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RESEARCH ARTICLE

Pathologic Findings of Anthraco-silicosis in the Lungs of One Humped Camels *(Camelus dromedarius)* and Its Role in the Occurrence of Pneumonia

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Summary

Pneumoconiosis is an occupational lung disease which occurs by the accumulation of inhaled organic and inorganic particles in the pulmonary parenchyma. These particles interfere with the defense mechanisms of the lungs. This study was designed to describe the gross and histopathological features of pneumoconiosis in the lungs and its relation with occurrence of pneumonia in camels. 150 pneumonic and 100 healthy lungs were examined for pneumoconiosis in pathologic level. Macroscopic lesions of pneumoconiotic lungs were related to pneumonia and no remarkable features of pneumoconiosis were observed in the examined tissues. Pneumoconiosis was diagnosed in 43 out of 150 lungs with pneumonic lesions. Grossly healthy lungs did not show pneumoconiosis. Out of 43 pneumoconiotic lungs, 93% (n=40/43) were associated with interstitial pneumonia. Also, pneumoconiosis occurred in 2.32% (n=1/43) and 4.65% (n=2/43) of lungs with suppurative bronchopneumonia and bronchointerstitial pneumonia, respectively. Histopathologic findings related to pneumoconiosis were characterized by varying degrees of diffuse to nodular fibrosis as well as mild to heavy accumulations of mixed carbon and silicon dusts. Interlobular and interalveolar septa of alveoli were thickened by fibrosis and infiltration of mononuclear inflammatory cells. Crystalline silicon particles were in different shapes including filamentous, oblong and polygonal. Anthraco-silicosis particles were observed inside and outside of macrophages more adjacent to the vessels and bronchioles as well as lymphocytes infiltration. The color of particle aggregations was different from scant gray to brown or dense black, depending on the amount of silicon or carbon dust deposition. In the lungs, areas with prominent deposition of crystalline silica rather than carbon were gray to light brown and focal interstitial fibrosis was occurred. The present study suggests a positive causal relationship between pneumoconiosis and pneumonia. It seems these particles predispose animals to pulmonary diseases especially various type of pneumonia.

Keywords: *Pneumoconiosis, Pathology, Silicosis, Anthracosis, Camel*

Tek Hörgüçlü Develerde *(Camelus dromedarius)* Anrako-silikozisin Akciğerlerdeki Patolojik Bulguları ve Pömoni Oluşumundaki Rolü

Özet

Pnömokoniozis pulmoner parankimde solunan organik ve inorganik partiküllerin birikmesi ile oluşan bir akciğer hastalığıdır. Bu partiküller akciğerlerin savunma mekanizmalarını bozarlar. Bu çalışma develerde pnömokoniozisin makroskopik ve histopatolojik özelliklerini tanımlamak ve pnömoni oluşumu ile ilişkisini ortaya koymak maksadıyla yapılmıştır. Çalışmada 150 pnömonili ve 100 sağlıklı akciğer pnömokoniozis yönünden incelendi. Pnömokoniotik akciğerlerdeki makroskopik lezyonlar pnömoni ile ilişkilendirildi ve incelenen dokularda pnömokoniozisin belirgin hiçbir özelliği gözlemlenmedi. Pnömoni lezyonlu 150 akciğerden 43'ünde pnömokoniozis tespit edildi. Makroskopik olarak sağlıklı akciğerlerde pnömokoniozis gözlemlenmedi. 43 adet pnömokonitik akciğerin %93'ü (n=40/43) intersitisyal pnömoni ile ilişkili idi. Pnömokoniozis %2.32 oranında suppuratif pnömoni ile %4.65 oranında da bronkointersitisyal pnömoni ile gözlemlendi. Pnömokoniozis ile ilişkili histopatolojik bulgular diffuzdan nodülere değişen derecede fibrozis ve ortadan şiddetliye değişen oranda miks karbon ve silika tozlarının birikmesi ile karakterize idi. İnterlobular ve interalveolar septumlar fibrozis ve mononüklear yangı hücrelerinin infiltrasyonu ile kalınlaşmıştı. Kristalize silika partikülleri filamentöz, oblong ve poligonal şekilleri içeren değişik şekillerde idi. Antrako-silika partikülleri damar ve bronşiyol duvarlarına özellikle yakın olmak üzere makrofajların içinde ve dışında, lenfosit infiltasyonu ile birlikte gözlemlendi. Partikül birikintilerinin rengi açık griden kahverengi siyaha değişen renklerde silika ve kadron birikim miktarına bağlı olarak gözlemlendi. Akciğerlerde kadrondan daha ziyade silika kristallerinin belirgin birikimi olan alanlar gri açık kahverenginde olup fokal intersitisyal fibrozis ile birlikte gözlemlendi. Bu çalışma pnömokoniozis ve pnömoni arasında belirgin bir ilişki olduğunu ortaya koymaktadır. Bu partiküllerin çeşitli tip pnömonilere karşı predispoze ettiği kanısına varıldı.

Anahtar sözcükler: *Pnömokoniozis, Patoloji, Silikozis, Antrakozis, Deve*

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INTRODUCTION

Pneumoconiosis is an occupational lung disease which causes by the accumulation of inhaled particles in the pulmonary parenchyma. These particles escape from the mucociliary defense mechanisms of the upper respiratory systems and deposit in the alveoli. After phagocytosis of particles by pulmonary macrophages, they are transferred to the peribronchial and perivascular regions [1,2]. These particles persist within the macrophages and induce a tissue reaction in the lungs by stimulating inflammatory mediators [2]. Pneumoconiosis may be clinicopathologically classified as fibrotic (lead to nodular or diffuse fibrosis) or nonfibrotic (including particle-laden macrophages, with minimum or no fibrosis) [3]. Some subgroups of pneumoconiosis such as silicosis, anthracosis or coal worker pneumoconiosis, asbestosis, berylliosis, and talcosis are fibrotic forms of pneumoconiosis. Siderosis (from iron oxide), stannosis (from tin oxide), and baritosis (from barium sulfate) are nonfibrotic forms of the disease [4]. Anthracosis or black pigment discoloration causes by coal dusts or environmental pollutions. Lungs and regional lymph nodes are target tissues. In human, extrathoracic anthracosis are rarely reported in the esophagus [5] and liver [6]. This condition is more common in human than animals because they are not in exposure to the occupational conditions in contrast to human cases [2,7]. In animals, Mild pulmonary anthracosis is a common incidental finding in crowded city or those cohabite with cigarette smokers. Also, those animals living in the desert have pneumoconiosis and crystals accumulate be more with increasing the age [8]. Therefore, spontaneously occurring pneumoconiosis in the animals is rarely reported [2,8]. This study describes the gross and histopathological features of pneumoconiosis in the lungs and its relation with the occurrence of pneumonia in camels.

MATERIAL and METHODS

Sample Collection

This study was performed in the local abattoir of Najaf-Abbad, Esfahan province, central part of Iran from November 2010 to April 2011. In this abattoir, ruminants including sheep, goat, cow and camels are slaughtered daily. The camels originated from east, south and south east parts of Iran that these areas have warm, dry and windy weather. For this study, 150 lungs of slaughtered camels with macroscopic pneumonia lesions and 100 apparently normal lungs (2 to 7 years old) inspected and some specimens of the lungs were taken for histopathologic study.

Pathological Investigation

The collected samples in 1 cm^3 thicknesses of the pneumonic and normal lungs were fixed in 10% neutral buffered formalin for histopathological examination. The samples were then dehydrated in graded ethanol and embedded in paraffin. Sections of 5 µm in thickness were stained with hematoxylin and eosin and then, examined by an ordinary light microscopy.

RESULTS

In this study, pneumoconiosis was diagnosed in 43 out of 150 lungs with the pneumonic lesions in histopathologic level and the apparently healthy lungs did not show any deposition of particles in the parenchyma. Observed macroscopic lesions of the affected lungs were related to pneumonia and no remarkable features of pneumoconiosis were visible in the examined tissues. Out of 43 pneumoconiotic lungs, 93% (n=40/43) were associated with interstitial pneumonia. Affected lungs were enlarged, rubbery in consistency, diffusely red to pale appearance and rib impressions were seen on the costal surfaces of the diaphragmatic lobes. They failed to collapse with pressure and no evidence of exudates was detected in the cut surfaces and airways *(Fig. 1)*. Also, pneumoconiosis was occurred in 2.32% (n=1/43) and 4.65% (n=2/43) of lungs with suppurative bronchopneumonia and bronchointerstitial pneumonia respectively. The lungs with suppurative pneumonia showed consolidation of the cranioventral and accessory lobes. The affected lobes were dark red with firm consistency *(Fig. 2)*.

Histopathologic findings of pneumoconiosis were characterized by varying degrees of diffuse to nodular fibrosis associated with mild to heavy accumulations of mixed carbon and silicon dusts. Diffuse interstitial fibrosis was prominent feature. Interlobular and interalveolar

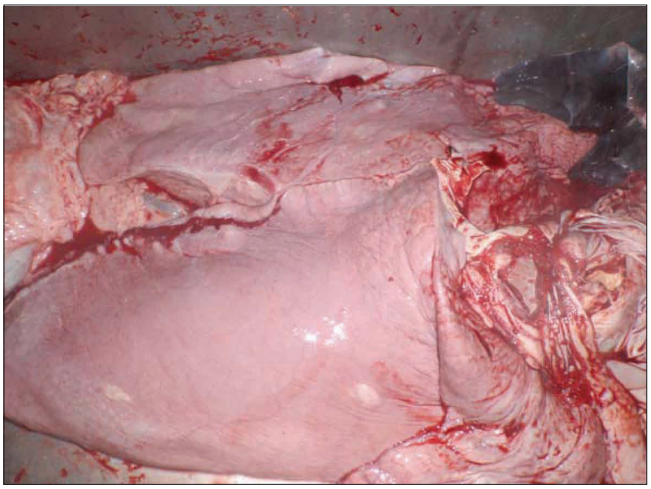


Fig 1. Macroscopic appearance of interstitial pneumonia. Affected lungs are enlarged, pale and uncollapsed

Şekil 1. İntersitisyal pnömoninin makroskopik görüntüsü. Akciğerler büyümüş, açık renkli ve kollabe olmamış

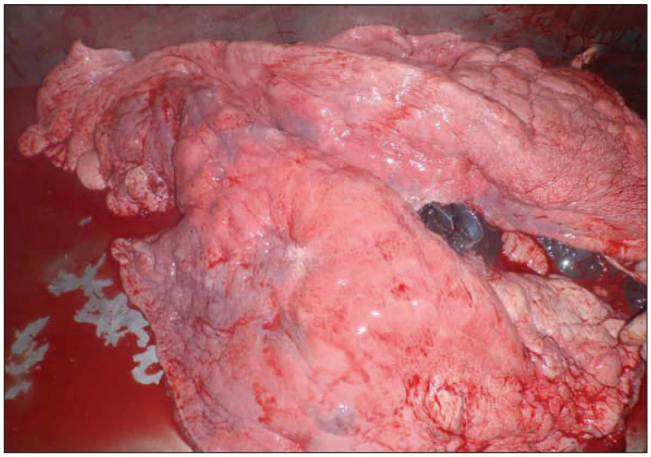


Fig 2. Macroscopic appearance of suppurative bronchopneumonia showed consolidation of the cranioventral and accessory lobes

Şekil 2. Kranioventral ve aksesor loblarda konsalidasyon ile karakterize Suppuratif bronkopnömoninin makroskopik görüntüsü

septa of alveoli were thickened by fibrosis and infiltration of mononuclear inflammatory cells. Emphysema and atelectasis were visible. Anthraco-silicosis particles were observed inside and outside of macrophages more adjacent to the vessels and bronchioles as well as lymphocytes infiltration (*Fig. 3* and *Fig. 4*). The color of anthraco-silicosis particles had variety from scant gray to brown or dense black, depending on the amount of silicon or carbon dust. Silicon particles were crystalline in different shapes including filamentous, oblong and polygonal *(Fig. 5)*. The areas with prominent deposition of crystalline silica rather than carbon were gray to light brown and focal interstitial fibrosis was occurred. In suppurative bronchopneumonia, in addition pneumoconiosis lesions, neutrophil-rich exudates were noted in the alveolar spaces and lumens of the airways, and in some occasions an admixture of cell debris, mucus, fibrin, and macrophages were observed in these areas.

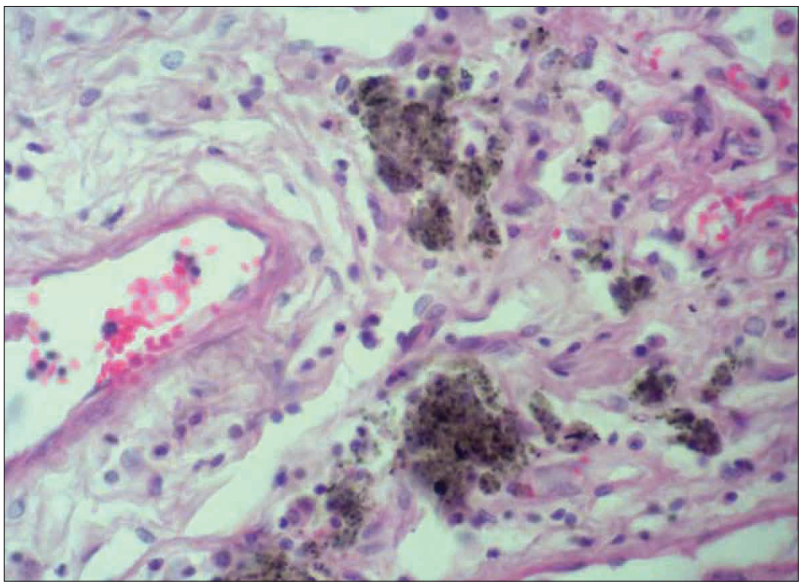
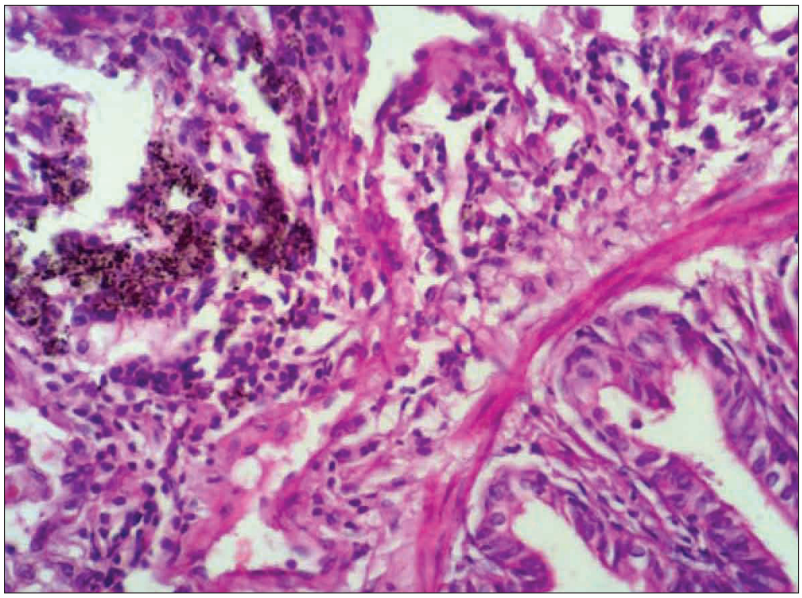


Fig 3. Histopathologic section shows black color of pulmonary parenchyma due to deposited carbon in the cytoplasm of macrophages and free in the parenchyma with fibrosis and lymphocytes infiltration adjacent to the vessel (H and E, ×400)

Şekil 3. Makrofajların sitoplazmasında ve boşlukta karbon birikimi nedeniyle pulmoner parankimde siyah renk ve fibrozis ile birlilkte dammar duvarına yakın lenfosit infiltrasyonu

Fig 4. Peribronchiolar deposition of carbon particles associated with lymphocytes infiltration (H and E, ×400)

Şekil 4. Lenfosit infiltrasyonu ile birlikte peribronşioler karbon partiküllerinin birikimi (HE, ×400)



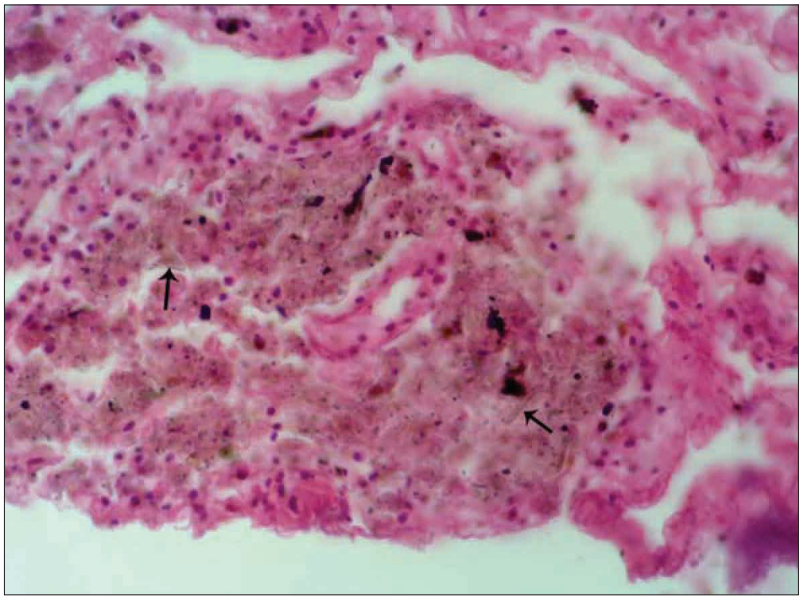


Fig 5. Gray to light brown pulmonary tissue due to crystalline silicon particles in different shapes including filamentous, oblong and polygonal (arrows) (H and E, ×400)

Şekil 5. Filamentöz, oblong ve polygonal şekilleri içeren değişik şekillerde kristal silika partikülleri nedeniyle gri açık kahverengindeki pulmoner doku

DISCUSSION

Accumulation of organic and inorganic particles in the pulmonary parenchyma causes a disease named pneumoconiosis [1,9]. Silicosis, antharcosis, and asbestosis are the most common types of pneumoconiosis, whereas siderosis, berylliosis, stannosis, and baritosis are uncommon forms [4]. Anthracosis and silicosis are terms that used for lesions induced by deposition of carbon and silicon in affected tissues. Carbon originates from coal mines, smoking, air pollution and fuels used for general usages such as cooking or home heating [10-13]. The breathing of fine crystalline silicon dioxide (silica) leads to pulmonary silicosis [14]. Occupational conditions such as mining, quarrying, and tunneling are associated with silicosis [15].

In animals, pneumoconiosis is not common and considerable. Those animals such as camels that live in arid or desert areas are in exposure to atmosphere rich in silicates due to bellowing the "sand wind" in deserts and carry a lot of dust particles into the respiratory system. Silica causes aggressive pneumoconiosis with fibrotic and nodular lesions [8].

Previously, the pathologists had belief that the carbon dusts are neutral and harmless pigments. Nowadays, it is demonstrated that carbon lead to fibro-inflammatory response in the tissues alone or in combination with other dust particles such as silica which causes anthraco-silicosis [16]. In human, anthracosis causes the obstruction of the large bronchioles and can make severe respiratory symptoms including dyspnea, coughing and susceptibility to infections [17]. Although, anthracosis has usually used for coal miners and rarely in city dwellers, but the experiences of some physicians have shown infectious diseases and malignancies are implicated in a background of anthracosis [18].

Diagnostic techniques for pneumoconiosis are including radiography and observation of small rounded or irregular opacities by chest X-rays and computed tomography (CT) [4,9]. Anthracosis can be recognized simply by light microscope as black-colored pigments along the respiratory airway or lymph nodes [19]. The minerals could be visible as birefringent crystals by polarized light [1].

In the present study, anthraco-silicosis was detected in 43 (28.7%) of 150 pneumonic lungs of slaughtered camels. The most common pneumonia associated with pneumoconiosis was interstitial pneumonia and then, bronchointerstitial and suppurative bronchopneumonia. Gross lesions related to anthraco-silicosis were not evident. Histopathologic lesions were different from diffuse to focal pulmonary fibrosis and mixed pigments of carbons and silicons. These particles were inside the cytoplasm of macrophages or as extracellulary around the bronchioles and vessels and, lesser in the pulmonary parenchyma. In the areas with prominent deposition of crystalline, affected tissues showed gray to light brown color and focal interstitial fibrosis.

Bekele [20] identified pneumoconiosis in the lungs and associated lymph nodes of 36 (34.62%) camels. Microscopic examination of lesions was varied from focal aggregates of dust-laden macrophages with diffused fibrosis (58.1%) to fibrous granuloma (41.9%). The fibrous granulomas were as firm nodules on palpation that located deeply in the lung parenchyma. About 48.4% of the pneumoconiotic lesions in the lungs were associated with the peribronchiolar or perivascular fibrosis and 39% revealed chronic pleuritis. 36.11% and 8.33% of pneumoconiotic lungs had emphysema and bronchopneumonia respectively. Aggregations of dust-laden macrophages were also visible in the bronchial lymph nodes. This researcher described that pneumoconiosis is a common environmental dangerous health that predisposes camels to secondary infections. These particles cause peribronchiolar fibrosis

and granulomas that disturb the normal airflow of the conducting systems and interfere with the defense mechanisms of the lungs [20]. This point is in agreement with the present study that pneumoconiosis was associated with the pneumonic lungs especially interstitial pneumonia and did not observe in the normal ones. In the previous reports of pneumoconiosis in camels, described pathologic features were similar to our results. Also, in a study by Hansen *et al*.[21] chronic interstitial pneumonia and fibrosis were observed in the pneumoconiotic lungs.

Xuanren [22] studied pneumoconiosis in the lungs and regional lymph nodes of 48 Bactrian camels by the light and electron microscope, electronic probe microanalysis technique and the mineralogical analysis. Pneumoconiosis lesions were diagnosed only in the lungs (n=13) and, the lungs with the regional lymph nodes (n=35). The pulmonary lesions were characterized by multifocal interstitial pneumonia with accumulation of dust laden macrophage. In the lymph nodes, affected cells showed the vacuolar mitochondria, swollen endoplasmic reticula and completely damage of the cytoplasmic organelles. Dust particles were mainly composed of aluminium silicate, the main substances of the sand dusts. They concluded that the camels may expose to sand dust particles for a long time and suffer from aluminium silicate pneumoconiosis [22]. Similar results were reported in a study on pneumoconiosis in camel by Zliuo *et al*.[23].

Beytut [24] studied gross and histopathological findings of anthracosis in lungs and local lymph nodes of sheep and its potential role in the occurrence of pneumonia in Turkey. Anthracosis was diagnosed in the lungs and regional lymph nodes of 45 (2.25%) out of 2000 sheep. Grossly, only 12 (26.6%) out of 45 antharcotic lungs showed patchy areas of dark red consolidation in the caudal and cranial lobes. No prominent pigmentation was detected in the other pneumoconiotic lungs. The lymph nodes were greyish to black in appearance. In histopathlogic examination, similar to the present study, focal accumulation of carbon-laden alveolar macrophages had been visible mainly around the terminal respiratory bronchioles. The cytoplasm of sinusoidal macrophages located in the medulla of the lymph nodes filled with phagocytized particles.

Özcan & Beytut [25] reported pneumoconiosis in 3.85% (n=27/700) of lungs and regional lymph node of slaughtered cattle in Turkey. In the lungs, black pigments areas observed grossly on the pleural surface and around the bronchioles bifurcation. Also, entire surface of the local lymph nodes were covered by carbon pigments. In agreement with our study, carbon particles accumulated freely in the peribronchiolar and perivascular tissues or in the cytoplasm of macrophages. Polluted environments impair the alveolar clearance and cause peribronchiolar and perivascular deposition of particles [1]. Perillo *et al*.[26] described lesions due to minerals deposition in the lung and regional lymph nodes of 60 out of 183 slaughtered cattle. Alveolar septa were thickened and foci of fibrosis and bronchopulmonary inflammation occurred. Silicon, aluminium, titanium, iron, carbon and small quantity of the other metals were detected by energy-dispersive X-ray microanalysis. They concluded that the cattle living in the polluted areas may be helpful in estimation of the environmental contaminants risk for human in exposure.

Roperto *et al*.[27] described silicate pneumoconiosis in 4 pigs lived near chalk quarries. The pulmonary lesions were comprised of the thickened alveolar septa and small foci of initial fibrosis. Free and intracytoplasmic dusts were present in the bronchiolar, alveolar and interstitial tissues. An energy dispersive X-ray microanalysis with a scanning electron microscope detected that these dust were composed mainly of silicon, calcium, potassium, sulphur, aluminium and iron. The same elements were found in the local lymph nodes. These researchers stated that domestic animals in polluted environments are an important biological source that gives helpful data for evaluation of human health risks.

Similar to pneumoconiosis in animals, nodular fibrosis adjacent to the vessels and bronchioles were reported in human [28-30]. In patients affected with coal worker pneumoconiosis, two morphologic features including coal macules and progressive massive fibrosis were observed. The size of coal macules ranged from 1 to 5 mm and was characterized by solid anthracotic pigmentation without intervening fibrotic tissue. Progressive massive fibrosis was identified by the presence of a fibrotic mass with diameter of more than 1 cm and carbon pigmentation. The fibrotic masses were constructed of arranged collagen with numerous pigment-laden macrophages and a lot of free pigments especially in their central regions [31,32]. Coal dusts inhalation lead to chronic obstructive pulmonary disease and ultimately death in patients [33,34]. Also, the risk of tuberculosis increases in coal worker pneumoconiosis [33]. Brambilla *et al*.[8] described that deposited crystals are associated with different pulmonary lesions, eg, pneumonia.

In animals, only clinically important pneumoconiosis is silicosis in horses and, rarely, dogs. It occurs in certain geographic areas with the soil containing crystalline silica. Particles in 0.5-5 µm size are the most likely particles that trappe in the distal airways, and filamentous particles are more injurious than amorphous ones. The clinical signs are including exercise intolerance, weight loss, and dyspnea. Miliary lesions are distributed throughout the lungs, and consist of fibrosis containing multifocal granulomatous with necrosis and mineralization in their centers. Birefringent, eosinophilic, or brown crystals are not clearly visible in the affected tissues. They are detectable by electron microscopy and X-ray spectroscopy [2].

The present study suggests a positive causal relationship between pneumoconiosis and occurrence of pneumonia. It seems these particles predispose animals to

pulmonary diseases especially various types of pneumonia. Further researches are necessary for understanding the pathogenesis of various particles and their effects on immune system of the lungs and occurrence of pulmonary diseases.

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RESEARCH ARTICLE

Physiological Effects of Water Temperatures in Swimming Toy Breed Dogs [1]

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Summary

The purpose of the present study was to examine the effect of water temperature on heart rate and respiratory rate during swimming, as well as changes in rectal temperature, blood glucose and blood lactate before and after swimming. Twenty-one small breed dogs (male = 9, female = 12) were used as subjects of this study. Dogs swam for 20 min in different water temperatures: 25°C, 33°C and 37°C. Heart rate and respiratory rate were monitored every 5 min during swimming. Blood samples were obtained before and after swimming for analysis of glucose and lactate levels. Rectal temperature was measured before and after swimming. The results showed that dogs that swam in 25°C water had the highest heart rate and serum glucose level (significant difference, $P<0.05$). The highest respiration rate was found in dogs that swam in 37°C water ($P<0.05$). Serum lactate significantly ($P<0.05$) increased after 20 min swimming at all water temperatures. Thus, the dogs should swim in 33°C water to prevent tachycardia, hyperventilation and hyperthermia.

Keywords: *Dog, Heart rate, Lactate, Respiration rate, Water temperature, Swimming*

Su Sıcaklığının Yüzdürülen Küçük Cüsseli Köpeklerdeki Fizyolojik Etkileri

Özet

Bu çalışmanın amacı su sıcaklığının yüzme öncesi ve sonrası kalp atım ve solunum sayılarına, rektal sıcaklığa, kan glikoz ve laktat seviyelerine olan etkisini araştırmaktır. Yirmi bir küçük cüsseli köpek (erkek=9, dişi=12) bu çalışmada denek olarak kullanıldı. Köpekler 20 dakika farklı ısılardaki suda (25°C, 33°C ve 37°C) yüzdürüldü. Kalp atım ve solunum sayıları yüzme süresince 5 dakikada bir ölçüldü. Glikoz ve laktat ölçümleri için yüzme öncesi ve sonrasında kan örnekleri alındı. Rektal sıcaklık yüzme öncesi ve sonrası ölçüldü. Sonuçlar 25°C'de yüzdürülen köpeklerde kalp atım sayısı ve serum glikoz seviyesinin en yüksek olduğunu ortaya koydu ($P<0.05$). En yüksek solunum sayısı 37°C suda yüzdürülen köpeklerde tespit edildi ($P<0.05$). Serum laktat seviyesi 20 dakika yüzme sonrasında tüm su sıcaklıklarında anlamlı derecede artma gösterdi ($P<0.05$). Sonuç olarak köpekler taşikardinin, hiperventilasyonun ve hiperterminin engellenmesi amacıyla 33°C suda yüzdürülmelidir.

Anahtar sözcükler: *Köpek, Kalp atım sayısı, Laktat, Solunum sayısı, Su sıcaklığı, Yüzme*

INTRODUCTION

Swimming is one of the most popular exercises, and has many therapeutic benefits as well. It can increase muscle strength and endurance, and improve cardiovascular system function. Swimming exercise increases blood flow, resulting in increased oxygen supply to the muscles and a higher rate of release of waste products from muscles into the bloodstream [1]. Moreover, exercising in water can be beneficial by minimizing weight-bearing forces, thus reducing pain and allowing improved range of joint motion and muscle strength [2,3]. The therapeutic properties

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of water include buoyancy, resistance, and hydrostatic pressure.

However, swimming in a pool also can result in some complications. Nganvongpanit and Yano [4] reported on the side effects in 412 dogs (male = 219 and female = 193) from swimming in a chlorinated swimming pool; these included dry hair (20.63%), dry skin (18.93%), and abrasion wounds at the armpit (15.78%), all of which increased with greater frequency of swimming. Other adverse effects were red eye (13.59%), otitis (6.31%), and a small number of respiratory problems (0.49%).

The functional and metabolic responses to swimming are influenced by duration, intensity, frequency, and environmental conditions. Water temperature during swimming is one of the most important factors involved in these physiological changes. Previous studies have proven that water possesses higher specific heat and thermal conductivity than air, by approximately 25-fold [1,5]. Hence, exercise in water places a much more severe thermal load on the body compared to exercise in air [6-8].

Many researchers have studied the effects of water temperature in animal models and in humans [1,9-16]. Those reports found that low water temperature close to body temperature resulted in longer exercise compared to higher water temperature close to body temperature and very low temperature (lower than 20°C), respectively. However, as yet there have been no reports on the effect of water temperature on physiological changes in dogs during swimming. Swimming for the exercise and rehabilitation of dogs has become of increasing interest in the field of veterinary medicine; however there is still only a limited amount of basic data. For this reason, this study has investigated the effects of water temperature on some physiological changes in dogs during swimming, including heart rate, respiratory rate, blood glucose and lactic acid.

MATERIAL and METHODS

Animals

Twenty-one small breed dogs (male = 9, female = 12) including Poodle (n = 1), Chihuahua (n = 15) and Pomeranian (n = 5) served as subjects in this study. All animals were healthy, age 55.6±12.05 months, weight 3.10±1.01 kg. The dogs were examined by a veterinarian through a physical examination, blood evaluation (cell count and blood chemistry) and analysis of radiographic images of the heart and lungs. The experimental protocol was approved (2012) by the Faculty of Veterinary Medicine and the Ethics Committee, Chiang Mai University, Thailand.

Experimental Design

All animals were trained to swim on at least 5 or 6 occasions prior to the start of the experiment, in order to prevent overly excited behavior during swimming which could affect the results [17]. During the experiment, dogs were allowed to swim 20 min for collection of data [17]. This study used a mobile whirlpool (V.S. Engineering, Bangkok, Thailand). Three different water temperatures were used in this study: 37±2°C, 33±2°C and 25±2°C. All dogs are swam in three different temperatures 1 week interval.

Data Collection

Physiological data - including heart rate, respiratory rate, body temperature, blood glucose, and blood lactic acid - were measured. Heart rate during the experiment was recorded real time using a pulse watch (CHF-100-1VDR; Casio, Tokyo, Japan), while respiration rate was monitored by two veterinarians using counting machine (SDI 1055, Thailand). Rectal temperature was detected using a rectal thermometer. Two ml of blood was collected from the cephalic vein two times, pre- and post-swimming; these samples were used to evaluate glucose and lactic acid levels. Blood glucose was measured using blood glucose electrodes (Optium Xceed, MediSense®; Abbott Diabetes Care, Doncaster, Australia). Blood lactate was analyzed using an automated high-throughput system (DiaSys Diagnostic Systems, Holzheim, Germany) at a diagnostic laboratory at Maharaj Nakorn Chiang Mai Hospital, Faculty of Veterinary Medicine, Chiang Mai University, Thailand.

Statistical Analysis

The heart and respiration rates of subjects were measured at 5 min intervals, from pre-swimming (0 min) to the end of the testing period (20 min). This data, as well as rectal temperature and levels of blood lactate and glucose pre- and post-swimming, are presented as means. Relative changes of rectal temperature, blood lactate and glucose pre- and post-swimming were also calculated to compare between the three groups. Differences in mean values between two or more experimental groups were tested using ANOVA, followed by multiple pairwise comparisons using a *t*-test. Differences of $P<0.05$ were considered to be significant. All data were analyzed using SPSS version 17.0.1 software.

RESULTS

Heart rate increased during 20 min of swimming in all three water temperatures *(Fig. 1)*. However, heart rate in water temperature of 25°C showed a significantly higher increase ($P<0.05$) compared to the other two temperatures (33 and 37°C), beginning after 5 min of swimming and continuing through the end of the testing period (20 min). After 20 min, the heart rate of dogs swimming in 33°C water was significantly higher ($P<0.05$) than that of dogs swimming in 37°C water.

Respiration rate increased during 20 min of swimming only in dogs swimming in a water temperature of 37°C

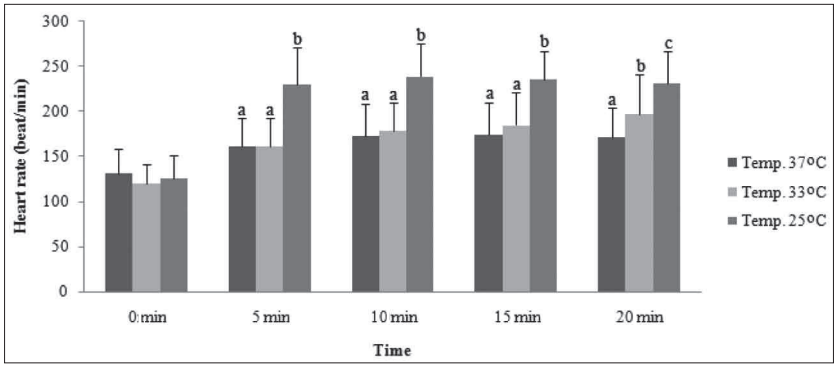
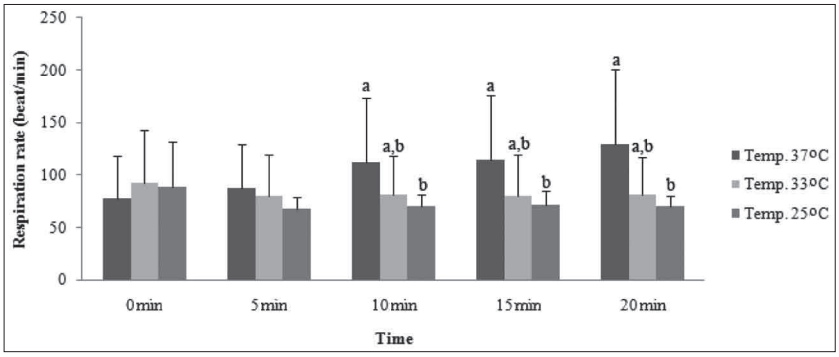


Fig 1. Mean (± SD) change in heart rate during swimming in different water temperatures. Individual bars show the mean ± SD. Significant differences (P<0.05) between the three temperatures at the same time are indicated by superscripts (a,b,c) above the bars

Şekil 1. Değişik sıcaklıklardaki suda yüzme sırasında kalp atım sayısının ortalama (± Standart sapma) değişimi. Bağımsız sütunlar ortalama ± standart sapmayı göstermektedir. Aynı zamanda üç sıcaklık için anlamlı derecedeki farklar (P<0.05) sütunların üzerinde üst simge olarak (a,b,c) belirtilmiştir

Fig 2. Mean (± SD) respiration rate change during the experiment in different water temperatures. Individual bars show the mean ± SD. Significant differences (P<0.05) between the three temperatures at the same time are indicated by superscripts (a,b) above the bars

Şekil 2. Değişik sıcaklıklardaki suda yüzme sırasında solunum sayısının ortalama (± Standart sapma) değişimi. Bağımsız sütunlar ortalama ± standart sapmayı göstermektedir. Aynı zamanda üç sıcaklık için anlamlı derecedeki farklar (P<0.05) sütunların üzerinde üst simge olarak (a,b) belirtilmiştir



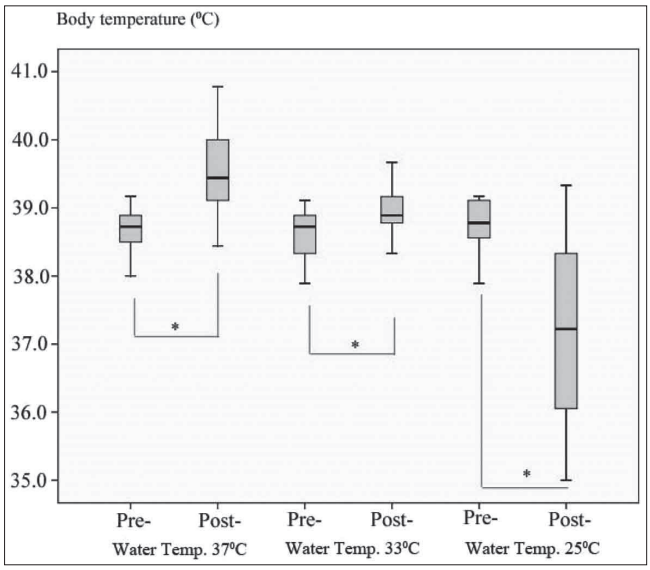


Fig 3. Comparative body temperature between pre- and post-swimming in different water temperatures. A significant difference (P<0.05) was found between pre- and post-swimming at the same water temperature (*)

Şekil 3. Değişik sıcaklıklardaki suda yüzme öncesi ve sonrası orantısal vücut sıcaklıkları. Aynı sıcaklıktaki suda (*) yüzme öncesi ve sonrası anlamlı derecede fark (P<0.05) tespit edildi

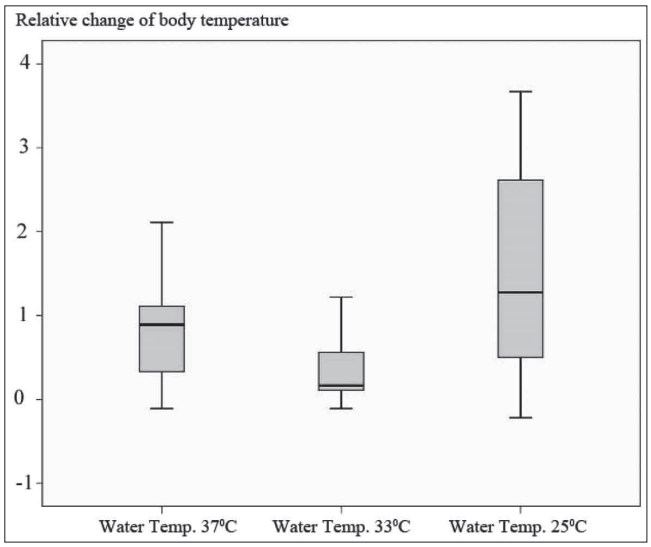


Fig 4. Relative change of body temperature in dogs swimming in different water temperatures

Şekil 4. Değişik sıcaklıklardaki suda yüzen köpeklerde orantısal vücut sıcaklığı değişimi

(Fig. 2). When dogs swam in water temperatures of 33 and 25°C, their respiration rate decreased. The respiration rate of dogs swimming in 25°C water was significantly lower (P<0.05) than that of dogs swimming in 37°C water after 10, 15 and 20 min of swimming.

Body temperature after swimming was significantly different (P<0.05) compared to pre-swimming *(Fig. 3)*. After swimming in 37°C water, body temperature increased from 38.68±0.32°C to 39.54±0.63°C. After swimming in 33°C water, body temperature increased from 38.59±0.38°C to 38.92±0.47°C. Conversely, swimming in 25°C water resulted in a decrease in body temperature, from 38.74±0.34°C to 37.22±1.27°C. However, the relative changes of body temperature after swimming in all water temperatures were not significantly different *(Fig. 4)*: 0.86±0.63, 0.33±0.47 and 1.52±1.22 in water temperatures of 37, 33 and 25°C, respectively.

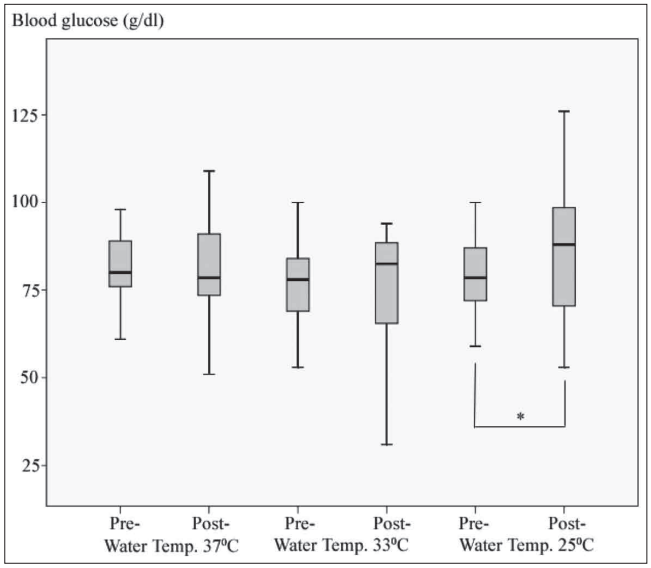


Fig 5. Comparative blood glucose levels between pre- and post-swimming in different water temperatures. A significant difference (P<0.05) was found between pre- and post-swimming at the same water temperature (*)

Şekil 5. Değişik sıcaklıklardaki suda yüzme öncesi ve sonrası orantısal kan glikoz seviyeleri. Aynı sıcaklıktaki suda (*) yüzme öncesi ve sonrası anlamlı derecede fark (P<0.05) tespit edildi

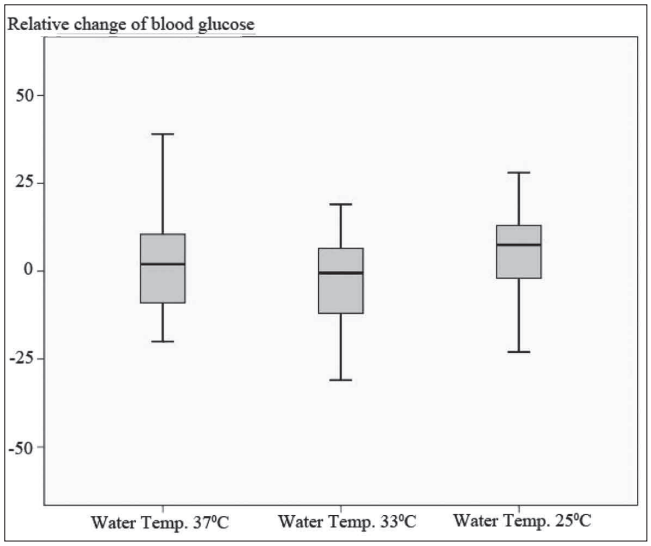


Fig 6. Relative change of blood glucose in dogs swimming in different water temperatures

Şekil 6. Değişik sıcaklıklardaki suda yüzen köpeklerde orantısal kan glikoz değişimi

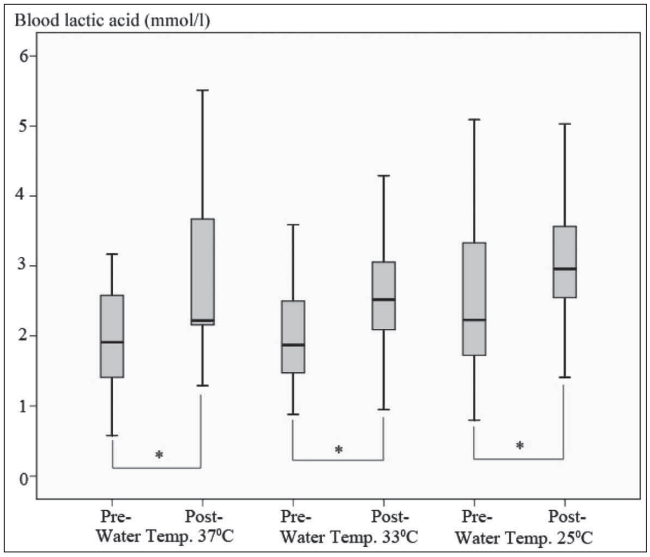


Fig 7. Comparative blood lactic acid levels between pre- and post-swimming in different water temperatures. A significant difference (P<0.05) was found between pre- and post-swimming at the same water temperature (*)

Şekil 7. Değişik sıcaklıklardaki suda yüzme öncesi ve sonrası orantısal kan laktik asit seviyeleri. Aynı sıcaklıktaki suda (*) yüzme öncesi ve sonrası anlamlı derecede fark (P<0.05) tespit edildi

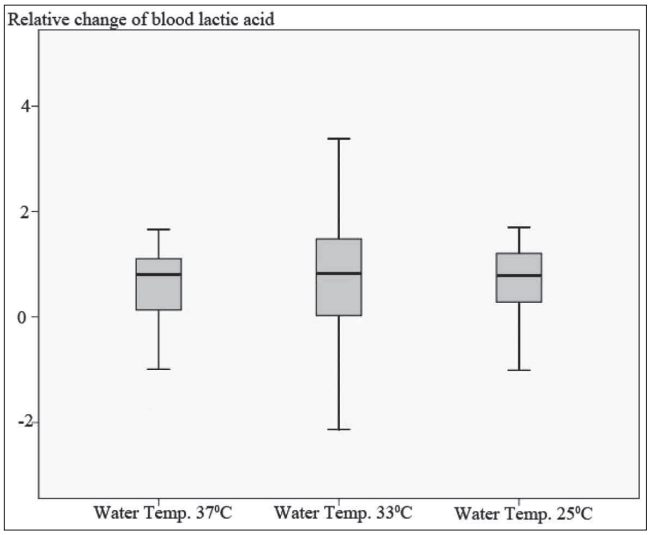


Fig 8. Relative change of blood lactic acid in dogs swimming in different water temperatures

Şekil 8. Değişik sıcaklıklardaki suda yüzen köpeklerde orantısal kan laktik asit değişimi

After swimming in 37°C water, blood glucose level slightly increased, from 81.35±10.61 g/dl to 81.90±19.69 g/dl. After swimming in 33°C water, blood glucose increased from 76.90±12.47 g/dl to 79.11±14.26 g/dl. But swimming in 25°C water resulted in a significant increase (P<0.05) in blood glucose, from 79.05±11.16 g/dl to 86.05±19.09 g/dl *(Fig. 5)*. However, the relative changes of blood glucose after swimming in different water temperatures were not significantly different *(Fig. 6)*: 0.85±20.44, 0.94±10.53 and 7.00±14.39 in water temperatures of 37, 33 and 25°C, respectively.

