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Ana Metin

Özet:Özet 300 kelimeyi aşmamalıdır ve kaynak kullanımından kaçınılmalıdır. Türkçe sunulan makalelerde ayrıca İngilizce Özet (Abstract) bölümü hazırlanmalıdır.

Giriş:Bu bölümde sonuçların anlaşılabilmesi ve yorumlanabilmesi için o konu ile ilgili (daha önceden) yapılmış olan çalışmalar hakkında bilgilere yer verilmelidir. Giriş'te çalışmanın hipotezi belirtilmelidir. Çalışmanın amacı bu bölümün en sonunda açık olarak yazılmalıdır. Sadece gerekli olan kaynaklara atıf yapılmalıdır.

Materyal ve Metot: Bu bölümde deneysel çalışmalar diğer araştırmacılar tarafından tekrarlanabilecek yeterlilikteki detayı ile verilmelidir. İstatistik çalışmalar ayrı bir alt başlık şeklinde sunulabilir. Uluslararası indekslerde yer alan dergilerde yayınlanmış bir makalede açıklanan bir teknik yeniden kullanıldığında, metodun çok kısa açıklanması ve ilgili orijinal makaleye atıf yapılması yeterlidir. Hayvan deneylerini içeren çalışmalarda yerel etik kurul kararlarına uyulmalıdır.

Sonuçlar:Araştırma bulguları açık ve anlaşılabilir şekilde, Materyal ve Metot bölümünde verilen sıra ile sunulmalı, tartışma veya yorumlara yer verilmemelidir. Bulgular tablo ve şekillerle desteklenerek kısa olarak sunulmalıdır.

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Yayına kabul edilmiş ama basılmamış çalışmalar referanslarda "(basımda)" olarak belirtilmelidir. Carrau RL, Khidr A, Crawley JA, Hillson EM, Davis JK, Pashos CL. The impact of laryngopharyngeal reflux on patient-reported quality of life. Laryngoscope. In press.

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RESEARCH ARTICLES

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Effect of Intravenously Injected Arachidonic Acid on Electrocardiography in Rats

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Abstract

Arachidonic acid (AA), which is released from phospholipids in the cell membrane by a variety of stimuli, has physiological and pathophysiological roles in the cardiovascular system. The current study was designed to determine the effect of intravenously (iv) injected AA on the electrocardiography (ECG) of the anesthetized rats.

The ECG waves obtained from the lead II were written by placing electrodes on limbs of the ketamine and xylazine mixture (50 mg/kg/20 mg/kg; im) in anesthetized adult Spraque Dawley rats. AA (3 mg/kg; iv) statistically significantly ($p<0.05$) caused to prolong of the ECG waves and intervals, resulting in a decrease in the heart rate of the rats.

The current findings herein present the effect of the most abundant endogenous unsaturated fatty acid AA on ECG. The results clearly showed that AA could produce to bradycardia response by increasing the ECG waves and interval durations.

Keywords: Arachidonic acid, Electrocardiography, Intravenous, Heart rate.

Introduction

Arachidonic acid (AA) is the most common polyunsaturated fatty acid in the body. In response to various physiological and pathological stimuli, it is released from cell membrane phospholipids under the effect of phospholipase enzymes and is metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) pathways into many biologically active products in the body.[1] AA and its metabolites have a role in the cardiovascular homeostasis.[2] In the peripheral, AA and its metabolites cause the relaxation in vascular smooth muscle, including coronary arteries.[3-5] It was reported that iv injection of AA caused first a rapid fall of arterial pressure followed by a brief rise and a subsequent prolonged fall.[6] The same report also indicated that peripherally injected AA decreased the heart rate of the rats along with its blood pressure effect.[6] In another report, that intravenously injected AA produced a dose-dependent fall in blood pressure in normotensive and spontaneously hypertensive rats was showed.[7] Moreover, central administration of the AA has many autonomic potentials including being able to affect the cardiovascular system.[8,9] We previously reported that intracerebroventricular injection of AA caused the pressor and bradycardic responses in normotensive rats by activating central COX[8] and LOX[9] pathways.

Although the previous reports explain that AA has a potential role on blood pressure and heart rate as cardiovascular parameters, there is no clear report about the effect of AA on electrocardiography (ECG), reflecting the electrical activity of the heart. Therefore, the purpose of the present study was to investigate the role of iv AA injection on the ECG waves.

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Material and Methods

3-4 months old, having 275-325 g body weight, 10 Sprague–Dawley rats were used in the experiments approved by The Animal Care and Use Committee of Bursa Uludag University (2020-03/05).

In the study, animals were used individually under ketamine/xylazine (50 mg/kg 20 mg/kg; im) mixture anesthesia. The electrocardiogram (ECG) was measured at leads II level in the anesthetized rats by using the MP36 system and the AcqKnowledge software (BIOPAC Systems Inc.). For this reason, ECG electrodes (SS2L, BIOPAC Systems Inc. California, USA) were inserted limbs of rats. The P and the T waves duration, the QRS complex duration, and the P-R, the Q-T and the R-R intervals duration were used as ECG parameters in the present study. The heart rate (HR) of the rats was calculated by using the R-R intervals duration formula.

The animals were divided into 2 groups which included 5 rats in each group, as control and experimental groups. The animals in the control group and experimental group were treated with saline (1 mL/kg; iv) and AA (3 mg/kg; iv), respectively, via the tail vein of the rats. After the treatments, the ECG of the rats was recorded for 60 min. AA purchased from Sigma-Aldrich Co. (Deisenhofen, Germany) was freshly solved in saline on the day of the experiment. The dose of AA was chosen from the previous study.[7]

Sigma Stat 3.5 software (CA, USA) was used for the statistical analysis of data. For Statistical analysis, repeated-measures analysis of variance (ANOVA; two-way) and the post-ANOVA test of Bonferroni were preferred. The data given as mean ± standard error of the mean (SEM) in the graphs were considered significant at $p<0.05$.

Results

Before the treatments, the P wave duration (Fig. 1A), the T wave duration (Fig. 1B), the QRS complex duration (Fig. 1C), the P-R interval duration (Fig. 1D), the Q-T interval duration (Fig. 1E), the R-R interval duration (Fig. 1F), and HR (Fig. 2) baseline levels of the anesthetized rats (n=10) were measured as 0.025 sec, 0.052 sec, 0.022 sec, 0.052 sec, 0.070 sec, 0,290 sec, and 226 bpm, respectively.

It was observed that iv injected AA group animals (n=5) had an increase in the P wave duration (Fig. 1A), the T wave duration (Fig. 1B), the QRS complex duration (Fig. 1C), the P-R interval duration (Fig. 1D), the Q-T interval duration (Fig. 1E), and the R-R interval duration (Fig. 1F) when compared to the control group animals (n=5). The maximum delayed effect was observed as 0.001 sec for P wave duration (Fig. 1A), 0.0102 sec for the T wave duration (Fig. 1B), 0.0012 for the QRS complex duration (Fig. 1C), 0.0011 sec for the P-R interval duration (Fig. 1D), 0.0102 sec for the Q-T interval duration (Fig. 1E), and 0.026 sec for the R-R interval duration (Fig. 1F). Also, iv injected AA caused a decrease in HR (Fig. 2) of the anesthetized rats (n=5). The maximum bradycardic effect was detected as 12.4 bpm for HR (Fig. 2) While iv injected AA caused to prolong the rate of the electrical activity of the heart, it failed to change in the ECG waveforms, amplitude, and also isoelectric line.

Discussion and Conclusion

The obtained findings demonstrated that iv administered AA caused a decrease in HR of the anesthetized rats by increasing the P wave duration, the T wave duration, the QRS complex duration, the P-R interval duration, the Q-T interval duration, and the R-R interval duration.

ECG presents an important understanding into the functional and structural characteristics of the myocardium by reflecting the electrical activity of the heart. Action potential of heart generating in the sinoatrial node, like a natural pacemaker of the heart, reaches ventricular cardiomyocytes through subsequently the atrioventricular node, His bundle, His bundle branches, and Purkinje fibers. An ECG record includes P wave, QRS complex, and T wave which have been reflecting atrial depolarization, ventricular depolarization, and ventricular repolarization, respectively.[10,11] Findings showed that IV injection of AA increased the duration of the waves and the intervals without changing the ECG waveforms, amplitude, and also isoelectric line. The increased duration in the waves and intervals with AA injection caused the bradycardia overall in the heart. It is well known that sympathetic and vagal nerves have chronotropic, inotropic, and dromotropic effects in the heart to provide heart rate homeostasis.[12] It was reported that activation of the COX pathway of AA had a potential role in the attenuation of sympathetic influences on the heart.[13] That the attenuation of sympathetic influences on the heart with AA created bradycardia by producing chronotropic and dromotropic effects could explain the findings. In addition, stimulation of the sympathetic nervous system causes an increase in intracellular Ca^{2+} and an increase in the contraction of both atria and ventricles due to the inotropic effect.[14] Attenuation of sympathetic stimulation by AA may also cause the weakening of L-type voltage-gated calcium channel activity.

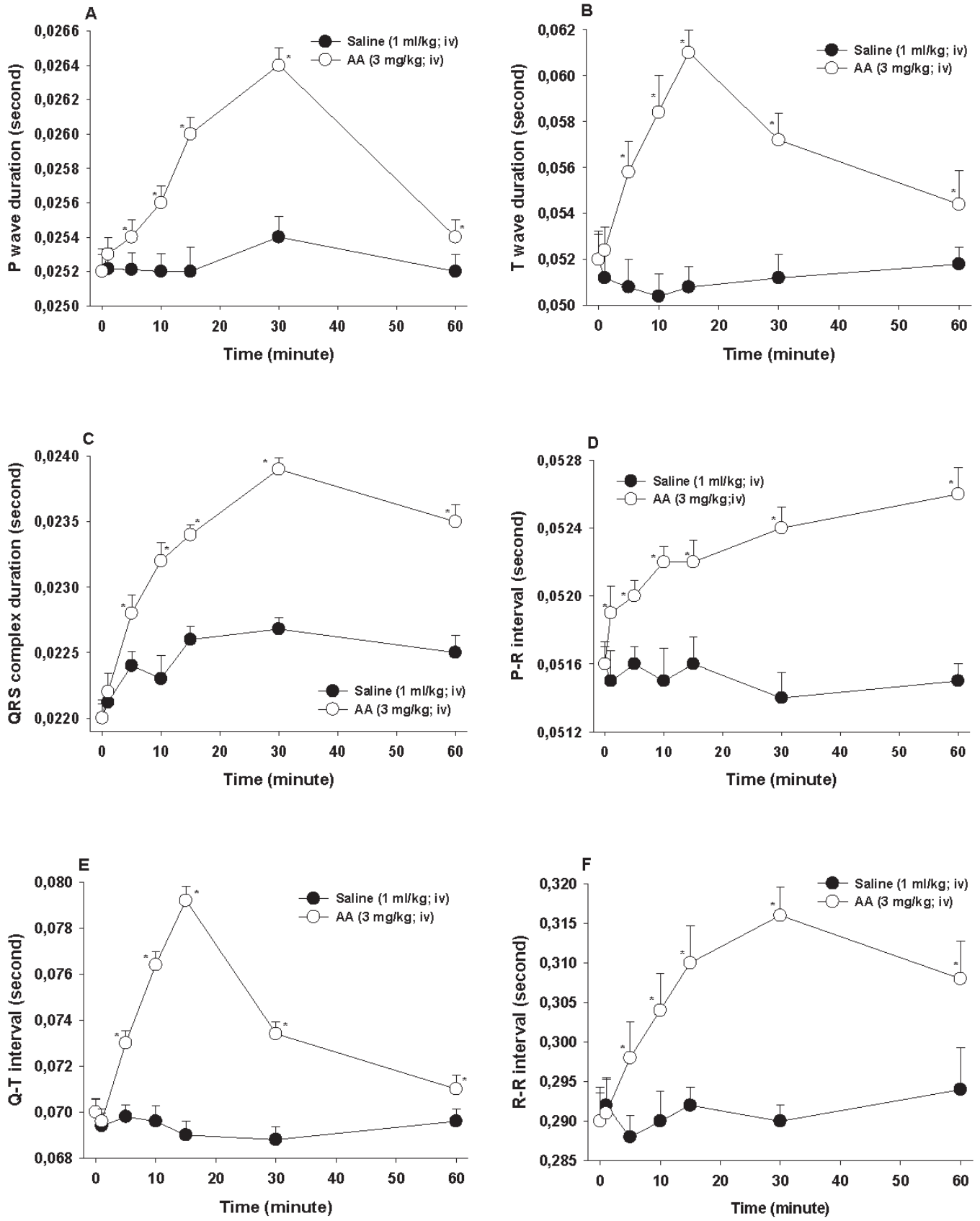

Figure 1. Effect of iv injected AA on ECG waves and intervals duration in the anesthetized rats. Saline (1 ml/kg; n=5) or AA (3 mg/kg; n=5) was iv injected to the rats. After injections, ECG was monitored for the next 60 min. The duration of the P wave (A) the T wave (B), the QRS complex (C), the P-R interval (D), the Q-T interval (E), and the R-R interval (F) measurements were obtained from the ECG. Statistical analysis was performed using two-way RM-ANOVA with a post hoc Bonferroni test. *$p<0.05$, significantly different from the value of the saline-treated group.

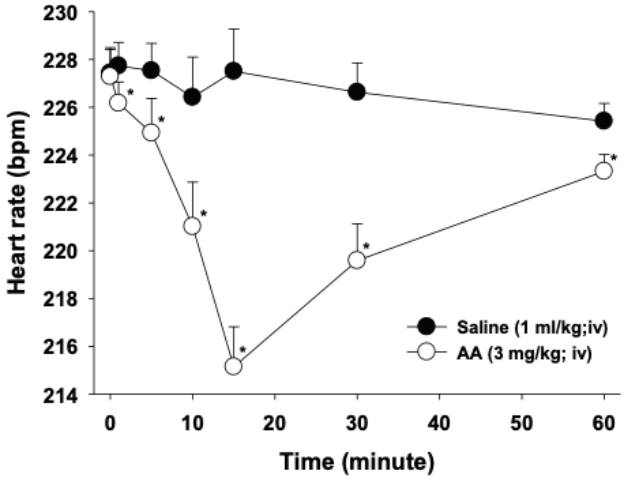

Figure 2. Effect of iv injected AA on HR in the anesthetized rats. Saline (1 ml/kg; n=5) or AA (3 mg/kg; n=5) was iv injected to the rats. After injections, ECG was monitored for the next 60 min. HR measurements obtained from the ECG. Statistical analysis was performed using two-way RM-ANOVA with a post hoc Bonferroni test. *p<0.05, significantly different from the value of the saline-treated group

Previous reports about the effect of AA on heart rate was contradictive. It was shown that IV AA injection in anesthetized rats caused bradycardia in accordance with our findings.[6] Another study reported that AA caused an increase on heart rate in the isolated perfused rat heart.[15] The contradiction may arise from the response of the organism as a whole since there was no neural control on the heart in the study made in heart rate on the isolated perfused rat heart. The neural control of the heart with sympathetic and parasympathetic neurons is more effective on the heart rate than the local mechanisms that affect the heartbeat. Moreover, it is known that AA decreases the sympathetic neural activity on the heart.[13]

AA, as an active cardiovascular neuromodulator is plenty in the central nervous system and also has roles on various autonomic controls.[16] The exogenously central injection of AA causes pressor and bradycardic responses through the COX[17] and LOX[9] pathways. Moreover, centrally injected AA-evoked pressor and bradycardic responses were mediated by COX metabolites such as TXA_2,[17] PGD, PGE, and $PGF_{2\alpha}$.[18] It is known that AA is able to cross the brain-blood barrier.[19] AA-induced bradycardia due to the delay in waves and intervals of the ECG observed in the findings may also be due to the central effect of AA by crossing the blood-brain barrier.

In summary, the present findings suggest that iv administration of AA produces prolong the rate of the electrical activity of the heart by increasing the duration of the ECG waveforms without affecting the amplitude and isoelectric line. As a result of the prolonged time of ECG waveforms, iv injected AA leads to the bradycardic response. The bradycardic response caused by AA may be due to the fact that AA may directly affect the heart or indirectly activate the central nervous system.

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Prevalence of *Nosema ceranae* in North and South Regions of Azerbaijan

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Abstract

Nosemosis is one of the most dangerous infectious diseases of honeybees in Azerbaijan and the world. *Nosema ceranae* is the dominant species in Azerbaijan, and this study aimed to detect the prevalence of the infection in the country. For this purpose, an average of 100 honeybee samples were collected from 64 hives, 24 from three regions in the north and 40 from five areas in the south. In the lab, the abdomens of 50 bees from each group were dissected and crushed in a container, adding 50 ml of distilled water. According to obtained data after microscopic examination, the *N. ceranae* spores were found to have a high-level prevalence in northern regions (45.8% average) than in the southern areas (22.5% average) in Azerbaijan. Molecular diagnoses of Nosema-positive samples have been performed with PCR and *N. ceranae* has been detected from all regions. Data show us that the Nosemosis is common in Azerbaijan and is a significant threat in the beekeeping industry.

Keywords: *Nosema ceranae*, prevalence, honeybee, Azerbaijan

Introduction

Beekeeping is one of the important economic sectors in the world, as well as in Azerbaijan. In recent years, the country has been making significant progress with approximately 500.000 colonies and annual honey production of 6.000 tons[1].

The productivity of the bees depends on both physiological characteristics and health levels. In case the disease occurs in the bee colonies, the functional ability of the bees falls, and the losses become happening in the bee colonies. Nosema is one of the microsporidian species and is becoming a dangerous honeybee pathogen with its two species: *Nosema apis* and *N. ceranae*[2,3]. These parasites infect the digestive system of adult bees and cause significant bee losses worldwide[4-7]. The first reported experimental infection of *A. mellifera* by *N. ceranae* clearly showed that this parasite was highly pathogenic to its new host[2]. It induced significantly higher bee mortality than *N. apis*[8].

The existence of *Nosema apis* in Azerbaijan has been noted in many scientific publications and conferences, and reported one the most prevalent diseases among the honeybees[9,10]. Recently, the existence of *N. ceranae* has been recorded in our preliminary research before[11].

This study aimed to determine the prevalence of *N. ceranae* infection of honeybees in southern and northern regions in Azerbaijan.

Material and Method

The study was conducted in May-June 2019. Since honeybees are invertebrates, there was no need for an ethics

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committee report. An average of 100 worker bee samples per hive was collected from three regions in the north of Azerbaijan (Guba, Gusar, Shabran) and five in the south (Astara, Lankaran, Masalli, Jalilabad, Bilasuvar). Samples were collected from 64 hives, eight from each region in different places. The samples were put into specific plastic boxes and were brought to the laboratory to keep cold.

To determine the Nosema spores in the sampled bees, 50 bees were dissected from each group. The abdominal parts of the bees were cut and crumbled by adding 50 ml of distilled water in a large tube. After the resulting suspension was filtered through the filter, 0.1 ml of liquid was taken, dripped on a hemocytometric slide, covered with a cover glass and examined under an ×400 magnified light microscope. The total number of spores (N) per bee is found with this formula: $N= S\times4\times10^6 / 80$; where 'S' is the total spores in five cytometric areas on the slide[12,13]. The average number of spores was determined in these areas, and the total number of spores for an individual was estimated.
PCR analyses of Nosema-positive samples were conducted in the preliminary study before this study[11], in which the samples were taken at the same time. A multiplex-PCR method and 16S rRNA genes have been used[11].

Results and Discussion

As a result of PCR analysis on samples in our previous study, all species have been identified as Nosema ceranae[11]. It was found that it had a high-level prevalence in northern (12.5-62.5%) than in southern in Azerbaijan (0-37.5%).
The study examined 64 samples from eight hives of eight sites, finding 20 (31.25%) hives Nosema-positive in total. According to the data in the Table 1, the highest infection rate of Nosema was detected in Quba and Qusar localities (62.5%), the least in Bilasuvar and Shabran localities (12.5%). No infection was seen in the Masalli area. If reviewed by data, the infection in the northern region of the country (45.8% average) appears to be greater than in the south (22.5% average).

Table 1. Prevalence of N. ceranae according to regions in Azerbaijan

Regions	Number of Colonies	Positive colonies	Minimum number of spores	Maximum number of spores	Percentage (%)
Astara	8	3	100.000	800.000	37.5
Lankaran	8	3	250.000	5.150.000	37.5
Masallı	8	0	0	0	0
Jalilabad	8	2	50.000	1.200.000	25
Bilasuvar	8	1	50.000	750.000	12.5
Shabran	8	1	50.000	200.000	12.5%
Quba	8	5	500.000	5.700.000	62.5%
Qusar	8	5	50.000	1.800.000	62.5%
Total	**64**	**20**	**1.050.000**	**15.400.000**	**31.25%**

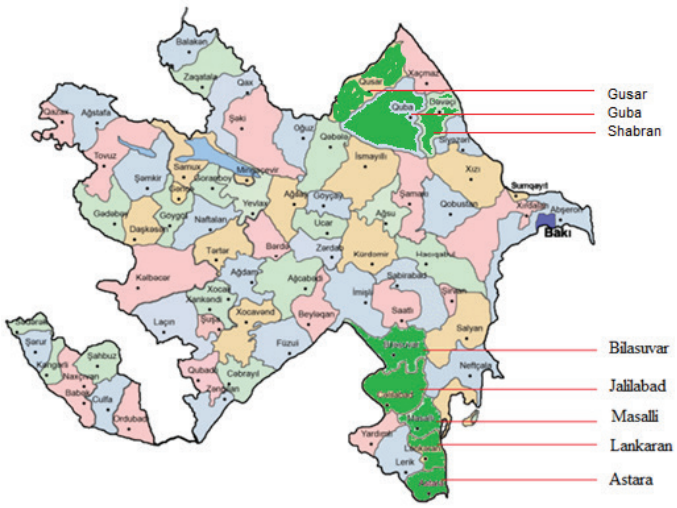

Figure 1. The location of the Azerbaijani regions in the map from which the samples were taken.

Nowadays, infection with *N. ceranae* has been proved almost on all continents. Recent reports have confirmed changes in the clinical and epidemiological patterns of Nosemosis, suggesting that *N. ceranae* has been one of the most prevalent pathogens in honeybees around the world[5]. The prevalence of *N. ceranae* in Iran's Azerbaijan climatic zones was significantly different, and the highest majority was found in the semi-humid climate (71.00%), followed by very humid (68.10%) and humid (53.80%) climates[14]. The number of Nosema spores per bee and climatic conditions seems to be related to the clinical signs and mortality. Obvious clinical signs have been higher during the rainy months, which we also observed[15]. As a result of the laboratory studies, it was defined that the prevalence level of the disease is higher in the districts of the region in which the humidity is relatively high. Based on these results, it should be noted that there is a proportion between the moisture and the Nosema disease. However, not only the humidity factor but also the other influential factors should be indicated as the causes of the disease. These possible factors include the lack of proper care for the bees, dirty water feeding, excessive treatment with a high dose of Varroa mite, and other environmental factors. It appears that *N. ceranae* is better adapted to complete its endogenous cycle with a higher biotic index at different temperatures reflecting the epidemiological differences between both microsporidian species in field conditions and at the colony level[16].

The highest indicators in the northern region were mentioned in Quba and Qusar regions. During the collection of material from those regions, pesticides have been used in the surrounding areas, especially in the apple and cherry gardens. In the Southern part, the highest indicators of the disease belong to the Astara region. The high humidity level in that region possibly leads to making available conditions for creating this disease.

In previous years, during the studies of the national scholars, the existence of *N. apis* species has been detected[9]. Tahirov and Hüseynov[17] reported that the Nosemosis is a widespread disease, with the prevalence of *N. apis* at 24.9-48.3% in low coastal areas, 29.8-63.2% in a mountainous region and 58.2-87.0% in Zengezur mountainous region of Nakhichevan Autonomous Republic, only via microscopic examination. Although there was no molecular diagnosis in those studies, it was possible to see such species of this disease in many regions of Azerbaijan. The presence of *N. ceranae* in the literal materials was noted in previous studies in the northwest part of Iran (close to the Azerbaijan border) as 48.2%[18]. In our survey conducted in 2019, the proliferation of *N. ceranae* in the southern part of Azerbaijan was described at the molecular level[11].

This study showed that *N. ceranae* is a severe and widespread problem in Azerbaijani honeybees. *Nosema apis* has not been detected in the samples taken from all eight provinces. Whether in the north or south of the country, the disease is common in the apiaries and necessary measures should be taken about the disease nationwide.

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Embriyo Transferi Yapılan Taşıyıcı İneklerde Progesteron Seviyesinin Gebelik Başarısı Üzerine Etkisi

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Özet

Bu çalışmada embriyo transferi sırasında taşıyıcı Holstein ırkı ineklerin kan progesteron seviyelerinin gebelik oranları üzerine etkisinin belirlenmesi amaçlandı. Bu amaçla, 5 üstün genetik özelliklere sahip donör inek ve 36 taşıyıcı inek hayvan materyali olarak kullanıldı. Donörlerde süperovulasyon östrusun 9. gününde başlayan 12 saat ara ve 4 gün süreyle uygulanan FSH hormonu ile sağlandı. Uterus yıkaması bir hafta sonra gerçekleştirildi. Taşıyıcı hayvanlar 11 gün ara ile iki kez uygulanan prostaglandin enjeksiyonu ile senkronize edildi. Transfer günü taşıyıcı ineklerden kan örnekleri alındı ve kan progesteron seviyelerine göre taşıyıcılar üç gruba (Grup 1 (<4 ng/ml), Grup 2 (4-8 ng/ml), Grup 3 (>8ng/ml)] ayrıldı. Çalışmada sadece birinci kalite blastosist (Grade I) evresindeki embriyolar kullanıldı. Gebelik muayeneleri embriyo transferi sonrası 30. günde ultrasonla yapıldı. Sonuçların istatistiksel olarak değerlendirilmesinde SPSS programı (SPSS 23, Chicago, IL, USA) kullanıldı. Embriyo transferi zamanında taşıyıcı hayvanların kan progesteron seviyesinin ölçülmesinin gebelik oranlarının geliştirilmesi için önemli olduğu belirlendi (P<0.05). Özellikle >8ng/ml kan progesteron seviyesine sahip olan hayvanlara yapılacak embriyo transferlerinin gebelik oranlarını artıracağı tespit edildi.

Anahtar sözcükler: Embriyo transferi, taşıyıcı inek, progesteron

The Effect of Progesterone Level on Pregnancy Success in Embryo Transfer Carrier Cows

Abstract

In this study, it was aimed to determine the effect of blood progesterone levels on pregnancy rates of recipient Holstein cows during embryo transfer. For this purpose, 5 donor cows with superior genetic characteristics and 36 recipient cows were used. Superovulation in donors was achieved with FSH, which was administered for 4 days, with an interval of 12 hours, starting on the 9th day of estrus. Flushing was made one week later. Recipients were synchronized with two prostaglandin injections administered 11 days apart. Blood samples were collected from the recipients on the day of transfer and the recipients were divided into three groups (Group 1 (<4 ng/ml), Group 2 (4-8 ng/ml), Group 3 (>8ng/ml)] according to their blood progesterone levels. Blastocyst (Grade I) stage embryos were used in the study. SPSS program (SPSS 23, Chicago, IL, USA) was used for statistical analysis. The measurements of blood progesterone level of recipients at the time of embryo transfer were found important to improve pregnancy rates (P<0.05). It was determined that transfers of embryos to recipients with a blood progesterone level of >8ng/ml would increase the pregnancy rates.

Keywords: Embryo transfer, recipient cow, progesterone

Giriş

Ülkemiz sahip olduğu sığır sayısı bakımından (17.975.482 sığır- TÜİK 2020) dünyada üst sıralarda yer almasına rağmen, damızlık sığır sayısı açısından kendi ihtiyacını maalesef karşılayamamaktadır. Bunun en önemli nedeni üstün genetik özelliğe sahip hayvan sayısındaki eksiklik ve bu hayvanlardaki döl verimi kaybıdır.[1,2,3,4] Hayvanlardaki ve-

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rimliliğin artırılması ve genetiğin iyileştirilmesinde biyoteknolojik yöntemlerin kullanılması ve yaygınlaştırılması elzemdir.

Sığır yetiştiriciliğinde embriyo transferi, yüksek verim yeteneğine sahip damızlık ineklerden bir yılda birden fazla yavru alınarak çekirdek damızlık sürüde yüksek verim yeteneğine sahip damızlık sayısının arttırılmasıyla genetik ilerlemenin hızlandırılmasına katkıda bulunan en önemli ıslah metodudur.[1] Bu amaçla donörlerde süperovulasyon işlemi kullanılarak çok sayıda embriyo elde edilmeye çalışılır ve bu embriyolar taşıyıcılara nakledilerek çok sayıda yavru alınması amaçlanır.[2] Fakat son yıllarda süt sığırcılığı endüstrisinde üreme fizyolojisindeki değişiklikler nedeniyle süt verimi artarken gebelik oranları gerek suni tohumlamada gerekse embriyo transferinde düşmektedir.[5] Buzağı verimi ve laktasyon sayısının düşmesi ciddi ekonomik kayıplara neden olmaktadır. Bu nedenle gebelik oranlarını artırmak ve ekonomik kayıplarının önüne geçebilmek amacıyla yeni yöntemlerin ve uygulamaların geliştirilmesi gerekmektedir.

Sığırlarda embriyonik ölümlerin etiyolojisindeki en önemli faktörlerden birinin progesteron hormonunun düzeyinin düşüklüğü ile sonuçlanan luteal yetmezlik olduğu bilinmektedir.[6,7] Tohumlama sonrası erken dönemdeki progesteron düzeyi ve profilinin, embriyonal yaşama gücü açısından geç döneme göre daha önemli ve pozitif bir ilişki taşıdığı bilinmektedir.[8,9,20] Özellikle ovulasyon sonrası progesteron konsantrasyonunun erken embriyonik ölümlerle (fertilizasyon sonrası 8-16. Gün) ilişkili olduğu bildirilmektedir.[9,10,11]

Kan progesteron düzeyi üreme döngüsü ve gebelik esnasında farklı seviyelerde bulunmaktadır. Süt sığırlarında kan progesteron seviyesinin kızgınlıkta en düşük (0.21 ng/ml) olduğu, 7. günde arttığı (1.5 ng/ml) ve 14. günde en yüksek seviyede olduğu (2.21 ng/ml) ve daha sonra 21. günde düştüğü (0.38 ng/ml) belirtilmektedir.[12] Bu nedenle embriyo transferi yapılacak gündeki kan progesteron seviyesinin gebelik başarısını etkileyeceği düşünülmektedir.

Sunulan çalışmada, embriyo transferi sırasında taşıyıcı ineklerin kan progesteron seviyesinin gebelik oranları üzerine etkisinin belirlenmesi amaçlanmaktadır.

Materials and Methods

Hayvan Materyali

Bu çalışmada 5 adet üstün genetik özelliklere sahip donör inek ve 36 adet taşıyıcı inek hayvan materyali olarak kullanılmıştır. Donör inekler; 600-650 kg ağırlığında, 5 yaşlı, vücut kondisyon skoru 3.5, üçüncü laktasyonda olan, düzenli östrus aktivitesi gösteren ve klinik yönden sağlıklı hayvanlardan seçildi. Taşıyıcı inekler ise düzenli östrus gösteren, 3-4 yaşlı, sağlıklı ve daha önce tohumlanmamış hayvanlar arasından seçilmiş ve gruplar arası eşit dağılım sağlanmıştır (Etik Kurul No:2022/02-03).

Donör ve Taşıyıcı Hayvanlara Uygulanan Süperovulasyon ve Senkronizasyon Protokolü

Günler	*DONÖR HAYVANLAR*			*TAŞIYICI HAYVANLAR*
	Uygulama		**Uygulama Dozu**	**Uygulama**
-3				PGF
0	CIDR + GnRH		2,5 ml	
7	FSH	Sabah	2 ml	
		Akşam	2 ml	
8	FSH	Sabah	1,5 ml	
		Akşam	1,5 ml	PGF
9	FSH+PGF	Sabah	1 ml	
	FSH+CIDR Uzaklaştır	Akşam	1 ml	
10	FSH	Sabah	0,5 ml	
		Akşam	0,5 ml	
11	Östrusta 12 saat ara ile en az 2 kez suni tohumlama			Östrusların taranması
18	Uterus yıkaması			Embriyo transferi

Çalışmada tohumlamadan sonraki 7. günde, embriyolar uterusun cerrahi gerektirmeyen yöntem ile yıkanmasıyla toplandı. Bu amaçla öncelikle epidural anestezi 4 ml lidokain uygulandı. Sonrasında foley kataterı serviksten geçirilip kornu uteri içinde balonu şişirilerek sabitlendi. Yıkama solüsyonu olarak %1,5 oranında fötal buzağı serumu (FCS) ilave edilmiş 1 litrelik laktatlı ringer solüsyonu kullanıldı. Yıkama solüsyonu ilk olarak kornunun 2/3'ü dolacak miktarda aktarıldıktan sonra, tekrar filtre sistemine bağlı yoldan geri alındı. Aynı işlem 4-5 kez kornu uteri tam doldurulup boşaltılarak tekrarlandı. Aynı uygulama diğer kornu uteride de yapıldı.[2]

Yıkama Sonrasında Embriyoların Aranması

Yıkama sonrası filtrede tutulmuş olan embriyolar, filtrenin kapağı çıkarıldıktan sonra alt kısmında bulunan yıkantı solüsyonunun stereo mikroskop altında incelenmesiyle tespit edildi. Tespit edilen embriyolar zaman kaybetmeden içerisinde TL- HEPES bulunan petri kaplarına aktarıldı[2] Embriyolar IETS (International Embryo Transfer Society) tarafından tanımlanan kalite değerlendirme kriterlerine göre değerlendirildi. Blastosist aşamasında olan 1. kalitedeki embriyolar 0,25 ml'lik payetler içerisine çekildi. Gruplar arası eşit dağılım sağlandı.

Taşıyıcı Hayvanların Hazırlanması ve Embriyoların Taşıyıcılara Transferi

Senkronize edilen taşıyıcı ineklerde transfer günü ultrason yardımıyla ovaryum muayeneleri yapıldı. Korpus luteumların hangi ovaryumlar üzerinde yer aldığı belirlendi. İçerisine embriyo aktarılmış payet embriyo transfer kataterine yerleştirildi. Katater üzerine geçirilen naylon kılıf serviks girişinde yırtıldı ve serviks geçildi. Katater corpus luteumun bulunduğu taraftaki kornu içerisine ilerletildi ve pistole itilerek embriyo transfer işlemi tamamlandı.[2] Embriyo transferi yapılan hayvanlara fluniksin meglumin uygulaması yapılmıştır. [15,16]

Taşıyıcı hayvanların kan progesteron seviyelerinin belirlenmesi

Kan progesteron düzeyini belirlemek amacıyla taşıyıcı ineklerden embriyo transfer günü Na-EDTA içeren vakumlu tüplere kuyruk venasından kan örnekleri toplandı. Alınan kan örnekleri bekletilmeden 5000 devir/dakika hızda 10 dakika santrifüj edilerek plazmaları ayrıldı. Plazma örneklerinde progesteron düzeyleri (ng/ml) Access Beckmann Coulter (USA) cihazında Access Progesterone kiti kullanılarak Radioimmunoassay (RIA) yöntemi ile ölçüldü. Elde edilen sonuçlarına göre gruplar belirlendi.

Taşıyıcı Hayvanlarda Gebeliklerin Belirlenmesi

Taşıyıcı hayvanlarda embriyo transferi sonrası 32. günde ultrason yardımıyla gebelik muayeneleri gerçekleştirilmiştir.

İstatistiksel Analiz

Sonuçların istatistiksel olarak değerlendirilmesinde SPSS (Windows için SPSS 23.0; SPSS, Chicago SPSS 23) programından yararlanılmıştır. Gruplar arasındaki gebelikler Ki-kare (Chi-square) testi kullanılarak karşılaştırıldı.

Bulgular

Çalışmada grupların ortalama P4 seviyeleri (mean ± SD) Grup 1'de 3.21±1.12, Grup 2' de 6.13±1.08 ve Grup 3'te 10.02±1.42 elde edilmiştir. Tablo 1'de elde edilen gebelik oranları belirtilmiştir.

Tablo 1: Progesteron (P4) seviyelerine göre taşıyıcı hayvanların gebelik oranları

Gruplar	n	Gebelik (+)	Gebelik (-)	Gebelik oranı (%)
Grup 1 (<4 ng/ml)	11	**3**	**8**	27.27[a]
Grup 2 (4-8 ng/ml)	13	**5**	**8**	38.46[ab]
Grup 3 (>8ng/ml)	12	**9**	**3**	75[b]

Tartışma Sonuç

Sığırlarda embriyo transferi, üstün genetik özelliklere sahip donör hayvanlardan alınan embriyoların sağlıklı taşıyıcı hayvanlara transfer edilmesiyle sonuçlanan biyoteknolojik bir yöntemdir.[13] Yetiştiricilik açısından önemli avantajlar sağlamasına rağmen gebelik başına ekonomik maliyeti nedeniyle sadece üstün genetik özelliğe sahip hayvanlarda kullanılmalı ve taşıyıcılarda gebelik oranlarının yükseltilmesi amaçlanmalıdır. Çalışmamızda sürü içerisinde genetik kapasitesi üst seviyede olan donör hayvanlardan alınan embriyolar progesteron seviyelerine göre gruplara ayrılmış taşıyıcı hayvanlara nakledilmiştir.

Transfer edilebilir kalitede bir embriyonun transferi sonrası elde edilecek gebelik oranını etkileyen en önemli faktör, taşıyıcının reprodüktif bakımdan uygunluğudur. Taşıyıcı hayvanların östruslarının doğru tespiti, siklus yaşının izlenebilmesi ve kan progesteron seviyesinin doğru belirlenebilmesi elde edilecek gebelik oranı açısından çok önemlidir.[14,15] Çalışmamızda taşıyıcı ineklerin embriyo transfer günü kan progesteron seviyelerinin gebelik oranları üzerine etkisinin belirlenmesi amaçlanmıştır.

