

4. Bortell R, Asquith RL, Edds GT, Simpson GF: Acute experimentally induced aflatoxicosis in the weanling pony. *Am J Vet Res*, 44, 2110-2114, 1983.

5. Chao TC, Maxwell SM, Wong SY: An outbreak of aflatoxicosis and boric acid poisoning in Malaysia: A clinicopathological study. *J Pathol*, 164, 225-233, 1991. DOI: 10.1002/path.1711640307

6. Bastaki SA, Osman N, Kochiyil J, Shafiullah M, Padmanabhan R, Abdulrazzaq YM: Toxicokinetics of aflatoxin in pregnant mice. *Int J Toxicol*, 29, 425-431, 2010. DOI: 10.1177/1091581810369565

7. Bailey DG: Fruit juice inhibition of uptake transport: A new type of food-drug interaction. *Brit J Clin Pharmacol*, 70, 645-655, 2010. DOI: 10.1111/j.1365-2125.2010.03722.x

8. Abdallah HM, Al-Abd AM, El-Dine RS, El-Halawany AM: P-glyco-protein inhibitors of natural origin as potential tumor chemosensitizers: A review. *J Adv Res*, 6, 45-62, 2015. DOI: 10.1016/j.jare.2014.11.008

9. An G, Mukker JK, Derendorf H, Frye RF: Enzyme- and transporter-mediated beverage-drug interactions: An update on fruit juices and

green tea. *J Clin Pharmacol*, 55, 1313-1331, 2015. DOI: 10.1002/jcph.563

10. Van Herwaarden AE, Wagenaar E, Karnekamp B, Merino G, Jonker JW, Schinkel AH: Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. *Carcinogenesis*, 27, 123-130, 2006. DOI: 10.1093/carcin/bgi176

11. Robey RW, To KKK, Polgar O, Dohse M, Fetsch P, Dean M, Bates SE: ABCG: A perspective. *Adv Drug Deliver Rev*, 61, 3-13, 2009. DOI: 10.1016/j.addr.2008.11.003

12. Loe DW, Stewart RK, Massey TE, Deeley RG, Cole SP: ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. *Mol Pharmacol*, 51, 1034-1041, 1997.

13. Löscher W, Potscka H: Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Prog Neurobiol*, 76, 22-76, 2005. DOI: 10.1016/j.pneurobio.2005.04.006

14. Sharom FJ: 2008: ABC multidrug transporters: Structure, function and role in chemoresistance. *Pharmacogenomics,* 9, 105-127, 2008. DOI: 10.2217/14622416.9.1.105

15. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM: International transporter consortium. Membrane transporters in drug development. *Nat Rev Drug Discov*, 9, 215-236, 2010. DOI: 10.1038/nrd3028

16. Eisenblatter T, Galla HJ: A new multidrug resistance protein at the blood-brain barrier. *Biochem Biophys Res Commun*, 293, 1273-1278, 2002. DOI: 10.1016/S0006-291X(02)00376-5

17. Aszalos A: Role of ATP-binding cassette (ABC) transporters in interactions between natural products and drugs. *Curr Drug Metab*, 9, 1010-1018, 2008. DOI: 10.2174/138920008786927776

18. Zhou SF: Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*, 38, 802-832, 2008. DOI: 10.1080/00498250701867889

19. Durmus S, Xu N, Sparidans IRW, Wagenaar E, Beijnenb JH, Schinkel AH: P-glycoprotein (MDR1/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) restrict brain accumulation of the JAK1/2 inhibitor, CYT387. *Pharmacol Res*, 76, 9-16, 2013. DOI: 10.1016/j.phrs.2013.06.009

20. AOAC (Association of Official Analytical Chemist): Official method of analysis. Natural Toxins. Vol. 2, 17th edn., 20-22, Association of Official Analytical Chemist, Gaithersburg, MD, USA, 2000.

21. Chiavaro E, Cacchioli C, Berni E, Spotti E: Immunoaffinity clean-up and direct fluorescence measurement of aflatoxins B1 and M1 in pig liver: Comparison with high-performance liquid chromatography determination. *Food Addit Contam*, 22, 1154-1161, 2005. DOI: 10.1080/02652030500307115

22. Hooper DG, Bolton VE, Guilford FT, Straus DC: Mycotoxin detection in human samples from patients exposed to environmental molds. *Int J Mol Sci*, 10, 1465-1475, 2009. DOI: 10.3390/ijms10041465

23. Gangolli S: The dictionary of substances and their effects. **In,** Gangolli S (Ed): The Royal Society of Chemistry. 2nd edn., Cambridge, England, 1999.

24. Takano M, Yumoto R, Murakami T: Expression and function of efflux drug transporters in the intestine. *Pharmacol Ther*, 109, 137-161, 2006. DOI: 10.1016/j.pharmthera.2005.06.005

25. Liu Y, Liu H, Yang H, Wen T, Shang Y, Liu X, Xie L, Wang G: Impaired expression and function of breast cancer resistance protein (Bcrp) in brain cortex of streptozocin-induced diabetic rats. *Biochem Pharmacol*, 74, 1766-1772, 2007. DOI: 10.1016/j.bcp.2007.08.021

26. Juvale K. Wiese M: 4-Substituted-2-phenylquinazolines as inhibitors of BCRP. *Bioorg Med Chem Lett*, 22, 6766-6769, 2012. DOI: 10.1016/j.bmcl.2012.08.024

27. Takara K, Yamamoto K, Matsubara M, Minegaki T, Takahashi M, Yokoyama T, Okumura K: Effects of α-adrenoceptor antagonists on ABCG2/BCRP-mediated resistance and transport. *PLoS One* 7 (2): e30697. 2012. DOI: 10.1371/journal.pone.0030697

28. Tanaka Y, Slitt AL, Leazer TM, Maher JM, Klaassen CD: Tissue distribution and hormonal regulation of the breast cancer resistance protein (Bcrp/Abcg2) in rats and mice. *Biochem Biophys Res Commun*, 326, 181-187, 2004. DOI: 10.1016/j.bbrc.2004.11.012

29. González-Lobato L, Real R, Prieto JG, Alvarez AI, Merino G: Differential inhibition of murine Bcrp1/Abcg2 and human BCRP/ABCG2 by the mycotoxin fumitremorgin C. *Eur J Pharmacol*, 644, 41-48, 2010. DOI: 10.1016/j.ejphar.2010.07.016

30. Zong J, Pollack GM: Modulation of P-glycoprotein transport activity in the mouse blood-brain barrier by rifampin. *J Pharmacol Exp Ther*, 306, 556-562, 2003. DOI: 10.1124/jpet.103.049452

31. Cetin G, Tras B, Uney K: Effects of dexamethasone and fexofenadine on the penetration into brain and testes tissues of levofloxacin, a P-gp substrate. *12th International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT 2012),* July 8-12, Noordwijkerhout-Netherlands, 2012.

32. Enokizono J, Kusuhara H, Ose A, Schinkel AH, Sugiyama Y: Quantitative investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) in limiting brain and testis penetration of xenobiotic compounds. *Drug Metab Dispos*, 36, 995-1002, 2008. DOI: 10.1124/dmd.107.019257

33. Zhou L, Schmidt K, Nelson FR, Zelesky V, Troutman MD: The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl) propanoic acid (PF-407288) in mice. *Drug Metab Dispos*, 37, 946-955, 2009. DOI: 10.1124/dmd.108.024489

34. Cripe LD, Uno H, Paietta EM, Litzow MR, Ketterling RP, Bennett JM, Rowe JM, Lazarus HM, Luger S, Tallman MS: Zosuquidar, a novel modulator of P-glycoprotein, does not improve the outcome of older patients with newly diagnosed acute myeloid leukemia: A randomized, placebo-controlled trial of the Eastern Cooperative Oncology Group 3999. *Blood*, 116, 4077-4085, 2010. DOI: 10.1182/blood-2010-04-277269

35. Mittapalli RK, Vaidhyanathan S, Sane R, Elmquıst WF: Impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on the brain distribution of a novel BRAF inhibitor: Vemurafenib (PLX4032). *J Pharmacol Exp Ther*, 342, 33-40, 2012. DOI: 10.1124/jpet.112.192195

36. De Lange EC, Marchand S, van den Berg D, van der Sandt IC, de Boer AG, Delon A, Bouquet S, Couet W: *In vitro* and *in vivo* investigations of fluoroquinolones: Effects of the P-glycoprotein efflux transporter on brain distribution of sparfloxacin. *Eur J Pharm Sci*, 12, 85-93, 2000. DOI: 10.1016/S0928-0987(00)00149-4

37. Tamai, I, Yamashita J, Kido Y, Ohnari A, Sai Y, Shıma Y, Naruhashi K, Koizumi S, Tsuji A: Limited distribution of new quinolone antibacterial agents into brain caused by multiple efflux transporters at the blood-brain barrier. *J Pharmacol Exp Ther*, 295, 146-152, 2000.

38. Demeule M, Jodoin J, Beaulieu E, Brossard M, Beliveau R: Dexamethasone modulation of multidrug transporters in normal tissues. *Febs Letters*, 442, 208-214, 1999. DOI: 10.1016/S0014-5793(98)01663-9

39. Drescher S, Glaeser H, Mürdter T, Hitzl M, Eichelbaum M, Fromm MF: P-glycoprotein-mediated intestinal and biliary digoxin transport in humans. *Clin Pharmacol Ther*, 73, 223-231, 2003. DOI: 10.1067/mcp.2003.27

40. Kirby GM, Chemin I, Montesano R, Chisari FV, Lang MA, Wild VP: Induction of specific cytochrome P450s involved in aflatoxin B1 metabolism in hepatitis B virus transgenic mice. *Mol Carcinogen*, 11, 74-80, 1994. DOI: 10.1002/mc.2940110204

41. Van Vleet TR, Klein PJ, Coulombe RA: Metabolism of aflatoxin B1 by normal human bronchial epithelial cells. *J Toxicol Environ Health Part A*, 63, 525-540, 2001. DOI: 10.1080/15287390152410156

42. Yang X, Lu H, Li Z, Bian Q, Qiu L, Li Z, Liu Q, Li J, Wang X, Wang S: Cytochrome P450 2A13 mediates aflatoxin B1-induced cytotoxicity and apoptosis in human bronchial epithelial cells. *Toxicology*, 300, 138-148, 2012. DOI: 10.1016/j.tox.2012.06.010

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Research Article

The Effects of Swimming Exercise and Probiotic VSL#3 on Zonulin and Some Inflammatory and Oxidative Parameters in Rats [1]

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Abstract

Moderate exercise stimulates immune system whereas intensive exercise may display immune-suppressive effect associated with the disruption of intestinal barrier. With this study, we tested the effects of moderate and intensive swimming exercises on some cytokines and oxidant variables and zonulin, an intestinal barrier marker, in rats. We also tested possible ameliorative effects of probiotic VSL#3 in both moderate and intensive exercise regimens. Twenty eight rats were randomly divided into 4 equal groups: Control-C, Probiotic-P, Exercise-E, Probiotic+Exercise-PE. The rats in group E and PE underwent moderate swimming exercise for 5 weeks. Following this period, intensive swimming exercise was performed for 5 days. The rats in group C and group P were sedentary. Probiotic VSL#3 was given to group P and PE in the water. At the end of the experiments, serum zonulin, TNF-α, IL-6, IL-10, TGF-β, MDA, and protein carbonyl levels were determined. Evidences obtained from present study indicate that moderate swimming exercise improves barrier integrity of intestine and decreases oxidative stress. During the moderate swimming experiment, probiotic VSL#3 supplementation may also improve inflammatory response. On the other hand, intensive exercise does not led changes in the inflammatory response and oxidative stress, but beneficial responses of moderate exercise on the selected parameters probably disappear due to the intense exercise-induced mild stress.

Keywords: *Cytokine, Oxidative parameters, Probiotic, Rat, Swimming exercise, Zonulin*

Ratlarda Yüzdürme Egzersizi ve Probiyotik VSL#3'ün Zonulin İle Bazı Yangısal ve Oksidatif Değişkenlere Etkileri

Özet

Yoğun egzersizler intestinal bariyerin bozulmasıyla ilişkili olarak immun-supresif etki oluşturabilirken, ılımlı egzersizler immun sistemi stimule eder. Bu çalışma ile ratlarda ılımlı ve yoğun yüzdürme egzersizlerinin bazı sitokinlere, oksidan değişkenlere ve intestinal bariyer belirteci zonuline etkilerini değerlendirdik. Ayrıca probiyotik VSL#3'ün hem ılımlı hem de yoğun yüzdürme periyodundaki olası iyileştirici etkilerini test ettik. Yirmi sekiz rat, rasgele 4 eşit gruba ayrıldı: Kontrol-C, Probiyotik-P, Egzersiz-E, Probiyotik+Egzersiz-PE. E ve PE grubundaki ratlara 5 hafta süreyle ılımlı yüzdürme egzersizi ve ılımlı yüzdürme periyodunu takiben 5 gün süreyle yoğun yüzdürme egzersizi yaptırıldı. C ve P grupları ise sedanter grupları oluşturdu. P ve PE gruplarına içme suyu ile probiyotik VSL#3 verildi. Deneylerin sonunda serumda zonulin, TNF-α, IL-6, IL-10, TGF-β, MDA ve protein karbonil düzeyleri belirlendi. Sonuçlar ılımlı yüzdürme egzersizinin bağırsak bariyer bütünlüğünü koruduğunu ve oksidatif stresi azalttığını göstermektedir. Ilımlı egzersiz sürecinde probiyotik VSL#3 alınması yangısal yanıtı da olumlu etkileyebilir. Diğer taraftan yoğun egzersiz herhangi bir yangısal yanıta ve oksidatif strese neden olmamıştır. Fakat ılımlı egzersizin ortaya çıkardığı faydalı yanıtlar muhtemelen yoğun egzersizin oluşturduğu hafif şiddetteki stres nedeniyle ortadan kalkmıştır.

Anahtar sözcükler: *Sitokin, Oksidatif değişkenler, Probiyotik, Rat, Yüzdürme egzersizi, Zonulin*

INTRODUCTION

Cytokines are involved in the maintaining of many physiological processes besides fighting pathogens entering the body. They have significant roles against the changes in internal environment caused by various stress factors such as exercise [1]. Moderate exercise stimulates immune system, but intense or exhaustive exercise may cause immune-suppressive effect [2]. Intensive exercise may also cause upper respiratory infections and some

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gastrointestinal complaints such as cramp, diarrhea, bloating, nausea or bleeding [3,4]. The prevention of such disorders occurring after strenuous training or competition are among priorities of exercise scientists, athletes, and trainers [3,5]. These problems created by intense exercise are associated with the weakness of intestinal barrier and the alterations in the intestinal permeability [4]. Intestinal ischemia-induced oxidative damage and some pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 activated by oxidative damage and nuclear factor kappa (NFκB) are responsible for the alterations in the barrier integrity during and after intensive exercise [6,7]. Intestinal barrier weakness, also called leaky gut, can lead to absorption of pathogens and toxins into the blood and tissues. One of the main components of gastrointestinal permeability is the tight junctions controlling the passage of the molecules from paracellular area among the intestinal epithelial cells. The integrity of the tight junctions is regulated by complex interactions between the gut microbiota and their products, intestinal epithelial cells, and immune cells [8]. Zonula occludens toxin (ZOT) derived from Vibrio cholera causes reversible disruption in the structure of tight junction and increases intestinal permeability. An analogue of this protein, zonulin also known pre-haptoglobin 2, has recently been discovered and isolated from fetal and adult human gut. When intestinal barrier is disrupted, zonulin levels increase in serum, gut and stool, leading to reversible deterioration of tight junction [9].

Probiotics have favorable effects on the intestinal barrier integrity and immune system. They are major supplements for intestinal and overall health among athletes [4,10]. Probiotics reduce the incidence and the severity of respiratory infections [11-13], and duration of gastrointestinal complaints in athletes [13,14]. Probiotics also attenuate the inflammatory response [15] and enhance the plasma antioxidant levels [16]. Probiotic supplementation for 14 weeks leads to a reduction in fecal zonulin and plasma TNF-α levels and this also ameliorates the exercise-induced protein oxidation in training athletes [15]. However, information on the effects of probiotic on intestinal barrier, inflammatory, and the oxidative response in moderate and intensive exercise is limited [4]. We aimed to investigate whether moderate and intensive swimming exercises affect intestinal barrier and the exercise-associated oxidative and inflammatory processes. We also investigated the ameliorative effects of probiotic VSL#3 on these processes both in sedentary and exercised rats.

MATERIAL and METHODS

Animals and Experimental Design

The present study was approved by the Adnan Menderes University Animal Ethics Committee (ADÜ-HADYEK-Approval No: 64583101/2013/096). A total of 28 early adult male Sprague-Dawley rats were housed in individual cages at 22±2°C and 55-65% humidity. Standard mouse chow and tap water were given *ad libitum*. The rats were divided into 4 groups equally (n=7). Two groups underwent moderate swimming exercise on weekdays for the first 5 weeks. They were then subjected to intensive swimming exercise on weekdays for one additional week following moderate swimming. The other two groups of the rats were not subjected to exercise (sedentary groups). Probiotic VSL#3 (Sigma Tau Pharmaceuticals, Inc. MD, USA) in tap water was given to one of sedentary groups and one of exercise groups. The rats were then swum with a lead load of 5% body weight attached to the tail in water at 32±1°C and 70 cm depth in plastic bucket, which was 80 cm in length, 50 cm in width and 90 cm in depth between 10:00 a.m. and 12:00 p.m. for 1 h a day, 5 days a week for 5-week moderate swimming exercise program [17,18]. Adaptation to swimming with load was performed by gradually increasing swimming time (10 min. every day). At day 5 of first week of moderate exercise, the rats were swum for 10 min. free and for 50 min. with load. The swimming exercise procedure continued as 10 min. free and for 50 min. with load for the next 5 weeks. The intensive swimming program was performed as 3 times (1 h each) a day for the next 1 week following the moderate swimming exercise. In this period, rats were allowed to rest for 150 min between exercise sessions. To eliminate the effects of water stress, sedentary groups left into shallow water without load at the same temperature for 30 min once a day during moderate exercise and for 30 min. 3 times a day during intensive exercise. After the rats were taken out from the water, they were dried with towel and returned to their cages. For adaptation to water, the rats were allowed to rest in the buckets filled with water for 30 min before the experiments started *(Table 1)*.

Probiotic Supplementation

Probiotic VSL#3 in tap water (20 mg dissolved in 75 mL tap water/rat/day) was given on weekdays for 6 weeks [19]. Probiotic was prepared daily. Each unflavored VSL#3 sachets

Table 1. *Experimental design*
Tablo 1. *Deney dizaynı*

<table>
<tr><th colspan="6">Groups [Sedantary Groups; Control (C) and Probiotic (P), Swimming Exercise Groups; Exercise (E) and Probiotic+Exercise (PE)]</th></tr>
<tr><th colspan="6">Weeks (0-6)</th></tr>
<tr><td>Water Adaptation</td><td>Moderate Swimming Exercise</td><td rowspan="3">Blood collection</td><td>Intensive Swimming Exercise</td><td rowspan="3">Blood collection</td></tr>
<tr><td>0</td><td>5 weeks</td><td>6th week</td></tr>
<tr><td>30 min/a day</td><td>1 h/ a day for 5 days/ a week</td><td>3 times, 1 h/a day with 150 min interval for 5 days/a week</td></tr>
</table>

(2.5 g) contain corn starch and 450 billion live freeze-dried bacteria; one strain of *Streptococcus Thermophilus*, three strains of *Bifidobacterium* and four strains of *Lactobacillus*. The rats consumed mean 60 mL water per day *(Fig. 3)*, corresponding to about 16 mg probiotic or 2.88 billion bacteria.

Blood Collection and Analysis

Body weight, food and water consumption were recorded weekly throughout the experiment. Blood samples were collected from the tail vein at the end of week 5 (immediately after last moderate exercise) and week 6 (immediately after intensive exercise) under ether anesthesia. Serum was separated by centrifugation at 3.500 rpm for 15 min and stored at -20°C until analyzed. Zonulin, TNF-α, IL-6, IL-10, TGF-β, and MDA and protein carbonyl concentrations were measured by ELISA except MDA. A microplate spectrophotometer (Thermo Scientific, Multiscan GO, Finland) was used for determining the absorbance of all ELISA tests.

Cytokine Analysis

TNF-α, IL-6, IL-10, and TGF-β levels were measured by ELISA method using commercial kits (eBioscience Rat Platinum Sandwich ELISA kits). All test protocols described by the manufacturer for the cytokine analyses were similar. Each cytokine present in sample or standard coating specific anti-rat cytokine antibodies was adsorbed onto microwells. A biotin conjugated anti-rat cytokine antibody was added and bond to rat cytokine captured by the first antibody. Following incubation, unbound biotin conjugated anti-rat cytokine antibody was removed with washing steps. Streptavidin-HRP was added and bond to the biotin-conjugated anti-rat cytokine antibody. Following incubation, the unbound Streptavidin-HRP was removed with washing steps and substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of rat TNF-α, IL-6, IL-10 or TGF-β present in sample or standard. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from 7 specific standard dilutions and each cytokine concentration was determined. Concentrations of cytokines in serum were expressed as pg/mL.

Zonulin Analysis

Zonulin analysis was assessed using the Rat Zonulin ELISA kit (MyBioSource). Principle of the assay based on the competitive enzyme immunoassay technique utilizing a monoclonal anti-ZON antibody and a ZON-HRP conjugate in pre-coated plate for 1 h. After the incubation period, the wells are decanted and washed 5 times. Wells were incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction formed a blue colored complex. Finally, a stop solution was added and blue colored solution turned to yellow. The intensity of the color was measured at 450 nm. The intensity of color was inversely proportional to the ZON concentration since ZON from samples and ZON-HRP conjugate compete for the anti-ZON antibody binding site. A standard curve was prepared relating the intensity of the color to the concentration of standards and ZON concentration in each sample was determined. Results were expressed as ng/mL.

Analysis of Protein Carbonyl and Malondialdehyde

Protein Carbonyl was measured using the Oxiselect™ Protein Carbonyl ELISA Kit (Cell Biolabs, Inc). BSA standards or protein samples (10 µg/mL) were adsorbed onto micro-wells at 4°C overnight. The protein carbonyl present in sample or standard was derivatized to DNP hydrazine and probed with an anti-DNP antibody, followed by HRP conjugated secondary antibody. Reaction was stopped after incubation with the substrate solution. Absorbance of each well was measured at 450 nm. The protein carbonyl content in unknown sample was determined by comparing with a standard curve that was prepared from predetermined reduced or oxidized BSA standards. Results are expressed as nmol carbonyl content/mg protein.

Serum MDA levels were determined thiobarbituric acid (TBA) method described by Ohkawa et al.[20]. Principle of analysis based on the measurement of pink-colored pigment produced with reaction between TBA and MDA in acidic pH and hot environment. The absorbance was measured spectrophotometrically at 532 nm and results were expressed as µmol MDA/mL.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (*Version 22.0, SPSS Inc., Chicago, IL, USA*). Data are expressed as mean ± standard error of mean (SEM). Distribution of data was assessed by the Shapiro-Wilk test. The data distributed normally were analyzed by ANOVA with Duncan *post hoc* test for multiple comparisons after moderate and intensive swimming exercises. The data violated normality assumptions were analyzed by Kruskal-Wallis ANOVA with Bonferroni-adjusted Mann-Whitney-U *post hoc* test. Body weight, food and water consumption were analyzed by repeated measures test. P-values <0.05 were considered to be statistically significant.

RESULTS

Body weight, food and water consumption increased over time (P<0,001), but there was no intervention effect *(Fig. 1, 2* and *3)*.

It was determined that moderate exercise or probiotic separately has reducing effect on zonulin concentration after 5 weeks. Zonulin concentration decreased in exercise and sedentary probiotic groups when compared with the control group (P=0.011). Moderate exercise and probiotic

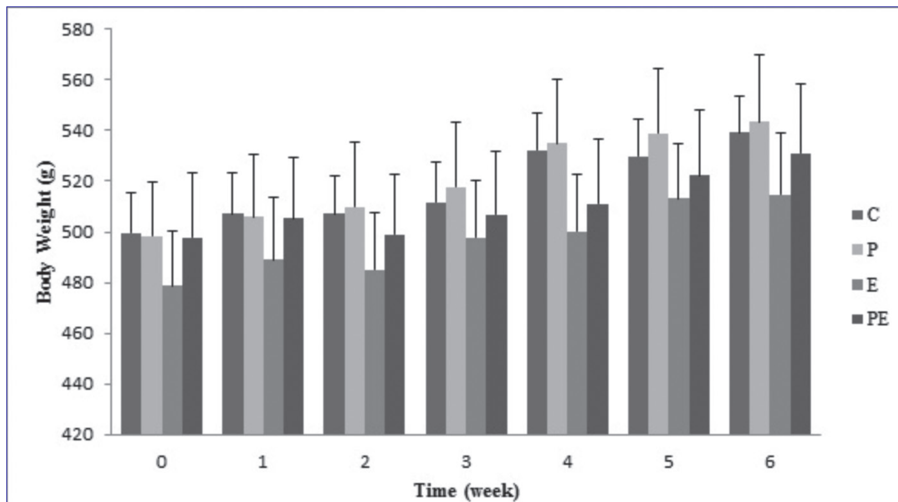

Fig 1. Body weight of rats

Şekil 1. Ratların vücut ağırlıkları

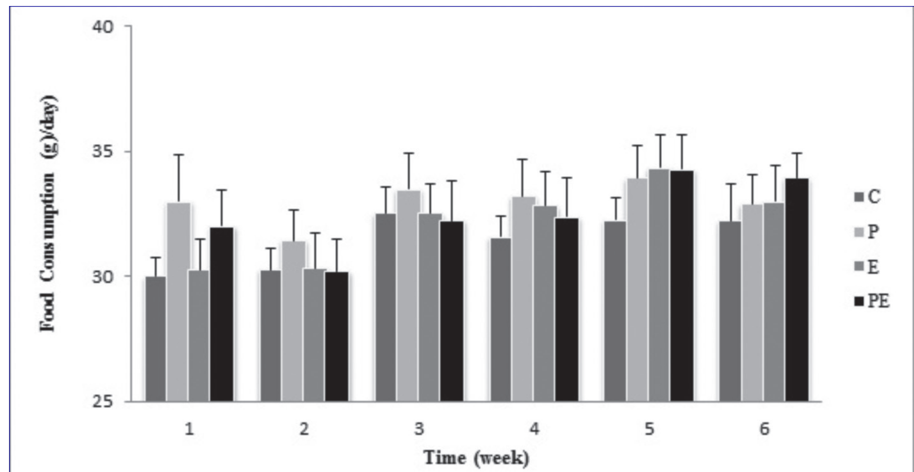

Fig 2. Food consumption of rats

Şekil 2. Ratların yem tüketimleri

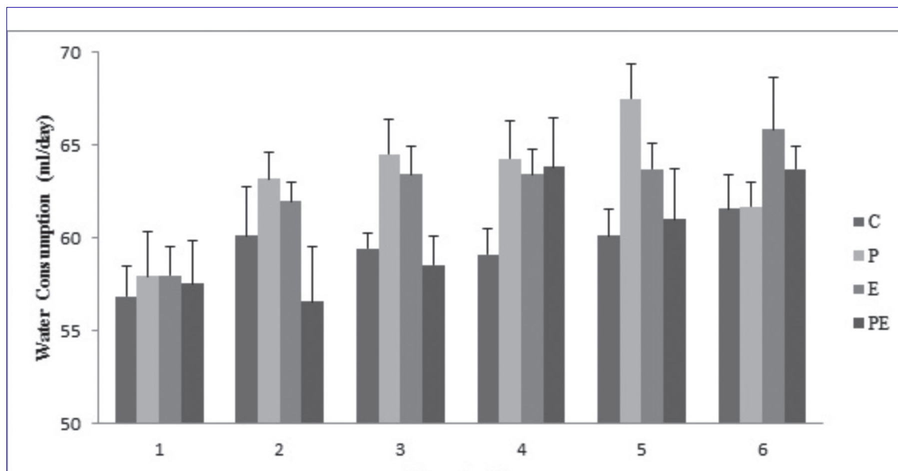

Fig 3. Water consumption of rats

Şekil 3. Ratların su tüketimleri

VSL#3 supplementation together led to non-significant reduction in zonulin levels, in contrast to the expectations, indicating that no synergistic effect between exercise and probiotic VSL#3 supplementation. After intense exercise, zonulin levels were still low in the exercise group, but this was not statistically significant (P=0.072) *(Table 2)*.

The effects of moderate exercise on the cytokine and oxidant variables were found to be more prominent than the intense exercise (*Table 2* and *Table 3*, respectively). Moderate exercise and probiotic VSL#3 supplementation together decreased IL-6, TNF-α, and TGF-β levels compared with the one or more of other groups (P=0.022, P=0.003 and P=0.040 respectively) *(Table 2)*. However, the mean levels of IL-6, TNF-α, and TGF-β in sedentary probiotic or exercise groups were not different from the control group.

Table 2. *Zonulin and cytokine levels after moderate (M) and intensive (I) swimming exercise periods*
Tablo 2. *Ilımlı (M) ve yoğun (I) yüzdürme egzersizi sonrası zonulin ve sitokin düzeyleri*

Zonulin and Cytokines	Groups				P
	Control	Probiotic	Exercise	Probiotic + Exercise	
Zonulin/M (ng/mL)	321.26±15.43^{a}	217.01±31.48^{b}	198.05±29.73^{b}	271.88±24.53^{ab}	0.011
Zonulin/I (ng/mL)	334.94±30.81	308.46±37.61	235.76±40.29	383.84±39.18	0.072
IL6/M (pg/mL)	107.82±4.15^{ab}	112.03±3.27^{ab}	114.35±2.93^{a}	97.60±4.07^{b}	0.022
IL6/I (pg/mL)	110.39±3.75	114.53±5.10	111.98±6.54	105.49±4.56	NS
TNF-α/M (pg/mL)	151.67±4.29^{b}	140.39±2.37^{ab}	151.99±6.06^{b}	128.86±3.37^{a}	0.003
TNF-α/I (pg/mL)	141.76±4.98	134.64±5.80	139.63±3.85	131.34±6.87	NS
TGF-β/M (pg/mL)	520.22±24.08^{ab}	554.63±31.83^{b}	576.62±32.09^{b}	449.31±31.31^{a}	0.040
TGF-β/I (pg/mL)	488.53±40.60	540.88±53.20	519.39±43.40	493.68±30.38	NS
IL10/M (pg/mL)	193.97±20.57	188.05±34.66	188.44±19.12	180.47±22.69	NS
IL10/I (pg/mL)	127.91±11.09	106.76±12.39	149.79±12.51	139.70±20.50	NS

*Data are shown as means ± SEM; ^{a,b} The means in the different row with differing letters differ from each other, **NS:** Not significant*

Table 3. *The levels of oxidant variables after moderate (M) and intensive (I) swimming exercise periods*
Tablo 3. *Ilımlı (M) ve yoğun (I) yüzdürme egzersizi sonrası oksidan değişkenlerin düzeyleri*

Oxidant Parameters	Groups				P
	Control	Probiotic	Exercise	Probiotic + Exercise	
PC/M (nmol/mg prot)	0.62±0.03	0.78±0.08	0.83±0.07	0.62±0.07	NS
PC/I (nmol/mg prot)	0.71±0.03	0.55±0.11	0.70±0.10	0.73±0.03	NS
MDA/M (μmol/mL)	0.40±0.05^{ab}	0.43±0.01^{b}	0.37±0.01^{a}	0.36±0.03^{a}	0.010
MDA/I (μmol/mL)	0.47±0.01^{b}	0.48±0.02^{b}	0.43±0.01^{a}	0.44±0.01^{ab}	0.035

*Data are shown as means ± SEM; ^{a,b} The means in the different row with differing letters differ from each other, **NS:** Not significant*

There were no significant changes for IL-10 levels between groups after both moderate and intensive exercises *(Table 2)*.

Oxidant variables including protein carbonyl and MDA showed different responses to exercise *(Table 3)*. Protein carbonyl levels tended to increase in exercise groups and probiotic supplementation returned protein carbonyl levels to the control levels (P=0.097). Both moderate and intensive exercises led to a reduction in MDA levels. MDA levels in the both exercise groups were lower than the sedentary groups after 5-week moderate exercise, but differences were confirmed only for between exercise groups and sedentary probiotic group (P=0.010). MDA levels of sedentary probiotic group were not different from those of the control group. After intense exercise, MDA levels significantly decreased only in exercise group compared with the control and probiotic groups (P=0.035).

DISCUSSION

Exercise leads to several endocrine, immunological and oxidative responses. Regular and moderate exercise regimens induce immune response beneficially and are effective in prevention and in the treatment of various diseases. In contrast, intensive or strenuous exercise can have immune suppressive effect and cause some upper respiratory and gastrointestinal complaints which are associated with disruption of the intestinal barrier [3,21,22]. Based on these evidences, we hypothesized that moderate exercise and/or probiotic supplementation decrease intestinal barrier marker, zonulin, levels and selected cytokines such as pro-inflammatory (e.g.TNF-α), inflammation responsive (e.g. IL-6), and mechanical stress-induced (e.g. TGF-β1) whereas increase anti-inflammatory cytokines (e.g. IL-10). We also hypothesized that intense swimming exercise causes the opposite effects of moderate exercise on cytokine and zonulin levels and the usage of probiotics can improve this situation.

We preferred cocktail VSL#3 as probiotic supplementation because VSL#3 is one of the most beneficial recommendations for ulcerative colitis therapy [23] and leads to a reduction in colonic expression of pro-inflammatory cytokines and mucosal damage and stimulates barrier integrity in different cases of colitis [24] or in common bile duct ligation induced inflammatory response [25]. We here show that 5-week moderate exercise and probiotic VSL#3 supplementation together led to reductions in some cytokine levels whereas only moderate exercise or probiotic VSL#3 supplementation does not, suggesting that usage

of probiotic VSL#3 during moderate exercise improves immune system, at least in part with these experimental conditions. In general, exercise can exclusively affect cytokine levels depending on exercise type, duration, and intensity [21,26]. Several exercise protocols performed in animals or humans have reported different findings regarding cytokine levels [15,27-31]. Mechanical stimulation of some tissues such as muscle, vascular smooth muscle, connective tissue, and bone leads to change in TGF-β1 production. It seems like TGF-β1 levels transiently increase at the beginning of the exercise regimen and then normalization occurs due to the adaptation [32]. It has also been determined that exercise led to a reduction in TNF-α by stimulating IL-6 levels and increase in IL-1ra and IL-10 and antioxidant levels [22,27-30]. On the other hand, there is evidence that moderate and overtraining swimming exercise does not induce any significant differences in TNF-α and IL-6 levels, but moderate exercise decreases TBARS levels in rats, suggesting that the intensive exercise periods or overtraining may not cause detrimental effects which consistent with our results [31]. Together, our results obtained from exercise group are partly consistent with previous studies. More importantly, probiotic VSL#3 and moderate exercise together make significant changes in immune response which may be important or more effective for curing some diseases such as colitis.

Many studies indicate that moderate endurance exercises decrease oxidative stress whereas improved anti-oxidative mechanisms [28,33-35]. Our results support these evidences that moderate and intensive exercise exert to decrease oxidative stress, but probiotic supplementation neither improve nor exacerbate this notion which suggests that there is no synergistic effect between probiotic and exercise in terms of MDA levels. Probiotics have anti-oxidative effects by reducing oxidative stress induced by mutagen, doxorubicin, diabetes or common bile duct ligation in different animal models [25,36-38]. However, this effect does not appear in healthy state [37,38]. The administration of probiotic fermented beetroot juice [38] or kefir [37] does not have effect on MDA levels in rats which is consistent with our result. In addition, it seems like protein oxidation does not affect from probiotic supplementation, exercise or both as evidenced by no change in protein carbonyl (a product of protein oxidation) levels which is supported by the estimates of Wadley et al.[39]. The effects of exercise on protein carbonyl levels may vary depending on intensity and duration of exercise due to balance between production and clearance of protein carbonyl product [39].

Probiotics are live micro-organism that regulates gut microbiota, mucosal barrier, and immune system. There is limited number of studies regarding relationship between probiotics, exercise, and zonulin levels [15,35]. Zonulin is an important marker that modulates the permeability of the intestinal barrier [9]. Probiotic supplementation causes a reduction in zonulin levels both *in vivo* and *in vitro* [15,40-42]. Chronic exercise training may cause a mild increase in the intestinal permeability by slightly enhancing zonulin levels in human [15,43]. On the other hand, there is evidence that suggest 10-day treadmill exercise (60 min/day) may led to alterations in zonulin mRNA expression and this could be related to intestinal barrier disruption [35]. Probiotic supplementation and chronic exercise together also caused to a reduction in zonulin levels in feces [15] which suggest that exercise and probiotic supplementation can beneficially affect the intestinal barrier. In the present study, we show that either probiotic supplementation or moderate exercise improve intestinal barrier integrity by decreasing blood zonulin levels. However, we did not determine additive effect between probiotic supplementation and exercise, as evidenced by non-significant reduction in zonulin levels of the rats that subjected to exercise and probiotic supplementation.

In conclusion, moderate swimming exercise decreases oxidative stress and zonulin levels that are important for the integrity of the intestinal barrier. Moderate swimming exercise and probiotic supplementation together improve immune and oxidative response and display beneficial tendencies regarding barrier integrity. However, these beneficial changes in moderate exercise and/or probiotic supplementation disappear in case of intensive swimming exercise possibly due to a mild stress in this period. The lack of any significant alterations in barrier disruption and inflammatory response suggest that exercise-adapted rats could tolerate this intensive regimen.

REFERENCES

1. Cannon JG: Inflammatory cytokines in nonpathological states. *News Physiol Sci*, 15, 298-303, 2000.

2. Mackinnon LT: Chronic exercise training effects on immune function. *Med Sci Sports Exerc,* 32 (Suppl.): S369-S376, 2000. DOI: 10.1097/00005768-200007001-00001

3. West NP, Pyne DB, Peake JM, Cripps AW: Probiotics, immunity and exercise: A review. *Exerc Immunol Rev*, 15, 107-126, 2009.

4. Lamprecht M, **Frauwallner A:** Exercise, intestinal barrier dysfunction and probiotic supplementation. *Med Sport Sci,* 59, 47-56, 2013. DOI: 10.1159/000342169

5. Kumaş C, Maden M: Evaluation of the dynamic (overground) endoscopy procedure in the diagnosis of upper respiratory tract diseases affecting performance of race horses. *Kafkas Univ Vet Fak Derg*, 19 (Suppl. A): A55-A60, 2013. DOI: 10.9775/kvfd.2012.7707

6. Gloire G, Legrand-Poels S, Piette J: NF-kappa B activation by reactive oxygen species: Fifteen years later. *Biochem Pharmacol*, 72, 1493-1505, 2006. DOI: 10.1016/j.bcp.2006.04.011

7. Lambert GP: Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J Anim Sci*, 87 (Suppl.): E101-E108, 2009. DOI: 10.2527/jas.2008-1339

8. Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C: Bacteria and host interactions in the gut epithelial barrier. *Nat Chem Biol,* 8, 36-45, 2011. DOI: 10.1038/nchembio.741

9. Fasano A: Zonulin and its regulation of intestinal barrier function: The biological door to inflammation, autoimmunity, and cancer. *Physiol Rev*, 91, 151-175, 2011. DOI: 10.1152/physrev.00003.2008

10. Pyne DB, West NP, Cripps AW: Probiotics and immune response to exercise. *Am J Lifest Med,* 7, 51-59, 2013. DOI: 10.1177/1559827612449599

11. Cox AJ, Pyne DB, Saunders PU, Fricker PA: Oral administration of the probiotic *Lactobacillus fermentum* VRI-003 and mucosal immunity in endurance athletes. *Br J Sports Med*, 44, 222-226, 2010. DOI: 10.1136/bjsm.2007.044628

12. Gleeson M, Bishop NC, Oliveira M, Tauler P: Daily probiotic's *(Lactobacillus casei* Shirota) reduction of infection incidence in athletes. *Int J Sport Nutr Exerc Metab,* 21, 55-64, 2011.

13. West NP, Pyne DB, Cripps AW, Hopkins WG, Eskesen DC, Jairath A, Christophersen CT, Conlon MA, Fricker PA: *Lactobacillus fermentum* (PCCW) supplementation and gastrointestinal and respiratory-tract illness symptoms: A randomised control trial in athlets. *Nutr J,* 11, 10-30, 2011. DOI: 10.1186/1475-2891-10-30

14. Kekkonen RA, Vasankari TJ, Vuorimaa T, Haahtela T, Julkunen I, Korpela R: The effect of probiotics on respiratory infections and gastrointestinal symptoms during training in marathon runners. *Int J Sport Nutr Exerc Metab*, 17, 352-363, 2007.

15. Lamprecht M, Bogner S, Schippinger G, Steinbauer K, Fankhauser F, Hallstroem S, Schuetz B, Greilberger JF: Probiotic supplementation affects markers of intestinal barrier, oxidation, and inflammation in trained men: A randomized, double-blinded, placebo-controlled trial. *J Int Soc Sports Nutr,* 9, 45, 2012. DOI: 10.1186/1550-2783-9-45

16. Martarelli D, Verdenelli MC, Scuri S, Cocchioni M, Silvi S, Cecchini C, Pompei P: Effect of a probiotic intake on oxidant and antioxidant parameters in plasma of athletes during intense exercise training. *Curr Microbiol*, 62, 1689-1696, 2011. DOI: 10.1007/s00284-011-9915-3

17. Santos RV, Caperuto EC, Costa Rosa LF: Effects of acute exhaustive physical exercise upon glutamine metabolism of lymphocytes from trained rats. *Life Sci*, 80, 573-578, 2007. DOI: 10.1016/j.lfs.2006.10.015

18. Lima FD, Stamm DN, Della-Pace ID, Dobrachinski F, de Carvalho NR, Royes LF, Soares FA, Rocha JB, González-Gallego J, Bresciani G: Swimming training induces liver mitochondrial adaptations to oxidative stress in rats submitted to repeated exhaustive swimming bouts. *PLoS One,* 8 (2): e55668, 2013. DOI: 10.1371/journal.pone.0055668

19. Dai C, Zhao DH, Jiang M: VSL#3 probiotics regulate the intestinal epithelial barrier *in vivo* and *in vitro* via the p38 and ERK signaling pathways. *Int J Mol Med*, 29, 202-208, 2012. DOI: 10.3892/ijmm.2011.839

20. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*, 95, 351-358, 1979. DOI: 10.1016/0003-2697(79)90738-3

21. Pedersen BK, Hoffman-Goetz L: Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev,* 80, 1055-1081, 2000.

22. Petersen AM, Pedersen BK: The anti-inflammatory effect of exercise. *J Appl Physiol* 98,1154-1162, 2005. DOI: 10.1152/japplphysiol.00164.2004

23. Chibbar R, Dieleman LA: Probiotics in the management of ulcerative colitis. *J Clin Gastroenterol,* 49 (Suppl. 1): S50-55, 2015. DOI: 10.1097/MCG.0000000000000368

24. Orel R, Trop TK: Intestinal microbiota, probiotics, and prebiotics in inflammatory bowel disease. *World J Gastroenterol*, 20, 11505-11524, 2014. DOI: 10.3748/wjg.v20.i33.11505

25. Rashid SK, Idris-Khodja N, Auger C, Alhosin M, Boehm N, Oswald-Mammosser M, Schini-Kerth VB: Probiotics (VSL#3) prevent endothelial dysfunction in rats with portal hypertension: Role of the angiotensin system. *PLoS One*, 9 (5): e97458, 2014. DOI: 10.1371/journal.pone.0097458

26. Karsten K, Frank-Christoph M, Christian P: The immunomodulatory effects of physical activity. *Curr Pharm Des*, 22, 3730-3748, 2016. DOI: 10.2174/1381612822666160322145107

27. Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK: Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol*, 515 (Pt1): 287-291, 1999. DOI: 10.1111/j.1469-7793.1999.287ad.x

28. Hoffman-Goetz L, Pervaiz N, Guan J: Voluntary exercise training in mice increases the expression of antioxidant enzymes and decreases the expression of TNF-alpha in intestinal lymphocytes. *Brain Behav Immun*, 23, 498-506, 2009. DOI: 10.1016/j.bbi.2009.01.015

29. Cechella JL, Leite MR, Dobrachinski F, da Rocha JT, Carvalho NR, Duarte MM, Soares FA, Bresciani G, Royes LF, Zeni G: Moderate swimming exercise and caffeine supplementation reduce the levels of inflammatory cytokines without causing oxidative stress in tissues of middle-aged rats. *Amino Acids,* 46, 1187-1195, 2014. DOI: 10.1007/s00726-014-1679-1

30. Kesherwani V, Chavali V, Hackfort BT, Tyagi SC, Mishra PK: Exercise ameliorates high-fat diet induced cardiac dysfunction by increasing interleukin 10. *Front Physiol,* 6, 124, 2015. DOI: 10.3389/fphys.2015.00124

31. Stanojevic D, Jakovljevic V, Barudzic N, Zivkovic V, Srejovic I, Parezanovic Ilic K, Cubrilo D, Ahmetovic Z, Peric D, Rosic M, Radovanovic D, Djordjevic D: Overtraining does not induce oxidative stress and inflammation in blood and heart of rats. *Physiol Res,* 65, 81-90, 2016.

