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Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü
36040 - Kars / TÜRKİYE
Phone: +90 474 2426807-2426836/5228
Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

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Research Article

İç Anadolu Bölgesi Konya Bölümü Folklorunda Ruminantların Paraziter Hastalıkları ve Tedavi Yöntemleri [1][2]

Aşkın YAŞAR [1] Çağrı Çağlar SİNMEZ [2] Gökhan ASLIM [3]

[1] *Bu çalışma, "İç Anadolu Bölgesi Konya Bölümünde (Aksaray, Karaman ve Konya) Folklorik Veteriner Hekimliği ve Hayvancılık Üzerine Araştırma" başlıklı ve 112O428 numaralı TÜBİTAK-TOVAG araştırma projesinden yararlanılarak hazırlandı*

[2] *Bu çalışmanın özeti 29 Eylül-5 Ekim tarihleri arasında Denizli'de düzenlenen 18. Ulusal Parazitoloji Kongresi'nde sunuldu ve özet kitabı s.158'de yayımlandı*

[1] Selçuk Üniversitesi, Veteriner Fakültesi, Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı, TR-42003 Konya - TÜRKİYE
[2] Erciyes Üniversitesi, Veteriner Fakültesi, Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı, TR-38039 Kayseri - TÜRKİYE
[3] Aksaray Üniversitesi, Veteriner Fakültesi, Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı, TR-68100 Aksaray - TÜRKİYE

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Özet

Çalışmada, ruminantların paraziter hastalıklarına ve alternatif tedavi uygulamalarına yönelik folklorik verilerin İç Anadolu Bölgesi Konya Bölümündeki (Aksaray, Karaman ve Konya) çeşitliliğinin belirlenmesi ve yeni tedavi modelleri açısından komparatif bir tartışma ortamı oluşturmasına fırsat sunulması, halk arasında paraziter hastalıklar için tedavi amacıyla kullanılan bitkisel, hayvansal ve madensel kökenli ilaç hammaddelerinin etkinliğinin ve yan etkilerinin güncel tıp bilgileri doğrultusunda araştırılması ve tedavi arayışlarına yardımcı olunması amaçlandı. Çalışmanın materyalini, İç Anadolu'nun Konya Bölümü içerisinde yaşayan halk hekimliği uygulayıcıları, hayvan sahipleri ve hayvancılıkla uğraşan 177 kaynak kişiden "*bilgi derleme formu*" yoluyla elde edilen yazılı verilerle birlikte sözlü ve görsel veriler oluşturdu. Çalışmada, İç Anadolu Bölgesi Konya Bölümünde yapılan folklor çalışmasında ruminantlarda bit, pire, kene, nokra, uyuz, coenurus cerebralis, fasciolozis, mide-bağırsak parazitleri ve babesiozis gibi paraziter hastalıklara rastlandığı ve alternatif tedavi uygulamaları arasında; hidroterapi (su ile tedavi), fitoterapi (bitki ile tedavi), jeoterapi (kil ve çamur ile tedavi) gibi yöntemlerin kullanıldığı belirlendi. Bununla birlikte paraziter hastalıkların tedavisinde katran, bit otu, termiğe tohumu, tütün, kül, karamuk bitkisi kökü, yumurta, kükürt, tuz, göktaşı ve kil gibi bitkisel, hayvansal ve madensel ilaç hammaddeleri ve terkiplerinin kullanıldığı tespit edildi. Sonuç olarak, İç Anadolu Bölgesi Konya Bölümünde yapılan folklor çalışmasında ruminantlarda bit, pire, kene, nokra, uyuz, coenurus cerebralis, fasciolozis, mide-bağırsak parazitleri ve babesiozis gibi paraziter hastalıklara rastlandığı; parazit tedavisinde kullanılan katran, bit otu, termiğe tohumu, tütün, karamuk bitkisi kökü, kükürt, tuz, göktaşı ve kil gibi ilaç hammaddelerinden modern tıpta da yararlanıldığı; ancak genel olarak uygulamaların dinî-sihrî, ampirik ve rasyonel nitelikler taşıdığı söylenebilir.

Anahtar sözcükler: *Alternatif tedavi, Folklor, Konya Bölümü, Ruminantların paraziter hastalıkları*

Ruminant Parasitic Diseases and Treatment Methods at Folklore of Konya Area in Central Anatolia Region

Abstract

The aim of this study was to reveal the diversity of folkloric data in Konya Area (Aksaray, Karaman, Konya) of Central Anatolia Region regarding alternative treatment application to parasitic diseases of ruminant and, to provide an oppurtunity to comparative discussion media for the new treatment model, to reveal the effectiviness and side effect of herbal, animal and mineral based drug raw materials by actual medicine information and, to help treatment quest in today. The study material consisted of visual, oral and written data by "*information collection form*" from the 177 source person dealing with animal husbandry, animal owner sand folk medicine practitioners lived in Konya Area. Alternative treatment methods in this study varied as hydrotheraphy, phytotherapy, jeotherapy and it has been determined herbal, animal and mineral oil, tar, delphinium, lupine, tobacco, oak ash, corncockle, egg, salt, sulfur, copper sulphate and clay based on raw materials of herbal, animal and mineral drugs. In conclusion, in terms of parasitic diseases of ruminant Konya Area of Central Anatolia Region it has been identified such as lice, flea, tick, warble, scabies, coenurus cerebralis, fasciolozis, gastro-intestinal parasites, babesiozis. Furthermore, used to treat parasites such as tar, delphinium, lupine, tobacco, barberry plant root, sulfur, salt, copper sulphate and clay are also used in modern medicine. It is concluded that practices have generally religious-magic, empirical and rational roots.

Keywords: *Alternative treatment; Folklore; Konya Area; Parasitic diseases of ruminant*

İletişim (Correspondence)
+90 382 2882942
gokhanaslim@aksaray.edu.tr

GİRİŞ

Halkın geleneğe bağlı maddi ve manevi kültürünü, kendine has yöntemlerle derleyen, araştıran, sınıflandıran, çözümleyen ve halk kültürü üzerine değerlendirmeler yapan bir bilim olarak tanımlanan folklorun bir kısmını "*veteriner hekimliği folkloru*" oluşturmaktadır. Birçok mesleğin halk tarafından uygulanan bir biçimi, kendine has gelenek, görenek ve inanışları mevcuttur. Buna o mesleğin folkloru adı verilmektedir. Bu bağlamda veteriner hekimliği mesleğinin de kendine has gelenek, görenek ve çeşitli inanışları vardır. Bu da "*veteriner hekimliği folkloru*" olarak ifade edilmektedir [1].

İnsanoğlu, birçok bitkisel, hayvansal ve madensel kökenli drogları tedavi edici özelliklerinden dolayı çok eski çağlardan beri hastalıkların sağaltımında kullanmıştır. İlaç uygulamaları ve ilaçların hazırlanma yöntemleri, zamanla ampirik bilgilerden uzaklaşmış, bilimsel temellere dayandırılarak günümüz uygulamalarındaki yerini almıştır. Son yıllarda, sentetik kimyasalların sebep olduğu çevre kirliliğinin önlenmesi için farklı bitki ekstraktlarıyla yeni zararlılar üzerinde çalışmaların yapılması ve tedavide tıbbi bitkilerin doğal şekilleriyle ilaç olarak kullanımı yeni inceleme ve araştırma konularının ortaya çıkmasına neden olmuştur [2,3]. Artun'a göre [4], geçmişte kalan, deneme-yanılma usulüyle gelişmiş fakat tekrar tekrar kullanılaran ve herkesin bildiği faydalı halk ilaçlarının ve değişik tedavi yöntemlerinin, modern tıbbın ışığında yeniden incelenmesi gerekmektedir.

Çalışmada, ruminantların paraziter hastalıklarına ve alternatif tedavi uygulamalarına yönelik folklorik verilerin İç Anadolu Bölgesi Konya Bölümündeki (Aksaray, Karaman ve Konya) çeşitliliğinin belirlenmesi, yeni tedavi modelleri açısından komparatif bir tartışma ortamı oluşturmasına fırsat sunulması, halk arasında paraziter hastalıklar için tedavi amacıyla kullanılan bitkisel, hayvansal ve madensel kökenli ilaç hammaddelerinin etkinliğinin ve yan etkilerinin güncel tıp bilgileri doğrultusunda araştırılması ve tedavi arayışlarına yardımcı olunması amaçlandı.

MATERYAL ve METOT

Çalışmanın materyalini, İç Anadolu Bölgesinin Konya Bölümünde (Aksaray, Karaman ve Konya), halktan elde edilen yazılı, sözlü ve görsel veriler oluşturdu. Yazılı materyali, Sinmez'den [5] yararlanılarak hazırlanan *"bilgi derleme formu"* kullanılarak elde edilen veriler; sözlü materyali ses kayıt cihazı kullanılarak, inceleme yapılan yerlerdeki halk hekimliği uygulayıcıları, hayvan sahipleri, hayvancılıkla uğraşan kişiler ve veteriner hekimlerden elde edilen veriler; görsel materyali ise hayvancılık uygulamaları ya da hayvan hastalıklarının tedavilerinde kullanılan bazı folklorik unsurların görüntüleri oluşturdu.

Çalışma kapsamına, sosyal bilimlerdeki nitel araştırma yöntemlerinden olan "*kaynak kişilerle görüşme tekniği*" ile toplam 177 kaynak kişi ile görüşüldü. Görüşmeler 06.11.2012-08.07.2013 tarihleri arasında gerçekleştirildi. Kaynak kişiler, bulgular bölümünde metin içinde yer alma sıralarına göre dipnot şeklinde, birden fazla bilgi veren kaynak kişilerin ismi ise sadece yer aldığı ilk dipnotta belirtildi.

BULGULAR

Dış Parazitler ve İlgili Hastalıklar

Bit ve Pire: Kış aylarında koyunların zayıflamasına sebep olan koyun yapağı bitine "*kırkışlı*" adı verilir (KK 1) [1]. Tedavide, danaların boyun ve sırtlarının kılları arasında beyaz renkte görülen bit yumurtaları elle seçilerek temizlenir (KK 2-7) [2]. Mandaları bitlerden korumak amacıyla bezir yağı ve katran kaynatılır, sıcak olarak bitli deriye sürülür (KK 8) [3].

Keçi kılı yağda kaynatılıp bit olan deriye sürülür. Ahır ve kümeslerde bit ve pireleri öldürmek için yaş ot, "*bit otu*" *(Delphinium staphisagria* L.), "*hışır*" (arpa-saman sapı), talaş, mazot veya Dikloro Difenol Trikloroethan (DDT) yere dökülerek yakılır ve dumanı ile ağıldaki bit ve pireler öldürülür (KK 2-7). Koyun ve keçi üzerindeki bitler "*termiğe*" *(Lupinus albus* L.) suyu ile temizlenir (KK 9-13) [4]. Saman, ekin ilacı (bambıl tozu) veya kükürt yakılıp tütsülenir (KK 14-16) [5]. Kaynatılan tütün, banyo şeklinde koyunların sırtına uygulanır (KK 4-5).

Kene: Hayvanların üzerinde çıplak gözle görülen keneler *(yavsı)* elle koparılmak suretiyle uzaklaştırılır. "*Keneci*", "*üssükçü*", "*ocak*" adı verilen bir kişi keneleri dişleri arasında ısırır ve suyun içine tükürür, bir tas suyla koyun sürüsünün üzerine dökerek afsunlar ve hayvanların keneden korunması sağlanır (KK 17-30) [6].

Kükürt, DDT, meşe külü, katran, bezir hayvanların sırtına sürülmek ya da dökülmek suretiyle kullanılır. Kenelerin bulunduğu ahırların duvarlarına sönmüş kireçle badana yapılır. Kenelerle doğal yoldan mücadele için ahırlarda güvercin ve tavuk beslenir ya da koyun ve keçilerde olduğu gibi "*kırkım makası*" ile yünler kırkılır (KK 31-47) [7]. Ahır kapısı

[1] KK 1: Halil DURMUŞ (KONYA)
[2] KK 2: Mustafa ARSLAN, KK 3: İbrahim ERTEK (KONYA), KK 4: Koçaş EKİNCİ, KK 5: Mehmet ER, KK 6: Asil ATEŞ, KK 7: Mevlüt BEKTAŞ (AKSARAY)
[3] KK 8: Cüneyt DEMİR (AKSARAY)
[4] KK 9: Ramazan KAR, KK 10: Osman KÖYGI, KK 11: Mehmet KARASU, KK 12: Ömer GÜDEKOĞLU, KK 13: Ali SARIGÜL (KONYA)
[5] KK 14: Mahmut AKTÜRK, KK 15: Süleyman KAÇAR (AKSARAY), KK 16: Kerim GÜZEL (KARAMAN)
[6] KK 17: H. Ömer ÇOPANOĞLU, KK 18: İrfan YARALI, KK 19: Atıf AKKAN, KK 20: Mehmet AYVACI, KK 21: Nazmi ÇOLAK, KK 22: Yakup ZEYBEK, KK 23: Burhan DOĞAN KK 24: Mevlüt GÜLTEKİN (KONYA), KK 25: Müctehit ATUĞ, KK 26: Osman MUTLU, KK 27: Döne ÜNLÜ (AKSARAY), KK 28: Özcan KOÇ, KK 29: Namık ARPINAR, KK 30: H. İbrahim AYDIN (KARAMAN)
[7] KK 31: Mevlüt ÇİFTÇİ, KK 32: Bekir ALIM, KK 33: Ömer ZEYBEK, KK 34: Ahmet SARIBAŞ, KK 35: İbrahim SARI, KK 36: Mustafa ATISOĞLU, KK 37: Hüseyin BAKIRCI, KK 38: Mevlüt ÖZÇELİK, KK 39: Kemal ÇETİN, KK 40: Mehmet GÖNÜLKIRMAZ, KK 41: Hayrettin YORGUN, KK 42: Abdurrahman SAYIN, KK 43: Hüseyin ALDEMİR (KONYA), KK 44: Süleyman BÖLÜKBAŞ (AKSARAY), KK 45: Mehmet YAMAN, KK 46: Ahmet BOZDEMİR, KK 47: Ali KARPUZ (KARAMAN)

kapatılarak naftalin, katran, saman, çaput, lastik karışımı yakılarak tütsü yapılır (KK 15). Söğüt yaprağı kazanlarda kaynatılır ve üzerine katran dökülür. Karışım soğuduktan sonra bu suyla hayvanlara banyo yaptırılır (KK 47).

Nokra (Hypodermosis): Sığırların sırt derisi altında oluşan küçük yuvarlak şişliklere "*okra*", "*yarkın kurdu*", "*yaz kurdu*", "*gökbaş*", "*büvelek*" adı verilir (KK 26, KK 39, KK 48-53 [8]).

Nokralı hayvanın tedavisinde, şişkinliklerin elle sıkılarak içlerinden "*beyaz kurtlar*" *(Hypoderma bovis* larvası) çıkartılır. Sıkılan yerlere katran, kızgın tuğla, bezir ya da tereyağı sürülür (KK 54-55) [9]. Bezir yağı, mandaların derilerini (sarı renkten koyu siyaha dönüştürür) karartarak güneşin yakıcı etkisinden koruması ve "*büvelek tutmasını*" engellemesi için sürülür (KK 50, KK 56-58 [10]).

Uyuz (Scabies): Hastalık, "*kellik*", "*gicimik*", "*kermecik*" isimleriyle bilinir (KK 59-63) [11]. Hayvanın boyun derisi kalınlaşmış, tüyleri dökülmüş ve kıvrımlı bir hal almıştır. Bu görünüşe "*kellik*" adı verilir. Deri kepekle örtülü görünüştedir. Hayvan boynunu duvar ve ahır direklerine sürer (KK 31, KK 64 [12]).

Tedavi olarak, Arap sabunu ya da kükürtlü bir sabunla hayvan yıkanır. Tereyağı veya bitkisel yağlar (ayçiçeği ve zeytinyağı), yoğurt, otomobil atık yağı, gres yağı, gazyağı veya tuz hastalıklı alana sürülür (KK 35, KK 65-82 [13]). Meşe külü suyla karıştırılarak hastalıklı bölgeler yıkanır (KK 46, KK 83-84 [14]).

Küçükbaşların bacaklarına tavuk pisliği sürülür. Katran, bezir, tereyağı ve tuz kaynatılır. Elde edilen karışım ılıklaşınca hastalıklı bölgelere sürülür (KK 85-86) [15]. Sarımsak (*Allium sativum* L.) ve tuz karıştırılarak uyuzlu deriye sürülür. Hasta hayvanlar uyuz suyuna ya da gölüne götürülerek yıkanır (KK 87-91) [16]. Ağıl içinde "*kerme*" (gübre) yakılarak, dumanıyla tütsüleme yapılır (KK 92) [17]. Tütün bitkisi (*Nicotina tabacum* L.*)* kaynatılır ve suyuyla hayvanlara banyo yaptırılır (KK 93-94) [18].

İç Parazitler ve İlgili Hastalıklar

Coenurus cerebralis: Köpeklerin gaitasını veya "*karakasap otu*", "*çalgan otu*", "*anız*" yiyen koyunlarda görülen bu hastalık "*delibaş*", "*cinlenme*", "*dönelek*" olarak bilinir. Koyunlar olduğu yerde dairesel hareketler yaparak döner. Hastalanan hayvan sürünün gerisinde kalır. Hastalığa, dişi kuzu ve toklularda daha çok rastlanır (KK 40, KK 61, KK 95-109 [19]).

Tedavide, "*sayacak*" adı verilen demir ateşte kızdırılmak suretiyle hasta koyunların kafası dağlanır. Koyunların kulakları ve burunları kesilerek kan akıtılır (KK 20, KK 97, KK 107, KK 110-119 [20]). Hastalanan hayvanlar kesilerek köyün dışında bir yere gömülür (KK 46).

Hastalıktan korunmak için, her yıl damızlık amacıyla kullanılan koç değiştirilir. Buradaki amaç koçların kendi soyundan olan koyunlarla (bir nevi akraba evliliği) çiftleşmesini engellemektir. Hastalıktan korunmak amacıyla hasta bir koyun ikiz çocuk doğurmuş bir kadının bacak arasından geçirilir (KK 120-121) [21].

Fasciolozis: "*Kelebek*", "*gebelek*" olarak bilinen bu hastalığa bataklıklar *(kara su)* sebep olmaktadır. Kelebekli koyunların çene altları *(gıdıkları)* sallanır görünümde ve şiş olup, öksürük mevcuttur. Hayvanların tüyleri birbirine karışmıştır ve yürümekte zorluk çekerler (KK 19, KK 59, KK 122-123 [22]).

Tedavide, katran, tuz ve göktaşı (Bakır sülfat) karıştırılarak koyunlara aç karnına içirilir. Bu uygulama ayda iki sefer yapılır **(**KK 68, KK 124-126 [23]). "*Karamuk (Berberis crataegina* L.*) kökü*" ve göktaşı veya katran sulandırılarak hayvana aç karnına içirilir. Koyunlara 1 g, kuzulara 0.5 g göktaşı verilir (KK 67, KK 127-129 [24]). Hasta hayvanlara "*gökbaş otu*" *(Centaurea cyanus* L.) yedirilir (KK 130) [25].

Mide ve Bağırsak Parazitleri: Tedavide, buzağıların solucan *(Nematod)* ve kıl kurtlarını *(Trichostrongylus)* düşürmek için kırmızı pul biber yedirilir. Sonbahar döneminde karamuk bitkisinin kökü kesilerek kaynatılır ve suyu içirilir. Özellikle abdest bozan şerit *(mirt, iplicek)* tedavisinde bu yöntem kullanılır (KK 25, KK 66-67, KK 89, KK 131-136 [26]).

[8] KK 48: Mehmet OĞURLU, KK 49: İsmail ÇOK, KK 50: Adil ACAR, KK 51: Salim SARPKAYA, KK 52: Bülent BATTIR (KONYA), KK 53: Musa KARTAL (KARAMAN)

[9] KK 54: Mustafa SELVİ, KK 55: Rafet TAŞLITEPE (KONYA)

[10] KK 56: İdris ATAR (KONYA), KK 57: Akit KUTLU, KK 58: Dede TÜRKOĞLU (AKSARAY)

[11] KK 59: İsmail BECERİK, KK 60: Muammer ESER, KK 61: Şaban TÜRKSEVER, KK 62: Mustafa DURSUN (KONYA), KK 63: Hüseyin BAL (AKSARAY)

[12] KK 64: Ali Rıza KOÇ (KONYA)

[13] KK 65: Ömer TATLI, KK 66: Mehmet ÖZKARABIYIK, KK 67: Hüseyin ÜNLÜ, KK 68: Ali DİLEK, KK 69: Mustafa AKBIYIK, KK 70: Ali GÜLOĞLU, KK 71: Lütfi ERDOĞAN, KK 72: Şakir AKSU, KK 73: Mehmet ATA, KK 74: Mustafa ULUDAĞ (KONYA), KK 75: Yaşar SEVİNÇ, KK 76: Murat BOZ (AKSARAY), KK 77: Ahmet ÖZTÜRK, KK 78: Şaban KOÇ, KK 79: Rahmi SOLAK, KK 80: Bahtiyar AYDIN, KK 81: Süleyman İNCE, KK 82: Nurhan KURT (KARAMAN)

[14] KK 83: Mehmet KÜÇÜK, KK 84: Ömer ŞAHİN (KONYA)

[15] KK 85: Ali BASMAZ, KK 86: Mustafa MUTLU (KARAMAN)

[16] KK 87: Mehmet AĞIRKAN, KK 88: Mehmet YÜKSEL,KK 89: Mehmet KÜÇÜKHAŞHAŞ, KK 90: Hasan GÜNGÖR (KONYA), KK 91: Ramazan TURAL (KARAMAN)

[17] KK 92: Haşim KAYA (KONYA)

[18] KK 93: Osman DOĞAN, KK 94: Akif GÜNAL (KONYA)

[19] KK 95: Muammer İNAL, KK 96: Emine KIL, KK 97: Adil ÖZARDIÇ, KK 98: Sami YALDIR, KK 99: Servet TOSUN, KK 100: Musa HORZUM, KK 101: Bayram BAYSAL, KK 102: Ali MUTLU, KK 103: Mustafa ATOL, KK 104: Abdullah KORKMAZ (KONYA), KK 105: Tekin AYDIN, KK 106: İbrahim KALKAN, KK 107: Bayram SARIKAYA, KK 108: Kudret AKDOĞAN (AKSARAY), KK 109: İsmail AYDINLI (KARAMAN)

[20] KK 110: Murat UYAR, KK 111: Hüseyin ÜNLÜBALTACI, KK 112: Ahmet BALCI, KK 113: Niyazi DEMİRTAŞ, KK114: Cumhur ADIAY, KK 115: Sadık POYRAZ, KK 116: Aykut BARAN, KK 117: Hüseyin KÜÇÜKASLAN (KONYA), KK 118: Mehmet OĞUZ, KK 119: Remzi YİĞİT (KARAMAN)

[21] KK 120: Hasan BOYAR, KK 121: Lütfi AKTAŞ (KONYA)

[22] KK 122: Engin ERDOĞAN, KK 123: Hacı ERCAN (KONYA)

[23] KK 124: Hakan CENGİZ, KK 125: Mustafa KARAASLAN, KK126: Ömer UNCU (KONYA)

[24] KK 127: Süleyman SERÇE, KK 128: Mustafa ÜĞDÜL, KK129: Ali ZEYBEK (KONYA)

[25] KK 130: Süleyman BAŞARAN (KONYA)

[26] KK 131: Bekir GENCER, KK 132: Yaşar ÇOLAKLAR (KONYA), KK 133: Adnan BALALİOĞLU, KK 134: Derviş ÖZOĞLAN (AKSARAY), KK 135: Kemal YAVUZ, KK 136: Kerim BAŞKARA (KARAMAN)

Tablo 1. *Konya Bölümü folklorunda veteriner paraziter hastalıklarda kullanılan bitkiler*
Table 1. *The plants used in veterinary parasitic diseases at folklore of Konya Area*

Bitkinin İsim ve Familyası	Bitkinin Kullanılan Yeri	Yerel İsmi	Teropatik Etkisi	Preparasyon/ Hazırlama	Uygulama	Literatürde Bildirilen Kullanımları
Acroptionrepens L. –*Asteraceae*	Çiçekleri	Kekre	Mide-bağırsak parazitleri	İnfuzyon	İnternal	Antimikrobiyel, antipiretik [6]
Alliumsativum L.- Alliaceae	Soğanı	Sarımsak	Uyuz Babesiozis	Ezme Hap	Eksternal İnternal	Antiparaziter, antiseptik, mukolitik [7,8]
Berberis crataegina L. -Berberidaceae	Kökü	Karamuk, Şam püremi	Fasciolozis Mide-bağırsak parazitleri	İnfuzyon	İnternal	Antelmintik, antienflamatuar, antibakteriyel [9,10]
Brassica oleracea L.- *Brassicaceae*	Yaprakları	Lahana	Mide-bağırsak parazitleri	Salamura	İnternal	Kurt düşürücü, sürgüt etkili [11,12]
Capsicum annuum L.- Solanaceae	Meyvesi	Kırmızı biber	Mide-bağırsak parazitleri	İnfuzyon	İnternal	Antibakteriyel [13]
Centaurea cyanus L.- Asteraceae	Gövde ve çiçekleri	Gökbaş, Kökbaş	Fasciolozis	Doğrama	İnternal	Aperatif, antienflamatuar, antimikrobiyel, tonik ve diüretik [11]
Delphinium staphisagria L.- *Ranunculaceae*	Gövdesi	Bit otu	Bit ve pire	Tütsü	Eksternal	Antiparaziter [11,14]
Gypsophila L.- Caryophyllaceae	Gövde ve çiçekleri	Çöğen, Çöven	Mide-bağırsak parazitleri	İnfuzyon	İnternal	Antipiretik, Ekspektorant, Diüretik [11]
Juniperus oxycedrus L.- Cupressaceae	Dalı Odunu	Ardıç	Mide-bağırsak parazitleri Bit ve pire Kene Nokra Uyuz Fasciolozis Mide ve bağırsak parazitleri Babesiozis	İnfuzyon Katran Katran Katran İnfuzyon Katran KatranKatran	İnternal Eksternal Eksternal Eksternal Eksternal İnternal İnternal İnternal	Antiseptik ve Antiparaziter [11,14]
Linum usitatissimum L.- Linaceae	Tohumu	Keten	Bit ve pire Kene Nokra Uyuz	Bezir yağı Bezir yağı Bezir yağı Bezir yağı	Eksternal Eksternal Eksternal Eksternal	Antiparaziter [12]
Lupinus albus L.- Fabaceae	Tohumu	Termiğe	Bit ve pire	İnfuzyon	Eksternal	Antiparaziter [11,14]
Nicotina tabacum L.- Solanaceae	Taneleri	Tütün	Bit ve pire Uyuz	İnfuzyon İnfuzyon	Eksternal Eksternal	Antiparaziter [11,14]
Prunus divaricata var. divaricata- Rosaceae	Meyvesi	Dağ Eriği	Mide-bağırsak parazitleri	İnfuzyon	İnternal	Antidiyarel [13]
Quercus L.- Fagaceae	Kabuğu	Meşe	Kene Uyuz	Yakma/Külü Yakma/Külü	Eksternal Eksternal	Antiparaziter [15]
Salix alba L. -*Salicaceae*	Yaprağı Dalı	Söğüt	Kene Mide ve bağırsak parazitleri	İnfuzyon İnfuzyon	Eksternal İnternal	Antiparaziter [15]
Triticum L. -Poaceae	Kabuk	Buğday	Mide ve bağırsak parazitleri	Kepek hali	İnternal	Antikonstipan [11]
Viscum album L. *Subsp.austriacum* - Santalaceae	Yaprak ve dalları	Gövelek, Ökse otu	Mide ve bağırsak parazitleri	Doğrama	İnternal	Antikanserejonik, immun sistemi kuvvetlendirici ve yüksek kan basıncını azaltıcı [6,11]
Vitis L.- *Vitaceae*	Meyvesi	Üzüm	Mide ve bağırsak parazitleri Babesiozis	Şurup/pekmez	İnternal	Kan yapıcı, enerji verici [11]

Şerit düşürmek için karamuk kökü ve dağ eriği *(Prunus divaricata* var. *divaricata)* kaynatılır ve suyu içirilir (KK 53, KK 137-139 [27]). Aynı amaçla göktaşı, kil suyu veya tuz da içirilir (KK 20, KK 98, KK 104, KK 133, KK 140-147 [28]). Göktaşı tuz ile karıştırılır ve hayvan başına 1 g verilir (KK 131, KK 148-150 [29]). "*Kekre otu*" kaynatılır ve suyu içirilir (KK 125). "*Şam püremi*" *(yeşil pürem)* ismi verilen karamuk kökü, ardıç ve söğüt dalları karışımı kaynatılır ve suyu içirilir (KK 151-154) [30]. Çöğen otu *(Gypsophila* L.*)* kaynatılarak içirilir (KK 155) [31].

Tuz ve sıvı yağ içirilerek hayvan ishal edilir. Lahana turşusu da kurt düşürücü olarak içirilir. Manda yavrularına ve buzağılara doğar doğmaz yumurta içirilir (KK 156-157) [32]. Badem ve armut ağaçlarının üzerinde bulunan "*gövelek otu*" *(Viscum album* L.) yedirilir (KK 158-159) [33]. "*Topalak*" adı verilen katran ve un karışımı oğlaklara hap şeklinde yutturulur (KK 160) [34]. 1 kg kepek, katran ve 200 g pekmez karışımı kıl kurtlarını düşürmek için içirilir (KK 161) [35].

Kan Parazitleri ve İlgili Hastalıklar

Babesiozis (Babesiosis): "*Ağrıma*", "*gün çalması*", "*burmaca*", "*sarılık*" olarak bilinir. Hastalık, bahar aylarında yağmurun çok yağdığı ve çiçeklerin açtığı dönemde, özellikle "*kannık otu*", "*ölemez otu*"nun yenilmesi ve kene tutması neticesinde, koyunların kırkım zamanında, Haziran ayında *(gün dönmesi)* veya otların kurumaya başladığı dönemlerde görülür. Hayvanın yüzü diken diken olur. Hayvanın idrarı ve sütü kanlıdır (KK 18, KK 29, KK 83, KK 106, KK 144, KK 146, KK 162-171 [36]). Hayvan dişlerini gıcırdatır ve yürümede zorluk çeker (KK 172) [37].

Tedavide, üzüm pekmezi hayvana kan vermesi için içirilir. Her gün 1 litre pekmez suyla karıştırılarak üç dört gün süreyle içirilir (KK 173) [38]. Hayvanların kulakları kesilerek kan akıtılır. Sarımsaklı ayran, yumurta veya katran içirilir (KK 174-176) [39]. Hasta hayvanların sırtları çamurla sıvanır (KK 177) [40]. Koyunların kuyruk altına sarımsaklı yoğurt sürülür, sırtlarına da yoğurt suyu dökülür (KK 89).

Korunmada, hayvanlar kırkımdan sonra 15 gün süreyle güneşe çıkarılmamalı, sabahları soğuktan korunmalı ve kuru yem verilmelidir (KK 162).

TARTIŞMA ve SONUÇ

Halk arasında insan ve hayvanların kaşıntısını belirtmek için söylenen "*gicişti*" kelimesi, Divanu Lügati't-Türk'te [16,17] "*kiçidi*" şeklinde yer almakta; Anadolu halk dilinde uyuz, "*kaşıntı*", "*gecimik*" ya da "*gicimek*" olarak adlandırılmaktadır [18]. Çalışmada, hastalığın "*kellik*", "*gicimik*", "*kermecik*" olarak adlandırıldığı ve yukarıdaki literatürlerle benzerlik gösterdiği söylenebilir. Uyuz ile ilgili olarak baytarnamelerde uyuzlu atın hamamda su ve sabunla yıkandığı sonra duvardan uzak güneşe bağlanıp katranlı ilaç sürüldüğü ve koyun uyuzunda yağ ve kükürtlü ilaçların kullanıldığı bildirilmektedir [19,20]. Anadolu'da yapılan folklorik çalışmalarda [1,5,21-23] Arap sabunu, kükürtlü sabun, kül, tütün, benzin, gazyağı, çam katranı, tereyağı, bezir yağı, ardıç katranı, sarımsaklı sirke, tuz, tavuk pisliği ve otomobil atık yağı gibi çeşitli maddelerin uyuz tedavisinde kullanıldığı görülmektedir. Sucu [13] ve Erdemir [24], çok eski çağlardan günümüze, uyuz ve deri hastalıklarına karşı kullanılan kükürtün, deride sülfür, alkalik disülfür ve pentationik asit şekline geçerek antiparaziter ve fungisit etki gösterdiğini; Baytop [11], Koruk ve ark.[25] ve Kaya [14], ardıç katranının antiseptik ve antiparaziter etkilerinin, bileşimindeki fenol türevlerinden (gaiakol, etil, kreosol) ileri geldiğini ve ekstratlarının tıp ve veteriner hekimliğinde deri hastalıklarının (uyuz vb.) tedavisinde haricen kullanıldığını bildirmektedirler. Ayrıca sarımsı-esmer renkli, koruyucu bir yağ olan bezir yağının *(Oleum Lini)* antiparaziter etkisinin, iyot sayısı yüksek doymamış yağ asitleri (linoleik, linolenik ve oleik asitler) ihtiva etmesine bağlı olduğu bildirilmektedir [12]. Çalışmada uyuz hastalığı tedavisindeki pratiklerin, baytarnameler, Anadolu'daki uygulamalar ve günümüz hekimliği ile benzerlikler gösterdiği, ancak yoğurt, tereyağı, bitkisel yağlar, otomobil atık yağı, gres yağı, gaz yağı ve tuz kullanımının uyuz tedavisinde etkisinin olmadığı söylenebilir.

Baytop [11] ve Kaya [14], bit otu *(Delphinium staphisagria* L.), termiğe tohumu *(Lupinus albus* L.) ve tütünün, bileşiminde bulunan alkaloitlerden (veratrin; lupinin; nikotin) dolayı, baş ve vücut bitlerinde infuzyon şeklinde uygulandığını bildirmektedir. Dinçer [1] ve Sinmez [5,22], tütünün, Anadolu'da hayvanların paraziter hastalıklarda antiparaziter olarak kullanıldığını belirtmektedir. Mimioğlu [26], pirelerin kontrolünde %5'lik DDT çözeltisinin hayvanlara uygulandığını söylemektedir. Çalışmada ise bit ve pire tedavisinde, yağda kaynatılan keçi kılı ve tütünün bit olan deriye sürüldüğü, ahır ve kümeslerde bitleri öldürmek için bit otu veya DDT tütsüsünün yapıldığı, koyun ve keçi üzerindeki bitleri temizlemek için termiğe ve tütün suyu banyolarının

[27] KK 137: Süleyman ÖZCAN, KK 138: Ömer DAĞ, KK 139: İzzet TEKE (KONYA)
[28] KK 140: Ali DOĞANAY, KK 141: Ali KARABUCAK, KK 142: Osman YAZGAN, KK 143: Erhan YILMAZ, KK 144: Hüseyin IŞIK (KONYA), KK 145: Muhittin AKBAŞ, KK 146: M. Ali BOZDAĞ (AKSARAY), KK 147: Yüksel DÖLEK (KARAMAN)
[29] KK 148: Mehmet POÇANOĞLU, KK 149: Niyazi AKBAY, KK 150: İrfan SARAÇ (KONYA)
[30] KK 151: Hasan SEVİNÇ, KK 152: Mustafa KIRCI, KK 153: Recep GÜNAY (KONYA), KK 154: Şaban AKIN (AKSARAY)
[31] KK 155: İsmet GÜR (AKSARAY) KK 156: Yakup BEZGEN, KK 157: Mahir AKIN (AKSARAY)
[32] KK 156: Yakup BEZGEN, KK 157: Mahir AKIN (AKSARAY)
[33] KK 158: Ramazan CANER, KK 159: Fatih NADAR (KONYA)
[34] KK 160: Davut BÜYÜKKOÇAK (KONYA)
[35] KK 161: Hikmet REMET (KARAMAN)
[36] KK 162: Salih BAYRAM, KK 163: Bayram ÇAM, KK 164: Ali ALÇAM, KK 165: Ali Ramazan BAĞCI, KK 166: Osman GÜLLÜ, KK 167: Hüseyin ASKER (KONYA), KK 168: Süleyman ŞAHİN, KK 169: Celal GÜLBEYAZ, KK 170: Melikşah YILDIZ (AKSARAY), KK 171: Zekeriya SARAY (KARAMAN)
[37] KK 172: Kerim GÜZEL (KARAMAN)
[38] KK 173: Önder YILDIZ (AKSARAY)
[39] KK 174: Kemal KOTUK, KK 175: Muzaffer OK, KK 176: Mahmut VURULMAZ (KONYA)
[40] KK 177: İrfan BİRİCİK (KONYA)

yaptırıldığı tespit edildi. Buradan bit otu, DDT, termiğe tohumu ve tütün uygulamalarının Baytop [11], Kaya [14] ve Mimioğlu'nun [26] bulgularına benzerliği düşünüldüğünde bit ve pire tedavisinde elde edilen folklorik bilgilerin günümüz hekimliği ile benzerlikler gösteren rasyonel uygulamalar arasında yer aldığı ileri sürülebilir.

Coenurus cerebralis hastalığına ilişkin, Durmuş [27], ateşle kızdırılan bir demir ile hastalığın olduğu yerin dağlandığını; Araz [28] ve Güngör [29], demirin "*kutsiyeti*" ve "*koruyuculuğu*" ile ateşin "*kurtarıcılığı*" yönündeki inancın, demir ve ateşe bağlı eski Türk inançlarının izlerini taşıdığını ifade etmektedirler. Anadolu'da yapılan folklorik veteriner hekimliği araştırmalarında, hastalığın "*dönek*", "*delibaş*", "*dönme hastalığı*", "*dönere düştü*" ve "*yıldız ağmış*" adlarıyla bilindiği, tedavide kızgın demirle başın dağlandığı, hayvanın gözünün ön tarafında bulunan damardan kan akıtıldığı [1,5,22,23,30] görülmektedir. Güçlü ve ark.[31], *Coenurus cerebralis*'in beyine yapmış olduğu basınçtan dolayı hayvanlarda kendi etrafında dönme, diş gıcırdatması, inkoordinasyon ve tortikolis gibi sinirsel semptomların ortaya çıktığını bildirmektedir. Çalışmada, hastalığın, "*cinlenme*", "*dönelek*" olarak tanındığı, hastalığa köpek gaitası, "*karakasap otu*", "*çalgan otu*" veya "*anız*"ın sebep olduğu, tedavide kızgın demir ile koyunların kafasının dağlandığı, kulak ve burunlarının kesilip kan akıtıldığı veya hayvanların kesilerek köyün dışına gömüldüğü ve hastalıktan korunma amacıyla tabii aşımda kullanılan koçların her yıl değiştirildiği belirlendi. Bu bulgular özelinde, yukarıdaki literatür bulgularına paralel olarak demirin ateşte kızdırılmak suretiyle hasta koyunların kafasının dağlanmasının, kulak ve burunlarının kesilerek kan akıtılması gibi folklorik tedavi uygulamalarının geleneksel Türk inançlarının izlerini taşıdığı; ayrıca hastalığın iki farklı ismi, etiyolojisi ve hastalıktan korunma yollarına ilişkin bilgilerin ilk kez bu çalışmayla ortaya çıkarıldığı söylenebilir.

Veteriner parazitoloji alanında yürütülen çalışmalarda, tanen içeren yemlerin koyunlardaki nematodların yaşam siklusunu kırabildikleri, otlakların enfektif larvalarla kontaminasyonunu azaltabilecekleri, dolayısıyla ruminantların antelmintik ilaç kullanım sıklığını azaltabileceği düşünülmektedir [32]. Meşe kabukları %10-20 ve söğüt dalları %15 oranında tanen içermekte olup, yapılarındaki tanenlerin direkt antiparaziter etkileri ile sindirim kanalı parazitlerinin larval gelişimini engelledikleri [15]; karamuk *(Berberis crataegina* L.) kökü infuzyonunun antelmintik, antienflamatuar, antibakteriyel, analjezik, ekspektorant ve diüretik etkili olduğu ifade edilmektedir [9,10]. Sarımsağın antigiardial, antelmintik, antiprotozootik ve protoskolekslere karşı etkisinin, bileşiminde bulunan allisininden kaynaklandığı [7,33] ve köpeklerde görülen sarkoptik uyuzunda, sarımsak ekstraktının lokal uygulanmasında iyileşme oranının %54 olduğu [6]; tavuklarda görülen kenelere karşı %10'luk sarımsak suyunun üç hafta süreyle sprey tarzında uygulanması sonucunda tavuklara yerleşen kenelerin azaldığı tespit edilmiştir [8]. Lahana *(Brassica oleracea* L.) yaprakları ve tohumunun da kurt düşürücü ve sürgüt etkilerinin bulunduğu bildirilmiştir [11,12]. Çalışmada, sarımsağın uyuz hastalığı ve babesioziste, karamuk kökünün fasciolozis hastalığında ve mide-bağırsak parazitlerinde, lahananın mide ve bağırsak parazitlerinde kullanılmasının yukarıdaki literatür bilgilerine benzerlik gösterdiği ve bu bitkilerin tüketimiyle sindirim kanalı parazitlerinin sayısının azalabileceği; dolayısıyla hayvanların performansında artış meydana geleceği ileri sürülebilir. Külün ise bit, pire ve kene gibi ektoparazitlerin stigma adı verilen organlarını tıkayarak, parazitlerin solunumunu engelleyici özellikte olduğu düşünüldüğünde, uygulamaların rasyonel nitelik taşıdığı söylenebilir. Bu bağlamda, İç Anadolu Bölgesi Konya Bölümü folklorunda elde edilen bitkilerin kimyasal ve biyolojik aktivitelerinin tespitinin etnomedikal bilim dalları tarafından yapılması önerilebilir.

Göktaşı olarak bilinen bakır sülfatın, bazı deri hastalıklarında kurutucu ve antiseptik olarak [24], Anadolu folklorunda ise hayvanların bağırsak parazitlerine karşı kullanıldığı [1,5,22]; deneysel olarak yapılan bir çalışmada bazı trematod, serker ve metaserkerler üzerine öldürücü etkisinin olduğu [34]; ayrıca, hayvanlarda parazit yükünün kontrol altına alınabilmesi amacıyla da kullanıldığı bildirilmektedir [35]. Tarihin ilk çağlarında yaraları iyileştirmek ve cildi temizlemek amacıyla kullanılan kil minerallerinin, deriyi film şeklinde kaplayarak dışarıdan gelecek olan fiziksel ve kimyasal ajanlara karşı koruma sağladığı, emici özellikleriyle de deri salgılarını emerek deride bakteri gelişimini engellediği bilinmektedir [36]. Tappeh ve ark.[37], %20'lik hipertonik tuzun, tüm hidatik kist protoskolekslerini öldürdüğünü bildirmektedir. Çalışmada, uyuz, fasciolozis ve mide-bağırsak parazit mücadelesinde kullanımının, tuzun ozmotik basınç dengesini bozarak parazitlerin hücrelere invazyonuna engel teşkil edebileceği düşünüldüğünde, buna ek olarak bakır sülfat ve kil mineralinin modern tıptaki endikasyonları da hesaba katıldığında Konya Bölümü folklorunda göktaşı, kil ve tuz kullanılmak suretiyle gerçekleştirilmeye çalışılan terapilerin rasyonel nitelikte oldukları ileri sürülebilir.

Çalışmada, paraziter hastalık tedavilerinde kullanılan halk ilaçlarının terkibinde 18'i bitkisel (%48.65), 13'ü madensel (%35.13) ve 6'sı (%16.22) hayvansal kaynaklı olmak üzere toplam 37 farklı drog kullanıldığı belirlendi. Ayrıca çalışmada, kekre *(Acroption repens* L.) otu, çöğen otu *(Gypsophila* L.), dağ eriği *(Prunus divaricata* var. *divaricata*), gökbaş otu *(Centaurea cyanus* L.*)* kırmızı biber *(Capsicum annuum* L), gövelek otu *(Viscum album* L.) gibi bitkisel ilaç hammaddelerinin kodekslerde kayıtlı etkilerinin dışında kullanıldığı belirlendi.

Sonuç olarak, İç Anadolu Bölgesi Konya Bölümünde yapılanan folklor çalışmasında ruminantlarda bit, pire, kene, nokra, uyuz, coenurus cerebralis, fasciolozis, mide-bağırsak parazitleri ve babesiozis gibi paraziter hastalıklara rastlandığı ve tedavi yöntemleri arasında hidroterapi, fitoterapi, jeoterapi gibi yöntemlerin yer aldığı; parazit

tedavisinde kullanılan katran, bit otu, termiğe tohumu, tütün, karamuk bitkisi kökü, kükürt, tuz, göktaşı ve kil gibi ilaç hammaddelerinden modern tıpta da yararlanıldığı; ancak genel olarak uygulamaların dinî-sihrî, ampirik ve rasyonel nitelikler taşıdığı söylenebilir.

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Research Article

Comparison of Disinfection Activities of Nicotine with Copper Sulphate in water Containing *Limnatis nilotica* [1]

Mahmoud BAHMANI [1]
Ebrahimkhalil BANIHABIB [1]
Mahmoud RAFIEIAN-KOPAEI [2]
Majid GHOLAMI-AHANGARAN [3]

[1] *This research was supported by research grant (No. 189/03) from Deputy for Research and Technology, Iran*
[1] Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, IRAN
[2] Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, IRAN
[3] Poultry Diseases Department, Veterinary Medicine Faculty, Islamic Azad University, Shahrekord Branch, Shahrekord, IRAN

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Abstract

In this study, we investigated the potential use of nicotine in controlling water polluted by leeches. The nicotine and copper sulphate LC50 values were also determined following 30 min exposure. The anti parasitic effect of nicotine was also compared with that of copper sulphate as positive control. The anti-leech effect of nicotine was evaluated against L. nilotica in which the number of dead and alive leeches in each utensil was counted for 30 min. The positive control group was copper sulphate and the negative control was distilled water. Our data showed that the LD_{50} value for nicotine was $6/10^3$ ppm with mean death time of 1.25±0.45 min while the LD50 value for copper sulphate was $637/10^2$ ppm with a mean death time of 12.00±3.69 min. Based on the obtained results nicotine is highly effective on leeches and might be used for disinfection purposes.

Keywords: *Disinfection assay, L. nilotica, Nicotine, Copper sulphate, LC_{50}*

Limnatis nilotica Bulunan Suda Dezenfeksiyon Amaçlı Olarak Nikotinin Bakır Sülfat İle Karşılaştırılması

Özet

Bu çalışmada, sülük ile kontamine suda nikotinin kirliliği kontrol altında tutmak amaçlı olarak potansiyel kullanımı araştırılmıştır. Nikotin ve bakır sülfatın LC50 değerleri 30 dakikalık maruz bırakmada hesaplanmıştır. Nikotinin anti-parazitik etkisi control olarak kullanılan bakır sülfat ile karşılaştırılmıştır. Nikotinin anti-parazitik etkisi L. nilotica'ya karşı 30 dakika süresince ölü ve canlı sülüklerin sayılması ile gerçekleştirildi. Pozitif kontrol olarak bakır sülfat ve negatif kontrol için ise distile su kullanıldı. Çalışmanın sonuçları nikotin için LD_{50} değerinin $6/10^3$ ppm, ortalama ölüm zamanının 1.25±0.45 dakika olduğunu, bu değerlerin bakır sülfat için ise sırasıyla $637/10^2$ ppm ve 12.00±3.69 dakika olduğunu gösterdi. Elde edilen sonuçlar nikotinin sülüklere karşı oldukça etkili olduğunu ve dezenfeksiyon maksatlı kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Dezenfeksiyon testi, L. nilotica, Nikotin, Bakır sülfat, LC_{50}*

INTRODUCTION

Contamination of superficial and supernatant water and the need for access to new sources are the biggest problems in developing countries, focused by international studies. With the growth of population and a decline in water supplies, clean water sources are more urgently needed [1]. Poor quality of water, environmental sanitation, and hygiene kill 1.7 million people worldwide annually. The mortality rate due to contaminated water is 3.1 million deaths in the world [2].

According to statistics released by the World Health Organization, 75 percent of diseases of human are due to the lack of access to safe water with hygienic (swimming, bathing, etc.) and drinking purposes [3]. Water sources are contaminated with various chemical pollutants such as heavy metals, germs, bacteria, and parasites. Leeches are parasitic elements of water contamination.

So far, 650 species of aquatic and terrestrial leeches have been identified. Leeches have been found in different parts of the human bodies, such as membranes, conjunctiva,

İletişim (Correspondence)
+98 381 3330709
rafieian@skums.ac.ir

nose, larynx, pharynx, esophagus, urethra, vagina, and anus [4]. Parasitic infestation with leeches happens through contaminated water supplies.

Disorders such as anemia, chest pain, coughing, difficulties in swallowing, breathing, fever, vomiting, bloody diarrhea, and vaginal bleeding complications occur with aquatic leeches [5].

There are several ways to disinfect water supplies. Disinfection of water suppliess contaminated with *Giardia lamblia* might be done by electricity flows [6], sunshine [7], gamma rays [8,9], hydrogen peroxide-silver complex and chlorine [10].

A group of medicinal plants or their active ingredients are used to improve or prevent human and animal diseases [11]. One type of plants by-products (nitrogenous) are alkaloids. These compounds are the largest group of plant secondary compounds. Traditional uses of alkaloids by human go back to over 3000 BC. Nowadays, the alkaloids from certain plants have a great value in the treatment of certain diseases as well as pharmaceutical industries [11]. There are several reports on human infections with leeches and some reports exist on the positive effects of medicinal plants on these parasites. Nicotine or 3-(1-Methyl-2-pyrrolidinyl) pyridine, (S)-(-)-Nicotine ($C_{10}H_{14}N_2$) with the following formula is an alkaloid of tobacco plant [12].

In the present study the potential use of nicotine in controlling water supplies polluted by leeches was investigated.

MATERIAL and METHODS

Taxonomy and Species of the Leeches

In this study 30 *L. nilotica* leeches were used. These species have morphological characteristics such as dark-green color surface with yellowish-orange rows and green spots on yellowish-orange dorsal surface [13].

Chemical Components

In this interventional screening study, nicotine (Merk, Germany) was prepared and then tested with copper sulphate (Sahand, Iran) ($CuSO_4$) as positive group.

Evaluation of the anti Annalida Activities

To investigate the effects of treatment, the method of Bahmani et al.[14] was used. The *L. nilotica* was placed in the plastic utensil containing water. Then, nicotine with compactness of $6/10^{+3}$ ppm was added to the utensil. The experiment was carried out in three replicates for each compound. The number of dead and alive leeches in each utensil was counted for 30 min. The positive control group was copper sulphate and the negative control was distilled water. The leeches were considered dead if they did not exhibit any internal or external movement when stimulated with a needle in the needle test [14].

Statistical Analysis

The differences between control and treatment groups were analyzed using one-way ANOVA statistical method by Sigma State 2.0 software.

RESULTS

During the 30 min of screening, the number of alive and dead leeches was enumerated. The results of the leech lethality trial are presented in *Table 1*. The LC_{50} values for nicotine as bioactive component less than the ones of positive (copper sulphate) or negative (distilled water) control groups.

There was a significant difference between the treatment and control groups ($P<0.05$). Normality failed with a median of 1, 11, and 30 for nicotine, $CuSO_4$, and water respectively.

Table 1. *Compounds, LC_{50} and effective dose for L. nilotica*
Tablo. *Maddeler, L. nilotica için LC_{50} ve etki dozu*

Compounds	LC_{50} (Mean±SD)	Dose (ppm)
Nicotine	1.25 ± 0.45^a	$6/10^{+3}$
$CuSO_4$	12.00 ± 3.69^b	$637/10^{+2}$
Distilled water	30 ± 0^c	10^{+2}

DISCUSSION

In this study, the potential use of nicotine in controlling water polluted by leech was investigated. The nicotine and copper sulphate LC_{50} values were also determined following 30 min exposure. Nicotine showed an anti-leech activity with LD_{50} value of $6/10^3$ ppm with a mean death time of 1.25±0.45 min while the LD_{50} value for copper sulphate was $637/10^2$ ppm with a mean death time of 12.00±3.69 min. Based on the obtained results, nicotine is highly effective on leeches and might be used for disinfection purposes. Several studies have investigated the efficacy of chemical and natural anti leech drugs. Considering the importance and frequency of contamination of surface water with leeches, study on the effects of different combinations of drugs is essential. Bahmani et al.[15] reported that garlic methanol extract (*Allium sativum* L.) had the anti immature *L. nilotica* effect. In another study Gholami-Ahangaran et al.[16] reported that *Vitis vinifera* L. and grape methanolic extracts, ivermectin, and niclosamide on *L. nilotica* had anti parasite activity against *L. nilotica*. Eftekhari et al.[17] investigated the anti *L. nilotica* effect of *A. sativum* L. extract and Levamisole on mature *L. nilotica*. Their results demonstrated that garlic methanol extract had a mean dead time of 144.55±57.217 min. In another study the disinfection effects (LC_{50s}) of *Nicotiana tabacum* extract, copper sulphate, and ammonium

chloride on *L. nilotica* were found to be 13/10^4, 8/10^4, and 370/10^4 ppm, respectively. In previous studies, effective and positive effects of grapes, olives, ginger has been demonstrated to leech [18-21].

Nicotine is a highly toxic compound for some animals [12]. Due to the strong effects of nicotine in cleaning water supplies polluted with leech, it could be a natural compound to be used in the treatment of contaminated water supplies. The results of a study showed that LCD_{50} for nicotine was 1.25±0.45 min., which is a reasonable dead time and acceptable for clearing water supplies polluted by leeches. Another study showed that high doses of Harmal methanol extract had no effect on mortality of leeches, which are not consistent with the results of this study [14].

In the present study nicotine had a very good dead time against *L. nilotica*, therefore, it might be beneficial in controlling water supplies polluted with leeches. Although this compound is derived from a plant and the plants are usually safer than synthetic ones, its safety profile should be tested, in the same way as other compounds have been tested [22- 25].

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Research Article

Effects of Cage Stocking Density on Egg Quality Traits in Japanese Quails

Mahmoud Salah EL-TARABANY [1] Tamer Mohamed ABDEL-HAMID [1]
Hesham Hosny MOHAMMED [2]

[1] Department of Animal Wealth Development, Faculty of Veterinary Medicine, Zagazig University, EGYPT
[2] Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, EGYPT

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Abstract

This study was conducted to investigate the effects of different stocking densities on external and internal egg quality traits and to measure the phenotypic correlation coefficients between external and internal egg quality traits. Two hundred and sixteen birds of Japanese quail at 14th week of age were used in this experiment. The birds were divided randomly into three groups, 60, 72 and 84. Each group subdivided into 4 replicates, where the cages floor spaces were 200, 167 and 143 cm^2/bird; respectively. The obtained results revealed that birds housed at 200 cm^2/bird laid heavier egg weight (12.24 g), with significant ($P<0.05$) higher external quality traits, including shell weight (1.27 g), eggshell ratio (10.55%), shell thickness (0.23 mm) and egg shell density (48.70 mg/cm^2). Similarly, internal quality traits, including yolk height (10.72 mm), albumen height (5.67 mm), yolk diameter (24.97 mm), yolk index (43.49%) and Haugh unit (92.77). Shell weight positively correlated with yolk height ($P<0.01$), albumen height ($P<0.01$), albumen weight ($P<0.05$) and haugh unit ($P<0.01$). It was concluded that, housing Japanese quail at low cage floor space associated with depression in external and internal egg quality traits. Moreover there is an economical hazard of housing quails at cage floor space lower than 200 cm^2/bird.

Keywords: *Cage, Egg quality, Stocking density, Quail*

Bıldırcınlarda Kafes Yoğunluğunun Yumurta kalitesi Üzerine Etkileri

Özet

Bu çalışma değişik kafesleme yoğunluklarının iç ve dış yumurta kalitesi üzerine etkisini araştırmak, iç ve dış yumurta kalitelerinin fenotipik korelasyon katsayısını ölçmek amacıyla yapılmıştır. Çalışmada 14 haftalık toplam 216 bıldırcın kullanılmıştır. Hayvanlar rastgele olarak 3 gruba (60, 72 ve 84) ayrıldı. Her bir grup taban alanları sırasıyla 200, 167 ve 143 cm^2/bıldırcın olacak şekilde ayrıca 4 tekrarlı olmak üzere ayrıldı. Çalışma bulguları sonucunda 200 cm^2/bıldırcın olan grupta bıldırcınların daha ağır yumurta verdikleri (12,24 g), anlamlı derecede daha yüksek dış özellikler gösterdikleri; kabuk ağırlığı (1.27 g), kabuk oranı (%10.55), kabuk kalınlığı (0.23 mm) ve kabuk yoğunluğu (48.70 mg/cm^2) belirlendi. Benzer olarak iç kalite özellikleri; yumurta sarısı yüksekliği (10.72 mm), yumurta akı yüksekliği (5.67 mm), yumurta sarısı çapı (24.97 mm), yumurta sarısı indeksi (%43.49) ve Haugh birim (92.77) olarak belirlendi. Kabuk ağırlığı pozitif olarak yumurta sarısı yüksekliği ($P<0.01$), yumurta akı yüksekliği ($P<0.01$), yumurta akı ağırlığı ($P<0.05$) ve Haugh birim ($P<0.01$) ile ilişkiliydi. Çalışma bulguları; bıldırcınların küçük tabanlı alanda yerleştirilmesinin dış ve iç yumurta kalitesini olumsuz etkilediği ortaya koymuştur. Bıldırcınların kafes taban alanının 200 cm^2/bıldırcından daha düşük olmasının ekonomik kayıp oluşturduğu sonucuna varılmıştır.

Anahtar sözcükler: *Kafes, Yumurta kalitesi, Kafes yoğunluğu, Bıldırcın*

INTRODUCTION

Japanese quail (*Coturnix coturnix japonica*) is distributed in many parts of the world, also in Egypt where it could be used as a source of meat and eggs. It has also depicted worldwide usage as laboratory animal [1]. Advantages of Quail as meat animal are: low housing costs and smaller body weight so low floor and/or cage space requirements. In addition to, outcome of waste is low compared with other conventional livestock enterprises, and thus it is not so detrimental to the environment [2].

Overall egg quality is important for both poultry breeders and for consumers. Poor quality results in substantial economic losses to the worldwide egg industry. For example, losses due to poor eggshell quality have been

İletişim (Correspondence)
+20 122 3668785; Fax: +20 205 52283683
mmohamedibrahim@zu.edu.eg; mahmoudtarabany2887@yahoo.com

calculated to be in the order of 6-8% [3]. The egg industry is more dependent on high stocking densities for laying hens and quails either during the rearing or laying phases as a tool to reduce housing and equipment costs per bird. The much reduction in the available cage floor space per bird, moreover, feeder and drinker space per bird, may have depressive effects on growth and later performance, so feed intake might decrease which had a worse effect on live weight, and muscular and bone development. Body weight and feed intake are key factors in bird development, egg production, egg size and feed conversion [4].

The phenotypic correlation between any two quantitative traits explained the degree to which individuals above average for one trait tend to be above, below or near the average for the other traits [5]. Knowledge of correlations among productive traits is essential for the construction of selection indices designed to maximize the rate of genetic improvement. Phenotypic characteristic for egg quality traits of external and internal type is important mainly for interest of economy of production [6]. The eggs weight is directly correlated with egg shell quality which has a positive correlation with shell thickness and shell weight [7]. Kul and Seker [8] estimated the phenotypic correlation between internal and external quality traits of eggs in quails. Positive phenotypic correlation was recorded between egg weight and other egg biometrical traits. Ayasan et al.[9] demonstrated high feed conversion ratio, live weight and carcass traits in female quails managed at low stocking density.

The economical importance of stocking density comes from its depressive effect on gain per bird housed and too small profit margins as poultry industry depends on intensive production [10]. Therefore, the objectives of the present investigation were to evaluate the effects of cage density on external and internal egg quality traits and to measure the phenotypic correlation coefficients between external and internal egg quality traits.

MATERIAL and METHODS

Experimental procedures were conducted in accordance with the Zagazig University Animal Ethics Committee guidelines (ANWD-206). Two hundred and sixteen birds of Japanese quails at 14th week of age were used with average initial body weight (173.46 g±1.38). The birds were obtained from experimental unit belonging to Faculty of Agriculture, Zagazig University. The birds were divided randomly into three groups, 60, 72 and 84. Each subdivided into 4 replicates, where the cages floor spaces were 200, 167 and 143 cm^2/bird; respectively. The measures of each cage were 60 x 50 x 37 cm (L x W x H).

Fourteen hours of light per day were provided for quails to maintain maximum egg production and fertility. Supplementary lighting was provided as required to maintain the production [11]. Basic quail's diet was provided ad libitum containing 19.5% crude protein and 2852 k.cal ME/kg. The calculated analysis and ingredients of laying diet were demonstrated in *Table 1*. To assess the egg quality parameters, a total of 240 fresh eggs were randomly collected in 4 sequent weeks, 80 eggs from each stocking density group. Eggs were individually labeled after collection and the following measures of egg quality were taken.

Egg weight measured using the Sartorius 1202 MP balance with accuracy 0.01 g. Egg length and width (mm) measured using an electronic digital caliper. Egg shape index: calculated as egg width/egg length x 100 [12]. Egg shell thickness (mm): measured using an electronic digital caliper, taken as the mean of measures from the equator and both ends of the egg. Egg shell weight (g) was measured, as well as egg shell percentage as shell weight/egg weight x 100. Egg surface area cm^2: calculated as $3.9782W^{0.7056}$, where W = egg weight [13]. Egg shell density mg/cm^2: calculated as shell weight (mg)/egg surface area [14].

The measurements of the internal qualities were obtained by gently broken the egg using a scalpel and the contents were taken on flat surface. The yolk was carefully separated from the albumen for weighing. The albumen weight was calculated by subtracting the weight of yolk and shell from the weight of whole egg. The albumen and yolk height and width (mm) were measured using electronic caliper [15]. Yolk weight ratio (%) = yolk weight (g)/egg weight (g) x 100. Similarly, albumen weight ratio

Table 1. *Diet composition in the laying period*
Tablo 1. *Yumurtlama periyodunca kullanılan diyetin içeriği*

Ingredients	**Laying diet**
Yellow corn	64.50
Soybean meal (44%)	20.50
Concentrate (52%)	10.00
Di- calcium phosphate	2.31
Limestone	0.96
DL- methionine	0.09
Lysine	0.08
Vitamin and trace mineral	0.30
Premix	1.06
Coccidostate	0.10
Antioxidant	0.10
Calculated Analysis	
ME (K.cal/kg)	2852
Crude protein (cp %)	19.50
Calcium %	2.33
Available phosphorus %	0.66
Lysine %	1.04
Methionine	0.52

(%) = albumen weight (g)/egg weight (g) x 100. Yolk index (%) = yolk height (mm)/yolk diameter (mm) x 100 [16]. Haugh Unit = 100 log (albumen height (mm) + 7.57 - 1.7 x egg weight (g) $^{0.37}$ [17].

Data were analyzed using SAS statistical analysis system package [18]. One way ANOVA was performed using the following model

$Y_{ij} = \mu + L_i + eij$

Where Y_{ij} = egg quality trait; L_i = effect of i^{th} stocking density and e_{ij} = residual. The preliminary effects of replicates were non-significant. Differences among means were compared statistically using Duncan's multiple range tests [19]. Pearson correlations were performed to compute the relationship of the external and internal egg quality traits.

RESULTS

Means ± standard error of external and internal egg quality traits at different stocking densities (200, 167 and 143 cm²/bird) are shown in *Table 2*. It clearly shows that different stocking densities had significant effect on most of egg quality traits.

Phenotypic correlation coefficient between external and internal egg quality are presented in *Table 3*. As it was seen, there were significant correlations among the different egg quality traits.

DISCUSSION

The primary objective of this study was to investigate the effect of cage stocking density on egg quality traits in Japanese quails and to explore the correlations between external and internal egg quality traits. Birds housed at 200 cm²/bird, cage floor space, recorded significant (P<0.05) higher estimates for egg weight (12.24 g), shell weight (1.27 g), eggshell ratio (10.55%), shell thickness (0.23 mm) and egg shell density (48.70 mg/cm²), compared to birds housed at lower cage floor space (167 and 143 cm²/bird). Results revealed that, stocking density had a non-significant effects (P>0.05) on egg length, where the longest egg length had been measured for quails kept at cage floor space of 200 cm²/bird. Birds housed at 200 cm²/bird, cage floor space, had significant (P<0.05) higher estimates for yolk height (10.72 mm), albumen height (5.67 mm), yolk diameter (24.97 mm), yolk index (43.49%) and Haugh unit (92.77), compared to birds housed at lower cage floor space (167 and 143 cm²/bird). However, results cleared that stocking density had a non-significant effects (P>0.05) on yolk weight, yolk ratio, albumen weight and albumen ratio.

The linear lowering in egg weight with each increase in

Table 2. *Effects of different cage stocking densities on the external and the internal egg quality traits of Japanese quails*
Tablo 2. *Bıldırcınlarda kafes yoğunluğunun dış ve iç yumurta kalitesi üzerine etkileri*

Trait	Cage Stocking Density (cm²/bird)			F	P value
	200	167	143		
Egg weight (g)	12.24±0.16^{a}	11.87±0.12^{ab}	11.74±0.14^{b}	3.16	0.04
Egg Length (mm)	33.11±0.19	32.80±0.18	32.95±0.16	0.74	0.47
Egg Width (mm)	25.92±0.14^{a}	25.50±0.15^{b}	26.12±0.13^{a}	4.80	0.00
Egg shape index (%)	78.41±0.48^{ab}	77.87±0.55^{b}	79.35±0.38^{a}	2.43	0.09
Shell weight (g)	1.27±0.01^{a}	1.08±0.01^{b}	1.06±0.01^{b}	59.98	0.00
Eggshell ratio (%)	10.55±0.19^{a}	9.22±0.11^{b}	9.16±0.14^{b}	25.91	0.00
Shell thickness (mm)	0.23±0.009^{a}	0.20±0.003^{b}	0.20±0.002^{b}	39.68	0.00
Egg surface area (cm²)	23.26±0.21^{a}	22.78±0.17^{ab}	22.59±0.19^{b}	3.10	0.04
Eggshell density (mg/cm²)	48.70±0.90^{a}	44.92±0.51^{b}	44.37±0.64^{b}	11.01	0.00
Yolk height (mm)	10.72±0.22^{a}	9.13±0.12^{b}	8.38±0.14^{c}	48.41	0.00
Albumen height (mm)	5.67±0.13^{a}	4.15±0.06^{b}	3.95±0.05^{b}	38.12	0.00
Yolk diameter (mm)	24.97±0.29^{a}	23.86±0.24^{b}	23.58±0.28^{b}	6.99	0.00
Yolk weight (g)	4.31±0.13	4.31±0.13	4.27±0.11	0.03	0.96
Yolk ratio	35.46±1.08	36.57±1.13	36.86±1.01	0.47	0.62
Albumen weight (g)	6.64±0.17	6.41±0.15	6.26±0.15	1.38	0.25
Albumen ratio	53.98±1.10	54.15±1.12	53.34±0.98	0.15	0.85
Yolk index (%)	43.49±1.09^{a}	38.52±0.63^{b}	35.97±0.77^{c}	19.79	0.00
Haugh unit	92.77±0.40^{a}	87.35±0.33^{b}	86.29±0.35^{c}	32.03	0.00

Means within the same row having different superscripts are significantly different

Table 3. *Correlation coefficients among the external and internal egg quality traits in Japanese quails*
Tablo 3. *Bıldırcınlarda dış ve iç yumurta kaliteleri arasındaki korelasyon katsayıları*

Traits	Egg Weight (g)	Egg Length (mm)	Egg Width (mm)	Egg Shape Index (%)	Shell Weight (g)	Eggshell Ratio	Shell Thickness (mm)	Egg Surface Area (cm^2)	Eggshell Density (mg/cm^2)
Yolk height (mm)	0.004	-0.03	-0.026	0.001	0.244**	0.222**	0.314**	0.004	0.136*
Albumen height (mm)	-0.031	-0.091	-0.175**	-0.087	0.371**	0.380**	0.262**	-0.032	0.265**
Yolk diameter	0.356**	0.244**	0.233**	0.00	0.220**	-0.058	0.174**	0.357**	-0.037
Yolk weight (g)	0.237**	0.026	0.132*	0.105	0.007	-0.164*	0.058	0.237**	-0.138*
Yolk ratio	-0.169*	-0.274**	-0.096	0.153*	-0.103	0.024	0.004	-0.180**	0.003
Albumen weight (g)	0.671**	0.579**	0.336**	-0.190**	0.135*	-0.338**	0.046	0.671**	-0.267**
Albumen ratio	0.225**	0.298**	0.108	-0.161*	0.013	-0.144*	-0.011	0.226**	-0.119
Yolk index (%)	-0.172*	-0.155*	-0.139*	0.007	0.119	0.242**	0.206**	-0.181**	0.151*
Haugh unit	-0.121	-0.196**	-0.182**	-0.001	0.311**	0.380**	0.299**	-0.139*	0.255**

*** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed)*

density from 200 to 143 cm^2 per bird agreed with findings of Faitarone et al.[20], this can be explained by depression of feed intake and more energy expenditure may be accountable for the reduction in egg weight of quail housed at higher cage stocking density. Davami et al.[21] concluded that hens in lower density cages were allowed more movement within the cage, which may results in less stressful conditions. Food is portioned between body functions, including maintenance, growth, reproduction and health. In stress, most of the consumed food is used to cope with unpleasant conditions [22]. In the present investigation, this condition may explain the reduction in the egg weight in groups with high densities.