Embriyo transferi uygulamalarında taşıyıcılara yapılan östrüs senkronizasyonunun amacı östrüslerin kısa bir zaman aralığında toplulaştırılması yanında taşıyıcının siklusunun senkronize edilerek embriyo transferi için uygun zamanın tespitine yönelik bir ön hazırlık yapmaktır. Embriyo transferi, embriyonun corpus luteumun (CL) bulunduğu ovaryum tarafındaki uterus kornusuna bırakılması ile gerçekleştirilmektedir. Sunulan çalışmada birinci kalitede olan blastosist aşamasındaki taze embriyolar ultrason yardımıyla tespit edilen CL'nin bulunduğu kornu uterilere transfer edilmiştir. Ancak, transfer sırasındaki manipülasyonun PGF2α salınımına neden olarak embriyonun gelişimini olumsuz yönde etkileyebileceği ve gebelik oranını düşürebileceği bazı araştırmacılar tarafından belirtilmektedir.[16,17,18] Bu nedenle taşıyıcı hayvanlarda embriyo transfer günü güçlü bir non-steroidal anti-inflamatuar madde (NSAID) olan flunixin meglumin (FM) uygulaması yapılarak endometriumdan PGF2α salgılanmasının baskılanması,[15,16] bu sayede de gruplar arası manipülasyona bağlı oluşabilecek embriyonik ölümlerin önüne geçilmesi hedeflenmiştir.

İneklerde gebeliğin devamlılığı açısından progesteron konsantrasyonunun büyük öneme sahip olduğu birçok araştırmacı tarafından belirtilmektedir.[6,7,19] Özellikle tohumlama sonrası yedinci gün düşük progesteron seviyesinin embriyonik ölüm oranını arttırdığı bildirilmiştir.[20,21] Çalışmamızda da düşük progesteron seviyesine sahip olan hayvanlarda gebelik başarısı yüksek progesteron seviyesine sahip hayvanlara göre daha düşük bulunmuştur ($p<0.05$).

Elde edilen sonuçlar değerlendirildiğinde; embriyo transferi zamanında taşıyıcı hayvanların kan progesteron seviyelerinin ölçülmesinin gebelik oranlarının geliştirilmesi açısından önemli olduğu görülmüştür. Özellikle >8ng/ml kan progesteron seviyesine sahip olan hayvanlarda yapılacak transferlerin olumlu katkı sağlayacağı belirlenmiştir. Sonraki çalışmalarda transfer sayılarının arttırılmasına ve başarıyı arttıracak faktörlerin geliştirilmesine odaklanılacaktır.

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Effects of Keel Bone Deviation on Post-Peak Egg Production in a Commercial Laying Hen Flock With Different Breast Condition

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Abstract

This study was made to investigate the effects of breast condition and keel bone deviations on post-peak egg production of a commercial laying hen housed in a multi-tier conventional battery cage. The birds divided into two groups according to presence of keel bone deviation at first. Then the birds further divided into two groups according to breast condition as well developed or relatively well developed. The laying hens were kept under identical management conditions for commercial laying hens during the study. Data about daily egg production, feed intake, mortality and egg weight was collected from 62 to 77 weeks of age in the groups. There were no significant effects of keel bone deviation on body weight and egg weight of the layer hens. The initial and final body weight of the birds are significantly different between the breast condition groups (P<0.001). The birds with well developed breast condition had significantly better hen-housed and hen-day egg production (P<0.001). The significant keel bone deviation x breast condition interaction for egg production revealed that presence of keel bone deviation was effective in birds only had relativelly well developed breast condition (P<0.001). Daily feed intake per hen and survival rate between the groups were not affected by presence of keel bone deviation and breast condition of the birds. Results from this study indicated there was a link among breast condition, presence of keel bone deviation and egg performance of laying hens.

Key words: Laying hen, keel bone deviation, breast condition, performance.

Introduction

The presence of keel bone damages and plumage damages in commercial egg production represents one of the greatest challenges due to the negative impact on the health and welfare of laying hens [1, 2, 3]. British Farm Animal Welfare Council is determined that the keel bone damages are one of the most important welfare issues in commercial layer flocks[4, 5]. An EU Cost action which involved a large number of scientists and institutions from Europe and that the overall goal understood the causes of keel bone damage and to reduce its occurrence, has been completed recently (Keel Bone Damage-Cost project, 2016-2021). The keel bone deviations result from a prolonged pressure on the keel especially during perching [6, 7, 8] and prolonged pressure might cause hematomas, wounds and fractures of the surrounding tissue of the keel bone [9]. It was reported that the prevalance of fractures in layer flocks was exceeding 80% in different countries[10] and it was highly prevalent at the beginning of lay up to 8% of a flock [6, 11, 12, 13,14]. In organic and conventional layer flocks in Austria and Germany, it was found no difference between the flocks, with 28% and 27% of the birds showing keel bone deviations or fractures [15]. In another study, Bestman and Wagenaar[16] reported keel bone deformations in 4–48% of the hens, with an average of 21%, including 49 flocks of organic layers in Denmark. Jung et al[17] showed that type of housing systems, natural daylight inside the house, proportions of underweight hens and laying performance were significantly affected the development of keel bone damage in organic layer flocks in

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several European countries.

The bird age, housing condition, nutrition, the age at first egg, ossification time of the keel bone, bone diseases, inactivity of the birds and genetic stock health could be predisposing factors for presence of keel bone hazards[3,18,19, 20, 21]. In modern production, commercial layers have been selected for greater egg numbers and optimized egg quality over the years[23, 24]. In general, high level egg production is assumed one of the major contributing factors for keel bone deviation and fractures in laying hens[25, 26]. Although higher egg numbers per hen and extended calcium resorption from cortical bone are believed to be the major factor of development of bone fragility and increased keel bone damage susceptibility, it is not clear how a layer's productivity is affected after a keel bone damage has occurred. Despite the egg production being affected by the breast condition of the birds[27], until now, no specific studies have yet been identified the effects of keel bone deviation and breast condition x keel bone deviation interaction on laying hen performance. The objective of this study was to investigate the effects of breast condition and presence of keel bone deviation on laying hen performance under commercial condition.

Material and Methods

The data was collected from a commercial layer flock housed in three-tier conventional battery cages with 3500 laying hens (Lohmann LSL) at Research and Production Farm of Bursa Uludag University. The experimental procedures were employed in accordance with the principles and guidelines set out by the Committee of Bursa Uludag University on animal care and management. This study doesn't require ethical permission according to Animal Experiments Ethics Committees Regulation on Working Procedures and Principles, Article 8 19-k [28].

Management

At the beginning of the experiment, the birds were divided into two groups according to presence of keel bone deviation with keel bone palpation. Keel bone status of the birds was recorded on a scale from 0 to 1 (1: moderate to severe deformity; 0: slight or no deformity). Then, the birds in each deviation groups further divided into according to breast condition as score 0 or score 1 by photographic specifications based on palpating the protuberance of the keel bone, and the development of the layer breast muscles (29). According to this, the breast condition score 0 is indicated a well-developed round breast muscle with limited protuberance at the keel bone, while score 1 showed relatively well-developed breast muscle with a distinct protuberance at the keel bone.

The layer barn was furnished with a commercial battery cages with 3 tiers. Animal density was 4 hens/per cages (625 cm2 per hen). During the 105 days of the experiment (from 62 weeks of ages) all laying hens in the groups were kept under identical management conditions for commercial egg production [30]. A 16 h lighting including daylight and artificial light were provided during the experiment. Hens in all groups were fed ad libitum with a second phase of soybean and maize based commercial layer feed.

Data collection

Data collection started after the formation of the groups and a 15-day adaptation period. Daily egg production, weekly feed consumption and number of dead birds when it occurred were recorded from 62 and 77 weeks of age. Egg production traits were calculated on the basis of hen-housed and hen-day, whereas survival rate and feed intake were calculated on hen-housed basis [30]. Initial and final live body weights with initial and final egg weights of the hens was recorded at 62 and 77 weeks of age.

Statistical tests

The statistical tests for the traits measured were performed using SPSS® Computer Software 13.00[31]. ANOVA test was used to analyze the effects of, and interactions between breast condition and presence of keel bone deviation on egg production, feed intake and egg weight [32]. The model used in the analyses was the following:

$Yij = \mu + Ai + Bj + AxB + eij$

A; breast condition, B; presence of kell bone deviation; A×B; an interaction; also i:1,2 (1: well developed breast condition, 2: relatively well developed breast condition), j:1,2 (1:presence of keel bone deviation, 2:without kell bone deviation), μ; constant; e; error term.

Results

The effects of presence of keel bone deviations and breast condition on initial and final live body weight and egg weight were showed in table 1. There were no significant effects of keel bone deviation on initial and final live body weight and egg weight of the birds in the groups. Breast condition of the birds was significantly affected initial and final body weight of the laying hens between the groups ($P<0.001$, $P<0.001$). Keel bone deviation x breast condition interaction for initial body weight and final egg weight were found significantly important ($P<0.048$, $P<0.015$).

Table 1 : Effect of presence of keel bone deviation (KBD) and breast condition (BC) on body weight and egg weight in laying hens.

Groups	Body Weight		Egg Weight	
	Initial	Final	Initial	Final
Keel Bone (KB)				
With deviation (D)	1608±19	1577±25	62.01±0.66	62.22±0.85
Without deviation (WD)	1581±17	1566±18	60.34±0.70	62.86±0.59
Breast Condition (BC)				
Well developed (WD)	1693±15	1635±21	63.01±0.53	62.69±0.59
Relatively well developed (RWD)	1495±21	1508±22	59.34±0.80	62.40±0.85
BC x KB				
WD x D	1681±21	1610±24	63.44±0.76	61.09±0.80[b]
WD x WD	1705±22	1659±25	62.59±0.75	64.49±0.86[a]
RWD x D	1534±32[a]	1543±37	60.58±1.07	63.35±1.23[a]
RWD x WD	1456±27[b]	1473±34	58.07±1.19	61.45±1.17[b]
ANOVA				
KB	0.279	0.728	0.084	0.529
BC	0.001	0.001	0.001	0.782
KB x BC	0.048	0.053	0.395	0.015

a-b; represent a significant BC x KB interaction (RWDXD and RWD X WD) for initial body weight and final egg weight within columns.

Table 2: Data on egg production, feed intake and survival rate in the groups

Groups	Egg Production				Survival rate	Daily feed intake/hen/d
	Hen-Day		Hen-Housed			Hen-Housed
	%	Number	%	Number	%	g
Keel Bone (KB)						
With deviation (D)	79.05±0.6	83.00±0.6	73.34±0.59	77.07±0.64	87.50	105.78±1.54
Without deviation (WD)	76.56±0.7	80.35±0.7	65.80±0.61	69.09±0.63	88.75	108.83±1.45
Breast Condition (BC)						
Well developed (WD)	81.03±0.7	85.08±0.7	74.51±0.62	78.24±0.63	85.45	106.25±1.56
Relatively well developed (RWD)	74.51±0.6	78.31±0.6	64.68±0.61	67.92±0.64	94.00	108.35±1.49
BC x KB						
WD x D	79.84±0.89	83.84±0.89	73.92±0.86	77.62±0.90	87.27	105.30±2.54
WD x WD	82.21±0.81	86.32±0.81	75.10±0.85	78.86±0.91	83.63	107.21±2.00
RWD x D	78.25±0.93[a]	82.16±0.93[a]	72.88±0.86[a]	76.52±0.91[a]	88.00	106.25±2.42
RWD x WD	70.91±0.92[b]	74.45±0.92[b]	56.49±0.84[b]	59.31±0.92[b]	100.00	110.46±2.04
ANOVA						
KB	0.005	0.005	0.001	0.001	n.s	n.s
BC	0.001	0.001	0.001	0.001	n.s	n.s
KB X BC	0.001	0.001	0.001	0.001	n.s	n.s

a-b; represent a significant BC x KB interaction (RWDXD and RWD X WD) for egg production within columns.

The egg production percentage, number of eggs during the study, feed intake per birds and survival rate in the groups were presented in table 2. It was found that the effects of keel bone deviation and breast condition on hen-housed and hen-day egg production traits were significantly important between the groups (P<0.001, P<0.005). The hen-day egg ratio in well developed and relatively well developed breast condition gropus were found 81.03 and 74.51% whereas the hen-housed egg production were 74.51 and 64.68%, respectively. Hen-housed daily feed intake per hen and survival rate in both keel bone and breast condition groups were found similar.

Discussion

In this study the relationship between breast condition and presence of keel bone deformations on egg production traits in a white layer hens at post-peak production period were investigated. As expected, the birds with well developed breast condition had significantly greater initial and final live weight than birds had a relatively well developed breast condition[33]. Compare to the birds with relatively well developed breast condition, the birds with well developed breast condition had significantly greater hen-housed and hen-day egg production. The birds with relativelly well developed breast condition without keel bone deviation had significantly lower egg production compare to the other groups (Table 1). Breast condition is an important indicator of body condition in poultry. The breast muscle development may also be a good indicator of protein mobilisation for egg production and subsequent muscle atrophy[34]. In practice, the body condition scores in animal are used for evaluating the adequacy of nutrition, assessing the health status of individual animals. Jung et al.[17] have showed that heavier birds are less likely to develop fractures than lighter birds in organic layer flocks. Dunn et al.[35] reported that some traits such as genetic, some environmental and management factors can positively impact the overall quality of the skeleton of layer hens.

In this study, the egg production of the birds with keel bone deviation was significantly higher than the birds without keel bone deviation. This was probably due to the influence of other factors affecting egg production. To support this, Dunn et al. [35] showed that no evidence for a relationship between post-peak egg production and bone quality, and the longer laying periods will not adversely affect the bone quality of the birds. Similarly, Eusemann et al.[25] showed that there was no relationship between egg production and keel bone deviations in a layer line. In another study, it was reported that the layer stock with the highest egg production had better bone quality [12]. Fleming et al. [36] showed that selection for better bone quality was possible without reducing egg production. As in the current study, Podisi et al. [37] reported that there was limited evidence for a pheno-

typic association between egg productivity and bone stability among the layer genotypes. Baldinger and Bussemas[38] reported that 34–45% of the dual-purpose layer showed keel bone damage at the end of the laying period and dual-purpose crosses housed in floor system had less keel damage and low laying performance. In general, the end of layer hens have eggs with poor eggshell and bone quality[39, 40]. It is clear that high level of egg calcium requirements for eggshell makes hens more susceptible to skeletal problems such as osteoporosis[18]. Keel bone hazards can occur in all types of poultry housing systems and in all types of commercial layer hens[21]. In comparison with conventional cage systems, osteoporosis is not as prevalent in hens that are non-cage housing system, due to greater exercise opportunities in these systems[41]. A significant keel bone deviation x breast condition interaction for egg production revealed that presence of deviation was effective in birds only had relativelly well developed breast condition (P<0.001).

High egg production and high calcium requirements for egg shell formation make layers more susceptible to the skeletal problems[2]. Birds with keel bone hazards experience pain[42, 43], reduced egg production, reduced egg size and egg quality, increased feed and water consumption [44, 45, 46]. In other side, Gebhardt-Henrich and Fröhlich[47] observed no correlation between keel damage and egg production in laying hens. Non-laying layer hens show increased keel density and a lower risk of fractures than do hens producing eggs, but there is no similar relationship reported for keel deviations[25]. In another study, Eusemann et al.[48] reported that there was a significantly lower prevalence of deviations and fractures in the low performing brown layers when compared to the high performing brown layers. Whereas, Alfonso-Carrillo et al.[40] reported that egg production, bone and eggshell quality traits are independent each other and can be improved separately. They also showed that laying hens with low egg production and poor eggshell quality had better bone quality.

In this study, daily feed intake of the birds and survival rate in all groups were not affected by breast condition or presence of keel bone deviation. But, it might be possible that presence of deviation in keel bone affected feed intake where affected hens consumed more feed in response to increased metabolic demands.

Conclusion

On the basis of the results of this study, the effects of breast condition on egg production traits are very clear but no significant associations were found between flock level prevalence of keel bone deviation. Further research especially in more dense populations including different housing systems, genetic material, environmental condition etc. would be very helpful to understand the role of keel bone damages on egg production traits.

Competing interests

The authors declare that they have no conflict of interest for this manuscript.

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First Isolation And Characterization Of Bovine Herpesvirus 1.2b (BoHV-1.2b) Strain From Upper Respiratory Tract Of Cattle In Türkiye

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Abstract

Analyzing fingerprints by Restriction Length Polymorphism (RFLP) analysis of complete virus genome which is laborious and time-consuming classifies Bovine Herpervirus-*1* (BoHV-1) strains into three subtypes, BoHV-1.1, 1.2a, 1.2b. These subtypes can also being referred according to clinical futures, however, no clear relation was shown. Mostly BoHV-1.2b is associated with genital disease conditions. In this study, BoHV-1 isolate was obtained in a nasal swab sample taken from respiratory tract disease. In this study also, phylogenetic analysis which was targeting UL44 (Glycoprotein C) region of the genome, subtyping was carried out by a recently developed multiplex PCR targeting UL39 and US3 region followed by a RFLP analysis using Hind III enzyme. Also, success of isolation was compared in two continuous cell lines. SFT-R cell line found more susceptible for BoHV-1 field sample isolation than MDBK. The obtained isolate (ID:8640) was serologically undistinguishable from Cooper strain while molecular analysis classified as BoHV-1.2b. Current study reports the first isolation of BoHV-1.2b in Turkey as well as infrequent BoHV-1.2b isolation from clinical case of respiratory illness. Results also highlights the efficiency of PCR based RFLP analysis for easy and quick subtyping but demonstrates the requirement of more investigation to reveal differences based on genetic diversity of BoHV-1 field isolates.

Keywords: BoHV-1 subtyping, molecular characterization, restriction endonuclease analysis, virus isolation.

Introduction

Bovine herpesvirus type 1 (BoHV-1) is associated with various clinical disorders in cattle comprising respiratory and reproductive systems. Clinical signs of the infection involves nasal discharge, conjunctivitis, depression, fever, milk yield reduction, genital disorders like abortion and vulvovaginitis in female cattle and balanoposthitis in bulls. Although several European countries have successfully eradicated BoHV-1[1], introduction of the virus to all the continents had been reported[2] and has been found to be the cause of mass economical losses in cattle industry [3].

BoHV-1, classifies in the *Varicellovirus* genus of the subfamily *Alphaherpesvirinae* of *Herpesviridae*. Virus has an enveloped icosahedral symmetry containing double stranded viral DNA which is about 135 kb in length and codes for 33 structural proteins. At least 13 of mentioned are associated with the viral envelope [4]. According to composition and restriction site analysis of the genome, BoHV-1 strains are classified into three subtypes. BoHV-1.1 is generally responsible for infections in respiratory tissues as well as genital tract. Thus, the respiratory disease called infectious bovine rhinotracteitis (IBR) is generally caused by this subtype [5]. Subtype BoHV-1.2, generally accepted to be responsible for genital infections known as "infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB)". BoHV-1.2 subtype also further divided into two groups of variants (genotype) namely BoHV-1.2a and BoHV-1.2b, based on genetic and antigenic differences. BoHV-1.2a strains can cause abortion in cattle while BoHV-1.2b is not assumed to be abortifacient [6] despite causing common genital infections. Possibility of respiratory infections caused by BoHV-1.2b strains that is less virulent compared to BoHV-1.1, is also suggested [5,7]. A former

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subtype (BoHV-1.3) causing encephalitis in cattle is currently classified as a distinct virus species of BoHV-5 .[8] Despite genetic variations, BoHV-1 subtypes are closely related to each other in serological testing. Since BoHV-5, BoHV-1 and its subtypes share antigenically very similar epitopes, determination of virus species and subtypes by serological differention is laborious. Differentiation of BoHV-1 subtypes was entirely performed by restriction site analysis using different DNA digestion enzymes[9]. Though this analysis are still employed for the same purposes, so far, there are many sequences from different parts of BoHV-1 genome deposited in the GenBank for various strains of the virus [10]. Hence, phylogenetic analysis on the basis of selected genes is a reliable method for subtyping BoHV-1 isolates. One of the most common part of viral DNA used for characterization and differentiation of BoHV-1 isolates is the gene coding for glycoprotein C (gC, UL44) encode for envelope glycoprotein [11] which is responsible for attachment of the virions onto cells.

The agent, BoHV-1, leads to a latent infection localized in sensory ganglia after primary infection that persists for lifelong. Clinical symptoms and virus shedding are induced in latently infected animals affected by various stressors or subjected to corticosteroid application [2]. Laboratory diagnosis for virus detection can be achieved using different methods including PCR, antigen ELISA and virus isolation in cell culture. Cell lines from different tissue origin are susceptible for BoHV-1 propagation with visual cytopathogenic effect (CPE) [2].

The aim of this study was to present the first isolation of BoHV-1.2b from natural case of bovine respiratory infection, and its molecular characterization consisting of both phylogenetic analysis and PCR-RFLP method which is less preferred in BoHV isolates. At the same time, the success of the BoHV-1 isolation from field samples in two continuous cell lines, MDBK and SFT-R was compared.

Material and Methods

Samples: In September 2014, non-clothed blood samples from 37 beef cattle with lumpy skin disease (LSD)-like findings were submitted for laboratory diagnosis. There was no history of BoHV-1 vaccination in the herd. Skin samples from 2 of these animals and additional nasal swab sample (sample ID: 8640) from one animal exhibiting nasal discharge was also submitted. Thus, the total number of samples handled in this study was 39. All the samples were subjected to nucleic acid isolation for testing LSD virus by PCR. In the meantime, inocula prepared from samples for the purpose of virus isolation in cell culture were centrifuged at +4 ºC, 3000 rpm for 10 minutes and the supernatants obtained were filtered via 0,2 µm and treated with antibiotics (penicillin 100 IU /ml and streptomycin 1 µg /ml) and antifungals (amphotericine B 2,5 µg /ml) solutions. All the samples and inoculum were either directly included in the test protocol or stored at -80 ºC up to testing.

Cell line and the test virus: Madin Darby bovine kidney (MDBK) and Sheep fetal thymus (SFT-R) cell lines were used for virus inoculation and serum neutralization studies. Both of the cell line were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf sera. Cell lines and calf sera were tested by PCR and virus isolation protocols to be free of *Pestivirus* contamination before use. BoHV-1 strain Cooper propagated in MDBK cell line was used as positive control virus in PCR and neutralization tests.

Virus isolation: All the samples taken from total of 36 animals were inoculated onto MDBK and SFT-R cells prepared in 24 well plates. Cells cultivated in 75 cm^2 flasks were suspended as containing $1x10^5$ cells /mL and 1 mL of this suspension was distributed to each well of 24 well plates for using in virus isolation procedure. Culture media were changed after 24 h and samples were inoculated. All the wells were daily screened under inverted light microscope during 3 blind passages each for 7 days. The culture media from the wells in which cytopathogenic effect (CPE) was observed was individually collected and stored at -80 ºC. At the end of each blind passage (day 7 pi), inoculated cell cultures in 24 well plates were freeze-thawed for 3 times and the media from each well was subjected to following test step.

ELISA: The sample (8640) producing CPE in cell culture was subjected to commercial antigen ELISA protocols detecting BoHV-1 (Pulmotest, Bio-X diagnostics, Belgium) and bovine viral diarrhea virus (Herdcheck, IDEXX, Switzerland). While applying the commercial BVDV ELISA method originally validated for detection of persistently infected carriers, for confirmation of the validity of applied method, BVDV NADL strain produced in cell culture in the laboratory was also used as an external control, apart from the positive control included in the kit and it was found to comply with the validation criteria.

Both of ELISA protocols were applied as described by the manufacturers. The test plates were read on the ELISA reader (Thermo-Multiskan EX, Finland) at a wavelength of 450 nm.

Polymerase chain reaction: Because the sample 8640 produced positive result in BoHV-1 antigen ELISA it was further confirmed in PCR. For that purpose both the original swab sample directly taken from the animal and cell culture supernatant from 4th passage level were subjected to testing. Viral nucleic acid was isolated using a commercial kit (NucleoSpin Virus, Macharey-Nagel, Germany). The primer pair P1-forward (5'-CACGGACCTGGTGGACAAGAAG-3') and P2-revers (5'-CTACCGTCACGTGCTGTGTACG-3') targeting the glycoprotein B (gB, UL26) gene of BoHV-1 [12] was employed. The 50 µL of reaction mixture was consisted of nuclease free water (39,8 µL), $MgCl_2$ (25mM, 2 µL), 10 x PCR buffer (5 µL), forward and revers primers (50pmol, 0,5 µL each), dNTPs (10mM each, 1 µL), Taq DNA polymerase (0,2 U) and sample DNA (1 µL). Applied thermal profile was as follows: 94°C for 5 min; 35 cycles of 94°C for 1 min, 61°C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min. The 468 bp PCR products were visualized in 2% agarose gel electrophoresis by Red-Safe™ (ABC Scientific, CA, USA) staining.

Serum neutralization assay: For serological identification, viral isolate from sample 8640 was tested in serum neutralization assay using polyclonal serum yielded against BoHV-1 Cooper strain (BoHV-1.1). Animal experiments to obtain hyperimmune sera were conducted according to rules by national and local ethical commitee (HADYEK). Briefly, 2 series of 2 fold diluted hiperimmunised sera were prepared in DMEM as duplicates of 96 well plates in a volume of 50 µL. BoHV-1 Cooper strain and viral isolate from sample 8640 were prepared in the titer of $100TCID_{50}$ and 50 µl volume of virus suspension was added onto each step of the serum dilutions in different series. After incubation at 37°C in a 5% CO_2 atmosphere for 2 hours, 50 µl of MDBK cell suspension including $3x10^5$ cells /ml was added to each well and incubated in the same conditions for 5 days by daily evaluation. Inhibition of the cytopathogenic virus growth was recorded as positive reaction for neutralization and the antibody titer was evaluated as the highest dilution where the last positive result was observed.

Sequencing and phylogenetic analysis: For genetic characterization of the isolate, a different PCR protocol targeting gC region of the genome was used. Test protocol including the primer pairs PF (5'-CGGCCACGACGCTGACGA-3') and PR (5'-CGCCGCCGAGTACTACCC-3') were applied as described elsewhere [11]. Reaction conditions were applied as given above. The 572-575 bp PCR products were visualized in 1% agarose gel electrophoresis by Red-Safe™ (ABC Scientific, CA, USA) staining. PCR products were submitted for sequencing (Genmar, Turkey) and obtained sequence data were aligned by ClustalW Multiple alignment tool using BioEdit software [13]. Phylogeny analysis was applied on the sequences truncated as to be coding region. Neighbor-Joining method and bootstrap analysis with 1000 replicates were chosen in Mega 7 software. For phylogeny tree, reference BoHV-1 and BoHV-5 sequences were obtained from GenBank. Accession numbers for the reference strains are shown on Fig.1.

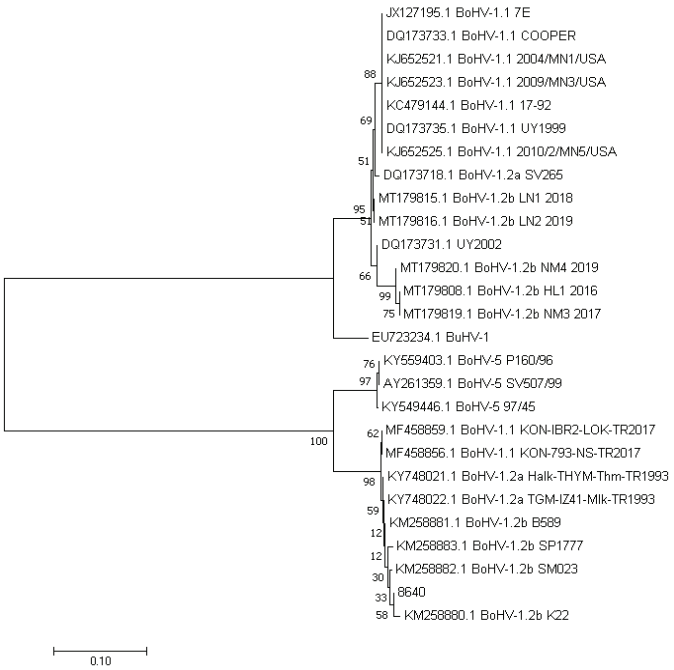

Fig. 1 Phylogenetic tree based on maximum likelihood method for glycoprotein C gene of BHV-1 field strain of 8640

The tree was displayed using Mega10 software. The numbers at branches indicates the frequency after 1000 bootstrap evaluation. Other sequences compared with 8640 isolate were obtained from the NCBI Pubmed website.

Restriction Fragment Length Polymorphism (RFLP): Restriction site analysis were performed as described by Maidana et al. [14]. Multiplex PCR was carried out by using two different primer sets targeting UL39 open reading frame and the US3 upstream intergenic regions. Primers set RS1 (US3; F: 5'-TCGTCGAAGAGCGTCCACACA-3', R: 5'-ACCGCGCTGTACCGGCAGCT-3') and RS2, (US3, F: 5'-TACAAATCGGCGGCGCCAAA-3', R: 5'-TTGTTGACGGCCAAGTATAA-3') amplified the fragments 493 bp and 700 bp, respectively. The multiplex PCR amplification was performed in a final volume of 50 µL, containing; 25 µL Maxima Hot Start 2x Green PCR Master Mix (Thermo Scientific™, K1061), 2,5 µL RNAse/DNAse free water, 5 µL of 10 pmol each sense and antisense primers, 1 µL extracted DNA and 1,5 µL DMSO. PCR products were directly cleaved with Hind III enzyme according to recommended conditions (FastDigest HindIII, Thermo Scientific™) without a purification step. The digested products were separated in 1,5 % agarose gel using 1xTAE buffer. BoHV-

1.1 reference strain Cooper was used as control virus both for multiplex PCR and digestion.

Results

Virus isolation and ELISA: Although samples refer to the laboratory with LSD-like clinical findings, characteristic CPE form encountered in cell cultures and number of days for CPE detection suggested bovine herpesvirus infection. By virus isolation studies conducted on 39 samples, the nasal swab sample (ID: 8640) and one blood sample (data not shown) produced positive result as indicated by visual CPE characterized by rounding, aggregation and lysis of the infected cells in the culture. Those of effects were observed both in MDBK and SFT-R cell cultures at 5th day pi in the first round of blind passages, while an obvious virus growth was recorded starting from 24th hours post infection by the second passage (Fig.2). Further 5 passages were achieved to confirm cytopathogenic growth of the isolates. The isolate produced a higher viral titer ($DKID_{50}$ $10^{5.75}$) in the culture of SFT-R cells comparing to MDBK cells ($DKID_{50}$ $10^{5.25}$). All the final confirmatory tests were performed on cell culture supernatant from 4th passage of the original sample and positive results obtained in BoHV-1 Ag ELISA (OD:0.248). Beside sample was found negative by BVDV ELISA in which BVDV NADL strain produced in cell culture in the laboratory was used as an external control.

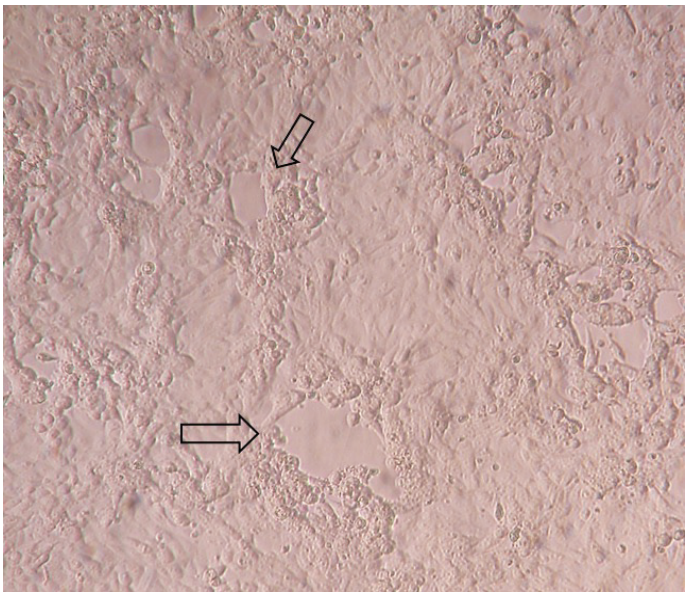

Fig. 2 Virus propogation in SFT cells
Arrows: Foci from virus growth areas (cytopathogenic effect, isolate 8640, 2nd passage, d.1 pi)

PCR and Phylogenetic analysis: BVDV negatif status of the sample was further confirmed by RT-PCR. LSD PCR test performed on the samples was determined negative. However, 486 bp amplicons were yielded by PCR targeting gB region of BoHV-1 genome. Nucleotide sequence analyses of gC region conducted to identify the genetic similarities between 8640 isolate (Access no. MW207646) and with subtype classification realized BoHV-1 viruses. BoHV-5 and Bubaline alphaherpesvirus-1 (BuHV-1) sequences were also included in phylogenetic analyses. Aligned nucleotide sequences revealed high degrees of identity in herpesviruses included in the assessment, but not for the BoHV-1 subtype characterization. The phylogenetic analysis showed the BoHV-1, BoHV-5 and BuHV-1 isolates grouped in distinct branches (Fig.1). The strains of BoHV-1.1 and BoHV-1.2 grouped together in two different branches. The sequences analyzed for the BoHV-1.2a and BoHV-1.2b strains are also intertwined in two different branches. Isolate 8640 revealed between BoHV-1.2 isolates but mostly similar with K22 strain (BoHV-1.2b) subtype and SM023 (BoHV-1.2b) subtype. Genomes alignment was also verified using BLAST database (NCBI, nucleotide sequence blast). BLAST analysis revealed that this sequence homologies in the context of query cover was mostly seen between strain 8640, strain B589 (BHV-1.2b), and strain K22 (BHV-1.2b), respectively. While nucleotide sequence of strain 8640 was compared, glycoprotein C region was mostly conserved according to sequence of BoHV-1.2b K22 reference strain (Fig.3). Multiple sequence alignments showed single substitutions at nucleotide position 17589 which differs from other aligned sequences of BoHV-1.2. Also another substitution revealed at 17360 position identified only in K22 strain sequence.

Fig. 3 Multiple nucleotide sequence alignment of the isolate 8640 to strains from BoHV-1 subtypes
Multiple alignment was conducted between isolates 8640 detected in this study and 6 other strains retrieved from GeneBank [BoHV-1.2b K22 reference strain (KM258880.1), BHV-1.2b B589 strain (KM258881.1), BHV-1.2b SM023 (KM258882.1), BHV-1.2b SP1777 (KM258883.1), BHV-1.2a Halk-THYM-Thm-TR1993 (KY748021.1), BoHV-1.1 Cooper (DQ173733)]. Numbers on the sequence indicate nucleotide positions in the glycoprotein C gene for each virus. Dots indicate that nucleotides in that line are identical in all sequences in the alignment.

Restriction Fragment Length Polimorphism (RFLP): For RFLP; multiplex PCR products were identical for BoHV-1.1 cooper strain. Both the UL39 and the US3 fragments were amplified as it was expected, and the isolate 8640 was detected positive for UL39 region. However, several repeats were conducted in different reaction conditions, no amplification was observed for 8640 isolate by using RS2 primer set. The restriction patterns obtained by Hind III digestions are shown in Fig.4. The fingerprints identical to BoHV-1.1 Cooper strain showed the RS1 and RS2 fragments without cleavage. For the isolate 8640, RS1 amplicon was cleaved into 2 sub-fragments. According to recommended RS1 sub-fragment patterns the field isolate 8640 was determined as BoHV-1.2b.

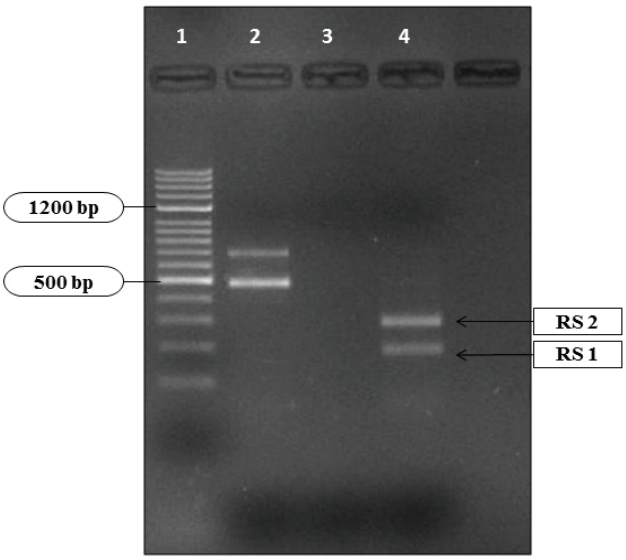

Fig. 4 Genomic fingerprints of the strain 8640 obtained after RFLP analysis of PCR products from UL39

Line 1: ladder; Line 2: BHV-1.1 strain Cooper undigested PCR amplicons of both primer sets (RS1 = amplicon of 493 bp; RS2 = amplicon of 700 bp) Line 3: strain 8640, digested amplicons of RS1; Line 4: negative control (DNase, RNase free water).

Virus neutralization assay: The viral isolate taken from nasal swab sample (BoHV-1 8640/Tur/2014) was also identified as to be BoHV-1 using serum-virus neutralization assay beside BoHV-1 antigen ELISA and PCR. Serum neutralization assay resulted with a neutralization reaction up to 1:32 serum dilution similar to BoHV-1 strain Cooper used as control virus.