For all three water temperatures, blood lactic acid level after swimming significantly (P<0.05) increased compared to pre-swimming *(Fig. 7)*. Swimming in 37°C water resulted in a blood lactic acid increase from 1.23±1.26 to 2.83±1.07 mmol/l; in 33°C water, from 2.08±0.82 to 2.89±1.25 mmol/l; and in 25°C water, from 2.68±1.22 to 3.48±1.58 mmol/l. However, the relative changes of blood lactic acid after swimming in different water temperatures were

not significantly different *(Fig. 8)*: 0.60±1.07, 0.81±1.29 and 0.79±1.05 in water temperatures of 37, 33 and 25°C, respectively.

DISCUSSION

Swimming has become increasingly popular in small animal medicine for purposes such as exercise and rehabilitation. This work is the first to report on the effect of water temperature on physiological changes in dogs. This study found that there were significant changes in heart rate, respiratory rate, body temperature, serum lactate and glucose level after swimming compared to pre-swimming. The results also showed a significant difference in heart rate and respiratory rate between the three water temperatures used in the experiment. However, there were some limitations of this study. Because the necessary instruments were not available, heart function, blood pressure, oxygen consumption and carbon dioxide production could not be measured.

Previous studies in rats and mice [9,11,18] as well as humans [13-15,19] have shown a decreased heart rate during swimming in cold water. In humans, a water temperature slightly lower than body temperature was found to decrease heart rate [20], while water temperature higher than body temperature resulted in an increased heart rate [16]. This can be explained by the fact that swimming in water with a temperature lower than body temperature can cause peripheral vasoconstriction, resulting in increased blood pressure. Higher blood pressure affects the baro-receptor reflex, causing a decreased heart rate with a consequent reduction in blood volume and, finally, decreased blood pressure [21]. When swimming in lower water temperature, the body's muscles require additional blood supply. This can result in a decrease in the amount of venous blood returning to the heart, which inactivates the baroreceptor reflex, causing an increase in heart rate. While swimming in a water temperature that similar to body temperature results in vasodilatation, which decreases blood pressure. This causes an increase in the heart rate in order to increase blood volume; however, this increase is less than when swimming in lower water temperature [10,12,22]. However, there is some evidence that conflicts with the changes mentioned above. A report by Graham [23] found no difference in heart rate among women during exercise in air temperatures of 5°C and 22°C. There are several factors that can influence changes in heart rate during exercise: level of exercise, relative workload, stress, shivering, increased muscle tone, and the sympathetic nervous system [12,23].

The present study found that swimming in 25°C water resulted in an increased heart rate, compared to the other two water temperatures; this result differed from other previous reports, as mentioned above [7,9,14]. Three possible explanations could account for the elevation of heart rate in dogs during swimming in 25°C water. First, the levels of catecholamine (norepinephrine, epinephrine and dopamine) are higher during exercise in cold air and water [12]. For this reason, it is possible that the increased heart rate in cold water is an effect of catecholamine. It may also be noteworthy that the level of glucose in serum after swimming in 25°C water was found to be significantly elevated, while the other two groups showed no significant change. Second, a dog's hair can prevent vasoconstriction of the peripheral vessels on the skin; thus the blood pressure does not change, resulting in non-stimulation of the baroreceptor reflex and consequently no decrease in heart rate. In humans it was found that skin temperature was closely related to water temperature during swimming [24]; however this has not yet been proven in dogs. Finally, dogs began shivering while swimming in lower water temperature, which resulted in increased heart rate [25]. However, to conclusively confirm the reason that heart rate was elevated in dogs during swimming in lower temperature water, further experiments must be performed: for example, measuring differences in blood pressure during swimming in lower or higher water temperature than used in this study, and measuring some related hormones (i.e. adrenaline, noradrenaline, cortisol, insulin and glucagon).

In this study, the respiration rate in dogs swimming in 37°C water was found to be significantly increased compared with the other two water temperatures, 33°C and 25°C, in which the respiration rate decreased. Since dogs, unlike humans, do not have sweat glands on the skin, their body heat is decreased primarily from respiration and secondly from evaporation. Hence, swimming in higher water temperature can cause increased body heat, resulting in increased respiration rate [22]. The present study found that after 20 min of swimming in 37°C water, respiration rate was elevated by 67% compared to the resting period. Swimming in low water temperature resulted in lower heat production. Dogs were able to reduce body temperature via evaporation, resulting in decreased respiration rate. This study also found that respiration rate was down-regulated by 20% compared to the resting period after 20 min of swimming in lowest rate [12].

Animal hair functions to protect against environmental temperature [22]. To prevent the effect of the hair coat from interfering with accurate measurement of body temperature, the hair of all long-haired breeds in this study, i.e. Poodles and Pomeranians, was clipped to a length of 1 to 3 cm, similar to that of Chihuahuas.

Rectal temperature after swimming in 33°C and 37°C water was significantly increased compared to pre-swimming (1% and 1.5%, respectively). But after swimming in 25°C water, body temperature was significantly decreased (3%). The reason for the increase in body temperature during swimming in water that is close to body temperature is that dogs are not able to release heat from the body

when swimming in a water temperature similar to body temperature. But heat can be conducted from the body when dogs swim in low water temperature (25°C), resulting in a significant decrease in body temperature. Previous studies [9,26] have suggested that a change in body temperature during swimming is an important determinant of swimming capacity. The present results indicate that healthy dogs should swim in lower temperature water in order to prevent hyperthermia, as opposed to swimming in water close to body temperature.

Blood glucose after swimming in 33°C and 37°C water was not significantly different compared to pre-swimming; but after swimming in 25°C water, blood glucose was significantly higher (9%). However, the relative changes of blood glucose after swimming in the three different water temperatures were not significantly different. During exercise, epinephrine and norepinephrine inhibit insulin function, preventing glucose uptake into cells; they also activate glycolysis in the liver to increase the glucose level in blood [27]. Other studies in rats have also shown the same result [28-30]. However, this effect might be evident only during the first 20-30 min of exercise. Galbo and collages [13] reported that humans swimming in lower water temperature had higher serum glucose level during the first 20-30 min; after that time through the end of study (75 min), the level of serum glucose in humans swimming in higher temperature water was up-regulated to a greater extent than in the other groups. That study also found that the serum insulin level during the first 20-30 min in humans swimming in lower temperature water was higher compared to those swimming in higher temperature; but after that time through the end of study, the level of serum insulin in those swimming in higher temperature water was up-regulated to a greater extent than in the other groups.

Serum lactate can be used as an indicator of anaerobic metabolism during exercise [28]. In a rat model, serum lactate level was significantly increased after swimming in 25°C, 33°C and 37°C water [28]. This study also found a significantly higher level of serum lactate after swimming in every water temperature. The relative changes of serum lactate after swimming in the three different water temperatures were not significantly different. This was in accordance with a report by Mougios and Deligiannis [7], who found no significant difference in serum lactate between swimming in different water temperatures (20°C, 26°C and 32°C). However, other studies have shown a contrasting result [13,14]. Holmér and Bergh [14] reported significantly increased lactate when swimming in lower water temperature (18°C) compared to higher temperature (24°C and 34°C). They concluded that the higher lactate level from swimming in lower water temperature resulted from shivering. Another published report suggested that lactate level in the blood increased because of decreased blood supply to muscles due to vasoconstriction [31]. Other studies have suggested that because the heart rate decreases in lower water temperature, the reduction in cardiac output results in decreased oxygen supply throughout the body, which in turn increases anaerobic metabolism [9,11]. Galbo and colleages [13] also reported that during 75 min swimming, the serum lactate level in humans who swam in 21°C water was higher compared to those who swam in 33°C water. The peak level of serum lactate in humans was found after 5-15 min [13], depending on various factors: for example, type of exercise, intensity, level of exercise, and environmental temperature.

No reports have been published on serum lactate level in dogs during exercise, although several studies have been conducted in humans and rats [7,13,28,31]. The changes of serum lactate level during swimming are able to represent metabolic activity (anaerobic or aerobic metabolism), which can help the dog trainer or veterinarian in designing an appropriate exercise program. Future studies to determine serum lactate level during exercise under various conditions would also be useful for this purpose.

Therefore, the results of this study could increase veterinarians' understanding of the physiological changes in dogs during swimming in water at varying temperature. Most of the physiological responses in dogs during swimming are similar to those of others mammals. Based on the results of this study, swimming in a water temperature between 25-33°C is recommended for older dogs or for dogs with heart and/or respiratory disease in order to prevent tachycardia, hyperventilation and hyperthermia.

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RESEARCH ARTICLE

Accumulation of Nonessential Potentially Toxic Trace Elements (PTEs) in the Some Economically Important Seafood Species of Mediterranean

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Summary

This study was conducted to determine the levels of some nonessential potentially toxic trace elements (PTEs) (Ag, Al, As, Ni, Sn) of some economically important seafood species *(Mullus barbatus*, Linnaeus, 1758, *Mugil cephalus,* Linnaeus, 1758, *Panaeus semisulcatus,* De Haan, 1844) caught in the Gulf of Antalya Mediterranean Sea by using ICP-OES (Inductively Coupled Plasma-Optic Emission Spectrophotometer). The averages and the standard deviations of the results of the samples were determined as Ag (0.030±0.017 mg/kg), Al (12.163±7.298 mg/kg), As (0.269±0.121 mg/kg), Ni (0.084±0.067 mg/kg), Sn (0.022±0.003 mg/kg) for the Red Mullet *(M. barbatus)*, Ag (0.038±0.024 mg/kg), Al (11.120±4.019 mg/kg), As (0.140±0.082 mg/kg), Ni (0.060±0.050 mg/kg), Sn (0.022±0.003 mg/kg) for the Grey Mullet *(M. cephalus)*, Ag (0.032±0.029mg/kg), Al (20.924±9.829 mg/kg), As (0.249±0.116 mg/kg), Ni (0.124±0.102 mg/kg), Sn (0.026±0.004 mg/kg) for the Green Tiger Prawn *(P. semisulcatus)*. The determined levels of trace elements were below the available daily intake limits except Al. Also evaluation of other studies conducted in the Gulf of Antalya, Al levels are higher. These results showed that, although increasing the legal rules about environmental pollution, seafood species which caught from the Gulf are exposed to Al in a higher rate.

Keywords: *Gulf of Antalya, Mediterranean Sea, Potentially toxic trace element, Seefood*

Akdeniz'in Bazı Ekonomik Deniz Ürünü Türlerinde Esansiyel Olmayan Potansiyel Toksik İz Elementlerin Birikimi

Özet

Bu çalışma Akdeniz Antalya Körfezi'nde avlanan ekonomik öneme sahip bazı deniz ürünleri türlerindeki *(Mullus barbatus,* Linnaeus, 1758, *Mugil cephalus,* Linnaeus, 1758, *Panaeus semisulcatus,* De Haan, 1844) bazı esansiyel olmayan potansiyel toksik iz elementlerin (PTEs) birikim düzeylerinin ICP-OES (İndüktif Eşleşmiş Plazma-Optik Emisyon Spektrometresi) kullanılarak belirlenmesi için yapılmıştır. Örneklere ait belirlenen sonuçların ortalama ve standart sapmaları Barbunya *(M. barbatus)* için Ag (0.030±0.017 mg/kg), Al (12.163±7.298 mg/kg), As (0.269±0.121 mg/kg), Ni (0.084±0.067 mg/kg), Sn (0.022±0.003 mg/kg) olarak; Kefal *(M. cephalus*) için Ag (0.038±0.024 mg/kg), Al (11.120±4.019 mg/kg), As (0.140±0.082 mg/kg), Ni (0.060±0.050 mg/kg), Sn (0.022±0.003 mg/kg) olarak ve Yeşil Kaplan Karidesi *(P. semisulcatus)* için Ag (0.032±0.029 mg/kg), Al (20.924±9.829 mg/kg), As (0.249±0.116 mg/kg), Ni (0.124±0.102 mg/kg), Sn (0.026±0.004 mg/kg) olarak belirlenmiştir. İz elemetlerin belirlenen düzeyleri Al dışındakilerde ulaşılabilen günlük alım limitlerinin altında kalmıştır. Ayrıca Antalya Körfezi'nde yapılmış diğer çalışmalar değerlendirildiğinde Al düzeyleri yüksektir. Bu sonuçlar çevre kirliliği konusundaki yasal yaptırımların artmasına rağmen körfezden avlanan deniz ürünlerinin Al'a yüksek oranda mağruz kaldığını göstermiştir.

Anahtar sözcükler: *Akdeniz; Antalya Körfezi; Deniz ürünü; Potansiyel toksik iz element*

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INTRODUCTION

Intense human activities (urbanisation, tourism, coastal population, agriculture, maritime traffic, industry, nuclear power stations, mining and the influence of fisheries) in regions surrounding enclosed and semi-enclosed seas such as the Mediterranean Sea always produce, in the long term, a strong environmental impact in the form of coastal and marine degradation and a heightened risk of more serious damage [1-4]. There is a large range of different activities scattered all around the Mediterranean basin and a number of hot-spots are concentrated mainly in the north-east like *Gulf of Antalya*, generated by tourism, urbanization, agriculture, industry and commercial harbours [5,6]. The degree and extent of heavy metal pollution has been one of the main topics studied in environmental geochemistry [7]. The term *heavy metal* has been widely used and inadequately described in scientific literature over the past two decades. The term is often defined as metals and metalloids that have been associated with contamination or potential toxicity to an environment. However, there is no authoritative definition of this term in the relevant literature and recommended a new classification based on the periodic table that reflects an understanding of the chemical basis of toxicity [8]. However, no such a classification has been accepted at this time. Therefore, to use the term *potentially toxic elements* would be more scientific instead of the term *heavy metals*. When a heavy metal enters a food web, organisms can react to its bioavailability in different ways. Some organisms may discriminate against the uptake of one or more potentially toxic metals. Others may incorporate the metal(s) in their soft or hard parts in proportion to the concentration(s) in the growth environments, excreting any excess. Still other organisms may be tolerant of heavy metals and will accumulate concentrations greatly in excess amounts in a growth environment without any damage. Once a metal is incorporated, it is distributed to tissues (muscle) and organs (kidney, lung) [9,10]. Although excretion typically occurs through the kidneys and digestive tract, metals tend to persist in sites like the liver, bones, and kidneys for years or decades. Individual variability in vulnerability to metal toxicity remains a subject of investigation. Low-level metals exposure likely contributes to chronic disease and impaired functioning [9]. Non-essential elements where human toxicity has been reported includes Pb, Cd, Hg, As, Al, Ba, Li, Pt, Te, Ti, Sb, Be, Ga, In, V, Ni, Sr, Sn, Ge, Ag, Au, Bi, Tl, and U [11,12]. Determination the levels of trace elements is extremely important in terms of despite are needed for some physiological process but are toxic over specific doses on this processes, uses to determine the extent of exposure to environmental pollution, gives information about the body nutrisiyonel status, plays a role in the diagnosis and treatment of some diseases its relationship with occupational diseases. As mentioned above, determination trace elements levels is a methot for used to in order to understand with which concentrations organisms exposed to environmental pollution. The increase in the amount of environmentally induced diseases parallel with increasing environmental pollution as a result of developing technology, industrialization and urbanization is extremely worryingin [13].

The present study was conducted to determine the levels of some nonessential potentially toxic trace elements (Ag, Al, As, Ni, Sn) of some economically important seafood caught in the Gulf of Antalya Mediterranean Sea.

MATERIAL and METHODS

Sampling and Study Areas

In this study, 35 Red Mullet Red *(Mullus barbatus,* L., 1758), 35 Grey Mullet *(Mugil cephalus*, L., 1758) and 35 Green Tiger Prawn *(Panaeus semisulcatus*, De Haan, 1844) species collected and used for analysis which caught in the Gulf of Antalya Mediterranean Sea. Total 105 samples of muscle tissues nonessential potentially toxic trace elements (Ag, Al, As, Ni, Sn) levels were determined. The fish and prawn samples collected between 2011-2012 from Kemer, Center of Antalya, Manavgat, Alanya and Gazipaşa stations. After fish and prawns height and weight measure, muscle tissues were taken with sterile dissection tools. And the samples were stored in plastic bags and kept at -20°C in deep freezer until analyzed.

Trace Element Analysis

In order to prevent contamination of samples, all the materials used in study were washed in HNO_3 (10%) and dried (70°C). 0.5 g of homogenized each sample get into porcelain crucibles and were mixed with 0.5 ml magnesium acetate (2 mg/g). After dryed at 100°C for 3-4 h, the samples were ashed at 600°C for 6-8 h. The ash was extracted with nitric acid (HNO_3) 2N and was diluted to 15 ml. This filtrate content of trace elements were analyzed by using ICP-OES according to the EPA metot 200.7 [14]. The accuracy of the instrument was periodically checked with a known standard. Calibration curves were prepared using dilutions of stock solutions. The results were read three times and the mean values and the relative standard deviations were computed.

Statistical Analysis

Data are statistically analysed using the statistics software (SPSS® 17.0.0) by one-way analysis of variance (ANOVA) and presented as min-max values and mean ± standard deviation (SD) *(Table 1)*. When significant treatment effects were detected, DUNCAN'S multiple range test was used to identify specific differences between the metal accumulation means at a probability level of $P<0.05$.

RESULTS

The averages and the standard deviations of the results of the samples were determined as Ag (0.030±0.017 mg/kg), Al (12.163±7.298 mg/kg), As (0.269±0.121 mg/kg), Ni (0.084±0.067 mg/kg), Sn (0.022±0.003 mg/kg) for the Red Mullet *(M. barbatus)*, Ag (0.038±0.024 mg/kg), Al (11.120±4.019 mg/kg), As (0.140±0.082 mg/kg), Ni (0.060±0.050 mg/kg), Sn (0.022±0.003 mg/kg) for the Grey Mullet *(M., cephalus)*, Ag (0.032±0.029 mg/kg), Al (20.924±9.829 mg/kg), As (0.249±0.116 mg/kg), Ni (0.124±0.102 mg/kg), Sn (0.026±0.004 mg/kg) for the Green Tiger Prawn *(P. semisulcatus)*. The results are shown in *Table 1* as the average, standard deviation, minimum and maximum values of nonessential potentially toxic trace element concentrations in the muscle tissues of Red Mullet *(Mullus barbatus)*, Grey Mullet *(Mugil cephalus)* and Green Tiger Prawn *(Panaeus semisulcatus)*.

DISCUSSION

It was concluded that determined Ag, Al, As, Ni, Sn, levels of Red Mullet (*M. barbatus*), Grey Mullet *(M. cephalus)* and Green Tiger Prawn *(P. semisulcatus)* caught in the Gulf of Antalya Mediterranean Sea generally were below the other studies of Mediterranean *(Table 2)*. Al, Ni levels of

Tablo 1. *Nonesansiyel toksik iz elementlerin konsantrasyonları (mg/kg)*
Table 1. *Concentrations of nonessential potentially toxic trace elements (mg/kg)*

Element	Species		
	Red Mullet *(Mullus barbatus)* **Mean±SD Min. – Max.**	**Grey Mullet *(Mugil cephalus)*** **Mean±SD Min. – Max.**	**Green Tiger Prawn *(Panaeus semisulcatus)*** **Mean±SD Min. – Max.**
Silver (Ag)	0.030±0.017 0.006-0.070	0.038±0.024 0.006-0.131	0.032±0.029 0.006-0.169
Aluminum (Al)	12.163±7.298 6.890-41.600	11.120±4.019 6.610-22.300	20.924±9.829 8.570-49.900
Arsenic (As)	0.269±0.121 0.61-0.563	0.140±0.082 0.042-0.391	0.249±0.116 0.102-0.562
Nickel (Ni)	0.084±0.067 0.021-0.336	0.060±0.050 0.018-0.295	0.124±0.102 0.032-0.524
Tin (Sn)	0.022±0.003 0.016-0.031	0.022±0.003 0.014-0.032	0.026±0.004 0.018-0.039

Tablo 2. *Diğer çalışmalarda belirlenen nonesansiyel PTEs konsantrasyonları*
Table 2. *Determinated nonessential PTEs concentrations in other studies*

Species	Element	Concentration	Location	Reference
Mullus barbatus	Al	6.67 mg/kg	Gulf of İskenderun, Turkey	[15]
	Ni	LOD		
	As	27.01 ppm	Channel of Sicily, Italy	[16]
	Ni	0.042 ppm		
	As	10.35-23.71mg/kg	Iberian, Spain	[17]
	Al	6.676 µg/g	Gulf of İskenderun, Turkey	[18]
	Ni	0.001 µg/g		
	Al	2.60 µg/g	West Black Sea Coasts, Turkey	[19]
	Ni	0.63 µg/g		
	Al	8.384 µg/g	Black Sea and Mediterranean Coast, Turkey	[20]
	Ni	0.663 µg/g		
Penaeus semisulcatus	Ag	1.5-2.8 µg/g	Gulf of İskenderun, Turkey	[21]
	Ni	0.6-3.6 µg/g		
Mugil cephalus	Ni	0.73-1.34 µg/g		[22]
	As	3.0 µg/g	Lake Macquarie, Australia	[23]
	Al	1.273 mg/kg	Gulf of İskenderun, Turkey	[24]
	Ni	1.174 mg/kg		
	Ni	0.61 mg/kg	Northern East Mediterranean, Turkey	[25]
	As	4.18 µg/g	Anpin Horbor, Taiwan	[26]
Mugil spp.	Ni	2 µg/g	Red sea, Iran	[17]

Red Mullet *(M. barbatus)* were exceeded levels of some other studies [15,18]. The Ag concentrations found in muscle tissue of Green Tiger Prawn *(P. semisulcatus)* is lower than the other study done in the Northern East Mediterranean Sea [18]. The Al concentrations found in muscle tissue of Red Mullet *(M. barbatus)* and Grey Mullet *(M. cephalus)* are higher than the other study done in the Mediterranean Sea and the Black Sea [15,18-20,25]. The As concentrations found in muscle tissue of Red Mullet *(M. barbatus)* and Grey Mullet *(M. cephalus)* are lower than the other study done in the Mediterranean Sea and the others [16,17,23,26]. The Ni concentrations found in muscle tissue of Red Mullet *(M. barbatus)* and Green Tiger Prawn *(P. semisulcatus)* are higher [15,16,18-20], Grey Mullet *(M. cephalus)* fall in the range of studies done in the Mediterranean Sea and others, or are lower [22,24,25,27]. Evaluation of other studies conducted in the Gulf of Antalya, Al levels are higher. These results showed that, although increasing the legal rules about pollution, seafood which caught from the Gulf are exposed to Al in a higher rate. Green Tiger Prawn *(P. semisulcatus)* Al levels are determined as over the tolerable intake (6 mg/day for children and 6-14 mg/day for teenagers and adults) and Red Mullet *(M. barbatus),* Grey Mullet *(M. cephalus)* Al levels determined as closed. Red Mullet *(M. barbatus)* and Green Tiger Prawn *(P. semisulcatus)* As levels are determined as closed the tolerable intake (0.015 mg/kg b.w.) [28]. Differences between the nonessential potentially toxic trace elements levels of seafood species is due to their habitats. Red Mullet *(M. barbatus)* (demersal) (As) and Green Tiger Prawn *(P. semisulcatus)* (benthic) (Al, Ni, Sn) are live and feed on or near the bottom of the sea. Therefore exposed to more intense and accumulate the nonessential potentially toxic trace elements is understood. The study results showed that; deterrmined levels of trace elements were below the legal limits and available daily intake limits.

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RESEARCH ARTICLE

Expression Profiles of Interferon-Tau Stimulated Genes (ISGs) in Peripheral Blood Leucocytes (PBLs) and Milk Cells in Pregnant Dairy Cows [1]

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Summary

In previous reports, it was indicated that measurement of activity of Interferon-tau Stimulated Genes (ISGs) in Peripheral Blood Leucocytes (PBLs) may be used as an alternative early pregnancy detection method in dairy cows. However, there are no data showing the expression profiles of ISGs in other body fluids containing leucocytes such as milk. In the present study, it was hypothesized that leucocytes in milk samples may reflect the increases in expression profiles of ISGs as shown in PBLs. For this purpose, nine pregnant lactating Holstein cows were used. Insemination day was accepted as day zero (day 0). Blood and milk samples were collected on day 0 and 18 after insemination for cell isolation. Total RNA was extracted from isolated cells and converted to cDNA. Steady state levels of Interferon-tau Stimulated Gene 15 (ISG15), Myxovirus (influenza virus) resistance 1 (MX1) and 2 (MX2) mRNA transcripts were assayed by using real-time reverse transcriptase PCR. Relative Expression Software Tool (REST2009) was used for statistical analyses. There was no statistical significant difference for expression levels of ISG15, MX1 and MX2 mRNAs between days 0 and 18 in milk samples. However, when compared to day 0, levels of ISG15 and MX2 transcripts were increased 6.97±0.68 fold and 5.84±1.27 fold on day 18 in PBLs in pregnant cows, respectively ($P<0.05$). According to this result, it may be suggested that milk cells are not suitable measurement of expression profiles of ISGs to detect early pregnancy in lactating dairy cows.

Keywords: *Early pregnancy detection, ISGs, qPCR, Milk, Cow*

Sütçü Gebe İneklerde Periferal Kan Lökositleri (PBLs) ve Süt Hücrelerindeki Interferon-Tau Stimulated Genlerinin (ISGs) Ekspresyon Profili

Özet

Önceki çalışmalarda sütçü ineklerde periferal kan lökositlerindeki interferon-tau stimulated genlerindeki (ISGs) aktivite ölçümünün alternatif erken gebelik teşhis yöntemi olarak kullanılabileceği gösterilmiştir. Bununla birlikte süt gibi lökosit içeren diğer vücut sıvılarında ISGs'lerin ekspresyon profilindeki değişimleri inceleyen bir veri bulunmamaktadır. Sunulan çalışmada hipotez olarak süt örneklerindeki lökositlerde de, PBLs'lerde gösterildiği gibi ISGs'lerin ekspresyon profilini arttırabileceği düşünüldü. Bu amaç için dokuz (n=9) sağmal Holştayn ırkı gebe inek kullanıldı. Tohumlama günü sıfırıncı gün (0. gün) olarak kabul edildi. Kan ve süt örnekleri hücre izolasyonu için 0. ve 18. gün alındı. İzole edilen hücrelerden total RNA elde edildi ve cDNA'ya dönüştürüldü. Interferon-tau Stimulated Gene 15 (ISG15), Myxovirus (influenza virus) resistance 1 (MX1) and 2 (MX2) genlerinin mRNA transkriptlerinin seviyeleri real-time reverse transcriptase PCR tekniği ile ölçüldü. İstatistiksel analizler için Relative Expression Software Tool (REST2009) programı kullanıldı. Süt örneklerinin 0 ve 18. günleri arasında ISG15, MX1 ve MX2 mRNA'larının ekspresyon seviyelerindeki farklılık istatistiki olarak önemli olmadı. Bununla birlikte, gebe ineklerin 18. gün PBLs'deki ISG15 ve MX2'nin transkript seviyeleri 0. gün ile karşılaştırıldığında sırasıyla 6.97±0.68 ve 5.84±1.27 kat arttı ($P<0.05$). Bu sonuçlara göre sağmal ineklerde süt hücrelerindeki ISGs ekspresyon seviyelerinin ölçülmesinin erken gebelik teşhisi için uygun olmadığı önerilebilir.

Anahtar sözcükler: *Erken gebelik teşhisi, ISGs, qPCR, Süt, İnek*

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INTRODUCTION

Detection of pregnancy as early as possible in dairy cows is an important factor to sustain the farm management. In practice, the bovine pregnancy can be easily detected around day 30 after ovulation by using transrectal ultrasonography [1]. However, day 30 means one missed valuable cycle to reschedule non pregnant cows for re-insemination. Furthermore, one missed cycle for each day/cow costs about 5 USD for dairy farms in TURKEY [2]. Besides economical effect of late detection of pregnancy, using ultrasonography requires skilled large animal veterinarians [3]. All these factors put pressure on the scientists to develop alternative early pregnancy detection methods in cows. Nowadays, molecular biology techniques such as PCR, RIA and ELISA are major candidates as alternative pregnancy detection methods in cow. Early pregnancy factor on day 2, expression profile of ISGs after day 18, remaining elevated progesterone level (>1 ng/ml) between day 18 and day 21 of pregnancy, pregnancy associated glycoproteins (PAGs) on day 28 are accepted as alternative pregnancy detection markers for cows [1,3-5].

Studies have shown that the bovine embryo signals its presence to the dam by a glycoprotein, interferon -tau, which is secreted by embryonic trophoblastic cells [6]. During the maternal recognition of pregnancy, embryonic interferon-tau abolishes endometrial luteolytic mechanism by specifically causing down regulation of endometrial estrogen receptor alpha and oxytocin receptor expressions [7]. Without an embryo, pulsatile releasing of endometrial PGF results in functional and structural demise of the CL (corpus luteum) in cows [8]. Therefore, elongated tropho-blastic cells of the embryo must secrete enough amounts of interferon-tau in a timely manner to maintain the CL, source of progesterone [9]. Embryonic interferon-tau not only causes inhibition of luteolytic mechanism but also stimulates expression of its target genes both in intra-uterine and extra-uterine tissues [10]. Those genes are named as Interferon-tau Stimulated Genes (ISGs) [11,12]. Being a cytokine, interferon-tau can stimulate leukocyte activity. Expression profiles of ISGs in PBLs have been studied in detail and a significant increase in some ISGs in PBLs of early pregnant cows compared to those of non-pregnant cows were reported [12,13].

We hypothesize that immune cells present in milk may also reflect the changes in expression profiles of ISGs as shown in PBLs and milk would be an easily collectable candidate sample for a possible use of early pregnancy detection in cows. For this purpose, we sought to elucidate expression profiles of ISG15, MX1, MX2 mRNAs in milk cells collected from lactating cows.

MATERIAL and METHODS

All animal experimental procedures were approved by ethical committee of Dicle University, Diyarbakır (#2012-51), Turkey and the experimental procedures were performed in the Dairy Farm of Cukurova University, Adana, Turkey. Twenty lactating Holstein dairy cows (3-5 years old, 137±7 DIM, 35 kg/day milk) were synchronized using standard ovsynch protocol (day -10 GnRH, day -3 PGF2 alpha, day -1 GnRH, day 0 Timed AI). Cows were free of any health problems, specifically of mastitis. Evidence of mastitis was eliminated by indirect Somatic cells count (Califronia Mastitis Test, CMT) and direct Somatic cells count measurements (DeLaval Cell counter DCC, DeLaval, Tumba, Sweden). Blood (10 mL) and milk (50 mL) samples were collected from cows on days of 0 and 18. Nine cows were diagnosed pregnant on day 35 by using transrectal ultrasonography. PBLs were isolated according to Kurar et al.[14]. Cells from milk samples were isolated according to the following protocol. Fifty mL milk sample was centrifuged at 4.000 RPM (+4°C) for 10 min. Cell pellet was washed three times with chilled PBS. After each washing step cell suspension was centrifuged at 4.000 RPM (+4°C) for 5 min. Immediately after washing, the isolated cells were snap frozen and stored at -80°C until RNA isolation.

RNA isolation kit (RNeasy Mini Kit, Qiagen) was used for total RNA isolation from milk samples. Kit protocol was followed accordingly. Briefly, frozen milk cells were re-suspended in Lysis buffer passed through a 25 G needle for homogenization. Following 10 min incubation at room temperature (RT), the homogenized sample was centrifuged at 10.000 x g for 5 min at RT. Upper phase of the solution was carefully transferred into a new sterile tube and was mixed with 70% ethanol. The mixture was loaded on a filter cartilage and centrifuged for 15 sec at 8.000 x g at RT. Subsequently, the filter cartilage was washed once with Buffer RW1 and twice with Buffer RPE. Following each washing step, the filter cartilage was centrifuged for 1 min at 8.000 x g, and for one min to remove residual fluid from the filter during the last wash. Finally, filter was transferred to a new collection tube and 50 µL RNAse, and DNase free water was applied to the center of the filter and centrifuged for 30 sec at max speed to recover total RNA.

For total mRNA isolation from PBLs, a protocol described by Kurar et al.[14] was followed. Concentrations and purity of total RNA were determined by using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Palo Alto, CA). Total RNA from PBLs and milk cells were treated with DNAse I to clean gDNA contamination and was then reverse transcribed in the presence of both random hexamer and oligo dT primers in equal volume by using Revert Aid First Strand cDNA Synthesis Kit (Fermentas Life Science, USA) according to the manufacturer's protocol.

Primers for MX1, MX2, and ISG15 mRNAs were derived from Gifford et al.[12] and Boerboom et al.[15]. The primer pair sequences and product sizes are shown in *Table 1*. qPCR reactions were set up as follows: 5 µL SYBR Green Master Mix (2X, Fermentas Life Science, USA), 2.5 pMol

Table 1. *Primers for Interferone-tau stimulated genes (ISGs) used in Real-time PCR*
Tablo 1. *Real-time PCR'da kullanılan İnterferon-tau tarafından uyarılan genlerin primerleri*

Locus	Primer Sequence	Reference	Access Code
ISG15	F 5'- ggtatccgagctgaagcagtt -3' R 5'- acctccctgctgtcaaggt -3'	Gifford et al.[12]	NM_174366
MX1	F 5'- gtacgagccgagttctccaa -3' R 5'- atgtccacagcaggctcttc -3'	Gifford et al.[12]	AF047692
MX2	F 5'- cttcagagacgcctcagtcg -3' R 5'- tgaagcagccaggaatagtg -3'	Gifford et al.[12]	NM_173941
GAPDH	F 5'- atcaccatcttccaggagcgaga-3' R 5'- gtcttctgggtggcagtgatgg-3'	Boerboom et al.[15]	XM_001502360

of each primer, 0.5 µL cDNA, and ddH_2O to bring final volume to 10 µL. Thermal cycling was done by initially incubating the mixture at 50°C for 2 min with subsequent denaturation at 95°C for 10 min. This was followed by 40 cycles of denaturation, annealing, and amplification (95°C 30 sec, 60°C 30 sec, 72°C 30 sec). All reactions were done on the Real-Time PCR System (Applied Bioscience Stepone plus, Foster City, CA). In each run, negative controls with no cDNA template and RT negative controls were included. All samples were evaluated in duplicate for each cDNA. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA primer was derived from Boerboom et al.[15] and used as a housekeeping gene to normalize the expression of ISG15, MX1 and MX2. Mean threshold of cycle values from day of 0 was used as a reference point and Ct values for each days (day 0 and day 18) were used to calculate the fold change from this reference point using reference point Ct values according to $2^{-\Delta\Delta}$Ct method described by Livak and Schmittgen [16]. Expression levels of ISG15, MX1 and MX2 transcripts were compared by using algorithm (Relative Expression Software Tool 2009) in which the mathematical model is based on the PCR efficiency of each gene investigated and the mean deviation in Ct between groups [17]. The expression ratios were considered statistically significant at $P<0.05$.

RESULTS

Total amounts of RNA isolated from 50 ml milk and 10 ml blood samples were 148.6±9.6 ng and 30±8 µg, respectively. Optical densities of 260/280 UV in Nanodrop measurement were 1.7±0.1 for milk isolated total RNA, 2.0±0.1 for PBLs isolated total RNA. Amplification products were verified by separation on a 2% agarose gel *(Fig. 1)*.

Expression levels of ISG15, MX1 and MX2 mRNA were not statistically significant between comparison days (days 0 and 18) for milk cells *(Fig. 2)*. However, when compared to day 0, steady state levels of ISG15 and MX2 transcripts were up regulated as 6.97±0.68 fold and 5.84±1.27 fold on day 18 in PBLs, respectively (*Fig. 3*, $P<0.05$). MX1 mRNA expression did not change between days 0 and 18 in PBLs *(Fig. 3)*.

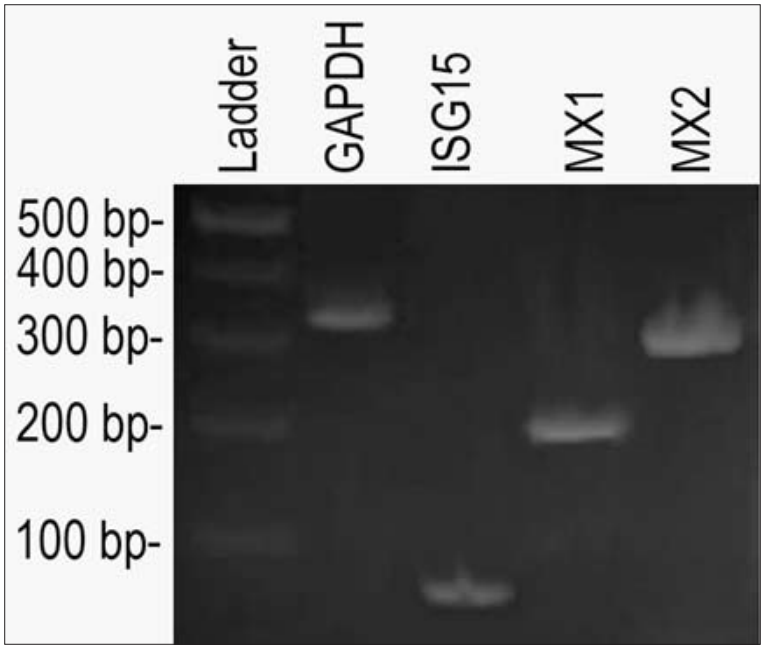


Fig 1. Agarose gel electrophoresis of GAPDH (341 bp), ISG15 (87 bp), MX1 (197 bp) and MX2 (232 bp) PCR Amplification products

Şekil 1. GAPDH (341 bp), ISG15 (87 bp), MX1 (197 bp) ve MX2 (232 bp) PZR ürünlerinin agaroz gel elektroforezi

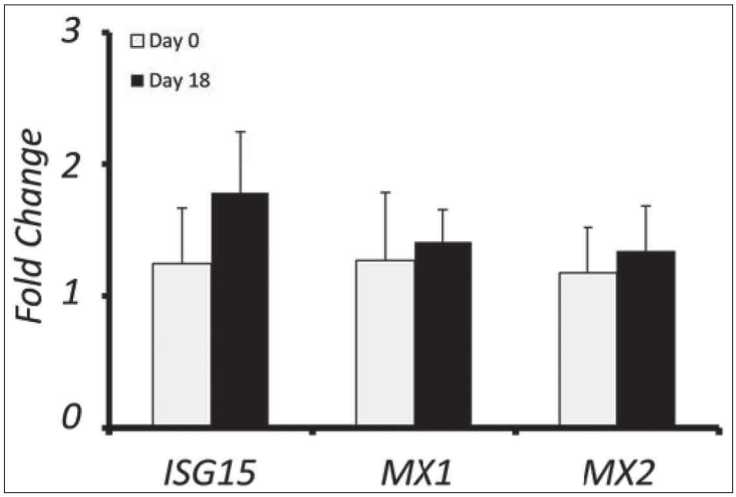
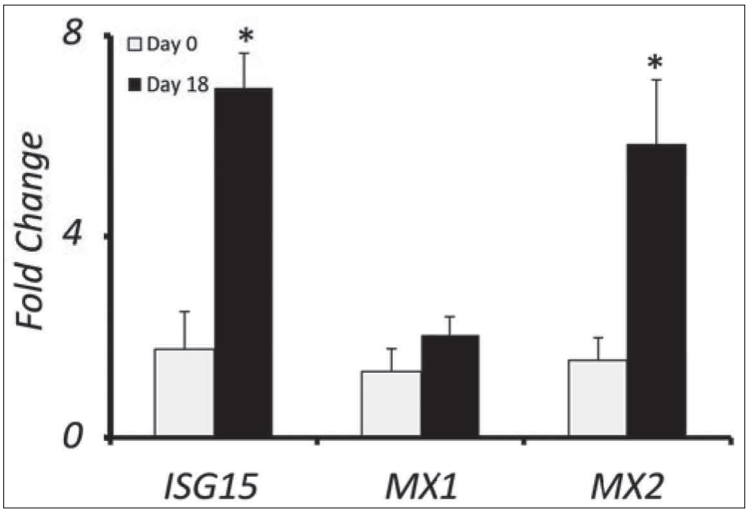


Fig 2. The expression of ISG15, MX1 and MX2 mRNAs (± SEM) in bovine milk samples (n=9 for each day)

Şekil 2. İnek süt örneklerinde ISG15, MX1 ve MX2 mRNAs (± SEM)'ların ekspresyonu (Her gün için n=9)

Fig 3. The expression of ISG15, MX1 and MX2 mRNAs (± SEM) in bovine Peripheral Blood Leucocytes (n=9 for each day); * indicate statistically significant differences between days (P<0.05)

Şekil 3. İnek periferal kan lökositlerinde ISG15, MX1 ve MX2 mRNAs (± SEM)'ların ekspresyonu (Her gün için n=9); * Günler arasında istatistiksel önemli farklılıkları göstermektedir (P<0.05)



DISCUSSION

Apart from the endometrium, the CL and PBLs in which the effect of interferon-tau were very well documented [10,18,19], there is no available data that demonstrates presence and profile of ISGs expression in the other body fluids containing leucocytes such as milk. Therefore, the present study aimed to detect the presence of ISGs in milk cells and their expression profile in search of any possible change in expression levels due to pregnancy.

In the present study, expression profile of ISGs in lactating pregnant cow blood and milk cells on day 18 was demonstrated by qPCR. MX1, MX2 and ISG15 mRNA transcripts showed an up regulated profiles due to early pregnancy in both pregnant ewes and cows in previous studies [12,20] Therefore, these genes were chosen as candidate pregnancy detection genes for milk samples from among ISGs. As internal control for ISG expression on day 18, day 0 samples collected from each individual animal were used. Fold changes on day 18 for each ISGs were calculated according to day 0 values. This model allows us to eliminate individual animal effect on day 18 ISGs expression. Compared to isolated PBLs from 10 mL blood, very small amount of cells were isolated from the 50 mL milk sample. As expected, isolated total RNA from milk cells were also less abundant than that of PBLs. This may be explained by the number of cells from blood (7-10x 10^6/mL) and milk (1-2x10^5/mL) from a healthy cow [21].

Yankey et al.[20] reported an increased MX1 expression in PBLs of pregnant ewes and Gifford et al.[12] also indicated that PBLs produces ISGs due to embryonic interferon-tau during the early pregnancy in cow. This effect was also confirmed by intrauterine interferon-tau infusion studies [22]. Despite a significant increase in ISGs transcripts (MX2 and ISG15 mRNAs) [12] on day 18 in PBLs in the present study, we could not see any significant changes for ISGs mRNA expression in milk cells. This might indicate that INF-tau is not responsible for activation of ISGs in milk immune cells or the amount and effect of INF-tau is not enough to detect any expression changes for ISGs.

Milk cells are composed mostly of leukocytes (95%) and udder epithelial cells (5%) and these are called as somatic cells [23]. The number of somatic cells is accepted as a gold standart for udder health [24]. Milk progesterone level is

also used for detection of open-cow [25]. Previous reports were shown that the effect of IFN-tau can be monitored by ISGs expression in endometrium and CL and PBLs [10,20]. Interferon-tau, like other type I interferons, is a chemokine and has immunomodulatory activities [26]. Both in vivo and in vitro studies were clearly demonstrated that interferon-tau proliferates leucocytes [12,13]. Interferon-tau mediated expression of more than 100 ISGs as has been reported by [27] but only a few increase in PBLs due to pregnancy [28,29]. The measurement of ISGs in PBLs provides an alternative method to follow early pregnancy in cows. However, according to present study result, milk cells may not be suitable for monitoring early bovine pregnancy.

Moreover, healthy milk mostly contains macrophages (70%) and they are accepted as resident cell population. Compared to macrophages, number of neutrophil comprise only 5-20% of total cells in healthy milk [23]. However, if inflammation occurs, neutrophiles invade into the udder from blood and cell composition changes in favor of the neutrophils (70-80%) [30]. Furthermore it was demonstrated that the neutrophils are more sensitive to INF-tau compared to other peripheral blood mononuclear cells [13]. We suppose that our method does not reflect the same changes in ISGs mRNA expression as in PBLs, since the expression was assessed in healthy pregnant animal milk cells. In the latter, the predominant cells are macrophages.

ISGs expression (MX1, MX2 and ISG15) profiles in PBLs and milk cells were followed to detect any changes due to early pregnancy by the present study. Although expression of MX2 and ISG15 mRNA transcripts showed a significantly increased expression profile in PBLs on day 18 compared to day 0, MX1, MX2 and ISG15 did not change in milk samples during early pregnancy. According to this result, we may suggest that milk cells are not suitable for following ISGs expression profiles to detect early pregnancy in lactating dairy cow.

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RESEARCH ARTICLE

Determination of Propofol and Isoflurane Anesthesia Depth with Bispectral Index Monitorization in Dogs Undergoing Ovariohysterectomy Procedure

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Summary

In this study, 12 bitches were used to compare anesthetic depth by bispectral index (BIS) monitoring and vital parameters under propofol and isoflurane anesthesia. The animals were randomly divided into two groups (n=6) for ovariohysterectomy. All animals were premedicated with atropine sulphate (0.04 mg/kg, sc) and midazolam (0.3 mg/kg, iv). The induction of anesthesia was achieved by propofol (5 mg/kg, iv) in groups. General anesthesia was maintained with propofol (0.4 mg/kg/min.) in first group (PRO group) and 2% isoflurane in second group (ISO group). Non-invasive systolic (SAP), diastolic (DAP), mean arterial blood pressure (MAP), heart rate (HR), respiratory rate (RR), haemoglobin oxygen saturation (SpO_2) and BIS were measured before anesthesia (0 min, T0), maintenance of anesthesia (T5, T10, T15), end of anesthesia (T30) and additionally BIS also measured at 5 min after the terminating of anesthesia (T35). HR, RR, SAP and DAP did not significantly differ between groups. The MAP value did not show any significant difference within both groups. However, the MAP value detected on T5, T10, T15 and T30 was higher in PRO group than those detected in ISO group ($P<0.05$). The BIS values detected at all measured times in PRO and ISO groups were lower than those detected before anesthesia. The BIS value detected on T5 and T10 was higher in ISO group, whereas the value determined at T15 was higher in PRO group ($P<0.05$). In conclusion, it is suggested that bispectral index is a useful tool for monitoring depth of anesthesia in veterinary practice and total intravenous anesthesia with propofol may be used as an alternative to isoflurane anesthesia.

Keywords: *Bispectral Index, Dog, Isoflurane, Propofol*

Ovariohisterektomi Yapılan Köpeklerde Propofol ve İzofloran Anestezisinin Derinliğinin Bispektral İndeks Monitörizasyonu ile Belirlenmesi

Özet

Bu çalışmada, propofol ve izofluran anestezisinde anestezi derinliğinin bispektral indeks (BIS) ve vital parametrelerin izlenmesi ile karşılaştırılması için 12 adet dişi köpek kullanıldı. Hayvanlar ovariohisterektomi operasyonu için rastgele iki gruba bölündü (n=6 her grupta). Hayvanlar atropin sülfat (0.04 mg/kg, sc) ve midazolam (0.3 mg/kg, iv) ile premedike edildi. Anestezi indüksiyonu gruplarda propofol (5 mg/kg, iv) ile sağlandı. Genel anestezi ilk grupta propofol (PRO grubu) ile ikinci grupta %2 izofluran (ISO grubu) ile devam ettirildi. Non-invaziv sistolik (SAP), diastolik (DAP), ortalama arteriyel kan basıncı (MAP), nabız sayısı (HR), solunum sayısı (RR), hemoglobin oksijen saturasyonu (SpO_2) ve BIS anestezi öncesi (0. dakika, T0), anestezi boyunca (T5, T10, T15) ve anestezi sonunda (T30) ölçülürken BIS ölçümü anestezi sonlandırıldıktan 5 dakika sonra (T35) da ölçüldü. HR, RR, SAP ve DAP değerleri gruplar arasında farklılık göstermedi. MAP değeri her iki grup içinde istatiktiksel olarak farklılık oluşturmadı. Bununla birlikte, T5, T10, T15 ve T30 ölçüm zamanlarında PRO grubundaki MAP değerinin ISO grubuna göre yüksek olduğu belirlendi ($P<0.05$). PRO ve ISO gruplarında tüm ölçüm zamanlarındaki BIS değerlerinin anestezi öncesi değere göre daha düşük olduğu izlendi. BIS değerinin ISO grubunda T5 veT10 zamanında, PRO grubunda ise T15 zamanında yüksek olduğu gözlendi ($P<0.05$). Sonuç olarak, bispektral indeksin veteriner pratikte anestezi derinliğinin ölçümünde faydalı olduğu ve propofol ile yürütülen total intravenöz anestezinin izofluran anestezisine alternatif olarak kullanılabileceği ileri sürülmektedir.