32. Hering S, Jost C, Schulz H, Hellmich B, Schatz H, Pfeiffer H: Circulating transforming growth factor beta1 (TGFbeta1) is elevated by extensive exercise. *Eur J Appl Physiol*, 86, 406-410. 2002. DOI: 10.1007/s00421-001-0537-5

33. Rosa EF, Silva AC, Ihara SS, Mora OA, Aboulafia J, Nouailhetas VL: Habitual exercise program protects murine intestinal, skeletal, and cardiac muscles against aging. *J Appl Physiol*, 99, 4, 1569-1575, 2005. DOI: 10.1152/japplphysiol.00417.2005

34. De Lira CA, Vancini RL, Ihara SS, da Silva AC, Aboulafia J, Nouailhetas VL: Aerobic exercise affects C57BL/6 murine intestinal contractile function. *Eur J Appl Physiol,* 103, 215-223, 2008. DOI: 10.1007/s00421-008-0689-7

35. Holland AM, Hyatt HW, Smuder AJ, Sollanek KJ, Morton AB, Roberts MD, Kavazis AN: Influence of endurance exercise training on antioxidant enzymes, tight junction proteins, and inflammatory markers in the rat ileum. *BMC Res Notes,* 8, 514, 2015. DOI: 10.1186/s13104-015-1500-6

36. Amaretti A, di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A: Antioxidant properties of potentially probiotic bacteria: *In vitro* and *in vivo* activities. *Appl Microbiol Biotechnol*, 97, 809-817, 2013. DOI: 10.1007/s00253-012-4241-7

37. Punaro GR, Maciel FR, Rodrigues AM, Rogero MM, Bogsan CS, Oliveira MN, Ihara SS, Araujo SR, Sanches TR, Andrade LC, Higa EM: Kefir administration reduced progression of renal injury in STZ-diabetic rats by lowering oxidative stress. *Nitric Oxide,* 37, 53-60, 2014. DOI: 10.1016/j.niox.2013.12.012

38. Klewicka E, Zduńczyk Z, Juśkiewicz J, Klewicki R: Effects of lactofermented beetroot juice alone or with N-nitroso-N-methylurea on selected metabolic parameters, composition of the microbiota adhering to the gut epithelium and antioxidant status of rats. *Nutrients,* 7, 5905-5915, 2015. DOI: 10.3390/nu7075260

39. Wadley AJ, Turner JE, Aldred S: Factors influencing post-exercise plasma protein carbonyl concentration. *Free Radic Res*, 50, 375-384, 2016. DOI: 10.3109/10715762.2015.1131824

40. De Angelis M, Rizzello CG, Fasano A, Clemente MG, De Simone C, Silano M, De Vincenzi M, Losito I, Gobbetti M: VSL#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for celiac sprue. *Biochim Biophys Acta*, 1762, 80-93, 2006. DOI: 10.1016/j.bbadis.2005.09.008

41. Liu ZH, Huang MJ, Zhang XW, Wang L, Huang NQ, Peng H, Lan P, Peng JS, Yang Z, Xia Y, Liu WJ, Yang J, Qin HL, Wang JP: The effects of perioperative probiotic treatment on serum zonulin concentration and subsequent postoperative infectious complications after colorectal cancer surgery: A double-center and double-blind randomized clinical trial. *Am J Clin Nutr,* 97, 117-126, 2013. DOI: 10.3945/ajcn.112.040949

42. Liu Z, Li C, Huang M, Tong C, Zhang X, Wang L, Peng H, Lan P, Zhang P, Huang N, Peng J, Wu X, Luo Y, Qin H, Kang L, Wang J: Positive regulatory effects of perioperative probiotic treatment on postoperative liver complications after colorectal liver metastases surgery: A double-center and double-blind randomized clinical trial. *BMC Gastroenterol,* 15, 34, 2015. DOI: 10.1186/s12876-015-0260-z

43. Lamprecht M, Bogner S, Steinbauer K, Schuetz B, Greilberger JF, Leber B, Wagner B, Zinser E, Petek T, Wallner-Liebmann S, Oberwinkler T, Bachl N, Schippinger G: Effects of zeolite supplementation on parameters of intestinal barrier integrity, inflammation, redox biology and performance in aerobically trained subjects. *J Int Soc Sports Nutr,* 12, 40, 2015. DOI: 10.1186/s12970-015-0101-z

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Research Article

Effects of Different Fattening Systems on Fattening Performance, Slaughter and Carcass Characteristics of Male Tuj Lambs [1] [2]

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Abstract

The purpose of this study were to determine effects of fattening systems on fattening performance (n=39) and slaughter and carcass characteristics (n=24) in male Tuj lambs. Three different fattening groups were formed as extensive (n=13), semi-intensive (n=13) and intensive (n=13). Lambs in the extensive group were grazed in pasture. In addition to pasture, concentrated feed was given to lambs in the semi-intensive group. High quality fodder and concentrated feed were given to those in the intensive group. The study was completed in 90 d. Final live weights of extensive, semi-intensive and intensive groups were 31.19, 41.22, and 40.56 kg (P<0.001), respectively, and for daily live weight gain were (DLWG) 117.52, 229.66, and 221.11 g (P<0.001), respectively. Feed conversion rates (FCR) were 3.05 and 5.16 respectively in the semi-intensive and intensive fattening. It was found that hot carcass weight was 13.41, 19.51, and 19.89 kg (P<0.001) in extensive, semi-intensive, and intensive fattening groups respectively. Hot carcass yield was 43.11, 46.95, and 49.77% (P<0.001) in extensive, semi-intensive, and intensive fattening groups respectively. Consequently; leg, foreleg, shoulder, neck, flank, and kidney percentages, and spleen and full stomach weights of lambs in the extensive group were higher than in semi-extensive and entensive groups. Although there was no statistical difference between semi-extensive and entensive groups in respect to fattening performance, slaugher weight, hot and cold carcass weights, entensive group was higher than the other groups in respect to hot and cold carcass yield.

Keywords: *Tuj lambs, Fattening systems, Fattening performance, Slaughter and carcass characteristics*

Farklı Besi Sistemlerinin Erkek Tuj Kuzularında Besi Performansı, Kesim ve Karkas Özelliklerine Etkisi

Özet

Bu araştırma, erkek Tuj kuzularında besi sistemlerinin besi performansı (n=39), kesim ve karkas özelliklerine (n=24) etkisini belirlemek amacıyla yapılmıştır. Araştırmada ekstansif (n=13), yarı entansif (n=13) ve entansif (n=13) olmak üzere 3 değişik besi gruba oluşturulmuştur. Ekstansif grup merada otlatılmıştır. Yarı entansif gruba meraya ilaveten konsantre yem verilmiştir. Entansif gruba ise kaliteli kuru ot ve konsantre yem verilmiştir. Araştırma 90 günde tamamlanmıştır. Ekstansif, yarı entansif ve entansif besi gruplarında besi sonu ağırlığı sırasıyla 31.19, 41.22 ve 40.56 kg (P<0.001), günlük canlı ağırlık artışı sırasıyla 117.52, 229.66 ve 221.11 g (P<0.001) olarak belirlenmiştir. Yarı entansif ve entansif beside yemden yararlanma oranı sırasıyla 3.05 ve 5.16 olarak tespit edilmiştir. Ekstansif, yarı entansif ve entansif beside sıcak karkas ağırlığı 13.41, 19.51 ve 19.89 kg (P<0.001) olarak belirlenmiştir. Sıcak karkas randımanı aynı sıra ile %43.11, 46.95 ve 49.77 (P<0.001) olarak tespit edilmiştir. Sonuç olarak; ekstansif besi grubundaki kuzuların but, kol, omuz, boyun, etek ve böbrek oranları ile dalak ve dolu mide ağırlığı, yarı entansif ve entansif besi gruplarından yüksek bulunmuştur. Yarı entansif ve entansif gruplar arasında besi performansı, kesim ağırlığı, sıcak ve soğuk karkas ağırlıkları bakımından istatistiki bir fark bulunmamasına rağmen, sıcak ve soğuk karkas randımanı bakımından entansif grup, diğer gruplardan yüksek belirlenmiştir.

Anahtar sözcükler: *Tuj kuzusu, Besi sistemleri, Besi performansı, Kesim ve karkas özellikleri*

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INTRODUCTION

Sheep breeding occupies an important place in terms of its contribution to economy and human nutrition in the world. This significance is due to the sheep's ability to utilize lower quality grasslands and meadows, field crops and vegetation from which the cattle could not utilize and to convert them to animal products [1,2].

Despite having a significant place in terms of the number of sheep, Turkey has a lower place considering the yields obtained. According to the data of 2015, there were 31.507.934 sheeps in Turkey. 92.47% of these sheeps consisted of native breeds, and the remaining 7.53% were cultivated breeds [3]. The number of sheeps slaughtered was 5.008.411 and the amount of meat obtained was 100.021 tons. Carcass weight per animal obtained from sheep in Turkey varies between 13.00-19.97 kg [3]. The reasons for lower carcass weights may be native breeds with low yield, a great number of premature lamb slaughtered, and slaughtering of animals following the pasture fattening without intensive fattening [1,4,5].

Lamb breeding has been performed in various ways based on numerous factors such as establishment structure, genetic level of breed, pasture status, care and feeding methods, market conditions, livestock policy of the country. Profitability level of lamb breeding depends on production of high quality lamb meat in large amounts within a short period and inexpensive production. The quality and quantity of lamb meat are determined by some factors such as fattening systems, fattening period, final weight. There are various lamb breeding methods in which such factors can be regulated in different ways. It is possible to classify them as suckling lambs, pasture (extensive), intensive, and yearling breeding [1,5,6].

Suckling lamb fattening is based on the principle that lambs' attaintment of slaughter weight in a short period as a result of feeding lambs with milking, roughage and concentrated feed. In this system lambs are slaughtered when they are weaned, 3.5-4 months old, and have a 30-35 kg live weight. Pasture fattening is applied in regions with good pasture and in systems in which nomadic sheep breeding is performed. A certain slaughter weight has not been targeted and condition of lambs can be good or bad. This breeding type continues for long time, in which lambs reach to slaughter age late and they are marketed at low slaughter weight. The intensive fattening is applied more on lambs which are raised by using early weaning and motherless breeding methods. Lambs are fed with qualified concentrated feed and roughage after weaning when they are 2.5-3 months old, in order to obtain an increase of 250-300 g in weight in sheep fold for 2.5-3 months. Lambs can have 36-42 kg in 4 months [1,5].

This study was conducted for the purpose of comparing male Tuj lambs fed under extensive, semi-intensive, and intensive fattening conditions in terms of fattening performance, slaughter and carcass characteristics.

MATERIAL and METHODS

The ethical committee approval of Kafkas University (Official form date and number: 03.03.2011 and 2011-005) was obtained in order to conduct this study. The study was conducted at the Application and Research Farm of the Faculty of Veterinary Medicine, Kafkas University. The lambs were weaned at 3 months age old. After 10 d subsequent to their adaptation to pasture and concentrate mixture, the study was started. Medication against internal and external parasites was given lambs prior to the study. Three fattening groups were formed as extensive, semi-intensive, and intensive. Each group involved 13 lambs.

The lambs in the extensive and semi-intensive fattening groups were fed with pastures on a daily basis. Lambs in the semi-intensive group were fed *ad libitum* with both pastures and concentrated feed. Concentrated food contained 17.1% CP (crude protein) and 2710 kcal/kg ME (metabolic energy) [7]. Composition of concentrated feed is present in *Table 1*. Also information regarding to nutrient contents of concentrated feed and roughage is given in *Table 2*. A private feed factory prepared the concentrated feed whereas the Farm of Veterinary Faculty provided the roughage. FCR was determined with the concentrated feed consumption. Electronic scales having capacity of 150 kg and sensitivity of 10 g were used to weigh the feed. Clean water was given to lambs in the extensive and semi-intensive fattening groups at least three times a day. On the other hand, those in the intensive fattening group always drank clean water. The experiment took 3 months (June 5-August 5 in 2012).

Table 1. *Composition of the mixed feed used in intensive and semi-intensive fattening*

Tablo 1. *Entansif ve yarı entansif beside kullanılan karma yemin bileşimi*

Ingredient	%	Crude Protein (%)	Metabolic Energy (kcal/kg)
Barley	32.00	12.00	3110
Maize bran	10.00	9.20	2740
Maize	18.00	10.00	3300
Vegetable oil	2.60	-	7070
Sunflower cake	6.00	37.00	2250
Cotton seed cake	6.00	34.00	2300
Soy cake	14.00	48.00	3200
Molasses	8.50	7.80	2580
Lime stone	2.00	-	-
Sodium bicarbonate	0.20	-	-
Salt	0.50	-	-
Vit.-min. premix	0.20	-	-

The natural nutrient contents at various mowing times of the pastures, where the animals grazed, have been given in *Table 3*. For this purpose, samples were taken from various four locations of the pasture 3 times once a month (between June 5 and August 5) and the fodder of an area of 50 cm^2 of pasture was cut with a weed trimmer from 1 cm above the soil level. The pasture sample's dry matter (DM), organic matter (OM), crude protein (CP), crude ash (CA), crude cellulose (CC), crude fat (CF), and nitrogen free extract (NFE) levels were determined according to AOAC [8].

Eight fattening lambs from each group (24 lambs in total) were slaughtered to determine slaughter and carcass characteristics. Before they were slaughtered, nothing was given them for 12 h other than water. Their slaughter live weights were registered. They were slaughtered at Kafkas University Veterinary Faculty slaughtering house. Then, their head, skin, feet, offal, and gastro intestinal tract were weighed and their hot carcass weights were recorded. The carcass was chilled at +4°C for 24 h before dissecting and taken on the intact cold carcass. Subsequently, carcasses were longitudinally cut into two parts. Remaining parts were divided into six pieces (shoulder, neck, foreleg, flank, leg, and back-loin) according to the method specified by Colomer-Rocher et al.[9]. The calliper was used to measure carcass fat thickness between 12th-13th ribs, the musculus longissimus dorsi (MLD) area was taken onto the acetate sheet, and measurement of its surface area was performed by the digital planimeter.

Statistical package for the social sciences (SPSS 20.0) [10] software program was used for analysis of variance for the purpose of determining the impact of different fattening systems on fattening performance, slaughter and carcass characteristics. Significance of the difference among the groups was assessed with Duncan's multiple range test.

Table 2. *Nutrient contents of concentrate feed and roughage*
Tablo 2. *Konsantre ve kaba yemin besin madde içeriği*

Ingredient	Concentrate Feed	Roughage
Dry matter (%)	88.80	90.69
Crude protein (%)	17.10	10.35
Crude cellulose (%)	5.70	32.38
Crude fat (%)	3.50	2.00
Crude ash (%)	6.40	8.86
Metabolisable energy (kcal/kg)*	2710	2000

**It was determined by calculation made over values of the table*

RESULTS

The fattening performance of lambs in the extensive, semi-intensive, and intensive fattening groups is shown in *Table 4*. The initial weights of fattening process were 20.62, 20.55 and 20.66 kg, and the final weights at the end of the 90 d fattening period were 31.19, 41.22 and 40.56 kg for extensive, semi-intensive, and intensive fattening groups, respectively. At the end of 90 d, the daily live weights gain (DLWG) was 117.52, 229.66 and 221.11 g for extensive, semi-intensive, and intensive fattening groups, respectively.

The daily concentrated feed consumption in semi-intensive and intensive fattening groups was 0.70 and 1.14 kg, respectively and the feed conversion rate was 3.05 and 5.16 kg, respectively.

Table 5 presents the slaughter characteristics of lambs in the extensive, semi-intensive, and intensive fattening groups. There were statistically significant differences among the groups in terms of slaughter weights, hot and cold carcass weight, hot and cold carcass percentage, head, feet, skin, liver, lung, omental, full and empty small intestinal weights ($P<0.001$); full stomach weight ($P<0.01$); heart and MLD area ($P<0.05$). The carcass characteristics of different fattening groups with regard to the slaughter weight are shown in *Table 6*. There were significant differences among the groups in terms of leg, shoulder, tail percentages ($P<0.001$); foreleg, kidney-pelvic fat percentages ($P<0.01$) and flank percentages ($P<0.05$).

DISCUSSION

Extensive group was lower than DLWG other groups. Final weight and DLWG values determined for extensive group in this study were lower compared to values stated by Işık and Kaya [11] in Tuj lambs for final weight (34.24 kg) and DLWG (181.60 g), reported by Sarı et al.[12] in Hemşin lambs for final weight (33.32 kg) and DLWG (121.11 g), stated by Kaya et al.[13] in Morkaraman and Tuj lambs for final weight (34.23 kg) and DLWG (207.74 g) in pasture (extensive) group. However, final weight at the end of fattening and DLWG determined in the extensive group of this study were higher compared to values stated by Sarıçiçek et al.[14] in Karayaka lambs for final weight (22.34 kg) and DLWG (78.14 g) in the pasture group. The reason for differences between studies was caused from differences in breed,

Table 3. *Natural nutrients of the pasture at various mowing times, %*
Tablo 3. *Çeşitli biçim zamanlarında meranın doğal besin içeriği, %*

Pasture Mow/Month	DM	OM	CA	CP	CF	CC	NFE
I. mow	26.25	23.85	2.30	3.55	0.69	8.40	11.35
II. mow	32.35	30.10	2.30	2.70	0.99	9.70	16.68
III. mow	36.40	33.90	2.75	3.50	1.05	12.66	16.70

Table 4. *The fattening performance of lambs in different fattening groups (X±Sx)*
Tablo 4. *Farklı besi gruplarında kuzuların besi performansı (X±Sx)*

Characteristics	**Fattening Systems**			
	Extensive (n = 13)	**Semi Intensive (n = 13)**	**Intensive (n = 13)**	**P**
Initial weight (kg)	20.62±0.76	20.55±0.74	20.66±0.73	-
Final weight (kg)	31.19±0.88[b]	41.22±1.55[a]	40.56±1.49[a]	***
Daily live weight gain (g)	117.52±4.26[b]	229.66±12.01[a]	221.11±12.96[a]	***
Daily concentrated feed consumption (kg)	NC	0.70±0.04	1.14±0.06	***
Feed conversion ratio	NC	3.05±0.18	5.16±0.25	***

*-: P>0.05; *** P <0.001; **a, b:** The differences between the means of groups carrying various letters in the same row are significant (P<0.05). **NC:** Not converged*

Table 5. *The slaughter characteristics of lambs in different fattening groups (X±Sx)*
Tablo 5. *Farklı besi gruplarında kuzuların kesim özellikleri (X±Sx)*

Characteristics	**Fattening Systems**			
	Extensive (n = 8)	**Semi Intensive (n = 8)**	**Intensive (n = 8)**	**P**
Slaughter weight (kg)	31.13±0.68[b]	41.55±1.14[a]	39.85±1.21[a]	***
Hot carcass weight (kg)	13.41±0.32[b]	19.51±0.61[a]	19.89±0.87[a]	***
Cold carcass weight (kg)	12.93±0.33[b]	19.03±0.59[a]	19.35 ±0.86[a]	***
Hot carcass percentage (%)	43.11±0.69[c]	46.95±0.58[b]	49.77±0.74[a]	***
Cold carcass percentage (%)	41.58±0.59[c]	45.80±0.59[b]	48.42±0.76[a]	***
Head weight (g)	1826.20±25.07[b]	2144.40±47.52[a]	2071.90±68.40[a]	***
Feet weight (g)	806.25±9.99[b]	974.25±26.41[a]	920.12±29.22[a]	***
Skin weight (g)	3400.00±440.78[b]	5370.90±137.78[a]	5433.80±267.39[a]	***
Heart weight (g)	170.00±5.35[b]	198.89±6.76[a]	186.12±7.52[ab]	*
Liver weight (g)	447.50±10.98[c]	766.00±35.49[a]	642.50±31.76[b]	***
Lung weight (g)	387.50±14.36[b]	492.38±15.84[a]	425.12±17.04[b]	***
Spleen weight (g)	47.50±7.26	52.00±2.51	46.63±2.92	-
Omental weight (g)	27.50±1.64[b]	80.13±19.52[b]	149.00±28.71[a]	***
Full stomach weight (g)	5508.80±143.83[a]	5096.20±441.81[a]	4046.00±121.12[b]	**
Empty stomach weight (g)	1082.50±56.18[b]	1466.10±65.38[a]	1365.00±62.62[a]	***
Full small intestine weight (g)	1362.50±43.78[b]	2120.40±115.24[a]	1395.00±38.94[b]	***
Empty small intestine weight (g)	885.00±51.27[b]	1212.50±44.17[a]	944.12±30.84[b]	***
Full large intestine weight (g)	1493.80±67.43	1713.40±116.49	1522.10±57.19	-
Empty large intestine weight (g)	456.25±22.03	525.00±31.34	475.00±25.00	-
Fat thickness (mm)	4.04±0.36	4.84±0.26	5.04±0.48	-
M. longissimus dorsi area (cm^2)	12.42±0.54[b]	14.84±0.39[a]	13.30±0.71[ab]	*

*-: P>0.05, * P<0.05, ** P<0.01, *** P<0.001. **a, b, c:** The differences between the means of groups carrying various letters in the same row are significant (P<0.05)*

initial weight, pasture quality, fattening period, final weight, care and feeding.

Even though final weight at the end of fattening period stated in this study for semi-intensive group was lower than final weights (44.92 and 46.88 kg) of Tuj lambs in the groups which were given with 200 and 400 g concentrated feed along with pasture in the study conducted by Kaya et al.[15], DLWG was higher than DLWG values (160.57 and 183.14 kg) stated by the same researchers. DLWG in semi-intensive group in this study was higher than DLWG values (98 and 118 g) in groups fed additionally in the study conducted by Saatcı et al.[16].

Live weights gain (221.11 g) determined for intensive group in this study was lower than DLWG (250.00 g) stated by Altın et al.[17] in Kıvırcık intensive group lambs and DLWG (235.56 g) reported by Macit et al.[18] in Tuj intensive group lambs and DLWG (270.4 g) reported by Sen et al.[19] Karayaka male intensiv group lambs. It was similar to DLWG (211 g) reported by Yıldırım et al.[20] in Karayaka male intensive group lambs; Final weight at

***Table 6.** The carcass characteristics of lambs in different fattening groups (X±Sx)*
***Tablo 6.** Farklı besi gruplarında kuzuların karkas özellikleri (X±Sx)*

Characteristics	Fattening Systems			
	Extensive (n = 8)	Semi Intensive (n = 8)	Intensive (n = 8)	P
Leg (%)	33.40±0.32[a]	30.12±0.54[b]	29.95±0.46[b]	***
Foreleg (%)	17.40±0.19[a]	16.06±0.39[b]	15.53±0.54[b]	**
Back-loin (%)	13.27±0.39	13.39±0.72	13.24±0.39	-
Shoulder (%)	7.12±0.32[a]	5.42±0.36[b]	5.12±0.35[b]	***
Neck (%)	6.47±0.14	6.12±0.28	5.88±0.25	-
Flank (%)	11.34±0.36[a]	10.39±0.28[ab]	9.81±0.50[b]	*
Tail (%)	9.48±0.59[b]	17.14±1.12[a]	18.82±1.23[a]	***
Kidney (%)	0.78±0.03	0.76±0.02	0.77±0.03	-
Kidney-pelvic fat (%)	0.78±0.03[a]	0.62±0.07[b]	0.89±0.05[a]	**

***-:** P>0.05, * P<0.05, ** P<0.01, *** P <0.001. **a, b, c:** The differences between the means of groups carrying various letters in the same row are significant (P<0.05)*

the end of fattening determined for intensive group in this study was higher than final weight (34.70 and 29.92 kg) reported by Altın et al.[17], for Kıvırcık and Karya lambs and lower than final weight (44.00 kg) stated by Macit et al.[18] for Tuj lambs. While FCR (5.16) in intensive group in this study was similar to FCR (5.30) stated by Macit et al.[18] for Tuj lambs and by Altın et al.[17] for Kıvırcık lambs, it was lower than FCR (6.25) specified by Altın et al.[17] for Karya lambs. Differences observed between studies were due to differences in breed, initial weight, fattening period, final weights, care and feeding.

Slaughter weight stated in the extensive group in this study was lower than slaughter weights (32.00, 34.70 and 33.70 kg) stated by Ulusan et al.[21] for Morkaraman, Tuj, and their cross-breed yearlings in pasture group and slaughter weight reported by Sarı et al.[22] for Tuj lambs in pasture group. Slaughter weight specified for semi-intensive group in this study was similar to slaughter weights (41.36 and 42.48 kg) by Kırmızıbayrak et al.[23] for Tuj and Morkaraman lambs, and also similar to slaughter weights (39.30 and 40.97 kg) stated by Öztürk et al.[24] in Morkaraman and Kıvırcık x Morkaraman (F_1) lambs under semi-intensive conditions.

Hot and cold carcass weights for all three fattening groups in the study were higher than hot carcass (11.79, 12.35 and 12.15 kg) and cold carcass (11.52, 12.03 and 11.89 kg) weights stated by Yaralı and Karaca [25] for Karya lambs in pasture, pasture+feed, and intensive groups and those reported by Carrasco et al.[26] for Churra Tensina light lambs reared under grazing, grazing+supplement, drylot lambs with rationed graz-dams and drylot lambs with dams fed in confinement. Hot and cold carcass weight values determined for intensive group in this study were lower than those reported by Macit et al.[18] for Tuj intensive group lambs. Cold carcass weight values determined for intensive group in this study was lower than the value reported by Bjelanovic et al.[27].

Carcass dressing percentage is a singificant factor for carcas quality in meat production [28]. Hot and cold carcass dressing percentage in all three fattening groups in this study were between 41.58-49.77%, the highest yield was determined in the intensive fattening group. Hot and cold carcass dressing percentage determined in the extensive group were higher than those (40.00% and 38.00%) stated by Ulusan et al.[21] for Tuj lambs grazed in pasture. Hot carcass dressing percentage determined in the semi-intensive group was lower than the value (49.09%) reported by Macit et al.[29], for Tuj lambs under semi-intensive conditions, similar to the values under semi-intensive conditions (46.78%) stated by Kırmızıbayrak et al.[23] for Tuj lambs and (47.56% and 47.64%) stated by Sarı et al.[22]. The highest cold carcass dressing percentage was in intensive group and it is in agreement with the results in intensive group observed by Carrasco et al.[26], Aksoy and Ulutaş [30], Akçapınar et al.[31], and Joy et al.[32]. Cold carcass dressing percentages (41.58-48.42%) of Tuj male lambs of the present study were similar to values of the different fatting systems reported by Aksoy and Ulutaş [30] (45.34-48.44%), Akçapınar et al.[31] (47.15%) and Koçak et al.[33] (44.17-45.57%).

While weights of skin, spleen, omental fat, full and empty stomach, empty small and large intestine reported in the extensive fattening in this study were lower than those values under extensive fatting system stated by Ulusan et al.[21], value of MLD area in this study was higher than the value stated by the same researchers. Weights of head, feet, hearth, liver, and lung determined in the semi-intensive group were higher than those determined by Macit et al.[29] for Tuj semi-intensive group lambs. This difference could be resulted from origin, initial weight, fattening period, final weight of lambs and different environmental conditions. Weights of feet, skin, empty stomach and MLD area specified in the intensive group in this study were similar to those reported by Macit et alfor Tuj lambs in

the intensive fattening. Weights of head, feet, skin, empty stomach, empty small intestine and full large intestine in the intensive group in this study were higher than weights stated by Aksoy [34] for Tuj intensive group lambs in 40 kg weight group, lower than weights of spleen, omental, full stomach and empty large intestine and MLD area reported by the same researcher.

Carcasses of lambs are mostly sold by separating them into parts, and different dishes are prepared by using different carcass parts. Leg, and back-loin are considered as valuable parts. Leg, back-loin, kidney and kidney-pelvic fat percentages reported in the extensive fattening in this study were higher than percentages of leg (31.45%), back-loin (11.60%), kidney (0.62%), kidney fat (0.48%), stated in the study conducted by Ulusan et al.[21] on male Tuj yearlings fed in pasture and similar to percentages of foreleg (17.45%) and tail (10.33%). Percentages of leg and foreleg determined in all three groups in the study were lower than leg percentages (34.71, 34.28 and 34.12%) and foreleg rates (21.02, 21.07 and 20.19%) determined by Yaralı and Karaca [25] for Karya lambs in pasture, pasture + feed, and intensive groups.

Consequently; fattening performance and the other slaughter characteristics of lambs in intensive and semi-intensive groups were higher than extensive group. Although there was generally no statistical difference between semi-intensive and intensive groups in terms of fattening performance, slaughter weight, hot and cold carcass weights, intensive group had higher hot and cold carcass yield compared to the other groups. However, leg, foreleg, shoulder, neck, flank, and kidney percentages, and spleen and full stomach weights of lambs in the extensive group were higher than in semi-extensive and entensive groups.

Lamb breeding in Kars is generally performed according to pasture, and the period when pastures are green and nutritious is a very short like 3-4 months [35]. Other than this season, pastures largely lose their valuableness and live weight loss, decrease in carcass amount and quality, and significant economic loss occur since lambs cannot feed enough and are slaughtered early [12]. Even though this situation changes depending on factors such as nutrient contents of pasture, grazing, climate, flora, etc.[36], results obtained from this study indicate that feeding along with pasture will be more suitable. Therefore, considering the demand for red meat in the country and the producer preferences, beside intensive system, semi-intensive feeding system can be recommended for fattening performance, slaughter weight, hot and cold carcass weights, hot and cold carcass yield for male Tuj lambs.

REFERENCES

1. Akçapınar H: Koyun Yetiştiricilği. Genişletilmiş 2. Baskı, İsmat Matbacılık, Ankara, 2000.

2. Kaymakçı M, Özder M, Karaca O, Torun O, Baş S, Koşum N: Turkey breeding sheep strategy. *Uludag Univ Zir Fak Derg*, 23, 67-77, 2009.

3. TUİK: Türkiye İstatistik Kurumu. 2016. http://www.tuik.gov.tr/PreTablo.do?alt_id =1002, *Accessed:* 07.04.2016.

4. Ekiz B, Yılmaz A, Özcan M, Kaptan C, Hanoğlu H, Erdoğan I, Yalçıntan H: Carcass measurements and meat quality of Turkish Merino, Ramlic, Kıvırcık, Chios and Imroz lambs raised under an intensive production system. *Meat Sci*, 82, 64-70, 2009. DOI: 10.1016/j.meatsci.2008.12.001

5. Tekin ME: Kasaplık Kuzu Üretimi. Selçuk Universitesi Basımevi, Konya, 2009.

6. Zervas G, Tsiplakou E: The efect of feeding system on the characteristics of products from small ruminants. *Small Ruminant Res*, 101, 140-149, 2011. DOI: 10.1016/j.smallrumres.2011.09.034

7. NRC: Nutrient Requirements of Sheep. 6th rev. edn., National Academy Press, Washington, DC, USA, 1985.

8. AOAC: Official Methods of Analysis. 14th edn., Arlington, Virginia, USA, 1990.

9. Colomer-Rocher F, Morand-Fehr P, Kirton AH, Delfa R, Sierra-Alfranca I: Standardized methods for the study of the quantitative and qualitative characters of sheep and goat carcasses. Ministerio de Agricultura, Pesca y Alimentacion. *Cuadernos/NIA*, No. 17, 41, 1988.

10. SPSS: IBM SPSS for Windows, Version 20.0. IBM corporation, Armonk, NY, USA, 2011.

11. Işık S, Kaya İ: The Effect of vegetation period on the qualilty of pasture and performance of grazing Tujhin sheep and lambs. *Kafkas Univ Vet Fak Derg,* 17, 7-11, 2011. DOI: 10.9775/kvfd.2010.1964

12. Sarı M, Önk K, Aydın E, Tilki M, Tufan T: Effects of different fattening systems on fattening performanceand body measurements of Hemsin male lambs. *Kafkas Univ Vet Fak Derg*, 20, 209-215, 2014. DOI: 10.9775/kvfd.2013.9823

13. Kaya İ, Saatcı M, Ünal Y, Öncüer A, Kırmızıbayrak T: Effect of grazing on green growing pasture and concentrate supplementation on pasture quality and growth, rumen ph, total volative fatty acids, ammonium nitrojen in Morkaraman and Tuj. *Lalahan Hay Araşt Enst Derg,* 44, 33-39, 2004.

14. Sarıçiçek BZ, Ocak N, Erener G: The effects of different concentrates on pasture on fattening performance of Karakaya lambs. *J Agric Sci*, 2, 27-31, 1996.

15. Kaya İ, Şahin T, Elmalı Aksu D, Ünal Y: The effects of grazing and concentrate supplementation at pasture on performance and rumen parameters in lambs. *Kafkas Univ Vet Fak Derg*, 17, 693-697, 2011. DOI: 10.9775/kvfd.2011.3699

16. Saatci M, Yıldız S, Kaya I: New rearing systems for Tuj (Tushin) lambs. *Small Ruminant Res,* 50, 23-27, 2003. DOI: 10.1016/S0921-4488(03)00115-9

17. Altın T, Karaca O, Cemal I, Yılmaz M, Yılmaz O: The fattening and carcass characteristics of Kıvırcık and Karya lambs. *J Anim Prod,* 46, 19-29, 2005.

18. Macit M, Karaoğlu M, Yaprak M, Kopuzlu S: Determination of the fattening performance, and slaughtering and carcass characteristics of Tushin lambs under intensive farm conditions. *Atatürk Üniv Ziraat Fak Derg*, 28, 64-73, 1997.

19. Şen U, Şirin E, Ulutaş Z, Kuran M: Fattening performance, slaughter, carcass and meat quality traits of Karayaka lambs. *Trop Anim Health Prod,* 43, 409-416, 2011. DOI: 10.1007/s11250-010-9707-y

20. Yıldırım A, Ulutaş Z, Ocak N, Kaptan M: Effects of birth weight and feeding system on fattening performance and feedin behaviour of karayaka male lambs. *Ital J Anim Sci*, 12, 546-550, 2013. DOI: 10.4081/ijas.2013.e89

21. Ulusan HOK, Aksoy AR, Uzun N, Karabulak C, Laçin E: Slaughter and carcass traits of Morkaraman, Tuj and their crosses (Morkaraman x Tuj) male lambs fedon natural pasture. *Kafkas Univ Vet Fak Derg,* 2, 28-33, 1996.

22. Sarı M, Aksoy AR, Tilki M, Kaya I, Işık S: Effect of different fattening methods on slaughter and carcass characteristics of Tuj male lambs. *Archiv Tierzucht*, 55, 480-484, 2012.

23. Kırmızıbayrak T, Saatcı M, Aksoy AR: Slaughter and carcass characteristics of Tushin and red Karaman lambs raised in semi intensive conditions. *Kafkas Univ Vet Fak Derg*, 9, 75-78, 2003.

24. Öztürk Y, Küçük M, Karslı MA: Yarı entansif şartlarda yetiştirilen Morkaraman ve Kıvırcık x Morkaraman (F_1) kuzularda büyüme, kesim ve karkas özelliklerinin arştırılması. *Kafkas Univ Vet Fak Derg*, 18, 1-6, 2012. DOI: 10.9775/kvfd.2011.4351

25. Yaralı E, Karaca O: The fattening performance, slaughtering and carcass merits of Karya lambs fattened in different systems. *J Anim Prod,* 52, 1-9, 2011.

26. Carrasco S, Ripoll G, Sanz A, Alvarez-Rodriguez J, Panea B, Revilla R, Joy M: Effect of feeding system on growth and carcass characteristics of Churra Tensina light lambs. *Livest Sci*, 121, 56-63, 2009. DOI: 10.1016/j.livsci.2008.05.017

27. Bjelanovic M, Grabez V, Vucic G, Martinovic A, Lima LR, Markovic B, Ehelandsdal B: Effect of different production systems on carcass and meat quality of sheep and lamb from Western Balkan and Norway. *Biotechnol Anim Husb,* 31, 203-221, 2015. DOI: 10.2298/BAH1502203B

28. Tufan M, Akmaz A: Slaughter and carcass traits of Güney Karaman, Kangal Akkaraman and Akkaraman lambs at different slaughter weights. *Turk J Vet Anim Sci,* 25, 405-504, 2001.

29. Macit M, Şahin S, Esenbuğa N, Karaoğlu M: Growth and carcass characteristics of three fat-tailed pure breeds under grazing with concentrate supplementation. *Turk J Vet Anim Sci*, 27, 331-337, 2003.

30. Aksoy Y, Ulutaş Z: Effect of different slaughter weights on slaughter carcass traits of Male Karayaka lambs reared under intensive production systems. *TURJAF,* 3, 406-412, 2015.

31. Akçapınar H, Atasoy F, Ünal N, Aytaç M, Aylanç A: The feeding and carcass characteristics of Bafra (Chois x Karayaka (B_1) lambs. *J Lalahan Livest Res Inst,* 42, 19-28, 2002.

32. Joy M, Ripoll G, Delfa R: Effects of feeding system on carcass and non-carcass composition of Churra Tensina light lambs. *Small Ruminant Res,* 78, 123-133, 2008. DOI: 10.1016/j.smallrumres.2008.05.011

33. Koçak Ö, Ekiz B, Yalçıntan H, Yılmaz A: Slaughter and carcass characteristics of Chios x Tahirova crossbred lambs under intensive, traditional and organic production system. *Ankara Univ Vet Fak Derg*, 63, 187-193, 2016. DOI: 10.1501/Vetfak_0000002728

34. Aksoy AR: Fattening performance, slaughtering and carcass characteristics of Morkaraman and Tushin ram lambs at different slaughtering weights. *Ankara Univ Vet Fak Derg*, 42, 15-23, 1995. DOI: 10.1501/Vetfak_0000000799

35. Kaya I, Karademir B: The role of grassland in livestock animal nutrition and performation-contamination of diseases in Kars district. *Lalahan Hay Arast Enst Derg*, 42, 59-66, 2002.

36. Holmes W: Grass Its Production and Utilization. Blackwell Scientific Publications, London, 1994.

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Research Article

The Relationship between Body Dimensions and Fat Deposits in Herik Lambs [1]

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Abstract

The aim of this research was to determine the relationships between some body measurements and fat deposits in the Herik lamb, reared in the central Black Sea Region of Turkey. In this study, data on 20 single-born male Herik lambs, namely 10 with short, round, fat tail, and 10 with long, semi-fat tails, were used. They were finished for an average of 105 days under intensive management after weaning and slaughtered at an average body weight of 40 kg. Several body measurements were taken before slaughter. Carcasses were cut into sections which were separated into meat, bone and fat. Weights of tail, carcass and non-carcass fat were recorded and used in the calculation of the total body fat weight. The highest correlation coefficient was between tail fat weight and the upper tail circumference (r= 0.937, P<0.01), and the lowest was between the fat tail weight and tail length (r=0.059). The upper and lower tail circumferences explained 88% of total variation in the tail weight, and 71% of total variation in total body fat, respectively. In conclusion, lower and upper tail circumference can be used to predict fat tail weight, as well as total body fat, in Herik lambs. More detailed studies are needed for the determination of the relationship between body measurements and fat deposits and to improve the carcass characteristics of Herik lambs.

Keywords: *Body measurement, Carcass, Fat, Herik, Lamb, Meat*

Herik Kuzularında Bazı Kuyruk ve Beden Ölçüleri ile Yağ Depoları Arasındaki İlişki

Özet

Bu araştırmanın amacı, Türkiye'de Orta Karadeniz Bölgesinde yetiştirilen Herik kuzularında bazı beden ölçüleri ile yağ depoları arasındaki ilişkiyi belirmekti. Bu çalışmada 10 baş kısa yuvarlak yağlı kuyruklu ve 10 baş uzun yarım yağlı kuyruklu kuzu olmak üzere 20 erkek tekiz doğmuş Herik kuzusu kullanıldı. Kuzular sütten kesimden sonra ortalama 105 gün entansif koşullarda beslendi ve ortalama 40 kg da kesildi. Kesimden önce bazı beden ölçüleri alındı. Karkaslar parçalara ayrıldı. Karkas parçaları et, kemik ve yağa ayrıldı. Kuyruk, karkas ve karkasa ait olmayan yağlar tartıldı ve toplam beden yağ ağırlığı belirlendi. En yüksek korelasyon kuyruk yağı ağırlığı ile kuyruk kökü çevresi arasında (r=0.937, P<0.01), en düşük korelasyon ise kuyruk yağı ağırlığı ile kuyruk uzunluğu arasında bulundu (r=0.059). Kuyruk kökü ve en geniş kuyruk çevresi, kuyruk yağı ağırlığındaki varyasyonun %88'ini, toplam beden yağı ağırlığındaki varyasyonun ise %71'ini açıkladı. Sonuç olarak, Herik kuzularında kuyruk kökü ve en geniş yerindeki kuyruk çevresi hem kuyruk yağı ağırlığını hem de toplam beden yağı ağırlığını tahmin etmede kullanılabilir. Herik kuzularında kuyruk ölçüleri ile yağ depoları arasındaki ilişkiyi belirlemek ve Herik kuzularının karkas özelliklerini geliştirmek için daha fazla çalışma yapılmalıdır.

Anahtar sözcükler: *Beden ölçüleri, Karkas, Yağ, Herik, Kuzu, Et*

INTRODUCTION

The fat tailed sheep is characterized by an accumulation of fat in the tail which provides an energy source when nutrition is insufficient [1,2]. The fat tailed sheep, which can walk long distances and overcome harsh environmental conditions such as high temperatures, is traditionally raised for meat production in arid or semiarid areas [2,3].

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Turkey's sheep population is 31.1 million head and approximately 90-95% of all the sheep are fat and semi-fat tailed sheep [4]. Two tail types are seen in Herik sheep, namely short, round and long, semi-fat. The long, semi-fat tail is wide at the base and narrows towards the end [5] and the other tail type is short and round. Herik sheep are produced by crossbreeding the White Karaman and Red Karaman with Karayaka rams and are raised in the central Black Sea Region of Turkey, especially in Amasya Province [6].

Many authors have focused on the relationship between body weight and several body measurements in breeding strategies for small ruminants [7-9]. *In vivo* measurements, especially fat tail measurements, are commonly used in order to estimate carcass composition and fat deposits in breeding programs [10]. There have been many studies on the relationship between tail measurements and the fat deposits of sheep [2,11-14]. The aim of this research was to investigate the relationships between some tail and body measurements and fat deposits in Herik sheep in Turkey.

MATERIAL and METHODS

Animals and Measurements

This study was conducted on a private farm in Atakum, Samsun Province, Turkey. The farm is about 171 m above the sea level, and is located at a latitude of 41°24'N and a longitude of 36°08'E. The average temperature and humidity at the site were 22°C and 76% during the study. Fifty five millimetres of rain fell during the study period. A total of 20 single-born, male, Herik lambs, namely 10 short, round, fat-tailed lambs and 10 long, semi-fat tailed lambs, were used in this study. The lambs were born in April 2015 and kept with their dams until approximately 2.5 months of age. At weaning, the lambs were weighed (average 20.78±0.51 kg), and then kept in a finishing pen. The lambs were checked twice a day for health problems during the finishing period. Anthelmintics were administered for internal and external parasites and they were vaccinated against clostridia disease. The lambs were weighed weekly during the finishing period and slaughtered after reaching 40 kg slaughter weight (SW). The lambs were finished with two different feed concentrates for an average of 105 days. Diet 1 (18% CP, 2650 kcal/kg ME) was provided ad libitum until the lambs reached a body weight of 30 kg and Diet 2 (16% CP, 2650 kcal/kg ME) was provided ad libitum until the lambs reached the SW of 40 kg. In addition, the lambs were provided with 300 g of alfalfa hay per lamb per day, unlimited access to water and a mineral lick. The tail and body measurements were obtained before slaughter.

The body measurements included were withers height (WH), rump height (RH), heart girth (HG), chest depth (CD), body length (BL) and rump width (RW). WH was measured as the distance from the ground to the withers, RH was measured from the ground to the top of the rump, HG was measured as the circumference of the chest, CD was measured between the top of the withers and the lowest point of the chest, BL was measured from the articulatio humeri to the tuber ischii, and RW was measured between the outer edges of the major hip bones on the right and left sides. The tail dimensions included the upper circumference (UC), lower circumference (LC), upper tail thickness (UTT), upper width (UW), lower width (LW) and length of the fat-tail (TL). "Upper' and 'lower' measurements were taken at the base and the widest part of the fat-tail, respectively, as described by Safdarian et al.[14]. Circumference and width were measured with an ordinary flexible tape measure. The depth was measured with calipers and the length was measured with a ruler. All measurements were obtained while the animal was held in a standing position.

Slaughtering Procedures and Carcass Composition

The lambs were not fed but were provided ad libitum access to water in the 12 h immediately before slaughter. Lambs were slaughtered near their pen by severing the major blood vessels in the neck. Following skinning and evisceration, the hot carcass, including the tail fat, perinephric fat and kidneys, was weighed. Omental, mesenteric, scrotal and pericardial fats were separated from hot carcass fat and weighted. Cold carcass weights were recorded after storage at 4°C for 24 h. Weight of the tail, perinephric and pelvic fats were obtained from the cold carcass. The combined weights of omental, mesenteric, scrotal, pericardial, perinephric and pelvic fats were then recorded as the total non-carcass fat (NCF). The carcasses were split along the median plane into two equal halves with a band saw. The weights of the right and left halves were obtained. Subcutaneous fat depth was measured with calipers between the 12th and 13th ribs. Four measurements were taken, two on each side of the carcass, and their average was recorded as the subcutaneous fat depth. The left halves were sectioned according to a procedure reported by Akçapınar [15]. Each section (leg, foreleg, back, loin, neck, breast + flank) was then completely dissected into bone, fat (subcutaneous and intermuscular) and trimmed meat (TM), including the nerves and connective tissue, which were weighed separately. The amounts of subcutaneous and intermuscular fats (carcass fat, CF), muscle and bone were doubled to obtain the total carcass muscle, bone and fat. Total body fat (TBF) was calculated as NCF plus CF and FTW.

Statistical Analysis

The SPSS program was used for statistical analyses including descriptive statistics, Pearson's correlation coefficients and, multiple linear regression analyses, including residual standard deviations (RSD) [16].

RESULTS

The body measurements of the 20 Herik sheep included in the present study are presented in *Table 1*. Average body

***Table 1.** Means and standard errors (SE) for body measurements of Herik lambs*

***Tablo 1.** Herik kuzularının beden ölçülerinin ortalama ve standart hataları (SE)*

Characteristics	n	Mean	SE
Heart girth (HG), cm	20	69.08	0.40
Chest depth (CD), cm	20	25.93	0.52
Withers height (WH), cm	14	60.04	1.08
Rump height (RH), cm	14	55.86	0.92
Rump width (RW), cm	20	17.83	0.27
Body length (BL), cm	20	77.78	0.60
Upper circumference (UC), cm	14	43.57	2.68
Lower circumference (LC), cm	14	46.07	2.77
Lower tail width (LW), cm	14	26.54	8.03
Upper tail width (UW), cm	14	26.07	8.45
Tail length (TL), cm	14	32.86	0.67
Upper tail thickness (UTT), mm	14	39.95	2.36

***Table 2.** Means and standard errors (SE) for certain carcass characteristics, carcass composition and fat deposits in Herik lambs.*

***Tablo 2.** Herik kuzularının bazı karkas özellikleri, karkas kompozisyonu ve yağ depolarının ortalama ve standart hataları (SE)*

Characteristics	n	Mean	SE
Slaughter weight, kg	20	40.30	0.35
Hot carcass weight, kg	20	18.95	0.22
Cold carcass weight, kg	20	18.55	0.25
Dressing percentage, %	20	47.03	0.50
Back fat thickness, mm	20	4.30	0.24
Weight of fat deposits			
Total carcass fat, kg	14	5.01	0.15
Total non-carcass fat, kg	14	1.27	0.07
Tail fat weight, kg	20	1.71	0.14
Total body fat, kg	14	8.11	0.26
Proportions of fat deposits			
Total carcass fat, %	14	62.02	1.22
Total non-carcass fat, %	14	15.81	1.06
Tail fat, %	14	22.17	1.83
Weight of dissected tissues, kg			
Trimmed meat, kg	14	9.11	0.24
Bone, kg	14	3.01	0.12
Subcutaneous fat, kg	14	3.66	0.15
Intermuscular fat, kg	14	1.35	0.07
Proportions of dissected tissues			
Trimmed meat, %	14	48.37	1.18
Bone, %	14	15.98	0.54
Subcutaneous fat, %	14	19.46	0.78
Intermuscular fat, %	14	7.19	0.41
Tail fat, %	20	9.00	0.65

length, wither height, heart girth and chest depth were 77.78, 60.04, 69.08 and 25.93 cm, respectively. Carcass composition and fat deposit percentages are presented in *Table 2*. Average cold carcass weight was 18.6 kg, composed of 48.37% muscle, 26.65% fat and 15.98% bone. Correlation coefficients among some body measurements and carcass composition traits are shown in *Table 3*. LC had a positive and significant correlation with FTW ($r=0.937$, $P<0.01$), which was positively correlated with TBF ($r=0.806$, $P<0.05$).

In the present study, the tail fat percentage was lower than in fat tailed breeds. However subcutaneous and inter-muscular fat percentages were higher than those of fat tailed breeds. Multiple linear regression equations were produced for the relationships between tail fat and total body fat, and LC and UC, with:

FTW = - 998.16 + 70.30 (LC) - 5.09 (UC), $R^2 = 0.879$, ($P<0.001$), RSD = 243.03

TBF = 4494.23 - 31.49 (LC) + 108.19 (UC), $R^2 = 0.714$, ($P<0.001$), RSD = 517.94

A significant multiple linear regression was also determined for the relationship between TM, and SW and RW, with:

TM = - 3529.64 + 0.07 (SW) + 558.17 (RW), $R^2 = 0.554$, ($P<0.05$), RSD = 594.48

DISCUSSION

There are no published reports for the carcass characteristics of Herik lambs. Body measurements, carcass composition and fat deposits were therefore compared with reports for other sheep breeds.