Discussion and Conclusion

BoHV-1 was first recognized in Germany in early 19th century, characterized by clinical signs associated with genital tissues, known as "infectious pustular vulvovaginitis" (IPV) in cows and "infectious pustular balanoposthitis" (IPB) in bulls [15]. Strain K22 was the first isolate [16] and today subtyped as BoHV-1.2. After half of the 19th century, respiratory form has recognized in North America. Acute respiratory disease form, known as Infectious bovine rhinotracheitis (IBR) quickly spread to Europe [15]. Causative agents of those two different clinical conditions were not antigenically separated from each other by cross neutralization assays [17]. Analyzing fingerprints by restriction endonuclease of viral DNA allowed subtype classification. Due to the mostly isolation of BoHV-1.1 from respiratory tract disease or abortion and BoHV-1.2 in genital lesions, these subtypes can also being referred according to clinical futures [8]. However, no clear relation was shown between tropism of respiratory or genital tract infections and subtypes [18,19]. Although BoHV-1.2b mostly associated with genital diseases in accordance with some of previous reports [5,10,20], the present study also reveals the molecular characterization of BoHV-1.2b isolate obtained from nasal swab sample at respiratory disease.

In this study, the phylogenetic analysis based on gC gene region clearly grouped the BoHV-1, BoHV-5 and BuHV-1 isolates into different clusters. Unlike the results of some researches which also targets gC region [11,21,22] the phylogeny tree constructed based on nucleotide sequences in this study did not show consistent branches between BoHV-1 subtypes. The isolate 8640 sequence identity was found mostly close to BoHV-1.2b reference strain K22 and strain SM023 (BoHV-1.2b). In a study [23] some of the Chinese BoHV-1.2b isolates have been shown to be grouped separately in phylogenetic investigations and suspected as "atypical" BoHV-1.2 strains with UY2002 strain [23]. The closest sequence of those three samples (MT179808, MT179819, MT179820) was also identified as UY2002. But mentioned Chinese sequences which was referred as BHV-1.2b, has fallen into a different branch separated from 8640.

In subtyping BoHV, it is accepted as the gold standard to examine the complete genome of the virus using the Hind III enzyme by RFLP method. BoHV-1, BoHV-5 differentiation and even subtype restriction endonuclease analysis profiles has distinct fingerprints [24–26]. But this technique needs large quantity of purified viral DNA to display patterns after virus isolation which is laborious and time consuming. Besides this classic application, alternative methods which enables restriction of BoHV PCR products have been developed for alphaherpesviruses [14,27–29]. PCR-RFLP method is promising for rapid and easy implementation of detection and differentiation of BoHV-1 subtypes. In this study, in addition to PCR sequencing assay, one of those proposed techniques was used for subtyping. PCR-RFLP performed successfully for BoHV-1.1 strain Cooper. But isolate 8640 showed amplification only for the RS1 primer

pair. Failure of amplification using RS2 primers was proposed for BoHV-5 and BuHV-1 strains by the authors [14]. However RS1 cleavage was only proposed for BoHV-1.2b subtypes, not for the BoHV-1.1, BoHV-1.2a, BoHV-5 and BuHV. Comparing with the phylogenetic analysis, results was not surprising. Although it is not considered a valid method for subtype characterization, the results of phylogenetic analyses show that the isolate 8640 is not BoHV-5 or BuHV-1. And partial RFLP results obtained in this study supports digestion of RS1 amplicons, which classifies isolate 8640 as subtype BoHV-1.2b. It could be assumed that possible mutations can be responsible for the failure of RS2 amplification. Though restriction patterns for BoHV-1 by PCR-RFLP or selected amplification regions further needs to be investigated in order to enable the evaluation of possible mutations between field isolates.

BoHV infections are common in Turkey where the studies mainly involve serological investigations and limited number of molecular detections [30,31], and only one covers detailed characterization [21]. Beside the predominant prevalence of BoHV-1.1 or few BoHV-1.2a detection, according to our knowledge BoHV-1.2b strain has not been reported in Turkey so far. BoHV-1.2b has been isolated in Europe, America, Australia and lately in China [10,23]. Hence the isolate 8640 is the first BoHV-1.2b isolate reported from Turkey. Our results do not provide to determine the origin of the current isolate, however, it should be noted that live animal importation can be an effective route to introduce such new viral subtypes in to country.

We also compared the cross neutralization antibody reactions between hyperimmune sera for Cooper strain and isolate 8640 (BoHV-1.2b). There was no difference between the neutralization capacities of hyperimmune serum against both the viruses tested. It was also previously reported to recognize BoHV1.2b isolate by BoHV-1.1 hyperimmune sera [21] and due to this cross reactivity, antibody titers do not allow to subtype differentiation[32].

MDBK is the commonly preferred cell line for BoHV isolations and propagation in practice. Besides, adaptation of BoHV-1 to propagate in embryoned chicken eggs [33] and susceptibility of a sheep kidney derived cell line (FLK-N3) [34] were established as an alternative to primary and secondary cell cultures. We obtained BoHV-1 field isolates and propagated not only in MDBK cells but also in SFT-R cell culture at first inoculation. Following 5 passages in each cell line, viral titers obtained in both cell lines were compared. Surprisingly, the virus titer of the field isolate reached higher levels in the SFT-R cells. In a previous study which reports genomic characterization of 13 field strains only 4 could be adapted to the MDBK cell line as intended quantity (3). Regarding the data from the current study SFT-R cell lines can be realized as satisfied alternative to MDBK cell line for BoHV-1 isolation.

In the present study, the isolation and molecular characterization of BoHV-1 strain 8640 were performed from nasal swab sample of a cattle. These findings are valuable for ensuring interior or cross-country epidemiological tracking. But further research are needed to determine the prevalence of BoHV-1.2b subtype in the field. Though standardization of laborless molecular techniques are also needed to provide opportunity for extensive research in this direction. Increased complete genome sequence investments may reflect the genetic diversity and contribute diagnostic improvements.

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Ethical Statement

This study was approved by the Bursa University Animal Experiments Local Ethics Committee (2020 – 09 /10).

Conflict of Interest

The authors declare that they have no conflicts of interest.

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RESEARCH ARTICLE

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Effect of Extenders Including High Concentrations Dimethyl Sulfoxide (DMSO) on Post-Thaw Rabbit Sperm Parameters

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Abstract

Cryoprotectants have critical roles to prevent cell damages during cryopreservation. However, the adjustment of cryoprotectant concentration is also very crucial to protect cells from cryoprotectant toxicity. The present study was designed to investigate the effect of extenders including high concentration dimethyl sulfoxide (DMSO) on post-thaw rabbit sperm quality. Pooled rabbit semen samples (n=7) were diluted and cryopreserved in extenders including 250 mmol/L Tris, 88 mmol/L citric acid, 47 mmol/L glucose, 1% sucrose and different concentrations of DMSO (8%, 10%, 12% and 14%). The presence of high concentration DMSO (12% and 14%) in extender decreased sperm total and progressive motility ($P < 0.01$). The 8% and 10% DMSO supplementations in extender increased live spermatozoon rates ($P < 0.01$). Live and intact acrosome or intact membrane spermatozoon rates were detected higher in 8 and 10 DMSO groups ($P < 0.05$). Although total intact membrane spermatozoon rates were similar in all groups, total intact acrosome spermatozoon rate was higher in 8 DMSO group compared to 12 DMSO group ($P < 0.05$). In conclusion, when 12% and 14% DMSO additions in extender adversely affected post-thaw sperm parameters, the presence of 8% DMSO in extender provided the highest post-thaw sperm quality.

Keywords: sperm, rabbit, cryopreservation, dimethyl sulfoxide.

Introduction

Recently, rabbit production has gained importance as an alternative meat sources in many countries. Thus, assisted reproductive techniques such as sperm cryopreservation[1], artificial insemination[2], etc. have started to study and use more commonly in this species. Unfortunately, the rabbit sperm freezing technology has not reached a sufficient level compared to farm animals, yet. Therefore, different cryoprotectants[3], proteins[4], sugars[5] and antioxidants[6] were supplemented in freezing extenders to improve post-thaw rabbit sperm quality. The cryoprotectants are the most important components of freezing extenders. Either permeable (glycerol, dimethyl sulfoxide (DMSO), ethylene glycol) or non-permeable (saccharides and lipoproteins) cryoprotectants are supplemented in cryopreservation extenders to freeze semen of various animals.[7] When permeable agents bind intracellular water and suppress the production of ice crystals, non-permeable agents enhance extracellular osmolality and trigger cell dehydration.[8] Although both glycerol and DMSO are common cryoprotectants, glycerol is not proper for freezing of rabbit sperm due to its low water permeability and high activation energy.[9] Additionally, DMSO was found more efficient than dimethylacetamide (DMA) for the freezing of the rabbit sperm.[10] Iaffaldano et al.,[10] also reported that 8% DMSO supplementation in freezing extender provided better post-thaw sperm quality than 4% and 6% DMSO supplementations. The post-thaw motile and membrane intact sperm rates were found higher in presence of 10% DMSO when compared with 5% DMSO.[3] The previous studies have indicated that sperm freezing extenders including 8% and 10% DMSO might be good option for rabbits.[3,10] However, the effect of higher concentrations of DMSO on post-thaw

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rabbit sperm parameters has been needed to search. Thus, the present study was designed to investigate the effect of extenders including high concentrations dimethyl sulfoxide (DMSO) on post-thaw rabbit sperm parameters.

Materials and Methods

Animals, Sperm Collection and Cryopreservation

The present study was approved by the Ethical Committee of Aydın Adnan Menderes University (64583101/2022/010). A total of five mature White New Zealand rabbits were used as sperm donors in this study. Semen samples were collected with artificial vagina. Collected semen samples were pooled after initial semen evaluation. The semen samples were higher than 70% motility and less than 25% abnormal sperm rate were used for pooling process. The pooled semen samples (n=7) were separated four equal groups. The sperm samples belongs to each group were diluted at a ratio of 1:3 (to a final concentration of approximately 120 × 10^6 spermatozoa per ml) in a sperm freezing extender including 250 mmol/L Tris-hydroxymethylaminomethane, 88 mmol/L citric acid, 47 mmol/L glucose, 1% sucrose[10] and different concentrations of DMSO (8%, 10%, 12% and 14% DMSO groups). The final DMSO concentrations of the groups were respectively adjusted to 8%, 10%, 12% and 14% DMSO with two-step dilution technique. After dilution process, sperm samples were filled in 0.25 mL straws (IMV Technologies) and then cooled from 36 °C to 4 °C in 90 min with a programmable incubator (Nüve ES 120). The straws were incubated further 10 min at 4 °C for equilibration. Eventually, the cooled straws were frozen with liquid nitrogen vapor 6-7 cm above the surface for 10 min, just before directly plunging into the liquid nitrogen.[11] The frozen sperm samples were stored in liquid nitrogen until the start of sperm analyses.

Evaluation of Sperm Motility Parameters by CASA

A phase contrast microscope (Olympus CX 41) connected to the computer assisted sperm analyzer system (SCA®-Sperm Class Analyzer, Microptic) were used to evaluate total and progressive motility parameters. Thus, 3 µL sperm sample from each experimental group was placed on a pre-warmed (37 °C) glass slide for the evaluation. Three different fields from each slide was assessed at 100× magnification by the system.[11]

Evaluation of Sperm Viability and Acrosome Integrity

The sperm viability and acrosome integrity were assessed with propidium iodide (PI) and Lectin from Arachis hypogaea (peanut) fluorescein isothiocyanate conjugate (FITC-PNA) staining procedure.[11] Briefly, 50 µL sperm sample was incubated and stained with 2.5 µL PI (500 µg/mL) and 5 µL FITC-PNA (200 µg/mL) for 10 min at room temperature. The stained 3 µL sperm sample was placed on slide and covered with cover slip. The image of spermatozoa in same field were captured and evaluated with epifluorescence microscope (Olympus BX53) equipped with a differential interference contrast (DIC) and multiple-fluorescence filters (DM505, U-FBW, BA510IF, BP460-495) and epifluorescent optic (×400 magnification) using a digital camera (DP26- Olympus 5.0 MP). Spermatozoa were classified in four categories: live spermatozoon with intact acrosome (no fluorescence), dead spermatozoon with intact acrosome (only red fluorescence), live spermatozoon with damaged acrosome (only green fluorescence) and dead spermatozoon with damaged acrosome (green and red fluorescence together).

Evaluation of Sperm Membrane Integrity

To evaluate sperm membrane integrity hypo-osmotic swelling test (HOST) in combination with eosin-nigrosin (EN) staining procedure was used.[12] Thus, semen sample (25 µL) were added to 475 µL fructose solution (100 mOsm/L). The samples were incubated in 35°C water bath for 20 min. The samples were stained with EN stain and the membrane integrity of the spermatozoon was determined with under bright field microscope at 400× magnification. Spermatozoa were classified in two groups: live-intact (unstained sperm heads and coiled tails) and total intact (stained and unstained sperm heads with coiled tails).

Statistical Analyses

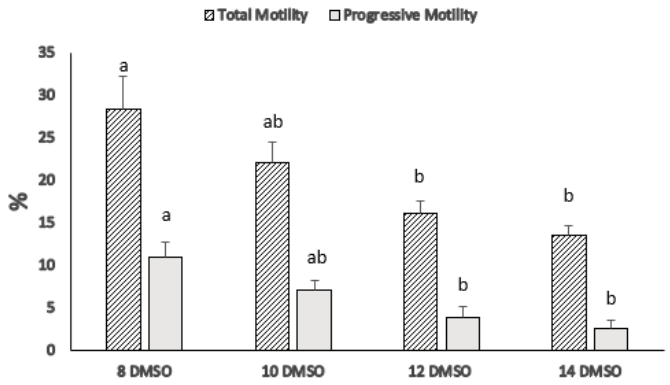
The results of present study (sperm motility, viability, acrosome and membrane integrity) were analyzed with ANOVA General Linear Model (IBM, SPSS Statistics). Significance was determined by fixing probability level at $P < 0.05$. When ANOVA found a significant difference between groups, groups were compared with pairwise multiple comparison post hoc test (Tukey). All the results were presented as mean ± S.E.M.

Results

The total and progressive motility results were higher in 8 DMSO group in comparison with 12 DMSO and 14 DMSO groups ($P < 0.01$) as well as there was no significant difference between 10 DMSO group and other groups (Figure 1). The 8 DMSO and 10 DMSO groups had higher live sperm rates than 12 DMSO and 14 DMSO groups (Figure 2, $P < 0.01$). Similarly, live and acrosome-intact sperm rates of 8 DMSO and 10 DMSO groups were higher than 12 DMSO and 14 DMSO groups (Figure 3, $P < 0.01$). Eight DMSO group had higher total acrosome-intact spermatozoon rate compared to the 12 DMSO group ($P < 0.05$), there was no significant difference between other groups (Figure 3). The live and membrane-intact sperm rate was better in 8 DMSO group than 12 DMSO and 14 DMSO groups (Figure 4, $P < 0.01$). Additionally, 10 DMSO group

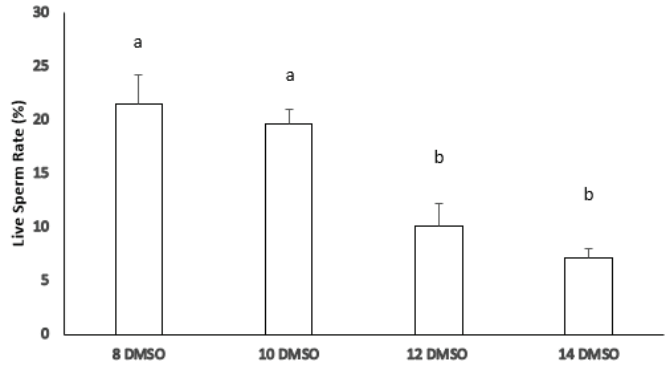
provided higher live and membrane-intact sperm rate than 14 DMSO group ($P < 0.05$). Finally, the total membrane-intact spermatozoon rates were similar amongst all the groups (Figure 4).

Figure 1. Effect of different DMSO concentrations on post-thaw motility parameters of rabbit semen.



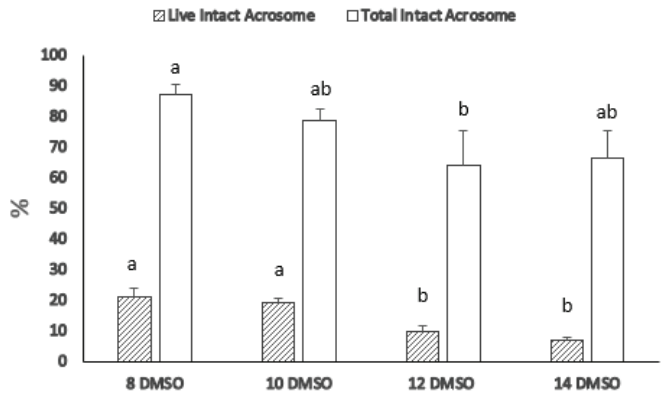
Different superscripts a, b indicates significant differences between groups ($P < 0.01$).

Figure 2. Effect of different DMSO concentrations on post-thaw viability of rabbit semen



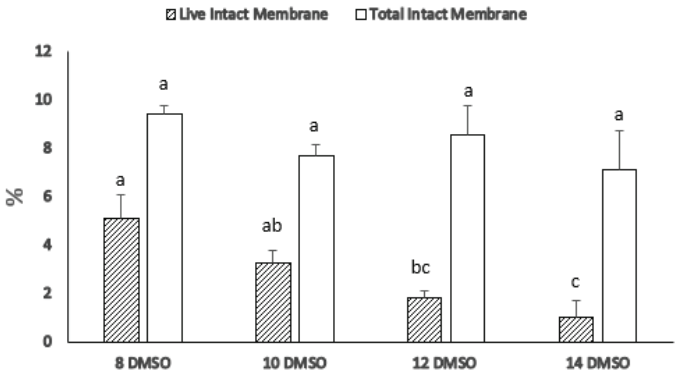
Different superscripts a, b indicates significant differences between groups ($P < 0.01$).

Figure 3. Effect of different DMSO concentrations on post-thaw acrosome integrity of rabbit semen.



Different superscripts a, b indicates significant differences between groups ($P < 0.05$)

Figure 4. Effect of different DMSO concentrations on post-thaw membrane integrity of rabbit sperm



Different superscripts a, b, c indicates significant differences between groups ($P < 0.05$).

Discussion and Conclusion

Cryopreservation technology is an important tool for storage and transfer of sperm cells and subsequently to improve the livestock production and genetic capacity.[13] Although cryoprotective agents are necessary to minimize injuries during cryopreservation process, high concentrations of these agents cause toxic effects on cells.[13] Eight or 10 percent DMSO supplementations were found more beneficial than less concentration of DMSO for rabbit semen cryopreservation in previous studies.[3,10] The effect of higher DMSO concentrations on rabbit semen freezing technology were evaluated in the present study. The present data indicated that the addition of high concentrations of DMSO (12% and 14%) in freezing extender dramatically decreased post-thaw rabbit sperm quality (Figure 1, 2, 3 and 4). Briefly, the high concentration DMSO (12% and 14%) supplementations in freezing extender decreased both total and progressive motility parameters compared to the presence of 8% DMSO (Figure 1, $P < 0.01$). Similarly, Si et al.,[14] reported that 15% DMSO treatment decreased sperm motility compared to 10% DMSO treatment in monkeys. The better motility results in lower concentrate (8% and 10%) DMSO groups were possibly the reflection of higher live sperm rates in these groups (Figure 2, $P < 0.01$). Higher viability rates in eight and 10 percent DMSO groups supported by another study in which the post-thaw Vero cell viability was detected higher in 10% DMSO group in comparison 15% DMSO group.[15] Additionally, the live and acrosome-intact or membrane-intact sperm rates were higher in lower DMSO concentrations (Figure 3 and 4, $P < 0.05$). When total membrane-intact spermatozoon rates were similar between all the groups, the total acrosome-intact sperm rates were higher ($P < 0.05$) in 8 DMSO group than 12 DMSO group (Figure 3 and 4). Likewise, acrosome and membrane integrity rates of the monkey semen were unfavorably effected in the presence of high DMSO con-

centration.[14] Interestingly, when the results of both studies examined carefully, acrosome looks more vulnerable than the plasma membrane of the spermatozoon against high DMSO concentrations. Finally, high concentration DMSO supplementations in freezing extender dramatically decreased the both viability and motility as well as adversely affected the both acrosome and membrane integrity of the spermatozoon. In conclusion, while the addition of 8% DMSO in cryopreservation extender provided the best post-thaw semen quality, the higher DMSO concentrations (12% and 14%) detrimentally effected post thaw rabbit sperm parameters under the present experimental conditions. The presence of 8 percent DMSO in the present extender composition was found optimal to freeze rabbit semen.

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Changes in the Gene Expression of Pyruvate Dehydrogenase Kinase Isoenzymes During Early Differentiation of Mouse Embryonic Stem Cells

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Abstract

The embryonic stem cells (ESCs) are pluripotent, self-renewing cells that are able to differentiate into any of the germ layers involved in embryogenesis. However, the molecular mechanisms that control ESC pluripotency and differentiation remain poorly understood. The family of Pyruvate dehydrogenase kinase (PDK1-4), inactivates the mitochondrial pyruvate dehydrogenase complex via phosphorylation, plays a crucial role in the control of glucose homeostasis. In the current study, gene expression levels of PDK isoenzymes were analyzed on undifferentiated mouse embryonic stem cells (mESCs) and compared to mESCs induced to differentiate by removal of leukemia inhibitory factor (LIF) for 1, 3 and 5 days. Besides, gene expression analysis of several genes related to pluripotency and differentiation were performed by real-time quantitative PCR. In addition, glucose uptake rates in early differentiated and undifferentiated mESCs were determined using a colorimetric assay kit. Differently expression level of PDK isoenzymes in pluripotent and LIF-depleted mESCs suggest that they may have roles in differentiation and pluripotency of ESCs. Furthermore, this study lays the foundation for detailed investigation of molecular mechanisms underlying the roles of PDKs in the pluripotency and transition to differentiated state of ESCs.

Keywords: Stem cell, Pyruvate Dehydrogenase Kinase, Differentiation

Introduction

Pluripotent embryonic stem cells (ESCs) are characterized by their unique ability to both unlimited self-renewal and differentiation. Pluripotency describes the capacity of a cell to differentiate into all cell lineages derived from the three germ layers of the embryo including endoderm, mesoderm, and ectoderm[1,2]. Mouse and human ESCs are isolated from the inner cell mass (ICM) of pre-implantation embryos (blastocysts stage). The early stages of embryonic development take place in a hypoxic environment[3]. Owing to the hypoxic environment in which they reside, ESCs that are highly proliferative cell types utilize glycolysis-based metabolism. Highly proliferative cells need energy, however they also need nucleotides, amino acids and lipids for biosynthesis of cellular components of daughter cell generation. Although oxidative phosphorylation (OXPHOS) is more efficient than glycolysis for generating ATP, using all available glucose solely for the purpose of generating ATP would be limiting for a proliferating cell. Instead, some glycolytic intermediates redirect into the pentose phosphate shunt. It is perhaps for this reason that ESCs, show mainly aerobic glycolytic activity, glycolysis that results into lactate generation, instead of mitochondrial oxidation of pyruvate, regardless of oxygen availability[4,5]. During the early stages of embryonic development, a metabolic shift exists from OXPHOS to glycolysis and oxidative metabolism is reinstituted at post implantation stage. Zhou et al.[6] reported that ESCs rely mostly on anaerobic glycolysis for energy supply and the mitochondria of the cells are inactive during the first stages of spontaneous differentiation. Hypoxia stimulates faster glucose consumption in an attempt to maintain ATP generation via less efficient anaerobic glycolysis.

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In a hypoxic environment, hypoxia-inducible factor 1, or HIF1α transcription factor, is the main regulatory protein, which play a crucial role in stem cell homeostasis. It was demonstrated that HIF-1α is a key factor in regulating embryonic morphogenesis and an important compensator of increased glycolytic activity in the early stages of differentiation[7,8]. HIF-1α up regulation induces the expression of glycolytic genes including pyruvate dehydrogenase kinase 1 (PDK1), lactate dehydrogenase (LDH), hexokinase (HK)[9,10]. ESCs, display Warburg effect, a shift from oxidative phosphorylation to aerobic glycolysis characteristic of cancer cells. Recent data implicate that the Warburg effect, enhanced glycolysis, is also involved in cellular immortalization and sensitizing cells to oncogenic transformation[11]. Therefore, understanding the roles of the major metabolic pathways in the maintenance and acquisition of the pluripotency and in the integration of pluripotency and glycolysis can contribute to a better understanding of molecular basis of stem cells and cancer biology.

Mitochondrial multienzyme pyruvate dehydrogenase complex (PDC) catalyzes irreversible step of the oxidative decarboxylation of pyruvate to acetyl-CoA. Pyruvate dehydrogenase kinase (PDK) enzyme family inactivates the enzyme pyruvate dehydrogenase by the phosphorylation of three seryl residues in the pyruvate decarboxylase subunit, E1 and redirecting metabolism of pyruvate into lactate. PDK has four biochemically distinct isoforms (PDK1, PDK2, PDK3, and PDK4), which have diverse tissue-specific distributions and functions[9,10]. PDK isoforms are up regulated in several types of cancer including breast cancer[12], ovarian cancer[13], hepatocellular carcinoma[14], pancreatic cancer[15], leukemia[16], lymphoma[17]. Most cancer cells use glycolysis as the primary energy source and are characterized through a shift from a mitochondria-based glucose oxidation to glycolysis or lactate fermentation regardless of the presence of oxygen (Warburg effect)[18]. PDK isoforms are the key players of this metabolic shift[13,19]. Besides, stem cell metabolism which mostly relies on glycolysis, shows certain parallelism with that of cancer cells. However, due to the limited literature about the functions of PDK isoenzymes in stem cell metabolism, it is not fully known whether there are possible roles in maintaining pluripotency and there still is a lack of study how PDK family genes are expressed in pluripotent and differentiated stem cells. The current study aimed to investigate the changes of PDK isoenzymes under conditions that induce self-renewal of mESCs (in the presence of essential pluripotency mediator, Leukemia inhibitor factor-LIF), and under conditions that induce differentiation for 1, 3 and 5 days (in the absence of LIF).

Material and Method

Cell culture

Mouse embryonic stem R1 cell line (ATCC) was cultured on tissue culture dishes (Corning, Amsterdam, The Netherlands) coated with 0.1% gelatin (Sigma, Munchen, Germany) in a Dulbecco's modified Eagle's medium (DMEM) (Sigma, Munchen, Germany) supplemented with 15% ES cell-qualified fetal bovine serum (Sigma, Munchen, Germany), 0.1 mM 2-mercaptoethanol (Sigma, Munchen, Germany), 0.1mM MEM non-essential amino acids (Sigma), L glutamine (Sigma), 100 U/ml penicillin-100 ug/ml streptomycin mix and 1000 units/ml of recombinant mouse LIF at 37 °C in a humidified atmosphere with 5% CO2. Cells were passaged every 3 or 4 days using trypsin EDTA. Optimal cell seeding density was conserved at 40,000 cells/cm2 in mouse ESCs to maintain its pluripotent characteristics.

To induce spontaneous differentiation for five days, the ESCs were washed with 1× PBS (Sigma, Munchen, Germany) twice 12 h after the culture and then cultured in the same DMEM and supplements without LIF. Media were changed every 2 days.

Real-time quantitative PCR (RT qPCR)

Total RNA was isolated using a commercial kit (Thermo Fisher Sci.) and reverse-transcribed using cDNA synthesis kit (Thermo Fisher Sci.) according to manufacturer's directions. To analyze total PDK1, PDK2, PDK3, PDK4, SOX2, Brachyury (T2), Nestin (NES) mRNA expressions, real-time quantitative PCR was performed with StepOnePlus (Thermo Fisher Sci., NY, USA) using TaqMan probes (Thermo Fischer Sci., Cat #: PDK1, Mm00554300_m1; PDK2, Mm00446681-m1; PDK3, Mm00455220-m1; PDK4, Mm01166879-m1; SOX2, Mm03053810_s1; T2, Mm00436877_m1; NES, Mm00450205_m1; GAPDH, Mm99999915_g1). GAPDH was used as housekeeping gene control for normalization of cDNA. While Brachyury and Nestin were analyzed as differentiation markers, SOX2, was evaluated as pluripotency marker.

Glucose uptake assays

The glucose uptake colorimetric assay kit (BioVision, Milpitas, CA, USA) was used to detect relative glucose uptake of differentiated and pluripotent ESCs in accordance with the manufacturer's instructions. The principle of this assay is briefly as follows: 2-deoxyglucose (2-DG) metabolized to 2-DG-6-phosphate (2-DG6P) Which cannot be further metabolized, and thus accumulates in the cells. The accumulated 2-DG6P is directly proportional to 2-DG (or glucose) uptake by cells. 2-DG6P is oxidized to generate NADPH, which can be determined by an enzymatic recycling

amplification reaction.

Statistical analysis

The Statistical Package for the Social Sciences version 23.0 (SPSS, Chicago, IL, USA) was used to analyze the gene expression ratios and relative glucose uptake. Cycle threshold (CT) values were taken from qPCR reactions and regulation of genes of interest (up/down) was determined by the ΔΔCT method, using GAPDH as internal control gene, and with undifferentiated mESCs as a baseline. Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Student`s t-test and $p < 0.05$ was considered significant.

Results

LIF withdrawal induces the expression of differentiation genes but suppresses the expression of pluripotency gene SOX2

The cytokine LIF plays a crucial role to sustain prolonged proliferation of mESCs while maintaining their undifferentiated state (self-renewal) and pluripotency. First, the morphological characteristics of mESCs R1 cell line culturing in the absence versus presence of LIF were demonstrated. The morphology of early differentiating cells (5 days after removal of LIF) showed some morphological changes compared to undifferentiated mESCs. mESCs cultured in the absence of LIF were independently growing cells, less compact and more flattened while undifferentiated mESCs grew as compact dome-shaped colonies (Figure 1a).

qRT-PCR data showed that the removal of LIF leads to a reduction in the expression levels of pluripotency gene marker SOX2 within 3 day ($p<0.01$) (Figure 1b). Concomitantly with downregulation of SOX2 gene expression, Nestin ($p<0.01$) (Figure 1c), a neuroectodermal stem cell marker, and brachyrury ($p<0.01$) (Figure 1d), earliest marker of mesodermal and also endodermal differentiation, progressively upregulated.

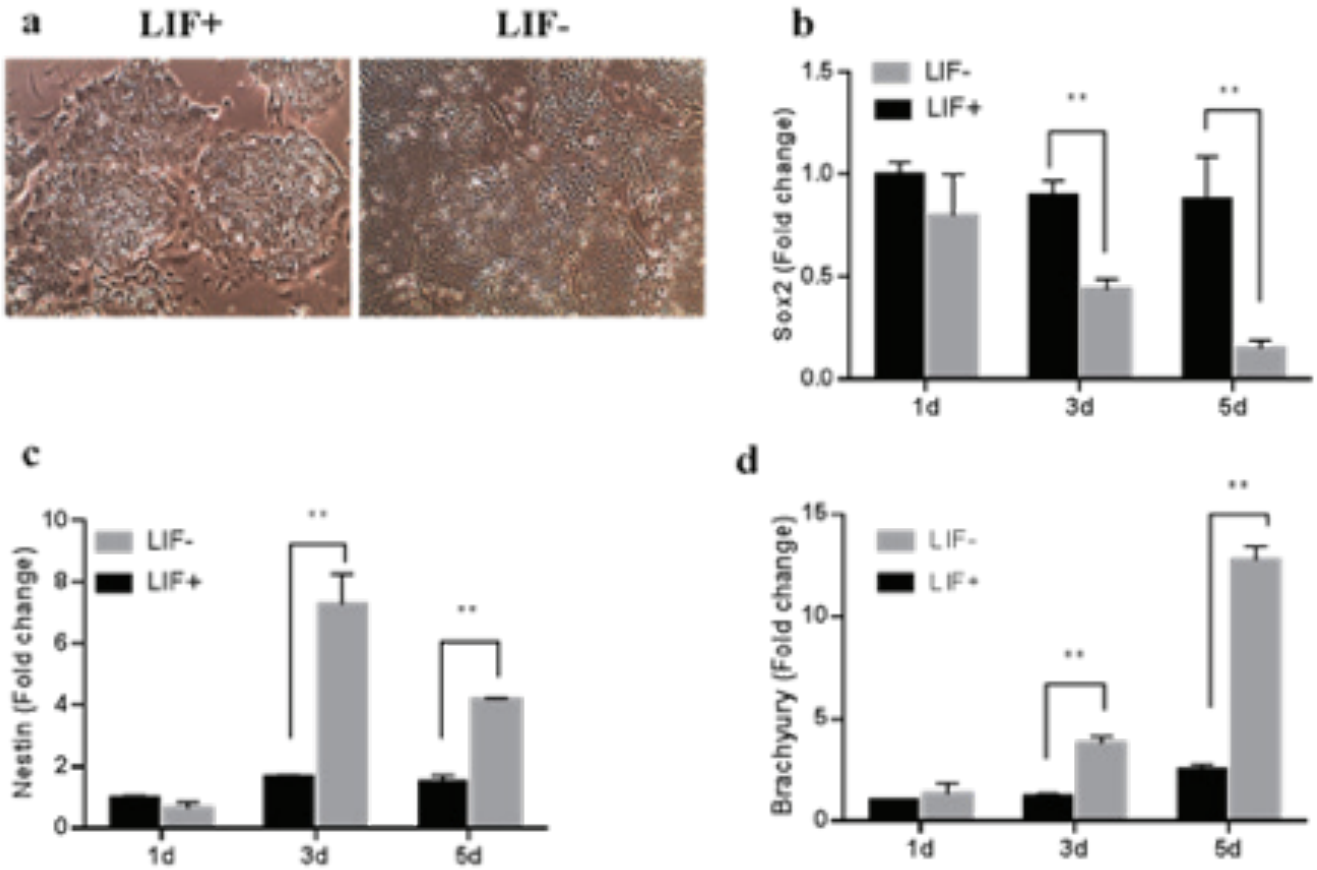

Figure 1 LIF withdrawal induces the expression of differentiation genes but suppresses the expression of pluripotency gene SOX2 **(a)**Morphology of mESCs grown in the presence (LIF+) and absence (-LIF) of LIF for 5 days. **(b)** mRNA expression levels of SOX2 gene in undifferentiated (+LIF) and LIF-depleted (LIF-) for 1, 3, 5d mESC. **(c)** mRNA expression levels of Nestin gene in undifferentiated and LIF-depleted for 1, 3, 5d mESC. **(d)** mRNA expression levels of brachyury gene of mESCs grown in the presence of LIF and without LIF for 1, 3, 5d

PDK isoenzymes are expressed differently in LIF-supplemented and LIF-depleted mESCs.

Next, the changes in the expression of the four PDK genes of mESCs during the first 5 days of differentiation were investigated. Quantitative gene expression analysis demonstrated that LIF withdrawal for 5 days, PDK1 mRNA levels decreased gradually ($p<0.05$, on day 3 and 5) (Figure 2a). Contrastly, LIF withdrawal led to an increase in the mRNA level of PDK2 gene on day 3 and 5 of differentiation ($p<0.05$) (Figure 2b). Expression of PDK3 was also increased at day 5 in the differentiated mESCs compared with undifferentiated mESCs (Figure 2c). The early differentiated mESCs showed lower PDK4 expression levels compared with undifferentiated mESCs but this difference was only statistically significant at day 3 ($p<0.01$) (Figure 2d).

LIF withdrawal decreases glucose uptake in mESCs

In addition, the effect of LIF withdrawal on glucose uptake which is the indicator of glucose metabolism was evaluated. Early differentiated ESCs (for 5 days) showed a decrease in terms of glucose uptake ($P < 0.05$ vs. their undifferentiated counterparts) (Figure 3).

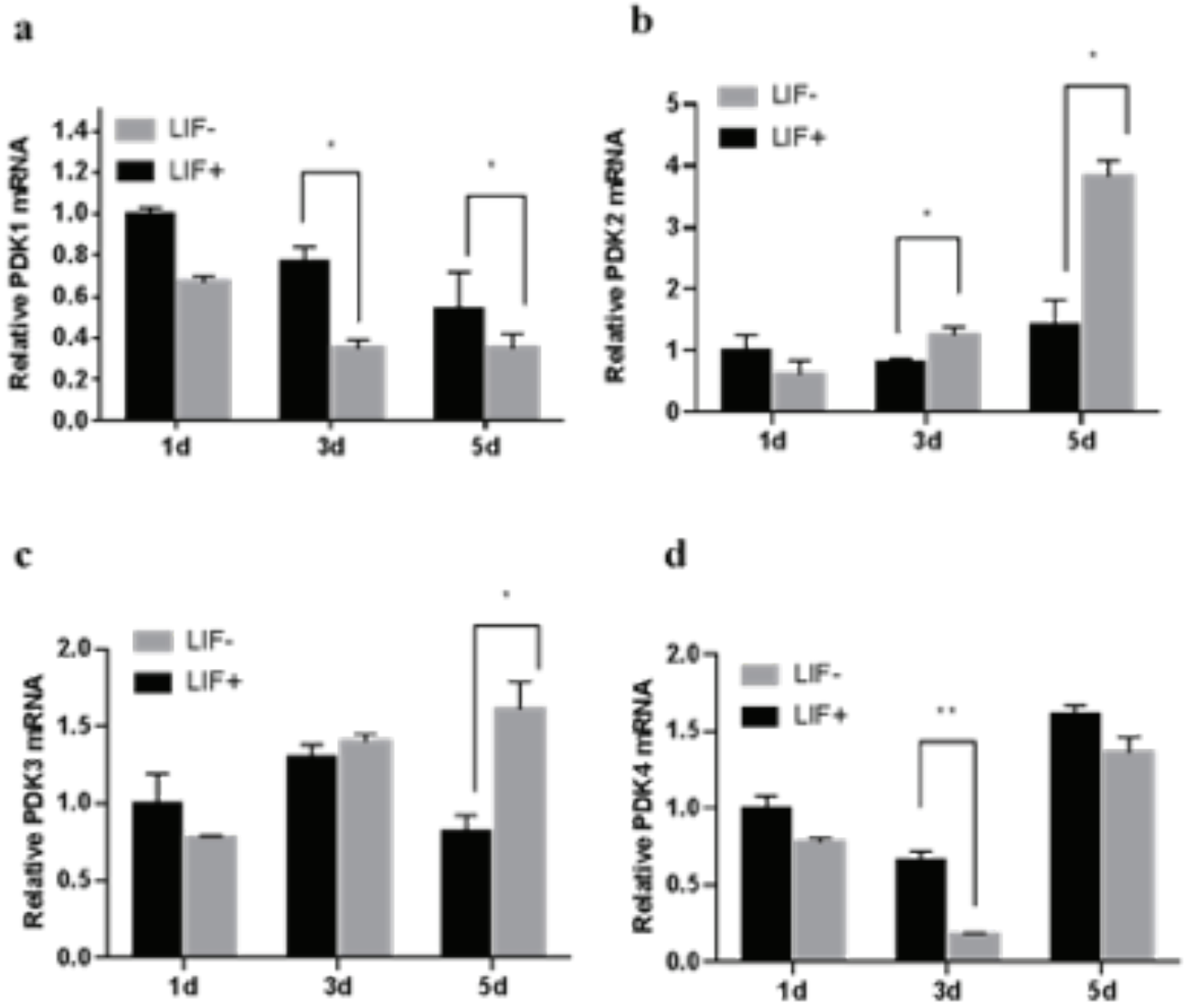

Figure 2 PDK isoenzymes are expressed differently in LIF-supplemented (LIF+) and LIF-depleted (LIF-) mESCs. **(a)** mRNA expression levels of PDK1 gene of mESCs grown in the presence of LIF and without LIF for 1, 3 and 5d. **(b)** mRNA expression levels of PDK2 gene of mESCs grown in the presence of LIF and in LIF-depleted for 1, 3 and 5d. **(c)** mRNA expression levels of PDK3 gene of mESCs grown in the presence of LIF and in LIF-depleted for 1, 3 and 5d. **(d)** mRNA expression levels of PDK4 gene of mESCs cultured in the presence of LIF and without LIF for 1, 3 and 5d.