Anahtar sözcükler: *Bispektral İndeks, Köpek, izofloran, Propofol*

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INTRODUCTION

Inhalation anesthetics are widely used in veterinary medicine and the basic advantage of these anesthetics is their independent hepatic and renal system elimination. Therefore, the biotransformation of inhalant anesthetics and morbidity and mortality is very low, as compared to other anesthetics [1,2].

The application of total intravenous anesthesia (TIVA), an alternative to inhalant anesthetics, has an increasing popularity with the advent of the infusion pump technology to induce anesthesia in small animal practice [3]. TIVA is a general anesthesia technique which is used to induction of anesthesia by intravenously given drugs and maintains the anesthesia [4]. Propofol (2,6-diisopropylphenol) is an intravenously using anesthetic agent in alkyl phenol family [4,5].

Recently, bispectral index (BIS) monitoring has been developed to utilize the depth of anesthesia by estimating electroencephalogram (EEG) signals [1,6,7]. BIS, numerical value of EEG derivative is used for evaluation of depression of central nervous system (CNS) in human medicine. The depressive effect of sedative and anesthetic agents on CNS in human is correlated to BIS [8]. It has been reported that BIS is used for the evaluation of CNS depression in dogs anesthetized with isoflurane, sevoflurane or propofol [6,9,10], however there are limited reports in veterinary medicine. It has been indicated that the frequency of nociceptive stimuli and the sensitivity of anesthetics affects the BIS value [11]. Therefore, this study was designed to compare anesthetic depth by BIS monitoring and vital parameters in ovariohysterectomized dogs anesthetized by propofol and isoflurane.

MATERIAL and METHODS

All procedures were approved by local ethical committee of Experimental Animal Research Center, Afyon Kocatepe University (Reference No: 65-11, Date: 03.10.2011) and the approval of the owners were also received.

Animals

In this study, 12 bitches which referred to animal hospital for elective ovariohysterectomy, in different breeds, aged between 1 and 6, weighing at 22±4 kg were used. Clinical aspect, hematologic and biochemical parameters of the animals were examined. Dogs having abnormal hematologic and biochemical parameters were excluded from the study. The animals were not allowed to eat 12 hours before the surgery, however water intake was allowed until four hours prior to ovariohysterectomy.

Anesthesia

The dogs were randomly divided into two groups (n=6). The left *v. cephalica accessoria* were catheterized in all dogs using a 20 gauge catheter for administration of intravenously given fluids in both groups. An additional 20 gauge catheter was placed in the right *v. cephalica accessoria* for infusion of anesthetics.

Atropine sulphate (Atropin 2%, Vetaş, Turkey) at 0.04 mg/kg was administered subcutaneously (sc) approximately 45 min before general anesthesia. The dogs were premedicated with midazolam (Dormicum, Roche, Turkey) at a dose of 0.3 mg/kg intravenously. When the sedation was achieved, the induction of anesthesia in both groups was performed with propofol (Propofol 1%, Fresenius Kabi, Germany) which was slowly administered at a dose of 5 mg/kg intravenously by manual injection over a period of about 60 sec to effect. General anesthesia was induced with the infusion pump (Medifusion DI-2000, Korea) of propofol at a dose of 0.4 mg/kg/min in the first group (PRO group). Immediately after tracheal intubation, the dogs in second group (ISO group) were connected to anesthesia machine (SMS 2000 Classic Anesthesia System with Vent-V Automatic Ventilator. SMS, Turkey). General anesthesia was maintained with 2% isoflurane (Forane Liquid, Abbott Company USA). All general anesthesia procedure was ended after 30 min in both groups. The dogs in group ISO was extubated at the end of the anesthesia. During general anesthesia, lactated ringers solution was infused at a dose of 10 ml/kg per hour in both groups. Moreover, $PaCO_2$ and pH values were stabilized between 35-45 mmHg and 7.35-7.45, respectively in groups (Gastat Mini, Japan).

Surgery

Ovariohysterectomy was performed by a median line laparatomy and ended in 30 min in both groups. The induction of anesthesia in groups was terminated after the measurement of last data at the end of the surgery. However, the study was ended after the measurement of BIS that was performed 5 min after ending of infusion in group PRO, and 5 min after terminating of induction of anesthesia in group ISO.

Skin incision was performed in 5 min after the induction of general anesthesia. Ovaries and uterine body were removed in a routine manner. The closure of incision line was achieved in approximately 25 min after the skin incision and the anesthesia was terminated. Dexketoprofen Trometamol (Arveles, UFSA, Turkey) at a dose of 1 mg/kg was administered intravenously to control prophylactic postoperative pain in both groups.

Measuring of Vital Parameters

Before premedication (0 min, T0) and every 5 min during anesthesia (T5, T10, T15) and end of anesthesia (T30), non-invasive systolic (SAP), diastolic (DAP), mean arterial blood pressure (MAP), heart rate (HR), respiratory rate (RR), haemoglobin oxygen saturation (SpO_2) were recorded in both groups using a multi-channel monitor

(KMA PETAS 800 multi-channel monitor, PETAS, Turkey). However, the RR in ISO group was 14 per minute due to mechanic ventilation. In PRO group, ovariohysterectomy was performed under spontaneous ventilation; thereby RR per minute was recorded. During SpO_2 measurement, SpO_2 probe was placed to the tongue of dogs and the probe was connected to the monitor for recording the data.

For ECG monitoring, ECG electrodes were attached to all four extremities of dogs and, ECG tracings from lead II were monitored and recorded on the multi-channel monitor.

Measurement of BIS

Before general anesthesia, frontal-temporal part of cranium was shaved and the skin was defatted by ether. BIS electrodes using in human medicine were adapted to the dogs. The sensors for assessing the BIS (Quatro, Aspect Medical Systems International B.V., Netherlands) were attached by 5 electrodes in a frontal-temporal configuration. Three electrodes were connected to frontal area, whereas electrode 4 and 5 was placed into temporal area in front of left and right ear, respectively. Before premedication (0 min, T0), every 5 min during anesthesia (T5, T10, T15) and end of anesthesia (T30) and 5 min after the terminating of anesthesia (T35), BIS activity was continuously recorded by the BIS monitor (Bispectral Index Monitor, A-2000 PIN: 185-0070, BIS XP Platform Aspect Medical Systems Inc., USA) following the monitor-sensor connection.

Statistical analysis

Data were analyzed with the SPSS 16.0 (SPSS Inc, for Windows) software package. A one-way ANOVA test was used to compare the groups. Test significance levels within and between groups were checked using Duncan's test. Descriptive results are expressed as means ± standard deviation. For all comparative tests, a value of $P<0.05$ was considered significant.

RESULTS

The values of HR, RR, SAP, DAP, MAP, SpO_2 and BIS in group PRO and ISO are given in *Table 1*.

The RR did not differ significantly at measured times in both groups. The RR rate was stable in ISO group due to mechanic ventilation, whereas it did not show any significant difference at measured times in PRO group.

The SAP and DAP in group PRO and ISO showed no significant changes at measured times.

The MAP in group ISO decreased (P<0.05) at T5, T10, T15 and T30, as compared to T0 (P<0.05) *(Table 1, Fig. 1)*. However, no decrement was observed in PRO group.

Oxygen saturation exhibited normal values in both PRO and ISO groups.

The BIS values detected at all measured times in PRO and ISO groups were lower than those detected before anesthesia. When the BIS values were compared between groups, it was observed that the value detected on T5 was higher in ISO group, whereas the value determined at T15 was higher in PRO group (P<0.05) *(Table 1, Fig. 2)*.

DISCUSSION

The present study clearly demonstrated the application of BIS monitoring in ovariohysterectomized dogs

Table1. *Heart rate, respiratory rate, systolic, diastolic and mean arterial blood pressures, SpO_2 and BIS values in propofol and isoflurane groups. (Mean ± SD) (n=6)*

Tablo 1. *Propofol ve izofluran gruplarında kalp frekansı, solunum sayısı, sistolik, diastolik ve ortalama arteriyal kan basınc ile SpO_2 ve BİS değerleri. (Ort ± SD) (n=6)*

Time (min)	Group	HR Pulse/min	RR	SAP (mmHg)	DAP (mmHg)	MAP (mmHg)	SpO_2 %	BIS
T0	PRO ISO	120.3±15.0 115.6±14.3	24.8±2.2 26±4.0	162.5±14.4 153.1±20.6	92.1±17.6 85.1±20.4	130.8±11.8^{a} 110.1±23.0	98.1±0.7 98.1±0.7	100±0.0^{a} 100±0.0^{a}
T5	PRO ISO	133±1.6 125±7.6	21.1±3.8 14±0.0	160.6±7.8 139.3±15.1	86.6±14.5 71.1±15.8	123.5±7.4^{ab*} 94.8±9.7	97.8±0.9 98.1±0.4	59.3±14.9^{cd*} 70±13.2^{bc}
T10	PRO ISO	129.5±7.6 124.6±18.3	21.1±3.6 14±0.0	159.8±10.5 135.3±18.8	86.6±20.7 72.6±21.3	123.8±11.4^{ab*} 97.5±15.1	97.3±0.8 97.1±0.7	56.3±3.6^{d} 59.5±13.7^{cd}
T15	PRO ISO	131.3±8.7 121.8±12.2	19.3±2.4 14±0.0	163.8±14.6 139.1±25.1	88.5±19.6 74.6±15.6	121.5±10.5^{abc*} 102.5±23.8	98±0.8 98±0.8	56.1±7.5^{d*} 47.5±10.6^{de}
T30	PRO ISO	130.5±15.8 119.3±13.9	21.5±3.8 14±0.0	148.1±26.6 139.6±21.8	91.3±19.1 80.1±20.2	117.3±16.7^{abcd*} 104.3±15.6	97.6±0.8 97.5±0.5	51.3±10.1^{de} 42.5±9.2^{e}
T35	PRO ISO							75.0±3.7^{b} 77.6±8.2^{b}

** There is significant difference between groups (P<0.05). The values with different letters in the same column have significant difference (P<0.05)*
HR: *heart rate,* ***RR:*** *respiratory rate,* ***SAP:*** *systolic arterial blood pressure,* ***DAP:*** *diastolic arterial blood pressure,* ***MAP:*** *mean arterial blood pressure,* ***SpO_2:*** *haemoglobin oxygen saturation,* ***BIS:*** *bispecteral index*

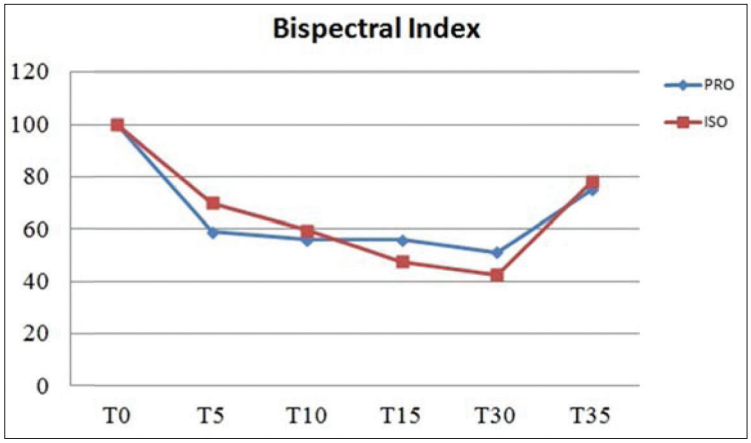
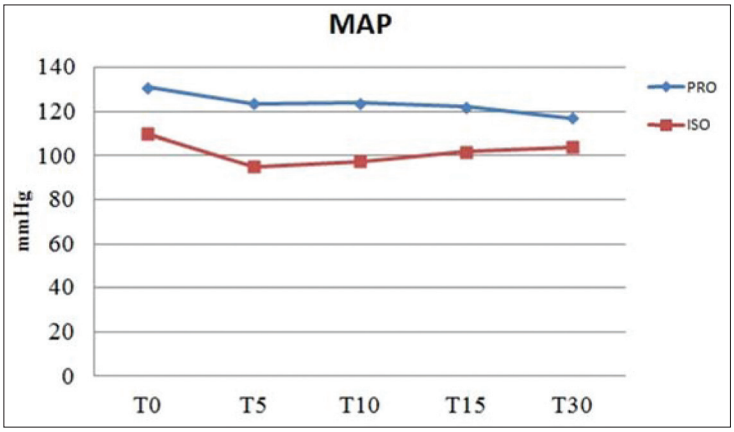


Fig 1. BIS value of dogs in PRO and ISO groups

Şekil 1. PRO ve ISO grubu köpeklerde BİS değerleri

Fig 2. Mean arterial pressure (MAP) of dogs in PRO and ISO groups

Şekil 2. PRO ve ISO grubu köpeklerde ortalama arteriyel kan basıncı



anesthetized with propofol and isoflurane anesthesia. In human medicine, bispectral monitoring has been used to quantify the degree of hypnosis as a result of amount of used anesthetic [1,12] and is also described in dogs and other species [1,8,13-15]. In the human, a BIS value of 90 or higher indicates wakefulness, while a score under 50 is ideal for surgical procedures [16]. Compagnol et al.[17] reported that BIS value was 66, 63, and 47 under 1.5%, 2.3% and 3% isoflurane anesthesia, respectively and a weak correlation was evident between BIS value and the concentration of anesthetic drug. Moreover, Mattos-Junior et al.[1] studied BIS values during ovariohysterectomy operation under halothane, isoflurane and sevoflurane anesthesia in dogs premedicated with acepromazine or acepromazine + meperidine. In their study, BIS values were measured 15 min after premedication, 10 min after general anesthesia, during ligation of pedicle of right ovary, closure of muscles, closure of skin and 10 min after termination of general anesthesia. They also reported that total anesthesia was maintained longer than 60 min and BIS value in halothane group was lower than those in isoflurane group and higher than those in sevoflurane group. In this study, it was found that MAP and BIS values detected at except T5 in ISO group was lower than those in PRO group.

Ibrahim et al.[12] indicated that the measurement of BIS was more effective to detect the level of hypnosis in patients sedated with propofol as compared to isoflurane. Hatshbach et al.[18] compared the BIS value in ovariohysterectomized dogs under propofol anesthesia or propofol anesthesia combined with remifentanyl and the BIS value was found between 73 and 79. In the present study, BIS value throughout the general anesthesia in PRO group was measured between 51 and 59. In PRO and ISO groups, premedication was performed by midazolam and induction was conducted by propofol, however TIVA was maintained by propofol at a dose of 0.4 mg/kg per min in PRO group.

Propofol which is characterized by tranquilising and fast anesthesia induction has been reported to have short duration of recovery when it is used as constant rate infusion (CRI) for maintenance of anesthesia and decreases HR and MAP under therapeutic dose range [4]. In current study, SAP and DAP values in PRO and ISO groups were compared, no significant difference was observed. However, it was determined that MAP value detected during premedication in PRO group was higher than those detected during other measured times but MAP value decreased all measured times in ISO group. Unlike Suarez et al.[4] reported a decrement in HR, it was observed that HR value did not show any significant difference between groups. The researchers also indicated that the infusion of propofol and alphaxolane had adequate anesthesia for ovariohysterectomy in dogs premedicated with acepromazine and morphine however, ventilatory support

was needed due to long-term hypoventilation caused by using of these anesthetic agents [4]. In the present study, all ovariohysterectomie procedure was ended in 30 min and SpO_2 value in PRO group which TIVA was used, was in normal range throughout the surgery. Therefore, ventilatory support was not needed in this study.

Mattos-Junior et al.[1] stated that a change in anesthetic depth was based on ocular movements and the presence or absence of palpebral reflexes and slow intravenous infusion of Fentanyl bolus was administered, when HR and MAP value increased at 20%. In this study, reflex was disappeared after the induction of anesthesia and no narcotic agent was used. Moreover, the depth of surgical anesthesia was clearly achieved in both groups, since the BIS values confirmed this observation. Furthermore, increasing salivation, vomiting and excitation findings were in agreement with Matos-Junior et al.[1].

BIS is a quantitative method to detect the depressive effect of anesthetics such as sevoflurane, isoflurane or propofol on central nervous system (CNS) in dog [6,9,10,19]. However, BIS may not reflect changes in depth of anesthesia in dogs anesthetized with isoflurane in the absence of noxious stimulation [6,17]. In current study, the depth of anesthesia changed parallel to BIS and MAP values during surgery.

Yamashita et al.[20] reported that BIS monitoring was an indicator to detect intraoperative awareness but did not show the degree of CNS depression. In this study, BIS value decreased parallel to depth of anesthesia and no reflex response was present related to anesthesia either in dogs of ISO group or PRO group during surgical procedure.

In a study which was compared the propofol and isoflurane anesthesia without any surgical approach in rabbits premedicated with dexmedetomidin by Saritas et al.[21], BIS value was found to parallel to MAP and HR values but depth of anesthesia was higher in propofol group. Moreover, it was emphasized that general anesthesia in isoflurane group induced by glove-mask method altered the depth of anesthesia.

It was concluded that BIS monitorization should be taken into consideration together with vital parameters, especially MAP for monitoring of anesthetic depth. Moreover, it is suggested that total intravenous anesthesia with propofol may be used as an alternative to isoflurane anesthesia.

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RESEARCH ARTICLE

The Morphology and Proliferation Rate of Canine and Equine Adipose Derived Mesenchymal Stem Cells Cultured with Flunixin Meglumine-*in vitro*

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Summary

The regenerative medicine in animals is a rapidly growing field, especially when therapies with stem cells are applied. Currently, stem cells are used for treatment of orthopedic diseases occurring both in small and large animals. Non-steroidal anti-inflammatory drugs (NSAIDs) are routinely used and are often accompanied with adipose derived mesenchymal stem cells (ADMSCs) therapy. Therefore, it is reasonable to monitor morphology of cells and the proliferation status of canine and equine ADMSCs cultured with NSAIDs. We focused on the analysis of the above mentioned parameters. Morphology of investigated cells was monitored using an epifluorescence microscope and scanning electron microscope (SEM). Moreover, SEM analysis was carried out for determination of microvesicles secretion. Proliferation activity of AdMSCs was evaluated with a resazurin-based test. Our research showed that the lowest concentration of Flunixin meglumine (0.01 mg/ml) had a stimulating effect on canine AdMSCs proliferation, while the same concentration significantly slowed down equine stem cell growth. Interestingly, the 0.01 mg/ml concentration of Flunixin meglumine did not effect morphology of the investigated stem cells population. Thus, results obtained from multilevel research allow us to conclude that the lowest concentration of Flunixin meglumine may be accompanied with canine adipose derived mesenchymal stem cells in orthopedic treatment.

Keywords: *Adipose-derived mesenchymal stem cells, Non-steroidal anti-inflammatory drugs, Flunixin meglumine, Proliferation rate, Morphology, Canine, Equine*

Flunixin Meglumine İle *in vitro* Kültüre Edilen Köpek ve At Yağ Kökenli Mezenkimal Kök Hücrelerinin Morfolojisi ve Çoğalma Hızı

Özet

Hayvanlarda özellikle kök hücrelerinin kullanıldığı rejeneratif (yenilenebilir) tıp hızla gelişim gösteren bir alandır. Günümüzde hem büyük hem de küçük hayvanların ortopedik hastalıklarında kök hücre tedavisinden faydalanılmaktadır. Klinik pratikte rutin olarak kullanılan non-steroidal anti-inflamatuar ilaçlar, adipoz kökenli mezenşimal kök hücresi tedavisine de dahil edilmektedir. Bu çalışmada köpek ve atlardan elde edilmiş adipoz kökenli mezenşimal kök hücrelerinin, yaygın kullanılan bir non-steroidal anti-inflamatuar ilaç (flunixin meglumine) ile kültüre edilmesinin, hücre morfolojisi ve proliferasyonu üzerine etkileri araştırıldı. Hücrelerin morfolojik takibinde epifuloresans mikroskop ve taramalı elektron mikroskobu (SEM) kullanıldı. Hücrelerin mikrovezikül sekresyonları da SEM aracılığıyla izlendi. Adipoz kökenli mezenşimal kök hücrelerinin proliferasyon aktiviteleri resazurin-temelli bir test yardımıyla değerlendirildi. Araştırma sonuçlarına göre fluniksin meglumin'in en düşük konsantrasyonlarının (0.01 mg/ml) köpeklerden elde edilen adipoz kökenli mezenşimal kök hücrelerinde proliferasyonu uyardığı; ancak aynı konsantrasyonun at kökenli hücrelerde proliferasyonu belirgin düzeyde yavaşlattığı gözlendi. Enteresan bir sonuç olarak 0.01 mg/ml konsantrasyondaki flunixin meglumine'in, incelenen kök hücre populasyonunun morfolojilerine etki etmediği belirlendi. Çok aşamalı bu araştırmadan elde edilen bulgular ışığında, köpeklerden elde edilen adipoz kökenli mezenşimal kök hücrelerinin ortopedik tedavilerde kullanımı sırasında, flunixin meglumine'in en düşük konsantrasyonlarda kullanılmasının yararlı olacağı sonucuna ulaşıldı.

Anahtar sözcükler: *Adipoz-kökenli mezenşimal kök hücreleri, Non-steroidal anti-inflamatuar ilaçlar, Flunixin meglumine, Proliferasyon oranı, Morfoloji, Köpek, At*

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for orthopedic patients that suffer from pain and inflammation. The group of drugs is officially classified by the World Health Organization (WHO) as a therapy of choice for mild pain treatment [1]. Its usage is particularly appropriate and has great importance in the postoperative period. The NSAIDs are also applied as an accompanying therapy in infectious diseases and musculoskeletal disorders, and are often indicated for potential oedema [1].

The role of NSAIDs involves limitation of prostaglandins (PGs) formation, which is responsible for inflammation, swelling, pain and fever [2]. The NSAIDs inhibit the activity of cyclooxygenase (COX) and therefore the production of prostaglandins (PG) and tromboxanes. Consequently, NSAIDs application leads to reduction of releasing inflammation mediators such as histamine and/or bradykinin [3].

The undisputed advantage of NSAIDs is non-addictive action; therefore prolonged application of non-steroidal anti-inflammatory drugs is preferable by many veterinarians. Moreover, NSAIDs do not cause sedation or respiratory depression. Numerous studies support this thesis that non-steroidal, anti-inflammatory drugs have satisfactory analgesic effect on small and large animals. However, all that glitters is not gold - some *in vivo* research and clinical observations have reported the side effects of NSAIDs application, *e.g.* gastric ulcers, nephrotoxycity and coagulation disturbance [1,3].

Musculoskeletal and locomotor system disorders in dogs and horses are the most commonly occurring therapeutic problems, which leads to limitations of physiological activities, and in extreme cases may cause serious clinical complications [3,4]. These disorders require immediate veterinary intervention and foremost, the application of all NSAIDs drugs. Nevertheless, this medical strategy may improve the patient's condition, but only for a short time. Therefore, modern veterinary medicine is looking for new treatment methods that would work efficiently and improve permanently the patient's clinical outcome. Regenerative medicine is a fast developing branch of veterinary care. Its main goal is to replace degenerative tissue and as a consequence, the whole organ, with a new forming structure that has complete functionality [5-8]. Regenerative medicine provides new solutions in the field of joint and ligament disorders treatment and gives hope as an alternative or complementation to other drug therapies. One of the most promising approaches is stem cell therapy [4-6,8-11]. Many research groups reported positive effects in cases of osteoarthritis (OA) and degenerative joint disorders (DJD) when adult stem cells were applied [8-12]. Adipose-derived mesenchymal stem cells (AdMSCs) together with those isolated from bone marrow (BMMSCs), seems to be populations that are the most often used in clinical practice. At today's level of scientific knowledge, we are still unable to answer the question of, which among those populations are more efficient in the treatment of particular disorders. However, clinical studies performed with the use of AdMSCs bring many positive effects and confirms the thesis that adipose stem cells are a sterling approach in veterinary regenerative medicine [5,8-10]. It was proven that these populations possess two essential features of stem cells i.e. multi-potential character and the ability of self-renewal of the population. Moreover, when introduced into an inflammatory environment, they significantly reduce inflammation process [6]. This mechanism was also clearly described by Gonzalez et al.[12], which showed AdMSCs acting as a natural blocker of pro-inflammatory cytokines.

Therapeutic effects of stem cells are probably related to their pararcine action. That is why evaluation of localization, size and density of mesenchymal stem-cells microvesicles (mMVs) seems to be crucial in the assessment of their AdMSCs' potential in terms of the regeneration process. As it was reported by Lai et al.[14], mMVs shed by adult stem cells are rich in a broad spectrum of growth factors and cytokines e.g. VEGF (Vascular Endothelial Growth Factor), HGF (Hepatocyte Growth Factor), FGF (Fibroblast Growth Factor), IGF-I (Insulin-like Growth Factor 1), MCP-1 (Monocyte Chemoattractant Protein-1) and BMPs (Bone Morphogenetic Proteins), which exert effects on cells in their vicinity. Additionally, evaluation of cytonemes and the presence of tunneling nanotubules are very important because it may directly correlate with particular culture physiological activity. The applying of stem cells in clinical treatment requires their detailed morphological and ultrastructural evaluation at every stage of culture [7,8].

The goal of this research was to evaluate the *in vitro* influence of commonly used NSAID - flunixin meglumine on canine and equine adipose derived mesenchymal stem cells. In this research we investigated morphology, viability and cytophisiological activity of canine and equine AdMSCs cultured with flunixin meglumine.

MATERIAL and METHODS

Ethical Approval

The experiment was conducted with the approval of Bioethical Commission, as stated by the Second Local Bioethical Commission at the Department of Biology and Animal Breeding, at University of Environmental and Life Sciences in Wroclaw, Chełmonskiego 38C, Poland (dec. number 177/2010 from 11.15.2010).

Isolation of AdMSCs

Mesenchymal stem cells were isolated from sub-

cutaneous fat tissue under local anesthesia, from dogs and horses. Owners of the animals provided proper agreement for the procedure. All stages of the procedure were performed as previously described [16,17]. Fat tissue biopsies (2 g) were washed in Hank's Balanced Salt Solution (HBSS) from tissue contaminations and digested in type I collagenase (5 mg/ml). Next, samples were centrifuged at 1200×g for 10 min. After centrifugation, stromal vascular fraction (SVF) was suspended in culture media and placed into culture dishes.

After 24 h, non-adherent cells were removed. For the primary culture, Ham's F12 medium was applied, and for the secondary culture, Dulbecco's Modified Eagle's medium (DMEM) ith 4500 mg/L concentration of glucose was used. Media were supplemented with 10% of fetal bovine serum (FBS). Additionally, 1% of antibiotic/antimycotic solution was used in the culture as a prophylaxis against potential infections. A constant condition of the culture was maintain during the experiment (5% CO_2, 95% humidity, at 37°C). The culture media was changed every two days.

Non-steroidal anti-inflammatory drug (NSAIDs)

Flunixin meglumine solution (Intervet®, 50 mg/mL, Istanbul, Turkey) was tested *in vitro* as an additive to culture medium at the following concentrations: 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL. The *in vitro* evaluation of funixin meglumine effect on canine and equine AdMSCs was performed in 24-well plates. The initial inoculum concentration was $2x10^4$ cells in 0.5 ml of medium per well. First dosages of the investigated drug were added after 6 h, when adhesion of the cells was observed. Untreated stem cells were used as a control for comparison with the investigated culture. All samples were prepared in duplicate.

The medium was changed 48 h after the cells' propagation. Morphology and viability of the cells were evaluated after 24, 72, and 120 h, on the basis of studies made by Nuzzi et al.[19].

Cytotoxicity Test

Metabolic activity of living cells was determined using resazurin - resorufin system (*in vitro* toxicology assay, AlamrBlue® assay, Invitrogen, USA). In order to perform the assay, supernatants were removed and replaced with a medium containing 10% of dye. Cultures were incubated for 2 h in CO_2 incubator. After the defined time, supernatants were collected and transferred into a microplate. Absorbance of supernatants was measured spectrophotomertically at a wavelength of 600 nm and with a reference wavelength of 690 nm. For population doubling time (PDT) estimation, a standard curve from different a range of cell concentrations ($2×10^4$, $4×10^4$, $8×10^4$ and $16×10^4$) was performed.

Morphology and Cell Activity Evaluation

Morphology of cells was evaluated under an inverted phase contrast microscope (Zeiss®, Axio Observer A.1). Additionally, scanning electron microscopy (SEM, Zeiss Evo LS 15) was applied in order to observe mMVs. For analysis of cellular composition, diamidino-2-phenylindole (DAPI) was used for nuclei staining, whereas phalloidin was used for actin filaments visualization. Prior to staining, cells were fixed with 4% ice cold paraformaldehyde for 15 min at room temperature. After fixation, cells were washed and permeabilized with 0.1% triton X-100 for 15 min at room temperature. Cells were washed again and stained with atto-488-labeled phalloidin for 30 min and then counterstained with DAPI for 5 min. Next, samples were washed three times and observed with an inverted, epi-fluorescence microscope. For scanning electron microscopy, cells were fixed in 2.5% glutaraldehyde (1:1 in DMEM) for 30 min at room temperature, then triple washed with phosphate buffered saline (PBS), and dehydrated in alcohol series (from 50% to 100% every 10%). Cells were observed with a SE1 detector, at 10 kV of filament tension.

RESULTS

Effects of Flunixin meglumine on Proliferation of Canine and Equine AdMSCs

The proliferation rate of canine and equine AdMSCs, depended on the concentration of flunixin meglumine used in the experiment. At 0.01 mg/ml and 0.1 mg/ml concentration the drug exerted a slight or non-toxic effect, whereas application of the drug at 1 mg/ml concentration had a strong toxic effect. An increase in proliferation activity was observed only in the canine culture after 48 h of propagation in the presence of flunixin meglumine at 0.01 mg/ml concentration *(Fig. 1)*. The same concentration of the drug did not cause enhancement of equine proliferation *(Fig. 2)*. The analysis of population doubling time revealed that the application of flunixin meglumine at concentration 0.01 and 0.1 mg/ml elongated the PDT value of CaAdMSC, in respect to the control culture. In the case of EqAdMSCs culture, the PDT value after the addition of 0.01 mg/ml flunixin meglumine was approximate to the PDT of the control culture. However, expansion rate of the culture affected with 0.1 mg/ml was higher compared to the control *(Table 1)*.

Morphology and Cell Activity of Canine and Equine AdMSCs

The lowest concentration of flunixin meglumine (0.01 mg/ml) did not affect the canine AdMSCs morphology during the entire experimental period *(Fig. 3)*. Investigated cells were characterized by a spindle shape and

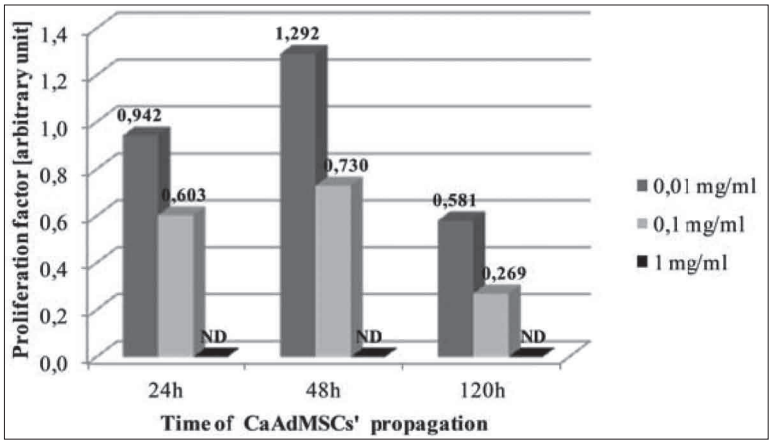
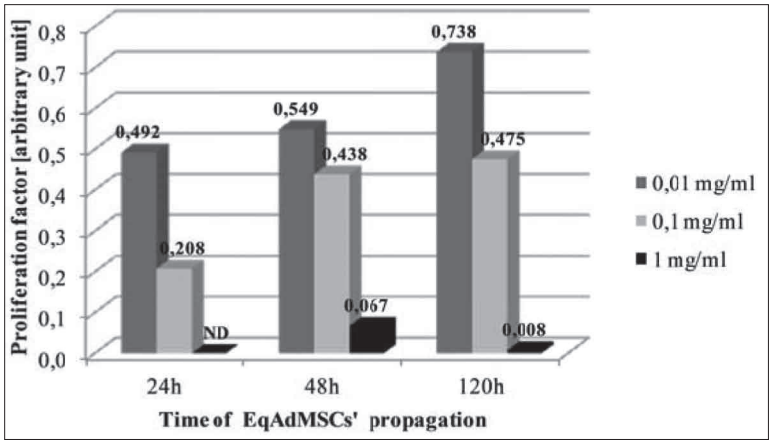


Fig 1. Proliferation ratio of canine AdMSCs treated with defined concentration of flunixin meglumine. The factor was expressed as a arbitrary unit. Abbreviation ND refers to not detectable proliferation activity of cells

Şekil 1. Değişik flunixin meglumine konsantrasyonları ile muamele edilen köpek AdMSC'lerinin proliferasyon oranı. Faktör birimi keyfi olarak belirlendi. ND hücrelerin tespit edilemeyen proliferasyon aktivitesinin kısaltmasıdır

Fig 2. Proliferation ratio of equine AdMSCs treated with defined concentration of flunixin meglumine. The factor was expressed as a arbitrary unit. Abbreviation ND refers to not detectable proliferation activity of cells

Şekil 2. Değişik flunixin meglumine konsantrasyonları ile muamele edilen at AdMSC'lerinin proliferasyon oranı. Faktör birimi keyfi olarak belirlendi. ND hücrelerin tespit edilemeyen proliferasyon aktivitesinin kısaltmasıdır



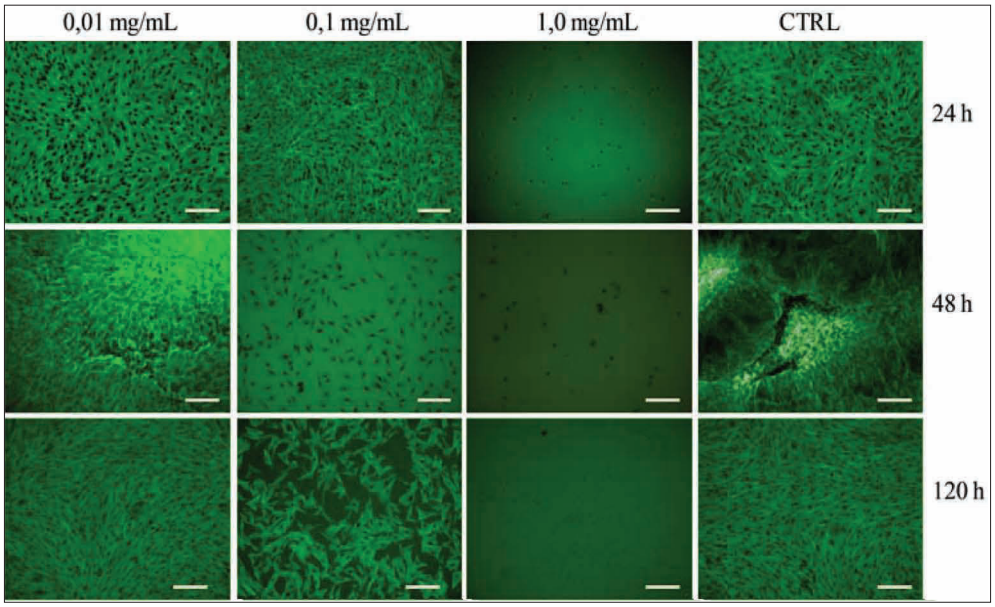


Fig 3. Morphological changes of canine AdMSCs' treated with different concentration of flunixin meglumine; Scale bar = 200 mm

Şekil 3. Farklı flunixin meglumine konsantrasyonları ile muamele edilen köpek AdMSC'lerindeki morfolojik değişimler; Ölçek birimi: 200 µm

uniform growth pattern, typical for stem cells. Higher incorporation of flunixin meglumine (0.1 mg/ml) caused equal distribution of canine cells in the culture however, single apoptotic bodies were noticed. While analyzing the highest concentration of flunixin meglumine (1 mg/ml), the first day of research revealed that apoptotic cells were predominated.

In cultures of equine AdMSCs, only the lowest concentration of the investigated drug did not affect the cells morphology. Higher incorporation of flunixin meglumine to the equine cultures induced morphological changes and promoted occurrences of apoptotic bodies on the second day of research. Untypical and incorrect morphology was observed after application of flunixin

Table 1. *The results of PDT evaluation*
Tablo 1. *PDT değerlendirilmesinin sonuçları*

Cell Culture Type	Flunixin meglumine Dosage (mg/mL)	PTD
CaAdMSCs	0.01	118.69
	0.1	347.4
	1	NA
	0	58.37
EqAdMSCs	0.01	65.38
	0.1	96.71
	1	NA
	0	70.86

meglumine at 1 mg/ml concentration. In this particular case, the presence of numerous death cells in the culture was noticeable *(Fig. 4)*.

Cell Activity Expressed by the Synthesis of Mesenchymal Microvesicles

Concentration of flunixin meglumine 0.01 and 0.1 mg/ml, had a stimulating effect on mMVs synthesis in canine and equine mesenchymal stem cells (*Fig. 5* and *6*). It was maintained until the 120th h of the experiment. The higher concentration of flunixin meglumine 1 mg/ml caused abnormal cell morphology in both species, thus scarce mMVs was observed. Also, when distribution of mMVs is considered, especially in regard to the equine culture,

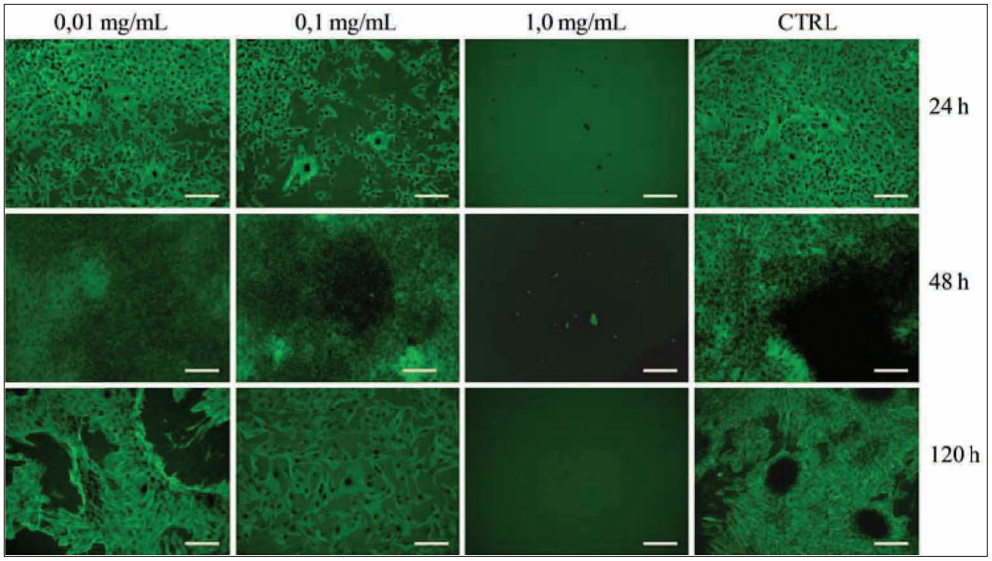
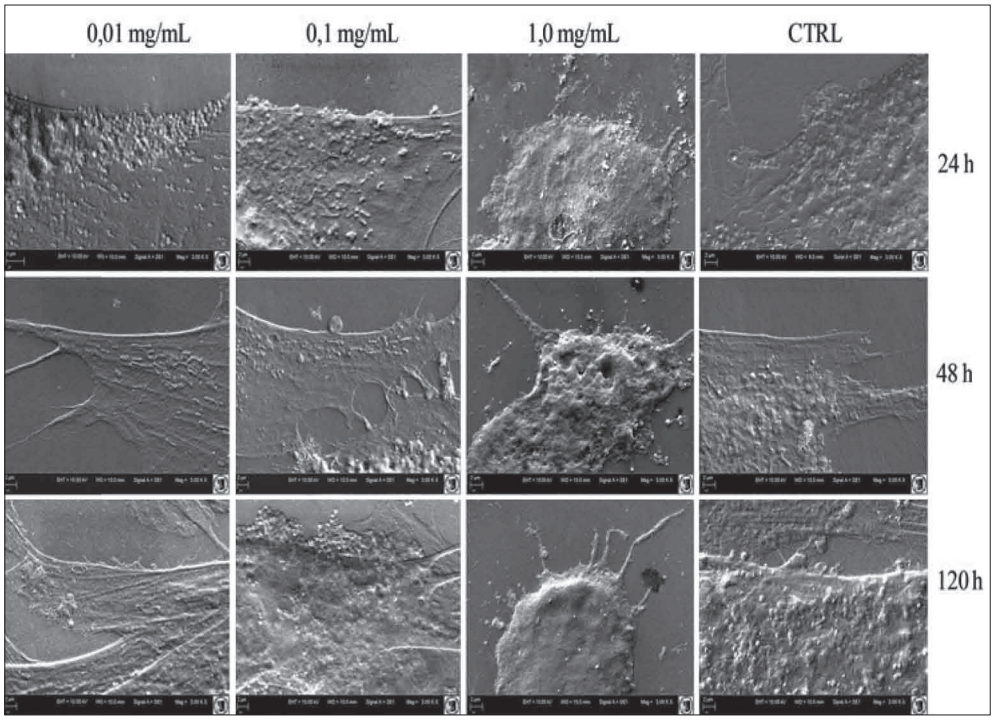


Fig 4. Morphological changes of equine AdMSCs' treated with different concentration of flunixin meglumine; Scale bar = 200 mm

Şekil 4. Değişik flunixin meglumine konsantrasyonları ile muamele edilen at AdMSC'lerindeki morfolojik değişimler; Ölçek birimi: 200 µm

Fig 5. Microphotographs of canine AdMSCs treated with flunixin meglumine; Scale bar = 2 mm.

Şekil 5. Flunixin meglumine ile muamele edilen köpek AdMSC'lerinin mikrofotoğrafları; Ölçek birimi: 2 µm



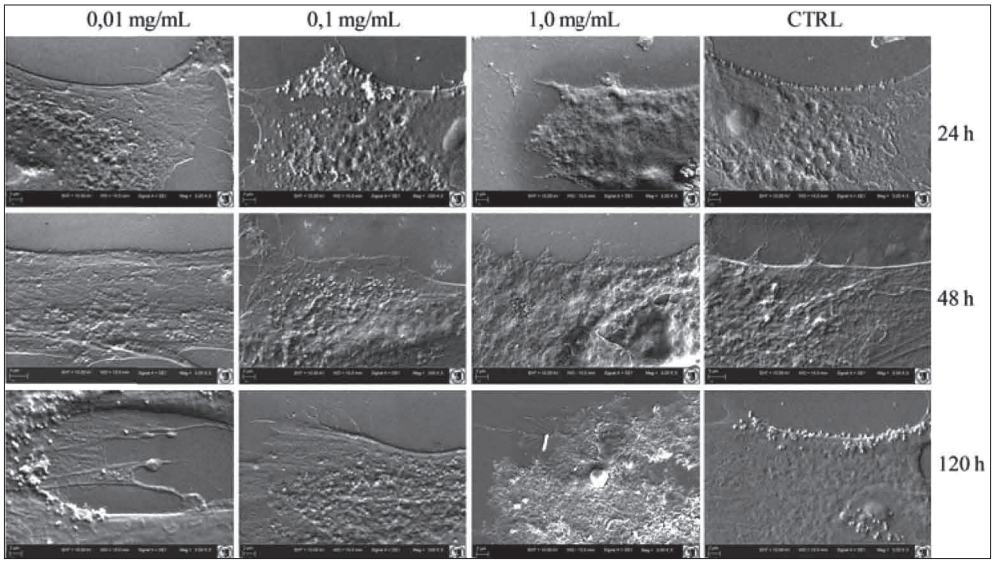


Fig 6. Microphotographs of equine AdMSCs treated with flunixin meglumine; Scale bar = 2 mm

Şekil 6. Flunixin meglumine ile muamele edilen at AdMSC'lerinin mikrofotoğrafları; Ölçek birimi: 2 µm

only the intracellular microvesicles location was noticed. A slight degree of extracellular distribution of mMVs in the canine culture was observed when 0.1 mg/ml of flunixin meglumine was used on the fifth day of the experiment.

DISCUSSION

In recent years, there has been an increased demand for effective anti-inflammatory and anti-pain therapy in veterinary medicine. This is mainly due to intensification of sport horse-riding activities, as well as hereditary diseases of many select dog breeds [19]. These factors lead to more frequent occurrence of diseases closely correlated with the locomotive and musculoskeletal systems. Due to advancement of diagnostic methods, disorders such as tendon and ligament injuries, rheumatoid arthritis (RA), osteoarthritis (OA) and hip joint dysplasia are more effectively diagnosed. As a consequence, treatment may be undertaken in the early stages of the disease. For many years, NSAIDs have been the one and only alternative for pain treatment [20]. Patients that manifest strong pain and inflammation issues require constant veterinary intervention. There are studies which demonstrate the positive effects of NSAIDs *in vivo,* especially when pain management is considered. However, there are reports indicating that prolonged NSAIDs usage causes many side effects [1,21-22]. This *status quo* forces the development of medical alternatives, which may lead to better anti-inflammatories and may even contribute to the re-creation of damaged tissue. Such alternatives entail regenerative medicine. This new branch of medicine is strongly developing, especially in the field of veterinary orthopedics. In the last decade, scientists devoted more attention to applied stem cells both in the case of dogs and horses.

Our previous studies [10,11] demonstrated the positive effects of stem cells application for tendon disorders and spar treatment in horses. These results were also confirmed by other research groups, which showed regenerative potential of adult stem cells in a dog's osteoarthritis treatment [23]. In the course of a clinical procedure, NSAIDs application is routinely applied, especially in the early stages of treatment [24]. Therefore, in this research we decided to investigate the influence of flunixin meglumine on the morphology and proliferation of canine and equine adipose-derived mesenchymal stem cells. Our findings showed a stimulating effect of 0.01 mg/ml of the investigated drug on canine stem cells proliferation, but only for the first 48 h of the experiment. In further stages, after 120 h, we observed a significant slowdown of CaAdMSCs proliferation rate when compared to the control culture.

Higher concentrations of flunixin meglumine (0.1 mg/ml) resulted in a lower proliferation rate but surprisingly, canine stem cells revealed typical morphology only when this concentration was applied. When 0.01 and 1.0 mg/ml of flunixin meglumine was investigated, untypical, clustered growing patterns were observed.