In studies of White Karaman lambs slaughtered between 36.7-41.6 kg, body lengths were between 57.8 and 67.8 cm, wither heights between 64.3 and 65.3, heart girths between 80.2 and 85.5 cm, and chest depths between 25.3 and 31.6 cm [13,15,17]. The reported values for body lengths were between 58.7 and 67.2 cm, wither heights between 55.0 and 64.9 cm, heart girths between 88.0 and 89.9 cm, and chest depths between 28.3 and 30.5 cm for Karayaka lambs slaughtered at 41.0-44.7 kg [18-20]. Body length, heart girth and chest depth of Red Karaman lambs slaughtered at 40 kg were 67.7 cm, 78.7 cm and 29.00, respectively [21]. In the present study, body length, wither height, heart girth and chest depth were 77.78, 60.04, 69.08 and 25.93 cm, respectively. That meant that the body length of Herik lambs was greater than that of White Karaman and Red Karaman lambs, while the withers height, heart girth and chest depth were shorter.

The reported values for tail circumferences were between 59.1 and 63.1 cm, and tail widths between 26.2 and 32.9 cm, for White Karaman lambs slaughtered at

Table 3. *Correlation coefficients among some body measurements and carcass composition traits*
Tablo 3. *Beden ölçüleri ile karkas kompozisyon özellikleri arasındaki korelasyon eşitlikleri*

Characteristics	Tail Fat Weight (FTW)	Back Fat Thickness	Trimmed Meat (TM)	Non Carcass Fat (NCF)	Carcass Fat (CF)	Subcutaneous Fat	Intermuscular Fat	Total Body Fat (TBF)
Slaughter weight (SW)	0.276	-0.527*	0.578*	0.065	-0.004	0.030	-0.071	0.195
Heart girth (HG)	-0.105	0.126	0.410	0.159	0.020	0.196	-0.366	-0.077
Chest depth (CD)	0.176	0.225	0.476	-0.187	-0.214	-0.055	-0.330	-0.116
Withers height (WH)	-0.368	-0.223	0.229	-0.055	-0.489	-0.388	-0.206	-0.567*
Rump height (RH)	-0.346	-0.447	0.439	-0.121	-0.550*	-0.485	-0.130	-0.605*
Rump width (RW)	0.174	-0.365	0.738**	-0.160	-0.349	-0.321	-0.056	-0.182
Body length (BL)	-0.178	0.123	-0.323	-0.008	-0.168	-0.214	0.096	-0.351
Lower circumference (LC)	0.937**	0.127	-0.283	-0.419	0.427	0.516	-0.190	0.806**
Upper circumference (UC)	0.911**	0.170	-0.365	-0.285	0.455	0.552*	-0.205	0.842**
Lower tail width (LW)	-0.264	0.018	0.018	0.162	-0.221	-0.152	-0.142	-0.273
Upper tail width (UW)	-0.283	-0.021	0.063	0.210	-0.242	-0.204	-0.078	-0.285
Tail length (TL)	0.059	0.203	-0.050	0.106	-0.103	-0.230	0.266	0.013
Upper tail thickness (UTT)	0.785**	-0.219	0.097	-0.305	-0.026	0.096	-0.252	0.465
Tail fat weight (FTW)	1.000	-0.016	-0.189	-0.467	0.264	0.315	-0.108	0.743**

** P<0.05; ** P<0.01*

36.7 kg [17], 31.9 kg [22] and 41.6 kg [13]. In that study, the tail width, tail length and tail thickness of crossbred lambs (Chios x White Karaman F_1) was 17.80, 25.5 cm and 25 mm, respectively. In the present study, the LC, UC, LW, UW, TL and UTT were 43.57, 46.07, 26.54, 26.07, 32.86 cm and 39.95 mm, respectively. Also in the present study, the tail circumferences of Herik lambs were less than in their studies, while the tail length and tail width results of our study were in accordance with the results of their studies [13,17,22].

Yardımcı et al.[13] reported the dressing percentage, and percentages of tail fat, muscle, fat and bone as 48.4%, 15.29%, 48.9%, 15.08% and 19.6%, respectively, for White Karaman lambs slaughtered at 41.6 kg. Tufan and Akmaz [23] reported the dressing percentage, and percentages of tail fat, muscle, fat, bone and back fat thickness as 50.3%, 16.20%, 44.7%, 21.4%, 15.3% and 3.75 mm, respectively for White Karaman lambs slaughtered at 40.17 kg. Kadak [24] reported the dressing percentage, and muscle, fat and bone percentages of White Karaman lambs at 42 kg SW to be 51.3%, 58.5%, 18.4% and 19.0%, respectively. Arslan [21] measured cold carcass weight, and the percentages of tail fat, muscle, fat, bone and back fat thickness as 21.2 kg, 22.0%, 45.7%, 17%, 14.4% and 4.48 mm, respectively for Red Karaman lambs at 40.0 kg. Macit [25] reported cold carcass weight, dressing percentage, tail fat weight, tail fat percentage and fat thickness as 19.6 kg, 48.9%, 3.2 kg, 16.3% and 2.7 mm, respectively, for the Red Karaman breed at 40.5 kg body weight. Öztürk [26] reported dressing percentage and tail fat percentage of 48.4% and 15.72%, respectively, for the Red Karaman at 39.83 kg. Aydoğan [27] reported cold carcass weight, and percentages of muscle, fat and bone as 17.5 kg, 49.8%, 31.5% and 16.6% respectively, for Karayaka at 35.2 kg body weight.

In the present study, the average body weight of the lambs at slaughter was 40.3 kg. Mean cold carcass weight was 18.6 kg, with a composition of 48.37% muscle, 26.65% fat and 15.98% bone. Moreover, the fat thickness and tail fat percentage were 4.30 mm and 9.00%, respectively. The carcass fat percentage in Herik lambs was higher than for the fat tailed breeds (White Karaman and Red Karaman), but tail fat percentages in White Karaman and Red Karaman lambs were higher than in Herik lambs. In comparison with the fat tailed breeds, the tail fat percentage decreased, but the carcass fat percentage increased in Herik lambs.

In the current study, some tail dimensions were significantly and closely correlated with FTW and TBF. The highest correlation coefficients were observed between circumference measurements and tail thickness. The LC had a positive and significant correlation with FTW (r=0.937, P<0.01) which was positively correlated with TBF (r=0.806, P<0.05). UC was also positively and significantly correlated with FTW (r=0.911, P<0.01), which was positively correlated with TBF (r=0.842, P<0.01).

Zamiri and Izadifard [11] reported that in Ghezel rams. LC had the highest correlation with FTW (r=0.84). Safdarian et al.[14] reported a high correlation between the FTW and UC in Torki-Ghashghaii sheep (r=0.88). Yardımcı et al.[13] reported that tail circumference was positively and significant correlated with FTW (r=0.88, P<0.01), which was also significantly correlated with TBF (r=0.72, P<0.01) in White Karaman lambs. Atti and Hamouda [12] reported that UC and middle tail circumference were highly correlated with FTW, with r=0.87 and r=0.85, respectively, in Barbarine lambs. In the present study, the correlations between tail circumference measurements and tail fat weight were higher than in these studies. The LC and UC could therefore be used to predict the FTW and TBF.

Safdarian et al.[14] reported a high correlation between the FTW and TBF (r=0.70) and Atti and Hamouda [12] reported that FTW was significantly and highly correlated with TBF (r=0.91). In the present study, a high, positive correlation coefficient (r=0.743, P<0.01) was determined between the FTW and TBF. The results of our study are in accordance with the results of their studies.

Safdarian et al.[14] reported correlation coefficients of r=0.62 and r=0.66 the upper thickness and lower thicknesses of the tail, respectively, with FTW. Atti and Hamouda [12] reported correlations of the upper tail depth and middle tail depth with FTW of r=0.62 and r=0.54, respectively. Furthermore, Zamiri and Izadifard [11] reported that FTW was positively and significantly correlated with upper and lower fat tail thickness in Mehreman rams, with r=0.40 and r=0.37, respectively and also in Ghezel rams, with r=0.25 and r=0.37, respectively. In the present study, we found a significant correlation between the UTT and FTW (r=0.785, P<0.01). This result was in agreement with the results of earlier studies [11,12,14].

The most remarkable result of the present study was obtained for FTW, subcutaneous fat and intermusclar fat percentage. Yardımcı et al.[13] reported that the tail fat, subcutaneous fat and intermuscular fat percentages were 15.29%, 10.2% and 4.88% in White Karaman sheep of 41.6 kg body weight. Kashan et al.[2] reported that in Chaal (fat tailed), Zandi (fat tailed), Zel x Chaal (semi-fat tailed) and Zel x Zandi (semi-fat tailed) sheep, tail fat percentages were 13.6, 15.8, 7.2 and 7.5%, respectively, that subcutaneous fat percentages were 13.8, 14.9, 17.6 and 18.5%, respectively, and that intermuscular fat percentages were 5.7, 8.1, 9.3 and 9.4%, respectively. Kyanzad [28] reported that as the tail fat percentage decreased, the subcutaneous fat and inter-muscular fat percentage increased in thin tailed x fat tailed crossbred lambs. In the present study, the tail fat percentage, subcutaneous fat percentage and intermuscular fat percentage were 9.00, 19.46 and 7.19%, respectively.

Safdarian et al.[14] reported that the UC and LC constituted 85% of the variation in FTW in Torki-Ghashghaii sheep and Zamiri and Izadifard [11] reported that the LC accounted for 70% of the total variation of the FTW in Ghezel rams. Yardımcı et al.[13] reported an R^2 value of 0.81 for the prediction of FTW in White Karaman sheep, based on tail dimensions. In addition, Atti and Hamouda [12] reported that the FTW (R^2=0.75) and TBF (R^2=0.69) of Barbarine sheep could be estimated by using only tail measurements as independent variables. In the present study, LC and UC accounted for 88% and 71% of the total variation in FTW and TBF, respectively. The FTW and TBF prediction equations obtained in the current study were more accurate than the equations reported by Atti and Hamouda [12].

In conclusion, **s**ome tail dimensions, especially UC, LC and UTT, were significantly correlated with FTW and TBF. Moreover, UC and LC accounted for 88% of total variation in FTW and 71% of total variation in the TBF. Therefore, our results indicate that FTW and TBF in live Herik lambs can be estimated from the UC and LC. However, further work with a larger number of Herik lambs is required to fully understand the relationships.

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REFERENCES

1. Atti N, Bocquier F: Adaptation des brebis Barbarine à l'alternance sous-nutrition-réalimentation: Effets sur les tissus adipeux. *Ann Zootech*, 48, 189-198, 2009.

2. Kashan NE, Manafi Azar J, Afzalzadeh GH, Salehi A: Growth performance and carcass quality of fattening lambs from fat-tailed and tailed sheep breeds. *Small Ruminant Res*, 60, 267-271, 2005. DOI: 10.1016/j.smallrumres.2005.01.001

3. Pourlis Aris F: A review of morphological characteristics relating to the production and reproduction of fat-tailed sheep breeds. *Trop Anim Health Prod*, 43, 1267-1287, 2011. DOI: 10.1007/s11250-011-9853-x

4. FAO: Sheep population in Turkey. http://faostat3.fao.org/browse/Q/QA/E, 2015.

5. Yarkın I: Herik koyunu yapağisi üzerinde arastirmalar. *Ankara* Üniv. *Zir. Fak. Yıllığı*, 14, 133-149, 1964.

6. TAGEM: Türkiye Evcil Hayvan Genetik Kaynakları Tanıtım Kataloğu, www.tagem.gov.tr, 2009.

7. Eyduran E, Waheed A, Tariq MM, Iqbal F, Ahmad S: Prediction of live weight from morphological characteristics of commercial goat in Pakistan using factor and principal component scores in multiple linear regression. *J Anim Plant Sci*, 23, 1532-1540, 2013.

8. Jahan M, Tariq MM, Kakar MA, Eyduran E, Waheed A: Predicting body weight from body and testicular characteristics of Balochi male sheep in Pakistan using different statistical analyses. *J Anim Plant Sci*, 23, 14-19, 2013.

9. Khan MA, Tariq MM, Eyduran E, Tatliyer A, Rafeeq M, Abbas F, Rashid N, Awan MA, Javed K: Estimating body weight from several body measurements in Harnai sheep without multicollinearity problem. *J Anim Plant Sci*, 24, 120-126, 2014.

10. Vatankah M, Talebi MA: Genetic parameters of body weight and fat-tail measurements in lambs. *Small Ruminant Res*, 75, 1-6, 2008. DOI: 10.1016/j.smallrumres.2007.06.012

11. Zamiri MJ, Izadifard J: Relationship of fat-tail weight with fa t-tail measurements and carcass characteristics of Mehraban and Ghezel rams. *Small Ruminant Res*, 26, 261-266, 1997. DOI: 10.1016/S0921-4488(97)00013-8

12. Atti N, Ben Hamouda M: Relationships among carcass composition and tail measurements in fat-tailed Barbarine sheep. *Small Ruminant Res*, 53, 151-155, 2004. DOI: 10.1016/j.smallrumres.2003.08.016

13. Yardımcı M, Şahin EH, Çetingül İS, Bayram İ, Altunbaş K, Şengör E: Estimation of carcass composition and fat dopts by means of subcutaneous adipocyte area and body and tail measurements in fat tailed Akkaraman lambs. *S Afr J Anim Sci*, 38, 282-289, 2008.

14. Safdarian MM, Zamiri MI, Hashemi M, Noorolahi H: Relationships of fat-tail dimensions with fat-tail weight and carcass characteristics at different slaughter weights of Torki-Ghashghaii sheep. *Meat Sci*, 80, 686-689, 2008. DOI: 10.1016/j.meatsci.2008.03.007

15. Akçapınar H: Dağlıç, Akkaraman ve Kıvırcık kuzuların entansif beside büyüme ve yemden yararlanma kabiliyeti üzerinde karşılaştırmalı araştırmalar. *Ankara* Üniv *Vet Fak Derg*, 28, 112-129, 1981. DOI: 10.1501/Vetfak_0000000912

16. SPSS: Statistical package for the social sciences, Release 22.0, SPSS Inc, Chicago, Il, USA, 2013.

17. Ünal N: Survival rate, growth characteristics and some body measurements of Akkaraman and Sakız (Chios) x Akkaraman F_1 lambs. *Turk J Vet Anim Sci,* 26, 109-116, 2002.

18. Özcan H: Devlet üretme çiftliği Merinos x Karayaka melezlerin beden ölçüleri ve yapağı vasıfların üzerinde araştırmalar. Ankara Üniv. Vet. Fak. Yayınları, No: 122, Ege Matbaası, Ankara, 1960.

19. Arıtürk E, Utkanlar N, İmeryüz F, Müftüoğlu Ş, Öznacar K: Karaköy Harasında yetiştirilen üç yaşlı Karayaka koyunlarında beden ölçüleri, canlı ağırlık, yapağı verim ve özellikleri. *Lalahan Zoot Araşt Enst Derg*, 3, 9-27, 1963.

20. Atasoy F, Ünal N, Akçapınar H, Mundan D: Karayaka ve Bafra (Sakız x Karayaka G_1) koyunlarında bazı verim özellikleri. *Turk J Vet Anim Sci*, 27, 259-264, 2003.

21. Arslan M: Saf ve Melez (Dorset Down x Morkaraman (F_1), Corriedale x Morkaraman (F_1)) Morkaraman kuzuların besi performansı, kesim ve karkas özellikleri. Doktora Tezi, Yüzüncü Yıl Üniv Sağlık Bil Enst, 1998.

22. Unal N, Akcapınar H, Atasoy F, Aytac M: Some reproductive and growth traits of crossbred genotypes produced by crossing local sheep breeds of Kivircik x White Karaman and Chios x White Karaman in steppe conditions. *Arch Tierz*, 49, 55-63, 2006.

23. Tufan M, Akmaz A: Slaughter and carcass traits of Guney Karaman, Kangal-Akkaraman and Akkaraman lambs at different slaughter weights. *Turk J Vet Anim Sci*, 25, 495-504, 2001.

24. Kadak R: Akkaraman, Morkaraman ve Ivesi ırkı kuzuların farklı kesim ağırlıklarında besi performansı ve karkas özelliklerinin karşılaştırılması. Doktora Tezi, Fırat Üniv. Sağlık Bil. Enst., 1983.

25. Macit M: Growth and carcass characteristics of male lambs of the Morkaraman breed. *Small Ruminant Res*, 43, 191-194, 2002. DOI: 10.1016/S0921-4488(01)00268-1

26. Öztürk Y, Küçük M, Karslı MA: A study on growth, slaughter and carcass traits of Morkaraman and Kivircik x Morkaraman (F_1) lambs in semi-intensive condition. *Kafkas Univ Vet Fak Derg*, 18, 1-6, 2012. DOI: 10.9775/kvfd.2011.4351

27. Aydoğan M: Karayaka, Ile De France x Karayaka (F_1) ve Sakız x Karayaka (F_1) kuzularının büyüme, besi performansı ve karkas özelliklerinin karşılaştırılması. *Ankara Üniv Vet Fak Derg*, 32, 111-130, 1985. DOI: 10.1501/Vetfak_0000000333

28. Kyanzad MR: Crossbreeding of the three Iranian sheep breeds with emphasis on growth and carcass characteristics of the lambs. *PhD Thesis.* University of Utra, Malaysia, 2001.

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Research Article

The Volatile Compounds, Free Fatty Acid Composition and Microbiological Properties of Sepet Cheese Packaged with Different Modified Atmosphere Conditions [1][2]

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Abstract

The objective of this present study was to investigate traditional sepet cheese samples which were packaged with different modified atmosphere conditions (MAP). The volatile compounds, free fatty acid composition and microbiological properties of Sepet cheeses were analyzed on 1., 45., 90. and 180. day of the storage period at +4°C. In packaking of cheese, three different modified atmosphere packaging conditions were tried. These cheeses were packaged into polystyrene packages that include 100% N_2 (N), 80% N_2 + 20% CO_2 (NC), 100% CO_2 (C). In accordance with volatile compounds of sepet cheese samples, it was shown that control cheese sample was different from MAP samples in terms of aroma fractions. MAP samples showed variability in preservation of aroma fractions. When fatty acid composition during the storage period was researched, it was found that there was a significant difference in all samples. When microbiological properties were investigated statistically, it was determined that there was not a significant difference in samples during storage. In general, it could be said that sepet cheeses that were packaged in different modified conditions protected their specialities better.

Keywords: *Sepet cheese, Modified atmosphere packaging, Cheese quality*

Farklı Modifiye Atmosfer Koşulları ile Paketlenen Sepet Peynirinin Aroma Bileşenleri, Serbest Yağ Asitleri Kompozisyonu ve Mikrobiyolojik Özellikleri

Özet

Çalışmamızın amacı farklı modifiye atmosfer koşullarında paketlenen geleneksel sepet peyniri örneklerini incelemektir. +4°C' de depolanan sepet peynirlerinin aroma bileşenleri, serbest yağ asitleri kompozisyonu ve mikrobiyolojik özellikleri depolamanın 1., 45., 90. ve 180. günlerinde incelenmiştir. Peynirlerin paketlenmesinde 3 farklı modifiye atmosfer koşulu denenmiştir. Üretilen peynirler 100% N_2 (N), 80% N_2 + 20% CO_2 (NC), 100% CO_2 (C) içeren atmosfer koşullarda polistiren ambalajlarda paketlenmiştir. Sepet peynirlerinin aroma maddeleri komposizyonu incelendiğinde kontrol grubunun MAP grubundan farklılık gösterdiği tespit edilmiştir. MAP örnekleri aroma fraksiyonları bakımında çeşitlilik göstermiştir. Yağ asitleri komposizyonu tüm sepet peyniri örneklerinde farklılık göstermiştir. Mikrobiyolojik özellikler istatistiksel olarak değerlendirildiğinde depolama boyunca meydana gelen farklılığın önemli olmadığı tespit edilmiştir. Genel olarak bakıldığında farklı modifiye koşullarda paketlenmiş olan sepet peynirlerinin özelliklerini daha iyi muhafaza ettiği görülmüştür.

Anahtar sözcükler: *Sepet peyniri, Modifiye atmosfer paketleme, Peynir kalitesi*

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INTRODUCTION

There are various cheese types in the Aegean region of Anatolia various cheese types with different shape, color, and taste. Cheese is one of the important traditional foods in the Turkish cuisine. Many cheese types produced only in certain regions are consumed locally in large quantities in Turkey [1-3]. The Sepet cheese is a traditional semi-hard cheese produced in the Mediterranean region of Turkey. Some cheese types have begun to sink into oblivion and such products are sold only at local markets and produced within families [4]. The Sepet Cheese is produced in small towns close to the shore in the Aegean region, especially in Ayvalik and also in Dikili, Burhaniye, Foça, Çesme, Urla, Karaburun, Ödemis and Söke [5].

Technological advances in processing and packaging of dairy foods have an impact on the consumers' trends. Dairy products with enhanced nutrition and specific product functionalities are particularly demanded by the consumers [6,7]. Reduction of the chemical and microbial overload in foods have been especially recommended by the food safety regulatory authorities. The food industry has begun to search for ways to develop new packaging concepts due to these strict regulations and the consumer preferences for natural and healthy products free of preservatives with extended shelf lives. It was determined that, one of these packaging concepts, modified atmosphere packaging (MAP), in combination with refrigeration, is successful in extending the shelf life of milk and milk products [8-11]. Modified atmosphere packaging, elimination of oxygen from inside package and filled with different concentrations of CO_2 and N_2 [12].

The objective of this study is to investigate technological, microbiological and biochemical aspects of Sepet cheese during manufacture and ripening, and to consider future work on certain specific topics. For this purpose, sepet cheese was compared with other brined cheeses.

MATERIAL and METHODS

Goat milk was used in the production of these sepet cheeses. Production of cheese has been made in a local manufacturer. Cheese was produced as follow: Without any heat treatment, raw goat's milk was heated up to 58-60°C and immediately cooled to renneting temperature. Coagulation occured in 2 h using animal origin rennet (1/16.000 MCU/ml). The curd's temperture was increased to 36-38°C by pourin hot water on for easier and faster draining. Meanwhile, curd subsided and seperated from whey. The curd at the bottom of container, was collected with hand, given a ball shape and stuffed in optinally 1, 3 or 5 kg capacity baskets made of reed stems and top surface was flattened. The cheese in the basket was left to draining spontanously without putting any weight on the basket. In the meantine curd took the basket's shape during draining. Cheese have been turned upside down in order to make the top surface take the basket's shape. Cheese had been taken out of the basket 15 min after the whey started to drain. Top and bottom surfaces were salted with thin salt and relocated in the basket for further percolation. Approximately 18 h later, when it became a single firm hoop, it was taken out of the basket and put on a wooden surface in a shady place. Cheeses were rubbed with salt every other day in a total of 15 days. Then the cheeses were packaged with polystyrene material in different modified atmospheric conditions. The sepet cheese (about 200 g) were packaged in expanded polystyrene (EPS) (0.2 mm thickness, oxygen transmission rate 2.600-7.700 cm^3/m^2/day bar, CO_2 transmission rate 10.000-.26.000 cm^3/m^2/day bar at 25°C) trays placed in gas-barrier bags under three different atmosphere conditions (100% nitrogen, 80% nitrogen + 20% carbondioxide, 100% carbondioxide). The cheeses were ripened for 6 months at +4°C and were analysed at 1., 45., 90. and 180. day of the storage period. K, N, NC and C codes were given to the cheese samples Control, 100% N_2, 80% N_2 + 20% CO_2, 100% CO_2, respectively.

The volatile compounds of Sepet cheeses were determined with a solid-phase-microextraction (SPME) method using a fiber (57348-U, Supelco Inc., Bellefonte, PA, USA) coated with the sorbent material, divinylbenzene/carboxen/polydimethylsiloxane. Volatile compounds of Sepet cheeses were determined with using gas chromatography (GC) (Clarus 600, Pelkin Elmer Inc., Massachusetts, USA) equipped with flame ionization detector. The temperature of GC oven was programmed as follows: held at 40°C for 6 min, then the temperature was raised to 100°C with 5°C/min and held for 2 min. and finally the temperature was reached to 250°C (10°C/min, held for 4 min). Carrier gasses were He with 1 ml/min and H_2 with 1 ml/min flow rates. The analysis was performed in duplicate. The identification of chromatographic peaks were carried out with comparison of the retention times of appropriate standards (Sigma Chemical Company, St. Louis, MO, USA) [13].

Lipids were extracted using purified kieselgurh (Fluka Chemie GmbH, Buchs, Switzerland) and diethyl ether (Riedel-de Haën, Germany) as described by Renner [14]. Approximately 50 g of sample was measured with 6-8 g of kieselgurh, and then mixed with 200 ml diethyl ether. Fatty acid methyl esters were prepared according to AOCS Official Method Ce 2-66 [15]. Fatty acid composition was determined by a Hewlett-Packard Gas Chromatography (model 6890, Avondale, PA, USA equipped with Supelco SP-2380 fused silica capillary column (100 m × 0.25 mm i.d., 0.2 μm film thickness; Supelco Inc., Bellefonte, PA, USA) [16].

For each cheese sample, 10 g was weighed, diluted aseptically in 90 mL of citrate buffer (2%, wt/vol), and homogenized in a sterile polyethylene bag using a Stomacher 400 (Seward Laboratory, London, UK) for 1.5 min. Serial dilutions were made in sterile ringer solution and all

determinations were made in duplicate. The enumeration of *Lactobacillus* ssp. (MRS Agar; Merck Darmstadt, Germany) at 37°C for 72 h [17], *Lactococcus ssp.* (M17 Agar; Merck) at 37°C for 48 h [17], and *Enterococcus* ssp. (Kanamycin esculin Agar; Merck) at 37°C for 48 h [18] were performed during the ripening of Sepet Cheese.

The experiment was carried out in duplicate. Data were analyzed using the general linear model procedure of the SPSS software (version 20; SPSS Institute Inc., Chicago, IL). Analysis of variance for each set of data was conducted and Duncan's multiple range tests were used to compare the means when the effect was sinificant (*P<0.05*). In addition statistical significances ar the 95% confidence interval (*P<0.05*) as being considered statistically significant.

RESULTS

Total of 24 volatile compounds were determined in Sepet cheeses packaged under different modified atmosphere conditions during 6 months of storage *(Table 1)*. In all sepet cheese samples, free fatty acids were the most abundant volatile compounds of total identified fraction such as hexanoic acid, octanoic acid, and butyric acid which had the highest percentage values, respectively. There were statistical differences in fatty acids results (*P<0.05*). Free fatty acids reduced during storage in sample K. However, only butyric acid increased during storage in this sample. Butyric acid was found with the highest percentage in all samples during storage. Hexanoic acid had also high percentages in all samples. The esters in the volatile fraction of Sepet cheeses were ethyl hexanoate, ethyl decanoate, ethyl octanoate, and ethyl butyrate. Some differences were observed in ethyl butyrate percentage in all samples during storage (*P<0.05*).

Some aldehydes such as octanal, decanal, 2,6-nonadienal were also found in low percentage in all samples. When cheese samples were compared with aldehydes amount, it was seen that percentage of aldehydes in C, NC and C cheese samples were higher than sample K. In addition some ketones as a diacetyl, acetoin and 2-nonanone were detected during storage. Other important aromatic compounds for cheese, ethanol and acetaldehyde, were also found in some samples during storage. In sample N and NC, acetaldehyde percentage was higher than in sample C and K.

The unsatureted and satureted fatty acid composition of Sepet cheeses ripened different modified atmosphere conditions are given *Table 2*. Butyric acid (C4:0) is a fatty acid which has an important role in formation of flavour in brine-cured cheeses and in formation of rancid taste. As seen in *Table 2*, butyric acid content of the cheeses changes between 1.71% and 2.50%. Compared to control sample, N, NC and C cheeses were found to have lower butyric acid content on the 45th day of storage. But on the further days of storage results are found to be closer. The avarage amount of caproic (C6:0) and caprilic (C8:0) acids, among total fatty acids were between 1.50-1.73% and 0.94-1.15% respectively. In many types of cheese similar to white cheese butyric, caproic and caprilic acids are the indicators of starter oriented lipolytic activity. Also these fatty acids may easily influence flavour. Among the fatty acids, the amount of capric (C:10), lauric (C:12) and myristic (C:14) acids were changed between 2.09-2.58%, 2.54-2.99% and 9.96-11.89% respectively. In the N, NC and C cheese samples, fatty acid content were higher than the control sample, as seen in *Table 2*.

In *Table 2*, among the long chain fatty acids (C16-C20), palmitic acid (C16:0) was found to have a bigger ratio with an avarage of between 28.05% -33.15%. In the control sample, palmitic acid content was higher on the last day of storage, where the other samples had lower palmitic acid content. The NC cheese sample was found to contain a higher avarage palmitic acid content than the other samples. Among the total fatty acids the amount of stearic and oleic acids were 14.81-26.30% and 16.15-27.26%, respectively. The avarage ratios of palmitoleic acid among the fatty acid composition, which was said to give information on the level of lipolysis and causing off flavour and aroma, were between 0.06% and 0.69%. Another significant fatty acid in the composition was linoleic acid (C18:2 *cis*-9,12) and these values were ranged between 1.42 - 2.69% in all samples.

The microbiological characteristics of Sepet cheeses were studied during 180 d of ripening *(Table 3)*. In all cheeses, the highest levels of *Lactobacillus* ssp. were found at the first stage of ripening, and counts decreased until 90 d. However, the counts increased sharply at the end of ripening (180 d), and the final values ranged from 6.56 to 7.08 log cfu/g in all samples. The counts of *Lactobacillus ssp.* were significantly affected by storage period in control sample (*P<0.05)*. It was seen that *Lactococcus* ssp. counts changed between 7.67 to 9.78 log cfu/g. Statistical differences were seen in K, N and C samples (*P<0.05*). The all samples had high level of *Lactococcus* ssp. on the first day of storage, but their count decreased until 180 d. *Enterococcus* ssp. count changed between 3.23 and 4.96 log cfu/g. It was seen that *Enterococcus ssp.* count increased on the further days.

DISCUSSION

Aroma is the one of the most important parameters in food quality while influencing selection and consumption of food. Several volatile compounds such as fatty acids, esters, hydrocarbons, alcohols, aldehydes, ketones, lactones, sulphur- and nitrogen-containing compounds contribute to cheese flavor [19]. Esters, which are responsible for the fruity flavor in cheese, are formed through 2 enzymatic reactions, esterification and alcoholysis [2,20,21]. Esterification is the

Table 1. *Volatile compounds during production and ripening of Sepet Cheese in different atmosphere packaking Condition*[1]

Tablo 1. *Farklı Atmosfer Koşullarında Paketlenen Sepet Peynirlerinin Üretim ve Olgunlaşma Aşamasında Aroma Maddeleri*[1]

Chemical	RT (min)	Cheese											
		K			N			NC			C		
		Storage Days			Storage Days			Storage Days			Storage Days		
		1	45	180	1	45	180	1	45	180	1	45	180
Free Fatty acid													
Butyric Acid	24.48	29.82±0.07^{a}	27.47±1.27^{a}	34.97±0.31^{bY}	30.68±2.32^{a}	24.57±5.92^{a}	49.00±1.66^{bZ}	32.86±1.63^{a}	33.02±4.89^{a}	5.33±0.98^{bX}	32.99±1.78^{a}	23.42±1.54^{b}	49.89±4.69^{aZ}
Hexanoic Acid	27.20	37.14±3.82^{bX}	35.96±0.77^{bY}	25.12±3.18^{aY}	23.62±4.21^{abY}	29.86±4.21^{bY}	13.10±0.55^{aX}	21.66±0.83^{Y}	21.15±0.78^{X}	29.87±5.62^{Y}	21,29±2.71^{Y}	10.23±0.38^{Z}	19.81±3.86^{XY}
Heptanoic Acid	28.72	0.13±0.03	0.15±0.01	0.18±0.09	0.24±0.02	0.30±0.13	0.07±0.06	0.15±0.08	0.54±0.37	0.17±0.16	0.14±0.01	0.36±0,12	0.16±0.13
Octanoic Acid	28.99	16.14±1.39^{cX}	9.83±1.04^{bX}	6.14±0.66^{aY}	0.81±0.48^{aY}	2.89±1.36^{aY}	5.76±0.54^{bY}	4.72±0.78^{Y}	4.15±1.08^{Y}	2.19±0.68^{X}	14.03±3,06^{bX}	3.05±1.24^{aY}	1.36±0.83^{aX}
4-Methy octanoic Acid	30.79	0.49±0.08^{X}	0.79±0.29	0.29±0.20	0.31±0.03	0.41±0.15^{X}	0.35±0.13	0.48±0.13^{X}	0.50±0.22	0.57±0.02	1.10±0.16^{c}	0.62±0.00^{bY}	0.16±0.16^{a}
Decanoic Acid	31.40	0.14±0.04	0.22±0.17	0.15±0.01	0.15±0.04	0.18±0.09	0.21±0.04	0.16±0.05	0.09±0.01	0.17±0.00	0.11±0.00	0.22±0.17	0.20±0.01
Dodecanoic Acid	32.75	0.53±0.04^{cY}	0.32±0.04^{b}	0.13±0.00^{a}	0.68±0.38^{X}	0.18±0.04	0.40±0.24	0.22±0.19^{X}	0.74±0.19	0.30±0.25	0.09±0.00^{X}	0.62±0.87	0.17±0,00
Ketone													
Diacetyl	4.42	0.58±0.06^{X}	3.85±0.63^{XY}	2.17±0.90	12.13±1.97^{bY}	1.67±1.51^{aX}	4.02±1.56^{a}	0.06±0.09^{aX}	1.56±0.09^{aX}	7.99±1.98^{b}	9.42±1.92^{Y}	7.06±2.00^{Y}	2.56±1.67
Acetoin	15.01	1.33±0.29^{X}	0.66±0.48	0.29±0.31^{X}	5.94±0.94^{Y}	5.73±1.07	2.02±1.51^{X}	10.92±0.99^{Z}	4.77±0.99	12.54±3.18^{Y}	0.00±0.00^{aX}	4.41±1.17^{b}	1.01±0.54^{aX}
2-Nonanone	18.29	2.79±0.56	2.07±0.15	3.92±2.26	7.38±1.81	1.79±0.91	0.00±0.00	0.00±0.00	3.63±0.01	0.00±0.00	0.95±0.44	4.39±1.37	1.91±1.13
Aldehyde													
Acetaldehyde	3.24	1.25±0.01^{X}	6.49±2.89^{Y}	3.52±0.28	7.42±1.50^{aY}	11.07±1.63^{Y}	8.01±0.07	1.48±0.6^{aX}	7.57±1.51^{bY}	7.72±0.00^{b}	4.66±1.75^{XY}	0.00±0.00^{X}	2.02±1.09
Octanal	15.65	0.09±0.12^{X}	0.14±0.20^{X}	0.41±0.19^{X}	1.63±2.31^{aX}	0.76±1.07^{bX}	0.91±0.73^{bX}	12.48±2.32^{bY}	1.78±2.32^{aXY}	9.55±3.01^{bX}	3.23±1.60^{X}	4.14±0.82^{Y}	2.58±0.24^{Y}
Nonanal	18.87	0.34±0.23^{Y}	4.23±2.57	0.68±0.95	0.00±0.00^{Z}	0.00±0.00	0.04±0.05	1.15±0.76^{aX}	0.00±0.00^{b}	0.72±1.02^{a}	0.00±0.00^{XY}	0.00±0.00	0.02±0.03
Decanal	21.65	0.00±0.00^{aX}	0.07±0.04^{a}	0.75±0.29^{b}	0.06±0.08^{X}	0.96±0.89	0.54±0.18	0.10±0.13^{aX}	0.23±0.02^{a}	2.68±0.76^{b}	0.39±0.03^{Y}	1.58±0.39	2.21±0.39
E-2. Z-6 Nonadienal	23.80	0.08±0.11	0.64±0.49^{X}	1.51±0.67	0.36±0.51^{a}	3.17±1.52^{X}	0.36±0.36	0.03±0.04^{a}	0.88±0.21^{bX}	0.08±0.00^{a}	0.04±0.01^{a}	7.94±2.22^{bY}	0.80±0.72^{a}
E-2 Decenal	24.96	0.20±0.16	0.59±0.41	0.12±0.13	0.54±0.77^{a}	3.29±2.74	0.13±0.18	0.52±0.74^{a}	7.72±0.65^{b}	0.41±0.09^{a}	0.34±0.10^{a}	5.23±1.69^{b}	0.76±0.50^{a}
Ester													
Ethyl butyrate	7.57	0.16±0.22^{X}	0.17±0.24	0.54±0.05	0.00±0.00^{aX}	0.00±0.00^{a}	0.58±0.23^{a}	3.72±1.00^{bY}	0.00±0.00^{a}	0.12±0.17^{a}	0.00±0.00^{aX}	0.0±0.00^{a}	0.25±0.02^{b}
Etyhl hexanoate	14.00	0.50±0.08	0.42±0.07^{X}	3.00±1.21	2.86±0.15	3.33±0.88^{Y}	1.29±0.17	0.01±0.02^{a}	0.28±0.02^{aX}	7.99±2.60^{b}	4.91±1.22	0.54±0.14^{X}	2.96±1.30
Ethyl octanoate	22.32	0.26±0.28	0.16±0.07	0.35±0.04	0.22±0.31	0.00±0.00	0.04±0.03	0.08±0.04	0.00±0.00	0.16±0.23	0,27±0.01	0.00±0.00	0.30±0.22
Ethyl decanoate	25.95	1.38±0.52	0.95±0.19^{XY}	0.23±0.00	0.38±0.53	0.00±0.00^{X}	0.99±0.39	0.36±0.03	0.13±0.03^{X}	1.10±0.39	0.92±0.01	1.88±0.67^{Y}	1.09±0.74
Other													
Ethanol	3.40	2.43±0.98^{bY}	0.00±0.00^{bX}	8.92±0.03^{cX}	3.16±1.12^{Y}	1.95±1.1.^{X}	0.00±0.00^{Y}	0.00±0.00^{aX}	6.55±0.96^{bY}	3.62±1.34^{bZ}	0.14±0.19^{X}	6.62±2.27^{Y}	2.77±1.73^{YZ}
D-Limonene	13.60	0.00±0.00^{X}	0.00±0.00	0.00±0.00	0.08±0.11^{X}	0.00±0.00	0.10±0.14	2.51±1.31^{bY}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{X}	0.00±0.00	0.59±0.83
1-Hexanol	17.85	1.45±0.44^{Y}	1.91±0.86	0.97±0.65	0.08±0.11^{a}	0.00±0.00^{aX}	4.61±1.22^{b}	1.71±1.87	0.00±0.00^{X}	2.03±1.68	2.92±0.18^{b}	0.00±0.00^{aX}	0.24±0.33^{a}
γ-Dodecalactone	32.10	0.04±0.05	0.04±0.05	0.14±0.19	0.00±0.00	0.00±0.00	0.18±0.14	0.07±0.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Unknown 1	16.32	1.72±0.40	0.90±0.85	2.13±2.55	0.00±0.00	3.83±3.42	2.36±0.92	0.00±0.00	0.43±0.61	0.00±0.00	0.00±0.00	6.68±2.31	1.81±0.97
Unknown 2	19.70	0.12±0.01	0.24±0.00	0.86±0.13	0.90±0.09	2.34±1.19	4.17±0.45	0.29±0.14	1.42±0.46	3.60±0.83	0.31±0.11	0.49±0.03	2.54±1.10
Unknown 3	21.25	0.89±0.05	1.70±0.70	2.37±2.71	0.30±0.43	1.65±1.17	0.43±0.02	4.16±0.33	2.84±1.63	1.00±0.80	1.73±0.38	10.47±0.27	1.82±0.28
Unknown 4	33.15	0.00±0.00	0.00±0.00	0.13±0.18	0.00±0.00	0.00±0.00	0.25±0.22	0.02±0.03	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.10	0.00±0.00

$^{a-d}$ Means ± SD within a row with no common superscript differ (P<0.05). (for compare storage days) $^{X-Z}$ Means ± SD within a row with no common superscript differ (P<0.05). (for compare between product difference) [1] Values are the means of 2 replicates. K refer to the cheeses ripened in normal condition ; N, NC and C refer to the cheeses ripened in 100% nitrogen, 80% nitrogen + 20% carbondioxide, 100% carbondioxide respectively

Table 2. *Unsaturated and saturated fatty acid composition of Sepet cheese in different atmosphere packaking condition*[1]

Tablo 2. *Farklı Atmosfer Koşullarında Paketlenen Sepet Peynirlerinin Doymamış ve Doymuş Yağ asitleri Kompozisyonu*[1]

Cheese	Unsaturated Fatty Acid Composition										
	Days	**C14:1**	**C15:1**	**C16:1**	**C17:1**	**C18:1 cis**	**C18:1 tr**	**C18:2 cis**	**C18:2 tr**	**C18:3**	**C20:1**	**C22:4**
K	1	0.54±0.29	0.01±0.00	0.06±0.03	0.32±0.06	17.74±4.68	0.60±0.20^{ab}	2.59±0.36	0.17±0.02	0.39±0.18	0.23±0.02	0.07±0.03
	45	0.66±0.01	0.01±0.00	0.08±0.05	0.33±0.01	23.28±9.79	0.74±0.03^{b}	2.77±0.46	0.16±0.04	0.31±0.08	0.31±0.09	0.04±0.01
	90	0.73±0.22	0.02±0.00	0.11±0.03	0.42±0.17	17.78±1.95	0.62±0.20^{ab}	1.80±0.94	0.21±0.02	0.18±0.07	0.18±0.07	0.07±0.01
	180	0.92±0.40	0.01±0.00	0.06±0.01	0.44±0.01	17.58±0.38	0.27±0.11^{a}	1.42±1.81	0.18±0.06	0.17±0.05	0.30±0.11	0.06±0.00
N	45	0.57±0.28	0.02±0.00^{b}	0.15±0.06	0.31±0.05	25.46±2.20^{b}	0.60±0.22	2.38±0.86	0.16±0.06	0.23±0.00	0.25±0.01	0.03±0.02
	90	0.56±0.24	0.02±0.00^{a}	0.69±0.47	0.33±0.07	16.15±1.43^{a}	0.60±0.43	2.45±0.77	0.16±0.06	0.23±0.12	0.24±0.04	0.06±0.00
	180	0.70±0.67	0.01±0.00^{a}	0.03±0.01	0.25±0.05	18.68±3.44^{ab}	0.58±0.12	2.58±0.10	0.18±0.02	0.26±0.12	0.18±0.11	0.08±0.05
NC	45	0.54±0.27	0.01±0.00	0.16±0.20	0.53±0.29	18.51±2.84	0.33±0.11	1.63±0.68	0.14±0.02	0.18±0.03	0.24±0.01	0.04±0.00^{ab}
	90	0.59±0.32	0.01±0.00	0.16±0.15	0.32±0.07	18.26±2.42	0.68±0.36	2.69±0.56	0.17±0.06	0.22±0.16	0.26±0.05	0.02±0.00^{a}
	180	0.52±0.27	0.07±0.00	0.55±0.36	0.28±0.05	22.17±4.06	0.64±0.15	2.23±0.72	0.14±0.03	0.29±0.08	0.24±0.00	0.07±0.01^{b}
C	45	0.94±0.20	0.03±0.01	0.17±0.13	0.69±0.52	27.26±3.44^{b}	0.64±0.38	1.51±0.72	0.10±0.00	0.24±0.00	0.24±0.05	0.05±0.05
	90	0.53±0.36	0.01±0.00	0.18±0.22	0.29±0.05	19.22±0.09^{ab}	0.57±0.20	2.43±0.48	0.15±0.07	0.24±0.08	0.23±0.03	0.08±0.07
	180	0.56±0.24	0.01±0.00	0.04±0.00	0.37±0.08	17.84±3.06^{a}	0.72±0.37	2.08±0.52	0.18±0.08	0.22±0.01	0.31±0.09	0.04±0.00

Cheese	Saturated Fatty Acid Composition											
	Days	**C4**	**C6**	**C8**	**C10**	**C11**	**C12**	**C13**	**C14**	**C15**	**C16**	**C17**	**C 18**
K	1	2.50±0.18	1.70±0.38	1.05±0.01	2.30±0.03	0.021±0.00	2.78±0.09	0.011±0.00	11.62±0.93	1.29±0.19	32.07±5.45	0.66±0.01	21.13±1.33
	45	2.15±0.38	1.57±0.22	1.04±0.21	2.33±0.54	0.017±0.00	2.54±0.29	0.021±0.01	9.96±0.46	1.40±0.02	28.72±2.75	0.58±0.11	20.94±5.75
	90	2.37±0.12	1.72±0.32	1.07±0.24	2.31±0.61	0.019±0.00	2.61±0.61	0.011±0.00	10.29±1.69	1.44±0.07	29.04±0.76	0.65±0.05	26.30±1.82
	180	2.17±0.32	1.50±0.16	0.94±0.04	2.09±0.07	0.016±0.00	2.57±0.30	0.037±0.03	11.09±1.90	1.30±0.20	33.15±5.29	0.62±0.02	23.09±5.60
N	45	1.71±0.06^{a}	1.41±0.02	0.94±0.03	2.27±0.01	0.019±0.00	2.79±0.01	0.017±0.00	11.89±0.73	1.42±0.00	31.85±4.39	0.62±0.09	14.81±1.41^{a}
	90	2.13±0.19^{ab}	1.64±0.19	1.08±0.18	2.42±0.40	0.023±0.00	2.95±0.35	0.014±0.00	11.84±0.28	1.36±0.45	31.69±3.28	0.66±0.15	22.60±2.60^{b}
	180	2.36±0.14^{b}	1.72±0.01	1.15±0.03	2.58±0.11	0.023±0.00	2.80±0.18	0.011±0.00	10.57±0.93	1.45±0.16	28.05±1.96	0.58±0.12	25.11±0.42^{b}
NC	45	1.95±0.56	1.54±0.28	1.02±0.16	2.29±0.28	0.02±0.00	2.81±0.12	0.012±0.00	11.49±0.73	1.32±0.33	31.94±4.84	0.66±0.07	22.49±6.78
	90	2.25±0.23	1.68±0.17	1.09±0.14	2.45±0.33	0.02±0.00	2.99±0.32	0.010±0.00	12.00±0.21	1.42±0.46	32.07±3.50	0.69±0.11	19.90±2.56
	180	2.45±0.76	1.73±0.34	1.08±0.15	2.38±0.24	0.13±0.14	2.79±0.12	0.033±0.02	11.42±0.79	1.39±0.09	30.61±5.13	0.65±0.06	17.95±1.96
C	45	2.1±0.49	1.62±0.45	1.052±0.27	2.34±0.57	0.02±0.00	2.76±0.40	0.021±0.01	11.22±0.37	1.254±0.34	30.73±1.63	0.632±0.07	19.06±8.41
	90	2.23±0.34	1.62±0.30	1.033±0.22	2.29±0.47	0.02±0.00	2.75±0.40	0.013±0.00	11.34±0.88	1.227±0.35	30.30±1.83	0.601±0.12	22.63±2.43
	180	2.05±0.30	1.62±0.17	1.070±0.08	2.41±0.12	0.02±0.00	2.85±0.06	0.015±0.00	11.60±0.97	1.464±0.10	31.76±3.70	0.622±0.07	22.61±6.28

$^{a–d}$ Means ± SD within a column with no common superscript differ (P<0.05).$^{x-z}$ Means ± SD within a row with no common superscript differ (P<0.05).[1] Presented values are the means of 2 replicate trials. K refer to the cheeses ripened in normal condition ; N, NC and C refer to the cheeses ripened in 100% nitrogen, 80% nitrogen + 20% carbondioxide, 100% carbondioxide respectively

Table 3. *Microbiological characteristics (in log cfu/g) of Sepet cheeses ripened in different atmosphere packaking Condition*[1]
Tablo 3. *Farklı Atmosfer Koşullarında Paketlenen Olgunlaşmış Sepet Peynirlerinin Mikrobiyolojik Özellikleri*[1] *(log cfu/g)*

Variable	Days	Cheese			
		K	N	NC	C
***Lactobacillus* ssp.**	**1**	8.11±0.05	8.11±0.05	8.11±0.05 ^{a}	8.11±0.05
	45	7.47±0.67	7.25±0.36	7.70±0.53 ^{a}	7.83±1.06
	90	7.24±0.33	7.30±0.36	7.27±0.19 ^{ab}	7.36±0.60
	180	6.98±0.03	7.08±0.01	6.59±0.16 ^{b}	6.56±0.73
***Lactococcus* ssp.**	**1**	9.78±0.01 ^{a}	9.78±0.01 ^{a}	9.78±0.01	9.78±0.01 ^{a}
	45	9.31±0.48 ^{ab}	9.26±0.49 ^{ab}	9.17±0.63	9.03±0.27 ^{ab}
	90	8.46±0.19 ^{bc}	8.60±0.09 ^{bc}	8.60±0.10	8.50±0.20 ^{b}
	180	7.67±0.52 ^{c}	8.41±0.25 ^{c}	8.06±0.54	8.06±0.02 ^{b}
***Enterococcus* ssp.**	**1**	3.28±0.28	3.28±0.28	3.28±0.28	3.28±0.28
	45	3.53±0.75	3.99±0.40	4.60±0.36	3.93±0.20
	90	4.71±1.01	4.96±0.16	4.91±0.30	4.67±0.50
	180	4.80±1.08	4.93±0.59	4.90±0.82	4.85±0.77

$^{a-d}$ Means ± SD within a column with no common superscript differ (P< 0.05). $^{x-z}$ Means ± SD within a row with no common superscript differ (P<0.05). [1] *Presented values are the means of 2 replicate trials. K refer to the cheeses ripened in normal condition; N, NC and C refer to the cheeses ripened in 100% nitrogen, 80% nitrogen + 20% carbondioxide, 100% carbondioxide respectively*

formation of esters from alcohols and carboxylic acids whereas alcoholysis is the production of esters from alcohols and acylglycerols or from alcohols or acyl-coenzyme A. Aldehydes are produced by the catabolism of fatty acids or aminoacid via decarboxylation or deamination [2,22]. Some aldehydes such as octanal, decanal, 2,6-nonadienal were also found in low percentage in all samples. According to free faty acids, esters, aldehydes results, our research show similarities with Sepet cheese produced from goat milk and Tulum cheese produced from ewe milk [2,23]. When all volatile results were analyzed, the butyric acid, hexanoic acid, octanoic acid, diacetly and acetaldehyde were found to be at the highest percentage in all of the samples during storage. The low percentages of ester contents were observed in all samples. When percentages of esters were compared in samples, it could be said that sample K and sample NC had the lowest percentages. In terms of volatile compunds, similar results were determined by Trobetas *et al.*[24].