High stocking density in the cages makes it very difficult for the birds to dissipate their heat [23]. Gou et al.[24] stated that, birds raised in small group size had a lower rectal temperature than those raised in big group size, indicating a facilitating effect of thermoregulation. The birds tend to distance themselves from one another to maximize sensible heat loss. The reduction in reproductive performance associated with heat stress is a well-known phenomenon in domestic birds. This is probably due to the direct debilitating effect of high heat stress on ovarian function in the birds or indirectly through reduction in blood flow to the ovary [25]. Also, Dhaliwal et al.[26] stated a higher stocking density adversely affected egg quality. Negotiated results were demonstrated [27]. They found that albumen index and fresh albumen weight were higher in birds in the lower stocking density than those in other stocking density. On the contrary, non-significant effects of stocking density on egg weight in Japanese quails were reported, and accounted these finding for smaller size of Japanese quails compared to Italian quails [28]. Kakimoto et al.[29] and Bovera et al.[30] enhances these finding, they concluded that, external and internal quality of egg remains unaffected by group size in laying hens. Nagarajan et al.[10] reported that shell thickness was influenced neither by age of the hen nor by stocking density, and the yolk index was superior in the lowest stocking density group. Bandyopadhyay and Ahuja [31] added that cage density significantly affected albumen index, yolk index and internal egg quality, but not egg weight, shape index or shell thickness. In general, yolk index and internal egg quality decreased with increasing cage density; albumen index did not show a definite pattern

Egg weight was positively correlated (P<0.05) with yolk diameter (0.356), yolk weight (0.237), albumen weight (0.671) and albumen ratio (0.225). Egg length was positively correlated with yolk diameter (0.244), albumen weight (0.579) and albumen ratio (0.298). Otherwise, egg weight was negatively correlated with yolk ratio (- 0.169), due to lower specific gravity of the yolk which has more lipid content. Shell weight was positively correlated with yolk height (0.244), albumen height (0.371), yolk diameter (0.220) and Haugh unit (0.311). Concretely, shell thickness was observed to be correlated significantly with yolk height (0.314), albumen height (0.262), yolk diameter (0.174), yolk index (0.206) and Haugh unit (0.299). Egg shell density was positively correlated with yolk height (0.136), albumen height (0.265), yolk index (0.151) and Haugh unit (0.255). On the contrary, egg length was negatively correlated (P<0.01) with yolk ratio (-0.274) and haugh unit (-0.196). Similarly, eggshell ratio and eggshell density were negatively correlated (P<0.01) with albumen weight (-0.338 and -0.267).

Results regarding the egg weight were positively correlated with yolk diameter, yolk weight, albumin weight, albumin ratio, which are supported by Zita et al.[32]. They measured positive phenotypic correlations of egg weight with yolk weight, albumen weight and egg shell weight. Also, their finding about negative correlation of egg weight with yolk ratio and egg shell proportion negotiated our experiment. In addition to, positive correlation of egg weight with albumen ratio and negative correlation of albumen weight with eggshell ratio. The findings of

Baumgartner [1] and Minvielle et al.[33] are in the same line with our investigation who estimated high positive correlations between egg weight and the weights of its contents but, Baumgartner et al.[34] found that the correlations between egg weight and the yolk, albumen and eggshell weight were non-significant, low positive estimates (0.432, 0.438 and 0.234; respectively). Yannakopoulos and Tserveni-Gousi [35] demonstrated that egg weight was positively correlated with albumen weight. Kul and Seker [6] and Ozcelik [36] stated that the increase in the egg weight will result in decreased yolk ratio. Kul and Seker [6] reported that yolk and shell ratios negatively correlated with the albumen ratio and albumen index. Ozcelik [36] demonstrated that, egg shape index was negatively correlated with albumen weight and yolk weight. We concluded that, housing Japanese quails at low cage floor space associated with depression in external and internal egg quality traits, reflecting the economical hazards of housing quails at cage floor space lower than 200 cm^2/bird.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Research Article

Effect of *in ovo* Feeding of Butyric Acid on Hatchability, Performance and Small Intestinal Morphology of Turkey Poults

Mehdi SALMANZADEH [1] Habib AGHDAM SHAHRYAR [2] Alireza LOTFI [3]

[1] Young Researchers and Elite Club, Shabestar Branch, Islamic Azad University, Shabestar, IRAN
[2] Department of Animal Science, Shabestar Branch, Islamic Azad University, Shabestar, IRAN
[3] Ilkhchi Branch, Islamic Azad University, Ilkhchi, IRAN

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Abstract

The aim of present study was to investigate effect of *in ovo* administration of butyric acid on hatchability, performance, and small intestinal morphology of turkey poults. Fertilized eggs were subjected to injections with butyric acid (10 mg, 20 mg and 30mg dissolved in 0.5 mL of deionized water) on the d 7 of incubation. Hatching traits, Body weight gain (BWG), feed intake, and feed conversion ratio (FCR) were determined during experiment.Small intestinal morphology included villus height (VH), crypt depth (CD), and villus width (VW) were measured at hatch and the end of each rearing periods. Finally, the results of the present study indicate that the weight of newly-hatched poults was significantly greater when butyric acid were administrated, in comparison with control groups.But, *in ovo* feeding (IOF) caused lower hatchability than in control group (not-injection eggs) ($P<0.01$). Poults from IOF showed better weight gain and FCR (0-42 day of age), when compared to poults hatched from control ($P<0.01$). The IOFB significantly increased VH for duodenum, jejunum and ileum at both hatch and starter period. It was concluded that IOFB may affect VH of intestine at hatch and starter (post-hatch) period in turkeys. Also, IOFB can improve performances.

Keywords: *Butyric acid, Feeding, Performance, Small intestinal morphology, Turkey poult*

In ovo Bütirik Asit Beslemesinin Hindilerde Yumurtadan Çıkma, Performans ve İnce Barsak Morfolojisi Üzerine Etkileri

Özet

Bu çalışmanın amacı *in ovo* bütirik asit beslemesinin hindilerde yumurtadan çıkma, performans ve ince barsak morfolojisi üzerine etkilerini araştırmaktır. Fertilize olmuş yumurtalara inkübasyonun 7. gününde 0.5 mL deiyonize su içerisinde çözdürülmüş 10, 20 veya 30 mg bütirik asit enjekte edildi. Yumurtadan çıkma, vücut ağırlık artışı (BWG), yem tüketimi ve yem konversiyon oranı (FCR) belirlendi. İnce barsak morfolojisini gösteren villus yüksekliği (VH), cript derinliği (CD) ve villus genişliği (VW) yumurtadan çıkma zamanında ve her bir yetiştirme periyodu sonunda ölçüldü. Çalışma bulguları kontrol grubu ile karşılaştırıldığında bütirik asit verilenlerde yumurtadan çıkma zamanında vücut ağırlıklarının anlamlı derecede büyük olduğunu gösterdi. Ancak *in ovo* besleme (IOF) kontrol grubu (enjeksiyon yapılmayan) ile karşılaştırıldığında daha düşük yumurtadan çıkmaya neden oldu ($P<0.01$). *In ovo* beslenen civcivler kontrol grubu civcivleriyle karşılaştırıldığında daha iyi vücut ağırlığı kazanımı ve yem konversiyon oranı (0-42 gün) gösterdiler ($P<0.01$). *In ovo* bütitik asit besleme duodenum, jejenum ve ileum villus yüksekliklerinde hem yumurtadan çıkma hem de starter döneminde anlamlı artışa neden oldu. Sonuç olarak hindilerde *in ovo* bütitik asit besleme yumurtadan çıkma ve starter dönemlerinde villus yüksekliğini etkilemektedir. Ayrıca *in ovo* bütitik asit besleme performansta iyileşmeye neden olabilir.

Anahtar sözcükler: *Bütitik asit, Besleme, Performans, İnce barsak morfolojisi, Hindi*

INTRODUCTION

Early post-hatch starvation has been associated with lower satellite cell development and decreased muscle growth in starved chicks than in fed controls throughout the experiment [1]. In this study, Chicks were either fed or starved for 48 h post-hatch (d 0-d 2, d 2-d 4 or d 4-d 6) and then refed for 41 d [1], whereas early post-hatch feeding stimulates satellite cells and muscle growth in turkey poults [2]. Thus, Uni *et al*.[3] hypothesized that the IOF solution contained 25 g/L maltose, 25 g/L sucrose, 200 g/L dextrin, 1 g/L HMB all dissolved in 5 g/L NaCl,

İletişim (Correspondence)
++98 461 2334218, Fax: +98 461 2456225
salmanzadeh_mehdi@ymail.com

probably enhanced myoblast development during the late embryonic stages in broilers. Also, IOF of amino acid as early feeding may provide poultry companies with an alternative method for increasing the weight of newly-hatched chicken and growth performance [4,5]. Moreover, anovel method of supplementing the (IO) nutritive of oviparous species, described as feeding (IOF) within the US Patent (6592878) of Uni and Ferket [6] was demonstrated to be an effective way to administer exogenous nutrient to support the development of the embryos and neonates in poultry.

Foye *et al*.[7] reported that IOF of egg white segment, β-hydroxy-β-methylbutyrate, and carbohydrates may help to overcome the constraint of limited egg nutrients in turkeys.

Overall, the organogenesis of important segments of the chick embryo are occurred at first week of incubation, in this regard, gastrointestinal organogenesis (includes foregut, midgut and hindgut) was reported at 4-7 days of incubation. In other hand, formation of most important organs includes ovary, ileum, femur, pancreas, gastrocnemius muscle, and duodenum are establish at day-9 of incubation. Because of these evidences, IOF of energy-supplemented nutrients in this critical period (day-7) can be efficient stimulator for optimal growth and development of organs [8]. In the past studies related to "*in ovo* feeding", almost all of the works were conducted at the days of 17-21, late-embryonic or pre-hatch stages [3,9-13], but in the present study, we evaluated the effects of IOF in early- embryonic life of turkey poult, and the IOF of nutrient supplementation in early embryonic life.

Short-chain fatty acids, such as butyric acidare considered the prime enterocytes energy source and it is necessary for the correct development of the gut-associated lymphoid tissue [14]. Butyric acid is considered as potential alternatives to antibiotic growth promoter with positive effects on gastrointestinal function and improvement feed conversion ratio [15-17]. In this regard, Pryde *et al*.[18] reported that butyric acid can be utilized as an energy source for growth and development of intestine epithelial cells in colon.

In poultry, butyric acid is considered as a selective foodstuff for optimizes growth performance during commercial rearing period [19]. The small intestine is the major site for digestion and absorption of nutrients [20-22].

In this regard, butyric acid is known as an energy source for growth and development of intestine which it was reported in Japanese quails [23].

These results suggest that butyric acid may affect the hatching traits, performance and small intestinal morphology of turkey poults by modifying the early energy status of embryogenesis. So, the main propose of the present study was to determine of effect of injection of butyric acid into turkey eggs at d 7of incubation on hatchability, post-hatching performance, and small intestinal morphology of turkey poults.

MATERIAL and METHODS

Incubation and Injection

Seven hundred twenty fertile eggs were collected from turkey breeder (Nicholasstrain) at 34 weeks of age. All eggs were collected from the same breeder flock, weighed and eggs with a weight of 82±1 g were selected for incubation (37.8°C and 63% RH). On the d 6 of incubation, the eggs were candled, and the infertile were removed. At d 7 of incubation, fertile eggs were divided into 5 treatments with 4 replicates per treatment and 36 eggs per replicate, based on completely randomized design. Treatments were includes: 1) without injection (control group), 2) *in ovo* infusion of 0.5 mL of deionized water (sham group), 3) *in ovo* infusion of butyric acid (10 mg dissolved in 0.5 mL of deionized water), 4) *in ovo* infusion of butyric acid (20 mg dissolved in 0.5 mL of deionized water), 5) *in ovo* infusion of butyric acid (30 mg dissolved in 0.5 mL of deionized water).

Then, each egg was candled to identify the location of the injection. Next, *in ovo* injection was conducted using a 22-gauge needle and 0.5 mL of IOFB solution into the yolk sac to a depth of 19 mm. The injection area on the egg shells was disinfected with an ethyl alcohol-laden swab, sealed with cellophane tape, and transferred to hatching baskets. During injection process, the control group (non-injected eggs) were removed from the incubator together with the treated groups, and kept in the same environment. Injected solutions containing butyric acid were prepared by directly dissolving butyric acid in the deionized water. Butyric acid was purchasedfrom Silo® Co (Silo Company, Italy) and contained 25 to 30% monoglycerides in the 1 or 3 positions, 50 to 55% diglycerides in the 1 or 3 positions, and 15 to 25% triglyceride.

Birds

After hatching, poults were transferred to experimental Farm of Islamic Azad University and reared for 42 days with same ration in according to NRC turkey ration [24], in according to *Table 1*. Each treatment group and poult was identified by neck tags. All treatments were randomly assigned to 1 of 20 pens. Each pen was bedded with soft pine wood shavings and equipped with automatic drinkers, and manual self-feeders. They had *ad libitum* access to feed and drinking water. Environmental conditions of housing were constant during the trial (temperature: 20±3°C, RH: 60%, and 23-h lighting).

Data Collection

On hatching, hatchability and hatching weight were measured. Weight of newly-hatched poults was determined

Table 1. *Composition and nutrient contents of the basal diet distributed turkey poults for 1- 21 and 22-42 days*

Tablo 1. *Hindilere 1-21 ve 22-42 günler arası verilen bazal diyetin kompozisyonu ve besin içerikleri*

Ingredients (%)	0- 21day	22-42 day
Corn	45.90	33.77
Wheat	10.00	25.00
Soybean meal (48% CP)	30.91	27.90
Corn gluten meal (52% CP)	6.00	6.00
Soybean oil	4.00	3.90
Dicalcium phosphate	1.00	1.90
Limestone	1.59	1.40
Salt	0.27	0.27
VMP[1]	0.50	0.50
L-Lysine HCl	0.32	0.29
DL-Methionine	0.10	0.08
Calculated composition (%)		
Metabolisable energy (kcal/kg)	3102	3089
Crude Protein	22.96	22.13
Lysine	1.50	1.5
Methionine	0.49	0.48
Calcium	1.00	1.00
Available Phosphorus	0.50	0.50
ME/CP	135	139.5

[1] Vitamin-mineral mixture provided (per kilogram of diet): vitamin A (all-trans-retinyl palmitate), 8.800 IU; cholecalciferol, 3.300 IU; vitamin E (all-rac-a-tocopheryl acetate), 40 IU; menadione, 3.3 mg; thiamin, 4.0 mg; riboflavin, 8.0 mg; pantothenic acid, 15.0 mg; niacin, 50 mg; pyridoxine, 3.3 mg; choline, 600 mg; folic acid, 1.0 mg; biotin, 220 mg; vitamin B_{12}, 12 mg; ethoxyquin, 120 mg; manganese, 70 mg; zinc, 70 mg; iron, 60 mg; copper,10 mg; iodine, 1.0 mg; and selenium, 0.3 mg

by weighing all hatched poults.Hatchability was calculated by considering the ratio of poults hatched to the live embryos after the treatment and expressed as a percentage of fertilized eggs. In each pen, bird body weight and feed intake were recorded on d 0, 21, and 42 post-hatch. Then, mean body weight gain, feed intake, and FCR were calculated for each pen (replicate) between 0 and 21, and 22 and 42 d. In each period, BWG was calculated and expressed as grams per bird. Feed intake (g of feed intake/bird) over the entire grow-out period was calculated by totaling feed consumption in each time interval between each bird sampling.

Morphometric Indices of the Duodenum, Jejunum, and Ileum

At hatching and the end of each rearing period, eight birds from per treatment were euthanized by cervical dislocation. Intestinal segment samples (each ~2.5 cm in length) of duodenum, jejunum, and ileum were excised and flushed with 0.9% saline to remove the contents. The intestinal segments were fixed in 10% neutral-buffered formalin. The intestinal segments collected were the loop of the duodenum, midpoint between the bile duct entry and Meckel's diverticulum (jejunum), and midway between Meckel's diverticulum and the ileo-cecal junction (ileum). The Samples were dehydrated, cleared, and paraffinembedded. Intestinal segments were sectioned at 5-µm thickness, placed on glass slides, and processed by hematoxylin and eosin stain for examination by light microscopy, according to Girdhar *et al*.[25]. Morphometric indices include villus height (VH) from the tip of the villus to the crypt, crypt depth from the base of the villi to the submucosa, villus width (VW; average of VW at one-third and two-third of the villus) were evaluated [26]. Morphometric measurements were performed on 16 villi chosen from each segment, using a table of random numbers and a computer-aided light microscope image with Openlab software (OpenlabVersion 2.2.5, Improvision, Waltham, MA [25]).

Statistical Analysis

Results were analyzed by ANOVA using the GLM procedure of SAS softwareVer. 9.1 [27]. Differences between treatments were detected by the Duncan's multiple range tests following ANOVA, and values were considered statistically different at P<0.05.

RESULTS

The weight of newly-hatched poults in butyric acid administrated groups was significantly higher than control groups. But hatchability significantly decreased in all injected eggs in compared to the non- injection group (P<0.01) *(Table 2)*.

At Hatching: VH of duodenum and CH of jejunum were significantly increased in birds hatched from injected eggs in compared with control groups (P<0.01) *(Table 3)*.

Starter Period (0-21 day of age): At the end of the starter period, significantly increased VH in the duodenum, jejunum and ileum was observed for the groups with IOF of butyric acid. There was no effect of the IOFB on CH and VW in the duodenum and ileum *(Table 4)*.

Grower Period (22-42 day of age): there was no significant difference in VH of duodenum between experimental groups, at the end of the grower period. No effect of injection was detected on CH and VW for

Table 2. *Effects of IOFB on hatchability and hatching weight in turkey*

Tablo 2. *Hindilerde in ovo bütirik asit beslemenin yumurtadan çıkma ve vücut ağırlığı üzerine etkileri*

Treatment	Hatchability (%)	Hatch Weight (g)
Control	89.8^{a}	54.67^{b}
Sham	78.9^{bc}	54.72^{b}
Butyric acid 10 mg	80.2^{bc}	55.62^{a}
Butyric acid 20 mg	77.8^{c}	55.24^{a}
Butyric acid 30 mg	81.5^{b}	55.48^{a}
P-Value	0.0001	0.0002
SEM	0.99	0.130

$^{a-c}$ Averages in a column with different superscript letters are significantly different

Table 3. *Effects of IOFB on small intestinal morphology of turkeys at hatching (d 0)*

Tablo 3. *in ovo bütirik asit beslemenin yumurtadan çıkma zamanında (0. gün) hindilerde ince barsak morfolojisi üzerine etkileri*

Treatment	(0 d)		
	Villus Height	Villus Width	Crypt Depth
Duodenum			
Control	141.36^{c}	22.90^{b}	32.90
Sham	140.91^{c}	22.98^{b}	31.48
Butyric acid 10 mg	150.52^{b}	23.14^{b}	29.80
Butyric acid 20 mg	156.79^{a}	26.05^{a}	31.33
Butyric acid 30 mg	153.97^{ab}	25.85^{a}	33.35
P-Value	0.0001	0.0004	0.476
SEM	1.44	0.51	1.46
Jejunum			
Control	116.65^{b}	25.33^{b}	31.83^{c}
Sham	122.66^{ab}	23.20^{c}	33.70b^{c}
Butyric acid 10 mg	125.31^{ab}	24.99^{b}	34.99^{b}
Butyric acid 20 mg	134.20^{a}	28.03^{a}	37.83^{a}
Butyric acid 30 mg	130.40^{a}	27.02^{a}	36.02^{ab}
P-Value	0.036	0.0001	0.002
SEM	3.69	0.49	0.86
Ileum			
Control	73.43^{b}	22.69	32.69
Sham	70.97^{b}	22.39	32.89
Butyric acid 10 mg	80.12^{a}	21.77	33.52
Butyric acid 20 mg	82.33^{a}	19.79	30.29
Butyric acid 30 mg	75.26^{b}	23.19	34.19
P-Value	0.0004	0.139	0.601
SEM	1.50	0.92	1.76

$^{a-c}$ Averages in a column with different superscript letters are significantly different

Table 4. *Effects of IOFB on small intestinal morphology of turkeys at the end of the starter period (0-21 day of age)*

Tablo 4. *in ovo bütirik asit beslemenin starter dönemi sonunda (0-21 günler arası) hindilerde ince barsak morfolojisi üzerine etkileri*

Treatment	21 d		
	Villus Height	Villus Width	Crypt Depth
Duodenum			
Control	864.18^{c}	52.98	101.01
Sham	853.48^{c}	54.40	92.18
Butyric acid 10 mg	914.53^{ab}	52.55	91.14
Butyric acid 20 mg	898.13^{b}	57.08	82.99
Butyric acid 30 mg	926.47^{a}	56.34	87.86
P-Value	0.0001	0.199	0.373
SEM	8.43	1.52	6.19
Jejunum			
Control	358.93^{c}	52.14^{b}	75.53
Sham	365.97^{c}	49.16^{b}	71.88
Butyric acid 10 mg	386.89^{b}	52.81^{ab}	68.14
Butyric acid 20 mg	402.66^{a}	57.39^{a}	81.49
Butyric acid 30 mg	390.15^{ab}	53.34^{ab}	77.09
P-Value	0.0001	0.029	0.477
SEM	4.56	1.55	5.29
Ileum			
Control	241.13^{bc}	49.64	68.92
Sham	233.47^{c}	52.90	65.84
Butyric acid 10 mg	252.72^{ab}	53.05	77.14
Butyric acid 20 mg	259.41^{a}	50.89	75.49
Butyric acid 30 mg	262.77^{a}	47.57	81.61
P-Value	0.0019	0.603	0.224
SEM	4.59	2.75	5.02

$^{a-c}$ Averages in a column with different superscript letters are significantly different

duodenum, jejunum and ileum at the end of the grower period *(Table 5)*.

As shown in *Table 6*, IOFB had no significant effect on feed intake (FI) between 0 and 21, and 22 and 42 d post-hatch. To the contrary, poults from IOF of butyric acid had improved body weight gain and feed conversion ratio (FCR), when compared with the not-injected and sham controls throughout the 22-42 day of ages.

DISCUSSION

Hatching Traits

In the Ipek *et al.*[29] study, *in ovo* administration of glucose did not have a positive effect on hatchability of broiler chickens. On the contrary, the examination injection into the yolk sac caused significant decrease of hatchability of the newly-hatched poults. Probably the decreasing rate of hatching was because of the injection into the yolk sac. Another reason by allergic cavity that is under the air sac had been causing the respiration of developing embryo to stop and die. Previous studies on IOF of hormones such as corticosteroids at embryonic d7 resulted in 35% decline of hatchability [30]. Some of reviewed reports on *in ovo* feeding especially in early embryonic life were not successful in terms of hatchability [30-34]. Also, in the present study, the IOFB into fertile turkey eggs at d 7 of incubation did not significant effect on hatchability of sham group (injected with 0.5 mL of deionized water) than other injected groups. In according to the past studies and our present observations, it seems that any IOF at early embryonic life can harmful for internal environment susceptibility and would have negative effect on hatching; this effect is largely independent from injected butyric acid (or any other feed) effect. Also, Ohta *et al.*[4] showed the effect of

Table 5. *Effects of IOFB on small intestinal morphology of turkeys at the end of the grower period (22-42 day of age)*

Tablo 5. *in ovo bütirik asit beslemenin büyüme dönemi sonunda (22-42 günler arası) hindilerde ince barsak morfolojisi üzerine etkileri*

Treatment	42 d		
	Villus Height	Villus Width	Crypt Depth
Duodenum			
Control	756.68^{ab}	48.15	62.26
Sham	745.98^{b}	54.40	82.12
Butyric acid 10 mg	772.03^{a}	52.57	72.46
Butyric acid 20 mg	777.38^{a}	44.58	67.74
Butyric acid 30 mg	763.22^{ab}	59.07	76.28
P-Value	0.034	0.673	0.370
SEM	7.43	7.27	7.12
Jejunum			
Control	531.44	64.04	74.78
Sham	503.48	54.16	73.14
Butyric acid 10 mg	494.39	61.57	93.40
Butyric acid 20 mg	555.16	57.39	86.50
Butyric acid 30 mg	547.66	53.34	75.35
P-Value	0.144	0.613	0.250
SEM	18.83	5.60	7.23
Ileum			
Control	261.13	54.65	76.42
Sham	283.47	49.90	91.84
Butyric acid 10 mg	252.73	58.37	76.89
Butyric acid 20 mg	249.42	52.15	95.49
Butyric acid 30 mg	306.28	47.57	81.61
P-Value	0.174	0.931	0.166
SEM	17.67	9.22	6.38

$^{a-b}$ *Averages in a column with different superscript letters are significantly different*

amino Acid IOF on chicken hatchability may be related to *in ovo* injection site. Leitao*et al*.[35] investigated the effect of the IOF into broiler breederseggs on the hatchability, reported that the IOF may decreased the hatching rate. Adriana *et al*.[36] found that decreased hatchability was observed when chickens embryo received IOF at d16 of incubation. Moreover, one of the important factors may affect embryo mortality is osmolality of solution, the maximum osmolality of solution was 500-600 miliosmol which suggested by Uni and Ferket [6]. Whereas osmolality of butyric acid solution in the present study was far lower than Uni and Ferket [6] recommendation.

Based on the findings of present study, the IOFB in the yolk sac can be an effective tool to increase the weight of newly-hatched poults. Also,this result generally agrees with our idea that exogenous nutrition provision can substitute for amino acids to provide energy. Thus, exogenous nutrients supply reduces the dependency of the embryo upon amino acids by improvement in embryo energy status and increases protein deposition, probably by attenuating muscle wasting to help increase the weight of newly-hatched poults. The present report confirms the earlier patent claims by Uni and Ferket [6] that IOF enhances chick energy status and gut maturation. Also, several studies stated that IOF can improve embryo energy status and hatch weight [3,9,28].

Morphometric Indices

The first barrier to nutrient metabolism in animals is the gastrointestinal tract, and its metabolic activity can have an effect on the nutrient supply of the whole animal. The nutrient utilization efficiency would be more if the nutrient loss at the gastrointestinal tract level could be minimized [37]. The integrity of the intestinal epithelium is important so as to utilize the nutrients to the maximum extent. The changes in the morphology of villi and reduction in absorptive surface area may reduce the nutrient absorption

Table 6. *Effects of IOFB on body weight gain (BWG), food intake (FI) and feed conversion ratio (FCR) of turkey poults in starter (0-21 day of age) and grower periods (22-42 day of age)*

Tablo 6. *Hindilerde in ovo bütirik asit beslemenin starter (0-21 günler) ve büyüme döneminde (22-42 günler arası) vücut ağırlık kazanımı (BWG), yem tüketimi (FI) ve yem konversiyon oranı (FCR) üzerine etkileri*

Treatment	0-21 Day of Age (g)			22-42 Day of Age (g)		
	BW	FI	FCR	BW	FI	FCR
Control	674.77^{b}	933.80	1.383^{a}	1541.26^{b}	3081.07	1.99^{a}
Group sham	671.07^{b}	930.25	1.386^{a}	1550.00^{b}	3084.06	1.98^{a}
Butyric acid 10 mg	683.27^{a}	919.33	1.345^{b}	1583.30^{a}	3073.59	1.94^{b}
Butyric acid 20 mg	688.11^{a}	922.28	1.340^{b}	1577.25^{a}	3068.68	1.94^{b}
Butyric acid 30 mg	686.83^{a}	915.73	1.333^{b}	1585.02^{a}	3070.63	1.93^{b}
P-Value	0.0001	0.524	0.006	0.0001	0.455	0.0001
SEM	1.76	8.26	0.01	3.14	6.79	0.005

$^{a-b}$ *Averages in a column with different superscript letters are significantly different*

and hence lead to reduced production performance. Considering the effect of butyric acid to improve the intestinal morphology and increase performance. In the present study, it was observed that the IOFB can increase growth in gastrointestinal tract and improve performance of poults.

As already reported by other authors [14,16,17,23,38-40] butyric acid is the major development promoter of the gastro-intestinal tract, as confirmed by our results about increase growth of gut.

Additionally, preliminary studies demonstrated that IOF significantly increased intestinal villus width, goblet cell density of jejunum villi and surface area in comparison with the controls in broiler chicks at hatch [28,41-43]. Also, Chen *et al*.[11] demonstrated that the IOF of glutamine and carbohydrates into duck eggs increased intestine weight.

Performances

Several authors concluded that IOF improved post-hatch growth [3,7,28]. These results are in agreement with that of Zhonghong and Yuming [44] who observed that the dietary sodium butyrate supplementation at the level of 500 mg/kg increased body weight gain from 0 to 21 days and improved FCR during the period from 0 to 42 days. Also, Antongiovanni *et al*.[38] reported positive beneficial effects of BA on production performance traits of broiler chickens. To the contrary, in another study Leeson *et al*.[16] stated that the dietary butyric acid had no effect on weight gain in starter, grower and finisher periods. But, birds consumed less starter feed when diets were supplemented with butyric acid relative to the control birds.

These results indicated that IOFB can contribute to cumulative effects of butyric acid on the morphology of the gastrointestinal tract, which may cause an increase in the absorption of the nutrients from the gut and improve performance. Also, to reduce the utilize liver glycogen reserves and the depletion of muscle protein, we hypothesized that the IOFB into the yolk sac prior to hatching would support the energy status of the hatching by elevating the glycogen reserves, moderating the use of muscle proteins, and thus contributing to enhanced post-hatch performance. As a result of these properties of butyric acid, IOFB may increase growth in the gastro-intestinal tract and can improve small intestinal morphology and performance. Tako *et al*.[28] demonstrated that body weight of *in ovo* fed-chicks were significantly greater than the controls at hatch through 10 days post-hatch. Also, Foye *et al*.[7] stated that IOF of egg white protein, β-hydroxy-β-methylbutyrate, and carbohydrates can elevates5 to 6% of turkey body weight.

In conclusion, results of the present study showed that the IOFB may improve development of the gastrointestinal tract and consequently increase the growth performance of turkey poults. Thus, this result generally agrees with our hypothesis that IOFB may provide energy for small intestine and poult embryo activity, in turn alleviating energy lack, sparing the pectoral muscle protein and increasing breast muscle mass and consequently, increase the growth performance. Finally, *in ovo* feeding of butyric acid can be suggested as a method for optimizing final weight in poultry farming. Overall, these results are in agreement with the finding of Salmanzadeh *et al*.[45] who, reported that, *in ovo* administration of L-carnitine into turkey eggs significantly improve hatch weight, growth performance (0-21 and 22-42 day of age) and carcass characteristics (42 day of age) whereas hatchability significantly depressed in all injected eggs compared to the not injected ones.

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Research Article

The Effect of Orally Administrated β-glucan and Dietary Restriction on Faecal Microflora in Rats [1]

Tülay ELAL MUŞ [1] Füsun AK SONAT [2]

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[1] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Uludag, TR-16059 Bursa - TURKEY
[2] Department of Physiology, Faculty of Veterinary Medicine, University of Uludag, TR-16059 Bursa - TURKEY

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Abstract

In this study we aimed to evaluate the effect of orally administered β-glucan and/or dietary restriction on *Lactobacillus* spp., coliforms, *Enterobacteriaceae* and enterococci counts in rat faeces. For this aim, rats were divided into three experimental groups: i) first group animals received normal diet for 6 months and administrated orally with β-glucan (20 mg/kg for bodyweigth) over the last 14 day of experiment, ii) second group was dietary restricted animals for 6 months and receiving β-glucan as those of first group animals, iii) last group was the control group rats receiving only *ad libitum* feed. Compared to control group, numeric increase was observed in the number of coliforms, *Enterobacriaceae* and lactobacillus counts in first group but this was not statistically important. The increase in coliforms and *Enterobacteriaceae* counts was nearly 2 log while this was 1 log for lactobacillus counts. Interestingly, dietary restriction + β-glucan administration had no significant influence on the increase of defined bacterial groups. The results of the present study showed that orally administration of the β-glucan, widely used as prebiotic, has the potential to modify faeces microbiota in rat model.

Keywords: *β-glucan, Probiotic, Prebiotic, Lactobacillus*

Oral Yolla Uygulanan β-glukan ve Diyet Kısıtlamasının Ratların Fekal Mikroflorasına Etkisi

Özet

Bu çalışmanın amacı oral yolla verilen β-glukan ve/veya diyet kısıtlamasının rat feçesindeki *Lactobacillus* spp., koliform, *Enterobacteriaceae* ve enterokok sayıları üzerine etkisini ortaya koymaktır. Bu amaçla ratlar üç deneysel gruba ayrıldı: i) birinci grup 6 ay süresince normal diyetle beslenen ve son 14 gün oral yolla β-glukan (20 mg/kg, canlı ağırlığa) verilen hayvanlar ii) ikinci grup 6 ay boyunca diyet kısıtlaması uygulanan ve ilk grupla aynı şekilde β-glukan verilen hayvanlar iii) yalnızca *ad libitum* beslenen kontrol grubu ratlardan oluşmaktadır. Kontrol grubu ile karşılaştırıldığında, ilk grupta koliform, *Enterobacteriaceae* ve laktobasil sayılarında artış gözlendi, fakat bu artış istatistiksel olarak önemli değildi. Koliform ve *Enterobacteriaceae* sayılarındaki artış yaklaşık 2 log iken, laktobasil sayılarında bu artış 1 log idi. İlginç olarak diyet kısıtlaması ile birlikte β-glukan uygulaması adı geçen bakterilerin artışında önemli bir etki oluşturmadı. Mevcut çalışmanın sonuçları, prebiyotik olarak geniş kullanım alanı bulunan β-glukan'ın oral yolla takviyesinin rat modelinde dışkıdaki mikroorganizmaları modifiye etme potansiyeline sahip olduğunu gösterdi.

Anahtar sözcükler: *β-glukan, Probiotik, Prebiotik, Laktobasil*

INTRODUCTION

Prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health" [1,2]. Today prebiotics have been widely used to enhance health in the human life [3]. Additionally they benefits on growth and performance of animals [4]. Probiotics and prebiotics are mostly consumed for strenghtening of friendly microflora of gut [5]. β-glucan, which is a novel prebiotic, has many health benefits. Some of these effects are immunomodulation, increasing the defence system to invading pathogens, anti-tumor activity, lowering the blood cholesterol level and beneficially affect to gut health [4,6]. Because of this effects, β-glucan

İletişim (Correspondence)
+90 224 2941229
fusunak@uludag.edu.tr

is considered as biological response changers and regard from the pharmaceutical and functional food industries [7]. In addition to these positive properties β-glucan has prebiotic effect by improving the growth of lactobacilli in gut microflora [8] and is one of the most studied prebiotics [9]. Degradation of β-glucan by microbiota resuted with some fermentation products such as short chain fatty acids (SCFA) which are important for the development of the colon microflora. Presence of SCFA reduce the colonic pH and increases the bacterial cultivation [10]. Production of SCFA also prevents growth of potential pathogens [11]. Many researchers reported that β-glucan has positive effects on probiotic bacteria such as lactobacilli and bifidobacteria in gut microflora by animal experiments [12-14]. But there is not enough research about impact of β-glucan to intestinal microflora such as *Lactobacillus*, coliform, *Enterobacteriaceae*, enterococci.

The composition of the gastrointestinal microrganisms is intensively influenced by several factors that include the main gut flora at birth, host genetics, immunological factors, antimicrobial consumption and dietary effects. Change the intakes of the carbonhydrates, proteins and fats can significantly modify the composition of the microflora [15].

Dietary restriction (DR) that underfeeding without malnutrition, extends rats life-span in comparison with *ad libitum* feeding [16,17]. DR is the only experimental orientation that has been presented to delay aging, decrease deseases, health risks and the occurence and proceeding of tumors [16]. DR delays onset of various age-related neurodegenerative diseases such as Alzheimer's disease and insuline resistance. DR is also ameliorate oxidative stres-releated impairment in tissues [17]. Altough these possitive effects there is not enough research about impact of dietary restriction to intestinal microflora.

The aim of this study was to evaluate the in vivo prebiotic potential of β-glucan and the efficiacy of long term dietary restriction supported with β-glucan on rat faecal microbiota such as *Lactobacillus*, coliform, *Enterobacteriaceae*, enterococci.

MATERIAL and METHODS

Animals and Management

Male, 3-months old Sprague-Dawley rats were used in this study. Rats were purchased from Experimental Animals Breeding and Research Centre of Uludag University. Thirty Sprague-Dawley rats were housed under controlled humidity (50-60%) and temperature (20-23ºC) with a 12 h light-dark cycle. Rats had free access to tap water, and fed with a laboratory chow diet. The composition of the diet was as follows: protein 18% (min), lipid 2.5% (min), fiber 4% (max), ash 5.5% (max), nitrogen free extract 57.0% (max), metabolic energy 2650 kcal/kg (min), water 13% (max) plus various amino acids, minerals and vitamins (data obtained from the supplier). This study was performed after approval of the Local Ethical Committee of Animal Experiments of Uludag University with decision number 2012-14/05.

Study Design

All rats were divided to 3 groups and each group consisted of 10 rats. All animals were cared during 6 months. First and third (control) groups were given tap water and standart laboratory chow diet with *ad libitum*. Also, first group was orally administreted by β-glucan. Second group rats was applied by DR (monday, wednesday and friday mornings) (the food hoppers were placed the following morning as an every-other-day feeding schedule) and tap water ad libitum [16,17]. During last 14 days of 6 months housing, daily β-glucan intake in first and second groups were 20 mg/kg dose by intragastric way.

Microbiological Analyses

Rat faecal samples were collected from cage litters at 1st, 7th, and 14th days under sterile conditions and stored at -20°C for microbiological analysis until the last required sample is collected. Approximately 5 g of each faecal sample were homogenised in 45 ml sterile peptone water (0.1%) solution using a stomacher for at least two minutes. Decimal dilutions were made in sterile pepton water (0,1%) and plated in duplicate on the selective agars. Coliforms were isolated on Violet Red Bile agar (CM0107, Oxoid, UK) with overnight (20-24 h) incubation at 35-37°C. For enterococci the culture was grown on Slanetz and Bartley medium (CM0377, Oxoid, UK) at 35-37°C for 48 h. *Enterobactericeae* count was performed by Violet Red Bile Glucose agar (CM0485, Oxoid, UK) and incubated 20-24 h at 35-37ºC [18]. *Lactobacillus* spp. was enumerated on de Man Rogosa Sharpe medium (CM0361, Oxoid, UK) and plates were incubated under anaerobic conditions at 35-37ºC for 72 h [19].

Statistical Analyses

Statistical analyses were performed by SPPS 20 programme by Kruskal-Wallis test. When differances among the groups were significant, Mann-Whitney test was used. For interpreting results $P<0.05$ significance level was used.

RESULTS

The results related to the counts of coliforms, *enterobactericeae, enterococci* and *Lactobacillus* spp. according to groups are summarized in *Table 1*. Increase in coliform counts was almost 2 logs in the first group, while this increase was sligtly lower in other groups with a change of 1 log. But there was no statistically significant difference ($P>0.05$) among the groups. Only the first group animals exhibited changes in term of *Enterobactericeae* counts which were increased 2 log in 2 weeks period. In

Table 1. *Lactobacillus spp., Coliform bacteria, Enterobacteriaceae and Enterococci counts (log_{10} cfu/g) according to groups and sampling days*
Tablo 1. *Gruplara ve örnekleme günlerine göre Lactobacillus spp., koliform bakteri, Enterobacteriaceae ve enterokok sayıları (log_{10} kob/g)*

Microorganism	Day 1			Day 7			Day 14		
	First (β-glucan)	Second (DR+ β-glucan)	Third (Control)	First (β-glucan)	Second (DR+ β-glucan)	Third (Control)	First (β-glucan)	Second (DR+ β-glucan)	Third (Control)
	Mean±SEM			Mean±SEM			Mean±SEM		
Lactobacillus spp.	8.67±0.07*	9.02±0.17*	8.92±0.15*	8.53±0.07	9.02±0.16	8.64±0.05	9.04±0.40	9.11±0.10	8.66±0.09
Coliform	4.62±0.13	5.13±0.59	4.27±0.28	5.11±0.27	5.17±0.31	5.01±0.38	6.37±0.37	5.36±0.42	5.01±0.30
Enterobacteriaceae	4.80±0.09	5.27±0.60	4.97±0.14	5.18±0.22	5.20±0.27	5.06±0.42	6.35±0.51	5.44±0.45	4.77±0.39
Enterococci	5.77±0.23	5.81±0.48	5.57±0.42	6.08±0.14	5.83±0.12	5.75±0.07	6.26±0.22	5.34±0.23	6.07±0.38

** Values sharing the same symbol in the same row are not significantly (P>0.05) different among groups*

other two (control and DR + β-glucan) groups, the counts of *Enterobacteriaceae* did not change. Although the higher counts of enterococci in first and third groups were detected, the faecal samples of the second group rats were not assigned any change for enterococci counts after 14 days period. For *Enterobacteriaceae* and enterococci counts, the differences among groups were not statistically significant (P>0.05).

Lactobacillus spp. population in first group were similar at 1st and 7th days. But on 14th day the counts of these bacteria were observed a 1 log increase. On the other hand the increase was not statistically significant (P>0.05). On 1st, 7th and 14th days, *Lactobacillus* spp. counts in second and third groups were not seen any difference.

DISCUSSION

This study was carried out to determine the effect on faecal microrganism counts of the feeding by β-glucan. The addition of β-glucan in the diet of rats did not significantly increased (P>0.05) the numbers of coliform, enterococci, *Enterobacteriaceae*, enterococci and *Lactobacillus* spp. in the faecal samples (1, 7 and 14 days after feeding).

In the present study, coliform counts increased at least one log at first (β-glucan) and third groups. On the other hand, Turunen et al.[10] reported that feacal coliform populations were reduced in the human faeces with β-glucan feed on 30th days. We found that feed with β-glucan caused a 2 log increase in *Enterobacteriaceae* counts. A similar result was reported by Murphy et al.[20] who suggested that oat based β-glucan rised *Enterobacteriaceae* populations 2 log in the porcine gastrointestinal tract. In second (DR+ β-glucan) and third (control) groups, *Enterobacteriaceae* counts didn't change on 14th day. Enterococci populations at first and third group rats increased in the end of the feed. In another study investigated of the effect on faecal microflora of β-glucan, Lowry et al.[21] reported that β-glucan as feed addictive significantly reduced the incidence of *Salmonella enterica* serovar *Enteritidis* organ invasion in immature chickens. In this study, cause of the increase in the number of coliforms and enterococcus bacteria in the group treated with β-glucan, may be due to lack of the dose of β-glucan or administration time.

In only β-glucan feed rats, *Lactobacillus* spp. populations showed an increasing at 1 log level. There are reports showing that a β-glucan was able to increase *Lactobacillus* spp. in rat faeces [14,22]. Again, Reiilly et al.[12] and Murphy et al.[20] assigned that β-glucan raised lactobacillus counts in porcine gastrointestinal tract. Also, a study conducted by Mitsou et al.[23] and Kuda et al.[13] exhibited that β-glucan significantly increased probiotic bacteria in human and rat faeces, respectively. Dietary restriction supplemented with β-glucan (second group) did not effect *Lactobacillus* spp. populations in the our study. This result could depends on the β-glucans efficacy inhibited by long time dietary restriction treatment. Therfore could not observed any increase in *Lactobacillus* spp. in second group rats. In our knowledge, the results of the present study represent the first data on the effect of dietary restriction + β-glucan on some selected faecal microorganisms in rats. On the other hand, several studies indicated that the addition of different dietary supplements such as sorbitol, heparin or heparosan at the diet increased *Lactobacillus* spp. population in gut microbiota [9,24].

In conclusion, we found that addition of prebiotic matter β-glucan has the potential to change at rat faeces microflora, and to increase, despite not a statistically significant, both probiotic lactobacillus and other micro-orgamisms including coliform, *Enterobacteriaceae* and enterococci populations. In the future we aim to find the exact β-glucan dose which can be used to reduce the colonization of pathogenic bacteria and to increase probiotic bacteria populations as lactobacillus in gastro-intestinal tract of animals.

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Research Article

Effects of Various Cryoprotective Agents on Post-Thaw Drone Semen Quality

Selim ALÇAY [1] Burcu ÜSTÜNER [1] İbrahim ÇAKMAK [2]
Selvinar ÇAKMAK [3] Zekariya NUR [1]

[1] Uludağ Üniversitesi, Veteriner Fakültesi, Dölerme ve Suni Tohumlama Anabilim Dalı, TR-16059 Görükle, Bursa - TÜRKİYE
[2] Uludağ Üniversitesi Arıcılık Geliştirme - Uygulama ve Araştırma Merkezi (AGAM), Mustafa Kemalpasa MYO, TR-16500 Bursa - TÜRKİYE
[3] Uludağ Üniversitesi Arıcılık Geliştirme - Uygulama ve Araştırma Merkezi (AGAM), TR-16059 Bursa - TÜRKİYE

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Abstract

The aim of the present study was to evaluate the effect of different cryoprotectants on post-thaw semen motility and plasma membrane integrity of drone spermatozoa. Semen was obtained from mature drones (16 days or older) harvested from four colonies. Collected semen was diluted to a final concentration of 1/5 (semen/extender) in 0% cryoprotectant (control), 6% glycerol, 6% Ethylene Glycol, 6% 1,2 propanediol or 6% DMSO using a two-step dilution method. The equilibrated semen was frozen in 0.25-ml straws. The percentage of sperm motility and swollen tails (HOST) spermatozoa were evaluated following dilution with extender A (non-cryoprotectant), equilibration and post-thaw stages. In terms of post-thaw motility and plasma membrane integrity recovery we can rank the used cryoprotectant as DMSO, Ethylene Glycol, Glycerol and 1,2 Propanediol; respectively. In conclusion, post-thaw sperm motility and plasma membrane integrity of the present study was significantly better when sperm was frozen in DMSO with respect to control, glycerol, ethylene glycol, 1,2 propanediol ($P<0.05$).

Keywords: *Drone spermatozoa, Cryoprotectants, Cryopreservation*

Farklı Kriyoprotektanların Eritme Sonrası Arı Sperm Kalitesi Üzerine Etkileri

Özet

Bu çalışmanın amacı, farklı kriyoprotektanların eritme sonrası arı spermasının motilite ve plazma membran bütünlüğü üzerine etkisini değerlendirmektir. Sperma dört koloniden bulunan olgun arılardan (16 gün ve üzeri) alındı. Toplanan sperma final konsantrasyonu 1/5 olacak şekilde %0 kriyoprotektan içermeyen (kontrol), %6 gliserol, %6 etilen glikol ve %6 1,2 Propanediol ile iki aşamalı sulandırıldı. Ekilibrasyon sonrası sperma 0.25 ml'lik payetlerde dondurulduu. Sulandırma sonrası, ekilibrasyon ve eritme sonrası aşamalarda motilite (%) ve HOS testi yapıldı. Eritme sonrası motilite ve plazma membran bütünlüğüne bakıldığında kullanım öncülüğüne göre kriyoprotektanları DMSO, Etilen Glikol, Gliserol ve 1,2 Propanediol olarak sıralayabiliriz. Sonuç olarak, eritme sonrası motilite ve plazma membran bütünlüğü bakımından; DMSO ile dondurulan sperma, kontrol, gliserol, etilen glikol, 1,2 propanediol gruplarıyla karşılaştırıldığında en iyi sonucu vermiştir ($P<0.05$).

Anahtar sözcükler: *Arı sperması, Kriyoprotektanlar, Dondurma*

INTRODUCTION

Honey bee breeder would like to obtain bee colonies that are tolerant or resistant to introduced or formerly unknown pathogens and parasites. Due to rapidly decreasing gene pool with many subspecies and ecotypes, *Apis mellifera* facing massive introgression of foreign genotypes [1].

Beekepers have little ability to control over the source of the semen that the queen stores in her spermatecha, unless they use isolated mating regimes or an area with desirable drone starins, ecotypes and races [2,3]. The development of the instrumentally insemination of the queen make it possible to control the number and genetic stock of drones involved in mating [4].

İletişim (Correspondence)
+90 224 2941345
nurzek@uludag.edu.tr

The development of a practical means to store drone semen would enhance the bee breeder's ability to select and maintain superior honey bee stocks. Storing drone semen for a long time of particular colonies, strains, and races would allow bee breeders and scientists to retrieve, conserve, and spread valuable genetic traits [5]. Also, cryopreserving drone semen would be very useful for conserving germplasm that is being lost due to the recent and alarming mortality rates of honey bee colonies in many countries, often referred to as colony collapse disorder [6].

There have been successful attempts to store honey bee semen *in vitro* for various periods on time [4]. A number of storage techniques have been tried in drone semen with some success, using neat [5], cooled [7] and frozen [8] semen.

Neat and cooled semen have limited life span compared to frozen form. Maximum storage life at room temperature for fresh drone semen is approximately two weeks [5] and the cooled drone semen maintain fertilizing ability approximately for one year [7].

Deep-freezing techniques, known as cryopreservation, have been applied to mammalian semen for many years [9,10]. This technique, however, has not been successfully applied for honey bee *(Apis mellifera)* spermatozoa.

Cryopreservation of honey bee semen for later use would make it possible to maintain or increase genetic diversity in selected honey bee stocks. Higher intra-colony diversity has been shown to increase productivity, fitness and disease resistance and to decrease severity of infection and parasite loads in honey bees [11,12]. Additionally, there is an urgent need for the cryopreservation of honey bee spermatozoa to counter allelic losses caused by the population declines due to varroa mites, colony collapse disorder and other future threats [13].

With the aim of avoiding harmful effect of the cryopreservation, the addition of cryoprotectant to the extender is extremely important [14,15]. DMSO, Ethylene Glycol, Glycerol and 1,2 Propanenediol are the most used cryoprotectans in mammalian and drone semen using different concentrations and freeze-thaw procedures [14].

Using cryopreserverd semen is necessary to inseminate queens [13]. Because of the performance of instrumentally inseminated queens is related to semen quality, it is likely that poor results in previous studies may be consequence of low sperm viability after thawing [16].

The hypo-osmotic swelling (HOS) test which developed by Jeyendran et al.[17] has been effectively used to assess the functional integrity of mammalian and honey bee sperm plasma [18].

Honey bee queens can only be inseminated with fresh semen, when the drones are present [16]. The development of cryopreservation protocols for drone semen is a multi-factorial problem, involving the optimization of freezing rate, thawing rate, the nature and concentrations of cryoprotectants, and the extenders composition [8]. Therefore, the aim of the present study was to monitor the effect of different cryoprotectants on post-thaw semen motility and plasma membrane integrity of drone spermatozoa.

MATERIAL and METHODS

Experiments were performed in the Laboratory of Andrology in the Department of Reproduction and Artificial Insemination at the Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey, in July.

Animals

Drones were reared in colonies established with a drone wax foundation. Mature drones (16 days or older) were collected from 4 colonies and brought to the laboratory.

Semen Collection and Dilution

Semen was collected from each drone by holding the head and thorax and gently squeezing the abdomen [4]. For semen collection at a 1/1 ratio, 0.8 mL of saline solution was drawn into a Schley syringe tip 1.10 (Schley Instrumental Insemination equipment, Lich, Germany) under a stereo microscope, followed by approximately 0.8 mL of semen (one drone). This process was replicated until a final volume of 10 mL of semen was obtained (approximately 12-14 drones) for each freezing groups.

Following semen collection, the samples were analyzed for sperm motility [16] and sperm plasma integrity using the water test [18].

Sperm Motility

Sperm motility was assessed by examining a drop of diluted ejaculate, covered with a cover slip under a phase-contrast microscope at 400x magnifications. Sperm motility was scored on a scale of 0 to 5 corresponding to 0%, 20%, 40%, 60%, 80% and ≥95% of the observed population being motile, respectively [16].

Water Test

A volume of 1.0 mL of semen was added to 250 mL of distilled water and incubated at room temperature for 5 min. Immediately after the incubation, one drop of semen was placed on a glass slide, covered and evaluated under a phase-contrast microscope (400x). Microscope fields were selected randomly. At least 100 spermatozoa were evaluated per slide, and the percentages of swollen tail spermatozoa were calculated [18].

Semen Dilution and Freezing

This study was adopted form one step dilution

mammalian semen freezing procedures. The composition of extenders was prepared according to Taylor et al.[16] diluents IV: (g/100 ml distillated water): Na Citrate 2.43, $NaHCO_3$ 0.21, KCl 0.04, Amoxicillin 0.03 and Catalase 200 mL). Prepared extender Ph' was adjusted to 8.1 and divided to five groups (Control group (cryoprotectant free), 6% Ethylene Glycol, 6% 1,2 6% Propanenediol and 6% DMSO)

Pooled semen (10 mL) was diluted with one of the extender at ratio of 1/5 semen/extender and cooled to 5°C within 1 h in water bath and than equilibrated at 5°C for 2 h. Equilibrated semen samples were filled into the center of 0.25 straws. The appropriate extender for each group was filled into one end of the straw, followed by an air space (~ 10 mm), then the equilibrated semen sample, followed by another similar-sized air space, and finally, the appropriate extender was added to the other end of the straw *(Fig. 1)*. Semen filled straws were sealed with different colors of the polyvinylalcohol (PVA) sealing powder for post-thaw straw identification *(Fig. 1)*. Semen filled straws were frozen in liquid nitrogen vapour at -110°C for 10 min and then plunged into liquid nitrogen at -196°C, where they were stored for at least one month.

At least three straws from each group were thawed at 37°C for 30 s in a water bath to evaluate post-thaw semen motility and plasma membrane integrity (5x3=15 straws). The procedure was repeated 5 times for each group.

Statistical Analyses

The Kruskal Wallis–Mann Whitney U test was used to compare the mean percentages of motile spermatozoa, and swollen spermatozoa obtained with water tests. All data were analyzed with the SPSS statistical package (SPSS 10.0 for Windows; SPSS, Chicago, IL, U.S.A), and a difference was considered significant at the $P<0.05$ level.

RESULTS

The mean observed semen motility and plasma membrane integrity in function of cryoprotectant at three stage were presented in *Table 1*. Sperm motility was progressively reduced through cooling and the freeze-thaw process ($P<0.001$). The motility of diluted semen in control group was significantly lower than those in the glycerol, Etilene glychol, 1,2 propanediol and DMSO ($P<0.05$). There are no significant differences among groups in terms of sperm plasma membrane integrity of diluted and equilibrated semen ($P>0.05$).

The sperm motility and plasma membrane integrity of the equilibration stage were lower than after dilution stage ($P<0.01$). The mean of sperm motility of equilibrated spermatozoa was higher in DMSO supplemented group as compared to control group ($P<0.05$).

Post-thaw semen motility and sperm plasma membrane integrity were lower than the equilibration and after dilution stages ($P<0.001$). All used cryoprotectants support post thaw semen motility and plasma membrane integrity to some degree. Post-Thaw semen motility and plasma membrane integrity was better in DMSO supplemented group than the glycerol, Etilene glychol, 1,2 propanediol and control groups ($P<0.05$).

DISCUSSION

The freeze-thaw process is detrimental to mammalian sperm motility and functional integrity [14,19,20], and to drone semen viability and motility [16]. Cryopreservation of honey bee semen has potential for long-term preservation of germplasm, however several factors need to be studied further to optimize post-thaw survival rates. Various extenders and cryoprotective agents have been developed for the cryopreservation of mammalian [14,21,22] and drone [16]

Fig 1. Straws filled and sealed semen with different colors of the polyvinylalcohol (PVA) powder for identification on freezing rack

Şekil 1. İçinde sperma doldurularak farklı renkli polyvinyl-alcohol (PVA) ile kapatılmış payetlerin dondurma tarağı üzerindeki görüntüsü

Table 1. *The mean (x±Sx) of studied sperm parameters in the function of cryoprotectant*

Tablo 1. *Kriyoprotektanlara göre elde edilen spermatolojik bulguların ortalamaları (x±Sx)*

Stage	Cryoprotectant	n	Motility (%) x±Sx	HOST (%) x±Sx
After dilution	Control	5	3.3±0.3^{a}	88.4±2.5
	Glycerol	5	3.9±1.9^{ab}	86.0±3.2
	Ethylene Glycol	5	4.1±0.1^{b}	89.4±2.4
	1,2 Propanediol	5	3.8±0.1^{ab}	87.0±3.2
	DMSO	5	4.0±0.0^{b}	92.8±0.7
Equilibrated	Control	5	2.2±0.6^{a}	81.0±4.3
	Glycerol	5	3.0±0.3^{ab}	74.6±5.4
	Ethylene Glycol	5	3.7±0.1^{b}	87.0±2.0
	1,2 Propanediol	5	3.4±0.3^{ab}	85.8±2.4
	DMSO	5	3.8±0.2^{b}	89.2±1.2
Post-thaw	Control	15	0.5±0.0^{a}	30.7±2.1^{a}
	Glycerol	15	2.0±0.2^{bc}	51.8±3.6^{b}
	Ethylene Glycol	15	2.3±0.2^{b}	55.9±4.0^{b}
	1,2 Propanediol	15	1.5±0.2^{c}	49.3±5.4^{b}
	DMSO	15	3.1±0.2^{d}	69.5±3.2^{c}

semen. In the present study, we evaluated the effects of different cryoprotectants on post-thaw semen motility and plasma membrane integrity of drone spermatozoa frozen in a Na citrate based extender.

The motility of semen diluted in Na citrate based extender in control, glycerol, Etilene glychol, 1,2 propanediol and DMSO group were 3.3, 3.9, 4.1, 3.8 and 4.0 and the percentage of intact sperm with plasma membrane functional integrity 88.4%, 86.0%, 89.4%, 87.0% and 92.8%; respectively. The sperm motility and plasma membrane integrity are similar to Nur at al.[18] used neat drone semen.

The development of drone semen cryopreservation protocols is a multi factorial problem, involving the optimization of cooling and freezing rate, thawing rate, the kind and concentrations of cryoprotectants, and extender composition. Like this study, the drone semen freezing protocols of many studies have been adopted from mammalian semen freezing procedures. Semen extension procedure of the present study was adopted form one step dilution mammalian semen freezing procedures [15]. Equilibrated semen quality is a sign of cryosurvivability in farm animals and included in many study related on semen freezing. Unfortunately there are no documented results related to equilibrate drone semen. Equilibrated semen motility and plasma membrane integrity in control, glycerol, Etilene glychol, 1,2 propanediol and DMSO group were 2.2, 3.0, 3.7, 3.4 and 3.8, and 81.0, 74.6, 87.0, 85.8 and 89.2; respectively.

The extender composition, extension temperatures, dilution rate, cooling rates to 5°C and presence of cryoprotectant have an effect on ram [14,15,18] bull [20] and drone [16] semen quality. As expected, the sperm motility and plasma membrane integrity of the equilibration stage were lower than after dilution stage (P<0.01). The mean of sperm motility of equilibrated spermatozoa was higher in DMSO supplemented group as compared to control group (P<0.05). It was observed that DMSO is good choice for drone semen cooling.

The equilibrated semen motility was better in all cryoprotectant supplemented groups compared to control group. This difference was observed in percentage of swollen tail spermatozoa generally except glycerol supplemented group. Glycerol containing diluents lead to cell death prior freezing stage [13]. For the criyoprotectant supplemented groups, it was observed that the lowest sperm motility and plasma membrane integrity were obtained in glycerol supplemented group.

The success of cryopreservation depends on many factors other than the freezing rate, such as species, breed, or variation among individual animals. Also the nature of the cryoprotectant [14,16], thawing temperature [18], sperm concentration [16] and variations in methodology [15] effect the post-thaw sperm recovery. Different animal species exhibit different sperm membrane compositions, such as different cholesterol/phospholipid ratios and degrees of hydrocarbon chain saturation, which can affect how the sperm responds to cooling and, subsequently, confer different sperm cryosensitivities across various species [23]. Despite the advances in cryopreservation techniques for mammalian spermatozoa, the success achieved with the cryosurvival of farm animal sperm has not been obtained from drone spermatozoa at the same success rate.

The main cryoprotective effect of cyoprotectant is visible at post-thaw stage. Post-thaw semen motility and plasma membrane integrity in control, glycerol, Etilene glychol, 1,2 propanediol and DMSO group were 0.5, 2.0, 2.3, 1.5 and 3.1, and 30.7, 51.8, 55.9, 49.3 and 69.5; respectively. All cryoprotectant supplemented groups were yield better motility and plasma membrane integrity. Post-thaw semen motility and sperm plasma membrane integrity were lower than the equilibration and after dilution stages (P<0.001).

The extenders composition assists in stabilizing the cell during the freezing and thawing process [14,19]. Post-thaw plasma integrity was unbroken for some degree in control group. These findings indicated that used extender has a protecting capability on drone plasma membrane integrity. Also the better survivability of post-thaw plasma membrane integrity than motility means that drone sperm plasma membrane integrity is more resistant to the cryopreservation related damages.

In terms of post-thaw motility and plasma membrane integrity recovery we can rank the used cryoprotectant as

DMSO, Ethylene Glycol, Glycerol and 1,2 Propanenediol; respectively. Although the presence of glycerol and 1,2 propanedidiol in freezing extenders protect sperm motility and plasma membrane integrity, they were incapable of preserving sperm plasma functional integrity. 1,3 Propanediol is a very poor glass-former [24] and a low dose of 1-3 Propanediol is likely to be insufficient for the efficient cryopreservation of drone sperm. It is unlikely to be suitable as single-component CPA for honey bee drone semen, but could be interesting as one component of a CPA cocktail [25]. It was observed that 6% 1,3 propanediol supplemented group post-thaw motility and plasma membrane integrity was the lowest in cryoprotectant supplemented groups.

The cryoprotective agent most frequently used with drone semen freezing is dimethyl sulfoxide (DMSO), although this substance has been suspected of causing genetic damage in sperm [25]. Due to the DMSO led to higher post-thaw survival [16], different concentration of DMSO has became the main choice for many studies on drone semen preservation up to the present [8,16]. Post-thaw sperm motility and plasma integrity of the present study were significantly better when sperm was frozen in DMSO with respect to control, glycerol, Ethylene glycol, 1,2 propanediol.

It could be concluded that DMSO supplemented extenders are a better choice for drone semen freezing compared to Glycerol, Ethylene Glycol, and cryoprotectant free control group for post-thaw motility and plasma membrane integrity. Further studies on combination of CPA with some sugars should be done for the overcome the detrimental effect of CPA and cryopreservation related damages on motility and plasma membrane integrity.

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Research Article

Effect of Bull Exposure on the Post-partum Reproductive Efficiency in Cholistani Cows

Muhammad Saleem AKHTAR [1] Syed Israr HUSSAIN [2] Muhammad Mazhar AYAZ [1] Laeeq Akbar LODHI [3] Muhammad AMEEN [4] Saleem ULLAH [5] Mushtaq Hussain LASHARI [6] Abdul Asim FAROOQ [1] Masood AKHTAR [1]

[1] Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, PAKISTAN
[2] Department of Animal Sciences, Allama Iqbal Open University, Islamabad, PAKISTAN
[3] Faculty of Veterinary Sciences, University of Agriculture, Faisalabad, PAKISTAN
[4] 4 DDLO Khanewal, Livestock and Dairy Development Department, Punjab, PAKISTAN
[5] 5 Veterinary Officer, Livestock and Dairy Development Department, Punjab, PAKISTAN
[6] 6 Department of Life Sciences, The Islamia University of Bahawalpur, PAKISTAN

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Abstract

The present study was conducted with the objective to determine the effect of bull exposure on postpartum resumption of ovarian activity, first behavioural oestrus and conception rate in Cholistani cows. A total of 24 Cholistani cows kept at Shadabad Cooperative Livestock Farms Cholistan, Pakistan during September-2012 to January-2013, were divided into two groups. Bull exposed (BE) cows (n=18) were exposed to mature bull throughout the study period whereas bull not exposed (BNE) cows (n=6) were not. Both groups were kept under similar feeding and management conditions. The mean interval from the calving to resumption of ovarian activity was 39.75±3.19 days in the BE and 49.75±2.75 days in BNE animals (P<0.05). The mean interval from the calving to the first behavioural oestrus was 46.93±0.48 days in BE and 57.5±1.29 days in BNE animals (P<0.05). During the study period, more (P<0.05) cows from the BE group (16/18 = 88.88%) showed behavioural oestrus as compared to the BNE group ones (4/6=66.66%). The conception rate in BE and BNE cows were 66.66% and 33.33%, respectively (P<0.05). It was concluded that, cows exposed to the presence of bulls at early postpartum periods showed reduced intervals from the calving to resumption of ovarian activity, first behavioural oestrus and resumed cyclicity earlier.

Keywords: *Bull exposure, Reproductive efficiency, Cholistani cow*

Cholistani İneklerde Boğa Maruziyetinin Doğum-sonrası Üreme Etkinliği Üzerine Etkisi

Özet

Sunulan çalışma, Cholistani ırkı ineklerde doğum-sonrası ovaryum aktivitesinin yeniden başlaması, ilk östrus davranışı gösterme ve gebe kalma oranı üzerine boğa maruziyetinin etkisini belirlemek amacıyla yapıldı. Bu amaçla, Eylül 2012 - Ocak 2013 tarihleri arasında Shadabad Hayvancılık Kooperatifi Çiftlikleri, Cholistan, Pakistan'da barındırılan toplam 24 baş Cholistani ırk ineği iki gruba ayrıldı. Boğaya maruz bırakılmış (BE) inekler (n=18) çalışma boyunca ergin boğaya maruz bırakılırken, boğaya maruz bırakılmamış (BNE) inekler (n=6) böylesi bir maruziyete bırakılmadı. Her iki grup aynı besleme ve yönetim koşullarında tutuldu. Ortalama buzağılama-ovaryum aktivitesinin yeniden başlama aralığı, BE'de 39.75±3.19 gün iken, BNE hayvanlarda 49.75±2.75 gün idi (P<0.05). Ortalama buzağılama-ilk östrus davranışı gösterme aralığı, BE'de 46.93±0.48 gün iken, BNE hayvanlarda 57.5±1.29 gün idi (P<0.05). Çalışma periyodu boyunca, BNE grubundakilere (4/6=66.66%) kıyasla, BE grubunda daha fazla (P<0.05) sayıdaki inek (16/18 = 88.88%) östrus davranışı gösterdi. Gebe kalma oranı, BE ve BNE ineklerinde sırasıyla 66.66% and 33.33% idi. (P<0.05). Sonuç olarak; doğum-sonrası erken dönemde boğa varlığına maruz bırakılan ineklerde, buzağılama-ovaryum aktivitesinin yeniden başlaması ve ilk östrus davranışı gösterme aralığının kısaldığı ve siklisitenin daha erken (yeniden) başladığı saptandı.