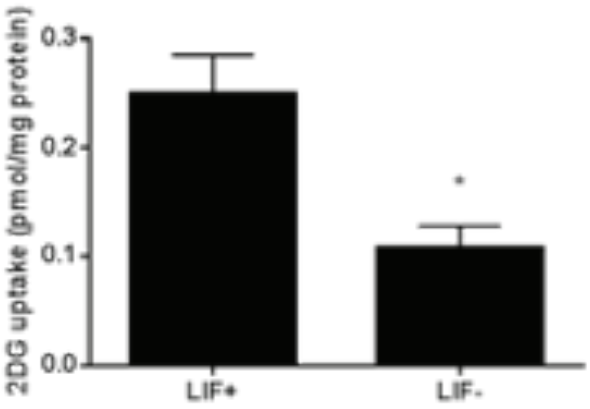

Figure 3 2-DG uptake of mESCs grown in the presence (LIF+) and absence of LIF (LIF-) for 5 days.

Discussion and Conclusion

PDK gene family is involved in regulation of pyruvate dehydrogenase complex (PDC). PDC catalyzes the irreversible conversion of pyruvate into acetyl-CoA, and then participates in the krebs cycle in the mitochondria[20]. Similarly to cancer cells, metabolic activities in embryonic stem cells rely mostly on glycolysis for energy supply[21] in the consistency with our study in which we demonstrated higher glucose uptake in pluripotent mESCs than in early differentiated cells. Furthermore, mitochondria in these cells are rather inactive and generation of acetyl-CoA is suppressed because PDH is phosphorylated[22,23]. The phosphorylation of PDH is catalyzed by a highly specific PDK isoenzymes which leads to a shift from OXPHOS to glycolysis[24]. Tokmakov et al.[25] demonstrated that the expression of PDK isoforms increased several-fold in the embryogenesis following mid-blastula transition in clawed frog, Xenopus laevis. Also they showed that PDK3 increased by about 3-fold during maturation while other PDK family genes are being at their lowest expression levels in the early embryos, grown-up oocytes and matured eggs. The molecular mechanisms underlying the glycolytic energy metabolism of pluripotent stem cells and pathways related to differentiation of them remain unclear. On the basis of these data, we hypothesized that PDK isoenzymes could play a possible role on pluripotency and differentiation of mESCs. Therefore we aimed to comparatively evaluate the expressions of PDK isoenzymes on pluripotent and early differentiated mESCs.

ESCs are derived from the inner cell mass (ICM) of blastocysts of embryo. Retention of pluripotency of these cells relies on expression of transcription factors including KLF4, SOX2, OCT4 and NANOG[26]. SOX2 is an essential factor in the formation of early pluripotent embryonic cells[27]. Consistent with this data, lower level of SOX2 gene expression during early differentiation was determined in this study. Furthermore, the decrease of pluripotency was accompanied with an increase of differentiation markers; Nestin and Brachyury.

In the present study, compared with undifferentiated ESCs, early differentiating ESCs displayed a lower expression of PDK1 gene. This result is in accordance with previous report that PDK1 expression is higher at the protein level in LSK cells, an early form of mouse hematopoietic stem cells (HSCs) compared with differentiated cells[28]. In addition, Halvarsson et al.[29] demonstrated that LT-HSCs (long term HSCs), ST-HSCs (Short term HSCs) and MPPs (multipotent progenitors) expressed higher levels of PDK1 compared to more differentiated cells. Takubo et al.[30] also reported that expression of murine PDK1 was higher in LT-HSCs. Interestingly, another study reported that there was no increase in PDK1 gene expression in pluripotent human embryonic stem cells vs. differentiated cells, although protein levels were higher in pluripotent lines[21].
The current study detected a certain similarity in the expressions of PDK2 and PDK3 isoenzymes; both PDKs were higher expressed in early differentiated mESCs (especially on 5th day) than pluripotent mESCs. Consistent with this result, a recent study showed that there was a positive role of PDK2 in chondrogenic differentiation of mesenchymal stem cells[31]. Contrary, Halvarsson et al.[29] showed that PDK2 and PDK3 expressions were higher in LT-HSCs than in their differentiated progeny. Klimmeck et al.[28] also showed that the protein levels of PDK3 were higher in the LSK cells compared to more differentiated cells. Takubo et al.[30] demonstrated that the expression of PDK2 is important for the quiescence and function of mouse HSCs.

It has previously been shown that the expression of PDK4 is higher in pluripotent LT-HSCs when compared to differentiated cells. Furthermore, it has also been revealed that loss of both PDK4 and PDK2 in mice suppressed glycolysis in HSCs, while overexpression of PDK in glycolysis-defective HSCs reestablished glycolysis and stem cell capacity. Thus, they revealed that PDK4 and PDK2 play a pivotal role in the transition from proliferation to differentiation of the HSCs[30]. Liu et al.[32] showed that PDK4 is the highest expressed isoform of PDKs in placental trophoblast cells and PDK4 expression is dramatically down-regulated during syncytialization. Thus, they proved that PDK4 plays a critical role during syncytiotrophoblast differentiation. Besides, it was demonstrated that PDK4 increases cell stemness properties and stem-associated genes expression in ovarian cancer cells[13]. Consistent with these studies, this

study also determined that PDK4 expression was higher (especially on 3rd day) in pluripotent mESCs than in their early differentiating counterpart. Based on obtained comparative mRNA expression profiling of PDK isoforms in mESCs and early differentiating cells, differently expression profile of both PDK2 and PDK3 from other two isoforms may be attributed to compensatory upregulation of these genes.

Current study comparatively revealed the expression levels of PDK isoforms in pluripotent mESCs (R1 cell lines) and their early differentiated progeny (for 5 day), for the first time. Given that the results indicate expression of PDK isoforms at different levels in pluripotent mESCs and LIF depleted cells, it can be envisaged that PDK isoforms may be important markers for pluripotency and early differentiation of mESCs.

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The Relationship Between Sod1 And Hsp70 Expression in Broiler Ileum Throughout Post-Hatching Development

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Abstract

Heat shock proteins (Hsps) are molecular chaperones that play critical functions in the survival and development of cells. Hsps influence adaptive and innate immune responses and may promote cross-talk between the two systems. Superoxide dismutases (SODs) are metalloenzymes that play an essential role in the body's defense against oxidative stress by efficiently removing excess reactive oxygen species. This study is an experimental study that was conducted to determine the relationship between SOD1 and Hsp70 expression in the ileum during the post-hatching development of the broiler. In the study, samples were taken from ileum tissue of 0-, 21- and 42-day-old broilers were used as material. While the Hsp70 immunoreactivity observed in the epithelial cells was specific to a few cells on day 0, it was detected in more villus epithelial cells on days 21 and 42. The Hsp70 expression in the ileum increased from the age 0 to up to day 42, especially in villus epithelial cells. In sections stained by SOD1, the ileum's villus epithelial cells and smooth muscle cells showed an intracytoplasmic reaction. From day 21 to day 42, a regular increase in SOD1 expression was detected in the crypt and villus epithelial cells. As a remarkable finding, a more intense intracytoplasmic staining was detected in villus epithelial cells located at the apex of intestinal villi. In conclusion, it was observed that SOD1 and Hsp70 expression increased in the ileum tissue throughout post-hatching development in broilers with a positive correlation with age. Based on the histological findings, it can be concluded that SOD1 and Hsp70 play a critical protective role in the small intestine after hatching and contribute to the rapid development of the intestine.

Keywords: Hsp70, ileum, post-hatching period, SOD1.

Introduction

From birth to adulthood, the gastrointestinal tract gradually achieves its mature structure and function[1]. The ability of intestinal villus epithelial cells to communicate with immune and neurological systems against foreign substances plays an important role in the tissue defense system[2]. Also, the intestinal mucosa's histological layers work together to resist, avoid, and, if required, repair harm[3].

Heat shock proteins (Hsps) are molecular chaperones that play critical functions in the survival and development of cells[4]. Hsps are cellular structural proteins that serve a key role in removing and modifying denatured cellular proteins and preventing aggregation of those proteins[5]. Thus, this chaperone broadly impacts protein homeostasis by regulating protein quality and turnover in both normal and stressful situations[6]. Furthermore, to maintain the cytoskeleton structure, Hsps in cells assist in correctly folding proteins and refolding misfolded proteins[7]. Hsps influence both adaptive and innate immune responses and may promote cross-talk between the two systems[8]. It has also been

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reported that Hsps contribute to the pathogenesis of autoimmune and chronic inflammatory diseases by inducing proinflammatory cytokine production[9]. Hsps consist of many different families differentiated by their molecular weight. The Hsp 70 is one of the well-known chaperones of the Hsp family[10]. Hsp70 plays a protective part in the inflammatory and immune response in case of tissue damage[11].

Reactive oxygen species (ROS) are a product of normal redox reactions in organisms and are important determinants of cellular signaling[12]. On the other hand, excessive ROS production can harm lipids, proteins, and DNA by oxidizing them. As a result, to ensure proper cellular function and survival, a tight balance in ROS levels must be maintained[13]. Superoxide dismutases (SODs) are metalloenzymes that play an essential role in the body's defense against oxidative stress by efficiently removing excess ROS[14]. Enzymatic defense systems such as SOD, catalase, and glutathione peroxidases control the immediate detoxification of intracellular ROS under physiological conditions[15]. SOD, an enzyme system made up of three isozymes: SOD1, SOD2, and SOD3, are one of the most well-known antioxidant defense systems. SOD1 is the most widely distributed in the tissues, accounting for 90% of total SOD activity[16]. SOD1 is required to counteract superoxide generation during mitochondrial respiration and is the first line of defense against oxidative damage. It also offers direct regulation of cellular ROS levels[17].

Our study aimed to determine the immunohistochemical expression of SOD1 and Hsp70 throughout post-hatching development in the ileum, a vital tissue in gastrointestinal immunity. It was also to examine the probable relationship between SOD1 and Hsp70 cytokines.

Materials and Methods

Tissue samples

Broiler eggs were incubated in a forced draft poultry incubator at 50-60% relative humidity at 35°C and incubated under appropriate conditions. (Eggs were supplied from Beypiliç A.Ş., Bolu, Turkey). Three groups were formed, each with six animals. Newly hatched (0 days old), 21, and 42-day post-hatching. After the animals were sacrificed under anesthesia, the ileum was sampled for histochemical investigation. A 10 percent formaldehyde solution was used to fix tissue samples for 24 hours. The tissues were kept in a running water bath for 24 hours to remove the formalin, then placed in 70%, 80%, and 96 percent alcohol for 1 hour each. After that, pure alcohol and xylol were applied three times for one hour each. To embed the tissue samples, paraplast was employed. Ankara University, the local animal ethical committee, approved all experimental procedures (2013-5-38).

Immunohistochemistry

Using the Streptavidin biotin complex method, 5µm ileum sections were stained immunohistochemically using rabbit polyclonal Hsp70 (1/200 dilution, Santa Cruz Biotechnology, sc-33575) and rabbit polyclonal SOD1 (1/200 dilution, Bioss Antibodies, bs-10216R) primary antibodies[18]. The secondary antibody was Histostain Plus (Zymed kit: 85-6743, United States). Following deparaffinization, endogenous peroxidase activity was blocked with H2O2 3% in absolute methanol for 15 min. The sections were rinsed with phosphate-buffered salines (PBS, pH:7.2). Sections were heated in a microwave oven at 700 W for antigen retrieval in a citrate buffer (pH:6) solution. The tissues were incubated in a 3% hydrogen peroxide solution to block endogenous peroxidase activity. After washing with phosphate buffer solution, serum was dripped from the kit to prevent non-specific protein binding in sections. The primary antibody was applied, and the samples were kept at +4 ^{0}C overnight. In the negative control group, only the PBS solution was used. After washing, sections were instilled with biotinylated secondary antibody and incubated at streptavidin-horseradish peroxidase complex. The final stage involved using 3,3'-diaminobenzidine (DAP) as a chromogen and covering the slides with entellan after hematoxylin counterstaining.

Immunohistochemical examination

The histological score was determined as follows: Slides were analyzed at a 20 x magnification under the light microscopy (A Nikon digital-sight imaging system was used with a Nikon Eclipse 50i microscope). The intensity of positive staining in the immunohistochemical examination was examined in 10 different areas[19]. Semi-quantitative scoring was used to determine the staining intensity as follows: 0 = no expression; 1 = mild; 2 = moderate; and 3 = intense[20].

Statistical Analysis

All statistical analyses were performed using the IBM SPSS Statistics Version 22.0 statistical software. Comparisons between groups were made with the independent Student's t-test for parametric data. Results were presented as mean ± SEM (standard error of mean) and statistical significance was accepted at $p < 0.05$.

Results

In Hsp 70 immunostaining

Hsp70 protein was mostly found in the cytoplasm of villus epithelial cells, according to immunostaining. While the Hsp70 immunoreactivity observed in the epithelial cells was specific to a few cells on day 0, it was detected in more villus epithelial cells on days 21 and 42. Also, no immunoreaction was observed in goblet cells. It was observed that Hsp70 immunoreactivity was mainly localized to the apical compartment of both villus epithelial and crypt epithelial cells. The Hsp70 expression in the ileum increased from the age 0 to up to day 42. especially villus epithelial cells. In addition, it was seen that the smooth muscle cells forming the tunica muscularis gave a positive reaction (Figure 1). When the effect of the post-hatching period in tunica muscularis, it was determined that the differences amongst study groups were statistically insignificant. Table 1 summarizes and evaluates the immunohistochemical results.

Table 1. The histological score of HSP70 and SOD1 immunoreactivity in the broiler ileum throughout post-hatching development.

	HSP70			SOD1		
Groups (n=6)	Villous epithelial cell	Crypt epithelial cell	Tunica muscularis	Villous epithelial cell	Crypt epithelial cell	Tunica muscularis
Day 0	1 ± 0.26	0 ± 0	1.33 ± 0.21	0.83 ± 0.17	1.33 ± 0.21	2.17 ± 0.17
Day 21	1.83 ± 0.31	1.17 ± 0.17*	1.33 ± 0.33	1 ± 0	1.37 ± 0.17	2.33 ± 0.21
Day 42	2.83 ± 0.17*,#	2.17 ± 0.17*,$	1.33 ± 0.21	2.17 ± 0.31*,&	2.17 ± 0.31#	2.17 ± 0.31

Data presented as mean ± SEM (Student's t-test).

*p<0.001 when compared to Day 0.

#p<0.05, &p<0.01, and $p<0.001 when compared to Day 21.

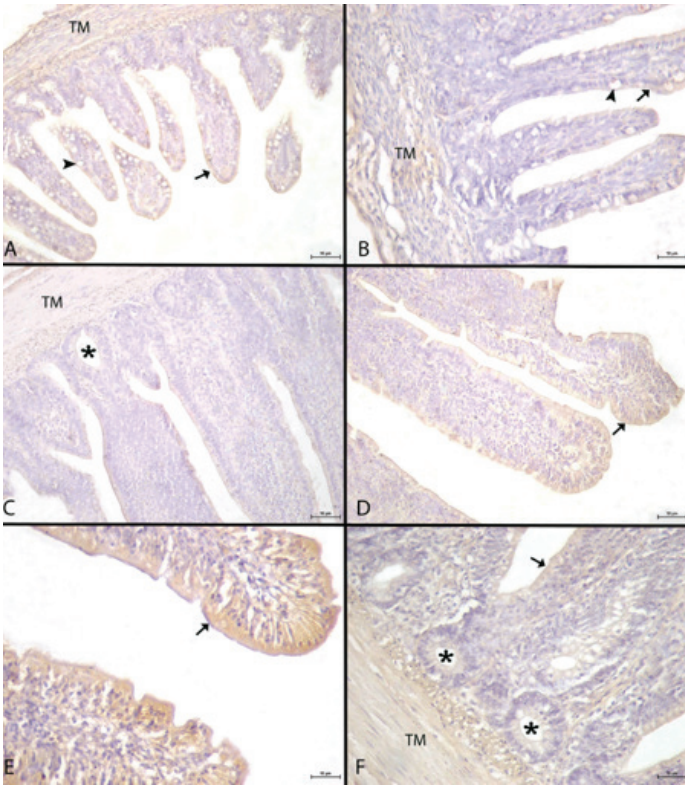

Figure 1. Hsp70 immunostaining in different stages of post-hatching (brown precipitate). A,B: (Day 0), C,D: (Day 21), E,F: (Day 42). Arrow: (villus epithelial cells), arrowhead: (goblet cell), asterix: (intestinal crypt), TM: (tunica muscularis); range bar, 10 µm.

SOD1 immunostaining

In sections stained by SOD1, the ileum's villus epithelial cells and smooth muscle cells showed an intracytoplasmic reaction. On day 0, SOD1 expression in epithelial cells was limited to a few cells; however, by days 21 and 42, it was seen in a more significant number of cells. In addition, there was no SOD1 immunoreactivity found in goblet cells. Furthermore, we found a weak intracytoplasmic SOD1 expression in crypt and villus epithelial cells on days 0 and 21. As a notable finding, after day 21, crypt and villus epithelial cells showed a steady increase in SOD1 expression. Moderate staining was observed on day 42 in SOD1 immunoreactivity. Furthermore, as a remarkable finding, a more intense intracytoplasmic staining was detected in villus epithelial cells located at the apex of intestinal villi (Figure 2). In addition, an increase was observed in the number of stromal cells that reacted positively with SOD1 on the 42nd day. It was determined that smooth muscle cells forming the tunica muscularis were moderate-stained with SOD1 in all groups. In contrast to epithelial cells, smooth muscle cells did not exhibit any differences in SOD1 expression intensity over the post-incubation period. The results of the immunohistochemistry are presented in Table 1.

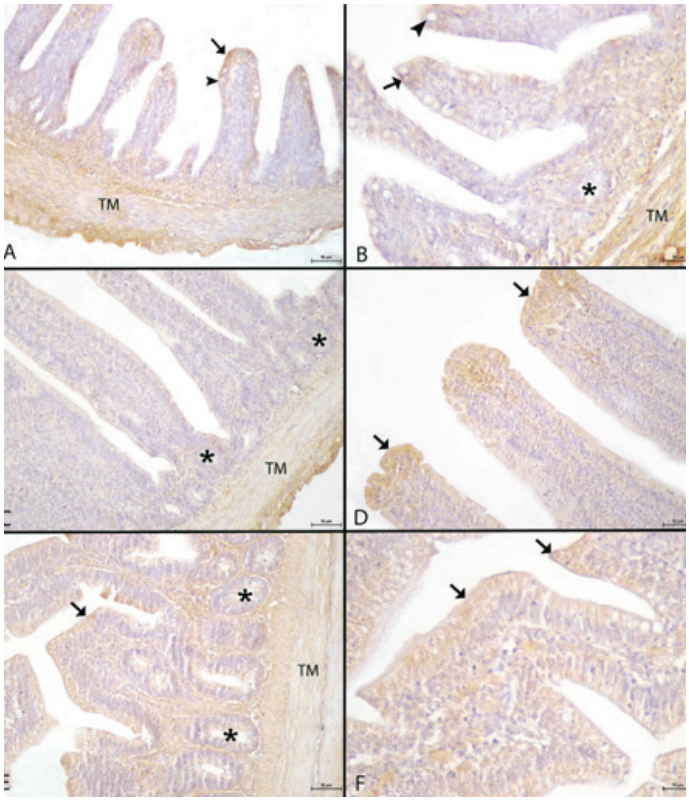

Figure 2. SOD1 immunostaining in different stages of post-hatching (brown precipitate). A,B: (Day 0), C,D: (Day 21), E,F: (Day 42). Arrow: (villus epithelial cells), arrowhead: (goblet cell), asterix: (intestinal crypt), TM: (tunica muscularis); range bar, 10 µm.

Discussion and Conclusion

Cytokines/chemokines are soluble polypeptides with a low molecular weight secreted by immune cells and other cell types. They are produced in response to microorganisms and other antigens, regulate immunological and inflammatory responses, and function in paracrine and autocrine ways[21]. In addition to aiding the transport, digestion, and absorption of nutrients, the small intestines' large surface area acts as a regulatory barrier for microbes and exogenous pathogens[22]. Also, It is known that intestinal epithelial cells can produce proinflammatory cytokines and function as antigen-presenting cells[23].

It is well known that oxidative stress increases the expression of Hsp70 and SOD1 and that the regulation of these two cytokines is positively correlated[24]. There are studies in the literature examining the interaction between SOD1 and Hsp70. For example, it was shown that Hsp70 and SOD1 expression increase in retinal ganglion cells when intraocular pressure is applied to rats[25]. Another study has revealed that decreased Hsp70 and SOD1 levels due to gastric mucosal damage were increased with aucubin administration in ethanol-induced gastric mucosal injury in mice[26].

Hsps expression is stimulated during the development and differentiation of tissues to protect against physiological stress[27]. Hsps have critical functions in embryogenesis, tissue maintenance, and injury response. The Hsp70 protein is essential for cell viability[28]. Its important function is to aid in protein folding and cycling. Quality control systems in normal cells prevent the accumulation of toxic misfolded protein species[29]. Hsp70 is known to protect intestinal epithelial cells, which are easily injured by numerous stressors, from toxic chemicals and ulcerogenic conditions in the gastrointestinal mucosa[30]. Upregulation of Hsps, particularly Hsp70, is also thought to be a protective mechanism because it can inhibit the expression of pro-inflammatory cytokines[31]. Heat shock factors (Hsf) regulate Hsp expression and mediate the multifaceted response to stress. Stress is known to initiate Hsf phosphorylation and trimerization, and these Hsf trimers bind to heat shock elements. Up-regulated Hsf increases Hsp70 expression in the ileum, contributing to cell functions under stress conditions[32]. Hsp70 is primarily known for its cytoprotective function, but it has also been proposed that it regulates cell growth via modulating certain signaling pathways. Under normal conditions, one of the leading roles of Hsps is to maintain the appropriate embryonic and postnatal development of different tissue and organ systems, particularly the neurological system[10]. It is well known that age-related processes or disease conditions can disrupt cellular protein homeostasis. During aging, the accumulation of misfolded proteins increases, possibly leading to elevated Hsp levels[33]. During the development of skeletal muscle myoblasts, the Hsp70 protein is also involved in the formation of myosin thick filaments[34]. Zhong et al.[35] reported that Hsp70 expression appeared predominantly localized in the epithelial cell cytoplasm in the ileum. Our work agrees with previous reports that Hsp70 expression in the ileum. In addition, we also observed that age might have an effect on Hsp70 expression in the ileum. Hsp70 expression in the ileum gradually increased from the age 0 to up to day 42 in villus epithelial cells. We thought that Hsp70, which is known to play an important role in protein folding and cycling, may be necessary for the functional integrity of cells in the broiler ileum during the post-incubation period.

SOD1 plays an important role in protecting tissues against oxidative stress by catalyzing the conversion of superoxide radicals to hydrogen peroxide, which can be reduced to water[36]. By generating cytokines or creating direct cell-cell contact, intestinal immune cells can influence mucosal barriers[37]. SOD1 is thought to regulate the coordinated balance between inflammation, mucosal immune cells, and various soluble factors[38]. In a study on chick embryos, SOD1 immunoreactivity was observed in ectodermal origin tissues and the cauda mesoderm during the gastrulation to neurulation. Also, it has been reported that SOD1 might indirectly regulate cell proliferation during gastrulation[39]. It was found in the study conducted by van der Loo et al.[40] that there was an increase in SOD1 expression in the aorta depending on age in rats. Of this find, they suggested that this increase may be an essential determinant for overcoming oxidative stress as a critical factor in a series of events that appear to be a physiological consequence of aging. A previous study shows that SOD1 expression is observed in the epithelial layer, submucosa, and muscle layer of the ileum tissue. Furthermore, it has been reported that SOD1 was highly expressed in mature epithelial cells compared to immature cells present in the intestinal crypt[41]. In our study, we observed SOD1 expression in smooth muscle cells, crypt and villus epithelial cells in accordance with previous findings. Furthermore, we noted that SOD1 expression in villus epithelial cells was limited to a few cells, it was seen in more cells on days 21 and 42. Also, the expression of SOD1 in the crypt and villus epithelial cells increased steadily from day 0 to day 42. We speculated that SOD1 might be necessary for the ileum's antioxidant defense systems and immune system in the post-hatching period.

In conclusion, we tried to determine the expression of SOD1 metalloenzyme and Hsp70 chaperone in the developmental stage of ileum tissue after post-hatching in broilers. The expression of SOD1 and Hsp70 was observed to increase in a positive correlation with age. Based on the histological findings, it can be concluded that SOD1 and Hsp70 play a critical protective role in the small intestine after hatching and contribute to the rapid development of the intestine.

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Comparison of the Efficacy of Enrofloxacin and Lactobacillus Plantarum Cell-Free Supernatant Treatments on Vaginitis in Ewes

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Abstract

The aim of the study was to investigate the effect of different intravaginal treatment strategies on the vaginal discharge score, vaginal microbiota, bacterial and Enterobacteriaceae counts in nulliparous Merino ewes. All ewes (n=45) received intravaginal sponges containing 60 mg medroxyprogesterone acetate for 7 days and allocated into three equal groups (n=15). Sponges were injected Lactobacillus plantarum cell-free supernatant (SUPER), enrofloxacin (ENRO), or physiologic saline (CON) prior to sponge insertion. At sponge removal, 500 IU equine chorionic gonadotropin (eCG) were administered in all ewes. For the detection of vaginal microbiota, bacterial and Enterobacteriaceae counts, samples were collected prior to sponge insertion, at sponge withdrawal, and 48 h later after sponge withdrawal. Vaginal discharge score was not different in ENRO (2.26±0.18) and SUPER (2.20±0.14) compared to CON (2.46±0.16). The time-dependent alteration was significant for the mean bacterial and Enterobacteriaceae count in all groups (P<0.05). Bacterial counts were found to be lower in ENRO (5.50±0.17) than SUPER (6.31±0.19) and CON (6.07±0.15) at sponge removal (P<0.05). In addition, SUPER (3.74±0.21) and ENRO (3.49±0.27) had lower Enterobacteriaceae counts compared to CON (4.78±0.21) at sponge removal (P<0.01). The most frequently isolated bacteria species were Trueperella pyogenes (28.9%) and Escherichia coli (46.7%). In conclusion, treatment with enrofloxacin or cell-free supernatant decreased the Enterobacteriaceae counts in ewes. Comprehensive studies are needed to assess the effectiveness of lactic acid bacteria as an antibiotic-free treatment strategy on vaginitis in ewes that were synchronized with progesterone impregnated intravaginal sponge.

Keywords: Antibiotic, Bacterial count, Lactobacillus plantarum, Sheep, Vaginitis

Introduction

Reproduction of small ruminants is commonly controlled by using the progesterone impregnated intravaginal sponges to achieve optimal lamb production.[1] Intravaginal sponge generates local inflammation with histological and cytological alterations in the vaginal flora.[2,3] Narrow vaginal lumen of nulliparous ewes increase the severity of vaginal inflammation compared to multiparous ewes.[4] These alterations are explained by the proliferation of pathogenic bacteria and the presence of abnormal mucus accumulation at sponge withdrawal.[3,5] In addition to vagina inflammation, the collection of mucus impaired sperm quality in rams[6] and decreased the sexual attractiveness of ewes to rams.[7] Therefore, vaginitis after using intravaginal sponges leads to the reduction of pregnancy rate.[8]

Vaginal bacterial load number increases with vaginitis at sponge removal compared to sponge insertion in ewes.[2] Escherichia coli, Staphylococcus spp., and Trueperella spp. constitutes the majority of the vaginal microbiota in ewes

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with vaginitis.[5,8–13] Efficient antibiotic therapy such as enrofloxacin was recommended to reduce the detrimental effects of vaginitis in recent studies.[8] However, controlling vaginitis by adding antibiotics into sponge has not been preferred due to the resistance of microorganisms[8,14] and drug residues.[15,16] Considering the resistance of microorganisms to antibiotics and residue for human health, antibiotic-free treatment options should be investigated.

Recent studies reported that Lactobacillus species were commonly used as antibiotic-free therapy to treat bacterial vaginosis in women.[17,18] These bacteria secrete many natural antimicrobial metabolites such as bacteriocin, phenyl lactic acid, organic acids, and hydrogen peroxide.[18] The inhibitory effect of lactic acid bacteria on pathogenic bacteria is likely associated with the antimicrobial compounds produced by lactic acid bacteria. However, Quereda et al. (2020)[9] reported that infusion of lactic acid bacteria did not decrease bacterial counts in ewes that had vaginitis. L. plantarum is one of the most effective probiotic agents that inhibit bacterial pathogens.[19,20] The objective of this study was to compare the effectiveness of L. plantarum cell-free supernatant or enrofloxacin on vaginal discharge score, vaginal microbiota, the vaginal bacterial load, and the number of Enterobacteriaceae in nulliparous ewes.

Materials and Methods

Animals and Management

The experimental procedures were approved by the Balıkesir University Animal Care Committee (Approval no: 2021/3-4). The study was carried out during non-breeding season (March-May) in a farm located in Bursa (40° 13' N, 29° 29' E) in Turkey. Forty-five nulliparous Merino ewes, 10-13 month age and body condition score of 3.0-3.5, were used in the study. Ewes were grazed on natural pasture and received an average 200 g alfaalfa (Medicago sativa) per ewe/day and 1000 g concentrate barley per ewe/day during the experiment. Ewes had ad libitum access to good-quality drinking water.

Experimental design

Intravaginal sponges containing 60 mg medroxyprogesterone acetate (MAP; Esponjavet®, HIPRA, Turkey) were inserted and remained in situ for 7 days in all ewes. Ewes were randomly divided into three groups (n=15 for each group) receiving two treatment groups and a control group as follows; L. plantarum cell-free supernatant (SUPER; 1 mL/sponge), Enrofloxacin (ENRO; 0.5 mL, 10%, Enrolen®, Alke, Turkey) diluted with 0.5 mL 0.9% NaCl (the solution was applied totally as 1 mL in each sponge) or Control (CON; 1 mL/sponge, 0.9%, NaCl, Polifarma®, Turkey). All applications were injected at six different points on the sponge after the sponge was placed on the applicator to prevent the overflow of solutions. All materials were disinfected with 1% benzalkonium chloride (Zefirol, Dermosept®, Turkey) before each application. Ewes received one intramuscular injection of 500 IU equine chorionic gonadotropin (eCG; Gonaser®, HIPRA, Turkey) at the time of sponge removal. Following the removal of sponge, oestrus detection was performed with five teaser rams with crayon marks at 12-h intervals for 5 days.

Preparation of cell-free supernatants and antimicrobial activity

L. plantarum (DSM 1055) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ®, Germany). L. plantarum strain was grown in De Man Rogosa and Sharpe Broth (MRS Broth®, Merck, Germany) media at 37° C for 48 h. Bacterial suspension was transferred to sterile 10 milliliters falcon tubes. Bacterial cells were removed by centrifugation at 5000 g for 10 min. Cell-free supernatant was concentrated by either 50% or 75% with evaporator (55-60 °C/0.75 atm) to increase the concentration of antimicrobial activity. Cell-free supernatant was obtain after concentrated supernatant was filtered through a 0.22µ filter. The cell free supernatant was prepared 2 days before the application of sponge.

The three different concentrations (0%, 50% and 75%) of cell-free supernatant from L. plantarum and enrofloxacin were assessed their antimicrobial capability against E. coli (American Type Culture Collection, ATCC 25922) and collection of coliform bacteria in the Department of Public Health in of Balıkesir University. Agar well diffusion assays were performed on 90 mm Petri dishes containing Mueller Hinton agar media (Merck, Germany).[21] The agar plates were cut for 4 mm depth to place 50 µl of supernatant or enrofloxacin into each well. The plates were incubated at 37 °C for 48 hours. Following incubation, the diameters of the growth inhibition zones were measured in millimeters to the nearest 0.1 mm using electronic callipers.[21] Each stage was repeated two times. For the antimicrobial activity of enrofloxacin and three concentrations of cell-free supernatant, the mean diameters of inhibition zones are presented in Table 1.

Table 1. Zone diameters of Lactobacillus plantarum cell-free supernatant (SUPER) at different concentrations and enrofloxacin (ENRO) against bacteria on Mueller Hinton Agar

Bacteria	SUPER	SUPER %50	SUPER %75	ENRO
Escherichia coli	20.66 ± 0.57	24.33 ± 0.57	33.00 ± 1.00	40.00 ± 1.00
Coliform bacteria	19.66 ± 0.57	23.33 ± 0.57	32.00 ± 1.00	41.33 ± 0.57

SUPER: *Lactobacillus plantarum* cell-free supernatant; ENRO: Enrofloxacin

Evaluation of vaginitis

Vaginitis mucus were scored according to vaginal discharge characteristics (amount and aspect) as follows; score 0: negligible or no discharge, score 1/moderate: clear and some amount of discharge, and score 2/severe: abundant purulent or hemorrhagic discharge.[22] The odour of vaginal discharge (score 0 = none, score 1 = mild, and score 2 = abundant) was determined at sponge removal according to Viñoles et al. (2011)[13] following the sponge removal, sponges were weighted to determine the accumulation of mucus secretion.2 Vaginal pH is determined using pH indicator strips (Merck®, Germany) at the sponge insertion and sponge removal.

Collection of Vaginal Samples

All samples were collected on the day of sponge insertion (day 0), at sponge removal (day 7), and 48 h later (day 9). The vulvar area of ewes was disinfected with 1% benzalkonium chloride (Zefirol®, Dermosept) and the vaginal lips were opened with one veterinarian to avoid contamination. Samples were collected using sterile stuart transport swab (Fıratmed®, Turkey) which was rotated over the anterior vaginal mucosa by direct contact for bacterial examination. For the counting of colony forming units (log CFU/mL) and Enterobacteriaceae, the second samples were collected using sterile plastic stick swab in the same technique as the bacterial samples and swab was placed in a 9 mL of Maximum Recovery Diluent (MRD, Merck®, Germany) at neutral pH. The samples were transported at 4°C to the laboratory after 2 h following collection.

Bacterial Count

Tubes, diluted in 9 mL of MRD at neutral pH, was homogenized by vortex for 1 min to suspend the bacteria. Serial dilutions were performed using MRD and dilutions were plated in Plate Count Agar (PCA, Merck®, Germany) for counting total bacterial count (CFU/mL). Dilutions were seeded on Violet Red Bile Dextrose agar medium (VRBD, Merck®, Germany) for Enterobacteriaceae count. Bacterial colonies were counted after incubation of PCA and VRBD agar for 48 h at 37°C.

Identification of Bacterial Population

Vagina swab samples were submitted to the microbiology laboratory and processed within 2 hours of retrieval form ewes. The samples were cultured for bacteria with 5% defibrinated sheep blood and incubated at 37°C. After incubation for 24-48h, plates were examined for growth. Colonies are stained with Gram stain. BBLTM CrystalTM biochemical tests were used according to manufacturers' instructions for identification of colonies.

Statistical Analysis

All statistical analyses were performed using SPSS® 23.0 package software (IBM Corporation, NY, USA). Total bacterial count (CFU/mL) and Enterobacteriaceae count were analyzed with analyses of variance (ANOVA) for repeated measurements after logarithmic transformation to normalize the data. Age, vaginal pH, vaginitis discharge score, vaginal odour score, and sponge weight were analyzed using ANOVA. Oestrus response and percentage of vaginal discharge score was analyzed using chi-square test Statistical significance level of $P<0.05$ was considered as significant, and statistical tendencies were defined as $0.05<P<0.10$.

Results

The mean age of ewes 11.84±0.48 month and there was no difference ($P>0.05$) for the age of ewes among the groups (CON; 11.72±0.83, ENRO; 11.76±0.91, and SUPER; 12.03±0.81) at the beginning of study. Oestrus response was 46.7% (21/45) and there was no significant difference among the groups, as follows CON; 53.3%, ENRO; 33.3% and SUPER; 53.3%. Vaginal discharge of varying severity was detected following the sponge removal in all ewes. The overall percentage of negligible discharge (Score=0; 8.9%, 4/45) was lower than moderate (Score=1; 51.1%, 23/45) and severe ones (Score=2; 40.0%, 18/45) irrespective of groups ($P<0.01$). The percentage of negligible, moderate and purulent/hemorrhagic vaginal discharge were 6.7%, 40.0%, 53.3% for CON, 13.3%, 46.7%, 40.0% for ENRO, and 6.7%, 66.6%, 26.7% for SUPER, respectively, data was shown at Table 2. When evaluated the mean vaginitis score among the groups, both of the two treatments (ENRO; 2.26±0.18 and SUPER; 2.20±0.14) did not affect ($P>0.05$) on the vaginitis score compared to that of CON (2.46±0.16).