Compared to the control sample, linoleic acid content of N, NC and C cheese samples were lower on the 45th day of storage period, where on the further days the levels of linoleic acid dropped even below the other samples. Temiz [25] stated that predominant short-chain fatty acid was lauric acid, the most abundant medium-chain fatty acid was palmitic acid and long-chain fatty acid was oleic acid in sliced Kashar cheeses which packaged different modified atmosphere conditions.

According to *Lactobacillus* ssp. count, a similar trend was reported by Demir *et al.*[11], Ercan [13] and Ercan *et al.*[26] in sepet cheese samples. However, counts of *Lactobacillus* ssp. at the end of the storage were lower than our study results. All samples had high level of *Lactococcus* ssp. on the first day of storage, but their count decreased during 180 d. Lactococci counts of all samples on the 45th day of the storage period were found close to each other. On the further days, Lactococci counts decreased in all sample. However the highest reduction was seen in control sample during the storage days. This difference may be due to the packaging conditions and packaging may effect the consistency in lactococci count. Ercan *et al.*[26] reported that the lactococci count of Sepet cheeses changed between 5.49 and 8.89 log cfu/g. In our study, counts were found to be higher. This difference could be due to the starter microflora. *Enterococcus* ssp. count on the first days of storage was low where on further days an increase in counts were observed in all Sepet cheese samples. The highest rising count were observed in NC sample. Ercan *et al.*[26] stated that the *Enterococcus* ssp. counts were between 5.44 and 8.87 log cfu/g. These values are considerably higher than the values found in our study.

In this study, volatile, fatty acid composition and microbiological properties of Sepet cheese were investigated during manufacture and ripening. For this purpose, the cheeses were compared with control sample. When Sepet cheese samples were compared to the control sample, it was found that packaging in different conditions had made a significant difference between samples regarding volatile compounds, fatty acid compositions and microbiological characteristics. The values were found to be statistically close and similiar. For the local community, Sepet cheese, with its high nutritional values is an important source of food for the families' protein needs. There is no standard method for production. Standard and industrial production technologies of traditional cheeses must be necessarily

conserved. Local cheeses should be manufactured in high capacities using modern technologies. Also, there is vital importance in choosing the appropriate packaging material for the preservation of its characteristics and immediate packaging following their production. This study showed that cheeses ripened in 100% nitrogen (N) and 80% nitrogen + 20% carbondioxide (NC) maintained their properties during the storage period. In the further studies, Sepet cheese and other similar regional cheeses should be scientifically researched and found the methods for production to gain standard products complying food safety for public.

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REFERENCES

1. Turkoglu H, Ceylan ZG, Dayisoylu KS: The microbiology and chemical quality of orgu cheese produced in Turkey. *Pak J Nutr,* 2, 92-94, 2003. DOI: 10.3923/pjn.2003.92.94

2. Hayaloglu AA, Fox FP, Guven M, Cakmakci S: Cheeses of Turkey: 1. Varieties ripened in goat-skin bags. *Lait*, 87, 79-95, 2007. DOI: 10.1051/lait:2007006

3. Kamber U: The traditional cheeses of Turkey: The Aegean Region. *Food Rev Int,* 24, 39-61, 2008. DOI: 10.1080/87559120701762195

4. Karakas R, Korukoglu M: Geleneksel bir peynirimiz: Sepet peynirinin kimyasal ve mikrobiyolojik özellikleri. *Gıda* 31 (3): 169-172, 2006.

5. Unsal A: Süt uyuyunca "Türkiye Peynirleri". 6. Baskı, 151-153, Yapı Kredi Yayınları, İstanbul, 1997.

6. Henning DR, Bear RJ, Hassan AN, Dave R: Major in concentrated and dry milk products, cheese and milk fat-based spreads. *J Dairy Sci,* 89, 1179-1188, 2006. DOI: 10.3168/jds.S0022-0302(06)72187-7

7. Singh P, Wani AA, Karim AA, Langowski HC: The use of carbon dioxide in the processing and packaging of milk and dairy products: A review. *Int J Dairy Technol,* 65, 161-176, 2011. DOI: 10.1111/j.1471-0307.2011.00744.x

8. Rodriguez-Aguilera R, Oliveira JC, Montanez JC, Mahajan PV: Effect of modified atmosphere packaging on quality factors and shelf life of surface mould ripened cheese: Part I - Constant temperature. *LWT-Food Sci Technol*, 44, 330-336, 2011. DOI: 10.1016/j.lwt.2010.06.015

9. Rodriguez-Aguilera R, Oliveira JC, Montanez JC, Mahajan PV: Effect of modified atmosphere packaging on quality factors and shelf life of surface mould ripened cheese: part II varying storage temperature. *LWT-Food Sci Technol*, 44:337–342, 2011b. DOI: 10.1016/j.lwt.2010.06.014

10. Pretti S, Wani AA, Karim AA, Horst-Christian L: The use of carbon dioxide in the processing and packaging of milk and dairy products: A review. *Int J Dairy Technol,* 65, 161-177, 2011. DOI: 10.1111/j.1471-0307.2011.00744.x

11. Demir P, Öksüztepe G, İncili GK, İlhak Oİ: Vakum paketli Şavak tulum peynirlerinde potasyum sorbatın kullanımı. *Kafkas Univ Vet Fak Derg*, 23-30, 2017. DOI: 10.9775/kvfd.2016.15707

12. Oğuzhan P, Angiş S: Effect of salting and packaging treatments on fresh rainbow trout *(Oncorhynchus mykiss)* fillets during storage at refrigerator temperatures. *Kafkas Univ Vet Fak Derg* 18, 443-448, 2012. DOI: 10.9775/kvfd.2011.5633

13. Ercan, D: Quality Characteristic of Traditional Sepet Cheese. The Graduate School of Engineering and Sciences of İzmir Institute of Technology, *Phd Thesis*, Izmir, Turkey, 2009.

14. Renner E: Milchpraktikum Skriptum zu den Übüngen. pp.76, Jestus Liebig Universitat, Giesen, Germany, 1993.

15. AOAC: Official Methods and Recommended Practices.1996-1997 edn., Am, 3-96, Association of Official Analytical Chemists, Arlington, 1997.

16. Ergonul B, Ergonul PG, Seckin AK: Chemical and textural attributes of Hellim (Halloumi) cheese marketed in Turkey. *Mljekarstvo*, 61 (2): 168-174, 2011.

17. Mucchetti G, Remagni CM, Ghiglıetti R, Locci F, Barzaghi S, Francolino S, Perrone A, Rubiloni A, Campo P, Gatti M, Carminati D: Influence of cheese-making technology on composition and microbiological characteristics of Vastedda cheese. *Food Control,* 19, 119-125, 2008. DOI: 10.1016/j.foodcont.2007.02.011

18. Dolci P, Alessandria V, Rantsiou K, Rolle L, Zeppa G, Cocolin L: Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. *Int J Food Microbiol*, 122, 302-311, 2007. DOI: 10.1016/j.ijfoodmicro.2007.12.018

19. Vítová E, Loupancová B, Zemanová J, Štoudková H, Březina PL, Babák L: Solid-phase microextraction for analysis of mould cheese aroma. *Czech J Food Sci,* 24 (6): 268-274, 2006.

20. Liu, S-Q, Holland R, Crow VL: Esters and their biosynthesis in fermented dairy products: A review. *Int Dairy J,* 14, 923-945, 2004. DOI: 10.1016/j.idairyj.2004.02.010

21. Frank DC, Owen CM, Patterson J: Solid phase microextraction (SPME) combined with gas-chromatography and olfactometry-mass spectrometry for characterization of cheese aroma compounds. *Food Sci Technol-LEB*, 37, 139-154, 2004. DOI: 10.1016/S0023-6438(03)00144-0

22. Liu S-Q, Holland R, Crow VL: The potential of dairy lactic acid bacteria to metabolise amino acids via non-transaminating reactions and endogenous transamination. *Int J Food Microbiol,* 86, 257-269, 2003. DOI: 10.1016/S0168-1605(03)00040-0

23. Buyukyoruk, S: Isolation of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains from traditionally manufactured Izmir Tulum Cheese and biochemical and PCR identification. *PhD Thesis*, Uludag Univ., Inst. Health Sci., 2007.

24. Trobetas A, Badeka A, Kontominas MG: Light-induced changes in grated Graviera hard cheese packaged under modified atmospheres. *Int Dairy,* 18, 1133-1139, 2008. DOI: 10.1016/j.idairyj.2008.06.002

25. Temiz H: Effect of modified atmosphere packaging on characteristics of sliced Kashar Cheese. *J Food Process Pres* 34, 926-943, 2010. DOI: 10.1111/j.1745-4549.2009.00431.x

26. Ercan D, Korel F, Orsahin H: Microbiological quality of artisanal Sepet Cheese. *Int J Dairy Technol,* 67, 384-393, 2014. DOI: 10.1111/1471-0307.12125

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Research Article

Effects of Activated Lactoperoxidase System on Microbiological Quality of Raw Milk

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Abstract

The poor microbiological quality of raw cow milk in Turkey is a major concern. It has been speculated that less activation of naturally present lactoperoxidase system in the milk is the reason for its poor microbiological quality. Hence, the objective of this study was to investigate the effects of activation of the lactoperoxidase (LP) system on microbiological quality of the raw milk. The milk samples collected from a dairy farm were analyzed in the laboratory by dividing into two equal parts as activated (experimental) and control group. The experimental group was activated by treatment with equal concentration of sodium thiocyanate and hydrogen peroxide (20:20 mg/kg) whereas the control sample remained unactivated. All samples were stored at 4°C during 12 h. The microbial load in all the samples was quantitatively determined at 0, 3, 6, 9 and 12 h. The quantitative changes in each microbial species in both growth were recorded and statistically analyzed. The initial count of total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae and yeast number were 7.10, 5.14, 6.42, 5.93 and 4.31 log cfu/mL, respectively, and at the end of 3 h the counts were 0.43, 2.23, 1.09, 0.93 and 0.37 log cfu/mL, respectively, were lower than controls. Significant (P<0.05) differences were observed for microbial count of activated and control samples except in case of lactic acid bacteria. The results of this study indicate that the addition of thiocyanate and hydrogen peroxidase to the milk activated lactoperoxidase enzyme already present in the milk and slowed down the microbiological growth, especially of the reducing proteolytic *Pseudomonas* spp. On comparison, the results for total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae and yeast were statistically significant (P<0.05) and no significant change was observed in case of lactic acid bacteria.

Keywords: *Lactoperoxidase system, Thiocyanate, Milk quality, Microbiology*

Laktoperoksidaz Sistem Aktivasyonunun Çiğ Sütün Mikrobiyolojik Kalitesi Üzerine Etkileri

Özet

Türkiye'de çiğ inek sütünün mikrobiyolojik kalitesinin düşük olması, bir sorun olarak önemini korumaktadır. Sütte doğal olarak bulunan laktoperoksidaz sistemin yetersiz aktivasyonunun düşük mikrobiyolojik kalitenin nedeni olduğu ileri sürülmektedir. Bu nedenle, bu çalışmanın amacı, çiğ sütün mikrobiyolojik kalitesi üzerine laktoperoksidaz (LP) sisteminin aktivasyonu etkisini araştırmaktır. Bir mandıradan toplanan süt örnekleri aktive edilmiş (deneysel) ve kontrol grubu olarak iki eşit parçaya ayrılmış ve laboratuvarda analiz edilmiştir. Aktive edilmiş grubta, süt örnekleri eşit konsantrasyonda (20:20 mg/kg) sodyum tiyosiyanat ve hidrojen peroksit ile muamele edilmiş, kontrol numunesine ise aktivasyon işlemi yapılmamıştır. Tüm örnekler 4°C'de 12 saat süresince muhafaza edilmiştir. Bütün örneklerde toplam mezofilik aerobik bakteri, psikrotrof bakteri, *Pseudomonas* spp., Enterobacteriaceae, laktik asit bakteri ve maya yükü, muhafazanın başlangıç, 3., 6., 9. ve 12. saatlerinde niceliksel olarak sayılmıştır. Soğuk muhafaza sırasında her türde meydana gelen niceliksel değişimler kaydedilmiş ve sonuçlar istatistiksel olarak analiz edilmiştir. Başlangıç toplam mezofilik aerobik bakteri, psikrotrof bakteri, *Pseudomonas* spp., Enterobacteriaceae ve maya yükü, sırasıyla 7.10, 5.14, 6.42, 5.93 ve 4.31 log kob/ml olarak belirlenmiştir. Soğuk muhafazanın 3. saatinde, toplam mezofilik aerobik bakteri, psikrotrof bakteri, *Pseudomonas* spp., Enterobacteriaceae ve maya sayıları, aktive edilmiş olan örneklerde, kontrol örneklerine göre, sırasıyla, 0.43, 2.23, 1.09, 0.93 and 0.37 log kob/mL daha düşük çıkmıştır. Aktive edilmiş ve kontrol örneklerinin mikrobiyal sayıları arasında, laktik asit bakterileri dışında, önemli farklılıklar (P<0.05) gözlenmiştir. Bu çalışmanın sonuçları, süte ilave edilen tiyosiyanat ve hidrojen peroksitin, sütte mevcut bulunan laktoperoksidaz enzimi ile birlikte çalışmasıyla, mikrobiyal gelişmeyi yavaşlattığını, özellikle proteolitik *Pseudomonas* türlerinde azalmaya sebep olduğunu göstermektedir. Sonuçlar karşılaştırıldığında, toplam mezofilik aerobik bakteri, psikrotrofik bakteri, *Pseudomonas* spp., Enterobacteriaceae ve maya sayılarındaki değişimler istatistiksel olarak önemli (P<0.05) bulunurken, laktik asit bakteri yükünde anlamlı bir değişiklik tespit edilmemiştir.

Anahtar sözcükler: *Laktoperoksidaz sistem, Tiyosiyanat, Süt kalitesi, Mikrobiyoloji*

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INTRODUCTION

Milk contains important nutrients, including proteins, carbonhydrates, unsaturated fats, minerals, and vitamins, hence it satisfies people's nutritional needs [1]. However, it is observed to be a food source, which likely exposes humans to major pathogenic microorganisms [2]. Some microorganisms present in the milk adversely affect the quality of milk and dairy products during process of collection, storage, and transportation [3]. This leads to food poisoning and causes economic losses [4]. Studies conducted in Turkey revealed that milk and dairy products are contaminated with enteric bacteria such as *Escherichia coli* [5]. Therefore, monitoring the bacterial load in milk is extremely important for maintaining the good quality of product and to ensure food safety and good human health.

In Turkey, the milk produced is of low microbiological quality, which is a serious issue. In the past decade, advanced production and processing technologies have been extensively used. However, studies related to the milk quality during its processing indicate that the microbiological quality of milk may be poor in Turkey and other countries [6,7].

Although, cold storage inhibits the growth of some microorganisms, like lactic acid (LA) bacteria, psychrotroph bacteria can cause souring of milk at low temperatures. Psychrotroph bacteria is a microflora with proteolytic and lipolytic enzyme activities and can grow dominantly well in cold conditions [8]. This situation leads to the growth of microorganisms, causing deterioration of the milk and dairy product.

Milk contains lactoperoxidase (LP) system a naturally occuring enzymatic system that inhibits the microbial growth in the milk. This system is effective against many gram-positive and gram-negative bacteria. Gram-negative bacteria are more sensitive to the lactoperoxidase system than the gram-positive ones [8]. The LP system consists of three major components, including lactoperoxidase enzyme, thiocyanate ion (SCN-) and hydrogen peroxide (H_2O_2). The LP system becomes completely active when these three components come together [9]. The enzyme in the presence of H_2O_2, catalyzes oxidation of thiocyanate (SCN-) to form an intermediate product hipothiocyanate, which exhibits bactericidal or bacteriostatic effect on the bacteria [10]. The formed intermediate product exerts antibacterial effect by oxidation of essential sulfydryl groups of the enzymes or proteins present in the bacteria. Although the milk proteins too have a small number of sulfydryl groups, however these are not oxidized by hypothiocyanate [3].

Depending both on the type of bacteria and animal characteristics, the lactoperoxidase enzyme is naturally present in the milk obtained from all the mammals [8]. Hydrogen peroxide is formed by lactic acid bacteria of the milk microflora [11]. The amount of thiocyanate may change depending on several factors such as species, genus and lactation period of the animals. Especially, the amounts of thiocyanate in milk depend on the feeds consumed by the dairy cows [12]. Nowadays, naturally-feeding husbandry produce milk with significantly less amounts of thiocyanate than the roughage-feeding cows. Thus, the decrease in the amounts of thiocyanate in milk, may cause insufficient activation of the lactoperoxidase system [13]. Therefore, several researchers suggested that LP system could be sufficiently activated by externally adding thiocyanate to the raw milk to improve its microbiological quality [1,8,14]. Even Food and Agriculture Organization (FAO) and World Health Organization (WHO) recommend the use of lacto-peroxidase system to control the microbiological quality of raw milk in some regions with poor hygienic standards [13].

The objective of this study was to investigate the effects of activated lactoperoxidase system on the microbiological quality of raw milk sample collected from dairy farms. We determined the effect of LP system only on few microorganisms such as mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, and yeast.

MATERIAL and METHODS

Sample of Raw Cow Milk

A total of 15 L of fresh raw cow milk was collected in the morning from dairy farms located in Çatalca, İstanbul. The collected stock was equally distributed in sterile sampling boxes in a thermobox at 4°C. The samples were brought to the laboratory on the same day and quickly subjected to the microbiological analysis.

Activation of the Lactoperoxidase System

One liter of raw cow milk was divided equally into two equal parts, each of 500 mL, under aseptic conditions. One part was used as a control, whereas the other part was subjected to the activation process. For activation of lactoperoxidase system, 20 mg/kg NaSCN (FlukaAnalytical 71938-250G, St. Louis, USA) and 20 mg/kg H_2O_2 (Merck 1.07210.1000, Darmstadt, Germany) were added to the experimental fraction and the samples were stirred thoroughly and further stored at 4°C for 12 h. The milk samples were then microbiologically examined for the presence of microflora including total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, lactic acid bacteria (LAB) and yeast at different time intervals of 0, 3, 6, 9 and 12 h.

Microbiological Analysis

Each sample, 1 mL, was homogenized in 9 mL sterile physiological saline solution, pH 7.0 (Merck) according to

the Guidelines of ISO 6887-1 [15] by serial dilutions. Different microorganisms were grown on their specific selective media. Plate Count Agar (LAB149, Lancashire, UK) for total mesophilic aerobic bacteria [16] and psychrotroph bacteria [17], Pseudomonas Agar (Merck 1.07620.0500, Darmstadt, Germany) including C-F-C Selective Supplement (Merck 1.07627.0001, Darmstadt, Germany) for *Pseudomonas* spp.[18], Violet Red Bile Glucose Agar (LAB088, Lancashire, UK) for Enterobacteriaceae [19], Man-Rogosa-Sharpe Agar (MRS) (Merck 1.10660.0500, Darmstadt, Germany) for lactic acid bacteria [20] and Yeast Glucose Chloramphenicol Agar (Merck 1.16000.0500, Darmstadt, Germany) for yeast [21] were used. For growth of total mesophilic aerobic bacteria, psychrotroph bacteria, and lactic acid bacteria, 1 mL of each decimal serial dilutions was transferred onto the corresponding sterile plates, then appropriate media were poured into each plate and incubated at 30°C for 72 h, at 7°C for 5-7 d and at 37°C for 48 h, respectively. As for *Pseudomonas* spp., Enterobacteriaceae, and yeast, 0.1 mL of each dilutions were spread over the prepared plates and then incubated at 25°C for 48 h, at 37°C for 24 h and at 25°C for 5 d, respectively. After incubation, the grown colonies in the plates were counted and recorded as cfu/mL for each decimal serial dilution.

Statistical Analysis

All experiments were repeated thrice by conducting them on different days and the results were converted into logarithmic values. Statistical analyses were performed by SPSS ver. 22.0 (SPSS INC., USA). The comparison of the effects of lactoperoxidase system on microbiological quality in the experimental group was evaluated by one-way ANOVA ($P<0.05$). The statistical significance of differences between the group means was analyzed by Duncan's test.

RESULTS

The results in *Table 1* show the comparison between the bacterial count in raw cow milk sample in control and activated milk sample [treated with equal concentration of sodium thiocyanate and hydrogen peroxide (20:20 mg/kg)]. At 0 h, the initial counts of total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, lactic acid bacteria, and yeast were found to be 7.10, 5.14, 6.42, 5.93, 6.92 and 4.31 log cfu/mL, respectively, in both samples. At the end of 3 h of cold storage of the activated raw milk, the log-reduction measurements for total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, lactic acid bacteria, and yeast were found to be 0.50, 2.09, 1.04, 0.97, 0.39, and 0.45 log cfu/mL, respectively. On comparison, the results for total mesophilic aerobic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, and yeast were statistically significant ($P<0.05$) and no significant change was observed in case of lactic acid bacteria.

DISCUSSION

In this study, we investigated and compared the microbiological quality of the nonactivated control and lactoperoxidase activated raw milk at different time intervals. We found that certain bacteria in LP activated milk were decreased in count as compared to the control raw milk.

Table 1. *Microbial counts in control and LP activated raw milk during cold storage (log cfu/mL)*
Tablo 1. *Soğukta muhafaza sırasında kontrol ve LP aktive edilmiş sütlerdeki mikroorganizma sayıları (log kob/mL)*

Microorganism	Group	Storage Periods (hour)				
		0.	3.	6.	9.	12.
TMAB	CON	7.10 ± 0.23^{a}	7.03 ± 0.21^{a}	6.94 ± 0.16^{a}	7.00 ± 0.22^{a}	7.14 ± 0.19^{a}
	ACT	7.10 ± 0.23^{a}	6.60 ± 0.20^{b}	6.67 ± 0.20^{b}	6.66 ± 0.21^{b}	6.70 ± 0.26^{b}
PSI	CON	5.14 ± 0.13^{c}	5.28 ± 0.15^{c}	5.34 ± 0.12^{bc}	5.59 ± 0.14^{ab}	5.78 ± 0.15^{a}
	ACT	5.14 ± 0.13^{a}	3.05 ± 0.24^{c}	3.28 ± 0.26^{c}	3.44 ± 0.21^{bc}	3.71 ± 0.26^{b}
PSE	CON	6.42 ± 0.28^{a}	6.47 ± 0.33^{a}	6.52 ± 0.26^{a}	6.58 ± 0.30^{a}	6.67 ± 0.30^{a}
	ACT	6.42 ± 0.28^{a}	5.38 ± 0.47^{b}	5.34 ± 0.30^{b}	5.33 ± 0.26^{b}	5.42 ± 0.23^{b}
ENT	CON	5.93 ± 0.27^{a}	5.89 ± 0.32^{a}	6.00 ± 0.29^{a}	6.09 ± 0.28^{a}	6.14 ± 0.29^{a}
	ACT	5.93 ± 0.27^{a}	4.96 ± 0.24^{b}	4.84 ± 0.23^{b}	4.86 ± 0.23^{b}	4.80 ± 0.20^{b}
LAB	CON	6.92 ± 0.40^{a}	6.75 ± 0.23^{a}	6.78 ± 0.24^{a}	6.89 ± 0.24^{a}	6.98 ± 0.32^{a}
	ACT	6.92 ± 0.40^{a}	6.53 ± 0.20^{a}	6.39 ± 0.29^{a}	6.66 ± 0.17^{a}	6.45 ± 0.33^{a}
Yeast	CON	4.31 ± 0.19^{a}	4.23 ± 0.19^{a}	4.43 ± 0.16^{a}	4.34 ± 0.22^{a}	4.42 ± 0.24^{a}
	ACT	4.31 ± 0.19^{a}	3.86 ± 0.22^{b}	3.60 ± 0.20^{b}	3.87 ± 0.24^{b}	3.85 ± 0.27^{b}

^{abc} *The differences between the means shown with same superscript in the same row is not significant ($P>0.05$);* ***TMAB:*** *Total Mesophilic Aerobic Bacteria;* ***PSI:*** *Psycrotrophic Bacteria;* ***PSE:*** *Pseudomonas spp.;* ***ENT:*** *Enterobacteriaceae;* ***LAB:*** *Lactic Acid Bacteria;* ***CON:*** *Control Group;* ***ACT:*** *LP Activated (Experimental) Group*

The bacteria, which can grow in mesophilic and aerobic conditions and can be saprophytic or pathogenic, are one of the most important indicators of the microbiological quality of milk and milk products [6]. Our study pointed that in the raw milk activated with LP system, which was kept in cold storage at 4°C, there was a change in the total number of mesophilic aerobic bacteria at the end of 3 h. The initial total mesophilic aerobic bacteria count (at 0 h) of 7.10 log cfu/mL was decreased to 0.43 log cfu/mL at the end of 3 h and this difference was statistically significant ($P<0.05$). This change remained constant until the end of 12 h of cold storage. In contrast, no change was observed in the total number of mesophilic aerobic bacteria in the control-raw milk (non-activated) at the end of 3 h, and it remained at an average value of 7.10 log cfu/mL. Further, there were no significant changes in the control group (non-activated) during the 12 h cold storage. Thus, our results suggested that external addition of thiocyanate increased activation of LP system in the raw milk, which reduced the microbial count and increased the quality of milk. Similar results have also been reported by other studies, where the activation of lactoperoxidase system significantly reduced the total count of mesophilic aerobic bacteria in raw milk [1,8,22]. One of these studies, added different quantities of thiocyanate and hydrogen peroxide to the raw milk and performed microbiological analysis at 24 h and reported reduction between 26% and 45% in all three activated groups. While another study performed the activation of LP system using raw cow milk at ambient temperature [23,24], and reported a reduction of 0.23 log cfu/mL in the activated group and an increase of 0.84 log cfu/mL in the control group at 7 h.

Cold storage of raw milk and dairy products is a prerequisite for the dairy industry. This is also done at farms and in processing plants to reduce the deterioration caused by mesophilic microorganisms. However, it cannot prevent the spoilage of raw milk by psychrotroph microorganisms [25]. Our study obtained similar results for psychrotroph bacteria as were observed for mesophilic aerobic bacteria. We observed that their total counts in the LP activated raw milk were reduced nearly to 2 log cfu/mL in the first three h. Furthermore, same results were observed at 6 and 9 h and a tendency to rise in numbers was seen at 12 h of cold storage. No significant change was observed in the control-raw milk. The results indicate that activation of the lactoperoxidase system occurs within the first three h. A study reported that activation of the lactoperoxidase system in raw milk is quite effective against psychrotroph bacteria, since it increases the shelf life of cold stored milk for several days [26]. As reported by another study, the initial psychrotroph bacterial load in the raw cow milk was 5.5×10^{3}/mL, and in the activated group, it reduced to 2.6×10^{6}/mL on 6th day. While in the control group, the count remained the same until the end of the 2nd day [27]. The results of these studies indicate that activation of lactoperoxidase system showed both bactericidal and bacteriostatic effect on psychrotroph bacteria.

Pseudomonas species are the most common psychrotroph microorganisms that cause spoilage of raw milk. The most important features of these bacteria are their ability to grow in the cold refrigerator conditions [28]. Generally, *Pseudomonas* spp. are inhibited by the process of pasteurization. However, before the heat treatment, if they are present in high counts in the raw milk, they release heat resistant hydrolytic enzymes, which remain active and cause deterioration during storage of pasteurized product [29]. Especially, when *Pseudomonas* spp. produce a protease enzyme that breaks down the milk protein casein imparting a grayish color and bitter taste to the milk. Furthermore, the lipases produced by these bacteria hydrolyze milk fat to release low molecular weight fatty acids, which give bitter taste and sour and soapy flavor to raw milk [30]. In our study, during cold storage at 4°C the initial *Pseudomonas* spp. load in raw milk was noted as 6.42 log cfu/mL, while there was a slight increase in the control group, it reached 6.67 log cfu/mL at 12 h. Lactoperoxidase system activation caused 1.04 log cfu/mL reduction in *Pseudomonas* spp. count in milk within the first three h and later remained constant (with no increase) in the cold storage. According to a study, lactoperoxidase system activation shows both bactericidal and bacteriostatic effect on *Pseudomonas* spp. in milk [31]. In agreement with our study, Zapico *et al.*[32] reported that the raw goat milk after LP system activation showed 1.69 log cfu/mL reduction in *Pseudomonas* spp. count during the first 24 h. In a study by Saad *et al.*[33], after 2 h of the treatment, there was no reduction in *Pseudomonas* spp. count in the activated group. However, significant differences were observed when compared with the control group. Thus, our results differed from those of Saad *et al.*[33], since they reported that the activation of lactoperoxidase system has a bactericidal effect on *Pseudomonas* spp.

Enterobacteria virtually infect human and all animals as well as the environment all over the world. Within this family, *E. coli* is increasingly becoming a pre dominant form worldwide [34]. Enterobacteriaceae is a family of gram-negative, non-spore forming bacteria and is one of the most important bacterial group known to humankind [35]. The most common pathogens that belong to this family are the coliforms [36]. Coliforms are also known as hygiene indicators, which produce acid and gas and disrupt milk proteins by fermenting lactose to cause rapid deterioration of milk [37]. In our study, we examined the effect of activated lactoperoxidase system on the Enterobacteriaceae load in milk and compared it with the nonactivated raw milk. Initially, the number of Enterobacteriaceae was determined as 5.93 log cfu/mL and further no significant increase was observed in the control group during cold storage. While Enterobacteriaceae count in the lactoperoxidase system activated raw milk was reduced to 0.97 cfu/mL log in the first three h and this reduction was found to be statistically significant ($P<0.05$). During subsequent storage period, there was no significant change in the count. Similarly,

Erginkaya *et al.*[38] reported that the activation of the LP system caused a decrease in the Enterobacteriaceae load at 4℃. Likewise, Niguse and Seifu [24] also reported that the coliform count, in the activated milk sample was 1.73 log cfu/mL lower than in the control milk sample up to seven h of storage at ambient temperature.

Lactic acid bacteria play an important role in the development of desirable characteristics of fermented dairy products; and they are intentionally added to milk for production of these products [39]. However, they may also cause deterioration in the raw milk and certain dairy products [40]. They are particularly responsible for the usual souring of milk [41]. In our study, lactoperoxidase system activation did not show any significant impact on the lactic acid bacteria load *(Table 1)*. Odabaşı *et al.*[42], in their study regarding preparation of white cheese, reported that the LP system slows down the growth of lactic acid bacteria. Similarly, Ndambi *et al.*[43], reported that during incubation lactoperoxidase system activation reduced lactic acid formation by 12.50% in yogurt as compared to the control group. Thus indicating that LP system had bacteriostatic effect on the lactic acid bacteria. In our study, due to the short storage time, we could not observe the bacteriostatic effect of lactoperoxidase system on growth of these bacteria.

Yeasts are an essential component of the microflora of lactic acid production and important cause of spoilage of yogurt and fermented milk products, where the low pH provides a suitable environment for their growth [28]. High yeast load may indicate a lack of hygiene during the process of milking and storage [44]. We determined the effect of lactoperoxidase system on the yeast in the raw cow milk. During cold storage, initial yeast load was 4.31 log cfu/mL and no significant change was observed in the control group throughout the storage period. After three h, the lactoperoxidase system activated raw milk stored in cold showed a count of 3.86 log cfu/mL with 0.45 log reduction ($P<0.05$). Atamer *et al.*[45] reported that LP system has inhibitory effect on the yeast-mold growth. Another study on the raw sheep milk revealed that the yeast and mold load of 0.71 log cfu/mL in activated samples were low than control samples at 7°C at 6 h of activation [8].

Our study revealed that the Lactoperoxidase system activation led to a significant reduction in the number of aerobic mesophilic bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae, and yeast. This reduction occurred within the first three h. The length of this time depends on intermediate products formed as a result of oxidation of thiocyanate by the presence of equal concentration of hydrogen peroxide. The time may be extended by increasing the concentration of hydrogen peroxide and thiocyanate. However, this may slow down the activity of the starter culture in further processed products [42].

According to Jooyandeh *et al.*[46] lactoperoxidase system activation is more effective against gram negative bacteria than gram positive bacteria because gram positive bacterial cell wall is more resistant to hypothiocyanite ions.

In general, hydrogen peroxide can alone be used for the preservation of milk. However, in order to show the antibacterial effect, hydrogen peroxide should be incorporated up to 800 mg/kg in the raw milk. This amount is much higher than the amount used for activation of lactoperoxidase system [47]. Since we used very low amount, the reduction in bacterial load reported of our study cannot be correctly connected to hydrogen peroxide.

In summary, we found that external addition of thiocyanate and hydrogen peroxide to the raw cow milk to improve activation of the Lactoperoxidase system, a natural antimicrobial defense system, reduced the initial microbial load and slowed down the growth of aerobic mesophilic total bacteria, psychrotroph bacteria, *Pseudomonas* spp., Enterobacteriaceae and yeast in the first three h after the treatment. In future, feeding the cow with thiocyanate rich feed can be tried, which in turn will increase the thiocyanate content of raw milk and will improve its microbiological quality.

Conflicts of Interest

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

REFERENCES

1. Hamid OIA, Musa ZAB: Effect of different levels of sodium thiocyanate and percarbonate for activation of lactoperoxidase on the keeping quality of raw milk. *J Adv Sci Res*, 4, 27-30, 2013.

2. Özpınar H, Tekiner İH, Karaman O, Kurt Y: Investigation of *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) in fecal and bulk milk samples from dairy farms in European Thrace Region of Turkey. *Kafkas Univ Vet Fak Derg*, 21, 247-252, 2015. DOI: 10.9775/kvfd.2014.12212

3. Kassa F, Yilma Z, Assefa G, Bekele T, Gojam Y, Nebiyu R, Kassa B: Evaluation of lactoperoxidase system as raw milk preservative at different storage temperature conditions in The Central Highlands of Ethiopia. *Livestock Res Rural Dev*, 25 (4): 2013.

4. Akıllı A, Atıl H, Kesenkaş H: Çiğ süt kalite değerlendirmesinde bulanık mantık yaklaşımı. *Kafkas Univ Vet Fak Derg,* 20, 223-229, 2014. DOI: 10.9775/kvfd.2013.9894

5. Özpınar H, Turan B, Tekiner İH, Tezmen G, Gökçe İ, Akıneden Ö: Evaluation of pathogenic *Escherichia coli* occurrence in vegetable samples from district bazaars of İstanbul using real time PCR. *Lett Appl Microbiol*, 57, 362-367, 2013. DOI: 10.1111/lam.12122

6. Kesenkaş H, Akbulut N: İzmir ilinde satılan sokak sütleri ile orta ve büyük ölçekli çiftliklerde üretilen sütlerin özelliklerinin belirlenmesi. *Ege Üniv Ziraat Fak Derg*, 47, 161-169, 2010.

7. Tolosa T, Verbeke J, Piepers S, Tefera M, Getachew Y, Supre K, DeVliegher S: Milk production, quality, and consumption in Jimma (Ethiopia): Facts and producers', retailers', and consumers' perspectives. *Prev Vet Med*, 124, 9-14, 2016. DOI: 10.1016/j.prevetmed.2015.12.016

8. Saad MSA, El Zubeir IEM, Elseed AMAF: Effect of lactoperoxidase enzyme system and storage temperature on the keeping quality of sheep milk. *Livestock Res Rural Dev*, 25 (6): 2013.

9. Campbell RE, Kang EJ, Bastian E, Drake MA: The use of lactoperoxidase for the bleaching of fluid whey. *J Dairy Sci,* 95, 2882-2890, 2012. DOI: 10.3168/jds.2011-5166

10. Panwar H: Biologically active components of human and bovine milk as potent antimicrobial agents. *J Innov Biol*, 1, 97-104, 2014.

11. Gaya P, Medina M, Nunez M: Effect of the lactoperoxidase system on *Listeria monocytogenes* behavior in raw milk at refrigeration temperatures. *Appl Environ Microbiol*, 57, 3355-3360, 1991.

12. Seifu E, Buys EM, Donkin EF: Significance of the lactoperoxidase system in the dairy industry and its potential applications: A review. *Trends Food Sci Technol*, 16, 137-154, 2005. DOI: 10.1016/j.tifs.2004.11.002

13. Codex Alimentarius: Milk and milk products first edition, guidelines for the preservation of raw milk by use of the lactoperoxidase system. CAC/GL 13-1991, 183-189, 2007.

14. Tayefi-Nasrabadi H, Hoseinpour-fayzi MA, Mohasseli M: Effect of heat treatment on lactoperoxidase activity in camel milk: A comparison with bovine lactoperoxidase. *Small Ruminant Res,* 99, 187-190, 2011. DOI: 10.1016/j.smallrumres.2011.04.007

15. ISO 6887-1: Preperation of test samples, initialsuspension and decimaldilutions for microbial examination. Part-1 General rules for preparation of dilutions for microbial examination. Geneva, Switzerland, 1999.

16. ISO 4833: Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms - Colony-count technique at 30°C. Geneva, Switzerland, 2003.

17. Harrigan WF: Laboratory Methods in Food Microbiology. 3rd edn., 534, Academic Press, London, 1998.

18. ISO 13720: Meat and meat products - Enumeration of presumptive *Pseudomonas* spp. Geneva, Switzerland, 2010.

19. ISO 21528-2: Microbiology of food and animal feding stuffs - Horizontal method for the detection and enumeration of *Enterobacteriaceae*. Part 2, Colony-count technique. Geneva, Switzerland, 2004.

20. ISO 15214: Microbiology of food and animal feedingstuffs - Horizontal method for enumeration of Mesophilic Lactic Acid Bacteria. Geneva, Switzerland, 1998.

21. ISO 7954: General guidance for enumeration of yeasts and moulds. Colony-count technique at 25°C. Geneva, Switzerland, 1988.

22. Musa ZA, Hamid OIA: Effect of Lactoperoxidase activation on the keeping quality of raw milk kept at refrigeration temperature. *Am J Res Commun*, 1 (2): 22-32, 2013.

23. Magdoub MNI, El-Gendy MH, Kamel YM, El-Nawawy MA: The effect of lactoperoxidase system on enhancing the microbiological quality of goat milk and yoghurt. ISAH-2007 Tartu, Estonia.

24. Nigussie H, Seifu E: Effect of the lactoperoxidase system and container smoking on the microbial quality of cows' milk produced in Kombolcha woreda, eastern Ethiopia. *Livestock Res Rural Dev,* 19 (10): 2007.

25. Correa APF, Daroit DJ, Velho RV, Brandelli A: Hydrolytic potential of A psychrotroph *Pseudomonas* isolated from refrigerated raw milk. *Braz J Microbiol,* 42, 1479-1484, 2011. DOI: 10.1590/S1517-83822011000400034

26. Ben Moussa O, Mankai M, Ben Fekih A, Hassouna M: Effect of the lactoperoxidase system on proteolysis and physicochemical changes in ultra high temperature milk during storage. *Afr J Biotechnol*, 12, 2041-2050, 2013. DOI: 10.5897/AJB2012.3033

27. Abdallah MIM: A new trial for preservation of cow's milk at 30°C and 5°C by activation of the natural lactoperoxidase system. *The 4th International Science Conference*. The Role of Veterinary Medicine for Community, Application of Biotechnology in Veterinary Medicine, Mansoura, Egypt, 2005.

28. Ledenbach LH, Marshall RT: Microbiological spoilage of dairy products. **In,** Sperber WH, Doyle MP (Eds.): Compendium of the Microbiological Spoilage of Foods and Beverages. 41-67, Springer Science+Business Media, New York, 2009.

29. Rajmohan S, Dodd CER, Waites WM: Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. *J Appl Microbiol*, 93, 205-213, 2002. DOI: 10.1046/j.1365-2672.2002.01674.x

30. Çaylak-Taş T, Yuvalı Çelik G, Onbaşılı D: Investigation of proteolytic, lipolytic activities and antibiotics susceptibility of some *Pseudomonas* bacteria isolated from raw milks. *Turk Hij Den Biyol Derg*, 70, 147-152, 2013. DOI: 10.5505/TurkHijyen.2013.42275

31. Björck L, Rosen CG, Marshall V, Reiter B: Antibacterial activity of the lactoperoxidase system in milk against *Pseudomonas* and other gram-negative bacteria. *Appl Microbiol,* 30, 199-204, 1975.

32. Zapico P, Gaya P, Nuñez M, Medina M: Activity of goats' milk lactoperoxidase system on *Pseudomonas fluorescens* and *Escherichia coli* at refrigeration temperatures. *J Food Prot*, 58, 1136-1138, 1995.

33. Saad AH: Activation of milk lactoperoxidase system for controlling *Pseudomonas* in cow's milk. *Int J Dairy Sci*, 3, 131-136, 2008.

34. Tekiner İH, Özpınar H: Occurrence and characteristics of extended spectrum beta-lactamases-producing *Enterobacteriaceae* from foods of animal origin. *Braz J Microbiol*, 47, 444-451, 2016. DOI: 10.1016/j.bjm.2015.11.034

35. Baylis C, Uyttendaele M, Joosten H, Davies A: The *Enterobacteriaceae* and their significance to the food industry. *Inter Life Sci Insti*, 1, 4-52, 2011.

36. El-Ziney MG, Al-Turki AI: Microbiological quality and safety assessment of camel milk *(Camelus Dromedaries)* in Saudi Arabia (Qassim Region). *App Ecology and Environ Res*, 5, 115-122, 2007.

37. Lu M, Shiau Y, Wong J, Lin R, Kravis H, Blackmon T, Pakzad T, Jen T, Cheng A, Chang J, Ong E, Sarfaraz N, Wang NS: Milk spoilage: Methods and practices of detecting milk quality. *Food Nutr Sci*, 4, 113-123, 2013. DOI: 10.4236/fns.2013.47A014

38. Erginkaya Z, Güven M, Karaca OB: Laktoperoksidaz sistemin aktivasyonuyla ve soğutularak korunan sütlerin mikrobiyolojik özellikleri. *Gıda*, 26, 367-373, 2001.

39. Daba H, Saidi S: Detection of bacteriocin-producing lactic acid bacteria from milk in various farms in north-east Algeria by a new procedure. *Agronomy Res,* 13, 907-918, 2015.

40. Florou-Paneri P, Christaki E, Bonos E: Lactic Acid Bacteria-R&D for Food, Health and Livestock Purposes. Chapter 25: Lactic acid bacteria as source of functional ingredients. 589-614, 2013. DOI: 10.5772/47766

41. Olatunji EA, Jubril A E, Okpu EO, Olafadehan OA, Ijah UJ, Njidda A: Bacterial assessment and quality analysis of raw milk sold in gwagwalada area Council of the Federal Capital Territory (FCT) Abuja, Nigeria. *J Food Sci Qual Manage*, 7, 1-4, 2012.

42. Odabaşı S, Gürsoy A, Çimer A, Atamer M: Laktoperoksidaz/Tiyosiyanat/Hidrojen Peroksit sisteminin aktivasyonu ile korunmuş sütlerden üretilen beyaz peynirlerin bazı kalite özellikleri üzerine araştırmalar. *Gıda,* 24, 327-335, 1999.

43. Ndambi OA, Kamga PB, Imele H, Mendi SD, Fonteh FA: Effects of milk preservation using the lactoperoxidase system on processed yogurt and cheese quality. *Afr J Food Agric Nutr Dev*, 8, 358-374, 2008. DOI: 10.4314/ajfand.v8i3.19198

44. Spanamberg A, Ramos JP, Leoncini O, Alves SH, Valente P: High frequency of potentially pathogenic yeast species in goat's raw milk and creamed cheese in Southern Brazil. *Acta Sci Vet*, 37, 133-141, 2009.

45. Atamer M, Yamaner N, Odabaşı S, Tamuçay B, Çimer A: Laktoperoksidaz/Tiyosiyanat/Hidrojen Peroksit (LP) sisteminin aktivasyonuyla korunmuş sütler ile bunlardan üretilen teleme ve kaşar peynirlerinin mikrobiyolojik özellikleri. *Gıda*, 22, 317-325, 1997.

46. Jooyandeh H, Aberoumand A, Nasehi B: Application of lactoperoxidase system in fish and food products: A review. *American-Eurasian J Agric Environ Sci,* 10, 89-96, 2011.

47. Fernandez O, Marrero E, Capdevila JZ: Safety considerations on lactoperoxidase system use for milk preservation. *Rev Salud Anim*, 27, 186-189, 2005.

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Research Article

Histological and Immunological Changes in *Uterus* During the Different Reproductive Stages at Californian Rabbit *(Oryctolagus cuniculus)*

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Abstract

Rabbit is the third most commonly used animal model in different fields of scientific research, such as reproductive biology, fertility and embryo transfer studies, and immunology. This animal species, often used in antibodies production, has minority of scientific records about the immunological status of its reproductive organs. The aim of this study was to find histological and immunological changes in rabbit female reproductive tract during different reproductive stages. The study was carried out on female rabbits, divided in three groups, according to the following stages of reproductive cycle: Estrous, ovulation and pregnancy. Histological and immunohistochemical stains for T- and B-cells were performed on tissue samples of cornu uteri and cervix. T lymphocytes were predominant in all anatomical parts of the uterus, in all stages of the cycle. The highest number of those cells was recorded at estrous, while the lowest was recorded at pregnancy. Cervix expressed more immunological activity than cornu uteri. The distribution and the number of immune positive cells in the rabbit female reproductive tract depend on its hormonal status.