Anahtar sözcükler: *Boğa maruziyeti, Üreme etkinliği, Cholistani inek*

İletişim (Correspondence)
+92 314 7890797
drsaleem46@hotmail.com

INTRODUCTION

Early pregnancy is the key to success of livestock production. A cow becoming pregnant in time may give birth to 7-8 calves along with that much lactation as compared to 2-3 calves per cow in her lifetime that could not conceive well in time. To reduce the calving interval, likely to be peculiarly long in some cases, pregnancy within 90 days is desired. By reducing the calving interval maximum, production and profit can be obtained thereby. The mean length of the calving intervals of a cow determines, to the large extent, her total productivity. Lesser number of days required by the cow to conceive after parturition is one of the best criteria for determining the reproductive ability under the range conditions. When active and fertile bulls are present, the average interval from parturition to the conception of a viable embryo reflects the postpartum interval to an ovulatory oestrus and the fertilisation and embryonic survival rate. This interval thus determines the length of the breeding season necessary to obtain a satisfactory calf crop [1].

The biostimulatory effect of male on oestrus and subsequent ovulatory response has been reported in many species. For a superior herd management, a bull is kept along with buffaloes resulting in the reduction of postpartum interval to oestrus [2]. Several studies similarly reported that cows come into oestrus earlier by exposing with a vasectomized bull in comparison to cows that were not exposed to Bull et al.[3,4].

A postpartum cow exposed to bull by physical contact or separated by fence-line [5] results in an early ovarian activity. This phenomenon is called as "biostimulatory effect of bull" and is interacted by pheromones secreted in the environment through the excretory products of male [3].

The biostimulatory effect of bull on the cow's ovarian activity also depends upon the duration of daily exposure. Fernandez et al.[6] reported that the interval from calving to resumption of ovarian activity was not hastened in cows exposed to bulls for 2 h after every 3rd day for 18 days starting 33 days after calving as compared to cows exposed to bulls continuously. Though, postpartum anoestrus was decreased in cows that were exposed by the excretory products of bulls daily for 12 h [7].

There is limited information regarding the biostimulatory effect of bull on the postpartum resumption of ovarian activity in Cholistani cows. The present study was therefore designed with the objective to know the effect of bull exposure on the postpartum ovarian activity, postpartum oestrous behaviour and conception rate in Cholistani cows.

MATERIAL and METHODS

The study was conducted at Shadbad Cooperative Livestock Farms (SCLF), Cholistan, Division Bahawalpur. Cholistan is a vast desert located between latitude 29.41 and longitude 72.24. The study was completed in 150 days. The experimental period extended from September-2012 to January-2013.

A total of 24 post partum Cholistani cows were used in this experiment. The animals aged between 5-8 years with body weights (post-calving) varied from 400 to 470 kg. Cows were in their 2nd lactation without having any previous calving problems and with similar gestational lengths. These cows were randomly divided into two groups. Eighteen post partum cows were exposed continuously to the presence of bull (BE) while six of them were not exposed to the bull (BNE). The cow to bull ratio provided to the BE cows was 18:1 while the BNE cows were kept as control (no bull). It was also assured that no bull should be present in 5 km radius of the BNE cows. The BE cows were kept at Shadbad Cooperative Livestock Farms, Chapu, whereas the BNE cows were kept at Shadbad Cooperative Livestock Farms, Mouj Garh. Distance from both locations was approximately 38 km. Both groups were provided with free access to water and salts. Housing conditions at both sites were similar. Both groups were kept in semi stall fed type shed. The environmental conditions were the same at both locations, whereas feeding practices were also kept the same during this period. Both groups were provided with the same feed (two kg concentrates and grazing on the same type of pasture i.e. *Medicago sativa*). The experiment was initiated one week after the parturition. In the BE cows, the intact bull remained present in the herd while grazing and being at shed throughout the study period. In these cows the bull was restricted not to serve the cows by tying his fore legs in a traditional manner. The BNE cows served as control and had no contact with bull or its excretory products.

Postpartum oestrus detection was initiated 5 days after the start of experiment in each group. Oestrus detection was performed twice daily (7:00 am and 6:00 pm) by visual examination to observe behavioural signs during the experiment. After the detection of oestrous symptoms, cows were inseminated artificially. The artificial insemination (AI) was done by "am and pm" rule. Frozen-thawed semen from a single bull belonging to Semen Production Unit, Qadirabad was used in this study and all the cows in oestrus were inseminated by the same technician. Inseminated cows in the BE and BNE were checked for pregnancy after 60 days for a possible re-insemination, through rectal palpation.

The following parameters were studied and recorded during the experiment:

1. Intervals from the calving to resumption of ovarian activity (days),
2. First behavioural oestrus (days),

3. Number of cows showing oestrus/group,

4. Conception rate (%).

The following criteria were used to characterize the resumption of postpartum oestrous cycles:

1) Displaying behavioural oestrus.

2) Presence of a palpable corpus luteum.

Daily blood samples were taken from the BE and BNE cows through jugular venipuncture and its serum was then aspirated. To determine the interval from the calving to resumption of ovarian activity, serum progesterone concentrations were measured through ELISA by using commercially available kit (BioCheck, Inc. Foster City, USA). The limit of sensitivity was 0.3 ng/ml as indicated by the manufacturer. Inter- and intra-assay coefficients of variations were 12.6 and 7.1%, respectively. All samples were processed in one batch; thus, the coefficient of variance inter-assay was zero. Once a rise of progesterone above 1 ng/ml was determined and a luteal structure was observed by rectal palpation, an interval from the calving to the corresponding day of the progesterone rise was calculated.

Pregnancy diagnosis was confirmed by presences of foetus and corpus luteum. Detection of oestrous was performed by visual exposure of oestrous symptoms.

Statistical Analysis

Data regarding the resumption of ovarian activity and first behavioural oestrus were determined by ANOVA whereas the conception rate was determined by Chi square test [8].

RESULTS

The mean interval from the calving to resumption of ovarian activity was 39.75±3.19 days in the BE and 49.75±2.75 days in the BNE cows (P<0.05). The mean interval from the calving to first behavioural oestrus was 46.93±0.48 days in the BE and 57.5±1.29 days in the BNE animals (P<0.05) *(Table 1)*.

During the study period (approximately 150 days) more cows from the BE group (16/18=88.88%) showed behavioural oestrus signs as compared to the BNE group (4/6=66.66%). In the present study, the conception rate in the BE and BNE cows were 66.66% and 33.33%, respectively (P<0.05) *(Table 2)*.

Table 1. *Interval (Mean ± SE) from the calving to the resumption of ovarian activity and the interval from calving to the first behavioural oestrus in the BE and BNE cows*

Tablo 1. *BE ve BNE ineklerde buzağılama ve ovaryum aktivitesinin yeniden başlaması ile buzağılama ve ilk östrus davranışı aralıkları (Ortalama ± SE)*

Parameters Studied	BE (n=18)	BNE, Control (n=6)
Interval from the calving to the resumption of ovarian activity(days)	39.75^{a}±3.19	49.75^{b}±2.75
Interval from calving to the first behavioural estrus (days)	46.93^{a}±0.48	57.5^{b}±1.29

Values with different superscripts in a row differed significantly (P<0.05)

Table 2. *Conception rate (%) in the bull exposed (BE) and the bull not exposed (BNE) cows*

Tablo 2. *Boğaya maruz bırakılan (BE) ve bırakılmayan (BNE) ineklerde gebe kalma oranı (%)*

Parameter Studied	BE (n=18)	BNE, Control (n=6)
Conception rate (%)	66.66^{b}	33.33^{a}

Values with different superscripts in a row differed significantly (P<0.05)

DISCUSSION

There was a marked difference between the BE and BNE cows for the interval from calving to resumption of ovarian activity. These findings are in agreement with those of Fike et al.[9] and Gifford et al.[10] while differed from those of Sipka and Ellis [11] who reported that in high-producing multiparous Holstein dairy cows, post-partum ovarian reactivation occurred later in bull-exposed treatments as compared to the cows not exposed to bull treatment. The inconsistency between the present study with the findings of Shipka and Ellis [11] may be due to high nutritional demands of dairy breed [12] and the intensity of bull exposure [9].

Further marked difference was observed between the BE and BNE cows for interval from calving to first behavioural oestrus. These findings corroborated with those of Barman et al.[13]. Findings of the present study are also in agreement with those of Naasz and Miller [14], who reported that spring calving beef cows exposed to the bull returned to oestrus at 42.2±3.1 days after calving, whereas in cows not exposed to bull the interval was 58.3±4.7 days. In their study, the bull exposed cows returned to oestrus 16 days earlier than cows not exposed to bull.

Knowledge about the mechanisms by which bull-exposure may reduce the intervals from calving to resumption of reproductive cyclicity and to first post-partum oestrus remains limited. A pheromonal signal driven out by body fluids or excretory products has been emphasized to be responsible for triggering the biostimulatory effect in cattle [3,15]. Although pheromonal signals from urine and sebaceous glands have been reported in other species [16], in cattle, both the route of emission and mechanism of chemo-signal remain unknown. The reception and transport of the pheromonal signal is probably initiated via olfaction through the

vomeronasal organ [17]. However, the mechanisms involving the posterior internalization and translation of the pheromonal signal into the endocrine system currently remain unknown.

Hernandez et al.[18] also reported that greater proportion of cows exposed to bull showed oestrus behaviour (97%) in comparison with control cows (80%). The findings of the present study are in agreement with those of Rekwot et al.[15]. Possible reason for these findings depends on the biostimulatory effect of bull-presence to resume the cyclicity early [18].

In the present study, the conception rate in the BE cows was markedly higher than that of the BNE cows. Berardinelli et al.[7] also reported 57.6% and 35.6% conception rate in cows exposed to bull and controls. However, contrary to the present study, Ahmad et al.[19] reported that the conception rate did not differ significantly between male-treated and control groups. However, they exposed the cow with bull's urine (treatment group) and with water (control group) instead of the exposure of cows directly with bull. Undoubtedly the, stimulatory effect of bull is also influenced by the intensity of contact (the frequency of contact, duration of contact as well as the quantity of stimuli) of pheromones fabricated by bulls [5].

It is concluded that cows exposed to the physical presence of bulls at early postpartum periods showed reduced intervals from the calving to resumption of ovarian activity, first behavioural oestrus and resumed cyclicity earlier. There appeared favourable effect of bull exposure in improving the reproductive efficiency in Cholistani cows. Therefore, it was suggested that male animals have to be provided in the herd to improve the reproductive potential of Cholistani cows.

Some breeders consider male animals as a burden to their economical livestock production due to their feeding and management cost and consider the AI as an economical substitute to the male animal. Nevertheless, it appears that the presence of bull(s) would also be profitable for the breeder in terms of reproductive efficiency of cows. In this sense, both bull-exposure (without mating) and practising AI would likely to be worth combining to see if any superior outcome might be achieved thereby.

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Research Article

The Presence of *Listeria* Species in Corn Silage and Raw Milk Produced in Southeast Region of Turkey

Hisamettin DURMAZ [1] Mehmet AVCI [2] Osman AYGÜN [3]

[1] Yuzuncu Yıl University, Veterinary Faculty, Food Hygiene and Technology Department, TR-65080 Van - TURKEY
[2] Harran University, Veterinary Faculty, Animal Nutrition Department, TR- 63200 Sanliurfa - TURKEY
[3] Programme of Food Technology, Keban Vocational School, Firat University, TR-23700 Keban/Elazig - TURKEY

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Abstract

The objective of this study was to investigate the presence of *Listeria* species in the corn silage and raw milk samples. A total of 140 raw milk samples obtained from cows, sheeps and goats fed with silage and 90 corn silage samples collected from 10 dairy farms in South-Eastern Region of Turkey were analyzed for *Listeria* spp. In the result, *L. monocytogenes* and *L. innocua* were isolated from 2 (2.2%) and 5 (5.5%) silage samples and from 3 (2.1%) and 5 (3.5%) raw milk samples, respectively. The results indicates that these are a potential risk for animals and public health. Prevention of growth of *L. monocytogenes* in silage will also contribute to reduction of *Listeria* spp. in milk.

Keywords: *Listeria spp., L. monocytogenes, Raw milk, Silage*

Türkiye'nin Güneydoğu Bölgesi'nde Üretilen Mısır Silajı ve Çiğ Sütlerde *Listeria* Türlerinin Varlığı

Özet

Bu çalışmanın amacı, mısır silajında ve çiğ süt örneklerinde bulunan *Listeria* türlerinin varlığının araştırılmasıdır. Türkiye'nin Güneydoğu Bölgesi'nde bulunan 10 süt çiftliğinde mısır silajıyla yemlenen inek, koyun ve keçilerden elde edilen toplam 140 çiğ süt ve 90 mısır silajı örnekleri *Listeria* türleri bakımından analiz edildi. Silaj örneklerinin ikisinde (%2.2) ve çiğ süt örneklerinin üçünde (%2.1) *L. monocytogenes,* silaj örneklerinin beşinde (%5.5) ve süt örneklerinin de beşinde (%3.5) ise *L. innocua* izole edildi. Elde edilen sonuçlar, mısır silajları ve çiğ süt örneklerinin hayvan ve insan sağlığı açısından potansiyel bir risk taşıdığını göstermektedir. Silajlarda *L. monocytogenes*'in çoğalmasının önlenmesi, aynı zamanda sütlerdeki *Listeria*'ların azalmasına da katkıda bulunacaktır.

Anahtar sözcükler: *Listeria spp., L. monocytogenes, Çiğ süt, Silaj*

INTRODUCTION

Listeria spp. are widely distributed in the farm and in the industrial and human food environment (soil, plants, silage, faecal material, sewage and water) and they frequently contaminate foods [1,2]. Listeriosis is cause by generally *Listeria monocytogenes* and rarely by *Listeria ivanovii* in humans. It is well known that human listeriosis is largely attributable to foodborne transmission of *L. monocytogenes* [1-4]. In general, mild symptoms including headache, fever, diarrhoea and myalgia are seen in the majority of cases [5]. However severe symptoms including septicaemia, meningoencephalitis, abortion and stillbirth are also seen in humans and animals, primarily in certain risk groups, such as, pregnant, new-borns, and immuno-compromised individuals in the cases of invasive listeriosis [2,3].

The quality of silage depends on the competition between different groups of microorganisms. Lactic acid bacteria, responsible for the silage fermentation process, usually dominate the silage microflora. However a number of undesirable microorganisms existing at low levels on fresh plant materials may also grow during the storage of silage and lead to anaerobic or aerobic spoilage. Yeasts are generally responsible for the initiation of aerobic spoilage. These microorganisms oxidize the preservative acids present in silage. Then the pH rises and other aerobic microorganisms start to proliferate. This secondary aerobic

İletişim (Correspondence)
+90 432 2251128/1556
hisamettindurmaz@yahoo.com

spoilage flora consists of *Listeria*, moulds, bacilli and *Enterobacteriaceae*. The aerobic flora not only decreases the nutritional value of the silage, but also presents a risk to animal health and the quality and safety of milk [6,7].

An association between listeriosis and the feeding of silage to dairy cattle has been well documented, with most cases resulting from the consumption of low quality and improperly fermented silage with a pH of 4.0. Reports of bovine listeriosis from silage feeding and of subsequent asymptomatic shedding of *L. monocytogenes* in milk are of obvious concern to the dairy industry [8]. It has been reported that listeriosis in cattle is mainly feed-borne and *Listeria* spp. have been detected in a ratio of between 1.2% and 60% from the silage samples [9-11]. Furthermore, Taşçı et al.[12] reported that 6.6% of silage samples and 1.17% of milk samples obtained from cows fed with silage were contaminated with *L. monocytogenes*. Fenlon [13] has stated that 29-31% of cattle started to shed *L. monocytogenes* after silage feeding.

Therefore it is important to have information on the presence of this pathogen in milk and silage which may be a source of contamination and infection for animals and humans with *L. monocytogenes*. However, there are few local studies on the presence of this pathogen in the raw milk obtained from animals fed with silage and especially in silage in Turkey [12,14]. The aim of this study was to investigate the presence of *Listeria* spp. in the raw milks obtained from cows, sheeps and goats fed with silage and in silages produced in Southeastern Region of Turkey.

MATERIAL and METHODS

Sampling Procedure

Total 140 raw milk samples obtained from cows (50), sheeps (75) and goats (15) fed with corn silage and 90 corn silage samples were collected from 10 farms in Sanliurfa (6) and Adiyaman (4) regions in Turkey. Milk samples were taken from the bulk storage tanks in the same farms. Silage samples were taken from the surface, interior of silos and in manger. All samples were kept at 4°C until examination.

Isolation and Identification

Listeria spp were isolated according to standard method recommmended by Food and Drug Administration [15]. Twenty five gram silage or 25 mL of milk sample was homogenized in a stomacher with 225 mL Listeria Enrichment Broth (Oxoid, CM0862) supplemented with Listeria Selective Enrichment Supplement (Oxoid, SR0141) and incubated at 30°C for 48 h. A loopful of the enriched culture was streaked onto Oxford Agar (Oxoid, CM0856) supplemented with Listeria Selective Supplement (Oxoid, SR0140) and incubated at 35°C for 48 h. Five selected colonies were confirmed by streaking cultures onto Tryptone Soya Agar (Oxoid, CM0131) and testing isolated colonies for catalase production and for the following characteristics: tumbling motility at 25°C, carbonhydrate fermentation (maltose, dextrose, mannitol, xylose and rhamnose), nitrate reduction, Methyl Red-Voges Proskauer reactions, umbrella motility in SIM medium at 25°C, β-hemolysis and Gram staining.

Physicochemical Analysis

A total of 25 g of fresh corn silage was macerated with 100 mL distilled water with a high-speed blender. The macerated silage samples were filtered through two layers of cheesecloth and the pH values of the filtrates were measured with a laboratory pH meter (Model 890, Nel Instruments Inc., Ankara, Turkey).

Statistical Analysis

Data were statistically analyzed by a one-way analysis of variance, and the means were compared by the Duncan's multiple-range test by using the software package [16].

RESULTS

The results of the study are presented in *Table 1* and *Table 2*. The analysis showed that 5.7% of raw milk and 7.7% of corn silage samples were contaminated with *Listeria* spp. *L. monocytogenes* and *L. innocua* were isolated from 2 (2.2%) and 5 (5.5%) silage samples and from 3 (2.1%) and 5 (3.5%) raw milk samples, respectively (*Table 1* and *2*).

DISCUSSION

The presence of *Listeria* spp. in silage samples was examined by several studies [10-12,14]. In a study, *Listeria*

Table 1. *The presence of Listeria spp. isolated from silage samples*
Tablo 1. *Silaj örneklerinde bulunan Listeria türleri*

Parameters	Silage Interior	Silage Surface	Silage in Manger	Total
Number of samples	30	30	30	90
pH	4.05±0.16*	5.77±0.12*	5.57±0.04*	-
L. monocytogenes	nd**	1 (3.3%)	1 (3.3%)	2 (2.2%)
L. innocua	nd**	3 (10.0%)	2 (6.7%)	5 (5.5%)
Total	nd**	4 (13.3%)	3 (10%)	7 (7.7%)

** Different letters show significant differences between the rows (P<0.001),* ***** nd:*** *not detected*

Table 2. *The presence of Listeria spp. isolated from raw milk samples*
Tablo 2. *Çiğ süt örneklerinde bulunan Listeria türleri*

Parameters	Cow's Milk	Sheep's Milk	Goat's Milk	Total
Number of samples	50	75	15	140
L. monocytogenes	1 (2.0%)	2 (2.7%)	nd*	3 (2.1%)
L. innocua	nd*	5 (6.7%)	nd*	5 (3.5%)
Total	1 (2.0%)	7 (9.3%)	nd*	8 (5.7%)

* **nd:** *not detected*

spp. (*L. monocytogenes, L. innocua* and *L. welshimeri*) were isolated from 10% of corn silage samples, 28% of hay silage samples, and 60% of grass silage samples [10]. These values were higher than the results for *Listeria* spp. in silage samples obtained from our study *(Table 1)*. In the same study [10], *L. monocytogenes* was isolated from 2.3% of corn samples and 2.6% of hay silage samples, which are similar with our results. Vilar et al.[11] detected *Listeria* spp. (*L. monocytogenes, L. innocua, L. welshimeri, L. grayi* and *L. seeligeri*) in 33.7% of total 83 grass and corn silage samples and *L. monocytogenes* in 6.0% of silage samples. Taşçı et al.[12] isolated *L. monocytogenes* in 6.66% of silage samples. These results on *L. monocytogenes* in silage samples are higher than the result obtained from our study. Şahin et al.[14] detected *Listeria* spp. (*L. welshimeri* and *L. grayi*) but not *L. monocytogenes* in silage samples. These authors have stated that the low prevalence of *L. monocytogenes* in silage samples may be attributed to the high-quality of the silage (as indicated by pH ≤4.0). It has been reported that *L. monocytogenes* rapidly disappear under strictly anaerobic conditions and at a pH value lower than 4.4 [7]. Therefore the growth and survival of *Listeria* spp. in silage depends on the degree of anaerobiosis and on the pH value of the silage. The results obtained by our study on the *Listeria* spp. in interior, manger and surface silage samples *(Table 1)* support this suggestion. Şahin et al.[14] indicated an inhibitory effect of the cold seasons on the growth of *L. monocytogenes*.

In present study, *L. monocytogenes* was detected in one milk sample (2.0%) obtained from cows and two milk samples (2.7%) obtained from sheep, while in goat's milk samples *L. monocytogenes* was not detected *(Table 2)*. Soyutemiz et al.[18] have found three positive samples (3%) from 100 raw milk samples for *L. monocytogenes* in West Anatolia. Sağun et al.[17] determined that 2.4% of raw milk samples obtained in Van province located in eastern Turkey, were positive for *Listeria* spp., where *L. monocytogenes* was found in 1.2%, *L. innocua* and *L. welshimeri* in 0.4% of those samples. Vilar et al.[11] detected *Listeria* spp. in 16.3% of bulk-tank milk samples, where *L. monocytogenes* was found in 6.1%, *L. innocua* in 7.1%, *L. welshimeri* in 1.0% and *L. grayi* in 2.0% of those samples. They emphasized a relationship between low silage quality by high pH and high prevalence (33.7%) of *Listeria* spp. in silage. In a study [12], *L. monocytogenes* was not found in milk samples obtained from cows not fed with silage, however, *L. monocytogenes* was isolated from 1.17% of milk samples obtained from cows fed with silage. In another study [14], *L. welshimeri* and *L. grayi* were isolated from milk samples obtained from cows fed with silage, whereas *L. monocytogenes* was not isolated in the milk samples. The authors have reported that *Listeria* spp. began to be seen in the milk samples together with the occurrence of *Listeria* spp. in silage samples. On the other hand, Aygun and Pehlivanlar [21] found one (2.12%) positive sample for *L. ivanovii* and *L. grayi* among 47 raw milk samples. Faecal or environmental contamination during milking, storage and transport, infected cows in dairy farms and poor silage quality have been reported [7,14,17,19-21] as contamination sources of *Listeria* spp. to raw milk. It was reported that the poor quality silage is one of the primary sources of contamination of raw milk by *L. monocytogenes* which presents a serious risk to the quality and safety of milk and animal health [7]. In present study, the contamination of raw milk samples with *Listeria* spp. may be due to the reasons mentioned above, especially poor quality silages.

Infection of animals with *L. monocytogenes* has been associated most frequently with poor-quality silage [11,12]. Low-quality silage with a pH value higher than 5.5 supports the growth of *Listeria* spp.[10]. In our study, the pH values of the *Listeria* spp.-positive silage samples ranged from 4.05 to 5.77 and the pH value of the silage samples contaminated with *L. monocytogenes* was higher than 5.5. In other studies, pH values were reported between 5.1 to 8.3 by Taşçı et al.[12], 3.8 - 5.2 by Rea et al.[22], <4 - 5.89 by Ryser et al.[10] and 4.47 - 6.97 by Vilar et al.[11]. However, Ryser et al.[10] could not identified *L. monocytogenes* in 3 *Listeria* spp.-positive grass silage samples were all of poor quality, ranging in pH from 5.78 to 5.89. It could say that one of the contamination sources of *Listeria* spp. was the consumption of low-quality silage with pH values higher than 5.5 by milking animals in parellel with studies by Driehuis and Oude Elferink [7], Vilar et al.[11] and Taşçı et al.[12].

In conclusion, the isolation of *L. monocytogenes* from corn silage and raw milk examined by this study indicates that these are a potential risk for animals and public health. To prevent the growth of the bacteria in silage it is important the controll of the silage fermentation process with a low pH value (<5.5). Application of special cultures of lactic acid bacteria or chemical additives can aid silage fermentation and improve aerobic stability of silage.

Prevention of growth of *L. monocytogenes* in silage will contribute to reduction of *Listeria* spp. in milk. Infected animals in dairy farms should be insulated, the milking animals not fed with poor-quality silage and the hygienic conditions should be improved in order to minimize the contamination risk of *L. monocytogenes* in raw milk during the milking, storage and transport process in farm and dairy plant.

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Research Article

The Efficacy of Thymol and Oxalic Acid in Bee Cake Against Bee Mite (*Varroa destructor* Anderson&Trueman) in Honey Bee (*Apis mellifera* L.) Colonies

Berna EMSEN [1] Ahmet DODOLOĞLU [1]

[1] Atatürk Üniversitesi, Ziraat Fakültesi, Zootekni Bölümü, TTR-25240 Erzurum - TÜRKİYE

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Abstract

In the present study, two organic compounds (thymol crystals and oxalic acid crystals in bee cake) with pollen supplement feeding were used to treat colonies against *Varroa destructor*. The organic compounds were applied as follows: (1) 10 g of powdered thymol was added to the bee cake prepared with fat- free soy flour, pollen and 1:1 sugar syrup (TBC), (2) 4 g of oxalic acid was prepared using the same cake recipe described above (OBC), (3) untreated colony (CC). 60 g of cakes from both treatments applied on the top of the frames in colonies. Treatments were carried out in three applications and mite fall were counted weekly during the experiment. The best result was obtained with thymol-bee cake (92.85%). The efficacy of oxalic acid treatment was recorded as 66.72%. There was no significant difference in the level of parasitization inside cells, bee population and brood area among treatments. However there was a significant reduction in mite infestation on adult bee with thymol-bee cake. This result indicates that thymol added to the bee cake is an effective, easy and safe alternative fall treatment for varroa mites.

Keywords: *Varroa destructor, Honey bee, Thymol, Oxalic acid, efficiency, Bee cake*

Bal Arısı Kolonilerinde (*Apis mellifera* L.) Arı Akarı (*Varroa destructor* Anderson&Trueman) İle Mücadelede Arı Kekine Katılan Thymol ve Oksalik Asidin Etkisi

Özet

Çalışmada, *Varroa destructor* akarına karşı polen içerikli iki organik bileşik (thymol kristal ve oksalik asit kristal içerikli arı keki) kullanılmıştır. Organik bileşikler aşağıdaki gibi uygulanmıştır : (1) 10 g thymol yağsız soya unu, polen ve 1:1 şeker şurubu ile hazırlanmış arı kekine katılmıştır (TBC), (2) 4 g oksalik asit yukarıda bahsedilen aynı kek tarifi kullanılarak hazırlanmıştır (OBC), (3) Muameleye tabi tutulmamış koloniler (CC). Her iki uygulamadan hazırlanan 60 g kek kolonilerde çerçevelerin üzerine yerleştirilmiştir. Tedaviler üç uygulamada gerçekleştirilmiş ve akar düşüşü deneme süresince haftalık sayılmıştır. En iyi sonuç (%92.85), thymol-arı kekinde elde edilmiştir. Oksalik asidin etkinliği %66.72 olarak kaydedilmiştir. Petek gözlerde akar oranı, arı populasyonu ve yavrulu alan bakımından gruplar arasındaki fark önemsiz bulunmuştur. Ancak, thymol-arı keki grubunda ergin arılar üzerinde akar bulaşıklığında önemli derecede azalma tespit edilmiştir. Bu sonuçlar, arı kekine katılan thymolun sonbaharda varroa akarına karşı etkili, kolay ve güvenilir alternatif bir mücadele yöntemi olabileceğini göstermektedir.

Anahtar sözcükler: *Varroa destructor, Bal arısı, Thymol, Oksalik asit, Etkinlik, Arı keki*

INTRODUCTION

The mite *Varroa destructor,* a parasite of *Apis mellifera,* has to be controlled by the regular use of acaricides in order to maintain honeybee colonies [1]. Synthetic miticides such as coumaphos, amitraz, fluvalanite and flumethrin have the disadvantage of leaving residues in honey and wax, being a hazard to handle, having some level of toxicity to bees and humans and also because mites quickly develop resistance to their active ingredient [2]. On the other hand, the use of organic compounds such as thymol and oxalic acid that does not show any toxic effects on human and honey bees can be very effective solution in the fight against varroa mite. Thymol is a

İletişim (Correspondence)
+90 442 2311988
bemsen@atauni.edu.tr

phenolic monoterpene extracted from garden thyme *(Thymus vulgaris)* and toxic to varroa mites [3]. The efficacy of thymol has been tested in previous studies and recommended that the product is a well-known effective compound against the varroa infection which can be used in place of chemical treatments such as coumaphos and amitraz [4,5]. The uses of organic substances containing thymol and oxalic acid have been studied extensively in controlling the varroa mite [6].

Therapeutic medicines may be incorporated in bee cake for treatment and control of bee disease. However, medicines in cake or syrup should not be given to colonies during a honey flow or 5 weeks prior to honey flow [7].

In our experiment, we evaluated the efficacy of thymol and oxalic acid added to bee cake to control *V. destructor* infestations in honey bee colonies during early fall in eastern Turkey.

MATERIAL and METHODS

A total of 24 honeybee colonies were placed in Langstroth hives and headed by Caucasian queen bees raised in Erzurum. Before the trial, colonies were initially equalized to contain a similar amount of brood and bee population and managed in the same way until the miticide trails initiated.

The natural mite mortality was monitored four times, between July 22 and August 21, prior to the treatments. The level of infestation of the experimental colonies was determined using sticky bottomboards three weeks before starting the miticide trials. The colonies were then randomly divided into three groups and treated for the varroa mites.

Treatments began on September 9, 2013. For thymol treatment, 10 g of powdered thymol was added to the bee cake using 20 g of pollen, 1.5 g of fat-free soy flour and 100 ml of sugar syrup (one part water one part sucrose) (TBC). Sixty grams of small cake was applied over the combs of brood chamber. Treatment 2 consisted of 4 g of oxalic acid was added to the bee cake using same mixture described above (OBC). All treatments applied weekly during 3 occasions. Cakes were replaced with new cakes every week. Control colonies (CC) received only 60 gram of miticide free bee cake.

To estimate the mite infestation levels of sealed worker brood, 5×5 cm pieces of comb containing brood were cut with a knife and put in plastic bags. Before and after the treatment, the number of mite from treated and control hives was counted in 300 cells/brood sample.

Adult bee infestation level was determined using a wire net. Approximately 200 workers were collected from each hive in small plastic jars which were filled with water and detergent. The mixture was shaken couple minutes while then adult bees were separated from varroa mite using double layer filter honey sieve under water stream. Mites and bees were counted to establish an infestation rate (number of mites/number of bees x 100) [8].

The capped brood area in each colony was estimated with digital photography. A picture of each side of brood frames was taken with a digital camera. The capped brood area from the digital pictures of each colony was measured to the nearest cm^2 using Adobe Photoshop® CS2 9.0 with a computer as per [9].

The degree of effectiveness of the treatments tested was determined by their percentage of control in relation to the synthetic miticide (Perizin®). At the end of the third week of the experimental trials, each colony was treated with two Perizin® treatments according to the instructions of the manufacturer. The percent efficacy of each treatment was calculated by dividing the number of mites that fell during the period of experimental treatment, by the total number of mites (mite drop of experimental and finisher treatment with Perizin®). The resulting figure was then multiplied by 100.

Data on percent efficacy were analyzed by analysis of variance (ANOVA) after (arcsine $\sqrt{y}/100$), arcsine transformation in the case of percentages, to reduce the heterogeneity of the variance. Means were separated applying the Duncan test ($P<0.05$) [10].

RESULTS

Mite levels in colonies were not different among the treatment groups prior to the treatments (34.25±8.0; $F_{2,21} = 0.28$; $P = 0.76$). The miticide efficacy of the TBC method (92.85±0.7%) was different to that of the OBC method (66.72±2.4%) and control (42.14±2.7%) *(Fig. 1)*.

The number of varroa mites fallen during the treatments with thymol cakes per beehive were higher than treatments with oxalic acid cakes *(Table 1)*. There was a significant difference between colonies in weekly mite fall. The lowest mite fall (49.25±4.9) after Perizin application was recorded in the group of TBC.

There was no significantly differences between the experimental groups and control groups regarding the size of sealed brood area ($F_{2,21}$=1.402, P=0.268; $F_{2,21}$=0.039, P=0.962, respectively), bee population ($F_{2,21}$=0.309, P=0.738; $F_{2,21}$=0.516, P=0.604) and the infestation level inside cells ($F_{2,21}$=0.413, P=0.667; $F_{2,21}$=0.970, P=0.395) before and after the treatments *(Table 2)*. However, treatments reduced the infestation compared to control. The infestation rate decreased from 10.20±0.46% to 7.41±0.26% and 9.37±1.04% to 8.12±0.47% for TBC and OBC groups, respectively.

Adult bee infestation rate did not show any difference between groups before the treatment. However, mite infestation decreased significantly from 16.07±0.72% to 7.82±0.70% in TBC group and 15.93±0.67% to 11.15±0.54% in OBC group. The lowest infestation rate (7.82±0.70%) was obtained in TBC group after the treatment *(Table 2)*.

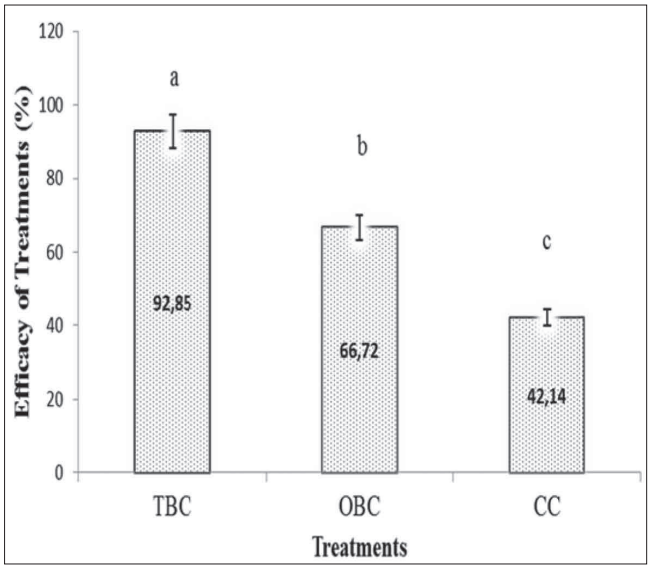

Fig 1. Efficacy of treatments against V. *destructor* infestation
a,b,c Means with different letters are significantly different (P<0.01)

Şekil 1. *Varroa* mücadelesinde kullanılan ilaçların etkinlik derecesi
a,b,c Farklı harfi taşıyan ortalamalar arasındaki fark önemlidir (P<0.01)

DISCUSSION

Treatments effects on varroa mite mortality were significant in colonies. The differences were found significantly important (P<0.01) between thymol and oxalic acid. Thymol bee cakes showed the highest efficacy against varroa control. In our study, the result of mite mortality averaged 92.85% in colonies receiving the thymol-bee cake was higher than the findings, 83.15% and 75.4% reported by different researchers [11,12], respectively. On the other hand, the efficacy of oxalic acid and Apilife-VAR® was recorded as 98.3% and 68.7%, respectively [13]. The results of different authors suggest that application of thymol can be effectively used as an "alternative *V. destructor* control" [5,14].

The efficacy of oxalic acid cake was low compared to the thymol-bee cake treatment. The result of oxalic acid treatments investigated in previous experiments was also evident in our studies [6,15].

The bee population decreased in all treatments at the end of the experiment but no treatment was significantly different than the others. This result was confirmed in

Table 1. *Number of fallen mites during treatments*
Tablo 1. *İlaç uygulamaları sırasında kartonlara dökülen ortalama varroa sayıları*

Treatments	n	N. of Mite Fall per Week			Total Mite Fall	Total Mite Fall with Perizin
		1st week	2nd week	3rd week		
TBC	8	359.75±50.56 a	171.62±19.83 a	111.00±8.82 a	642.38±60.42 a	49.25±4.9 a
OBC	8	150.00±25.93 b	83.62±15.65 b	89.25±6.76 ab	322.88±37.71 b	165.25±9.7 b
CC	8	73.37±15.19 c	52.37±12.53 c	73.50±6.41 b	199.25±78.69 c	293.37±23.6 c

a,b,c Means with different letters are significantly different (P<0.01) measured by One-Way ANOVA followed by Duncan's test for multiple comparisons

Table 2. *Effects of treatments on brood area, bee population, infestation rate inside cells and infestation on adult bee*
Tablo 2. *İlaç uygulamalarının kapalı yavru üretimi, arı varlığı, gözlerdeki bulaşıklık oranı ve ergin arılarda bulaşıklık oranı üzerine etkisi*

Treatments	n	Sealed Brood Area (cm²)		t	Number of Bee Population		t
		BT	AT		BT	AT	
TBC	8	2743.80±154.18 ns	999.49±56.32 ns	ns	40582.6±1373.7 ns	28977.0±743.0 ns	ns
OBC	8	3052.75±86.03	1003.54±25.94	ns	40463.8±1436.8	27862.5±962.6	ns
CC	8	2553.60±323.50	993.23±31.47	ns	41845±1323.0	27119.5±124.9	ns
Treatments	**n**	**Infestation Rate in Brood Cells (%)**		**t**	**Infestation Rate on Bees (%)**		**t**
		BT	**AT**		**BT**	**AT**	
TBC	8	10.20±0.46	7.41±0.26 a	ns	16.06±0.7 ns	7.82±07 a	**
OBC	8	9.37±1.04	8.12±0.47 a	ns	15.93±0.6	11.15±0.5 b	**
CC	8	11.04±0.73	12.75±0.53 b	ns	14.36±0.7	17.01±0.6 c	**

a,b,c Means with different letters are significantly different (P<0.05) measured by One-Way ANOVA followed by Duncan's test for multiple comparisons, ***BT:*** *before treatment,* ***AT:*** *after treatment and ns: not significant,* ***t:*** *comparison between before and after treatments*

previous trials by numerous researchers used different thymol and oxalic acid treatments [16-19]. On the other hand, it was reported that a 4.6% oxalic acid dehydrate solution had negative effects on bee population [19].

Infestation on adult bees decreased using two organic products (TBC and OBC) after the treatments. In a study, oxalic acid treatment reduced infestation from 33.6% to 7.7% [20]. Haggag and El-Badawy [21] reported 89.6%-94.1% reduction in infested brood after the 3rd treatment with thymol, camphor, garlic and thymol+garlic, respectively.

Using thymol-bee cake is inexpensive, easy to use and efficient against varroa mite. Both organic products decreased mite infestation on adult bees and did not cause any damage on colony development.

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Research Article

Effect of Dietary Zinc (II) Chelate Complex and Zinc (II) Enriched Soybean Meal on Selected Parameters of *in vivo* Caecal Fermentation in Laying Hens (Lohman Brown) [1]

Maciej JANECZEK [1] Mariusz KORCZYNSKI [2] Katarzyna BASINSKA [2]
Ewa PECKA [1] Katarzyna CHOJNACKA [3] Aleksander CHROSZCZ [1]
Albert CZERSKI [1] Nezir Yaşar TOKER [4]

[1] *The research was granted by Polish Ministry of Science and Higher Education, grant no. NN 209068140*

[1] Department of Biostructure and Animal Physiology, Wrocław University of Environmental and Life Sciences, POLAND
[2] Department of Animal Hygiene and Welfare, Wrocław University of Environmental and Life Sciences, POLAND
[3] Institute of Inorganic Technology and Mineral Fertilizers, Wrocław University of Technology, POLAND
[4] Istanbul Üniversitesi, Veteriner Fakültesi, Biyokimya Anabilim Dalı, TR-34320 İstanbul - TÜRKİYE

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Abstract

The study was conducted on 18 laying hens from Lohman Brown at 23 weeks of age. There were two research groups and one control group with six animals each. In the last four weeks of age, the animals were fed follows: Control group (Group C) complete diet; Group I diet containing zinc added via the biosorption process and; Group II: diet containing zinc as an organic chelate complex. The levels of short chain fatty acids (SCFAs) such as acetate, propionate, iso-butyrate, butyrate, iso-valeriate and valeriate were determined. It was observed that using a diet with additives obtained via biosorption does not have a negative influence on fermentation processes in the hen's caecum.

Keywords: *Laying hen, Caecum, Short-chain fatty acid, Biosorption, Soybean meal, Zinc*

Yumurtacı Tavuklarda (Lohman Brown) *in vivo* Körbarsak Fermentasyonunda Sindirilebilir Çinko (II) Şelat Kompleksi ve Çinko (II) İle Zenginleştirilmiş Soya Fasulyesi Küspesi İlavesinin Seçilen Parametrelere Etkisi

Özet

Çalışma 23 haftalık, 18 adet Lohman Brown ırkı yumurtacı tavuklarda yürütülmüştür. Araştırma altışarlı iki deney ve bir kontrol grubuyla yapılmıştır. Hayvanlar süreç içerisinde son dört haftada, aşağıdaki gibi beslenmiştir: Kontrol grubu (C) sadece yem; grupl çinko içeren biosorpsiyon prosesi ile, Grup II: organik şelat kompleksi olarak çinko içeren yemlerle beslenmişlerdir. Asetat, propiyonat, izo-bütirat, bütirat, izo-valeriat ve valeriat gibi kısa zincirli yağ asitlerinin düzeyleri belirlenmiştir. Burada katkı maddeleri ilave edilmiş yem kullanarak oluşturulan biozorpsiyonun tavuk körbarsağında fermantasyon prosesine olumsuz bir etkisinin olmadığı gözlemlenmiştir.

Anahtar sözcükler: *Yumurtacı tavuk, Körbağırsak, Kısa-zincirli yağ asidi, Biozorpsiyon, Soya fasulyesi küspesi, Çinko*

INTRODUCTION

The deficiency of micronutrients is a serious problem in human as well as veterinary science [1,2]. It is believed that zinc deficiencies impact 1/3 of the human population [3]. It seems that an integration of micronutrient-rich foods such as vegetables, fruits and animal products into the diets is the most practical and sustainable way to alleviate micronutrients deficiency [4,5]. In the recent years, research on the possibility of supplementing the micronutrients, including zinc, with the use of biofortified food were conducted [6,7]. In this way, the concentration of desired micronutrients in the food could be precisely controlled. Zinc is traditionally supplied as chelate complexes or

İletişim (Correspondence)
+48 71 3205888
basinskakatarzyna@wp.pl

inorganic salts. However, it is known that micronutrients supplied as inorganic salts have very little bioavailability and have a kind of a transit character [8-10]. The high supply of inorganic salts in diet causes their significant accumulation in the environment [11-14]. Conversely, using a much lower level of organically complexed minerals in diets, instead of inorganic forms, have no negative influence on haematological and biochemical parameters in broilers [15]. The chelate complexes have higher bioavailability than inorganic salts but lower than products obtained using biosorption. With respect to the above, studies on the use of biosorption in enriching the animals' diet with minerals were undertaken [16-18]. The products of animal origin obtained this way have a desired concentration of certain minerals with very high bioavailability that limits their emission into the environment [7]. Nonetheless, the influence of potential feed additives obtained with the biosorption on the processes occurring in the hens' caecum, which is of great importance to the health and productivity of the animals, is still not known. The studies conducted *in vitro* shows that feed additives obtained this way do not have a negative influence on fermentation processes occurring in the caecum of laying hens and should not have a negative influence on the animals' health [19-21]. In this study, we present the results of *in vivo* research on the fermentation process in the caecum of laying hens fed with the addition of zinc (II) brought in via the biosorption method, and compared it with the zinc (II) chelate complex.

MATERIAL and METHODS

Methods of Manufacturing Diet Supplements with Microelements

The experiment was carried out according to Local Bioethic Comitee Permission No. 129/2010. The soybean meal (Vetos, Zębowice) was enriched with zinc (II) ions by biosorption using inorganic salt ($ZnSO_4 \cdot 7H_2O$) permitted by law as a feed additive (POCh, Gliwice). The biosorption was conducted using a column reactor with a bed of volume of 0.1 dm^3. Water of pH 5.0 demineralized using 0.1M NaOH/HCl (POCh, Gliwice) was applied to the process. The water reaction was controlled using Mettler-Toledo pH-meter (Seven Multi, Switzerland) equipped with an InLab413 electrode with temperature compensation [21]. The biosorption process was conducted in a temperature of 20°C until bed saturation, while also controlling the concentration of solution flowing out of the column. The enriched biomass was air dried for 48 h.

The zinc concentration in the samples taken was determined using ICP-OES plasma spectrometer (Varian Vista-MPX; Varian, PaloALto, USA) in the Chemical Laboratory of Multi-elemental Analysis at Wrocław University of Technology, accredited by ILAC-MRA and the Polish Accreditation Centre (PCA) (No AB 696) *(Table 1)* [22].

The control group (C) and two experimental groups (I and II) were distinguished. In the first group (I) the zinc assimilation from a product obtained using biosorption was studied, and in the second group (II) the zinc absorption from organic chelate complex was examined. The biological preparation and the zinc chelate complex were separately added to the prepared diet (NJT-214, Tasomix). Compounds of diet mix were the same for all the groups *(Table 2)*. The diet composition was established not to contain the zinc addition, and the requirement for other micronutrients was assured by the addition of inorganic salts *(Table 3)*. The requirement for zinc in group I was fulfilled in 100% with the biological preparation and in group II – in 100% with the zinc chelate complex (Glystar Forte, Agsol). The digestibility of both the biological prepared zinc group (I) and zinc chelate complex (group II) was compared to the Control group, which was fed with

Table 1. *The zinc content of soybean meal before and after biosorption*
Tablo 1. *Biozorpsiyondan önce ve sonra soya küspesinin çinko içeriği*

Parameter	Non-enriched Soybean Meal ($\bar{x}$±SD, N=3)	Enriched Soybean Meal ($\bar{x}$±SD, N=3)
Zn (mg/g)	0.054±0.005	14.088±0.403

Table 2. *The ingredients of experimental diet (Tasomix®)*
Tablo 2. *Deney yemin bileşenleri (Tasomix®)*

Ingredients	%
Ground corn	29.92
Triticale	15.00
Soybean meal	13.70
Wheat	12.00
Calcium carbonate	8.42
Decoction wheat-corn	6.00
Triticale mix	4.50
Sunflower meal	4.20
Fats	2.30
Vitamin-mineral premix	2.00
Dried full blood	1.80
Mycofix selected (mineral sorbents)	0.05
L-Lysine	0.02
Biosorbent addition for	0.07
Lucantin pigment – red	0.02

[a] The composition of standard feed was established by the producer. Provides in kg of diet: vitamin A (retinyl acetate), 10.000 IU; cholecalciferol, 2.000 IU vitamin E (DL-α-tocopheryl acetate), 20.000 IU, vitamin K (bisulfite menadionsodium) 1.5 IU, vitamin B_1 (thiamine mononitrate) 3.99 IU, vitamin B_2 (riboflavin) 4.0 IU, Vitamin B_3 (nicotinic acid) 20.0 IU, Vitamin B_5 (D-calcium pantothenate) 8.0 IU, Vitamin B_6 (pyridoxine hydrochloride) 1.5 IU, Vitamin B_{12} (cyanocobalamin) 15.0 IU, biotin (D-biotin) 50.0 IU, folic acid (folic acid) 0.8 IU, choline (choline chloride) 999.6 IU. IU-international unit;
[a] Provides (mg/kg of diet): Cu, 8 (as $CuSO_4 \cdot 5H_2O$); Fe, 45 (as $FeSO_4 \cdot H_2O$); Mn, 85 (as MnO_2); Zn, Control group 60 mg/kg of diet (as ZnO); Group I Zinc (II) enriched soybean meal - 4.259 g/kg of diet; Group II Zinc (II) enriched chelate complex - 0.375 g/kg of diet

Table 3. *The chemical composition of the experimental diet (Tasomix®)*
Table 3. *Deneme yeminin kimyasal kompozisyonu (Tasomix®)*

Chemical Composition of Diet	g/kg
Dry matter	889.5
Crude ash	123.7
Crude protein	166.9
Crude fiber	32.00
Crude fat	46.3
Calcium	36.5
Total phosphorus	4.7
Metabolic energy (kcal/kg)	2821.45

a diet containing all of the micronutrients given as inorganic salts. In Group I, a preparation of 4.259 g of Zn (II) was used, and in the group II 0.375 g of zinc organic chelate complex was added.

Animals

The research material consisted of the caecum content collected from 18 hens from Lohman Brown at 23 weeks of age. The animals were held in the furnished Battery Cage System (one hen per cage) under microclimate controlled conditions. The animals from, 19 to 23 weeks of age, were fed with obtained diet mixes *(Table 3)*. Three groups of animals were selected: group C – the Control group fed with complete diet (n=6), group I – fed with diet containing zinc added with the biosorption process (n=6), group II – fed with diet containing zinc as an organic chelate complex (n=6).

SCFAs Analysis

The caecum content from each animal was collected during autopsy. It was subsequently mixed with the buffer of pH=7.3 in the ratio of 1 to 8, and underwent homogenisation. The pH of obtained suspension was measured with CP-401 pH-meter (ELMETRON, Zabrze, Poland) equipped with EPP-3 electrode and temperature sensor. The solution was centrifuged for 15 min with 13.000 rpm. The concentrated formic acid was added to the obtained liquid (0.1ml per 2ml of the solution) to stop the fermentation processes.

The samples of liquid were analysed using gas chromatograph (Agilen Technologies 7890A GC System) with FID detector to determine the total concentration of SCFAs and the percentage of particular acids: acetate, propionate, iso-butyrate, butyrate, iso-valeriate and valeriate. The identification and level of production of volatile fatty acids in the analysed samples was carried out by comparing to the retention time and the under-peak-area from the Supelco standard in ChemStation programme. The relationship between concentration of acetate and propionate and between concentration of propionate and butyrate were also evaluated.

Statistical Analysis

Data were tested for normality (Shapiro–Wilk's test). Statistical analysis was done by multivariate analysis of variance using Statistica 9.0 software (StatSoft Poland, Krakow, Poland) [23]. Significant differences were determined using Duncan's test. Differences with probability of P≤0.01 were considered significant.

RESULTS

The higher production of all the volatile fatty acids in the caecum content from hens fed with the zinc organic chelate complexes (II) was observed. The total amount of the produced volatile fatty acids was 20% higher than in the control group *(Table 4)*.

The results obtained in the samples from animals fed with the addition of biological preparation (I) were similar to the results from the group C, excluding acetic, propionic and isovaleric acids. Additionally, a lower concentration of acetic acid in the caecum content from hens fed with zinc added as a biological preparation (I) compared to the caecum content from hens fed with zinc added as an organic chelate complex (II) was noted. The lowest production of propionic acid (8.98 µmol/g) and butyric acid (5.91 µmol/g) was observed in hens from the group C.

The relationship between concentrations of particular volatile acids in the caecum content samples from hens from different groups varied. A reduced (P≤0.01) percentage of acetic acid in the caecum content from hens receiving zinc added by biosorption (I) as compared to other groups (C and II) was noted. The opposite dependence was observed for propionate and iso-valeriate, where was noticed a statistically significant differences between groups C and I (P≤0.01). The use of zinc as an organic chelate complex (II) caused the increase of percentage of propionate compared to the group C. The lowest level of butyrate production was observed in the caecum content from hens fed with zinc (II) organic chelate complex compared to group C and group I (P≤0.01).

The reduction of the ratio between the concentration of acetate and the concentration of propionate (A:P) in the group of hens fed with zinc added as a biological preparation (I) compared to group C (P≤0.01) was observed *(Fig. 1)*. The opposite tendency was observed in the ratio between the concentration of propionate and the concentration of butyrate (P:B) in the caecum content from hens fed with zinc added as an organic chelate complex (II) compared to other groups. The highest A:P ratio and the lowest P:B ratio was noted in the caecum content from hens in the Control group compared to hens fed with zinc enriched diet (I and II).

The pH of the caecum contents *(Fig. 2)* increased (P≤0.01) when using zinc obtained via biosorption and zinc organic chelate complex (adequately: 0.72 and 0.52 of pH value)

Table 4. *Effects of zinc supplementation on caecal SCFAs production (µmol/g and mol %) in laying hens (mean± SD, n=6)];[a] statistically significant differences (P≤0.01*

Tablo 4. *Seçilmiş mikroelementlerin ilavesinin tavuklarda (SD±, n=6) körbarsak içeriğinde kısa zincirli yağ asidi (µmol/g ve % mol) üretimi üzerine etkisi;[a] istatiksel açıdan önemli farklılık (P≤0.01*

SCFAs	Statistical	Group		
		C	I	II
µmol/g				
Acetate	$\bar{x} \pm SD$	31.89±11.04	24.79±7.46	40.15±17.02
Propionate	$\bar{x} \pm SD$	8.98±3.71	10.05±3.48	13.15±5.90
izo-Butytrate	$\bar{x} \pm SD$	0.77±0.70	0.74±0.63	1.05±0.56
n-Butytrate	$\bar{x} \pm SD$	5.91±1.27	6.05±2.65	5.92±2.91
iso-Valeriate	$\bar{x} \pm SD$	0.26±0.10	0.39±0.23	0.33±0.23
n-Valeriate	$\bar{x} \pm SD$	0.62±0.13	0.53±0.21	0.68±0.21
Total	$\bar{x} \pm SD$	48.43±15.38	42.55±14.20	61.29±25.81
mol%				
Acetate	$\bar{x} \pm SD$	65.41±2.46[a]	58.78±3.72[a]	65.57±4.45[a]
Propionate	$\bar{x} \pm SD$	18.34±2.48[a]	23.66±1.84a	21.41±2.15
izo-Butytrate	$\bar{x} \pm SD$	1.42±0.78	1.58±2-0.86	1.72±0.58
n-Butytrate	$\bar{x} \pm SD$	12.95±3.58	13.83±1.88[a]	9.57±2.49[a]
iso-Valeriate	$\bar{x} \pm SD$	0.54±0.08[a]	0.89±0.29[a]	0.55±0.24
n-Valeriate	$\bar{x} \pm SD$	1.33±0.27	1.25±0.18	1.19±0.36

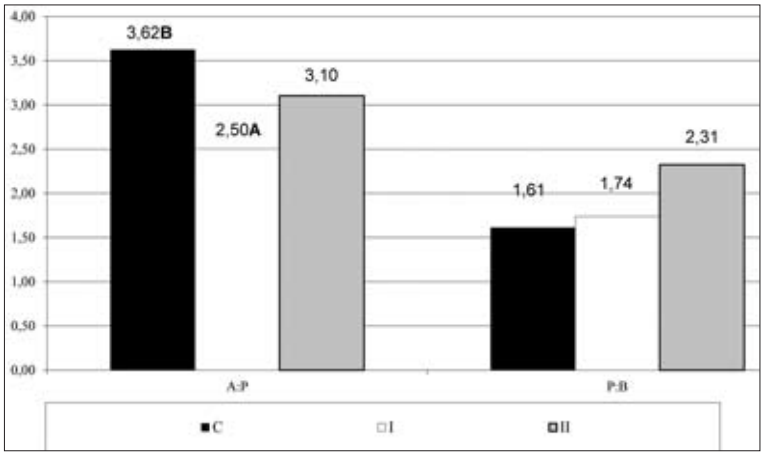
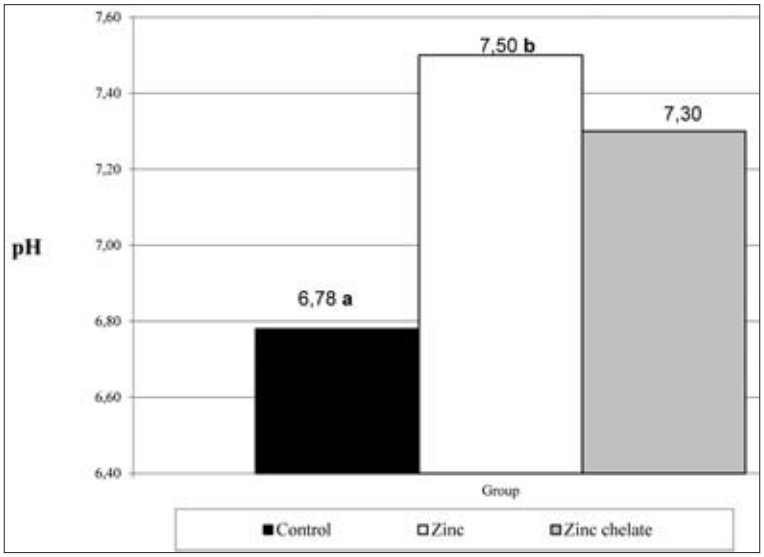


Fig 1. Concentration ratio between the acetate-propionate-butyrate
[A-B] statistically significant differences (P≤0.01)

Şekil 1. Asetat-Propionat-Butirat arasındaki konsantrasyon oranı
[A-B] istatiksel açıdan önemli farklılık (P≤0.01)

Fig 2. The level of the active acidity in caecal content
[a, b] highly statistically significant differences (P≤0.01)

Şekil 2. Körbarsak içeriğinin aktif asit seviyesi
[a, b] istatiksel açıdan yüksek önemli farklılık (P≤0.01)



as compared to the control group. The highest pH level of caecum contents was observed in the group of hens fed with zinc obtained via the biosorption method (7.5) and the lowest level was found in the Control group (6.78).

DISCUSSION

The fermentation processes in the hen's caecum are influenced by various factors. The use of reduced calcium level (800 mg/kg) and increased zinc content (110 mg/kg) in the diet decreases the level of production of volatile fatty acids (SCFAs) in the hens' caecum [24]. Because of their acid properties, SCFAs cause a decrease in the pH value in ceacum contents, therefore, the increased level of zinc in diet can cause the increase in the caecum content pH value [25]. In the analysed samples, zinc was added to the diet as an inorganic salt in the control group and as a preparation after biosorption (I) or as an organic chelate complex (II) in the research groups (60 mg/kg). An increase of pH value in the caecum content from hens receiving organic forms of zinc along with a simultaneous increase of about 12.89 µmol/g of the level of SCFAs (II) was noted, however, a decrease of about 5.88 µmol/g of produced SCFAs in Group I as compared to the Control group was observed. The obtained results can show a better digestibility of zinc given as a part of preparation obtained via biosorption. Nonetheless, the organic chelate complex causes an increase in the level of SCFAs, what is beneficial to the poultry. The diets used did not in negatively influence the pH value of the hens' caecum content, which reached the level of 6.78 to 7.50 in all of the research groups. Other studies obtained similar pH values [26-28].

It is believed that during the fermentation process in the caecum, the ratio between the acetate, propionate and butyrate should amount to 3-5:2:1 [29-32]. It is known that oversupply of zinc in diet causes an increase in acetate and propionate values and a decrease in the butyrate value [24]. In our study, a negative influence of given diet on the ratio between mentioned acids was not noted. The most beneficial ratio between the acetate and propionate (A:P) and between the propionate and butyrate (P:B) was noted in the caecum content from hens fed with zinc as an organic chelate complex (II) and amounted to 3:1 and 2:3.

The diversity of bacteria in a hen's caecum is relatively stable [24]. The bacterial fermentation leads to the formation of SCFAs, which are necessary in the metabolism of the intestine epithelium [32]. The acetic acid is characterised by the lowest energy value (0.876 MJ/mol) as compared to the propionate (1.536 MJ/mol) and the butyrate (2.194 MJ/mol), and their mutual ratio plays a role in determining the amount of energy delivered to the organism [33]. In our study the highest level of acetate, propionate and butyrate was noted in the caecum content from hens fed with zinc given as an organic chelate complex (II), which suggest the highest level of energy production.

It was shown that volatile fatty acids such as propionate and butyrate have an influence on the inhibition of pathogenic bacterial flora development [24,25,31,32,34-36]. A higher concentration of those acids was noted in the caecum content from hens fed with zinc as an organic and biological preparation as compared to the inorganic form. With reference to above, we surmise that using zinc in diet as an organic chelate complex or as a preparation obtained via biosorption can have an influence on limiting the pathogenic bacteria population and increasing the amount of energy obtained during fermentation processes. The influence of SCFAs on a microbial population requires further investigation.

The *in vitro* studies shown that using zinc as an organic chelate complex or as a preparation obtained via biosorption have a better influence on fermentation products in a hen's caecum than inorganic salts [20,21]. Our results confirm studies conducted by other authors who noted that micronutrients fed to hens as organic compounds have better digestibility than inorganic salts [37]. In *in vivo* studies zinc used as an organic chelate complex had the best influence on the level and profile of volatile fatty acids compared to other forms of this mineral, though it should be mentioned that using biological preparation obtained via biosorption did not have a negative influence on the processes studied. Taking into consideration the benefits following the use of supplements obtained via biosorption, it should be deemed appropriate to introduce them into livestock production, as this could have a significant influence on the possibilities of supplementing micronutrients deficient in human nutrition.

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Research Article

Estimating the Body Weight of Byzantine Dogs from the Theodosius Harbour at Yenikapı, Istanbul [1]

Vedat ONAR [1] Maciej JANECZEK [2] Gülsün PAZVANT [1] Nazan GEZER INCE [1]
Hasan ALPAK [1] Altan ARMUTAK [3] Alexander CHRÓSZCZ [2] Zeynep KIZILTAN [4]

[1] *This work was supported by the TUBITAK (Project No: 107O518)*

[1] Osteoarchaeology Laboratory, Department of Anatomy, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Istanbul - TURKEY
[2] Department of Biostructure and Animal Physiology, Wrocław University of Environmental and Life Sciences, PL-50375 Wrocław - POLAND
[3] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Istanbul - TURKEY
[4] Istanbul Archaeological Museums, Gülhane, TR-34122 Istanbul - TURKEY

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Abstract

In the present study, humeral and femoral midshaft circumferences were used in the weight estimation of dogs from the ancient site of the Theodosius Harbor in Istanbul. According to the calculations taken on each humerus and femur, body weight distribution of the Byzantine dogs from the Theodosius harbour was observed to be 7.953-22.385 kg. The relative ease to accommodate Terrier-size dogs in urban environments may have led to a preference for such breeds in Constantinople. It is possible that these 'light- and medium-sized mesocephalic dogs' were also used as 'alarm' guards in Constantinople. We suggest that the presence of several bones in the Yenikapı excavation area may indicate that dogs were simply buried or dumped as rubbish after death in everyday life in Constantinople.

Keywords: *Body weight, Byzantine dogs, Theodosius harbour, Yenikapı*

İstanbul Yenikapı Theodosius Limanından Bizans Köpeklerinin Vücut Ağırlığı Tahmini

Özet

Bu çalışmada, humeral ve femoral orta şaft çevreleri kullanılarak İstanbul Theodosius antik limanından elde edilen köpeklerin vücut ağırlıkları tahmin edildi. Her bir humerus ve femur'dan yapılan hesaplamalara göre, Theodosius limanı Bizans köpeklerinin vücut ağırlığı dağılımı 7.953-22.385 kg oldukları tespit edildi. Şehirleşmiş bölgelerde Terrier ebatında (büyüklüğünde) köpeklerin beslenmesinin nispeten daha kolay olduğunun bilinmesi gerçeği Konstantinapolis'de bu ırklara benzer köpeklerin bakılmasının tercih edildiği fikrine ulaşmamızı sağlayabilir. Muhtemelen Konstatinapolis'te bu "küçük ve orta büyüklükteki mezosefalik köpekler" bekçi köpeği olarak kullanıldılar. Yenikapı kazı alanındaki fazlaca kemik varlığının Konstantinapolis'in günlük yaşantısında köpeklerin ölümlerinden sonra basitçe gömülmüş veya çöp olarak atılmış olduğuna işaret ettiğini düşünmekteyiz.

Anahtar sözcükler: *Vücut ağırlığı, Bizans köpekleri, Theodosius limanı, Yenikapı*

INTRODUCTION

The body size of an animal is one of the most important ecological factors and crucial with respect to the mechanical properties of the skeleton in animals [1,2]. Besides, it is related to biomechanical and physiological demands [3]. Many life-history traits of animal species are correlated with body size [2,4]. Therefore, the interspecific frequency distribution of animal body sizes has long been a subject of interest [4].

Most analyses of body size relations begin by converting or transforming observed values into their logarithms. It has been reported that logarithmic transformation is a

İletişim (Correspondence)
+90 212 4737070/17193
nazan@istanbul.edu.tr

simple device to ease and improve diagrammatic and statistical descriptions of the effect of body size on other attributes [2].

The morphological appearance of animals has a marked effect on an animal's life history. Besides, body size is of major importance in the morphologies of animals [2,5]. Regressions of postcranial dimensions and various skeletal measurements relative to body mass have been used for estimating body size in a wide variety of mammals [3]. Therefore, it has been possible to form a logical estimate of the body weight and size and morphologies of animals [6-10]. Although dental and craniometric measurements, which are more intensively studied and easily available in archaeological sites, have been more frequently used [11,12], especially in both extant and extinct carnivores, it has been accepted that osteometric measurements of the long bones provide more reliable estimates of body mass [6]. Various scholars have used different formulations based on diameters and circumference of the long bones [1,6,8,10,13-18]. Therefore, body weight has been estimated by using these measures.

The Yenikapı excavation area covers an area of 58.000 square meters located 1.5 km inland from the Marmara Sea *(Fig. 1)*. In 2004, preliminary archaeological excavations conducted under the auspices of the Istanbul Archaeological Museum at the Yenikapı unearthed the remains of Constantinople's Theodosius Harbour [19-22]. The harbour was built by emperor Theodosius I (A.D. 379-395) to sustain the growing capital of the eastern Roman Empire.

Excavations at Yenikapı provided skeletal remains belonging to a large variety of aquatic and terrestrial species including, notably, horses, then dogs, cattle, sheep, dolphins, pigs, camels [21,23]. Animal bones are usually in a fragmentary condition and scattered across the excavation site.

In this study, further to our previous study performed on the Yenikapı Byzantine dogs [20]; we examined humeral and femoral circumferences, and tried to estimate the body weight of those dogs. Thus, we aimed to provide information about the morphological evaluation of the Byzantine dog population.

MATERIAL and METHODS

A total of 500 skulls of Yenikapı Byzantine dogs had previously been examined with to respect to typology [20]. In this study we used long bones (humerus and femur) of the dogs unearthed from the Yenikapı Metro and Marmaray Excavations which dates the time period to between the Early Byzantine (4th-7th centuries) and the Late Byzantine periods (15th century) [24].

As the first step in estimating body weights, osteometric measurements (humeral and femoral midshaft circumference measurements) of the long bones were taken and the calculation was carried out with the aid of formulations proposed by other authors for estimation of the body weight of carnivores [6,10,17]. As it is considered a reliable method, the "Anyonge equations" were used in estimating body weights in this study [6], as quoted by Onar [17].