Comparing morphological features of Canine (Ca-) and Equine (Eq-) AdMSCs, the infulence of 0.1 mg/ml flunixin meglumine resulted in a properly maintained phenotype of cells. It proves that the use of flunixin meglumine yields similar results for both stem cell populations. However, when proliferation rates are considered, EqAdMSCs, in the presence of all investigated drug concentrations, revealed significant inhibition of their activity. This can be explained by different mechanisms of drug metabolism pathways of canine and equine stem cells. Our results partially correlate with Muller et al.[2] findings where positive effects of NSAIDs (in lower concentrations) in EqAdMSCs was observed. Those findings strongly correlate with the presence and localization of mMVs on the AdMSCs' surface. When cytophisiological activity of Ca and Eq AdMSCs is considered, the lowest investigated concentration of flunixin meglumine caused a beads-like distribution of

mMVs. The rest of the drug concentration applied in the experiment caused poor secretion and distribution of mMVs.

Taken together, we conclude that clinical application of flunixin meglumine combined with stem cells in canine treatment may have a positive effect, but only when 0.01 mg/ml concentration is used. These conclusions are opposite to the observations made for EqADMSc. On the basis of the obtained results, we suggest that flunixin meglumine may significantly inhibit AdMSCs activity; therefore combined therapy for equine treatment may lead to an unsatisfactory clinical effect.

However, even 0.01 mg/ml concentration of flunixin meglumine resulted in lower viability after 120 h of the canine culture when compared to the control.

Our results provide practical information for veterinary clinicians concerning combined therapy of AdMSCs and a widely used NSAIDs drug, flunixin meglumine. We showed that the applied drug is not inert for AdMSCs and even in low doses, affects the cells proliferation ratio. However, at concentration 0.01 mg/ml, no significant changes in cell morphology was observed. Based on the obtained results, we conclude that flunixin meglumine would be a more advantageous approach to canine treatment.

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RESEARCH ARTICLE

Effects of Different Fattening Systems on Fattening Performance and Body Measurements of Hemsin Male Lambs [1]

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Summary

This study was conducted to determine effect of different fattening systems on fattening performance and body measurements of Hemsin male lambs. The materials of the study were consisted 39 male lambs weaned at 3 months of age (approximately live weight of 23 kg). In this study, Hemsin lambs were used in three group of extensive (n=13), semi-intensive (n=13) and intensive (n=13). The experiment was conducted for 90 days. Final live weights of groups of extensive, semi-intensive and intensive were 33.32, 41.16 and 42.09 kg, respectively, and for daily live weight gain (DLWG) were 121.11, 201.89 and 213.00 g, respectively. For semi-intensive and intensive of group, feed conversion ratios were 3.44 and 5.35 kg, respectively. As a result, intensive and semi-intensive groups of fattening performance were higher than that of extensive group. The differences among intensive and semi-intensive groups of fattening performance were not statistically significant (P>0.05). The results of this study suggest that semi-intensive group might be appropriate for fattening male Hemsin lambs. Hemsin lambs had a generally similar or lower fattening performance compared to local sheep breeds.

Keywords: *Hemsin lamb, Fattening systems, Fattening performance, Body measurements*

Hemşin Erkek Kuzularında Farklı Besi Sistemlerinin Besi Performansı ve Vücut Ölçülerine Etkisi

Özet

Bu araştırma, Hemşin erkek kuzularında besi performansı ve vücut ölçülerine farklı besi sistemlerinin etkisini belirlemek için yapılmıştır. Araştırmanın hayvan materyalini, 3 aylık yaşta sütten kesilmiş, 39 baş Hemşin erkek kuzu oluşturmuştur (Yaklaşık 23 kg canlı ağırlığında). Araştırmadaki kuzulara ekstansif (n=13), yarı entansif (n=13) ve entansif (n=13) olmak üzere 3 farklı besi yapılmıştır. Araştırma 90 günde tamamlanmıştır. Ekstansif, yarı entansif ve entansif besi gruplarında besi sonu canlı ağırlıkları sırasıyla 33.32, 41.16 ve 42.09 kg, günlük canlı ağırlık artışları sırasıyla 121.11, 201.89 ve 213.00 g olarak belirlenmiştir. Yarı entansif ve entansif besi gruplarında yemden yararlanma oranları ise sırasıyla 3.44 ve 5.35 kg olarak tespit edilmiştir. Sonuç olarak, entansif ve yarı entansif besi gruplarının besi performansı, ekstansif besi grubundan yüksek bulunmuştur. Besi performansı bakımından entansif ve yarı entansif besi grupları arasında istatistiki bir fark bulunmamıştır (P>0.05). Bu sonuçlara göre Hemşin erkek kuzularında yarı entansif besinin en uygun olacağı ortaya çıkmıştır. Besi performansı bakımından, genel olarak Hemşin erkek kuzuların diğer yerli ırklara göre benzer ve biraz düşük olduğu söylenebilir.

Anahtar sözcükler: *Hemşin kuzusu, Besi sistemleri, Besi performansı, Vücut ölçüleri*

INTRODUCTION

Sheep breeding is performed in many regions of the world, especially regions with extensive pastures, meadows and suitable climates. However, sheep breeding is performed at pastures and meadows with low quality and areas with vegetation not utilized for crops or cattle breeding. Sheep obtain a large proportion of their nutrition

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from pastures. They are resistant to diseases and adverse environmental conditions, easily manageable, and have low costs [1,2].

In the world there are around 200 sheep breed and this number is around 20 for Turkey. Some of them are Akkaraman, Morkaraman, İvesi, Kıvırcık, Dağlıç, Karayaka and Sakız. Many scientific studies have been and are being conducted on these breeds. Furthermore, there are also important many sheep breeds that are bred locally. There is literature information about their yield performance. Hemsin sheep is one of them. However, scientific studies on the Hemsin sheep are not adequate. The grazing area of the Hemsin sheep covers an extensive area, where there is a dominant Black Sea climate consisting of the eastern parts of Rize, Artvin, and northern district of Erzurum, particularly Artvin and its surroundings. This region is rich in terms of meadows and plateaus and utilized very well by the Hemsin sheep. During snowy periods the sheep are accommodated in open or semi open sheepfolds. Feeding consists of heavily roughage in the region [3,4].

The Hemsin sheep is a breed that is demanded by breeders and known for the quality and taste of its meat and preferred more by people in the region compared to other breeds in the region. Thus, it is necessary to study the fattening performance and body measurement, which are among the efficiency properties of the Hemsin breed to make a contribution to the literature. The total number of Hemsin sheep is 54 924 head [5] and each passing day their amount is decreasing due to various reasons. If measures are not taken urgently, they will be faced extinction [6]. The decrease in the number of sheep can be listed as the enterprises being small, scattered and unorganized, the inadequacy of the level of productivity of existing breeds, feeding being based on ever poor pastures, and the use of new production techniques and technology at the lowest level based on structural and economic factors [7].

No studies have been conducted for the purpose of determining the fattening performance and body measurements of Hemsin lambs. Studies have been only conducted for the purpose of determining the growth and development characteristic while being bred by locals. In the study conducted with locals, Hemsin lambs were taken to the pasture when they were 3 months of age. At the beginning of pasture overall, male and female weights of Hemsin lambs were determined to be 28.09, 28.41, and 27.79 kg, respectively and at the end of pasture weights were determined to be 39.27, 39.95 and 38.58 kg, respectively. The pasture beginning weights of lambs obtained from elite, under elite and base flocks were determined to be 27.20, 29.28 and 27.82 kg, respectively and their pasture end weights were determined to be 39.14, 37.96 and 40.72 kg, respectively [8].

This study was conducted for the purpose of determining the effect of different fattening systems on the fattening performance and body measurements of Hemsin lambs.

MATERIAL and METHODS

This study was undertaken after ethical approval of Kafkas University (Official form date and number: 03.03.2011 and 2011-005). The study was conducted at the Application and Research Farm of the Faculty of Veterinary Medicine, Kafkas University in 2012. The lambs were purchased from a breeder at the Bereket village at the district of Ardanuç in the province of Artvin. A total of 39 three month of age Hemsin ram lambs, with an average live weight of 23 kg were used and randomly allocated into three groups. The lambs were divided into the 3 different fattening groups of extensive, semi intensive, and intensive, with 13 head lambs in each group. Before the beginning of the study, the lambs were applied medication against internal and external parasites. The study was started after 10 days of adaptation of lambs to assess of pasture and concentrate mixture.

In extensive and semi intensive fattening the lambs were grazed on the pasture 8 h in a day. The semi-intensive group was grazed on pasture and additionally was given concentrated feed available ad libitum. The concentrated feed was given to the intensive group available ad libitum and 270.00 g of grass hay per lamb per day. Concentrated food was consisted of 17.10% CP and 2710 kcal/kg ME [9]. The composition of the concentrated feed has been given in *Table 1* and the nutrient contents of concentrated feed and roughage has been given in *Table 2*. The concentrated feed has been prepared in a private feed factory and the fodder was obtained from the Faculty of Veterinary Medicine Farm. The analysis of the feed was performed at the Animal Nutrition and Nutrition Diseases at the Faculty of Veterinary Medicine of the Kafkas University. Clean water was provided as constantly for intensive fattening group lambs and it was ensured that the lambs of extensive and semi intensive feeding groups drank clean water at least three times a day. The feed was determined by means of a digital scale sensitive to 1 g.

The natural nutrient contents at various mowing times of the pastures, where the animals grazed, have been given in *Table 3*. For this purpose, samples were taken from various four locations of the pasture 3 times once a month (between June 5 and August 5) and the fodder of an area of 50 cm^2 of pasture was cut with a weed trimmer from 1 cm above the soil level. The pasture sample's DM (Dry Matter), OM (Organic Matter), CP (Crude Protein), CA (Crude Ash), Crude Cellulose (CC), Crude Fat (CF), and NFE (nitrogen free extract) levels were determined according to A.O.A.C. [10].

The live weights and body measurements of lambs were determined fortnightly. All lambs were weighed after

Table 1. *Compositon of the mixed feed used in semi intensive and intensive fattening process*
Tablo 1. *Yarı entansif ve entansif beside kullanılan karma yemin bileşimi*

Ingredient	%	Crude Protein (%)	Metabolic Energy (kcal/kg)
Barley	32.00	12.00	3110
Maize bran	10.00	9.20	2740
Maize	18.00	10.00	3300
Vegetable oil	2.60	-	7070
Sunflower cake	6.00	37.00	2250
Cotton seed cake	6.00	34.00	2300
Soy cake	14.00	48.00	3200
Molasses	8.50	7.80	2580
Lime stone	2.00	-	-
Sodium bicarbonate	0.20	-	-
Salt	0.50	-	-
Vit.-min. premix	0.20	-	-

Table 2. *Nutrient contents of concentrated feed and roughage, %*
Tablo 2. *Konsantre ve kaba yemin besin içeriği, %*

Ingredient	Concentrated Feed	Roughage
Dry matter (%)	88.80	90.69
Crude protein (%)	17.10	10.35
Crude cellulose (%)	5.70	32.38
Crude fat (%)	3.50	2.00
Crude ash (%)	6.40	8.86
Metabolic energy (kcal/kg)*	2710	2000

**It was determined through calculation over the table values*

being fasted for 12 h. At the end of the 90 day fattening process they were measured and their final live weights were measured. Weights were determined by means of a digital scale sensitive to 1 g.

In order to determine the effects of different fattening systems on fattening performance, variance analysis method was employed using SPPS 12.0 statistical package software [11]. Duncan multiple range tests were used to evaluate the significance of the difference among the groups.

RESULTS

The average live weights of lambs in the groups during various periods and the periods in fattening have been given in *Table 4*, the daily live weight gain (DLWG) has been provided in *Table 5*, the amount of daily consumed feed (DCF) has been provided in *Table 6*, and the feed conversion ratio (FCR) has been provided in *Table 7*.

The live weights at the beginning of fattening process for extensive, semi intensive, and intensive fattening were determined to be 22.42, 22.99 and 22.92 kg, respectively, and the live weights at the end of the 90 day fattening period were determined to be 33.32, 41.16 and 42.09 kg, respectively. In extensive, semi intensive, and intensive fattening process the DLWG for weights at the end of 90 day fattening were determined to be 121.11, 201.89 and 213.00 g, respectively. The daily consumed concentrated feed in semi-intensive and intensive fattening were determined to be 0.70 and 1.14 kg, respectively and the concentrated FCR was determined to be 3.44 and 5.35 kg, respectively.

Table 3. *Natural nutrients of the pasture at vairous mowing times, %*
Tablo 3. *Çeşitli biçim zamanlarında meranın doğal besin içeriği, %*

Pasture Mow/Month	DM	OM	CA	CP	CF	CC	NFE
I. mow	26.25	23.85	2.30	3.55	0.69	8.40	11.35
II. mow	32.35	30.10	2.30	2.70	0.99	9.70	16.68
III. mow	36.40	33.90	2.75	3.50	1.05	12.66	16.70

Table 4. *Live weight (kg) changes in groups based on periods (Mean±SE)*
Tablo 4. *Dönemlere göre gruplardaki ortalama canlı ağırlık (kg) değişimleri (Ortalama±Standart hata)*

Days	Extensive	Semi-intensive	Intensive	Significance
0	22.42±0.56	22.99±0.54	22.92±0.55	-
14	23.92±0.49	24.99±0.72	24.13±0.70	-
28	26.42±0.62^{b}	28.99±0.82^{a}	25.35±0.60^{b}	***
42	27.42±0.62^{b}	30.72±0.81^{a}	29.33±0.71^{ab}	***
56	29.21±0.64^{b}	33.95±0.94^{a}	33.96±0.75^{a}	***
70	31.86±0.56^{b}	37.45±1.13^{a}	37.00±0.79^{a}	***
84	33.05±0.66^{b}	40.36±1.22^{a}	40.29±1.04^{a}	***
90	33.32±0.64^{b}	41.16±1.36^{a}	42.09±1.09^{a}	***

*-: P>0.05, *** P<0.001,* ***a, b:*** *The difference between groups in the same line with different letters is significant (P<0.05)*

Table 5. *Daily live weight gain (g/day) in the groups according to periods (Mean±SE)*
Tablo 5. *Dönemlere göre gruplardaki günlük canlı ağırlık artışı, (g/gün)*

Days	Extensive	Semi intensive	Intensive	Significance
0-14	107.14±18.55	142.86±17.35	86.43±26.38	-
15-28	178.57±18.53^{b}	285.71±23.59^{a}	87.14±33.30^{c}	***
29-42	71.43±13.14^{b}	123.57±29.16^{b}	284.29±30.28^{a}	***
43-56	127.86±9.17^{c}	230.71±24.56^{b}	330.71±25.44^{a}	***
57-70	189.29±17.34	250.00±35.56	217.14±28.23	-
71-84	85.00±14.44^{b}	207.86±38.86^{a}	235.00±27.80^{a}	**
85-90	19.29±15.48^{b}	57.14±34.69^{b}	128.57±18.65^{a}	***
0-90	121.11±6.25^{b}	201.89±13.84^{a}	213.00±9.86^{a}	***

*-: P>0.05, ** P<0.01, *** P<0.001,* ***a, b, c:*** *The difference between groups in the same line with different letters is significant (P<0.05)*

Table 6. *Daily feed consumption (kg) per animal according to periods (Mean±SE)*
Tablo 6. *Dönemlere göre hayvan başına tüketilen yem miktarları (kg) (Ortalama±Standart hata)*

Days	Concentrate Feed			Roughage	Intensive Total Feed
	Semi Intensive	Intensive	Significance	Intensive	
0-14	0.14±0.02^{b}	0.36±0.02^{a}	***	0.36±0.04	0.72
15-28	0.26±0.02^{b}	0.62±0.01^{a}	***	0.30±0.03	0.91
29-42	0.68±0.02^{b}	1.04±0.04^{a}	***	0.30±0.02	1.34
43-56	0.97±0.01^{b}	1.47±0.01^{a}	***	0.24±0.01	1.71
57-70	1.01±0.01^{b}	1.56±0.01^{a}	***	0.23±0.001	1.79
71-84	1.08±0.01^{b}	1.76±0.01^{a}	***	0.23±0.001	1.99
85-90	0.77±0.001^{b}	1.23±0.001^{a}	***	0.15±0.001	1.39
0-90	0.70±0.04^{b}	1.14±0.06^{a}	***	0.27±0.01	1.48

*** P<0.001,* ***a, b:*** *The difference between groups in the same line with different letters is significant (P<0.05)*

Table 7. *Feed utilization rates according to periods, feed conversion ratio*
Tablo 7. *Dönemlere göre yemden yararlanma oranları*

Days	Semi Intensive	Intensive	
	Concentrate Feed	Concentrate Feed	Roughage
0-14	0.98	4.18	4.15
15-28	0.91	7.00	3.40
29-42	5.47	3.64	1.06
43-56	4.19	4.44	0.73
57-70	4.04	7.19	1.06
71-84	5.21	7.48	0.98
85-90	13.46	9.57	1.20
0-90	3.44	5.35	1.26

The body measurements of the lambs at different phases of the study are presented in *Table 8* and *Table 9*.

DISCUSSION

In fattening process of lambs, shortest fattening period, the best fattening systems and highest feed conversion ratio were preferred. In this research, the DLWG of the fattening in the first 0-14th and 15th-28th day periods are ordered as the semi intensive, extensive, and intensive groups. This can be the result of the animals in the intensive group not being completely used to the feed and environment. At the end of fattening period, the DLWG in groups of extensive, semi intensive and intensive was found 121.11, 201.89 and 213.00 g, respectively. The reason of the DLWG in the extensive group being so low may be the result of the pasture quality and nutritional value decreasing each day. Because the lambs were grazed between 08.00 in the morning and 17.00 in the evening and the lambs were affected by the heat during these hours. In terms of DLWG, it was determined that in the periods between days 29-42, 43-56, 71-84 and 85-90 that of the intensive group is higher than the semi intensive and extensive groups. This is due to the animals in the intensive group getting used to the feed in this period, the amount of concentrated feed being high for feed consumed for a 1 kg increase in live weight, the temperature being high during these periods and the quality and nutrition of the pasture decreasing after the solstice on June 21.

In this study, the initial and final live weights, and DLWG in the extensive group were found to be lower than those reported by Sezgin et al.[8] for Hemsin lamb possessed by

Table 8. *Changes in body measurement (cm) accoriding to periods (Mean±SE)*
Tablo 8. *Dönemlere göre vücut ölçülerindeki (cm) değişiklikler (Ortalama±Standart hata)*

Traits	Body Length	Withers Height	Chest Depth	Chest Girth	Circumference of Cannon Bone Forelimb	Circumference of Cannon Bone Hindlimb
0. day	-	-	-	-	-	-
Extensive	54.08±1.06	53.85±0.82	20.68±0.29	62.98±0.79	7.32±0.08	8.33±0.06
Semi intensive	55.12±0.88	54.22±0.66	20.92±0.45	64.14±0.81	7.33±0.06	8.38±0.05
Intensive	55.89±0.76	53.36±0.65	21.04±0.24	63.92±0.72	7.39±0.08	8.44±0.08
14. day	-	-	-	-	-	-
Extensive	57.65±0.72	55.37±0.87	21.81±0.23	64.90±0.70	7.67±0.03	8.57±0.04
Semi intensive	59.24±0.59	56.45±0.45	22.13±0.28	65.82±0.89	7.72±0.05	8.68±0.05
Intensive	57.68±0.54	55.10±0.69	21.99±0.30	66.22±0.89	7.67±0.06	8.65±0.04
28. day	***	-		*	-	-
Extensive	59.68±0.59^{b}	58.19±0.71	22.44±0.63	68.69±0.77^{b}	8.04±0.04	9.04±0.03
Semi intensive	61.40±0.52^{a}	58.32±0.69	22.76±0.67	70.99±0.72^{a}	8.09±0.03	9.11±0.04
Intensive	58.69±0.39^{b}	57.48±0.55	21.17±0.25	68.70±0.70^{b}	8.12±0.02	9.15±0.02
42. day	-	-	-	***	-	-
Extensive	61.77±0.57	59.02±0.60	22.72±0.68	69.74±0.56^{b}	8.13±0.03	9.13±0.03
Semi intensive	62.42±0.50	60.46±0.63	23.07±0.18	72.85±0.54^{a}	8.21±0.03	9.17±0.03
Intensive	61.11±0.44	58.75±0.56	22.79±0.22	71.47±0.60^{a}	8.21±0.04	9.24±0.04

a, b: The difference between groups in the same column with different letters is significant (P<0.05), **-:** P>0.05, * P<0.05, *** P<0.001

Table 9. *Changes in body measurement (cm) accoriding to periods (Mean±SE)*
Tablo 9. *Dönemlere göre vücut ölçülerindeki (cm) değişiklikler (Ortalama±Standart hata)*

Traits	Body Length	Withers Height	Chest Depth	Chest Girth	Circumference of Cannon Bone Forelimb	Circumference of Cannon Bone Hindlimb
56. day	-	-	*	**	**	**
Extensive	63.49±0.29	60.25±0.60	23.52±0.25^{b}	73.95±0.80^{b}	8.31±0.05^{b}	9.32±0.06^{b}
Semi intensive	64.25±0.47	61.04±0.56	24.31±0.20^{a}	76.66±0.66^{a}	8.65±0.08^{a}	9.65±0.09^{a}
Intensive	64.49±0.47	60.68±0.32	24.32±0.20^{a}	78.14±0.95^{a}	8.49±0.06^{ab}	9.43±0.05^{b}
70. day	**	-	*	***	***	***
Extensive	63.89±0.17^{b}	60.85±0.53	24.45±0.14^{b}	76.61±0.73^{b}	8.60±0.05^{b}	9.54±0.04^{c}
Semi intensive	66.53±0.66^{a}	61.85±0.54	25.22±0.19^{a}	81.96±1.03^{a}	9.22±0.08^{a}	10.09±0.09^{b}
Intensive	66.22±0.61^{a}	61.65±0.27	24.92±0.22^{ab}	81.69±0.88^{a}	9.32±0.10^{a}	10.35±0.09^{a}
84. day	***	-	***	***	***	***
Extensive	64.27±0.17^{b}	61.73±0.38	24.65±0.13^{b}	81.69±1.27^{b}	8.90±0.05^{b}	10.03±0.09^{b}
Semi intensive	66.99±0.61^{a}	62.32±0.54	25.50±0.16^{a}	87.00±1.16^{a}	9.43±0.07^{a}	10.56±0.08^{a}
Intensive	66.81±0.55^{a}	62.38±0.32	25.42±0.11^{a}	88.00±1.07^{a}	9.43±0.10^{a}	10.59±0.07^{a}
90. day	***	-	***	***	***	***
Extensive	64.67±0.17^{b}	62.13±0.38	24.80±0.13^{b}	84.09±1.27^{b}	9.00±0.05^{b}	10.18±0.09^{b}
Semi intensive	67.39±0.61^{a}	62.76±0.54	25.65±0.16^{a}	89.37±1.13^{a}	9.53±0.07^{a}	10.71±0.08^{a}
Intensive	67.21±0.55^{a}	62.78±0.32	25.57±0.11^{a}	90.40±1.07^{a}	9.53±0.10^{a}	10.74±0.07^{a}

-: *P>0.05, * P<0.05, ** P<0.01, *** P<0.001,* ***a, b, c:*** *The difference between groups in the same column with different letters is significant (P<0.05)*

locals (initial live weight 28.09 kg, final live weight 39.27 kg, and DLWG 220.64 kg). These differences result from the differences between initial live weight, the quality of the pasture, fattening period, and care and feeding.

The DLWG in the extensive group was found to be higher than those reported by Saricicek et al.[12] reporting a DLWG of 78.14 g for Karayaka lambs feed on pasture. Again, in this study, the DLWG in the semi intensive group

was found to be higher than those reported by Saricicek et al.[12] reporting a DLWG of 145.43, 152.29 and 166.71 g for Karayaka lambs feed on pasture and additionally barley, barley + the fattening feed for lamb-calf (25%) and barley + the fattening feed for lamb-calf (50%), respectively. The reason for differences observed between studies are due to breed, pasture beginning and pasture end weight, fattening period, pasture quality, care and nutrition programs, and various environmental conditions.

In this study, the pasture end weight obtained at the end of the 90 day pasture fattening period and the DLWG values were found to be lower than the values determined by Isik and Kaya [13] in the study conducted for the purpose of determining the fattening performance of Tuj breed lambs grazing at a pasture (pasture initial live weight 8.78 kg, pasture end weight at the end of a 140 day pasture fattening period 34.24 kg, DLWG 181.60 g for ram lambs).

Values regarding the fattening performance reported in the semi intensive and intensive group in this study were found to be lower than the values of the pasture grazing, and the groups receiving 200 and 400 g concentrated feed in addition to pasture of Tuj lambs in the study of Kaya et al.[14] (initial live weight 30.35, 30.47, and 30.40 kg, fattening end weights at the end of the 90 day fattening period of 44.10, 44.92 and 46.88 kg, and the DLWG of 152.78 g in the pasture group) and higher than the DLWG of groups receiving 200 and 400 g concentrated feed in addition to pasture (160.57 and 183.14 kg).

The final live weight and DLWG in the extensive group were found to be lower than those reported by Kaya et al.[15] reporting a final live weight of 34.23 kg and DLWG of 207.74 g for Tuj lambs feed on pasture. But, in this research, the final live weights in the semi intensive group was found to be higher than those reported by Kaya et al.[15] reporting a final live weight of 37.02 kg for Tuj lambs feed on pasture+concentrate. Again, in this study, the DLWG determined for the extensive group were higher than those reported by Saatci et al.[16] reporting a DLWG of 77.00, 98.00 and 118.00 g for Tuj lambs in group T, group TC and group C, respectively.

In the present study, the FCR determined in the intensive group found to be similar reported by Altin et al.[17] reporting a FCR of 5.30 kg for Kıvırcık lambs. The same weight values of FCR were found to be lower than those reported by Altin et al.[17] reporting a FCR of 6.25 kg for Karya lambs. This difference between researchers is due to breed, fattening start weights, fattening periods, and care and nutrition.

In this study, the FCR in intensive group found to be similar reported by Kucuk et al.[18] reporting a FCR 5.38 and 5.75 kg for Morkaraman and Kıvırcık×Morkaraman (G_1) crossbreed lambs in intensive fattening. However, the DLWG in this study was found to be lower than those reported in the same study reporting a DLWG of 272.00 and 324.00 g.

In this study, the body measurement values reported at 56 days of age were found to be higher than those reported by Bayram and Odabasioglu [19] reporting a body length (61.00 and 64.17 cm), withers height (55.25 and 55.00 cm), circumference of cannon bone forelimb (8.25 and 8.50 cm) for Kıvırcık and Kıvırcık×Morkaraman F_1 lambs at the end of the 60 day fattening period in the pasture and pasture + feed group, but in this study the chest depth and chest girth lower than those reported by Bayram and Odabasioglu [19] reporting a chest depth (26.83 and 26.67 cm) and chest girth (84.50 and 84.75 cm).

The body measurement values reported for Hemsin lambs in the 56 day of age in extensive and semi intensive groups were found to be lower than those reported by Arik et al.[20] reporting a withers height (64.83 and 66.08 cm), chest depth (26.43 and 26.80 cm) and chest girth (85.30 and 81.90 cm) for Malya and Akkaraman lambs at the end of the 56 day fattening period in fattened with full rations based on barley and formed with various roughages.

In this study, the body measurement determined on day 70 of intensive fattening were found to be lower than those reported by Karabacak et al.[21] reporting a withers height (64.40, 60.00, 62.70, 64.60 and 63.40 cm) and chest girth (84.10, 79.80, 83.70, 88.50 and 86.30 cm) for Akkaraman, Dağlıç, Kıvırcık, Malya and Karacabey Merinos lambs at the end of fattening periods in intensive fattening, but in this study the chest depth were found to be similar reported by Karabacak et al.[21] reporting a chest depth (24.60, 23.80, 24.00, 24.80 and 24.50 cm).

In the 90 day fattening study conducted on Hemsin lambs DLWG was found to be the lowest in the extensive group. This varies according to the quality of the pasture, where fattening was conducted, temperature and the average period of lambs in the pasture. No statistical difference was determined between the semi intensive and intensive groups in terms of DLWG. The DLWG was found to be the highest in the intensive fattening group. Due to animals grazing in pastures in the extensive and semi intensive groups, the amount of roughage that they consumed was not determined. The amount of concentrated feed consumed daily in semi intensive fattening corresponds to nearly half of the amount of feed consumed daily. Thus, due to there being no statistical difference between semi intensive and intensive groups in terms of DLWG, it appears that semi-intensive fattening would be more appropriate. Furthermore, it can be said that the fattening performance of the Hemsin lamb in extensive, semi intensive and intensive fattening is similar or a slightly lower than indigenous breeds.

The statistical differences in the body measurements of Hemsin lambs were generally observed on day 56 of

fattening. On the ninetieth day the extensive group was found to have the lowest body measurement values and no statistical difference was observed between the body measurements of the semi intensive and intensive fattening groups. When the body length values reported in various fattening groups of Hemsin lambs are compared with other studies conducted with different breeds, the obtained values were found to be higher or similar. When the values obtained in various fattening groups in terms of withers height, chest girth, and chest depth are compared with other studies conducted with different breeds, they were found to be lower or similar. This is due to the Hemsin breed having a longer and narrower body shape compared to other breeds.

In conclusion, the fattening performance and body measurement in intensive and semi intensive groups were found to be higher than that of the extensive group. No statistical difference was determined between semi intensive and intensive groups in general in terms of fattening performance and body measurements. Thus, it was revealed that semi intensive would be more appropriate.

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RESEARCH ARTICLE

Detection of DNA Markers in Dogs with Patellar Luxation by High Annealing Temperature - Random Amplified Polymorphic DNA Analysis [1]

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Summary

Patellar luxation is one of the orthopaedic disorders found mostly in small-breed dogs. It can be inherited by the next generation, causing continuing problems in the dogs' health. However, if it can be detected, the affected dogs will not be selected for breeding, and hence the incidence will be less. In this study, the objective was to find a DNA marker representing dogs with patellar luxation which can be detected when first born. High annealing temperature-random amplified polymorphic DNA (HAT-RAPD) technique was used to amplify 39 dog blood samples (16 unaffected and 23 affected). It was also used to develop the polymorphic fragments capable of distinguishing patellar-luxation-affected dogs from those unaffected. Three candidate fragments were sequenced and found to be parts from three different chromosomes (10, 36 and X) after comparison with the GenBank dog genome database using the BLAST algorithm. Association analysis was performed using a chi-square test. The results showed that the fragment (generated by the OPB05 primer) from chromosome 36 was potentially related to the two groups of dogs, with a P value of 0.042. This is the first finding of a gene which related to canine patellar luxation, and merits further investigation.

Keywords: *Patella luxation, Dog, HAT-RAPD, DNA marker*

Patella Çıkığı Olan Köpeklerde DNA Belirteçlerinin Yüksek Bağlanma Sıcaklıklı RAPD Analizi ile Belirlenmesi

Özet

Patellar luksasyon küçük ırk köpeklerde en yaygın gözlenen bozukluklardan birisidir. Sonraki nesillere aktarılmak suretiyle köpeklerde süregelen bir problem olabilir. Ancak tespit edilmesi durumunda etkilenmiş köpekler üretim amaçlı kullanılmaz ve böylece görülme sıklığı azaltılabilir. Bu çalışmada, patellar luksasyon ile doğan köpeklerin tespitinde kullanılmak amacıyla bir DNA markır bulmak amaçlanmıştır. High annealing temperature–random amplified polymorphic DNA (HAT-RAPD) tekniği ile 39 köpeğin (16 normal, 23 patellar luksasyonlu) kan örnekleri kullanıldı. Üç aday parçacık (fragment) sekanse edildi ve BLAST algoritması kullanılarak Den Bank köpek genomu ile karşılaştırıldığında bunların üç farklı kromozomdan (10, 36 ve X) bölümler olduğu tespit edildi. Asosiasyon analizi chi-kare testi kullanılarak yapıldı. Sonuçlar 36. kromozomdaki parçacığın (OPB05 primeri ile üretilen) potansiyel olarak iki grup köpek ile ilgili olduğunu ortaya koydu (P=0.042). Bu çalışma köpek patellar luksasyon ile ilgili bir gene ilişkin ilk çalışma olup gelecek araştırmalara ihtiyaç vardır.

Anahtar sözcükler: *Patella Luksasyon, Köpek, HAT-RAPD, DNA belirteci*

INTRODUCTION

Various diseases and ailments are frequently found in dogs; some are fatal, while others can cause pain and affect their daily lives. Some breeds of dogs are prone to certain genetic ailments, such as elbow or hip dysplasia,

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blindness, and patellar luxation [1]. Especially in recent years, patellar luxation problems appear to be getting worse in Pomeranian and other small breeds. It has also been reported that this can be inherited polygenically [2,3], making it a serious problem that small dog breeders should address so that future generations of dogs will have strong patellas and hind limbs. Based on a recent health survey from 1974-2011, 40% of Pomeranians were diagnosed with patellar luxation [4]. Small dogs (weighing less than 9 kg) are said to be 12 times more likely to be affected by patellar luxation than medium, large or giant dogs. It can also be a heartbreaking problem for puppy purchasers. As a consequence, it is highly recommended that dogs with this disorder should not be used for breeding purposes [5]. However, because affected dogs rarely show symptoms until they enter middle age, at an early stage of patellar luxation it might be difficult to prognosticate whether some dogs will develop the disorder [6]. In cases of congenital patellar luxation, dogs carrying this trait can theoretically be detected by genetic markers, leading to a reduction in its incidence. Thus, the objective of this study was to construct a sequence-characterized amplified region (SCAR) marker derived from DNA polymorphisms in the genome of dogs with patellar luxation for discrimination and prediction of this disorder.

MATERIAL and METHODS

Blood Samples

Thirty-nine canine blood samples *(Table 1)* were kindly provided by a small animal clinic in Chiang Mai province. All samples were collected from a variety of small breeds and ages and were kept at −20°C, without any anticoagulant, prior to the experiment.

DNA Extraction

The isolation of total genomic DNA for molecular marker analysis was carried out utilizing the phenol-chloroform method of Taş [7].

HAT-RAPD Analysis

Genomic DNA from each sample was diluted to a concentration of 10 ng/ml. The amplifications followed the protocol of Anuntalabhochai et al.[8] and Liu et al.[9]. PCR was performed in a total volume of 25 µl containing: 1X reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, 1 mg/ml BSA, and 100 mM $(NH_4)_2SO_4$; RBC Bioscience, Taipei, Taiwan); 2 mM $MgCl_2$ (RBC Bioscience); 0.2 mM dNTP (Vivantis Technologies, Malaysia); 0.4 µM primers *(Table 2)* (Operon Technologies, Alameda CA, USA); 1 U *Taq* DNA polymerase (RBC Bioscience); 10 ng/ml genomic DNA; and deionized distilled water. PCR was performed using an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Hercules CA, USA) with the following cycling profile: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 30 sec, 45°C for 1 min and 72°C for 1.5 min; and a final cycle at 72°C for 5 min. After PCR was completed, the amplified samples were kept at 4°C prior to agarose gel electrophoresis.

Cloning of DNA Fragments

Candidate fragments were excised, using a clean scalpel, and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) following the manufacturer's recommended protocol for DNA extraction from agarose gel. The candidate fragments were ligated with the RBC TA Cloning Vector Kit using T_4 DNA ligase enzyme (RBC Bioscience), following the manufacturer's recommended protocol. The fragment-ligated vectors were transformed into HIT-DH5α competent cells (RBC Bioscience) following the manufacturer's recommended protocol. The mixture was incubated on ice for 10 min and poured immediately onto previously prepared luria broth (LB) agar. Positive colonies were selected by blue/white colony selection technique and were confirmed for the inserted DNA fragments by colony PCR using M13 forward and reverse primers. A colony that gave the correct DNA fragment length was inoculated in LB broth with vigorous shaking (200 rpm) at 37°C overnight or until the OD_{600} reached 0.5-0.7. The plasmid was extracted using the PureYield™ Plasmid Miniprep System (Promega, Fitchburg WI, USA) following the manufacturer's recommended centrifugation protocol.

Sequencing and Sequence Identification

The sequences of all fragments were commercially analyzed by an automated sequencer (1st BASE, Singapore) and compared with the GenBank dog *(Canis lupus familiaris)* genome database using the BLAST algorithm [10]. The likelihood was considered to correspond to the E-value, which had to be less than 1^{-30} to indicate a significant similarity.

Association Analysis

The relationships between the polymorphic fragments and the two populations were analyzed using a chi-square test. The appearance of each polymorphic fragment was scored as 0 or 1 for its occurrence or absence, respectively. The data were used to calculate the χ^2 value, a *P* value of <0.05 was considering significantly difference.

RESULTS

Polymerase chain reaction (PCR)-based high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) was performed using 16 primers *(Table 2)* on pooled DNA of 39 canine blood samples to select high polymorphic primers. The results are shown in *Fig. 1* and *Table 2*. These primers yielded a total number of 91 amplified fragments and 294 polymorphic fragments. The fragments ranged in size from 300 to 3.500 base pairs. In a search

Table 1. *Data from 39 canine blood samples (Numbers in parentheses represent patellar luxation grading; M = male, F = female)*
Table 1. *39 adet köpek kan örneğine ait veriler (parantez içerisindeki numaralar patellar luksasyon derecesini göstermektedir;* M = erkek, F = dişi)

Sample Code	Breed	Sex	Age (months)	Weight (kg)	Patellar Luxation
PL01	Chihuahua	F	6	3.6	Affected (3/4)
PL02	Chihuahua	F	7	2.5	Affected (2/4)
PL03	Chihuahua	M	7	3.2	Affected (3/4)
PL04	Chihuahua	F	8	1.2	Affected (2/4)
PL05	Chihuahua	F	9	2.25	Affected (2/4)
PL06	Chihuahua	M	9	3	Affected (3/4)
PL07	Chihuahua	M	13	2.3	Affected (4/4)
PL08	Chihuahua	F	14	2	Affected (3/4)
PL09	Pekingese	M	5	5.4	Affected (3/4)
PL10	Pomeranian	M	6	1.2	Affected (3/4)
PL11	Pomeranian	M	7	4	Affected (3/4)
PL12	Pomeranian	F	8	1.4	Affected (4/4)
PL13	Pomeranian	M	8	5	Affected (4/4)
PL14	Pomeranian	F	11	3	Affected (4/4)
PL15	Pomeranian	M	14	3.5	Affected (3/4)
PL16	Pomeranian	F	14	14	Affected (3/4)
PL17	Poodle	F	8	3.2	Affected (3/4)
PL18	Poodle	F	8	3.5	Affected (2/4)
PL19	Poodle	F	10	3.4	Affected (3/4)
PL20	Poodle	F	12	1.9	Affected (2/4)
PL21	Poodle	F	13	1.2	Affected (4/4)
PL22	Poodle	F	18	2.1	Affected (2/4)
PL23	Yorkshire Terrier	F	7	1.2	Affected (3/4)
N01	Chihuahua	F	19	1.4	Unaffected
N02	Chihuahua	F	25	2.4	Unaffected
N03	Chihuahua	F	32	2.4	Unaffected
N04	Chihuahua	M	54	3.9	Unaffected
N05	Chihuahua	M	60	1.8	Unaffected
N06	Chihuahua	F	70	2.4	Unaffected
N07	Chihuahua	F	98	3	Unaffected
N08	Pomeranian	F	54	9.2	Unaffected
N09	Pomeranian	F	75	2.8	Unaffected
N10	Pomeranian	F	83	3	Unaffected
N11	Pomeranian	M	98	3	Unaffected
N12	Poodle	M	13	7	Unaffected
N13	Poodle	F	23	2.85	Unaffected
N14	Shih Tzu	M	42	3.2	Unaffected
N15	Shih Tzu	F	84	5.8	Unaffected
N16	Yorkshire Terrier	F	32	1.85	Unaffected

of polymorphic fragments in two sample groups (non-affected and affected by patellar luxation), PCR-based HAT-RAPD was done with 16 randomly chosen primers of arbitrary nucleotide sequences on two DNA templates, pooled from both groups of samples. The results are shown in *Fig. 2* with arrows indicating two polymorphic fragments found. Two primers (OPB05 and R105) that gave the polymorphic fragments were chosen to amplify all 39 DNA samples. The results are shown in *Fig. 3* and *4*, respectively. The P values of the relationship between the two populations and the OPB05 and R105 primer-generated polymorphic fragments were 0.042 and 0.112,

Table 2. *List of primers and their sequences used in HAT-RAPD analysis, number of fragments and size ranges*

Table 2.*HAT-RAPD analizinde kullanılan primerlerin ve sekanslarının, parçacık numaraların ve boyutlarının listesi*

Primer	Sequence (5′ 3′)	Total Number of Amplified Fragments	Fragment Size Range (bp)
1. A4	GCATCAATCT	7	300-3.000
2. AP42	AACGCGCAAC	6	200-3.000
3. OPB04	GGACTGGAGT	4	500-2.000
4. OPB05	TGCGCCCTTC	5	600-3.000
5. OPB06	TGCTCTGCCC	3	800-2.000
6. OPB07	GGTGACGCAG	7	550-3.500
7. OPB08	GTCCACACGG	6	500-3.000
8. OPB10	CTGCTGGGAC	5	700-3.000
9. OPB17	AGGGAACGAG	9	300-4.000
10. OPB18	CCACAGCAGT	7	200-4.000
11. OPS11	AGTCGGGTGG	8	200-3.000
12. OPS16	AGGGGGTTCC	3	600-2.000
13. OPW09	GTGACCGAGT	5	450-2.500
14. R37	GAGTCACTCG	5	800-2.500
15. R55	CGCATTCCGC	5	220-3.800
16. R105	GCACCGAACG	6	550-2.200
Total		91	200-4.000

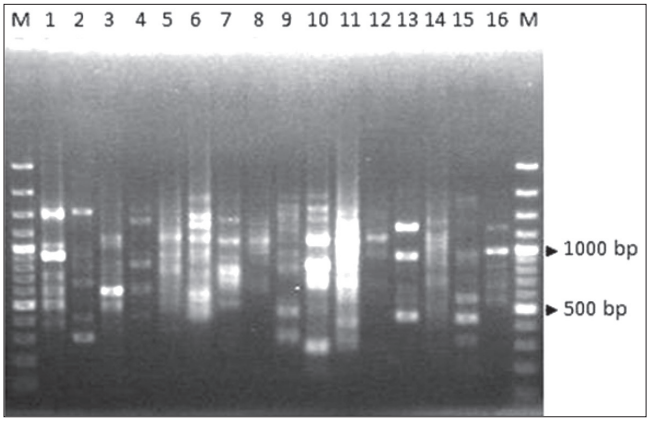

Fig 1. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows bands from random primer amplifications by 16 decanucleotide primers (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, and 16-R105)

Şekil 1. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. 16 dekanükleotid primerler ile elde edilen rastgele primer amplikonlarını gösterir bantlar (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, ve 16-R105)

respectively, when calculated by a chi-square test (the test of independence) with a degree of freedom of 1. Based on a 95% confidence interval, the OPB05-generated fragment was significantly related to the disorder. The

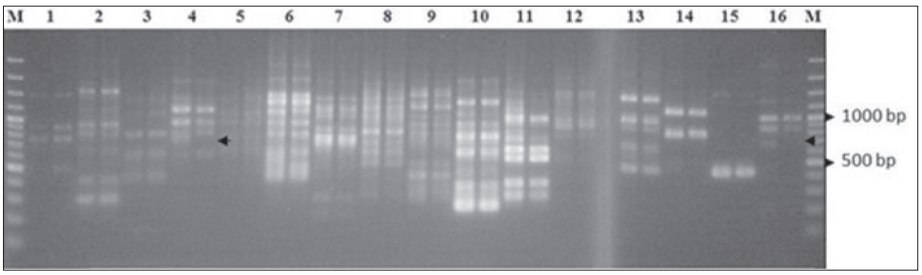

Fig 2. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows fragments from HAT-RAPD reactions with 16 randomly chosen primers (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, and 16-R105) of arbitrary nucleotide sequences on two DNA templates, pooled from samples non-affected and affected by patellar luxation. Arrows show polymorphic fragments that might be used to distinguish the two groups of samples

Şekil 2. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. Patellar luksasyonlu ve normal hayvanların iki DNA eşi üzerindeki rastgele nukleotid sekanslarına ait 16 rastgele seçilmiş primer (1-A4, 2-AP42, 3-OPB04, 4-OPB05, 5-OPB06, 6-OPB07, 7-OPB08, 8-OPB10, 9-OPB17, 10-OPB18, 11-OPS11, 12-OPS16, 13-OPW09, 14-R37, 15-R55, ve 16-R105)kullanılarak HAT-RAPD reaksiyonu ile elde edilen fragmentler

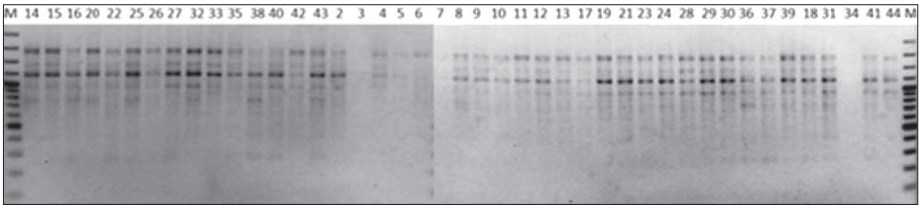

Fig 3. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows fragments amplified by HAT-RAPD reaction with OPB05 primer in DNA samples

Şekil 3. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. DNA örneklerinde OPB05 primeri kullanılarak HAT-RAPD reaksiyonu ile amlife edilmiş fragmentler

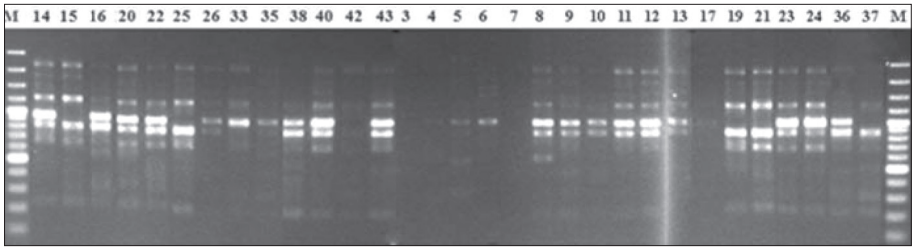

Fig 4. Two percent agarose gel stained with ethidium bromide and photographed by a UV transilluminator under UV light shows fragments amplified by HAT-RAPD reaction with R105 primer in DNA samples

Şekil 4. Etidiyum bromit ile boyanan %2'lik agar jel UV ışık altında fotoğraflanmıştır. DNA örneklerinde R105 primeri kullanılarak HAT-RAPD reaksiyonu ile amlife edilmiş fragmentler

sequences after alignment of three fragments from OPB05 (KF146953), R105-1 (KF146954) and R105-2 (KF146955) are shown in Additional file 1. Those three fragments were shown to be the genomic scaffolds from chromosomes 36, X and 10, respectively.

DISCUSSION

In this study, three different DNA fragments were found in the domestic dog genome, using HAT-RAPD analysis with 16 decanucleotide primers in an attempt to find molecular markers for canine patellar luxation. One fragment showed a significant relationship to the disorder, with a *P* value of 0.042. It has been suggested that canine patellar luxation is inherited [2], and patellar luxation has been demonstrated to be polygenic in its inheritance [3]. Although Soontornvipart et al.[11] showed 15 pedigrees of families of patellar luxation-affected dogs, no mode of inheritance has been confirmed as responsible for the penetrance of the phenotype. In addition, from the same study, microsatellite markers linked to the specifically collagen-related genes were used to identify the responsible polymorphism; however, the result indicated a non-involvement in the pathogenesis of patellar luxation, which supported the conclusion of Chase et al.[3] that patellar luxation is polygenic. Thus, the HAT-RAPD marker was chosen to identify the potential candidate polymorphisms accounting for the pathogenesis of patellar luxation, considering the ability of conventional RAPD to simultaneously screen several loci in the genome [12] and the high reproducibility of newly adapted HAT-RAPD [8].