Keywords: *Rabbit, Uterus, Cervix, B-cell, T-cells*

Kaliforniya Tavşanının *(Oryctolagus cuniculus)* Farklı Üreme Dönemlerinde Uterustaki Histolojik ve İmmunolojik Değişiklikler

Özet

Tavşan; üreme biyolojisi, üretkenlik ve embriyo transferi çalışmaları ve immünoloji gibi farklı alanlardaki bilimsel çalışmalarda en sıklıkla kullanılan hayvan modelleri arasında üçüncü sırada yer almaktadır. Antikor üretiminde kullanılan bu hayvanların üreme organlarının immünolojik durumu hakkındaki raporlar oldukça kısıtlıdır. Bu çalışmanın amacı farklı üreme devrelerinde bulunan dişi tavşanların üreme organlarında şekillenen histolojik ve immünolojik değişiklikleri belirlemektir. Dişi tavşanlar östrus, ovulasyon ve gebelik olmak üzere üç gruba ayrıldı. Cornu uteri ve serviks dokularında histolojik incelemeler ile T ve B-hücreleri için immunohistokimyasal boyamalar yapıldı. T lenfositler uterusun tüm anatomik bölümlerinde ve tüm dönemlerde baskın hücre olarak belirlendi. Bu hücreler en yüksek oranda östrusta ve en düşük gebelikte tespit edildi. Servikste cornu uteriden daha fazla immünolojik aktivite belirlendi. Immun pozitif hücrelerin dağılım ve sayısının dişi tavşanların üreme organlarında hormon durumu ile ilişkili olduğu sonucuna varıldı.

Anahtar sözcükler: *Tavşan, Uterus, Serviks, B-hücre, T-hücre*

INTRODUCTION

Although the rabbit is one of the most commonly used models for scientific researches in fundamental sciences and medicine [1], more immunological studies about local immune system in the female reproductive tract (FRT) have been taken on the rodent model. Even though the lack of available reagents for characterization of immune cells in the rabbit FRT could be the reason for choosing rodents for investigation, the similarity of Order Lagomorpha with

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humans has often been the crucial factor for using the rabbit model. This similarity is most easily distinguishable in very similar gene sequences [2] and in embryo and fetal differentiation. Limited information about changes in local immunity of the rabbit FRT during different reproductive stages was another reason to carry out this investigation using the rabbit model. Rabbit possesses *uterus duplex*, with two separate *cervices* and two *canalis cervicis uteri* in each *cervix* [3,4] with elongated uterine horns [5]. The topographic position of uterus is approximately at the level of IV to VII lumbal vertebra, for *cornu uteri*, and for *cervix* along to I sacral vertebra, at the lining of abdominal to pelvic cavity, depending of reproductive stage and hormonal status [6-8]. Receptive doe exhibits a higher estrogen level and a higher number of large ovarian follicles [5], resulting in clinical signs of estrous: reddish and edematous vulva and *lordosis* [9]. As a reflexively ovulating species, rabbit preovulatory surge of luteinizing hormone (LH) is induced by sensory and neuroendocrine stimuli. LH concentrations began to raise 30 min *post coitum* [10]. Immediate release of LH from the anterior pituitary reaches the maximum after 1 h, which results in ovulation [11]. After the ovulation in rabbits has been induced [1,12,13], it starts 10-12 h later [14], and until the *corpora lutea* are formed, estrogen is dominant ovarian hormone [15]. Epithelial lining of cervical mucosa is a simple columnar epithelium with ciliated cells and goblet cells. Cervical mucosa forms the primary, secondary and tertiary folds, which do not change their appearance during the reproductive stages. Ciliated cells show small apical microvilli, between the cilia, while goblet cells are mucous secreting cells, with large membrane bound vesicles and dense cytoplasm [16]. Columnar epithelium in the upper FRT has strong network of tight junction, but high levels of estrogens lead to its relaxation and increase the permeability of the epithelium and possible degradation of its barrier function [10,17]. That morphological event creates higher susceptibility to infection and requires tissue and immune remodeling. Estrogen receptors in lymphocytes mediate estrogen induced proliferation of T lymphocytes and NK cells, but not B lymphocytes [18]. Endometrial growth is significant for this proliferative phase, but maximal endometrium development is evident in progestational phase. *Lamina propria mucosa* consists of a connective tissue network with fever glands and vascular and lymphoid vessels. The endocervical glands produce mucus, which make a physical barrier to pathogens and potential ascending infection [19]. The products of their secretory activity are dissimilar trough the stage of reproductive cycle: estrogenic mucus is thin and watery, with low viscosity, while progestational mucus is thick and viscous [17]. This different consistence of the mucus facilitates sperm movement to the upper FRT, or impedes the movement of material from low to upper part of this track, on the other hand. Morphological feature of uterine glands also differs through the stage [20-22]. At active, secretory state uterine glands assume highly coiled form. Lumen of the glands increase and presence of glycoprotein secretory products indicates preparation for expected fertilization and implantation. Progesterone is an immunomodulatory molecule, which activity is connected with activation induced appearance of progesterone binding sites in the lymphocytes [23]. Unique immune cell phenotypes in FRT are different from those at other mucosal sites; even they are a part of mucosal immune system. Immune system throughout FRT is regulated by sex hormones, which directly or indirectly modulate recruitment and activation of lymphocytes [24]. The level of ovarian hormones significantly influences the distribution and the number of immune cells in FRT of rodents [25], rabbits [26] and humans [19,27,28].

The aim of this study was to investigate the histological changes and the changes in localization and number of immune cells in rabbit female reproductive tract following different reproductive stages.

MATERIAL and METHODS

Our study was carried out on female Californian rabbits aged 4-6 months and weighing 3500-4200 g. Animals were kept in an individual cage system, under the same environmental conditions, with free access to water and feeding *ad libitum*. Three groups with five animals in each were formed. Unmated female rabbits with clinical signs of estrous (red-colored vulva and *lordosis*) were selected as group I. Rabbits with clinical signs of estrous which were mated in order to induce ovulation formed group II and were sacrificed 12 h after mating. Group III consisted of gravid rabbits that were sacrificed on day 15 of gravidity, where day of mating was counted as day 0 and the next day as the 1st day of gravidity. Tissue samples of ovaries, *cornu uteri* and *cervix* (each side of organs) were collected, fixed in neutral 10% buffered formalin solution, embedded in paraffin, following the standard procedure, sectioned at 5 µm thick (10 transverse sections/organ) and stained with standard H&E technique. The phase of reproductive cycle was confirmed according to the ovarian morphology, as estral phase, post-ovulation phase and gravidity *(Fig. 1)*.

Immunohistochemistry was performed on serial cryosections of the lymph node, which were used as the positive control, while stained *cervix* and uterine horns tissue samples were used for detecting immune cells. Tissue samples, frozen in liquid nitrogen, were cut at cryotome (Leica), in transverse cryosections of 5 µm, 10 section/organ, at -24°C. After that, the samples were air dried for 12-16 h, at 21°C, fixed in cold acetone for 10 min and stored at -20°C until further use. For detection of T- and B-cells immunoperoxidase technique was used. T-cells were detected using mouse anti-rabbit T-lymphocytes monoclonal antibody *(Mouse anti Rabbit T Lymphocytes, clon KEN-5+, Catalog Number: MCA800G, AbD Serotec, A Bio-rad Company, Raleigh, North Carolina, USA)*, which recognizes rabbit T cells and binds to CD5+, whereas B-cells

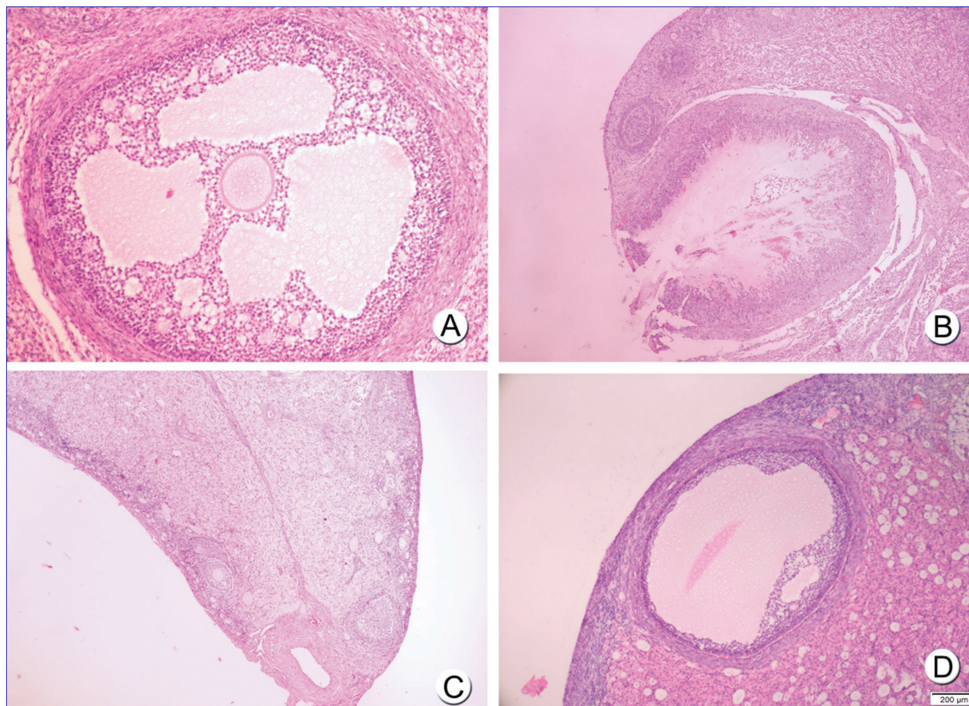

Fig 1. *Ovarium* at different reproductive stages

A: estrous, H & E, x400, B: ovulation, H & E, x40, C: pregnancy, H & E, x40, D: ovulatory follicle with diameter approximately up to 1100 µm, H & E, x40

Şekil 1. Farklı üreme dönemlerinde ovaryum

A: östrus, H & E, x400, B: ovulasyon, H & E, x40, C: gebelik, H & E, x40, D: çapı yaklaşık 1100 µm olan ovulasyon folikülü, H & E, x40

were detected with mouse anti-rabbit IgM monoclonal antibody *(Mouse anti Rabbit IgM-B cell Marker, clon NRBM, Catalog Number: MCA812GA, AbD Serotec, A Bio-rad Company, Raleigh, North Carolina, USA)*. Polyclonal goat anti-mouse IgG antibody conjugated with peroxidase was used as secondary antibody *(R&D Systems)*. For visualization the antigen-antibody complex, the slides were incubated with the peroxide substrate, *(DAB Peroxidase Substrate Tablet set, Sigma-Aldrich)*. Slides were lightly counterstained with Mayer's haematoxylin and cover slipped using Kaiser's glycerol gelatin as mounting media. Cryosections of *uterus* and *cervix*, stained without primary antibody incubation were used as negative control.

Histological and morphometric analyses were done by light microscopy (Olympus BX53), with compatible digital camera (Olympus UC50). Morphometric measurement of epithelial height was taken with computer-assisted image analysis system Olympus cellSens microscope imaging software, with magnification x40. At every section 15 measurements were taken, resulting in 150 measurements/animal. Reference area for T- and B-cell counting was defined using an ocular micrometric scale and microscopic ratio (RA=17.200 µm^2) with the magnification x40 at 50 areas (5 defined area/section). The results were represented as the number of positive cells/area. The number of immunopositive cells was presented as mean ± standard deviation (SD). Means of the number of T-cells in *uterus,* during the different stages of reproductive cycle, were analyzed statistically by Kruskal-Wallis method, followed by *post-hoc* Dunn's test, using Statistica v.6.0 (Statsoft USA). The differences between mean values were considered statistically significant if the P value was below 0.05. The investigation was done with the Permission of the Ethics Committee of Faculty of Veterinary Medicine, University of Belgrade, No 01-14/7.

RESULTS

Lamina epithelialis mucosae uteri varied in its thickness and height from stage to stage of reproductive cycle, depending also on anatomical part of *uterus*. A tall columnar ciliated and non-ciliated epithelial cells differed in their morphological appearance. Non-ciliated cells had dense cytoplasm in their basal portion, while secretory material occupied their apical processes formed as protrusion. The morphological profile of ciliated cells differed to previous one with slightly staining cytoplasm and larger size. The morphological change of *gll. uterinae* was also evident considering the shape, which lenghten within endometrial stroma and changed their initial straight tubular configuration to more sinous one.

The height of epithelial cells were increased *(Table 1)*, and endometrium became greatly thickened in post-ovulatory phase. Ciliated cells were the more prominent type of cells. Curled endometrial glands were spread through the loosely arranged connective tissue to the

Table 1. *Epithelial height in uterus, at different reproductive stages*
Tablo 1. *Farklı üreme dönemlerinde uterus epitel uzunluğu*

Uterine Segment	Estrous	Ovulation	Pregnancy
Cornu uteri (µm)	14.086±3.337^a	16.339±2.219^b	15.512±2.090^c
Cervix (µm)	32.709±5.446^a	37.520±5.166^b	20.137±2.227^c

Different superscript letters ^{abc} in the same row represent significantly different value (P<0.05)

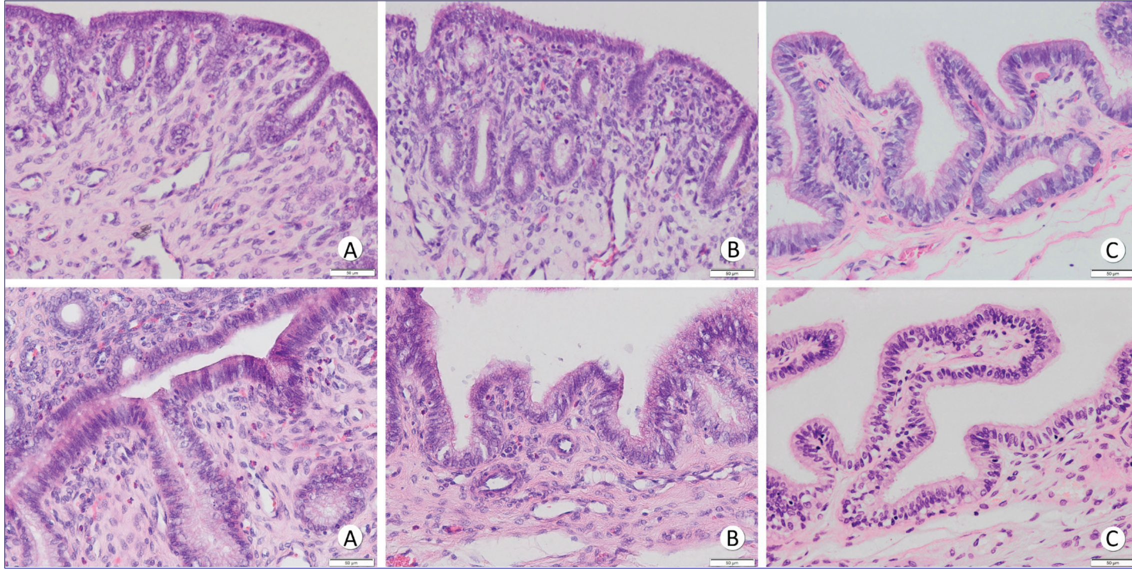

Fig 2. *Uterus* at different reproductive stages
Upper row: *cornu uteri*, **Lower row:** *cervix uteri,* A: estrous, B: ovulation, C: pregnancy, Scale bar 50 µm, H & E

Şekil 2. Farklı üreme dönemlerinde uterus
Üst sıra: cornu uteri, **Alt sıra:** serviks uteri, A: östrus, B: ovulasyon, C: gebelik, Ölçek 50 µm, H & E

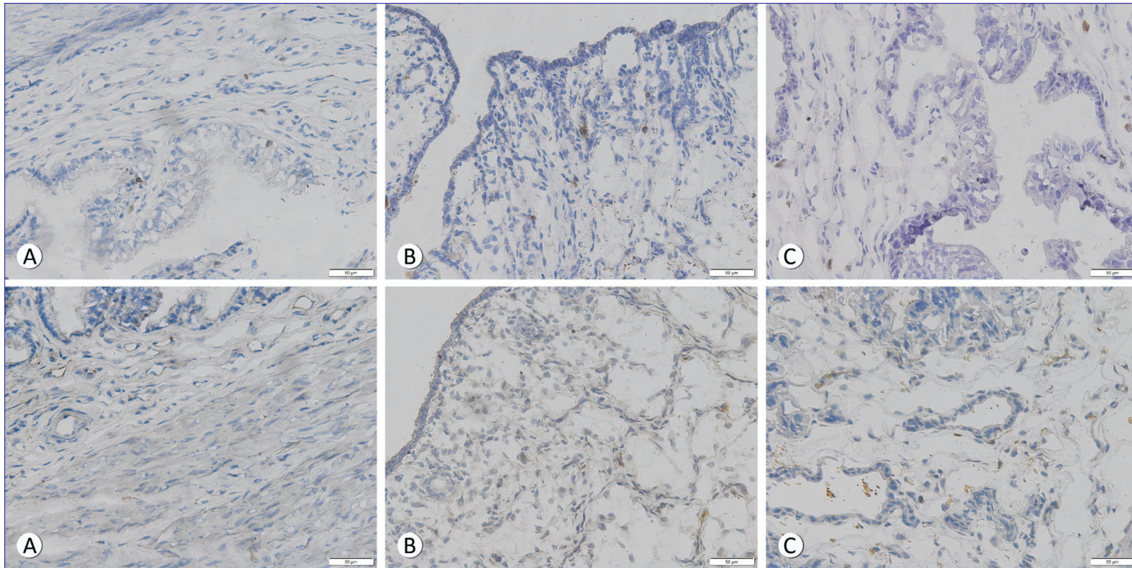

Fig 3. Distribution of T- and B-cells at *cornu uteri*
Upper row: T-cells, **Lower row:** B-cells at *cornu uteri,* A: estrous, B: after ovulation, C: pregnancy. Higher density of immunopositive cells was recorded for T-cells in estrous and post ovulatory stage. Scale bar 50 µm, chromogen diaminobenzidine, counterstain hematoxylin

Şekil 3. T ve B hücrelerinin cornu uterideki dağılımı
Üst sıra: T hücreler, **Alt sıra:** cornu uteride B hücreler, A: östrus, B: ovulasyon sonrası, C: gebelik. Östrus ve ovulasyon sonrası dönemlerde yüksek yoğunlukta T hücreleri gösterir immunpozitivite. Ölçek 50 µm, kromojen diaminobenzidine, zemin boyaması hematoksilen

myometrium, enlarged in their size and with dilated lumen. At this stage, the presence of lymphocytes in stroma was noticed. During pregnancy, epithelium became highly columnar and glandullar cells increased in height *(Fig. 2)*.

Cervical epithelium, consiting of cilliated cells, larger in size and with centrally or apically located nucleus, and secretory cells, narrow and with basally located nucleus, showed activity changes as a consequence of hormonal status. During ovulation, secretory cells increased their activity and secretory granules were noticed on their surfaces. During pregnancy, cervical mucosa formed the primary, secondary and tertiary folds; its columnar epithelium was edematous and the capillary network was particularly expressed *(Fig. 2)*.

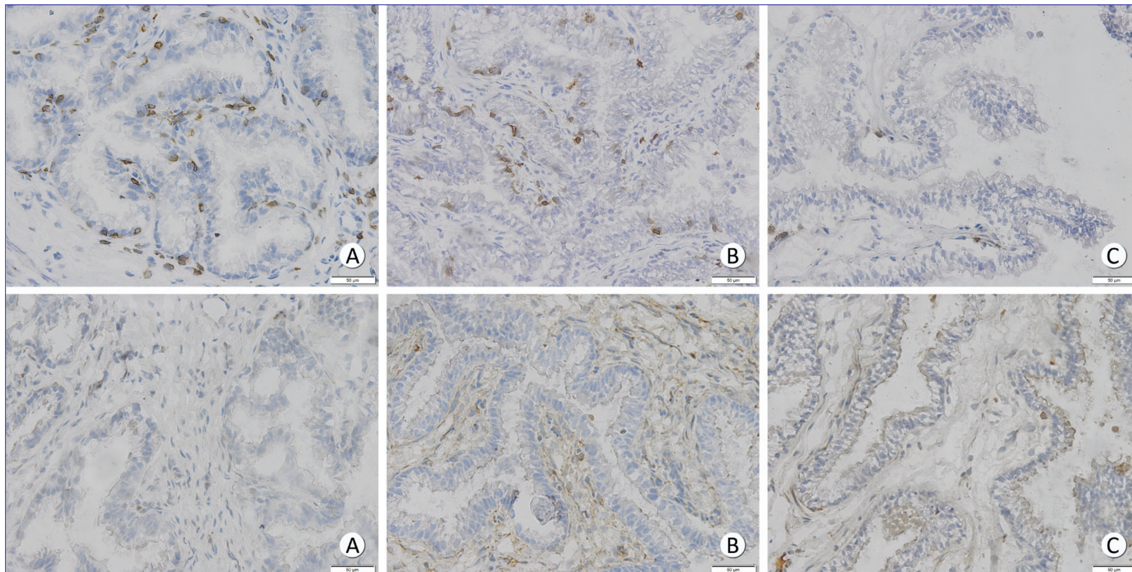

Fig 4. Distribution of T- and B-cells at *cervix uteri*
Upper row: T-cells, **Lower row:** B-cells at *cervix uteri,* A: estrous, B: after ovulation, C: pregnancy. The highest number of labeled immunopositive cells was noticed for T-cells in estrous. Scale bar 50 µm, chromogen diaminobenzidine, counterstain hematoxylin

Şekil 4. T ve B hücrelerinin serviks uterideki dağılımı
Üst sıra: T hücreler, **Alt sıra:** serviks uteride B hücreler, A: östrus, B: ovulasyon sonrası, C: gebelik. En fazla immunpozitif hücreler östrusta T hücrlerdi. Ölçek 50 µm, kromojen diaminobenzidine, zemin boyaması hematoksilen

Table 2. *Number of T and B lymphocytes per RA in cervix and cornu uteri, at different reproductive stages*

Tablo 2. *Farklı reproduktif dönemlerinde serviks ve kornu uteridekiher bir RA'da T ve B lenfosit sayıları*

Uterine Segment		Estrous	Ovulation	Pregnancy
Cornu uteri	T	$1.280 \pm 0.131^{a,1}$	1.440 ± 0.107^{a}	$0.200 \pm 0.057^{b,1}$
	B	0.280±0.075	0.800±0.128	0
Cervix	T	$2.120 \pm 0.123^{a,2}$	1.280 ± 0.107^{b}	$1.260 \pm 0.106^{b,c,2}$
	B	0.040±0.028	0.520±0.082	0.320±0.078

Different superscript letters ^{abc} in the same row represent significantly different value (P<0.05), different superscript numbers ^{1,2} in the same column represent significantly different value (P<0.05)

Following estrous, T-cells, round in shape, were located in subepithelium of *cornu uteri*, while B-cells were present around the blood and lymphatic vessels in the stroma. After ovulation, only a few T-cells were found separately in epithelium, while the majority was found at *lamina propria mucosae* and around blood vessels, along with scant B-cells around the blood and lymphatic vessels. During the pregnancy, the number of T-cells decreased and the presence of B-cells was only barely detected *(Fig. 3)*.

The number of T-cells in *cornu uteri* at estrous and in post-ovulatory stage was significantly higher (P<0.05) compared to their number in pregnancy *(Table 2)*.

During estrous, T-cells were present separately or in cell clusters under the epithelium, in the stroma of mucosal folds in *cervix* and around lymphatic vessels. This T-cell distribution was similar to the one in the post-ovulation phase. During pregnancy, both T- and B-cells were located under the cervical epithelium in very small numbers *(Fig. 4)*.

Although the localization of T-cells was similar in estrous and after ovulation, their number differed significantly (P<0.05). The number of T-cells was higher in estrous compared to pregnancy (P<0.05). Number of T-cells in *cervix* at estrous showed significant difference compared to the number in *cornu uteri* (P<0.05), and also in pregnancy. After ovulation, there was no significant difference in cell numbers between these regions *(Table 2)*.

DISCUSSION

The maintenance of the immune system in FRT is important not only in prevention of potential diseases, but also in limiting exposure to seminal male antigens, deposited during mating. Transforming growth factor β (TGFβ), contained in seminal plasma, stimulates post-coital immune response to pathogens and induces immune tolerance to those male antigens [29,30]. Although the *uterus* is not an immunologically privileged site, the immune response of genital tract could be modified toward immune tolerance [31,32]. Partutition is, on the contrary, characterized by influx of immune cells, that promote the re-establishment of the immune process [33].

Normal healthy FRT possesses various immunocompetent cells, such as macrophages, neutrophils, natural killer cells, Langerhans cells, lymphocytes, with T-cells and scarce B-cells [24,33,34]. A significant population of leukocytes in endometrium consists of T-cells (about 50%), where 2/3 of these cells are $CD8^{+}$ T cells [35,36]. $CD4^{+}$ and $CD8^{+}$

T-cells are located in stroma of lower genital tract as single cells or cell clusters, whereas in *uterus* these cells are organized as lymphoid aggregates. In human and mouse reproductive tracts, T-cells also represent the most numerous lymphocyte cell population [25,35]. In this study, we analyzed the presence of T- and B-cells in rabbit FRT. We found that CD5$^+$ T-cells in rabbit endometrium, as a single cells or clusters, were located beneath epithelium. Higher number of these cells was observed at endometrial stroma. In cervical folds and its stroma, CD5$^+$ T-cells were also detected. These findings are in correlation with previous publishing data [23,26]. Regarding the distribution of B-cells, we found that these cells were sparsed through endometrium stroma and around blood vessels in *cervix* and uterine horns, which is in correlation with findings in mice [37] and humans [36].

It is well known that the immune system in genital tract is regulated by sex hormones, produced by ovary during the reproductive cycle, so as to encompass the reproductive process [24]. The number of immunocompetent cells depends on the hormonal status and increases during proliferative phase. The number of T-cells in humans increases from the beginning of the menstrual cycle and reaches its maximum at the end of the proliferative stage [31,36]. Estrogen, as a regulator of this mechanism [38], provides *uterus* with better resistance to infection at estrous, but not in diestrous [39], by regulating the number, function and distribution of immune cells and antigen presentation in genital tract. The presence of major histocompatibility complex (MHC II$^+$) molecules was expressed more prominently in uterine body of mares than in the *cornu uteri* during the estrous [39]. In this study, we found a significantly higher number of T-cells in *cervix*, at estrous, as compared to other phases of the reproductive cycle. This finding differs from other findings in rabbits [26]. The increase in T-cells after ovulation could be a result of a differently defined control group. That group consisted of unmated, healthy female rabbits, with no clinical signs at estrous [26], which differs from the control group in our study.

Progesterone, also known as a natural immunosupresor, is essential for adequate maternal immune response to the fetus. Progesterone-mediated immunosupresive effect is achived by the downregulation of CD80, CD86 and MCH II$^+$ molecules on B-cells, and by supressing its antigen presentation. According to findings in humans and rodents, lower numbers of immune cells such as CD4$^+$ T-cells and dendritic cells are detected in *uterus* than in *vagina* during the dioestrous, and this could be a consequence of progesterone activity [25,33,37,40]. The presence of a low number of IgM plasma cells at rabbit *cervix* was recorded under the epithelium, or in stroma, while those cells were located mostly around blood vessels and in endometrial stroma [26]. It is known that the number and the localisation of IgM positive cells are not affected by the reproductive stage. Our study, along with a previous one [26], supports these findings. However, the lowest number of B-cells in gravid uterine horns, coupled with the lowest number of T-cells, described in our study, indicated a possible existence of immune tolerancy. Considering the findings that CD5 is overexpressed on regulatory T-cells (Treg) and regulatory B-cells [41], and that αβTCR$^+$CD4$^-$CD8$^-$ T- cells with regulatory function are dominant in mouse genital tissue [32], the presence of T-cells in *cervix* suggests that they may represent Treg cells. As αβTCR$^+$CD4$^-$CD8$^-$ T-cells are regulatory cells, and only a small proportion of rabbits B cells expresses CD5 [42], cells positive for CD5 staining in this investigation could be Treg cells. Those cells modulate immune reactivity in homeostatic and inflamatory conditions and lead to an enhanced immune response. The lower number of T-cells in post-ovulatory stage suggests that immune response in uterus gives way to immune tolerance, expecting a possible gestation. The assumption mentioned above remains to be confirmed in our further studies.

The presence of immunocompetent cells also depends on the anatomical compartments of reproductive tract [40]. Cervical and vaginal epithelial cells, together with macrophages and neutrophiles, present the first line of defense against pathogens [25]. *Cervix*, as the anatomical part of reproductive tract, has a more intense immunological activity in humans [19], due to the highest number of macrophages, CD4$^+$, and especially CD8$^+$ T-cells [35]. At cervical transformating zone, the accumulation of CD8$^+$ and TIA$^+$ cells indicated that this anatomical site is a strong immune barrier for ascending pathogens. Our finding of a significant number of T-cells in rabbit *cervix* under the epithelium and along the stroma suggests a possible migration of these cells into epithelium and implicates significant cervical reactivity, compared with uterine horns.

Lamina epithelialis mucosae and *lamina propria mucosae uteri* were exquisitely responsive to steroid hormonal stimulation or deprivation, which resulted in different morphological profiles through the reproductive phases. The number of T- and B-cells also showed considerable variation according to the reproductive stage. The findings that this immunological activity was significantly different in estrous compared to other reproductive stages indicates the principal role estrogen has in the regulation of adaptive immune response in rabbits. Similar findings were described in humans [24,34,36].

Conflict of Interest Statement

The authors declare that that there is no conflict of interest.

REFERENCES

1. Fischer B, Chavatte-Palmer P, Vielbahn C, Navarrete Santos Anne, Duranthon Veronique: Rabbit as a reproductive model for human health. *Reproduction*, 144, 1-10, 2012. DOI: 10.1530/REP-12-0091

2. Graur D, Duret L, Goury M: Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). *Nature*, 379, 333-335, 1996. DOI: 10.1038/379333a0

3. Kanayama K, Sankai T, Narial K, Endo T, Sakuma Y: Implantation in both uterine horns after the unilateral intrauterine insemination in rabbits. *J Vet Med Sci*, 54, 1199-1200, 1992. DOI: 10.1292/jvms.54.1199

4. Koning HE, Liebich HG: Ženski spolni organi (organa genitalia feminina). **In,** Koning HE, Liebich HG (Eds): Anatomija domaćih sisavaca. 3rd ed., 435-452, Naklada Slap, Republika Hrvatska, 2009.

5. Rebollar PG: Factors affecting efficacy of intravaginal administration of GnRH analogues for ovulation induction in rabbit does. *Giornale di Coniglicoltura ASIC*, 35-45, 2011.

6. Aragona P, Puozzolo D, Micali A, Ferreri G, Bretti D: Morphological and morphometric analysis on the rabbit connective goblet cells in different hormonal conditions. *Exp Eye Res*, 66, 81-88, 1998. DOI: 10.1006/exer.1997.0406

7. Bai W, Oliveros-Saunders B, Wang Q, Acevedo-Duncan M, Nicosia S: Estrogen stimulation of ovarian surface epithelial cell proliferation, In vitro. *Cell Dev Biol*, 36, 657-666, 2000. DOI: 10.1290/1071-2690(2000)036<0657:ESOOSE>2.0.CO;2

8. Hernandez J, Sanchez J, Perz-Martinez M: Morphometric characteristics of female reproductive organs of New Zealand rabbits with different body weight in peripuberal period of transition. *Vet Mex*, 41 (3): 211-218, 2010.

9. Hoffman KL, Gonzales-Mariscal G: Relevance of ovarian signaling for the early behavioral transition from estrus to pregnancy in the female rabbit. *Horm Behav*, 52, 531-539, 2007. DOI: 10.1016/j.yhbeh.2007.07.007

10. Rebollar PG, Boscon DA, Millan P, Cardinalli R, Brecchia G, Sylla L, Lorenzo PL, Castellini C: Ovulating inducing methods in rabbit does: The pituitary and ovarian responses. *Theriogenology*, 77, 292-8, 2012. DOI: 10.1016/j.theriogenology.2011.07.041

11. Bakker Julie, Baum JM: Neuroendocrine regulation of GnRH Release in Induced Ovulators, *Front Neuroendocrin*, 21, 220-262, 2000. DOI: 10.1006/frne.2000.0198

12. Tadakuma H, Okamura H, Kiotaka M, Iyama K, Usuku G: Association of immunolocalization of matrix metalloproteinase 1 with ovulation in hCG treated rabbit ovary. *J Reprod Fertil,* 98, 503-508, 1993. DOI: 10.1530/jrf.0.0980503

13. Sohn Joane, Couto AM: Anatomy, Physiology and Behavior. **In,** Suckow, Stevens Karla, Wilson R (Eds): The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. 1st edn., 195-213, Elsevier Inc, Academic Press, London, 2012.

14. Ju JC, Chang YC, Huang WT, Tang PC, Cheng SP: Superovulation and transplantation of demi- and aggregated embryos in rabbits. *Asian-Aust J Anim Sci,* 14, 455-461, 2001. DOI: 10.5713/ajas.2001.455

15. Arce SRA, Gaona HV, Perez-Martinez M: Variation in distribution of interstitial and epithelial lymphocytes in female uterine tubes of rabbit during early stages of pregnancy. *Tec Pecu Mex,* 46, 333-344, 2008.

16. Tyler KR: Histological changes in the cervix of the rabbit after coitus. *J Reprod Fert*, 49, 341-345, 1977. DOI: 10.1530/jrf.0.0490341

17. Wira CR, Rodriguez-Garcia Marta, Patel MV: The role of sex hormones in immune protection of the female reproductive tract. *Nat Rev Immunol*, 15, 217-230, 2015. DOI: 10.1038/nri3819

18. Pierdominici M, MaselliA, Colasanti T, Giammarioli AM, Delunardo F, Varcirca D, Sanchez M, Giovannetti A, Malorni W, Ortona E: Estrogen receptor profiles in human periphelial blood lymphocytes. *Immunol Lett,* 132, 79-85, 2010. DOI: 10.1016/j.imlet.2010.06.003

19. Trifonova RT, Lieberman J, van Baarle D: Distribution of immune cells in the human cervix and implications for HIV transmission. *Am J Reprod Immunol,* 71, 252-264, 2014. DOI: 10.1111/aji.12198

20. Hafez ESE, El-Banna AA, Yamashita T: Histochemical characteristic of cervical epithelium in rabbits and cattle. *Acta Histochem*, 39, 195-205, 1971.

21. Riches WG, Rumery RE, Eddy EM: Scanning electron microscopy of the rabbit cervix epithelium. *Biol Reprod*, 12, 573-583, 1975. DOI: 10.1095/biolreprod12.5.573

22. Suzuki H, Tsutsumi Y: Morphological studies of uterine and cervical epithelium in pseudopregnant rabbit. *Fac Agr Hakkaido Univ*, 60, 124-132, 1981.

23. Huerta XZ Veronica, Gaona HV, Arce SRA, Perez-Martinez M: Reduction of total lymphocyte migration to the uterus during the first days of pregnancy and pseudopregnancy of the rabbit *(Orictolagus cuniculus). Vet Mex,* 36, 63-74, 2005.

24. Wira CR, Fahey JV, Ghosh M, Patel V, Hickey DK, Ochiel DO: Sex hormone regulation of innate immunity in the female reproductive tract: The role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *Am J Reprod Immunol*, 63, 544-565, 2010. DOI: 10.1111/j.1600-0897.2010.00842.x

25. Zhang L, Chang KK, Qing LM, Jin LD, Yao XY: Mouse endometrial stromal cells and progesterone inhibit the activation and regulate the differentiation and antibody secretion of mouse B cells. *Int J Clin Exp Pathol,* 7, 123-133, 2014.

26. Gu W, Janssens P, Holland M, Seamark R, Kerr P: Lymphocytes and MHC class II positive cells in the female rabbit reproductive tract before and after ovulation. *Immunol Cell Biol*, 83, 596-606, 2005. DOI: 10.1111/j.1440-1711.2005.01375.x

27. Givan Alice, White Hillary, Stem Judy, Colby Esther, Guyre P, Wira C, Gosselin E: Flow cytometric analysis of leukocytes in the human female reproductive tract: Comparison of fallopian tube, uterus, cervix and vagina. *Am J Reprod Immunol*, 38, 350-359, 1997. DOI: 10.1111/j.1600-0897.1997.tb00311.x

28. Safwat el-Din MD, Habib FA, Oweiss NY: Distribution of macrophages in the human Fallopian tubes: an immunohistochemical and electron microscopic study. *Folia Morphol,* 67, 43-52, 2008.

29. Robertson A Sarah, Ingman WV, O'Leary S, Sharkey DJ, Tremellen KP: Transforming growth factor β- a mediator of immune deviation in seminal plasma. *J Reprod Immunol*, 57, 109-128, 2002. DOI: 10.1016/S0165-0378(02)00015-3

30. Sharkey DJ, Macpherson AM, Tremellen KP, Mothershead DG, Gilchrist RB, Robertson SA: TGFβ mediated proinflammatory seminal fluid signaling in human epithelial cells. *J Immunol*, 189, 1024-1035, 2012. DOI: 10.4049/jimmunol.1200005

31. Yeaman GR, Collins EJ, Fanger MW, Wira CR: CD8+ T cells in human endometrial lymphoid aggregates: Evidence for accumulation of cells by trafficking. *Immunology*, 102, 434-440, 2001. DOI: 10.1046/j.1365-2567.2001.01199.x

32. Johansson Marina, Lycke N: A unique population of extrathymically derived $\alpha\beta TCR^{+}CD4^{-}CD8^{-}$ T cells with regulatory functions dominated the mouse female genital tract. *J Immunol*, 170, 1695-1666, 2003. DOI: 10.4049/jimmunol.170.4.1659

33. Mor G, Cardenas Ingrid: The immune system in pregnancy: A unique complexity. *Am J Reprod Immunol*, 63, 423-433, 2010. DOI: 10.1111/j.1600-0897.2010.00836.x

34. Hafner LM, Cunningham K, Beagley KW: Ovarian steroid hormones: Effect on immune responses and *Chlamydia trachomatis* infections of the female genital tract. *Mucosal Immunology*, 6, 859-875, 2013. DOI: 10.1038/mi.2013.46

35. Pudney J, Quayle A, Anderson D: Immunological microenvironments in the human vagina and cervix: Mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod,* 73, 1253-1263, 2005. DOI: 10.1095/biolreprod.105.043133

36. Hickey DK, Patel MV, Fahey JV, Wira CR: Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: Stratification and integration of immune protection against the transmission of sexually transmitted Infections. *J Reprod Immunol*, 88, 185-194, 2011. DOI: 10.1016/j.jri.2011.01.005

37. Takeuchi T, Yoshida M, Shimizu T, Asano A, Shimokawa T, Nabeta M, Usui T: Differential expressions of toll-like receptor genes in the vagina of pregnant mice. *J Vet Med Sci,* 75, 561-565, 2013. DOI: 10.1292/jvms.12-0458

38. Eren U, Sandikci M, Kum S, Eren V: MHC Class II+ (HLA-DP-like) cells

in the cow reproductive tract: I. Immunolocalization and distribution of MHC Class II+ Cells in uterus at different phases on the estrus cycle. *Asian-Aust J Anim Sci*, 15, 35-41, 2008. DOI: 10.5713/ajas.2008.70224

39. Tunon AM, Rodrigez-Martinez H, Nummijarvi A, Magnusson U: Influence on age and parity on the distribution cells expressing major histocompability complex class II, CD4, or CD8 molecules in the endometrium of mares during estrus. *Am J Vet Res, 60*, 1531-1535, 1999.

40. Lopez Maria, Stanley Margaret: Cytokine profile of mouse vaginal and uterus lymphocytes at estrus and diestrus. *Clin Dev Immunol,* 12, 159-164, 2005. DOI: 10.1080/17402520500141010

41. Soldevila Gloria, Raman C, Lozano F: The imunomodulatory properties of the CD5 lymphocyte receptor in health and disease. *Curr Opin Immunol*, 23, 310-318, 2011. DOI: 10.1016/j.coi.2011.03.003

42. Pospisil R, Kabat J, Mage G Rose: Characterization of Rabbit CD5 isoforms. *Mol Immunol,* 46, 2456-2464, 2009. DOI: 10.1016/j.molimm.2009.05.026

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Research Article

An Evaluation of Post-operative Urinary Incontinence in Dogs with Intramural Ectopic Ureter Treated with Neoureterocystostomy [1]

[1] *A part of this study was presented as oral presentation in 1st International Turkey Veterinary Surgery Congress, 11-14 May 2016, Erzurum, Turkey*
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Abstract

The main motivation in the treatment of ectopic ureter (EU) is achieving complete urinary continence. Although, new neoureterostomy techniques were developed for the surgical treatment of intramural ectopic ureters, it was determined that postoperative incontinence scores achieved with the latest techniques have no distinct superiority over those achieved with older techniques. Therefore, we aim to determine the post-operative urinary incontinence scores in dogs with intramural EU that were treated with neoureterocystostomy and compared with older reports which neoureterostomy techniques were used. Seven female dogs which were brought to our clinic with a complaint of constant urine dribbling since they were born or adopted were included the study. The radiographic diagnosis was made by excretory urography in four dogs, excretory urography and retrograde vagino-urethrography in 2 dogs and MRI in 1 dog. Definitive diagnosis was made by cystotomy. Ectopic ureters were intramural character in all cases. Neoureterocystostomy technique was used for the surgical treatment and incontinence scores at post-operative 1st and 2nd months were recorded. For the purpose of complete elimination of the postoperative urinary incontinence, phenylpropalamine were used in 4 cases and oxybutynin were used in 1 case as additional medical therapy. Urinary incontinence was completely disappeared, 5 out of 7 patients (71%) at the end of the study. In conclusion, the results suggest that EU is most successfully treated with a surgical method that does not involve urethrotomy and it should be performed simultaneously with a surgical method used in the treatment of urethral sphincter mechanism incompetence and should be combined with a treatment involving the use of post-operative alpha adrenergic medications.

Keywords: *Dog, Ectopic ureter, Intramural, Neoureterocystostomy, Urinary incontinence*

Neoüreterosistostomi Yöntemiyle Tedavi Edilen İntramural Ektopik Üreter Hastası Köpeklerde Postoperatif Üriner İnkontinensin Değerlendirilmesi

Özet

Ektopik üreterin cerrahi tedavisinde ana motivasyon üriner inkontinensi ortadan kaldırmaktır. Özellikle intramural tipteki ektopik üreterin cerrahi tedavisi için farklı neoüreterostomi teknikleri geliştirilmesine rağmen, postoperatif üriner inkontinens skorlarının, eski teknikler kullanılarak elde edilenlere önemli bir üstünlüğü olmadığı tespit edilmiştir. Çalışmamızda, neoüreterosistostomi tekniğiyle tedavi edilen intramural ektopik üreter hastası köpeklerde postoperatif üriner inkontinens skorlarının, neoüreterostomi teknikleri kullanılarak elde edilen eski skorlarla karşılaştırılması amaçlanmıştır. Çalışmaya kliniğimize, doğduğundan veya sahiplenildiğinden beri sürekli damla damla idrar kaçırma şikayeti olan 7 dişi köpek dahil edildi. Radyolojik tanı için 4 hastada ekskretör ürografi, 2 hastada ekskretör ürografi ve retrograd vajinoüretrografi, bir hastada ise magnetik rezonans görüntülemeden (MRG) faydalanıldı. Kesin tanı sistotomi ile kondu. Hastaların tümünde ektopik üreter intramural karakterde idi. Hastalar neoüreterosistostomi tekniği kullanılarak sağaltıldı ve postoperatif 1. ve 2. ay inkontinens skorları kaydedildi. Postoperatif dönemde, üriner inkontinensin tamamen ortadan kaldırılması için, ek medikal tedavi olarak 4 hastada fenilpropalamin, bir hastada ise oksibutininden faydalanıldı. Çalışmanın sonunda, yedi hastanın 5 tanesinde (%71) üriner inkontinens tamamen ortadan kalktı. Elde ettiğimiz sonuçlar, farklı neoüreterostomi teknikleri kullanılarak elde edilenlerle benzer bulundu. Sonuçlar, intramural ektopik üreterin tedavisinde, üretrotomi içermeyen bir cerrahi yöntemin, üretra sfinkteri mekanizması yetersizliğinin cerrahi tedavisinde kullanılan bir yöntemle birlikte, eş zamanlı olarak uygulanması ve postoperatif dönemde alfa adrenerjik ilaçların kullanılmasını içeren bir kombine terapiyle en başarılı şekilde tedavi edilebileceğini düşündürdü.

Anahtar sözcükler: *Köpek, Ektopik üreter, İntramural, Neoüreterosistostomi, Üriner inkontinens*

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INTRODUCTION

The ureters are fibromuscular tube-like channels that transport urine from the pelvis renalis of the kidneys to the bladder [1]. Normally, they reach the dorsolateral surface of the bladder just cranial to its neck and enter the bladder obliquely at the trigonum vesicae after a short intramural course. Ectopic ureter (EU) is defined as an abnormality where one or both of the ureters opens into an area distal to the bladder neck. Common sites of the termination of the ectopic ureter include the urethra, vagina and the uterus. This congenital anomaly is caused by the disembryogenesis of the ureteric bud [2] and EU is observed more frequently in females than males [3].

Even though there are different classifications, ectopic ureters are classified into two categories; extramural and intramural [2,4-7]. With extramural EU, the ureter completely bypasses the urinary bladder to insert more distally into the urogenital system. With intramural EU, the ureter attaches to the dorsal or dorsolateral part of the bladder, running submucosally through the bladder wall and opens into the bladder neck, urethra or vagina, instead of opening its normal position [8]. The major clinical sign of EU is urinary incontinence [9,10]. Peri-vulvar dermatitis, cystitis and pyelonephritis secondary to ascending infection can also occur [2]. Hematology and serum biochemistry findings are usually normal [8].

Urinary incontinence is defined as involuntary passage of urine through the urethra [11]. Even though continuous or intermittent urinary incontinence is reported as primary complaint since birth in the history [5], urinary incontinence is generally continuous character in EU patients [11-13].

Excretory urography, retrograde vagino-urethrography, positive-contrast cystography, double-contrast cystography, pneumocystography [14], ultrasonography [8], fluoroscopy [2], cystoscopy [15] contrast-enhanced computer tomography [4], transurethral cystoscopy [16] and magnetic resonance imaging (MRI) [12] are used in the diagnosis of EU. Although transurethral cystoscopy is considered the most reliable method of diagnosis because it allows direct imaging of urethra and bladder trigone, definitive diagnosis can be made using cystotomy or postmortem examination [5,15].

It is recommended that the decision about the choice of which surgical method will be used in the treatment of EU is based on the location and morphological characteristics of the ectopy and the presence of secondary lower and upper urinary system abnormalities [4]. In the surgical treatment of extramural EU, the ureter is transected from the dorsal wall of the urethra, vagina or uterus and reimplanted in the urinary bladder through a point between the bladder neck and apex. This procedure is called neoureterocystostomy or ureteroneocystostomy [4,17]. With intramural EU, a new stoma is usually created in the mucosa of the urinary bladder in the normal anatomical position of the ureter orificium and the distal submucosal ureteral tunnel is ligated. This technique is called neoureterostomy [2,4,17]. Cystoscopic-guided laser ablation has also been used in the treatment of intramural EU [18].

The correlation between the morphological classification of EU and the preferred surgical method with the prognosis has not been established [2]. Different neoureterostomy techniques have been developed, especially for the treatment of intramural EU [13,18,19]. The primary objective of these studies is to minimize the presence of post-operative incontinence. However, it was determined that incontinence scores achieved with the latest techniques have no distinct superiority over those achieved with older techniques [13]. Therefore, we aim to evaluate the presence of post-operative urinary incontinence in dogs with intramural EU that we treated with neoureterocystostomy.