Fig 1. Yenikapı excavation area [23]

Şekil 1. Yenikapı kazı alanı [23]

The following formulae were used:

Body weight in grams = $10^{(2.88 \times \log (f)) - 3.4}$

Body weight in grams = $10^{(2.47 \times \log (h)) - 2.72}$

Log (f): femoral circumference taken at the midpoint on the long axis.

Log (h): humeral circumference taken at a point 35% back from the distal end of the humerus.

The long-bone measurements obtained are shown in *Fig. 2*.

The body weight obtained were then compared with values from contemporary canine breed [25,26], and other mediaval [27] and Iron-Age archaelogical sites [17]. This was how we obtained data that would give an idea of the size and morphologies of the Yenikapı Byzantine dogs.

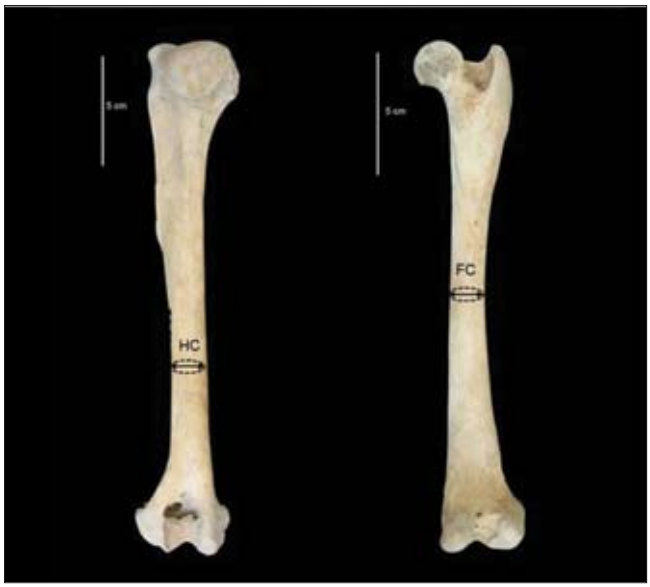

Fig 2. Long-bone measurements. Left: humerus (posterior view); right: femur (posterior view); HC - humeral circumference; FC - femoral circumference

Şekil 2. Uzun kemik ölçümleri. Sol: humerus (arkadan görünüş); sağ: femur (arkadan görünüş); HC - humerus çevresi; FC - femur çevresi

RESULTS

Humerus and femur mid-shaft circumferences were measured for both the right and left bones unearthed from the Yenikapı Excavations. A total of 97 humeri and 94 femurs were used in this study. Body weights were considered in six groups to better understand the distribution (5-10 kg; 10-15 kg; 15-20 kg; 20-25 kg; 25-30 kg and 30 kg and upper). Osteometric data obtained from the humerus and femur of Yenikapı Byzantine dogs and estimated body weights are given in *Table 1* and *Table 2*.

According to the calculations conducted on each humerus and femur, body weight distribution was observed to be within the 11-15 kg and 16-20 kg ranges. The curves showing this distribution are given below *(Fig. 3)*.

Our results were calculated where the right and left bones are not separated. As a result of the calculations on the humeral and femoral midshaft circumference, it was observed that the occurrence of heavy-bodied dogs (31 kg and upper) is less common.

DISCUSSION

In the present study, humeral and femoral midshaft circumferences were used in weight estimation. These measurements are highly correlated with an animal's body weight for living terrestrial vertebrates [6,13]. The skull typology of Byzantine dogs from the Theodosius Harbour at Yenikapı had been determined in an earlier study [20]. Craniometric data for these dogs were used for comparison with modern breeds in that study. However, body conformation has not been considered. It is believed that this calculation method for body weight offers a clearer picture of the dog's conformation. The body weight distribution of the Byzantine dogs from the Theodosius harbour was observed to be within the range of 7.953-22.385 kg (according to the femoral calculations). Results show that the majority (84.05%) of the Byzantine dogs from the Yenikapı excavations were in the above range. This range shows similarities with the medieval mesocephalic

Table 1. *Estimated body weight according to humerus midshaft circumference*
Tablo 1. *Humerus orta şaft çevresine göre vücut ağırlığı tahmini*

Statistical Values	Body Weight					
	5-10 kg	11-15 kg	16-20 kg	21-25 kg	26-30 kg	31 kg and upper
N	32	54	42	32	15	16
Min	4.733	10.248	15.208	19.473	25.039	31.120
Max	9.948	14.718	19.488	24.476	28.496	46.993
Mean	7.955	12.054	17.221	22.253	26.406	36.556
%	16.75	28.27	21.99	16.75	7.85	8.38

Table 2. *Estimated body weight according to femur midshaft circumference*
Tablo 2. *Femur orta şaft çevresine göre vücut ağırlığı tahmini*

Statistical Values	Body Weight					
	5-10 kg	11-15 kg	16-20 kg	21-25 kg	26-30 kg	31 kg and upper
N	20	28	17	14	6	9
Min	4.733	10.248	15.215	20.155	25.250	31.120
Max	9.820	14.660	19.488	24.476	26.360	45.386
Mean	7.953	11.884	16.992	22.385	25.830	36.466
%	21.28	29.79	18.09	14.89	6.38	9.57

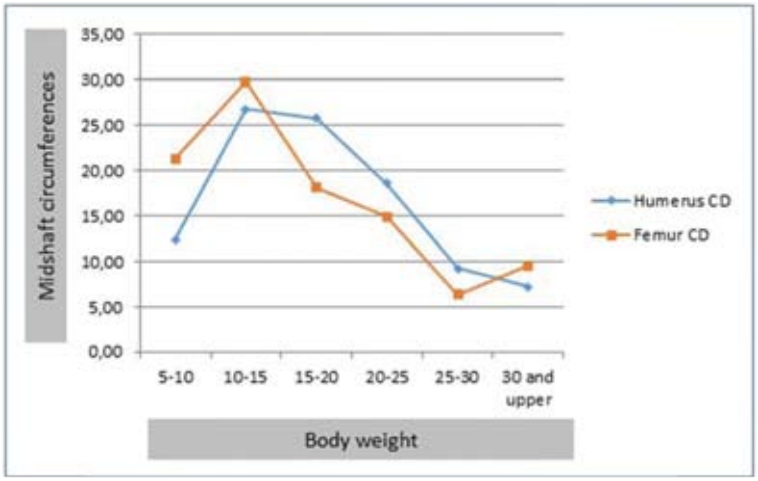

Fig 3. The distribution curve of body weight calculated from humerus and femur midshaft circumferences

Şekil 3. Humerus ve femur orta şaft çevrelerinden hesaplanan vücut ağırlığının dağılım eğrisi

dogs from the excavations at Novgorod in Russia [27]. In that study, by using humeral and femoral circumferences on medieval dogs from Novgorod, Russia (X-XIV century), it has been reported that these dogs range from size of the modern Finnish Spitz (6.8 kg) to that of the Harrier (23.1 kg), and this research showed that the "classical" light- and medium-sized mesocephalic dogs were the most widespread in that city [27]. Body weight distribution of the Van-Yoncatepe dogs unearthed from the necropolis of the Van-Yoncatepe Castle in Eastern Anatolia, which dates back to the beginning of the 1st millennium BC (Early Iron Age), was observed to be in the range of 20.963-28.105 kg. These dogs were considered to form part of the group of large-size dolichocephalic dogs [17].

When we compared the data obtained from the estimated body weight of the Yenikapı Byzantine dogs with that of today's dog breeds [25,26] and other archaeological sites [17,27], we concluded that the Yenikapı Byzantine dogs were close to the light- and medium-sized mesocephalic breeds. In addition, it is thought that the remains from the Yenikapı excavations generally represent various mesocephalic breeds growing slightly larger than Terrier breeds. The relative easy accommodation of Terrier-size dogs in urban environments may have led to a preference for such breeds in Constantinople. It is possible that these 'light- and medium-sized mesocephalic dogs' were also used as 'alarm' guards in Constantinople; given that these dogs need less food for maintenance in everyday life than larger breeds. For this reason, it has been possible to assume that, while the light-sized mesocephalic might have been used as pets, larger individuals served as 'alarm' guard partners.

In conclusion, we discussed the results of body conformation by using body weight estimations on adult dogs from Constantinople's Theodosius harbour in the present paper. There is no evidence that the dog's meat was consumed in Constantinople. We suggest that the presence of several bones in the Yenikapı excavation area may indicate that in everyday life dogs were simply buried or dumped as rubbish after death in Constantinople.

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Research Article

Effects of Cooling Rate on Membrane Integrity and Motility Parameters of Cryopreserved Ram Spermatozoa [1]

Kamber DEMİR [1] Gül BAKIRER ÖZTÜRK [2] Ümit CİRİT [3] H. Hakan BOZKURT [4]
Abit AKTAŞ [4] Sema BİRLER [1] Kemal AK [1] Serhat PABUCCUOĞLU [1]

[1] *This study was supported by TÜBİTAK (Project Number: 107 G 093)*

[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY
[2] Institute of Experimental Medicine, Department of Laboratory Animals Biology and B.A., Istanbul Univ, TR-34393 Fatih, Istanbul - TURKEY
[3] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Dicle University, TR-21280 Diyarbakir - TURKEY
[4] Department of Embryology and Histology, Faculty of Veterinary Medicine, TR-34320 Avcılar, Istanbul - TURKEY

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Abstract

In this study we aimed to determinate the effects of three different cooling rates from +26°C to +5°C at (0.3°C/min 0.6°C/min and 0.9°C/min) on spermatologic and ultrastructure properties of ram semen. For this purpose semen from 6 rams was collected by electroejaculator and was pooled in a +26°C waterbath. Pooled semen was diluated with tris based extender and divided into three equal parts according cooling rates (0.3°C/min., 0.6°C/min. and 0.9°C/min). Cooled semen was reextended with extender B +5°C in the second step. Diluated samples were equilibrated for 1 h and then were loaded in 0.25 mL straws and freezed in liquid nitrogen vapor. After each freezing stage semen was evaluated motility with computer-assisted semen analysis (CASA). Electron microscobic evaluation was done for pooled and chilled samples. It has been observed that 0.3°C/min. cooled group had meaningfully higher values of motility and progressive motility at +5°C after equilibration and post-thaw stages when compared with the 0.9°C/min. group (P<0.05). When compared to the 0.6°C/min., the 0.3°C/min. cooled group had higher total motility values at after cooling to +5°C (P<0.05), equilibration (P<0.05) and post thaw stages (P>0.05) and had higher progressive motility at after cooling to +5°C (P<0.05), equilibration (P>0.05) and post-thaw stage (P<0.05). The TEM evaluation showed that at cooling to the +5°C increases the total damaged spermatozoa in all groups (P<0.05). In conclusion, cooling the ram semen to +5°C with a rate above 0.3°C/min. affected negatively the spermatological characteristics. Reaching the cooling rates of 0.6 and 0.9°C/min. increasingly deteriorated the post-thaw motility and progressive motility values. Also, low temperature related to ultrastructural damage was observed at the first dilution step and localized at different regions of the sperm head depends upon the processes and cooling rates.

Keywords: *Ram, Spermatozoa, Cooling rate, Ultrastructure*

Koç Spermasının Dondurulmasında Kullanılan Soğutma Oranlarının Membran Bütünlüğü ve Motilite Özelliklerine Etkisi

Özet

Bu çalışmada koç spermasının 26°C'den +5°C'ye indirilmesinde farklı soğutma hızlarının (0.3°C/dk., 0.6°C/dk. ve 0.9°C/dk.) eritme sonrası spermatolojik özellikler ve spermatozoonların ultrastrüktürel yapısı üzerindeki etkilerinin incelenmesi amaçlanmıştır. Altı adet koçtan elektro ejakülatörle alınan spermalar 26°C'daki su banyosunda pooling işlemine tabii tutuldu. Tris bazlı sulandırıcıyla sulandırılan birleştirilmiş sperma üç eşit hacme bölünerek 3 farklı hızda (0.3, 0.6 ve 0.9°C/dk.) +5°C 'ye soğutuldu. Sperma iki basamakta sulandırıldı, gliserol sperma ısısının +5°C'ye indiği ikinci basamakta katıldı. Sulandırma sonrası sperma 1 saat ekilibre edildi daha sonrasında 0.25 ml payetlere çekilerek sıvı azot buharında dondurulduu. Sperma pooling, sulandırma, soğutma, ekilibrasyon ve eritme sonrası gibi tüm aşamalarında motilite değerleri Bilgisayar Destekli Analiz Sistemleri (CASA) ile değerlendirildi. Pooling ve soğutma sonrasında elektron mikroskop incelemeleri gerçekleştirildi. 0.3°C/dk. soğutma grubunun, spermanın +5°C'ye soğutma, ekilibrasyon ve eritme sonrasındaki hem total motilite hem de progressive motilite değerleri 0.9°C/dk. soğutma grubuna göre önemli derecede yüksek bulundu (P<0.05). Bu grup 0.6°C/dk soğutma hızı ile karşılaştırıldığında ise, 0.3°C/dk. soğutma grubunun soğutma ve ekilibrasyon sonrasındaki total motilite değerleri yüksek bulundu ancak eritme sonrası gruplar arasında fark bulunmadı (P>0.05). Soğutma ve eritme sonrasında ise progressive motilite değerleri daha yüksek bulunurken(P<0.05), ekilibrasyon aşamasında progresif motilite değerleri arasında fark bulunmadı(P>0.05). Yapılan TEM incelemesinde, tüm soğutma hızı gruplarında eritme sonrasında tespit edilen toplam hasarlı spermatozoit oranı, pooling sonrasına göre önemli derecede yüksek bulunmuştur (P<0.05). Sonuç olarak koç spermasının dondurulması öncesinde +5°C'ye soğutulmasında 0.3°C/dk.'nın üzerinde soğutma hızlarının kullanılmasının sperma kalitesini olumsuz etkilediği ve soğutma hızı 0.6 ve 0.9°C/dk.'ya arttırıldıkça eritme sonrası total ve progresif motilitenin artan oranlarda etkilendiği sonucu çıkarılmıştır. Ayrıca, koç spermasında düşük sıcaklara bağlı olarak oluşan ultra strüktürel hasarların ilk sulandırma aşamasından itibaren başladığı ve ultra strüktürel hasarların, spermanın gördüğü işleme ve soğutma hızlarına göre başın farklı bölgelerinde lokalize olma eğiliminde olduğu sonucu çıkarılmıştır.

Anahtar sözcükler: *Koç, Spermatozoa, Soğutma oranı, Ultrastrüktür*

İletişim (Correspondence)
+90 212 4737070/17264
kamberdemir@gmail.com

INTRODUCTION

Semen cryopreservation and artificial insemination (AI) have been principal reproductive technologies in cattle industry for so many years. Unfortunately, suboptimal semen preservation methods in combination with the difficulty in passing through the cervix during AI are the major obstacles to the extensive use of cooled or cryopreserved ram semen in AI programs. One of the most integral components of AI programs is semen processing which often requires extenders with ingredients that provide protection to the spermatozoa against cold shock stress [1]. Morphological changes which reduces the fertilization ability of the spermatozoa, occurs during the cooling of spermatozoa and increases during the cryopreservation process [2-4].

Diluted semen is cooled to a temperature close to 0°C. Cooling is a proceses adapts spermatozoa to reduced metabolism. The cooling rate of diluted semen from temperatures above 0°C can significantly influence the post - thaw survival of spermatozoa. Rapid cooling of extended semen from +30 to about +15°C may have no effect on survival of spermatozoa, but fast cooling from +30°C to +10°C, +5°C or 0°C decreases the post-thaw motility of spermatozoa [5]. These damages ultimately can alter functional integrity of spermatozoa and reduces fertililysing ability [3].

Although approximately 40-60% of ram spermatozoa preserve their motility after freeze–thawing, only about 20-30% remains biologically undamaged. Cold shock can harm spermatozoa in various subcellular levels [2,6]. The basic damage to spermatozoa may be ultrastructural (physical), biochemical, functional or DNA integrity [7,8]. Ultrastructural damage occurs to the plasma and acrosome membranes, the acrosome, the mitochondrial sheath and the axoneme. It is well established that ram spermatozoa are relatively more sensitive to cold shock injury than other livestock species' spermatozoa and ultrastructural damage generally is more severe for ram than bull spermatozoa [2,4]. A spermatozoon may be motile, but damaged, in which case it is doubtful if such a cell will fertilise the egg. After both slow and fast freezing of ram semen, motility is better preserved than the morphological integrity of spermatozoa [9].

Any ultrastructural changes like total or acrosomal plazma membrane seperation, partial destruction or fibril defects in the axonemma can only be detected by high resolution Transmission Electron Microscopy (TEM) [10,11].

Early studies showed that cooling spermatozoa to +5°C with high cooling rates negatively affects both the post-thaw sperm quality and motility. However in our knowledge there are no studies on the effects of cooling rates on sperm ultrastructure. The aim of the present study was to determine the effects of different cooling rates (0.3°C/min, 0.6°C/min. and 0.9°C/min.) from +26°C to +5°C on ultrastructure properties and post-thaw motility of ram spermatozoa.

MATERIAL and METHODS

All chemicals used in this study were analytically qualified. Except Ethilen alcohol, Gluteraldehide Merck (Darmstadt, Germany), Osmium Tetraoxide, Proplene Oxide, Epon 812, Uranile Asetate and Lead Asetate, Ladd Research Institute (Vermont USA), all the chemicals were acquired from Sigma Aldrich (St. Louis, MO, USA).

The experiment was performed in accordance with guidelines for animal research from Istanbul University Veterinary Faculty Ethics Commite on Animal Research (2007/183).

Semen Collection

The study was conducted out of the breeding season (May-July). Two to five years old Hemşin rams (n=6) were housed at the Faculty of Veterinary Medicine in Istanbul University under the surveillance of health and nutritional programmes. Ram semen was collected by an electroejeculator (P-T Electronics, Oregon and USA). Implemented electrical stimulatons intervals were 5 second. Semen samples were obtained from all rams following a maximum of 3 or 4 electrical stimuli [12]. The ejaculate was kept in an insulated Styrofoam box containing warm heat pads (30°C) and transported to the laboratory immediately, good quality (volume: ≥0.5 mL; mass motility: ≥4; motility: ≥70%, sperm concentration: ≥2 × 10^9/mL) were pooled [13].

Sperm Collection and Pre-evaluation

A tris-based extender (tris 27.1 g/L, citric acid 14 g/L, fructose 10 g/L, egg yolk 15% (v/v), pH 6.8) was employed to semen. The base extender was divided into two parts and marked as fraction A and B. Then 10% glycerol (v/v) was added to the fraction B, at a final glycerol concentration of 5%. A two-step dilution (with fractions A and B) was used and the glycerol was added in the second step [14]. The pooled semen sample was diluted slowly with fraction A (without glycerol) to final concentration 80×10^6 sperm/ml in a water bath at +26°C. Then, sperm motility and velocity were evaluated by CASA. Then, the pooled semen sample was divided into three equal aliquots (study groups) and cooled to +5°C according to 0.3°C/min, 0.6°C/min., 0.9°C/min cooling rates.

After gliserization and equilibration semen was frozen in liquid nitrogen vapour, 4 cm above the liquid nitrogen level, for 10 min. by using 0.25 mL straws and then were immersed into liquid nitrogen for storage.

Evaluation of Sperm Motility and Kinetic Parameters with CASA System

The pooling, cooling, equilibration and post-thaw motility and kinetic parameters of the sperm samples were measured with computer assissted sperm analysis system (CASA 12.3 IVOS, Hamilton - Thorne Biosciences, Beverly, MA, USA). The sperm analyser was set-up as follows: phase contrast; frame rate – 60 Hz, minimum contrast – 60 Hz, low and high static size gates – 0.6 to 4.32; low and high intensity gates – 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size 5 pixels; default cell intensity-55, VAP (Path Velocity, µm/s), cutoff 20 µm/s, progressive minimum VAP cutoff 50 µm/s, VSL (Progressive Velocity, µm/s) cutoff 30 µm/s. After the CASA system was set to evaluate ram sperm, sperm samples from different stages of cryopreservation process were diluated with tris based diluator and loaded to +37°C heated glass slides (Leja 4, Leja products, Luzernestraat B.V., Holland). Total motility (TM%), progressive motility (PM%), VAP, VSL and other sperm kinetic parameters were measured under 100X magnification and approximetally 600-800 sperm cells in 10 different areas were evaluated [15].

Transmission Electron Microscopy

Pooled and cooled semen were evaluated by the transmission electron microscopy for the ultrastructural defects. The 75 µl of semen from each group were fixated in 0.1 M Phosphate Buffer Saline (pH 7.4) containing 2.5% Gluteraldehide at +4°C, for 4-6 h. After fixation, samples were washed with PBS (pH 7.4) for two times. Then samples were centrifuged at 300 g, for 15 min. and were incubated at +4°C for 24 h. Pellets at the bottom of the tube were transferred to 1% Osmium Tetraoxide (OsO_4) PBS and were incubated at +4°C for 3 h. Following the second incubation peroid, samples were dehidraed through 70%, 80%, 90% and 100% alcohol series for 10 min per each, respectively. Dehidrated samples were washed in Propylene oxide (PPO) for three times for 10 min. to remove alcohol leftovers. Washed samples were blocked by embeding in epon with using Epon Solidication Kit (Fluka Chemie Gmbt, Swiztzerland) in 48 h. Five mm deep bullet shaped blocks were obtained after the blocks were hardened. Sections of 90 nm thickness were cut from those blocks with ultramicrotome (Super Nova Reichert - Yung Austria). Sections were dyed with 3% uranile acetate and lead acetate. Cell membrane distruptions were detected in the sample sections by using JEM – 1010 (Jeol Tokyo) Electron Microscobe and photographed. Differences between groups were evaluated by recording the damages in different areas [16,17].

Statistics

The Kruskal Wallis Test was used for comparation of motility and other kinetic parameters when comparing the cooling rate groups. Mann-Whitney Test was used for evaluation of the ultrastructural findings of the groups after cooling process. In all tests $P<0.05$ was accepted value for statistical significance.

RESULTS

Total and progressive motility values at +5°C of the 0.3°C/min. group was better when compared to 0.6 and 0.9°C/min ($P<0.05$). For the 0.6 and 0.9°C/min. groups motility and progressive motility values were found similar after cooling to +5°C ($P>0.05$). The total and progressive motility of equilibrated spermatozoa of the 0.3°C/min. cooling rate group were significantly higher than 0.9°C/min. rate cooling group's ($P<0.05$). Also the total motility values of the 0.3°C/min. cooling rate were higher than 0.6°C/min. ($P<0.05$) *(Table 1)*. VAP, VSL,VCL, BCF and LIN values were similar in all groups after cooling and equilibration stages ($P>0.05$). Post-thaw 0.3°C/min. group had higher total motility and STR values than 0.9°C/min. group ($P<0.05$). The progressive motility and VCL of the 0.3°C/min. group had ranked significantly higher values than 0.6°C/min. and 0.9°C/min. groups both ($P<0.05$). Moreover the post-thaw VAP values of 0.3°C/min. group was meaningfully higher than 0.6°C/min. group ($P<0.05$) *(Table 2)*.

Such as seperations of the plasma membranes which covers the head in partially or totally were observed in pooled spermatozoa *(Fig. 1A)*. Similar seperations were detected in the cooling groups as were seen in the pooled semen *(Fig. 1 B, C, D)*. In the TEM evaluation, total defected spermatozoa ratio was meaningfully higher in all cooling rate groups when compared to the pooled

Table 1. *Total and progressive motility rates of cooling groups (n= 10)*
Tablo 1. *Soğutma gruplarının toplam ve progresif motilite oranları(n=10)*

Cooling Groups	After Cooling		After Equilibration		Post-Thaw	
	MOT (%)	PMOT (%)	MOT (%)	PMOT (%)	MOT (%)	PMOT (%)
0.3°C/min.	76.1±2.29^{b}	53.3±2.45^{b}	77.1±4.80^{b}	53.9±5.23^{b}	29.8±2.76^{b}	17.8±2.02^{b}
0.6°C/min.	67.8±1.61^{a}	45.3±1.61^{a}	69.0±3.29^{a}	48.8±2.04^{ab}	22.6±2.96^{ab}	11.5±0.99^{a}
0.9°C/min.	60.8±3.32^{a}	40.9±2.92^{a}	68.9±2.16^{a}	44.3±3.21^{a}	16.6±2.94^{a}	10.9±2.30^{a}

^{a,b} *Different superscripts in the same column denote significant differences statistically among Total Motility and Progressive Motility ($P<0.05$);* ***MOT*** *= Total motility,* ***PMOT*** *= Progressive motility*

Table 2. *According to the kinematic values of semen cooling group (n=10)*
Tablo 2. *Soğutma gruplarının sperm kinematik değerleri (n=10)*

Groups		VAP (µm/sn)	VSL (µm/sn)	VCL (µm/sn)	ALH (µm/sn)	BCF (Hz)	STR (%)	LIN (%)
After Cooling	0.3°C/min.	114.3±2.84	100.4±2.74	180.2±2.23	6.3±0.12	40.0±0.91	83.8±0.91	55.2±1.29
	0.6°C/min.	103.7±4.66	90.9±4.62	170.2±5.30	6.3±0.17	39.5±0.85	83.9±1.17	52.9±1.72
	0.9°C/min.	102.4±5.18	90.2±5.30	168.0±6.28	6.1±0.16	40.1±1.18	84.3±1.20	53.5±1.59
After Equilibration	0.3°C/min.	112.1±2.69	97.6±3.14	174.9±1.62	6.1±0.16	40.7±0.96	83.1±1.72	55.6±1.99
	0.6°C/min.	105.7±3.74	93.5±5.06	165.7±5.84	6.0±0.16	40.5±0.93	85.5±1.96	56.5±1.62
	0.9°C/min.	114.0±5.40	99.6±5.11	179.1±4.96	6.3±0.13	42.2±1.21	82.2±2.26	54.8±1.66
Post -Thaw	0.3°C/min.	91.1±2.39^{b}	79.0±2.49	153.1±2.06^{b}	5.7±0.16	41.7±0.62	81.0±1.10	50.8±1.33
	0.6°C/min.	84.9±6.00^{a}	73.8±6.03	147.7±7.97^{a}	6.0±0.15	40.7±0.92	81.5±1.15	49.6±1.21
	0.9°C/min.	88.3±4.23^{ab}	78.4±4.44	145.8±5.66^{a}	5.3±0.14	41.4±0.81	83.9±1.12	53.2±1.67

^{a,b} Different superscripts in the same column denote significant differences statistically among parameters (P<0.05); ***VAP****= Average path velocity,* ***VSL****= Straight linear velocity,* ***VCL****= Curvilinear velocity,* ***ALH****= Lateral head amplitude,* ***BCF****= Beat cross frequency,* ***STR****= Straightness,* ***LIN****= Linearity (Ratio of VSL:VCL)*

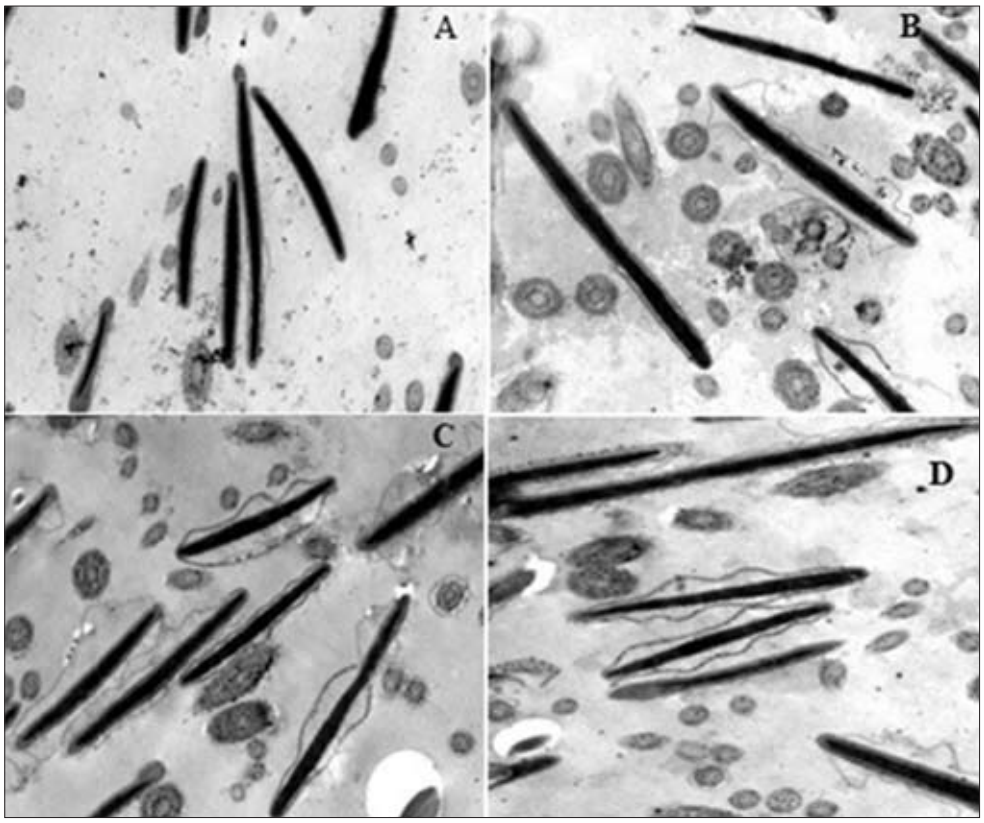

Fig 1. Transmission electron microscopy (TEM) images of Pooling (A) and Cooled (B,C,D) Hemşin ram sperm. In the head of spermatozoa in the plasma membrane ultrastructural changes that occur in different types. Magnification 10.000x

Şekil 1. Pooling (A) ve Soğutma (B, C, D) sonrası Hemşin ırkı koç spermatazoonlarının Transmisyon Elektron Mikroskop (TEM) görüntüleri. Spermatazoonların baş bölgesini saran plazma zarında farklı tipte oluşan ultrastrüktürel değişimler, x10.000 büyütme

semen (P<0.05). This ultrastructural damage in spermatozoa had a tendency to localize at different parts of the spermatozoa according to the cooling rate. When the cooling rate groups were compared with the after pooling stage had lower ratios of ultrastructural damage at the acrosomal region's plasma membrane but on the other hand had higher ratios of damages at the post acrosomal part (P<0.05). The swallowing damage at the plasma membrane covering the acrosome was found higher in 0.3°C/min. cooling group, than the 0.6°C/min. and 0.9°C/min. groups (P<0.05). However in 0.6°C/min and 0.9°C/min. groups, the ultrastructural defects at the whole head region was significantly higher than in the 0.3°C/min. cooling and the pooling stage groups (P<0.05) *(Table 3)*.

DISCUSSION

One of the major causes of reduced motility after freeze thawing is cold shock [18], which often results in swelling and blobbing of the acrosomal membrane and disruption and/or increased permeability of the plasma membrane [9,19]. The morphological damages that occur

Table 3. *In fresh semen (after pooling) and cooled with different cooling rates, ultrastructural damages in spermatozoa that were determined by TEM*
Tablo 3. *Taze (pooling sonrasında) ve farklı soğutma hızları ile soğutulan spermatazoonlarda TEM ile tespit edilen ultrastrüktürel hasarlar*

Groups	Ultrastructural Damages in Cells Based on the Observed Region (%)				
	Post Acrosomal Region	Acrosomal Region	Entire Head	Other Regions	Total Damaged Spermatozoa
Fresh Semen (Pooling)	24[a]	13[a]	20[a]	8[a]	65[c]
0.3°C/dk.	0[b]	54[b]	20[a]	16[a]	90[ab]
0.6°C/dk.	2[b]	28[c]	51[b]	15[a]	96[a]
0.9°C/dk.	2[b]	27[c]	45[b]	12[a]	86[b]

[a,b,c] Different superscripts in the same column denote significant differences statistically among parameters (P<0.05)

at the cell and acrosomal membrane of the spermatozoa during the cooling and freezing processes, reduces the pregnancy rates [20,21]. Cooling the spermatozoa to +15 or +5°C is important, for protecting spermatozoa from cold shock [22]. Dhami et al.[23] have reported post-thaw motility of bull spermatozoa were influenced by cooling rate from +30°C to +5°C. Bacinoglu et al.[24] reported that the detrimental effect of glycerol on post-thaw semen motility was compensated by two step cooling rate regimes. Ak et al.[25] have suggested that, cooling rate from +30°C to +5°C to have controversal effect on semen parameters up to equilibration time but not on post-thaw semen parameters in rams.

In this study the cooling rate of the spermatozoa to +5°C affected both pre and post-thaw quality of the ram spermatozoa. The 0.3°C/min. cooling group had significantly better total and progressive motility values than 0.9°C/min group in cooling to 5°C, equilibration and post-thaw stages. When compared to the 0.6°C/min. group, 0.3°C/min. group had higher total motility values in cooling and equilibration stages and progressive motility values at post-thaw stage (P<0.05). According to the results of the study, 0.6°C/min. cooling rate negatively affected total or progressive motility during different stages of the cryopreservation process, but when the cooling rate was raised to 0.9°C/min in all stages of the process the total and the progressive motility were affected negatively. Similar to our findings, Jones [26] showed that raising the cooling time of the spermatozoa from 30 to 5°C from 1 h to 2 and 3 h, obtained better post-thaw motility (respectively 36.9%, 44.8% and 47.9%, P<0.001).

According to the cooling rate that was used, we have detected morphological ultrastructural changes besides the changes in movement competence of the spermatozoa by evaluating the samples taken from both pre and post cooling stages under TEM. The morphological structural changes occuring during the cooling or freezing of the spermatozoa change the acrosome integrity and also make ultrastructural changes that can not be detected neither under light microscobe nor with flouresance probs [27]. Those morphological changes result lower pregnancy rates at inseminations made with frozen thawed spermatozoa than made with fresh spermatozoa even having the same number of spermatozoa and the same motility values with the frozen thaw samples [9]. The first negative morphological changes in spermatozoa were seen during the gradually cooling stage to 5°C, especially at the outer acrosomal membrane and plasma membrane. Most of the damages occur at the membrane part of the head of the spermatozoa [3,8].

During the cooling stage of spermatozoa due to the temperature changes, lipid molecules in the cell membrane change place with lateral phase transition and these results with destruction of membrane integrity [21]. Fisher and Fairfull [5] reported that there were quite less or non significant destruction was seen in cell membranes when high cooling rate (2°C/min.) were used in ram spermatozoa from 30°C to 15°C. Drobnis et al.[28] indicated that destruction in the spermatozoa cell membrane due to the tempareture began during the cooling stage from 15°C to +5°C. However Holt ve North [29] reported that temperature related to cell membrane changes in the ram spermatozoa as a result of the lateral phase transition due to the temperature change are developed mostly between +17 to +22°C. Wolf et al.[30] declared that the lateral phase transition in cooling the ram spermatozoa occured at 26°C. In this research we have detected that, allthough the motility values have not been affected negatively when pooled at 26°C waterbath, cell membranes had some morphological damages that reduce fertilisation capacity, propably. Most of the morphological damages were seen generally as swelling or seperation of the membrane that covers the head of the spermatozoa, especially at the postacrosomal area, acrosome or the whole membrane itself. Our finding are similar to those Armengol et al.[27], in which detected swelling and seperations at the cell membrane that cover the head part at 30°C after pooling. Detection of 65% cell membrane damage in ram spermatozoa samples pooling indicates that ram spermatozoa are very sensitive to cold damage and these damages can ocur in 26°C in an opposite manner to Drobnis et al.[28].

When the morphological structure changes after

pooling and cooling stages are investigated under electronmicroscope, it is seen that there was a high ratio of swelling and separations at the cell membrane covering the head part. Our electronmicroscopic findings about the ultrastructurel changes in the spermatozoa were similar to the Armengol et al.[27].

We have observed that the ultrastructural damage in spermatozoa was localized in different parts of the head according to the cooling rate. Despite the cell membrane seperations at the acrosome part were the most in the 0.3°C/min. cooling rate group, in the 0.6°C and the 0.9°C/min. groups, these seperations were at the entire cell membrane of the head. The cell membrane that covers the acrosome of the spermatozoa binds with the outer acrosome membrane and reveals the acrosomal enzymes out during the acrosome reaction (AR) while the cell membrane that covers the equatorial segment provides spermatozoa to attach to the oocyte during the fertilisation process. Apart from that, the cell membrane provides to maintain the inner cell ion, pH equilibrium and enzyme activities with its semipermeable structure [10]. The structural integrity of the cell membrane which is at the center of the events occurs synchronously with ovulation like capasitation, acrosome reaction and hypermotility, is irrevocable for fertilisation. The free lipids and proteins that diffuse in between the classical double layered protein/phospholipid structure of the cell membrane are found in the acrosomal, equatorial, postacrosomal, mid piece and last part regions in different concentrations. This differences of dispertion of the lipid mollecules in diferent parts of regional cell membranes cause spermatozoa to show different levels of morphological changes to the cooling rates [31].

In conclusion, it has been investigated out that when ram spermatozoa are cooled to +5°C before cryopreservation, cooling rates above 0.3°C/min. affected the sperm quality negatively and if cooling rates were increased to 0.6 and 0.9°C/min. total and progressive motility at the post-thaw stage was affected with the increasing rate. In this study, we determined that the ultrastructural damages in ram spermatozoa have started to occur at the first dilution stage, and localized at different parts of the head due to the process and cooling rate.

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Research Article

PCR Detection of *Coxiella burnetii* in Fetal Abomasal Contents of Ruminants

Elçin GÜNAYDIN [1] Hamit Kaan MÜŞTAK [2] Barış SAREYYÜPOĞLU [2] Zafer ATA [3]

[1] Veterinary Control Central Research Institute, Breeding Disease Diagnosis Laboratory, TR-06020 Etlik, Ankara - TURKEY
[2] Ankara University, Faculty of Veterinary Medicine, Department of Microbiology, TR-06110 Dıskapi, Ankara - TURKEY
[3] Military Veterinary School and Educational Central Commandership, TR-16600, Gemlik, Bursa - TURKEY

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Abstract

This study was carried out on abomasal contents of aborted fetuses of 102 cattles, 45 sheep and 5 goats sent to the laboratory from Central Anatolia, and Black Sea Region of Turkey to investigate the presence *Coxiella burnetii (C. burnetii)* by trans-PCR. Out of 152 abomasal contents, 11 (7.23%) were detected to contain *C. burnetii* DNA. Four (3.92%) out of 102 cattle, 5 (11.11%) out of 45 sheep, and 2 (40%) out of 5 goat abomasal contents harbored *C. burnetii* DNA. As a result, PCR detection of *C. burnetii* DNA in fetal abomasum contents is a significant finding of a possible *C. burnetii* related abortion.

Keywords: *Coxiella burnetii, Fetal abomasal contents, Trans-PCR*

Ruminantların Fötal Abomasal İçeriklerinde *Coxiella burnetii*'nin PCR ile Tespiti

Özet

Bu çalışma, İç Anadolu ve Karadeniz Bölgesi'nden laboratuvara gönderilen 102 sığır, 45 koyun ve 5 keçi atık fötus mide içeriğinde trans-PCR ile *Coxiella burnetii (C. burnetii)* varlığını araştırmak için yapıldı. Yüz elli iki abomasal içeriğin, 11'inin (%7.23) *C. burnetii* DNA'sı içerdiği tespit edildi. 102 sığırın 4 (%3.92)'ünün, 45 koyunun 5 (%11.11)'inin ve 5 keçinin 2 (%40)'sinin abomasal içeriklerinde *C. burnetii* DNA'sı tespit edildi. Sonuç olarak, fetal abomasal içeriklerde PCR ile *C. burnetii* DNA'sı tespit edilmesinin, *C. burnetii* ilişkili abortlar için önemli bir bulgu olduğu düşünüldü.

Anahtar sözcükler: *Coxiella burnetii, Fötal abomasal içerik, Trans-PCR*

INTRODUCTION

Q fever is a highly contagious zoonotic disease caused by *Coxiella burnetii*, a Gram-negative obligate intracellular bacterium, which has an ability to survive in phagolysosomes and resist against unfavorable environmental conditions [1]. Cattle, sheep, and goat are the primary reservoirs for *C. burnetii* [2]. *C. burnetii* can infect broad spectrum of susceptible hosts including wildlife, and even non-mammalian species comprising ticks, birds, and reptiles. Any infected animal has the potential to transmit the pathogen via bacterial shedding in their body secretions. Transmission occurs mainly through the inhalation of aerosols formed during parturition or at slaughter [3].

Routine diagnosis of Q fever is usually established by serological tests such as, immunofluorescence, complement fixation and enzyme-linked immunosorbent assay (ELISA) [4]. However, seroconversion typically occurs 7-15 days after symptoms appear. For a definitive diagnosis in the early stages of acute Q fever, serologic testing in combination with PCR is recommended [5]. Isolation of *C. burnetii* is restricted to specialized laboratories and not preferred in veterinary medicine, since *C. burnetii* does not grow on standard laboratory bacteriological media and its isolation is time consuming, laborious, hazardous to perform and requiring biosafety level 3 laboratories [6]. PCR-based diagnostic assays have been developed for the detection of *C. burnetii* DNA and these have been used primarily for clinical samples [7,8]. Recently, other types

İletişim (Correspondence)
+90 312 3260090/261
elcin_gunaydin@hotmail.com

of PCR assays, like nested PCR [9,10] and quantitative PCR (qPCR) [11,12] have been developed, and used sometimes in combination with high-throughput capabilities [13].

Several PCR-based methods have been developed targeting the isocitrate dehydrogenase gene; *icd* [14], the superoxide dismutase gene; *sod* [15], the outer membrane protein-coding gene; *com1* (10), and a transposon-like repetitive region; *IS1111* [16]. A PCR performed with primers based on a repetitive, transposon-like element (Trans PCR) [16] has proved to be highly specific and sensitive for the laboratory diagnosis of *C. burnetii* infections, as even very few copies of a specific DNA sequence can be detected.

The purpose of this study was to investigate *C. burnetii* in the abomasal contents of aborted fetuses of ovine, caprine and bovine origin by conventional PCR (Trans-PCR) targeting transposon-like repetitive element.

MATERIAL and METHODS

Samples

Between the years 2009-2011, a total of 152 abomasal contents of the aborted fetuses consisting of 102 cattles, 45 sheep, 5 goats were sent to the laboratory under sterile conditions and cold chain. The distribution of the abomasal contents sent to the laboratory among the ruminants and provinces were mentioned in *Table 1*. At the time of arrival, DNAs were extracted from all samples.

DNA Extraction

DNA extracted from positive strain containing the gene coding phase II antigen was kindly obtained from Department of Microbiology, Faculty of Veterinary Medicine, Fırat University, and, DNAs from all abomasal contents were extracted by commercial DNA isolation kit (DNeasy Tissue Kit, Qiagen, Germany) according to the manufacturer's instructions. DNAs were stored at -20°C until used.

Primers

Trans-1 and trans-2 primers, specific to the *IS1111* fragment, a transposon-like repetitive region were targeted for the detection of *C. burnetii* by Trans-PCR. Primers as previously described by Hoover et al.[16] consisted of the following sequences: Trans 1; 5'-TAT GTA TCC ACC GTA GCC AGT C-3' and Trans-2; 5'-CCC AAC AAC ACC TCC TTA TTC-3'. Expected amplicon size was 687 bp *(Fig. 1)*.

Trans PCR

Each reaction had a volume of 25 µl including, 22 µl reaction mixture containing 2.5 µl 10× PCR buffer (without $MgCl_2$), 0.5 µl dNTP (10 mM), 1.5 µl $MgCl_2$ (25 mM), a 1 µl of each primer (10 pmol/µl), 0.25 µl Taq DNA polymerase (5 U/ µl) (Fermantas, Vilnius, Lithuania), 15.25 µl deionized

Table 1. *The distribution of the abomasal contents sent to the laboratory among ruminants and provinces*

Tablo 1. *Laboratuvara gönderilen mide içeriklerinin ruminantlar ve iller arasında dağılımı*

Provinces	Cattle	Sheep	Goat
Ankara	12	22	2
Yozgat	-	2	1
Eskişehir	1	4	-
Çankırı	68	5	-
Çorum	3	2	1
Bartın	3	-	-
Bolu	9	3	1
Kayseri	-	1	-
Kastamonu	3	1	-
Kırşehir	3	3	-
Kırıkkale	-	1	-
Nevşehir	-	1	-
Total	102	45	5

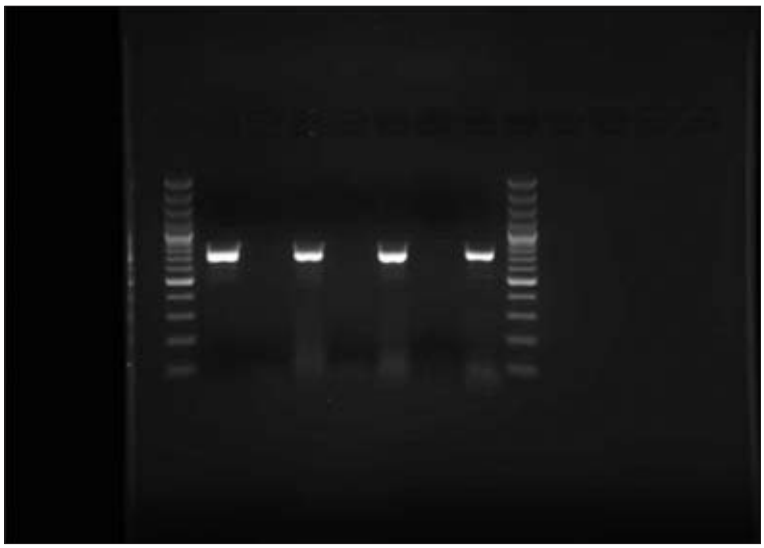

Fig 1. The figure of examined samples for *C. burnetti* by Trans-PCR (Left to right)
Lane1: Marker; 100 bp DNA ladder, Lane 2: DNA extracted from positive strain containing the gene coding phase II antigen (positive control), Lane 3: Negative control (deionized water), Lane 4-6-8: *C. burnetii* positive abomasal contents, Lane 5-7: *C. burnetii* negative abomasal contents, Lane 9: Marker; 100 bp DNA ladder

Şekil 1. Trans-PCR ile *C. burnetii* yönünden incelenen örnekler (Soldan sağa)
Kuyucuk 1: Marker; 100 bp DNA merdiveni, Kuyucuk 2: Faz II antijenini kodlayan geni içeren pozitif suştan ekstrakte edilen DNA (pozitif kontrol), Kuyucuk 3: Negatif kontrol (deionize su), Kuyucuk 4-6-8: *C. burnetii* pozitif mide içeriği, Kuyucuk 5-7: *C. burnetii* negatif mide içeriği, Kuyucuk 9: Marker; 100 bp DNA merdiven

water and 3 µl template. Cycling parameters were as follows: initial denaturation at 95°C for 2 min followed by 5 cycles of denaturation at 94°C for 30 sec, 66 to 61°C (the temperature was decreased by 1°C between consecutive steps) for 1 min, extension 72°C for 1 min and final extension 72°C for 10 min. Trans-PCR was performed using Thermal Cycler (Arktik, Thermoscientific, Germany).

Agarose Gel Electrophoresis

PCR products were electrophoresed on a 1.5% agarose gel in TBE buffer (Thermo Scientific, Vilnius, Lithuania) containing 0.5 µ/ml of ethidium bromide at 100 V for 45 min and visualized under UV light.

RESULTS

In this study, 152 abomasal contents of the aborted fetuses were sent to Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, from the provinces of Central Anatolia, and Black Sea Region of Turkey. Out of 152 abomasal contents, 11 (7.23%) were determined as *C. burnetii* positive. Four (3.92%) out of 102 cattle, 5 (11.11 %) out of 45 sheep, and 2 (40%) out of 5 goat abomasal contents were found to harbor *C. burnetii* DNA *(Table 2)*. Of the determined *C. burnetii* positive samples, 2 positivity each belonging to 1 cattle and 1 goat were detected in the samples sent from the provinces of Black Sea Region, Bartın and Bolu, respectively *(Table 3)*. Nine out of 11 positivity were detected in abomasal contents of the aborted fetuses delivered to the laboratory from provinces of Central Anatolia Region. Four (1 sheep and 3 cattle) out of 11 *C. burnetii* positive abomasal contents were from Çankırı. Two out of 11 positivity were determined in the abomasal contents of aborted sheep fetuses from Ankara. The other 2 sheep abomasal contents harboring *C. burnetii* DNA were from Eskişehir and Yozgat. The last positive sample belonged to a goat from Çorum *(Table 3)*. Remaining 141 (92.76%) abomasal contents were found to be negative for *C. burnetii (Table 2)*.

DISCUSSION

A few serological surveys investigating seroprevalence of Q fever in human, sheep, cattle, and goat were implemented, but PCR-based studies were limited in Turkey [17-21]. There were few PCR-based studies, conducted on detection of *C. burnetii* in animal clinical specimens such as blood and milk. Kirkan et al.[22] detected *C. burnetii* DNA in 6 of 138 cattle blood samples by PCR. Ongor et al.[23] found that, of the examined 400 sheep milk samples, 14 (3-5 %) were *C. burnetii* positive by IMS-PCR. Dogru et al.[24] could not detect any *C. burnetii* DNA in milk samples belonging to seropositive cattle and sheep. To our knowledge, till now, neither vaginal, placental, birth fluids nor abomasal contents of aborted fetuses had been investigated for the presence of *C. burnetii* by PCR in Turkey. Nevertheless, other researchers from Turkey investigated *C. burnetii* DNA in the abomasal contents of aborted ruminant fetuses in a study conducted in Cyprus [25]. To sum up, this study was the first for the detection of *C. burnetii* from abomasal contents of aborted fetuses from ruminants by Trans-PCR in Turkey.

Table 2. *The results of the examined samples for C. burnetii by Trans-PCR*
Tablo 2. *Trans-PCR ile C. burnetii yönünden incelenen örneklerin sonuçları*

Sample Type	Number of examined Sample	Number of Positive Samples (%)	Number of Negative Samples (%)
Cattle	102	4 (3.92%)	98 (96.07%)
Sheep	45	5 (11.11%)	40 (88.88%)
Goat	5	2 (40%)	3 (60.00%)
Total	152	11(7.23%)	141(92.76%)

Table 3. *Distribution of C. burnetii positive samples among ruminants and provinces*
Tablo 3. *C. burnetii pozitif örneklerin ruminantlar ve iller arasında dağılımı*

Provinces	Cattle	Sheep	Goat
Ankara	-	2	-
Yozgat	-	1	-
Eskişehir	-	1	-
Çankırı	3	1	-
Çorum	-	-	1
Bartın	1	-	-
Bolu	-	-	1
Total	4	5	2

Various clinical samples such as milk [26], feces [7], urine [27], abomasal contents [25,28], vaginal mucus [29], blood [22,30], serum [31] and semen [32] are used for PCR investigation of *C. burnetii* DNA. *C. burnetii* in veterinary matrices are found in birth materials, like amniotic fluids and placentas [33,34], and in lower quantities in milk [35] and blood [9].

Before sampling, the stage of the infection and the sample type has to be well decided. In abortion, infection in placenta could spread to the fetus by the amniotic-oral route. This occurs after the penetration of placenta when the bacteria contaminate the amniotic fluid and become aspirated/swallowed by the fetus. Haematogenous spread through the umbilical vessels could be another cause of bacterial presence in fetuses and the following abortion [36].

Therefore, in this study, abomasal contents of 152 aborted fetuses comprising sheep, cattle, and goat were preferred as the material. And also, the superiority of the abomasal contents is due to comprising all of the vaginal, placental and even birth fluids [28]. Solely abomasal contents rather than other clinical samples (vaginal swabs, placental tissues, fetal lung, fetal liver, etc.) were sampled and PCR investigated in this study, therefore we cannot comment on possible presence of the agent in other tissues or samples. Similar to our study, in a study conducted in Cyprus, the researchers collected both abomasal contents and placental cotyledons from the aborted ruminants representing cattle, goat, sheep and performed trans-PCR and CB-PCR [25]. The positivity of 32.2% from placental cotyledons and 37% from abomasal contents were found by both trans-PCR and CB-PCR. The difference was attributed to the contamination of placental tissues (cotyledons) with fecal material during abortion and the positive results by both trans-PCR and CB-PCR from abomasal contents were accepted for the final decision. Parallel to our study, Dehkordi et al.[28], preferred the abomasal contents from the aborted fetuses in order to diagnose Q fever since, those were not depend on the sampling time and also as aforementioned, abomasal contents was declared harboring all of the vaginal, placental and even birth fluids. The same authors found 98 (12.53%) and 122 (16.39%) out of 782 and 744 ovine and caprine aborted fetuses were positive for the presence of *C. burnetii* by nested PCR with com1 primers, respectively. They analyzed the same samples by real-time PCR and showed that real-time PCR with trans-F and trans-R primers was 1.5 times sensitive than nested PCR [28].

The epidemiology of Q fever in Turkey is essentially unknown and to the authors' knowledge, the prevalence rate of *C. burnetii* in ruminant's aborted fetuses in Turkey has not been reported yet. Although our study was not a prevalence study, we compared Q fever positive results from the abomasal contents of aborted fetuses detected by trans-PCR with the results of similar studies from the aborted fetuses. The prevalence rate of *C. burnetii* in aborted sheep in this study in Turkey (8.88%) was found to be lower than Iran (15.47%) [28], Italy (10%) [34], Netherlands (80%) [37], Cyprus (33%) [25] and approximately the same with northern Spain (9%) [38]. While the prevalence rate of *C. burnetii* in aborted goat fetuses in this study (40%) was higher than Iran (20.43%) [28], Italy (21.5%) [39], United Kingdom (25%) [40], it was found to be lower than Netherlands (up to 80%) [37], Cyprus (50%) [25]. *C. burnetii* in aborted cattle fetuses in this study (3.92%) was found to be lower than Italy (11.6%) [39] and Cyprus (35%) [25].

In our study, 2 out of 11 positivity were determined from Bartın (1 cattle) and Bolu (1 goat), the provinces of Black Sea Region who has a Q fever history among the human. In 2002, in the Black Sea Region of Turkey, Gozalan et al.[19], declared the human Q fever outbreak and attributed the outbreak to sheep and goat parturitions. Nine out of 11 positivities were detected from provinces of Central Anatolia Region. Although Q fever is commonly seen in the mountainous regions whose climate is tropical, Bartin and Bolu, located in west Black Sea Region have a similar climatic features such as rainy-moderate in the coastal area, dry-continental inward of the mountains and geographical features such as semi-mountainous. The climatic and geographic feature of Central Anatolia Region is continental and flat, respectively. Therefore, 11 positivity were attributed to resistance of *C. burnetii* spores to environmental conditions such as dry, harsh weather and dry atmosphere might enhance the dispersion of aerosols in those regions [41].

As a conclusion, to our knowledge this is the first study on molecular investigation of *C. burnetii* presence in stomach contents of aborted fetuses of ruminants in Turkey to date. We recommend fresh and aseptically sampled fetal stomach contents as the most appropriate materials to investigate either presence or prevalence of abortifacient pathogens, since other materials like placenta, amniotic fluid, etc. are prone to contamination by both genital flora bacteria and environmental bacteria which can lead to misidentification of causative bacteria. As previously stated by Sargison [42], amniotic fluid and fetal stomach content of healthy ewes and fetuses are sterile, so identification of bacteria in smears or culture of stomach contents of recently aborted fetuses indicates placental infection. Thus, PCR detection of *C. burnetii* DNA in fetal abomasum contents is a significant finding of a possible *C. burnetii* related abortion.

For the further studies, the relationship among the *C. burnetii* isolates detected in different geographical regions and/or the necessity of the epidemiological studies depending on the circulation of *C. burnetii* isolates within different ruminants in different and/or the same geographic regions should be taken into consideration.

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Research Article

Koyun ve Keçilerde Bulaşıcı Agalaksi Hastalığının Bakteriyolojik ve PCR Metotları ile Araştırılması [1]

Hüban GÖÇMEN [1] Mihriban ÜLGEN [2] K.Tayfun ÇARLI [2] Kaan ÖNAT [3]
Serpil KAHYA [2] Ümit ÖZDEMİR [4] Burak MAT [5]

[1] *Bu çalışma Uludağ Üniversitesi Bilimsel Araştırma Projeleri Birimi tarafından desteklenmiştir (Proje No: 2011/57)*
[1] Uludağ Üniversitesi Sağlık Bilimleri Enstitüsü, Veteriner-Mikrobiyoloji Anabilim Dalı, TR -16059 Nilüfer, Bursa - TÜRKİYE
[2] Uludağ Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı, TR-16059 Nilüfer, Bursa - TÜRKİYE
[3] Manyas İlçe Gıda Tarım ve Hayvancılık Müdürlüğü, TR- 10470 Manyas, Balıkesir - TÜRKİYE
[4] Pendik Veteriner Kontrol ve Araştırma Enstitüsü, TR-34890 Pendik, İstanbul - TÜRKİYE
[5] Selçuk Üniversitesi Veteriner Fakültesi Hayvancılık Ekonomisi ve İşletmeciliği Anabilim Dalı, TR- 42003 Selçuklu, Konya - TÜRKİYE

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Özet

Bu çalışmada, koyun ve keçilerde Bulaşıcı Agalaksi hastalığının varlığını bakteriyolojik ve moleküler yöntemler ile teşhis etmek amaçlandı. Bursa, Balıkesir, Çanakkale ve Edirne illerine ait koyun ve keçilerden toplanan 339 adet örnek bakteriyolojik ve moleküler yöntemlerle incelendi. Örneklerin 162 adedini süt örneği, 147 adedini göz svabı, 15 adedini eklem sıvısı, 11 adedini burun svabı ve 4 adedini de akciğer dokusu oluşturmuştur. Bakteriyolojik incelemede 29 izolat *Mycoplasma* sp. olarak değerlendirildi. Uygulanan biyokimyasal testler ve üreme inhibisyon testleri sonucunda, 29 (%8.55) izolatın 25'i (%7.37) Mycoplasma agalactiae olarak, 2 (%0.58)'si *Mycoplasma ovipneumoniae* ve 2 (%0.58)'si de *Mycoplasma arginini* olarak identifiye edildi. Moleküler teşhiste ise, *pol*C-PCR sonucunda %9.14 oranında *M. agalactiae* pozitif bulundu. PCR bulguları ile bakteriyolojik bulgular karşılaştırıldığında, 5 süt örneği ve 1 akciğer örneği *pol*C-PCR ile *M. agalactiae* pozitif bulunurken, kültür ile negatif bulundu. *Pol*C-PCR sonuçlarına göre, süt örnekleri %14.19 oranı ile, eklem sıvı örnekleri %13.33 oranı ile, göz svabı örnekleri %2.72 oranı ile ve akciğer örnekleri %50 oranı ile pozitif bulunurken, burun svabı örnekleri negatif bulundu. Bu çalışmada, Bulaşıcı Agalaksi hastalığının varlığı bakteriyolojik ve moleküler yöntemler ile araştırılmış ve başlıca hastalığa neden olan etkenin *M. agalactiae* olduğu tespit edilmiştir, hastalığa neden olan diğer mikoplazma etkenlerine rastlanılmamıştır.

Anahtar sözcükler: *Bulaşıcı Agalaksi, Mycoplasma agalactiae, Bakteriyoloji, PCR, Koyun, Keçi*

Investigation of Contagious Agalactia by Bacteriological and PCR Methods in Sheep and Goats

Abstract

The aim of this study was diagnosis that occurrence of Contagious Agalactia by bacteriological and molecular methods in sheep and goats. A total of 339 samples from sheep and goats in Bursa, Balıkesir, Çanakkale and Edirne provinces were examined by bacteriological and molecular methods. The samples were 162 milk samples,147 eye swabs, 15 joint fluids, 11 nasal swabs and 4 lung tissue. In bacteriological examination, 29 isolates were evaluated as *Mycoplasma* sp.. As a result of biochemical tests and growth inhibition tests, 29 (8.55%) *Mycoplasma* sp. were identified as 25 (7.37%) *Mycoplasma agalactiae*, 2 (0.58%) *Mycoplasma ovipneumoniae* and 2 (0.58%) *Mycoplasma arginini.* In molecular diagnosis, *pol*C gene-PCR results could be detected *M. agalactiae* positive with 9.14% rate. As a result of this, 5 milk samples and 1 lung tissue sample were detected positive by *pol*C-PCR while negative by bacteriological examination. The results of *pol*C-PCR detected *M. agalactiae* positive with 14.19% rate of milk samples, 13.33% rate of joint fluids, 2.72% rate of eye swabs and 50% rate of lung tissue samples but nasal swabs were detected as negative. In this study, presence of Contagious Agalactia were investigated by bacteriological and molecular methods and *M. agalactiae* was detected as a main agent which cause disease however other *Mycoplasma* species which cause disease were not observed.

Keywords: *Contagious Agalactia, Mycoplasma agalactiae, Bacteriology, PCR, Sheep, Goat*

İletişim (Correspondence)
+90 224 2940818
hubangocmen@gmail.com

GİRİŞ

Bulaşıcı Agalaksi hastalığı halk arasında 'süt kesen hastalığı' olarak bilinmektedir. Koyun ve keçilerde mastitis, süt kesilmesi, keratokonjunktivitis, artritis gibi tipik semptomlara sahiptir ve abort, genital lezyonlar, solunum sistemi rahatsızlıkları gibi atipik bulgularla da karşımıza çıkabilmektedir. Hastalığın başlıca etkeni *Mycoplasma agalactiae* (Ma) olmakla birlikte *Mycoplasma capricolum* subsp. *capricolum* (Mcc), *Mycoplasma mycoides* subsp. *capri* (Mmc) ve *Mycoplasma putrefaciens* (Mp) türleri de hastalığa neden olmaktadır. Bulaşıcı agalaksi hastalığı, Dünya Hayvan Sağlığı Örgütü'nün (OIE) bildirilmesi zorunlu hastalıklar listesinde yer almaktadır [1]. Bulaşıcı agalaksi, hayvanlarda süt üretiminde azalmaya, genç hayvanlarda ölüme, gebelerde abortuslara neden olduğu için önemli ekonomik kayıplar meydana getirmektedir [2,3]. Bulaşıcı Agalaksi ile infekte hayvanlardan etken başlıca süt, göz-burun akıntısı, açılmış eklemlerin akıntıları ile saçılmakta ve ayrıca dışkı, idrar ve genital sistem akıntıları da etken kaynağı olabilmektedir. Etken, klinik belirtiler ortadan kalkana kadar sütle minimum 12 ay saçılmakta, hayvanların klinik olarak iyileşmesinden sonra da etken bir yıldan fazla vücutta kalabilmektedir. Sürülerde böyle asemptomatik taşıyıcıların bulunması ciddi bir risk olarak görülmektedir. Bu asemptomatik hayvanlar etkeni genellikle dişilerde erkeklerden daha fazla olmak üzere genital yollarda ve daha az sıklıkla da dış kulak kanalında taşımaktadırlar. Bu olağan dışı bölgelerde konakçı savunma mekanizması iyi çalışmadığı için etkene avantaj sağlamaktadır. Gerek infekte gerekse taşıyıcı hayvanlardan duyarlı hayvanlara etken meme, konjuktiva, sindirim, solunum, genital ve deri gibi birçok yolla bulaşabilmektedir. Özellikle meme, sindirim ve solunum en önemli bulaşma yollarıdır. İntensif yetiştiricilik yapılan işletmelerde solunum semptomları ile birlikte damlacık infeksiyonu daha fazla önem kazanmaktadır [4-7] .

Bulaşıcı Agalaksi hastalığının laboratuvar teşhisinde infekte hayvanlardan alınan süt örneği, göz, kulak ve burun svapları, eklem sıvısı örneklerinin; bakteriyolojik, serolojik ve moleküler metodlar ile incelenmesi sonucu hastalığa neden olan etkenler ortaya konulmaktadır. Türkiye'de mikoplazmaların enfekte hayvanlardan izolasyonu ile ilgili birçok çalışma mevcut olmasına rağmen [8-12], Bulaşıcı Agalaksi Hastalığı üzerindeki çalışma sayısı sınırlıdır [13-15]. Çetinkaya ve ark.[13], Doğu Anadolu bölgesinde yaptıkları çalışmada Bulaşıcı Agalaksi hastalığı etkeni *M. agalactiae*'nın yaygınlığını kültür ve PCR metodları ile %81.7 olarak bulduklarını belirtmişlerdir. Özdemir ve Türkaslan [14] ise Marmara, Ege ve Akdeniz bölgelerinde Bulaşıcı Agalaksi salgınlarından *M. agalactiae*'yı %36.8 oranında izole etmişler ve ayrıca Bulaşıcı Agalaksi hastalığına sebep olan diğer türlerden Mmc (%4.16) ve Mcc (%3.47)'un da düşük oranlarda izole edildiğini bildirmişlerdir.

Bu çalışmada, Marmara bölgesinde Bulaşıcı Agalaksi hastalığının varlığını bakteriyolojik ve moleküler yöntemler ile araştırmak ve hastalığa neden olan mikoplazma türlerinin saha suşlarını ortaya koymak amaçlanmıştır.

MATERYAL ve METOT

Materyal Toplanması ve Örnekleme

Sunulan çalışma sırasında hayvanlardan alınan süt, eklem sıvısı ve göz ve burun svap örnekleri için gerekli izin Uludağ Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından 10.05.2011 tarihli ve 2011-05/03 no'lu karar ile onaylandı.

2010 ve 2012 yılları arasında Bursa, Balıkesir, Çanakkale ve Edirne illerinde Bulaşıcı Agalaksi semptomları gösteren ve semptom göstermediği halde anemneze göre şüphe edilen koyun ve keçilerden 335 adet örnek toplandı. Ayrıca Uludağ Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı laboratuvarına gönderilen pnömoni bulgusu taşıyan 4 adet akciğer örneği de incelemeye alındı. Buna göre toplam 339 örneğin; 190'ı keçi, 57'si koyun, 19'u oğlak, 6'sı teke, 27'si kuzu ve 6'sı koç'a ait numunelerdir. Toplanan örneklerin 162 adedi süt, 147'si göz svabı, 11'i burun svabı, 15'i eklem sıvısı ve 4 adedi ise akciğer örneğidir *(Tablo 1)*. Tüm örnekler asepsi antisepsi kurallarına uyularak alındı ve soğuk zincirde laboratuvara ulaştırıldı.

Bakteriyolojik İncelemeler

İzolasyon amacıyla laboratuvara getirilen süt örnekleri 3.000 rpm'de 15 dk santrifüj edildikten sonra üst sıvı besiyerine ekim için kullanıldı. Süt örnekleri, eklem sıvıları 10^{-1}'lik dilüsyonları hazırlanarak, akciğer örnekleri, göz ve burun svapları ise direkt olarak sıvı ve katı besiyerlerine ekildi. Katı besiyeri olarak Mycoplasma Selective Supplement (Oxoid SR0059) eklenmiş Mycoplasma Agar Base (Oxoid CM0401), sıvı besiyeri olarak ise yine Mycoplasma Selective Supplement eklenmiş Mycoplasma Broth Base (Oxoid CM0403) kullanıldı. İnokule edilen sıvı besiyerleri ve agarlar 37°C'de %5 CO_2'li nemli ortamda 5-7 gün inkübe edildi. Bu süre sonunda stereomikroskopta x 35 büyütme ile incelenen katı besiyerlerinde mikoplazma şüpheli koloniler 3 kez pasajlandı ve ayrıca L formlarından ayırımı amacıyla kanlı agara (%5 koyun kanlı) pasajlandı [14].

Mycoplasma sp. şüpheli izolatları *Acholeplasma* sp.'den ayırmak için dijitonin duyarlılık testi, *Ureaplasma* sp.'den ayırmak için de üreaz testi yapıldı. Üç kez pasajlanarak saf kültürü hazırlanan ve *Mycoplasma* sp. olarak değerlendirilen izolatların tür identifikasyonu için glikoz fermentasyonu, arjinin hidrolizi, fosfataz aktivitesi, tetrazolium redüksiyon testleri ve film ve spot oluşumu gibi biyokimyasal testler ve ayrıca Üreme İnhibisyon testi uygulandı [1]. Üreme İnhibisyon testinde kullanılan Ma, Mmc, Mcc, Mp antiserumları Pendik Veteriner Kontrol ve Araştırma Enstitüsü'nden sağlandı.