The mean weight of the sponge at the time of removal was significantly heavier ($P<0.01$) in the ENRO (6.92±0.59 g) compared to those of SUPER (5.14±0.22 g) and CON (4.92±0.17 g). However, there was a tendency ($P=0.08$) for reduced odour score of vaginal discharge in ENRO

Table 2. Comparison of different parameters following different treatment with medroxyprogesterone acetate plus eCG in nulliparous ewes

Parameters	OVERALL	CON	ENRO	SUPER	*P*
Age (months)	11.84 ± 0.48	11.72 ± 0.83	11.76 ± 0.91	12.03 ± 0.81	>0.05
Vaginal discharge score 1 %, (n/n)	8.9% (4/45)	6.7% (1/15)	13.3% (2/15)	6.7% (1/15)	>0.05
Vaginal discharge score 2 %, (n/n)	51.1% (23/45)	40.0% (6/15)	46.7% (7/15)	66.7% (10/15)	>0.05
Vaginal discharge score 3 %, (n/n)	40.0% (18/45)	53.3% (8/15)	40.0% (6/15)	26.7% (4/15)	>0.05
Vaginal discharge score (mean ± S.E.M)	2.31 ± 0.09	2.46 ± 0.16	2.26 ± 0.18	2.20 ± 0.14	>0.05
Odour score of vaginal discharge (mean ± S.E.M)	2.18 ± 0.12	2.40 ± 0.19^{a}	1.80 ± 0.22^{b}	2.33 ± 0.19^{a}	0.08
pH at sponge insertion	7.00 ± 0.06	7.05 ± 0.06	7.03 ± 0.10	7.10 ± 0.11	>0.05
pH at sponge removal	7.21 ± 0.05	7.19 ± 0.63	7.18 ± 0.97	7.24 ± 0.90	>0.05
Sponge weight (g)	5.66 ± 0.25	4.92 ± 0.17^{a}	6.92 ± 0.59^{b}	5.14 ± 022^{a}	<0.01

^{a,b} Values within a row with different superscripts differ significantly at $^{**}P<0.01$; $^{*}0.05<P<0.10$ (tendency)

OVERALL: The mean value of all groups; CON: Control; ENRO: Enrofloxacin; SUPER: *Lactobacillus plantarum* cell-free supernatant

(1.80±0.22) compared to CON (2.40±0.19) and SUPER (2.33±0.09). The mean vaginal pH (mean±S.E.M) was not different (P>0.05) at sponge insertion (7.05±0.06, 7.03±0.10, 7.10±0.11) and following sponge removal (7.19±0.63, 7.18±0.97, 7.24±0.90) among the groups (CON, ENRO and SUPER, respectively).

Table 3. Comparison of total bacterial count (log CFU/mL) and Enterobactericea count (ENTERO) at sponge insertion (day 0), sponge removal (day 7), and 48 later after sponge removal (day 9) following different vaginal treatment protocol with synchronization eCG in ewes

Bacterial Count	OVERALL	CON	ENRO	SUPER	P
CFU at day 0	4.44 ± 0.14	4.60 ± 0.25	4.12 ± 0.20	4.58 ± 0.25	>0.05
CFU at day 7	5.96 ± 0.11	6.07 ± 0.15[a]	5.50 ± 0.17[b]	6.31 ± 0.19[a]	<0.05
CFU at day 9	5.32 ± 0.13	5.40 ± 0.23	5.17 ± 0.25	5.39 ± 0.20	>0.05
ENTERO at day 0	1.99 ± 0.13	2.03 ± 0.23	1.96 ± 0.24	1.98 ± 0.18	>0.05
ENTERO at day 7	4.00 ± 0.16	4.78 ± 0,21[a]	3.49 ± 0,27[b]	3.74 ± 0,21[b]	<0.01
ENTERO at day 9	3.35 ± 0.17	3.85 ± 0.30	3.04 ± 0.30	3.15 ± 0.26	>0.05

a,b Values within a row with different superscripts differ significantly at *P<0.05; **P<0.01;

OVERALL: The mean value of all groups; CON: Control; ENRO: Enrofloxacin; SUPER: Lactobacillus plantarum cell-free supernatant

The mean bacterial count (log CFU/mL) was 4.44±0.14 at sponge insertion regardless of groups. The mean bacterial count significantly increased (5.96±0.11) at sponge removal and it decreased (5.32±0.13) at 48 h later following the sponge removal (P<0.01). The log CFU/mL in ENRO (5.50±0.17) was lower compared to SUPER (6.31±0.19) and CON (6.07±0.15) at sponge removal (P<0.05). Similar to log CFU/mL, the time-dependent alteration was also significant in the number of Enterobacteriaceae (P<0.05). The mean value of the Enterobacteriaceae count was 1.99±0.13 at sponge insertion, 4.00±0.16 at sponge removal, and 3.35±0.17 at 48 later after sponge removal. The number of Enterobacteriaceae was not significant at sponge insertion and 48 h later after sponge removal among groups. However, the Enterobacteriaceae counts in SUPER (3.74±0.21) and ENRO (3.49±0.27) was lower (P<0.01) at sponge removal when compared to CON (4.78±0.21) group (Table 3). Additionally, the bacterial count and Enterobacteriaceae at two days later after sponge removal was not different (P>0.05) in ewes showed oestrus compared to ewes did not show oestrus.

The number of vaginal bacteria species isolated in different stages of synchronization was presented in Table 4. Irrespective of treatment groups, the number of Gram-positive bacteria was 7/45 (15.5%) of samples at sponge insertion, 35/45 (77.8%) at sponge removal, and 33/45 (73.3%) at 48 hours after sponge withdrawal. The number of Gram-negative bacteria was 4/45 (8.9%) of samples at sponge insertion, 22/45 (48.9%) at sponge removal, and 18/45 (40.0%) at 48 hours after sponge withdrawal. Irrespective of the groups and timing of sampling, 16 Gram-positive and 3 Gram-negative bacteria species were isolated. The most frequently isolated bacteria species were T. pyogenes (28.9%) and E. coli (46.7%) at sponge removal. For the experimental groups, the number of T. pyogenes was 4/15 (26.7%) for SUPER, 0/15 (0%) for ENRO, and 9/15 (60.0%) for CON at sponge removal. The number of E. coli was 11/15 (73.3%) for SUPER, 4/15 (26.7%) for ENRO, and 9/15 (40.0%) for CON at sponge removal.

Table 4. Distributions of vaginal bacteria on different sampling days according to different treatment strategies in nulliparous ewes

	SUPER			ENRO			CON		
	Days of sampling								
	0	7	9	0	7	9	0	7	9
Gram-positive bacteria									
Staphy scheleiferi	-	1/15	1/15	-	-	-	-	-	-
Enterococcus faecium	-	1/15	-	1/15	2/15	-	-	-	-
Enterococcus faecalis	-	-	-	-	1/15	1/15	-	-	-
Trueperella pyogenes	1/15	4/15	1/15	-	-	2/15	-	9/15	8/15
Streptecoccus acidominimus	-	1/15	-	-	2/15	2/15	-	-	-
Enterococcus durans	-	-	1/15	-	-	-	-	1/15	1/15
Staphylococcus xylosus	-	-	-	-	1/15	2/15	-	-	-
Streptecoccus uberis	1/15	2/15	1/15	1/15	2/15	1/15	-	1/15	1/15
Aerococcus viridans	-	-	-	-	-	-	-	1/15	-
Staphylococcus equorum	-	-	-	-	-	-	1/15	1/15	4/5
Staphylococcus kloosii	-	-	-	1/15	1/15	1/15	-	-	-
Streptecoccus pneumonie	-	-	-	-	1/15	1/15	-	-	-
Streptecoccus galloly ssp. galloly	-	1/15	-	1/15	1/15	1/15	-	-	-
Enterococcus hirae	-	-	1/15	-	-	-	-	-	-
Staphylococcus lentus	-	-	1/15	-	1/15	-	-	-	-
Staphylococcus gallinarum	-	-	-	-	-	-	-	-	1/15
Overall	2/15	10/15	6/15	4/15	12/15	11/15	1/15	13/15	15/15
Gram-negative bacteria									-
Escherichia coli	1/15	11/15	7/15	1/15	4/15	5/15	1/15	6/15	4/15
Proteus mirabilis	-	-	1/15	-	-	-	-	-	-
Alcaligenes faecalis	-	-	-	1/15	1/15	1/15	-	-	-
Overall	1/15	11/15	8/15	2/15	5/15	6/15	1/15	6/15	4/15

CON: Control; ENRO: Enrofloxacin; SUPER: *Lactobacillus plantarum* cell-free supernatant

Discussion and Conclusion

The vaginal discharge rate was 100% in nulliparous ewes as a response of vaginal mucosa to the presence of the sponge in this study. The increment of vaginal discharge rate could resulted from narrow vaginal lumen of nulliparous ewes[4]. Previous studies reported that vaginal discharge rate was varied from 98.5% to 100% after use of progesterone impregnated intravaginal sponge in multiparous ewes.[22,23] Local treatment with tetracycline and penicillin seems to be the widespread choice rather than enrofloxacin in previous studies.[12,13] However, enrofloxacin was reported as the most effective antibiotic to control the vaginitis due to high sensitivity to vaginal microorganism in a recent study.8 Although most of previous studies reported the treatment strategies of vaginitis with antibiotics, vaginal discharge score was not evaluated.[5,8,13,24,25]

The severity of vaginal discharge after sponge removal did not differ among groups in this study. Additionally, the bacterial count of vaginal flora was not different prior to sponge insertion among three groups. The presence of

sponge as a foreign body and bacterial load causes the infiltration of leucocytes into the vagina.[26] Similar to previous studies.[5,26] bacterial count significantly increased at sponge removal in all groups in the present study. The increment of bacterial counts at sponge removal was significantly lower in ENRO group than other groups. Besides, this benefit was consistent with decreased the odour of vaginal discharge in ENRO compared to other groups. Viñoles et al. (2011)[13] reported the decreased odour of sponges after spraying sponges with antibiotics. According to our observation, mucus accumulation was more fluid in ENRO and this effect may have resulted in a higher sponge weight in ENRO. Our results was consistent with the previous studies which recommended fluoroquinolones such as enrofloxacin[8] and ciprofloxacin[10,25] to control the vaginitis in ewes.

Several studies reported that original microbiota at sponge insertion was recovered at 48 h later after sponge removal.[2,5,27] Bacterial count decreased at 48 h later after sponge removal in all groups compared to that of sponge removal. However, bacterial count at 48 h later after sponge removal did not return similar values at sponge insertion in this study.[8,11] Bacterial count at 48 h later after sponge removal is significant, as ewes show oestrus at that time and they mate with rams.[28] Furthermore, overall oestrus detection rate was 46.7% and bacterial count at two days later after sponge removal was not changed depending on oestrus expression in all groups. Previous studies indicated that local vaginal conditions vary depending on not only different stages of the oestrus cycle but also vaginal bacterial species.[11,29]

The predominant bacteria species were T. pyogenes and E. coli in this study. These results are in agreement with those isolated bacteria related to vaginitis in ewes in previous studies.[11,13,29] Continual contamination of the sponge string by faeces, urine and the retention of intravaginal foreign bodies leads to higher isolation of T. pyogenes and E. coli in vaginal mucus.[13] These important pathogens cause the severe vaginal discharge, suppression of estradiol production.[29] When comparing the previous studies[4,28], the lower oestrus reponse could be resulted from the presence of T. pyogenes and E. coli in this study. Consistent with the our results, many studies reported that the mostly isolated dominant pathogen was Enterobacteriaceae at sponge removal.[8,9,12,26] The number of Enterobacteriaceae at sponge removal was lower in ENRO compared to that in CON in this study. Enrofloxacin inhibits the bacterial DNA gyrase and is more effective on Gram-negative bacteria.[8] The reduction in bacterial counts mainly could have resulted from the high sensitivity to enrofloxacin of vaginal bacteria in this study.[30] Antibiotic resistance and residue is a major challenge to control vaginitis in ewes.[15,25] For this reason, lactic acid bacteria or their cell-free supernatant was a plausible strategy to control vaginitis by preventing the high vaginal pH which is required for pathogenic bacteria to growth.[9] Lactic acid bacteria are well known as natural preservatives for their antimicrobial activity against pathogens in foods.31 Similarly, previous reports revealed the potential beneficial effects of lactic acid bacteria in cows with reproductive tract infections.[32,33]

Quereda et al. (2020)[9] firstly reported the efficacy of commercial probiotic bacteria mix of 60% Lactobacillus crispatus, 20% Lactobacillus brevis and 20% Lactobacillus gasseri on vaginitis in ewes. The authors reported that the use of lactic acid bacteria had no beneficial effect on bacterial counts and the number of Enterobacteriaceae. Consistent with previous study[9], the administration of cell-free supernatant of L. plantarum prior to sponge insertion did not change the bacterial count in this study. However, the use of cell-free supernatant significantly reduced the number of Enterobacteriaceae at sponge removal. In aggreement with previous report in cows[33], the use of cell-free supernatant of lactic acid bacteria as antimicrobial substances had a beneficial effect on Enterobacteriaceae in ewes that had vaginitis after using progesterone impregnated intravaginal sponge.

In conclusion, progesterone impregnated intravaginal sponge insertion leads to vaginal discharge and increased bacterial count in all ewes. The most frequent bacteria were T. pyogenes and E. coli. The addition of enrofloxacin into sponge reduced the total bacterial and Enterobacteriaceae count. Cell-free supernatant was effective for reducing the Enterobacteriaceae count. Normal vaginal flora composition partially recovered at 48 h after sponge removal in all groups. As an alternative strategy for the use of antibiotics, further studies are needed to evaluate the effectiveness of antimicrobial substances (cell-free supernatant) on vaginitis in ewes.

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Histological and Molecular Evaluation of Raw Meatball Products

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Abstract

Meat is an important source of protein with high biological value. Due to this importance, the production and consumption of meat and meat products such as minced meat, salami, sausage, meatballs, and roasted meat have been increasing rapidly in recent years. In order to earn more money, some companies sell animal meats that are not consumed by society by mixing them into meat products without considering the health, habits, ethical and cultural values of people. In this study, it is aimed to examine whether there are undesirable tissues and muscle tissue belonging to different species in meat products by making histological and molecular evaluations in raw meatball products offered for sale. Evaluations were made by taking samples of raw meatball products offered for sale by 6 different well-known supermarkets. In the results we obtained, it was observed that the integrity of the muscle tissue was not impaired in sample 1, but the presence of cartilage fragments in places. In sample 2, different sizes of tendon fragments and nerve fiber bundles were detected, with less muscle tissue. In sample number 3, it was observed that the integrity of the muscle tissue was not impaired and the spice pieces were excessive. In sample number 4, it was observed that the integrity of the muscle tissue was partially damaged. In sample 5, large tendon fragments and abundant connective tissue were detected. In sample 6, an image was detected, suggesting that it was caused by too much use of the additive. In the RT-PCR results, no tissue belonging to different species was found in any of the 6 samples we received. These results, in addition to being inappropriate in terms of public health and consumer rights, also appear as attempts to gain unfair financial gain.
Key words: Raw meatball, histology, Rt-Pcr.

Introduction

Meat has an important place among the nutrients of animal origin in terms of nutrition. First of all, meat is an important source of protein. Other than that, meat; contains a significant amount of lipids, mineral substances and, vitamins (A and B complex). After water, protein is the most important nutrient required for the body's growth, development, and protection from diseases. Because every living thing necessarily contains protein. While protein controls the water balance and acid-base balance of the organism, it also helps the formation of hormones. Another important task is to ensure the production of hemoglobin. Hemoglobin is the blood protein that carries oxygen to cells and takes up carbon dioxide.[1,2]

Meat protein is a protein with high biological value. The reason why the proteins in meat are of high quality is that they contain all of the exogenous amino acids necessary for human nutrition. The absorption of these proteins in the body is 97 – 98%. In other words, it is used almost completely in the body. Even if the person has taken the protein that should be taken daily with vegetable proteins, this does not mean that the protein needs of the body are met. Because vegetable proteins are poorer in terms of exogen-amino acids. The amino acids found in meat and their amounts are almost the same in the meat of all animal species.[2,3]

Generally; meat is defined as "edible animal tissues obtained from healthy animals (bovine, ovine, poultry, and

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aquatic animals) that have reached sufficient maturity in accordance with the technique". According to the Turkish Food Codex Meat Products Communiqué; it is possible to define meat as "all parts suitable for human consumption obtained from domestic ruminants, poultry, rabbits and pigs". [4] For meatball; it is a ready-to-cook red meat mixture or a cooked meat product prepared in various ways by adding the fats of the same animal, flavorings, and one or more of the other food ingredients, if desired, to the mixture of one or more minced bovine and ovine animals.[4]

Meat consumption in our country is incomparably lower than in developed countries (7 kg/p in 2020).[5] Protein deficiency brings with it developmental disorders and health problems. The reason for growth retardation problems in Turkey is the inability to be fed with protein-rich foods. Since 90% of brain development is completed by the age of 3, energy and protein deficiency also negatively affect mental development. After a certain age, it is necessary to take enough white meats together with lean red meats. However, children and young people should consume more red meat.[1]

Due to the importance of meat, the production and consumption of meat and meat products such as minced meat, salami, sausage, meatballs, hamburger meatballs, and roasting have been increasing in recent years. Although a significant increase has been achieved in the quality of these products thanks to the developing production technologies, there are significant problems in compliance with the standards and quality control. As a matter of fact, some manufacturers resort to various tricks in production in order to make more profit by reducing the production cost and use low-quality meats and internal organs with residues that should not be added to production, contrary to the Food Products Regulations. This situation not only leads to unfair competition among companies that make quality products following standards but also deceives consumers and threatens public health.[3,6]

Meat and meat products are highly priced due to animal production policies. Therefore, the demand of low-income consumers for cheap meat and meat products is increasing. Some companies, taking advantage of this opportunity, to earn more money, without considering people's habits, ethics, culture, and religious values, especially by obtaining animal meat, which is not consumed by society, in a very cheap way, mixed into meat products and offered for sale. Some problems arise with the introduction of such meats or meat products for consumption.[7]
Considering these reasons, it is necessary to distinguish the tissues or organs of different animal species added to meat products that may threaten public health. Especially in products such as meatballs, sausages, salami, etc. which are prepared from a mixture, the rate of cheating increases even more.[8]

To summarize the tricks made for this purpose;

- The use of animal meats that are not allowed to be used in meat products,
- The use of various internal organ parts in the production of meat products,
- Incorporation of meat product residues into new product dough,
- Addition of plant origin substances to meat products,
- The addition of chemicals that are not allowed to be used in meat products.

Apart from the muscle and fat tissue determined by the food product registrations, very different textures can be mixed into meat products. Undesirable but most frequently encountered and used textures; cartilage tissue, bone tissue, nervous tissue, large arteries and veins, salivary glands, internal organ parts (thymus, lung, etc.), mammary glands, hair.[8,9]

In our study, we aimed to examine the presence of undesirable tissue parts and the presence of muscle tissue of different animal species, by making histological evaluation technics and RT-PCR, in raw meatball products offered for sale by well-known supermarkets.

Materials and Methods

Histological evaluation and RT-PCR were performed on samples taken from meatball products ready for consumption (6 different supermarkets), offered for sale by well-known and preferred big supermarkets. All histological procedures were carried out in Bursa Uludağ University Faculty of Veterinary Medicine Histology-Embryology Laboratory. RT-PCR evaluations were carried out in Bursa Food and Feed Control Central Research Institute, Animal Products Department Laboratory.

Histological Analysis

To obtain a more homogeneous product, a mixture was made by combining the purchased raw meatballs (for each supermarket). Samples were taken from 5 different points of the meatball content (6x5=30 samples) and placed in the fixation solution. Buffered neutral formalin was used as a fixation solution. A routine histological procedure was applied to the samples, which were kept in fixation liquid for approximately 48 hours, and the tissues were embedded in paraffin. 5µ thick sections were taken from the obtained

paraffin blocks and adhered to the slide. Samples from different supermarkets were randomly numbered from 1 to 6. Crossman's Triple Staining was then applied to the tissues.[10]

Tissues whose histological procedure and staining was completed were evaluated using a Nikon Eclipse 80i microscope and photographs were taken. In the histological evaluation, the presence of undesirable tissues in meat products, therefore in meatball products, and the integrity of the existing muscle tissue were taken into consideration.
Real Time-PCR Analysis

RT-PCR technique was used in raw meatballs to determine whether there is a tissue belonging to a different species other than beef, as specified in the label information. After DNA isolation from the samples, purity and amount controls were made with a spectrophotometer. Species-specific commercial kits were used to compare the DNA isolates obtained and the manufacturer's recommended protocol was followed. Evaluation was made for five different species. Horse species identification (SNP Biotechnology, 301R-10-01, Ankara, Turkey), pig species identification (SNP Biotechnology, 302R-10-01, Ankara, Turkey), donkey identification (SNP Biotechnology, 303R-10-01, Ankara, Turkey), and chicken species identification (SNP Biotechnology, 312R-10-01, Ankara, Turkey).

Results

The presence of undesirable tissues was examined histologically, and the presence of tissues belonging to different animal species was examined by PCR technique, in raw meatball products offered for sale by 6 different well-known and preferred supermarkets.

Since the raw meatball products offered for sale are made from beef on the sales labels, evaluations have been made on animal meats such as chicken, which is not prohibited but should not be included in these products.

Histology

In sample No: 1, histologically, the integrity of the muscle tissue was not impaired, the general appearance was good, but the presence of cartilage pieces in places (Figure 1).

In sample No: 2, less muscle tissue amount, different sizes of tendon fragments, nerve fiber bundles, and some kind of food additives were detected (Figure 2). It was not possible to determine the types of food additives, histologically.

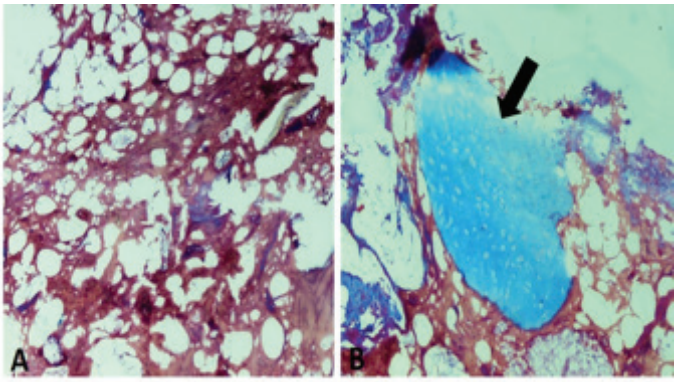

Figure 1. Histological section of sample No:1. (A) General view of muscle tissue. (B) big piece of cartilage tissue (arrow) (Triple Stain 100x).

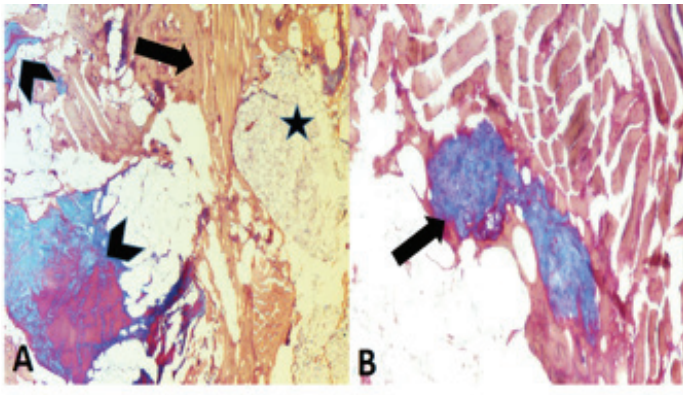

Figure 2. Histological section of sample No:2. (A)Muscle tissue (arrow), tendon pieces (arrowhead) and food additive (star). (B) Nerve fiber bundle (arrow) (Triple Stain 100x).

In sample No: 3, it was observed that the integrity of the muscle tissue was not impaired and the lots of spice pieces were excessive. Again in this sample, visceral parts that were suspected to be thymus or hemal nodes, but could not be determined histologically, were found (Figure 3).

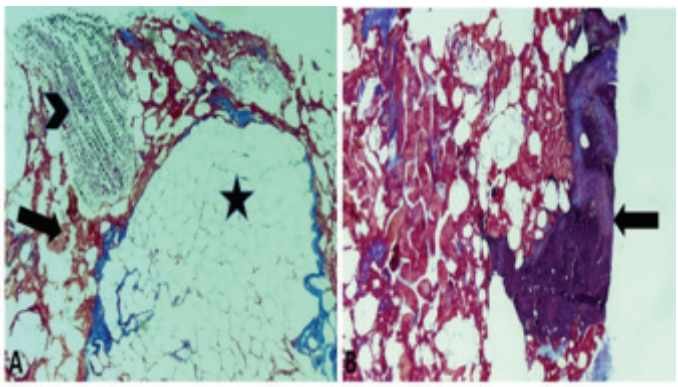

Figure 3. Histological section of sample No:3. (A) Muscle tissue (arrow), adipose tissue (star) and spice (arrowhead). (B) visceral part that was suspected to be thymus or hemal node (arrow) (Triple Stain 100x).

In sample No: 4, the integrity of the muscle tissue was partially damaged, nerve fiber bundles and large pieces of tendon were detected in large pieces of connective tissue (Figure 4).

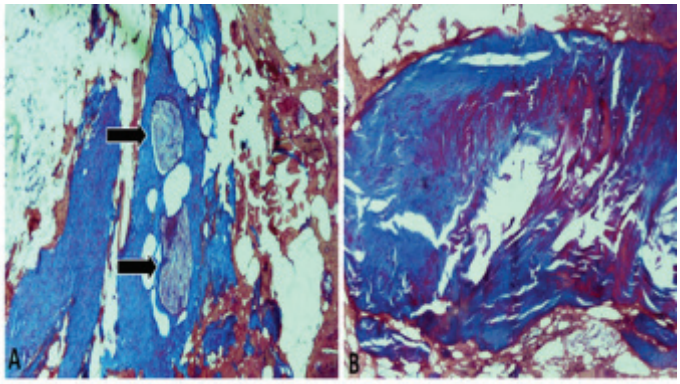

Figure 4. Histological section of sample No:4. (A) Nerve fiber bundles in a large connective tissue (arrow). (B) Large piece of a tendon (Triple Stain 100x).

In sample No: 5, large tendon fragments and abundant connective tissue were detected. In addition, the general appearance of the muscle tissue in this sample, as well as the staining intensity and quality, made us think that it might be an old-dated or expired product (Figure 5).

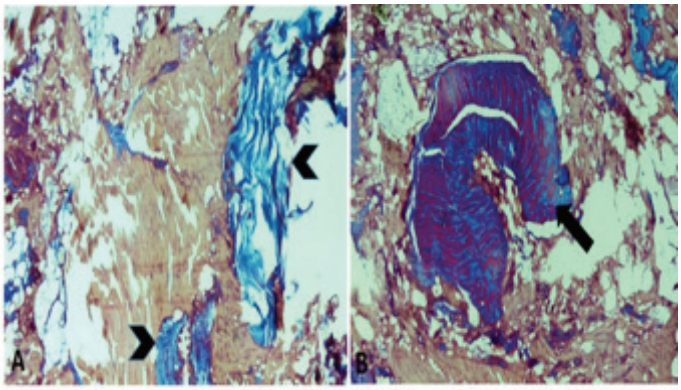

Figure 5. Histological section of sample No:5. (A) General view of muscle tissue and connective tissue pieces (arrowhead). (B) Piece of a tendon (arrow) (Triple Stain 100x).

In sample No: 6, we detected a result that we think is caused by using too much of the food additive(s). While the muscle tissue was seen in very small amounts, the additive(s) were detected in very large areas. It was not possible to determine the types of food additives, histologically (Figure 6).

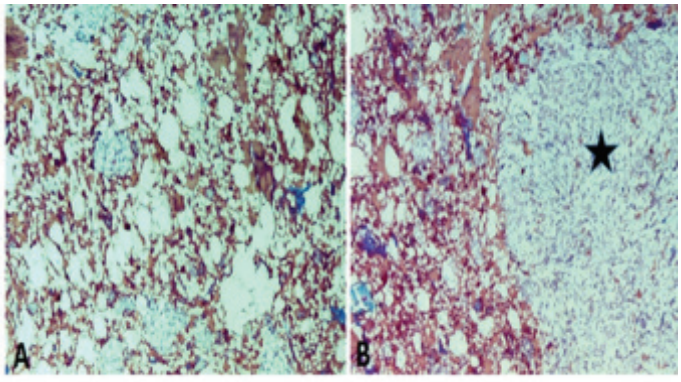

Figure 6. Histological section of sample No:6. (A) General view of muscle tissue. (B) Large amount of food additive (star) (Triple Stain 100x).

Real Time - PCR

It was written on the sales labels of the raw meatball packages that we used in our study that they were made from beef. As a result of RT-PCR performed in our samples to determine species identification, it was determined that there was no mixture of horse, pig, donkey and, chicken meat in any sample (Figure 7).

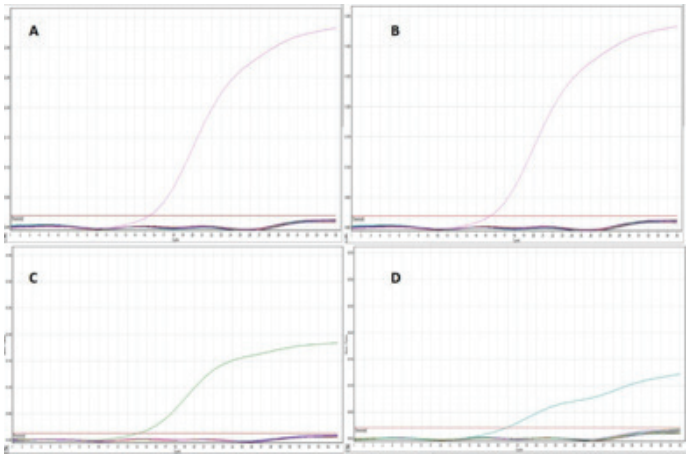

Figure 7. Readings registered by RT-PCR. (A) Horse DNA, (B) Pig DNA, (C) Donkey DNA, (D) Chicken DNA (detection limit 10-3).

Discussion and Conclusion

To determine the quality of meat and meat products, physical, chemical, microbiological, and histological evaluations can be made. In the histological evaluations, the appropriate method should be selected and evaluations should be made by considering the structural integrity and staining characteristics of the tissues and organs in the meat products. In our study, raw meatball products were evaluated using the paraffin embedding method, which is frequently used and known to have more positive results.[11]

Although the label information of the samples we received stated that they were completely beef, the fact that we encountered tissues and organ parts other than muscle tissue in some meatball samples makes us think that these samples were not prepared in accordance with the 'Meat and Meat Products Communiqué'.[4] The cartilage, tendon pieces, visceral pieces, large connective tissue pieces, and nerve fiber bundles we encountered reinforce this idea. In addition, the morphological structure and staining of the muscle tissue seen in sample No: 5, suggest that the meat quality is low or that the product has completed its shelf life. It is possible to see spices or additives (soy, etc.) in such samples (meatballs, sausage, salami, etc.).[4] However, in some samples, especially in sample No: 6, images suggesting that it was used in excessive amounts were encountered. It is not possible to reveal a numerical ratio histologically. However, in terms of being the subject of future research, it would be appropriate to evaluate chemical tests

and samples from this point of view as well.

Similar situations are encountered not only in meatball products but also in meat products such as salami and sausages. Epithelial tissue, seromucous gland epithelial cells, cartilage, and bone tissues were found in salami produced locally and bought from markets in Kars city.[12] In another study conducted in Konya city, tissue and visceral parts that should not normally be found were found in 18 of 48 salami samples.[13] Yıldız et al[14] reported that they found connective tissue pieces, inedible tissue, and offal pieces in meatball samples in another study they conducted in Istanbul city. Such samples can be increased especially for products such as salami and sausage. In our research, foreign tissues or visceral parts were found in all 6 samples, although at varying rates. Therefore, it is, unfortunately, possible to frequently see textures that should not be included in meat products, regardless of the type of product.

Another important issue in the evaluation of meat and meat products is which animal type muscle tissue is in the product. Although it is not possible to detect this with routine histological staining, it is possible to detect it with special immunologic staining, ELISA, or PCR method. For this purpose, species determination was made by the RT-PCR method in our study. It is a positive result that no tissue belonging to different species was found in any of the 6 samples we received.

Raw meatballs are among the preferred meat products due to their practicality. Although it is stated in the Meat and Meat Products Communique, cartilage, tendon, visceral parts, and similar tissues that are not suitable for human consumption and do not have nutritional value were found in the samples we examined histologically. These results, in addition to being inappropriate in terms of public health and consumer rights, also appear as attempts to gain unfair financial gain.

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RESEARCH ARTICLE

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Promising Effects of Vinasse Use on Bone Strength in Laying Hens

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Abstract

This study was carried out to determine the effects of vinasse on bone strength and some material properties in laying hens with specific amounts of vinasse added to their diets. The study was the first to determine the mechanical properties of laying hens fed a diet supplemented with vinasse. The study was conducted on tibiotarsi of two hundred 94-week-old laying hens which were selected randomly from a commercial farm. Morphometric measurements were made using a digital scale and caliper, and mechanical data were collected from tibiotarsi using a three-point bending test with a compression-tension machine. The laying hens fed with vinasse had higher tibiotarsal length and weight than those in Control group. The groups fed with vinasse were significantly stiffer and had a higher breaking force than Control group (P=0.002, P<0.001). In conclusion, significant and promising bone material properties were obtained from the laying hens fed with vinasse.

Keywords: Bone strength, Laying hens, Tibiotarsus, Vinasse.

Introduction

Intensive selection for rapid growth rate or high egg production in the poultry industry production systems has a negative effect on the musculoskeletal structures of broilers and layer hens.[1,2] Although the exact etiology of leg problems is unknown, weakness of bone tissue may exacerbate leg problems by increasing the tendency for bone deformation and fragility.[3] In laying hens, especially during the laying period, bone fractures are observed due to mineral weakness in the bone structure. This is also of economic importance, especially regarding commercially produced chickens.[4,5] Osteoporosis-related bone fractures caused severe welfare problems in laying hens. Although osteoporosis depends on genetic and environmental factors, the adverse effects of osteoporosis can be reduced or eliminated by improving nutritional conditions.[6,7] Modern hybrid laying hens have been subjected to special selection for maximum egg production. Those chickens produce one egg per day for a period of approximately 50 weeks, and bone stores meet the calcium requirement of the shell glands. This calcium requirement is compensated by the medullary bone, which is formed mainly during the laying period. In this period, only medullary bone is formed, while normal structural lamellar bone formation stops. Numerous active osteoclasts are involved in resorbing and mobilizing calcium from the medullary bone, and unfortunately, cortical and trabecular bones are also resorbed at this time. Especially in older chickens (>40 weeks), the risk of osteopenia and osteoporotic fractures increases.[8] Supplementing poultry rations with microbial cultures aids in the nutrient absorption of beneficial bacteria and maintains microbial balance in the poultry digestive tract. Therefore, probiotics and by-products are used to eliminate stress-induced abnormalities in the gastrointestinal tract, so intestinal activity continues normally. Some studies have reported that probiotics support intestinal microbial balance and may help improve bone development and growth at the same time.[9–11]

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Vinasse is a by-product from the industrial production of yeasts, alcohol, citric acid, or other substances by fermentation of molasses.[12–14] Vinasse has a lower sugar concentration and higher ash and crude protein content than molasses, as most sugar is consumed during fermentation.[15,16] Modified dry vinasse from the baker's yeast industry is rich in protein and energy.[16] The chemical composition of beta vinasse consists of 35% crude protein and 31% betaine.[17] In many countries, researchers have started using vinasse as a valuable protein source in animal nutrition instead of an expensive food product, soybean.[16] Vinasse is widely used in the diet of ruminant animals since it mainly contains dissolved yeast residues, abundant soluble protein-mostly non-protein nitrogen, plenty of vitamin B, and high mineral content.[18–20] The ratio of minerals such as iron, calcium, and phosphorus in the content of fermented vinasse is also convenient for animals.[14] Because of these properties, beta vinasse can be considered a good source of nutrients and minerals. Moreover, the mineral content of vinasse may play a crucial role in developing the musculoskeletal system[21], increasing bone strength, and preventing losses due to disorders related to the musculoskeletal system in laying hens. However, the effect of vinasse on bone strength in laying hens has not yet been discovered.

The aim of this study was to determine the effects of vinasse on bone strength and some material properties in laying hens with certain amounts of vinasse added to their diets. The study was the first to determine the mechanical properties of laying hens supplemented with vinasse.

Material and Methods

Animals and feeding

This study was carried out in the Department of Anatomy with the poultry unit of Animal Health and Animal Production Application and Research Center of Bursa Uludag University Faculty of Veterinary Medicine.

In this study, two hundred 94-week-old Nick Chick laying hens were used, which were selected randomly from a commercial farm. Laying hens were randomly divided into one control and three experimental groups, each of which had 50 laying hens. Each group was divided into 10 subgroups consisting of 5 layers (40 replicates). The study consisted of four groups fed with a corn- and soy-based basic diet (0% Vinasse) and 1.0%, 3.0%, and 6.0% Vinasse added to this basic diet, respectively.

The raw nutrient analyses of the feeds used in the research were carried out in the Laboratory of the Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, according to the methods reported in the Association of Official Analytical Chemists (AOAC).[22] Laying hen diet composition was calculated according to National Research Council (NRC).[23] Animals in the study were fed a basic ration containing an average of 16.50% crude protein and 2800 kcal/kg of metabolizable energy. Unlimited (ad libitum) food and water were provided, and the study was terminated on day 90.