MATERIAL and METHODS

Patient Selection

The material of our study consisted of 7 female dogs, brought to our clinic between March 2010 and July 2015 *(Table 1)*. Common complaint of the dogs included in the study was the constant urine dribbling, every 10-30 seconds since they are born or adopted. The hair coat on the distal vulva was moist or wet, and they had peri-vulvar dermatitis of varying severities in all dogs. Anamnesis revealed that dogs rarely voluntarily urinate and both the amount of urine passed was smaller and the urination time was shorter than those of other dogs.

Clinical Examination

Before starting the examination, questions about whether or not the dog was sterilized, history of trauma, history of urinary system disease, as well as when and how often it had urinary incontinence were asked and recorded. In the inspection, the presence of neurological findings such as ataxic gait, coordination disorder, nystagmus, and anisocoria was investigated. The perineal and bulbospongiosus reflexes, patellar reflex response and conscious proprioception were evaluated. In the next step, the peri-vulvar region of the patient was examined for dermatitis and continuous urine dribbling while the patient was standing. The size of the urinary bladder (large, normal or small) was evaluated through abdominal palpation during the clinical examination. The urine sample collected by cystosynthesis was sent for complete urinalysis and urine culture test.

Excretory Urography

Excretory urography was used to diagnose EU in 6 patients (Case No. 1,2,3,4,5 and 7) *(Fig. 1)*. A 20G intravenous catheter was placed in the vena cephalica antebrachii. Direct LL and VD abdominal X-rays were taken before

Table 1. *Signalment, diagnostic procedures, features of ureteral ectopia, urinary tract anomalies and postoperative 30th and 60th day incontinence scores of the patients*

Tablo 1. *Hastaların eşgali, tanı için kullanılan prosedürler, üreteral ektopinin özellikleri, üriner sistem anomalileri ve postoperatif 30. ve 60. gün inkontinens skorları*

Case No	Breed	Age (month)	Sex	Type	Diagnosis	Side	Surgical Procedure	Renal Hypoplasia	Ureter Dilatation	Tortuous Ureter	Pelvic Bladder	Hypoplastic Bladder	Post-op 30th day Incontinence Score	Post-op 60th day Incontinence Score
1	Golden Ret.	5	IF	Intramural	EU + RVU + Cystotomy	Unilateral Left	Ureteroneocystostomy	-	-	-	-	-	1	1
2	Golden Ret.	5	IF	Intramural	EU + RVU + Cystotomy	Unilateral Left	Ureteroneocystostomy	-	-	-	-	+	3	1
3	Boston Terrier	11	IF	Intramural	EU + Cystotomy	Unilateral Right	Ureteroneocystostomy	-	-	-	-	-	1	1
4	Golden Ret.	4	IF	Intramural	EU + Cystotomy	Unilateral Left	Ureteroneocystostomy	-	+	+	+	-	3	2
5	Golden Ret.	36	SF	Intramural	EU + Cystotomy	Unilateral Right	Ureteroneocystostomy	Right	+	+	-	-	4	3
6	Golden Ret.	36	SF	Intramural	EU + MRI + Cystotomy	Bilateral	Right Nephroureterectomy Left Ureteroneocystostomy	Left	+	+	-	-	5	5
7	Golden Ret.	19	SF	Intramural	EU + Cystotomy	Unilateral Left	Ureteroneocystostomy	-	+	+	+	-	3	2

*Abreviations: **IF**, intact female; **SF**, spayed female; **EU**, excretory urography; **RV**, retrograde vagino-urethrography; **MRI**, magnetic resonance imaging*

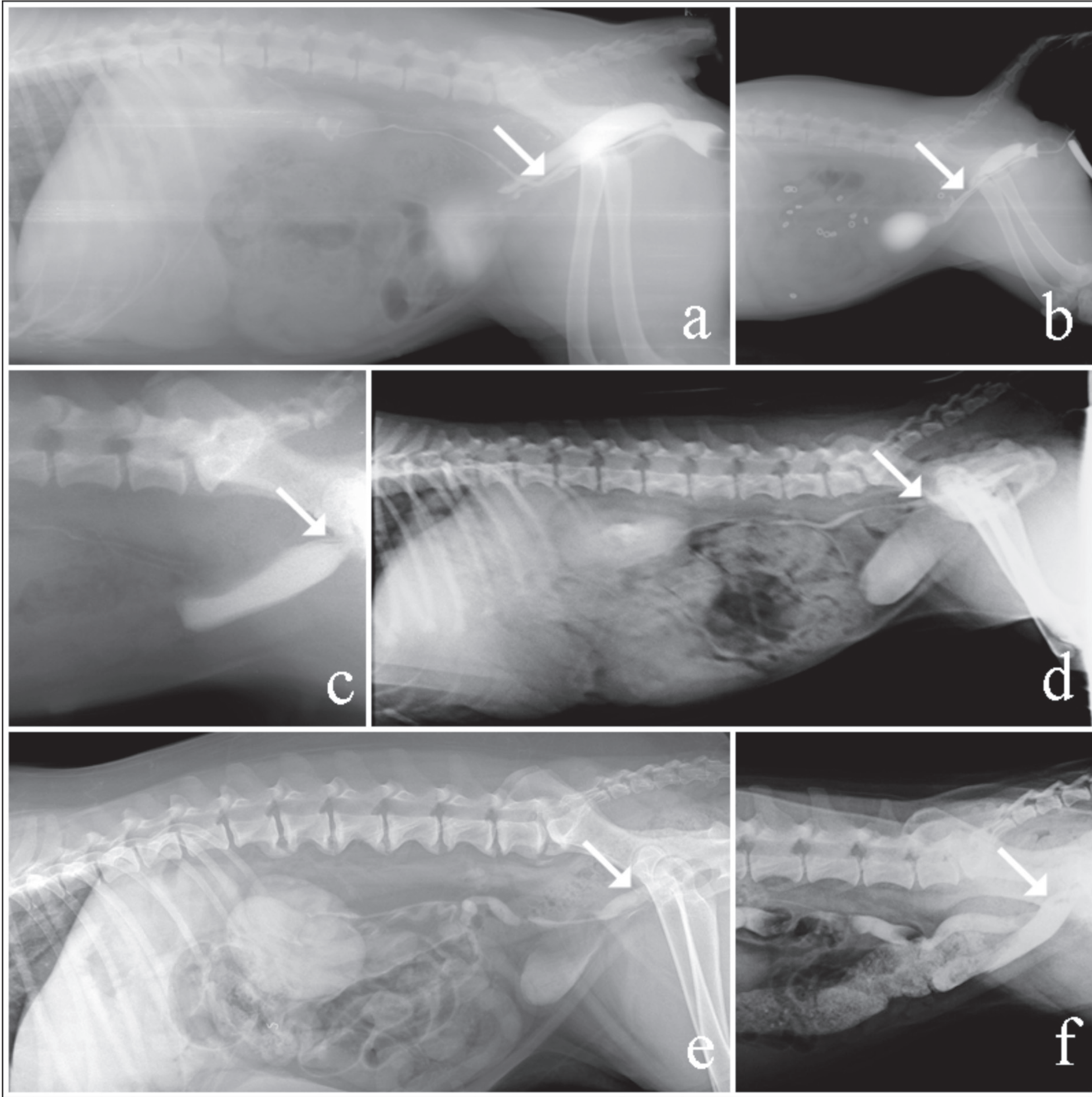

Fig 1. Lateral radiographic view of excretory urography and retrograd vagino-urethrography procedures of case no.1, ectopic ureter *(white arrow)* **(a),** Lateral radiographic view of excretory urography and retrograd vagino-urethrography procedures of case no.2, ectopic ureter *(white arrow)* **(b),** Lateral radiographic view of excretory urography of case no.3, ectopic ureter *(white arrow)* **(c),** Lateral radiographic view of excretory urography of case no.4, ectopic ureter *(white arrow)* **(d),** Lateral radiographic view of excretory urography of case no.5, ectopic ureter *(white arrow)* **(e),** Lateral radiographic view of excretory urography of case no.7, ectopic ureter *(white arrow)* **(f)**

Şekil 1. Bir numaralı olgunun ekskretör ürografi ve retrograd vajinoüretrografi işlemi esnasında çekilmiş LL grafisi, ektopik üreter *(beyaz ok)* **(a),** İki numaralı olgunun ekskretör ürografi ve retrograd vajinoüretrografi işlemi esnasında çekilmiş LL grafisi, ektopik üreter *(beyaz ok)* **(b),** Üç numaralı olgunun ekskretör ürografi esnasında çekilmiş LL grafisi, ektopik üreter *(beyaz ok)* **(c),** Dört numaralı olgunun ekskretör ürografi esnasında çekilmiş LL grafisi, ektopik üreter *(beyaz ok)* **(d),** Beş numaralı olgunun ekskretör ürografi esnasında çekilmiş LL grafisi, ektopik üreter *(beyaz ok)* **(e),** Yedi numaralı olgunun ekskretör ürografi esnasında çekilmiş LL grafisi, ektopik üreter *(beyaz ok)* **(f)**

excretory urography. Contrast medium containing 800 mg/kg iodine (Urografin® 76%, 370 mg iodine/mL, Bayer) was administered to the patients as IV bolus. The kidneys, ureters, and bladder were evaluated with radiographs taken at post-injection 1, 5, 10 and 20 min. Because digital fluoroscopy was not used, additional radiographs were taken between 5-10 min post-injection to obtain clear images of the distal parts of the ureters. In radiographs taken in the cystogram phase, whether or not the bladder neck lies caudally to the cranial border of the pubis (the presence of pelvic bladder) and the presence of persistent urachus was examined and recorded. In cases where a combination of excretory urography and retrograde vagino-urethrography is used (Case 1, Case 2), the procedures were performed under general anesthesia. General anesthesia was induced by IV administration of 5 mg/kg ketamine HCI (Ketalar® 10% vial, Pfizer) after pre-medication with 1 mg/kg xylazine HCL (Rompun® 2%

vial, Bayer) IM. Compression bandage was not applied to the abdomen during the procedure, and enema was not performed on any of the patients.

Retrograde Vagino-Urethrography

Retrograde vagino-urethrography was used to diagnose EU in 2 patients (Case No. 1 and 2) *(Fig. 1)*. For positive-contrast retrograde vagino-urethrography, a 12 FR 2-way latex Foley catheter (Galena®) was inserted in the vaginal opening, and the balloon was inflated. During the administration of contrast medium, curved intestinal forceps was placed in the distal vulva to prevent the falling out of the Foley catheter and contrast medium leakage. Presence of pain response during the placement of the intestinal forceps was checked, because it was reported that, if anesthetic depth is inadequate, the passage of the contrast medium to the urethra fails [6]. In the next step, contrast medium was administered intravenously as explained above, and excretory urography was started. When the passage of contrast medium to the ureters was established by radiography, contrast medium (Urografin® 76%, 370 mg iodine/mL, Bayer) was administered through the Foley catheter at a dose of 1 mL/kg.

Magnetic Resonance Imaging (MRI)

MRI was used to diagnose EU in 1 patient (Case No. 6) *(Fig. 2)*. MRI was performed using 1.5-T Signa Excite (GE Medical Systems, Milwauke, WI, USA) MRI system under general anesthesia. General anesthesia was induced by IV administration of 5 mg/kg ketamine HCl (Ketalar® 10% vial, Pfizer) after pre-medication with 1 mg/kg xylazine HCL (Rompun® 2% vial, Bayer) IM. The patient was positioned in right lateral and dorsal recumbency. MRI was performed with heavely T2-weighted (T2-W) 2D turbo-spin echo (TSE), sagittal sequences (TR 3572 ms, TE 78 ms, 24 slices, section thickness 4 mm) and coronal sequences (TR 924 ms, TE 75 ms, 21 slices, section thickness 5.0 mm).

Surgical Procedure

In all patients, CBC and serum biochemical parameters were examined pre-operatively. Pre-anesthesia was induced with IV administration of 0.4 mg/kg diazepam (Diazem® Deva). Anesthesia was induced with IV administration of 5 mg/kg ketamine HCl (Ketalar®, Pfizer). After intubation, anesthesia was maintained with 2-3% isoflurane (Isoflurane®, Adeka) and 100% oxygen. The patients were positioned in dorsal recumbency, and ventral and lateral parts of the abdomen were shaved and disinfected. Access to the abdominal cavity was obtained with median celiotomy. The urogenital system was inspected. The kidneys were evaluated for size and shape, and ureters for dilatation and tortuosity. A stay suture was then placed in the apex of the urinary bladder, and access to the urinary bladder was obtained with ventral incision. The course of the ureters and the position of the ureteral orifices were evaluated, and EU were classified as unilateral, bilateral, extramural or intramural. After accessing the bladder, whether or not the ureters open into the bladder from the normal anatomic position was checked by inserting a closed-end tomcat-style catheter (Buster®, Kruuse) to both ureters. By this means, absolute classification of ureteral ectopia as intramural or extramural could be established.

Surgical treatment was performed with neoureterocystostomy in all patients with EU. In this procedure, the EU was dissected after ligating at the point where it enters the urinary bladder using 2/0 monofilament absorbable suture material (Monocryl®, Ethicon). A longitudinal incision measuring 2-3 mm in length was then made on the distal ureter, after pulled into the bladder from a point between the trigone and apex of the bladder, and the mucosa of the ureter was attached to the mucosa of the urinary bladder with a 5/0 monofilament absorbable suture (Monocryl®, Ethicon) using a simple interrupted pattern. The incision made on the urinary bladder was closed without passing through the mucosa by using a simple interrupted suture technique as the first layer with 2/0 monofilament absorbable suture material (Monocryl®, Ethicon). Continuous Cushing suture technique were used for the second layer with the same suture material. In two cases (Case No. 4 and 7), pelvic bladder was diagnosed and cystopexy was performed before closing the abdomen.

In one case (Case No. 6), it was determined that the right kidney was severely atrophic, and nephroureterectomy was performed. In this procedure, the kidney was removed from the retroperitoneal space using blunt dissection and it was reflected medially. After that, the fat on the renal hilus was dissected, and the vascular pedicle was exposed. The arteria and vena renalis were ligated at a point as close as possible to the aorta abdominalis and vena cava caudalis respectively. The ureter was then separated from the ureteral fascia and it was ligated and cut at the point attached to the urinary bladder.

Post-Operative Assessment

After the patients were awakened in the reanimation room, they were discharged from hospital. Amoxicilline-clavulanic acid (Synulox®, Pfizer) 8.75 mg/kg IM for 10 days and carprofen (Rimadyl®, Pfizer) 2 mg/kg PO for 5 days were prescribed to the patients. No medical treatment was administered to the patients between post-operative 10th and 30th days. All patients were called to come in for follow-up at post-operative 30th and 60th days and were evaluated for urinary incontinence although more frequent follow-ups and phone calls were carried out for some patients. Urinary incontinence at post-operative 30th and 60th days was scored between 1-5. The patients in which complete urinary continence was achieved without any need for additional medical treatment scored 1; the patients in which complete urinary continence was achieved with phenylpropanolamine use scored 2; the patients in

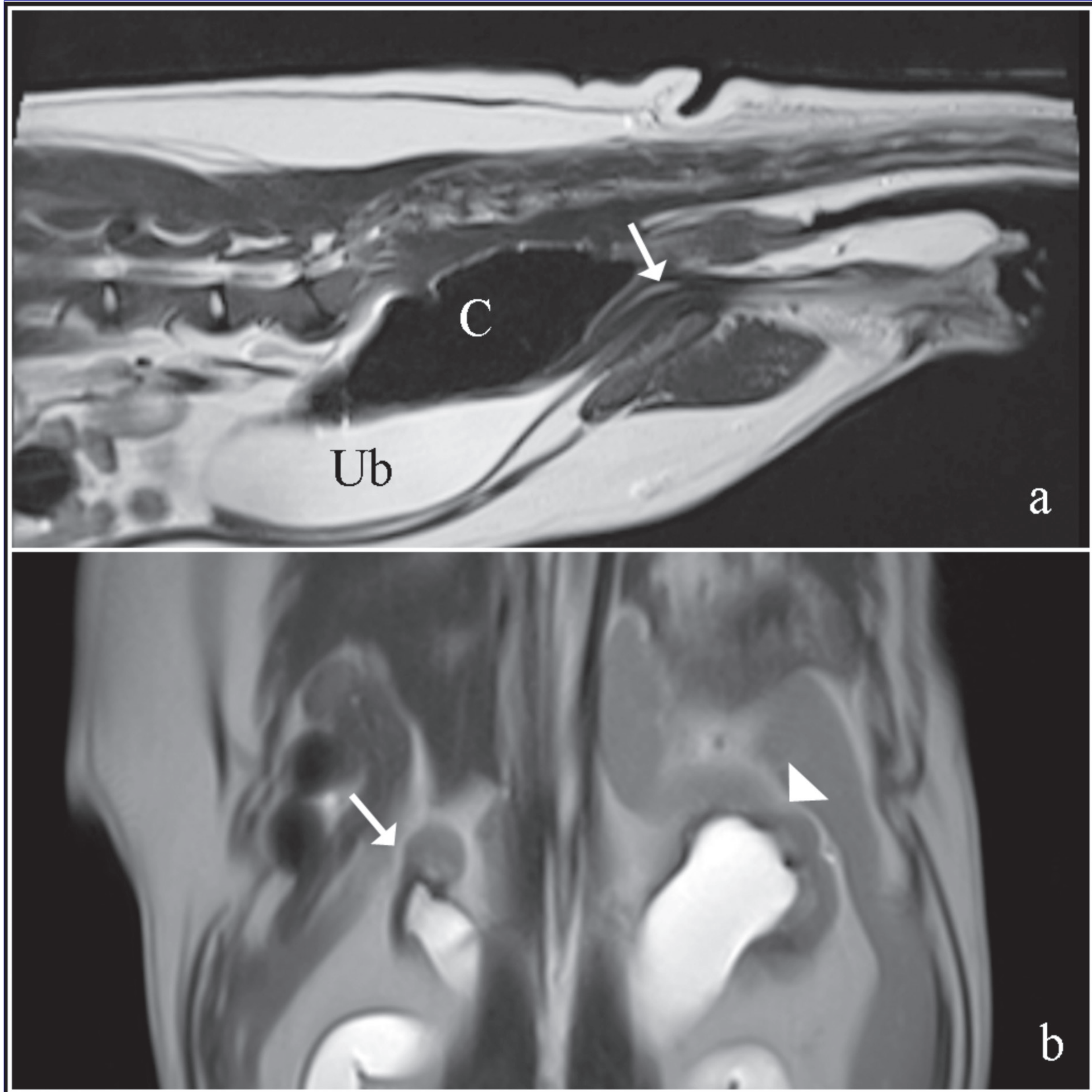

Fig 2. MRI images of case no. 6. Saggital t2 view, ectopic ureter suspicion *(white arrow)* **(a),** Coroner t2 view of the same patient, right atrophic kidney *(white arrow)*, left hypoplastic kidney *(arrow head)* **(b),** C, colon; Ub, urinary bladder

Şekil 2. Altı numaralı olgunun magnetik rezonans görüntüleri. Sagital t2 görüntüsü, ektopik üreter şüphesi *(beyaz ok)* **(a),** Koroner t2 görüntüsü, sağ atrofik böbrek *(beyaz ok)*, sol hipoplastik böbrek *(ok başı)* **(b),** C, kolon; Ub, idrar kesesi

which the complaint of urinary incontinence diminished but still occurred once or twice a week scored 3; the patients in which urinary incontinence persists at the same frequency but at a lower quantity scored 4; and the patients with no regression of urinary incontinence scored 5. Prognosis was considered successful in patients that scored 1 and 2 [15].

RESULTS

Six of our patients were female Golden Retriever and one was a female Boston Terrier. Their ages varied between 4 and 36 months (average 16.5 months). Three of our patients (43%) had been sterilized, and 4 (53%) were intact. In sterilized patients, the owners did not know the date of sterilization because they were adopted after the spaying procedure. The history revealed that all dogs had a complaint of continuous urine dribbling. Their neurological examination findings were normal. Clinical examination revealed that all dogs had peri-vulvar dermatitis of varying severities and their urinary bladders were small or normal in size. Four of our patients were diagnosed with left EU, two with right EU and one with bilateral EU. Four patients were diagnosed with ipsilateral ureter dilatation and tortuous ureter, two with renal hypoplasia and pelvic bladder, one with renal atrophy and one with urinary bladder hypoplasia. Reimplantation of the ureter to the urinary bladder was made using neoureterocystostomy in

seven out of eight EU cases. In one EU case, where atrophy developed in the ipsilateral kidney, nephroureterectomy was performed. None of the cases had renal agenesis, ureterocele or persistent urachus *(Table 1)*.

The average urea concentration of our patients was 30.8 mg/dL (reference range 10-55 mg/dL), and the average creatinine concentration was 1.2 mg/dL (reference range 0.6-1.4 mg/dL). Pre-operative urinary culture showed *E. coli* growth in 2 patients (Case 5 and 6) and *Pseudomanas spp.* growth in one patient (Case 7). These patients were administered 8.75 mg/kg IM amoxicillin/clavulanate (Synulox®, Pfizer) and 5 mg/kg IM enrofloxacine (Baytril®, Bayer) for 14 days before the surgery, and the urine culture was repeated. When the urine culture results were negative, the surgery was performed.

In two cases (Case No. 1 and 3) it was determined that complete continence was achieved at post-operative 30th day control. Urinary incontinence persisted in varying severities in cases number 2, 4, 5, 6, and 7 *(Table 1)*. Phenylpropanolamine (Proin®, PRN Pharmacal) was prescribed at the dose of 1 mg/kg three times a day for 1 month in cases, number 4, 5, 6, and 7. Oxybutynin (Uropan®, Kocak Farma) was prescribed for case number 2 at the dose of 5 mg/kg (splitting the total dose in half) PO two times a day for 1 month.

It was determined that, urinary continence was completely achieved in cases, number 2, 4, and 7 at post-operative 60th day control. At the end of the study, complete continence was achieved in 5 out of 7 patients (71%). The treatment failed in cases number 5 and 6 (29%) *(Table 1)*.

DISCUSSION

The decision about which surgical technique will be used for the treatment of EU in dogs is made based on the morphology of the EU [20]. Generally, neoureterostomy is preferred in the surgical treatment of intramural EU, and neoureterocystostomy is the preferred surgical technique for the treatment of extramural EU [2,4,17]. However, it is also known that this is not a hard and fast rule, which means that in some cases, neoureterocystostomy can be used for the surgical treatment of intramural EU [5,21]. For example, intramural EU is hard or sometimes impossible to treat with neoureterostomy in male dogs with prostatomegaly, small dog breeds in which catheterization of the distal ureteral segment is difficult [5] and in cases where EU opens into the distal urethra and vagina [2]. In neoureterostomy, surgical intervention to the distal ureteral segment can be performed while a new ureter stoma is created in the normal anatomic position. These surgical interventions can be classified as ligating the distal ureteral segment [2], complete resection of the distal submucosal ureteral tunnel [19] or resection of the intravesical part of the distal submucosal ureteral tunnel [13] have also been described. In addition to these techniques performed in combination with celiotomy and cystotomy, cystoscopic-guided laser ablation was also used for the same purpose [18]; however, it has certain disadvantages such as requiring special equipment and experience [15].

The main motivation in the treatment of EU is achieving complete urinary continence. The persistence of urinary incontinence in the post-operative period is attributed to urethral sphincter mechanism incompetence (USMI), recanalization of the distal ureteral segment despite ligation, insufficient urethral closure due to failure to remove the distal ureteral segment or poorly developed trigone [13]. At this point, it is beneficial to analyze the relationship between the surgical method to chosen in the treatment of EU and post-operative incontinence scores. The reason for efforts to describe new neoureterostomy techniques over time is that efforts have been made to improve post-operative incontinence scores.

It is believed that the more intact the urethra sphincter is, the less likely post-operative urinary incontinence is, particularly in female dogs [15]. Given that EU most commonly terminate at the urethra, structural degenerations of the urethra and USMI can occur because it is continuously exposed to urine and is open to bacterial infections until the EU diagnosis is made. Therefore, one might think that, performing a surgical intervention to the distal ureteral segment in urethra that is already damaged to varying degrees, may not provide a significant improvement in post-operative incontinence scores [13].

Reichler et al.[13] used the neoureterostomy technique for the treatment of intramural EU but resected only the part of the distal ureteral segment that is in the bladder wall and did not touch the part in the urethra. The reason for this is they wanted to avoid further damage to the sphincter by urethrotomy because USMI is frequently observed in EU patients. At the end of the study, they found no difference between the post-operative incontinence scores achieved in studies where neoureterostomy was performed without resection of the distal ureteral segment and those achieved following neoureterocystostomy and their own results, which forced them to conclude that resection of the distal ureteral segment is unnecessary. In other words, they demonstrated that the effect of recanalization or removal of the distal ureteral segment on the elimination of urinary incontinence in the post-operative period is not as important as thought.

As a matter of fact, these results demonstrate that an observation made years ago is still valid: 'There is no study which successfully establishes the correlation between classifying EU by their morphological characters and choosing a method specific to this morphology and the results achieved with the recommended surgical methods' [2]. The rate of persisting urinary incontinence following surgical treatment of intramural EU using different neoureterostomy

techniques varies between 42% and 78% [19]. The urinary incontinence scores achieved with cystoscopic-guided laser ablation, which has begun to be used recently, is similar to those achieved with neoureterostomy and neoureterocystostomy [15].

Another reason why urinary incontinence persists in the post-operative period in dogs with EU is reported to be USMI [5,13,20]. We were unable to conduct a pre-operative urethral pressure profile measurement in our study. However, it is a fact that no clear relationship was found between the measurement of maximal urethral closure strength by pre-operative urethral pressure profilometry and post-operative incontinence prognosis [19].

Although the definitive diagnosis of EU can be made by cystotomy, we generally need to use an additional imaging technique to support our suspicion about the presence of ureteral ectopia in pre-operative period. As we mentioned above, several techniques can be used to diagnose EU, excretory urography and retrograde vagino-urethrography are simple and cost effective techniques and can be used where ever x-ray machine available. In excretory urography, it is possible to diagnose ureteral ectopia at 5-10 min post-injection of IV contrast medium and we successfully used excretory urography 6 of 7 patients. But in one case (Case No. 6), ureteral ectopia could not be diagnosed even at 45th min post injection of contrast medium and just ureteral dilation was determined. In other words, excretory urography did not provide enough information about presence of EU. In this case, the age of the patient was 3 and information about medical history were pure since the dog was adopted from an animal shelter a couple of weeks ago. Because of these reasons, it was thought that, using an advanced imaging technique before laparotomy could be useful. Although transurethral cystoscopy is considered most reliable method, we could not use because of appropriate cystoscopy system was not available. MRI was used as advanced imaging technique to diagnose ectopic ureter in this case although we could be used computed tomography. The main reason to prefer MRI was, it could be achieved to provide more detailed information about other soft tissue structures in abdomen at the same time when compared to computed tomography. Also a distal ureteral segment was determined at sagittal section due to MRI *(Fig. 2-b)*.

Estrogen or phenylpropanolamine could be use in the treatment of USMI. Estrogen increases the closure pressure of the urethra by synthesizing new alpha receptors. On the other hand, phenylpropanolamine like alpha adrenergic agonists increases the closure pressure of the urethra by stimulating these receptors [22]. Phenylpropanolamine is usually used in the treatment of post-operative urinary incontinence, and it has gained a sit even in scoring post-operative incontinence [15]. In our study, we used phenylpropanolamine for 1 month in 4 patients with persistent urinary incontinence between post-operative 30th and 60th days and it was observed that complete continence was achieved in 2 patients.

Peri-vulvar dermatitis is a clinical examination finding that encountered due to continuous contact with urine of the peri-vulvar region and is frequently observed in dogs with EU [2,5]. Although we observed peri-vulvar dermatitis in all of our patients, this condition was very severe and dermatitis in a much more extensive area than peri-vulvar region, including the posterior part of the hindlimbs was determined in two cases (Case No. 5 and 6). In these two cases we were not achieved to stop urine dribbling despite of the phenylpropanolamine use. Even though it could not be evaluated objectively, the prognosis should be accepted doubtful in female dogs with urinary incontinence due to EU when dermatitis is so severe and constant urine dribbling causes a large part of the coat in the posterior area of the hindlims to be considerably moist.

Case number 2 was diagnosed with urinary bladder hypoplasia during the pre-operative radiography and intra-operative examination. In this case, persistence of urinary incontinence at post-operative 30 days was attributed to the urinary bladder hypoplasia, and only oxybutynin was prescribed. Oxybutynin is an anti-muscarinic agent that increases the urine storage capacity of the bladder [11]. Complete continence was achieved following oxybutynin use for 1 month in this case. We did not choose to use phenylpropanolamine in this case because the patient had not had its first estrus cycle. Our prediction that the estrogen that would be secreted during the first estrus cycle would raise the alpha-receptor count in the urethra and thus increase the closing strength of the urethra sphincter proved to be correct.

However, we preferred phenylpropanolamine in case number 4 even though she also had not had her first estrus cycle. The reason for this was that unlike cases who had not reached puberty, this case had dilated and tortuous ureter and pelvic bladder. Even though the bladder neck was reflected into the abdomen via cystopexy during the operation, phenylpropanolamine was used to eliminate the adverse effects of pelvic bladder on the urethra sphincter mechanism, and complete continence was achieved at the follow-up at post-operative 60 days.

Excretion of urine directly without accumulation in the bladder makes the urinary system susceptible to infections. A study reported that the prevalence of urinary system infections in dogs with EU is 64% [23]. We found urinary system infections in 3 of our patients (43%) in our study. The negative effect of bacterial toxins on the tonus of smooth muscles [8] causes USMI in dogs with EU and thus can be considered one of the factors that cause the incontinence to persist in the post-operative period. However, urinary system infections are not considered the primary cause of persistent post-operative urinary incontinence [1]. In our study, 3 cases were diagnosed with pre-operative urinary

system infection. Bacterial culture analysis revealed *E. Coli* growth in 2 of our patients, and *Pseudomanas spp.* growth in 1 of them. Urinary continence could not be achieved in 2 of our patients in which we isolated *E. coli.* The treatment was successful in our patient with *Pseudomanas spp.* growth. This result was consistent with a previous study which reported that post-operative urinary incontinence is quite hard to cure in patients with *E. coli* growth [1].

On the other hand, complete continence is reportedly achieved after the first estrous cycle in approximately 50% of female puppies suffering from this condition without surgical intervention [5]. In the light of this argument, postponing surgical treatment in dogs diagnosed with EU before the first estrus cycle might be considered. However, it is recommended that surgical treatment should be administered as soon as possible to prevent the development of secondary abnormalities such as hydroureter and hydronephrosis, which may cause ascending urinary system infections or urinary tract obstruction, even in cases where complete continence can be achieved with medical treatment [17]. Three of the cases (cases number 1, 2, and 4) were puppies which have not had their estrous yet, and we achieved successful results in these patients.

Several breeds are reported to be predisposed to EU. These include Siberian Husky, Miniature and Toy Poodles, Golden Retriever, Labrador Retriever, Shetland Sheepdogs, Spaniels, West Highland White Terrier, Wire Fox Terrier, Bulldog and Newfoundland [3,8]. Of these breeds, Labrador Retriever dogs are reported to have a better prognosis of post-operative urinary incontinence compared to other breeds [13,24]. Studies show that Golden Retriever breed dogs are related to Labrador Retriever dogs and they have similar chromosome structures [25]. Of the 7 dogs diagnosed with EU in our study, 6 (86%) were Golden Retrievers, and we achieved complete urinary continence in 4 (67%) of them. This is important for showing that the results obtained in this study and previous studies evaluating the results of surgical treatment of EU in dogs may vary based on the breed distribution of the population.

EU is usually accompanied by other urinary and urogenital system abnormalities such as renal agenesis, dysplasia and hypoplasia, hydronephrosis, ureter dilatation, tortuous ureter, pelvic bladder, hypoplastic bladder, urachal remnant, paramesonephric remnant, vaginal septum and dual vagina [2,18,26-28]. Paramesonephric remnant, vaginal septum and dual vagina is quite hard to diagnose without cystoscopy [18]. Other abnormalities can be diagnosed by excretory urography, ultrasonography and celiotomy [2,8,13].

In dogs with EU, ureter dilatation is frequently observed [10,21,29,30]. It is thought that this condition develops congenital or secondary to functional or physical ureteral obstruction or chronic pyelonephritis. However, its definitive pathogenesis has not been established. Experience shows that partial ureteral obstructions impair ureteral functions but peristaltic contractions can still continue. However, ureteral contractions reportedly disappear and ureteral functions are impaired in the presence of chronic ureteral obstruction accompanied by infection [21,31]. Ureteral dilatation is observed in 59% - 76% of dogs with EU [32]. We found ureteral dilatation and tortuous ureter in four of our cases (57%). However, post-operative changes in ureteral dilatation could not be observed because long-term post-operative follow-up could not be carried out.

Cases 5 and 6 were diagnosed with renal hypoplasia and their treatment failed. In addition, dermatitis, induced by continuous urinary incontinence affected not only the peri-vulvar region but a much more extensive area including the skin surface and coat on the posterior part of the femur in these two cases. These patients also had bacterial growth in their pre-operative urine cultures. Right nephroureterectomy was performed in case number 6, which we diagnosed with bilateral EU, because the right kidney was completely atrophic, but neoureterocystostomy was performed despite the presence of left renal hypoplasia.

In conclusion, various factors such as age, breed, gender, sterilization, the presence of urinary system infections, location of the bladder and other urinary system abnormalities accompanying EU may affect the result of the surgery in dogs with EU. Additionally, the results obtained with different surgical techniques in different populations where the aforementioned factors were heterogeneous did not provide clear guidance to veterinarians for selecting the appropriate technique. On the other hand, the post-operative incontinence scores that we achieved with neoureterocystostomy in the surgical treatment of 7 dogs with intramural EU are similar to those achieved with different neoureterostomy techniques. The results we achieved suggest that EU can be most successfully managed with a surgical method that does not involve urethrotomy and it should be combined with a surgical method which is used in the treatment of USMI and post-operative alpha adrenergic medications.

REFERENCES

1. McLaughlin R, Miller CW: Urinary incontinence after surgical repair of ureteral ectopia in dogs. *Vet Surg*, 20, 100-103, 1991. DOI: 10.1111/j.1532-950X.1991.tb00315.x

2. McLoughlin MA, Chew DJ: Diagnosis and surgical management of ectopic ureters. *Clin Tech Small Anim Pract,* 15, 17-24, 2000. DOI: 10.1053/svms.2000.7302

3. Novellas R, Stone J, Pratschke K, Hammond G: Duplicated ectopic ureter in a nine-year-old Labrador. *J Small Anim Pract*, 54, 386-389, 2013. DOI: 10.1111/jsap.12056

4. McLoughlin MA, Bjorling DE: Ureters. **In,** Slatter D (Ed): Textbook of Small Animal Surgery. Vol. 2. 3rd edn., 1619-1628, Saunders, Philadelphia, 2003.

5. Hoelzler MG, Lidbetter DA: Surgical management of urinary

incontinence. *Vet Clin Small Anim Pract*, 34, 1057-1073, 2004. DOI: 10.1016/j.cvsm.2004.03.003

6. Holt PE: Urinary incontinence. **In,** Holp PE (Ed): Urological Disorders of the Dog and Cat Investigation, Diagnosis and Treatment. 134-142, Manson, London, 2008.

7. Gultekin C, Ozgencil E, Seyrek-Intas D: The effect of deslorelin acetate in the treatment of persistent urinary incontinence after operation of ectopic ureter in a Golden Retriever bitch. *Kafkas Univ Vet Fak Derg*, 22, 465-468, 2016. DOI: 10.9775/kvfd.2015.14628

8. Oglesby PA, Carter A: Ultrasonographic diagnosis of unilateral ectopic ureter in a Labrador dog. *JSAVA*, 74, 84-86, 2003. DOI: 10.4102/jsava.v74i3.517

9. Hayes HM: Ectopic ureter in dogs: Epidemiologic features. *Teratology,* 10, 129-132, 1974. DOI: 10.1002/tera.1420100206

10. Holt PE, Gibbs C, Pearson H: Canine ectopic ureter a review of twenty nine cases. *J Small Anim Pract,* 23, 195-208, 1982. DOI: 10.1111/j.1748-5827.1982.tb01658.x

11. Fischer J, Lane IF: Micturation Disorders. **In,** Bartges J, Polzin DJ (Eds): Nephrology and Urology of Small Animals. 755-778, Wiley Blackwell, Malaysia, 2011.

12. Sach J, Möller S: Ectopic ureter in a seven-week-old Tibetan terrier using MRI imaging. *Kleintierpraxis*, 54, 688-694, 2009.

13. Reichler IM, Specker CE, Hubler M, Alois B, Haessig M, Arnold S: Ectopic ureters in dogs: Clinical features, surgical techniques and outcome. *Vet Surg*, 41, 515-522, 2012. DOI: 10.1111/j.1532-950X.2012.00952.x

14. Mason LK, Stone EA, Biery DN: Surgery of ectopic ureters: pre and postoperative radiographic morphology. *JAAHA,* 26, 73-79, 1990.

15. Smith AL, Radlinsky MG, Rawlings CA: Cystoscopic diagnosis and treatment of ectopic ureters in female dogs: 16 cases (2005-2008). *JAVMA*, 237, 191-195, 2010. DOI: 10.2460/javma.237.2.191

16. Cannizzo KL, McLoughlin MA, Mattoon JS, Samii VF, Chew DJ, Dibartola SP: Evaluation of transurethral cystoscopy and excretory urography for diagnosis of ectopic ureters in female dogs: 25 cases (1992-2000). *JAVMA*, 223, 475-481, 2003. DOI: 10.2460/javma.2003.223.475

17. Fossum TW: Surgery of the kidney and ureter. **In,** Fossum TW (Ed): Small Animal Surgery. 549-568, Mosby, China, 2012.

18. Berent AC, Weisse C, Mayhew PD, Todd K, Wright M, Bagley D: Evaluation of cystoscopic-guided laser ablation of intramural ectopic ureters in female dogs. *JAVMA*, 240, 716-724, 2012. DOI: 10.2460/javma.240.6.716

19. Mayhew PD, Lee KCL, Gregory SP, Brockman DJ: Comparison of two surgical techniques for management of intramural ureteral ectopia in dogs: 36 cases (1994-2004). *JAVMA,* 229, 389-393, 2006. DOI: 10.2460/javma.229.3.389

20. Newman M, Landon B: Surgical treatment of a duplicated and ectopic ureter in a dog. *J Small Anim Pract*, 55, 475-478, 2014. DOI: 10.1111/jsap.12227

21. Steffey MA, Brockman DJ: Congenital ectopic ureters in a continent male dog and cat. *JAVMA,* 224, 1607-1610, 2004. DOI: 10.2460/javma.2004.224.1607

22. Reichler IM, Hubler M: Urinary incontinence in the bitch: An update. *Reprod Dom Anim*, 49 (Suppl. 2): 75-80, 2014. DOI: 10.1111/rda.12298

23. Stone EA, Mason LK: Surgery of ectopic ureters: types, method of correction, and postoperative results. *JAAHA*, 26, 81-88, 1990.

24. Wiegand U, Rickel RF, van den Brom WE: Prognostic factors in the treatment of canine ectopic ureters. *Kleintierpraxis*, 41, 157-167, 1996.

25. Sutter NB, Eberle MA, Parker HG, Pullar BJ, Kirknes EF, Kruglyak L, Ostrander EA: Extensive and breed-specific linkage disequilibrium in *Canis familiaris*. *Genome Res*, 14, 2388-2396, 2004. DOI: 10.1101/gr.3147604

26. Agut A, Fernández del Palacio MJ, Laredo FG, Murciano J, Bayon A, Soler M: Unilateral renal agenesis associated with additional congenital abnormalities of the urinary tract in a Pekingese bitch. *J Small Anim Pract,* 43, 32-35, 2002. DOI: 10.1111/j.1748-5827.2002.tb00007.x

27. Taney KG, Moore KW, Carro T, Spencer C: Bilateral ectopic ureters in a male dog with unilateral renal agenesis. *JAVMA,* 223, 817-820, 2003. DOI: 10.2460/javma.2003.223.817

28. Yoon HY, Mann FA, Punke JP, Jeong SW: Bilateral ureteral ectopia with renal dysplasia and urolithiasis in a dog. *JAAHA,* 46, 209-214, 2010. DOI: 10.5326/0460209

29. Dean PW, Bojrap MJ, Constantinescu GM: Canine ectopic ureter. *Compend Contin Educ Pract Vet*, 10, 146-157, 1988.

30. Holt PE, Moore AH: Canine ureteral ectopia: An analysis of 175 cases and comparison of surgical treatments. *Vet Rec*, 136, 345-349, 1995. DOI: 10.1136/vr.136.14.345

31. Rose JG, Gillenwater JY: The effect of chronic ureteral obstruction and infection upon ureteral function. *Invest Urol,* 11, 471-478, 1974.

32. Samii VF, McLoughlin MA, Mattoon JS, Drost WmT, Chew DJ, Dibartola SP, Woodard SH: Digital fluoroscopic excretory urography, digital fluoroscopic urethrography, helical computed tomography, and cystoscopy in 24 dogs with suspected ureteral ectopia. *J Vet Intern Med,* 18, 271-281, 2004. DOI: 10.1111/j.1939-1676.2004.tb02545.x

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Research Article

Effects of Aqueous Artichoke *(Cynara scolymus)* Leaf Extract on Hepatic Damage Generated by Alpha-Amanitine [1]

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Abstract

Approximately 90% of mushroom poisoning deaths in the world is caused by types of *Amanita phalloides*. Alpha-amanitine has a bicyclic octapeptide structure, which is the main structure responsible for these poisoning cases. In the present study, it was aimed to investigate effects of leaf extracts with artichoke extract on this toxicity. In the study, 28 male rats of Sprague-Dawley species were randomized to 4 groups. The groups were designed as control; receiving serum physiological solution of 0.1 mL intraperitoneally (ip), alpha-amanitine; receiving 3 mg/kg single dose ip, artichoke leaf extract; receiving 1.5 g/kg orally for 14 d, and treatment group¸ receiving alpha-amanitine 3 mg/kg single dose ip+artichoke leaf extract 1.5 g/kg orally for 14 d. It was determined that alpha-amanitine increased hepatic malondialdehyde (MDA) levels, and decrease superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) activities with decreasing glutathione (GSH) levels. The supplementation with extract with artichoke, decreased MDA levels, it improved antioxidant parameters, and histopathological findings, so it was decided that extract with artichoke juice might be beneficial in alpha-amanitine related hepatic damage.

Keywords: *Artichoke, Alpha-Amanitine, Amanita phalloides, Antioxidant, Hepatotoxicity*

Alfa-Amanitin İle Oluşturulmuş Karaciğer Hasari Üzerine Enginar *(Cynara scolymus)* Sulu Yaprak Ekstresinin Etkileri

Özet

Dünyada mantar zehirlenmelerine bağlı ölümlerin yaklaşık 90%'ına *Amanita phalloides* türü mantarlar neden olmaktadır. Alfa-amanitin, bisiklik oktapeptid yapıda bu zehirlenmelerden sorumlu ana yapıdır. Bu çalışmada, enginar sulu yaprak ekstresinin oluşturulan toksisite üzerine etkilerinin araştırılması amaçlanmıştır. Çalışmada, Sprague-Dawley cinsi 28 erkek rat rastgele 4 gruba ayrıldı. Gruplar, kontrol grubu (serum fizyolojik 0.1 mL ip), alfa-amanitin (3 mg/kg tek doz ip), enginar yaprak ekstresi (oral 1.5 g/kg, 14 gün) ve tedavi uygulanan (alfa-amanitin 3 mg/kg tek doz ip+ enginar yaprak ekstresi oral 1.5 g/kg, 14 gün) olmak üzere belirlendi. Alfa-amanitinin karaciğer malondialdehit (MDA) düzeyini arttırdığı, süperoksit dismutaz (SOD), glutatyon peroksidaz (GSH-Px), katalaz (CAT) aktiviteleri ile glutatyon (GSH) düzeyini düşürdüğü tespit edilmiştir. Enginar sulu yaprak ekstresi takviyesinin MDA seviyesini düşürürken antioksidan parametrelerde ve histopatolojik bulgularda iyileşmeye neden olduğu saptanmıştır. Çalışma sonucunda, alfa-amanitin kaynaklı karaciğer hasarına karşı enginarın sulu ekstresinin kullanımının, tedaviye destek olarak kullanılabileceği düşünülmektedir.

Anahtar sözcükler: *Enginar, Alfa-Amantin, Antioksidan, Amanita phalloides, Hepatotoksisite*

INTRODUCTION

Of approximately 1.5 million types of mushrooms distributed worldwide, 2000 types are toxic, and the ones causing mortality have cyclopeptide structures. *Amanita* species are the mushrooms containing these structures and that are responsible for 90% of death cases worldwide [1].

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Among amanita species, the most common death cases are reported with *A. phalloides* mushroom. It is known that approximately 50 g of this type is lethal. It contains octapeptides such as amanitines and phalloidines, which are the main sources of toxicity and can dissolve in liquid environment, but resistant to high temperatures and against digestive enzymes. It has been reported that lethal doses for amanitines are 0.1 mg/kg, and for phalloidines are 1-2 mg/kg [2-4].

It has been indicated that the primary cause of toxicity in amanita species alpha-amanitine which exits among amanitine derivatives. Alpha-amanitine is absorbed and distributed to tissues within 1.5-2 h in the body. As it is not bound to circulating proteins, toxicity is rapid and severe. Mechanism of toxicity acts through binding of alpha-amanitine to RNA polymerase II enzyme, and inhibiting of DNA transcription. Therefore, it is reported that necrosis is observed in hepatocytes, in which excessive protein synthesis is performed [5].

Currently, there is no specific antidote present for *A. phalloides* intoxication. For treatment, supportive treatments such as gastric lavage, activated charcoal, and balancing liquid and electrolyte losses are performed. In addition to these, silybilin, which is the active ingredient of intravenous crystallized penicillin G and *Silybum marianum* herb, is being performed. It has been shown in different studies that administration of silybin may be lifesaving [6,7].

Artichoke *(Cynara scolymus)* is a vegetable, which is grown in countries with borders to the Mediterranean Sea, like our country, and is known to be rich in antioxidants and has preserving effects on liver. It has cynarine and chlorogenic acid which are caffeoylquinic acid derivatives, and flavonoids (luteonin, apigenin) in its structure. It has been reported in the literature that leaves of artichoke may be beneficial for liver intoxication, and it may also be antimicrobial and has cholesterol-lowering effects. Additionally, it has been reported that it can prevent hepatic damage by its strong antioxidant structure. Because of its peppery taste, and dissolution capacity of its active substances in water, liquid extracts of dried leaves and teas are being used medically [8-11].

In the present study, it was aimed to investigate effects of artichoke that was endemically grown in our country, on hepatic toxicity.

MATERIAL and METHODS

The study dated 04.04.2013 was performed, with 45 numbered countenance, providing approval of the Local Ethics Committee for Experimental Animal at Firat University Experimental Research Center (FUDAM). The study was performed on 28 male Sprague-Dawley species rat, which were 8 weeks old, and body weights were between 200 and 220 gram. Before and during the study, all rats were fed in 12 h light and 12 h darkness photo periods at 22-24°C fixed room temperature with standard rat feed *ad libitum*. 28 rats were randomized to four groups, so that each group was consisted of seven rats.

Alpha-amanitine, used in the study, has been supplied at least 95% purity from AppliChem (ABD) company. Artichoke leaf extract was taken from Arı Engineering (Ankara). Extracts 6.5% caffeoylquinic acid (3.7% monocaffeoylquinic acid and 2.6% dicaffeoylquinic acid named of cynarine) that is containing is cited from the company. Both of these materials can dissolve in water.