Moleküler İncelemeler

DNA Ekstraksiyonunda Roche High Pure PCR Template Preparation Kit, DNA Ekstraksiyon Kiti olarak kullanıldı. Kitin kullanma kılavuzunda belirtilen sıvı kültür ve örneklerden DNA ekstraksiyon yöntemi prosedürlerine göre *M. agalactiae* AIK suşunun DNA'sı ve direk olarak 339 adet şüpheli örnekten etken DNA'ları ekstrakte edildi. Şüpheli örnekler, PCR metodu ile sadece *M. agalactiae* yönünden test edildi.

Mycoplasma agalactiae spesifik primerleri, *pol*C geninden (MAPol-1F: 5-CAT TGA ACC TCT TAT GTC ATT TAC TTT G-3; MAPol-5R: 5-CTA TGT CAT CAG CTT TTG GGT GA-3) seçildi ve 265 baz çifti büyüklüğündeki ürünlerin amplifikasyonu hedeflendi. Toplam 25 µl'lik miktarlarda hazırlanan reaksiyon için; 2.5 µl 10X PCR Reaksiyon Buffer, 5 U/ µl *Taq* DNA polimeraz, dNTP'ler (300 µM dATP, dTTP; 150 µM dGTP, dCTP), 2 mM $MgCl_2$, 10 pmol primerlerden konuldu [17]. Tüm reaktifler Roche FastStart *Taq* DNA Polymerase, dNTPack paketinden kullanıldı. Negatif kontrol olarak *M. putrefaciens* (NCTC 10155) ve deiyonize su, pozitif kontrol olarak Pendik Veteriner Kontrol ve Araştırma Enstitüsü'nden sağlanan *M. agalactiae* AIK suşunun ekstrakte edilen DNA'ları kullanıldı. Tüm örneklerin amplifikasyonu Gradient Thermal cycler (Techne TC-3000G, Bibby Scientific, UK) cihazında gerçekleştirildi.

Reaksiyonda kullanılan DNA amplifikasyon parametreleri ise; 94°C'de 2 dakika ön ısıtmayı takiben 94°C'de 30 saniye denatürasyon, 49°C'de 30 saniye bağlanma, 72°C'de 30 saniye uzama (sentez) olmak üzere 30 döngü olarak gerçekleştirildi ve son sentezleme 72°C'de 5 dk. olarak tamamlandı [17]. Elde edilen PCR amplikonları, %2 agaroz jel kullanılarak 110 voltta 60 dk elektroforezde (Thermo Scientific Owl Easycast B2) yürütüldü. Süre sonunda agaroz jel, etidyum bromür ile 15 dk süre ile boyandı ve Vilber Lourmat Quantum 1100 marka Jel Görüntüleme ve Dökümantasyon Sistemi ile beyaz ışık ve UV ışığı altında DNA'ların görüntülenmesi sağlandı ve kayıtları alındı.

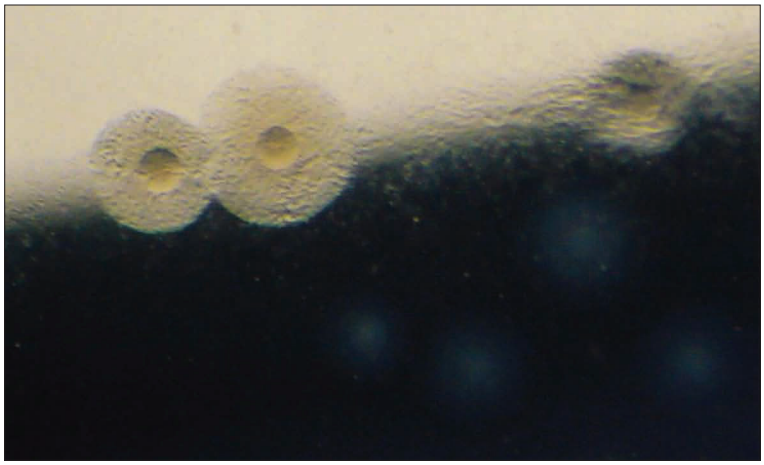
BULGULAR

Bakteriyolojik Bulgular

Toplanan 339 örneğin 29 (%8.55)'undan *Mycoplasma* sp. izole edildi. Mikoplazma kolonilerinin stereomikroskopta (x35) tipik sahanda yumurta görünümünde ortası düğmeli, kenarları yuvarlak ve küçük oldukları görüldü *(Şekil 1)*. Yirmidokuz *Mycoplasma* sp.'nin 18 (%11.11)'i süt örneğinden, 4 (%2.72)'ü göz svablarından, 2 (%13.33)'si eklem sıvılarından, 3 (%27.27)'ü burun svablarından ve 2 (%50)'si akciğer örneklerinden izole edildi *(Tablo 1)*.

Şekil 1. *Mycoplasma* sp.'nin koloni morfolojisi

Fig 1. Colony morphology of *Mycoplasma* sp.

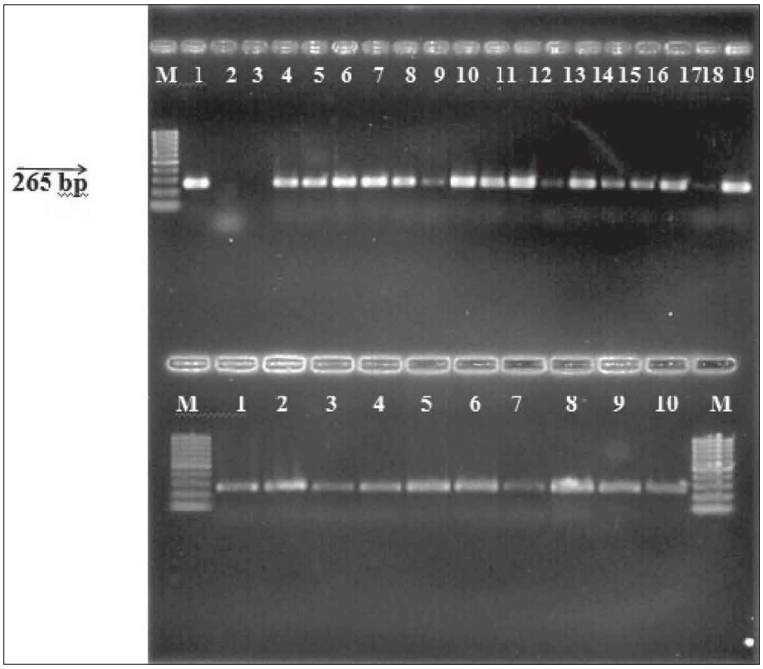


Tablo 1. *Koyun ve keçilerden toplanan örneklerden elde edilen bakteriyolojik bulgular ve M. agalactiae polC-PCR sonuçları*
Table 1. *Bacteriological findings and M. agalactiae-polC-PCR results obtained from samples of goats and sheep*

Örnek Türü	Örnek Sayısı	*Mycoplasma* sp.-Bakteriyolojik Bulgular		*M. agalactiae* - Kültür Bulguları		*pol*C-PCR Bulguları	
		+ Örnek Sayısı	Oran (%)	+ Örnek Sayısı	Oran (%)	+ Örnek Sayısı	Oran (%)
Süt	162	18	11.11	18	11.11	23	14.19
Göz svabı	147	4	2.72	4	2.72	4	2.72
Eklem sıvısı	15	2	13.33	2	13.33	2	13.33
Burun svabı	11	3	27.27	-	-	-	-
Akciğer	4	2	50	1	25	2	50
Toplam	339	29	8.55	25	7.37	31	9.14

Tablo 2. *İzole edilen mikoplazma suşlarının biyokimyasal özellikleri*
Table 2. *Biochemical properties of mycoplasma strains as isolated*

İzole Edilen Suşlar (N)	Glikoz Fermentasyonu	Arjinin Hidrolizi	Fosfataz Aktivitesi	Tetrazolium Redüksiyonu (aerob/anaerob)	Film/Spot Oluşumu
M. agalactiae (25)	-	-	+	+/+	Değişken
M. ovipneumoniae (2)	+	-	-	+/+	-
M. arginini (2)	-	+	-	-/+	-



Şekil 2. *M. agalactiae polC*-PCR ürünlerinin agaroz jel elektroforez (%2) görüntüsü. Üst sıradan M: Markır (100 baz çifti) (Thermo Scientific, SM0242), 1. kuyucuk: *M. agalactiae* pozitif kontrol (AIK suşu, Pendik Veteriner Kontrol ve Araştırma Enstitüsü), 2. kuyucuk: negatif kontrol-(*M. putrefaciens*-NCTC 10155 suşu), 3. kuyucuk: negatif kontrol (deiyonize su), 4-19 arası kuyucuklar: pozitif örnekler. Alt sıradan; M: Markır, 1-10 arası kuyucuklar; pozitif örnekler

Fig 2. Agarose gel electrophoresis (2%) image of *M. agalactiae polC*-PCR products. From top; M: Marker (100 base pair) (Thermo Scientific, SM0242), Lane 1: *M. agalactiae* positive control (AIK strain from Pendik Veterinary Control Institute), Lane: 2 negative control: (*M. putrefaciens*-NCTC 10155 strain), Lane 3: negative control (PCR grade), Lane 4 to 19: positive samples. From bottom; M: Marker, Lane 1 to 10; positive samples

Biyokimyasal testler ve Üreme İnhibisyon testi sonucunda izolatların 25 (%7.37)'i *M. agalactiae* olarak identifiye edildi. Ayrıca sadece biyokimyasal test sonuçlarına göre Bulaşıcı Agalaksi hastalığı etkeni olmayan 2 izolat *M. ovipneumoniae* ve diğer 2 izolat da *M. arginini* olarak identifiye edildi *(Tablo 2)*. *M. agalactiae* izolatlarının 18'i süt örneğinden, 2'si eklem sıvısı örneğinden, 1'i akciğer örneğinden ve 4'ü de göz svabından izole edildi. *M. ovipneumoniae* izolatlarının 1'i akciğer örneğinden ve 1'i burun svabından izole edilirken; *M. arginini* izolatlarının 2'si de burun svabından izole edildi *(Tablo 1)*.

Polimeraz Zincir Reaksiyonu (PCR) Bulguları

*Pol*C-PCR sonucunda %9.14 oranında *M. agalactiae* pozitif bulundu *(Şekil 2)*. *Pol*C -PCR sonuçlarına göre, 162 süt örneğinden 23 (%14.19)'ü, 15 eklem sıvı örneğinden 2 (%13.33)'si, 147 göz svabı örneğinden 4 (%2.72)'ü ve 4 akciğer örneğinden 2 (%50)'si *M. agalactiae* pozitif bulundu. Şüpheli örneklerin amplifiye DNA'larına uygulanan PCR sonucunda, PCR ürünleri 265 baz çifti olarak hesaplandı ve *M. agalactiae* pozitif kabul edildi. Burun svabı örneklerinden *polC*-PCR ile *M. agalactiae* saptanmadı. PCR bulguları ile bakteriyolojik bulgular karşılaştırıldığında, 5 süt örneği ve 1 akciğer örneği *polC*-PCR ile Ma pozitif bulunurken, kültür ile negatif bulundu *(Tablo 1)*.

TARTIŞMA ve SONUÇ

Bulaşıcı agalaksi, hayvanlarda süt üretiminde azalmaya, genç hayvanlarda ölüme, gebelerde abortuslara neden olarak önemli ekonomik kayıplar meydana getirir [2,3]. Bulaşıcı Agalaksi, Akdeniz ülkeleri, Asya ve Afrika'da endemik, Amerika'da sporadik olarak seyretmekte ülkemizde ise endemilerle kendini göstermektedir. Abtin ve ark.[18], İran'da Bulaşıcı Agalaksi semptomları gösteren hayvanlardan topladıkları örnekleri bakteriyolojik ve PCR metodları ile incelemiş ve sonucunda 102 örneğin 19 (%32.2)'unda *M. agalactiae* identifiye etmişlerdir. De La Fe ve ark.[19] ise, İspanya'da süt, eklem ve kulak svabı örnekleri topladıkları 28 adet keçi sürüsünün %40'ında *M. agalactiae* saptamışlardır.

Türkiye'de en güncel koyun ve keçi mikoplazmaları üzerine araştırmaları Çetinkaya ve ark.[12], öncelikle Keçilerin Bulaşıcı Plöropnömonisi hastalığının etkeni olan *Mycoplasma capricolum* subsp. *capripneumoniae* 'nin Türkiye'deki yaygınlığı üzerine yapmıştır. Aynı araştırıcılar daha sonra mikoplazma aşı stratejileri geliştirmek amacıyla Bulaşıcı Agalaksi hastalık etkenlerinin Doğu Anadolu bölgesindeki illerde yaygınlığını araştırmışlardır. Süt örneklerinin bakteriyolojik ve Ma spesifik PCR

ile incelenmesi neticesinde %81.7'sinde *M. agalactiae* saptamışlardır [13]. Özdemir ve Türkaslan [14] ise yaptıkları çalışmada, Bulaşıcı Agalaksi'den şüpheli 22 adet koyun ve keçi sürüsünden toplam 144 örnek üzerinde çalışmışlar ve 53 (%36.8) örnekten *M. agalactiae,* 6 örnekten Mmc (%4.16) ve 5 örnekten de Mcc (%3.47) izole edildiğini bildirmişlerdir. Ayrıca coğrafik olarak, Marmara bölgesinde hastalığın Ege ve Akdeniz bölgelerine göre daha yoğun olduğu da aynı çalışmada vurgulanmıştır. Bu çalışmada ise Marmara bölgesindeki çeşitli illerden toplanan 339 örnekten 25 (%7.37) *M. agalactiae*, 2 (%0.58) *M. ovipneumoniae* ve 2 (%0.58) *M. arginini* izole edilmiştir. Buna göre Marmara Bölgesindeki koyun ve keçilerde Bulaşıcı Agalaksi hastalığına neden olan mikoplazma türü olarak *M. agalactiae* belirlenmiş, Bulaşıcı Agalaksi'nin diğer etkenleri olan Mmc, Mcc ve Mp türlerine rastlanmamıştır. Koyun ve keçilerde yapılan çalışmalarda Bulaşıcı Agalaksi Hastalığına neden olan mikoplazma türleri dışında, *M. ovipneumoniae* ve *M. arginini* türleri de dikkat çekmektedir [10,20-22]. Türkiye'de Bulaşıcı Agalaksi hastalığına yönelik spesifik çalışma sayısı çok az olmakla birlikte mevcut çalışmalar ile karşılaştırıldığında çalışmamızdaki etken izolasyon oranının düşük olduğu görülmektedir. Bunun nedeni örnekleme zamanı veya sürülerde uygulanan tedaviler olabilir [9]. Nitekim sürü sahiplerinden sürülerde uygulanan antibiyotik tedavileri ile ilgili net yanıtlar alınamamıştır.

Mikoplazmaların identifikasyonunda glikoz fermentasyonu, arjinin hidrolizi, fosfataz aktivitesi ve tetrazolium redüksiyonu gibi biyokimyasal testlerden yararlanılır [7,23-25]. Bu çalışmada izole edilen mikoplazma suşları digitonin duyarlılığı, glikoz fermentasyonu, arjinin hidrolizi, fosfataz aktivitesi, tetrazolium redüksiyonu, film ve spot oluşumu özellikleri yönünden incelenmiş. Tüm *Mycoplasma* sp. izolatlarının digitonine duyarlılığı olduğu ve üreaz aktivitesi testinin negatif olduğu belirlenmiştir. *M. agalactiae* izolatlarının hepsinin glikoz ve arjinin testlerine negatif, fosfataz ve tetrazolium testlerine pozitif yanıt verdiği ancak film ve spot oluşumunda değişkenlik olduğu saptanmıştır. Çalışmamızda uygulanan Üreme İnhibisyon testinde, 25 izolatın üremesi *M. agalactiae* antiserumları ile inhibe olmuş ve inhibisyon alanları net olarak gözlenmiştir. Çalışmalarımız sonucunda identifiye edilen *M. agalactiae* saha suşlarının literatürlerde belirtilen biyokimyasal özellikleri ile uyumluluk gösterdiği, atipik biyokimyasal veri gösteren izolat bulunmadığı gözlenmiştir [5,6].

Bulaşıcı Agalaksi hastalığına neden olan *M. agalactiae* etkeninin bakteriyolojik ve serolojik yöntemlerle izolasyon ve identifikasyonu zaman alıcı prosedürler olduğu için daha hızlı teşhis ve identifikasyon aracı olan moleküler metotlara başvurulmaktadır. Özellikle mikoplazmaların kültürde nazlı ve yavaş üremeleri, serolojik teşhislerde en erken infeksiyondan 10-14 gün sonra sonuç vermesi, kronik vakalarda yetersiz sensitivite ile karşılaşılması ve yanlış pozitifliklerin meydana gelmesi, immunosupresyon ve antibiyotik tedavileri geleneksel diyagnostik mikrobiyolojik analizlerde önemli sorunları ortaya çıkartmaktadır.

Mikoplazmaların hızlı teşhisi ve identifikasyonu için PCR tabanlı teşhis analizleri geliştirilmiş olup, amplifikasyon analizlerinde hedef gen, hem korunan hem de değişken sekansları içeren 16 S rRNA (*rrs*) genleri olmuştur [26]. PCR ile yakın türler arasında ya da alt tipler arasında ayrım yapılabilir ve direkt klinik örneklerden hızlı teşhis mümkün olmaktadır. PCR metodunda örneklerin kültürlerinden ya da direkt klinik örnekten DNA ekstraksiyonları yapılabilir. Tola ve ark.[27] direkt koyun sütlerinden guanidium thiocyanate [28] ekstraksiyon metodunu uygulamış ve 357 örnekten 153'ünde *M. agalactiae*-PCR pozitif bulmuştur. Direkt ekstraksiyon yöntemi kullanılan diğer bir çalışmada 58 koyun ve keçi süt örneğinde %81.7 oranı ile *M. agalactiae* identifiye edilmiştir ve kültür sonuçları ile uyumlu sonuç alınmıştır [13]. Çalışmamızda ise direkt örneklerden uygulanan ekstraksiyon metodu sonucunda elde edilen PCR pozitiflik oranı diğer çalışmalara göre daha düşüktür. Ancak çalışmamızda kültür sonuçları (%7.37) ile karşılaştırıldığında PCR da elde edilen *M. agalactiae* pozitiflik oranının (%9.14) yüksek olduğu gözlenmiştir.

M. agalactiae'nın moleküler teşhisinde kullanılan farklı hedef gen bölgelerini baz alan spesifik primerler ile PCR metotları uygulanmıştır [12,29,30]. Marenda ve ark.[17] yaptığı çalışma ile *uvr*C-PCR amplifikasyonunda bağlanma sıcaklıklarındaki farklılıkların yanında her iki metotta da *M. agalactiae* ve *M. bovis* suşları arasındaki filogenetik yakınlığa rağmen aynı duyarlıklıkta saptama oranını sağlayabilmişlerdir. Fakat standart şartlar altındaki laboratuvarlar arasında uygulanan *uvr*C-PCR metodunun sonuçları uyumluluk göstermezken, *pol*C-PCR metodunun sonuçları açık ve kesin şekilde uyumluluk göstermiştir. Yaptığımız çalışmada, *M. agalactiae pol*C geni ile yapılan PCR sonucunda %9.14 oranında *M. agalactiae* pozitif bulunmuştur.

Sonuç olarak bu çalışmada, Bulaşıcı Agalaksi hastalığının varlığı bakteriyolojik ve moleküler yöntemler ile araştırılmış ve bölgemizde hastalığa neden olan başlıca etkenin *M. agalactiae* olduğu saptanmıştır. Koyun ve keçilerden toplanan 162 adet süt örneği, 147 adet göz ve 11 adet burun svabı, 15 adet eklem sıvısı ve 4 adet akciğer örnekleri olmak üzere toplam 339 örneğin 29'undan *Mycoplasma* sp. izole edilmiş ve izolatların 25 (%7.37)'i *M. agalactiae*, 2 (%0.58)'si *M. ovipneumoniae* ve 2 (%0.58)'si *M. arginini* olarak identifiye edilmiştir. PCR sonucunda ise *M. agalactiae*'nın *pol*C-PCR metodu uygulanarak %9.14 saptama oranı sağlanmıştır.

Türkiye'de Bulaşıcı Agalaksi hastalığına neden olan tüm etkenlerin yaygınlığı ve dağılımının tespit edilmesine, saha suşlarının ortaya konulmasına ve varsa diğer ülkelerdeki suşlar ile genetik yakınlıklarının belirlenmesine, aşıların bu yerel suşları içerecek şekilde üretilmesine yönelik çalışmalara ihtiyaç vardır.

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Research Article

The Effects of L-Ergothioneine, N-acetylcystein and Cystein on Freezing of Ram Semen [1][2]

Savaş YILDIZ [1] Yavuz ÖZTÜRKLER [1] Umut Çağın ARI [1]
Necdet Cankat LEHİMCİOĞLU [1] Emine ATAKİŞİ [2] Recai KULAKSIZ [1]

[1] *This study was supported by the TÜBİTAK-Tovag (Project No: 109O236)*
[2] *This study was presented as poster at the VII. National Reproduction and Artificial Insemination Science Congress on 1-4 July 2013, Kars, Turkey*
[1] Department of Artificial İnsemination, Faculty of Veterinary Medicine, University of Kafkas, TR36100 Kars - TURKEY
[2] Department of Biochemistry, Faculty of Veterinary Medicine, University of Kafkas, TR36100 Kars – TURKEY

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Abstract

The aim of this study was to determine the influence of different doses of L-Ergothioneine (LE), cystein and N-acetylcystein (NAC) on post-thawing semen parameters in rams. Ejaculates collected from four Tushin Rams were evaluated and pooled at 35°C. Semen samples were diluted with skim-milk based extender containing LE (5, 10 mM), Cystein (5, 10 mM), NAC (5, 10 mM) or not containing any antioxidant (control) and loaded to 0.25 ml French straws. Straws were cooled to 5°C for 2 h, frozen in liquid nitrogen vapour (aprox. -120°C) for 15 min and then being stored in liquid nitrogen until thawing process. Straws were thawed in water bath (37°C for 1 min). The percentages of motility, viability, abnormal acrosome, total abnormalities, membrane integrity (hypoosmatic swelling test, HOST) were statistically assessed. Also total antioxidant capacity (TAC) and total oxidatif stres (TOS) were evaluated in samples from replications. It was seen that LE was superior to N-acetylcystein and cystein in motility, viability, defected acrosome, total morphological abnormalities, HOST and TAC, ($P<0.05$) except cystein in motility. Neverthless, there was not any statistically difference between LE and control groups ($P>0.05$). It was conlcluded that there was not any beneficial or detrimental effects of LE on post thawing semen parameters in rams while it was determined that cystein and NAC may have been some detrimental effects on post thawing semen parameters in rams. These results warrants future scientific studies on LE, NAC and cystein in ram semen cryopreservation.

Keywords: *Ram semen, Cryopreservation, L-Ergothioneine, Cystein, N-acetylcystein, Tushin*

Koç Spermasının Dondurulması Üzerine L-Ergothionin, N-asetil sistein ve Sisteinin Etkileri

Özet

Bu çalışmanın amacı, koçlarda L-Ergothionin (LE), sistein ve N-asetil sisteinin (NAC) farklı dozlarının çözüm sonu sperma parametreleri üzerine etkisini belirlemekti. Dört Tuj ırkı koçtan alınan ejakülatlar değerlendirildi ve 35°C'de karışım yapıldı. Sperma örnekleri, LE (5, 10 mM), Sistein (5, 10 mM), NAC (5, 10 mM) içeren veya antioksidan içermeyen (kontrol), yağsız süt temelli sulandırıcıyla sulandırılarak, 0.25 ml'lik payetlere doldurulduu. Payetler 5°C'ye 2 saat süreyle soğutuldu, sıvı azot buharında (yaklaşık -120°C) 15 dak. süre ile donduruldu ve çözme işlemine kadar sıvı azot içinde saklandı. Payetler su banyosunda (37°C'de 1 dak.) çözüldü. Motilite, canlılık, anormal akrozom, toplam anormalite ve membran bütünlüğü (hipoozmotik şişme testi, HOST) yüzdeleri istatistiksel olarak değerlendirildi. Ayrıca, toplam antioksidan kapasite (TAK) ve toplam oksidatif stres (TOS), replikasyonlardan elde edilen örneklerde değerlendirildi. LE'nin motilite (sistein hariç), canlılık, akrozom hasarı, total morfolojik anormalite, HOST ve TAK oranları bakımından, NAC ve sisteine üstünlük sağladığı görüldü ($P<0.05$). Yine de, LE ve kontrol grubu arasında istatistiksel bir fark yoktu ($P>0.05$). Sistein ve NAC'ın koçlarda çözüm sonu spermatolojik parametreler üzerine bazı olumsuz etkileri belirlenirken, LE'nin olumlu ya da olumsuz etkisinin olmadığı sonucuna varıldı. Bu sonuçlar, koç spermasının dondurulmasında, LE, NAC ve sistein kullanılması üzerine yeni bilimsel çalışmaların yapılması gerektiğini göstermektedir.

Anahtar sözcükler: *Koç sperması, Dondurma, L-Ergotiyonin, Sistein, N-asetilsistein, Tuj*

İletişim (Correspondence)
+90 474 242 6836/5247 GSM: +90 505 2635755
sfyzyildiz@gmail.com

INTRODUCTION

Artificial insemination with frozen semen does not yet give satisfactory results in sheep. Therefore, for increasing fertility rates are needed in the development of new semen extenders or new compositions. In semen, cooling, freezing, thawing and premature activation in genital tract cause deterioration in membrane integrity and decreases the success of artificial insemination [1,2].

It is well known that ram semen is susceptible to freezing and thawing processes. So, in recent years, various antioxidants have been used in the freezing of ram semen. In ram semen, despite the positive effects of antioxidants on the motility and membrane integrity, previous results are not satisfactory to achieve in artificial insemination. Hence, the many studies have been continuing on this subject. Although there are many studies on effect of antioxidants, the avaliable information on the antioxidant capacity and lipid peroxidation in frozen ram semen is limited [1-3]. The investigations on the selection of the best diluent, antioxidant and dose have been continued by researchers [4-8]. Some literatures documented have shown that antioxidants supplemented to semen extenders have a positive effect on the success in cryopreservation [9-11]. Even synthetic antioxidants on sperm have been investigated [2]. Not only investigation and detection of ram semen characteristics, also the measuring the activity of antioxidant enzymes in ram semen has recently gained importance in researches. The measurement of antioxidant activity for better understanding of the effects of antioxidants gives important clues in terms of the success of freezing [9,10].

Recently, a thiol compound, LE, has been used to determine the antioxidant activity in in the storage of semen and shown to have positive effects on storage of ram semen [1]. However, the studies regarding use of antioxidants in the storage of semen is very limited. Nevertheless, it has been reported that N-acetylcysteine reduce reactive oxygen species and DNA damage in human sperm [11,12]. Also, It has been shown that L-Cystein hidrochloide, reducing oxidative stress in semen, is one of major thiol compounds which is involved in glutathione structure and decreases chromatin damage and also enhances the sperm viability [13].

The oxidative damage during freezing and thawing is induced by the reactive oxygen species (ROS) produced by cellular metabolism and sperm components. Hence, ROS is one of the main causes of decrease of motility and potential fertility in spermatozoa and comprises primary factor comprising oxidative stress. For these reasons, addition of antioxidant compounds to the culture media have become widespread in recent times [14,15].

However, according to current literature knowledged, it is seen that LE which has been much more important than the other antioxidants upto now and superior features of LE have not been effectively used in culture medium of oocytes and embryos. It has been known that LE is a fungal metabolite with antioxidant function in mammalian cells [16]. The preventing of oxidative stress induced by hydrogen peroxide in the presence of catalase and glutathione (GSH) consumption stability regulated by N-ethylmaleimide is realized by intracellular LE [17]. It had been investigated the effectiveness of LE on prevention of DNA damage and revention of cell deaths caused by hydrogen peroxyde. In this study, researches had concluded that LE could act as an *in vivo* non-toxic thiol buffer antioxidant and provide the oxidative stability in pharmaceutical preparations [18]. LE is known as an unique and single antioxidant amongst antioxidants which chelates heavy metals and protects of the cells (primarily erythrocytes) from ROS damage. Further, LE is shown as a single and powerful antioxidant which induces lipid peroxidation alike thiol antioksidants in the presence of ferric acid antioxidants and neutralizes the hydroxyl radicals, peroxynitrite hypochloroik acid and peroxynitrite [19-21].

In reproductive studies, the significant protective effects of LE has been demonstrated especially on semen. LE is the predominant sulphidryl in human, equine and porcine semen. It has been reported that LE explicitly protects the semen from oxidatif stress. As a result of the antioxidant properties of LE, while it increases sperm viability during storage, eliminates the harmful effects of hydrogen peroxide on viability of spermatozoa and vital impact [1,22]. The aim of in this study was to investigate the effects of LE, NAC and Cystein on the frozen ram semen.

MATERIAL and METHODS

The experiment was performed in accordance with guidelines for animal research from the National Instutites of Health and all procedures on animals were approved by the Kafkas University Ethics Committee on Animal Research in current study (approval date/number: 2009/02).

Chemicals

All chemicals were provided from Sigma-Aldrich® (Germany) if not stated.

Animals

The animals were housed at the Kafkas University, Faculty Of Veterinary Medicine, Education Research and Practice Farm and kept under uniform feeding, housing and lighting conditions.

Semen Extension, Freezing and Thawing

Semen samples were obtained from four Tushin rams (2 and 4 years of age) and all of ejaculates were collected from the rams with artificial vagina twice a week during

the breeding season. The ewes in oestrus were used as phantom. Ejaculates were evaluated. Only ejaculates with in volume, spermatozoa with >80% progressive motility and concentration higher than 2×10^9 spermatozoa/ml were pooled, balancing the sperm output of each male to compansate individual differences [23-25]. Each pooled ejaculate was splitted into seven equal aliquots and diluted at 37°C with skimmed milk extender (prepared from 10% milk powder w/v, 0.9% glucose w/v; and added 10% egg yolk v/v, 5% glycerol v/v; pH adjusted to 6.8 with 1N NaOH) supplemented with LE (5 and 10 mM), Cystein (5 and 10 mM), N-acetylcystein (5 and 10 mM) and no-antioxidant (control), respectively, and a total of seven experimental groups were established. After dilution, final concentration was adjusted to of approximately 4×10^8 spermatozoa/ml. Diluted semen samples were aspirated into 0.25 ml French straws, sealed with polyvinyl alcohol powder and equilibrated at 5°C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen (-120°C), for 15 min. Thereafter, the straws were plunged into the liquid nitrogen for storage. The frozen semen in straws were thawed at 37°C for 1 min in a water bath for post-thawing evaluation immediately after thawing.

Analysis of Standard Semen Parameters

Semen Volume: The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals.

Motility: Progressive spermozoa motility was estimated using phase-contrast microscopy (400×; Nikon Eclipes e400, Japan), with a warm stage kept at 37°C. For motility assessment, one drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm-motility estimations were performed in three different microscopic fields for each semen sample. The mean (SEM ±) of the three successive estimations was recorded as final motility percentage (%).

The Percentage of Live and Dead Sperm: The percentage of live sperm in the sample was assessed by means of a nigrosin–eosin staining method [26]. In examination, the 2% solution of eosin stain powder prepared with 3% Sodium Citrate were used for staining. The stain fixation of sperm were prepared by mixing a drop of semen with two drops of the stain on a warm slide and spreading the stain immediately with a second slide. The viable sperm rate was assessed by counting 200 cells under bright field microscope (400× magnification; Olympus CX21, Japan). Spermatozoa showing partial or complete purple colorization was considered non-viable or dead and only sperm showing strict exclusion of the stain were considered to be alive.

The Percentage of Sperm Abnormalities: For the assessment of sperm abnormalities, one drop of each sample were added to Eppendorf tubes containing 0.5 ml of Hancock solution (62.5 ml of 37%, formalin 150 ml sodium saline solution, 150 ml buffer solution and 500 ml double-distilled water) [27]. The mixing samples were stored at refrigerator (+4°C). After providing fixation of samples, one drop of this mixture was put on a slide and covered with a cover slip. The percentage total sperm abnormalities were determined by counting a total of 400 spermatozoa under phase-contrast microscopy (magnification 1000×/oil immersion object). Further, sperm abnormalities were classified as acrosome, head, midpiece and tail (AHMT), and each category were recorded seperately.

Sperm Concentration: The sperm concentration was measured by using a haemocytometer [28]. The obtained results were recorded as 10^9/ml.

The Hypo-osmotic Swelling Test (HOST): The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, basedon curled and swollen tails. This procedure was carried out by incubating 30 µl of semen with 300 µl of a 100 mOsm hypo-osmotic solution (9.0 g fructose + 4.9 g sodium citrate in 1.000 ml of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spreaded with a cover slip on a warm slide. Four hundred sperms were evaluated (magnification 400×) with bright-field microscopy (Olympus CX21, Japan). Sperm with coiled tails were recorded as membrane intact [29,30].

Biochemical Assays

Biochemical assays were performed on the sperm samples immediately after thawing and without washing by using spesific kits. Total antioxidant capacity (TAC) in samples was assayed by following the procedures as described in recent years [31]. After semen samples were centrifuged in rapid of 3.000 rpm/5 min, seminal plasma were obtained. TAC and TOC levels were measured by using kit (Rel Assay Diagnostic, Turkey). The test named as blue-green ABTS radical cation which is based on the principle of color loss. In this method, 2,2'-azino-di (3-sulfonate to etlylbenzthiozol=ABTS) reacts with peroxidase and hydrogen peroxide. At the end of this reaction, radical cation ABTS positive (+) is formed and a blue-green color is obtained spectrophotometrically. This color was measured by spectrophotometry at 600 nm [32].

Statistical Analysis

The study was replicated seven times. The means of results obtained from seven different experimental groups were analyzed. Results were expressed as the mean±S.E.M mean were analyzed using a one-way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences in all the parameters between groups using the SPSS/PC computer program (version 14.0, SPSS, Chicago, IL). Differences with values of $P<0.01$ were considered to be statistically significant [33].

RESULTS

The mean±S.E.M average of motility, viability, total sperm abnormalities, acrosomal anormalities and the values of HOST, TAC and TOS values in frozen ram semen dilueted skimmed milk powder with supplemented of different antioxidants in different doses are shown in *Table 1*. The results of spermatological parameters, the values of TAC and TOS in all experimental groups were not superior with the addition of the anti-oxidants, when compared to the control. However, LE supplemented groups were superior to the other antioxidant supplemented groups (P<0.01) *(Table 1)*.

DISCUSSION

The evaluation of the data obtained from control, N-acetyl cystein, cystein and LE groups pointed out that motility rates of LE groups were statistically superior to the other experimental groups except control in current study. Viability rate in control and LE groups was found as numerically superior to other groups. Acrosome and total morphological defects rates in the control group was statistically significant lower than other groups, except LE group. On the other hand, the acrosomal defects in LE group were lower than the other groups, this lowerness was found as statically signficant compared to NAC5 and NAC10 groups, except control. From these results, acrosome defect rate and total morphological defect rates were understood to be parallel with each other.

In present study, the best membrane integrity (HOS Test) results were obtained from groups of LE5 and LE10, similar to rates in other groups. These findings demonstrated that there was consistency among sperm parameters evaluated in current study. In the examinig of TAC, although the LE group was relatively more successful, there were no any differences between groups in TOS values. Considering all the assessments mentioned above, it might be suggested that LE tried for the first time, Cystein and N-acetylcystein in the freezing of semen did not give succesful results compared to control. Unlike, regarding with spermatological parameters (motility, viability, total morphological abnormalities, intact acrosome and HOST) LE group have been successfully compared to other anti-oxidant groups. According to the literatures documented, as it has not been found any literature except for two studies (Çoyan et al.[34] and Ari et al.[35]) regarding with LE and the other antioxidants investigated in the present study, the main theme of the experiment could not be done subject-specific discussion in detail. However, in different number of mammalian species, analyzing the results of different or similar antioxidants with some studies [36,37] especially, motility results obtained in the present study is lower and in those of some others is similar [38,39]. In the same study, in the group added 2 mM LE subjective, CASA and progressive motility values were 81.3%, 57.9% and 31.0%, while in the group added 4 mM, these scores were 80.6%, 65.2% and 32.4% respectively. It was observed that the percentage of motility in LE group was significantly higher cystein group and the more addition dose was increased the success of study increased in LE group. The motility percentages were significantly lower than those of the results in the study of Çoyan et al.[34] similarly, in the present study, LE group was higher than cystein group. Moreover, Ari et al.[35] determined that 10 mM LE improved motility and membrane integrity in replication with poor freezability compared with 0, 1, 2, 5 mM LE. However, they could not determine any beneficial effects of LE in replication with good freezabillity.

In the present study, the viability percentages in the groups added LE and cystein were similar to the results obtained from some studies carried out in various animal species [39,40], while the results of the group supplemented with N-acetylcystein were lower than the results of some investigators [40,41]. On the other hand, the percentages

Table 1. *The mean±S.E.M average of motility, viability, total sperm abnormalities, acrosomal anormalities and the values of HOST, TAC and TOS values in frozen ram semen dilueted skimmed milk powder with supplemented of different antioxidants*

Tablo 1. *Farklı antioksidanlar içeren yağsız süt tozu sulandırıcısında dondurulmuş koç spermasında ortalama motilite, canlılık, toplam anormalite, akrozom bozuklukları, HOST, TAK ve TOS değerleri*

Parameters	Control	LE5	LE10	NAC5	NAC10	C5	C10
Motility (%)	26.28±3.16^{a}	24.71±2.85^{a}	26.14±4.13^{a}	4.00±2.69^{b}	2.85±2.42^{b}	15.14±3.28^{c}	16.00±4.38^{c}
Viability (%)	32.28±4.10^{a}	28.71±3.12^{a}	26.85±4.21^{ab}	17.57±3.56^{bc}	14.00±3.92^{d}	24.71±2.40^{ab}	25.00±3.40^{ab}
Total Morphological Abnormalities (%)	46.14±3.48^{a}	49.42±2.56^{ab}	51.71±3.80^{ab}	78.42±2.92^{c}	82.57±3.24^{c}	57.00±3.47^{b}	59.28±3.65^{b}
Acrosome anormalities (%)	42.00±3.92^{a}	45.85±2.74^{ab}	49.00±3.60^{ab}	73.14±3.45^{c}	77.57±4.08^{c}	53.71±4.50^{b}	55.42±3.62^{b}
HOST (%)	30.28±3.25^{a}	29.57±2.62^{a}	28.57±2.66^{a}	15.50±3.80^{b}	14.80±4.31^{b}	24.14±3.13^{a}	23.00±3.75^{a}
TAC (mM/lt)	155.14±2.72^{a}	173.89±5.09^{a}	250.00±28.0^{ab}	328.01±17.2^{b}	357.08±17.8^{b}	356.69±23.5^{b}	359.82±19.2^{b}
TOS (mM/lt)	66.61±18.5	61.93±12.7	61.83±20.5	45.57±23.6	49.91±21.4	73.12±10.9	70.00±11.9

$^{a, b, c}$ *Different letters within the same column are significantly different (P<0.01)*

LE5: *5 mM L-Ergothionin supplementation,* ***LE10:*** *10 mM L-Ergothionin supplementation,* ***NAC5:*** *5 mM N-Acetyl Cystein supplementation,* ***NAC10:*** *10 mM -Acetyl Cystein supplementation,* ***C5:*** *5 mM Cystein supplementation,* ***C10:*** 10 mM Cystein supplementation

of viability in a study which has been added 6 mM N-acetylcystein to dog semen [37] had been higher than those of the present study's results.

The percentages of acrosomal defects in the present study were lower than some studies's results [24] while were higher than those of some studies [37,38,40,41]. However, the percentages of acrosomal defects in a study [38] which was subjected to addition of cystein in bull semen were highly lower than those of the present study. It also suggests that bull semen is more resistance than ram semen to thawing procedure. On the other hand, the acrosomal defects rates of the groups supplemented with N-acetyl cystein and cystein in the present study were higher than those of the other experimental groups (LE and control groups) and some other studies [24,40,41].

In present study, it was seen that total morphological abnormalities showed similar pattern with the percentage of acrosome disorders, so in the same way, these rations has shown similar differences with other studies [24,37,38,40,41].

Compared with Çoyan et al.[34] study in ram semen, the findings obtained from control and LE groups of the present study were higher and similar to those of N-acetylcysteine and cysteine added.

Our findings about LE doses was similar with findings from Ari et al.[35] when all replications considered. However, Ari et al.[35] pointed out that higher doses of LE (i.e. 10 mM) improved post-thaw motility and membrane integrity in poor freezability replications, while lower doses of LE (1, 2, 5 mM) did not show any beneficial effects on post thawing sperm parameters neither in poor nor in good freezability replications.

Ari et al.[42] determined that higher doses of NAC (i.e. 0.75 mM) had some detrimental effect on post-thawing motility and other semen parameters on ram semen diluted with milk based extender. Similarly, we found that 5 mM and 10 mM doses of NAC detrimentally affected post thawing parameters in ram semen.

Compared HOST results obtained from present study in all groups with other studies [35-37,39] are seen to below.

TAC and TOS relevant results, obtained from the present study, have not been able to discuss in detail with the results from other studies because limited literatures found. However, Çoyan et al.[34] reported that LE and cystein did not affect significantly the superoxide dismutase (SOD) and gluthation peroxidase (GPx) activity. In TOS terms, similar situation was observed in the present study. On the other hand, in the same study, it was shown that the motility rates were positively affected in the groups of the addition of LE compared with control.

Differences between results in our study and the other studies might have been orginated from different methods and sheep breeds or species. Because it has been pointed out that the different methods and breeds or species used may cause variations in sensitivity to oxidative stres in sperm [39]. In addition, it is also known that individual characteristics of rams, season, native quality of semen, extenders/diluents, cryoprotectants, procedure of cryopreservation and also thawing procedure can directly affect results of post-thawing semen quality in rams [40].

In conclusion, in this study, while the 5 and 10 mM doses of LE were superior to the 5 and 10 mM doses of NAC in the view of the percentages of motility, viability, acrosomal defects, total morphological abnormalities, membrane integrity (HOST) and TAC values, except motilty rates in the 5 and 10 mM doses of cystein. However, 5 mM dose of LE were more succesful than all doses of NAC and cystein, considered TAC values. Neverthless there were not statistically significant differences between LE and control groups. The results of current study and limited literature knowledge about LE, described as unique and strong antioxidant, warrants future studies on ram semen cryopreservation.

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Research Article

Isolation of Vancomycin Resistant Enterococci from Animal Faeces, Detection of Antimicrobial Resistance Profiles and Vancomycin Resistant Genes [1] [2]

Arzu FUNDA BAĞCIGİL [1] Serkan İKİZ [1] Seyyal AK [1] Naciye YAKUT ÖZGÜR [1]

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[1] Istanbul University, Faculty of Veterinary Medicine, Dept. of Microbiology, Avcılar, TR-34320 Istanbul - TURKEY

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Abstract

Infections caused by Vancomycin Resistant Enterococci (VRE) are important in human medicine in terms of treatment difficulties. Molecular studies in the last years revealed that VRE occurrence in animals might be important in epidemiology of infections in human. This study aims to detect VRE occurrence in various animals, examine antibiotic resistance profiles phenotypically, and determine the distribution of the vancomycin resistant genes, *vanA, vanB, vacC1, vanC2/C3*. For this purpose, rectal swabs were collected from farm and companion animals; and cloacal swab or litter were collected from chickens and they were processed for VRE isolation. Following the identification of the isolates, antimicrobial susceptibilities of the isolates were determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) standards. Distribution of the vancomycin resistant genes; *vanA, vanB, vanC1* and *vanC2/C3* among enterococcus species and different animal species were determined by multiplex PCR. VRE were isolated from 17% of the feline samples, 20% of each of the other species, and 19% of all the samples. Those isolates were identified as *E. casseliflavus* (n=39), *E. gallinarum* (n=55) and *E. faecium* (n=3) as a result of multiplex-PCR. According to the antimicrobial susceptibility tests, most of the isolates were found to be resistant to penicillin G, ciprofloxacin and erythromycin. Eighteen (18.8%) of the isolates were found to be resistant against two antibiotic groups, while 69 (71 %) of them were resistant to three or more antibiotics.

Keywords: *Antimicrobial resistance, Enterococcus spp., animal faeces, vancomycin resistance*

Hayvan Dışkılarından Vankomisin Dirençli Enterokokların İzolasyonu, Antimikrobiyal Direnç Profillerinin ve Vankomisin Direnç Genlerinin Saptanması

Özet

Vankomisin dirençli enterokoklardan (VRE) kaynaklanan infeksiyonlar, tedavide karşılaşılan zorluklar nedeniyle insan hekimliğinde önemli bir yer tutmaktadır. Son yıllarda yapılan genetik düzeydeki çalışmalar, hayvanlardaki VRE varlığının da insanlardaki infeksiyonunun epidemiyolojisinde önemli olacağını vurgulamaktadır.Bu çalışmada farklı hayvan türlerinde VRE varlığı ve türlerinin dağılımının saptanması; antibiyotiklere duyarlılıklarının belirlenmesi; vankomisin direncinin kodlayan v*anA, vanB, vacC1, vanC2/C3* genlerinin dağılımının araştırılması amaçlanmıştır. Bu amaçla evcil hayvanlardan ve çiftlik hayvanlarından rektal svab ve tavuklardan kloakal svab/altlık örnekleri (n=500) toplandı ve VRE yönünden bakteriyolojik olarak incelendi. İzolatların identifikasyonun takiben, antimikrobiyal duyarlıkları Clinical and Laboratory Standards Institute (CLSI) standartlarına uygun yöntemlerle saptandı. Vankomisine direnç ile ilişkili *vanA, vanB, vanC1* ve *vanC2/C3* genlerinin *Enterococcus* türleri arasındaki dağılımı multipleks-PCR ile araştırıldı. Kedilerin %17'sinden, diğer gruplardaki hayvanların herbirinin %20'sinden, toplamda örneklerin %19'undan VRE izole edilmiştir. Yapılan multiplex PCR sonucunda izolatların 39'u *E. casseliflavus,* 55'i *E. gallinarum* ve 3'ü *E. faecium* olarak tanımlanmıştır. Antimikrobiyal duyarlılık testi sonuçlarına göre izolatların büyük çoğunluğu penicillin G, siprofloksasin ve eritromisine dirençli bulundu. İzolatların 18 (%18.6)'inin iki antibiyotik grubuna ve 69 (%71)'unun 3 ve daha fazla sayıda antibiyotik grubuna dirençli oldukları saptandı.

Anahtar sözcükler: *Antimikrobiyal direnç, Enterococcus spp., hayvan dışkıları, vankomisin direnci*

İletişim (Correspondence)
+90 212 4737070/17047
ser@istanbul.edu.tr

INTRODUCTION

Enterococci are a part of normal human and animal faecal flora. On the other hand, they can cause septicemia, endocarditis, meningitis, urinary and genital tract infections as opportunistic pathogens; and they have emerged as an increasingly important cause of nosocomial infection since 1980s. These bacteria have clinical importance because of their increasing acquired antimicrobial resistance along with intrinsic resistance [1-4]. In the last decade, studies on examination of nosocomial infectious agents such as methicillin resistant staphylococci, vancomycin resistant enterococci in different animals started to have clinical concern. The emergence of resistance to vancomycin has presented an increasingly important problem in treatment [1,2,5-10]. Various genes including *vanA, vanB, vanC, vanD*, *vanE, van G, vanN* and *vanM* encode vancomycin resistance among enterococci. There are two types of vancomycin resistance. The first is intrinsic resistance demonstrated as low-level resistance to vancomycin, which is observed among *E. gallinarum, E. casseliflavus* and *E. flavescens* species. These strains carry *vanC* gene and are susceptible to teicoplanin. The second one is acquired and inducible resistance, which is mostly observed in *E. faecium* and *E. faecalis*. These strains often carry transferable *vanA* or *vanB* genes. Strains with *vanA* genotype display inducible high-level resistance to both vancomycin and teicoplanin; while strains with *vanB* genotype have resistance only to vancomycin [2,4,8-11]. Although vancomycin resistant *E. gallinarum* strains commonly carry *vanC* gene, strains carrying both *vanA* and *vanC* genes have been reported [8,9]. Therefore, it is clinically and epidemiologically important to determine the gene encoding resistance and then to detect vancomycin resistance phenotypically [8,9]. There are various studies reporting the existence of VRE species in various animal species [2,4,6,11,12]. Recent molecular epidemiological studies suggest that VRE residing the gastrointestinal flora of animals can be a source of infection for human. Many researchers report that those VRE can be transmitted to human via contaminated raw or insufficiently treated food, or after a physical contact with a companion animal such as cats and dogs [7,10,12,13]. This study aims to detect VRE occurrence in various animals, to examine antimicrobial resistance profiles phenotypically, and to determine the distribution of vancomycin resistant genes, *vanA*, *vanB*, *vacC1*, *vanC2/C3*.

MATERIAL and METHODS

Fecal Samples

The animal species in this study were divided into three groups. The first group included farm animals (cows and sheep); the second group included companion animals (cats and dogs); the third group included poultry animals. One hundred rectal swabs were collected from each species in group one and two (totally 400 samples). In group three, rectal swabs or litter samples were collected from each flock. Samples from group-1 were collected from the farms were located mainly in Istanbul and Çatalca, Maşukiye, Tekirdağ. In those farms the most common antimicrobials were enrofloxacin, amoxicillin clavulanic acid, oxytetracycline, penicillin G and erythromycin. All of the canine and feline samples were collected from the animals in Istanbul. The most common antimicrobials were cephalosporins, aminoglycosides particularly gentamicin, azithromycin and enrofloxacin. Poultry samples were collected from Marmara region mainly, Istanbul, Balıkesir, Bandırma. Erythromycin, neomycin and tylosin were used in those flocks. Other information about the animals is shown in *Table 1*.

Culture

Swabs were inoculated into tubes containing Bile Esculin Azide Broth (BD BBL 212207) supplemented with 6 µl/ml vancomycin hydrochloride (Molekula) and incubated for 24 h at 37˚C. Five grams from the litter samples were homogenized in 45 ml saline water and 5 ml of it was transferred into Bile Esculin Broth supplemented with 6 µl/ml vancomycin hydrochloride (Molekula). Cultures

Table 1. *Animals included in the study*
Tablo1.*Çalışmaya dahil edilen hayvanlar*

Animal Species	Samples	Age		Antibiotic Usage		
		<1 year	≥1 year	Used	Not Used	Not Known
Sheep	100 rectal swabs	28	72	1[a]	8[a]	0
Cattle	100 rectal swabs	8	92	32	52	16
Cat	100 rectal swabs	27	73	37	32	31
Dog	100 rectal swabs	24	76	14	47	39
Poultry	3 layer flocks, 45 cloacal swabs	-[c]	-[c]	3[a]	0	0
	53 broiler flocks, 53 litter samples	-[c]	-[c]	26	27	0
	2 individual samples[b]	-[c]	-[c]	0	2	0

[a] The numbers indicate the farm/flock number; [b] Intestinal content from a pigeon and a layer chicken after necropsy; [c] Layers: between 3 to 11 months-old; broilers: between 10 to 45 days-old; individual samples: 4 months-old pigeon and 30 days-old broiler chicken

with colour change were subcultured onto Bile Esculin Agar (BD BBL 299068) and incubated for 24 h at 37˚C. Presumptive *Enteroccocus* spp. with black colour was subcultured onto Nutrient agar (BD Difco 269100) plates supplemented with 7% sheep blood to achieve pure cultures. Catalase negative, aesculin hydrolysis positive and growth of 6.5% in NaCl positive colonies were evaluated as presumptive *Enterococcus* species. Further identification was performed through API 20 STREP along with pigment production; and by methyl-α D-glucopyranoside acidification test and motility test. Due to the inadequacy of API 20 STREP test in differentiation of some strains, the final identification was completed after the multiplex PCR results [9,11,12,14,15].

Detection of Vancomycin Resistance Genes

After phenotypical confirmation of vancomycin resistance of the isolates by macro-dilution method [17], vancomycin resistant enterococci were further examined by multiplex PCR according to Kariyama et al.[15] for the detection of genes encoding vancomycin resistance. Primers suggested by Elsayed et al.[16] were used in order to detect *vanB* gene. Fifty µl from VRE cultures from Tryptic Soya Broth after 24 h of incubation at 37˚C were mixed with the equal volume of 7.5% Chelex 100 (BioRad). The mixture was heated for 10 minutes at 100˚C and centrifuged; and a 2.5 µl volume of the supernatant was then used for PCR amplification. Primer sets shown in *Table 2* were included into the reaction mixtures as follows: 5 pmol of *vanA* primers, 2.5 pmol of each, *vanC1*, *vanC2/C3* and *rrs* primers, 7.5 pmol of *E. faecalis* specific primers, 1.25 pmol of *vanB, E. faecium* specific primers. The multiplex PCR was performed in a total volume of 25 µl containing 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM per deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.625 U *Taq* DNA polymerase (TaKaRa Taq, 250 U). DNA amplification was carried out according to the following protocol: initial denaturation at 94˚C for 5 min, 30 cycles of amplification (denaturation at 94˚C for 1 min, annealing at 54˚C for 1 min, extension at 72˚C for 2 min) and final extension at 72˚C for 10 min in a (MaxyGene Gradient Therm-1000) system. PCR products were analyzed on a 1.5% Agarose B Low EEO (Bio Basic Inc.) with 0.5 x Tris-borate-EDTA buffer.

Control strains which were kindly provided by Dr. Luca Guardabassi (Life University, Copenhagen), including *E. faecium* BM4147 (VanA), *E. faecalis* V583 (Van*B*), *E. gallinarum* BM4174 (VanC1), *E. casseliflavus* DSMZ 20680 (VanC2/C3), *E. faecium* CCUG542 (vancomycin susceptible) were used in PCR assays.

Antimicrobial Susceptibility Test

The isolates were examined by disc diffusion method according to the standards of Clinical and Laboratory Standards Institute (CLSI) for detection of penicillin (10 mg), ampicillin (10 mg), erythromycin (15 mg), tetracycline (30 mg), ciprofloxacin (5 mg), doxycycline (30 mg) and rifampicin (5 mg) susceptibilities. In addition, Minimal Inhibition Concentration (MIC) values for teicoplanin were determined by broth macro dilution method. To detect high level of aminoglycoside resistance (HLAR), the growths in gentamicin (600 mg/ml) and streptomycin (1.000 mg/ml) were evaluated. In order to detect β-lactamase producing isolates, beta-lactamase (Nitrocefin) disks (Bio Chemika, Fluka) were used. *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 strains were used as control strains in antimicrobial susceptibility tests [17].

RESULTS

Isolation and Identification of Vancomycin Resistant Enterococci

Of 500 fecal samples 97 (19%) VRE were isolated. Isolation rate of VRE were 17% for cats, 20% for each of

Table 2. *Multiplex PCR primers*
Tablo 2.*Multipleks PCR primerleri*

Target Gene	Size of PCR Product	Primer Pair Sequence	Reference
vanA	1.030 bp	5′-CATGAATAGAATAAAAGTTGCAATA -3′ 5′-CCCCTTTAACGCTAATACGATCAA -3′	[14]
vanB	536 bp	5′-AAGCTATGCAAGAAGCCATG -3′ 5′-CCGACAATCAAATCATCCTC -3′	[10]
vanC1	822 bp	5′-GGTATCAAGGAAACCTC -3′ 5′-CTTCCGCCATCATAGCT -3′	[14]
vanC2/C3	484 bp	5′-CGGGGAAGATGGCAGTAT -3′ 5′-CGCAGGGACGGTGATTTT -3′	[14]
E. faecalis	941 bp	5′-ATCAAGTACAGTTAGTCTTTATTAG -3′ 5′-ACGATTCAAAGCTAACTGAATCAGT -3′	[14]
E. *faecium*	658 bp	5′-TTGAGGCAGACCAGATTGACG -3′ 5′-TATGACAGCGACTCCGATTCC -3′	[14]
rrs (16SrRNA)	320 bp	5′-GGATTAGATACCCTGGTAGTCC -3′ 5′-TCGTTGCGGGACTTAACCCAAC -3′	[14]

the other groups. Biochemical tests and API results were sufficient only for the identification of the isolates in the genus level, but the differentiation of *E. faecalis, E. gallinarum* or *E. casseliflavus* was performed after multiplex PCR assay. Final identification results are shown in *Table 3*.

Group 1 - Farm Animals: VRE were isolated from 20% of sheep samples examined. The samples were collected from six different farms; in only one of these farms, antimicrobial treatment was applied to the animals, and six VRE were isolated from the sheep from that farm. The VRE isolation rate for the cow was 20%. Twenty percent (n=4) of those isolates were from the farms in which antibiotic treatment had been applied and 60% were from the antibiotic unused farms. Among these 20 isolates, 20% of them were isolated from cattle that had an antimicrobial treatment within one year, while 60% of them from non-antimicrobial used animals. For the remaining four VRE positive animals, the farmers gave no information about antimicrobial usage.

Group 2 - Companion animals: VRE were isolated from 20% of the dogs. The isolation rate was 29% in dogs with antimicrobial therapy history within one-year period, and was 19% in dogs with non-antimicrobial therapy background. VRE were isolated from 4 of 24 dogs that were younger than 1-year age; 16 of 76 one-year age and older dogs. Of dogs from which VRE were isolated, 75% were owned dogs; remaining 25% were from kennels. VRE were isolated from 17% of the sampled cats. The isolation rate was 24% in the cats with antimicrobial therapy history within one-year period, and was 19% in cats without any therapy. VRE were isolated from 10 of 27 cats that were younger than 1-year age; 7 of 73 one-year age and older ones. It was remarkable that VRE were isolated from five of the six cats that were younger than one-year and with antimicrobial therapy history.

Group 3 - Poultry animals: VRE were isolated from 12 of 46 layers, 7 of 53 litter samples and one pigeon. It was informed that in 26 of the 53 broiler flocks antimicrobial agents were being used and VRE were isolated from 6 of those flocks. In 29 flocks no antimicrobial therapy were applied and VRE were isolated only from one of those flocks. Antimicrobial agents were used in all of the layers.

Antimicrobial Susceptibility Test Results

Group 1 - Farm animals: The MIC values of ovine isolates were 8-16 µg/ml and 0.5-1 µg/ml for vancomycin and teicoplanin, respectively. All the ovine isolates were susceptible to tetracycline, and none of them showed high level of aminoglycoside resistance, however, 30% of the isolates were multidrug resistant (resistant to three or more antimicrobial agents).

MIC value was 512 µg/ml for vancomycin and 64 µg/ml for teicoplanin for one bovine isolate *(Table 3)* and the values of the remaining bovine isolates were 8-32 µg/ml and 0.5-16 µg/ml for vancomycin and teicoplanin, respectively. None of the bovine isolates had high level of aminoglycoside resistance, and 60% of them were multidrug resistant.

Group 2 - Companion animals: For canine isolates; 18 isolates had 8-16 µg/ml and 0.5-1 µg/ml MIC values, while two high level vancomycin resistant isolates *(Table 3)* had 512 µg/ml and 128 µg/ml MIC values for vancomycin and teicoplanin, respectively. Among the isolates, 75% were multidrug resistant, and more than 50% of those isolates were resistant to penicillin, rifampicin, ciprofloxacin and erythromycin. High level of gentamicin resistance were observed in one isolate and both streptomycin and gentamicin resistance in two isolates. Two *E. gallinarum* isolates with high level of vancomycin resistance were also resistant to all other antimicrobial tested by disc diffusion; and in addition, one of them had HLAR.

MIC values of feline isolates were 16-32 µg/ml and 0.25-1.0 µg/ml for vancomycin and teicoplanin respectively. Ninety four percent of the isolates were multidrug resistant. High level of aminoglycosides (both gentamicin and streptomycin) was observed in five isolates, only streptomycin resistance was observed in three isolates.

Group-3: Poultry animals: The MIC values of 17 avian isolates were between 8-16 µg/ml and 0.5-2 µg/ml for vancomycin and teicoplanin, respectively. The remaining three isolates showed high level of vancomycin and teicoplanin (256-512 µg/ml and 32-128 µg/ml) *(Table 3, Table 5)*. All avian isolates were multidrug resistant. Among 20 isolates, in 10 (50%) only streptomycin, in five (25%)

Table 3. *Distribution of vancomycin resistant enterococci species*
Tablo 3. *Vankomisin dirençli enterokok türlerinin dağılımı*

Source	*E. faecium & vanA*	*E. casseliflavus*	*E. gallinarum*	Total
Dog	0	6	14[a]	20
Cat	0	3	14	17
Cow	0	19[a)]	1	20
Sheep	0	11	9	20
Poultry	3	0	17	20

[a] High level of vancomycin resistance (MIC value = 512 µg/ml) was detected in two canine and one bovine isolates, but vanA or vanB genes were not detected

only gentamicin and in five (25%) both streptomycin and gentamicin resistance were detected.

Isolates with High Level of Vancomycin Resistance

We detected high level of vancomycin resistance in two canine *E. gallinarum* isolates and one bovine *E. casseliflavus* isolate, however, we did not detect *vanA* or *vanB* genes by PCR. The two dogs were from a kennel and from the same cage. Since they were street dogs, there were no information about their previous health status, and antimicrobial therapy history.

The other isolate, *E. casseliflavus* with *vanC* gene was from a 3 to 6 years old cattle raised in a semi intensive system. Ten animals from the same farm were examined and VRE were isolated from 5 (50%) of them, however, high level of vancomycin resistance were observed from only one animal. The farmers informed us that oxytetracycline and penicillin products had been used in this farm in the sampling year, but they had not been applied to this animal.

There were three *E. faecium* isolates carrying *vanA* gene, two of them were isolated from the litters of two different flocks including 90-day-old layers in a breeding unit. The other isolate was from a litter of a 10 to 15 day-old broiler flock in which enrofloxacin application was being performed at the sampling time.

Antimicrobial resistance rates of the all vancomycin resistant enterococci and of the isolates with high level of vancomycin resistance were shown in *Table 4* and *Table 5*, respectively.

DISCUSSION

There are various studies on occurrence of VRE in different animal species or their products. The different isolation rates or diversity of species can be resulted from different breeding facilities, management procedures and environmental factors [2,4,10-12,18-20]. The presence of VRE in companion animals is becoming a high clinical concern due to the high transmission risk of VRE via close contact with their owners. Herrero et al.[12] have examined randomly selected 87 dogs for the presence of VRE for 5 years, and have isolated VRE from 15 samples and have reported that *vanA* originated glycopeptides resistance was common among the canine *E. faecium* isolates. Boynukara et al.[6] have detected vancomycin resistance in 91.3% of *Enterococcus* species isolated from human, dog and cat faeces. Lopez et al.[10] have sampled 126 canine faecal samples and have not detected VRE with acquired resistance. In the present study, no *Enterococcus* species carrying *vanA* and/or *vanB* genes were isolated; however, *E. gallinarum* and *E. casseliflavus* with *vanC1* and *vanC2/3* genes were detected. Besides, in two dogs, *Enterococcus* species representing VanA phenotypic resistance (high-level resistance to vancomycin and teicoplanin) were detected.

De Leener et al.[13] have reported that combination of different resistance against two and more antimicrobial agents detected more frequently from cats and dogs from kennels than the ones from private owners. There are some reports documenting the presence of VRE in dogs living on farms where VRE were present among the other farm animals. However, Herrero et al.[12] have reported that ten of the eleven VRE harbouring dogs did not have any contact with farm animals. Abbott et al.[1] have described a high-level gentamicin resistant and vancomycin resistant *E. faecium* in a dog. The authors have suggested that the origin of the agent was from an external source, possibly from the oral cavity or faeces of an attacking dog, a veterinary health care profession, the owner or the environment. In the present study, 75% of the VRE positive dogs were owned dogs and they did not have any direct contact with any other animals. The VRE isolation rate

Table 4. *Antimicrobial resistance rates of vancomycin resistant enterococci based on animal species*
Tablo4. *Vankomisin dirençli enterokokların hayvan türlerine gore antimikrobiyal dirençlilik oranları*

Source	Number (%)	P10	AM 10	E15	T30	CIP5	DO30	RA5	GM-HLAR	S-HLAR	B-LACTAMASE
CAT (n=17)	n	17	9	13	8	12	7	6	5	8	-
	%	100	53	76	47	71	41	35	29	47	-
DOG (n=20)	n	18	6	12	9	10	7	11	2	3	-
	%	90	30	60	45	50	35	55	10	15	-
COW (n=20)	n	16	2	12	3	13	1	11	-	-	-
	%	80	10	60	15	65	5	55	-	-	-
SHEEP (n=20)	n	16	2	4	-	14	3	12	-	-	-
	%	80	10	20	-	70	15	60	-	-	-
POULTRY (n=20)	n	20	8	20	19	12	15	14	10	15	-
	%	100	40	100	95	60	75	70	50	75	-
TOTAL (n=97)	n	87	27	61	39	61	33	54	17	26	-
	%	90	28	63	40	63	34	56	18	27	-

P10 *= penicillin (10 mg),* ***AM 10*** *= ampicillin (10 mg),* ***E15*** *= erythromycin (15 mg),* ***T30*** *= tetracycline (30 mg),* ***CIP5*** *= ciprofloxacin (5 mg),* ***DO*** *= 30 doxycycline (30 mg),* ***RA5*** *= rifampicin (5 mg),* ***GM-HLAR*** *= gentamicin-high level of aminoglycoside resistance,* ***S-HLAR*** *= streptomycin- high level of aminoglycoside resistance*

Table 5. *Antimicrobial susceptibilities of the five Enterococcus species with high level of vancomycin resistance*
Tablo 5. *Yüksek vankomisin direncine sahip beş Enterokok türünün antimikrobiyal duyarlılık profili*

Sample Number	Species and Resistant Gene	MIC-Van (ug/ml)	MIC-Tei (ug/ml)	P10	AM 10	E15	T30	CIP5	DO30	RA5	GM-HLAR	S-HLAR	B-LACTAMASE
132[a]	*E. faecium, VanA*	256	64	R	R	R	O	S	S	S	R	S	N
135[a]	*E. faecium, VanA*	256	32	R	S	R	S	S	S	S	R	S	N
61[a]	*E. faecium, VanA*	512	128	R	S	R	R	S	R	S	S	R	N
147[b]	*E. gallinarum VanC1*	512	256	R	R	O	R	O	O	R	S	S	N
148[b]	*E. gallinarum VanC1*	512	128	R	R	R	R	R	O	R	R	R	N
82[c]	*E. casseliflavus VanC2*	512	64	R	S	O	O	S	S	R	S	S	N

MIC-Van *= Minimal İnhibitory Concentration for vancomycin,* ***MIC-Tei*** *= Minimal İnhibitory Concentration for teicoplanin,* ***P10*** *= penicillin (10 mg),* ***AM 10*** *= ampicillin (10 mg),* ***E15*** *= erythromycin (15 mg),* ***T30*** *= tetracycline (30 mg),* ***CIP5*** *= ciprofloxacin (5 mg),* ***DO*** *= 30 doxycycline (30 mg),* ***RA5*** *= rifampicin (5 mg),* ***GM-HLAR*** *= gentamicin-high level of aminoglycoside resistance,* ***S-HLAR=*** *streptomycin- high level of aminoglycoside resistance; [a] avian isolate; [b] canine isolate; [c] bovine isolate*

among the dogs treated with an antimicrobial therapy within a year was 29%, while it was 19% among the dogs without any therapy. Besides, it was remarkable that the two isolates with high-level vancomycin and teicoplanin resistance were both from two dogs sharing the same box in a kennel. There was no information, for example, about the antimicrobial therapy background of those two dogs. However, as they are sharing the same box, horizontal transmission of the agent is highly possible.