After slaughtering the animals by cervical dislocation as recommended by the Bursa Uludag University Animal Experimentation Local Ethics Committee (Approval No: 87799839-325.04.02-E.5641), the tibiotarsus of the left leg of each animal was separated from the body. Bones were cleaned entirely from the surrounding tissues and individually wrapped in gauze soaked with 0.9% saline solution, packed, and stored at -20°C until morphometric and mechanical measurements.[24] For the morphometric and mechanical analysis, the bones were kept at +8°C overnight to be thawed. Then morphometric and mechanical measurements were made at least one hour after the bones were kept at room temperature. Tibiotarsi weighed using a digital scale (Precisa XB4200C, Precisa Instruments Ltd., Switzerland). The tibiotarsal length of each bone was measured from the proximal end to the distal end using a digital caliper (Mitutoyo CDN-20C, Mitutoyo Corp., Kawasaki, Japan).

Mechanical measurements

Tibiotarsi specimens were submitted to three-point bending tests with the low-strength material testing machine designed and manufactured by Tufekci et al.[25] (Figure 1). Force and corresponding displacement were measured by a load-cell (100 N, Tedea Huntleigh Malvern, USA) and a Linear Variable Differential Transformer (LVDT) (10-mm stroke, Novotechnik Tr10, Germany), respectively. In addition, an oscilloscope (Nicholet-Oddysey XE, USA) recorded force and displacement data at the rate of 50 data/sec. The length between supports was adjusted to 70 mm, and the load was applied at the mid-point of the diaphysis until the bones were broken with a continual loading speed of 10mm/min.

Statistical Analysis

Statistical analysis was performed by One-way Analysis of Variance using the Statistical Package for the Social Sciences (SPSS) (SPSS, Version 23.0; Chicago, IL, USA). Differences among the groups were analyzed using the Duncan's multiple-range test.

Probabilities (P) were considered significant at $P<0.05$.

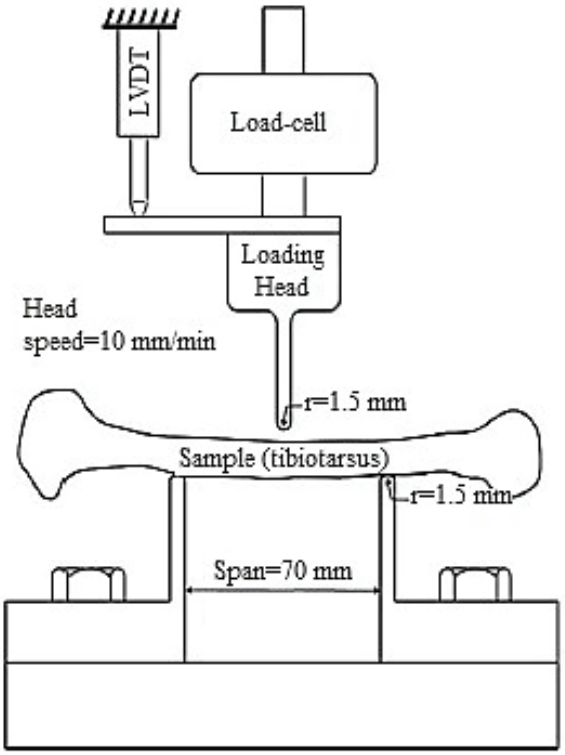

Figure 1. Schematic presentation of three-point bending test on tibiotarsus (modified from Suzer et al.26)

Results

The tibiotarsal length, weight and mechanical measurements of the laying hens were shown in Table 1. It was observed that vinasse had positive effects on bone weight and length in animals. The mean tibiotarsal length was 110.049±2.715 mm, and the tibiotarsal weight was 8.081±0.475 gr for Control group. Hens fed with vinasse had longer bone lengths than those not fed vinasse. The mean tibiotarsal length was 112.811±2.726 mm, and the tibiotarsal weight was 8.226±0.375 g for 1.0% Vinasse group. The mean tibiotarsal length was 113.946±2.513 mm, and the tibiotarsal weight was 8.724±0.701 g for 3.0% Vinasse group, and the length was 114.683±2.158 mm, and tibiotarsal weight was 8.507±0.444 g for 6.0% Vinasse group. It was observed that bone length and weight increased in direct proportion to the vinasse added to the diet. No significant differences were observed in terms of bone lengths between the groups fed with vinasse.

The three-point bending tests were performed on the bone samples, and stiffness was identified. The stiffness for the bones in Control group was 87.394±13.763 N/mm. Stiffness for the other groups was found 92.640±11.578 N/mm fed with 1.0% vinasse diet, 115.400±14.268 N/mm fed with 3.0% vinasse diet, 113.560±18.328 N/mm fed with 6.0% vinasse diet. It was seen that diets with vinasse added had a positive effect on bone stiffness (Figure 2). The average fracture force for those fed with a standard diet (0% Vinasse)

Table 1. The tibiotarsal length, weight and mechanical measurements in control and laying hens were fed different doses of vinasse.

	Breaking Deflection (mm)	Stiffness (N/mm)	Breaking Force (N)	Bone Length (mm)	Bone Weight (g)
Control	2.627±0.276^{a}	87.394±13.763^{a}	122.153±16.216^{a}	110.049±2.715^{a}	8.081±0.475^{a}
1% Vinasse	2.355±0.183^{ab}	92.640±11.578^{a}	143.082±19.213^{ab}	112.811±2.726^{ab}	8.226±0.375^{ab}
3% Vinasse	2.227±0.219^{b}	115.400±14.268^{b}	155.721±24.641^{b}	113.946±2.513^{b}	8.724±0.701^{b}
6% Vinasse	2.255±0.264^{b}	113.560±18.328^{b}	165.590±34.099^{b}	114.683±2.158^{b}	8.507±0.444^{ab}
P-value	0.005	0.002	>0.001	0.001	0.038

- Values in the table were presented as Mean ± Standard deviation of the mean.
- The mean significant difference level was 0.05.
- Different superscripts stated the significant differences between groups.

was 122.153±16.216 N. Forces were 143.082±19.213 N, 155.721±24.641 N, 165.590±34.099 N for groups fed with a 1.0%, 3.0% and 6.0% vinasse diet respectively and overall P values less than 0.001 were considered statistically significant. It was observed that Control group was statistically significantly more displaced, more ductile, and fractured at a lower force than the groups fed with vinasse (Figure 3). According to this result, it could be said that Control group had less compressive strength. It was observed that the groups fed with vinasse were significantly stiffer, and fractured at a higher strength than Control group. This showed that it had a higher compressive strength and tends to break without much deformation (bending).

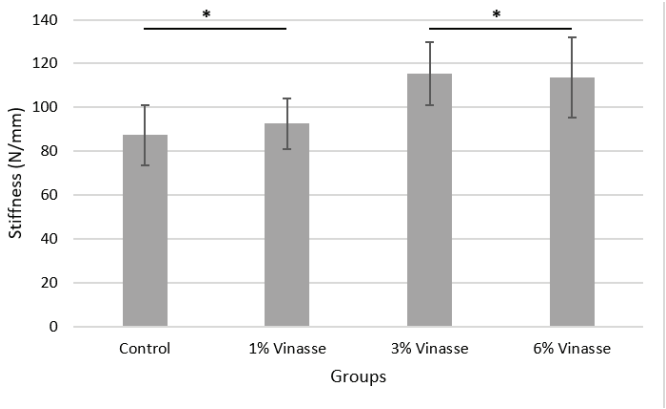

Figure 2. Comparison of stiffness in control and laying hens were fed different doses of vinasse.

* indicates the significant differences between groups.

In addition, no significant difference was found between the groups fed with different doses of vinasse in terms of breaking deflection, stiffness, and breaking force. However, it was observed that the amount of breaking force that the bone could carry without breaking tends to increase in direct proportion to the increase in the dose of vinasse.

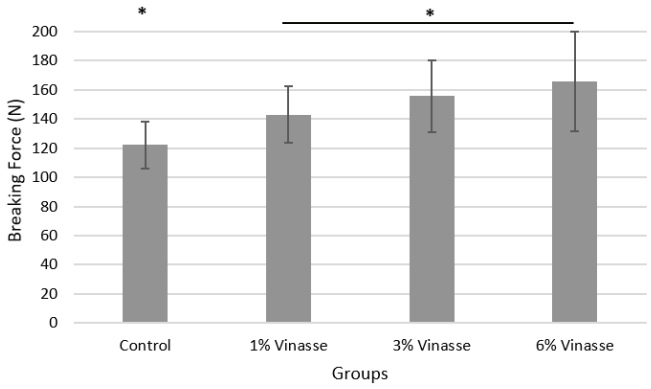

Figure 3. Comparison of breaking force in control and laying hens were fed different doses of vinasse.

* indicates the significant differences between groups.

Discussion and Conclusion

Vinasse obtained from molasses, a by-product of the sugar industry, is an important alternative for other protein feeds in the feed industry with its high betaine, mineral and crude protein content.[27] In the present study, when Control and groups fed with vinasse were compared, it was seen that the laying hens in the Control group had a shorter tibiotarsal length. Additionally, although there was significant difference between Control group and all experimental groups, no significant difference was observed in terms of bone lengths between the groups fed with vinasse. However, the bone length increased in direct proportion to the vinasse amount added to the diet, although it was not statistically significant (P>0.05). Similarly, Bilal et al.[27] reported that there were no significant changes in tibiotarsal length and diameter in broilers supplemented with 2.5% and 5% vinasse. Similar to the bone length results of our study, we found that tibial weight was higher in animals fed with vinasse compared to Control group. It was also observed that tibiotarsal bone weight increased in direct proportion to the vinasse-supplemented diet. However, there was no significant difference in terms of bone weight between the groups fed with vinasse.

In the present study, significant differences were found in breaking force between Control and vinasse groups. The present study showed that hens fed with vinasse appeared to have higher strength than those not fed vinasse. According to that result, it could be said that Control group had less bending strength. It was observed that the groups given vinasse were significantly stiffer and fractured at a higher force than Control group. This showed that vinasse groups had a higher breaking force and tended to break without much deformation. In addition, no significant difference was found between the groups given different doses of vinasse in terms of breaking deflection, stiffness and breaking force. However, it was observed that the amount of breaking force that the bone could carry without breaking tended to increase in direct proportion with the dose of vinasse.

In our literature search to date, we could not find a reference to compare our study data, as no bone strength publications were studied with vinasse. However, we thought that the positive effects of vinasse on bone strength were due to the high mineral and betaine content of vinasse. Consistent with this argument, Cetin et al.[17] reported that the chemical composition of beta vinasse consists of 31% betaine. Those researchers also mentioned that betaine caused an increase in mineral substance absorption in the intestines. Similarly, it was also reported that dietary betaine supplementation in laying quails might increase the digestibility of dry matter, crude protein, crude fiber, and crude ash, which positively affects the intestinal mineral absorption and digestive capacity of the intestinal epithelium.[28,29] Gerimipour et al.[21] also reported that vinasse contained betaine and the calcium concentration of vinasse was also high. The positive effects of vinasse on mineralization was observed by Cetin et al.[17] that egg shell thickness and shell breaking resistance increased in quails fed with the addition of vinasse compared to quails without vinasse, and it was reported that this positive effect may be due to the effect of betaine on the increase of mineral substance absorption in the intestines. Consistent with those researchers, Maidin et al.[30] reported that betaine supplemented diet reduced plasma homocysteine concentrations in blood and improved bone strength.

In conclusion, the present study was the first report measuring the effects of vinasse on some biomechanical traits of tibiotarsus in laying hens. Since tibiotarsal breaking strength is a good indicator and has a high correlation with other bone properties[31], we observed that there was a significant and promising results that the tibiotarsus had stronger and better properties in the laying hens fed with vinasse. Besides, the addition of dietary vinasse increased bone strength in laying hens, which could be used as an alternative feed additive to reduce welfare concerns related to leg problems in laying hens.

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Kars Bölgesinde Yetiştirilen Linda Irkı Kazlarda Nisan-Haziran Ayları Arasında Spermatolojik Verilerin Değerlendirilmesi

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Özet

Sunulan çalışmada Kars ilinde yetiştiriciliği gerçekleştirilen Linda ırkı kazlarda Nisan ve Haziran ayları arasında sezona bağlı spermatolojik değişimler izlenmiştir. Ülkemizde yoğun olarak Kars, Ardahan ve Muş illerinde yetiştiriciliği gerçekleştirilen kazların; genellikle etinden, tüyünden ve karaciğer gibi yan ürünlerinden faydalanılmaktadır. Kaz yetiştiriciliği karlı ve avantajlı olmasına rağmen, kazların sezona bağlı üreme faaliyetlerinde görülen düşüşler ise yetiştiriciliği kısıtlamaktadır. Etkin bir şekilde yardımcı üreme tekniklerinden faydalanılabilmek için, öncelikle mevsimin üreme üzerine etkilerinin incelenmesi gerekmektedir. Bu amaçla çalışmamızda üç yaşında beş kaz sperma alınması için materyal olarak kullanıldı. Kazlardan sperma, haftada iki kez olmak üzere dorso-abdominal masaj yöntemiyle alındı. Alınan taze sperma motilite yönünden incelendi. Taze sperma örneklerinin aylara göre ortalama motilite değerleri sırasıyla 16.1±5.48, 1.09±0.99, 0.0±0.0 olarak bulundu. Motilite değerlerinin Nisan ayından sonra aniden düştüğü görüldü ($P<0.05$). Mayıs ve Haziran ayları arasında ise istatistiki bir fark bulunmadı ($P>0.05$). Sonuç olarak Kars bölgesinde yetiştiriciliği yapılan Linda ırkı kazlarda, Nisan ayı itibariyle spermatolojik verilerin kalitesinde bir düşüş olduğu ve üreme döneminin gerilemeye girdiği tespit edildi. Kaz yetiştiriciliğinin hem bölgesel önemi hem de ihracat ürünü potansiyeli düşünüldüğünde, bu konu üzerinde daha derin çalışmalar gerçekleştirmesi gerekliliği ciddiyetle değerlendirilmelidir.

Anahtar sözcükler: Kaz, sperma, üreme.

Abstract

Evaluation of Spermatological Data in Linda Breed Geese Raised in Kars Region Between April and June

In the present study, seasonal spermatological changes were observed in Linda breed gander bred in Kars province between April and June. Of the geese, which are intensively bred in Kars, Ardahan and Muş provinces in Türkiye; Generally, meat, feathers and by-products such as liver are used. Although goose breeding is profitable and advantageous, decreases in seasonal breeding activities of geese restrict breeding. In order to benefit from assisted reproductive techniques effectively, first of all, the effects of the season on reproduction should be examined. For this purpose, in our study, five three-year-old ganders were used as material for semen collection. The semen from the gander was collected by dorso-abdominal massage method twice a week. Fresh semen taken were examined for motility. The mean motility values of fresh semen samples by months were found to be 16.1±5.48, 1.09±0.99 and 0.0±0.0, respectively. It was observed that the motility values suddenly decreased after April ($P<0.05$). There was no statistical difference between May and June ($P>0.05$). As a result, it was determined that there was a decrease in the quality of spermatological data in Linda breed geese bred in the Kars region as of April, and the reproductive period began to decline. Considering both the regional importance and export product potential of goose breeding, the necessity of carrying out more in-depth studies on this issue should be seriously evaluated.

Keywords: Gander, semen, reproduction.

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Giriş

Hayvan yetiştiriciliği, yeterli ve dengeli beslenmenin sağlanması yanında birçok ekonomik değeri olan yan ürün de sağlaması sebebiyle oldukça önemli bir konuma sahiptir. Kanatlı hayvan yetiştiriciliğine konu olan kaz yetiştiriciliği faaliyetleri; en başta etinden, tüyünden faydalanmak amacıyla ve karaciğer gibi diğer ürünleri sebebiyle gerçekleştirilmektedir[1]. Kaz yetiştiriciliği Çin başta olmak üzere pek çok ülkede önemli bir yere sahiptir[2].

Türkiye'de kaz yetiştiriciliği; Kars ili başta olmak üzere, Ardahan, Muş illerinde yoğun olarak görülmektedir[3,4]. Ancak ülkemizde kaz yetiştiriciliği modelinin genel olarak aile işletmeleri bazında kalması sebebiyle ülke genelinde profesyonel anlamda bir karşılığı tam olarak bulamamıştır[3].

Bütün iklim tiplerine kolayca uyum sağlayabilen kazlarda hastalıkların görülme sıklığı da düşük olarak bildirilmektedir[5,6]. Ancak ülkemiz kaz yetiştiriciliğinde görülen en önemli problemlerden birisi döllenmiş yumurta oranının azlığıdır[5]. Kazlar mevsime bağlı üreme özelliği göstermektedirler. Genel olarak; erkek kazlarda Aralık ayının sonu itibariyle üreme faaliyetleri gözlemlenmeye başlamakta ve Nisan ayında ise üreme sezonu bitmektedir[7,8]. Üremenin mevsime bağlı olmasına bağlı olarak, sürü genelinde görülen dölveriminin düşük olması kaz üretimini sınırlandırmaktadır[7].

Kazlarda suni tohumlama hindi ve tavuklarda olduğu kadar yaygın olmamakla birlikte dondurulmuş sperma diğer evcil hayvanlardaki gibi pratikte kullanılmamaktadır. Ayrıca, kaz gibi bazı kanatlı türlerinde dondurulmuş-çözdürülmüş sperma düşük cinsel aktivitenin olduğu durumlarda ve dönemlerde döllenmiş yumurta oranlarını artırmak için de kullanılmaktadır[7].

Sunulan bu çalışmada: Ülkemizde kaz üretimini sınırlandıran faktörlerin daha iyi anlaşılması amacıyla, Kars ilinde Nisan, Mayıs ve Haziran aylarında Linda ırkı kazlardaki spermatolojik değişimler incelenmiştir.

Materyal ve Metot

Hayvan Materyali

Bu çalışmada Kafkas Üniversitesi Veteriner Fakültesi Prof. Dr. Ali Rıza Aksoy Eğitim Araştırma ve Uygulama Çiftliğindeki Linda (Çin kazı X Emden) ırkı 3 yaşlı 5 adet kaz kullanılmıştır. (Etik Kurul No: KAÜ-HADYEK/2021-136)."

Spermanın Alınması

Alınacak spermada idrar ve dışkı partikülleri gibi kirleticilerin varlığını engellemek amacıyla, sperma alımından 8 saat önce kazların yem ve su tüketimi tamamen engellendi. Kazlardan sperma, dorso-abdominal masaj yöntemiyle haftada iki kez alındı. Masaj yapılacak kaz, arka kısmı öne bakacak şekilde koltuk altında tespit edildi ve kuyruğu sırtına doğru yatırıldı. Kazı tespit eden elin baş ve işaret parmakları kloakanın her iki tarafına yerleştirildi. Diğer elin baş ve işaret parmakları ile abdomenin dorsal kesimine hızlı ve sürekli şekilde masaj uygulandı. Kontaminasyonun önlenmesi amacıyla, sperma toplama kadehi fallusta pulzasyonların hissedilmeye başlanması ve ereksiyonun gerçekleşmesinden sonra yerleştirilerek sperma elde edildi[9].

Spermanın Muayenesi

Motilite muayenesi, ısıtıcı (37°C) tablalı faz-kontrast mikroskopta (Nikon Eclipse-E400, Tokyo, Japonya) x40'lık objektif ile değerlendirildi. Bu amaçla, ısıtıcı tabla üzerinde önceden ısıtılmış lam üzerine bir damla serum fizyolojik ve bir damla sperma örneği damlatılarak karıştırıldı ve üzeri lamel ile kapatılarak en az üç farklı alan incelenerek, sonuç % olarak değerlendirildi. Değerlendirmede hızlı ve güçlü bir şekilde, başı yönünde herhangi bir yöne doğru giden spermatozoonlar motil olarak kabul edildi[10].

Ölü-Canlı Spermatozoon Muayenesi

Ejekulattaki ölü spermatozoon oranının incelenmesi eozin-nigrozin boyama yöntemi ile yapıldı. Preperat hazırlığı için ilk önce, lam üzerine bir damla eozin ve bir damla sperma damlatılarak birbiri ile karıştırıldıktan sonra, karışıma bir damla nigrozin eklenip sürme froti yapıldı. Froti ısıtma tablasında kısa sürede kurutuldu. Nigrozin fon boyası üzerinde eozin boyayı alan kırmızı-mor renkteki hücreler ölü, boya almamış renksiz görülen spermatozoonlar canlı olarak değerlendirildi[11].

Hazırlanan frotiler x40 büyütmede 333 spermazoit sayılarak değerlendirildi. Sayılan toplam 333 hücredeki canlı spermatozoit sayısı 3 ile çarpılarak elde edilen miktar 10'a bölündü ve elde edilen sayı spermadaki % canlı spermatozoon oranı olarak kaydedildi.

Morfolojik Muayene

Spermadaki anormal spermatozoonların oranı da yine eozin-nigrozin boyama yöntemi ile hazırlanan aynı frotilerde saptandı. Hazırlanan preparatlar ışık mikroskobu ile x100 büyütmede incelendi. Toplam 333 hücre sayılarak total morfolojik bozukluk gösteren spermatozoitler % olarak hesaplandı[11].

İstatistiksel Analiz

Sonuçlar ortalama ± standart sapma olarak gösterildi. Normallik testi için Shapiro-Wilk testi kullanıldı. Sperma parametreleri Kruskal-Wallis testi kullanılarak analiz edildi ve gruplar arasındaki istatistiksel farklılıklar Mann-Whitney U testi ile belirlendi. Çalışmada elde edilen tüm veriler SPSS (Windows için SPSS 20.0; SPSS, Chicago, IL, ABD) kullanılarak analiz edildi. P<0.05 değeri istatistiksel olarak anlamlı kabul edildi.

Bulgular

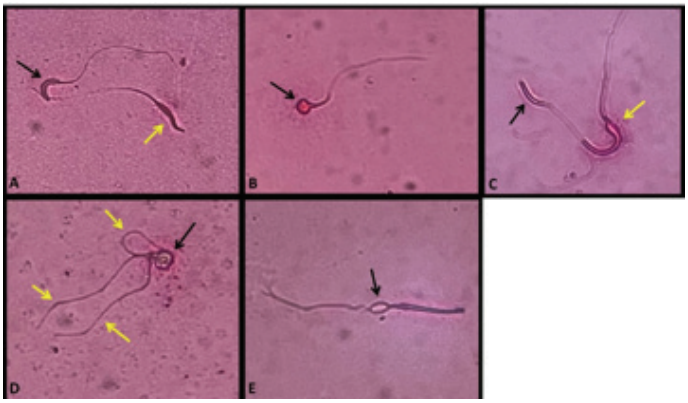
Çalışmada taze sperma örneklerinin aylara göre ortalama motilite değerleri (Tablo 1) sırasıyla 16.1±5.48, 1.09±0.99, 0.0±0.0 olarak bulundu. Nisan ayından sonra 5 hayvana ait ejakülatlarda, bir hayvana ait iki gün değerleri haricinde, speramatozoona rastlanılmamıştır. Motilitenin, Nisan ayından sonra sert bir şekilde düştüğü tespit edildi (P<0.05). Mayıs ve Haziran ayları arasında istatistiki bir fark bulunmadı (P>0.05).

Tablo 1: Farklı aylara göre motilite değerlerinin ortalaması

Sperma Alınan Ay	Motilite (%)
Nisan	16.1 ± 5.48^{a}
Mayıs	1.09 ± 0.99^{b}
Haziran	0.0 ± 0.0^{b}

Veriler ortalama ± S.S. olarak verilmiştir.

Farklı üst simgeler (a, b) istatistiksel farklılıkları göstermektedir (p<0.05).



Resim 1: Kaz spermasında ölü-canlı spermatozoa ve çeşitli morfolojik bozukluklar

Eozin boyama sonucu elde ettiğimiz ölü-canlı oranı ve morfolojik bozukluklara ait veriler Nisan ayına aittir (Resim 1). Sonraki aylarda alınan ejakülatlarda spermatozoona rastlanılmadığı için sonuçlar istatistiksel analiz gerçekleştirilmeden paylaşılmıştır (Tablo 2).

Tablo 2: Linda ırkı kaz spermasında Nisan ayında görülen canlı spermatozoa oranı ve morfolojik bozukluk oranları

	Minimum (%)	Maksimum (%)	Ortalama (%)	Standart Sapma
Canlı Spermatozoa Oranı	10,00	95,00	50,80	34,83
Baş Bozukluğu	1,00	10,00	4,00	2,96
Orta Kısım Bozukluğu	1,00	4,00	2,22	1,20
Kuyruk Bozukluğu	1,00	2,00	1,67	,50

Tartışma ve Sonuç

Kanatlı hayvan türlerinde, mevsime bağlı üreme özelliklerindeki değişiklikler kuşların hayatta kalma ve üreyebilmeleri açısından hayati öneme sahiptir[12,13,14]. Ülkemizde kaz yetiştiriciliğinin iyileştirilebilmesi amacıyla; kazların Kars ilinde Nisan ve Haziran aylarındaki değişimleri bir miktar daha derinlemesine incelediğimiz bu çalışmada, spermatolojik verilerin yaz aylarına doğru kötüleştiği görülmüştür. Gerçekleştirdiğimiz incelemelere göre ülkemizde bu anlamda bir çalışmaya rastlanılmamıştır.

1962 yılında Mehrota[8], Hindistan'da ve Anser Melanotus ırkı kazlarda yaptığı çalışmada epididimiste sezona bağlı meydana gelen değişimleri incelemiştir. Bu çalışmasındaki gözlemlerine göre üreme siklusunu 4 aşamaya bölmüştür. Bunlar; üreme dönemi (Ocak-Mart), gerileme dönemi (Nisan-Temmuz), refrakter dönem (Temmuz-Eylül) ve gelişme dönemi (Ekim-Aralık) olarak belirtilmiştir.

Zhuang ve ark.[14] Tayvan'da ve Germen ırkı kazlarda gerçekleştirdikleri çalışmada; üreme sezonu dışında (Temmuz) testis kütlesinin en düşük, üreme kabiliyetinin kazanılmaya başlanıldığı Aralık ayında yüksek olduğu, üreme sezonu olan Şubat ayında ise en yüksek testis kütlesinin gözlemlenildiği bildirilmiştir. Testis gelişimi ve spermatogenezis ile olumlu yönde bir korelasyon olduğu daha önceki çalışmalarda bildirilmiştir[14]. Bu bağlamda Zhuang ve ark.[14] sezona bağlı testis gelişimini değerlendirdikleri çalışmanın verileri ile araştırmamızdan elde edilen sonuçların uyumlu olduğu görülmektedir.

Gumulka ve Rozenboim[15]'in Polonya'da Şubat-Haziran ayları arasında gerçekleştirdikleri ve spermatolojik verileri de değerlendirdikleri çalışmada Nisan ve Mayıs aylarında motilite oranlarının önceki aylarla benzerlik taşıdığı (sırasıyla %62,6 ve % 62,7), Haziran ayında ise motilitenin düştüğünü bildirmişlerdir (%56,3). Araştırmamızda ise Kars ilinde Nisan ayında motilitenin daha düşük olması bu bölgede kazların bu ayda üreme anlamında gerileme dönemine girdiğini düşündürtmektedir. Gumulka ve Rozenboim[15] aynı çalışmada canlı spermatozoa oranını Nisan ayında %89,4 olarak, sağlam morfolojiye sahip spermatozoa oranını ise %44,9 olarak bildirmektedirler. Çalışmamızdaki ölü-canlı spermatozoa oranları ile farklılıkların yine sezona bağlı

olduğu düşünülmektedir ve morfolojik bozukluk oranları arasındaki farklılık ise kullanılan boyama yöntemlerinden kaynaklandığı düşünülmektedir.

Sonuç olarak Kars bölgesinde yetiştiriciliği yapılan Linda ırkı kazlarda, Nisan ayı itibariyle spermatolojik verilerin kalitesinde bir düşüş olduğu ve üreme döneminin gerileme dönemine girdiği görülmüştür. Bu bölgede kazlarda üreme sezonunun daha iyi anlaşılabilmesi için sene içerisinde daha detaylı analizlerin yapılması gerekmektedir.

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Ensuring the Reproduction of Gazelles, Whose Numbers are Decreasing in Türkiye and Whose Habitats are Confined to a Narrow Region, in New Habitats

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Abstract

The aim of this study was to resettle Gazella Marica, whose habitats are declining in Türkiye, to the foothills of Cudi Mountain, which is connected to the Silopi District of Şırnak Province, which was previously located within the natural habitat zone. The gazelles obtained from the 75th Year Gazelle Production Station were placed in individual crates of 100x36x90 cm made of plywood, with 51 numbers (24 females, 27 males) gazelles in 2020 and 40 numbers (19 females and 21 males) in 2021. Mass releases were made with a ceremony in an area with similar climatic characteristics, which is approximately 380 km away. 1 gazelle died in 2020, 6 gazelles died in 2021. During the post-release monitoring activities, the first reproduction records of gazelles released in 2020 were successfully recorded in 2021 and 23 new individuals were obtained. The current number of individuals reached 106. It has been observed that gazelles have adapted to the area in the 2 years. Illegal hunting is prevented as the region is within the borders of military security. Monitoring studies continue for the long-term management plan.

Key words: Gazella marica, habitat, reintroducing, breeding, observation

Introduction

Gazelles, which have a wide taxonomy and distribution area, are decreasing in number every day. Gazella Marica; It spreads from Yemen, Oman, Syria, Iraq, Saudi Arabia to Türkiye. Their number has been reported to be around 1750-2150 adults.[1] Two gazelle species have been reported in Türkiye. One of them is Gazella Marica, a subspecies of Goitered Gazelle, and the other is Gazella gazella, Hatay Mountain Gazelle.[2,3,4] At the beginning of the 20th century, it stretched from Çukurova to Eastern Anatolia (Iğdır and the Ararat Plains), but today, although its numbers are high in Şanlıurfa, it has remained in a narrow and limited area starting from Hatay and extending to Cizre along the southern border of Türkiye.[5,6] Gazella Marica females are hornless, males are horned. Adult females average 13.86 kg, males 19.39 kg. From the second birth of females, the rate of twin births is high according to the season and nutritional status.[7] The birth weight of the offspring is between 1.84 for females and 1.95 kg for males.[8] Birth seasons vary according to climate and species. Depending on the photoperiod, the mating season in our country is in the months of October-November-December, since it is located in the northern latitude. Births occur in late April and early May.[9,10] In the first weeks after birth, the cubs mostly hide under a stone or in the grass.[11] When baby gazellesstart walking and socializing, they gradually begin to communicate with their peers and later with the herd.[12] For gazelles grown indoors, alfalfa as roughage, black barley as concentrated feed, wheat, barley, superlac concentrate, etc. is given as adlibitum.[13] Diet in gazelles living wild in nature; They are the plants in the region and the season. The main forage sources are Chenopodiaceae, Poaceae plants belonging to the family wheatgrass. In addition, it consumes Ephedraceae, Tamariaceae, Zygopllaceae, Haloxylon, Ammodendron families.[14,15] Vegetation periods vary

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depending on the seasons of the plant species that it consumes. Since the plant species in each region is unique, the types of feed consumed by gazelles have a wide place. Since the plants it consumes are similar to other domesticated herbivorous mammals living in the region, the amount of feed consumed by gazelles during intensive grazing activities decreases, causing a lack of food, especially in winter.[14] For the first time, a hunting ban was enacted in 1957 for gazelles, whose numbers are decreasing day by day in Türkiye. [8,16] Despite the ban, the decline in numbers continued. Due to reasons such as the prevalence of illegal hunting, the opening of agricultural areas to settlement, the opening of pasture areas to agriculture, the living areas and numbers of gazelles have decreased. Due to these reasons, the natural habitats of gazelles have narrowed. In 2005, 86 Ceylan Wildlife Development Areas were released from the area near the Şanlıurfa Kızılkuyu village.[8] Within the borders of the same region (37˚02'N-38˚42'E), the 75th Gazelle Breeding Station with an area of 22.72 hectares surrounded by a wire fence was established. The purpose of the establishment of this station: Maintaining the existence of gazelles, increasing their production and releasing them to the Wildlife Development Area, leaving them to new settlement areas with old living areas, protecting cultural values, and bringing them to tourism.[17] Şanlıurfa Central Kızılkuyu Wildlife Development Area was declared on 5 October 2006 and its natural habitats were taken under protection.[18] IUCN[19] guidelines for reintroduction have been followed and efforts have been made to ensure that appropriate taxa of released gazelles are reintroduced and reintroduced in their former historical areas.

The aims of the study here: To ensure the resettlement of gazelles that had previously existed in the region. To examine the areas where the gazelles' biology spread and to protect its habitat in the area where the placement is made. Situations that may pose a threat should be identified and eliminated. To raise awareness of the people of the region. To contribute to ecotourism in the long-term process.

Material and methods

Study area and study group

Gazelles (37˚02'N-38˚42'E) were obtained from the 75. Year Gazelle Breeding Station, which consists of an area of 22.72 hectares surrounded by wire fences. The altitude varies between 545-600 meters. The average annual temperature is 18.5 °C (highest 46.8 °C lowest -12.4 °C) Annual precipitation average is 463.3 mm.20 Its location was measured using the Magellan explorist 610 model GPS and Google Eart. There are artificial water pools and feeders in both parcels for Gazelles in the Breeding Station. It is given once a day as 350 g of alfalfa and 250 g of black barley per animal. In 2020, 50 gazelles were released in the region whose first release area is located in the Üçağaç Village Gavita hamlet (37˚20'N-42˚22'E) of Şırnak Province Silopi District. In 2021, 40 gazelles were released in the area located in the Bestebelaka hamlet (37˚17'N-42˚23'E) of the second release Üçağaç Village. The average annual temperature is 15.1 C° (highest 40.4 C° lowest -14.5 C°) Annual precipitation average is 719.44 mm.[21] No predatory wildlife was observed in the region. There are no illegal hunting activities since the region is located within the military security borders.

The aim of this study is to ensure that Gazella Marica, which has a natural habitat along the borders of Şırnak province, will continue to exist in the region again. Before the release, feasibility studies were carried out in a 1-year period in terms of the climate of the region, the vegetation and the suitability of the area to be released, and then the placement processes started. The local people's ownership of the work and the government's support increased the chances of resettlement work.

Methods

All transplanted gazelles were obtained from the 75th year Gazelle Production Station. The gazelles were caught in a triangle trap of 2350 square meters, which was set up in an area of approximately 13 hectares of the Breeding Station, which consists of 2 parcels. Capture time We waited for the births to end and the offspring to reach a sufficient size in order to avoid possible abortions or death of the offspring. All inner trap wire walls are covered with a 10 cm thick sponge-covered tarpaulin and a net over the trap. The aim is to minimize trauma and death that will occur during capture. Due to the inclination of the station, a staff group of 15-20 people formed a parallel line from the highest peak of the station, and the animals were supplied to walk slowly from top to bottom, on the gazelles, and enter the trap correctly. This process was repeated several times. Two officers, who were hiding on the edge of the mouth open trap, closed the mouth of the trap with a net and wire wall with a sufficient number of animals entered, and the trapping processes started half an hour after the gazelles began to calm down. The gazelles in the trap were caught manually. Captures were made close to sunrise or sunset in the morning. This process was carried out in cool hours against the negativities that may occur from the heat. Captured gazelles were placed in wooden crates opened from both sides made of plywood 100x36x90 cm, 30-40 holes with 1 cm wide.[22,23] Individual ear tags were attached and recorded before all animals were caught and trans-

ferred to wooden crates. In the preventive drug application, only internal and external parasite applications were made. During loading, the gazelles were loaded onto the transport vehicle with their heads forward. The distance of approximately 380 km was reached in 5 hours. During the breaks, the condition of the animals was checked. All transplanted animals were kept together in the quarantine cage. The cage is 2 meters in height and has an area of 400 square meters, 20x20 meters wide, covered with a sponge canvas. The gazelles were given clover, black barley and water as forensic bitumen. There are natural water sources in the settlement area, and 3 more artificial water sources were built. Due to the steppe vegetation of the region, the need for additional feeding is done in the winter months.

Work plan 2020

First Transportation: The first transplant group consisting of 18 gazelles (11 male, 7 female) took place after the capture on 23.09.2020 in the afternoon. The release area was reached at night, and they were unloaded from the boxes and left in the quarantine cage prepared before release. It was detected that 1 female adult gazelle died after the transportation. (Photo 1), (Photo 2).

Photo 1

Photo 2

Second Transportation: 18 gazelles (8 males, 10 females) were caught close to sunrise on 25.09.2020 and transported on the same day. It is included in the quarantine cage. No deaths were observed during the transportation.

Third Transportation: 15 gazelles (8 males, 7 females) were caught close to sunrise on 28.09.2020 and transported on the same day. It is included in the quarantine cage. No deaths were observed during the transportation.

Release: All gazelles were kept in the same quarantine cage after transportation (Photo 1). In the region located in Üçağaç Village Gavita hamlet (37˚20'N-42˚22'E), headed by the Governor of Şırnak, on 29.09.2020, together with the Civil Administrative Chiefs, Şırnak University Rectorate, Şırnak Municipality, Ministry of Agriculture and Forestry III Under the responsibility of the Regional Directorate, the doors of the cage were opened with a ceremony 50 gazelles were released into their new habitats at the same time. The release process was successful (Photo 3).

Photo 3

Work plan 2021

First Transportation: 19 gazelles (10 males, 9 females) were caught close to sunrise on 05.10.2021 and transported on the same day. GPS is worn to 4 male gazelles. They were placed in a quarantine cage before release. No deaths were observed during transport.

Second Transportation: 15 gazelles (10 males and 5 females) were caught close to sunrise on 06.10.2021 and transported on the same day. They were placed in a quarantine cage before release. One adult male gazelle was found dead during transportation.

Third Transportation: 6 gazelles (1 male and 5 females) were caught near sunrise on 21.10.2021 2021 and transported on the same day. 1 male gazelle is worn with a GPS. They were placed in a quarantine cage before release. No deaths were observed during transportation.