The first group, as the control group, received serum physiological solution (0.1 mL) ip infusion. The second group received alpha-amanitine was administered at 3 mg/kg in single ip infusion. The third group received orally liquid form of artichoke leaf extract at 1.5 g/kg dose for 14 d. The last group received single dose of 3 mg/kg alpha-amanitine ip and then they were given liquid form of artichoke leaf extract at 1.5 g/kg dose for 14 d. All groups were decapitated under anesthesia at the end of day 14. Toxicity formation and administration route of the extract were defined according to previous studies in the literature [12-14].

Obtained hepatic samples were washed by 0.9% NaCl, and stored at -80°C until the day of study. Tissue homogenization was performed in a Teflon homogenizer with 1.15% KCl tamponade at 1:10 (w/v) ratio.

Following the euthanasia, some of samples were embedded in %10 formalin solution, and then they were passed through alcohol and xylol series and embedded in paraffin blocks. The blocks were cut at 5-µm thickness by using microtome, and prepared samples were stained by hematoxylin-eosin and they were evaluated under the light microscope for histopathological findings.

Hepatic MDA levels were determined according to method performed by Placer et al.[15], SOD activity was measured according to the method performed by Sun et al.[16], CAT activity was measured according to the method performed by Aebi [17], GSH-Px activity was measured according to the method performed by Matkovics et al.[18], and GSH level was determined according to the method performed by Sedlak et al.[19].

Statistically Analysis

In order to test statistical significant differences between intergroup means of MDA, SOD, GSH, GSH-Px and CAT values, One-Way Variation Analysis was performed. Groups were independent and measurement levels were quantitative. Levene homogeneity test and Shapiro-Wilk normality test in small samples were employed to determine whether one-way variation analysis could satisfy other assumptions.

Homogeneity assumption was satisfied between groups for all variables, and normality assumption was satisfied for all other variables except for MDA variable. When homogeneity was preserved between the groups, F testing was not extensively affected from deviation from normality assumption [20]. Therefore, performing one-way variation analysis was applicable. As the result of one-way variation analysis all differences between groups were statistically significant for all variables ($P<0.001$). Scheffe Test, which was one of the most conservative tests in errors of multiple comparisons, was used to determine which groups caused intergroup differences.

RESULTS

The results are shown in *Table 1*. *Fig. 1* shows the mean values between groups for each variable. Using the same letters indicate that there is no difference among variable in one line, whereas using different letters indicate that there are differences between groups.

When MDA means were compared between the groups, artichoke extract using group increased MDA level when compared to the control group. Increasing MDA values by administration of alpha-amanitine were nearly returned those observed in the control group by administration of artichoke treatment ($P<0.05$). While SOD activities of hepatic tissues were observed closer between control and artichoke receiving groups, it was observed that the activity lowered by administration of alpha-amanitine was increased after administration of artichoke extract ($P<0.05$). GSH levels were determined very different in four groups. While GSH level of the group receiving artichoke extract was higher than the control group, GSH levels were very decreased by administration of alpha-amanitine, but they were increased to the values of the control group after artichoke extract administration. When compared with the control group, artichoke extract group had differences only in MDA and GSH variables. While alpha-amanitine caused differences in all variables, alpha-amanitine+artichoke group had differences in SOD, GSH-Px and CAT values.

Histopathological Findings

In trial groups (alpha-amanitine and alpha-amanitine+ artichoke leaf extract receiving groups) various degrees of degenerations were observed in all hepatic samples (hydropic degeneration, fat degeneration, and blurred swelling), and prominent changes with single cell necrosis were observed. In the control group, no pathological change was observed. No necrotic areas or inflammatory changes were observed in both trial groups.

DISCUSSION

Currently as there is no antidote for alpha-amanitine, some treatment methods about elimination and blocking the toxic substance into cells are being performed. It was reported that silybin which was derived from *Silybum marianum* herb, and was water-soluble substance of silimarine derivative, decreased entrances of amatoxins into cells, so that they showed hepatoprotective effect [21].

Artichoke vegetable, which was used in our study, is still being used as a medication for liver and gallbladder. Cynarine, which is the biological active substance in artichoke is present in all of the vegetable, but mainly concentrated in its leaves. Therefore, artichoke containing drugs and extracts are generally prepared as liquid extracts from the leaves [22].

Cynarine, a component of phenolic acid, is a substance with hepatoprotective and regenerative effects. It is the main derivative of caffeoylquinic acid and stated that antiatherosclerotic, antioxidative, and cholesterol-lowering effects. Also cynarine was reported that it showed cholagogue and chlorotic effects, and it provided hepatic detoxification by bile secretion [23]. According to study on cell culture made by Gezer et al.[24], cell culture treatment of cancerous human cells in cynarine gave an affirmative answer, increases survival rates, decreased of oxidative stress. In 1987, Adzet et al.[25] reported in their study that they observed effects of cynarine in hepatic toxicity induced by carbon tetra chloride (CCl_4), and it had a hepatoprotective effect. In our study, hepatoprotective effects and oxidative results might based on cynarine was main and effective structure of caffeoylquinic acid derives.

Majeed et al.[26] analysed anti-inflammatory activities of artichoke leaf extract obtained with different methods.

Table 1. *Scheffe Test results*
Tablo 1. *Scheffe Test sonuçları*

Group/ Variable	Control	Artichoke	Alpha-amanitine	Aa+artichoke
MDA (nmol/g)	121.82±12.67 (a)	68.23±6.29 (b)	427.07±13.20 (c)	122.15±11.49 (a)
SOD (units/mg protein)	20.99±2.52 (a)	21.22±2.26 (a)	12.34±1.68 (b)	16.37±1.38 (c)
CAT (k/mg protein)	2.47±0,30 (a)	2,42±0,31 (a)	1.39±0,38 (b)	1.91±0,23 (c)
GSH-Px (units/mg protein)	3.39±0,37 (a)	3,63±0.48 (a)	1,01±0,19 (b)	2,08±0,24 (c)
GSH (nmol/g)	6.85±0.37 (a)	7.61±0.45 (b)	5.55±0.34 (c)	6.89±0.59 (a)

(a-c): *Different superscript letters in same row indicate statistical differences*

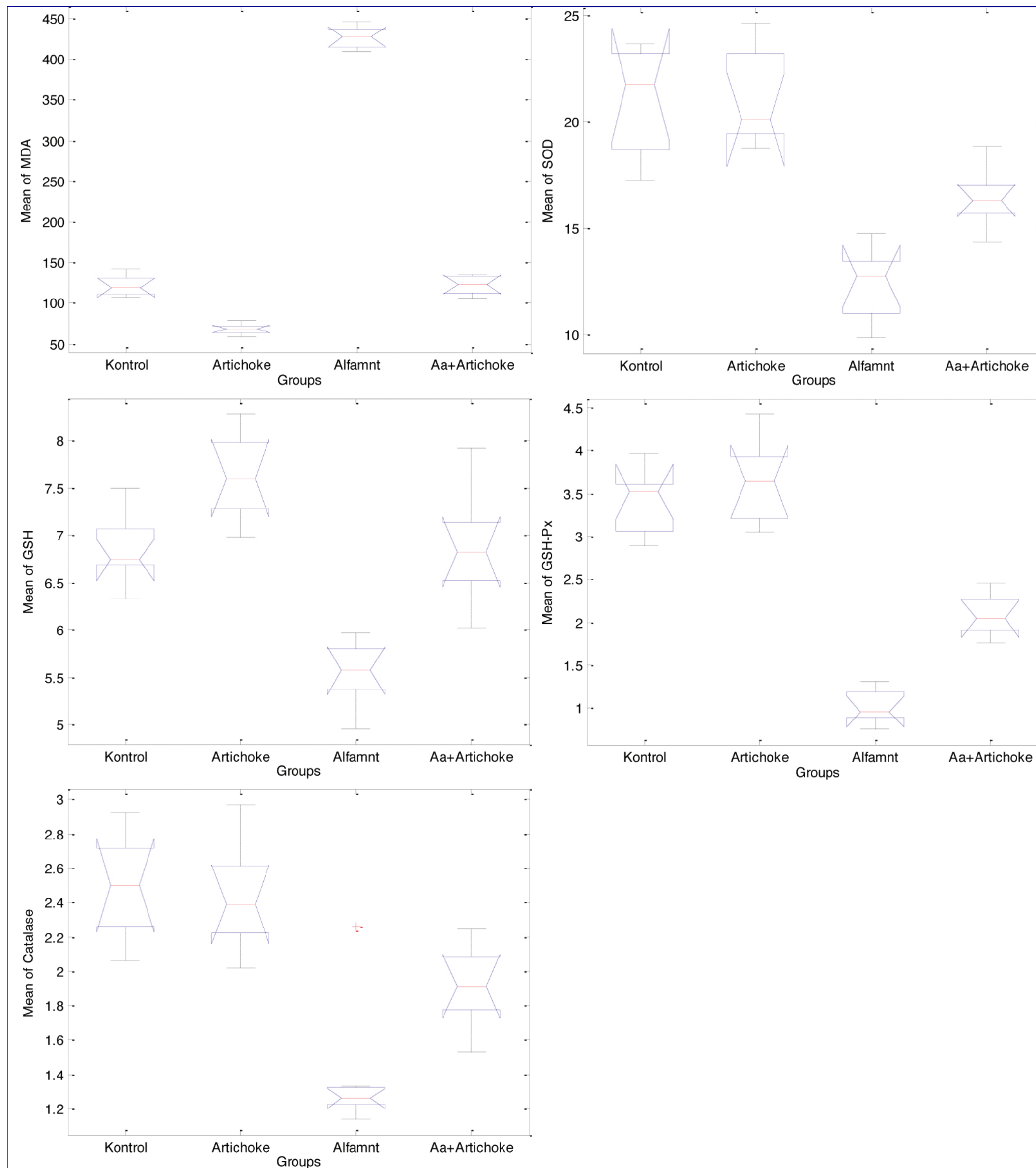


Fig 1. The mean values of all variables for the groups
Şekil 1. Tüm değişken grupların ortalama değerleri

They got close result from dexamethasone and diclofenac sodium group, chosen as pozitive control group, and got meaningful results from negative control implemented dimethylsulfoxide. Today's current treatment of caused from Alpha-amanitine, immune-suppressive and anti-inflammatory medicines are used as supportive medicines. According to Majeed at al.[26], supportive effect of artichoke leaf extract, used in our study, has shown that the parallelism.

In another study, Colak et al.[27] analyzed the artichoke's regenerated property on liver. CCl_4 induced liver damage studied by Colak et al.[27], applying artichoke leaf extract demonstrates that as like in our study also at antioxidant parameters how to reduce inflammation caused by to heal.

Antonio Jimeanez-Escrig et al. performed a study in which they extracted polyphenols form active substances of artichoke, and they tried them on rats. They reported that there were significant differences in SOD, GSH, GSH-Px and 2-aminoadipic semi aldehyde, which is a protein oxidation parameter, when compared to the control group [28].

Küçükgergin et al.[29] reported that hepatic and cardiac oxidative stress damage, which was caused in rats fed by high cholesterol diet, and caused by high cholesterol, could be decreased by artichoke leaf extract.

Under the light of these results, we aimed at determining whether artichoke leaf extract would be effective against *A. phalloides* intoxication that had no present antidote. As the results of our trial, it was observed that alpha amantadine caused oxidative stress and damage in all groups significantly. Significant changes were determined

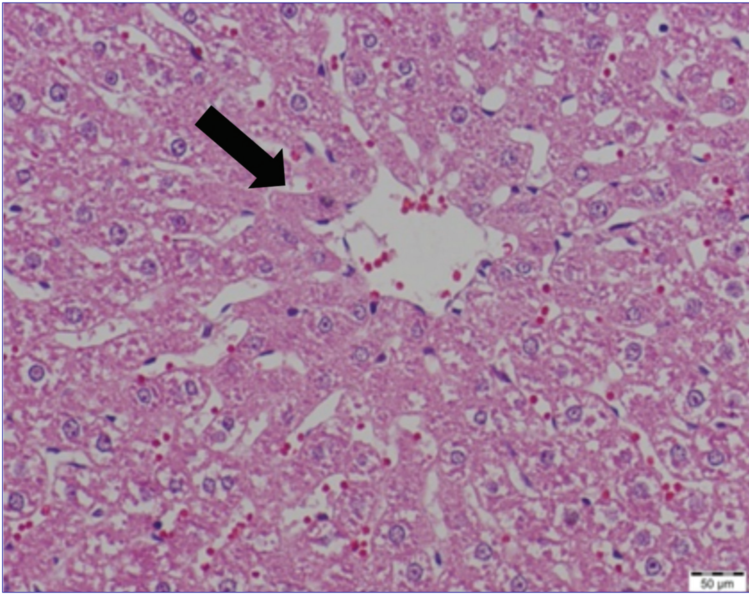
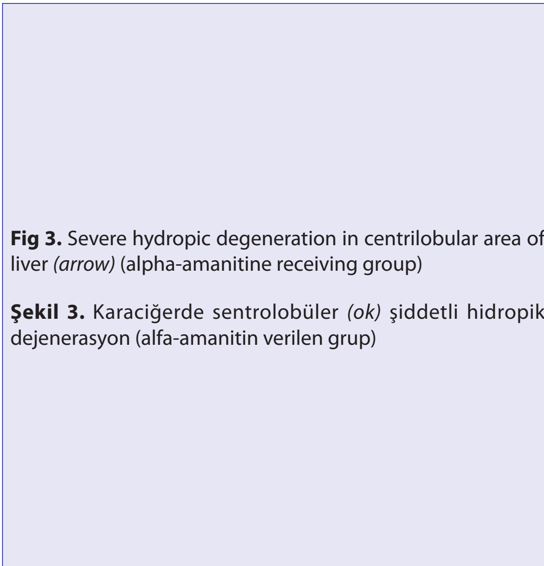


Fig 2. Mild-moderate blurred swelling in centrilobular area of liver *(arrow)* (alpha-amanitine+artichoke leaf extract receiving group)

Şekil 2. Karaciğerde sentrolobüler *(ok)* bölgede hafif- orta şiddette bulanık şişkinlik (alfa-amanitin+enginar yaprak ekstresi verilen grup)



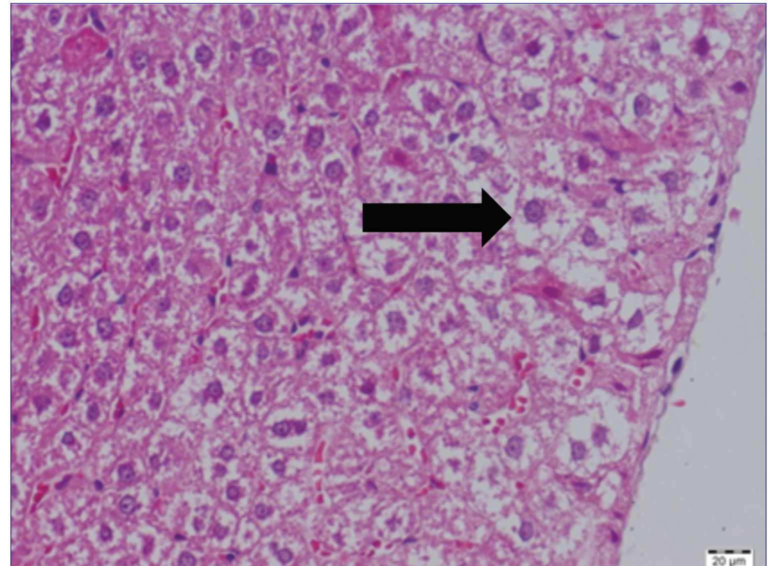


Fig 3. Severe hydropic degeneration in centrilobular area of liver *(arrow)* (alpha-amanitine receiving group)

Şekil 3. Karaciğerde sentrolobüler *(ok)* şiddetli hidropik dejenerasyon (alfa-amanitin verilen grup)

in all of investigated antioxidant parameters between alpha-amanitine and alpha-amanitine+artichoke leaf extract groups, so it was decided that the extract might have positive effects on toxicity, and it might have a protective effect on hepatocytes. Moreover, significant decreases observed in MDA and GSH levels in control and only in artichoke extract groups could indicate the antioxidant effect of artichoke. Also, statistical insignificances in especially MDA and GSH levels between control and alpha-amanitine+artichoke groups suggested the idea that artichoke might recover negative effects of the toxin. SOD, GSH-Px and CAT levels were significantly increased in alpha-amanitine group when compared with the control group, and they were significantly decreased after administration of the extract. This condition has suggested that artichoke may be a helpful treatment in this intoxication.

In conclusion, it is determined that liquid leaf extract of artichoke, which is endemically grown in our country, and had the antioxidant effect, has a healing effect on alpha-amanitine induced hepatic toxicity, and it has been decided that using artichoke as a supportive treatment will be beneficial in cases with alpha-amanitine induced hepatic toxicity.

REFERENCES

1. Flomenbaum NE, Goldfrank LR, Hoffman RS, Howland MA, Lewin NA, Nelson LS: Mushrooms. **In,** Goldfrank LR (Ed): Goldfrank's Toxicologic Emergencies. 8th edn., 1564-1569, Mc Graw-Hill Company, New York, 2006.

2. Vural N: Doğal Kaynaklı Zehirler. **In,** Vural N (Ed): Toksikoloji. 73th edn., 610-623, Ankara Üniv Ecz Fak Yayınları, Ankara, 1996.

3. Klein AS, Hart J, Brems JJ, Goldstein L, Lewin K, Busuttil RW: Amanita poisoning treatment and the role of liver transplantation. *Am J Med*, 86, 187-193, 1989. DOI: 10.1016/0002-9343(89)90267-2

4. Himmelmann A, Mang G, Schnorf-Huber S: Lethal ingestion of stored Amanita phalloides mushrooms. *Swiss Med Wkly*, 131, 616-617, 2001.

5. Nguyen VT, Giannoni F, Dubois MF, Seo SJ, Vigneron M, Kédinger C, Bensaude O: *In vivo* degradation of RNA polymerase II largest subunit triggered by alpha-amanitin. *Nucleic Acids Res*, 24, 2924-2929, 1996. DOI: 10.1093/nar/24.15.2924

6. Goldfrank LR, Flamenbaum NE, Lewin NA, Weisman RS, Hawland MA, Hoffman RS: Mushrooms: Toxic and hallusinogenic. **In,** Goldfrank LR (Ed): Goldfrank's Toxicologic Emergencies. 6th edn., 1207-1220, Mc Graw-Hill Company, New York, 1998.

7. Garcia J, Costa VM, Carvalho A, Baptista P, Pinho PG, Bastos ML, Carvalho F: Amanita phalloides poisoning: Mechanisms of toxicity and treatment. *Food Chem Toxicol*, 86, 41-55, 2015. DOI: 10.1016/j.fct.2015.09.008

8. Llorach R, Espin JC, Tomas-Barberan FA, Ferreres F: Artichoke *(Cynara scolymus* L.) byproducts as a potential source of health-promoting antioxidant phenolics. *J Agr Food Chem,* 50, 3458-3464, 2002. DOI: 10.1021/jf0200570

9. Aktay G, Deliorman D, Ergun E, Ergun F, Yesilada E, Cevik C: Hepatoprotective effects of Turkish folk remedies on experimental liver injury. *J Ethnopharmacol*, 73, 121-129, 2000. DOI: 10.1016/S0378-8741(00)00286-5

10. Speroni E, Cervellati R, Govoni P, Guizzardi S, Renzulli C, Guerra MC: Efficiency of different *Cynara scolymus* preparations liver complaints. *J Ethnopharmacol*, 86, 203- 211, 2003. DOI: 10.1016/S0378-8741(03)00076-X

11. Tanker M, Tanker N: Flavonozitler. **In,** Tanker M, Tanker N (Eds): Farmakognozi. 218-219, Ankara Üniversitesi Basımevi, Ankara, 1991.

12. Bergö M, Wu G, Ruge T, Olivecrona T: Down-regulation of adipose tissue lipoprotein lipase during fasting requires that a gene, separate from the lipase gene, is switched on. *J Biol Chem*, 277, 11927-11932, 2002. DOI: 10.1074/jbc.M200325200

13. Wieland T, Faulstich H: Amatoxins, phallotoxins, phallolysin, and antamanide: The biologically active components of poisonous *Amanita mushrooms*. *CRC Crit Rev Biochem*, 5, 185-260, 1978. DOI: 10.3109/10409237809149870

14. Mehmetcik G, Ozdemirler G, Toker NK, Cevikbas U, Uysal M: Effect of pretreatment with artichoke extract on carbon tetrachloride-induced liver injury and oxidative stress. *Ex Toxicol Pathol*, 60, 475-480, 2008. DOI: 10.1016/j.etp.2008.04.014

15. Placer ZA, Cushman L, Johnson BC: Estimation of products of lipid peroxidation (malonyl dialdeyhde) in biological fluids. *Anal Biochem*, 16, 359-364, 1966. DOI: 10.1016/0003-2697(66)90167-9

16. Sun Y, Oberley WL, Li Y: A simple method for clinical assay of superoxide dismutase. *Clin Chem*, 34, 497-500, 1988.

17. Aebi H: Catalase *in vitro*. *Methods Enzymol*, 105, 121-126, 1984. DOI: 10.1016/S0076-6879(84)05016-3

18. Matkovics B, Szabo L, Varga LS: Determination on enzyme activities in lipid peroxidation and glutathione pathways. *Laboral Diagn*, 15, 248-249, 1988.

19. Sedlak J, Lindsay RH: Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem*, 25, 192-205, 1968. DOI: 10.1016/0003-2697(68)90092-4

20. Ferguson GA, Takane Y: Repeated Measurements and Other Designs. **In,** Ferguson GA, Takane Y (Eds): Statistical Analysis in Psychology and Education. 6th edn., 347-390, McGraw-Hill Ryerson Ltd, Montreal, 2005.

21. Enjalbert F, Rapior S, Soulé JN, Guillon S, Amouroux N, Cabot C: Treatment of amatoxin poisoning: 20-year retrospective analysis. *J Toxicol Clin Toxicon,* 40, 715-757, 2002. DOI: 10.1081/CLT-120014646

22. Edwards SE, Heinrich M, Rocha I, Williamson ME: Artichoke. **In,** Edwards SE (Ed): Phytopharmacy: An Evidence-based Guide to Herbal Products. 29-31, John Wiley&Sons Ltd, Oxford, 2015.

23. Ben-Hod G, Basnizki Y, Zohary D, Mayer AM: Cynarin and chlorogenic acid content in germinanting seeds of globe artichoke *(Cynara scolymus* L.). *J Genet Breed*, 46, 63-67, 1992.

24. Gezer C, Yücecan S, Rattanc SIS: Artichoke compound cynarin differentially affects the survival, growth, and stressresponse of normal, immortalized, and cancerous human cells. *Turk J Biol*, 39, 299-305, 2015. DOI: 10.3906/biy-1407-67

25. Adzet T, Camarasa J, Carlos Laguna J: Hepatoprotective activity of polyphenolic compounds from Cynara scolymus against CCl_4 toxicity in isolated rat hepatocytes. *J Nat Prod*, 50, 612-617, 1987. DOI: 10.1021/np50052a004

26. Majeed MF, Numan IT, Noori M: Study the anti-inflammatory activity of artichoke (cynara scolymus) different extracts in experimentel models of acute inflammation in rats. *Pharmacie Globale (IJCP)*, 6, 4-5, 2015.

27. Colak E, Ustuner MC, Tekin N, Colak E, Burukoglu D, Degirmenci I, Gunes HV: The hepatocurative effects of *Cynara scolymus* L. leaf extract on carbon tetrachloride-induced oxidative stress and hepatic injury in rats. *Springerplus,* 5, 216, 2016. DOI: 10.1186/s40064-016-1894-1

28. Escrig AJ, Dragsted LO, Daneshvar B, Pulido R, Saura-Calixto F: *In vitro* antioxidant activities of edible artichoke *(Cynara scolymus* L.) and effect on biomarkers of antioxidants in rats. *J Agric Food Chem*, 51, 5540-5545, 2003. DOI: 10.1021/jf030047e

29. Küçükgergin C, Aydın AF, Özdemirler-Erata G, Mehmetcik G, Toker NK, Uysal M: Effect of artichoke leaf extract on hepatic and cardiac oxidative stress in rats. Fed on high cholesterol diet. *Biol Trace Elem Res*, 135, 264-274, 2010. DOI: 10.1007/s12011-009-8484-9

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Short Communication

Molecular Detection of *Theileria equi* and *Babesia caballi* Infections in Horses by PCR Method in Iran

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Abstract

The aim of the current study was to determine the prevalence of *Theileria equi* and *Babesia caballi* infections in horses in the Central and Southwest, Iran. Blood samples were collected from 53 and 37 horses settled in Isfahan and Shahrekord, respectively and a PCR method was used to detect the parasites in blood samples. The results showed that *Theileria equi* detected in 6 horses in Isfahan and in 4 horses in Shahrekord. Based on the findings, the prevalence of equine theileriosis was much higher than babesiosis and it occurred in both Isfahan and Shahrekord regions of the country. To the authors' knowledge, this is the first report of the molecular survey of *Theileria equi* and *Babesia caballi* infections in horses in Iran. This survey could provide further information on different parasitic infections in horses and its epidemiology.

Keywords: *Horses, Babesia, PCR, Theileria equi, Iran*

İran'daki Atlarda *Theileria equi* ve *Babesia caballi* Enfeksiyonlarının PCR Yöntemi İle Moleküler Tayini

Özet

Bu çalışma, İran'ın Orta ve Güneybatısındaki atlarda *Theileria equi* ve *Babesia caballi* enfeksiyonlarının sıklığını belirlemek amacıyla yapıldı. İsfahan ve Shahrekord'ta yaşayan atlardan sırasıyla 53 ve 37 adet kan numunesi toplandı ve kan örneklerinde parazitleri tespit etmek için bir PCR yöntemi kullanıldı. Sonuçlar, İsfahan'ki 6 at ile Shahrekord'taki 4 atta *Theileria equi* tespit edildiğini gösterdi. Bulgulara göre, at Theileriosis'in prevalansı babeziyoz'a göre çok daha yüksekti ve ülkenin hem İsfahan hem de Shahrekord bölgelerinde oluştu. Yazarların bildiği kadarıyla, bu çalışma *Theileria equi* ve *Babesia caballi* enfeksiyonların İran'daki atlarda moleküler olarak araştırıldığı ilk bildirimdir. Bu çalışma, atlardaki farklı parazit enfeksiyonları ve epidemiyolojisi hakkında ayrıntılı bilgi sağlayabilir.

Anahtar sözcükler: *Atlar, Babesia, PCR, Theileria equi, İran*

INTRODUCTION

The *Theileria* species infect a wide range of both domestic and wild animals and are transmitted by ixodid ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus*. Most of these ticks are renowned for the large economic losses they cause to the agricultural industry due to disease outbreaks, mortalities, damage to hide and poor production in domestic animals [1]. The genus *Theileria* is distinguished by infection of leukocytes by sporozoites, maturation of schizonts into merozoites and subsequent infection of red blood cells to form piroplasms [2].

Theileria equi is a protozoan of the phylum Apicomplexa that is biologically transmitted by ixodid ticks. It causes disease in equids characterized by fever, anemia, icterus, hepatosplenomegaly, intravascular hemolysis, hemoglobinuria and in some cases death can occur [3]. The

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disease has a worldwide distribution and is endemic in most tropical and subtropical areas as well as in some temperate zones of the world [3-6]. It has caused important economic losses in the horse industry, being a serious threat to the horse raising industry and international movement of horses [7].

Theileria equi and *Babesia caballi* is tick-borne haemo-parasites that may cause babesiosis of Equidae. Equine piroplasmosis is an economically important tick-borne protozoan disease of horses that has been reported worldwide [3]. In southern Europe *T. equi* is enzootic and infections may occur asymptomatically and more frequently than those due to *B. caballi* [8,9]. *Babesia* parasites destroy host erythrocytes and induce fever, anemia, and icterus in infected horses [10].

Theileria equi is a tick-borne heamoprotozoan parasite, which causes equine babesiosis [11]. The disease is endemic in most tropical and subtropical regions of the world, including Europe, Asia, Africa, America and Australia continents [12,13]. The diseased animal is characterized by anemia, fever, hemoglobinuria, icterus and in some cases death can occur [3].

Babesia caballi, like *T. equi*, is a tick-borne protozoan parasite, which causes fever, anemia, jaundice, and edema in the infected horses and sometimes results in death [3,14-16]. These equine babesioses are known to induce significant economic losses in the horse industry [6,16]. These parasites are usually detected in blood smears during the acute stage of infection but not easily in those of the recovered animals that remain carriers of the parasite [17]. Clinical signs are not specific diagnostic measures for babesiosis, especially in asymptomatic or mixed infection in endemic areas [9].

Therefore, detection of *T. equi* and *B. caballi* may be useful for developing effective strategies to treat and control different infections in horses. The diagnosis is routinely done by conventional parasitological techniques like Giemsa stained thin blood smear. Giemsa stained blood smear examination is not a sensitive method to demonstrate parasites in the blood mainly because of the periodically cryptic nature of the parasite. Recently, Polymerase chain reaction (PCR) is considered to be superior to parasite detection and antigen detection tests due to its sensitivity in detecting the prepatent and chronic phase of an infection [13,18]. The lack of molecular data severely hampers our efforts in studying molecular epidemiology of *T. equi* and *B. caballi*. Hence, the objective of this study was to determine the prevalence of *T. equi* and *B. caballi* infections in horses from Isfahan Central and Shahrekord Southwest, Iran using PCR molecular method.

MATERIAL and METHODS

Samples: This cross-sectional study was conducted between February and December, 2014. A total of 53 and 37 horses settled in Isfahan Central and Shahrekord Southwest, Iran, respectively, were randomly selected. The breed, sex and age of each of the horses was noted and appropriately recorded. Blood sample was collected by vein puncture from the external jugular vein from each of the horse using anticoagulant [ethylenediamine tetraceticacid (EDTA)]-containing vacutainer. The samples were transported aseptically in ice packs to the Biotechnology Research Center, and stored at -20°C until needed.

DNA Extraction: Genomic DNA in the blood samples were extracted using DNA extraction kit (Cinnagen, Tehran, Iran) following the manufacturer's instruction. Concentration of extracted DNA from each blood sample was measured spectro-photometrically at 260 nm optical density following the method described by Sambrook and Russell [19]. Extracted DNA samples were kept frozen at -70°C until needed.

Polymerase Chain Reaction: Polymerase chain reaction was performed on the genomic DNA of *T. equi* and *B. caballi*. The primers used for amplification are as shown in *Table 1*. The amplification of *Theileria equi* and *Babesia caballi* DNA was done using thermocycler (Eppendorf, Hamburg, Germany). Polymerase chain reaction products were run using 1.5% agarose gel in 1X TBE buffer at 80 V for 30 min, stained with ethidium bromide and the images were visualized in UVIdoc gel documentation systems (Uvitec, UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany).

RESULTS

Overall Prevalence

The PCR products for *T. equi* (241 bp) and *B. caballi* (180

Table 1. *Primer sequence used for detection of Theileria equi and Babesia caballi genes in horse's blood*
Tablo 1. *At kanındaki Theileria equi ve Babesia caballi genlerinin tespiti için kullanılan primer dizisi*

Organism	Primers Sequences	GenBank Accession Numbers	Annealing Temperature	Size (base pair)
Theileria equi	F: 5´ - GAGGAGCACATCGTCTACACTG - 3´	KC347577	60°C	241 bp
	R: 5´ - ACAAGACCTCCTGGTAGAACTCG-3´			
Babesia caballi	F: 5´ - CGGCTGCTATGGTTATTCAG - 3´	AB017700	60°C	180 bp
	R: 5´ - AGAGTGCAACCGAGCAATGC-3´			

F: *Forward;* ***R:*** *Reverse*

bp) were identified by 100 bp DNA size marker (Fermentas, Germany). A total of 53 samples from Isfahan and 37 from Shahrekord were collected, of these the results of the PCR assays showed that *T. equi* detected in 6 horses in Isfahan and in 4 horses in Shahrekord. In addition, 5 horses were infected by *B. caballi* in each states, Isfahan and Shahrekord. Differences in infection rates were statistically non significant between male and female horses and among different age groups *(Table 2, Table 3)*.

Prevalence According to Breed: The distribution of *T. equi* and *B. caballi* in different horse breeds in Isfahan and Shahrekord is indicated in *Table 4*. The result showed that of the 6 *T. equi* detected in Isfahan 2 were from Standardbred, 3 from Thoroughbred and 1 from Arab on the other hand in Shahrekord out of 4 identified *T. equi,* 1 were from Standardbred, 2 from Thoroughbred and 1 from Arab. In addition, of the 5 identified *B. caballi* in each states Isfahan and Shahrekord, 1 were from Standardbred, 2 from Thoroughbred, 1 from Arab and 1 also from Turkoman.

DISCUSSION

In the present study, the occurrence of *T. equi* infection in horses from Isfahan and Shahrekord was investigated by PCR. The results demonstrate that *T. equiis* widespread in the region studied, suggesting high levels of transmission, as there was found a high rate of positive horses by molecular methods. This is probably related to the prevalence and intensity of tick infestation in this region. Arslan et al.[20] and Friedhoff [21] reported that *Dermacentor marginatus* known to transmit *B. equi*. In addition, another study conducted in Hungary showed that the prevalence of *T. equi* infection among 101 horses was 49% with PCR [22]. These differences may be related to management practices and due to a difference in the prevalence of tick vector for *B. equi* between countries, where climatic factors such as temperature, humidity and rainfall influence the habitat for ticks.

Theileria equi is more common and pathogenic than *B.*

Table 2. *Distribution of Theileria equi and Babesia caballi among equine population in Isfahan and Shahrekord*
Tablo 2. *İsfahan ve Shahrekord'taki at nüfusu içinde Theileria equi ve Babesia caballi Dağılımı*

Sex	Number of Samples Collected		Number (%) of Horses Infected			
			Theileria equi		*Babesia caballi*	
	Isfahan	Shahrekord	Isfahan	Shahrekord	Isfahan	Shahrekord
Stallion	22	23	1 (4.5)	3 (13.0)	1 (4.5)	2 (8.7)
Mare	31	14	5 (16.1)	1 (7.1)	4 (12.9)	3 (21.4)
Total	53	37	6 (20.6)	4 (20.1)	5 (17.4)	5 (30.1)

Table 3. *Distribution of Theileria equi, Theileria evansi, Babesia caballi, and Habronema among different age groups of horses in Isfahan and Shahrekord*
Tablo 3. *İsfahan ve Shahrekord'taki farklı yaş gruplarındaki atlar arasında Theileria equi, Theileria evansi, Babesia caballi ve Habronema Dağılımı*

Age	Number of Samples Collected		Number (%) of Horses Infected			
			Theileria equi		*Babesia caballi*	
	Isfahan	Shahrekord	Isfahan	Shahrekord	Isfahan	Shahrekord
<1	7	3	1 (14.3)	0 (0)	0 (0)	1 (33.3)
1-2	11	4	1 (9.1)	0 (0)	0 (0)	1 (25.0)
2-3	11	6	1 (9.1)	1 (16.7)	1 (9.1)	1 (16.7)
>3	24	24	3 (12.5)	3 (12.5)	4 (16.7)	2 (8.3)
Total	53	37	6 (11.3)	4 (10.8)	5 (9.4)	5 (13.5)

Table 4. *Distribution of Theileria equi, Theileria evansi, Babesia caballi, and Habronema in different horse breeds in Isfahan and Shahrekord*
Tablo 4. *İsfahan ve Shahrekord'taki farklı at ırklarında Theileria equi, Theileria evansi, Babesia caballi ve Habronema Dağılımı*

Breed	Number of Samples Collected		Number (%) of Horses Infected			
			Theileria equi		*Babesia caballi*	
	Isfahan	Shahrekord	Isfahan	Shahrekord	Isfahan	Shahrekord
Standardbred	18	13	2 (11.1)	1 (7.7)	1 (5.6)	1 (7.7)
Thoroughbred	21	15	3 (14.3)	2 (13.3)	2 (9.5)	2 (13.3)
Arab	7	7	1 (0)	1 (0)	1 (0)	1 (0)
Turkoman	7	2	0 (0)	0 (0)	1 (0)	1 (0)
Total	53	37	6 (11.3)	4 (10.8)	5 (9.4)	5 (13.5)

caballi in endemic countries [23-25]. The results of the present study demonstrated that *T. equi* was more prevalent than *B. caballi*. Our findings were in agreement with the previous study in Iran [26]. A possible reason for the low prevalence of *B. caballi* could be associated with the earlier removal of the parasite after a short term of infection [27].

The results of molecular and microscopic examinations confirmed the simultaneous infection of horses in the study region with both equine Babesia species, which was consistent with findings of Seifi et al.[26] and Abedi et al.[28]. They reported mixed infection of *T. equi* and *B. caballi* in horses of Turkmen region in Iran. Results indicated that even subclinical and latent carrier infections diagnosed by molecular means are responsible for inducing pathogenicity. In our study no differences were observed between the *T. equi* and *B. caballi* prevalence in all age and sex groups of the horse examined. It may be due to high number of ticks in this area and continuous exposure of young and old horses to infected ticks [29].

The present study suggests the existence of *T. equi* and *B. caballi* in different horse breeds in Isfahan and Shahrekord. Thus, our results indicate that *T. equi* occurs more frequently than *B. caballi* in the investigated geographical region. However, no diversity was observed among the isolates within the studied regions. The detection of the pathogenic species of *T. equi* and *B. caballi* in asymptomatic horses indicates that the relationship between parasite species/subspecies and clinical signs of infection in horses deserves further investigation. We further recommend investigating the prevalence of *T. equi* and *B. caballi* in other domestic animals, like sheep and goats, living in the same environment.

Conflict of Interest

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

Authors' Contributions

All authors contributed equally to this work. All authors read and approved the final manuscript.

REFERENCES

1. Bishop R, Musoke A, Morzaria S, Gardner M, Nene V: *Theileria*: Intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. *Parasitol* 129, S271-S283, 2004. DOI: 10.1017/S0031182003004748

2. Uilenberg G: Babesia - A historical perspective. *Vet Parasitol*, 138, 3-10, 2006. DOI: 10.1016/j.vetpar.2006.01.035

3. Schein E: Equine babesiosis. **In,** Ristic M (Ed): Babesiosis of Domestic Animals and Man. 197-208, CRS Press, Boca Raton, Florida, 1988.

4. De Waal DT: Equine piroplasmosis: A review. *Br Vet J*, 148, 6-14, 1992. DOI: 10.1016/0007-1935(92)90061-5

5. Brüning A: Equine piroplasmosis an update on diagnosis, treatment and prevention. *Br Vet J*, 152, 139-151, 1996. DOI: 10.1016/S0007-1935(96)80070-4

6. Kizilarslan F, Yildirim A, Duzlu O, Inci A, Onder Z, Ciloglu A: molecular detection and characterization of *Theileria equi* and *Babesia caballi* in horses *(Equus ferus caballus)* in Turkey. *J Equine Vet Sci*, 35, 830-835, 2015. DOI: 10.1016/j.jevs.2015.08.002

7. Friedhoff KT, Tenter AM, Muller I: Haemoparasites of equines: Impact on internacional trade of horses. *Rev Sci Tech*, 9, 1187-1194, 1990.

8. Beck R, Vojta L, Mrljak V, Marinculić A, Beck A, Zivicnjak T, Cacciò SM: Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. *Int J Parasitol*, 39, 843-848, 2009. DOI: 10.1016/j.ijpara.2008.12.005

9. Bashiruddin JD, Camma C, Revelo E: Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. *Vet Parasitol*, 84, 75-83, 1999. DOI: 10.1016/S0304-4017(99)00049-7

10. Knowles DP: Control of *Babesia equi* parasitemia. *Parasitol Today*, 12, 195-198, 1996. DOI: 10.1016/0169-4758(96)10007-7

11. Holbrook AA, Johnson AJ, Madden PA: Equine piroplasmosis: Intra-erythrocyte development of *Babesia caballi* (Nuttal) and *Babesia equi* (Laveran). *Am J Vet Res*, 29, 297-303, 1968.

12. Kuttler KL: Chemotherapy of babesiosis. **In,** Ristic M (Ed): Babesiosis of Domestic Animals and Man. 227-243, CRC Press, Boca Raton, Florida, 1988.

13. Khamesipour F, Doosti A, Koohi A, Chehelgerdi M, Mokhtari-Farsani A, Chengula AA: Determination of the presence of *Babesia* species in blood samples of cattle, camel and sheep in Iran by PCR. *Arch Biol Sci*, 67, 83-90, 2015. DOI: 10.2298/ABS140410009K

14. Purnell RE: Babesiosis in various hosts. **In,** Ristic M, Krier JP (Eds): Babesiosis. 25-64, Academic Press, New York, 1981.

15. Friedhoff KT: The piroplasms of equidae. Significance for the international equine trade. *Berl Muench Tieraerztl Wochenschr*, 95, 368-374, 1982.

16. Brüning A, Phipps P, Posnett E, Canning EU: Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. *Vet Parasitol*, 58, 11-26, 1997. DOI: 10.1016/S0304-4017(96)01074-6

17. Holbrook AA: Biology of equine piroplasmosis. *Am J Vet Med Assoc*, 155, 453-454, 1969.

18. Brun R, Hecker H, Lum ZR: Trypanosoma evansi and *T. equiperdum*: Distribution, biology, treatment and phylogenetic relationship (a review). *Vet Parasitol,* 79, 95-107, 1998. DOI: 10.1016/S0304-4017(98)00146-0

19. Sambrook J, Russell DW: Molecular cloning: A laboratory manual. 10-120, Cold Spring Harbor Laboratory Press, New York, 2001.

20. Arslan MÖ, Umur Ş, Aydın L: Kars yöresi sığırlarında Ixodidae türlerinin yaygınlığı. *Türkiye Parazitol Derg*, 23, 331-335, 1999.

21. Friedhoff KT: Transmission of Babesia. **In,** Ristic M (Ed): Babesiosis of Domestic Animals and Man. 23-52, CRC Press, Boca Raton, Florida. 1988.

22. Farkas R, Tánczos B, Gyurkovszky M, Földvári G, Solymosi N, Edelhofer R, Hornok S: Serological and molecular detection of *Theileria equi* infection in horses in Hungary. *Vet Parasitol*, 192, 143-148, 2013. DOI: 10.1016/j.vetpar.2012.09.035

23. De Waal DT: Distribution, transmission and serodiagnosis of Babesia *equi* and Babesia *caballi* in South Africa. *PhD Thesis,* University of Pretoria, 1995.

24. De Waal DT: Global importance of piroplasmosis. *J Protozool Res*, 10, 106-127, 2000.

25. Zweygarth E, Lopez-Rebollar LM, Nurton J, Guthrie AJ: Culture, isolation and propagation of *Babesia caballi* from naturally infected horses. *Parasitol Res*, 88, 460-462, 2002. DOI: 10.1007/s00436-002-0609-4

26. Abedi V, Razmi GH, Seifi H, Naghibi A: Seroprevalence of Theileria equi and Babesia caballi infection in Turkoman breed horses in Iran. *IJVST*, 5 (2): 45-52, 2013.

27. Salim BO, Hassan SM, Bakheit MA, Alhassan A, Igarashi I, Karanis P, Abdelrahman MB: Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR. *Parasitol Res*, 103, 1145-1150, 2008. DOI: 10.1007/s00436-008-1108-z

28. Seifi HA, Mohri M, Sardari K: A mixed infection of *Babesia equi* and *Babesia caballi* in a racing colt: A report from Iran. *J Equine Vet Sci*, 20, 858-860, 2000. DOI: 10.1016/S0737-0806(00)80117-3

29. Razmi GR, Naghibi A, Aslani MR, Fathivand M, Dastjerdi K: An epidemiological study on ovine babesiosis in the Mashhad suburb area, province of Khorasan, Iran. *Vet Parasitol*, 108, 109-115, 2002. DOI: 10.1016/S0304-4017(02)00203-0

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Short Communication

Some Physiological, Hematological Values and ANAE-Positive Lymphocyte Rations of Domestic Donkeys *(Equus asinus)* in Kyrgyzstan

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Abstract

The aim of this study was to determine the physiological, hematological parameters and ANAE positivity of donkeys in Kyrgyzstan. Animals (n=24) were clinically examined and blood samples were taken. The average pulsation and respiration rates of male and female donkeys were measured as 52.25±9.27; 49.16±4.80 beats/minute and 18.41±4.21; 18.58±3.30 breaths/minute, respectively. HGB, MCV, MCH and MCHC values did not different in the groups. RBC and WBC values were higher in females. Mean ANAE-positive PBL ratio of donkeys was found as 42.90±1.18%. Consequently, some physiological, hematological values and ANAE-profile were determined and advised as reference values of donkeys in Kyrgyzstan.

Keywords: *Physiology, Hematology, ANAE- positivity, Donkey, Kyrgyzstan*

Kırgızistan'da Barındırılan Evcil Eşeklerde *(Equus asinus)* Bazı Fizyolojik ve Hematolojik Değerler ile ANAE Pozitif Lenfosit Oranının Belirlenmesi

Özet

Bu çalışmanın amacı, Kırgızistandaki eşeklerin fizyolojik, hematolojik değerleriyle ANAE-pozitiflik oranının belirlenmesidir. Hayvanların (n=24) genel klinik muayenesi yapıldı ve kan örnekleri alındı. Erkek ve dişi eşeklerin ortalama pulzasyon ve respirasyon oranları sırasıyla, 52.25±9.27; 49.16±4.80 atım/dk ve 18.41±4.21; 18.58±3.30 solunum/dk olarak ölçüldü. HGB, MCV, MCH ve MCHC değerlerinde gruplar arasında bir fark bulunamadı. RBC ve WBC değerleri dişilerde daha yüksek saptandı. Eşeklerin ANAE-pozitif PBL oranı %42.90±1.18 olarak belirlendi. Sonuç olarak, Kırgızistanda yaşayan eşeklere ait bazı fizyolojik, hematolojik ve ANAE-pozitiflik oranı belirlenmiş ve referans değer olarak sunulmuştur.

Anahtar sözcükler: *Hematoloji, ANAE pozitifliği, Eşek, Kırgızistan*

INTRODUCTION

Determination of the normal physiological, hematological and biochemical parameters help evaluating the clinical prognosis of many animal diseases [1,2]. In addition, alpha-naphthyl acetate esterase (ANAE) staining has been used as a useful tool to differentiate T and B lymphocytes and monocytes in some certain species including many

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animals and thought to be responsible for the cytotoxic effects of T lymphocytes [3,4].

The aim of this study was to determine the normal physiological and hematological parameters and ANAE positivity profile of donkey which live and freely fed in Tong region of Kyrgyzstan, did not referred before, and present the reference values.

MATERIAL and METHODS

Animal Selection and Sample Collection Procedure

The animals in different ages and weights (143-170 kg) were selected living in Tong region, Kyrgyzstan (42°18›32.41» N; 76°17›10.33» E, 5350 ft). Donkeys (male, n=12 and female, n=12) were divided in to three groups according to their ages. They were 6-24 months aged (4 male, 7 female), 2-15 years aged (6 male, 3 female) and >15 years aged (2 male, 2 female). The study was done in November and average weather temperature was recorded between minus 6±2.5°C and plus 12±2.0°C during all days according to weather forecast [5].

In order to detect healthy condition, general clinical examination methods were used and blood samples were taken.

Physiological and Hematological Parameters

Donkey's rectal body temperatures were measured by digital thermometers (DIGI-TEMP, Kruuse). After the reaching comfort (15 to 30 min), respiration was noted and pulsation rates also measured by statoscopes.

Blood samples were collected by jugular vein via needle (1.2 mm X 38 mm) to heparinized tubes. Red blood cell (RBC) count, white blood cell count (WBC), hemoglobin (HGB), hematocrit (HCT), blood clot cell count (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) values were measured with Auto Hematology Analyzer (Mindray BC-5300, China).