Seo et al.[4] have detected *vanA* gene in six of VRE isolates showing high-level vancomycin resistance (MIC: >256 µg/ml) from poultry farms, and four of those isolates were resistant to other antimicrobials in addition to vancomycin and teicoplanin. Ünal et al.[21] were isolated high-level vancomycin resistant *E. faecium* from one of 400 swab samples collected from commercial broiler farms. In a study in Brazil, Xavier et al.[18] have not isolated any *Enterococcus* species carrying *vanA* or *vanB* genes, but they have detected *vanC1* in 13% and *vanC2/C3* in 5.5% of the isolates. Kaya et al.[22] have reported that none of the 80 *Enterococcus* species from chicken intestinal content showed resistance to vancomycin and teicoplanin; however, they have detected that 17.5% of them were resistant to high-level aminoglycosides. In the present study, *vanA* carrying *E faecium* (n=3) and *vanC1* carrying *E. gallinarum* (n=19) were isolated from poultry samples. Two of the *E. faecium* isolates were from layer flocks having neomycin sulphate administration, the other one was from a broiler flock with enrofloxacin administration at the time of sampling. It was observed that VRE isolation rate was higher in the flocks with intense antimicrobial usage.

Kempf et al.[23] have reported that all *vanA* carrying avian originated *Enterococcus* spp. isolates were resistant to tetracycline, 66 % of them were resistant to erythromycin, but none of them was resistant to ampicillin or gentamicin. Herrero et al.[12] have reported that all vancomycin resistant *E. faecium* strains from dogs were highly resistant to vancomycin and harboured the *vanA* gene; moreover, 11 of those strains were resistant to tetracycline, and 10 were resistant to erythromycin. Kaya et al.[22] have detected resistance to erythromycin in 45% of the chicken VRE strains. In the present study, 55% of ovine, 60% of bovine, 75% of the canine, 94% of the feline and all of the avian isolates were resistant to three or more antimicrobial agents. It was remarkable that all the chicken isolates were resistant to penicillin and erythromycin and 95% of them were resistant to tetracycline. This resistance profiles especially in avian isolates, are good examples of adverse effect of antimicrobial usage for preventive purposes in poultry flocks.

Gentamicin is an antimicrobial agent used in combination with b-lactams or glycopeptide antibiotics for treatment of enterococcal infections in humans. However, this synergistic bactericidal effect is lost in case of high-level of gentamicin resistance. Transmission of gentamicin resistant enterococci from food-producing animals to human through food chain was discussed. Besides, it was also mentioned that enterococci from the intestinal microbiota of cats and dogs might act as a reservoir of resistance genes for animal and human pathogens. Therefore it is important to pay attention to this type of resistance and a well-considered use of this antibiotic in companion animals is needed [3,13]. High-level aminoglycoside resistance occurs in two mechanisms. The first one is the resistance resulted by alteration of aminoglycoside binding region on the ribosomes. This kind of resistance only causes high level of streptomycin resistance (S-HLAR) and is not transferable. In the second resistance mechanism, which is observed as transferable gentamicin resistance (GM-HLAR), adenyltransferase, phosphotransferase, acetyltransferase enzymes are involved. The strains with GM-HLAR are resistant to all other aminoglycosides except streptomycin [3]. In the current study, GM-HLAR was detected in five feline, two canine and 10 avian isolates. The contamination risk at the poultry slaughter houses or close contact of the companion animals with their owners increases the importance of the detection of this transferable resistance in this study. Furthermore, resistance to both streptomycin and gentamicin was detected in five canine, two feline and five avian isolates. In any case of transmission of such isolates to human, it

would be unavoidable to have some problems in the treatment of these cases.

E. casseliflavus and *E. gallinarum* represent significant percentage of the faecal enterococci population of various animal species [4,10,11]. Khan et al.[24] have isolated *E. gallinarum* from milk samples of animals with mastitis and litters from 28 different flocks. In addition to the intermediate level of vancomycin resistance, those isolates were resistant to 6 to 8 antimicrobial agents among 13 different antimicrobials. The researchers have commented that the situation occurs because of previous usage of those antimicrobial agents or transmission of some resistance markers from another bacterial species. Although isolates with low level of vancomycin resistance (MIC: 4-8 µg/ml) were evaluated as unimportant isolates some cases such as endocarditis, bacteremia caused by *E. gallinarum* and *E. casseliflavus* strains with *vanC* intrinsic resistance particularly in immunosuppressed people has been reported recently [4,9]. Moreover, Corso et al.[9] have revealed that the clones of two *E. gallinarum* isolates with *vanA* gene had successfully transferred their resistance gene to one previously vancomycin susceptible *E. faecium* strain. Haenni et al.[25] have reported the first isolation of *E. casseliflavus* S8702 strain with vanB/*vanA-vanC* complex resistance from three different calves. Lopez et al.[10] have recovered *E. gallinarum* and *E. casseliflavus* in 12% of dog and healthy human faecal samples. Çetinkaya et al.[7] have detected MIC values higher than 256 µg/ml for vancomycin and teicoplanin in *E. gallinarum*, *E. avium* isolates. There is no particular protocol suggested by CDC for the patients that are infected or colonized by *E. gallinarum*. However, most researchers emphasize that in spite of the lack of any instructions for those patients, the ability of *E. gallinarum* strains to catch the genes encoding the high level of vancomycin resistance and to transfer them to important clinical strains such as *E. faecium* should not be omitted [4,9,10,24].

In conclusion, in the present study, the isolation rate of *E. faecium* carrying *vanA* gene was low, however, we detected both *E. casseliflavus* and *E. gallinarum* isolates with multidrug resistance in both examined animal groups. When the close contacts between companion animals and the owners, or among farm animals and the farmers, or the cross contamination at the slaughterhouses from intestinal content through to carcasses are considered, the presence of those intrinsic vancomycin resistance and multidrug resistant *Enterococcus* species should never be ignored. Therefore, the importance of general hygiene and management rules as well as routine screening tests is increasing in different breeding facilities.

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Research Article

The Investigation by PCR and Culture Methods of Foulbrood Diseases in Honey Bees in South Marmara Region [1]

A. Ebru BORUM [1] Cüneyt ÖZAKIN [2] Ertan GÜNEŞ [3]
Levent AYDIN [4] Mihriban ÜLGEN [5] İbrahim ÇAKMAK [6]

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[1] Department of Microbiology, Faculty of Veterinary Medicine, Balıkesir University, TR-10145 Balıkesir - TURKEY
[2] Department of Microbiology and Infectious diseases, Faculty of Medicine, Uludag University, TR-16110 Nilufer, Bursa - TURKEY
[3] Vocational School of Technical Sciences, Uludag University, TR-16059 Nilufer, Bursa - TURKEY
[4] Department of Parasitology, Faculty of Veterinary Medicine, Uludag University, TR-16059 Nilufer, Bursa - TURKEY
[5] Department of Microbiology, Faculty of Veterinary Medicine, Uludag University, TR-16059 Nilufer, Bursa - TURKEY
[6] Vocational School of Mustafakemalpaşa, Uludag University, TR-16500 Mustafakemalpaşa, Bursa - TURKEY

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Abstract

American Foulbrood and European Foulbrood diseases of honeybees were examined in 725 beehives from 23 apiaries located in the South Marmara Region of Turkey. We determined that 19 apiaries were infected and the suspected clinical signs of foulbrood diseases were investigated in 102 beehives by PCR and cultural method. Broods and combs from colonies with suspected clinical symptoms of foulbrood diseases were collected and cultured for bacteriological examination. All of the specimens contaminated with bacteriae and 37 species of bacteriae were isolated such as *Stapylococcus epidermidis, Bacillus subtilis, Corynebacterium jeikum, Corynebacterium pseudotuberculosis, Bacillus* spp. All of these bacteria are related to human, animal and environmental origins. In this study, *Paenibacillus larvae* by PCR amplifying the 973-bp region PL1 and PL2 with 1f, *Melissococcus plutonius* amplifying the 973-bp region EFB-F and EFB-R gene were amplified. American Foulbrood causative agent *Paenibacillus larvae* and European Foulbrood causative agent *Melissococcus plutonius* were not detected in any sample examined by PCR and cultural methods. On the other hand, *Paenibacillus alvei* that is a seconder agent to European Foulbrood was found in two samples by cultural methods. In conclusion, the results showed that *P. larvae* and *M. plutonius* are not present in South Marmara Region. In this study, human, animal and environment originated agents were isolated.

Keywords: *Apis mellifera, Honeybee, Foulbrood, PCR*

Güney Marmara Bölgesindeki Bal Arılarının Yavru Çürüklüğü Hastalığı etkenlerinin PZR ve Kültürel Metodlar ile Belirlenmesi

Özet

Güney Marmara Bölgesinde 23 arılıktan 725 kovan Amerikan Yavru Çürüklüğü (AYÇ) ve Avrupa Yavru Çürüklüğü (AvYÇ) hastalığı açısından incelendi. 19 arılıktan AYÇ ve AvYÇ şüpheli ya da infekte 102 kovan PCR ve kültürel metodlar ile incelendi. Klinik olarak yavru çürüklüğü şüpheli kovanlardan petek ve larva örnekleri alınarak bakteriyolojik kültür için kullanıldı. Bütün örnekler 37 tür bakteri izole ve identifiye edildi. Bunlar, *Staphylococcus epidermidis, Bacillus subtilis, Corynebacterium jeikum, Corynebacterium pseudotuberculosis, Bacillus* spp. gibi insan çevre ve hayvan orjinli etkenlerdi. Bu çalışmada, *Paenibacillus larvae* 973 bp'lik PL1 ve PL2, *Melissococcus plutonius*'un 973 bp.'lik EFB-F ve EFB-R gen bölgeleri PCR ile amplifiye edildi. Hiçbir numunede *Paenibacillus larvae* ve *Melissococcus plutonius* üremesi olmadı. AvYÇ'nin sekonder etkeni *Paenibacillus alvei* kültürel yöntemle iki örnekte izole edildi. Bu çalışmada, Güney Marmara Bölgesinde *P. larvae* ve *M. plutonius* varlığı saptanmadı. Çalışmada yavru çürüklüğü şüpheli kovanlardan insan, çevre ve hayvan orjinli etkenler izole edildi.

Anahtar sözcükler: *Apis mellifera, Bal arısı, Yavru Çürüklüğü, PCR*

İletişim (Correspondence)
+90 266 6136692
ebruborum@balikesir.edu.tr

INTRODUCTION

Bacterial, viral and fungal originated brood diseases have been reported [1-3]. The most important diseases of bacterial origin are American Foulbrood (AFB) and European Foulbrood (EFB) in honeybees. AFB is caused by the bacterium *Paenibacillus larvae (P. larvae)*. AFB infection is one of severe bacterial diseases of honeybees and widely contagious. The disease is major problem for many beekeepers and causing great losses to the beekeeping industry. *P. larvae* spores are very highly resistant and could survive in the environment for 30-50 years [2,4-6].

Melissococcus plutonius (M. plutonius) causes EFB, an important disease, that affects brood of honeybees. Several bacteria may be associated with the cases of EFB. Larvae with EFB have a various microflora including *Paenibacillus alvei, Bacterium eurydice, Bacillus laterosporus, Enterococcus faecalis*, and *Paenibacillus apiarius* [7,8].

The accurate laboratory diagnosis of honeybee diseases is very important due to different control and protection methods for bacterial diseases. Traditional diagnosis of bacterial diseases is based on observation of clinical symptoms and microbial cultivation in infected and suspicious materials. Identification of bacterial pathogens of honeybees could be achieved by several methods. There are many sensitive and selective culture media exist to identify the bacterial agents in honeybees. Biochemical characteristics and microscopy of suspected materials are used for routine identification of bacterial agents [8-11].

Molecular techniques have also been developed for the identification of *P. larvae* and *M. plutonius*. PCR-based methods for detecting *P. larvae* have been described by several authors [1,8-13]. The sequences of detection primers were based on 16S rRNA gene of *P. larvae* and *M. plutonius* [8,10,12-14].

The objective of this study was to identify the bacteria that cause the symptoms of foulbrood diseases and the use of the PCR assay, cultivation, in the diagnosis, existence of *P. larvae* and *M. plutonius* from samples.

MATERIAL and METHODS

Larvae were taken from brood combs suspected with clinical signs of foulbrood diseases in apiaries. A total of 725 beehives from 23 apiaries located in South Marmara Region (Bursa, Bilecik, Çanakkale, Balıkesir, Yalova) were examined for AFB and EFB diseases in honey bees *(Table 1)*.

Table 1. *Locations and numbers of hives*
Tablo 1. *Kovan sayıları ve yerleşim bölgeleri*

Locations	Total Numbers of Hives	Numbers of Suspected Hives
Bursa	272	31
Bilecik	65	3
Balıkesir	207	45
Çanakkale	87	5
Yalova	94	18
Total	725	102

Cultivation of Bacteria

Larval remains from brood comb were collected with a sterile swab for the bacterial isolation and suspended in 5 ml of sterile distilled water [8,10,11,14]. The suspension was incubated at room temperature for 10 min and separated into two samples. Vegetative bacteriae in the first part of samples were killed by incubation at 80°C for 10 min. The second part of samples was not heated. The suspension (200 µl) was used for each medium. The suspensions were inoculated on to different mediums. *Paenibacillus larvae* agar (PLA) was prepared according to Schuch et al.[15]. MYPGP agar was prepared as reported by De Graaf et al.[10]. Brain-heart infusion agar (Oxoid CM375) with thiamin (BHIT) agar with 0.1 mg/L thiamine (Sigma T2645), basal medium for *M. plutonius* based on the original formulation has been described by Bailey [16], and Columbia Agar with 5% Sheep Blood (BD 221263). All culture plates were incubated for 24-72 h at 37ºC in aerobic and microaerophilic environment. All plates were examined on daily basis in order to control the growth of bacterial agents. The isolates were examined by light microscopy following gram and carbol fuchsin stain, catalase test and identified with BBL crystal system (BBL Crystal Enteric/Nonfermenter ID and Gram Positive ID Kits -Becton Dickinson and Company, USA) as previously reported authors [3, 8-11,16,17].

Polymerase Chain Reaction (PCR) Assay

We used brood samples for PCR. Primers of PCR were designed on the basis of the 16S rRNA gene of *P. larvae* and selectively amplify a 973-bp amplicon. The PCR primers were used from 16S rRNA gene of *M. plutonius* and 831-bp amplicon. All the negative culture samples for *P. larvae* and *M. plutonius* were subjected to PCR analysis.

The larvae suspected with clinical signs of foulbrood diseases were homogenized in 500 µl PBS. 100 µl homogenate was centrifuged at 14.000 g for 10 min and the obtained pellet was used for DNA isolation. Pellets were suspended in 200 µl enzyme solution (containing of 20 mg lysozyme per ml, 20 mM Tris-HCl in pH 8.0, 2 mM EDTA, and 1.2% Triton) incubated for 37°C for 1 h. After, 25 µl Proteinase K and 200 µl of buffer AL (Qiagen) was added, and the lysates were incubated at 56°C for 30 min and then at 96°C for 5 min. DNA was eluted with 200 µl of elution buffer and stored at -20°C. Bacterial DNA was isolated using the OIAamp DNA minikit (Qiagen) as instructed by the manufacturer [8,10-12,14,17].

Primers used to identify *P. larvae:*

Primer 1: 5'-AAGTCGAGCGGACCTTGTGTTTC -3'

Primer 2: 5-'TCTATCTCAAAACCGGTCAGAGG-3' [10-12].

Primers used to identify *M.plutonius*:

Primer 1: GAAGAGGAGTTAAAAGGCGC

Primer 2: TTATCTCTAAGGCGTTCAAAGG [8,13].

Reference strains of *P. larvae* (ATCC 9545 and NRRL B 3555) and reference strains of *M. plutonius* (ATCC 35311 and NCDO 2443) were used as positive control. Deionized water was used as a negative control.

P. larvae PCR protocol were performed with final volume of 50 µl PCR mixture contained; 5 µl template DNA, 5 µl PCR buffer (containing 2 mM $MgCl_2$), 10 nM of each dNTP, 50 pmol forward and reverse primer, 2 U *Taq DNA polymerase* enzyme (Fermentas).

PCR was performed at 95°C for 15 min followed by 40 cycles of denaturation at 93°C for 1 min, at 55°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR amplified products were examined on agarose (0.8%) gel electrophoresis. DNA amplified by PCR was stained with ethidium bromide and visualised with UV transilluminator [10-12,14,17,18].

M. plutonius PCR protocols were done with 50 µl reaction comprising; 4 mM $MgCl_2$, 200 µM of each dNTP, 100 ng of primers, 5 µl of 10 x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3 µg of *Taq DNA polymerase*.

PCR conditions were performed with initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 6°C for 15 s, primer extension at 72°C for 60 s and final extension cycle at 72°C for 5 min [8,13,19,20]. PCR products were analysed by agarose gel and stained with ethidium bromide. The amplification was performed in a Perkin-Elmer Gene Amp PCR System 2400 Thermocycler.

The amplicons were stored at +4°C until electrophoresis was carried out. Samples of the amplicons (5 µl) were electrophoresed on a agarose gel in Tris-boric acid-EDTA (TBE) gel at 75 V for 1 h. The gel was stained with ethidium bromide (0.5 µg/ml). The PCR products were visualized on a UV light. The sizes of bands were determined by comparing to a standard 100 bp DNA marker (Fermentas). *P. larvae* (ATCC 9545 and NRRL B 3555) and of *M. plutonius* (ATCC 35311 and NCDO 2443) were used as positive control.

RESULTS

After incubation, colonies with different morphologies were Gram and Carbol fuchsin stained and identified by CRYSTAL System (Becton Dickinson, Aalst, Belgium). All of the samples contaminated with bacteriae (100%).

The organisms were isolated in pure culture from 64 samples (62.74%), and isolated in mixed culture from 38 samples (37.25%). Thirty-seven species of bacteriae were isolated from 102 hives *(Table 2)*.

AFB causative agent, *P. larvae,* and EFB primer agent, *M. plutonius,* were not detected in any of the samples. However, *Paenibacillus alvei* was isolated and identified in two samples.

All AFB and EFB suspected samples were negative in PCR. Standard strains were found to be positive in PCR. The microorganisms identified belong to human, animal and environmental origins. All samples showed the clinical findings consistent with AFB and EFB.

Table 2. *Identified bacterial species.*
Tablo 2. *İdentifiye edilen bakteri türleri*

Bacteriae Species	**Strain Number Were Isolated**
Bacillus subtilis	25 (67.56%)
Corynebacterium jeikeium	19 (51.35%)
Staphylococcus epidermidis *Bacillus brevis*	10 (27.02%)
Corynebacterium aquaticum *Bacillus spp.*	9 (24.32%)
Corynebacterium pseudodiphteriticum *Corynebacterium pseudotuberculosis*	8 (21.62%)
Corynebacterium bovis *Staphylococcus pasteuri*	5 (13.51%)
Staphylococcus saprophticus, *Enterococcus faecalis* *Bacillus cereus*	4 (10.81%)
Bacillus pumilus *Aerococcus urinae* *Corynebacterium renale group* *Lactococcus lactis ssp. Cremoris* *Bacillus licheniformis*	3 (8.10%)
Staphylococcus simulans *Gemella morbillorum* *P. alvei*	2 (5.40%)
Staphylococcus warneri *Staphylococcus capitis ssp. capitis* *Alloicoccus otitidis* *Bacillus circulans* *Corynebacterium striatum* *Micrococcus luteus* *Rhodococcus equi* *Sphingomonas paucimobilis* *Corynebacterium spp.* *Providencia stuartii* *Escherichia coli* *Staphylococcus aureus* *Hafnia aluci* *Morganella morgani* *Acinetobacter iwoffii* *Klebsiella oxytoca*	1 (2.70%)

DISCUSSION

American foulbrood is the most dangerous and contagious of the infectious diseases in bees.

Diagnosing of AFB and EFB is time consuming, expensive and has difficult isolation and identification procedures concerning *P. larvae* and *M. plutonius*. PCR is rapid, easy and reliable method for *P. larvae* and *M. plutonius*. PCR is very important tool for diagnosing bee diseases.

In this study, cultural method and PCR assay were tested and existence of *P. larvae* and *M. plutonius* from samples were detected.

Different *P. larvae* and *M. plutonius* identification rates have been reported. Garrido-Bailón et al.[1] reported 1.6% prevalance of *P. larvae* and 0.5% *M. plutonius* in the honey bees. McKee et al.[20] detected *M. plutonius* 27.5% of these samples. Kılıç et al.[17] have identified *P. larvae* in 7% of the samples by the culture growth method and in 8% of the samples by the PCR method. Şimşek and Kalender [22] have isolated *P. larvae* in 32 samples out of 335 (9.55%) in Turkey. Ozakın et al.[3] did not detect AFB and EFB causative agents in Bursa, Turkey. *Paenibacillus alvei*, a seconder agent of EFB, was detected only in two samples. In this study, we did not isolate *P. larvae* and *M. plutonius* by cultivation in suspected larvae of South Marmara Region. Secondary factor of EFB, *P. alvei* were detected in only two samples.

In the study of Govan et al.[12] they used 16S rRNA gene of *P. larvae,* then, selectively amplified a 973-bp amplicon and revealed that this amplicon had high sensitivity. Dobbelaera et al.[21] reported that they used technique to detect *P. larvae* in the DNA extracts obtained from larvae remains infected with American foulbrood. Kılıç et al.[17] reported Af 6 and Af 7 primer pair is a highly sensitive primer pairs. Govan et al.[13] reported that PCR technique is a rapid and reliable method for identifying *M. plutonius* directly from diseased bee larvae. Djordjevic et al.[19] reported that MP1 and MP2 have been shown to be specific for *M. plutonius*. The detection of *M. plutonius* in larvae, in healthy and diseased hives by PCR provides a specific and sensitive method for epidemiological studies in EFB [13,20].

PCR technique is a quick and reliable method for the identification of *P. larvae* and *M. plutonius* directly from larvae samples. PCR procedure of takes approximately 24 hours, cultivation and identification of *P. larvae* and *M. plutonius* may take 3-7 days. The diseases of AFB and EFB cause serious colony losses, thus the short-term diagnosis would prevent spread of these diseases as well as the losses of beekeepers. Therefore, PCR is highly reliable and quick test in the diagnosis of AFB and EFB.

In this study, suspected samples were examined with cultural and PCR methods. In both methods, *P. larvae* and *M. plutonius* were detected as negative. Secondary agent of EFB, *Paenibacillus alvei* was detected as positive by cultural method.

We identified bacteriae related to human, animal and environmental origins. All hives showed similar clinical symptoms consistent with AFB and EFB. Minor brood diseases are less serious than foulbrood diseases. However, these agents are extremely contagious [2]. Clinical signs are similar. Thus, it is difficult to distinguish AFB and EFB. Beekeepers must recognise these signs, and distinguish them from foulbrood diseases. If beekeepers pay attention to basic hygienic practises, it is likely to prevent diseases. Lack of hygiene can cause serious losses. Therefore, accurate diagnosis of the foulbrood diseases prevents spreading diseases and consequent economic losses [23].

In conclusion, the results showed that *P. larvae* and *M.plutonius* are not present in South Marmara Region. Secondary agent of EFB, *Paenibacillus alvei* were detected in only two samples. AFB and EFB causative agents were not detected by cultural and PCR methods. In this study, human, animal and environment originated agents were isolated.

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Research Article

The Effects of Lichens Extracts in The Healthy Rats and The Medical Utility of These Extracts in The Prevention of Diabetes-Associated Multiple Organ Failures

Suat ÇOLAK [1] Fatime GEYIKOĞLU [2] Hasan TURKEZ [3] Murat BAKIR [2]
Mirkhalil HUSSEINIGOUZDAGANII [2] Serpil CAN [4] Gülşen ÇIĞŞAR [5] Ali ASLAN [6]

[1] Artvin Coruh University, Department of Biology, Faculty of Science and Arts, TR-08100 Artvin - TÜRKİYE
[2] Atatürk University, Department of Biology, Faculty of Science, TR-25100 Erzurum - TÜRKİYE
[3] rzurum Technical University, Department of Molecular Biology and Genetics, Faculty of Science, TR-25100 Erzurum - TÜRKİYE
[4] Kafkas University, Department of Physiology, Faculty of Medicine, TR-36100 Kars - TÜRKİYE
[5] Kafkas University, Department of Emergency Medical, Faculty of Medicine, TR 36100 Kars - TÜRKİYE
[6] Atatürk University, Department of Biology, Kazim Karabekir Education Faculty, TR-25100 Erzurum - TÜRKİYE

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Abstract

In the present study, we firstly assessed *Cetraria islandica* and Pseudevernia furfuracae to avoid detrimental effects on multiple tissues of rats. Diabetes mellitus (DM) with the subsequent generation of oxidative stress represents a major risk factor for organs. The second aim of this study is to investigate whether administration of both lichens could prevent type 1 diabetes (T1D)-induced organ dysfunctions. During two weeks, both control and diabetic rats were treated with aqueous lichen extracts. The metabolic changes were determined. On day 14, after animals were decapitated, required samples for biochemical and genetic analysis were collected. Oxidative damage of DNA was estimated by measuring the increase in 8-hydroxy-2'-deoxyguanosine formation. Biochemical parameters were used to observe and evaluate the functional changes in tissues. Experimental data showed that the increasing doses of lichens alone have not any detrimental effect on above parameters. Moreover, *C. islandica* decreased the diabetes-induced glucose and malondialdehyde (MDA) levels. Thus, it seemed that the antioxidant treatment has an important effect on the organ failure in ill rats. However, the protective effect of *C. islandica* was inadequate on diabetes-induced disorders and DNA damages. Lichens are a safe in the studied dose range but the power of *C. islandica* is limited because of intensive oxidative stress in essential organs of T1D rats.

Keywords: *Cetraria islandica, Pseudevernia furfuracae, Diabetes mellitus, Organ dysfunctions, Antioxidant capacity, 8-hydroxy-2'-deoxyguanosine*

Liken Ekstrelerinin Sağlıklı Ratlar Üzerindeki Etkileri ve Bu Ekstrelerin Diyabete Bağlı Çoklu Organ Yetmezliğinin Önlenmesinde Medikal Kullanımı

Özet

Bu çalışmada, ilk olarak *Cetraria islandica* ve *Pseudevernia furfuracae* türü liken ekstraktlarının sıçanlara ait çeşitli dokular üzerine zararlı etkilerinin olup olmadığı değerlendirilmiştir. Diabetes mellitusa (DM) bağlı oksidatif stres, organlar için büyük bir risk faktörü oluşturmaktadır. Çalışmamızın ikinci bölümünde ise her iki liken ekstaktı uygulamasının tip 1 diyabet kaynaklı organ bozukluklarına karşı koruyucu olup olmadıklarını araştırılmıştır. İki hafta boyunca hem kontrol hem de diyabetli ratlara sulu liken ekstreleri uygulanmıştır. Süre sonunda metabolik değişimler belirlenmiştir. Ondördüncü günde, hayvanların yaşamları servikal dislokasyonla sonlandırıldıktan sonra biyokimya ve genetik çalışmalar için gerekli örnekler alınmıştır. DNA'nın oksidatif hasarı 8-hidroksi-2'-deoksiguanosin oluşumunda meydana gelen artış ölçülerek hesaplanmıştır. Biyokimyasal parametreler dokularda meydana gelen fonksiyon değişimlerinin gözlenmesi ve değerlendirilmesi için kullanılmıştır. Deney sonuçları tek başına uygulanan liken ekstraktlarının artan dozlarda herhangi bir zararlı etkiye sahip olmadığını göstermiştir. Ayrıca, *C. islandica* diyabete bağlı artan kan glikoz ve malondialdehit (MDA) seviyesini kısmen düşürmüştür. Antioksidan tedavinin hasta ratlarda organ yetmezliğine karşı fayda sağlayacağı düşünülmektedir. Bununla birlikte, *C. islandica*'nın antioksidan etkisinin diyabet teşvikli bozukluklarda ve DNA hasarına karşı yetersiz olduğu anlaşılmıştır. Sonuç olarak, likenlerin çalışılan doz aralığında güvenli olduğu ve tip 1 diyabetin neden olduğu yaşamsal organlar üzerindeki güçlü oksidatif stres nedeniyle *C. islandica* sınırlı bir etkiye sahip olduğu gözlenmiştir.

Anahtar sözcükler: *Cetraria islandica, Pseudevernia furfuracae, Diyabetes mellitus, Organ bozuklukları, Antioksidan kapasite, 8-hidroksi-2'-deoksiguanosin*

İletişim (Correspondence)
+90 442 2314325
geyikogluff@yahoo.com

INTRODUCTION

DM is one of the most common endocrine metabolic disorders in developing countries. T1D affects millions of people worldwide and has an incidence that is increasing at a striking rate, especially in young children [1]. A large body of earlier investigations has examined the role of oxidative stress in insulin resistance in type 1 and type 2 diabetes and its associated complications [2]. The investigations have revealed that hyperglycemia degrades antioxidant enzyme defenses by allowing reactive oxygen species to damage cells and tissues [3]. The balance of glucose by the drugs has emerged as a novel therapeutic approach to disease that develop with high circulating glucose. Lichens have been used for various purposes such as dyes, perfumes and remedies in folk medicine indicating the pharmaceutical potential of lichens [4]. The lichens have also antioxidant, antimicrobial and anticancer properties [5,6]. It is documented that they effective in the treatment of tuberculosis [7], hemorrhoids and dysentery [8] and also induce apoptosis in colon [9,10] and prostate cancers [11]. Lichen species are very common in Turkey. Unique lichen flora has attracted many researchers on the systematical basis [12]. It is pointed that lichens may be easily accessible sources of natural drugs that could be used as a possible food supplement or in pharmaceutical industry [13].

Diabetic nephropathy (DN) remains the most common cause of end-stage renal disease [14]. Chronic kidney disease (CKD) is characterized by progressive decline in renal function [15]. To prevent the development of this disease and to improve advanced kidney injury, effective therapies are required. Although diabetic hepatopathy is potentially less common, it may be appropriate for addition to the list of target organ conditions related to diabetes [16]. Enzymology is a diagnostic indicator for diabetes in liver dysfunctions. Therefore, liver function tests (LFTs) are commonly used in clinical practice to screen the progression of disease, and monitor the effects of hepatic drugs. The most common LFTs include the serum aminotransferases as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and lactate dehydrogenase (LDH). A rational therapy rich in antioxidants reduces levels of metabolical disorders in diabetic individuals [17]. Here we used a strategy based on feasible alternative therapeutic for metabolically labile patients with T1D. So far, lichens have not been evaluated under controlled conditions suggesting improved glucose control and a reduced risk of hypoglycemia. This opens up an important strategy for therapy of diabetes and may provide a promising avenue for future approaches to lichens. In response to pharmacological activation or oxidative stress, we studied two well-known lichen species *Cetraria islandica (C. islandica)*, *Pseudevernia furfuracea (P. furfuracea)* in relation to the survival of liver, pancreas and kidney in model with T1D. In many countries, *C. islandica* is used medicinally e.g. for colds, bronchitis, and asthma [18] and *P. furfuracea* is studied for antioxidant properties *in vitro* conditions [19].

Although these lichens are popular around the world, their beneficial and/or adverse effects on human health have not been scientifically determined yet. Hence, our study firstly assessed *C. islandica* and *P. furfuracea* extracts to avoid detrimental effects on multiple tissues of rats *in vivo* conditions. Secondly, the aim of present study was to evaluate the effects of *C. islandica* and *P. furfuracea* on the development of diabetes-related organ dysfunctions, in relation to biochemical changes and also DNA damages in diabetic rats. The correlation of DNA damage with adverse health effects is important in evaluating the safety of various agents and prospective therapeutics. Many techniques exist that afford the ability to identify and measure cellular DNA damage upon exposure to a suspected genotoxic agent; however, the modified nucleoside 8-hydroxy-2'-deoxyguanosine (8-OHdG) is commonly used as a reliable and sensitive index of oxidative DNA damage [20].

There is a very little information regarding the biological activity of these two lichen species. Therefore, in this study, the effects of two lichen species at the different doses (250 and 500 mg/kg bw) were investigated in non-diabetic, diabetic and lichen-treated diabetic rats.

MATERIAL and METHODS

Lichen Extracts

Lichen species, *C. islandica* (L.) Ach. and *P. furfuracea* (L.) Zopf were collected from the Giresun, Erzurum and Artvin province in Turkey, during summer of 2011. The samples were identified using various flora books and papers [12,21]. Identified samples were air-dried and stored in the herbarium of Kazım Karabekir Education Faculty, Atatürk University. For water extraction of lichenes, 20 g sample was mixed with 400 mL distillated and boiling water using magnetic stirrer for 15 min. Then the extracts were filtered over Whatmann No. 1 paper. The filtrates were frozen and lyophilized in lyophilizator (Labconco, Freezone IL) [19].

Animals

Seventy adult male Spraque-Dawley rats (6 weeks old, weighing 200-250 g) obtained from Medical Experimental Application and Research Center, Atatürk University were used. Animals were housed inside polycarbonate cages in an air-conditioned room (22±2°C) with 12-h light-dark cycle. Standard rat feed and water were provided ad libitum. The rats were allowed to acclimatize to the laboratory environment for 7 days before the start of the experiment. All procedures were performed in conformity with the Institutional Ethical Committee for Animal Care and Use at Atatürk University (Protocol Number: ATADEM, B.30.2.ATA.0.23.85-11 and Date: 26.03.2010) and the Guide for the Care and Use of Laboratory Animal.

Experimental Induction of Diabetes

Streptozotocin (STZ) (single dose of 50 mg/kg bw) (purchased from Sigma, St Louis, MO, USA) dissolved in freshly prepared 0.01 M citrate buffer pH 4.5 was injected intraperitoneally in a volume of 1 ml/kg. STZ-induced animals exhibited massive glycosuria and hyperglycemia within 2 days. T1D was confirmed in STZ-induced rats by measuring the fasting blood glucose concentration 72 h after the injection of STZ. The rats with blood glucose level>200 mg/dl were considered to be diabetic and were used in the experiment [22].

Experimental Design

There was very little evidence in the literature about lichens and their uses on therapy and therefore the study included from 5 to 500 mg/kg large-dose range. Use of large-dose range of lichens provided a more convenient and potentially more effective strategy for assisting diabetic recovery. The effective doses were 250 and 500 mg/kg and thus the rats were randomly divided into ten groups (n=7) as follows *(Table 1)*:

The aqueous extracts of lichens were administered intraperitoneally daily for 14 days. After completion of the experiments, rats were killed via rapid decapitation without anaesthetic. The body weight, feed, and water intake in animals were measured. The blood and tissue samples of rats were collected for biochemical and oxidative DNA damage analyses.

Biochemical Analyses

The blood glucose levels were measured with a automatic glucometer (Accu-Chek GO®) in a tail blood before decapitation. The rat serum insulin levels were detected by commercial elisa kit purchased from Randox Laboratories Ltd Dialab Produktion und laborinstriementen Gessellschaftm.b.H. (A-1160 Wien-panikengasse, Germany). The serum samples were prepared by centrifugation at 1.600 g at 4°C for 15 min using a cooling centrifuge (Nüve, NF 400R, Turkey). The blood glucose and serum insulin levels were respectively expressed in mg/dL and µg/L.

Blood samples were collected into serum superetor tubes (Microtainer; Becton Dickinson, Franklin Lakes, NJ, USA), allowed to stand (75-90 min), centrifuged (11.000 g, 5 min), serum harvested, and stored at -20°C. The following parameters for liver injury were measured by an automated biochemical analyzer (OLYMPUS AU 2700) with Bayer testing kits (Bioclinica): AST, ALT and LDH. Renal impairment was assessed by blood urea nitrogen (BUN) and serum creatinine (Cr) and uric acid (UA) levels with an autoanalyzer (Syncron LX 20, Galway, Ireland) by using commercial Beckman Coulter diagnostic kits. After the 14 day treatment, the rats were fasted overnight and then decapitated. Thoracic cavities were incised open; pancreas, liver, and kidney tissues were removed and stored at −70°C. All defrosted tissues were homogenized in ice shower containing 4 mL of 0.2 M phosphate buffer at pH 7.4. Homogenates were centrifuged at 3.000 g for 15 min at 4°C to remove tissue remnants. Activities were determined in the supernatant. Total antioxidant capacity (TAC) levels were measured using the commercial kit (Rel Assay Diagnostic) Syncron LX autoanalyzer [23] Lipid peroxidation was determined by quantifying malondialdehyde (MDA) concentrations, which was spectrophotometrically measured by the absorbance of a red-colored product with thiobarbituric acid [24].

Nucleic Acid Oxidation

DNA was extracted from 100 mg tissue samples by commercially available DNA extraction kits (DNAzol® Life Technologies, Gaithersburg, MD, USA). Via using these kits up to approximately 5 µg of DNA/mg of tissue was obtained. Extracted DNA was dissolved in water at 1-5 mg/mL. Then DNA samples were converted to single-stranded DNA by incubating the sample at 95°C for 5 min and rapidly chilling on ice, and digested to nucleosides by incubating the denatured DNA with 5-20 units of nuclease P1 for 2 h at 37°C in 20 mM sodium acetate, pH 5.2, and following with treatment of 5-10 units of alkaline phosphatase

Table 1. *Experimental groups*
Tablo 1. *Deney grupları*

Non-diabetic	**Non-diabetic control**	**Group 1 (NC):** Non-diabetic control rats (n=7)
	Treated with CIAE	**Group 3 (CIAE-250):** Rats recevied 250 mg/kg bw aqueous extract *of Cetraria islandica* (n=7)
		Group 4 (CIAE-500): Rats recevied 500 mg/kg bw aqueous extract *of Cetraria islandica* (n=7)
	Treated with PFAE	**Group 5 (PFAE-250):** Rats recevied 250 mg/kg bw aqueous extract *of Pseudevernia furfuracea* (n=7)
		Group 6 (PFAE-500): Rats recevied 500 mg/kg bw aqueous extract *of Pseudevernia furfuracea* (n=7)
Diabetic	**Diabetic control**	**Group 2 (DC):** Diabetic control rats recevied 50 mg/kg bw single injection (ip) of STZ (n=7)
	Treated with CIAE	**Group 7 (CIAE-250):** Diabetic rats treated with 250 mg/kg bw aqueous extract *of Cetraria islandica* (n=7)
		Group 8 (CIAE-500): Diabetic rats treated with 500 mg/kg bw aqueous extract *of Cetraria islandica* (n=7)
	Treated with PFAE	***Group 9 (PFAE-250):*** Diabetic rats treated with 250 mg/kg bw aqueous extract *of Pseudevernia furfuracea* (n=7)
		Group 10 (PFAE-500): Diabetic rats treated with 500 mg/kg bw aqueous extract *of Pseudevernia furfuracea* (n=7)

for 1 h at 37°C in 100 mM Tris, pH 7.5 [25]. The levels of 8-OHG in the samples were then quantiated using a HPLC technique with electrochemical detection (HPLC-EC) by an adaptation of the method of Kasai et al.[26] as described previously [27]. Briefly we used Kontron HPLC pump for 420 as HPLC apparatus, Beckman ultrasphere (0.46 x 25 cm) as column, and 8% aqueous methanol containing 10 mM NaH_2PO_4 as eluent, the flow rate was 1 ml/min. Peaks gained with electrochemical (for 8-OHdG) and UV (for dO) detectors were integrated under a background noise corection loaded on an integrator. The levels of 8-OHdG were determined as numbers of 8-OHdGs per l06 dOs, by calibration against curves from runs of standard samples, containing known amounts of authentic 8-OHdG and dG. During the asays, light and air contamination were avoided as strictly as posible.

Statistical Analysis

For statistical analysis, we used SPSS for Windows 13.0 (SPSS Inc., Chicago, USA). The experimental data were analysed using oneway analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparisons. Results are presented as mean ± standart deviation (SD) and values $P<0.05$ were regarded as statistifically significant.

RESULTS

The diagnostic parameters of both control and experimental rats before and after 14 days of treatment with STZ are shown in *Fig. 1* and *Fig. 2*. According to figures, the feed and water intake in diabetic rats are significantly increased throughout the study period as compared with the normal control group. Intraperitoneal administration of two lichen species extracts with low doses did not have a positive effect on diagnostic and body weight compared with diabetic rats. Moreover, similar results were also observed with higher doses administration of lichen extracts (data not shown). Conversely, the weight loss of ill animals is prominent.

In addition, intraperitoneal administration of STZ into the rats causes significant diabetogenic response in subjects with significant increase in the level of blood sugar and a significant decrease in the level of insulin as compared with normal rats (*Fig. 3* and *Fig. 4*). The blood glucose level is increased from an avarage 100 mg/dL to about 450 mg/dL. And the insulin level is decreased to 4 µg/L from 9 µg/L. Following intraperitoneal injection of *C. islandica* and *P. furfuracea* extracts, the feed and water intake are not changed as compared with the diabetic untreated rats. Furthermore, the extracts alone have not any adverse effect the body weight of the animals, blood glucose and insulin levels (data not shown). In animals with T1D, the treatment with *C. islandica* does not show positive effect on above parameters except for glucose and insulin levels. It is established that the administration of lichen at the dose 250 mg/kg, the blood glucose reduces and insulin levels increases as compared with diabetic rats ($P>0.05$). Depending on the increasing dose, the responses are not enhanced and such changes go unnoticed in T1D rats. Unfortunately, above parameteres remain unchange with respect to 250 mg/kg dose of *P. furfuracea* in diabetic animals and also both lichen extracts do not show any evidence of dose-related effect. Often such change goes unnoticed in pictures, although it can affect preference.

Table 2 shows the effects of *C. islandica* and *P. furfuracea* on biochemical parameters in all experimental groups. As compared with the controls, the TAC level are markedly decreased in pancreas, kidney and liver of diabetic rats

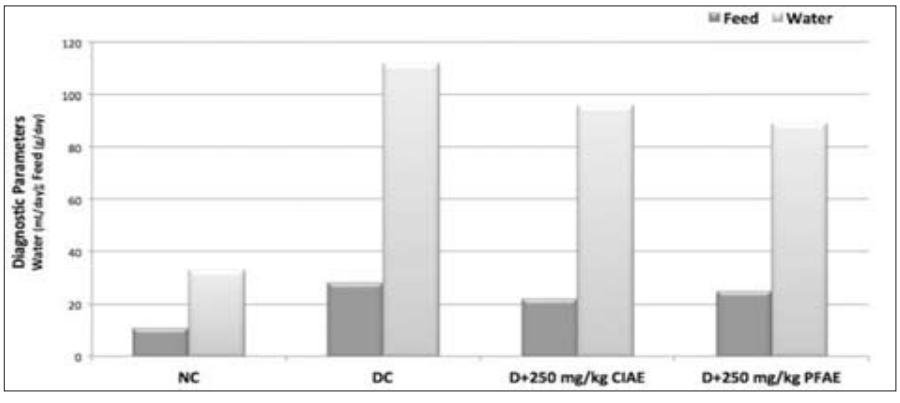


Fig 1. The effect of aqueous extracts of *C. islandica* (CIAE) and *P. furfuracea* (PFAE) on feed and water intake in control and diabetic rats (n=7); **NC:** Non-diabetic control group, **DC:** Diabetic control group, **D+CIAE:** Diabetic rats treated with *C. islandica* aqueous extract (250 or 500 mg/kg) and **D+PFAE:** Diabetic rats treated with *P. furfuracea* aqueous extract (250 or 500 mg/kg)

Şekil 1. Kontrol ve diyabetik sıçanlarda yem ve su alımı üzerindeki *C. islandica* (CIAE) ve *P. furfuracea* (PFAE) sulu ekstraklarının etkisi (n=7); **NC:** Non-diyabetik kontrol grubu, **DC:** Diyabetik kontrol grubu, **D+CIAE:** *C. islandica* sulu ekstresi ile tedavi edilen diyabetik sıçanlar (250 veya 500 mg/kg) ve **D+PFAE** islandica: *P. furfuracea* sulu ekstresi ile tedavi edilen diyabetik sıçanlar (250 veya 500 mg/kg)

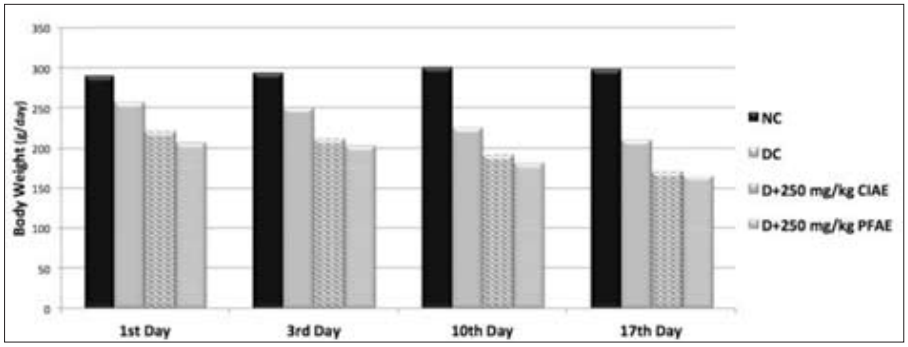
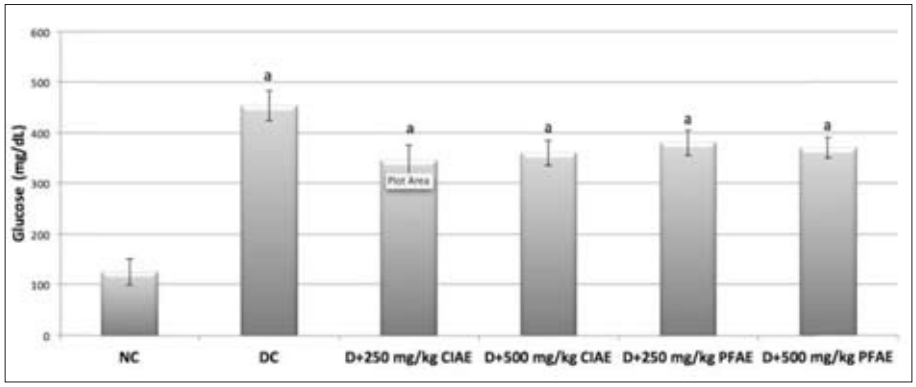


Fig 2. The effect of aqueous extracts of *C. islandica* (CIAE) and *P. furfuracea* (PFAE) on body weight in control and diabetic rats (n=7)

Şekil 2. *C. islandica* (CIAE) ve *P. furfuracea* (PFAE) sulu ekstraktlarının kontrol ve diyabetik sıçanların vücut ağırlığına etkisi (n=7)

Fig 3. The effect of aqueous extracts of *C. islandica* (CIAE) and *P. furfuracea* (PFAE) on blood glucose level in control and diabetic rats (n=7); Data are mean±SD values (n=7). They are significantly different between [a]NC and [b]DC group. (P>0.05; Tukey's multiple range test)

Şekil 3. *C. islandica* (CIAE) and *P. furfuracea* (PFAE) sıvı ekstraktlarının kontrol ve diyabetik sıçanların kan glukoz seviyesine etkisi (n=7); Veriler ort±SD değerlerdir (n=7). [a]NC ve [b]DC gruplar arasında anlamlı bir farklılık vardır. (P>0.05; Tukey çoklu karşılaştırma testi)



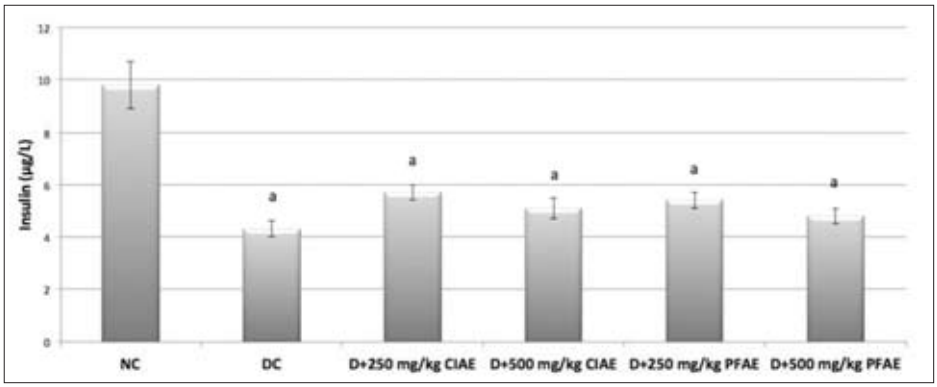


Fig 4. The effect of *C. islandica* (CIAE) and *P. furfuracea* (PFAE) extracts on serum insulin level in control and diabetic rats (n=7); Data are mean±SD values (n=7). They are significantly different between aNC and bDC group. (P > 0.05; Tukey's multiple range test)

Şekil 4. *C. islandica* (CIAE) and *P. furfuracea* (PFAE) ekstraktlarının kontrol ve diyabetik sıçanların serum insulin seviyelerine etkisi (n=7); Veriler ort±SD değerlerdir (n=7). [a]NC ve [b]DC gruplar arasında anlamlı bir farklılık vardır. (P> 0.05; Tukey çoklu karşılaştırma testi)

while MDA increased (P<0.05). In alone lichen treatments, MDA levels remain unchanged and lichens do not show any adverse effect with respect to increasing dose. Furthermore, the *P. furfuracea* extract increases the level of TAC at both dosage (250 and 500 mg/kg) but the best result is observed at the doses of *C. islandica*. However, the increase of TAC after supplementation with *C. islandica* is inadequate STZ-induced diabetic groups and MDA levels does not return to the control values (P>0.05). Moreover, the effect of extracts on these parameters is not dose related (data not shown).

In the rat kidney tissues, the levels of BUN and UA decrease in the *C. islandica* 250 and *C. islandica* 500 mg/kg groups when compared with the T1D *(Table 3)*; however, statistically significant differences were not seen between these groups and the control groups (P>0.05). Serum Cr is not reduced in T1D+*C. islandica* and T1D+*P. furfuracea* groups, whereas, all parameters are similar in experimental rats with *C. islandica* and *P. furfuracea* alone to controls.

In present study, activity levels of serum marker enzymes of liver are found elevated markedly in rats with T1D *(Table 4)*. No such changes are observed in control rat samples. As is evident from table, lichen doses alone (250 and 500 mg/kg) are not change the activity levels

Table 2. *The effect of C. islandica (CIAE) and P. furfuracea (PFAE) extracts on MDA and TAC levels in pancreas, kidney and liver tissues in normal and diabetic rats*

Table 2. *Kontrol ve diyabetik ratlarda pancreas, böbrek ve karaciğer dokuları TAC ve MDA seviyeleri üzerine C. islandica (CIAE) ve P. furfuracea (PFAE) ekstraktlarının etkisi*

Groups$^{\Psi}$ (mg/kg bw)	**MDA (nmol/g tissue)**			TAC (mmol Trolox Equiv/L)		
	Pancreas	**Liver**	**Kidney**	**Pancreas**	**Liver**	**Kidney**
Normal	4.33±0.97^{a}	3.46±0.43^{a}	6.61±0.32^{a}	1.51±0.12^{d}	1.81±0.76^{d}	1.63±0.3^{d}
Diabetic control	9.62±1.01^{b}	8.02±0.85^{b}	13.79±1.06^{b}	0.79±0.17^{f}	1.07±0.11^{f}	0.94±0.13^{f}
CIAE (250)	4.17±0.66^{a}	3.04±0.69^{a}	6.23±0.73^{a}	1.95±0.16^{a}	2.23±0.31^{a}	2.14±0.49^{a}
CIAE (500)	4.26±1.09^{a}	3.28±0.29^{a}	6.36±0.44^{a}	1.79±0.29^{b}	2.11±0.17^{b}	1.97±0.29^{b}
PFAE (250)	4.36±0.59^{a}	3.35±0.21^{a}	6.47±0.81^{a}	1.71±0.19^{b,c}	2.04±0.22^{b,c}	1.87±0.34^{b,c}
PFAE (500)	4.42±0.78^{a}	3.51±0.75^{a}	6.58±0.37^{a}	1.63±0.28^{c}	1.96±0.46^{c}	1.79±0.26^{c}
Diabetic+ CIAE(250)	9.17±0.85^{b}	7.65±0.39^{b}	13.39±1.52^{b}	0.99±0.37^{e,f}	1.26±0.43^{e,f}	1.13±0.33^{e,f}
Diabetic+ CIAE(500)	9.55±1.02^{b}	7.93±0.46^{b}	13.72±1.64^{b}	0.83±0.32^{f}	1.12±0.25^{f}	1.0±0.28^{f}
Diabetic+ PFAE(250)	9.59±0.88^{b}	7.98±0.37^{b}	13.76±1.73^{b}	0.81±0.14^{f}	1.11±0.21^{f}	0.98±0.13^{f}
Diabetic+ PFAE(500)	9.60±1.06^{b}	8.01±0.54^{b}	13.78±1.41^{b}	0.80±0.24^{f}	1.08±0.39^{f}	0.95±0.19^{f}

$^{\Psi}$*Values are mean±SD of seven rats in each group (n=7). Means of five measurements marked by different letters within each column present a statistical difference at P<0.05 CIAE: C. islandica aqueous extract (250 or 500 mg/kg) and PFAE: P. furfuracea aqueous extract (250 or 500 mg/kg)*

Table 3.*The effect of C. islandica (CIAE) and P. furfuracea (PFAE) extracts on serum BUN, UA and Cr levels in normal and diabetic rats*

Table 3. *Kontrol ve diyabetik ratlarda serum BUN, UA ve Cr seviyeleri üzerine C. islandica (CIAE) ve P. furfuracea (PFAE) ekstraktlarının etkisi*

Groups$^{\Psi}$	**BUN (U/L)**	**UA (U/L)**	**CRE (U/L)**
Normal	24.67±3.54	1.35±0.13	0.37±0.04
Diabetic control	91.11±9.17^{a}	3.87±0.89^{a}	0.84±0.08^{a}
CIAE (250 mg/kg)	23.94±4.15^{b}	1.17±0.54^{b}	0.48±0.05^{b}
CIAE (500 mg/kg)	24.60±2.42^{b}	1.42±0.61^{b}	0.39±0.02^{b}
PFAE (250 mg/kg)	24.43±5.01^{b}	1.21±0.49^{b}	0.34±0.01^{b}
PFAE (500 mg/kg)	23.27±7.16^{b}	1.33±0.44^{b}	0.36±0.02^{b}
Diabetic+ CIAE (250 mg/kg)	86.41±6.94^{a}	3.22±0.21^{a}	0.79±0.06^{a}
Diabetic+ CIAE (500 mg/kg)	90.17±6.82^{a}	3.79±0.38^{a}	0.81±0.07^{a}
Diabetic+ PFAE (250 mg/kg)	91.56±7.01^{a}	3.85±0.29^{a}	0.82±0.08^{a}
Diabetic+ PFAE (500 mg/kg)	91.87±5.37^{a}	3.91±0.41^{a}	0.85±0.07^{a}

$^{\Psi}$*Values are mean±SD of seven rats in each group (n=7).* ^{a}*Significant difference was detected in experimental groups comparing with normal group at P<0.05.* ^{b}*Significant difference was detected in experimental groups comparing with diabetic control group at P<0.05*

of AST, ALT and LDH (P>0.05). And the treatment with *C. islandica* can bring a decrease in the activity levels of these enzymes when compared to *P. furfuracea* group. Our results clearly reveal that *C. islandica* presents positive effects on the activities of enzymes without undepending on dose against T1D-induced liver damages. However, the levels of all ALT and AST enzyme samples do not show a significant similarity with control values after treatments with *C. islandica* in T1D. Moreover, the LDH enzyme activity is not normalized by doses of *C. islandica* exposure.

The status of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in studied tissues of control and experimental groups is presented in *Fig. 5*. Compared with the control group, there are significant increases in hepatic, kidney and pancreatic 8-OHdG levels in STZ-induced diabetic rats (P<0.05). Whereas, all doses of *C. islandica* and *P. furfuracae* extracts alone have not any effect on the level of 8-OHdG. However, post-treatment of *C. islandica* extracts can't significantly restore the oxidative DNA damage in the multiple tissues of diabetic rats without undepending on dose.

DISCUSSION

Increased throughput of any substrate involves a regional imbalance between ROS production and breakdown. Oxidative stress is a consequence of high circulating glucose levels in diabetic patients and rodents. Furthermore, a change in the balance of glucose substrate entails a degree of cellular oxidative stress [28]. In present study we assessed indicator of oxidative stress, MDA, in

Table 4. *The effect of C. islandica (CIAE) and P. furfuracea (PFAE) extracts on serum AST, ALT and LDH levels in control and diabetic rats*
Tablo 4. *Kontrol ve diyabetik ratlarda serum AST, ALT ve LDH seviyeleri üzerine C. islandica (CIAE) ve P. furfuracea (PFAE) ekstraktlarının etkisi*

Groups[Ψ]	AST (U/L)	ALT (U/L)	LDH (U/L)
Normal	167±26.57	51.15±6.27	1335.21±134.76
Diabetic control	371.1±43.63[a]	82.91±4.72[a]	2069.48±204.11[a]
CIAE (250 mg/kg)	172.9±24.71[b]	51.19±3.94[b]	1305.56±153.45[b]
CIAE (500 mg/kg)	174.3±27.91[b]	54.39±2.99[b]	1349.35±126.24[b]
PFAE (250 mg/kg)	169.7±35.07[b]	53.25±5.17[b]	1363.40±143.46[b]
PFAE (500 mg/kg)	170.5±24.03[b]	51.72±4.62[b]	1427.72±137.08[b]
Diabetic + CIAE (250 mg/kg)	316.4±36.24[a]	75.87±3.66[a]	1995.87±171.49[a]
Diabetic + CIAE (500 mg/kg)	341.7±48.91[a]	79.30±4.01[a]	2027.19±185.53[a]
Diabetic + PFAE (250 mg/kg)	360.2±34.27[a]	81.48±3.87[a]	1994.21±194.34[a]
Diabetic + PFAE (500 mg/kg)	368.4±29.06[a]	83.55±3.21[a]	2097.71±49.37[a]

[Ψ]Values are mean±SD of seven rats in each group (n=7). [a]Significant difference was detected in experimental groups comparing with normal group at P<0.05. [b]Significant difference was detected in experimental groups comparing with diabetic control group at P<0.05

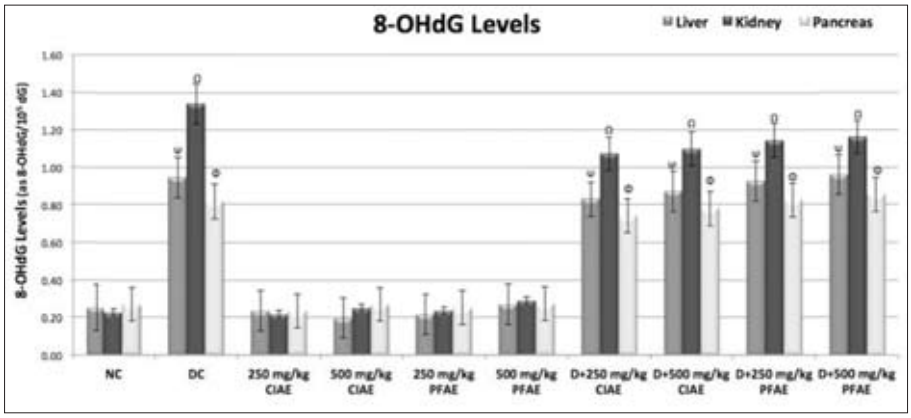


Fig 5. The effect of *C. islandica* (CIAE) and *P. furfuracea* (PFAE) extracts on 8-OHdG levels on liver, kidney and pancreas tissues in normal and diabetic rats (n=7); The symbols used for different tissues indicate the groups that are significantly difference from control group

Şekil 5. *C. islandica* (CIAE) ve *P. furfuracea* (PFAE) ekstraktlarının, normal ve diyabetik sıçanların karaciğer, böbrek ve pankreas dokularında 8-OHdG düzeyleri (n=7); Farklı dokular için kullanılan simgeler, kontrol grubu ile karşılaştırıldığında anlamlı olan grupları temsil eder

pancreas, kidney and liver cells. MDA is an important by-product of lipid peroxidation (LPO), and therefore high levels of MDA lead to oxidative damage to cell membrane lipids [29]. Regrettably, the results presented in this work showed that high glucose allowed the production of MDA effectively in multiple tissues of diabetic rats. It is reported that oxidative stress is a major mediator of tissue and cell injuries [30]. Hence, *in vivo* risk assessment for lichens needs exploration. This study assesses the oxidative effects of *C. islandica* and *P. furfuracea* extracts on pancreas, liver and kidney tissues of rats. Fortunately, MDA levels remained unchanged and lichens alone did not show any adverse effect with respect to increasing dose. Targets of accumulating ROS include proteins involved in antioxidant response. Thus, there is accumulating evidence that interaction of the antioxidant defense system as a regulator of disease development and oxidative stress generation in diabetic patients evokes diabetic organ complications, liver and kidney dysfunctions, and pancreatic β-cell apoptosis [30,31]. These organs contain a number of endogenous antioxidants, to restrict steady state ROS levels. The balance between ROS generation and their elimination by endogenous antioxidant mechanisms play a critical role in preserving organ functions; inappropriate levels of ROS likely precipitate impairment of functions and abnormalities in organ structure [32]. A variety of natural antioxidants exists to scavenge oxygen free radicals and prevents oxidative damage to biological structures. The natural products have antioxidant potential to protect the essential organs against the oxidative damage induced by diabetes [33]. Nowadays, lichens as a novel bioresource for natural antioxidant have been discovered [34]. *P. furfuracea* is shown to modulate the adverse effects of AFB(1) in human blood cells [19]. The antioxidant properties of *C. islandica* and *P. furfuracea* are explained *in vitro* [5,6,19], but not *in vivo*. Our results corroborate this antioxidant activity of lichens and extracts have attractive properties with increasing TAC levels in pancreas, liver and kidney tissues *in vivo*. In these tissues, *C. islandica* significantly increased TAC level and P. furfuracea presented moderate antioxidant supplement for tissues. TAC combines the activities of non-enzymes such as glutathione (GSH) and enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [35,36]. The most abundant intracellular antioxidant, GSH, lowers endogenous ROS and/or exogenous oxidative damage in animal cells GSH-Px enzyme metabolises peroxides such as H_2O_2 and protects cell membranes from LPO [37]. According to Ralph

et al.[38], amino acids may protect against toxicity by serving as precursors for GSH. GSH becomes depleted if cells are deprived of energy substrates. In a hepatopathy model, the effects of *C. islandica* extract are carried out on rats, treated intraperitoneally. It is reported that *C. islandica* extract has hepatoprotective and immuno-stimulating effects [39,40]. *C. islandica* determined adaptogenic-antistress effects, confirmes by its actions on oxidative stress parameters [40]. In this study, *C. islandica* and *P. furfuracea* might be a promising treatment in rats with T1D. Thus, lichen-based therapy is firstly used by this investigastion in treatment of T1D. The results of this study clearly demonstrated that STZ caused significant increases MDA levels in multiple tissues. Conversely, TAC levels in T1D rats were much lower than those healthy controls ($P<0.05$). It was crucial that our research focus on the high antioxidant ability of *C. islandica* for adequate treatment of tissues. After supplementation with *C. islandica* in diabetic animals, decreased in MDA levels and increased in TAC levels were found but the *in vivo* activation of antioxidant capacity by this lichen produced a limited and slight prevention of the tissue cell destructions. The antioxidant status after the *P. furfuracea* treatment was measured to evaluate the effectiveness of a therapy and unfortunately was not improved the clinical response. Again, it was established that there was not any an association between increasing lichen doses and above parameters. As a matter of fact, the high dose of lichens did not allow for a better evaluation of therapeutic effect because of their unchanged antioxidant properties.

The anti-diabetic extracts induce important metabolic changes in T1D [41]. Carbohydrate metabolism disorders in the form of T1D connect with an process connected with the increase of the insulin [42]. And the strict control of the blood glucose level is considered to be essential in order to delay and/or prevent the development of diabetic complications [42]. In this study, we investigated the responses elicited by lichens and how these responses may regulate T1D development. The experimental data showed that *P. furfuracea* lichen did not positively affected plasma insulin level and *C. islandica* extract (250-500 mg/kg) did not provide a significant advantage for T1D rats. Therefore, increase in blood glucose level unfortunately was not inhibited after administration of *C. islandica*. On the other hand, the most significant markers of T1D in both human and animal models are polydypsia and polyphagia [43]. As compared with the normal rats, we determined that the feed and water intake of the diabetic rats were significantly increased. Additionally, the insulin-induced the weight loss was observed after induction of STZ into the animals in present investigation. Animals treated with STZ exhibite continuous hyperglycemia, which coincided with a nearly complete loss of islet β-cells [44,45]. Two explanations for weight loss may be presented; firstly, due to polyuria and dehydration the body weight may be declined and, secondly, as the blood glucose level is high there is possibility of muscle breakdown in hyperglycemic rats [46]. Only, the treatment with the *C. islandica* enhanced pancreas function by improving glucose tolerance and increasing β-cell insulin reserve in rats. However, *C. islandica* seemed inadequate to prevent body weight loss in diabetic rats.

In current study, diabetic rats presented renal damages that were evidenced by the elevation in serum urea, uric acid, and creatinine levels, which were considered as significant markers of renal dysfunction [47]. Further our findings, it has been found that the liver was necrotized in diabetic rats due to the deficiency of insulin because of pancreas dysfunction. It is reported that insulin suppress the gene encoding gluconeogenic enzymes [48], ALT is a gluconeogenic enzyme and it is an indicator of impaired insulin signaling [49]. In present study, the activity of AST also increased in diabetic rats, this could be due to an increased release oxidative insult of diabetes lead to damage in hepatocytes [50]. Again, the LDH in serum as a biological marker for liver damage increases [51]. Cell necrosis leads to a rise in the concentration of the LDH enzyme in serum and tissue. The LDH released into the medium provides an index of cell death and membrane permeability to LDH, and an increase in LDH activity in the medium occurs as a result of cell membrane disintegration and enzyme leakage [52]. Thus, the increased activities of ALT, AST and LDH in serum is mainly due to the leakage of these enzymes largely from the liver cytosol into the blood stream [53], which gives an indication of the abnormal function of liver. This study also revealed that hepatocytes retained their capacity to normal function after lichen alone exposures. Moreover, *C. islandica* against T1D presented useful effects on the activities of enzymes without undepending on dose (ALT and AST except for LDH).

The renal impairment, hepatic and pancreatic damage in T1D may be associated with a number of genetic disorders [54]. The method used in the present study is 8-OHdG, which is known to be a sensitive marker of oxidative DNA damage and of the total systemic oxidative stress *in vivo* [55]. 8-OHdG is a product of oxidative DNA damage following specific enzymatic cleavage after the ROS-induced 8-hydroxylation of guanine bases in DNA [56]. Importantly, 8-OHdG appears to play a role in tissue cell injury via the induction of apoptotic cell death [57]. Levels of 8-OHdG have been found to be increased in the pancreatic β cells of STZ-diabetic rats [58,59]. Similarly, increased number of 8-OHdG–positive islet cells was found in our study. In addition, our data confirmed a previous report of the accumulation of 8-OHdG in the nuclear DNA in kidney and liver of diabetic rats. 8-OHdG levels are rapidly normalized by insulin treatment, suggesting the involvement of hyperglycemia in oxidative DNA damage [57-59]. In this study, we provided evidence that lichen extracts alone did not show the production of 8-OHdG during exposure. We also demonstrated that 8-OHdG accumulation in multiple tissues was reduced by the *C. islandica* on the association

between the glycaemic control and levels of 8-OHdG. This is in agreement with a recent study showing that treatment of rat tissues with antioxidants reverse glucose-mediated ROS production [60,61]. Thus, the measurement of 8-OHdG has been associated with potential antioxidant capacity of *C. islandica*.