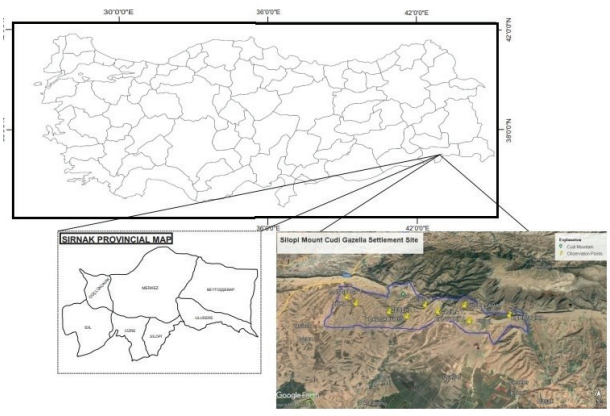
Release: Thanks to the successful release process in 2020, the same procedures (transfer, cage installation, release)

were carried out more practically and quickly in 2021.In the region located in the Bestebelaka hamlet of Üçağaç village (37˚17'N-42˚23'E), 40 gazelles on 07.10.2021, under the Presidency of the Governor of Şırnak, together with the Local Administrative Officers, under the responsibility of the Ministry of Agriculture and Forestry III. Regional Directorate, the doors of the cage were opened, and all the gazelles were released into the new living areas at the same time. The release processes were successful (Photo 4).

Results

Post-release tracking

In 2020, 1 female gazelle was found dead on 29.09.2021. In 2021, 6 gazelles (2 females and 4 males) died due to various reasons [1 (male) after being hit by a car, 2 (1 female 1 male) attack by a stray dog, 3 (2 male 1 female) gazelle found dead in the region].Monitoring studies were carried out using camera traps and focal animal observation.24 During the inventory work, it was observed from a distance with binoculars at 9 different locations by the same personnel between 06:30-08:30 in the morning and 16:00-18:00 in the afternoon. The width of the released area was approximately 6000 hectares, and the average distribution of the animals equipped with GPS was determined as 200 hectares. In the inventory studies, a total of 106 individuals were identified, of which 37 males 32 females 23 juveniles, 14 of which were uncertain. (Figure 1).(Table 1).



(Table 1:Some breeding characteristics in 2020 and 2021 of Gazella Marica reintroduced on the slopes of Mount Cudi in Şırnak's Silopi District in Türkiye.)

Breeding records of gazelles

	2020		2021	
	Male	Female	Male	Female
Transportation	27	24	21	19
Death		2	4	2
Newborn			23	

Births have been determined by observations made since May. In 11.12.2021, 9 observation points were made with 7 watchmen in an area of 5270.51 hectares, and as a result of the inventory study, 23 juvenile individuals were determined. There is a 10% margin of error in inventory studies.

Feed and Water source

It was observed that the freed gazelles consumed the grasses in nature, since the vegetation of the area where they were placed was steppe. Gazelles have a wide variety of diets as grass and shrub. 14,23 The presence of arable agricultural lands (grain forage crops, leguminous forage crops) and pastures within the borders of the area where they spread meets the feed requirement. Depending on the climate of the region, with the much rainfall in February - March and April the greening of the pastures is abundant with the compared to other months.21 Accordingly, it ensures that there is an abundant variety of plants. It provides access to abundant food at the beginning of the calving period. Supplementary feeding is done in times of snowfall in winter.25 Dry baled alfalfa (Medicago sativa) is given as feed. In addition to the region's natural water resources, 3 artificial water troughs were built at points far from the water source.

Discussion

In the resettlement study located in this study, the presence of animals of the same breed that previously existed in the region will facilitate the adaptation of the animal in the region. Compared to this study[26] a study for the same purpose was conducted. In order for the study to be successful, it should be investigated why the number of gazelles that previously existed in the region disappeared, and possible threats should be eliminated. The reintroduction of Gazella Marica in Şırnak province and its close follow-up over a two-year period will ensure that the management is successful and sustainable in long-term processes. When compared with the study[23] the number of animals sent to Şırnak province is higher, but the number of baby gazelles received is also higher. However, the spreading area is less.

On the other hand, it will make tracking the animals easier. Observation and follow-up performed by adequate and experienced personnel will provide more reliable data. Monitoring of animals attached with GPS will provide more information about their distribution within the region. Making a suitable grazing plan so that the rangelands it spreads will be productive, will ensure the continuity of the vegetation of the pastures. Raising the awareness of the local people and increasing the relations with the local people will have a great role in preserving the Ceylan existence. It has been suggested by the Governor's Office and the Ministry to increase the number of gazelles and to be a place where local and foreign tourists can visit.

Conclusion

No illegal hunting took place during the 2-year management period. The local people's adoption of the work done and their protection of the gazelle will increase the current population in a long-term process. In particular, Labor is regularly monitored by the Ministry of Agriculture and Forestry III. Regional Directorate and Şırnak Provincial Branch Directorate, and the studies are followed. Feeding studies and artificial water troughs are arranged according to the needs of the animals and their distribution areas.

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Yenidoğan Buzağı Ishal Olgularında Enterik Virusların (BRV, BCoV, BVDV, BToV) Çoklu Enfeksiyonu

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Özet

Neonatal dönemde en önemli mortalite sebebi olan buzağı ishalleri birçok faktöre bağlı olarak gelişmekte ve büyük ekonomik kayıplara sebep olmaktadır. Türkiye' de bugüne kadar yapılan epidemiyolojik çalışmalarda, viral etkenlerin yeni doğan buzağılarda önemli oranda neonatal ishal olgularına neden olduğu gösterilmiştir. Bu çalışmada şiddetli ishal ve neonatal ölümlerin görüldüğü buzağılarda çoklu viral enfeksiyonların rolü araştırıldı. Çalışma kapsamında tek bir işletmeden toplanan dışkı örnekleri (n=16) üç farklı hücre hattında (MDBK, HRT-18 ve MA-104) virus izolasyonuna alındı. Tüm örneklere 4 kör pasaj işlemi ve sonrasında IPMA testi uygulandı. Bu süreçte araştırılan enterik virusların (BRV, BCoV, BVDV, BToV) hücre kültüründe izolasyonu yapılamadı. Çalışma kapsamında dışkı örneklerinden PCR ve Ag ELISA testlerinin sonuçlarına göre, ishal semptomu gösteren 16 buzağıdan alınan dışkı örneklerinin 14 (%87,5) adedinde araştırılan enterik virusların en az bir tanesi tespit edildi. Araştırılan olgularda tekli BRV enfeksiyonuna rastlanmazken; 2 (%12,5) hayvanda tek BCoV, 2 (%12,5) hayvanda tek BVDV, 2 (% 12,5) hayvanda tek BToV enfeksiyonu saptandı. Diğer yandan 1 (%6,25) hayvanda BRV ve BVDV, 1 (%6,25) hayvanda BCoV ve BToV, 4 (%25) hayvanda BCoV ve BVDV, 1 (%6,25) hayvanda BVDV ve BToV içeren ikili enfeksiyonlar tespit edildi. 1 (%6,25) hayvanda ise BVDV, BToV ve BCoV olmak üzere enterik virusların oluşturduğu üçlü enfeksiyon saptandı. SDS-PAGE testinde akrilamid jel üzerinde RNA segmentlerinin bant profillerine göre (4/2/3/2) tespit edilen rotavirusun grup A'da yer aldığı gösterildi. Ayrıca çalışmada da PCR testi ile pozitif saptanan rotavirusun, G10P[11] genotipine sahip olduğu, genotip spesifik primerler kullanılarak tespit edildi. Bu araştırmada neonatal buzağı ishallerine sebep olabilen önemli viral etkenler olan BRV, BCoV, BVDV ve BToV' un aynı işletmede eş zamanlı olarak görülebileceği ve şiddetli hastalık bulguları ve kayıplara neden olabileceği gösterilmiştir.

Anahtar Kelimeler: Neonatal buzağı ishalleri, Çoklu viral enfeksiyon, Bovine Rotavirus (BRV), Bovine Coronavirus (BCoV), Bovine Viral Diarrhea Virus (BVDV), Bovine Torovirus (BToV)

Concurrent Infections of Enteric Viruses (BRV, Bcov, BVDV, Btov) in Calves with Neonatal Diarrhea

Abstract

Calf diarrhea, which develops in the neonatal period and is the most important cause of mortality, develops depending on many factors and causes significant economic losses all over the world. In the epidemiological studies conducted in Turkey to date, it has been shown that diarrhea often occurs after the calving period and causes great economic losses with growth retardation, treatment costs and deaths. Stool samples collected in the study were isolated in three different cell lines (MDBK, HRT-18 and MA-104), enteric viruses (BRV, BCoV, BVDV, BToV), which were intended to be obtained after four blind passages and IPMA test, could not be isolated in cell culture. According to the results of PCR and Ag ELISA tests from stool samples within the scope of the study, at least one of the investigated enteric viruses was detected in 14 (87.5%) stool samples taken from 16 calves with diarrhea symptoms. While no single BRV infection was found in the study; Single BCoV infection was detected in 2 (12.5%) calves, single BVDV infection in 2 (12.5%) calves, and single BToV infection in 2 (12.5%) calves. Dual infections of BRV and BVDV were detected in 1 (6.25%) calf, BCoV and BToV in 1 (6.25%) calf, BCoV and BVDV in 4 (25%) calves, and BVDV and BToV in 1 (6.25%) calf. A triple multible infection of enteric viruses, BVDV, BToV and BCoV, was detected in 1 (6.25%) calf. Rotavirus, which was detected according to the migration pattern of RNA segments (4/2/3/2) on acrylamide gel in the SDS-PAGE test, was shown to be in group A. In addition, in the study, it was determined by using genotype-specific primers that the rotavirus, which was detected positive by PCR test, had the G10P[11] genotype. In this study, it was shown that BRV, BCoV, BVDV and BToV, which are important viral agents that can cause neonatal calf diarrhea, can be seen simultaneously in the same farm and cause severe disease findings and losses.

Keywords: Neonatal calf diarrhea, Multible viral infections, Bovine Rotavirus (BRV), Bovine Coronavirus (BCoV), Bovine Viral Diarrhea Virus (BVDV), Bovine Torovirus (BToV)

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Giriş

Neonatal dönemin ilk 10 günlük döneminde en önemli mortalite sebebi olan buzağı ishalleri birçok enfeksiyöz ya da non-enfeksiyöz faktörlere bağlı olarak gelişmekte ve sığırcılık işletmeleri için önemli ekonomik kayıplara sebep olmaktadır [1]. Bu dönemde özellikle enfeksiyöz orijinli (bakteriyel, viral ve paraziter) ishaller ön plana çıkmakta ve bunlar arasında E. coli, Cryptosporidium, Rotavirus, Coronavirus, Bovine Viral Diarrhea ve Toroviruslar yer almaktadır[2–7]. Non-infeksiyöz sebeplere bağlı olarak gelişen ishaller ise alimenter faktörler, hazırlayıcı faktörler ve çevresel faktörleri kapsamaktadır [8]. Hastalıklara karşı savunma mekanizması gelişmemiş ve kolostrum almamış buzağılarda, bakım ve besleme şartları iyi düzeyde olsa dahi neonatal ishal nedenli kayıplar yaşanabilmektedir. Türkiye'de yılda yaklaşık 6 milyon buzağının doğduğu ve neonatal dönemde en az %15 oranında kayıp yaşandığı ön görülmektedir [9].

Buzağı ishallerinde sıklıkla karşılaşılan ve ilk olarak 1974 yılında elektron mikroskobu ile teşhis edilmiş olan *rotaviruslar* [10], *Reoviridae* ailesi içerisinde Rotavirus genusunda sınıflandırılan, zarfsız, kübik simetrili, çift iplikçikli ve 11 segmentli RNA genomuna sahip olan viruslardır. Viral genom 6 yapısal (VP1- 4, VP6, VP7) ve 6 yapısal olmayan protein (NSP1-6) olmak üzere toplam 12 protein kodlamaktadır [11]. VP6 gen bölgesinin genetik ve antijenik özelliklerine dayanarak *rotaviruslar* on gruba (tür) (A-J) ayrılmıştır [12]. Buzağı ishallerinde rotavirus A grubu ön planda yer almakla birlikte B ve C grubu *rotaviruslar* sığırlarda spontan olarak enfeksiyona neden olmaktadır [13]. Rotavirus suşları, dış kapsid proteinleri VP7 (glikoprotein, G) ve VP4 (proteaza duyarlı protein, P) genleri kullanılarak tiplendirilmektedir [14]. Genel olarak *rotaviruslar* 42 farklı G genotipi (G1-G42) ve 58 farklı P genotipine (P[1]-P[58]) sahiptir [15]. Saha çalışmaları sırasında tespit edilen sığır rotavirus genotiplerinin büyük çoğunluğu G6, G10 [11] ve daha az yaygın olan G8 olarak tespit edilmiştir [16]. Bu genotipler genellikle P[5], P[11] ve daha az sıklıkla P[1] ile ilişkilendirilmiştir [17].

İlk olarak 1970' lerde yenidoğan buzağılar arasında diyare salgını sırasında tanımlanmış ve bugüne kadar birçok ülkeden bildirimi yapılmış olan coronaviruslar, *Nidovirales* dizininde, *Coronaviridae* ailesinde, *Coronavirinae* alt ailesinde, *Betacoronavirus* genusunda yer almaktadır [18]. Zarflı, helikal simetrili, pozitif polariteli olan RNA viruslarıdır. Viral genom nükleokapsit (N) proteini, transmembran (M) glikoproteini, spike (S) glikoproteini, envelope (E) proteini ve hemaglütinin-esteraz (HE) glikoproteini olmak üzere beş adet yapısal protein kodlamaktadır. S glikoproteini S1 ve S2 olmak üzere iki alt birimden oluşmaktadır ve değişken bölge olduğu için coronavirusların genetik değişimlerinin tespitinde kullanılmaktadır [19].

BVD virusu ilk olarak 1946 yılında New York şehrinde sığırların yeni bir virusu olarak tespit edilmiş olup, hastalığın ekonomik öneminden dolayı birçok ülke kontrol ve eradikasyon programları uygulamaktadır [20]. Multisistemik bir enfeksiyona neden olan etkenin, buzağılarda ishale sebep olduğu yapılan çalışmalarda gösterilmiştir [4] . *Flaviviridae* ailesinde, *Pestivirus* genusunda yer alan BVD virusu pozitif anlamlı, 12.5 kb ve lineer tek iplikçikli RNA genomuna sahip olup; virion zarflı ve ikozahedral simetrilidir [21]. Genomda bulunan genler 5'→ 3' yönünde sırasıyla; N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A ve NS5B ' dir. BVDV-1 ve BVDV-2 genotipleri taksonomik ve epidemiyolojik çalışmalarda sıklıkla kullanılan 5'UTR bölgesine göre ayrılmış olup, yapılan filogenetik analizler sonucunda BVDV-1 türünün 21 (1a-1u) ve BVDV-2 türünün 4 (2a-2d) alt grubu olduğu tespit edilmiştir. Aynı zamanda, her iki virus türe de sitopatojen (cp) ve sitopatojen olmayan biyotipleri (ncp) barındırmaktadır [4,22].

Sığır torovirusların (BToV) ilk olarak ishalli buzağılarda 1979 yılında Amerika Birleşik Devletlerinde tanımlanmıştır [5]. BToV, *Nidovirales* dizininde, *Coronaviridae* ailesinde, *Torovirinae* alt ailesinde tanımlanan zarflı, tek sarmallı, pozitif polariteli bir RNA virusudur. 25-30 kb uzunluğunda olan viral genomdan iki adet yapısal olmayan protein (ORF1a ve ORF1ab) ve dört adet yapısal protein (spike (S), membran (M), hemaglutinin-esteraz (HE) ve nükleokapsid (N)) kodlanmaktadır [1]. M ve S hedef gen bölgelerine göre BToV' ların filogenetik çalışmaları bulunmaktadır [1,5].

Ülkemizde yenidoğan buzağı kayıplarında ishal kaynaklı mortalite oranları bölgelere ve çiftliklere göre değişmektedir ayrıca koruyucu veteriner hekimlik hizmetlerinin uygulandığı çiftliklerde dahi yüksek olduğu tespit edilmiştir [23–25]. Bu çalışmada bir sığırcılık işletmesinde yenidoğan buzağılarda ishale sebep olan önemli enterik viral etkenlerin (BRV, BCoV, BVDV ve BToV) birlikte veya eş zamanlı olarak enfeksiyon oluşturma durumları araştırılmıştır.

Materyal ve Metot

Örneklerin toplanması

Araştırmaya konu olan vakalar 2021 yılı Kasım ayında Edirne' de 40 tanesi ishal semptomu gösteren, 60 günlükten küçük 250 buzağı bulunan entansif bir süt işletmesinde önlenemeyen, tedaviye sınırlı düzeyde cevap veren ve ölümle

sonuçlanan neonatal buzağı ishal olgularını kapsamaktadır. Doğumdan 2 gün sonra ishalin başladığı işletmede, bir ay içerisinde 21 buzağı ölmüştür. İşletme yönetim sistemi dahilinde anaç sığır popülasyonunda BVD, BoHV-1, rotavirus ve coronavirus enfeksiyonlarına karşı düzenli aşılama uygulandığı teyid edilmiştir. Olgulara ilişkin örnekleme çalışmaları işletme veteriner hekimi tarafından yürütülmüştür. Bu kapsamda ishal bulgusu gösteren toplam 16 buzağıdan rektal yolla bireysel dışkı örnekleri alınarak teşhis amacıyla steril plastik kaplar içerisinde ve soğuk zincir altında laboratuvara ulaştırılmıştır. Örneklenen tüm buzağıların doğum sonrası 1 aylık dönemde olduğu ve işletmede kolostrum yönetiminin titizlikle takip edildiği kaydedildi.

İnokulum hazırlığı

Dışkı örnekleri 100 IU/ml penicilin ve 100µ/ml streptomisin içeren steril PBS ile 1:10 oranında sulandırılarak homojenize edildi ve 3000 rpm devirde 30 dakika süreyle santrifuj edildi. Santrifüj sonrası ayrılan süpernatant 0.45 µm ve 0.22µm por çaplı enjektör filtrelerden süzüldü. Elde edilen süzüntü test edileceği zaman kullanılmak üzere -70 °C' de muhafaza edildi.

Ag ELISA testi

Toplanan 16 adet ishalli buzağı dışkı örneğinden antijen tespiti için BRV (BioX Diagnostic BIO K 343/2), BCoV (BioX Diagnostic BIO K 392/2) ve BVDV (IDEXX BVDV Ag/Serum Plus Test) ticari Ag ELISA kitleri kullanıldı. Kullanılan kitlerdeki test protokolü üretici firmaların önerdiği şekilde uygulandı. Test pleytleri ELISA okuyucuda (Thermo-Multiskan EX, Finlandiya) 450 nm dalga boyunda okutularak sonuçlar değerlendirildi.

Hücre hattı ve virus izolasyonu

MDBK ve HRT-18 hücre hatlarının hazırlama aşamasında %10 fötal dana serumu (FDS) ilave edilen Dulbecco's Modified Eagle Medium (DMEM) (Sigma,) kullanıldı. MA-104 hücre kültürünün hazırlanması aşamasında ise %10 fötal dana serumu (FDS) ilave edilen Glasgow Minimum Essential Medium (GMEM) kullanıldı. Ayrıca hücre kültürlerinde olası bakteri ve mantar kontaminasyonunu önlemek amacıyla vasatlara 100 UI/ml Penisilin/Streptomisin ve 250 µl/ml Amfoterisin B solüsyonu eklendi. Hücre kültürleri ve FDS kullanım öncesinde RT-PCR ve immunoperoksidaz yöntemleriyle test edilerek *Pestivirus* kontaminasyonundan ari olduğu gösterildi. Ayrıca söz konusu hücre kültürleri mikoplasma kontaminasyonu yönünden periyodik olarak test edildi.

African Green Monkey Kidney (MA-104) hücre hattında virus izolasyon çalışmaları

Rotavirus izolasyonu amacıyla MA-104 hücre hattından hazırlanan 24 gözlü hücre kültür pleytlerinin her gözüne 100.000 hücre/ml oranında olacak şekilde MA-104 hücre kültürü süspansiyonundan 1 ml konuldu. Ertesi gün pleyt yüzeyinin >%80' ini kaplayan hücre tabakasının üzerindeki üst sıvı uzaklaştırıldı. Pleyt yüzeyini kaplayan hücrelerin üzeri FDS içermeyen GMEM ile yıkandı. Daha önce hazırlanmış olan stok pankreatin (100µl/ml) çözeltisi ve GMEM ile %10 oranında pankreatin içeren virus üretme vasatı hazırlandı ve MA-104 hücre monolayerinin bulunduğu pleyt gözlerine, hücre yüzeyini kaplayacak kadar ilave edildi. Pleytler 37°C'de, %5 CO_2 ihtiva eden inkübatörde 30 dk inkübasyona bırakıldı. Eş zamanlı olarak ekimi yapılacak inokulumlar %10 oranında pankreatin (100µl/ml) ile muamele edildi ve 37°C'de, %5 CO_2 ihtiva eden inkübatörde 30 dk inkübasyona bırakıldı. İnkübasyon sonrası her örnekten bir pleyt gözüne 200 µl inokulum eklenerek tekrar 37°C' de, %5 CO_2 ihtiva eden inkübatörde 1 saat inkübasyona bırakıldı. Süre sonunda içerik uzaklaştırılarak her göze 1 ml, %10 oranında pankreatin içeren virus üretme vasatı ilave edildi ve 37°C'de, %5 CO_2 ihtiva eden inkübatöre kaldırıldı. Ekim yapılan kültürlere iki günde bir % 10 oranında pankreatin ilave edildi. Pleytler 6 gün boyunca hücre morfolojisindeki sitopatolojik değişimleri saptayabilmek için invert mikroskop yardımıyla incelendi. Altı günün sonunda pleytler -80°C' ye kaldırılarak dondurulduve daha sonra 37°C' de çözdürüldü. Söz konusu işlem inokule edilen inokulumların seri pasajlanması için üst sıvıların kullanılması yoluyla üç defa daha tekrarlanarak toplam dört kör pasaj işlemi yapıldı.

Madin Darby Bovine Kidney (MDBK) ve Human Rectal Tumor (HRT-18) hücre hatlarında virus izolasyon çalışmaları

MDBK ve HRT-18 hücre hatlarından hazırlanan farklı 24 gözlü hücre kültür pleytlerinin her gözüne 100.000 hücre/ml oranında olacak şekilde MDBK ve HRT-18 hücre kültürü süspansiyonundan 1 ml konuldu. Ertesi gün pleyt yüzeyinin >%80' ini kaplayan hücre tabakasının üzerindeki üst sıvı uzaklaştırıldı ve pleyt gözlerinin yüzeyini kaplayan hücrelerin üzeri FDS içermeyen DMEM ile yıkandı. Olası enterik virusların izolasyonu amacıyla, dışkı örneklerinden hazırlanan inokulumlar adsorbsiyona bağlı virus inokulasyonu yöntemiyle hücre hatlarına inokule edildi. Bu amaçla her örnekten pleytin bir gözüne 200 µl inokule edilerek 37°C' deki, %5 CO_2 ihtiva eden inkübatörde bir saat inkübasyona bırakıldı. Süre sonunda test gözlerinden içerik uzaklaştırılarak 1 ml DMEM ilave edildi ve tekrar 37°C'deki, %5 CO_2 ihtiva eden inkübatöre kaldırıldı. Pleytler 6 gün

boyunca hücre morfolojisindeki sitopatolojik değişimleri saptamak amacıyla invert mikroskop yardımıyla incelendi. Altı günün sonunda pleytler -80°C' ye kaldırılarak donduruldu ve daha sonra 37°C' de çözdürüldü. Söz konusu işlem inokule edilen inokulumların seri pasajlanması için üst sıvıların kullanılması yoluyla üç defa daha tekrarlanarak toplam dört kör pasaj işlemi yapıldı.

İmmunoperoksidaz Monolayer Assay (IPMA)

Test protokolü daha önce yapılmış bir çalışmada belirtildiği şekilde gerçekleştirildi [26]. Nonsitopatojen karakterdeki virus üremelerinin teyidi için 100,000 hücre/ml oranındaki MDBK hücre süspansiyonundan 24 gözlü makro pleytlerdeki (Corning, ABD) her bir göze 1 ml aktarıldı. Bir gün inkübe edildikten sonra daha önceden kör pasaj işlemleri yapılmış olan örneklerden her bir göze 100 µl inokule edildi. Ardından 37°C'lik %5 CO_2'li etüvde 3 gün süreyle inkübasyona bırakılan pleytler 80°C'ye ayarlanmış etüvde 3 saat boyunca fikzasyona tâbi tutuldu. Ardından tüm gözler 1 kez PBS ile yıkandı. Her göze 200 µl olmak üzere Tween-PBS (1x PBS, %0.05 Tween 20 Merck, 822184) içerinde 1:300 oranında sulandırması yapılan monoklonal antikor (mAb 1/4/7) eklenerek 90 dakika 37°C'de inkübasyona bırakıldı. Aynı koşullar altında sırasıyla 1:300'luk sulandırması yapılan anti-fare konjugatı (Pierce, 31800, ABD) eklendi. İnkübasyon ve tüm gözleri 4 kez PBS ile yıkamayı takiben 1:400'luk sulandırması yapılan strepto-avidinle işaretli HRPO konjugatı (Pierce, 21124, ABD) eklendi ve 90 dk inkübe edildi. Süre sonunda 4 kez PBS ile yıkama yapıldıktan sonra substrat solüsyonu (%0,05 H_2O_2, 2 mg AEC (Sigma, A5754, ABD) / 3 ml di-methyl-formamid (Sigma-Aldrich, 40255, ABD), 4,7 ml Na asetat buffer) ilave edildi. Sonuçlar, substrat ilavesini takip eden 30 dakika içerisinde invert mikroskopta (Nikon Eclipse, TS100, Japonya) kırmızı kahverengi hücre içi boyanma görülmesine göre değerlendirildi.

PCR için nükleik asit izolasyonu ve cDNA eldesi

Viral nükleik asit ekstraksiyon işlemi ticari olarak temin edilen nükleik asit izolasyon kitiyle (Macherey-Nagel Nucleospin Virus, Almanya) ve üretici firmanın önerdiği şekilde uygulandı. Takip edilen protokolle elde edilen yaklaşık 30 µl hacmindeki nükleik asit PCR aşamasına kadar -80°C sıcaklıktaki derin dondurucuda saklandı. cDNA elde etmek amacıyla ticari olarak temin edilen cDNA kiti (Biorad, 170-8891), üretici firmanın önerdiği şekilde gerçekleştirildi. Reaksiyon sürecinde 42°C' de 30 dk ve 85°C' de 5 dk sıcaklık uygulandı.

Polimeraz Zincir Reaksiyonu

BRV RT-PCR protokolü

BRV için PCR karışımı toplamda 25 µl olacak şekilde 12,5 µl Maxima Hot Start Green PCR Master Mix (2x), 7,5 µl nükleaz ari su, 1 µl VP6-F forward primer (50 pmol) ve 1 µl VP6-R reverse primer (50 pmol) (Tablo 1) ve 3 µl cDNA eklenerek hazırlandı. Elde edilen PCR karışımı 95°C' de 4 dk ön denatürasyon; 35 siklus (95°C 1dk, 58°C 1dk sn, 72°C 2 dk) ve 72°C' de 10 dk son uzama olacak şekilde thermal-cycler programına tâbi tutuldu. BRV RT-PCR da pozitif sonuç veren örneğin tiplendirmesi amacıyla genotip spesifik RT-PCR uygulandı [27].

Tablo1. Çalışmada kullanılan PCR primerleri

Etken	Hedef Gen Bölgesi	Primerler	Pozisyon	Dizin (5'-3')	Beklenen ürün büyüklüğü	Kaynak
BRV	VP6	VP6-F	747-766	GACGGVGCRACTACATGGT	379	27
		VP6-R	1126-1106	GTCCAATTCATNCCTGGTGG		
	G Genotiplendirme	Beg9-F	1-28	GGCTTTAAAAGAGAGAATTTCCGTCTGG	715	16
		G(10)-R	715-697	TTCAGCCGTTGCGACTTC		
	P Genotiplendirme	Pgen-F	1064-1085	TTCATTATTGGGACGATTCACA	335	
		P[11]-R	1398-1377	TGCCTCATAATATTGTTGGTCT		
Pestivirus	5'UTR	p-324	108-128	ATGCCCWTAGTAGGCTAGCA	288	28
		p-326	395-375	TCAACTCCATGTGCCATGTAC		
BCoV	N	F1	21-40	GCAATCCAGTAGTAGAGCGT	730	29
		R1	731-750	CTTAGTGGCATCCTTGCCAA		
		F2	79-98	GCCGATCAGTCCGACCAATG	407	
		R2	467-485	AGAATGTCAGCCGGGGTAG		
BToV	M	F	98-700	TTCTTACTACACTTTTTGGA	603	30
		R		ACTCAAACTTAACACTAGAC		
		nF	152-560	TATGTACTATGTTTCCAGCT	409	
		nR		CCAACACAAATCCGCAACGC		

G(10) içi PCR karışımı toplamda 25 µl olacak şekilde 12,5 µl Maxima Hot Start Green PCR Master Mix (2x), 9,5 µl nükleaz ari su, 1 µl Beg9-F forward primer (10 pmol) ve 1 µl G(10)-R reverse primer (10 pmol) (Tablo 1) ve 1 µl DNA eklenerek hazırladı. Elde edilen PCR karışımı 95°C' de 5 dk ön denatürasyon; 40 siklus (94°C 1dk, 55°C 1dk, 72°C 2 dk) ve 72°C' de 7 dk son uzama olacak şekilde thermal-cycler programına tâbi tutuldu [16].

P[11] için PCR karışımı toplamda 25 µl olacak şekilde 12,5 µl Maxima Hot Start Green PCR Master Mix (2x), 9,5 µl nükleaz ari su, 1 µl Pgen-F forward primer (10 pmol), 1 µl P[11]-R reverse primer (10 pmol) (Tablo 1) ve 1 µl DNA eklenerek hazırladı. Elde edilen PCR karışımı 95°C' de 5 dk ön denatürasyon; 40 siklus (94°C 1dk, 50°C 1dk, 72°C 2 dk) ve 72°C' de 7 dk son uzama olacak şekilde thermal-cycler programı [16].

Pestivirus RT-PCR protokolü

Pan*Pestivirus* için PCR karışımı toplamda 50 µl olacak şekilde 31,8 µl nükleaz ari su, 5 µl $MgCl_2$ (25mM), 5 µl 10x

PCR buffer, 1,5 µl p324 primeri (10 pmol) ve 1,5 µl p326 primeri (10 pmol) (Tablo 1), 1 µl dNTPs (10mM), 0,2 µl Taq DNA polimeraz (5 U/ µl) ve 4 µl 30 cDNA eklenerek hazırlandı. Elde edilen PCR karışımı 94°C' de 2 dk ön denatürasyon; 35 siklus (94°C 1 dk, 56°C 1 dk, 72°C 1 dk) ve 72°C' de 7 dk son uzama olacak şekilde thermal-cycler programına tâbi tutuldu [28].

BCoV ve BToV Nested PCR protokolü

Örneklerde BCoV taraması için nested RT-PCR protokolü takip edildi [29]. Birinci aşama için PCR karışımı toplamda 50 µl olacak şekilde 37,7 µl nükleaz ari su, 2 µl $MgCl_2$ (25mM), 5 µl 10x PCR buffer, 1 µl F1 primeri (25 pmol) ve 1 µl R1 primeri (25 pmol) (Tablo 1), 1 µl dNTPs (10mM), 0,3 µl Taq DNA polimeraz (5 U/µl) ve 2 µl cDNA eklenerek hazırlandı. Elde edilen PCR karışımı 94°C' de 4 dk ön denatürasyon; 35 siklus (94°C 1 dk, 58°C 1 dk, 72°C 2 dk) ve 72°C' de 7 dk son uzama olacak şekilde thermal-cycler programına tâbi tutuldu. Elde edilen birinci evre ürününden 0,5 µl alınarak reaksiyonun ikinci evresinde kullanıldı. PCR karışım oranlarında değişiklik olmaksızın F2 ve R2 primerleri ile PCR protokolünün ikinci evresi hazırlandı ve aynı thermal-cycler programında 35 siklus olacak şekilde uygulandı.

BToV nested PCR birinci evresi için PCR karışımı toplamda 25 µl olacak şekilde 12,5 µl Maxima Hot Start Green PCR Master Mix (2x), 7,5 µl nükleaz ari su, 2 µl F forward primer (50 pmol) ve 2 µl R reverse primer (50 pmol) (Tablo 1) ve 1 µl cDNA eklenerek hazırlandı. Elde edilen PCR karışımı 94°C' de 5 dk ön denatürasyon; 35 siklus (94°C 1 dk, 49°C 1 dk, 72°C 2 dk) ve 72°C' de 7 dk son uzama olacak şekilde thermal-cycler programına tâbi tutuldu. Elde edilen birinci evre ürününden 1 µl ürün, 9 µl nüklease ari su ile karıştırılıp 1/10 luk sulandırma işlemi yapıldı ve reaksiyonun ikinci evresinde 5 µl ürün kullanıldı. PCR karışım oranlarında değişiklik olmaksızın nF ve nR primerleri ile PCR protokolünün ikinci evresi hazırlandı ve aynı thermal-cycler programında 35 siklus olacak şekilde uygulandı [30].

PCR ürünleri 1x TAE buffer içinde hazırlanan % 2'lik agaroz jelle yüklendi ve 30 dakika boyunca 200 volt elektrik uygulanarak yürütüldü. Yürütme işlemi sonunda jel görüntüleme sistemi aracılığıyla PCR ürünleri görüntülendi ve kayıt altına alındı.

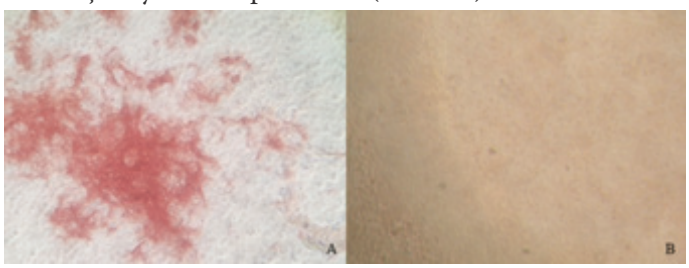
Poliakrilamid jel elektroforez (PAGE)

Toplanan 16 adet ishalli buzağı dışkı örneğinden BRV Ag ELISA ve BRV RT-PCR ile pozitif tespit edilen bir örnekte BRV nükleik asit segmentlerinin gösterimi PAGE yöntemiyle yapıldı. Bu aşamada dışkıdan uygulanan nükleik asit ekstraksiyonu ve PAGE yöntemi ayrıca jelde gümüş nitrat boyaması daha önce bildirilmiş bir protokolde sınırlı modifikasyonlar yapılarak uygulandı [31].

Bulgular

Toplanan dışkı örnekleri üç farklı hücre hattında (MDBK, HRT-18 ve MA-104) virus izolasyonuna alınmış olup, uygulanan dört kör pasaj işlemi sonrasında virus izolasyonu yapılamamıştır.

MDBK hücre hattında 4. pasaj sonrası, BVDV izolasyonu ve identifikasyonu amacıyla 5. pasaj işlemi yapıldı ve 24 gözlü pleyt 3 gün etüvde inkübe edildikten sonra IPMA testi uygulandı. BVDV spesifik substrat ilavesi sonrası pozitif kontrol hariç diğer gözlerde kırmızı-kahverengi hücre içi boyanma saptanmadı (Resim.1).



Resim 1. A: Pozitif virus kontrol (BVDV–FLK suşu)(x10 büyütme), B :Negatif kontrol (Enfekte edilmemiş MDBK hücre kültürü)(x10 büyütme)

Tablo 2. PCR ve Ag ELISA sonuçlarına göre tekli ve çoklu enfeksiyon bulunan örnek sayıları

Enterik Viruslar	Pozitif Örnek Sayısı (%)
Tek BRV	0 (%0)
Tek BCoV	2 (%12,5)
Tek BVDV	2 (%12,5)
Tek BToV	2 (%12,5)
BRV + BVDV	1 (%6,25)
BCoV + BToV	1 (%6,25)
BCoV + BVDV	4 (%25)
BVDV + BToV	1 (%6,25)
BVDV + BToV + BCoV	1 (%6,25)
Toplam	**14 (%87,5)**

16 buzağıdan alınan dışkı örnekleri BRV (BioX Diagnostic BIO K 343/2), BCoV (BioX Diagnostic BIO K 392/2) ve BVDV (IDEXX BVDV Ag/Serum Plus Test) antijen ELISA test kitleri kullanılarak test edildi. 16 örnekten 1 tanesi BRV pozitif, 8 tanesi BCoV pozitif ve 9 tanesi BVDV pozitif olarak tespit edildi (Tablo 3).

BRV, BCoV ve BVDV antijen ELISA ile pozitif tespit edilen örneklerin teyid edilmesi amacıyla aynı örneklere BRV, BVDV RT-PCR ve BCoV nested-PCR yapıldı. Ayrıca 16

örneğin hepsi BToV nested-PCR ile test edildi. BRV Ag ELISA ile pozitif tespit edilen 1 örneğin, BRV-RT PCR ile de pozitif olduğu doğrulandı. G(10) ve P(11) genotipine spesifik primerler ile RT-PCR yapılarak, pozitif tespit edilen BRV örneğinin G10P[11] genotipinde olduğu saptandı. BRV tiplendirmesi amacıyla uygulanan G6, G8, P(1) ve P(5) spesifik RT-PCR testlerinde ise negatif sonuç alındı. BCoV Ag ELISA ile pozitif tespit edilen 8 örnek BCoV nested PCR ile test edildi. PCR' ın 1. evresi sonrası 4 örnek pozitif tespit edilirken, 2. evresi sonrası 7 örneğin pozitif olduğu BCoV nested-PCR ile saptandı. BVDV Ag ELISA ile pozitif tespit edilen 9 örnek panpesti RT-PCR ile test edildi ve 9 örneğin hepsi negatif olarak tespit edildi. BToV varlığı açısından test edilen 16 örnekten 3 tanesi BToV nested PCR' ın 1. evresinde pozitif saptanırken, nested PCR ın 2. evresi sonrası toplam 5 örneğin BToV pozitif olduğu tespit edildi (Tablo 3).