Although HAT-RAPD has been used in plants [13,14] and platyhelminthes [15-17], it has never been used in mammals before; thus, the efficiency of using HAT-RAPD in the dog genome was analyzed. According to *Table 1*, HAT-RAPD showed a higher polymorphic ratio (16%) when compared with the use of 200 primers in conventional RAPD for identifying genetic markers associated with canine hip dysplasia (5%) [18], indicating that HAT-RAPD was suitable for producing DNA fragments in a domestic dog genome. This is in addition to its low initial investment, high speed of assay and simplicity, as described by [12,19,20].

To identify the potential candidate polymorphisms, HAT-RAPD was performed on two pooled DNA templates, patellar luxation-affected and patellar luxation-unaffected. Two polymorphic fragments from OPB05 (TGCGCCCTTC) and R105 (GCACCGAACG) primers were found (*Fig. 2*, as indicated by arrows), gel-purified, cloned and sequenced.

The results after aligning the three acquired sequences with the domestic dog *(Canis lupus familiaris)* genome in the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) algorithm showed that all fragments are parts of genomic scaffolds from three different chromosomes (10, 36 and X). Although none appeared to be the potentially responsible gene, these fragments can play important roles in monitoring the pathology of the disease, since an extragenic sequence can act as a promoter, enhancer, or even as an undiscovered gene, which can affect the expression of a gene or control protein functions [21]. However, when considering the flanking gene of the 659-nucleotide-long fragment located on chromosome X [KF146954], which is translated to the cAMP-dependent protein kinase catalytic subunit PRKX, the result was found to be in accordance with the findings of Wangdee et al.[22], that PL might be caused by malformation of the sartorius muscle, possibly due to the PRKX protein that functions in tissue formation, cellular differentiation and epithelial morphogenesis [23-25].

Furthermore, the 644-nucleotide-long fragment on chromosome 10 [KF146955] has one flanking uncharacterized protein [LOC403431] at 5.390 bases away on the 5' side, which can be its target of regulation since [21] stated that regulatory sequences affecting gene expression can be located both upstream and downstream of the regulatory target, with spacing up to tens of kilobases.

However, the number of chromosomes found in this study (10, 36 and X) was not consistent with chromosome 7, containing the single-nucleotide polymorphism (SNP) BICF234J1226, which has been reported to be the most significant SNP in an association analysis of 1.536 SNPs distributed over 38 autosomes and having an odds ratio of 13.6 [11]. There are 43 genes located in the 4 Mb region

surrounding the associated SNP, and none of these genes is known from other species to be involved in patellar luxation.

As a result of this study, the OPB05 primer-generated polymorphic fragment was significantly linked to patellar luxation. After alignment, the fragment was found to be genomic scaffold from chromosome 36 [KF146953] flanked by the ATP synthase gene at 398,900 bases away on the 5′ side, making the polymorphic fragment possibly linked to the gene, considering the 0.3 cM distance (less than 7 cM of linkage criteria) [26]. According to the etiology of the disorder, the patella luxates after the contraction of the quadriceps muscle. Together with the shallowness of the patellar groove, it might be possible that the luxation occurs as a result of the malfunctioning of ATP synthase, leading to the over-contraction stage of the quadriceps [27] and resulting in a floating (or luxating) patella.

Thus, HAT-RAPD has been proven to be capable of amplifying the dog genome and generating polymorphic fragments. The DNA fragment from chromosome 36 generated by the OPB05 primer (TGCGCCCTTC) showed a significant relationship with the disorder's occurrence, and is worthy of further investigation in more sample sizes with linkage analysis. In addition, further examining of three unconfirmed mutations found in the DNA fragment from the OPB05 primer should be performed. Primers specific to the region can be synthesized and used to amplify the fragment for mutation-confirmation processes, i.e. restriction enzyme cutting and sequencing. After the mutations are positively confirmed, genotyping can be performed by PCR-RFLP (restriction fragment length polymorphism) for SNP and by high-resolution polyacrylamide gel electrophoresis for deletions [28].

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RESEARCH ARTICLE

Çiğ Süt Kalite Değerlendirmesinde Bulanık Mantık Yaklaşımı [1]

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[1] *Bu çalışmanın bir kısmı Uluslararası Türk ve Akraba Toplulukları Zootekni Kongresi (11-13 Eylül 2012, Isparta, Türkiye)'nde sözlü olarak sunulmuştur*

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Özet

Gelişen toplum yapısı ile birlikte gerçek hayatta yaşanılan sorunlar ve olaylara bakış açıları da değişmektedir. İnsanlar sorunlarını sahip oldukları sözel ve sayısal verileri kullanarak çözmekte ve bunun için çeşitli yöntemlerden yararlanmaktadırlar. Matematiksel yöntemler insanlara kesinlik içeren durumlarda sorunların çözümlenmesinde sayısal verileri analiz ederek yardımcı olurken, belirsizlik içeren durumlarda yetersiz kalabilmektedir. Son yıllarda kalite değerlendirilmesi gibi belirsizlik içeren durumlarda ortaya çıkan problemlerin çözümünde sıklıkla kullanılan bulanık mantık, yapay zeka yöntemlerinden bir tanesidir. Klasik mantık teorisine göre daha esnek bir yapıya sahip olan bulanık mantık teorisi, olayları nesnelere "0" ve "1" arasında atadığı doğruluk dereceleri ile açıklamakta böylece sözel ve sayısal veriler arasında bir bağ oluşturmaktadır. Bu çalışmada, çiğ süt örneklerinin kalite sınıflarına ayrılmasını amaçlayan bulanık mantık tabanlı bir karar destek sistemi geliştirilmiştir. Sistemin girdileri çiğ süt örneklerine ilişkin toplam bakteri sayısı, somatik hücre sayısı ve protein miktarlarının ölçülen değerleridir. Tasarlanan bulanık sistemin çıktısı ise çiğ süt kalite değerlendirmesi şeklindedir. Yapılan analizin başarısını belirlemek amacıyla uzman kararları ile karşılaştırma yapılmış ve sistemin %80 değerinde başarılı olduğu görülmüştür. Sistemin modellenmesi Matlab (sürüm R2010b) programı kullanılarak yapılmıştır.

Anahtar sözcükler: *Bulanık mantık, Karar destek sistemi, Çiğ süt kalitesi*

Fuzzy Logic Approach in the Evaluation of Raw Milk Quality

Summary

The problems that faced with in real life and perspective of the events change with developing structure of society. The people in the face of problem use a variety of methods with their verbal and numerical data to find solution. Mathematical methods that including precision are sufficient in the analyses of numerical data while the modeling of verbal data may be insufficient in case of uncertainty. In recent years, fuzzy logic is one of the artificial intelligence methods that used in solution of the problems which are rosed from quality evaluation situations that consists of uncertainty cases. The fuzzy logic theory that has more flexible structure than the theory of classical logic, describe the events with degree of accuracy which is between "0" and "1" appointed to object. Fuzzy logic–based decision support system offers to people a more realistic and objective perspective in decision making. In this study, fuzzy logic base decision support system which aims to classify raw milk samples in quality has been developed. System inputs are; bacteria count for milk samples, somatic cell count and values for measured protein amounts. Designed fuzzy logic output is consist of raw milk quality value measurement; in order to calculate the success of the analysis, results have been compared to specialist's decisions and due to the comparison, it noticed that the system has 80% success rate. Modeling of the system has been made via Matlab (version R2010b) programme.

Keywords: *Fuzzy logic, Decision support system, Raw milk quality*

GİRİŞ

Üreticiler ve tüketiciler açısından çiğ süt kalitesi, işlenen süt ürünlerinin kalitesi ve raf ömrü uzunluğu ile hayvan sağlığı gibi konularda büyük bir öneme sahiptir. Ülkemizde Çiğ Süt ve Isıl İşlem Görmüş İçme Sütler Tebliği'nde, çiğ sütün ısıl işlem görmüş içme sütü, süt ürünleri ve süt bazlı ürünlerin imalatında kullanılan sütlerin tekniğine uygun ve hijyenik şekilde üretimi, depolanması, taşınması ve pazarlanmasını sağlamak üzere özelliklerinin belirlen-

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mesi amaçlanmıştır. Bu amaç doğrultusunda çiğ süt kalitesine ilişkin bir takım standartlar bulunmaktadır [1]. Süt üreticileri açısından verimliliğin en önemli unsurları temiz ve kaliteli süttür. Bu kapsamda çiğ süt kalitesinin değerlendirilmesinde kullanılan en önemli kriterlerden bir tanesi somatik hücre sayısıdır [2,3]. Somatik hücre sayısının takibi ve değerlendirilmesi, hayvanların bireysel ve sürü bazında sağlık durumlarının kontrolü için büyük önem taşımaktadır. Somatik hücre; kandan süte geçmiş beyaz kan hücreleri ve meme bezi epitel hücrelerinden oluşmaktadır [4]. Somatik hücre sayısındaki artışlar, klinik ve subklinik mastitis (meme iltihabı) gibi önemli hastalıkların belirtisidir. Klinik mastitiste hayvanın memesinde ve sütte oluşan değişiklikler gözle görülür şekilde belirgindir ancak subklinik mastitiste durum tam tersi olduğundan dolayı somatik hücre sayısının belirlenmesi hastalık tespiti için en önemli göstergedir. Beyaz kan hücreleri hayvanın memesinde ortaya çıkan mikroplarla savaşabilmek amacıyla meme dokusuna geçer. Memede ne kadar çok mikrop var ise somatik hücre sayısı o kadar artış gösterir. Bu durum süt ürünlerinde kalite hatalarına neden olmaktadır. Mastitis süt salgısı yapan hücreleri dolayısı ile dokuları yok eder. Yok olan dokular hastalık nedeni ile kendini yenileyemez ve bunların yerini bağ dokular alır. Somatik hücre takibi sağlıklı bir şekilde yapılmayıp gerekli önlemler alınmadığı takdirde ortaya çıkan mastitis hastalığı, süt verimini düşürüp ekonomik kayıplara neden olmakta ve aynı zamanda sürü sağlığını olumsuz yönde etkilemektedir [5,6].

Çiğ süt kalite değerlendirmesinde bir başka önemli kriter ise sütte bulunan bakteri sayısıdır. Ahır ve sağım koşulları ile hayvanın temizliği gibi çevre etkileri sütte bulunan bakteri sayısını gerekli önemler alınmadığı takdirde önemli ölçüde etkilemektedir. Bakteri sayısındaki yüksek miktarlı artışlar sütün bozulmasına neden olacağından, sütün besin değerini ve kalitesini olumsuz etkilemekte ve ekonomik anlamda ciddi kayıplara yol açmaktadır. Aynı zamanda bakteri içeren sütler tüketildiğinde insanlar ve hayvanlarda gıda zehirlenmelerine neden olabilmektedir [7-9].

Çiğ süt kalite değerlendirmesinde kullanılan bir diğer kriter olan proteinler, sindirimi kolay besin değeri yüksek insan beslenmesi ve yaşam kalitesi açısından oldukça büyük bir öneme sahiptir. Süt proteinlerinin içeriğinde insan vücudu tarafından sentezlenemeyen, mutlaka dışarıdan alınması gereken esansiyel amino asitler bulunmaktadır. Proteinler insanlar için hem enerji kaynağı hem de yapıtaşı olarak kabul edilmektedir. Proteinler süt teknolojisi açısından da büyük bir öneme sahiptir. Protein miktarı yoğurt, peynir vb. süt ürünlerinde kaliteyi oldukça yüksek düzeylerde etkilemektedir. Süt bileşiminde yer alan protein miktarı yetkili kurumlarca kontrol edilmekte ve yeterli koşullar altında üreticilere prim ödemeleri yapılabilmektedir [6].

Gerçekleştirilen mikrobiyolojik ve kimyasal analizlere ilişkin parametreler kullanılarak oluşturulan çeşitli modellemeler sayesinde çiğ süt kalitesine dair bilgiler ilgili kişilere sunulmakta ve farklı açılardan değerlendirme imkânı sağlanmaktadır. Bu modelleme yöntemlerinden bir tanesi son yıllarda tarımsal ürünlerin kalite değerlendirmelerinde sıklıkla karşılaşılmakta olan bulanık mantık yöntemidir.

Azeri asıllı bilim adamı Lotfi A. Zadeh olasılık dağılımı ile tanımlanamayan, bulanık (belirsiz) durumlar için farklı bir matematiğe ihtiyaç duyulduğunu belirtmiştir [10]. 1965 yılında bulanık mantık hakkında ilk makale olan "Fuzzy Sets" başlıklı çalışma Zadeh tarafından yapılmıştır. Zadeh, insanların düşünce yapısında çoğunlukla kesinlik taşımayan bulanık ifadelerin yer aldığını belirtmiştir. Klasik mantıktaki "0" ve "1" gibi kesin ayrımların yanı sıra, ara değerlerin de göz önünde bulundurulması gerekliliğine dikkat çekmiş ve olayların [0,1] aralığında belirli bir derece ile gösterildiğini ifade etmiştir [11]. Bulanık mantık teorisinin ortaya çıkışı ile birlikte özellikle insanların düşünce yapısındaki sözel verilerin işlenmesinde oldukça büyük gelişmeler sağlanmıştır. Aralık matematiği ve bulanık mantık kullanılarak oluşturulan bulanık sistemler sözel verilerden sayısal verilere geçişte bir köprü görevi görmektedir [12,13]. Kesin sayısal verilerle çalışıldığında klasik yöntemler kullanılabilmekte ancak belirsizlik ortamında ortaya çıkan sorunların çözümünde klasik yöntemler sağlıklı sonuçlar vermemektedir.

Günümüz teknolojisi özellikle yapay zeka tabanlı çeşitli yazılımlar sayesinde insanların sahip oldukları algılama yeteneğinin bilgisayarlar tarafından taklit edilebilmesine ve belli ölçülerde öğrenmesine imkan sağlamaktadır. Bulanık mantık, yapay sinir ağları, uzman sistemler ve genetik algoritmalar gibi yapay zeka yöntemleri başta mühendislik ve tıp olmak üzere farklı bilim dallarında başarı ile uygulanmaktadır [14-16]. Son yıllarda, bulanık mantık tabanlı yöntemler belirsizlik ve nesnelliğin olduğu durumlarda, mühendislik, tıp ve biyoloji alanlarında olduğu gibi tarım ve hayvancılık alanında da başarılı bir şekilde uygulanmaktadır. Tıp alanında bulanık mantık yardımıyla oluşturulan bulanık uzman sistemler ve karar destek sistemleri kanser ve tümör gibi ciddi hastalıkların teşhisinde ve değerlendirilmesinde karar almak amaçlı sıklıkla kullanılmaktadır [17-21]. Ülkemizde oldukça yeni kullanılmaya başlanan bulanık mantık teorisi, hayvancılık alanında da gerçekleştirilen birçok başarılı çalışmaya konu olmuştur. Örneğin hayvan ıslahı [22,23], kızgınlık tespiti [24-26], mastitis ve topallık gibi hastalıkların teşhisi [27-30] hayvan besleme [31,32] çeşitli verim özelliklerinin (süt, yumurta, canlı ağırlık vb.) tahmini ve hayvansal ürünlerin kalite sınıflandırması [33-37] gibi alanlarda kullanılabilmektedir.

Bu çalışmada, veri setini oluşturan çiğ süt örneklerinin mikrobiyolojik ve kimyasal özellikleri kullanılarak çiğ sütün yüksek kaliteli, orta kaliteli ve düşük kaliteli olmak üzere üç kalite sınıfına ayrılmasını hedefleyen bulanık mantık tabanlı bir karar destek sistemi geliştirilmesi amaçlanmıştır.

MATERYAL ve METOT

Materyal

Çalışmanın materyali, Ege Üniversitesi Ziraat Fakültesi Süt Teknolojisi Bölümü'nde gerçekleştirilmiş bir çalışmada çiğ süte ait kuru-madde, pH, yağ, protein, Soxhlet-Henkel (°SH) asitlik değerleri, toplam bakteri sayısı (TBS), somatik hücre sayısı (SHS) parametrelerinin kimyasal ve mikrobiyolojik analiz sonucu ölçülen değerlerine ait verilerinden oluşmaktadır [3]. Bu çalışma kapsamında tasarlanan bulanık sistemin girdi değişkenleri somatik hücre sayısı, toplam bakteri sayısı ve protein miktarı olarak belirlenmiştir. Tasarlanan bulanık sistemin çıktısı ise çiğ süt kalite değerlendirmesi şeklindedir. Çalışmanın analizleri Matlab (sürüm R2010b) paket programı kullanılarak gerçekleştirilmiştir.

Metot

Bulanık sistemler, bulanık "eğer-o halde" kuralları ile yapılandırılarak oluşturulan bilgi tabanlı sistemlerdir ve doğrusal olmayan bir fonksiyondan bilgi tabanına dönüşen sistematik bir süreç sağlamaktadır [10]. Bulanık mantık teorisi sayesinde, insan uzmanların sahip olduğu bilgiler "eğer-o halde" kuralları yardımıyla bilgisayar sistemlerine işlenmektedir. Böylece doğal dil yani insanların düşüncelerinde yer alan sözel veriler başarılı bir şekilde kullanılmaktadır. Bulanık sistemler bulanık kural tabanı, bulanık çıkarım motoru (karar verme birimi), bulanıklaştırıcı ve durulaştırıcı olmak üzere dört bileşenden oluşmaktadır. *Şekil 1*'de bulanık bir sistemin genel yapısı görülmektedir.

Bulanıklaştırma, gerçek bir değeri bulanık bir kümeye dönüştürücü olarak tanımlanmaktadır. Bunun için girdi değişkeni aralığının uygun evrensel kümeye dönüştürülmesi sağlanır ve böylece girdi değerleri uygun sözel değerlere dönüştürülmüş olur. Bulanıklaştırma aşamasında dışarıdan gelen verilerin, sistemin çıkarım mekanizması ile bulanık kural tabanındaki bilgiler kullanılarak işlenmesi amacıyla ön hazırlıklar yapılmaktadır. Bu amaçla bulanıklaştırma aşamasında çeşitli üyelik fonksiyonları kullanılmaktadır. Uygulamalarda en fazla kullanılan üyelik fonksiyonu tipleri Üçgen, Yamuk, Çan Eğrisi, Gauss, Sigmoidal, S ve Pi(π) üyelik fonksiyonlarıdır [10,38]. Üyelik fonksiyonlarının belirlenmesinde karınca kolonisi algortiması, klonal seçim algoritması, tabu arama algoritması, genetik algoritmalar ve yapay sinir ağları gibi yapay zeka yöntemleri ile sezgisel yöntemler araştırmacılar tarafından tercih edilmektedir [39-42]. Bu çalışmada ayrıntılı literatür incelemesi ve konusunda uzman kişinin görüşleri ışığında, üçgen ve yamuk üyelik fonksiyonları kullanılmıştır.

Bulanık çıkarım kısmında, bilginin sunumu için kullanılan kural tabanı ile birlikte bir çıkarım mekanizması bulunmaktadır. Bulanık kural tabanında sisteme gelen veriler işlenmeye hazır halde getirildikten sonra "eğer-o halde" şeklinde tanımlı kurallara göre çıkarım mekanizması tarafından işlenmektedir. Burada değişkenler, üyelik fonksiyonlarının sayısı ve kuralların sayısı yer almaktadır. Tanımlanan bu parametrelere göre yapısal bir öğrenme gerçekleşmektedir. Bulanık çıkarım mekanizmasında bilgi çeşitli yöntemler aracılığıyla modellenmektedir. Çıkarım yöntemleri adı verilen bu yöntemler Mamdani yöntemi, Larsen yöntemi, Tsukamoto yöntemi ve Tagaki-Sugeno-Kang yöntemi şeklinde ifade edilmektedir [13,36,43]. Bu çalışmada Mamdani çıkarım yöntemi kullanılmıştır. Bu yöntemin kural yapısı,

Eğer $X_1=A_1$ ve $X_2=B_1$ ise o halde $Z_1=C_1$

Eğer $X_1=A_2$ veya $X_2=B_2$ ise o halde $Z_2=C_2$

şeklinde gösterilmektedir. Burada X_1 ve X_2 girdi değişkenlerini, Z_1 ve Z_2 ise çıktı değişkenini temsil etmektedir. A_1, B_1, A_2 ve B_2 üyelik fonksiyonları, C_1 ve C_2 ise her kuralın sonucunda çıkan bulanık sonuç kümesidir. Mamdani çıkarım yönteminde kuralların eşik değerleri hesaplanırken önce "ve (kesişim)" daha sonra "veya (birleşim)" işlemcileri kullanılmaktadır.

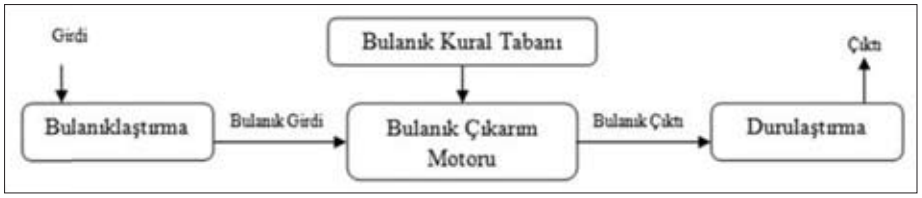
Durulaştırma kısmında, bulanık çıkarım motorunda elde edilen bulanık kümenin kesin bir değere dönüştürülmesi işlemi gerçekleşmektedir. Elde edilen bulanık kümenin, gerçek hayata tekrar uygulanması için sayısal bir değer olması gerekmektedir. En fazla karşılaşılan durulaştırma yöntemleri ağırlık merkezi yöntemi, en büyük üyelik ilkesi, ortalama en büyük üyelik, ağırlıklı ortalama yöntemi, en büyüklerin en küçüğü ve en büyüklerin en büyüğü yöntemleridir [15,38]. Bu çalışma kapsamında ağırlık merkezi yöntemi kullanılmıştır. Durulaştırma değeri,

$$y^* = \frac{\sum_{i=1}^{n} y_i . \mu_C(y_i)}{\sum_{i=1}^{n} \mu_C(y_i)}$$

formülü ile hesaplanmaktadır. Burada y_i tanımlı çıktı değişken değerini, $\mu_c(y_i)$ çıktı değişkeninin üyelik derecesini y* ise durulaştırma değerini temsil etmektedir.

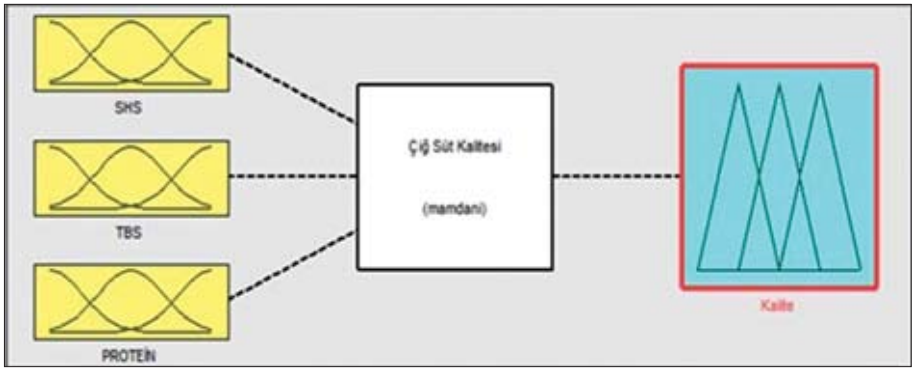
BULGULAR

Türk Kodeksi Çiğ Süt ve Isıl İşlem Görmüş Sütler Tebliği'ne göre [1] çiğ süt kalite standartlarında yer alan sütün



Şekil 1. Bulanık sistem yapısı

Fig 1. The structure of fuzzy system



Şekil 2. Tasarlanan bulanık sistem yapısı

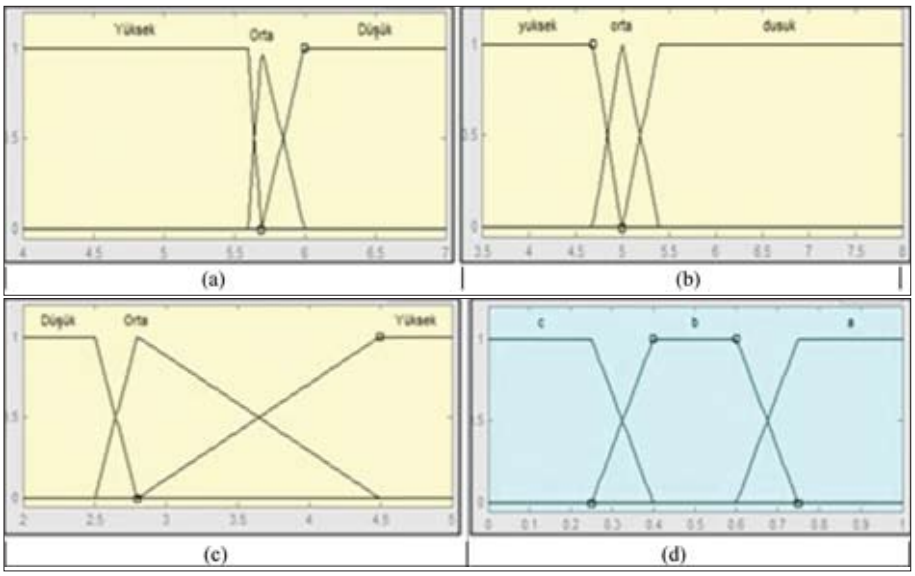
Fig 2. Designed fuzzy system structure

Tablo 1. *Girdi değişkenlerine ait kalite sınıf aralıkları*
Table 1. *Quality classes of input variable*

Kalite Sınıfları	Somatik Hücre Sayısı (SHS)	Toplam Bakteri Sayısı (TBS)	Protein (%)
Düşük	x>500.000	x>100.000	x<2.8
Orta	400.000≤ x ≤1.000.000	50.000≤ x ≤250.000	2.5≤ x ≤4.5
Yüksek	x<500.000	x<100.000	x>2.8

Şekil 3. a- Somatik hücre sayısı üyelik fonksiyonu, **b-** Toplam bakteri sayısı üyelik fonksiyonu, **c-** Protein üyelik fonksiyonu, **d-** Çiğ süt kalite değişkeni

Fig 3. a- Somatic cell count membership function, **b-** Total bacteri count membership function, **c-** Protein membership function, **d-** Raw milk quality variable



mikrobiyolojik ve kimyasal özellikleri ölçülen veriler, bulanık mantık terimleri ve karar destek sistemi yapısına göre oluşturulan kural tabanı bağlantıları ile yeniden biçimlendirilmiştir. *Şekil 2*'de Matlab programı kullanılarak tasarlanan bulanık sistemin genel görünümü yer almaktadır.

Bulanık sistem oluşumunun ilk aşaması olan bulanıklaştırma işlemine geçilmeden önce seçilen parametrelerin kalite sınıflarının ve sınıf aralıklarının belirlenmesi gerekmektedir. *Tablo 1*'de somatik hücre sayısı, toplam bakteri sayısı ve protein olarak belirlenen girdi değişkenlerinin kalite sınıfları ile sınıf aralıkları yer almaktadır. Hesaplamalar somatik hücre sayısı ve toplam bakteri sayısının logaritması alınarak yapılmıştır.

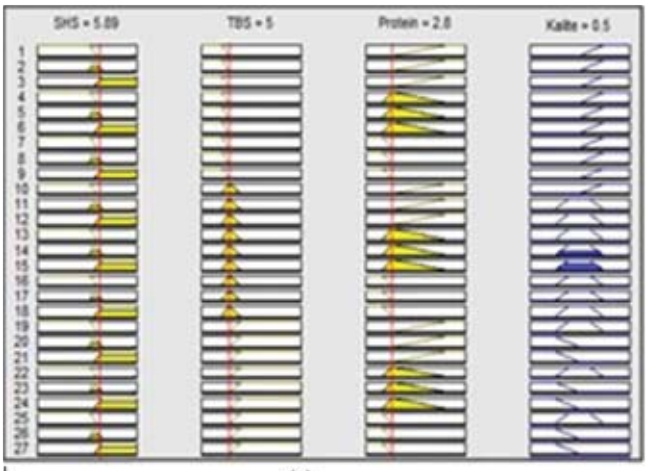
Şekil 3-a'da somatik hücre sayısı, *Şekil 3-b*'de toplam bakteri sayısı, *Şekil 3-c*'de protein miktarı girdi değişkenlerine ait üyelik fonksiyonlarının gösterimi yer almaktadır. *Şekil 3-d*'de ise çiğ süt kalite değişkenine ait çıktı fonksiyonu yer almaktadır.

Bu çalışma kapsamında çıkarım yöntemlerinden Mamdani yöntemi kullanılmıştır [36]. Çalışmada 27 adet "eğer-o halde" kuralı oluşturulmuştur. Kuralların sonucu kalite kararını bildirmektedir. *Tablo 2*'de girdi değişkenleri için oluşturulan kural tablosunun bir kısmı yer almaktadır.

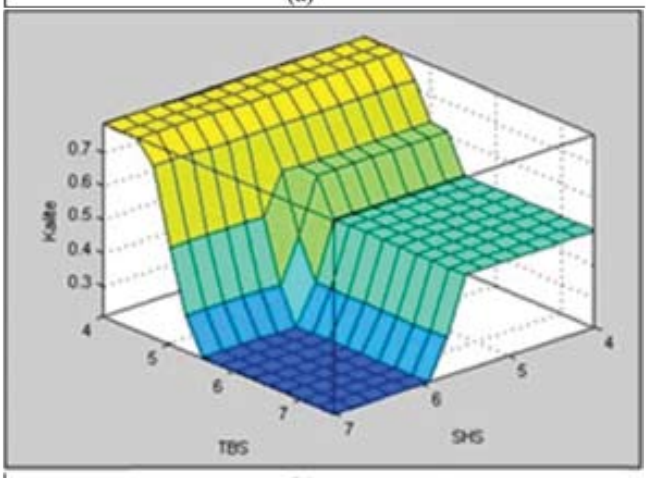
Şekil 4-a'da, sonucu öngörülmek istenen giriş parametreleri -örneğin SHS 5.89, TBS 5 ve protein 2.8- sisteme girildiğinde, durulaştırma işlemi neticesinde çıktı değeri 0.5 olarak elde edilmektedir. Konusunda uzman bir kişi ile oluşturulan *Tablo 2*'de yer alan bulanık kural tablosu incelendiğinde yukarıda belirtilen giriş parametrelerinin kural 14'ü etkilediği görülmektedir (*Şekil 3* ve *Tablo 2*).

Tablo 2. *Bulanık kural tablosu*
Table 2. *Fuzzy rule table*

Kural Numarası		TBS		SHS		Protein		Kalite
9		Yüksek		Düşük		Düşük		Düşük
10		Orta		Yüksek		Yüksek		Düşük
11	**Eğer**	Orta	**Ve**	Orta	**Ve**	Yüksek	**O halde**	Orta
12		Orta		Düşük		Yüksek		Orta
14		**Orta**		**Orta**		**Orta**		Orta
23		Düşük		Orta		Orta		Yüksek
27		Düşük		Düşük		Orta		Yüksek



(a)



(b)

Şekil 4. a- Kural gösterim penceresi, **b**- Yüzey gösterim penceresi
Fig 4. a- Rule viewer, **b**- Surface viewer

Bu kuralın sonucunda yer alan ifade çiğ süt örneğinin orta kalitede olduğunu belirtmektedir. Ayrıca, Matlab programında gerçekleştirilen analiz sonucunda da çıktı değerinin 0.5 olarak elde edilmesi, söz konusu örneğin orta kalite sınıfında yer aldığını göstermektedir. *Şekil 4-b*'de toplam bakteri sayısı ve somatik hücre sayısı girdi değişkenleri ile çıktı değişkeni arasındaki ilişki üç boyutlu olarak görülmektedir.

Matlab programı kullanılarak analiz edilen veriler, konusunda uzman kişi tarafından da değerlendirmeye alınmış ve sistem kararları ile karşılaştırmaya tabi tutulmuştur. *Tablo 3*'te, tasarlanan karar destek sisteminin kararları ile uzman kişinin kararlarının bir kısmı yer almaktadır.

Tablo 3. *Çiğ süt örneklerine ait veri seti ile uzman ve bulanık sistem kararları*
Table 3. *Raw milk sample's data set with expert's and fuzzy system's decisions*

TBS (log kob/ml)	SHS (log adet/ml)	Protein (%)	Sistem Kararı	Uzman Kararı
7.03	5.76	3.74	D	D
7.22	5.8	3.29	D	D
4.26	5.04	3.26	Y	Y
4.57	4.89	3.21	Y	Y
5.70	5.90	3.02	D	O
6.03	5.90	2.67	D	O

Gerçekleştirilen karşılaştırma sonucunda 50 adet çiğ süt örneğinin kalite sınıflarını belirlemek amacıyla oluşturulan karar destek sisteminin %80 değerinde başarı gösterdiği tespit edilmiştir. Veri setindeki örneklerde çiğ süt kalitesini etkileyen en önemli değişkenin toplam bakteri sayısı olduğu gözlenmiştir. Sistem ve uzman kararlarındaki %20 değerindeki uyuşmazlığın bakteri ve somatik hücre sayılarının aşırı derecede yüksekliğinden kaynaklandığı düşünülmektedir.

TARTIŞMA ve SONUÇ

Literatürde bulanık mantık yöntemi kullanılarak, çiğ süt kalitesi [33,37] ve dökme tank sütü kalitesinin [35] belirlenmesine yönelik çalışmalar mevcuttur.

Harris [33] çalışmasında, bulanık mantık yöntemini kullanarak çiğ süt kalitesini bileşimsel ve hijyenik açıdan ele almıştır. Bileşimsel kalite değerlendirmesi için tereyağı, yağsız kuru madde ve çiğ süt örneklerindeki toplam kuru madde miktarı; hijyenik açıdan değerlendirmede ise somatik hücre ve toplam bakteri sayısı girdi değişkenleri olarak kabul edilmiştir. İki farklı veri seti üzerinde çalışan araştırmacı dört farklı kalite sınıfı oluşturarak bulanık mantık yöntemini kalite değerlendirmesinde standart teknikler

ile karşılaştırmıştır. Bu çalışmada olduğu gibi üçgen ve yamuk üyelik fonksiyonlarını kullanılmış ve bulanık mantık yönteminin oldukça etkin olduğu sonucuna varılmıştır.

Cha ve ark.[35] çalışmasında dökme tank sütünün mikrobiyolojik test sonuçlarına dayalı olarak, süt sığırlarından oluşan sürünün sağım uygulamalarını ve kalitesini değerlendirmek için bulanık mantık yöntemini kullanmıştır. Bu çalışmadan farklı olarak Gauss ve yamuk üyelik fonksiyonlarını kullanan araştırmacılar çıkarım yöntemlerinden Mamdani çıkarım yöntemini kullanmışlardır. Girdi değişkenleri somatik hücre sayısı, bakteri sayısı, başlangıç inkübasyon sayıları, laboratuvar pastorizasyon sayımı, *agalaktik olmayan-Streptokok* ve Streptokok benzeri organizmalar ile *Staphylococcus aureus* şeklindedir. Değerlendirmeler sonucunda sütü mükemmel, iyi, soğutma problemi, temizlik problemi, çevresel mastitis veya mastitis-temizlik problemi olanlar şeklinde sınıflandırmışlardır. Uzman görüşleri ile gerçekleştirilen karşılaştırma sonucunda dört farklı çıktı kategorisinde %77, altı farklı kategoride ise %83 değerinde başarı sağlandığı görülmüştür. Ayrıca sınıflandırmada en etkin girdi değişkenlerinin somatik hücre ve bakteri sayısı olduğu görüşüne de varılmıştır.

Mahreban ve ark.[37] çiğ süt kalitesini bulanık mantık yöntemi ile mikrobiyolojik ve fizikokimyasal açıdan değerlendirmiştir. Bu amaçla kullanmış oldukları girdi değişkenleri toplam aerobik mikroorganizma sayısı, somatik hücre sayısı, koliform sayısı, yağ ve yağsız kuru madde yüzdeleridir. Çalışmalarında bu çalışmada olduğu gibi üçgen ve yamuk üyelik fonksiyonları, Mamdani çıkarım yöntemi ile ağırlık merkezi durulaştırma yöntemi kullanmışlardır. Beş farklı kalite sınıfı ile oluşturdukları bulanık sistemde 675 kural yer almaktadır. Sistemin performansını değerlendirmek amacıyla konusunda uzman araştırmacılar tarafından çiğ süt örneklerine kalite derecelendirmesi yapılmıştır. Bulanık sistem kararları ile gerçekleştirilen karşılaştırma sonucunda %82.5 değerinde başarı sağlandığı görülmüştür. Toplam bakteri sayısının diğerlerine göre kaliteyi belirlemede daha fazla önemli olduğu tespit edilmiştir. Bu çalışmada daha az girdi değişkeni ve bulanık kural ile bahsi geçen çalışmalara oldukça yakın bir sonuç elde edildiği görülmektedir. Çalışmaların sonuçları, bu çalışmanın sonucunda olduğu gibi, bulanık mantık yönteminin incelendiği alanlarda güvenle kullanılabileceğini göstermektedir.

Bu çalışmada tasarlanan bulanık mantık tabanlı karar destek sistemi %80 değerinde bir başarı sağlamış ve çiğ süt kalite değerlendirmesinde oldukça etkili olduğu sonucuna varılmıştır. Çiğ süt kalite değerlendirmesi, hayvancılık alanında karşılan belirsizlik problemlerinden birisidir. Son yıllarda bu tip problemlerin çözümünde sıklıkla kullanılan bulanık mantık yöntemi, çiğ süt kalite standartlarına klasik yöntemlere göre daha esnek bir yapı kazandırmakta ve değerlendirmelerde doğaya daha uygun bir bakış açısı sunmaktadır. Ayrıca insan uzmanların kısmen de olsa yerine geçerek zaman ve iş tasarrufu sağlamaya yardımcı olabilmektedir. Bu çalışmada ele alınan girdi değişkenlerinden toplam bakteri sayısının diğer girdi değişkenlerine göre çiğ süt kalitesini belirlemede çok daha etkin olduğu gözlenmiştir. Gerçekleştirilen çalışma bulanık mantık tabanlı karar destek sisteminin çiğ süt kalite analizinin belirlenmesinde oldukça başarılı olduğunu ve hayvancılık alanında güvenle kullanılabileceğini göstermektedir. İlerleyen dönemlerde bulanık mantık yöntemi ile diğer yapay zeka yöntemleri kullanılarak oluşturulacak entegre sistemlerin hayvancılık alanında çalışan araştırıcılara farklı bakış açıları ile değerlendirme imkanı sağlayacağı düşünülmektedir.

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RESEARCH ARTICLE

Et Tipi Ana Hattı Japon Bıldırcın Sürüsünde Çok Özellikli Seleksiyonun Fenotipik ve Genetik İlerlemelere Etkisi [1] [2]

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Özet

Bu çalışmada, et tipi bıldırcın ana hattı geliştirmek için karışık model eşitliklerinden yararlanarak çok özellikli seleksiyon programı uygulamanın fenotipik ve genetik ilerlemelere etkisi incelenmiştir. Araştırmada pedigri kaydı bulunan yaklaşık 2.000 birey kullanılmış olup, bıldırcın türünde çok özellikli birey modeli ile seleksiyon Türkiye'de ilk kez uygulanmıştır. Bir başlangıç sürüsünden (AHBS: ana hattı başlangıç sürüsü) şansa bağlı çiftleşmelerle 160 aileden oluşan (160 erkek - 480 dişi) ana hattı seleksiyon sürüsü (AHSS-1) elde edilmiştir. Sürüde ilk seleksiyon kriteri olarak 5 haftalık yaştaki canlı ağırlık (CA) seçilmiştir. Bunun yanında, ana hattında 20 haftalık yaşa kadar olan toplam yumurta verimi (YV), Gompertz büyüme eğrisinden tahmin edilen 5 haftalık yaştaki bağıl büyüme hızı (BBH) ve 10-11, 14-15 ve 17-18 haftalık yaşlarda toplanan yumurtalardan elde edilen döllülük oranı (DO) özellikleri seleksiyon kriterleri olarak kullanılmıştır. Çok özellikli BLUP değerleri elde edilmiş ve damızlık değerlerine göre en iyi %25 erkek ve dişi bıldırcın bir sonraki sürünün (AHSS-2) ebeveynleri olacak şekilde seçilmiştir. Seleksiyon sonrasında genetik parametre tahminleri, gerçekleşen genetik parametreler, seleksiyonla sağlanan genetik ilerlemeler ve genetik yönelimler elde edilmiştir. Kuşaklarda CA, BBH ve YV özellikleri bakımından istatistiksel olarak önemli genetik ilerlemeler sağlanmıştır. Çalışma sonuçları, negatif genetik ilişkili özelliklerin çok özellikli BLUP yöntemiyle seleksiyonda bir arada değerlendirilebileceğini ortaya koymuştur.

Anahtar sözcükler: *Karışık model eşitlikleri, BLUP birey modeli, Genetik ilerleme, Çok özellikli seleksiyon*

Effects of Multi-Trait Selection on Phenotypic and Genetic Changes in a Meat Type Dam Line of Japanese Quail

Summary

The present study in Japanese quail was aimed to develop multi-trait genetic selection program for meat type dam line utilizing the mixed-model methodology. In total, 2000 pedigreed quail were formed the basis of the research where a multi-trait animal model was performed for the first time in a selection study of quail in Turkey. A flock consisting of a total of 160 families (160 male- 480 female) was developed from the initial flock (AHBS: initial flock of dam line), so as to obtain a selection flock dam line (AHSS-1). Body weight at 5 weeks of age (CA) was chosen as a primary selection criterion in flock. The total egg number (YV) from the day of first lay to the 20 weeks of age, relative growth rate (BBH) at 5 weeks of age derived from Gompertz equation, and fertility rate (DO) were used as selection criteria in AHSS. Multi-trait BLUP methodology was carried out for genetic improvement of birds. In flock, 25 percent of males and females with highest breeding value were selected to produce next generations (AHSS-2). Genetic parameter estimates, realized genetic parameters, selection responses, and genetic trends were obtained. Significant ($P<0.01$) selection responses for CA, BBH and YV traits on generations were observed. The results of the study revealed that the negative genetic relationships exhibited between some studied traits had overcame by modern poultry breeding methods such as selection via multi-trait BLUP.

Keywords: *Mixed model equations, BLUP animal model, Response to selection, Multi-trait selection*

GİRİŞ

Et ve yumurta verimi oldukça yüksek olan Japon bıldırcını ticari üretimde her geçen yıl daha fazla önem kazanmaktadır. Uzak Doğu ve Asya ülkelerinde genellikle yumurta verimi için yetiştirilen bıldırcın, Avrupa ve Amerika'da ise daha

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çok et üretimi amacıyla yetiştirilmektedir [1,2]. Japon bıldırcınlarını konu alan bilimsel çalışmaların büyük kısmı türün model hayvan olarak değerlendirilmesiyle ilgili olmakla birlikte [3-7], gerçekleştirilen sınırlı sayıdaki ıslah çalışmasında da erken dönemlere ait yumurta verimi ya da sabit bir yaşa ait canlı ağırlık için kısa ve uzun dönemli seleksiyon uygulanmıştır [8-12]. Bu çalışmaların çoğunda modern ıslah araçları kullanılmamış olup fenotipik değerlere göre basit kitle seleksiyonu uygulanmıştır. Kısmi yumurta verimi ve haftalık canlı ağırlık özelliklerine ait kalıtım dereceleri yüksek olduğundan, kısa dönemli seleksiyon çalışmalarında bile oldukça başarılı sonuçlar alınmıştır [9]. Diğer yandan, söz konusu özellikler ile negatif genetik ilişkili olan döllülük ya da yumurta kalitesi gibi bazı karakterlerde ortaya çıkan gerilemelerden dolayı söz konusu çalışmalar sürdürülemez hale gelmiştir [8]. Bunun yanında ıslah çalışmalarının sonuçları ticari ürüne dönüştürülememiştir. Günümüzde ticari üretimde kullanılan bıldırcınlar genellikle ıslah edilmemiş ve mevcut potansiyellerinin çok daha altında ürün veren sürülerden oluşmaktadır [13].

Etlik piliç ıslahında başlıca seleksiyon ölçütleri olan büyüme hızı, yemden yararlanma ve karkas verimi özelliklerinin yanında büyüme eğrisi parametreleri, fiziksel ve teknolojik et kalite özellikleri, göğüs açısı gibi 20'nin üzerinde özellik kullanılmıştır [14,15]. Bıldırcınlarda ise kesimkarkas, büyüme eğrileri ve et kalite özellikleri için genetik parametre tahmini yapılan bazı çalışmalar bulunmakla birlikte, söz konusu özelliklerin dahil olduğu ana ve baba hatlarını geliştirmeye yönelik bir ıslah çalışması bulunmamaktadır [1,9]. Etlik piliç ıslahında genetik parametre tahminleri için kullanılan ebeveyn-yavru benzerliği ya da seleksiyon sonucu gerçekleşen kalıtım derecesi gibi yöntemler yerlerini sınıf-içi korelasyon temeline dayanan uygulamalara bırakmıştır [16]. Fenotipik kitle seleksiyonu terk edilmiştir ve damızlık değerler çeşitli karışık model eşitliklerinin çözümüyle elde edilmektedir [17-21].

Kanatlı hayvanların genetik iyileştirilme çalışmalarında uygulanan ıslah programları birbirini izleyen iki aşamadan oluşmaktadır. Bunlardan birincisi ana ya da baba ebeveyn sürülerin hat içi seleksiyonla belirli özellikler bakımından geliştirilmesi, ikincisi de belirli özellikler bakımından geliştirilmiş ebeveyn hatlarının melezlenmesidir [17]. Birinci aşamada eklemeli genetik etkiler kullanılarak akrabalık düzeyleri yüksek saf hatlar elde edilmesi amaçlanır. İkinci aşamada ise eklemeli olmayan genetik etkilerden de yararlanılarak daha üstün verim performansına sahip melez genotipler elde edilmesi hedeflenir. Hayvanlarda ölçülen özelliklerin geliştirilmesi, özellik ya da özellikler için genetik parametrelerin tahmini, uygun ıslah aracının seçilmesi, damızlık değerlerin tahmin edilmesi ve seleksiyon gibi uygulamaların tümü ilk aşamada yapılması gereken işlemlerdir [15]. Bu çalışmada et verimi amacıyla ana hattı olarak geliştirilmekte olan bir Japon bıldırcını sürüsünde çok özellikli BLUP (best linear unbiased prediction) yöntemiyle gerçekleştirilen seleksiyonun etkileri üzerinde durulmuştur. Seleksiyon kriterleri olarak 5. hafta canlı ağırlığı, 20 haftalık yaşa kadar olan toplam yumurta verimi, Gompertz büyüme eğrisinden tahmin edilen 5 haftalık yaştaki bağıl büyüme hızı, 10-11, 14-15 ve 17-18 haftalık yaşlarda toplanan yumurtalardan elde edilen döllülük oranı kullanılmıştır. Araştırmada çok özellikli seleksiyonla birlikte fenotipik ve genetik değişimlerin ortaya konulması, parametrelerden yola çıkarak gelecek kuşaklara yönelik öngörülerde bulunulması amaçlanmıştır.