To our knowledge, this report provides the first account of the *C. islandica* and *P. furfuracea* extracts *in vivo*. The increasing supplement of *C. islandica* and *P. furfuracea* alone do not show any oxidative and genotoxic effect in pancreas, kidney and liver of diabetic animals. Our opinion: these lichen species are a safe and findings also offer the possibility to early intervene in risk reduction of T1D by using *C. islandica*. As amatter of fact, *C. islandica* improve antioxidant defense system and presents anti-genotoxic effects but the power of *C. islandica* is limited because of intensive oxidative stress in multiple organs of T1D rats.

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Research Article

Seasonal, Geographical, Age and Breed Distributions of Equine Viral Arteritis in Iran

Shahin NEJAT [1] Hassan MOMTAZ [2] Mehrdad YADEGARI [1] Shayan NEJAT [3]
Farhad SAFARPOUR DEHKORDI [4] Faham KHAMESIPOUR [4]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord - IRAN
[2] Department of Microbiology, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord - IRAN
[3] Student of Veterinary Medicine, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord - IRAN
[4] Young Researchers and Elites Club, Shahrekord Branch, Islamic Azad University, Shahrekord - IRAN

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Abstract

The aim of this study was to estimate the prevalence of EVA in mare and stallion in various seasons, ages, breeds and regions of Iran. Totally, 470 blood samples which were taken from mares and stallions were tested for presence of EVA antigen and antibodies. Samples were collected in various seasons from various geographical parts of Iran. Blood and serum samples were taken from horses less than 1 year, 1-2 years, 2-3 years and more than 3 years old. Viral RNA and antibodies against EVA were detected in 4.46% and 4.04% of samples, respectively. Results showed that the EVA antigen and antibodies were higher in mare blood samples than stallion. Antibodies and antigen of EVA had the higher incidence in spring season and southern regions of Iran. Also, results showed that there were higher incidences of EVA antigen and antibodies in Standardbred breed and horses less than 1 year old. There were significant differences ($P<0.05$) for the incidence of EVA antigen and antibodies between mares and stallions, less than 1 year old and more than 3 years old horses, spring and winter seasons and finally, southern and northern parts of Iran. This study showed that incidences of Equine viral arteritis antigens and antibodies are related to sex and breed of horses, seasons of samples collection and geographic regions. As far as we know, this study is the first prevalence report of seasonal, geographical, age and breed distributions of EVA in stallions and mares in Iran.

Keywords: *Equine viral arteritis, Seasons, Regions, Breeds, Age, Sexual, Iran*

İran'da Atların Viral Arteritisi Enfeksiyonunun Mevsimsel, Coğrafi, Yaş ve Cins Yayılımı

Özet

Bu çalışmanın amacı İran'da Atların Viral Arteritisi Enfeksiyonunun (EVA) kısrak ve aygırlarda mevsimsel, coğrafi, yaş ve cins yayılımını araştırmaktır. Kısrak ve aygırlardan alınan toplam 470 kan örneği EVA antijen ve antikorlarının varlığı yönünden test edildi. Örnekler değişik mevsim ve coğrafi bölgelerden alındı. Kan ve serum örnekleri 1, 1-2, 2-3 ve 3 yaştan daha yaşlı hayvanlardan alındı. Viral RNA ve EVA antikorları sırasıyla %4.46 ve %4.04 örnekte tespit edildi. Elde edilen bulgular EVA antijen ve antikorlarının kısraklarda aygırlardan daha fazla oranda bulunduğunu ortaya koydu. EVA antikor ve antijenleri ilkbahar döneminde ve İran'ın güney kesiminde daha fazlaydı. Ayrıca sonuçlar EVA antijen ve antikorlarının Standartbred atlarda ve 1 yaşından daha gençlerde daha fazla olduğunu gösterdi. EVA antijen ve antikorlarının varlığı açısından kısraklarla aygırlar arasında, 1 yaşından gençler ile 3 yaşından daha yaşlılar arasında, ilkbahar ile kış mevsimleri arasında ve İran'ın güneyi ile kuzeyi arasında anlamlı farklar ($P<0.05$) saptandı. Bu çalışma Atların Viral Arteritisi antijenleri ve antikorlarının cinsiyet, at cinsi, örnek toplama mevsimi ve coğrafi bölge ile değişiklik gösterdiği tespit edildi. Bilgimiz dahilinde bu çalışma mevsimsel, coğrafi, yaş ve cinse göre atlarda EVA yaygınlığını İran'da tespit etmek amacıyla yapılan ilk çalışmadır.

Anahtar sözcükler: *Atların Viral Arteritisi, Mevsim, Bölge, Irk, Yaş, Cinsiyet, İran*

İletişim (Correspondence)
+98 381 7220836
shndfa@yahoo.com

INTRODUCTION

Equine Viral Arteritis (EVA) is an acute and contagious disease of both horses and donkeys which causes by *Equine Arteritis Virus* (*EAV*). It is characterized by fever, edema (especially of the scrotum, prepuce and limbs), nasal discharge, conjunctivitis, and infrequently death in young foals [1-3]. It may also result in abortion of pregnant mares and interstitial pneumonia or pneumoenteritis in young foals. Also, stallions can become persistently infected and shed virus into their semen [4,5].

Several outbreak of EVA have been reported previously from USA [6], UK [7], Spain [8], Denmark [9], South Africa [10] and Australia [11].

The two principal modes of transmission of *EAV* are horizontal, by direct contact with infectious respiratory tract secretions from acutely infected horses, and venereal, through natural breeding or artificial insemination with semen samples of persistently infected stallions [12]. Therefore, determination of the carrier stallion had critical epidemiological importance in the prevention and control of *EAV* infection [13].

Although EVA is a disease almost exclusively of equids, antibodies to EAV have been identified in donkeys [10]. Serological surveys have shown that EAV infection occurs among horses in North and South America, Europe, Australasia, Africa, and Asia [14] with considerable variation in seroprevalence of EAV infection among countries and within equine populations in some countries. There is only one known serotype of EAV, but geographically and temporally distinct strains of EAV differ in the severity of the clinical disease they induce and in their abortogenic potential [14,15].

The epidemiology and prevalence of EVA in mares and stallions is essentially unknown in Iran. Therefore, this present study was carried out in order to study the seasonal, geographical, age and breed distribution of EVA in mares and stallions blood samples using cell culture, Enzyme Linked Immunosorbent Assay (ELISA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) assays in Iran.

MATERIAL and METHODS

Samples

From December 2010 and December 2011 in various seasons of the year, blood and serum samples were collected randomly from stallions (n=220) and mares (n=250) (from 3 major breeds of Arab, Standardbred and Thoroughbred). Horses used in this study were evaluated as less than 1, 1 to 2, 2 to 3 and more than 3 years old which were settled in 4 different geographic regions of Iran (north, south, west and east).

The blood samples were collected into clot activator vacuum tubes and centrifuged at 800 × g for 10 min. The separated sera were kept at −20°C before testing. EDTA-blood samples (10 mL) were centrifuged at 18°C (1.400 × g) for 35 min. Buffy coat cells were re-suspended in 4 volumes of sterile 0.2% NaCl to lyse erythrocytes. After 1 min, 7.2% NaCl was added to reconstitute isotonicity. The cells were washed in phosphate-buffered saline and stored at −70°C.

RNA Extraction

The viral RNA was extracted by the guanidium isothiocyanate method. Briefly, 500 µL of 4 M guanidium isothiocyanate, 50 µL of 2 M sodium acetate (pH=4.0), 500 µL of water-saturated phenol, and 100 µL of chloroform were added to the sample, with thorough mixing after the addition of each reagent, and the mixture was left on ice for 15 min. Thereafter, the mixture was microcentrifuged for 20 min at 5°C, and the upper aqueous phase was transferred to a fresh tube. Viral RNA was precipitated with 500 µL of cold isopropanol for 1h at −20°C. After centrifugation, the pellet was resuspended in 150 µL of 4 M guanidium isothiocyanate. The suspension was then precipitated with isopropanol, and the resulting RNA pellet was washed with 75% ethanol, dried, and resuspended in 20 µL of diethylpyrocarbonate- treated water. The RNA samples were kept at −70°C until use.

Oligonucleotide Primers, cDNA Synthesis, and PCR

The oligonucleotide primers used for RT-PCR were from the 3′ end of ORF 1b of the *EAV* genome. Their sequences were as those described by other studies [16]:

PEV-10: 5′-GAGGATCCCACTTCATCT-3′

PEV-11: 5′-AATGGTCTGCACTGAGGT-3′

For cDNA synthesis, the reaction mixture was incubated at 70°C for 10 min and was chilled on ice prior to the addition of the reverse transcriptase enzyme and RNasin. RT of *EAV* RNA was carried out at 37°C for 15 min and at 42°C for 90 min in 1× Taq buffer (10× Taq buffer is 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1% Triton X-100) containing 0.5 mM (each) deoxynucleoside triphosphates and to which 3 µL of A buffer (400 mM Tris-HCl (pH 8.3), 50 mM $MgCl_2$), 8 U of avian myeloblastosis virus reverse transcriptase (Roche applied sciences), 17 U of RNasin (Fermentas), 4 µL of RNA sample, and 20 pmol of PEV-11 primer were added to a final reaction volume of 30 µL. Thereafter, the mixture was incubated at 95°C for 5 min to inactivate the avian myeloblastosis virus reverse transcriptase, and the following reagents were added: 25 pmol of each sense and antisense primer, 0.15 mM (each) deoxynucleoside triphosphates, 1.5 U of Taq polymerase (Fermentas), and 5 µL of 10× Taq buffer to a final reaction volume of 50 µL. The cDNA was then amplified by 30 successive cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and DNA chain extension at 72°C for 2

min with a programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Twenty µL of final PCR products were run on a 1.5% agarose gel containing ethidium bromide in 1X TBE buffer along with 100 bp DNA ladder (Fermentas, Germany).

Serological Test

Serum samples were tested for antibodies against *EAV* by the commercial ELISA kit (ID Vet Innovative Diagnostics, France), according to the manufacturer's instructions.

Statistical Analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using the SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA), Chi-square test analyses were performed, and differences were considered significant at values of $P<0.05$.

RESULTS

Table 1 presents the distribution of EVA antigen and antibody in mares and stallion blood samples. Totally, 21 out of 470 serum samples (4.46%) and 19 out of 470 blood samples (4.04%) were found to be positive for presences of EVA antibody and antigen, respectively. Also, 8 mares (3.63%) and 13 stallions (5.2%) serum samples were positive for antibodies against EAV. Besides, 7 mares (3.18%) and 12 stallions (4.8%) blood samples were positive for EAV antigen. There were significant differences about $P<0.05$ for presences of antibodies against virus and viral antigens between mares and stallions.

Table 1. *Distribution of EVA antigen and antibody in mare and stallion blood samples*
Tablo 1. *Kısrak ve aygır kan örneklerinde Atların Viral Arteritisi antijen ve antikorlarının yayılımı*

Type and Number of Samples	ELISA (%)	RT-PCR (%)
Stallion (220)	8 (3.63)	7 (3.18)
Mare (250)	13 (5.2)	12 (4.8)
Total (470)	21 (4.46)	19 (4.04)

Table 2 presents the seasonal and geographical distribution of antibodies against EVA and viral antigen in mare and stallion blood samples.

Results revealed that blood and serum samples which were collected from southern regions of Iran and samples which were collected in spring had the highest incidence of antibodies and antigen of EVA in mares and stallions. Statistical analysis were significant for the presences of EVA antibodies and antigens between the samples which were collected in spring with autumn and winter and also, between the samples which were collected from southern parts and northern and western parts of Iran ($P<0.05$).

Table 3 presents the age and breed distribution of EVA antigen and antibody in mare and stallion blood samples. Totally, the horses less than 1 year old and Standardbred horses had the highest incidence of EVA antigen and antibodies. There were significant differences ($P<0.05$) for incidences of viral antibodies and antigens between mares and stallions less than 1 year old and mares and stallions more than 3 years old. Also, statistical differences were significant for incidences of viral antigens and antibodies between Standardbred and Thoroughbred breeds ($P<0.05$).

DISCUSSION

The results of our present study indicated that there were effective seasonal and geographical distributions for incidences of EVA antigens and antibodies against EVA in

Table 2. *Seasonal and geographical distribution of EVA antigen and antibody in mare and stallion blood samples*
Tablo 2. *Kısrak ve aygır kan örneklerinde Atların Viral Arteritisi antijen ve antikorlarının mevsimsel ve coğrafi yayılımı*

Type and Number of Samples	Seasonal Distribution							
	Antibody (21 Positive)*				Antigen (19 Positive)**			
	Aut***	Spr	Sum	Wint	Aut	Spr	Sum	Wint
Stallion (220)	2	5	1	-	1	4	1	1
Mare (250)	3	8	2	1	4	6	1	1
Total (470)	5	12	3	1	5	11	2	2
Type and Number of Samples	**Geographical Distribution**							
	Antibody (21 Positive)*				**Antigen (19 Positive)****			
	South	North	East	West	South	North	East	West
Stallion (220)	5	-	2	1	4	-	2	1
Mare (250)	9	1	2	1	8	-	3	1
Total (470)	14	1	4	2	12	-	5	2

** Positive samples based on ELISA methods; ** Positive samples based on RT-PCR method; *** In this table Aut means autumn, Wint means winter, Spr means spring and Sum means summer*

Table 3. *Age and breeds distribution of EVA antigen and antibody in mare and stallion blood samples*
Tablo 3. *Kısrak ve aygır kan örneklerinde Atların Viral Arteritisi antijen ve antikorlarının yaş ve cinse göre yayılımı*

Type and Number of Samples	Age Distribution							
	Antibody (21 Positive)*				Antigen (19 Positive)**			
	1>	1-2	2-3	3<	1>	1-2	2-3	3<
Stallion (220)	5	-	-	-	2	1	1	-
Mare (250)	14	2	-	-	7	4	2	2
Total (470)	19	2	-	-	9	5	3	2
Type and Number of Samples	**Breed Distribution**					
	Antibody (21 Positive)*			**Antigen (19 Positive)****		
	Standardbred	**Thoroughbred**	**Arab**	**Standardbred**	**Thoroughbred**	**Arab**
Stallion (220)	4	1	2	3	1	2
Mare (250)	7	3	4	7	2	4
Total (470)	11	4	6	10	3	6

** Positive samples based on ELISA methods; ** Positive samples based on RT-PCR method*

mares and stallions. Samples which were collected from southern and eastern parts of Iran and also, samples which were collected in spring and summer seasons had the highest range of infection. The main reason for this finding is the fact that temperature and climate may have an effect on the prevalence of antigen and antibodies against EVA.

Spring and summer are breeding equine seasons in many parts of Iran especially southern and eastern regions. Therefore, in these parts and in these seasons, modes of transmission and infection rate become higher than other seasons and regions. The main reason for the highest prevalence of EVA in spring and summer seasons is the fact that during this time climatic events, heat, rain, and thunderstorms, as well as variation of barometric pressure has been changed and may have influence on the autonomic nervous system. These events caused reduction in the levels of animal immunity. Therefore, several infections have been occurred. Also, previous investigation showed that carrier stallions are the natural reservoir of EAV and the virus is maintained in equine populations between breeding seasons [4].

Also, survival of EAV in the environments is temperature dependant; although it may survive only 20-30 min at 56°C and from 2 to 3 day at 37°C, it can survive up to 75 day at 4°C but virus is so sensitive to temperature changes [17]. Therefore, this virus needs moderate constant temperature and especially between 20 to 4°C. After analyzing the average temperatures of these 4 seasons, it has been found that spring and summer had the most constant temperature. Besides, the most constant temperatures have been shown in southern and eastern parts of Iran. Therefore, the high prevalence of antigens and antibodies are seems reasonable in spring and summer seasons and southern and eastern parts of Iran.

Our results showed that there were strong age and breed distributions for the incidences of EVA antigens and antibodies in mares and stallions in Iran. The horses less than 1 year old followed by, those had 1-2 years old were the highest incidence of EVA antigens and antibodies. Previous studies showed that death in younger than 1 year old foal is one of the main complications of EVA [1-3].

This present study showed that Standardbred followed by, Arab horses had the highest incidences of EVA antigens and antibodies. Antigen and antibodies of EVA were less common in Thoroughbred horses. These findings are in harmony with previous investigations [14,18-20]. Infection is endemic among Standardbred but not Thoroughbred horses in the USA, with 77.5-84.3% of all Standard breds, but only 0-5.4% of Thoroughbreds being seropositive [14,18,20]. The seroprevalence of EAV infection in Warmblood stallions is also very high in a number of European countries, e.g., 55-93% of Austrian Warmblood stallions were positive for antibodies to EAV [21,22]. Although breed-specific differences might reflect inherent genetic differences that confer resistance to infection, they are more likely reflective of different cultural and management factors within horse populations and breeds. Previous studies have not demonstrated any breed-specific variation in susceptibility to EAV infection or in establishment of the carrier state [23] but our results for the first time showed that EVA antibodies and antigen had the highest incidence in Standardbreds and Arab breeds.

Extensive outbreaks of EVA were reported in North America and Europe [17]. Similarly, *EAV* infection of horses has been identified in countries including Australia, New Zealand, and South Africa, previously thought to be largely or completely free of the virus. Totally, serological surveys have demonstrated that *EAV* infection occurs in Europe, Australia, North and South America, Africa, and Asia [1,17]. Japan and Iceland are apparently free of the virus, whereas *EAV* infection is relatively common in horses in

several European and Asian countries. It seems that, the carrier state that occurs in persistently infected stallions constitutes the natural reservoir of EVA, with carrier stallions venereally transmitted EVA to susceptible mares by natural service or Artificial insemination (AI) [17]. Iran has a long history in breeding and maintaining of horses. Because the high frequency of horse trade with other countries in this area, it seemed so far that EVA infection did not exist.

The results of our study showed that 3.63% of stallion serum samples and 5.2% of mare serum samples were positive for antibodies against EVA. Higher prevalence of antibodies against EVA has been reported previously from Turkey (14.47% by ELISA test) [24]. Other study [3] reported that 51.1% of horses were serologically positive for EVA's antibodies. Another research [25] reported that 8.75% of Tunisian horse sera have antibodies to EVA. The seroprevalence of EVA have been reported previously as 11.3% (Switzerland) [26], 2.3% (UK) [27], 14% (Dutch) [15], 20% (Germany) [28] and 18.6% (USA) [29]. These high differences in prevalence of EVA in blood and serum samples maybe due the facts that type of samples (sex, breed and age of horses), number of samples, methods of sampling, method of experiment, geographical area and even climate of area which samples were collected are different in each investigation.

As far as we know, this present study is the first prevalence report of seasonal, age, breed and geographical distribution of EVA antigen and antibodies in stallions and mares in Iran. It seems that the main cause of prevalence of EVA infection in Iran is lack of diagnosis in the field conditions, and finally lack of programs for control and eradication of the disease by responsible organizations including veterinary organization. Therefore, several investigations should be done on prevalence of this viral disease in Iran. International transportation of carrier stallions or infected semen, play an important role in epidemiology of *EAV* infection. Therefore, horse transportation should be controlled firmly. More clinical cares should performed on less than 1 year old horses, standardbred horses and especially on southern and eastern parts of Iran on spring season.

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Short Communication

Transient Glomerular Dysfunction in Dogs Caused by *Dirofilaria immitis* Infection

Anton RUSENOV [1] Lazarin LAZAROV [1] Zvezdelina KIRKOVA [2]
Anton TONEV [2] Nikolina RUSENOVA [2] Francesca DILDA [3]

[1] Department of Internal Diseases, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, BULGARIA
[2] Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, BULGARIA
[3] Parco Tecnologico Padano, Via Einstein 26900 Lodi, ITALY

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Abstract

The present study aimed to examine two glomerular markers (urinary albumin, uALB; urinary C-reactive protein, uCRP) in healthy dogs and in dogs infected with *Dirofilaria immitis,* and to identify some possible changes in these markers after therapy with ivermectin and doxycycline. Twenty dogs with *D. immitis* infection positive by both the Knott method and SNAP 4Dx (IDEXX, USA) test were included in the research, as well as twelve clinically healthy dogs of similar age which served as controls. Glomerular biomarkers (mean, SD) increased significantly in dogs with heartworm disease (uAlB/Creatinine, Cr mg/g: 527.57±312.54; uCRP/Cr mg/g: 0.520±0.624), compared to control dogs (uAlB/Cr mg/g: 94.44±56.50; uCRP/Cr mg/g: below detection limit). Six months after the initial examination and the simultaneous treatment, all glomerular markers were considerably decreased and did not differ from those in healthy animals. In conclusion, the observed changes in glomerular biomarkers clearly indicated the transient nature of glomerular dysfunction caused by the heartworm infection in dogs, which may be of clinical relevance.

Keywords: *Dirofilaria immitis infection, Dogs, Nephropathy, Glomerular markers*

Köpeklerde *Dirofilaria immitis* Enfeksiyonu Nedenli Transient Glomerüler Disfonksiyon

Özet

Bu çalışmanın amacı sağlıklı ve *Dirofilaria immitis* ile enfekte köpeklerde ve ivermectin ve doxcycline ile tedavi sonrasında iki glomerüler markırdaki (üriner albumin, uALB; üriner C-reaktif protein, uCRP) muhtemel değişimleri araştırmaktır. Çalışmada Knott metodu and SNAP 4Dx (IDEXX, USA) testleriyle *D. immitis* ile enfekte olduğu tespit edilen yirmi köpek ile birlikte kontrol olarak aynı yaş grubunda klinik olarak sağlıklı 12 adet köpek kullanıldı. Kontrol grubuyla (uAlB/Cr mg/g: 94.44±56.50; uCRP/Cr mg/g: tespit limitinin altı) karşılaştırıldığında kalp kurdu olan köpeklerin glomerüler biomarkırlarında (ortalama, Standart sapma) (uAlB/Kreatin, Cr mg/g: 527.57±312.54; uCRP/Cr mg/g: 0.520±0.624) artma olduğu belirendi. İlk bakıdan sekiz ay sonra ve takip eden tedavi sonrasında tüm glomerüler markırların dikkati çeken derecede azaldığı ve sağlıklı olan hayvanların değerlerinden farklılık göstermediği belirlendi. Sonuç olarak, biomarkır değerlerinde gözlenen değişikliklerin köpeklerde kalp kurdu enfeksiyonu nedenli glomerüler disfonksiyonun değişken doğasını açıkça ortaya koyduğu ve bu durumun klinik değeri olabileceği gösterilmiştir.

Anahtar sözcükler: *Dirofilaria immitis enfeksiyonu, Köpek, Nefropati, Glomerüler markır*

INTRODUCTION

Dirofilaria immitis is the causative agent of Heartworm infection in dogs, which is accompanied by abnormalities in the cardiovascular system and is often fatal. Adult worms are mainly localized on the right side of the heart and the pulmonary artery, but microfilariae are circulating with the blood and could be found in the vessels of various organs, including kidneys [1], where they cause glomerular dysfunction manifested as mild to moderate proteinuria [2]. Early diagnosis of glomerular lesions allows for the selection of an appropriate therapeutic approach to prevent further progression of renal disease. Traditional diagnostic tests (serum creatinine - SCR and urea - BUN)

İletişim (Correspondence)
+359 88 5563476
vetroussenov@abv.bg

detect renal disease in an advanced stage of development, when more than 75% of functioning nephrons are irreversibly damaged [3,4].

Unlike conventional tests, in the recent years, more attention has been paid to the urinary proteins and enzymes as early markers of kidney injury in dogs. According to Clemo [5] and Price [6], urinary markers have the potential to determine the localization and severity of renal lesions in different parts of the nephron. They include proteins classified according to their molecular weight: high molecular weight (HMW), intermediate molecular weight (IMW) and low molecular weight (LMW) proteins [7,8]. According to Finco [4], the occurrence of urinary protein results from increased glomerular filtration rate or abnormal tubular function, leading to decreased reabsorption of naturally filtered protein. Glomerular dysfunction leads to the appearance of IMW (albumin) protein in the filtrate and, at a more advanced stage - to HMW (e.g. CRP) proteins [6,7,9]. Grauer et al.[10] determine microalbuminuria (albumin from 1 to 30 mg/dl), as the earliest indicator of glomerular impairment in dogs.

C-reactive protein (CRP) is a HMW acute phase protein whose serum concentration is increased during inflammatory processes [11,12]. Recently, two studies have assessed urinary CRP (uCRP) and CRP-to-creatinine ratio (uCRP/Cr) in dogs with pyometra and chronic renal failure, as indicators of glomerular damage [8,13]. To the best of our knowledge, investigations on urinary microalbuminuria and CRP in dogs with microfilariaemia have not been conducted until now, which motivated us to perform this particular study.

The aim of the present work was to examine two glomerular markers (uALB, uCRP) in healthy dogs and in dogs infected with *Dirofilaria immitis*, and to identify some possible changes in these markers after therapy with ivermectin and doxycycline.

MATERIAL and METHODS

Dogs

Twenty dogs with *Dirofilaria immitis* infection positive by both the Knott method and SNAP 4Dx (IDEXX, USA) test, patients of Small Animal Clinic of the Faculty of Veterinary Medicine, Trakia University, Stara Zagora were included in the study. Animals were of both sexes at a different age (1.5-6.7 years), of different breed and weight (14-31 kg). Twelve clinically healthy dogs were used as controls. All dogs were privately owned and admitted in the clinic between June 2010 and December 2012. The owners signed an informed consent form to allow participation of their dog in the study.

All patients were submitted to complete clinical examination and routine hematological and chemical analysis of blood and urine. During the clinical examination, the bioelectric heart activity was carried out by ECG registration.

Diagnosis was based on the history, clinical signs, ECG, blood test, chemical blood and urine analysis, as well positive results by both specific Knott assay and Snap 4Dx test.

Urine was collected by cystocentesis under ultrasound control to determine the specific gravity (USG), creatinine (uCr), total protein (UP), albumin (uALB), C-reactive protein (uCRP) and bacteriological culture. Urinary protein-to-creatinine ratio (UPC), albumin-to-creatinine (uALB/Cr) and C-reactive protein-to-creatinine (uCRP/Cr) ratios were calculated according to the conventional methods.

After the disease has been diagnosed, all dogs were assigned microfilaricide treatment with ivermectin - 0.06 mg/kg, sc (Cevamec 1%; Ceva-Phylaxia Veterinary Biologicals Co. Ltd., Budapest, Hungary, No 0604V1), once monthly (for 6 months) and daily oral administration of doxycycline - 10 mg/kg (Stada Arzneimittel-AG,-Germany) (for 4 weeks).

The control dogs were considered healthy from the absence of specific positive serological reaction and negative findings in Knott, as well as from blood and urine parameters within the normal range, including negative urine cultures. In 15 of 20 dogs initially diagnosed with heartworm infection, a secondary clinical examination, blood and urine tests were carried out, six months after the therapy. The remaining 5 dogs were excluded from the study because they died (2 dogs developed "caval syndrome", and 3 - decompensated heart failure).

Laboratory Methods

Routine Urinalysis: The urine specific gravity (USG) was determined on a refractometer. Routine analysis of the sediment was performed using dipstick, urine culture and microscopy. Urine was centrifuged (450 x g for 3 min), aliquoted and frozen at - 80°C until uCRP assay. The analysis was performed within 30 min after sampling on an automated biochemical analyzer and commercial kits for determination of urinary creatinine (uCr) and urinary albumin (uALB). The analysis of CRP was performed at the University of Milan, Italy, on a microplate spectrophotometric reader with species-specific commercial ELISA kit (TECO Medical Group, Switzerland) .

Statistical Analysis

The statistical analysis was performed using one way analysis of variance (ANOVA, software Statistica v. 7.0, StatSoft Inc., USA, 2004) and presented as mean (X) and standard deviation (±SD). The statistical significance of changes in parameters was determined in the LSD test at $P<0.05$.

RESULTS

Study Group Characteristics

Signalment and clinical data *(Table 1)* showed that reduced appetite and weight loss were among the commonest clinical signs, detected in 17 dogs. Cough was a frequent accompanying sign, observed in 14 out of 18 dogs. In 3 out of 20 dogs, ultrasound evidence for fluid in the abdomen and extremities swelling (2/20) were present. Seven dogs (35%) showed altered electric heart activity (ST segment depression, high or low amplitude of the ventricular complex). Ultrasound findings of kidneys showed signs specific for glomerulonephritis (enlarged kidney with hyperechoic and thickened renal cortex).

Insignificant changes in CBC parameters (Hb, Er and Hct) were established. A mild leukocytosis up to 17.31±6.21 10^9/L was observed. Sixteen out of 20 dogs with heartworm infection had hypoalbuminemia (<25 g/L), but none was hypoproteinemic (reference range: 25-37 g/L).

Routine Renal Tests in Healthy Dogs and Dogs with Dirofilariosis

Descriptive statistics of routine renal markers is shown in *Table 2*. Serum creatinine and urea concentrations in dogs with dirofilariosis before treatment and dogs with dirofilariosis included in the follow-up study after 6 months remained unchanged compared to control animals and reference ranges for dogs.

Average urinary protein-to-creatinine ratio (UPC) *(Table 2)* in control dogs was 0.29±0.11. Higher UPC values were established in dogs before treatment – 3.18±4.62 (P<0.05 vs control values). In dogs after treatment, UPC was restored to control and reference values.

Statistically significant changes were observed in mean urine specific gravity (USG). In group before treatment, USG attained 1.024±0.004 (P<0.001) as compared to control USG (1.033±0.008). In group after treatment, USG was recovered reaching 1.033±0.007 (P>0.05 vs control dogs).

Urinary Markers in Healthy dogs and Dogs with dirofilariosis

Urinary albumin-to-creatinine (uALB/Cr) and urinary C-reactive protein-to-creatinine (uCRP/Cr) ratio are presented in *Table 3*. The table shows that uALB/Cr markedly increased in dogs before treatment attaining 527.57±312.54 mg/g (110.8-1100.5 mg/g) at P<0.001 vs control values of 94.44±56.50 mg/g (36.05-112.01 mg/g). In dogs after treatment uALB/Cr normalized up to values similar to control ones.

In dogs with dirofilariosis, uCRP/Cr underwent considerable changes. In control dogs, no CRP (uCRP/Cr) was detected in urine. Significant changes in uCRP/Cr occurred in dogs before treatment, attaining 0.520±0.624 mg/g. Thirteen out of 15 treated dogs were negative for uCRP. Insignificant amounts of uCRP/Cr varying between 0.01-0.03 mg/g were detected in 2 animals.

Table 1. *Signalment and clinical signs, haematological and biochemical test results (mean±SD, n = 20) in dogs with dirofilariosis before treatment with ivermectin and doxycycline*

Tablo 1. *Ivermectin ve doxycyclin ile tedavi öncesi dirofilaria ile enfekte köpeklerin Klinik bulgu ve klinik belirtileri ile hematolojik ve biokimyasal test sonuçları (ortalama ± SD, n = 20)*

Signalment and Clinicals Signs		Haematological and Biochemical Results (mean ± SD)		Reference Range
Weight loss	17/20	Hb, (g/l)	127±36.76	120-180
Cough	13/20	Er, (10^{12}/L)	5.52±1.40	5.5-8.5
Ascites	3/20	Hct, (%)	37.0±9.40	37-55
Limbs oedema	2/20	Leuc, (10^9/L)	17.31±6.21	6-17
Retrograde pulse	2/20	TP, (g/L)	61.39±5.38	54-78
ECG changes	7/20	ALB, (g/L)	21.85±4.38	25-37

Table 2. *Routine parameters for renal function. Laboratory results in control dogs (n=12), dogs with dirofilariosis before treatment (n=20) and 6 months after treatment (n=15), (sCr, BUN, UPC and UPC, mean and SD)*

Table 2. *Renal fonksiyonların rutin parametreleri. Kontrol köpekleri, tedavi öncesi diroflariali köpekler (n=20) ve tedaviden 6 ay sonrasının (n=15) laboratuar sonuçları (sCr, BUN, UPC and UPC, ortalama ve SD)*

Parameter	Control (n=12)	Before Treatment (n=20)	After Treatment (n=15)	Reference Range
sCr (µmol/l)	79.67±16.41	85.45±22.05^{ns}	81.87±14.86^{ns}	<125
BUN (mmol/l)	4.35±0.92	5.41±2.21^{ns}	4.57±0.99^{ns}	3.3–8.3
UPC	0.29±0.11	3.18±4.62^{a}	0.27±0.11^{ns}	< 0.5
USG	1.033±0.008	1.024±0.004^{c}	1.033±0.007^{ns}	1.015-1.045

^{ns}P>0.05, ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001, ^{d}P<0.0001 - compared to control dogs

Table 3. *Urinary concentrations of glomerular markers: results in control dogs (n=12), dogs with dirofilariosis before treatment (n=20) and 6 months after treatment (n=15), (uALB/Cr, uCRP/Cr, mean and SD, BDL-below detection limit)*

Table 3.*Glomerüler markırların üriner konsantrasyonları: Kontrol köpekleri, tedavi öncesi diroflariallı köpekler (n=20) ve tedaviden 6 ay sonrasının (n=15) laboratuar sonuçları (uALB/Cr, uCRP/Cr, ortalama ve SD, BDL-tespit limitinin altı)*

Biomarker	Control Group	Before Treatment	After Treatment
uALB/Cr(mg/g)	94.44±56.50 36.05–112.01	527.57±312.54 [c] 110.8–1100.5	115.13±65.06 [ns] 39.54–248.6
uCRP/Cr(mg/g)	BDL (n=12)	0.520±0.624 0.142–3.012	BDL (n=13), 2 dogs (0.01–0.03)

[ns] P>0.05, [a] P<0.05, [b] P<0.01, [c] P<0.001 - compared to control dogs

DISCUSSION

In this preliminary study on dogs with heartworm disease a significant increase in glomerular markers, compared to control animals of similar age was established. Elevated uAlb and uCRP concentrations clearly show that dirofilariosis affects negatively the nephrons at a glomerular level. The appearance of urinary CRP in dogs with heartworm infection is an indication about glomerular membrane damage at an extent such that to allow filtration of high molecular weight proteins as CRP. The advantage of glomerular markers compared with routine renal parameters consists in their ability to detect the damage at an earlier stage [9]. The presence of 20 nonazotemic dogs with dirofilariosis, but with increased values of glomerular markers, as compared to healthy animals, clearly confirms this statement. According to IRIS, proteinuria is present when UPC values are greater than 0.5 [14]. In this study, the levels of the analyzed indicators were mildly to moderately elevated, but glomerular markers testing revealed significant differences between control animals and those with dirofilariosis. In addition, glomerular biomarkers (uALB, uCRP) provided guidelines for the origin of proteinuria, which is not possible with UPC.

Maddens et al.[13] reported a significant loss of ALB and CRP in the urine of dogs with pyometra, and determined uCRP as a glomerular indicator of unique diagnostic value. Along with glomerular markers, authors investigated some tubular biomarkers, and concluded that pyometra damages nephrons both at glomerular and tubular level. In a previous study Smets et al.[8] described a comparable increase in uALB and uCRP levels in dogs with chronic renal failure grade III and IV, but at a higher extent (uALB).

The most probable hypothesis explaining the significantly raised uAlb and uCRP levels is the chronic stimulation of the immune system by *Dirofilaria immitis* microfilariae causing formation of circulating immune complexes precipitated in glomeruli [15]. Morchón et al.[15] and Kramer et al.[16] consider that rickettsia of the genus Wolbachia (Rickettsiaceae) play an important role in the pathogenesis and immune response to microfilariaemia in dogs. Similar are the studies of Paes-de-Almeida et al.[17], who investigated electron microscopic renal lesions in dogs with experimentally induced heartworm disease. The authors suggest that the detected dense deposits of immune complexes associated to immature heartworms and microfilariae, as well eventually adult worms are causes for glomerulonephropathy.

In our study, six months after the initial examination and ongoing therapy, all glomerular markers were significantly decreased and did not differ from those in control animals. In two of the dogs slight traces of CRP in the urine were detected which may be due to an inflammatory response leading to increased plasma concentrations, as well as a slight damage of glomerular membrane.

In conclusion, the observed changes in glomerular biomarkers in this study clearly showed the transient nature of glomerular dysfunction caused by the heartworm infection in dogs. Future studies are needed to test additional glomerular and tubular biomarkers to evaluate the disease as a model of nephropathy.

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Short Communication

Prevalence, Hematology and Treatment of *Balantidium coli* among Small Ruminants in and Around Lahore, Pakistan

Mustafa JAMIL [1] Muhammad IJAZ [1] Muhammad MUDDASSIR ALI [2]

[1] Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences Lahore, PAKISTAN
[2] Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences Lahore, PAKISTAN

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Abstract

Sheep and goats of Lahore district were examined for the prevalence, hematology and treatment of *Balantidium coli*. A total of 752 sheep and goats were examined (n=376 sheep; n=376 goats). Sheep had a somewhat greater prevalence (3.99%) than goats (3.46%). In both species, decrease in hemoglobin (Hb) and increased packed cell volume (PCV) was observed in infected animals. Efficacy of Secnidazole was higher as compared to Oxytetracycline and Metronidazole in all experimental animals. Balantidiasis is rarely studied infection in small ruminants and this is the first report published on the disease from Pakistan. It is still unclear whether this is an emerging infection, or whether it is a newly reported endemic in the studied area.

Keywords: *Balantidium coli, Small ruminants, Treatment, Prevalence, Hematology, Oxytetracycline, Metronidazole*

Lahore, Pakistan ve Çevresinde Küçükbaş Geviş Getiren Hayvanlar Arasında *Balantidium coli*'nin Prevalansı, Hematolojisi ve Tedavisi

Özet

Lahore ilçesindeki koyunlar ve keçiler *Balantidium coli*'nin prevalansı, hematolojisi ve tedavisi açısından incelendi. Toplam 752 koyun ve keçi incelendi (n=376 koyun; n=376 keçi). Keçi (% 3.46) ile karşılaştırıldığında koyunda daha fazla *B. coli* prevalansı (3.99%) gözlenmiştir. Her iki türde, enfekte hayvanlarda hemoglobin (Hb) değerinde azalma ve packed cell volumde (PCV) artma gözlenmiştir. Tüm deney hayvanlarında Oksitetrasiklin ve metronidazol ile karşılaştırıldığında seknidazol etkinliği daha yüksekti. Blantidiasis küçükbaş geviş getiren hayvanlarda nadiren çalışılmış bir enfeksiyondur ve Pakistan'dan bu ilk rapordur. Bu enfeksiyonun bu bölgede endemik olup olmadığı ya da artarak gözlenip gözlenmediği henüz bilinmemektedir.

Anahtar sözcükler: *Balantidium coli, Küçükbaş geviş getiren hayvanlar, Tedavi, Prevalans, Oxytetracycline, Hematoloji, Metronidazole*

INTRODUCTION

Parasitism is not only a burden on small ruminant farmers nationwide but also a contributing factor in the reduction of per animal yield in Pakistan. It is one of the key threats for domestic animals, and is one of the primary obstacles to the development of a productive small ruminant agronomy [1]. Balantidiasis is a contagious malady worldwide [2]; it is most common in tropical and subtropical regions. In Tanzania, 4.8% *B. coli* prevalence was reported in goats [3], while in Kenya, its prevalence was reported to be between 2% and 3% in sheep and goats, mostly as mixed infection with other parasites [4].

B. coli infection creates zoonotic hazards [5]. This larges ciliated protozoon not only infects humans but nonhuman primates also [5]. Human balantidiasis is more common in poorly sanitized areas where human live in close contact with sheep, goats and pigs. It is chiefly spread through solid food and water that has been contaminated by human or animal feces containing *B. coli* cysts [6]. Hence it has also been considered as an emergent zoonotic pathogen [7].

There is a large body of knowledge on balantidiasis across pig raising nations but little research has been

İletişim (Correspondence)
+92 333 6252189
muddassir.ali@uvas.edu.pk

conducted on the parasite in small ruminants. This study elaborates the prevalence and chemotherapy of *B. coli* in small ruminants in Lahore district, Pakistan and will provide help in further studies on *B.coli* in small ruminants.

MATERIAL and METHODS

About 5 g of fecal sample was collected directly from the rectum of 752 animals (n=376 sheep; n=376 goats). Self-sealing polythene bags were used to place samples in; these were transferred to the laboratory in ice pack cooler. The Sedimentation and Direct Smear Methods were used to find the *B. coli* presence while cystic count was carried out by the McMaster Technique. Briefly, direct smear technique was performed by a small quantity of feces was mixed with the physiological saline solution in a Petri dish and a uniform suspension was made by stirring. A drop of suspension was placed on glass slide, covered with cover slip and examination low power magnification (10x) of microscope for the presence of *B. coli* cyst. Sedimentation method was done by 5 grams of feces was thoroughly mixed with forty times of its volume of water. The suspension was then filtered through the fine sieve in a centrifuge tube and was then centrifuged at 1.500 rpm for 5-10 min. The supernatant was discarded. A small quantity of the retained sediment was then withdrawn into a pipette and transferred to a glass slide and examined under low power (10x) of microscope. In Mac Master Technique, 3 grams of feces was put in a shaker jar having 42 ml saturated NaCl. The jar was shaken well then poured through a wire mesh with an aperture of 0.15 mm. The filtrate of feces was well mixed and sufficient amount was withdrawn with a pasture pipette and then run into one counting chamber. After further mixing, a second sample was withdrawn and run into other counting chamber. All the cycts under the two separate grids were counted. Since 3 g of feces produced 45 ml of suspension (1 g per 15 ml suspension) and the volume of suspension examined was 0.3 ml (0.15 ml under each grid of the counting chamber) the number of eggs per gram of feces was obtained by multiplying the total number of eggs under the two grids by 50.

Chemotherapeutic Trial

Of 24 small ruminants (n = 12 sheep; n = 12 goat) positive for *B. coli* were divided into three groups (A-C). Each group comprised of 8 (n = 4 sheep; n = 4 goat). Group A, B, and C were treated with a single oral dose of secnidazole at 10mg/kg, metronidazole at 25 mg/kg, oxytetracycline at 8mg/kg respectively. The fecal samples of all groups were collected at day 0 (pre-treatment) and then on day 2, 4, 6 and 12 (post-treatment). The efficacy of the drugs used was assessed on the basis of reduction in cystic count and was calculated as per formula of [8].

[(Pretreatment CPG - Post treatment CPG / Pretreatment CPG] * 100

Hematological Studies

Hemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), and total leukocyte count (TLC) were estimated by hematological analyzer. Blood samples (5ml) were taken from 10 animals (n=5 sheep; n=5 goat) positive for *B. coli* and 10 from healthy animals (n=5 sheep; n=5 goat) directly from the jugular vein in EDTA coated vaccutainers. The study was approved by the Ethics Committee of University of Veterinary and Animal Sciences Lahore Pakistan (Serial No. 171/2013)

Statistical Analysis

Prevalence of *B. coli* was estimated by Pearson's chi-square test for significance whereas data on hematology was analyzed by Student's *t*-test. Data on chemotherapy was analyzed by one-way ANOVA, using SPSS (statistical package for social science). $P<0.05$ was considered as significant.

RESULTS

Data on sex and breed vs. prevalence is shown in *Table 1*. Of 752 animals surveyed, balantidiasis were found in 3.72%. Specie-wise prevalence revealed that *B. coli* were more prevalent in sheep (3.99%) as compared to goat (3.46%). However species risk factor was statistically found insignificant ($P>0.05$). The prevalence of *B. coli* in female sheep and goats were higher as compared to male. Amongst sheep population a high prevalence of *B. coli* was found in mix breeds (4.9%), followed by Thali (4.12%), Kajli (3.88%) and Salt Range (2.7%), respectively while in goats the prevalence was highest in mix breeds (4.38%), followed by Teddy (3.25%) and Beetal (2.59%), respectively. Data regarding hematology is presented in *Table 2*. A significant ($P<0.05$) decrease in hemoglobin and increased PCV was observed in infected animals as compared to healthy ones. Secnidazole showed higher efficacy in sheep (86.66%) and goats (93.75%) as compared to metronidazole and oxytetracyclin at the end of treatment trial *(Table 3)*.

DISCUSSION

Prevalence of balantidiasis in sheep are in close agreement with the findings of Kanyari et al.[4] who recorded a 2% prevalence of *B. coli* in sheep and 3% in goats, reared in urban and peri urban areas of Africa. This slight variation in the prevalence might be due to the different geoclimatic and management conditions. However no significant difference ($P>0.05$) was observed among sexes in both species. Unlikely, Kanyari et al. noticed higher prevalence of balantidiasis in male small ruminants as compared to female ones [4]. However Khan et al.[9]. noted a statistically insignificant higher prevalence of *B. coli* in female donkeys as compared to males. In current study, mixed breeds showed higher prevalence of *B. coli*

Table 1. *Sex and Breed-wise prevalence of B. coli in sheep and goats*
Tablo 1. *Koyun ve keçilerde seks ve cinse göre B. coli'nin prevalansı*

Animals			Positive		Negative	95% CL	MH Chi-sq P Value	OR/Reciprocal
			n	%				
Sheep	Sex	Male	7	3.59	188	1.58- 6.97	-	**OR** = 0.81 [reciprocal = 1.24]
		Female	8	4.42	173	2.07- 8.22		
		Overall	15	3.99	361	2.34-6.35		
	Breed	Kajli	4	3.88	99	1.25-9.10	P = 0.934	-
		Salt range	2	2.70	72	0.46 to 8.64		
		Thali	4	4.12	93	1.32 to 9.65		
		Mix breed	5	4.90	97	1.82-10.53		
Goat	Sex	Male	7	3.27	207	1.44 to 6.36	-	0.88 [reciprocal = 1.14]
		Female	6	3.70	156	1.51 to 7.54		
		Overall	13	3.46	363	1.94 to 5.69		
	Breed	Teddy	4	3.25	119	1.04 to 7.66	P = 0.607	-
		Beetal	3	2.59	113	0.66 to 6.88		
		Mix breed	6	4.38	131	1.79 to 8.88		

Table 2. *Effect of Balantidium coli on various blood parameters in sheep and goats (Mean ± SD)*
Tablo 2. *Koyun ve keçilerde çeşitli kan parametreleri üzerine Balantidium coli'nin etkisi (Mean ± SD)*

Hematological Parameters	Healthy Sheep	Affected Sheep	*P-Value*	Healthy Goats	Affected Goats	*P-Value*
Hemoglobin (g/dl)	11.64±1.33	9.05±0.92	0.009*	11.30±1.01	9.49±0.43	0.012*
PCV (%)	32.01±1.14	34.05±0.64	0.012*	32.03±1.25	34.27±0.62	0.012*
TEC (10^{12}/l)	12.26±1.32	9.54±0.96	0.007*	12.36±1.04	9.736±0.46	0.003*
TLC (10^{9}/l)	9.48±1.24	10.50±0.46	0.143	8.44±1.02]	9.51±0.53	0.084

** Significant (P<0.05)*

Table 3. *Comparative efficacy of oxytetracycline, secnidazole and metronidazole at various days in sheep and goats (Mean ± SD)*
Tablo 3. *Koyun ve keçilerde çeşitli günlerde oksitetrasiklin, seknidazol ve metronidazol karşılaştırmalı olarak etkinliği (Ortalama ± SS)*

Group	Treatment	*B. coli* Cyst Count/g at Day in Sheep						*B. coli* Cyst Count/g at Day in Goats					
		0	2	4	6	12	*P-value*	0	2	4	6	12	*P-value*
A	Secnidazole	375±95.74	275±95.74	175±95.74	100±81.65	50±57.74	0.152	400±81.65	275±95.74	200±81.65	100±81.65	25±50	0.095
B	Metronidazole	400±81.65	325±50	300±81.65	275±95.74	225±125.83		450±57.74	350±57.74	325±95.74	250±129.01	225±95.74	
C	Oxytetracycline	375±95.74	300±81.65	250±57.74	150±57.74	125±95.74		375±50	300±81.65	175±95.74	150±57.74	100±81.65	

among sheep and goats. It was observed that the mix breeds are more susceptible to *B. coli* in both species, but breed was found to be an insignificant (P>0.05) risk factor in both species. Result of hematological values in this study suggests parasitic infestation induces blood loss via intestinal hemorrhaging. Similarly, these hematological signs have also been observed in *B. coli* infected pigs [10] and donkeys [9]. Blantidiasis causes intestinal pathological lesions, accompanied by severe hemorrhaging and bloody diarrhea. Analysis of CPG pre and post treatment in both species concluded that Secnidazole was the most effective at decreasing fecal cyst counts, followed by Oxytetracycline and Metronidazole, respectively. However, differences of the three drugs in lowering cyst counts was found statistically insignificant (P<0.05). There was lack of literature mentioning the reasons for variations observed in treatment of balantidiasis in small ruminants. However, therapeutic results are closely in line with that of Bilal et al.[11] who also recorded significant decrease of *B. coli* cyst count in a positive cattle group receiving Secnidazole followed by Oxytetracycline and Metronidazole, respectively. Lowering in cyst count using Secnidazole revealed its higher efficacy as compared to Oxytetracycline and Metronidazole in sheep and goats. Previous studies also have demonstrated that Secnidazole and Oxytetracycline are more efficacious against *B. coli* in donkeys [9], cattle [4], and Buffalos than Metronidazole [12,13].

In recent years, the zoonotic importance of *B. coli* has been well noted and is especially detrimental to those

humans suffering from HIV/AIDS [14]. In Pakistan and other countries like Pakistan, small ruminants are mostly reared along with other animals and most of times are in close contact with human population. So, small ruminants infections in unhygienic environments may easily result on *B. coli* outbreaks in other animals and humans. So, clean drinking water and separate housing for domestic ruminants is advised to avoid transmission of infection and for securing public health. This is the first report from the studied area so it is difficult to conclude that either this is an emerging infection, or an endemic one that has been recently uncovered.

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Short Communication

Kuzey Kıbrıs Türk Cumhuriyeti'nde İshalli Buzağılarda Grup A Rotavirus Tespiti ve Moleküler Karakterizasyonu

Feray ALKAN [1] Mehmet Özkan TİMURKAN [2] İlke KARAYEL [1]

[1] Ankara Üniversitesi, Veteriner Fakültesi, Viroloji Anabilim Dalı, TR-06110 Dışkapı, Ankara - TÜRKİYE
[2] Atatürk Üniversitesi, Veteriner Fakültesi, Viroloji Anabilim Dalı, TR-25240 Yakutiye, Erzurum - TÜRKİYE

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Özet

Bu çalışmada, Kuzey Kıbrıs Türk Cumhuriyeti'nde (KKTC) yerleşik bir sığır yetiştiriciliği işletmesinde bulunan ishalli buzağılarda saptanan Grup A rotavirusun moleküler karakterizasyonu bildirildi. İshalli buzağılardan sağlanan dışkı örnekleri antijen ELISA ve Grup A rotavirus VP6 proteini kodlayan gen bölgeleri esas alınarak yapılan RT-PCR ile test edildi. Daha sonra enfeksiyona neden olan virusun VP4 ve VP7 proteinlerini kodlayan gen bölgeleri, generik ve tip spesifik primerler kullanılarak çoğaltıldı. Elde edilen verilere dayanılarak söz konusu işletmede bulunan buzağılarda enfeksiyona neden olan rotavirusun G6P[11] genotipe sahip olduğu belirlendi. Bu çalışma, KKTC'de buzağılarda Grup A rotavirusların G ve P genotiplerinin bildirildiği ilk çalışmadır.

Anahtar sözcükler: *Rotavirus, Buzağı, İshal, Genotip, KKTC*

The Molecular Characterization and Detection of Group A Rotavirus From Calves with Diarrhea in Turkish Republic of Northern Cyprus

Abstract

In this study, the molecular characterization of the Group A rotavirus obtained from calves with diarrhea in a farm in TRNC was reported. Faces samples were detected as positive by ELISA and RT-PCR depending on the amplification of the VP6 gene of rotavirus. Then the aetiological agent was analyzed by RT-PCR with generic and type-spesific to the genome segments encoding VP4 and VP7 of rotavirus. Findings revealed that the rotavirus circulating within this farm belongs to G6 and P[11] genotype. This is the first study to report the G and P genotypes of Group A rotavirus from calves with diarrhea in TRNC.

Keywords: *Rotavirus, Calf, Diarrhoea, Genotype, TRNC*

GİRİŞ

Grup A rotaviruslar birçok türün yenidoğanlarında enteritis nedenlerinden olup, özellikle sığır yetiştiriciliğinde, enfekte hayvanların ağırlık kazanmasında azalma ya da ölüm, veteriner hekimlik hizmetlerine ilişkin giderler, vb. nedenlere bağlı olarak, önemli ekonomik kayıplara yol açmaktadırlar [1].

Rotavirus genomu 11 segmentli, çift iplikçikli olup, 6 yapısal ve 6 yapısal olmayan proteini kodlar. Bu proteinlerden dış kapsitte yer alan VP7 (glikoprotein) ve VP4 (proteaz duyarlı protein) nötralizan antikorların oluşumunu uyarır. Bu proteinleri kodlayan gen bölgelerinin farklılıkları esas alınarak, rotaviruslar sırasıyla G ve P genotipleri olarak sınıflandırılırlar [2]. Bugüne kadar insan ve farklı hayvan türlerinde 27 G ve 37 P genotip bildirilmiştir [3-11]. Sığırlarda sıklıkla bildirilen G genotipleri G6, G10 ve G8; P genotipleri ise P[1], P[5] ve P[11] [5-9] olmakla birlikte, bunların dışında G ve P genotipe sahip rotaviruslar da saptanmıştır [2,3,10,11].

Kuzey Kıbrıs Türk Cumhuriyeti'nde (KKTC) yeni doğan buzağı ishallerinin etiyolojik ajanı olarak rotavirusların varlığı, yaygınlığı ve moleküler karakterizasyonuna ilişkin olarak, bilindiği kadarıyla, bugüne dek bir çalışma bildirilmemiştir. Bu çalışmada, KKTC'de bulunan bir sığır yetiştiriciliği işletmesinde bulunan buzağılarda saptanan rotavirusun G ve P genotipi belirlenmiş olup, enfeksiyonunun epidemiyolojisine yönelik değerlendirmelerde bulunulmuştur.

İletişim (Correspondence)
+90 312 3170315/4363
falkan@ankara.edu.tr

MATERYAL ve METOT

Saha Materyalleri ve Referans Viruslar

Bu çalışmada KKTC'de bulunan bir sığır yetiştiriciliği işletmesinde yeni doğan buzağılarda görülen ishal olgusunun tanısı amacıyla -2008 yılında- laboratuvarımıza gönderilen ve ticari Antijen-ELISA (BioK 067, Bio-X Diagnostic, Belçika) kiti kullanılarak rotavirus antijen varlığı saptanan dışkı örneklerini (n=5) temsilen rastgele seçilen bir dışkı örneğinde bulunan rotavirusun G ve P genotipleri incelendi. Çalışmada pozitif kontrol olarak grup A sığır rotavirus referans suşları olan NCDV (G6P[1]), B223 (G10P[11]) ve UK (G6P[5]) suşları kullanıldı.

RNA Ekstraksiyonu

RNA ekstraksiyonu, High Pure Viral RNA Ekstraksiyon (Cat No:11858882001, Roche, Almanya) kiti kullanılarak, üretici firmanın belirttiği prosedüre uygun olarak yapıldı.

RT-PCR İle Genotiplendirme

Bu amaçla, rotavirus nükleik asidinin mutasyonel açıdan korunaklı VP6 gen bölgesi hedef alınarak uygulanan RT-PCR'da beklenen büyüklükte amplikon (379 bp) saptanmasını takiben, VP4 ve VP7 gen bölgeleri için spesifik generik primerler ve tip spesifik primerler kullanılarak *(Tablo 1)*, saha virusu G ve P genotipi yönünden analiz edildi. G ve P tiplendirme amacıyla generik primerler ile yapılan PCR (1. tur) sonrasında elde edilen amplikonlar, farklı G (G6, G8 ve G10) ve P (P[1], P[5] ve P[11]) genotiplerine spesifik primerlerin kullanıldığı 2. tur PCR işlemine alındı. G8, G10 ve P genotipleri (P[1], P[5] ve P[11]) için yapılan 2. tur PCR işleminde forward primer olarak, 1. tur PCR forward primerleri kullanıldı.

PCR öncesinde, ekstraksiyon işlemi ile elde edilen viral RNA kullanılarak, Revert Aid First Strand cDNA Sentez Kiti (#K1622, Thermo Scientific, Almanya) ve üretici firmanın belirttiği prosedür ile cDNA elde edildi. Hazırlanan cDNA, hedef gen bölgeleri için tasarlanan primerler ile karıştırıldıktan sonra, sekonder RNA yapılarının denatürasyonu amacıyla 95°C'de 5 dk bekletildi ve buzda soğutuldu. Bunların üzerine daha önce kokteyl olarak hazırlanmış olan reaksiyon tamponu, dNTP (her biri 10 mM), $MgCl_2$ (25mM) ve 5 IU Taq DNA polimeraz karışımından ilave edilerek, termal çeviricide ısı döngülerine (40 siklus 94°C'de 1 dk, 50°C'de 1 dk ve 72°C'de 2 dk) maruz bırakıldı. Elde edilen sonuçlar etidyum bromid boyası varlığında hazırlanan agaroz jel elektroforez işlemi ile UV-transilluminasyonu izlendi ve görüntülendi.

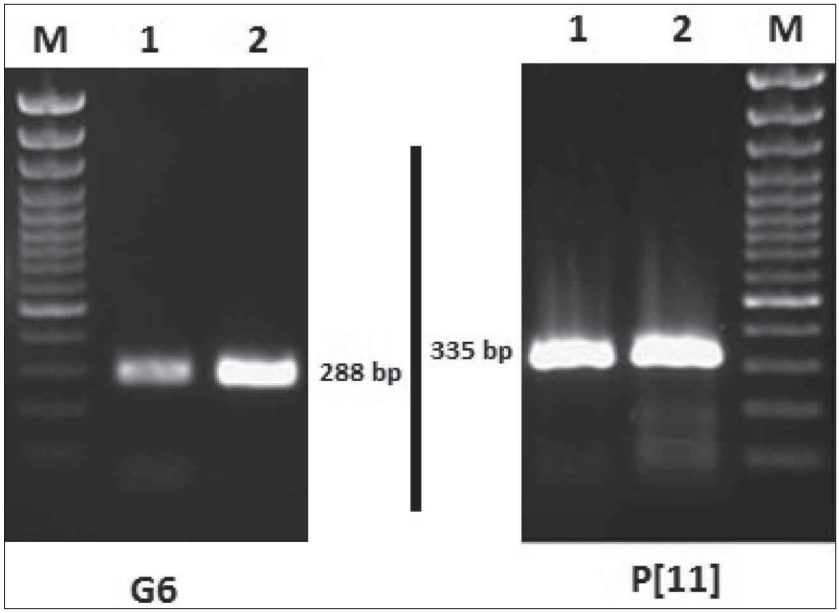
BULGULAR

Referans viruslar eşliğinde saha virusuna uygulanan RT-PCR sonrasında, VP4, VP6, VP7 kodlayan gen bölgesi için beklenen büyüklüklerde (sırasıyla 854 bp, 379 bp ve 1062 bp) ürün elde edildi. Tip spesifik primerler ile yapılan çalışma sonrasında ise VP7 gen bölgesi için referans G6 viruslar (NCDV ve UK) için 288 bp ve G10 virus (B223) için 715 bp büyüklüğünde; VP4 kodlayan gen bölgesi için referans viruslar NCDV, UK ve B223 için sırasıyla 463 bp (P[1] genotip spesifik), 662 bp (P[5] genotip spesifik) ve 335 bp (P[11] genotip spesifik) büyüklüğünde ürün elde edildi.

Araştırmada G ve P genotipleri sorgulanan saha virüsü ise G6 ve P[11] genotipleri ile uyumlu büyüklükte amplikon oluşturdu *(Şekil 1)*.

Tablo 1. *VP4, VP4 ve VP7 gen bölgelerinin çoğaltılması için kullanılan primer dizinleri*
Table 1. *Primers used for the amplification of VP4, VP6, VP7 gene regions of rotavirus*

Hedef	Primer	Primer Dizin (5'→3')	Gendeki Lokus	Ürün (bp)	Kaynak
VP6 Generik	VP6-F	GAC GGV GCR ACT ACA TGG T	747-766	379	[12]
	VP6-R	GTC CAA TTC ATN CCT GGT GG	1126-1106		
G Generik	Beg 9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	1062	[13]
	Eng 9	GGT CAC ATC ATA CAA TTC TAA TCT AAG	1062-1036		
	Eng 9 crw	GGT CAC ATC TTA CAG CTT TAA CCT	1062-1039		[14]
	End 9 deg	GGT CAC ATC DWM CAR YTC TAA YYH M	1062-1038		
P Generik	P-GenF	TTCATTATTGGGACGATTCACA	1064-1085	854	[7]
	P-GenR	CAACCGCAGCGGATATATCATC	1918-1897		
G genotiplendirme	G6-F	TGTATGGTATTGAATATACCAC	50-71	288	[15]
	G6-R	GGTATCAGCTATTTCGTTTGAT	336-315		
	G8-R	CGGTTCCGGATTAGACAC	274-256	274	[7]
	G10-R	TTCAGCCGTTGCGACTTC	715-697	715	
P genotiplendirme	P1-R	TTAAATTCATCTCTTAGTTCTC	1526-1505	463	[7]
	P5-R	GGCCGCATCGGATAAAGAGTCC	1725-1704	662	
	P11-R	TGCCTCATAATATTGTTGGTCT	1398-1377	335	



Şekil 1. Referans viruslar ve saha virusunun VP4 ve VP7 kodlayan gen bölgelerinin çoğaltılması görüntüsü. M; 100bp DNA Merdiveni, (Thermo Scientific, Almanya); Hat 1: ishalli buzağıda saptanan saha virüsü, Hat(2): Pozitif kontrol viruslar (G ve P tiplendirme için sırasıyla BRV NCDV (G6P[1]) ve BRV B223 (G10P[11])

Fig 1. The results of the amplification of genes coding VP4 and VP7 of reference viruses and of rotavirus field strain. M, 100 bp DNA ladder (Thermo Scientific, Germany); Lines 1, rotavirus detected in a calf with diarrhea; Lines 2, Positive controls (BRV NCDV (G6P[1]) and BRV B223 (G10P[11]) for G and P genotiping, respectively)

TARTIŞMA ve SONUÇ

Birçok hayvan türünün yeni doğanlarında ve çocuklarda ishal olgularının en önemli etiyolojik ajanlarından birisi olarak tanımlanan Grup A rotavirusların KKTC'de yenidoğan ishalli buzağılarda yaygınlığı, hastalığın neden olduğu ekonomik kayıpların boyutu ve rotavirus genotipleri konusunda herhangi bir bildirim bulunmamaktadır.

Bu çalışmada bir işletmede buzağılarda gözlenen ishal olgularından saptanan Grup A rotavirusun G ve P genotipleri sorgulanmıştır. Elde edilen veriler saha virusunun G6 ve P[11] genotipinde olduğunu ortaya koymuştur. Saptanan G ve P tipleri, dolayısıyla G/P genotip kombinasyonu, sığır rotavirusları için Türkiye [5] ve diğer bazı ülkelerde [9,11] sıklıkla tanımlanmıştır. Epidemiyolojik anlamda en önemli rotavirus G genotipleri G6 ve G10; P genotipleri ise P[1], P[5] ve P[11] olmakla birlikte [5,9-11,15], farklı genotip ya da genotip kombinasyonuna sahip rotaviruslar da [7,15] bildirilmiştir. Fodha ve ark.[8] Tunus'ta sığırlarda G6 ve G8 genotipleri ya da bunların her ikisinin birçok işletmede yaygın olduğunu bildirmişler ve ticari aşıların G6 içeriyor olması nedeniyle G8 genotipin ekonomik anlamada tehlikesine dikkat çekerek, G6/G8 divalent aşı kullanımını önermişlerdir. Bu çalışmada her ne kadar KKTC'deki bir işletmede buzağılarda oluşan ishale neden olan rotavirusun G ve P tiplendirme verileri sunulmuş ise de, belirlenen genotiplerin ve G/P genotip kombinasyonunun, buzağı ishallerinde sıklıkla saptanan G ve P genotipinde/genotip kombinasyonunda olması, tesadüfi bir bulgudan daha çok, KKTC'de buzağı ishallerinin en azından önemli bir kısmından sorumlu genotiplere işaret eden bir bulgu olarak değerlendirilmiştir. Bununla birlikte bu değerlendirmenin teyide muhtaç olduğunu ve daha çok sayıda enfekte buzağıdan sağlanan materyal kullanılarak yapılan serolojik ve moleküler karakterizasyon çalışmalarına ihtiyaç duyduğunu da hatırlatmakta yarar bulunmaktadır.

Sonuç olarak, bir ön bildirim niteliğinde olan bu çalışmada, KKTC'de en azından G6P[11] genotipli rotavirusların varlığı saptanmıştır. Planlanan yeni çalışmalar kapsamında KKTC'de enfeksiyonun yaygınlığı, ekonomik kayıpların düzeyi, farklı yerleşim yeri ve işletme tiplerinde bulunan buzağılarda saptanan rotavirusların moleküler karakterizasyonuna ilişkin bilgilerin edinilmesi sonrasında, enfeksiyondan korunmak için aşı kullanımı ve ticari aşı tercihleri konusunda bir yaklaşımda bulunulması da mümkün olabilecektir.

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Case Report

Bir Kedide *Mycoplasma haemofelis* Enfeksiyonu ve Etkenin Taramalı Elektron Mikroskopi ile Görüntülenmesi

Öznur ASLAN [1] İlknur KARACA BEKDİK [1] Esma Gamze İLGAR [2]

[1] Erciyes Üniversitesi, Veteriner Fakültesi, İç Hastalıkları Anabilim Dalı, TR-38039 Kayseri - TÜRKİYE
[2] Erciyes Üniversitesi, Veteriner Fakültesi, Genetik Anabilim Dalı, TR-38039 Kayseri - TÜRKİYE

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Özet

Bu olgu sunumunda, bir kedide *Mycoplasma haemofelis* enfeksiyonunun ve elektron mikroskopik görüntülenmesinin değerlendirilmesi amaçlandı. Olgunun materyalini iştahsızlık, halsizlik, dehidrasyon şikayeti olan, 3 yaşlı, erkek, sarman kedi oluşturdu. Kedinin yapılan klinik muayenesinde şiddetli dehidrasyon, apati, taşipne, kalpte üfürüm, taşikardi ve beden ısısının 38.2°C olduğu belirlendi. Kan örneklerinin sitolojik değerlendirilmesi ve polimeraz zincir reaksiyonu (PZR) ile *Mycoplasma haemofelis* tanısı kondu ve taramalı elektron mikroskobi ile görüntülendi. Sağaltım amacıyla oral yolla 14 gün süreyle 10 mg/kg/gün dozunda doksisiklin (Tetradox kapsül, Fako®) kullanıldı. Sağaltım sonucunda kedinin klinik durumunun düzeldiği, kan frotisinde etkenlerin sayısının azaldığı, kan hemogram ve serum biyokimyasal değerlerinin normale döndüğü belirlendi. Yapılan literatür araştırmasına göre ülkemizde *Mycoplasma haemofelis* ile doğal enfekte bir kedide etkenin taramalı elektron mikroskopi (TEM) yöntemiyle görüntülenmesi ilk kez gerçekleştirildi.