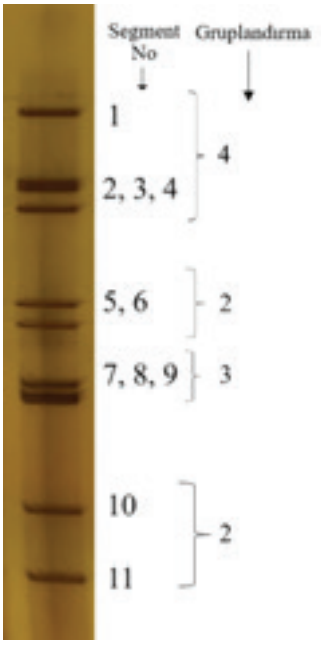
Tablo 3. İshalli dışkı örneklerinde elde edilen virolojik test sonuçları

Sıra No	Örnek ID	IPMA	Antijen (Ag) ELISA			PCR					
			BRV	BCoV	BVDV	BRV	BCoV 1.evre	BCoV 2.evre	Pestivirus	BoTV 1.evre	BoTV 2.evre
1.	**10410**	-	-	-	(+)				-	(+)	(+)
2.	**10411**	-	-	(+)	(+)		(+)	(+)		(+)	(+)
3.	**10412**	-	-	-	-					-	(+)
4.	**10413**	-	-	-	-					(+)	(+)
5.	**10414**	-	-	-	-					-	-
6.	**10415**	-	-	(+)	-		-	(+)		-	-
7.	**10422**	-	-	(+)	(+)		-	(+)	-	-	-
8.	**10423**	-	-	(+)	(+)		(+)	(+)	-	-	-
9.	**10424**	-	-	(+)	(+)		-	(+)	-	-	-
10.	**10425**	-	-	-	-					-	-
11.	**10426**	-	-	-	(+)				-	-	-
12.	**10427**	-	-	-	(+)				-	-	-
13.	**10428**	-	(+)	-	(+)	(+)			-	-	-
14.	**10429**	-	-	(+)	-		(+)	(+)		-	(+)
15.	**10430**	-	-	(+)	(+)		-	-	-	-	-
16.	**10431**	-	-	(+)	-		(+)	(+)		-	-

((+) : pozitif, (-) : negatif, IPMA: immunoperoksidaz testi, BRV: Bovine rotavirus, BCoV: Bovine coronavirus, BVDV: Bovine viral diarrhea virus, BToV: Bovine torovirus)

Çalışma kapsamında dışkı örneklerinden PCR ve Ag ELISA testlerinin sonuçlarına göre, ishal semptomu gösteren 16 buzağıdan alınan dışkı örneklerinin 14 (%87,5) adedinde araştırılan enterik virusların en az bir tanesi tespit edilmiştir. Çalışmada tek BRV enfeksiyonuna rastlanmazken; 2 (%12,5) hayvanda tek BCoV, 2 (%12,5) hayvanda tek BVDV, 2 (% 12,5) hayvanda tek BToV enfeksiyonu saptandı. 1 (%6,25) hayvanda BRV ve BVDV, 1 (%6,25) hayvanda BCoV ve BToV, 4 (%25) hayvanda BCoV ve BVDV, 1 (%6,25) hayvanda BVDV ve BToV ikili enfeksiyonları saptandı. 1 (%6,25) hayvanda BVDV, BToV ve BCoV olmak üzere enterik virusların oluşturduğu üçlü enfeksiyon saptandı (Tablo.2). Örnekleme yapılan 16 hayvandan 2 (%12,5) tanesinde çalışma kapsamında ele alınan enterik viruslara rastlanmadı.

SDS-PAGE testinde akrilamid jel üzerinde RNA segmentlerinin bant profillerine göre BRV segmentlerinin 4/2/3/2 şeklinde sıralandığı görüldü (Resim 2).



Resim 2. BRV pozitif örneğe ait RNA segmentlerinin akrilamid jelde bant profili

Tartışma ve Sonuç

Buzağı ishalleri dünyada, sığırcılık işletmelerinde yüksek morbitide ile seyreden en önemli sorunlardan biridir. Enfeksiyöz sebepleri oldukça karmaşık olan neonatal buzağı ishallerinde, dünyada enterik viral etkenler arasında BRV, BCoV, BVDV ve BToV' a sıklıkla rastlanılmaktadır [1,2,4,11,18]. Türkiye' de bugüne kadar yapılan epidemiyolojik çalışmalarda, buzağılama döneminden sonra sıklıkla ishallerin şekillendiği ve gelişme geriliği, tedavi masrafları ve ölümler ile büyük ekonomik kayıplara neden olduğunu tespit edilmiştir [9].

Enterik virusların hücre kültüründe izolasyonu oldukça zordur. BRV ile ilgili olarak MA-104 hücre hattı ve proteolitik bir enzim olan pankreatin kullanımının, BRV izolasyonu için uygun bir yaklaşım olduğunu bildirmiştir [32]. Virus izolasyon çalışmalarında BCoV ve BToV için HRT-18 hücre hattı [5] ve BVDV izolasyonu için MDBK hücre hattı kullanılmaktadır [26]. Bu doğrultuda yapmış olduğumuz çalışmada olası enterik virusların (BRV, BCoV, BVDV, BToV) tespiti için söz konusu hücre hatları (MA-104, MDBK ve HRT-18) kullanılmıştır.

Multisistemik bir enfeksiyon olarak buzağılarda ve erişkin hayvanlarda tespit edilen BVDV, buzağılarda ishale neden olup dışkı ile de saçılmaktadır [4]. Sahada sıklıkla karşılaşılan olası non-sitopatojen BVDV biyotiplerin tespiti amacıyla IPMA testinin uygulandığı birçok çalışma bulunmak-

tadır [26,33,34]. Bu çalışmada MDBK hücre hattında 5. kör pasaj işlemi sonrası yapılan IPMA testinde pozitif örnek saptanmamıştır. Ayrıca CPE tespitine dayalı olarak değerlendirme yapılan MDBK, HRT-18 ve MA-104 hücre hatlarında 4 kör pasaj sonrası 16 adet dışkı örneğinden olası bir enterik virus izolatı elde edilememiştir. Bu durumun Ag ELISA ve PCR testleri ile tespit edilen pozitifliklerin enfektivitesini kaybetmiş virus partiküllerinin bulunması veya virus izolasyon çalışmalarında üretilemeyen fakat duyarlı test yöntemleri ile varlığı gösterilebilen çok düşük sayıdaki viral partikülün bulunmasından kaynaklanabileceği değerlendirilmiştir. Yapılan her iki yöntemin (Ag ELISA ve PCR) diagnostik sensitivite değerinin (tespit alt sınırının) oldukça düşük böylece teşhis kapasitesinin yüksek olduğu daha önceki çalışmalarda gösterilmiştir [7,21,35]. Diğer taraftan örnekleme zamanının dışkıdan virus izolasyonunda oldukça önemli olduğu ve klinik bulguların başlangıç ve pik döneminde alınan örneklerde virus izolasyon şansının yüksek olduğu da bilinmektedir[7,24]. Bu çalışmada viral izolat elde edilememesinin bir nedeni de saha şartlarında örnek alım zamanlamasıyla ilgili olabileceği göz ardı edilmemelidir.

Yapılmış olan çalışmalarda PCR ve Ag ELISA testleri ile neonatal buzağı ishallerinde doğada sirküle olan enterik virusların (BRV, BCoV, BToV ve BVDV) tespiti bildirilmiştir[5,23,24,34,36]. Yapılan çalışmalarda aynı yöntemler ile tespit edilen tekli veya çoklu enfeksiyonlara sebep olan enterik patojenler ile bu çalışmada tespit edilen viral patojenler benzerlik göstermektedir.

Dünyada buzağılarda neonatal ishale sebep olan enterik virusların (BRV, BCoV, BToV ve BVDV) tekli ve çoklu enfeksiyonları yapılan çalışmada gösterilmiştir [2]. Ülkemizde buzağıların ishal olgularında, dışkıdan PCR metodu ile yapılmış olan çalışmalarda BToV tekli enfeksiyon oranı %4,7 - %16,7 arasında [5,37], tek BRV enfeksiyon yaygınlığı %6,1 - %37,27 [6,38], tek BCoV enfeksiyon yaygınlığı %10,8 - %37,27 [6,39] ve ikili BRV ve BCoV enfeksiyon yaygınlığı %4,16 - %18,18 [6,7] oranında tespit edilmiştir. ELISA metodu ile tek BRV yaygınlığı %17,80 - %71,1 [23,40] ve tek BCoV yaygınlığı %1.12 - %15.4 [23,41]; BRV ve BCoV ikili enfeksiyon yaygınlığı %13.4 oranında tespit edilmiştir [23]. Bu çalışmadan elde edilen sonuçlar, ülkemizde yapılmış diğer çalışmalar ile benzerlik göstermektedir. Türkiye' de araştırmada ele alınan enterik virusların (BRV, BCoV, BToV ve BVDV) tamamını kapsayan ve aynı hayvanda çoklu enfeksiyon varlığını gösteren veri bulunmamaktadır. Ayrıca ishalli buzağıların dışkı örneklerinde BVDV yaygınlığı gösteren prevalans çalışmaları kısıtlıdır [4]. Elde edilen sonuçların güncel olması ve çoklu enfeksiyon varlığının gösterilmesi, sonuçları değerli kılmaktadır.

Çalışmada Ag ELISA ile pozitif tespit edilen örneklerin teyidi için PCR yöntemi uygulanmıştır. Bu amaçla BVDV Ag ELISA ile pozitif tespit edilen örneklere, 5'UTR hedef gen bölgesine göre pan*Pestivirus* RT-PCR testi uygulanmıştır. Klinik örneklerde BVDV Ag ELISA ile tespit edilen pozitif örnek sayısının, RT-PCR testindeki pozitif örnek sayısından yüksek olduğu daha önce bildirilen bir veridir [33]. Bu çalışmada BVDV' nin tespitinde, BVDV Ag ELISA test sonucuna göre pozitif tespit edilen 9 örneğin tamamının pan*Pestivirus* RT-PCR ile negatif olması, dışkı örneklerinde BVDV tespitinde, BVDV Ag ELISA testinin analitik sensitivitesinin daha yüksek olduğunu teyit etmektedir. Diğer taraftan BCoV Ag ELISA ile pozitif tespit edilen 8 örnekten 7 tanesi BCoV nested-PCR ile pozitif tespit edilmiştir. BRV Ag ELISA ile BRV RT-PCR sonuçları da birbiri ile uyumludur.

*Rotavirusları*n farklı P ve G genotiplerinin tespiti, tip spesifik primerler kullanılarak PCR testi ile yapılmaktadır [11]. Dünya genelinde yapılan saha çalışmalarında tespit edilen sığır *rotavirusları*n büyük çoğunluğu G6 ve G10 genotipindedir [11,16]. Daha az yaygın olarak G8 genotipi de tespit edilmiştir [16]. Bu genotipler genellikle P[5], P[11] genotipleri ve daha az sıklıkla P[1] genotipi ile ilişkilendirilmiştir [17]. Türkiye' de ishalli buzağılarla yapılan saha çalışmalarda sıklıkla G10P[11] genotipine rastlanmaktadır [11]. Bu çalışmada da PCR testi ile pozitif saptanan rotavirus suşunun, G10P[11] genotipine sahip olduğu tespit edilmiştir.

SDS-PAGE testi dışkıda rotavirus gösterimi için kullanışlı bir yöntem olup 11 segmentili rotavirus genomunun PAGE ile gösterildiği birçok çalışma bulunmaktadır [42,43]. Akrilamid jel üzerinde RNA segmentlerinin bant profillerine göre yapılan çalışmalarda *rotavirusları*n A (4/2/3/2), B (4/2/2/3) ve C (4/3/2/2) gruplarına ayrıldığı gösterilmiştir [42]. Bu çalışmada tespit edilen segmentler 4/2/3/2 şeklinde sıralanmıştır (Resim 2). Bu sonuçlar, Ag ELISA ve PCR ile pozitif tespit edilen rotavirus örneğinin, SDS-PAGE testinde segmentlerin bant profillerine göre grup A'da olduğunu göstermektedir.

Sürü yönetiminde dikkatle takip edilmesi gereken hususlardan biri de hastalıktan korunma amacıyla biyogüvenlik tedbirleri ve aşı programlarının uygulanmasıdır. Bu çalışmaya konu olan örneklerin temin edildiği işletmede erişkin sığırlarda BVDV, BRV ve BCoV aşılama programları uygulanıyor olmasına rağmen buzağılarda bu denli yüksek oranlı pozitif vakaların tespit edilmesi beklenen

bir tablo değildir. Dolayısıyla bu gibi durumlarda özellikle kolostrum beslemesinin etkinliğinin sorgulanması ve buzağılarda pasif transfer yetmezliği sorununa odaklanılması yararlı olacaktır.

Ülkemizde neonatal dönemdeki buzağı kayıplarında ishal kaynaklı mortalite oranları çiftliklere ve bölgelere göre değişmekle birlikte, koruyucu veteriner hekimlik hizmetlerinin uygulandığı çiftliklerde dahi yüksek oranlara ulaşabilmektedir. Buzağı ishali ile ilgili hastalık kontrolü, genotip prevalansı, bulaşma koşulları, aşı etkinlik çalışmaları gibi epidemiyolojik çalışmaların sınırlı düzeyde olması nedeniyle, bu alandaki olguların incelenmesi ve raporlanması önem arz etmektedir. Bu araştırmada neonatal buzağı ishallerine sebep olabilen önemli viral etkenler olan BRV, BCoV, BVDV ve BToV' un aynı işletmede eş zamanlı olarak görülebileceği ve şiddetli hastalık bulguları ve kayıplara neden olabileceği gösterilmiştir.

Teşekkür

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In Vitro Cytotoxicity and Genotoxicity Screening of *Cuscuta Arvensis* Beyr. and *Achillea Wilhelmsii* C. Koch

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Abstract

Plant-based compounds have been used for medicinal purposes since ancient times, as easily accessible and low-cost treatment options. Despite the widespread belief that plants are quite safe and devoid of side effects, scientific studies have revealed the toxicity potential of active components of plants on healthy cells. The present study was designed to investigate in vitro cytotoxicity and genotoxicity potential of Achillea wilhelmsii C. Koch and Cuscuta arvensis Beyr., which are frequently used in traditional medicine. In this context, cytotoxicity evaluation of the extracts was performed by MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Our cytotoxicity results indicate that the extract from A. wilhelmsii did not affect the viability of fibroblasts at any tested concentrations, on the contrary, significantly stimulated cell proliferation from a concentration of 25 µg/mL. On the other hand, the extract from C. arvensis significantly reduced the viability of fibroblasts at all concentrations tested. In the second part of this research, the DNA damaging potential of the extracts was investigated by in vitro comet assay at non-cytotoxic concentrations. A. wilhelmsii extract caused a significant increase in the percentage of DNA in the tail (%TDNA), which is considered as an indicator of DNA damage, only at the highest concentration, while C. arvensis extract did not significantly affect %TDNA at concentrations tested. The results of the present study indicated that the methanolic extract from A. wilhelmsii may be considered safe up to a concentration of 100 µg/mL, however, the cytotoxicity potential of C. arvensis may be a factor limiting its safe use.

Keywords: Achillea wilhelmsii C. Koch, Cuscuta arvensis Beyr., Cytotoxicity, Genotoxicity

Introduction

Medicinal plants are the major reservoir for a wide variety of pharmacologically active phytocompounds and accordingly have been used for therapeutic purposes for many years.[1,2] According to the World Health Organization (WHO), up to 80% of the world's people rely upon plant-based treatment for primary health care due to its multiple advantages over conventional therapies (such as low cost, easy accessibility, low side effects, etc.).[3] Also from the perspective of the pharmaceutical industry, the active components of medicinal plants are considered to be a valuable resource for the development of new pharmacological agents.[4] Despite the profound beneficial effects of medicinal plants, it is well known that maintaining the balance between therapeutic and toxicological effects is the most crucial factor for a plant-based therapy can be considered safe.[5] This situation has led to the necessity of investigating the toxicity potential of medicinal plants and verifying their safe use with scientific evidence-based approaches. In this context, scientific studies have revealed that the genotoxicity and carcinogenicity potentials of some bioactive components in plants are extremely important factors limiting their safe and effective use.[6,7]

The flora of Turkey is very rich in the diversity of medicinal plants as a result of the geographical location of Turkey.[8] Species belonging to the genus *Cuscuta L.* (Cuscutaceae), which is represented by 21 species in the flora of Turkey, have been frequently used in traditional medicine due

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to their pharmacological activities.[9] Among these species, Cuscuta arvensis Beyr. has been mostly used in folk medicine for the treatment of jaundice in newborns and their mothers, in the southeastern part of Turkey.[9,10] The genus Achillea L. (Asteraceae), which has a wide distribution with approximately 42 species in the flora of Turkey, has been frequently used in traditional medicine due to its beneficial properties.[11] The extracts of Achillea species have been used for food supplements and therapeutic applications as an easily accessible source of natural antioxidants.[12] Infusion and decoction of *A. wilhelmsii* C. Koch have been reported to be used in Turkish folk medicine as diuretic, anti-hemorrhoidal, and against abdominal pain.[13] The biological activities of both *C. arvensis* Beyr. and *A. wilhelmsii* C. Koch have been confirmed by *in vitro* and *in vivo* scientific studies. In this context, it has been reported that *C. arvensis* Beyr. extracts with different polarities display antioxidant, anti-inflammatory, antinociceptive, and hepatoprotective properties.[9,14] Similarly, anti-inflammatory, antinociceptive, immunomodulatory, anxiolytic, hepatoprotective and antimicrobial activities of the extracts and essential oil from *A. wilhelmsii* have been previously demonstrated.[11,15-18] Although the pharmacological activities of *C. arvensis* Beyr. and *A. wilhelmsii* C. Koch have been extensively investigated, there are quite limited reports on the toxicological properties of these plants in the scientific literature.

The current study aimed to investigate the toxicity potential of extracts from *C. arvensis* and *A. wilhelmsii* in order to confirm the safety of their therapeutic use. It is well known that the determination of genotoxicity and cytotoxicity of a compound is one of the first steps in the biological evaluation process reported in ISO- 10993-5.[19] In this direction, the methanolic extracts were investigated in terms of their genotoxic and cytotoxic activities on healthy cells under *in vitro* conditions.

Materials and methods

Plant Samples and extraction process

A. wilhelmsii C. Koch was collected from Beyşehir, Derebucak district in Turkey during Spring of 2015. An authenticated voucher specimen (GUE 3490) was maintained in the Herbarium of the Faculty of Pharmacy (Gazi University, Turkey). *A. wilhelmsii* C. Koch (100 g) was extracted with pure methanol (3× 1250 mL) for 48 hours. The extract was dried by the evaporator, then the yield of the methanolic extract was calculated as 23.34%.

C. arvensis Beyr., which was a parasitic plant found on the host lentils, was purchased from a herbalist in Mardin, Turkey. The plant material was identified compared to the specimens (KHB-78) deposited in Kilis 7 Aralik University, Department of Biology. In the extraction process, *C. arvensis* (200 g) was powdered and extracted with methanol (3 × 1500 mL) for 48 hours. The extract was dried by using the evaporator, then the yield of methanolic extract was calculated as 20.67 %.

Cell culture and stock solutions

The 3T3-Swiss albino mouse fibroblast cell line (ATCC _CCL-92) was cultured in the Dulbecco's Modified Eagle's Medium- F12 (DMEM-F12) supplemented with fetal bovine serum (FBS, 10%) and penicillin/streptomycin solution (1%) in a humidified atmosphere containing 5% CO2 at 37 °C. The culture medium was changed twice a week. When cultures reached confluence, the adherent cells were detached from the culture flask through trypsinization (Trypsin-EDTA solution, 0.25%).

For toxicological assessment, the stock solutions of the plant extracts were prepared in dimethyl sulfoxide (DMSO) followed by further required dilutions with DMEM- F12. The highest concentration of DMSO was 0.3 % in the medium and the corresponding amounts of DMSO were added to the control cells. All stock solutions were stored at (-) 20°C until use.

Cytotoxicity assessment (MTT assay)

MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was performed to assess the cytotoxicity potential of *A. wilhelmsii* and *C. arvensis* methanolic extracts. In brief, the cells were seeded into triplicate wells of a 96-well plate at a density of 1x 10^4 cells/well and incubated overnight. One hundred microliters of the medium, with or without methanolic extracts of the plants (10- 200 µg/mL), were added to each well and the cells were incubated at 37 °C. After 24 h exposure, the cell viability was evaluated using the cell proliferation kit (MTT, Roche, Germany) and the absorbance of each well was read at 595 nm. The effect of each plant extract on cell viability was determined as percent cell viability where the vehicle (DMSO)-treated cells were received as 100% viable.

Genotoxicity assessment (Comet assay)

Treatment of cells with the extracts

The determination of the highest concentration for *in vitro* genotoxicity assessment is based on the results of cytotoxicity analysis.[19,20] Accordingly, the concentration ranges that yielded a cell survival rate of approximately 70% were selected for subsequent DNA damage analysis. [19,20] 3T3-Swiss albino mouse fibroblast cells were seeded in six-well

plates at a density of 1.5 x 10^5 cells /mL and incubated for 24 h. Then, the cells were treated with non-cytotoxic concentrations of the methanolic extracts (50, 100 and 200 µg/mL for *A. wilhelmsii*; 10, 25 and 50 µg/mL for *C. arvensis*) for 3 hours. Hydrogen peroxide (300 µM) was used as the positive control for DNA damage. At the end of the treatment period, the cells were washed twice with 4 mL of cool phosphate-buffered saline (PBS, pH 7.4), suspended by trypsinization, and collected into tubes. The cells were obtained by centrifugation at 200 g for 5 min and suspended in 300 µL of cold PBS for analysis.

Comet assay

The comet assay was performed under alkaline conditions (pH >13) using the method of Singh et al.[21] A 25 µL aliquot of the cell suspension was mixed with 75 µL of low melting point agarose in PBS at 37 °C (0.65%). This mixture was rapidly placed on microscope slides previously covered with normal melting point agarose (0.5%). Coverslips were added to each slide and the slides were maintained at 4 °C for 10 min to solidify. After removing the coverslips, the slides were left in a cold freshly prepared lysing solution (2.5M NaCl, 100mM Na-EDTA, 10mM Tris, pH: 10.0, DMSO (10%) and Triton X-100 (1%) mixed prior to use) at least 1 h, at 4°C. The slides were removed from the lysing solution and immediately incubated in a horizontal electrophoresis tank filled with a freshly prepared electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH >13), for 20 min. Subsequently, electrophoresis was performed at 25 V/300mA, for 30 minutes. The slides were neutralized with 0.4 M Tris buffer solution (pH: 7.5), fixed with ethanol for 2 minutes, and dried at room temperature. All slides were kept in a closed container until image analysis. All steps of comet assay were performed in the dark to avoid additional DNA damage.

Staining and image analysis

Before image analysis, each slide was stained with 50 µL of ethidium bromide solution (20 µg/mL). Two slides were prepared from each sample and the analysis was performed on randomly 50 cells per slide, using an Olympus BX51fluorescence microscope (Olympus Optical® Co. Ltd, Japan). Image analysis was performed by using image analysis software (Bs 200 Pro Software®, BAB Imaging System in Turkey). The percentage of DNA in the tail (TDNA %) was considered the indicator of DNA damage.

Statistical analysis

Data were expressed as the mean of triplicates ± standard deviation (SD). Statistical analysis of the results was performed by one-way ANOVA followed by Dunnett's multiple comparisons test by using Graphpad Prism® (version 9.1.0, Graphpad Software® Inc., CA, USA). All the results were considered significant at $p < 0.05$. The concentrations of extracts needed for 50% inhibition of cell viability (IC50) were determined by using the Biosoft CalcuSyn software (Biosoft, UK).

Results

Figure 1 represents the percentage of cell viability after treated with various concentrations (10-200 µg/ mL) of methanolic extracts from *A. wilhelmsii* and *C. arvensis*. As shown in the figure, *A. wilhelmsii* extract did not reduce the viability of fibroblasts at any concentrations tested, as well as significantly stimulated fibroblast proliferation from a concentration of 25 µg/ mL ($p<0.001$). The IC50 value could not be detected for *A. wilhelmsii* extract at the range of concentrations used, since it did not reduce the viability of fibroblasts. In contrast, *C. arvensis* extract reduced fibroblast viability by 27.4% to 60.4% compared to untreated cells (IC50: 176.07 µg/ mL).

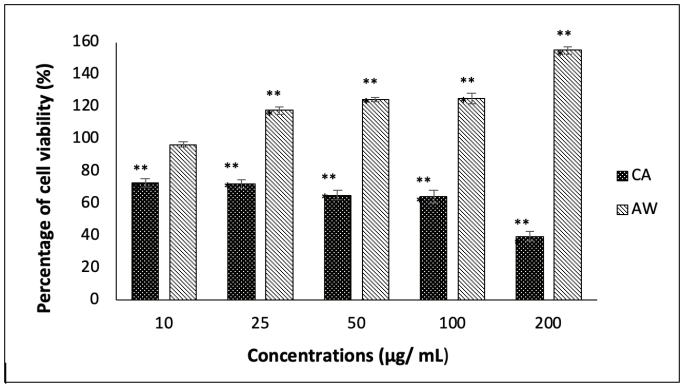

Figure 1. Cytotoxicity of the methanolic extracts from A. wilhelmsii and C. arvensis on Swiss-3T3 albino mouse fibroblasts. ***p<0.001, versus negative control. CA: Cuscuta arvensis Beyr.; AW- Achillea wilhelmsii C. Koch.

The results of the comet assay were shown in Figure 2 and the representative images were presented in Figure 3. The highest DNA damage was detected in cells treated with H2O2 (%TDNA 83.29 ± 1.72). Our findings demonstrated that *A. wilhelmsii* extract did not cause a significant increase in the %TDNA at concentrations of 50 and 100 µg/mL ($p>0.05$). Treatment of *A. wilhelmsii* extract induced DNA damage merely at the highest concentration, as shown by a %14 increase in %TDNA compared to control. (43.34 ± 1.57 versus 37.9 ± 1.63; $p < 0.05$). On the other hand, no significant increase was determined in %TDNA of cells treated with *C. arvensis* extract, indicating that this extract did not induce significant DNA damage at low concentrations ($p > 0.05$).

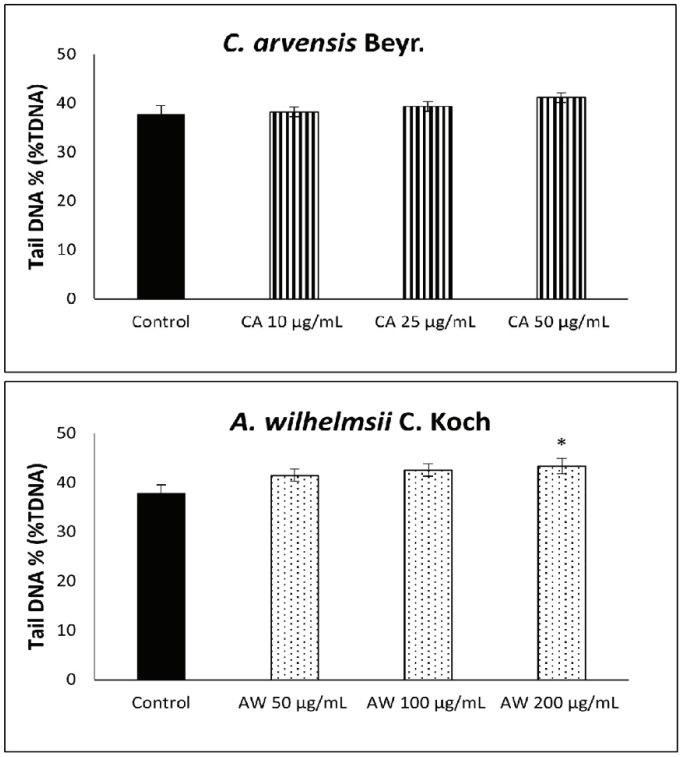

Figure 2. Percentage of tail DNA in cells after treatment with C. arvensis and A. wilhelmsii methanolic extracts. *p<0.05 versus control. CA- Cuscuta arvensis Beyr., AW- Achillea wilhelmsii C. Koch.

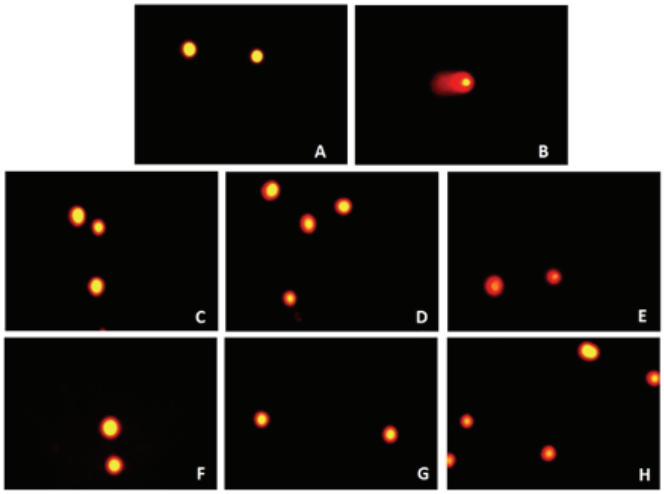

Figure 3. Comet images in fibroblasts treated with the methanolic extracts from A. wilhelmsii and C. arvensis (100x magnification). A) Control, B) Positive control- H2O2 (300 µM), C) A. wilhelmsii extract (50 µg/ mL), D) A. wilhelmsii extract (100 µg/ mL), E) A. wilhelmsii extract (200 µg/ mL), F) C. arvensis extract (10 µg/ mL), G) C. arvensis extract (25 µg/ mL), H) C. arvensis extract (50 µg/ mL). H2O2- Hydrogen peroxide.

Discussion

Evaluation of cytotoxic activity is a very important parameter in revealing the safety profiles of plant-based compounds.[22] Furthermore, since detected DNA damage can be a secondary effect of cytotoxicity, performing a cytotoxicity analysis is essential to avoid false interpretations of genotoxic activity.[22,23] In the present study, screening of cell viability was performed both to evaluate the cytotoxic activities of the extracts and to determine the concentrations to be used in studies on the DNA-damaging potentials of plant extracts.

Our results showed that methanolic extract from *A. wilhelmsii* did not adversely affect fibroblast viability even at high concentrations. Moreover, according to our results, this extract significantly stimulated fibroblast proliferation from a concentration of 25 µg/ mL ($p<0.001$). Previous studies on the biological activities of Achillea species confirm that phytocompounds contained in various extracts of these species can stimulate fibroblast proliferation. Similar to the result of our study, Ghobadian et al.[24] have suggested that the hydroalcoholic extract of A. millefolium stimulates proliferation of human skin fibroblasts at concentrations below 20 mg/mL. In another study investigating the effects of different Achillea species on the treatment of skin wounds, the stimulating effects of A. kotschyi extract on fibroblast proliferation have been reported (at the concentration of 2.5-20 µg/mL).[25] Contrary to the results of these studies, there are also reports indicating the cytotoxicity potential of Achillea species, especially on cancer cell lines. In this context, Sargazi et al.[26] demonstrated the significant antiproliferative activity of hydroalcoholic extract from *A. wilhelmsii* on HeLa cervical cancer cells. This can be explained by the difference in the sensitivity of cancer cells and healthy cells to the extracts or the differences in the phytochemical composition of various Achillea extracts.

According to International Standard Organisation (ISO)-10993-5, cell viability below 70% compared to untreated cells is considered a sign of cytotoxic effect.[19] In the present study, the viability of cells treated with *A. wilhelmsii* extract was above the standard level of cytotoxicity at all tested concentrations, indicating the non-cytotoxic behavior of this extract on mouse fibroblasts. On the other hand, methanolic extract of *C. arvensis* can be reported as cytotoxic in mouse fibroblasts according to the ISO 10993-5 standard, as it reduces cell viability below 70%, at concentrations higher than 25 µg/mL. Despite the widespread use and high therapeutic value of *C. arvensis*, no study was found in the scientific literature evaluating the toxicity potential of the extracts obtained from this plant. On the other hand, a limited number of studies evaluating the toxicity profile of other species from this genus have revealed the cytotoxic profile of Cuscuta extracts, similar to our findings. The findings of the study conducted by Abedini et al.[27] showed that ethanolic extract of C. epithymum exhibited

concentration-dependent cytotoxic activity on fibroblasts from a concentration of 312 µg/mL. In our study, the cytotoxic effect of *C. arvensis* extract was detected at lower concentrations (10-200 µg/mL) than the concentration reported by Abeni et al.[27] It is known that multiple factors such as the type of extraction solvent or extraction technique can affect the quantity of isolated bioactive compounds in plant extracts.[28] In our study, the observed effect on fibroblast viability even at low concentrations can be explained by the possibility that the methanolic extract may contain higher amounts of cytotoxic components. Similarly, it has been reported that chloroform extract of C. reflexa reduced the proliferation of RAW 264.7 murine macrophages and HEK 293 (human embryonic kidney) cells.[29] The antiproliferative activity of C. reflexa has been attributed to the presence of four compounds purified from the extract; scoparone, stigmasta-3,5-diene, p-coumaric acid, and 1-O-p-hydroxy cinnamoyl glucose.[30] On the other hand, in the study performed by Koca- Caliskan et al.[9], kaempferol-3-O-rhamnoside, a flavonol glycoside with antiproliferative activity, has been reported as the major component of *C. arvensis* methanolic extract.[31] In the present study, although no evaluation was carried out on the phytochemical composition of *C. arvensis*, it is possible that the cytotoxic effect on fibroblasts may be related to the phytocompounds with antiproliferative activity previously reported in this species.

Assessment of the potential genotoxicity of plant extracts is a very crucial issue as DNA damage can lead to critical mutations and thus increase the risk of cancer.[6] In the present study, we determined the genotoxicity potential of *A. wilhelmsii* and *C. arvensis* by comet assay, which is a highly sensitive method for assessing cell-specific DNA damage.[23] The increase in % TDNA value was considered to be correlated to the intensity of DNA damage occurring in the fibroblasts. Previous studies have reported that DNA damage can occur as a result of direct interaction of genotoxic compounds with DNA, or it can be an indirect result of oxidative stress induction or cytotoxicity.[32] In order to eliminate conflicting results related to cytotoxicity, we used non-cytotoxic concentrations of the extracts for the comet assay. Our results indicate that while *A. wilhelmsii* extract did not cause DNA damage at concentrations of 50 and 100 µg/mL, it induced DNA damage only at the highest concentration (200 µg/mL). The fact that *A. wilhelmsii* extract stimulated DNA damage (at a concentration of 200 µg/mL) without causing a concomitant increase in cytotoxicity may indicate that high concentrations of the extract may contain direct DNA-reactive compounds.

During the literature review, no study was found that evaluated the genotoxic activities of *A. wilhelmsii* extracts on healthy cells. On the other hand, the potential of *A. wilhelmsii* to cause DNA damage has been evaluated in only cancer cell lines. According to a previous report evaluating the genotoxicity potential of *A. wilhelmsii* in cancer cells, hydroalcoholic extract of *A. wilhelmsii* can cause DNA damage in HeLa cervical cancer cells (at 100 µg/mL concentration), by increasing the phosphorylation of H2AX as a very sensitive marker of unrepaired DNA damage.[26] Considering that *A. wilhelmsii* extract did not cause significant DNA damage up to 200 µg/mL concentration in our study, it can be concluded that this extract may be safe in healthy cells at concentrations reported to cause DNA damage in cancer cells. This can be interpreted as *A. wilhelmsii* extracts can be considered safe on healthy cells at the specified concentrations and can be a potential source for raw material in the pharmaceutical industry. On the other hand, to the best of our knowledge, no reports are available regarding the genotoxicity potential of the extracts from *C. arvensis*. In our study, the genotoxic potential of the methanolic extract from *C. arvensis* was examined in a very low concentration range (10-50 µg/mL), in order to avoid false positive results associated with cytotoxicity. Whereas, in a pharmacological activity study conducted with *C. arvensis*, it has been reported that the methanolic extract exhibited protective activity against acetaminophen-induced hepatotoxicity in the range of 125-250 mg/kg.[9] It is noteworthy that in our study, the non-genotoxic profile of *C. arvensis* extract was revealed at a lower concentration range than the concentrations at which it exhibited pharmacological activity. Upon also considering our cytotoxicity findings, it could be speculated that *C. arvensis* extracts may have toxicity potential on healthy cells, especially at higher concentrations. This view can be supported by the observation of Dokuparthi et al.[33] who reported the mutagenic activity of C. reflexa methanolic extract by Ames test, only at high concentrations (5000 µg/plate and 10000 µg/plate).

Conclusion

Natural products including medicinal plants are very important reservoirs for therapeutic applications and play a crucial role in the designing of new drugs. Despite a general perception that plant-based compounds are quite safe and devoid of side effects, the cytotoxicity/genotoxicity potentials of plant constituents are major factors limiting their use. The present study revealed the *in vitro* toxicological profile of methanolic extracts from *A. wilhelmsii* and *C. arvensis*. Our results demonstrated the cytotoxicity potential of *C. arvensis* extract and the safety profile of *A. wilhelmsii* extract (up to the concentration of 100 µg/mL) on mouse fibroblasts. Although the genotoxicity potential of *A. wilhelmsii* needs to be confirmed by different genotox-

icity assays especially at higher concentrations, this plant can be considered a safe therapeutic alternative at indicated concentrations.

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