MATERYAL ve METOT

Sürü Yönetimi

Çalışma Akdeniz Üniversitesi Zootekni Bölümü Kanatlı Hayvan Araştırma Merkezi'nde gerçekleştirilmiş ve Akdeniz Üniversitesi Deney Hayvanları Etik Kurulu 09/69-02.14.2011 sayılı kararı uyarınca yürütülmüştür. Başlangıç sürüsü oluşturmak amacıyla Akdeniz Üniversitesi ve Konya Selçuk Üniversitesi araştırma birimlerinde barındırılan, daha önce ıslah edilmemiş, şansa bağlı çiftleşen bıldırcınlardan toplam 1500 döllü yumurta alınarak, rastgele ve eşzamanlı biçimde kuluçkaya konulmuştur. Kuluçkadan çıkan civcivlere kanat numarası takılarak pedigri kayıtları başlatılmıştır. Civcivlerin 3 haftalık yaşta cinsiyetleri belirlenmiş ve rastgele seçilen 120 dişi-40 erkek bıldırcın ana hattı başlangıç sürüsü (AHBS) oluşturmak amacıyla bireysel damızlık kafeslerine yerleştirilmiştir. Her biri üç dişi ve bir erkek bıldırcından oluşan toplam 40 ailedeki bıldırcınlar 10 haftalık yaşa geldiğinde, 15 gün boyunca toplanan yaklaşık 1200 yumurta iki parti halinde kuluçka makinesine yerleştirilmiştir. Kuluçka süresi sonunda makineden çıkan civcivlere kanat numarası takılmış ve 21. günde tüylenmelerine göre cinsiyet tayini yapılana kadar, çevre kontrollü bir büyütme odasında yer alan ve her katında 96x43x21 cm boyutlarında bölmeler bulunan, altı katlı, ısıtıcılı büyütme kafeslerinde barındırılmıştır. Her bölmeye 50 adet civciv yerleştirilmiştir (82.56 cm^2/bıldırcın). Üçüncü hafta canlı ağırlık ölçümü sırasında şansa bağlı olarak toplam 160 erkek ve 480 adet dişi bıldırcın seçilmiş ve bireysel damızlık yumurtacı kafeslerine yerleştirilmiştir. Böylece 160 aileden oluşan birinci ana hattı seleksiyon sürüsü (AHSS-1) oluşturulmuştur. AHSS-1'de seleksiyon kriterleri olarak 5. hafta canlı ağırlığı (CA), 5. hafta bağıl büyüme hızı (BBH), 20 haftalık yaşa kadar olan yumurta verimi (YV) ve döllülük oranı (DO) özellikleri belirlenmiştir. AHSS-1'de yüksek döllülük sağlamak amacıyla, erkek bıldırcınlar 15 hafta boyunca her gün aile içindeki ayrı bir dişiyle çiftleştirilmiştir. Bıldırcınların çıkıştan itibaren haftalık canlı ağırlık tartımları bireysel olarak gerçekleştirilmiştir. Bireysel yumurta verimleri 20 haftalık yaşa kadar günlük kayıt edilmiş ve sayısal olarak (adet) değerlendirilmiştir. Döllülük oranlarının tespiti için 10., 11., 14., 15., 17., 18. haftalarda döllü yumurtalar toplanmış ve haftalık olarak kuluçka makinesine konularak kuluçkanın 15. gününde embriyonun durumuna bakılarak döllülük kontrolü yapılmıştır.

AHSS-1'de seleksiyon kriteri olan özelliklere ait ölçümler alındıktan sonra, damızlık değer tahminleri yapılmış ve sürüdeki en iyi 40 erkek ile 120 dişi bıldırcın, sonraki kuşağın (AHSS-2) ebeveynleri olarak seçilmiştir. AHSS-1'de gerçekleştirilen tüm işlemler AHSS-2'de de uygulanmıştır. AHSS, AHSS-1 ve AHSS-2 bıldırcınlarına büyütme döneminde %23 HP ve 2.900 kcal/kg ME içerikli karma yem, 21. günden deneme sonuna kadar %20 HP ve 2.800 kcal/kg ME içerikli karma yem *ad-libitum* olarak verilmiştir. Sürülere deneme boyunca günlük 18 saat aydınlatma yapılmıştır.

İstatistik

AHBS, AHSS-1 ve AHSS-2 bıldırcınlarında haftalık canlı ağırlık değerleri kullanılarak Gompertz büyüme modeli ile her bir bıldırcın için bireysel büyüme analizleri gerçekleştirilmiş ve büyüme eğrisi parametreleri, haftalık mutlak büyüme oranları, haftalık bağıl büyüme oranları belirlenmiştir. Büyümenin doğrusal olmayan regresyon analizinde aşağıda eşitliği sunulan Gompertz modelinden yararlanılmıştır [22].

$$y = \beta_0.\exp(-\beta_1.\exp(-\beta_2.t))$$

Modelde y; canlı ağırlığı, t; zamanı (gün), β_0; asimptotik ağırlığı, β_1; integrasyon sabitini, β_2 anlık büyüme hızını ifade etmektedir. Model parametrelerinin tahmini SAS 9.3 NLIN prosedürü kullanılarak Levenberg-Marquardt iterasyon yöntemiyle gerçekleştirilmiştir [23]. Gompertz büyüme modeli kullanılarak her bir bireye ait bağıl büyüme hızı $BBH=[\beta_2(\ln(\beta_0)-\ln(y_t))]$ eşitliğiyle hesaplanmıştır [22].

Özellikler için tanımlayıcı istatistiklerin elde edilmesinde SAS programının MEANS prosedüründen yaralanılmıştır [23]. Verilerin normalliği SAS programının UNIVARIATE prosedürü kullanılarak Kolmogorov-Smirnov testiyle sınanmış ve BBH, YV ve DO özelliklerinin normal dağılış göstermediği belirlenmiştir ($P<0.05$). Verilerin normal dağılışa uydurulması amacıyla parametrik dönüşüm yöntemlerinden Box-Cox [24] kullanılmış, ancak sadece YV özelliği normal dağılış göstermiş, ardından parametrik olmayan dönüşüm yöntemlerinden Rank dönüşümü [25] uygulanarak diğer özellikler için normal dağılış sağlanmıştır.

Genetik Parametre Tahminleri

Genetik parametrelerin tahmin edilmesinde kullanılan karışık doğrusal model (I) eşitliği aşağıda sunulmuştur [18]:

$$y = X\beta+Zu+e \quad (I)$$

Burada; *y*, gözlem değerlerini içeren vektör, *β* sabit etkiler vektörü, *u* ise şansa bağlı etkilere ilişkin vektördür. *X* ve *Z* sırası ile *β* ve *u* vektörlerine ilişkin desen matrisleridir. *e* hata terimlerine ait vektörüdür. *y* gözlem vektörünün y~MVN(Xβ, ZGZ`+R) ile çok değişkenli normal dağılış gösterdiği varsayılmaktadır. Ayrıca, *u* ve *e*'nin sırası ile u~MVN(0,G) ve e~MVN(0,G) ile çok değişkenli normal dağılışa sahip olduğu varsayılmaktadır. Burada, *I* birim matris ve *A* akrabalık matrisi olmak üzere, $G=G_0\otimes A$ (II) baba ve baba içi ana varyans-kovaryans unsurlarını içeren eklemeli genetik etkiler matrisi, $R=R_0\otimes I$ (III) ise hata terimlerini içeren varyans-kovaryans matrisidir.

Tahmin edilecek varyans-kovaryans parametreleri θ vektörü ile gösterilmek üzere, REML için olabilirlik fonksiyonu Eşitlik 4'teki gibi yazılır

$$l_R(\theta) = -\frac{1}{2}\log|V| - \frac{1}{2}\log|X'V^{-1}X| - \frac{1}{2}r'V^{-1}r - \frac{n-p}{2}\log(2\pi) \quad (IV)$$

Burada,

$$V = ZGZ' + R \quad \text{ve} \quad r = y - X\left(X'V^{-1}X\right)^{-}X'V^{-1}y = y - X\hat{\beta}$$

olup *p X* matrisinin rankıdır [23].

Çok özellikli BLUP değerlerinin elde edildiği karışık doğrusal model gösterimi V numaralı eşitlikte sunulmuştur.

$$\begin{bmatrix} X'R^{-1}X & X'R^{-1}Z' \\ Z'R^{-1}X & Z'R^{-1}Z + A^{-1}\otimes G^{-1} \end{bmatrix}\begin{bmatrix} \hat{b} \\ \hat{a} \end{bmatrix} = \begin{bmatrix} X'R^{-1}y \\ Z'R^{-1}y \end{bmatrix} \quad (V)$$

Eşitlikte $\hat{b}$ bilinmeyen sabit etkiler vektörünü; â tahmin edilmek istenen damızlık değerleri içeren, ortalaması sıfır, varyansı $A\sigma^2_a$ olan rastgele etkiler vektörünü ifade etmektedir. Eşitlikte *y* vektörü verimleri içermekte, *X* sabit etkiler için desen matrisi, *Z* şansa bağlı etkiler için dağılan birey desen matrisidir. Matrislerin yapısal durumlarına göre, karışık model eşitliklerinde farklı modeller oluşturulabilir, bu çalışmada birey (animal) modeli kullanılmıştır [18,26]. Modelde *G* eklemeli genetik varyans-kovaryans matrisi, *R* hata varyans-kovaryans matrisi, *A* akrabalık derecelerini içeren akrabalık matrisini temsil etmektedir. Akrabalık matrisleri SAS programının INBREED prosedüründe oluşturulmuş [27], çok özellikli karışık model eşitliklerinin çözümü aynı programın IML prosedüründe gerçekleştirilmiştir [23].

Seleksiyon

AHSS-1'de CA, BBH, YV ve DO özelliklerine ait veriler kullanılarak tüm bireyler için çok özellikli BLUP değerleri bir üst başlıkta açıklandığı şekilde tahmin edilmiştir [18]. Her hayvana ait bir indeks değeri oluşturmak üzere, dikkate alınan özelliklere ait BLUP değerleri eşit ekonomik ağırlık katsayılarıyla çarpılmıştır. Böylece her hayvana ait damızlık değerler çok özellikli bir indekse dönüştürülmüştür. Erkek ve dişi bıldırcınların en yüksek indeks değeri gösteren %25'lik kısmı bir sonraki kuşağın ebeveynleri olarak seçilmiştir.

BULGULAR

AHBS, AHSS-1 ve AHSS-2 sürülerinde CA, BBH, YV ve DO özellikleri için tanımlayıcı istatistikler *Tablo 1*'de sunulmuştur. Söz konusu tabloda dört özellik bakımından sürüler arasındaki farklılıklar sınanması amacıyla gerçekleştirilen varyans analizi ve Duncan çoklu karşılaştırma testlerinin

Tablo 1. *Özellikler için tanımlayıcı istatistikler ve hipotez testi sonuçları*
Table 1. *Descriptive statistics for studied traits and results of hypothesis tests*

Özellik	Kuşak	N	Ortalama	Standart Hata	Varyasyon Katsayısı (%)	En Düşük Gözlem	En Yüksek Gözlem	P
CA	AHBS	159	174.40[b]	1.54	19.46	133.40	229.20	0.005
	AHSS-1	624	174.66[b]	0.76	18.91	124.70	230.30	
	AHSS-2	640	178.30[a]	0.73	18.54	117.50	241.20	
BBH	AHBS	159	1.75[b]	0.02	37.06	-0.26	3.69	0.000
	AHSS-1	624	1.80[b]	0.02	34.34	-0.26	3.56	
	AHSS-2	640	2.04[a]	0.03	32.31	-0.22	4.10	
YV	AHBS	119	76.72[b]	0.65	21.43	9	96	0.004
	AHSS-1	624	76.16[b]	0.60	14.92	11	95	
	AHSS-2	640	78.70[a]	0.47	11.90	13	97	
DO	AHBS	159	75.77	0.56	22.66	0	100	0.079
	AHSS-1	624	78.88	0.86	21.42	0	100	
	AHSS-2	640	80.22	1.09	27.62	0	100	

CA: *5. hafta canlı ağırlığı, g;* ***BBH:*** *5 haftalık yaştaki bağıl büyüme hızı, %;* ***YV:*** *20 haftalık yaşa kadar olan toplam yumurta verimi, adet;* ***DO:*** *10-11, 14-15 ve 17-18 haftalık yaşlarda saptanan döllülük oranı, %;* ***[a-b]:*** *söz konusu özellik bakımından sürüler arasında istatistiksel farklılık bulunmaktadır, P<0.01*

Tablo 2. *Seleksiyon üstünlüğü, genetik ilerleme ve gerçekleşen kalıtım dereceleri*
Table 2. *Selection objectives, selection responses and realized heritabilities*

Özellik	Seleksiyon Üstünlüğü	Genetik ilerleme	Gerçekleşen Kalıtım Derecesi
CA	7.6348	3.6400	0.4768
BBH	0.0043	0.0024	0.5583
YV	5.5532	2.5400	0.4574
DO	7.6261	1.3400	0.1757

CA: *5. hafta canlı ağırlığı, g;* ***BBH:*** *5 haftalık yaştaki bağıl büyüme hızı, %;* ***YV:*** *20 haftalık yaşa kadar olan toplam yumurta verimi, adet;* ***DO:*** *10-11, 14-15 ve 17-18 haftalık yaşlarda saptanan döllülük oranı, %*

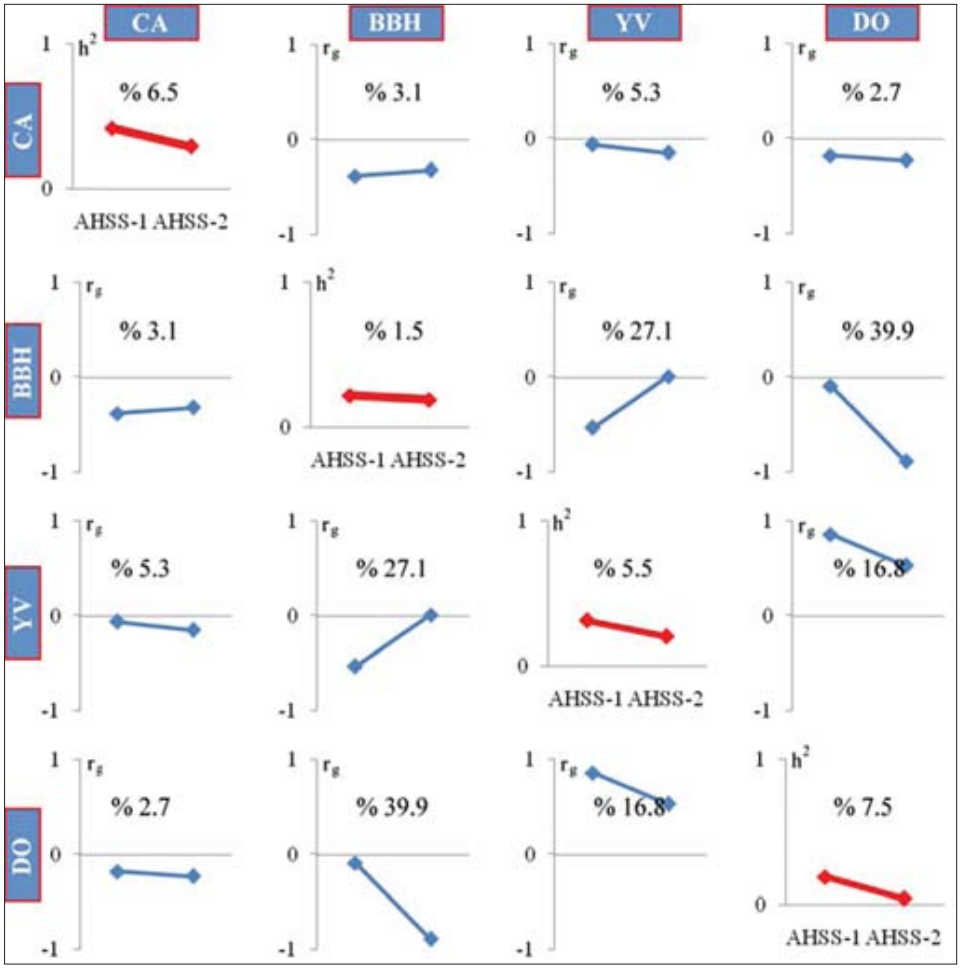
sonuçları yer almaktadır. CA, BBH ve YV özellikleri bakımından AHSS-2 için saptanan ortalamalar AHBS ve AHSS-1 sürülerine ait ortalamalardan istatistiksel olarak önemli derecede yüksek bulunmuştur (P<0.01). Bunun yanında, DO özelliği için gerçekleşirilen hipotez testi sonucuna göre her üç sürüye ait ortalamalar arasında önemli farklılık bulunmamıştır.

Her özellik için seleksiyon üstünlükleri, seleksiyonla sağlanan genetik ilerlemeler ve karakterler için seleksiyonla gerçekleşen kalıtım dereceleri *Tablo 2*'de gösterilmiştir. Gerçekleştirilen çok özellikli seleksiyon sonucunda eklemeli genetik varyans ve kovaryanslarda meydana gelen değişim ölçülerini içeren matris *Şekil 1*'de sunulmuştur. Kuşaklar boyunca her özellik bakımından bireyler için tahmin edilen BLUP değerleri ve bunların ortalamalarından yola çıkarak tahmin edilen genetik yönelimler de *Şekil 2*'de sunulmuştur. Genetik yönelimin tahmin edilmesi, yürütülen ıslah programının izlenmesi ve değerlendirilebilmesi açısından gereklidir. Islah programlarının uygulandığı populasyonlarda hayvanların damızlık değerlerinin giderek yükselmesi beklenmektedir. Araştırmada CA özelliği için AHBS, AHSS-1, AHSS-2 bıldırcınları için tahmin edilen BLUP değerlerinin ortalamaları sırasıyla -0.087, 0.053, 0.120 bulunmuştur. AHBS, AHSS-1, AHSS-2 sürülerinde BLUP ortalamaları BBH özelliği için 0.0109, -0.0013, 0.0043, EN özelliği için -0.399617, -0.015532, -0.068872, DO özelliği için -0.360538, -0.005145, -0.110020 olarak bulunmuştur.

TARTIŞMA ve SONUÇ

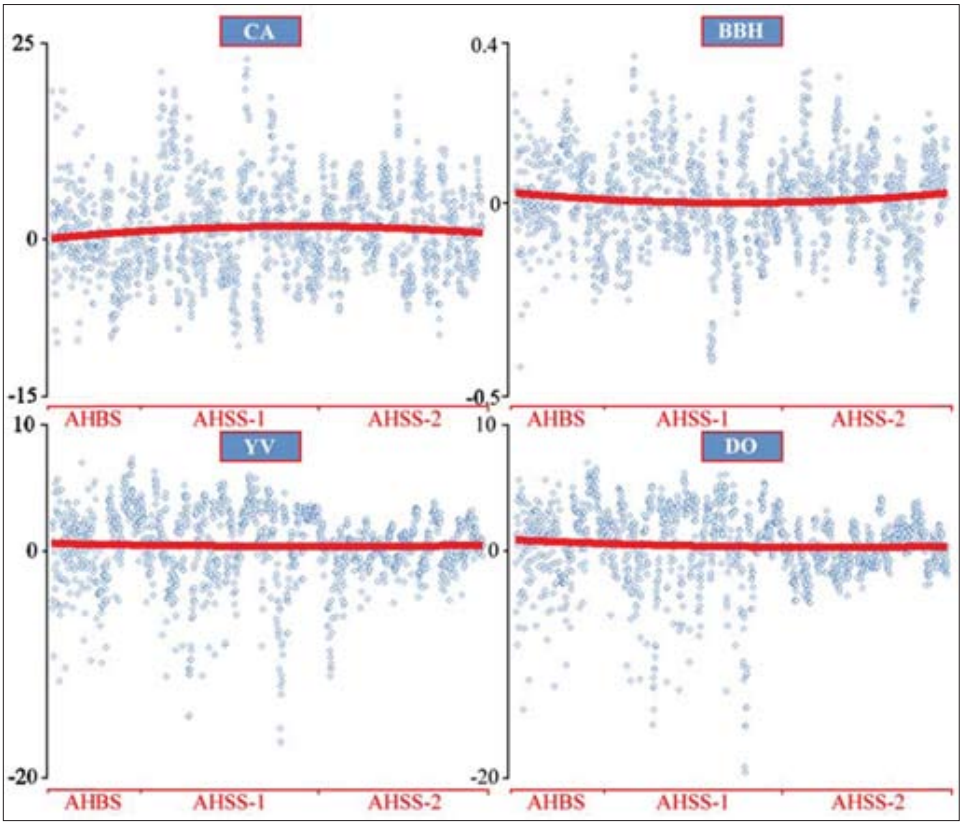
Fenotipik Değişimler

Araştırmada CA ortalamaları AHBS, AHSS-1 ve AHSS-2 kuşaklarında sırasıyla 174.40, 174.66 ve 178.30 g olarak bulunmuştur. Söz konusu değerler Toelle ve ark.[28] ve Sarı ve ark.[29] tarafından bildirilen değerlerle (170 g ve 176 g) uyumlu bulunmuştur. AHSS-2 bıldırcınlarına ait CA ortalamasının AHBS ve AHSS-1 bıldırcınlarından daha yüksek olduğu belirlenmiştir (P<0.01). Benzer şekilde Japon bıldırcınlarında canlı ağırlığı arttırmak için gerçekleştirilen seleksiyon çalışmalarının tümünde birinci kuşakta istatis-



Şekil 1. Eklemeli genetik varyans-kovaryans değişim (%) matrisi (köşegende kalıtım dereceleri, köşegenin altında ve üstünde genetik korelasyonlar yer almaktadır, her elemanın üzerindeki % mutlak varyans-kovaryans değişimini ifade etmektedir)

Fig 1. Change (%) matrix of additive variance-covariance (heritabilities on diagonal, genetic correlations above and below the diagonal, % values on each element represent absolute changes in the variance-covariance)



Şekil 2. Tahmin edilen BLUP değerleri ve genetik yönelimler

Fig 2. Estimated BLUP values and genetic trends

tiksel olarak anlamlı artışlar gözlenmiştir [8,10,12,30-33]. AHBS, AHSS-1 ve AHSS-2 sürülerinde BBH ortalamaları sırasıyla %1.75, 1.80 ve 2.04 olarak tahmin edilmiş ve AHSS-2'nin BBH ortalamasının diğer sürülerden önemli farklılık gösterdiği belirlenmiştir (P<0.01). Söz konusu BBH değerleri Narinç ve ark.[34] tarafından bildirilen değerlerle uyumlu bulunmuş, benzer şekilde Aggrey ve ark.[35] ile Hyankova ve ark.[11] bıldırcınlarda seleksiyonun BBH ortalamalarında değişime yol açtığını ortaya koymuşlardır. AHBS, AHSS-1 ve AHSS-2 bıldırcınlarının YV ortalamaları sırasıyla 76.72, 76.16, 78.70 yumurta olarak belirlenmiştir. Çalışma sonuçlarıyla uyumlu olarak Gildersleeve ve ark.[36] da 20 haftalık yaşa kadar yumurta sayısının 69-80 arasında olduğunu bildirmişlerdir. Literatürde bıldırcınların yumurta verimlerinin belirlendiği az sayıdaki çalışmada da farklı yaş aralıklarındaki yumurta verimleri üzerinde durulmuştur [37,38]. Çalışmada AHSS-2 bıldırcınlarının yumurta verim ortalamasının diğer iki sürüdeki ortalamalardan yüksek olduğu belirlenmiştir (P<0.01). Bıldırcınlarda canlı ağırlığı arttırmaya yönelik seleksiyon uygulayan birçok araştırıcı yumurta veriminde önemli gerilemeler gerçekleştiğini bildirmişlerdir [2,39]. Araştırmada DO ortalamaları AHBS, AHSS-1, AHSS-2 için sırasıyla %75.77, 78.88, 80.22 olarak bulunmuş ve sürüler arasında DO değerleri için anlamlı farklılık bulunmamıştır (P>0.01). Benzer şekilde Nestor ve ark.[37] dördüncü hafta canlı ağırlığı için yaptıkları çift yönlü seleksiyonun döllülük oranını etkilemediğini bildirmişlerdir.

Genetik Değişimler

Seleksiyon üstünlüğü, seleksiyonda dikkate alınan özellik sayısına, seleksiyon yoğunluğuna, üzerinde durulan özelliğin kalıtım derecesi ve eğer özellik birden fazlaysa aralarındaki eklemeli genetik kovaryansa bağlı olarak farklılık göstermektedir [45]. Farklı seleksiyon çalışmalarında aynı özellik için hesaplanan seleksiyon üstünlüklerini karşılaştırmak, şartlar aynı olamayacağından dolayı mümkün değildir. Tek özellikli seleksiyon yöntemleri (fenotipik kitle seleksiyonu, seleksiyon indeksi ya da BLUP) ile gerçekleşen genetik ilerlemenin çok özellikli yöntemler ile sağlanan ilerlemeden daha yüksek olduğu bilinmektedir [40]. Araştırmada CA özelliği için bir kuşaklık çok özellikli seleksiyon ile sağlanan genetik ilerleme 3.64 g (%2.08) olarak bulunmuştur *(Tablo 2)*. Yolcu [12] 5. hafta canlı ağırlığı için yaptığı 5 kuşaklık fenotipik kitle seleksiyonunda ilk kuşakta 7.6 g (%3.89) genetik ilerleme gerçekleştiğini bildirmiş, 5 kuşak sonunda ortalama genetik ilerlemenin 4.75 g/kuşak (%2.43) olduğunu bildirmiştir. Bıldırcınlarda 4. hafta canlı ağırlığı için uzun dönemli fenotipik kitle seleksiyonu yapan Marks ve Lepore [41] ilk 6 kuşakta ortalama genetik ilerlemenin %5.38, 15 kuşak sonunda %4.25 olduğunu bildirmiştir. Çalışmanın ilerleyen dönemlerinde (97 kuşak sonunda) ise genetik ilerlemenin çok düşük düzeylerde olduğunu bildirilmiştir [8]. Benzer bulgular bildiren Türkmut ve ark.[33], 4. hafta canlı ağırlığı için yapılan seleksiyonda genetik ilerlemelerin 1. ve 2. kuşaklarda sırasıyla 14.29 g ve 3.99 g olduğunu, Baylan ve Uluocak [42] ise 5. hafta canlı ağırlığı için yapılan seleksiyonda genetik ilerlemelerin 1. ve 2. kuşaklarda 8.78 g ve 5.80 g olduğunu bildirmişlerdir.

Seleksiyon denemelerinde ilk kuşaklarda sağlanan genetik ilerlemenin sonraki kuşaklardan yüksek olduğu çeşitli araştırıcılar [8,30,33] tarafından bildirilmiştir. Bu durum, seleksiyona konu olan özelliğin eklemeli genetik varyansındaki azalmadan kaynaklanmaktadır [40]. Oysa çok özellikli seleksiyon ile eklemeli genetik varyanstaki azalma daha yavaş olmaktadır ve bu durum da ıslah çalışmalarının sürdürülebilirliği açısından önem taşımaktadır [43]. Araştırmada seleksiyon kriterleri olan CA, BBH, YV ve DO özellikleri için eklemeli genetik varyans ve kovaryans değişimlerini içeren matris *Şekil 1*'de sunulmuştur. Bu çalışmada uygulanan çok özellikli seleksiyon ile CA, BBH, YV ve DO özelliklerinin eklemeli genetik varyanslarında sırasıyla %6.5, %1.5, %5.5 ve %7.5 azalma meydana gelmiştir. Beş kuşak fenotipik seleksiyon yapan Yolcu [12], deneme sonunda 5. hafta canlı ağırlığı için eklemeli genetik varyansta %17'lik bir azalma gerçekleştiğini bildirmiştir. Uzun süre bıldırcın ıslahı ile uğraşan Marks [8], 97 kuşaklık seleksiyon sonrasında genetik varyasyonun çok düşük olmasına rağmen devam ettiğini, ancak gerçekleşen kalıtım derecelerinin düştüğünü ve bunun eklemeli genetik varyanstaki azalmadan kaynaklandığını bildirmiştir.

Araştırmada CA, BBH, YV ve DO özellikleri için seleksiyonla gerçekleşen kalıtım dereceleri sırasıyla 0.48, 0.56, 0.46 ve 0.18 olarak hesaplanmıştır. Kalıtım dereceleri, bıldırcınları konu alan birçok ıslah çalışmasında seleksiyonla sağlanan genetik ilerlemeden faydalanılarak hesaplanmıştır [8,10,12,30]. Gerçekleşen kalıtım derecesi, yavru-ebeveyn regresyonu ya da kardeşler arasında sınıf içi korelasyon yöntemleriyle elde edilmektedir. Seleksiyonla gerçekleşen kalıtım derecesi, seleksiyonun tekrarlandığı denemelerde hesaplanabilmektedir ve kuşaklar üzerinden birikimli ilerleme ve seleksiyon üstünlüğü kullanılarak elde edilmektedir [40]. Gerçek kalıtım derecesi ise seleksiyonun her kuşağında dengeye ulaşıncaya kadar azalmakta olup, aynı durum seleksiyonda sağlanan ilerleme için de geçerlidir. Oysa birikimli hesaplamalarda seleksiyonla sağlanan ilerleme doğrusal olmamakta ve seleksiyonda i. kuşaktan i+1. kuşağa gerçekleşen kalıtım derecesi sadece i. kuşakta sapmasız olarak tahmin edilmektedir. Diğer kuşaklarda hesaplanan tüm kalıtım dereceleri sapmalı olmaktadır. Bu yüzden modern kanatlı ıslahında seleksiyonla gerçekleşen kalıtım derecelerinin kullanılması tavsiye edilmemektedir [26].

Uzun dönemli seleksiyon uygulayan Marks [8], 4. hafta canlı ağırlığı ile ileri yaşlara ait canlı ağırlık değerlerinin, ilk yumurta yaşının ve yumurta ağılığının pozitif genetik ilişkili, bunun aksine 4. hafta canlı ağırlığı ile döllülük, kuluçka randımanı ve yumurta verimi özelliklerinin negatif genetik ilişkili olduğunu bildirmiştir. Araştırmada CA-BBH, CA-YV, CA-DO ve BBH-YV ile BBH-DO genetik korelasyonlarının negatif yönlü olduğu ve seleksiyon sonrasında CA-YV, CA-DO, BBH-DO özellikleri arasındaki genetik korelas-

yonlarda artış olduğu belirlenmiştir. Eklemeli genetik varyanslarda gerçekleşen azalmalar ve özellikler arasındaki negatif yöndeki eklemeli genetik kovaryanslarda gerçekleşen artışların etkisiyle sürü belirli bir genetik platoya ulaşıldıktan sonra aynı seleksiyon yöntemiyle seçilen bireyler için hesaplanan seleksiyon üstünlüğünün sıfıra yakın olacağı ve genetik ilerlemenin mümkün olmayacağını söylemek olasıdır [40,43].

Bu çalışmanın sonuçlarına dayanarak, çok özellikli seleksiyon ile ana hattında genetik ilerleme mümkün olduğu kadar sağlandıktan sonra, sürüden 2 ya da 3 yeni hat oluşturulması tavsiye edilebilir. Elde edilecek yeni ana hatlarında CA, BBH, YV ve DO özellikleri için eklemeli genetik kovaryanslardaki değişimler dikkate alınarak yeni ağırlık katsayıları kullanılmalı ve farklı yönlerde genetik iyileştirme yapılmalıdır. Böylece eklemeli gen etkileri bakımından saflaştırılan ana hatları oluşturulabilir. Benzer yöntemle geliştirilecek baba hatlarının da katılacağı test melezlemeleri sonucunda elde edilecek 2'li, 3'lü ve 4'lü melez kombinasyonlarında eklemeli olmayan gen etkilerinin de dahil olmasıyla hibrit nitelikli melez genotiplerin elde edilmesine olanak sağlanabilir.

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RESEARCH ARTICLE

İçme Sularında Yüksek Nitrat Bulunan Akkaraman Koyunlarda Serum Seruloplazmin, Paraoksanaz ve Nitrik Oksit Düzeyleri [1]

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Özet

Nevşehir/Hacıbektaş ilçesinde kuyulardan alınan su örneklerinde, Ankara Refik Saydam Hıfzısıhha Merkezinde analizi neticesinde, sularda normalin üstünde nitrat olduğu anlaşılmıştır. İzleme çalışmaları kapsamında Yalnızağıl mevkiindeki 3 kuyudan alınan su numunelerinde 69.7 mg/L, 56.8 mg/L ve 59.4 mg/L oranlarında nitrata rastlanmıştır. Bu çalışmanın amacı, içme sularında yüksek düzeyde nitrat bulunan Akkaraman koyunlarında serum seruloplazmin, paraoksanaz ve nitrik oksit düzeylerindeki değişimleri araştırmaktır. Çalışmada bölgedeki Akkaraman koyunları materyal olarak kullanılmıştır. İçme sularında yüksek oranda nitrat bulunan koyunlardan kan örnekleri alınarak serum seruloplazmin, paraoksanaz ve nitrik oksit düzeyleri analiz edilmiştir. Serum seruloplazmin, paraoksanaz ve nitrik oksit düzeyleri spektrofotometrik olarak ölçülmüştür. Serum seruloplazmin, paraoksanaz ve nitrik oksit düzeyleri kontrol ve çalışma gruplarında sırasıyla 10.68±0.14 uM; 10.80±0.32 uM: 151.36±14.95 U/ml; 142.57±17.85 U/ml ve 2.01±0.30 U/ml; 0.99±0.11 U/ml bulunmuştur. Serum seruloplazmin düzeyleri çalışma grubunda kontrol grubuna göre istatiksel olarak önemli düzeyde düşük bulunmuştur. Serum paraoksanaz ve nitrik oksit düzeyleri gruplar arasında istatiksel önemli farklılık göstermemekle birlikte serum paraoksanaz düzeyleri çalışma grubunda daha düşük düzeylerde ölçülmüştür. Bu sonuçlar doğrultusunda, Akkaraman koyunlarda nitratın olumsuz etkilerinin mevcut konsantrasyonlarda meydana geldiği düşünülmektedir.

Anahtar sözcükler: *Seruloplazmin, Paraoksanaz, Nitrik oksit, Akkaraman, Nitrat*

Serum Ceruloplasmin, Paraoxanase and Nitric Oxide Levels in Akkaraman Sheep with High Nitrate in Drinkıng Water

Summary

The data obtained by Ankara Refik Saydam Hıfzısıhha center, in the scope of the routine monitoring works carried out at Nevşehir/Hacıbektaş regions' wells were found high nitrate content. Nitrate values of three well water sampling points in the province of Yalnızağıl obtained from the monitoring works 69.7 mg/L, 56.8 mg/L and 59.4 mg/L nitrate ratios were found. The purpose of this study is to investigate the changes of serum ceruloplasmin, paraoxonase and nitric oxide levels in Akkaraman sheep with high nitrate in drinking water. In this study, Akkaraman sheep which is the animal population of region were used as material. The blood samples collected from Akkaraman sheep were analyzed in terms of ceruloplasmin, paraoxonase and nitric oxide levels by spectrophotometry. Serum ceruloplasmin, paraoxonase and nitric oxide levels was found, 10.68±0.14 uM; 10.80±0.32 uM: 151.36±14.95 U/ml, 142.57±17.85 U/ml and 2.01±0.30 U/ml, 0.99±0.11 U/ml by means of control and study groups respectively. Statistically, Serum ceruloplasmin levels were significantly lower in the study group compared with control group. In addition, although paraoxanase and nitric oxide levels in serum were not show significant differences between groups, measurments reveal that paraoxanase levels were lower in study groups. These results suggest that adverse effects of nitrates on Akkaraman sheep occur at concentrations present in the fields.

Keywords: *Ceruloplasmin, Paraoxonase, Nitric oxide, Akkaraman, Nitrate*

GİRİŞ

Sularda yüksek düzeyde nitrat bulunması hem hayvan hem de insan sağlığı açısından problem oluşturmaktadır [1-4]. Sularda artan nitrat konsantrasyonu çevre ve sağlık problemlerine neden olduğundan Avrupa Birliği Komis-

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yonu 1991 yılında nitrat direktif uygulamalarını kabul etmiştir. Sularda 50 mg/l nitrat konsantrasyonu veya 10 mg/L nitrat-N nitrat kirliliği olarak kabul edilmektedir [5]. Hacıbektaş ilçesinin su ihtiyacının karşılandığı kuyulardan alınan su örneklerinin, Ankara Refik Saydam Hıfzısıhha Merkezinde analizi neticesinde, sularda normalin üstünde nitrat olduğu anlaşılmıştır. İlçenin su ihtiyacının karşılayan ve 15 kilometre uzaklıkta bulunan Yalnızağıl mevkiindeki kuyulardan alınan su numunelerinde 69.7 mg/L, 56.8 mg/L ve 59.4 mg/L oranlarında nitrata rastlanması üzerine bu kuyulardan şebekeye su verilmesi durdurulmuştur [6].

Seruloplazmin bir α-2 glikoprotein olup karbonhidrat içeriğini sialik asit oluşturur [7]. Seruloplazmin; plazmada bakır taşınmasında görevli temel protein olup toksik ferro demirin, toksik olmayan ferri demire oksitlenmesini sağlar. Aynı zamanda demirle ilişkili serbest radikal yaralanmalardan dokuları korur ve daha başka antioksidatif ve sitoprotektif aktivitelerde de görev alır [8].

Paraoksonaz (PON1), hem arilesteraz (E.C. 3.1.1.2) hem de paraoksonaz (E.C.3.1.8.1) aktivitesine sahip, glikoprotein yapısında olan kalsiyum bağımlı bir ester hidrolazdır [9]. PON1'in, LDL'nin hücre kaynaklı oksidasyonuna karşı koruyucu olduğu gösterilmiştir [10]. PON1'in bu antioksidan kapasitesinden 284. pozisyondaki serbest sisteinin sorumlu olduğu bildirilmiştir [11].

Nitrik oksit, çok önemli biyolojik fonksiyonları yerine getirmek üzere üretilen nitrojen merkezli bir radikaldir. Antioksidan ve antienflamatuvar etkileri yanında bir çok düzenleyici role de sahip olan NO'in, yüksek yoğunlukta bulunduğu durumlarda, DNA hasarına ve lipid peroksidasyonuna sebep olmak, antioksidanları tüketmek, enzim inhibisyonu yapmak ve bir çok toksik etkene duyarlılığı arttırmak gibi zararlı etkileri de mevcuttur. Düşük konsantrasyonlarda önemli fizyolojik fonksiyonlara sahip olan NO, stabil ürünleri olan nitrit ve nitrata okside olur [12].

Bu çalışmanın amacı, içme sularında yüksek düzeyde nitrat bulunan Akkaraman koyunlarında serum seruloplazmin paraoksanaz ve nitrik oksit düzeylerindeki değişimleri araştırmaktır.

MATERYAL ve METOT

Hayvan Materyali

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Çalışmada, belediyenin veteriner hizmetleri kapsamında Hacıbektaş yöresinde yüksek nitrat tesbit edilen kaynak sularını içme suyu olarak kullanan 30 adet Akkaraman koyunundan kan örnekleri alındı. Kontrol grubu olarak belediyenin veteriner hizmetleri kapsamında yaptığı analizlerde, sularında nitrat düzeyi düşük olarak tesbit edilen, aynı bölgedeki farklı köyden 10 adet sağlıklı Akkaraman koyundan kan alındı. Kan örnekleri, en kısa zamanda laboratuvara getirilerek 10 dak. 2.500 devirde santrifüj edilerek serumları çıkarıldı. Elde edilen serum örnekleri, seruloplazmin, paraoksanaz ve nitrik oksit analizlerinde kullanıldı.

Örneklerin Analizi

Seruloplazmin düzeyleri Ceron ve Suibela-Martinez tarafından bildirilen yöntemle ölçüldü [13]. pH 5.2 ve 37°C koşullarında asetat tamponunda hazırlanan P-fenilen diamin diklorid (PPD) serum örnekleri ile oluşan renkli ürünün absorbansı spektrometrede 550 nm'de okunarak serum seruloplazmin ölçümleri incelendi [14].

Paraoksanaz aktivitesi Armstrong tarafından bildirilen yöntemle ölçüldü [15]. Bu yöntemle, 1 mM $CaCl_2$ ve 4 mM paraokson ihtiva eden 50 mM glisin; pH 10.5 tamponu kullanılarak paraoksonazın enzimatik hidrolizi sonucu oluşan p-nitrofenolün 412 nm'deki optik dansiteleri ölçülerek, paraoksonaz aktiviteleri incelendi.

Nitrik-oksit konsantrasyonu Gilliam ve ark.[16] tarafından bildirilen yöntemle ölçüldü.

Toplam nitrit, her bir örneğin 550 nm'de optik yoğunluğunun okunmasıyla değerlendirildi. Nitrik oksit konsantrasyonu, bir potasyum nitrat standart eğrisiyle (0 ile 80 µM arasında) karşılaştırma yapılarak belirlendi [16].

İstatistiksel Analiz

Gruplar arası farklılık bağımsız gruplarda t testi kullanılarak incelenmiştir [17]. Elde edilen veriler aritmetik ortalama ve standart hata şeklinde verilmiştir. İstatistiksel olarak önemlilik $P<0.05$ şeklinde ifade edilmiştir.

BULGULAR

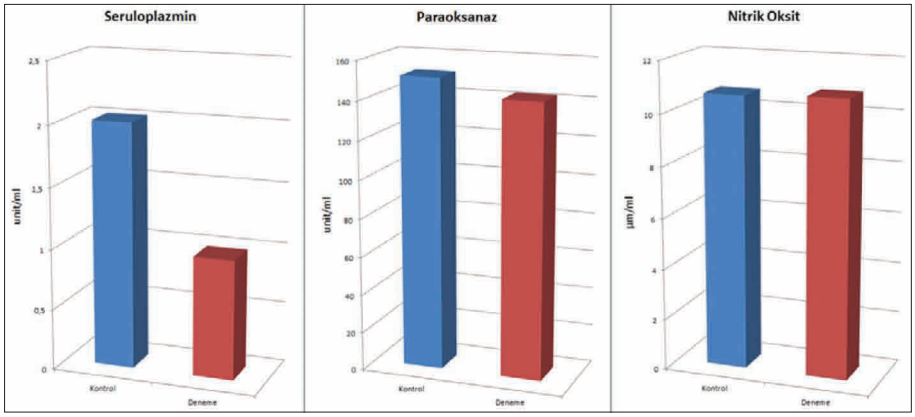
Serum seruloplazmin, paraoksanaz ve nitrik oksit düzeylerine ait sonuçlar *Tablo 1* ve *Şekil 1*'de gösterilmiştir.

Serum seruloplazmin düzeyleri, deneme grubunda 0.99±0.11U/ml; kontrol grubunda 2.01±0.30 U/ml olarak tespit edilmiş *(Tablo 1, Şekil 1b)* ve gruplar arasındaki fark, istatistiksel olarak önemli bulunmuştur ($P<0.001$). Serum paraoksanaz düzeyleri ise, deneme grubunda 142.57±17.85 U/ml; kontrol grubunda 151.36±14.95 U/ml olarak tesbit edilmiştir *(Tablo 1, Şekil 1c)*. Deneme grubu paraoksanaz düzeyleri kontrole göre düşük olmakla birlikte, gruplar arasındaki fark, istatistiksel olarak önemsiz bulunmuştur ($P>0.05$).

Serum nitrik oksit düzeyleri, deneme grubunda 10.80± 0.32 µmol/ml, kontrol grubunda 10.68±0.14 µmol/ml olarak tesbit edilmiştir *(Tablo 1, Şekil 1a)*. Gruplar arasındaki fark, istatistiksel olarak önemsiz bulunmuştur ($P>0.05$).

Tablo 1. *Kontrol ve çalışma grubu Akkaraman koyunlarına ait serum seruloplazmin, paraoksanaz ve nitrik oksit düzeyleri*
Table 1. *Serum ceruloplasmine, paraoxanase and nitric oxide levels of control and study group Akkaraman sheeps*

Ölçülen Parametre	Kontrol Grubu		Deneme Grubu		P
	n	X±Standard Hata	n	X±Standard Hata	
Seroplazmin (U/ml)	10	2.01±0.30	28	0.99±0.11	P<0.001
Paraoksanoz (U/ml)	10	151.36±14.95	28	142.57±17.85	P>0.05
Nitrik mg/mlasit	10	10.68±0.14	30	10.80±0.32	P>0.05



Şekil 1. Serum seruloplazmin, paraoksanaz ve nitrik oksit düzeyleri

Fig 1. Serum ceruloplasmine, paraoxanase and nitric oxide levels

TARTIŞMA ve SONUÇ

Dünyadaki yeraltı sularının en yaygın kimyasal kirleticisi nitrattır. Nitrat, genotoksik bileşiklerin bir sınıfı olan aynı zamanda hayvanlarda karsinojen etkili N-nitrozo bileşiklerin oluşumunda bir ön maddedir [18]. Nitrat, çiftlik hayvanlarında toksik etki göstermemekle birlikte, alınan nitrat rumen bakterilerince nitrite dönüştürülmektedir. Nitrit ise yüksek düzeyde toksik etkiye sahiptir ve daha ileri okside olarak amonyağa dönüşür. Yüksek düzeyde nitrat alımı sonucu kanda toksik düzeyde nitrit birikimi oluşur. Bunun sonucunda, hemoglobinin yapısında yer alan demir iyonu ferröz (Fe^{+2}) formdan ferrik (Fe^{+3}) forma dönüşür ve methemoglobin oluşur. Hemoglobindeki demirin Fe^{+3} şekli eritrositlerin oksijen taşıma kapasitesini düşürür. Nitrat tosisitesinin belirtileri hemoglobinin %20'sinin methemoglobine dönüştüğünde görülür. Rumende oluşan nitritin kan dolaşımına transferinde, alınan nitrat miktarı, yemlerin sindirim hızı, nitratın nitrite dönüşüm hızı ve rumenden nitritin absorpsiyonuna bağlı değişim gösterir. Nitrat/nitrit toksikasyonunda maruz kalma süresi ve hızı yanında bireysel tolerans ve metabolizma kapasitesi de etkilidir [19]. Sularda artan nitrat konsantrasyonu, çevre ve sağlık problemlerine neden olduğundan, Avrupa Birliği Komisyonu 1991 yılında nitrat direktif uygulamalarını kabul etmiştir. Sularda 50 mg/L nitrat konsantrasyonu veya 10 mg/L nitrat-nitrojen, nitrat kirliliği olarak kabul edilmektedir. Nevşehir'in Hacıbektaş ilçesindeki kuyulardan alınan su örneklerinin, Ankara Refik Saydam Hıfzısıhha Merkezindeki analizi neticesinde, sularda normalin üstünde nitrat olduğu anlaşılmıştır. İzleme çalışmaları kapsamında Yalnızağıl mevkiindeki 3 kuyudan alınan su numunelerinde 69.7 mg/L, 56.8 mg/L ve 59.4 mg/L oranlarında nitrata rastlanmıştır. Çalışmada, içme sularında yüksek nitrat bulunan Hacıbektaş yöresi Akkaraman koyunların serum örneklerinde antioksidan etkili seruloplazmin, paraoksanaz ve nitrik oksit aktiviteleri belirlenerek kontrol grubu ile karşılaştırılmıştır.

Kontrol ve çalışma grubu Akkaraman koyunlarında serum nitrik oksit düzeyleri bakımından, gruplar arasında istatistiksel olarak fark önemsiz bulunmuştur (P>0.05). Organizmada çok düşük yarı ömre sahip olan NO, stabil ürünleri olan nitrit ve nitrata okside olmaktadır [12]. Yüksek nitrat alımına bağlı serum nitrik oksit düzeylerinde beklenen artışın görülmemesi, yüksek nitrat alımına karşı koyunlarda fenotipik varyasyonların olduğu, bir çok karaciğer enziminin genetik ekspresyonunun nitrat toksikasyonuna farklı yanıtlar verdiği bildirilmektedir [20]. İçme sularına 100 mg/L, 250 mg/L ve 500 mg/L konsantrasyonlarında nitrat katılan ratlarla yapılan çalışmada, 250 mg/L ve 500 mg/L düzeyindeki yüksek dozlarda nitrat içeren içme sularını tüketen ratlarda NO düzeyi nitrat konsantrasyonundaki artışa paralel artış göstermiştir. Fakat 100 mg/L nitrat içeren sulardan tüketen ratlarda NO düzeyi değişim göstermemiştir [21]. Ratlarla yapılan deneysel çalışmanın NO sonuçları çalışmamızla uyumlu görülmektedir. Hacı Bektaş yöresi kaynak sularında nitrat düzeyi 100 mg/L geçmediği ve koyunların serum NO düzeyinde de önemli bir değişim olmadığı gözlenmiştir. Koyunlar, nitriti amonyağa dönüştürmede daha etkili olduklarından, sığırlara göre nitrit/nitrat zehirlenmesine daha az duyarlıdırlar [22].