Anahtar sözcükler: *Mycoplasma haemofelis, Taramalı elektron mikroskop, Doksisiklin*

Mycoplasma haemofelis Infection and Imaging of *Mycoplasma haemofelis* Using Scanning Electron Microscopy in a Cat

Abstract

In this case report was aimed to the evaluation of *Mycoplasma haemofelis* infection and electron microscopic imaging in a cat. The material of present case was included a cat, three years old, male, referred to a history of anorexia, lethargy and dehydration. Clinically were defined dehydration, apathy, tachypnea, 38.2°C body temperature, murmur and tachycardia at the auscultation of heart. *Mycoplasma haemofelis* was detected at the peripheral smear and using PCR assay, and viewed with scanning electron microscopy. The patient was treated with doxycycline (Tetradox kapsül, Fako®) at a dose of 10 mg/kg/day orally for 14 days. The clinical condition of the cat was improved after treatment, which reduced the number of *Mycoplasma haemofelis* in blood smears, hematological and serum biochemical values were determined to be normal. To the best of authors' knowledge, this case reports the first imaging of scanning electron microscopy of *Mycoplasma haemofelis* in cats that are naturally infected in Turkey.

Keywords: *Mycoplasma haemofelis, Scanning electron microscopy, Doxycycline*

GİRİŞ

Hemotropik mikoplazmalar eritrosit yüzeyinde bulunan, kültürü yapılamayan, gram negatif, obligat ve bakteri duvarı olmayan mikroorganizmalardır. Pek çok memeli türünde tespit edilen mikroorganizmalar kedilerde infeksiyöz anemiye sebep olmaktadır [1-3]. Son yıllarda anemili insanlarda da belirlenmesi nedeniyle zoonotik potansiyeli olduğu bildirilmektedir [4]. Kedilerde, "*Mycoplasma haemofelis*" "*Candidatus Mycoplasma haemominutum*" ve "*Candidatus Mycoplasma turicensis*" olmak üzere üç tür olup [5] bunların en patojeni *Mycoplasma haemofelis*'tir [6,7].

Komplikasyon gelişmeyen hastaların çoğu uygun antibiyotik sağaltımı ile iyileşmekte [8] fakat taşıyıcı olarak kalabilmektedir [9]. *Mycoplasma haemofelis* sağaltımında genellikle tetrasiklin ve türevi ilaçların kullanılmasının yanı sıra yan etkisinin az olması nedeniyle doksisiklin daha çok tercih edilmektedir. Doksisiklin oral yolla 1-3 mg/kg dozda 21 gün veya 10 mg/kg dozda 24 saate bir 14 gün süreyle kullanılmaktadır [8].

Hastalığın tanısı klinik ve hematolojik bulgularla birlikte, sürme kan preparatlarında eritrosit yüzeylerinde organizmaların görülmesiyle ya da polimeraz zincir reaksiyonu

İletişim (Correspondence)
+90 352 2076666/29627
oznuratalay@gmail.com

(PZR) ile konulabilmektedir [10]. Bunların yanı sıra *Mycoplasma haemofelis*, *Candidatus Mycoplasma haemominutum* ve *Candidatus Mycoplasma turicensis*'in TEM ve geçirimli elektron mikroskopi yöntemleriyle görüntülenmeleri ve morfolojik özelliklerinin belirlenmesi tanıda oldukça önemlidir [2,11].

Kedilerde hemotropik mikoplazmaların morfolojik görünümleri ile ilgili dünyada çok az sayıda kaynak olması ve bu olgu ile ülkemizde *Mycoplasma haemofelis*'in ilk kez TEM yöntemiyle görüntülenmesi nedeniyle bu olgunun sunulması amaçlandı.

OLGUNUN TANIMI

Bu olgunun materyalini iştahsızlık, halsizlik, dehidrasyon şikayeti olan, 3 yaşlı, erkek, sarman kedi oluşturdu. Kedide bir haftadır iştahsızlık, halsizlik, çevreye karşı ilgisizlik olduğu ve hızlı bir şekilde kilo kaybettiği, zaman zaman diğer kedilerle kavga neticesinde yüz ve boynunda apseler oluştuğu öğrenildi.

Kedinin rutin klinik muayeneleri yapılarak, sağaltım öncesi ve sonrası hematolojik-moleküler muayeneler için *V. cephalica antebrachii*'den etilen diamin tetra asetat'lı (EDTA) ve serum tüplerine kan örnekleri alındı. Tam kan sayımı (Mindray BC 2800®, Çin) ve serum glukoz, total bilirubin, kan üre nitrojen (BUN), kreatinin ve alanin aminotransferaz (ALT) değerleri (Fuji Dri-Chem NX500, Japan) ölçüldü. Tam kan örneği FeLV ve FIV enfeksiyonları açısından (Anigen Rapid FIV Ab/FeLV Ag Test Kit®, Korea) test edildi. Hemotropic *Mycoplasma spp.* belirlemek amacıyla EDTA'lı kan örnekleri PZR metodu ile değerlendirildi. Periferal kandan yapılan sürme preparat Giemsa boyama yöntemi ile boyanarak immersiyon objektifle ışık mikroskobunda incelendi. EDTA'lı kan örneklerinden DNA analizi için standart fenol-kloroform ekstraksiyon metodu kullanıldı [12,13]. *M. haemofelis* ile *Candidatus M. haemominutum*'un 16S rRNA bölgesinden sırasıyla 170 baz çifti (bp) ve 193 bp'lik bölgeleri çoğaltan 5'- ACG AAA GTC TGA TGG AGC AAT A-3' forward primer ve 5'- ACG CCC AAT AAA TCC GRA TAA T-3' reverse primerler kullanılarak PZR reaksiyonu kuruldu.

PZR reaksiyonunda Jensen ve ark.[14] tarafından geliştirilen metod kullanıldı. EDTA'lı kan örnekleri 5x10^3 eritrosit olacak şekilde kan serum fizyolojik ile sulandırılarak, 1/3 oranında fosfat tampon solüsyonu ile (pH 7.4) karıştırıldı ve 4.000 devirde 10 dk santrifüj edildi. Eritrositler fosfat tampon solüsyonu ile yıkanarak %1.25'lik gluteraldehit solüsyonu ile iyice homojenize edilerek 2 saat beklendikten sonra lamel üzerine yayma yapılarak kurutuldu. Tespiti yapılan frotiler TEM (Leo 440 Computer Controlled Digital) yöntemiyle incelendi.

Kedinin klinik muayenesinde apati, dehidrasyon, taşipne, kalbin oskültasyonunda üfürüm, taşikardi saptandı ve beden ısısının 38.2°C olduğu belirlendi. Kedinin sağaltım öncesi ve sonrası hematolojik ve biyokimyasal değerleri *Tablo 1*'de gösterildi. FeLV ve FIV test sonucunun negatif olduğu tespit edildi. Kan frotisinin mikroskobik muayenesinde eritrosit yüzeyinde *Mycoplasma* spp. etkenleri belirlendi ve Howell Jolley cisimciklerinin sayısının arttığı gözlendi. Hastanın periferal kanında PZR'da *Mycoplasma haemofelis*'e ait 16S rRNA gen amplifiye ürünlerinin %2'lik agaroz jel elektroforezinde 170 bmp'de band görülmesi ile *(Şekil 1) Mycoplasma haemofelis* belirlendi.

Taramalı elektron mikroskobik görüntüde *Mycoplasma haemofelis* eritrosit membranında fasulye, oval veya yuvarlak şekillerde ve yaklaşık 0.5 µm çapında belirlendi *(Şekil 2)*.

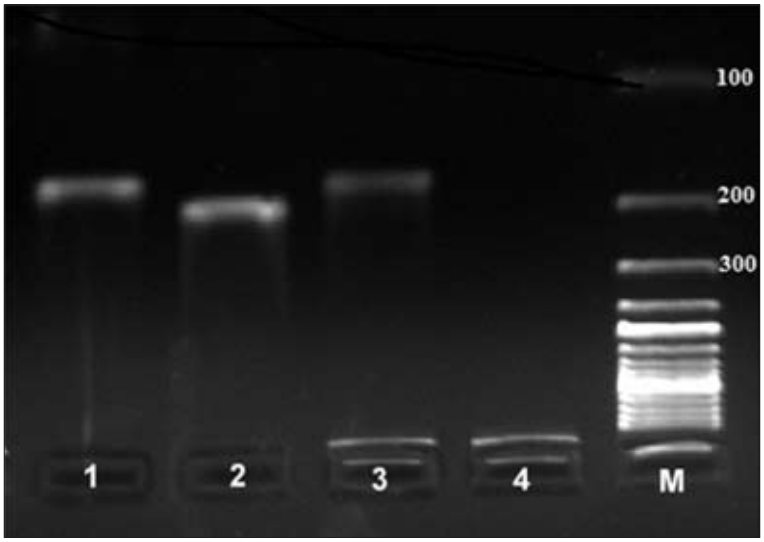
Hasta %5 dektroz (50 ml/gün, s.c.), NaCl izotonik (100 ml/gün s.c.), Fe ve B_{12} vitamini 0.5 ml s.c. (Ferdex B_{12}, Provet®) 5 gün ve doksisiklin (Tetradox kapsül, Fako®) 10 mg/kg/gün oral dozunda 14 gün süreyle sağaltım uygulandı.

Hastanın, sağaltımdan 14 gün sonra yapılan klinik muayenesinde iştahının normale döndüğü, çevresine karşı ilgisinin arttığı ve dehidrasyon durumunun düzeldiği, kan frotisinde ise etkenlerin sayısının azaldığı ancak kaybolmadığı gözlendi. Kan hemogram ve serum biyokimyasal değerlerinin referans sınırlar içinde olduğu belirlendi.

Tablo1. *Mycoplasma haemofelis ile enfekte kedinin sağaltım öncesi ve sonrası hematolojik ve biyokimyasal değerleri*

Table 1. *Hematological and serum biochemical parameters of cat with Mycoplasma haemofelis before and after the treatment*

Parametreler	Sağaltım Öncesi	Sağaltım Sonrası
WBC (x 10^3/µl)	6.7	8.4
Lenfosit (%)	70.3	45.1
Monosit (%)	3.1	4.8
Granülosit (%)	26.6	50.1
Eozinofil (%)	10.8	2.0
PLT (x 10^3/µl)	40	182
RBC (x 10^6/µl)	2.63	5.76
HGB (g/dl)	5.5	11.7
HCT (%)	14.1	30.5
MCV (fL)	53.8	53.0
MCH (pg)	20.9	20.3
MCHC (g/dl)	39	38.3
RDW (%)	20.9	17.3
BUN (mg/dl)	118	25.4
Kreatinin (mg/dl)	4.6	0.9
ALT (U/L)	134	57
Total Bilirubin (mg/dl)	1	0.2
Glukoz (mg/dl)	86	97

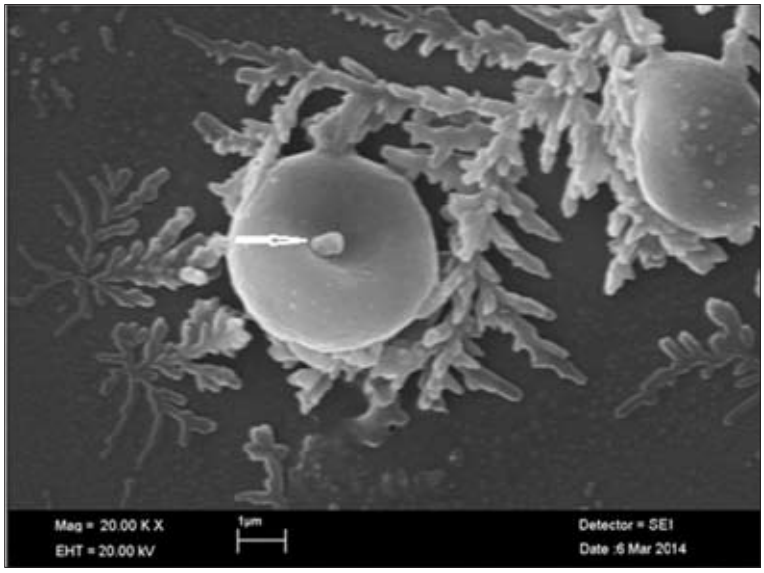


Şekil 1. Kedi periferal kanında Polimeraz Zincir Reaksiyonununa bağlı olarak *Mycoplasma haemofelis* (170 bp)'ten elde edilen DNA gen amplifiye ürünlerini gösteren Ethidium bromide ile boyanmış %2'lik agaroz jel elektroforez görüntüsü. M. 100bp'lik DNA cetveli, 1. *Mycoplasma haemofelis* pozitif kontrol, 2. *Candidatus Mycoplasma haemominitum* (193 bp) pozitif kontrol, 3. *Mycoplasma haemofelis* örnek, 4. Negatif kontrol (serum fizyolojik)

Fig 1. Ethidium bromide-stained agarose gel (2%) electropherogram demonstrating amplified DNA gene products obtained from *Mycoplasma haemofelis* (170 bp) using polymerase chain reaction from peripheral blood of cat. M. 100 bp DNA ladder. 1. *Mycoplasma haemofelis* positive control. 2. *Candidatus Mycoplasma haemominutum* (193 bp) positive control, 3. *Mycoplasma haemofelis* positive samples, 4. Negative control (isotonic saline)

Şekil 2. *Mycoplasma haemofelis* etkeninin taramalı elektron mikroskopik görünümü *(ok)*

Fig 2. Scanning electron microscopy image of *Mycoplasma haemofelis (arrow)*



TARTIŞMA ve SONUÇ

Hemotropik mikoplazmalara 4-6 yaşın altındaki, zayıf, anemik, kavga yaralanması ve/veya apsesi olan, rota virüs enfeksiyonlu, immun supresyonu bulunan, FeLV pozitif veya FeLV'e karşı aşılanmamış, dışarıyla ve çok sayıda kedi ile temas halinde olan kedilerde yakalanma riskinin yüksek olduğu bildirilmektedir [8,15,16]. Bu vaka da 3 yaşlı, erkek, kavga apseleri olan ve ev dışına çıkabilen bir kedi olmasıyla bildirimlerle uyumludur.

Mycoplasma haemofelis ile enfekte kedilerde yaygın olarak depresyon, iştahsızlık ve dehidrasyon geliştiği bazı kedilerde zayıflama görüldüğü, anemi nedeniyle, güçsüzlük, mukozalarda solgunluk, taşipne, taşikardi ve eğer enfeksiyon akut ve şiddetli seyrediyorsa nadir de olsa sinirsel semptomlar ve bayılma gibi belirtilerin ortaya çıkabileceği bildirilmektedir. Fiziksel muayenede şiplenomegali, kardiyak üfürüm, sarılık, bazı kedilerde ateş, terminal dönemde ise hipotermi gelişebildiği de belirtilmektedir [5]. Bu olguda da apati, iştahsızlık, şiddetli dehidrasyon ve kilo kaybı, anemi, mukozalarda solgunluk, taşikardi ve kardiyak üfürüm belirlenmiş olması bildirimlerle uyumludur.

Hastalarda genellikle makrositik-normokromik anemi saptandığı [8,17], *Mycoplasma haemofelis* ile enfekte kedilerde rejeneratif makrositik hipokromik hemolitik anemi gözlendiği [18] ve hastalığın başlangıcında dolaşımda fazla miktarda parazitli eritrositin bulunduğu olgularda hematokrit değerinin düştüğü bildirilmektedir [8]. Messick [19], köpeklerde *Mycoplasma haemocanis*'te nadiren de olsa trombositopeni gözlenebileceğini, ayrıca Aslan ve ark.[20] bir kedide haemobartonellozis olgusunda trombositopeni tespit ettiklerini bildirmişlerdir. Hematolojik muayenede; anizositozis, makrositik normokromik anemi, trombositopeni, hemoglobinemi, neutropeni, hematokrit değerde düşüş görülmesi bildirimlerle uyumludur. Klinik bulguların şiddeti ve hematokrit değerin düşüklüğü göz önüne alındığında enfeksiyonun akut formda olduğu düşünülmüştür.

Mycoplasma haemofelis ile enfekte kedilerde kan serumunda hepatik enzim düzeylerinde hafif artış, total serum proteini [8] ve bilirubin seviyesinde de artış olabileceği bildirilmektedir [21]. Ural [22] kedilerin sağaltım öncesi ortalama ALT, total bilirubin, total protein değeri ile sağaltım sonrası ALT, total bilirubin, total protein değeri arasındaki farkı istatistiksel olarak önemli bulmuş, AST, glukoz, kreatinin, üre ve GGT değerleri arasında anlamlı bir fark tespit etmemiştir. Bu olguda ise serum BUN ve kreatinin değerlerindeki artışın şiddetli dehidrasyondan, total bilirubin değerlerindeki artışın ise ekstra vasküler hemolizden kaynaklandığı düşünülmektedir.

Deneysel olarak yapılan bir çalışmada kedilerde *Candidatus Mycoplasma haemominutum* ve *Candidatus Mycoplasma turicensis* etkenlerinin yüksek çözünürlüklü TEM'de eritrositlerin yüzeyinde, 0.3 µm çapında ve *Mycoplasma haemofelis* etkenlerinin ise 0.5 µm çapında ve disk şeklinde oldukları bildirilmektedir [12]. Bu olguda da *Mycoplasma haemofelis* etkenlerinin eritrosit yüzeyinde fasulye, oval veya yuvarlak şekillerde ve yaklaşık 0.5 µm olarak belirlenmiş olması bildirimlerle uyumludur.

Sonuç olarak, değerlendirilen olgunun *Mycoplasma haemofelis* olduğu PZR yöntemiyle belirlendi ve 2 hafta süre ile doksisiklin sağaltımı uygulanarak klinik bulguların normale döndüğü ancak etkenin tamamen yok edilmesi için yeterli olmadığı görüldü. Ayrıca *Mycoplasma haemofelis* ile doğal enfekte bir kedide TEM yöntemiyle eritrosit yüzeyindeki görünümü ilk kez belirlendi.

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Case Report

Mitotane-Induced Hypoadrenocorticism in a Dog with Hyperadrenocorticism [1]

Ekrem Çağatay ÇOLAKOĞLU [1] Ali Evren HAYDARDEDEOĞLU [2]
Hadi ALİHOSSEİNİ [1] Arif KURTDEDE [1]

[1] *This case report has been accepted as poster presentation by the scientific committee of 5th Turkish Small Animal Veterinary Association (TSAVA) Congress, 2010, İstanbul, Turkey*

[1] Ankara University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-06110 Ankara - TURKEY
[2] Aksaray University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-68100 Aksaray - TURKEY

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Abstract

A 5-year-old, Terrier breed dog having the complaints of anestrus, polyuria, polydipsia and alopecia referred to Small Animal Veterinary Teaching Hospital. Paraclinical and clinical assessments confirmed the diagnosis of pituitary-dependent hyperadrenocorticism and Mitotane (25 mg/kg BID 5 days) prescribed. The dog was presented to clinic with the signs of vomiting, ataxia and weakness after two weeks. The owner reported that Mitotane therapy continued for 14 days without decreasing the dosage contrary to what is describe. Routin blood work and basal cortisol concentration revealed severe hypoglycemia and hypocortisolemia. Methylprednisolone acetate and supportive therapy were initiated. The dog died after three weeks according to owner's information. This case report emphasized that one of the possible complication of Mitotane therapy is hypoadrenocorticism.

Keywords: *Mitotane, Hypoadrenocorticism, Dog, Hyperadrenocorticism*

Hiperadrenokortisizmli Bir Köpekte Mitotan Kullanımına Bağlı Gelişen Hipoadrenokortisizm

Özet

5 yaşlı Terrier ırkı köpek, anöstrus, poliüri, polidipsi ve alopesi şikayetleriyle küçük hayvan hastanesine getirildi. Klinik ve paraklinik değerlendirmeler hipofiz kaynaklı hiperadrenokortisizmi doğruladı ve hastaya Mitotan (25 mg/kg BID 5 gün) reçete edildi. İki hafta sonra hasta; kusma, ataksi ve güçsüzlük şikayetleriyle getirildi. Hasta sahibi; herhangi bir dozaj azaltımı yapmaksızın, önerim dışı olarak Mitotan tedavisine 14 gün boyunca devam ettiğini belirtti. Rutin kan analizleri ve bazal kortizol ölçümlerinde şiddetli hipoglisemi ve hipokortisolemi belirlendi. Metilprednisolon asetat ve destekleyici sağaltıma başlandı. 3 hafta sonra hasta sahibinden köpeğin öldüğü bilgisi alındı. Bu olgu sunumu ile Mitotan tedavisinin olası komplikasyonunun hipoadrenokortisizm olabileceği vurgulanmıştır.

Anahtar sözcükler: *Mitotan, Hipoadrenokortisizm, Köpek, Hiperadrenokortisizm*

INTRODUCTION

Cushing's disease is a common endocrinological disorder of middle to old age dogs. A breed predisposition has been particularly noted in the Miniature Poodles, German Shepherds, Dachshunds, Boxers, Terriers and Beagles [1]. Cushing's Syndrome is characterised by the symptoms of polyuria, polydipsia, polyphagy, alopecia, pendulous abdomen, lethargy, recurrent urinary tract disease, hypotrichosis, hyperpigmentation, comedones and calcinosis cutis. Less common symptoms include hypertension, pulmonary thromboembolism, testicular atrophy, clitoral hypertrophy, anestrous, facial paralysis and corneal ulceration [2]. Pituitary-dependent hyperadrenocorticism (PDH) is responsible for 80% of all naturally occurred cases and more than 90% of dogs with PDH have a pituitary tumor. The pituitary tumors secreting excessive amounts of cortisol could be tiny (microadenoma) not easily identified by magnetic resonance imaging (MRI) [3].

Dogs with Cushing's disease have the stress leukogram,

İletişim (Correspondence)
+90 534 4304355
colakoglu@ankara.edu.tr

thrombocytosis (403.000-1.140.000 platelet/µl) and remarkable elevation of serum alkaline phosphatase level [4]. Hypercortisolemia is the most prominent sign for the Cushing's disease [5]. Thyroid hormone levels may be slightly low because of the suppression of glucocorticoids on TSH in dogs with Cushing's disease [3]. Adrenocorticotropic hormone stimulation and low-high dose dexamethasone suppression tests may be required for a definite diagnosis. Useful diagnostic tools to identify etiopathologic changes in Cushing's disease include abdominal radiography, ultrasonography and MRI. Options of surgery, radiation, adrenalectomy, hypophysectomy or medical therapy (Mitotane, Trilostane etc.) have been reported for treating hyperadrenocorticism in dogs [5].

Hypoadrenocorticism as an overlooked condition results from primary or secondary etiological reasons. Mitotane used for the control of PDH causes selective and progressive necrosis, cytotoxic effects on adrenal cortex and iatrogenic hypoadrenocorticism even when carefully procedure of dosing [6,7].

The purpose of this case report is to focus on the monitoring of Mitotane therapy in a dog with hyperadrenocorticism.

CASE HISTORY

A 5-year-old, 12 kg female, Terrier breed dog having the complaints of anestrus, polyuria, polydipsia and alopecia referred to Small Animal Veterinary Teaching Hospital. Alopecia, thin skin with hyperpigmentation, calcinosis cutis and vascularization and, pendulous abdomen were remarkable in physical examination *(Fig. 1-2)*. Initial diagnostic tests included complete blood count (CBC), liver function (Alkaline phosphatase, Alanin aminotranspherase, Aspartate aminotransferase, Gamma-glutamyl transferase), some electrolyte analysis (Sodium, Potassium, Chloride) and high dose dexamethasone

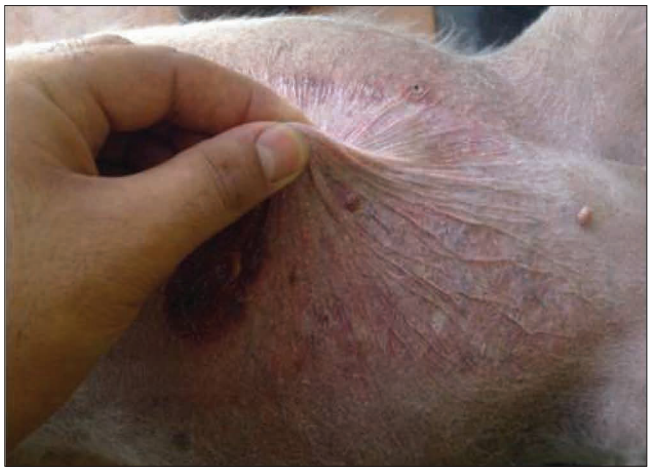

Fig 1. Alopecia, thin skin and vascularization
Şekil 1. Alopesi, incelmiş deri ve damarlaşma

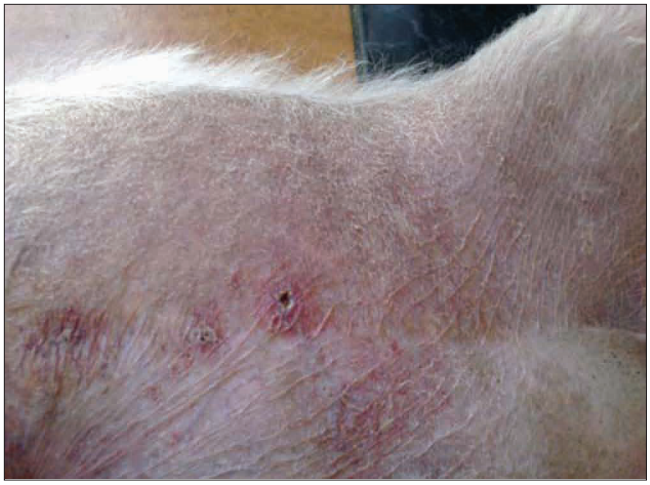

Fig 2. Calcinosis cutis
Şekil 2. Kalsinozis kutis

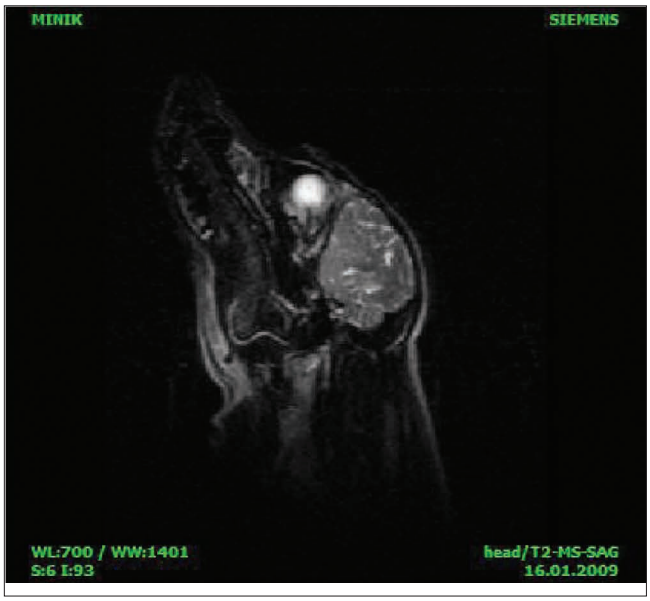

Fig 3. MRI imaging
Şekil 3. Hastanın MR görüntülemesi

suppression test. CBC revealed no abnormalities. High alkaline phosphatase (1301 IU/L; reference range [8], 20-156 IU/L), alanine aminotransferase (309 IU/L; reference range [8], 21-102 IU/L), glutamyl transferase (353 IU/L; reference range [8], 1.2-6.4 IU/L) and slightly elevated aspartate aminotransferase (93 IU/L; reference range [8], 23-66 IU/L) were noted. Serum sodium (149 mmol/l), potassium (3.9 mmol/l) and chloride (111 mmol/l) concentrations were obtained. The sodium:potassium (Na:K) ratio was 38 (reference range, 27-40 [8]). The analysis revealed total thyroxine (tT_4) and free thyroxine (fT_4) concentrations as 1.30 µg/dl and 7.00 pmol/L respectively (tT_4, reference range, 1.20-3.00 µg/dl; fT_4, reference range, 9.00-42.50 pmol/L). An increase in basal cortisol levels (28.50 µg/dl; reference range [8], 0.96-6.81 µg/dl) was also determined. The presence of hepatomegaly was

detected by abdominal ultrasonography. Any adrenal or pituitary masses and contour deformities were defined by ultrasonography and MRI *(Fig. 3)*. High dose Dexamethasone suppression test (0.1 mg/kg dexamethasone IV) was performed for the suspicion of hyperadrenocorticism. Post-dexamethasone cortisol concentrations were 6.1 µg/dl and 0.3 µg/dl at 4 and 6 h respectively. Paraclinical and clinical assessments confirmed the diagnosis of PDH. Mitotane (Lysodren®; 25 mg/kg BID 5 days) was prescribed for the dog. Fourteen days later the dog was presented to clinic with the signs of vomiting, ataxia and weakness. The owner reported that Mitotane therapy continued for 14 consecutive days in the same dose. Routin blood work and basal cortisol concentration revealed severe hypoglycemia (44 mg/dl; reference range [8], 70-120 mg/dl) and hypocortisolemia (<0.1 ng/dl; reference range [8], 1-6 µg/dl). In spite of optimal medical therapy with Methylprednisolone acetate (Prednol tablet®; 1 mg/kg) and supportive therapy, the dog died three weeks later.

DISCUSSION

Although new therapeutic options have been introduced, Mitotane is still the most common medical therapy for hyperadrenocorticism in dogs [9]. The therapeutic goal of the Mitotane as an adrenocorticolytic agent for dogs with PDH should be to provide the relatively hypoadrenal state without causing excessive Adrenocorticotropic hormone stimulation. For decreasing serum cortisol concentration, induction therapy with Mitotane is initiated at a dose of 25 mg/kg BID for 5 following days. Cortisol concentration falls in reference range during the induction phase. After induction therapy, Mitotane is administered for two times in a week with decreasing the dosage [10]. The adverse effects of Mitotane therapy associated with the gastrointestinal and neurologic signs can develop during the induction phase. The adverse effects include anorexia, episodes of vomiting, diarrhea, ataxy and weakness and decreases in water consumption. Long term Mitotane administration in induction phase may cause addisonian crisis [3]. In a study of 200 dogs treated with induction doses of Mitotane, adverse effects developped in 50 dogs [10]. In this case report, using Mitotane for 14 days by the owner instead of 5 days caused adverse effects and death in a dog with hyperadrenocorticism.

Veterinarians should be careful during the therapy period while using Mitotane in dogs. It is a necessity to follow the adverse clinical signs by the owners. In the third day of the induction phase (25 mg/kg BID), identifying the basal cortisol levels to prevent addisonian crisis may be lifesaving.

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Review

Hücrelerinden Arındırılmış Hayvansal Dokuların Rejeneratif Tedavilerde Kullanımı

Yavuz Emre ARSLAN [1] Meliha Merve HIZ [2] Tuğba SEZGİN ARSLAN [3]

[1] Çanakkale Onsekiz Mart Üniversitesi, Mühendislik Fakültesi, Biyomühendislik Bölümü, TR-17100 Çanakkale - TÜRKİYE
[2] Çanakkale Onsekiz Mart Üniversitesi, Fen-Edebiyat Fakültesi, Biyoloji Bölümü, TR-17100 Çanakkale - TÜRKİYE
[3] Çanakkale Onsekiz Mart Üniversitesi, Fen Bilimleri Enstitüsü, Biyomühendislik ve Malzeme Mühendisliğii Anabilim Dalı, TR-17100 Çanakkale - TÜRKİYE

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Özet

Çoklu potansiyele sahip adipoz mezenkimal kök hücreler, yağ dokusunda bulunan ve hematopoietik olmayan hücre hatlarıdır. Adipoz mezenkimal kök hücrelerin osteojenik, kondrojenik ve adipojenik fenotipler dâhil birçok soya farklılaşma yetenekleri vardır. Yüksek derecede plastisiteye sahip olmaları ve izolasyon kolaylığından ötürü doku mühendisliği ve rejeneratif tıp uygulamaları için büyük bir potansiyel taşımaktadırlar. Hücreleri, matrikse bağlı çözünür faktörleri ve iskele desteklerini kullanan doku mühendisliği yaklaşımı, fonksiyon gösteremeyen doku ve organların rejenerasyonu, tamiri veya replasmanı için umut vericidir. Üç-boyutlu iskeleler, transplante edilen hücrelerin yapışabileceği bir yüzey ve yeni doku veya organ oluşumuna rehberlik eden fiziksel bir destek olarak görev yaptığından dolayı gereklidir. Özellikle tüm doku ve organların hücrelerinden arındırılmasıyla elde edilen hücre dışı matrikslerin üç-boyutlu iskele olarak doku mühendisliği stratejilerinde kullanılması giderek ilgi görmektedir. Bu bağlamda, insan adipoz mezenkimal kök hücrelerin deselülerize tüm doku veya organlar üzerindeki davranışlarının incelenmesinin rejeneratif tıbba katkı sağlaması beklenmektedir. Bu derleme hayvansal dokuların deselülerizasyonu, elde edilen matriksler üzerinde kök hücrelerin davranışları ve bu matrikslerin insan/hayvan kliniği açısından potansiyel kullanımı hakkında güncel bir bakış açısı sunmaktadır.

Anahtar sözcükler: *Deselülerizasyon, Hücre dışı matriks, Adipoz kaynaklı mezenkimal kök hücre, Biyomalzeme, Doku mühendisliği, Rejeneratif tıp*

The Use of Decellularized Animal Tissues in Regenerative Therapies

Abstract

Human adipose-derived mesenchymal stem cells are nonhematopoietic cells found in the adipose tissue that have multipotent characteristics. Human adipose-derived mesenchymal stem cells have ability to differentiate into multiple lineages, including osteogenic, adipogenic and chondrogenic phenotypes. Because of their high degree of plasticity and ease of isolation, they have a great potential for tissue engineering and regenerative medicine applications. Tissue engineering, using cells, soluble matriks-bound factors and supporting scaffolds, is a promising approach for regeneration, repairing and replacement of malfunctioning tissues and organs. Three-dimensional scaffolds are essential to serve as an adhesive substrate for the transplanted cells and a physical support to guide the formation of new tissues or organs. Particularly, the use of extracellular matrices prepared by decellularized whole tissue and organ as three-dimensional constructs have drawn increasing attention as a tissue engineering strategy. In this context, it is expected that investigating the cellular behaviour of human adipose-derived mesenchymal stem cells on decellularized whole tissue and organ will have a positive impact on regenerative medicine. This review offers a current perspective about decellularization of animal tissues, stem cells' behaviors on obtained matrices and potential use of these matrices in human and/or animal clinic.

Keywords: *Decellularization, Extracellular matrix, Adipose derived mesenchymal stem cell, Biomaterial, Tissue engineering, Regenerative medicine*

GİRİŞ

Rejeneratif tıp, yaşlanma, hastalık veya travmaya bağlı zarar görmüş doku ve organların işlevselliğinin onarılmasını ya da iyileştirilmesini konu alan disiplinlerarası bir araştırma alanıdır. Rejeneratif tıbbın önemli bileşenlerinden biri olan doku mühendisliği ise hücre transplantasyonu, malzeme bilimi ve fonksiyonel biyolojik yapıların geliştirilmesinde

İletişim (Correspondence)
+90 286 2180018/2339
emre.arslan@comu.edu.tr

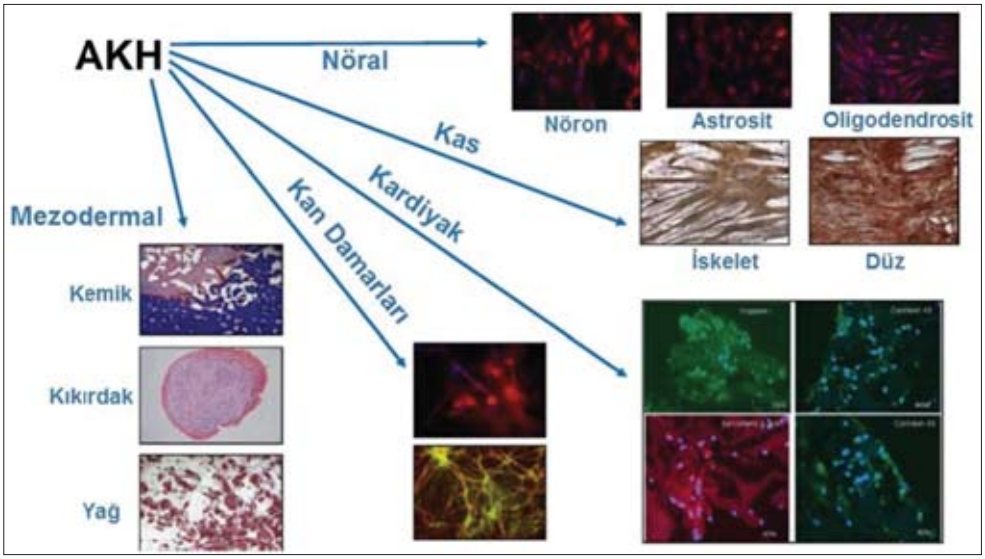
kullanılan mühendislik biliminin prensiplerini takip etmektedir. Doku mühendisliği stratejileri genel olarak hücrelerinden arındırılmış matriksler ve hücre/matriks yapıları olarak iki kategoride incelenebilir [1,2]. Hücre Dışı Matriks (HDM) bileşimine sahip biyolojik iskeleler klinikte sıklıkla birçok doku ve organın rejenerasyonu için kullanılmaktadır. Bileşiminde birçok biyoaktif molekül içeren HDM, dokuda hücrelerin bir arada tutulması ve dinamik hücresel davranışların düzenlenmesi gibi görevlerinin yanı sıra koruyucu ve destekleyici fonksiyonlara da sahiptir. Hücre dışı matrikslerin bileşiminde bulunan yapısal ve fonksiyonel moleküllerin karakterizasyonu tamamıyla yapılmamasına rağmen kollajen, elastin, laminin, fibronektin ve glikozaminoglikanlar gibi özel içerikler izole edilmişler ve birçok uygulamada kullanılmışlardır. Geniş ölçekte *in vivo* fonksiyona sahip HDM, doku-organ morfogenezi ve yara iyileşmesinde de görevlidir. Hücrelerinden arındırılmış HDM yapıları hücresel infiltrasyonu ve konak ile bütünleşmeyi destekler, yara izi oluşumunu önler ve işleme bağlı olarak immün yanıtın minimal düzeylerde kalmasını sağlar. Hücre Dışı Matriks temelli doku mühendisliği iskeleleri vücut içinde parçalanır ve matriksin yeniden şekillenmesini destekleyerek hasarlı veya nekroze olmuş dokuların rejenerasyonunu hızlandırırlar [3-5]. Kök hücreler ve HDM arasındaki etkileşimler, soy-spesifik farklılaşmanın indüklenmesi ve kimyasal/yapısal sinyaller sayesinde mezenkimal kök hücrelerin (MKH) biyolojik fonksiyonlarını sürdürebilmesi için gereklidir. Hücre Dışı Matriks tarafından oluşturulan biyofiziksel ve kimyasal sinyaller kök hücre adezyonunda, göçünde, yayılmasında, farklılaşmasında ve matriksin yeniden modellenmesinde kritik rol oynar [6]. Bu yüzden doku veya organların deselülerizasyon işlemleri sırasında takip edilen ve *Tablo 1*'de ayrıntıları verilen fiziksel, kimyasal ve enzimatik yöntemlerin hücre dışı matrikse en az zararla veya hiç zarar vermeyecek şekilde tasarlanması önemlidir.

Adipoz kaynaklı kök hücreler (AKH'ler), yağ dokusunun stromal vasküler fraksiyonunda bulunan MKH popülasyonlarıdır. Lipit depolama ve adipokin salgısı gibi iyi bilinen rolleri olan beyaz adipoz doku (BAD), yüksek miktarlarda bulunabilme ve izolasyon kolaylığı gibi avantajlarından ötürü kemik iliğine alternatif bir MKH kaynağı olarak karşımıza çıkmaktadır. AKH'ler BAD içerisinde perivasküler boşluklara yerleşmişlerdir ve artan enerji ihtiyacına yanıt olarak BAD büyümesini sağlamak amacıyla fizyolojik olarak adipogenezisi başlatırlar. AKH'lerin adipogenez dışında *Şekil 1*'de de görüldüğü üzere osteoblastlara, kondroblastlara, miyositlere, nöronlara ve diğer hücre tiplerine *in vitro* koşullarda farklılaşması indüklenebilmektedir [9].

Hayvanlar üzerinde yürütülen çalışmalarda, adipoz doku kaynaklı otolog mezenkimal kök hücreler kullanılarak hasarlı doku tedavisi gerçekleştirilmektedir. Nicpoń ve ark.[8], köpeklerde dirsek eklem bozukluğu tedavisinde adipoz doku kaynaklı otolog mezenkimal kök hücrelerin kullanıldığı grubun, non-steroid antienflamatuvar ilaçla tedavisi devam eden kontrol grubuna göre klinik semptomlarında daha hızlı bir gerilemenin olduğunu bildirmiştir. Otolog kök hücre tedavi sürecinde istenmeyen hiçbir yan etki görülmemesi, adipoz kaynaklı kök hücre tedavisinin insan kliniğine de güvenle uygulanabileceğini göstermektedir [8,9]. İlave olarak AKH'ler, diğer hücre tiplerine oranla indük-

Tablo 1. *Doku ve organları hücrelerinden arındırma teknikleri* [7]
Table 1. *The decellularization techniques of tissue and organs* [7]

Fiziksel	**Enzimatik**	**Kimyasal**
Mekanik Çalkalama	Tripsin	Alkali/Asit
Dondurma / Çözme	Endo-nükleazlar	Hipotonik ve Hipertonik Çözeltiler "*EDTA-EGTA*"
Sonikasyon	Ekzo-Nükleazlar	İyonik Olmayan Deterjanlar "*Triton X-100*"
		İyonik Deterjanlar "*Sodyum-Dodesil Sülfat (SDS)*"
		Zwitteriyonik Deterjanlar "*CHARPS*" "*Sülfobetanin -10 ve 16 (SB-10, SB-16)* "*Tri(n-butil) fosfat*"



Şekil 1. Adipoz kaynaklı kök hücrelerin çoklu-soy farklılaşma kapasitesi [13]

Fig 1. The multilineage differentiation capacity of adipose derived stem cells [13]

lenmiş pluripotent kök hücrelere (iPS) daha yüksek verimle yeniden programlanabilirler. Adipoz kaynaklı kök hücreler, immün ayrıcalıklı hücrelerdir ve doku tamirini kolaylaştıran immünomodülatör, anjiyojenik, antiapoptotik ve hematopoetik faktörleri salgılarlar. AKH'lerin çoklu-soy farklılaşma kapasitesi, eşsiz immünobiyolojik özellikleri ve sekrotomları rejeneratif tıp için büyük terapötik potansiyel sunmaktadır [9].

Doku mühendisliği, yara iyileşmesi ve doku yeniden modellenmesi sırasındaki biyolojik süreçleri taklit etmek amacıyla doku tamirinde anahtar bir faktör olarak HDM bileşenlerinin kullanılması hususuna dikkat çekmektedir. Hücre Dışı Matriks, hücreler için sadece yapısal destek sağlamakla kalmaz aynı zamanda hücre fizyolojisi ve fenotipi için önemli biyokimyasal ipuçlarını da bünyesinde barındırır [5,10,11]. Hücre-HDM ilişkisinin hücresel davranış üzerine etkisi primer hücre hatları için tüm yönleriyle tarif edilmesine rağmen, kök hücrelerin matriks üzerindeki davranışları ile ilgili çalışmalara yeni başlanmıştır. Bu ilişkilerin araştırılması, yönlendirmenin veya başka hücre hatlarına farklılaşmanın önlenmesi bakımından gereklidir. Örneğin; kollajen tip I üzerine ekilmiş embriyonik kök hücreler kendini yenileme kabiliyetini korurken erişkin MKH'ler osteojenik farklılaşmaya doğru gitmektedir. Bir başka çalışmada, kollajen tip I ve II bileşimli hidrojeller içerisine ekilmiş MKH'lerin kondrojenik farklılaşmaya gittiği gözlenmiştir. İlave olarak, kollajen tip IV ve laminin, glial hücre farklılaşmasını inhibe ederek nöral progenitör hücrelerin nöronlara farklılaşmasını tetikleyebilmektedir [12].

Bu yazı ile güncel rejeneratif tıp konuları içerisinde yer alan hayvansal dokuların deselülerizasyon teknikleri hakkında temel bilgilerin verilmesi ve elde edilen HDM'lerin biyolojik özelliklerinin irdelenmesi amaçlanmaktadır. Bu bilgiler ışığında insan/hayvan kök hücrelerinin bu matriksler üzerindeki davranışları (osteojenik, kondrojenik ve adipojenik) değerlendirilerek ortopedi/travmatoloji ve plastik cerrahi (onko-cerrahi vb.) gibi potansiyel disiplinlerarası klinik çalışmaların yaygınlaşmasına katkı sağlanması hedeflenmiştir.

KEMİK DOKU MÜHENDİSLİĞİ İÇİN DESELÜLERİZE MATRİKSLER

Kök hücrelerin kemik doku mühendisliğinde kullanılmasıyla birlikte, hem hayvan hem de insan hekimliğinde ortopedik hastalıkların tedavisinde yeni ufukların açılacağı düşünülmektedir [14-16]. ABD'de, enfeksiyonlar, travma, tümör rezeksiyonu, anormal gelişim ve konjenital malformasyon sonucunda oluşan kemik hasarlarının tamirinde yıllık 500.000'in üzerinde kemik grefti kullanılmaktadır. Bu sayının yaşlı nüfus ile doğru orantılı olarak artacağı tahmin edilmektedir. Klinikte, hastalıklı ya da hasarlı kemik dokusunun replasmanında osteojenik, osteo-kondüktif ve osteo-indüktif özeliklerinden dolayı otolog kemik greftleri sıklıkla kullanılmaktadır. Ancak bu yöntemin, donör saha morbiditesi, şiddetli kalıcı ağrı ve yeterince elde edilememe gibi olumsuz yönleri bulunmaktadır. Bu nedenlerle son yıllarda otolog kemik greftine alternatif olarak polimerler, kollajen süngerler, seramikler ve metallerin kullanılabilirliği yönünde araştırmalar yoğunlaşmaktadır. Nitekim bu amaçla yapılan çalışmalarda, potansiyel kemik greft malzemelerinin osteojenisitik özelliklerini araştırmak için osteo-progenitör hücreler *in vitro* koşullarda özellikli iskeleler üzerinde kültüre edilmiş ve iskelelerin ekili hücrelerin osteojenik farklılaşması ve yayılması üzerine etkileri değerlendirilmiştir. Nitekim HDM-titanyum örgü kompozit iskelelerin MKH'lerin osteojenik farklılaşması ve yayılmasını tetiklediği gösterilmiştir [17]. Kemik allogreftlerinde yeniden kolonileşmeyi destekleyen ve yeniden doku canlanmasını indükleyen yapıları üretmek için geliştirilmiş birçok yöntem bulunmaktadır. Bu nedenle MKH'ler yüklü kemik allogreftinden yapılmış kompozit bir iskele anjiyojenez ve osteo-indüktiviteyi tetikleyen ve büyüme faktörlerini salan bir sistem olarak önerilebilir [18]. Osteojenik faktörlerin ve MKH'lerin kullanıldığı biyolojik ajanlar ile kemik rejenerasyonu günümüzde klinik bir gerçeklik haline gelmiştir. Tuğlu ve ark.[16], erişkin Wistar sıçanlarında oluşturulan yapay defektlerin tedavisinde kök hücre ekili biyomalzemeler kullanmışlar ve kök hücre/biyomalzeme yapısının kontrol grubuna göre osteoid üretimini arttırması ile yeni kemik dokusu oluşumunu ve tedavi sürecini kısalttığını bildirmişlerdir. Hayvan modellerinde MKH transplantasyonunun heyecan verici bulgularının insan kliniği için de umut verici olduğu görülmektedir [16,19].

Büyük ölçekte kemik defektlerinin tamirinde kullanılan doku mühendisliği yapıları geliştirilirken üç önemli bileşenin anahtar rol oynadığı bilinmektedir. Bunlar;

I. MKH'ler

II. Osteo-kondüktif matriks

III. İndüktif faktörlerdir [20].

Kemik doku mühendisliğinde diğer önemli bir strateji ise kemik dokusu tamiri ve rejenerasyonunda görevli sinyal moleküllerini sağlayan ve mikroçevreyi taklit edebilen yapıların ortaya konulmasıdır [21,22]. Bu amaçla geliştirilen ve kemik grefti yedeği olarak kullanılan demineralize kemik matriksleri (DKM), oto ve allogreftlerin sınırlamalarını aşmak ve tedavi sürecini hızlandırmak açısından önemlidir.

Osteo-kondüktif özellikte DKM allojenik kemiğin asit ekstraksiyonu sonucunda üretilir ve yapısında büyüme faktörleri, kollajen olmayan proteinler ve tip I kollajeni barındırır. DKM'nin osteo-indüktif etkisinin hayvan çalışmalarıyla açık bir şekilde ortaya konulması ve klinikte DKM ürünlerine yoğun bir ilginin olmasına karşın insan hekimliğinde benzer uygulamaların sınırlı sayıda olduğu görülmektedir [23]. Ayrıca, son zamanlarda hücre temelli tedaviler için gözenekli hidroksiapatit iskeleleri çalışılmaya

başlanmıştır. Bu araştırmalar sonucunda etkin bir iskele geliştirebilmek için hücre tutunması, yayılması ve farklılaşmasına olanak sağlayan üç-boyutlu birbiri ile bağlantılı gözenekli yapıların gerekliliği vurgulanmaktadır [24].

KIKIRDAK DOKU MÜHENDİSLİĞİ İÇİN DESELÜLERİZE MATRİKSLER

Otolog kondrositlerin kullanıldığı kıkırdak doku tamiri prosedürleri, cerrahi müdahale öncesi defekt bölgesinde sıklıkla fibrokartilaj oluşumu ile sonuçlanan sağlıklı kıkırdak dokusu hasarları gibi birçok dezavantaj içerir. Bu nedenle, rejenerasyon kalitesini arttırmak için erişkin MKH'ler alternatif bir kaynak olarak karşımıza çıkmaktadır. MKH'lerin artiküler kıkırdak tamirinde kullanılması göz önüne alındığında hiyalin artiküler kıkırdakta bulunan hücreler gibi hipertropiye ve terminal farklılaşmaya karşı direnç gösteren kararlı kondrositlerin kök hücrelerden elde edilmesi büyük önem taşımaktadır [25]. Hücreler doku rejenerasyonunda hayati bir rol üstlenmektedir ve HDM gibi biyofiziksel sinyallerden etkilenirler. Hücrelerin özellikli ürünleri olan HDM, yalnızca fiziksel sinyaller oluşturmakla kalmayıp aynı zamanda biyolojik sinyaller de oluşturur. HDM bir sitokin deposu olarak görev yapabilir ve hücre-matriks etkileşimini ayarlayarak kök hücrelerin yaşamsal süreçlerini etkiler. Mühendisliği yapılmış doku ürünlerinin uzun dönem kararlılığı ve canlılığı doğal HDM bileşimine benzerliği ile doğru orantılıdır. Bu yüzden HDM, hücresel davranışların düzenlenmesinde ve doku rejenerasyonunda önemli bir rol oynar [26]. Kıkırdak HDM'den üretilen iskele, hücreleri küresel fenotipte tutabilen ancak kondrogenezin indüklenmesi için harici büyüme faktörlerine ihtiyaç duyan alginat gibi hidrojellerin aksine doğrudan kondrojenik farklılaşmayı etkileyen kondroindüktif bir çevre sağlamaktadır. Kıkırdak kaynaklı matriks (KKM) iskele insan AKH'lerin ve kemik iliği MKH'lerin çoğalmasını, kondrojenik farklılaşmasını ve kıkırdak matriksi biriktirmesini teşvik etmektedir. Harici büyüme faktörü yokluğunda KKM yapıları, domuz ve insan kondrositlerine benzer şekilde AKH'lerde kıkırdağa özgü genlerin ifadesini ve kıkırdak proteinlerinin sentezini arttırabilir [27]. Yumuşak doku rejenerasyonu ve konak doku ile implant uyumunu arttırmak amacıyla hücresel infiltrasyon ve canlılığı koruyan, yapısal olarak dayanıklı, hücre sinyalleşmesini teşvik edebilen ve yüksek miktarda gözenekli tipik biyo-iskeleler tasarlanmaktadır. Güncel çalışmalar birçok doku mühendisliği uygulamasına adapte edilebilen, düşük maliyetli, biyolojik olarak aktif ve yüksek ölçüde işlenebilir doğal kollajen köpükler üzerine odaklanmıştır. Dokuya özgü HDM kaynaklı köpükleri üretmek için insan HDM'nin kullanılmasına dair geniş ölçüde çalışmalar hala yoktur ve hücrelerinden arındırılmış insan dokularının biyo-iskele üretiminde ilginç bir kaynak olabileceği düşünülmektedir. HDM'in karmaşık mikroçevresi doku rejenerasyonu için kritik olan ve dokuya özgü canlılık, yayılma, göç etme ve farklılaşma gibi geniş çapta hücresel davranışa rehberlik edebilir [28].

PLASTİK VE REKONSTRÜKTİF CERRAHİ İÇİN DESELÜLERİZE MATRİKSLER

Meme kanseri tedavisinde lumpektomi veya mastektomi işlemleri sonucu oluşan yumuşak doku kayıplarının tekrar eş değeri ile doldurulması önemli bir cerrahi işlem durumundadır. Klinik raporlar ve temel araştırmalara göre de adipoz mezenkimal kök hücreler yağ greftleme işleminde önemli bir rol oynamaktadır. Hücresel bileşenler için mikroçevrenin oluşturulmasında HDM'in kullanılması ise adipoz doku tamiri için önemli diğer bir işlemdir. Plastik cerrahide kullanılan "benzeriyle değiştir" kuralına göre adipoz dokudan elde edilen HDM biyo-taklit bir iskele olarak fonksiyon gösterebilir [29]. Bu yüzden, özellikle yanık veya kanser tedavilerinde hasarlı dokunun rezeksiyonu sonucu oluşan boşluğu doldurmasının yanı sıra yağ dokusunun doğal şeklini alarak yeniden oluşumunu kolaylaştıran malzemelerin geliştirilmesi önemli bir klinik ihtiyaç haline gelmiştir. Klinisyenler, lipektomi sonucu elde edilen adipoz dokunun vücudun farklı bölgelerindeki deri altı boşluklara doğrudan transplantasyonu veya "lipotransfer" olarak adlandırılan bir yöntem ile bu sorunu çözmeye başlamışlardır. Son zamanlarda, biyokimyasal ve biyomekaniksel HDM ipuçlarının (sinyal molekülleri, yapısal proteinler ve glikozaminoglikanlar) kök hücrelerin farklılaşmasında uyarıcı bir rol oynadığı gösterilmesine rağmen, yapının adipogenezisi nasıl indüklediğine dair yeteri kadar araştırma yapılmamıştır [30]. Bu alanda yapılacak yeni çalışmaların plastik ve rekonstrüktif cerrahide önemli olacağı düşünülmektedir.

KÖK HÜCRE FARKLILAŞMASININ MOLEKÜLER DÜZEYDE İNCELENMESİ

Mezenkimal kök hücrelerin fizyolojik koşullarda osteojenik ya da kondrojenik farklılaşması hormonlar (paratiroid hormonu, östrojen, glukokortikoidler vb.) veya büyüme faktörleri [kemik morfogenetik proteini (BMP), dönüştürücü büyüme faktörü–β (TGF–β) ve fibroblast büyüme faktörü 2 (FGF2)] tarafından indüklenir [31]. Bununla birlikte osteojenik farklılaşma, harici bir uyaran (lityum) tarafından da indüklenebilir [32]. Mezenkimal kök hücrelerin uyarıcıya bağlı kondrojenik, osteojenik veya adipojenik farklılaşmasının yönünü sinyal molekülleri belirler. Adipojenez, kondrojenez ve osteojenez aşamalarında sırasıyla PPAR γ2 (peroksizom proliferatör-aktive edici reseptör-γ 2), Sox9 (SRY-kutu 9) ve Runx2 (Runt ilgili transkripsiyon faktörü 2) farklılaşmayı başlatan ana transkripsiyon faktörleridir [33-35]. MKH'lerin farklılaşmasında Wnt/β-katenin sinyal yolağında bulunan β-kateninin fosforlanarak yıkılması ya da sitoplazma konsantrasyonunun artmasıyla çekirdekte lokalize olması farklılaşmanın yönünü belirleyen temel mekanizmadır [33,34]. Wnt proteininin alt grupları hücre zarında kendine ait reseptörlere bağlanıp hücre dışı sinyalin hücre içi sin-

yale dönüşmesini ve hedef gen ifadesinin değişmesini tetikler [33-35].

Osteoprogenitör hücrelerin osteoblast ve kondrositlere dönüşümü Wnt/β-katenin sinyal yolağında β-katenin molekülünün konsantrasyonuna ve çekirdekte lokalizasyonuna bağlı olarak değişir [36,37]. Wnt sinyal yolağı üzerinden β-katenin'in düşük konsantrasyonu kondrositlere, yüksek konsantrasyonu ise osteoblastlara farklılaşmayı sağlar. Kondrojenik farklılaşma ve kıkırdak oluşumunu SRY (sex determining region Y)-box 9 (Sox9); osteojenik farklılaşma ve endokordiyal ossifikasyonunu ise TGF-ailesi üyesi olan BMP2 aktive eder [38]. Adipojenik farklılaşma için ise PPAR γ2 ifadesi sonucu β-katenin molekülünün yıkıcı kompleksle parçalanmasıyla Wnt/β-katenin sinyal yolağının inaktive olması gereklidir. Osteoblastlarda Wnt/β-katenin sinyal yolağında β-katenin ifadesinin kaybı osteoblastların adipositlere farklılaşmasına neden olur [39]. TGF-β ailesinin en büyük alt grubunu BMP oluşturmaktadır [40]. Osteoprogenitör hücrelerin osteoblast farklılaşması sırasında BMP-2, BMP-4, BMP-6 ve BMP-7 ifadelerinin artması Runx2'nin de aktivasyonunu sağlar [40,41]. Kondroosteoprogenitör hücrelerin BMP-2'nin immobilize edildiği alümina kaplı nano geçirgen yüzeylerde osteojenik farklılaşmaya gitmesi BMP-2'nin osteoblast farklılaşmasındaki ana kontrol noktası olduğunun göstergesidir [42]. BMP-2, heteromerik reseptörü üzerinden Smad proteinleri aktive ederek Runx2 gen ifadesini arttırmaktadır [43]. Runx2 ise osteoblast farklılaşmasından sorumlu olan gen bölgelerinin promotörlerinde bulunan OSE2 (osteoblast-specific cis-acting element 2) bölgelerine bağlanarak osteoblastlara özgü genlerin ifadesini başlatır [44]. Ayrıca, pre-osteoblastların olgun osteoblastlara ve osteositlere farklılaşmasını bir transkripsiyon faktörü olan Osterix (Osx) düzenler [45]. MKH'lerden osteoblastlara farklılaşma Runx2, alkalen fosfataz (ALP), Tip I Kollajen (Col I), osteopontin (OPN) ve osteokalsin (OC) gibi genlerinin ifadelerindeki artış ile karakterizedir [46-48]. Kondrogenezin erken aşamalarında hiyalüronik asit (HA) ve kondroitin sülfat (CS) kondrojenik bir transkripsiyon faktörü olan Sox9'un ifadesini arttırırken; kollajen tip-I (fibroblastik işaretçi) ifadesinin azalmasına neden olmaktadır. Öncül kondrositlerin belirlenmesinde ise Sox9 biyolojik bir işaretçi proteini olarak tercih edilmektedir. Kondrosit farklılaşmasında Sox9 ve β-katenin moleküllerinin fiziksel etkileşimleri sonucu kondrosit farklılaşması gerçekleşebileceği gibi osteoprogenitör hücreler de oluşabilmektedir. Bu noktada farklılaşmanın yönünü β-katenin çekirdek lokalizasyonu belirlemektedir. Kondrosit farklılaşmasında β-katenin çekirdek lokalizasyonu daha azdır ve kondrosite özgü olan CD44, CD151 yüzey işaretçileri ile agrekan, tip-2 kollajen, tip-4 kollajen gibi kondrositlere özgü proteinlerin ifadesi de artar. Sox9 proteininin miktarının azalmasıyla kondrositlerin proliferatif bölgeden hipertropik farklılaşması için Runx2/3 ifadesinin artması sonucu, β-katenin aracılı Wnt sinyal yolağının aktivasyonu ile [49,50] alkalen fosfataz ve kollajen tip-10 (Col X/Col10A1) ifadesi artmaktadır [51,52]. Terminal farklılaşmasını tamamlayan kondrositler apoptoza giderek kıkırdak dokusunun mineralizasyonu ile kemik yapısına dönüşebilir [53]. Kemik dokusuna farklılaşma sonucu kan damarlarının invazyonu için vasküler endotelyal büyüme faktörü (VEGF) ve kıkırdak dokunun parçalanması için matriks metallo proteinazlar salgılanır [54]. Adipojenik farklılaşmada ise kondrojenik ve osteojenik farklılaşmanın aksine Wnt sinyal yolağını aktive eden β-katenin ifadesinin azalması söz konusudur. Öncül adipositlerde Wnt10b, Wnt10a, ve Wnt6 hücre yüzeyinde bulunan Fzd ve LRP sinyal reseptörlerine bağlanarak β-katenin'in hipometile durumda kalmasını sağlayan GSK3β'yı inhibe ederek Wnt/β-katenin yolağı üzerinden T-hücre faktör (TCF) ile WNT hedef genlerinin ifade olmasını sağlar [55]. Adipositlerin olgunlaşmasında GSK3β tarafından aktive edilen proteozomal kompleksin β-katenin'leri yıkması gerekir. Bu işlem TNFα'nın hücre yüzeyindeki TNFα reseptörüne (TNFR1) ya da WNT5B'nin kendine ait hücre reseptörüne bağlanmasıyla β-katenin'lerin parçalanması sonucu gerçekleşir [56]. Preadipoz dokuda MAP kinaz ve GSK3β, bir transkripsiyon faktörü olan CCAAT/enhancer-binding protein β'nın (C/EBPβ) fosforlanması ve C/EBP-β'nın konformasyonel değişimiyle adipojenezden sorumlu genlerin PPARγ arttırıcı bölgelerine bağlanarak hedef genlerin transkripsiyonunu sağlar [57]. Peroksizom Proliferatör Cevap Elementleri (PPRE) olarak bilinen DNA dizisine ise transkripte olan PPARγ ve C/EBPα ile Retinoid X Reseptör (RXR) gibi koaktivatörlerin bağlanması ile adipojenik farklılaşmadan sorumlu genlerin ifadesi gerçekleşir [56]. Bu kompleks, adipositlerde ALBP (Adiponectin), FABP4 (Fatty Acid Transporter), Glut4 (Glukoz Transporter-4), LEP (Leptin) ve LPL (Lipoprotein Lipaz) genlerinin ifadesini arttırır [58].

SONUÇ

Doku mühendisliği uygulamaları, organ ve doku rejenerasyonu/tamirine gereksinim duyan hastalar için umut verici bir tedavi yaklaşımı olarak karşımıza çıkmaktadır. Bu bağlamda son zamanlarda giderek önemi artan hayvansal kaynaklı tüm doku ve organların hücrelerinden arındırılmasıyla elde edilen HDM üç-boyutlu iskelelerin terapötik uygulamalarda sıklıkla kullanılmaya başlandığı görülmektedir. Bahsi geçen yaklaşımda osteojenik, kondrojenik ve adipojenik fenotipler dâhil birçok soya farklılaşma yeteneğine sahip AKH'ler *ex vivo* şartlarda deselülerize edilen doku ve organlar üzerine ekildikten sonra özel şartlar altında yeniden farklılaştırılarak çoğaltılmakta ve oluşan doku benzeri yeni yapılar transplante edilmektedir. HDM bileşimine sahip biyolojik iskeleler çoğalma ve farklılaşma gibi hücresel davranışları düzenlerken aynı zamanda anjiyojenez ve parankimal hücrelerin tutunması için destek görevi de görmektedirler. Diğer taraftan deselülerize matrikslerden farklı yapısal, mekaniksel ve biyolojik özelliğe sahip hidrojeller üretilebilir. Koruyucu ve destekleyici fonksiyonlara sahip bu hidrojeller kemik ve kıkırdak doku mühendisliği çalışmalarında sıklıkla kullanılmaktadır. Ayrıca meme kanseri hastalarının tedavisi

için uygulanan lumpektomi veya mastektomi gibi cerrahi işlemler sonrasında meydana gelen yumuşak doku kayıplarının giderilmesinde kök hücrelerin ve otogreftlerin kullanılmasını konu alan çalışmalar kişiye özgü tedavi yaklaşımı açısından ilgi çekicidir. Memeli HDM'lerinin yapısal ve fonksiyonel özellikleri canlıda homeostazinin sağlanması için önemlidir ve bu yapıların klinikte başarılı bir terapötik ajan olarak kullanılmasının da anahtarıdır. HDM biyo-iskeleleri konu alan rejeneratif tıp stratejileri temel olarak etkili bir deselülerizasyon, kök hücrelerin bu yapılara ekimi, vaskülarizasyon, farklılaşmanın qRT-PCR ile karakterizasyonu ve hücre/iskele yapısının transplantasyonu basamaklarını içermektedir. Birçok hastalığın tedavisinde kullanılma potansiyeli olan bu yapıların üretilmesinde deselülerizasyon işleminin optimizasyonu kritiktir. Deselülerizasyon fiziksel, kimyasal ve enzimatik işlemlerin bir dizi kombinasyonu ile gerçekleştirilir. Yapısal ve fonksiyonel özelliklerini koruyan hücresiz HDM biyo-iskeleler elde etmek için ılıman koşulları sağlayan protokollerin oluşturulması beklenir. Doku veya organların deselülerizasyon işlemleri sırasında takip edilen fiziksel, kimyasal ve enzimatik yöntemler üretilecek iskelelerin mekaniksel davranış ve hücresel cevaplara ev sahipliği yapma gibi birçok önemli parametresini etkilemektedir. Deselülerizasyon işlemi sırasında adeziv proteinlerin ve glikozaminoglikanların zarar görmesi iskelenin kendi biyo-aktivitesini ve iskele üzerinde hücre göçünü yavaşlatabilmektedir. Kollajen ağının kırılması ile mekaniksel davranış ve iskelenin kollajen lif kinematiği değişebilmektedir. Bu durum da iskelelerin taşıma kapasitesini etkilemekte ve hücrelerin maruz kaldığı mekanik mikroçevreyi değiştirebilmektedir. Deselülerizasyon işlemlerine maruz kalan iskelelerin mekaniksel davranışı ve biyo-aktivitesiyle ilişkili diğer önemli bir faktör ise yapının bozulmasıdır. Sert kimyasal yöntemler, HDM iskelelerin *in vivo* koşullarda enzimatik bozulma direncini düşürmekte ve bu da iskelelerin mekaniksel gücünde azalmalara sebebiyet vermektedir. Rejeneratif tıpta kullanılmak üzere bir dizi onaylı deselülerizasyon protokolü ile üretilmiş HDM temelli biyo-iskele mevcuttur. Özellikle insan dermisi (Alloderm®, LifeCell, Corp.), domuz ince barsak altmukozası (SurgiSIS®, Cook Biotech, Inc.; Restore®, DePuy Orthopaedics, Inc.), domuz mesane (ACell, Inc.) ve domuz kalp kapakçıkları (Synergraft®, CryoLife, Inc.) göze çarpan önemli ticari ürünlerdir. Ayrıca yeni deselülerizasyon protokollerinin geliştirilmesi bu alana yönelik yapılan klinik çalışmalar için anlamlı ve önemlidir.

Son gelişmeler doku mühendisliğinin klinik uygulamalarda geniş bir yer tutabileceğini göstermekte ve yakın gelecekte ise kök hücre biyolojisi, doku mühendisliği ve çekirdek transfer teknikleri alanlarındaki büyük ilerlemeler sayesinde nitelikli rejeneratif tıp ürünlerinin raflarda yer alabileceği düşünülmektedir.

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