ANAE Demonstration and Evaluation

Air dried smears were fixed in phosphate buffered glutaraldehyde-acetone solution (pH 4.8) at -10°C for 3 min. ANAE demonstration was performed by according to Ozaydın et al.[6] The cells with lymphocyte morphology and having 1-3 large, reddish-brown granules were classified as ANAE-positive lymphocytes *(Fig. 1)* under the Nikon Eclipse 50i light microscope (Japan) by counting 200 lymphocytes.

Statistical Analysis

Data were analyzed using SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA) using sample t-tests to compare within groups and one way a nova 3 independent sample were used to compare between-group differences. A P-value <0.05 was considered statistically significant.

RESULTS

Physiological and Hematological Parameters

The average body temperature, pulsation values and respiration rates were determined as 37.60±0.62°C, 52.25±9.27 beats/minute, 18.41±4.21 breaths/minute for males and 37.03±1.34°C, 49.16±4.80 beats/minute and 18.58±3.30 breaths/minute for females, respectively. Similarly, RBC, WBC, HGB, HCT PLT, MCV, MCH and MCHC were analyzed for both sexes and statistical difference was not found within the groups *(Table 1, Table 2)*.

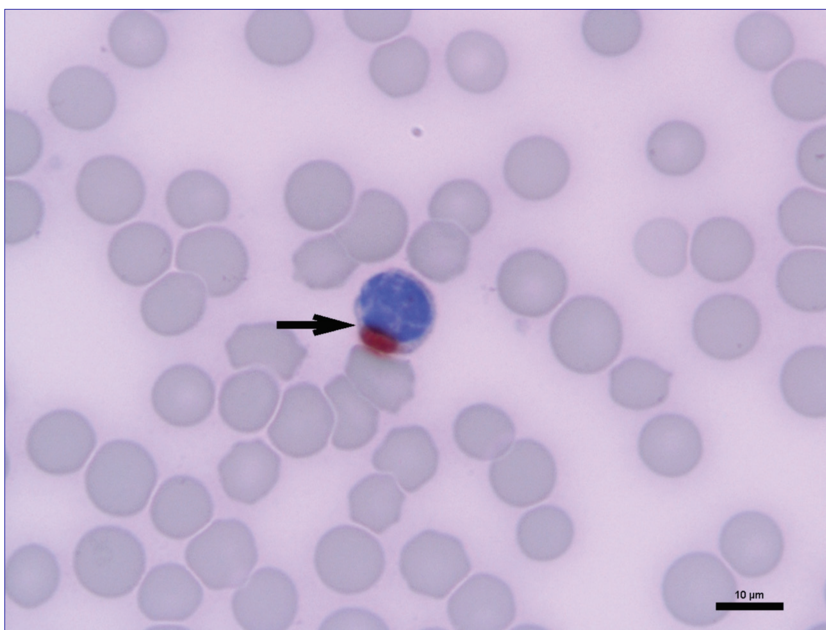

Fig 1. ANAE-positive lymphocyte in the peripheral blood of donkey *(arrow),* ANAE demonstration, Barr: 10 µm

Şekil 1. Eşek periferik kanında ANAE pozitif lenfosit *(ok),* Bar: 10 µm

***Table 1.** The average blood parameters of male donkeys in different ages*
***Tablo 1.** Farklı yaştaki erkek eşeklerde ortalama kan parametreleri*

Age	Male (n=12)							
	RBC (10^{12}/L)	WBC (10^9/L)	PLT (10^9/L)	HCT (%)	HGB (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
6-24 months aged	4.23	10.98	165.2	28.6	10.6	52.7	22.3	35.0
2-15 years aged	5.18	11.28	119.8	30.0	10.4	55.1	21.3	34.8
>15 years aged	5.42	10.37	158.5	29.8	11.1	57.8	20.0	35.7

***Table 2.** The average blood parameters of female donkeys in different ages*
***Tablo 2.** Farklı yaştaki erkek eşeklerde ortalama kan parametreleri*

Age	Female (n=12)							
	RBC (10^{12}/L)	WBC (10^9/L)	PLT (10^9/L)	HCT (%)	HGB (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
6-24 months aged	5.24	11.8	137.1	28.4	9.98	54.8	19.0	35.1
2-15 years aged	5.04	11.6	122.6	30.3	10.4	60.1	19.7	34.5
>15 years aged	5.41	11.75	121.5	31.9	11.65	59.6	18.85	36.4

ANAE Positivity

Mean ANAE-positive PBL *(Fig. 1)* ratio of donkeys was found as 42. 90±1.18%.

DISCUSSION

The average body temperature, pulsation values and respiration rates were determined as 37.60±0.62°C, 52.25±9.27 beats/minute, 18.41±4.21 breaths/minute for males and 37.03±1.34°C, 49.16±4.80 beats/minute and 18.58±3.30 breaths/minute for females, respectively. There was no statistical difference in the physiological parameters between and within the groups ($P>0.05$). Body temperature for many mammals under the normal conditions is regulated around 36-37°C [7,8]. The pulsation rate varies with age, size, and weight, activity or heavy physical work, excitement, anger and drug adminstration [9]. In our study, respiration rates of young male donkeys (6-24 months aged) was determined higher than the other groups but no statistical difference was found ($P<0.05$). Respiration rate can be changed according to the body weight, age, exercise, excitement environmental temperature, pregnancy, gastrointestinal fullness and diseases [10].

Many of our hematological data were supported by Laus et al.[11] WBC values were detected higher in female donkeys than males in our study similar with Babeker and Abdalbagi [12]. WBC values can be affected some intrinsic and extrinsic factors [13]. PLT and HGB values were determined higher in older groups (Above 15 years) in the present study. It was found that altitude is the most important factor affecting the reference value of the RBC and hematocrit [14].

MCV concentrations were observed higher in female groups than males. In contrary, MCH values were founded higher in male groups than females in our study. Besides, MCV and MCH values were determined higher in older donkeys (above 15 years) in other studies [2,11]. MCHC values were determined same between male and female age groups in our study. Hence many factors such as environmental condition, diet, fasting, drugs administration might be affected on our different datas [12,15].

Although there is no knowledge of whether ANAE positivity is specific for T-lymphocytes of the donkeys in Kyrgyzstan our histochemical results may be beneficial for further immunological and functional studies.

Consequently, the average body temperature, the average pulsation values, the average respiration rates, some hematological values and ANAE-profile was determined and advised as reference values in domestic donkeys *(Equus asinus)* in Tong region of Kyrgyzstan.

REFERENCES

1. Roubies N, Panousis N, Fytianou A, Katsoulos PD, Giadinis N, Karatzias H: Effects of age and reproductive stage on certain serum biochemical parameters of Chios sheep under Greek rearing conditions. *J Vet Med A Physiol Pathol Clin Med,* 53, 277-281, 2006. DOI: 10.1111/j.1439-0442.2006.00832.x

2. Etana KM, Jenbere TS, Bojia E, Negussie H: Determination of reference hematological and serum biochemical values for working donkeys of Ethiopia. *Vet Res,* 4, 90-94, 2011. DOI: 10.3923/vr.2011.90.94

3. Catowsky D: Practical Hematology, Churchill Livingstone, Edinburgh, UK, 1991.

4. Sur E, Celik I, Oznurlu Y, Aydin MF, Sen I, Ozparlak H: Enzyme histochemistry and AgNOR numbers in the peripheral blood leukocytes of 6 month-old Kangal bred Anatolian shepherd dogs. *Rev Med Vet,* 154, 591-598, 2003.

5. Gismeteo Weather Forecast: 2013. www.gismeteo.ru/diary/5208/2013/11/, *Accessed:* 20.01.2016.

6. Ozaydın T, Çelik İ, Sur E, Öznurlu Y, Uluışık D: Cytochemistry of peripheral blood leukocytes in thorougbred foals. *Biotec Histochem,* 88, 295-301, 2013. DOI: 10.3109/10520295.2013.782067

7. White CR, Phillips NF, Seymour RS: The scaling and temperature dependence of vertebrate metabolism. *Biol Letters,* 22, 125-127, 2006. DOI: 10.1098/rsbl.2005.0378

8. Clarke A, Rothery P: Scaling of body temperature in mammals and birds. *Funct Ecol,* 22, 58-67, 2008. DOI: 10.1111/j.1365-2435.2007.01341.x

9. Hughey M: Nursing Fundamentals II. 100th edn., Medical Education Division, Brookside Associates Publishing, San Antonio, Texas, 2007.

10. Reece WO: *Respiration* in *mammals*. **In,** Reece WO (Ed): *Dukes*' Physiology of Domestic *Animals*, 12th edn., Cornell University Press, Ithaca, NY, 2004.

11. Laus F, Faillace V, Serri E: Reference values for hematological and biochemical parameters of mixed breed donkeys *(Equus asinus)*. *Wulfenia*, 22, 294-304, 2015.

12. Babeker EA, Abdalbagi YM: Hematological profile of donkey *(Equus asinus)* breed in White Nile State, Sudan. *Univ Bakht Arruda Sci J,* 10, 1858-6139, 2014.

13. Carel RS, Eviatar J: Factors affecting leukocyte count in healthy adults. *Prev Med,* 14, 607-619, 1985. DOI: 10.1016/0091-7435(85)90081-7

14. Miao G, Qingsheng Y, Zhiyuan R, Hongxian Z, Yanfang Z, Shuyan Y: Reference value of presenile human hematocrit and geographical factors. *J Clin Lab Anal,* 16, 26-29, 2002. DOI: 10.1002/jcla.2051

15. Vecerek V, Strakova E, Suchy P, Voslarova E: Influence of high environmental temperature on production and haematological and biochemical indexes in broiler chickens. *Czech J Anim Sci,* 47, 176-182, 2002.

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Short Communication

Influence of *in ovo* Inoculation of Probiotic Strains on the Jejunal Goblet Cell Counts and Morphometry in Peri- and Post-hatching Chicks

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Abstract

The objective of the present study was to evaluate the effects of *in ovo* inoculation of different probiotic strains (*Bacillus subtilis, Enterococcus faecium*, and *Pediococcus acidilactici*) on jejunal goblet cells counts and morphometry in chicken. Probiotics were inoculated into the amniotic fluid of 480 eggs (day 17 of incubation), with four treatments and five replicates. At days 21 of incubation and 3 post-hatch, counts of goblet cells were 30% and 16% higher in the jejunum of group inoculated *Bacillus subtilis* as compared with the control group, respectively. Inoculation of *Enterococcus faecium,* and *Pediococcus acidilactici* had no effect (P>0.05) on goblet cells counts. Inoculation of *Bacillus subtilis* and *Pediococcus acidilactici* resulted in an increase of villus height, a decrease in crypt depth and a decrease in ratio of villus height to crypt depth compared with the control group (P>0.05), at days 8 and 28 of age. As a conclusion, various effects of different probiotic strains on goblet cells count and intestinal morphometry were observed. Among probiotic strains evaluated in this study, *Bacillus subtilis* has higher benefit effect on goblet cells counts in the early of life and morphometry of jejunum.

Keywords: *Incubation, Intestine, Morphology, Goblet cell, Probiotic*

Kuluçkadan Çıkış Öncesi ve Sonrası Civcivlerde Probiyotik Suşlarının *in ovo* Inokulasyonunun Jejunal Goblet Hücre Sayısı ve Morfometrisi Üzerine Etkisi

Özet

Bu çalışmanın amacı tcivcivlerde değişik probiyotik suşlarının (*Bacillus subtilis, Enterococcus faecium*, ve *Pediococcus acidilactici*) *in ovo* inokulasyonunun jejenum goblet hücre sayısı ve morfometri üzerine etkilerini araştırmaktır. Probiyotikler beş tekrar olmak üzere dört farklı uygulama olarak (uygulamanın 17. günü) 480 yumurtanın amniyotik sıvısı içine inokule edildi. Inokulasyonun 21. günü ve 3 post-yumurtadan çıkma, jejenum goblet hücre sayıları kontrol grubu ile karşılaştırıldığında *Bacillus subtilis* inokule edilenlerde sırasıyla %30 ve %16 daha yüksekti. *Enterococcus faecium* ve *Pediococcus acidilactici* inokulasyonlarının goblet hücre sayıları üzerine etkisi gözlenmedi (P>0.05). *Bacillus subtilis* ve *Pediococcus acidilactici* inokulasyonlarının 8 ve 28. günlerde villus boyunu artırdığı, kript derinliği ile villus boyu: Kript derinliği oranını ise kontrol grubuyla karşılaştırdığında azalttığı (P>0.05) belirlendi. Sonuç olarak, farklı probiyotik suşlarının goblet hücre sayıları ve barsak morfometrisi üzerine etkileri gözlemlendi. Çalışmada denenen probiyotik suşlarından *Bacillus subtilis'*in erken yaşta goblet hücre sayısı ve jejenum morfometrisi üzerine daha fazla yararlı etkiler sunduğu belirlendi.

Anahtar sözcükler: *İnkubasyon, Barsak, Morfoloji, Goblet hücresi, Probiyotik*

INTRODUCTION

In the modern poultry production, the contact between newborn chicks and hens is excluded, and colonization of bacteria in the gut depends on the type of bacteria present in the hatchery environment [1]. This condition exposes chicks to pathogenic bacteria colonization in the gut and causes a delay in desirable bacteria colonization [2]. The first contact of chicks with hatchery environment may include pathogen bacteria and leave gut colonization

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open to them, so it is necessary to inoculate desirable bacteria named probiotic. Different routes of early delivery of probiotics were examined, for example *in ovo* inoculation, immersion of eggs in probiotic medium, oral gavage, vent lip, and spraying of chick with probiotic solution [3-6]. In the previous studies that examined *in ovo* administration, probiotic solutions were injected into the air cell of eggs. This route of inoculation results in low hatchability. Chicken embryo swallow amniotic fluid during the last period of incubation; therefore, intra-amniotic inoculation of probiotic strains enables chicks to received desirable bacteria in the early of life without affecting the hatchability rate. Moreover, to our knowledge, in the literature the effect of intra-amniotic inoculation of different probiotic strains on intestinal characteristics of chicks was not evaluated. Therefore, the main objective of this study was to evaluate the effects of *in ovo* inoculation of different probiotic strains on the goblet cells counts and jejunal morphometry of broiler chicken at the peri- and post-hatch periods.

MATERIAL and METHODS

Chicks used in this study received human care based on criteria outlined in the Guide for the Care and Use of Laboratory Animals [7], and the experimental protocol was approved by the Research Committee of Islamic Azad University, Science and Research Branch (Approval date: 05.05.2013; No: 23874).

Fertile Cobb chicken eggs were obtained from a commercial hatchery from a flock with the 38 weeks of age. Eggs were incubated in a single-stage setter under the same condition of 37.6°C and 60% relative humidity and turned once per h. On day 17 of incubation, eggs with live embryo (No: 480, average weight of 58±1.1 g) were selected and weighed. In a completely randomized design, eggs were assigned to four experimental groups and five replicates of twenty four eggs per each. The four treatment groups that received *in ovo* 0.5 mL of sterile distilled water or probiotic mediums (10^7 cfu) into the amniotic fluid were: 1) sterile distilled water as control group, 2) B*acillus subtilis*, 3) *Enterococcus faecium* and 4) *Pediococcus acidilactici*. The *in ovo* inoculation procedure was performed as described by Tako et al.[8]. Solution was inoculated with a suitable needle inserted into the amniotic fluid, which was identified by candling. After inoculation, the hole in egg wall was sealed with cellophane tape, and eggs were placed in hatching trays. Upon hatching the chickens were allocated to related floor pens and raised for 6 weeks. Chickens management (water, feed, light program and pen environment) were based on Cobb 500 broiler chickens [9].

On days of 19 and 21 peri-hatch and days 1, 3, 8 and 28 post-hatch, two birds per each replicate were randomly selected, anesthetized with diethyl ether and caecal removed. The entry of caecal was sealed, removed and placed in ice and used for microbial assays. Also, samples of jejunum (3 cm) were taken and placed in buffered formalin solution (10%) for intestinal morphometry and goblet cells count. Histo-preparation was done according to the method described by Iji et al.[10]. Goblet cell count was determined by double-stained of samples with Periodic Acid-Schiff and hematoxylin according to the method of Horn et al.[11]. The goblet cells were counted in scale of 300 µm of epithelium length.

The normality of data was evaluated using Kolmogorov-Smirnov test. Then data were analyzed using the GLM procedure of SAS for Windows, version 9.1 (SAS Institute Inc., Cary, NC). Means were separated using Duncan's Multiple Comparison test (P<0.05).

RESULTS

There was a difference (P<0.05) between chicks received *Bacillus subtilis* and other treatments for goblet cells counts on day 21 of incubation and day 3 post-hatch *(Table 1)*. Differences among treatment for goblet cells count on day 19 peri-hatch and days 8 and 28 post-hatch were not significant statistically (P>0.05).

The means of jujunal villus height, crypt depth, and villus height: Crypt depth ratio are presented in *Table 2*. There were no differences (P>0.05) among treatment for mentioned traits on days 1 and 3 post-hatch, but on days 8 and 28 post-hatch differences were appeared among treatment (P<0.05). Inoculation of *Bacillus subtilis* and *Pediococcus acidilactici* resulted in increase of villus height and decrease in crypt depth and their ratio compared with the control group (P<0.05). There were no differences (P>0.05) for these traits between *Enterococcus faecium* and the control group.

DISCUSSION

Inoculation of probiotic bacteria via oral feeding is now recognized as a suitable route to reduce the risk of

Table 1. *Goblet cells counts (n per 300 µm of epithelium length) in the jejunum of chicks at different ages*

Tablo 1. *Farklı yaşlardaki civcivlerde jejenum goblet hücre sayıları (epitel uzunluğunun 300 µm'da bir n)*

Treatments	Peri-hatch		Post-hatch		
	19	21	3	8	28
Control	10.1	11.5 ^{b}	15.95 ^{b}	20.6	22.9
Bacillus subtilis	14.2	16.5 ^{a}	19.0 ^{a}	22.7	25.1
Enterococcus faecium	12.3	12.9 ^{b}	17.1 ^{ab}	22.1	23.2
Pediococcus acidilactici	12.4	13.4 ^{b}	17.8 ^{ab}	22.5	24.0
P value	0.258	0.001	0.033	0.744	0.463
SEM	1.43	1.85	0.62	1.38	2.01

^{a,b} *Means with different superscripts within the same column differ significantly (P ≤ 0.05)*

Table 2. *Villus height, crypt depth and their ratio in the jejunum of chicks at different ages*
Tablo 2. *Farklı yaşlardaki civcivlerde jejenum villus boyu, kript derinliği ve oranları*

Treatments	Days Post-hatch											
	Villus Height (µm)				Crypt Depth (µm)				Villus Height/Crypt Depth			
	1	3	8	28	1	3	8	28	1	3	8	28
Control	267	401	675 ^{b}	870 ^{b}	64	125	184 ^{a}	201 ^{a}	4.17	3.21	3.67 ^{b}	4.33^{b}
BS[*]	276	408	850 ^{a}	985 ^{a}	61	113	152 ^{b}	164 ^{b}	4.52	3.61	5.59 ^{a}	6.01^{a}
EF	282	391	721 ^{ab}	880 ^{ab}	59	115	165 ^{ab}	169^{b}	4.78	3.40	4.38 ^{b}	5.21^{a}
PA	274	405	832 ^{a}	985 ^{a}	65	104	155 ^{b}	169 ^{b}	4.22	3.89	5.37^{a}	5.83^{a}
SEM	26.7	56.3	45.6	34.7	4.8	6.3	8.2	6.5	0.405	0.525	0.330	0.266
P value	0.985	0.997	0.032	0.045	0.792	0.427	0. 054	0.004	0.753	0.778	0.002	0.002

^{a,b} *Means with different superscripts within the same column differ significantly (P≤0.05)* [*]***BS:*** *Bacillus subtilis;* ***EF:*** *Enterococcus faecium;* ***PA:*** *Pediococcus acidilactici*

intestinal infection by pathogenic bacteria [2]. An interesting study demonstrated that the time of initial intestinal colonization by desirable bacteria play an important role on the colonization of pathogens [1]. In the previous studies [4-6,8,12], the protection effects of *in ovo* inoculation or other route administration of probiotics against Salmonella infection were investigated, but the effects of inoculation of different probiotic strains on intestinal morphometry, and goblet cells count have not been attended. The main objective of this study was to evaluate the effect of three probiotic strains, *Bacillus subtilis, Pediococcus acidilactici* and *Enterococcus faecium,* on intestinal characteristics.

Chicks received *Bacillus subtilis* had higher goblet cells counts than the control group and those received other probiotic strains. An interesting study [13] showed that dietary factors and microbiota could affect goblet cell numbers. Feeding probiotic to the turkey poults has been reported to increase the goblet cell number in the small intestine, which can protect epithelia from pathogenic bacteria [14]. Mucin production is correlated with the goblet cells number and if a pathogen enters via the digestive tract, a thick mucus layer produced by goblet cells, will block the pathogen from penetrating the host's cells.

There were no differences among treatment for mentioned traits on days 1 and 3 post-hatch. In agreement to our finding, Santin et al.[15] with feeding *Saccharomyces cerevisiae* and Sieo et al.[16] with six Lactobacillus strains reported no differences in the small intestine morphometry. Probiotics strains were inoculated at day 17 of incubation and it seems that probiotics needs more times to affect the proliferation of intestinal cells.

Inoculation of *Bacillus subtilis* and *Pediococcus acidilactici* resulted in the increase of villus height and decrease in crypt depth and their ratio compared with control group on days 8 and 28 post-hatch. The increase in villus height due to the probiotic inoculation could be considered important and beneficial for the absorptive capacity of jejunum. An increase in the villus height suggests increase in the surface area capable of higher absorption of nutrients. *Enterococcus faecium* had no effect on intestinal morphometry parameters. In contrast, Chichlowski et al.[17] and Samli et al.[18] reported that inclusion of *Enterococcus faecium* increased the jejunal villus height and decreased the villus crypt depth as compared with the control group.

As a conclusion, various effects of different probiotic strains on goblet cells count and intestinal morphometry were observed. Among probiotic strains evaluated in this study, *Bacillus subtilis* has higher benefit effect on goblet cells counts in the early of life and morphometry of jejunum.

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REFERENCES

1. Sterzo E, Paiva J, Penha-Filho R, Berchieri-Junior A: Time required to protect the intestinal tract of chicks against *Salmonella enterica* serovar *Enteritidis* using competitive exclusion. *Braz J Poult Sci*, 7, 119-122, 2005. DOI: 10.1590/S1516-635X2005000200009

2. Cukrowska B, Lodinova-Zadnikova R, Enders C, Sonnenborn U, Schulze J, Tlaskalova-Hogenova H: Specific proliferative and antibody responses of premature infants to intestinal colonization with non-pathogenic probiotic *E. coli* strain Nissle 1917. *Scand J Immunol*, 55, 204-209. 2002. DOI: 10.1046/j.1365-3083.2002.01005.x

3. Schneitz C, Nuotio L, Mead G, Nurmi E: Competitive exclusion in the young bird: Challenge models, administration and reciprocal protection. *Int J Food Microbiol*, 15, 241-244, 1992. DOI: 10.1016/0168-1605(92)90055-8

4. Hashemzadeh Z, Karimi-Torshizi MA, Rahimi S, Razban V, Zahraei-Salehi T: Prevention of *Salmonella* colonization in neonatal broiler chicks by using different routes of probiotic administration in hatchery evaluated by culture and PCR techniques. *J Agric Sci Tech*, 12, 425-432, 2010.

5. Hosseini-Mansoub N, Vahdatpour T, Arjomandi M, Vahdatpour S: Comparison of different methods of probiotic prescription against *Salmonella* infection in hatchery broiler chicken. *Adv Environ Biol*, 5, 1857-1860, 2011.

6. Yamawaki RA, Milbradt EL, Coppola MP, Rodrigues JCZ, Andreatti Filho RL, Padovani CR, Okamoto AS: Effect of immersion and enoculation *in ovo* of *Lactobacillus* spp. in embryonated chicken eggs in the prevention of *Salmonella Enteritidis* after hatch. *Poult Sci*, 92, 1560-1563, 2013. DOI: 10.3382/ps.2012-02936

7. Clark JD, Gebhart GF, Gonder JC, Keeling ME, Kohn DF: Special Report: The 1996 Guide for the Care and Use of Laboratory Animals. *ILAR J*, 38, 41-48, 1997.

8. Tako E, Ferket PR, Uni Z: Effects of *in ovo* feeding of carbohydrates and beta-hydroxy-beta-methylbutyrate on the development of chicken intestine. *Poult Sci,* 83, 2023-2028, 2004. DOI: 10.1093/ps/83.12.2023

9. Cobb Broiler Performance & Nutrient Supplement Guide: Cobb-Vantress Inc., Siloam Springs, AR, USA, 2012. http://cobb-vantress.com/academy/product-guides; *Accessed:* 2 July 2015.

10. Iji PA, Saki AA, Tivey DR: Intestinal development and body growth of broiler chicks on diets supplemented with non-starch polysaccharides. *Anim Feed Sci Technol*, 89, 175-188, 2001. DOI: 10.1016/s0377-8401(00)00223-6

11. Horn NL, Donkin SS, Applegate TJ, Adeola O: Intestinal mucin dynamics: Response of broiler chicks and White Pekin ducklings to dietary threonine. *Poult Sci*, 88, 1906-1914, 2009. DOI: 10.3382/ps.2009-00009

12. de Oliveira JE, van der Hoeven-Hangoor E, van de Linde IB, Montijn RC, van der Vossen JMBM: *In ovo* inoculation of chicken embryos with probiotic bacteria and its effect on posthatch *Salmonella* susceptibility. *Poult Sci*, 93, 818-829, 2014. DOI: 10.3382/ps.2013-03409

13. Sharma R, Schumacher U: Morphometric analysis of intestinal mucins under different dietary conditions and gut flora in rats. *Dig Dis Sci*, 40, 2532-2539, 1995. DOI: 10.1007/BF02220438

14. Rahimi S, Kathariou S, Grimes JL, Siletzky RM: Effect of direct-fed microbials on performance and *Clostridium perfringens* colonization of turkey poults. *Poult Sci,* 90, 2656-2662, 2011. DOI: 10.3382/ps.2011-01342

15. Santin E, Maiorka A, Macari M, Grecco M, Sanchez J, Okada T, Myasaka A: Performance and intestinal mucosa development of broiler chickens fed diets containing *Saccharomyces cerevisiae* cell wall. *J Appl Poult Res*, 10, 236-244, 2001. DOI: 10.1093/japr/10.3.236

16. Sieo CC, Abdullah N, Tan W, Ho Y: Influence of beta-glucanase-producing *Lactobacillus* strains on intestinal characteristics and feed passage rate of broiler chickens. *Poult Sci*, 84, 734-741, 2005. DOI: 10.1093/ps/84.5.734

17. Chichlowski M, Croom WJ, Edens FW, MacBride BW, Qiu R, Chiang CC, Daniel LR, Havenstein GB, Koci MD: Microarchitecture and spatial relationship between bacteria and ileal, cecal and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poult Sci*, 86, 1121-1132, 2007. DOI: 10.1093/ps/86.6.1121

18. Samli HE, Senkoylu N, Koc F, Kanter M, Agma A: Effects of *Enterococcus faecium* and dried whey on broiler performance, gut histomorphology and microbiota. *Arch Anim Nutr*, 61, 42-49, 2007. DOI: 10.1080/17450390601106655

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Case Report

Immunohistochemical Diagnosis of Pseudorabies (Aujeszky's Disease) in a Cow in Van, Turkey

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Abstract

Aujeszky's disease, also known as pseudorabies, is caused by an alphaherpesvirus that infects the central nervous system and other system. Disease was first recognized as a fatal viral disease of cattle and dogs in 1902. The natural infections have been reported worldwide in swine, cattle, dogs and variety of wildlife species in more recent years. The study material was a 5 years old cow which was brought with symptoms such as skin itching, weakness, convulsions, fever and neurogical signs. As a result of the clinical examination the animal was suspected for Aujeszky's disease. Slaughter and necropsy were recommended. Subsequent to necropsy, tissues were fixed in formalin. Samples were evaluated histopathologically and immunohistochemically. According to the histopathological findings; diffuse, non-suppurative inflammation of brain, spinal cord and spinal nerves were determined. Brain lesions were detected commonly in cerebral and cerebellar cortexes. Grey and white matter were both affected. It was observed marked perivascular lymphoplasmocytic cell infiltration, glial cell proliferation, Hyperemia and hemoragia in veins in brain and light chromatolysis have been identified in some neurons. Intranuclear inclusions were observed in neurons. On the skin itching region of animal, serofibrinous inflammation in the dermis and subcutis was detected. According to the immunohistochemical findings; immunoperoxidase was detected strong positive in cytoplasms of both neurons and glial cells in areas with meningitis in brain. Consequently; after clinical, macroscopical and microscopical examination, this case was diagnosed as Aujeszky's disease. The aim of this study is presenting this disease immunochemically as it has never been reported in cattle in Turkey. We believe that it would be appropriate to present this case report since it is the first Pseudorabies case in cattle in Turkey.

Keywords: *Aujeszky's disease, Histopathology, Immunohistochemistry, Pseudorabies*

Van'da Bir İnekte Yalancı Kuduz (Aujeszky) Hastalığının İmmunohistokimyasal Teşhisi

Özet

Pseudorabies olarak da bilinen Aujeszky hastalığı, etkeni alphaherpesvirus olan ve merkezi sinir sistemi ile diğer sistemleri enfekte eden bir hastalıktır. Hastalık ilk olarak 1902 yılında sığır ve köpeklerde ölümcül bir viral hastalık olarak tanımlanmıştır. Hastalık yakın zamanda tüm dünyada domuz, sığır, köpek ve çeşitli yabani hayvanlarda bildirilmiştir. Bu çalışmanın amacı, Türkiye'de sığırlarda bildirimi olmayan bu hastalığı immunokimyasal olarak ortaya koymaktır. Çalışmanın materyalini, halsizlik, deride şiddetli kaşıntı, yüksek ateş, şiddetli konvulziyon ve sinirsel semptomlara sahip 5 yaşında bir inek oluşturdu. Klinik muayene sonucu hayvanda Aujeszky hastalığından şüphelenildi. Hayvana kesim ve nekropsi önerildi. Nekropsi sonrası alınan dokular formalinde fikse edildi. Dokular histopatolojik ve immunohistokimyasal olarak değerlendirildi. Histopatolojik olarak; beyinde spinal kortta ve spinal sinirlerde diffuz non-suppuratif meningoensefalitis belirlendi. Beyin lezyonları genellikle beyin ve beyinciğin korteksinde belirlendi. Beyinde hem beyaz madde hem de gri madde etkilenmişti. Beyin damarlarında hiperemi, hemoraji, belirgin bir perivasküler hücre infiltrasyonu ve gliozis görüldü. Bazı nöronlarda hafif kromatolizis ve bazı nöronlarda ise intranükleer inklüzyonlar belirlendi. Ayrıca hayvanda kaşıntının olduğu bölgedeki derinin dermis ve subkutis katmanlarında serofibrinöz yangı tespit edildi. İmmunohistokimyasal olarak; meningitisli bölgelerdeki nöronlarda, glia hücrelerinde ve bazı damar endotel hücrelerinde güçlü bir pozitiflik saptandı. Sonuç olarak; yapılan klinik, makroskobik ve mikroskobik muayenelerden sonra hastalığın Aujeszky hastalığı olduğu belirlendi. Türkiye'de bir sığırda görülen ilk klinik vaka olmasından dolayı, vakayı sunmanın yararlı olacağını düşünmekteyiz.

Anahtar sözcükler: *Aujeszky's hastalığı, Histopatoloji, Immunohistokimya, Yalancı kuduz*

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INTRODUCTION

Pseudorabies or Aujeszky is a disease caused by herpesvirus. Disease is fatal in many wild and domestic animals such as cattle, sheep, goats, cats, dogs, horses and mainly in swines. It is also a zoonotic disease in humans. It is an acute viral disease characterized by central nervous system symptoms [1-7]. The disease in cattle is commonly referred as *"mad itch"* because of the severe pruritus [2]. In 1902, it was first detected by Prof. Dr. Aladar Aujezsky in cattle and dogs in Hungary, therefore it is named as Aujeszky's Disease. Disease was found in many countries and its incidence gradually increased [1,3-5,8-10].

In disease transmission, domestic and wild swines are common natural hosts and the main infection sources [2,3,6,8,11,12]. However, domestic and wild carnivores, eat infected carcasses and constitute important infection sources for other species. Besides, rodents such as mice and rats may also have roles in transmission. Disease is transmitted from swines to carnivores and rodents orally or through the air. In cattle, disease is transmitted by direct contact with swines or keeping the cattle together with them. Transmission occurs especially through the air. Although it is rare, rodents or biting flies may also cause infection [4-6,8,11-14].

Due to the neurotropic characteristic of virus, viral production occurs on the bitten site in small quantities and reach to brain through nerves. It produces intensely in brain and causes encephalomyelitis. Thus, it leads to death in a short time [7,11].

Clinically in cattle, symptoms such as severe local pruritus, intensely licking the pruritic location and rubbing desire of this location to places, biting, plucking its own flesh (automutilation) are reported. Besides, symptoms such as increase in body temperature, discomfort, continuous bellowing, whirling around, convulsions, opisthotonus are also seen [2,11,12,14-16].

Pruritus occurs commonly on hooves, head and chest regions where the viral contamination is the most possible. And death occurs due to paralysis in 6-48 h after the first clinical symptoms appear [11-15].

In order to perform certain diagnosis; medical history, clinical findings, hematological and biochemical parameters are not sufficient. For laboratory diagnosis, various direct and indirect tests are used (immunofluorescence, immuno-peroxidase, PCR, virus neutralization, latex agglutination or ELISA). The most common diagnostic methods are immuno-fluorescence and immunoperoxidase [17]. As viral isolation is hard and time-consuming, immunoperoxidase is a mainly preferred method [9,11,14,17].

Even swine farms, wild swines and other infection resources are present in our country, there has never been a report according to this disease in cattle in our country. In our case, according to the clinical findings we suspected of Aujeszky's Disease. Subsequent to histopathological and immunochemical examination the case was determined as Aujeszky's Disease. As this case is the first Aujeszky's Disease case in cattle in Turkey, we thought it might be useful to present this case report.

CASE HISTORY

Animal material of this case was a 5 years old crossbred cow in city of Van in Turkey. According to medical history received from animal owner; animal had several neurological symptoms, severe pruritus and it was biting the pruritic location along with having tendency to bite foreign materials. The feed given to the animal was obtained from a mountain village. There were too many mice in the stable. According to the clinical examination; Pseudorabies (Aujeszky's Disease) was suspected in animal. Blood samples were obtained in order to perform routine haemo-tological and biochemical examinations. Approximately 12 h later; symptoms got more severe and the animal owner slaughtered the animal and necropsy was performed. Following necropsy; Brain, medulla spinalis, tonsilla, pharynx and skin tissue was obtained. Tissue samples were fixed in neutral buffered formalin and delivered to laboratory. In pathological examination, fixed tissue samples were processed routinely and embedded in paraffin. Then, thin sections were stained with hematoxylin-eosin (HE) for histopathological examination. The samples were examined in light microscope. Aujesky disease antibody was detected by avidin-biotin immunoperoxidase complex method (ABC). The reagents were used of commercial origin [VMRD catalog no. 3G9F3].

According to the physical examination; body temperature (40°C), respiratory (65/min) and heart rate (110/min) were increased. Clinical findings such as loss of appetite, dis-comfort, perspiration, hypersalivation and tympani were found. Besides, severe pruritus on the front left leg was found and animal was licking the pruritic location and rubbing it on objects. Animal had automutilation on the pruritic region *(Fig. 1A, 1B)*. They had neurological symptoms such as biting the foreign materials, teeth grinding (bruxism), convulsions and whirling around. According to routine haemotological and biochemical parameters, WBC (19.8x10^9/L) and serum CK (519 IU/L) values were determined as significantly increased, however other parameters were in usual reference values for cattle.

Erosions and ulcerations were found on the front leg skin due to severe pruritus and biting *(Fig. 1A, 1B)*. At necropsy, no characteristic gross lesion was observed.

According to histopathological examinations of the obtained tissue from the pruritic regions of skin; widespread subcutaneous edema, erosions, hemorrhage and necrosis descending to muscles were determined *(Fig. 1C)*. Diffuse, non-suppurative inflammation of brain, spinal cord and

spinal nerves were determined. Brain lesions were detected commonly in cerebral and cerebellar cortices. Grey and white matter were both affected. Marked perivascular lymphoplasmocytic cell infiltration, glial cell proliferation, hyperemia and hemorrhagia were observed in brain veins *(Fig. 2A, 2B, 2C)*. Light chromatolysis were identified in some neurons. Intranuclear inclusion bodies were determined in certain neurons *(Fig. 2D)*.

According to immunhistochemical findings, immunoperoxidase reactivity was occurred in areas of meningitis. The cytoplasm of neurons, glial cells and some vascular endothelial cells showed strong positivity *(Fig. 2E, 2F)*.

DISCUSSION

Pseudorabies or Aujeszky is a disease caused by herpesvirus. Disease is fatal in many wild and domestic animals such as cattle, sheep, goats, cats, dogs, horses and mainly in swines. It is an acute viral disease characterized by central nervous system symptoms [1-7].

In our case; increase in body temperature, various neurological symptoms, severe pruritus particularly on front leg and itching the pruritic location as if going to pluck it, tendency of biting the foreign materials were observed. Symptoms in our study were similar to those reported in other studies [12,15].

As Aujeszky's disease can be confused with toxication and several disorders progressing with neurologic symptoms, making certain diagnosis is extremely important [14]. In order to make certain diagnosis medical history, clinical findings, hematological and biochemical parameters are not sufficient, therefore laboratory diagnosis, various direct and indirect tests should be also performed. The most

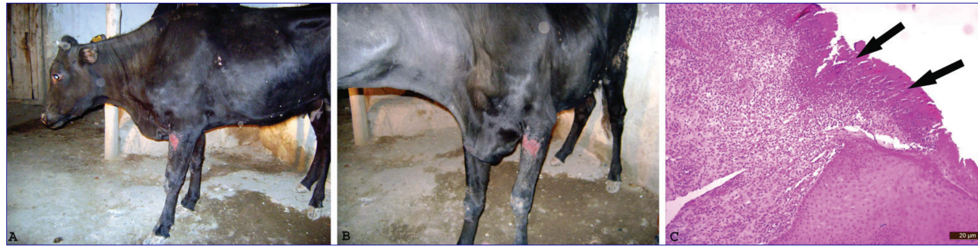

Fig 1. Severe pruritus in the front left leg and automutilation in animal (A, B), necrotic foci in skin (C), H&E, Bar: 20 µm

Şekil 1. Hayvanda sol ön bacakta şiddetli kaşıntı ve otomotilasyon (A, B), epidermiste nekrotik kitle (C), H&E, Bar: 20 µm

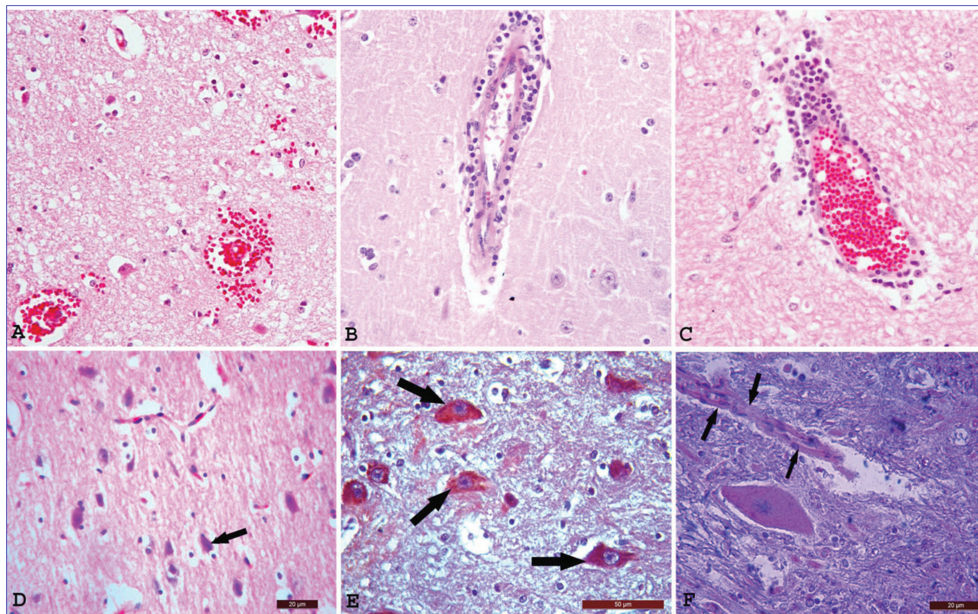

Fig 2. Hyperemia and hemorrhage in brain vein (A) and perivascular lymphoplasmocytic cell infiltration (B, C), intranuclear inclusion bodies in neurons *(arrow)* (D), H&E, Bar: 20 µm, cytoplasm of neurons *(arrows)* (E, Bar: 50µm), and vascular endothelial cells *(arrow)* demonstrated strong positive, immunoperoxidase (F, Bar: 20 µm)

Şekil 2. Beyinde hiperemi, hemoraji (A) ve perivasküler lenfositiktik hücre infiltrasyonu (B, C), nöronlarda intranükleer inklüzyon cisimciği *(ok)* (D), H&E, Bar: 20 µm, nöronlarda *(oklar)* (E, Bar: 50µm) ve damar endotel hücrelerinde immunperoksidaz güçlü pozitiflik *(oklar)*, (F, Bar: 20 µm)

common laboratory diagnostic methods are immunofluorescence and immunoperoxidase. As viral isolation is hard and time-consuming, immunoperoxidase is a mainly preferred method [9,11,17].

According to the immunohistochemical staining results of the obtained tissues, the cytoplasms of both neurons and glial cells showed strong positivity, thus certain diagnosis of Aujeszky's Disease was made. Histopathologic and immunohistochemical findings were similar to those previously reported [3,7,12,17].

Naturally, this disease is transmitted from swine to cattle. However in a previous study performed by Matsuoka et al.[3] Aujeszky's Disease was diagnosed in cattle living in a region which is close to a swine farm. Despite there are many case reports in many countries [2,3,15,17,18], there are not any reports in our country related to Aujeszky Disease in cattle. In our country the swine farms are rare and especially in our region there are not any swine farms. Therefore, this case report has importance as it is the first clinical case in our country and we thought that this disease might be transmitted from wild swine to cattle. In addition, the previous studies reported that wild animals and rats are also important for transmission [6,13]. We think that the transmission occurred due to mice and wild swine route because our case did not have direct contact with any domestic or wild swine and the feed was obtained from a mountain village and the stable had numerous mice. We diagnosed this case as Aujeszky Disease clinically, histopathologically and immunohistochemically.

As a conclusion; this is the first Aujeszky's Disease case in cattle in our country and there are not any swine farms in our city. We suggest that new studies should be performed related to this disease and veterinary practitioners should consider this disease during cattle examination.

REFERENCES

1. Matsuoka T, Iijma Y, Sakurai K, Konosu Y, Tamiya K, Oki M, Arai N, Koda M: Aujeszky's Disease in a dog. *Jpn J Vet Sci*, 50, 277-278, 1988.

2. Beasley VR, Crandell RA, Buck WB, Ely RW, Thilsted JP: A clinical episode demonstrating variable characteristics of pseudorabies infection in cattle. *Vet Res Commun*, 4, 125-129, 1980. DOI: 10.1007/BF02278490

3. Matsuoka T, Iijma Y, Sakurai K, Korihara T, Kounosu Y, Tamia K, Oki M, Haritani M, Imada T: Outbreak of Aujeszky's Disease in cattle in Japan. *Jpn J Vet Sci*, 49, 507-510, 1987. DOI: 10.1292/jvms1939.49.507

4. Schmidt SP, Pirtle EC, Hagemoser WA, Wages DP: A necrotizing pneumonia in lambs caused by Pseudorabies virus (Aujeszky's Disease Virus). *Can J Vet Res*, 51, 145-149, 1987.

5. Anonymous: Pseudorabies (Aujeszky's Disease). http://www.penpages.psu.edu/penpages_reference/29801/2980122, *Accessed:* 15.10.2015.

6. Richard E, Shope MD: Experiment on the epidemiology of Pseudorabies, I. mode of transmission of the disease in swine and their possible role in its spread to cattle. *J Exp Med*, 62, 85-99, 1935.

7. Marcaccini A, Pena ML, Quiroga MI, Bermudez R, Nieto JM, Aleman N: Pseudorabies virus infection in mink: A host-specific pathogenesis. *Vet Immunol Immunopathol*, 124, 264-273, 2008. DOI: 10.1016/j.vetimm.2008.03.013

8. Haddane B, Essalhi A: An outbreak of Aujezky's disease in the hunting dog (Lycaon Pictus). *European Association of Zoo-and Wildlife Veterinarians (EAZWV), Second Scientific Meeting*, May 21-24, 1998.

9. Anonymous: Aujeszky's Disease. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.02_AUJESZKYS_DIS.pdf, *Accessed:* 15.10. 2015.

10. Lee JYS, Wilson MR: A review of Pseudorabies (Aujeszky's Disease) in pigs. *Can Vet J*, 20, 65-69, 1979.

11. Anonymous: Aujeszky's Disease, the center for food security and public health, 2006. http://www.cfsph.iastate.edu/Factsheets/pdfs/aujeszkys_ disease.pdf, *Accessed*: 15.10.2015.

12. Schmidt SP, Hagemoser WA, Kluge JP, Hill HT: Pathogenesis of ovine Pseudorabies (Aujeszky's Disease) following intratracheal inoculation. *Can J Vet Res*, 51, 326-333, 1987.

13. Müller T, Teuffert J, Ziedler K, Possardt C, Kramer M, Staubach C, Conraths FJ: Pseudrabies in the European wild boar from Eastern Germany. *J Wildl Dis*, 34, 251-258, 1998. DOI: 10.7589/0090-3558-34.2.251

14. Kong H, Zhang K, Liu Y, Shang Y, Wu B, Liu X: Attenuated live vaccine (Bartha-K16) caused Pseudorabies (Aujeszky's Disease) in sheep. *Vet Res Commun*, 37, 329-332, 2013. DOI: 10.1007/s11259-013-9568-8

15. Szweda W, Janowski H, Grzechnik R, Brzeska E: Aujeszky's Disease of cattle in the Olsztyn Province in years 1980-1991. *Med Weter*, 13, 947-957, 2006.

16. Fukusho A: Aujeszky's Disease of cattle in the Olsztyn Province in years 1980-1991. *JARQ*, 16, 131-135, 1998.

17. Grieco V, Gelmetti D, Finazzi G, Brocchi E, Finazzi M: Immunohistologic diagnosis of Pseudorabies (Aujeszky's Disease) using monoclonal antibodies. *J Vet Diagn Invest*, 9, 326-328, 1997.

18. McCracken RM, McFerran JB, Dow C: The neural spread of Pseudorabies virus in calves. *J Gen Virol*, 20, 17-28, 1973. DOI: 10.1099/0022-1317-20-1-17

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup ve derleme türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve şekil ve tablo gibi görsel öğelerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.

Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.

Başvuru sırasında yazarlar yazıda yer alacak şekilleri online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.

3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- ***Makale Türleri***

Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.

Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir alan editörü ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

10- Yazarlara telif ücreti ödenmez.

11- Resim ve baskı masrafları için yazarlardan ücret alınır. Ücret bilgileri *http://vetdergi.kafkas.edu.tr/* adresinden öğrenilebilir.

12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

INSTRUCTIONS FOR AUTHORS

1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, and review and on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Original (full-length) Manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Abstract should contain 200±20 words.

Short Communication Manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the abstracts should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description (maximum 4 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

Case Reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow Introduction, Case History, Discussion and References without exceeding the total of 4 pages.

Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 2 pages.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow Introduction, Text, Conclusion, and References without exceeding 12 page.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

6- References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. ,339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

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