Kontrol ve çalışma grubu Akkaraman koyunları serum seruloplazmin düzeylerinde gruplar arasındaki fark istatistiksel olarak önemli bulunmuştur (P<0.001). Serum paraoksanaz düzeyleri ise, çalışma grubu paraoksanaz

düzeyleri kontrole göre düşük olmakla birlikte, gruplar arasındaki fark istatistiksel olarak önemli bulunmamıştır (P>0.05). Nitrat/nitrit toksikasyonlarında antioksidan etkili moleküllerde azalma/enzimlerde inhibisyon bildirilmektedir. Yüksek reaktivitede peroksinitrit oluşumunun antioksidan enzimlerde azalmaya neden olduğu bildirilmektedir [23]. İçme sularına yüksek konsantrasyonlarda nitrat katılan ratlarla yapılan çalışmada, oksidatif stres oluşturularak lipid peroksidasyon belirteçi olan MDA düzeylerine bakılmıştır. Serum MDA düzeyinde bir değişim gözlenmezken karaciğer doku MDA düzeyinde artış gözlenmiştir. Yüksek dozlarda nitrat alınımının serum MDA düzeyini etkilememesi, kan plazmasındaki mevcut antioksidan sistemlerinin serum MDA düzeylerini düşürmede etkili olabilecekleri ileri sürülmüştür [24]. Çalışmadaki antioksidan seruloplazmin ve paraoksanaz aktivitelerindeki düşüklük, içme sularındaki yüksek nitratın oluşturacağı oksidatif strese karşı kullanılmış olmalarından ileri gelebilir.

Sonuç olarak, Akkaraman koyunlarda nitratın olumsuz etkilerinin mevcut konsantrasyonlarda meydana geldiği düşünülmektedir.

Çiftlik hayvanlarında subakut ve kronik nitrat toksikasyonuna bağlı biyobelirteçlerin gelişimi için bir temel sağlaması açısından ve belirli sınırlarda nitrata maruz kalma ve ilgili sağlık etkileri değerlendirmek için daha kapsamlı ve kontrollü çalışmalara ihtiyaç vardır.

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RESEARCH ARTICLE

Isolation, Cloning and Sequence Analysis of Enolase Enzyme Encoding Gene from *Theileria annulata* for Assessment of Important Residues of This Enzyme [1]

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Summary

Drug resistance against one of the important antitheilerial drugs has been reported for the first time in 2010. For the aim of developing new antitheilerial drugs or vaccines, enolase gene was isolated from the genomic DNA of *Theileria annulata*, cloned for the first time in the literature and analyzed at nucleotide and amino acid levels by using different web based tools. These analyses showed that the gene was consisted of 1365 nucleotides including an intron sequence placed between residues 40-41. Restriction enzyme mapping analysis of the cloned gene showed that, base pair changes in *Ta*ENO Elazig strain caused differences on cutting and non-cutting restriction enzymes compared to the Ankara strain. These differences may help the identification of different strains by restriction mapping and it may be possible to determine the geological distribution of *T. annulata* strains in any region. As the comparison of enolase gene sequences from *T. annulata* and the muscle enolase isoform of the host *Bos taurus* was made, four different insertions in *T. annulata* enolase that do not exist in *B. taurus* enolase was reported as an important discovery of this study. The modeling studies on *T. annulata* enolase gene showed that these insertions constituted loops that do not exist in *B. taurus* enolase, suggesting that these loops could be specific binding sites for enzyme inhibitors.

Keywords: *Theileria annulata, Enolase, Strain identification, Antitheilerial drugs, Structure based drug design, Protein homology modeling*

Theileria annulata'nın Enolaz Enzimini Kodlayan Geninin, Önemli Rezidülerinin Değerlendirilmesi Amacıyla İzolasyonu, Klonlanması ve Dizi Analizinin Yapılması

Özet

Bu yıl ilk kez önemli bir antitheilerial ilaca karşı direnç geliştiği rapor edilmiştir. Yeni antitheilerial ilaçların veya aşıların geliştirilmesi amacıyla *Theileria annulata* genomik DNA'sından enolaz geni izole edilmiş, literatürde ilk kez klonlanmış ve web tabanlı araçlar kullanılarak hem nükleotid hem de amino asit seviyesinde analiz edilmiştir. Bu analizler genin 1365 nükleotidden oluştuğunu ve 40-41 rezidüleri arasında bir intron dizisi içerdiğini göstermiştir. Klonlanan genin restriksiyon enzim haritalama analizinde, *Ta*ENO Elazığ soyundaki baz çifti değişikliklerinin, Ankara soyu ile karşılaştırıldığında, geni kesen ve kesmeyen enzimlerde farklılıklar yarattığı gözlemlenmiştir. Bu farklılıklar, farklı soyların restriksiyon haritalama ile tanımlanmasına yardımcı olabilir ve *T. annulata* soylarının herhangi bir bölgedeki coğrafik dağılımının belirlenmesini mümkün kılabilir. *T. annulata* enolaz geni dizisi ile konak *Bos taurus*'un kas enolaz izoformu karşılaştırıldığında, *T. annulata*'da bulunan 4 farklı insersiyonun *B. taurus* enolazında bulunmadığı belirlenmiş ve bu çalışmanın önemli bir bulgusu olarak rapor edilmiştir. *T. annulata* enolazının modelleme çalışmaları bu insersiyonların *B. taurus*'ta bulunmayan halkalar oluşturduğunu göstermiş ve bu halkaların enzim inhibitörleri için spesifik bağlanma bölgeleri olabileceği önerilmiştir.

Anahtar sözcükler: *Theileria annulata, Enolaz, Soy tanımlama, Antitheilerial ilaçlar, Yapıya dayandırılmış ilaç tasarımı, Protein homoloji modelleme*

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INTRODUCTION

Tropical theileriosis is a serious and widespread disease transmitted by ticks from *Hyalomma* genus and caused by *Theileria annulata* which infects cattle and leads to major economic losses [1]. *Theileria parva* and *Theileria annulata* are the most pathogen species among Theileria species that are factors of theileriosis disease, characterized as lymphoproliferative and causing high morbidity and mortality [2]. Tropical theileriosis factor *T. annulata* is found in tropical and subtropical countries [3] and approximately 250 million animals are living in risky areas [4,5]. Strategies to control this disease can be listed as, acarisides against vector ticks, immunization by live attenuated cell line vaccines, chemotherapy [6,7] and antiprotozoal drugs like halofuginone, parvaquone and buparvaquone [8-10]. Buparvaquone (2-trans (4-t-butylcyclohexyl-methyl)-3-hydroxy-1,4-naphthoquinone) is found to be the most effective antitheilerial drug used against *T. annulata* born cattle theileriosis [4,8,10,11]. However, a resistance of *T. annulata* against buparvaquone was reported first time in 2010 in a study in Tunisia [3]. This situation indicates that, designing new antitheilerial drugs will be very essential in forthcoming years.

Basic metabolic pathways and biochemical features of apicomplexan parasites are potential chemotherapeutic targets for indicating antiparasitic drugs for the treatment of diseases caused by these parasites. For this purpose, the gene encoding enolase enzyme is chosen as a target. Enolase, is a metalloenzyme which catalyzes the inter-conversion of 2-phosphoglycerate (2-PGA) to phosphoenol-pyruvate (PEP) during glycolysis [12]. Since *Theileria* species do not have a proper citric acid cycle and produce their energy mainly via anaerobic respiration, enolase has a crucial importance in the parasites life because of its role in glycolysis.

Aim of this study was isolation, cloning, sequence analysis of *Theileria annulata* enolase encoding gene *(Ta*ENO) and homology modeling for prediction of the 3D structure of the protein. This cloning study will be the first one in literature for *Ta*ENO gene and information presented by it, may lead to a new antitheilerial drug design.

MATERIAL and METHODS

All general methods were applied according to Sambrook et al.[13] unless otherwise stated.

Bacterial Strain, Growth Media, Enzymes and Vector

Escherichia coli JM105 was used as the host bacterial strain to prepare DNA for cloning and sequencing in pGEM-T Easy vector (Promega, USA). The *E. coli* JM105 cells were cultured in 2xYT broth. Long PCR Enzyme Mix was obtained from Fermentas (Lithuania).

Parasite Isolate and Genomic DNA

Blood sample was taken from a 4 years old Brown Swiss cow showing the symptoms of acute tropical theileriosis in Elazig province, Turkey. The samples were kept in a tube containing the anticoagulant ethylene diamine tetra-acetic acid (EDTA). The clinical diagnosis was confirmed by observation of *T. annulata* piroplasms and schizonts on Giemsa-stained blood and lymph node biopsy smears at the Laboratory of Parasitology of the Veterinary School of Firat University, Elazig, Turkey. The Wizard genomic DNA purification system (Promega, USA) was used to prepare DNA according to the manufacturer's instructions.

Amplification of Theileria annulata ENO by PCR

The initial sequence of *Ta*ENO from Ankara strain (accession number XM948248) was obtained from NCBI. Two specific oligonucleotide primers complementary to the forward and reverse strands of the *Ta*ENO gene were designed using this sequence. PCR product was analyzed on a 1% agarose gel and a band of the expected size was observed. After confirmation of the product size, PCR was set up at the same conditions again and DNA band was extracted directly from the PCR product using Promega's Wizard SV Gel and PCR Clean-Up System.

Ligation and Transformation

Ligation and transformation were performed according to the supplier instructions (Promega, USA).

DNA Sequencing

Plasmid DNA was then prepared using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) and submitted for sequencing from both directions twice independently.

Database Analysis and Molecular Modeling

Enolase sequences of apicomplexan parasites, host *B. taurus* and plants were obtained from NCBI. Alignments of sequences at nucleotide level were performed by using ClustalW2 tool. Amino acid sequence alignment was performed manually by using catalytic residues as reference points to set up residue numbering correctly. Clustal W2 tool was used to align ENO from *T. annulata*, *B. taurus,* plants and some other apicomplexans. Modeling studies of *B. taurus* ENO-3 and *Ta*ENO conducted by SWISS-Model [14]. The nucleotide sequences used in this study, including *T. annulata* cloned in this study, with accession numbers: *T. annulata* HQ646253, *Plasmodium falciparum* U00152, *Eimeria tenella* AF353515, *Toxoplasma gondii* AF123457, *Lycopersicum esculentum* X58108, *Arabidopsis thaliana* AY092986 and *B. taurus* NM001034702.

Restriction Enzyme Analysis

*Ta*ENO gene was amplified by PCR again to prepare

template DNA for restriction enzyme analysis. This PCR product was then treated with BamH1 and Acl1 restriction enzymes.

RESULTS

Amplification, Cloning and DNA Sequencing of TaENO Gene

Amplification of *Ta*ENO gene was made by using two oligonucleotides (TaENO1 and TaENO2) and a fragment of about 1.3 kb length was obtained. This product was then purified *(Fig. 1)* following the PCR, inserted into the pGEM-T Easy plasmid vector and transformed into *E. coli* JM105 cells. This cloned sequence of *Ta*ENO was submitted to GenBank with the accession number HQ646253. This was the first time enolase gene was cloned from a *Theileria* species in literature. Sequence analysis of the cloned gene indicated that *Ta*ENO gene was consisted of 1365 base pairs, containing 2 open reading frames (ORF) of 40 bp and 1286bpwhich were divided by an intron sequence of 36bp conforming to the GT/AG rule at the splicing junctions.

Restriction Enzyme Analysis

Restriction enzyme mapping analysis showed that the 37 base pair changes in *Ta*ENO Elazig strain caused differences on cutting and non-cutting restriction enzymes compared to the Ankara strain. Four of these base pair changes generated four new enzyme cutting sites (AflIII, BamHI, BseRI and TstI) and 11 of these base pair changes abolished 11 enzyme cutting sites (AclI, ArsI, BplI, BsmI, BsrDI, DraII, HgaI, PflMI, PpuMI, TspGWI and XbaI) of Elazig strain. EtBr gel electrophoresis results showed clearly that BamH1 restriction enzyme can cut *Ta*ENO gene from Elazig strain while it can not cut *Ta*ENO of Ankara strain and also Acl1 restriction enzyme can not cut *Ta*ENO gene from Elazig strain but can cut *Ta*ENO of Ankara strain.

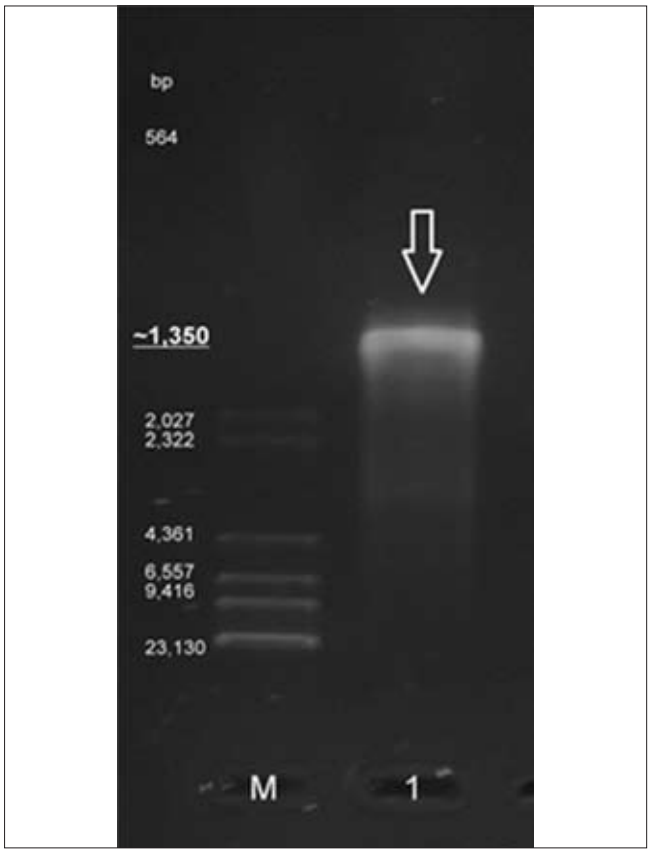

Fig 1. Agarose gel view of amplified and purified *Ta*ENO gene from genomic DNA of *T. annulata*. M: Marker, 1: *Ta*ENO

Şekil 1. *T. annulata* genomik DNA'sından amplifiye edilip saflaştırılmış *Ta*ENO geninin agaroz jel görüntüsü. M: Marker; 1: *Ta*ENO

Multiple Amino Acid Sequence Alignment and Phylogenetic Analysis of TaENO With Some Other Known Enolase Sequences

The amino acid sequence of *Ta*ENO (HQ646253) obtained in this study was first compared with host *B. taurus* muscle form enolase (*Bt*ENO3) (NM001034702). Alignment analysis showed the existence of a penta-peptide, a tripeptide and two dipeptide insertions in *Ta*ENO as $E_{103}W_{104}G_{105}Y_{106}C_{107}$, $T_{147}D_{148}$, $E_{262}K_{263}S_{264}$ and $K_{317}L_{318}$ respectively that do not exist in *Bt*ENO3. The same amino acid alignment comparison was also made with other apicomplexan parasite examples, *T. gondii* ENO1 (AF123457) and ENO2 (AY155668), *P. falciparum* (U00152) and *E. tenella* (AF353515) and a plant example *L. esculentum* (X58108). These comparisons showed that both apicomplexan parasites and plants had similar insertion sites as *Ta*ENO *(Fig. 2)*. Alignement analysis also showed that characteristic residues involved in the catalytic activity of the ENO were conserved [15] in all of the sequences presented in *Fig. 2*. Among these residues, E217 and E174 are involved in the dehydration step; D330, E303 and D252 required for the binding of the substrate; K355 and R384 interacts with the phosphate group and K406 and H383 with the carboxylic group of 2-PGA.

Comparison of T. annulata and Bos taurus ENO's by Homology Modeling and Potential Use of TaENO Gene In Structure Based Drug Design Studies

Structure based drug designing studies are mostly studied on *Plasmodium* sp. among apicomplexan parasites [16]. Because *Plasmodium* species do not have a functional Krebs cycle, as *Thelieria* species, they produce their energy via glycolysis [17,18]. Lactate dehydrogenase enzyme has a crucial role in this parasites life as it catalyzes the reduction of pyruvates to hydroxyls by oxidation of NADH to NAD^+ [16]. It is important to note that, Plasmodial LDH also has a pentapeptide insertion in the active site of the enzyme [19]. Crystallography studies on *Pf*LDH revealed that the pentapeptide insertion in an active site loop between 108. and 109. amino acids which constitutes a cleft on the surface of catalytic ring of the enzyme, but mammalian equivalent LDH does not contain this cleft as it does not have the pentapeptide insertion. From this point of view,

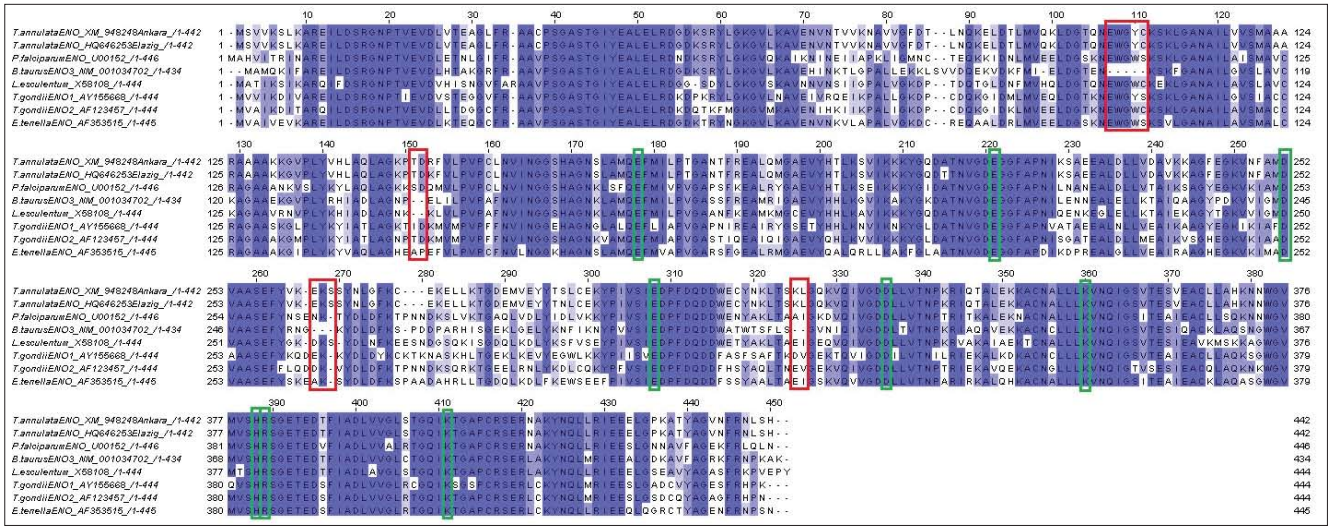

Fig 2. Comparison between amino acid sequences of enolase genes of *Theileria annulata* Ankara and Elazig strains, *Plasmodium falciparum, Bos taurus* ENO3, *Lycopersicum esculentum, Toxoplasma gondii* ENO1 and ENO2 and *Eimeria tenella,* from top to bottom respectively. Sites boxed in red, show the pentapeptide, the tripeptide and two dipeptide insertions. Sites boxed with green show the catalytic residues of enolase

Şekil 2. Yukarıdan aşağı sırayla,*Theileria annulata* Ankara ve Elazığ soyları, *Plasmodium falciparum, Bos taurus* ENO3, *Lycopersicum esculentum, Toxoplasma gondii ENO1 ve ENO2 ve Eimeria tenella*'nın enolaz genlerinin amino asit dizilerinin karşılaştırılması. Kırmızı ile kare içine alınmış bölgeler pentapeptid, tripeptid ve iki dipeptid insersiyonlarını göstermektedir. Yeşil ile kare içine alınan bölgeler enolazın katalitik rezidülerini göstermektedir

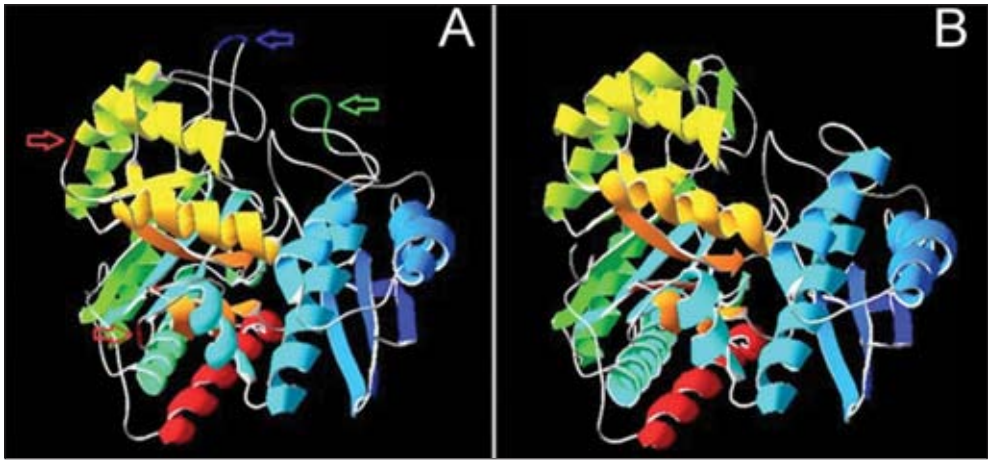

Fig 3. Homology modeling of enolase proteins for prediction of 3D structures of the protein, **A-** Homology model of enolase protein of *T. annulata* Elazig strain in ribbon style. Pentapeptide insertion site is marked with green, tripeptide insertion is marked with blue and dipeptide insertion sites are marked with red, **B-** Homology model of ENO-3 protein of host *B. taurus* in ribbon style

Şekil 3. Enolaz proteinin homoloji modelleme ile 3 boyutlu yapısının tahmini, **A-** Elazığ soyu *T. annulata*'nın enolaz proteininin kurdela modeli ile homoloji modellemesi; pentapeptid insersiyonu yeşil, tripeptid insersiyonu mavi ve dipeptid insersiyonları kırmısı ile işaretlenmiştir, **B-** Konak *B. taurus*'un ENO-3 proteininin kurdela modeli ile homoloji modellemesi

in vitro studies showed that azole based inhibitors can bind to active site of *Pf*LDH protein and stop both the enzyme activity and the parasite development in red blood cells. These compounds were selective against *Pf*LDH than human LDH and crystallography studies showed that the binding regions were also maintained in *P. berghei* LDH forms [16].

Considering these studies on *Plasmodium* species, it is aimed to investigate if it is possible to open a route to design new antitheilerial drugs by the evaluation of the data from isolation and cloning of enolase encoding gene from *T. annulata*. For this purpose, m-RNA sequence of cloned *Ta*ENO gene was compared to host *B. taurus* muscle enolase (ENO-3) using a web based tool to obtain a 3D model of both ENOs.

Amino acid sequence obtained from DNA sequencing of the cloned *Ta*ENO gene in this study was used to model 3D structure of *Ta*ENO by SWISS-MODEL workspace using the automatic modeling mode *(Fig. 3a)*. ENO-3 protein of the host *B. taurus* was also modelled by the same

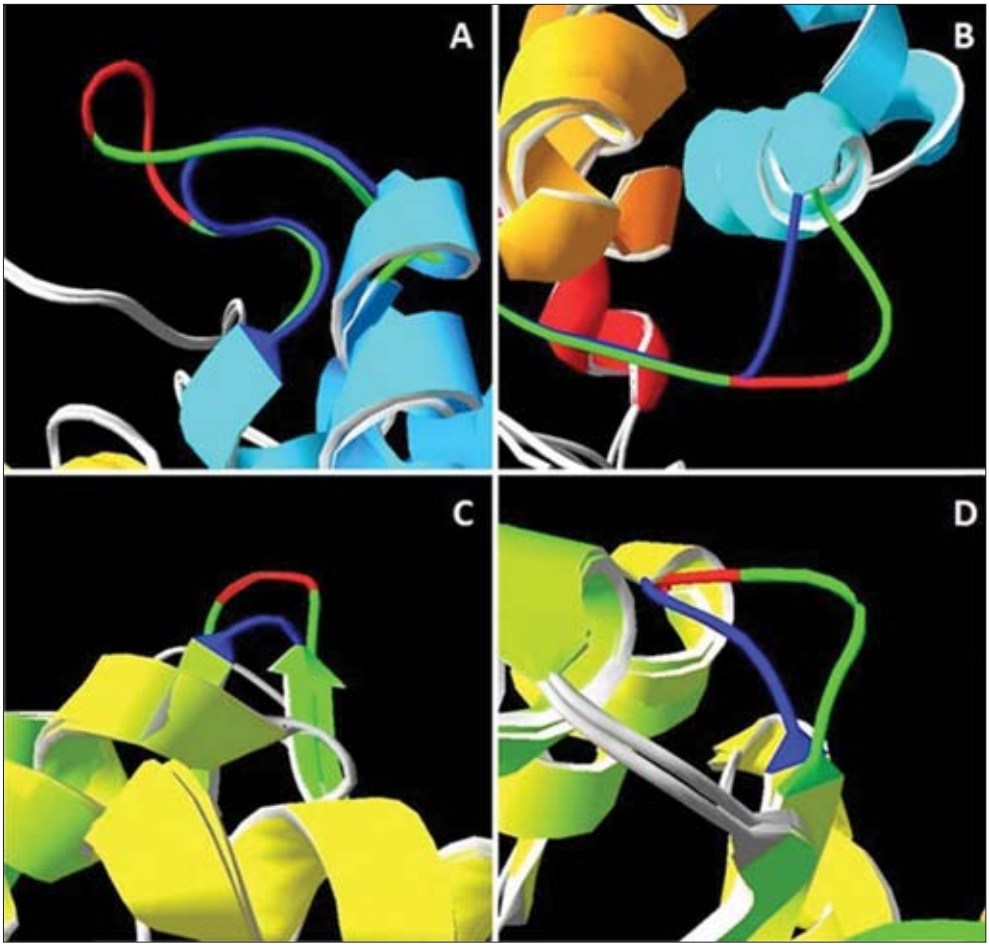

Fig 4. Overlay comparisons of insertion sites of *T. annulata* (green) and *B. taurus* (blue) enolases, **A-** The pentapeptide insertion of $E_{103}W_{104}G_{105}Y_{106}C_{107}$ in *Ta*ENO is shown in red, **B-** A dipeptide insertion ($T_{147}D_{148}$) in *Ta*ENO is shown in red, **C-** The tripeptide insertion ($E_{262}K_{263}S_{264}$) in *Ta*ENO is shown in red, **D-** Another dipeptide insertion ($K_{317}L_{318}$) in *Ta*ENO

Şekil 4. *T. annulata* (yeşil) ve *B. taurus*'un (mavi) enolazlarının insersiyon bölgelerinin (kırmızı) üst üste çakıştırılarak karşılaştırılması, **A-** *Ta*ENO pentapeptid insersiyonu ($E_{103}W_{104}G_{105}Y_{106}C_{107}$), **B-** *Ta*ENO dipeptid insersiyonu ($T_{147}D_{148}$), **C-** *Ta*ENO tripeptid insersiyonu ($E_{262}K_{263}S_{264}$), **D-** Diğer *Ta*ENO dipeptid insersiyonu ($K_{317}L_{318}$)

method using the amino acid sequence from GenBank (NM 001034702) for comparison with *Ta*ENO *(Fig. 3b)*. In detail formation of each loop caused by pentapeptide and dipeptide insertions in *Ta*ENO that do not exist in *Bt*ENO3 were clearly observed when the comparison of overlaid carbon backbone structures of the proteins was made *(Fig. 4)*.

DISCUSSION

Parvaquone [11] and buparvaquone [8] were used as drugs for treatment of tropical theileriosis without facing any resistance against these drugs since 1970's, although the high mortality rate of the disease because of the deficiency of treatment methods. However, a resistance of *T. annulata* against buparvaquone was first reported in Tunisia last year [3]. This situation indicates that designing antitheilerial drugs with different effects is essential. The gene encoding enolase enzyme from *T. annulata* was isolated, to our knowledge, cloned for the first time and analyzed in this study. Results indicated that, *Ta*ENO was consisted of 1365 base pairs including the stop codon and had and intron site of 36 base pairs. The homology modeling studies can give information leading to locate residues that are specific to the parasite and to inactivate the target enzyme of the parasite selectively [17]. As an important finding, four insertion sites, including a pentapeptide ($E_{103}W_{104}G_{105}Y_{106}C_{107}$), a tripeptide ($E_{262}K_{263}S_{264}$)and two di-peptide ($T_{147}D_{148}$and $K_{317}L_{318}$) insertions, were detected by comparison of homology models of *Ta*ENO and the equivalent in host *B. taurus,* muscle enolase (ENO3). It has been suggested that, the loops constituted on enolase by these four insertions of 5, 3 and 2 amino acids, do not exist in host *B. taurus* enolase and can be used as binding regions for specific enzyme inhibitors. Further kinetic, structural and mutagenic analysis of *Ta*ENO in comparison to the host ENO would be of great value towards the drug design studies against *T. annulata.*

Another important data obtained about insertion sites is that the region of the pentapeptide insertion may be an antigenic epitope. Effects on enzyme activity and structure, after removal of a pentapeptide insertion on a surface loop away from the active site by deletion in *P. falciparum* were studied by Vora and colleagues in 2009 [20]. It was reported that this deletion decreases the k_{cat}/K_m values for a 100 times and causes the dimeric form separate to monomers. The serum obtained from mice that were vaccinated by Pfen (*P. falciparum* enolase) and survived the parasite infection, gave a negligible reaction against the protein that carries the deletion, when compared to wild-type enolase. These results show that the insertion site is essential for enolase's proper activity and may constitute a preservative antigenic epitope in parasite's enolase [20]. A region, similar to *Plasmodium*s is also available in *Ta*ENO, which is cloned in this study, and it is suggested that this region may be an antigenic epitope for *Ta*ENO. In addition, virulence has been reported to vary across strains and cloned parasites of *T. annulata* as summarized in Tindih et al.[21]. As differences in ENO sequence of two strains of *T. annulata* from different territories are reported by this study, it may be possible to facilitate determination of virulence of different strains by identification of the strains via restriction enzyme analysis.

In this study isolation, cloning, sequence analysis in combination with homology modeling studies and restriction enzyme analysis were performed for enolase encoding gene from *T. annulata* (Elazig strain). This study is first to describe enolase sequence from this parasite and opens a route to structure based drug design studies after the first report of a drug resistance against a commonly used antitheilerial drug, buparvaquone, and vaccine studies as this study enables to distinguish strains from each other by a simple restriction enzyme analysis that may help to determine the virulence variation between different strains.

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RESEARCH ARTICLE

Diagnosis of *Mycoplasma bovis* Infection in Cattle by ELISA and PCR

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Summary

Mycoplasma bovis is one of the most pathogenic agents in the *Mycoplasma* species that cause disease in cattle. In particular, young calves at less than 4 months of age are a considerable risk from pneumonia caused by *M. bovis*. In this study, we investigated *M. bovis* from tracheal swabs and blood sera of cattle which showed respiratory symptoms. A total of 127 tracheal swab samples were collected from seven different farms in Turkey. In addition, a total 254 acute and convelance sera were collected from the same cattle at intervals 15 days. The materials were collected from cattle between 3-12 months of age that reported respiratory problems such as broncho-pneumonia with coughing, depression, lethargy and fever. *Mycoplasma bovis* was investigated in tracheal swab samples and sera collected from the cattle by using PCR and ELISA respectively. The PCR results showed that *M. bovis* infections were positive in 4 different farms. The rates ranged from 5.3% (1/19) to 37.5% (6/16). Out of the 127 cattle examined, 45 (35.4%) were positive for *M. bovis* antibodies, while 82 (64.6%) were found to be negative. All PCR positive cattle were also found to be positive by ELISA. However by using ELISA, *M. bovis* infections were positive in all farms and the ELISA positive rates ranged from 20% (2/10) to 68.8% (11/16). Considering these results, in especially chronic infections, ELISA is a more useful method than PCR to detect *M. bovis* infection.

Keywords: *Cattle, ELISA, Mycoplasma bovis, PCR*

Sığırlarda *Mycoplasma bovis* Infeksiyonunun ELISA ve PCR ile Teşhisi

Özet

Mycoplasma bovis, *Mycoplasma* etkenleri içerisinde sığırlarda infeksiyona neden olan en patojen etkenlerden biridir. Özellikle, 4 aylık yaşın altındaki genç buzağılarda, *M. bovis*'in neden olduğu pnömonilerde artan bir risk bulunmaktadır. Bu çalışmada solunum sistemi infeksiyonu semptomları gösteren sığırların trachea svapları ve kan serumlarından *M. bovis* infeksiyonunun teşhisi ve *M. bovis* teşhisi için serolojik ve moleküler metodların karşılaştırılması amaçlanmıştır. Türkiye'de bulunan 7 farklı çiflikten gönderilen 127 tracheal svap örneği ile 15 gün arayla aynı sığırlardan alınan 254 adet akut ve konvelesans serum örneği PCR ve ELISA yöntemleriyle incelendi. Bu örnekler 3-12 aylık yaşlar da olan ve bronkopnömoni, öksürük, depresyon, halsizlik ve ateş gibi solunum sistemi infeksiyonu semptomu gösteren sığırlardan toplandı. Tracheal svap örneklerinin PCR sonuçlarına göre 4 farklı çiftik *M. bovis* infeksiyonu yönünden pozitif bulundu. Oranlar %5.3 (1/19) ile %37.5 (6/16) arasında bulundu. *Mycoplasma bovis* antikorları yönünden incelen 127 sığıra ait serumlarda, 45 (%35.4) adeti pozitif olarak saptandı; 82 (%64.6) serum ise negatif olarak saptandı. PCR'da pozitif olarak saptanan tüm sığırlar ELISA yöntemiyle de pozitif olarak saptandı. *M. bovis* infeksiyonu tüm çiftliklerde pozitif olarak saptandı ve ELISA oranları %20 (2/10) ile %68 (11/16) arasında değişkenlik gösterdi. Bu sonuçlar göz önüne alındığında, özellikle kronik infeksiyonlarda *M. bovis* infeksiyonunun teşhisinde ELISA'nın, PCR yöntemine göre daha uygun bir yöntem olduğu sonucuna varıldı.

Anahtar sözcükler: *ELISA, Mycoplasma bovis, PCR, Sığır*

INTRODUCTION

Mycoplasma bovis is one of the most pathogenic agents in the *Mycoplasma* species that cause disease in cattle. *Mycoplasma bovis*-associated pneumonia occurs in cattle, including dairy and beef calves, beef cattle after arrival at a feedlot, and adults at any age [1]. *Mycoplasma bovis* is a particularly important cause of calf pneumonias [2,3]. Especially young calves under 4 months of age are at increased risk for pneumonia caused by *M. bovis* [2].

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Mycoplasma bovis infections can be explained as chronic and polymicrobial [1-5]. Animals can be infected via the respiratory system [2]. Respiratory tract and nasal secretions are important for epidemiology of infection [1,6]. Beside this, the importance of aerosols in calf-to-calf transmission of *M. bovis* is unknown but recently Maunsell et al.[1] reported that *M. bovis* has been isolated from air in shed containing diseased calves and calves may be experimentally infected by inhalation of *M. bovis*. Infected cattle spread *M. bovis* to enviroment through respiratory sectertions for many months as reservoir [4].

The severity of pneumonia could be varried depending on the maintenance and environmental conditions. The effects of antibiotics and vaccines are not known to have negligible effects in calves [3]. Control of infection is difficult and economic loses is imminent [7,8]. The first condition is to ensure a high level of hygiene in the prevention of *M. bovis* infection. Because of non-specific clinical manifestations, and a wide range of variation in the epizootiology and pathogenesis, all *M. bovis* infections are have to be had specific diagnostic and control procedures [7].

Mycoplasma bovis infections could be diagnosed by bacteriological culture and serological methods [2,6,9,10]. Nevertheless, these methods are time consuming and false-negative results could be common [9]. Serological methods are less time consuming than the cultural methods and also more samples can be investigated. Recently, new molecular identification methods were improved and used in diagnosis of *M. bovis* infections worldwide by several authors [11-13]. PCR is much shorter in comparison to the conventional culture method for the identification of *M. bovis* infection [12].

In Turkey there are few reports about the *M. bovis* infections in cattle [14]. In this study we investigated *M. bovis* from tracheal swabs and blood sera of cattle that showed respiratory symptoms for the situation of *M. bovis* in Turkey. Also we aimed to compare the efficiency of molecular and serological methods for detection of *M. bovis* infections.

MATERIAL and METHODS

A total of 127 tracheal swab samples and 254 acute and convelance sera (15 day intervals) were collected from 6-12 months age cattle located in seven different geographically distinct farms in Turkey that had respiratory problems such as broncho-pneumonia with symptoms coughing, depression, lethargy and fever *(Table 1)*. All farms were beef farms and the capacities were between about 100 and 14.000 cattle. All the samples were transported to the laboratory in cold chain and were stored at –20ºC.

Molecular Identification of Mycoplasma bovis

DNA extraction was performed by the boiling method from directly swab samples [14,15]. The swab samples were analyzed by PCR using *M. bovis* spesific primers derived from the *mb-mp*81 gene, as described by Foddai et al.[13,14]. *Mycoplasma bovis* spesific primers were used to amplify 447 bp of *mb-mp* gene of *M. bovis* (mb-mp1F: 5-TAT TGG ATC AAC TGC TGG AT-3; mb-mp1R: 5-AGA TGC TCC ACT TAT CTT AG-3). Amplification was performed in a total reaction volume of 50 µl, containing 5 µl 10x PCR buffer, 5 µl 25 mM $MgCl_2$, 250 µM of each dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each primer and 25 ng of template DNA. The reaction conditions were as follows: denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min for 30 cycles, followed by a final extension step at 72°C for 10 min. The amplified products were detected by staining with 10 mg/ml ethidium bromide after electrophoresis at 80 V for 2 h in 2% agarose gels [14]. The results were screened from agarose gel by the molecular imaging system (Gene Genius, Syngene, England). *Mycoplasma bovis* DNA, which is used as positive control in PCR tests, was obtained from Prof. Dr. Burhan ÇETİNKAYA from Fırat University Veterinary Faculty Department of Microbiology Elazığ/Turkey.

Table 1. *Origins of sera and swap samples*
Tablo 1. *Serum ve svap örneklerinin orijinleri*

Farm	Number of Samples		Age (Months)
	Swabs	Sera	
Farm 1	16	32	6-12
Farm 2	10	20	6-12
Farm 3	16	32	6-12
Farm 4	19	38	3-12
Farm 5	10	20	6-12
Farm 6	27	54	6-12
Farm 7	29	58	6-12

ELISA

Bio-X *M. bovis* ELISA kit (BIO K 260, Belgium) was used in the serological analysis. The test was carried out according to the manufacturer's instructions. After the test, the absorbance values were read at 450 nm with a Titertec Multiscan MS plate reader.

RESULTS

The PCR results showes that *M. bovis* infections were positive in 4 different farms. The rates ranged from 5.3% (1/19) to 37.5% (6/16). The overall percentage, with a mean of a 12.6% (16/127) *(Table 2, Fig. 1)*.

The ELISA results showed in *Table 3*. Out of the 127 cattle examined, 45 (35.4%) were positive for *M. bovis* antibodies, while 82 (64.6%) were found to be negative. All PCR positive cattle were also found positive by the ELISA. *Mycoplasma bovis* infections were positive in all farms and the ELISA positive rates ranged from 20% (2/10) to 68.8% (11/16). The

Table 2. *PCR findings of tracheal swab samples*
Tablo 2. *Tracheal svap örneklerine ait PCR bulguları*

Farm	Number of Samples	Positive Numbers (%)
Farm 1	16	6 (37.5)
Farm 2	10	0
Farm 3	16	0
Farm 4	19	1 (5.3)
Farm 5	10	3 (30)
Farm 6	27	0
Farm 7	29	6 (20.7)
Total	127	16 (12.6)

with both mastitis and respiratory problems. In this study, the PCR results shows that 12.6% (16/127) positive samples were detected and ELISA results showed 35.4% (45/127) positive rates. In this study, we found approximately same detection rates and this finding supports the Karahan et al.[14].

PCR was reported to have effective specificity and sensitivity in diagnosis of *M. bovis* infections [11]. Previous studies have shown that several species can be detected via two-stage nested PCR. However, in these procedures it is need to be looked carefully to their sensitivity characteristics. Sung et al.[12], reported that the optimization of primer sequences and the reaction conditions presented here

Fig 1. Agarose gel electrophoresis of PCR. Line 1, molecular weight markers (Fermentas); Line 2, positive control; L3-L20, swab samples

Şekil 1. PCR sonucu elde edilen agaroz jel elektroforez görüntüsü. Sıra 1, moleküler ağırlık marker'ı (Fermentas); Sıra 2, pozitif kontrol; L3-L20, svap örnekleri

Table 3. *ELISA results in comparison with PCR findings*
Tablo 3. *PCR bulguları ile karşılaştırmalı ELISA sonuçları*

Farm	Number of Cattle	PCR Positive (%)	ELISA Positive			
			Total (%)	++	+++	++++
Farm 1	16	6 (37.5)	11 (68.8)	0	5	6
Farm 2	10	0	2 (20)	2	0	0
Farm 3	16	0	9 (56.2)	5	4	0
Farm 4	19	1 (5.3)	4 (21.1)	1	2	1
Farm 5	10	3 (30)	4 (40)	1	0	3
Farm 6	27	0	7 (25.9)	6	1	0
Farm 7	29	6 (20.7)	9 (31)	0	3	6
Total	127	16 (12.6)	45 (35.4)	15	14	16

overall percentage was found positive as 35.4 % (45/127) *(Table 3)*.

DISCUSSION

This research showed that *M. bovis* infection is a common respiratory problem in cattle in Turkey. *Mycoplasma bovis* infections are causing various economic loses such as treatment, laboratory diagnosis and product for dairy and beef cattles.

In Turkey, Karahan et al.[14] were investigated a total of 148 samples (3 lungs, 4 eye swabs, 51 nasal swabs and 90 milk samples) from three different farms in Eastern Turkey. They found 23% (34/148) samples to be positive. These samples were 3 lung, 12 to 51 nasal swabs and 19 to 90 milk. Authors concluded that *M. bovis* was relatively common in eastern region of Turkey especially in cattle

enhanced the sensitivity and ensured the high degree of specificity of the two-stage nested PCR. It was revealed that the specificity of the method presented their study was sufficient for the discrimination of mycoplasma contamination from other probable contaminants, including *E. coli*, *S. aureus*, and budding yeasts. It was showed in this study that the use of PCR makes the identification of *M. bovis* infection much shorter comparing to the conventional culture method.

The other method for detection of *M. bovis* is serology. Serological tests shows increasing antibody titres ten to fourteen days after the onset of clinical symptoms. Consequently, the pathogen can not be detected during the incubation period [7,9]. Sachse et al.[7] reported that the authors developed an ELISA for *M. bovis* antibodies using whole-cell antigen of the agent for solid-phase coating. The assay has proved sufficiently specific and the sensitivity of detection (10^5-10^6 cfu/ml) was 100 times greater than

with other serological methods [7]. The critical problem in these methods is the serological cross reactions between *Mycoplasma* strains.

Comparison of the PCR and the ELISA results in this study showed that positive rates in PCR were less then the ELISA in all farms. The results can be explained as sampling: It must be taken into consideration with respiratory sampling that *M. bovis* can be better recovered from broncho-alveolar lavages than nasal swabs, although this method is much more diffucult [8]. All animals in these farms were treated with different antibiotics. PCR results could be affected negatively due to regular treatment with antibiotics at a high dosage (mycoplasma cell numbers in materials may be less then detectable limits). Antibodies to *M. bovis* persist for several months and can be detected easily with the ELISA.

Because of the lack of cell wall in *M. bovis*, certain groups of antibiotics do not effective [2]. These antibiotics are used to treat for the secondary bacterial infections but often ineffective to treat Mycoplasma infections [4]. Because of the difficulties of the treatment with antibiotics, vaccine is important for *M. bovis* infections. One experimental vaccine study in calf reported that a single dose of vaccine prepared from saponised *M. bovis* cell can provide effective control against mycoplasma induced calf pneumonia. Calves tested for 6 months after immunisation had high level of humoral immunity [4]. No vaccine is currently used against *M. bovis* infection in Turkey.

Considering these results, the ELISA was found to be more useful method than PCR to detect *M. bovis* infection because of the persistence of *M. bovis* antibodies especially in chronic infections and the results also induce a strong need for the effective vaccine development for Mycoplasma infections in Turkey.

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RESEARCH ARTICLE

Effects of Various Antioxidants on Cryopreserved Bull Sperm Quality [1] [2]

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Summary

The objective of this study was to assess the effects of antioxidant supplement (A), fetuin (F), aminoacid (AS) and cysteine (CY) on the sperm parameters, plasma membrane integrity, chromatin damage and antioxidant activities after freeze-thawing. Ejaculates were split into five aliquots and extended to a final concentration of $15x10^6$ spermatozoa/ml with the Tris base extender containing 0.5 ml A, 2 mg/ml F, 13% AS, 5 mM CY and no additive (C). The extended samples were equlibrated slowly to 4°C during 4 h and then frozen using a digital freezing machine.. Frozen straws were thawed individually in water bath at 37°C for 30 s to analyse progressive motility and sperm motion characteristics as well as membrane integrity. Biochemical assays were performed in a spectrophotometer using commercial kits. Chromatin damage was evaluated by Comet Assay. A, F, AS and CY did not show better result on the percentages of post-thaw sperm motilities. CY exhibited the greatest value of plasma membrane integrity ($P<0.05$). Total abnormalities were greater in C and F (17.5±0.57%; 15.5±1.98%, respectively; $P<0.05$). F had greater chromatin damage results ($P<0.05$). GPx activity was affected by type of antioxidant, notably CY yielded the lowest results when compared to the other groups ($P<0.05$). In conclusion, although using antioxidants does not have any influence on the sperm motility after thawing, A, AS and CY cause reduction at abnormal spermatozoa; CY exhibits the greatest cryoprotective activity on plasma membrane integrity and F caused an increase at chromatin damage.

Keywords: *Antioxidant activity, Bull sperm, DNA integrity, Oxidative stress, Sperm freezing*

Dondurulmuş Boğa Sperması Kalitesi Üzerine Değişik Antioksidanların Etkileri

Özet

Bu çalışmanın amacı, sperma sulandırıcısına ilave edilen antioksidan suplementi (A), fetuin (F), aminoasit (AS) ve sisteinin (CY) dondurma çözündürme sonrası spermatolojik parametreler, plazma membran bütünlüğü, kromatin hasarı ve antioksidan aktivite üzerine etkilerini değerlendirmekti. Ejakülatlar 5 eşit parçaya ayrıldı ve mililitrede $15x10^6$ spermatozoa olacak şekilde biri kontrol ve diğerleri 0.5 ml A, 2 mg/ml F, %13 AS ve 5 mM CY içeren Tris bazlı sperma sulandırıcısı ile sulandırıldı. Sulandırılan spermalar 4°C'de 4 saat süre ile ekülibre edildi ve otomatik sperma dondurma cihazı kullanılarak donduruldu. Dondurulan spermalar sıcak su banyosunda 37°C'de 30 saniye süre ile çözündürülerek plazma membran bütünlüğünün yanı sıra spermanın ileri yönlü hareketleri ve sperma hareket özellikleri değerlendirildi. Biyokimyasal analizler ticari kit kullanılarak spektrofotometrede yapıldı. Kromatin hasarı Comet Testi ile değerlendirildi. A, F, AS ve CY; ileri yönlü spermatozoa hareketi yönünden herhangi bir iyileştirici yönde sonuç göstermedi. Plazma membran bütünlüğü yönünden CY diğer gruplarla karşılaştırıldığında en yüksek değeri gösterdi ($P<0.05$). Toplam anormal spermatozoa oranı diğer gruplara oranla en yüksek C ve F (%17.5±0.57; %15.5±1.98, sırasıyla) gruplarında belirlendi ($P<0.05$). F en yüksek kromatin hasarını oluşturdu ($P<0.05$). GPx aktivitesi antioksidan tipinden etkilendi, diğer gruplarla karşılaştırıldığında özellikle CY en düşük sonuçları verdi ($P<0.05$). Sonuç olarak, kullanılan antioksidanlar dondurma çözündürme sonrası sperma motilitesi üzerine herhangi bir olumlu etki göstermemesine rağmen A, AS ve CY anormal spermatozoa oranının düşmesine neden oldu. CY plazma membran bütünlüğü yönünden en yüksek korumayı sağlarken, F kromatin hasarının artmasına neden oldu.

Anahtar sözcükler: *Antioksidan aktivite, Boğa sperması, DNA bütünlüğü, Oksidatif stres, Sperma dondurma*

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INTRODUCTION

Cryopreservation has been an invaluable technique for helping viability of spermatozoa [1,2]. However, the biggest obstacle in the preservation of semen is the occurence of lethal and sub-lethal damage on sperm structure during cryopreservation, causing poor fertility of preserved sperm [3]. Reactive oxygen species (ROS) have been generated by the cryopreservation processes. Spermatozoa have a high content of unsaturated fatty acids in their membranes, and have a lack of significant cytoplasmic component containing antioxidants [4]. Therefore, they are highly susceptible to oxidative injury and poorly equipped to fight ROS attack [5]. When ROS are produced excessively, they may display damaging effects on sperm motility [6,7], plasma membrane integrity [8], DNA integrity [9] and fertilizing capability [10].

Mammalian cells involve antioxidant systems to cope with oxidative stress and damage [11]. These antioxidant systems in sperm may be insufficient to prevent a decrease in motility and maintain sperm functions during freeze-thawing process [12]. Antioxidants have become increasingly important for the protection or management of oxidative stress and can be used as useful tools to protect from oxidative damage [6]. Cysteine (CY) is a ROS scavenger which stimulates glutathione synthesis and it prevents the loss of sperm functions during the freeze-thawing process [11]. Aminoacid (AS) is present at the extra-cellular level and improves sperm motility, acrosomal integrity and fertilizing capability after the freeze-thawing process [13,14]. Fetuin (F), which is a microheterogeneous protein, appears in fetal calf serum [15]. Several antioxidant agents have been tested *in vitro* and *in vivo* studies and there have been appeared beneficial effects [16,17] and contradictory results as well [18,19]. Most of these studies have suggested that further studies are required in order to obtain more concrete results.

Thus, the objective of this study was to assess the effects of antioxidant supplement (A), F, AS and CY on the sperm parameters, plasma membrane integrity, chromatin damage and antioxidant activities after freeze-thawing.

MATERIAL and METHODS

Animals and Semen Collection

Three Holstein bulls (3-4 years of age) with good quality semen characteristics (>80% forward progressive motility and concentrations of at least $1.0x10^9$ spermatozoa/ml) were selected to be the semen source. The bulls were clinically proven to be free from any general or genital diseases and were maintained at the Livestock Central Research Institute (Ankara, Turkey). Ejaculates were collected from the bulls with the aid of an artificial vagina twice a week. The ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the evaluated samples. The pooled semen sample was immersed in a water bath at 35.5°C until it could be assessed for total and progressive motility as well as sperm concentration. This study was replicated eight times for each group. The experimental procedures were approved by the Animal Care Committee of the Faculty of Veterinary Medicine, Istanbul University.

Semen Processing

The antioxidants used (antioxidant supplement A1345, fetuin from calf serum F2379, BME aminoacid solution B6766, L-Cysteine C-7352) were obtained from Sigma-Aldrich Chemical Co., USA. The total semen volume was determined from the graded collection tube soon after collection, and its concentration was determined using an Accucell photometer (IMV Technologie, L'Aigle, France). Progressive motility was evaluated subjectively using a phase contrast microscope (200x, Olympus BX43, Tokyo, Japan) at 37°C. A Tris-based extender (T) (30.7 g of Tris, 16.4 g of citric acid, 12.6 g of fructose, 20% v/v egg yolk, glycerol 6% (v/v) and 1000 ml of distilled water at a pH of 6.8) was used as the base for the experimental extenders. Pooled ejaculates were split into five aliquots and diluted to a final concentration of $15x10^6$ spermatozoa/ml with the base extender containing A (0.5 ml), F (2 mg/ml), AS (13%), CY (5 mM) and no additive (control; C). The extended samples were cooled slowly to 4°C and equilibrated for 4 h. They were then loaded into 0.25 ml French straws and frozen using a controlled rate freezer (Digitcool 5300 ZB 250, IMV, France) at 3 programmed rates: -3°C/min from +4°C to -10°C, -40°C/min from -10°C to -100°C, and -20°C/min from -100°C to -140°C. Thereafter, the straws were plunged into liquid nitrogen at -196°C.

Assessment of in vitro Sperm Quality

Subjective motility was assessed using a phase-contrast microscope (100x, Olympus BX43, Tokyo, Japan). A drop of semen was placed on a pre-warmed microscope slide and was subjectively assessed at 37°C for its percentage of progressive motility. In addition to estimating the subjective sperm motility, a computer-assisted sperm motility analysis program (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) was also used to analyse sperm motion characteristics. CASA was pre-adjusted for bovine sperm analysis. A semen sample was diluted 1:4 in Lactated Ringer solution, and the diluted semen sample was placed onto a pre-warmed 20 mm chamber slide (Leja 4, Leja Products BV, The Netherlands). The sperm motility characteristics were determined using a 10x objective microscope lens at 37°C. The following motility values were recorded: motility (%), progressive motility (%), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm/s), and beat cross frequency (BCF, Hz). A minimum of 10 microscopic fields

were analysed for each assessment, which included at least 300 cells. The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoal membranes. The HOS test was performed by incubating 30 µl of semen with 300 µl of a 100 mOsm hypo-osmotic solution at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spread on a warm slide with a cover slip and then was examined using a phase-contrast microscope (400x, Olympus BX43, Tokyo, Japan) [20]. The number of swollen spermatozoa out of 200 was counted; swelling is characterised by a coiled tail, indicating that the plasma membrane is intact. For the evaluation of sperm abnormalities, 10 µl of each sample was added to Eppendorf tubes containing 1 ml of Hancock solution [21]. One drop of this mixture was placed on a slide and covered with a cover slip. The percentages of acrosome, head, tail and total abnormalities out of 200 oil-immersed spermatozoa were determined using a phase-contrast microscope (1000x, Olympus BX43, Tokyo, Japan).

Assessment of Chromatin Damage

Sperm chromatin damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. Semen samples were immersed in water at 37°C for 30 s and then centrifuged at 600 g for 10 min at room temperature. The seminal plasma was removed, and the remaining sperm cells were washed with phosphate buffer solution (PBS; Ca^{2+} and Mg^{2+} free) twice to yield a concentration of $1x10^5$ spermatozoa/cm^3. Each microscope slide was pre-coated with a layer of 0.65% high melting-point agarose in distilled water and thoroughly dried at room temperature. Twenty-five µl of the sperm cell suspension were mixed with 75 µl of 0.65% low melting-point agarose at 50°C, and then a drop of the mixture was placed on a pre-coated slide and covered with a cover slip. The slides were allowed to solidify for 10 min at 4°C in a moist box. Then, the coverslips were removed, and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na^2–EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothreitol (pH 10) for 1 h at 4°C. The slides were then removed from the lysis buffer, drained, and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, which contained 300 mM NaOH and 1 mM EDTA (pH 13). The slides remained in the unit for 20 min to allow the DNA to unwind. Next, electrophoresis was performed at 25 V and an adjusted 300 mA for 20 min at room temperature. Subsequently, the slides were washed with a neutralising solution of 0.4 M Tris (pH 7.5) to remove the alkali and detergents. After neutralisation, the slides were stained with 65 µl of 20 µg/ml ethidium bromide and covered with a coverslip. All of these steps were conducted under dimmed lighting to prevent additional choromatin damage. The images of 100 randomly chosen nuclei were analysed using a fluorescent microscope at a magnification of 400 x (Zeiss, Germany). Nucleotide DNA extends under electrophoresis to form "comet tails," and the relative intensity of DNA in the tail reflects the frequency of DNA breakage. Thus, the percentage of the total DNA in the comet tail was taken as a direct measure of the DNA break frequency. Tail DNA (%) was assessed in 100 cells using the Comet Assay III image analysis system (Perceptive Instruments, UK). The analysis was performed blindly by one slide reader [22].

Biochemical Assays

Semen samples were centrifuged at 4°C and 1000 g for 15 min to separate out the spermatozoa. The pellet was washed 3 times using 0.5 ml of PBS. This final solution was homogenised 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 µl of the homogenate was mixed with 10 µl of 0.5 mM butyl hydroxyl toluene (BHT) and stored at -80°C until analysed. The rest of the homogenate was centrifuged at 8000 g for 15 min, and the supernatant was separated and stored at -80°C for a different enzyme analysis [22]. Enzyme levels were determined using commercial kits by spectrophotometry (Cintra 303-UV, GBC, Australia). Biochemical assay kits were obtained from Sigma-Aldrich Chemical (Interlab Ltd., Ankara, Turkey)

Statistical Analysis

Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared, using the SPSS/PC computer programme (version 14.1, Chicago, IL). The test revealed that the variances were homogeneous. After that, comparisons between the groups were made using analysis of variance with Duncan post hoc test. The results are expressed as means or proportions (±S.D.). The differences were considered significant at $P<0.05$.

RESULTS

As shown in *Table 1*, using A, F, AS and CY as antioxidants did not give better results on the percentages of sperm motility assessed subjectively or by CASA after thawing. Spermatozoa frozen in which containing CY exhibited the greatest value of VAP (120.5±1.97 µm/s), VCL (207.4±3.32 µm/s) and plasma membrane integrity (48.1±0.79%) compared to other groups ($P<0.05$). Although there were no significance differences in the percentages of acrosome abnormalities among treatment groups ($P>0.05$), total abnormalities were greater in C and F (17.5±0.57%; 15.5±1.98%, respectively) than the other groups ($P<0.05$).

As shown in *Table 2*, chromatin damage depending on the type of antioxidant; F caused greater chromatin damage than the other groups ($P<0.05$).

As shown in *Table 3*, as regards to antioxidant activity; although there were no significant differences in the GSH, CAT and total antioxidant activities, GPx activity was

Table 1. *Mean (±SE) sperm values in frozen thawed bull semen*
Tablo 1. *Boğa spermasının dondurma çözündürme sonrası ortalama spermatolojik değerleri*

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2 mg/ml)	Aminoacids (13%)	Cysteine (5 mM)	P
Subjective motility (%)	51.9±3.89	58.8±4.51	58.1±3.53	57.5±4.43	52.5±5.00	-
CASA motility (%)	48.3±4.34	53.1±5.56	50.8±6.59	49.4±4.52	53.3±5.03	-
Progressive motility (%)	20.8±2.40	22.4±3.79	21.4±3.55	20.5±2.43	18.1±3.73	-
VAP (µm/s)	100.8±1.19^{a}	102.6±1.78^{a}	100.6±2.05^{a}	102.0±1.86^{a}	120.5±1.97^{b}	*
VSL (µm/s)	77.1±1.16	78.6±1.16	79.5±1.30	79.5±1.19	81.5±1.87	-
VCL (µm/s)	176.0±1.79^{a}	178.5±4.70^{a}	169.5±5.23^{a}	175.6±3.81^{a}	207.4±3.32^{b}	*
ALH (µm/s)	7.9±0.12	7.9±0.24	7.6±0.31	7.7±0.51	8.5±0.24	-
BCF (Hz)	16.3±0.35^{a}	16.0±0.62^{a}	17.2±1.07^{a}	17.3±0.55^{a}	13.7±0.50^{b}	*
HOS T (%)	41.1±0.40^{a}	44.5±1.04^{b}	42.5±0.80^{ab}	41.6±0.53^{a}	48.1±0.79^{c}	*
Acrosome abnormalities (%)	5.8±0.45	4.3±0.75	4.3±0.59	3.9±0.61	3.4±0.53	-
Total abnormalities (%)	17.5±0.57^{a}	12.3±1.69^{bc}	15.5±1.98^{ab}	13.3±0.88^{bc}	9.8±0.80^{c}	*

^{a,b,c} *Different superscripts within the same row demonstrate significant differences (*P<0.05),* - *No significant difference (P>0.05)*

Table 2. *Mean (±SE) chromatin damage values in frozen thawed bull semen*
Tablo 2. *Boğa spermasında dondurma çözündürme sonrası oluşan ortalama kromatin hasarları*

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2 mg/ml)	Aminoacids (13%)	Cysteine (5 mM)	P
Tail intensity (%)	10.7±0.49^{a}	9.7±0.66^{a}	14.6±1.34^{b}	10.6±0.83^{a}	9.6±0.41^{a}	*
Tail moment (µm/s)	3.9±0.25^{a}	4.1±0.18^{a}	7.4±1.21^{b}	4.0±0.16^{a}	2.42±0.23^{a}	*

^{a,b,c} *Different superscripts within the same row demonstrate significant differences (*P<0.05),* - *No significant difference (P>0.05)*

Table 3 *Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioxidant activities in frozen thawed bull semen*
Tablo 3. *Boğa spermasında dondurma çözündürme sonrası ortalama glutatyon peroksidaz (GPx), lipit peroksidaz (LPO), redükte glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2 mg/ml)	Aminoacids (13%)	Cysteine (5 mM)	P
GPx (mU/ml-10^{9} cell/ml)	14.9±0.64^{c}	14.8±0.18^{c}	15.4±0.53^{c}	12.8±0.83^{b}	10.8±0.62^{a}	*
LPO (µm/ml-10^{9} cell/ml)	0.5±0.27	0.7±0.23	0.5±0.29	0.6±0.28	0.44±0.28	-
GSH (µm/ml-10^{9} cell/ml)	37.7±10.86	37.5±10.16	27.1±9.23	27.2±7.50	20.7±1.46	-
CAT (µm/ml-10^{9} cell/ml)	18.9±4.94	17.5±4.91	7.7±1.35	13.6±2.49	15.7±4.73	-
Total antioxidant activities (mmol/trolox/ml-10^{9} cell/ml)	15.2±3.30	14.2±3.27	7.7±0.90	11.6±1.66	13.0±3.15	-

^{a,b,c} *Different superscripts within the same row demonstrate significant differences (*P<0.05),* - *No significant difference (P>0.05)*

affected by the type of antioxidant, notably CY yielded the lowest results in comparison with the other groups (P<0.05).

DISCUSSION

The axoneme and associated dense fibers of the mid-pieces in sperm, which are responsible from the motility, are covered by mitochondria that generate energy by oxidative phosphorylation [23,24]. Large amounts of ROS can impair the sperm motility [9,25]. This study showed that using antioxidants do not give better results on the sperm's motility after thawing. However, their abnormal spermatozoa rates are lower than C except F. Spermatozoa in T containing CY exhibited the greatest percentages of plasma membrane integrity. Consistent with our study, Tuncer et al.[26] demonstrated that adding antioxidant to freeze the spermatozoa do not have any marked effects on the subjective and CASA motilities. Bucak et al.[18], reported that adding antioxidant to freeze the spermatozoa has positive effect on plasma membrane integrity but this situation does not prevent ROS formation and has no effect on total antioxidant activity. It has been suggested that using of glutathione and cysteine may improve the spermatozoa viability and functional integrity [17]. In contrast with our findings, it has been proposed that the antioxidants can be used successfully to improve boar [27], dog [28], goat [29] and rainbow trout [30] sperm motility. This study also contradicts the previous bull sperm study which cysteine (2.5 mM) was supplemented. Sperm motility and

acrosomal integrity rates were increased but these results did have no effects on fertility rates [31]. Bucak et al.[16] showed that bull sperm samples in which 7.5 mM carnitine and 7.5 mM inositol had been added, caused an increase on the spermatozoa motility. Their subjective motilities (61.9±1.3%; 51.3±1.6%) were similar, but CASA (41.6±2.9%; 34.9±2.0%) and progressive motilities (12.8±1.4%; 13.3±1.5%) were lower than those obtained in our study. Parallel to our findings, it was revealed that bull sperm samples with addition of cysteine (5 and 10 mM) and GSH (0.5 and 2 mM) did not have any further improvement on GPx activity and motility [19]. This study's progressive and CASA motilities were also similar with our findings, but plasma membrane integrity was greater and abnormal spermatozoa rate was lower than the values obtained in our study. Based on our results, we can hypothesize that the difference between the findings can be related with using different antioxidants or different doses of the same antioxidants or different antioxidant capacity of spermatozoa in the testing stage.

The excessive ROS production causes a damage on plasma membrane and DNA integrity of spermatozoa [9,32]. Chromatin damage, which is formed after dilution, freezing and thawing of sperm, can be prevented with the addition of antioxidants [33]. Chromatin damage on spermatozoa effects potantial embryo growing negatively [34] by reducing the fertilization rate [35]. In contrast to the results obtained, it has been shown that antioxidants added to sperm extender reduce the chromatin damage [36]. However, the antioxidants that we have used in our study did not provide any marked improvement at DNA integrity and F usage caused an increase in chromatin damage. These contradictory results can be hypothesized that chromatin damage may be related not only with oxidative damages but also with osmotic damages too.

Spermatozoa and seminal plasma involves ROS scavengers, including the enzymes such as SOD, GPx, and CAT [4,37]. In this study, it is stated that using antioxidant has no effect on the antioxidant activity and does not cause further improvement on motility values. Similar with our findings, Kasimanickam et al.[10] reported that there is no relationship between the antioxidant activity (GPx, LPO, SOD), DNA integrity and plasma membrane integrity. In a study on the sperm of ram and goat, it is found that using antioxidants does not have positive effect on LPO, GSH and GPx activities [18,38]. Different from our findings, it has been reported that GSH activity is decreased 80% while freezing and thawing of the bull sperm [6]. In another study, it is also reported that this potential reduction of GSH was originated from oxidative stress and the deterioration of the plasma membrane integrity, and accordingly this situation effects motility and viability [39]. It is proposed that cysteine (5 mM) added to sperm extender has a positive effect on endogenous antioxidant system and increases the GPx activity but does not reduce MDA levels. Increased GPx activity does not have any positive effects on sperm values [19]. Our findings indicate that changes in extender and its composition, animal species or breeds explain why antioxidant supplementation do not improve the sperm motility while some of them have effects on abnormalities and plasma membrane integrity positively.

In conclusion, although using antioxidants does not have any influence on the sperm motility after thawing, A, AS and CY cause reduction at abnormal spermatozoa; CY exhibits the greatest cryoprotective activity on plasma membrane integrity and F caused an increase at chromatin damage.

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RESEARCH ARTICLE

Estimating *In Situ* Effective Crude Protein Degradability with Cornell Net Carbohydrate and Protein System Parameters in Energy-Rich Feedstuffs for Ruminants [1]

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Summary

The objective of this experiment was to estimate *in situ* effective crude protein degradability (EPD) with Cornell Net Carbohydrate and Protein System (CNCPS) parameters [crude protein fractions (A, B_1, B_2, B_3 and C) and degradable protein intake value (DIP) values] of six energy-rich feedstuffs. Four cereals: maize, wheat, barley, rye and two wheat middling (WM-1 and WM-2) were tested. The *in situ* effective protein degradability (EPD) was calculated using the nylon bag method where the test feedstuffs incubated in the rumen of three Tahirova wethers. The EPD's were estimated as EPD2, EPD5 and EPD8 assuming rumen outflow rates of 0.02, 0.05 and 0.08 h^{-1}. The crude protein fractions *i.e.* A=NPN, B_1=fast, B_2=intermediate, B_3=slow and C=not fermented and unavailable to the animal were calculated using the soluble protein (SolP), the non-protein nitrogen (NPN, % of SolP), the neutral detergent insoluble protein (NDIP) and the acid detergent insoluble protein (ADIP=C) values of feedstuffs based on CNCPS. Then, DIP was calculated by using CNCPS crude protein fractions, degradation rate of B fractions (Kd) and coefficients of outflow rate on the different levels of dry matter intake (Kp): (DIP_{1X}=at 1x maintenance level of intake, DIP_{2x}=at 2x maintenance level of intake, and DIP_{3x}=at 3x maintenance level of intake). It was found that there was a significant multiple regression relation between the EPD8 (g/kg DM) and crude protein fractions (g/kg DM) (R^2=0.96, n=18, P<0.001), and simple regression relation between the EPD8 (g/kg DM) and DIP_{3X} (g/kg DM) (R^2=0.98, n=18, P<0.001). These regression relations did not improve when the different rumen outflow rates were used to estimate EPD. In conclusion, we claimed that *in situ* effective protein degradation (EPD) can be reliably and accurately predicted from CP fractions and DIP values in cereals and wheat middling.

Keywords: *Nylon bag method, CNCPS parameters, Protein degradation, Energy-rich feedstuffs*

Ruminantlarda Enerjice Zengin Yem Hammaddelerin *In Situ* Etkin Ham Protein Yıkımlanabilirliklerinin Cornell Net Karbonhidrat ve Protein Sistemi Kullanılarak Tahmin Edilmesi

Özet

Bu çalışma, enerjice zengin altı adet yem hammaddesinin ruminantlarda *in situ* etkin ham protein yıkımlanabilirliklerinin (EPD), Cornell Net Karbonhidrat and Protein Sistemi (CNCPS) parametreleri [ham protein fraksiyonları (A, B_1, B_2, B_3 and C) ve tüketilen parçalanabilir protein (DIP)] kullanılarak belirlenmesi amacıyla yapılmıştır. Çalışmanın yem materyali mısır, buğday, arpa, çavdar ile iki farklı buğday kepeğinden (WM-1 ve WM-2) oluşturulmuştur. Yem hammaddelerinin *in situ* etkin ham protein yıkımlanabilirlikleri (EPD), üç adet Tahirova koçu kullanılarak nylon kese tekniği ile belirlenmiştir. EPD değerleri (EPD2, EPD5 and EPD8) 0.02, 0.05 ve 0.08 s^{-1} rumenden geçiş hızı katsayılarında hesaplanmıştır. Ham protein fraksiyonları A=NPN, B_1=Hızlı, B_2=Orta, B_3=Yavaş ve C=yararlanılamayan protein, CNCPS ile tahıllar ve buğday kepeklerinin çözünebilir protein (SolP), protein tabiatında olmayan nitrojen (NPN, SolP'de %), nötral deterjanda çözünmeyen protein (NDIP) ve asit deterjanda çözünmeyen protein (ADIP=C) değerleri kullanılarak hesaplanmıştır. Daha sonra, DIP değerleri ham protein fraksiyonları, B fraksiyonlarının rumende parçalanma hızı katsayıları (Kd) ve farklı kurumadde tüketim düzeylerindeki rumenden geçiş hızı katsayıları kullanılarak hesaplanmıştır (DIP_{1X}=yaşama payı düzeyinde besleme, DIP_{2x}=yaşama payı düzeyinin iki katında besleme ve DIP_{3x}=yaşama payı düzeyinin üç katında besleme). Bulgular, EPD8 (g/kg KM) ve ham protein fraksiyonları (g/kg KM) (R^2=0.96, n=18, P<0.001) ile EPD8 (g/kg KM) ve DIP_{3X} değerleri (g/kg KM) (R^2=0.98, n=18, P<0.001) arasında önemli derecede regresyon ilişkileri olduğunu göstermiştir. Bu regresyon ilişkileri, EPD değerlerini tahminlemek için farklı rumen geçiş hızı katsayıları kullanıldığında geliştirilememiştir. Sonuç olarak, tahıllar ve buğday kepeklerinin *in situ* EPD değerlerinin ham protein fraksiyonları (A, B_1, B_2, B_3 and C) ve tüketilen parçalanabilir protein (DIP) değerleri ile tahmin edilebileceği ileri sürülebilir.

Anahtar sözcükler: *Naylon kese tekniği, CNCPS parametreleri, Protein yıkımlanabilirliği, Enerjice-zengin yem hammaddeleri*

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INTRODUCTION

The mathematical models include *in vivo, in situ* and *in vitro* methods have been used to determine the ruminal protein digestibility of the feedstuff [1-4]. Although, *in vivo* method is the most proper method for these mathematical models, surgicial preparation for animals with duedonal and rumen cannula and suitable markers for calculating flow rate of digesta make it risky, labour-intensive and expensive [5]. *In situ* Nylon Bag Method (NBM) is the most widely used research approach for measuring ruminal CP degradation [6]. This method also requires rumen cannulated animals, but it is relatively simple compared to *in vivo* method [1,7]. The CNCPS estimates the degradable proteins of the feedstuff using five CP fractions based on solubility in protein precipitant agents, buffer and detergent solutions: A represents the soluble non-protein nitrogen (NPN), B_1 (soluble true protein) is the albumins and globulins, B_2 is the most albumins and glutelins, B_3 is the prolamins, extension proteins and heat denatured proteins, and C is the unavailable N (N bound to lignin) [8-10]. These protein fractions present in each feedstuff were important factors influencing N solubility [11]. So far, no single methods has been accepted as being reliably accurate for predicting the rumen CP degradation. In recent years, the CNCPS as an alternative for estimation of degradable protein has become widely accepted in the studies [12-14], because CNCPS can be applied at the farm level and CP fractions could be measured easily in most feed analyses laboratories [15]. Morever, some researchers stated that *in situ* rumen degradability may be reliably and accurately predicted from CNCPS parameters [1,16,17]. Energy-rich feedstuffs are sources of rumen degradable protein and glutelin levels (B_2 fraction) are generaly high in cereals. In additon, there are no enough studies to compare the CP degradabilities of cereals and wheat middling in Turkey.

The objective of the this experiment was to estimate *in situ* effective protein degradability (EPD) with Cornell Net Carbohydrate and Protein System (CNCPS) parameters [crude protein fractions (A, B_1, B_2, B_3 and C) and degradable protein intake value (DIP)] in four cereals and two wheat middling offered to ruminant animals in Turkey.

MATERIAL and METHODS

Four cereals: maize, wheat, barley, rye and two wheat middling (WM-1 and WM-2) with three replicates were collected from feed factories in Western Anatolia Region. The chemical compositions: dry matter (DM), crude ash (CA), crude protein (CP) and ether extract (EE) were determined by Weende analyses method [18]. Ankom Fiber Analyzer (Ankom 200, Ankom Technology, Fairport NY) was used to determine neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses [19]. NDF analyses were carried out as alpha amylase pre-treated on test feedstuffs. All chemical analyses were carried out at least in dublicate. The chemical compositions are shown in *Table 1*. This study was approved by the internal ethical committee of Ege University (Approval no: 2002/06).

In Situ Nylon Bag Method

Three mature Tahirova wethers (average 50 kg body weighed) fitted with a rumen cannula (40 mm diameter) were used. The vaccination and parasite applications were performed based on veterinary recommendations. The wethers fed twice daily at 9:00 AM and 16:00 PM with 60% alfalfa hay and 40% concentrate with the 1.25 x of maintenance requirements. The alfalfa hay contained 145.0 g kg^{-1} of CP and 8.00 MJ kg^{-1} of metabolisable energy (ME), the concentrate contained 150.0 g kg^{-1} of CP and 11.50 MJ kg^{-1} of ME. Vitamin-mineral composition of concentrate consists of following: Vitamin A 7000 U/kg, Vitamin D_3 700 U/kg, Vitamin E 25 mg/kg, Ca 1.1%, P 0.4% and Na 0.25%. The animals were kept individually and had free access to fresh water. The CP degradability was determined according to the method of Bhargava and Orskov [20] using the nylon bag 9x14 cm in size with pore diameter of 40 µm. The feedstuffs were grinded using 2.5 mm sieve, weighed 5-6 g, and then incubated in the rumen for periods 4, 8, 16, 24, 48 h. The 72 h incubation

Table 1. *Chemical compositions of cereals and wheat middling (based on g/kg DM)*
Tablo 1. *Tahıllar ve buğday kepeklerinin kimyasal kompozisyonları (g/kg KM)*

Chemical Composition	Maize	Wheat	Barley	Rye	WM-1	WM-2	SE (±)
DM, g/kg	890.0	895.0	901.1	896.5	887.2	889.2	2.4
CA	14.9	17.7	27.5	21.5	61.5	49.8	1.4
CP	100.5^{c}	116.9^{c}	114.5^{c}	141.4^{b}	158.8^{b}	183.8^{a}	5.7
EE	38.7	15.3	22.8	19.6	32.2	44.4	2.5
NDF	134.9	245.0	329.6	329.6	457.5	401.6	27.7
NFC	711.0	605.1	505.6	487.9	290.0	320.4	28.1
ADF	32.5	39.0	60.4	49.8	153.4	126.3	4.6

Wheat middling (WM-1 and WM-2), ***DM:*** *Dry matter,* ***CA:*** *Crude ash,* ***CP:*** *Crude protein,* ***EE:*** *Ether extract,* ***NDF:*** *Neutral detergent fiber (alpha amylase pre-treated),* ***NFC:*** *Soluble carbohydrates in neutral detergent solution (1000 - CA - CP - EE - NDF),* ***ADF:*** *Acid detergent fiber, Different letters (a b, c) in the same row are statistically different for CP (P<0.05), SE, Standard error of mean*

period was only used with wheat middling. After removal from the rumen, the bags were rinsed in cold tap water. The washing losses were determined by measuring one hour incubation in 39°C water. Then, all bags were washed for 10 min in a washing machine, dried at 55-60°C for 48 h and weighed. Finally, the residues in the bags were used to determine CP degradability. Each feedstuff was tested using three animals with the three replicates (three bags per wethers). The CP degradability was determined by "$p=a+b(1-e^{-ct})$" model using Neway package program with the washing loss [21]. The (p) is the CP degradability at time t, a is the fraction of CP immediately soluble protein, b is the fraction of CP insoluble but degradable in the rumen, c is the rate constant of degradability of fraction b and t is the time of incubation on the model. The effective protein degradabilities (EPD2, EPD5 and EPD8) were calculated by "EPD=a+(bxc/c+k)" model. The (k) is the rumen outflow rates of 0.02, 0.05 and 0.08 h^{-1} on the model, which is representative for low, medium and high feeding levels, respectively [21].

The Cornell Net Carbohydrate and Protein System Parameters

The SolP, NPN (% of SolP), neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP) were determined standardized method of Licitra et al.[22]. The feedstuffs were grinded using 1 mm sieve. NDIP and ADIP were determined by filtering NDF and ADF residue on filter paper followed by Kjeldhal Method [18]. The CP fractions fractioned as a non-protein nitrogen (A fraction) and as true proteins (B and C fractions) [8,11]. Fraction A is rapidly degraded in the rumen. Fraction B can be divided into three subfractions (B_1, B_2 and B_3) based on the rate of ruminal degradation. Fraction B_1 is soluble true protein. The A + B_1 fractions generate the total soluble proteins (SolP). Total SolP was determined as the proportion of CP that is soluble in borate-phosphate buffer (pH = 6.7-6.8). Sodium azide solution was used to control microbial growth. The sample was filtered through Whatman#54 filter paper using several washes of buffer and the residue plus paper transferred into Kjeldhal tube for the estimation of N in residue. Tungstic acid was used as precipitating agent to determine Fraction A. B_2 is degraded in the rumen intermediate level. B_3 is the fraction with the slowest degradation rate. Fraction C (acid detergent insoluble protein = ADIP) is not fermented and unavailable to the animal. The following equations were used to calculate the CP fractions of feedstuff: A (% of CP) = SolP (% of CP) x (NPN (% of SolP)/100); B_1 (% of CP) = (SolP (% of CP) - A (% of CP)); C (% of CP) = ADIP (% of CP); B_3(% of CP) = (NDIP(% of CP) - ADIP(% of CP)); B_2 (% of CP) = (100 - Fractions (A+B_1+B_3+C)) (% of CP).

Degradable intake protein (DIP) was calculated by using the following equations: RDPA (% of CP) : rumen soluble protein, A fraction (NPN); $RDPB_1$ (% of CP): (B_1 x (Kd_{1x}/Kd_{1x} + Kp_{B1})) B_1 fraction (fast soluble protein); $RDPB_2$ (% of CP) : (B_2 x (Kd_{1x}/Kd_{1x} + Kp_{B2})) B_2 fraction (intermediate degradable protein); $RDPB_3$ (% of CP): (B_3 x (Kd_{1x}/Kd_{1x} + Kp_{B3})) B_3 fraction (slow degradable protein); RDP_{TOTAL} (% of CP) = RDPA +$RDPB_1$ + $RDPB_2$ + $RDPB_3$. RDP_{TOTAL} = DIP_{1X} (Degradable intake protein) according to dry matter intake fed at 1x maintenance level). In these calculations (DIP_{1X} = at 1x maintenance level of intake DIP_{2x} =at 2x maintenance level of intake, and DIP_{3x}=at 3x maintenance level of intake), the values stated in Fox et al.[8] and Sniffen et al.[11] were used for the degradation rate of B fractions (Kd) and the coefficients of outflow rate on the different levels of dry matter intake (Kp), respectively.

Statistical Analyses

The general linear model procedure of statistical package SPSS® was used one-way ANOVA on results [23]. The Duncan test was used to compare the means, when significant differences observed. Stepwise simple and multiple linear regressions were used to predict *in situ* EPD from CP fractions (A, B_1, B_2, B_3 and C) and DIP value based on CNCPS.

RESULTS

In Situ Effective Protein Degradability (EPD) Values

In situ CP degradability with the incubation time were ranged between 22.77-95.99% in cereals for 0-48 h, 50.15-92.81% in wheat middling for 0-72 h. The degradation parameters (a, b, c) and all EPD values were significantly affected by the feedstuffs *(Table 2)*. The WM-2 and rye had the highest EPD2 ($P<0.05$), while EPD2 values of WM-1, wheat and barley were similar. Maize had significantly the lowest EPD2 values. EPD5 and EPD8 had the same pattern that WM-2 had the highest in compare the others. Rye and WM-1 values of EPD5 and EPD8 were similar and higher than wheat and barley values. While wheat and barley were similar, maize had the lowest EPD5 and EPD8 values ($P<0.05$).

The Crude Protein Fractions and Degradable Intake Protein Values

CNCPS parameters of cereals and wheat middling were significanlty different *(Table 3)*. DIP values decreased in accordance with the increased feeding level of dry matter intake (1x, 2x and 3x). The A fraction results were following trend from the highest to lowest WM-2, WM-1 and wheat which were differ significantly. The rye, barley and maize had the similar A fractions, being the lowest one. Rye had the highest B_1 fraction compare to the others, in consequence, WM-2 and barley had the similar values and significantly higher than wheat. There was no significant differences between the wheat, WM-1, however, only wheat was significanlty higher than maize. B_2 fraction results showed no significant differences among maize, barley and wheat. At the same time, only wheat and barley were not significantly higher than WM-1. B_2 fraction of rye

Table 2. *In situ crude protein degradation characteristics of cereals and wheat middling (% of CP)*
Tablo 2. *Tahıllar ve buğday kepeklerinin in situ ham protein yıkımlanabilirlik özellikleri (HP'de, %)*

Degradation Characteristics	Maize	Wheat	Barley	Rye	WM-1	WM-2	SE(±)
Degradation parameters							
a	15.31^{d}	37.16^{cd}	29.84^{d}	48.45^{ab}	44.17^{bc}	53.79^{a}	2.63
b	54.93^{b}	58.93^{b}	67.17^{a}	46.03^{c}	46.46^{c}	38.74^{c}	2.65
c, h^{-1}	0.0588^{c}	0.0943^{b}	0.0860^{bc}	0.0999^{b}	0.1365^{a}	0.1486^{a}	0.012
RSD	1.47	1.27	1.50	1.56	1.64	1.04	0.14
Effective protein degradability							
EPD2	56.04^{c}	84.16^{b}	84.05^{b}	86.77^{a}	84.32^{b}	87.72^{a}	0.75
EPD5	44.71^{d}	72.48^{c}	71.99^{c}	79.11^{b}	77.67^{b}	82.51^{a}	1.07
EPD8	38.34^{d}	65.16^{c}	64.36^{c}	74.01^{b}	72.99^{b}	78.74^{a}	1.27

Wheat middling (WM-1 and WM-2), ***Degradation parameters:*** *a an intercept representing the proportion of CP solubilized at initiation of incubation time (soluble fraction), b the fraction of CP insoluble but degradable in the rumen, c the rate constant of degradability of fraction b,* ***RSD:*** *Residual standard deviation of equation, effective protein degradability (EPD) at rumen outflow rate k = 0.02, 0.05, and 0.08 h^{-1}. Different letters (a,b,c) in the same row are statistically different (P<0.05), SE, Standard error of mean*

Table 3. *CNCPS parameters of cereals and wheat middling (% of CP)*
Tablo 3. *Tahıllar ve buğday kepeklerinin CNCPS parametreleri, (HP'de %)*

CNCPS Parameters	Maize	Wheat	Barley	Rye	WM-1	WM-2	SE (±)
SolP	13.46^{d}	31.48^{bc}	25.74^{c}	55.53^{a}	35.43^{b}	54.12^{a}	2.91
NPN (% of SolP)	67.98^{a}	64.80^{a}	36.48^{b}	18.35^{c}	74.85^{a}	66.09^{a}	2.56
NDIP	15.74^{a}	5.70^{c}	9.97^{b}	9.08^{bc}	8.74^{bc}	7.05^{bc}	1.15
Crude protein fractions							
A = NPN	9.15^{d}	20.40^{c}	9.39^{d}	10.19^{d}	26.52^{b}	35.77^{a}	1.81
B_1	4.31^{d}	11.08^{c}	16.35^{b}	45.34^{a}	8.91^{cd}	18.35^{b}	1.58
B_2	70.80^{a}	62.82^{ab}	64.29^{ab}	35.39^{c}	55.83^{b}	38.83^{c}	3.19
B_3	13.76^{a}	3.77^{bc}	6.87^{b}	4.20^{bc}	4.89^{bc}	2.65^{c}	1.16
C (ADIP)	1.98^{c}	1.93^{c}	3.10^{bc}	4.88^{a}	3.85^{ab}	4.40^{ab}	0.51
Degradable intake protein							
DIP 1x	63.89^{d}	83.84^{b}	79.65^{c}	84.95^{ab}	83.94^{b}	87.65^{a}	0.97
DIP 2x	61.01^{d}	82.02^{b}	77.73^{c}	83.83^{ab}	82.15^{b}	86.40^{a}	0.98
DIP 3x	58.45^{d}	80.33^{b}	75.94^{c}	82.78^{ab}	80.50^{b}	85.24^{a}	1.00

Wheat middling (WM-1 and WM-2), ***SolP:*** *Soluble protein,* ***NPN:*** *non-protein nitrogen (% of SolP),* ***NDIP:*** *Neutral detergent insoluble protein, A fraction* ***(NPN):*** *non-protein nitrogen,* ***B_1:*** *fast soluble true protein,* ***B_2:*** *intermediate degradable protein,* ***B_3:*** *slow degradable protein,* ***ADIP (C):*** *acid detergent insoluble protein,* ***DIP:*** *Degradable intake protein fed at 1x maintenance level, at 2x maintenance level of intake, and at 3x maintenance level of intake, Different letters (a,b,c) in the same row are statistically different (P<0.05),* ***SE:*** *Standard error of mean*

Table 4. *The regression equations to predict in situ EPD values by using CNCPS parameters of cereals and wheat middling (n=18) according to level of feeding (g/kg DM)*
Tablo 4. *Tahıllar ve buğday kepeklerinin (n=18) yemleme düzeylerine göre in situ EPD değerlerini tahmin etmede kullanılan CNCPS parametreleri (g/kg KM)*

Regression Equations	R^2	SE (±)
EPD2 = - 25.495 + 1.035 A + 1.244 B_1+ 1.137 B_2 – 0.414 B_3 – 0.004 C	0.96	8.00
EPD5 = - 37.228 + 1.085 A + 1.259 B_1+ 1.112 B_2 – 0.329 B_3 – 0.049 C	0.96	8.08
EPD8 = - 42.987 + 1.098 A + 1.250 B_1+ 1.080 B_2 – 0.266 B_3 – 0.053 C	0.96	8.09
EPD2 = - 4.774 + 1.048 DIP1x	0.98	5.24
EPD5 = - 16.011 + 1.069 DIP2x	0.98	5.19
EPD8 = - 20.876 + 1.067 DIP3x	0.98	5.36

R^2: *Determination coefficient,* ***SE:*** *Standard error of the estimate, P<0.001 for each equation*

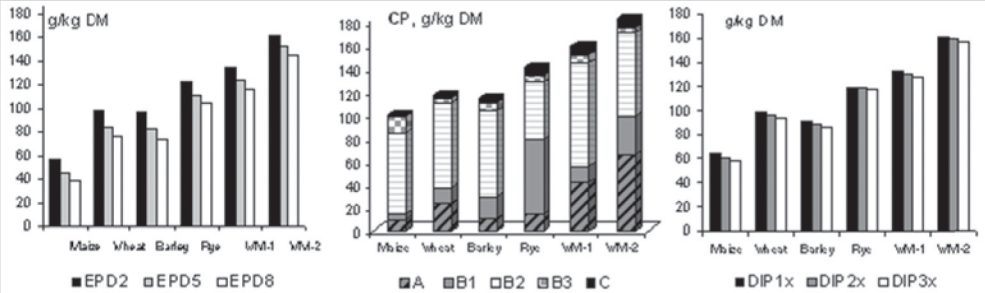

Fig 1. Effective protein degradability (EPD) and CNCPS parameters of cereals and wheat middling (g/kg DM)
Şekil 1. Tahıllar ve buğday kepeklerinin etkin protein yıkımlanabilirlik (EPD) ve CNCPS parametreleri (g/kg KM)

was similar to WM-2 and the lowest one compared to the others (P<0.05), while maize had the highest value. No significant differences were observed for barley, WM-1, wheat and rye in B_3 fractions, while barley was significanlty higher than WM-2. B_3 fraction of maize had the highest value compared to the others (P<0.05). In C fraction, there was no significant differences between rye, WM-2 and WM-1, however, rye was significanlty higher than barley, but not WM-1 and WM-2. The C fractions of maize and wheat had no significant differences with barley, but they were lower than the others (P<0.05). The results of DIP1x, DIP2x and DIP3x showed that there were no significant differences found between WM-2 and rye, while WM-2 was significanlty higher than WM-1 and wheat. The barley and maize were different each other, being maize had the lowest value (P<0.05).

The Prediction of Effective Protein Degradability (EPD) Values

The EPD2, EPD5 and EPD8 were predicted by using A,B_1,B_2,B_3,C and DIP1x, DIP2x, DIP3x values and these values were shown in *Fig. 1*. The regression equations to predict EPD's were shown in *Table 4*. The regression analysis indicated that there was significant multiple regression relationship between EPD values and CP fractions (A, B_1, B_2, B_3 and C) (R^2=0.96, n=18, P<0.001). And also, simple regression relationship is found to predict *in situ* EPD values from DIP values according to level of feeding (R^2=0.98, n=18, P<0.001).

DISCUSSION

The chemical compositions of energy-rich feedstuffs were varied widely *(Table 1)*, because the chemical compositions of feedstuffs are affected by soil type, fertilizing, climate and processing to by-product. The WM-2 had the highest CP in compared the cereals and WM-1. There were no significant differences between rye and WM-1, even they were higher than barley, wheat and maize (P<0.05). Van Soest [2] and Mc Donald et al.[24] stated similar values for cereals and wheat middling, except rye had higher content of CP (124-138 g/kg DM) compare to available literature.

Effective Protein Degradability (EPD)

The (a) (29.61%) and (b) (63.22%) parameters of barley were close to the our result in Woods et al.[25]. In consistent with Batajoo and Shaver [26], maize (0.041 h^{-1}) had the lowest, while WM (0.1710 h^{-1}) had the highest (c) parameter in our study. In comparison to our result for wheat and barley, Herrera-Saldana et al.[27] showed that the (c) parameter was lower in wheat (0.2536 h^{-1}) and barley (0.1778 h^{-1}). This difference could be attributed to microbial contamination of the feed residues as it stated in Varvikko and Lindberg [28], when estimating *in situ* degradability of CP in starchy feedstuffs. Also, microbial population inside the bag is restricted compared to normal digestion, thus *in situ* protein degradation rates could be lower than actual *in vivo* rates [3]. As the outflow rates (k) increased from rumen to abomasum (i.e from k=0.02-0.08 h^{-1}), the EPD values decreased *(Table 2)*. EPD values were similar in Cömert and Şayan [29] that maize's being lower than other feeds, higher in wheat middling than for other feedstuffs. It appears that the (c) parameter is important to determine the EPD values of any feedstuffs, because (c) parameter and EPD value were sorted to be the same for maize and wheat middling.

The Crude Protein Fractions and Degradable Intake Protein

The CP fractions and DIP values varied widely among feedstuffs *(Table 3)*, because CP fractions were affected by different protein structure in feedstuffs and processing to by-product. CNCPS parameters of study were compared with the values of Fox et al.[8] (CNCPS ver. 5 feedbank) and those determined by Fortina et al.[12]. The results of our analysis were generally agreed with Fox et al.[8]. However, some differences were observed for SolP of barley, NDIP of wheat middling and ADIP (C fraction) for maize. Fox et al.[8] (17%) reported that SolP of barley was lower than reported values of our study. However, the SolP value of barley reported in Fortina et al.[12] (21.2%) was close to our result. Fox et al.[8] reported that NDIP of wheat middling was lower compare the those of our study (at WM-1 8.74% and at WM-2 7.05% instead of 4%). Fox et al.[8] (5%) and Fortina et al.[12] (6.6%) reported that C fractions of maize was higher than reported values of ours. Regarding NDIP

and C (ADIP) fractions determinations, we suggested that the variability may be imputed to the use of different apparatus (Fibertec vs Ankom) in the laboratory. The results of A, B_1, B_2 and B_3 fractions were also in agrement with Fox et al.[8] except A fraction of barley. Fox et al.[8] reported that A fraction for barley (4.9%) was lower than our findings. The differences between the A fraction could be due to use of different reagents (tungstic acid vs trichloroacetic acid) and filtration methods [12]. Nikokyris and Kandylis [30] stated that wheat middling had a higher protein solubility than the unprocessed wheat protein, because soluble proteins such as albumin, globulin and NPN fractions have been increased by processing to by-products. This situation could be explained that A fraction had the highest in wheat middling. Because of the high prolamin and glutelin levels of the energy-rich feedstuffs [30], B_2 and B_3 fractions were high in cereals. And, B_2 fraction was the highest compare to A, B_1, B_3 and C fractions. The CP fractions of maize were better agreed with Fortina et al.[12], but barley and wheat middling were not close. B_2 fractions of our study were higher for barley (19%), B_3 fraction of our study were lower for barley (56.1%) and wheat middling (27.3%) than reported in Fortina et al.[12]. The variability of NDIP and ADIP values were caused the difference in B_2 and B_3 fractions of feedstuffs. Our study agreed with Sniffen et al.[11] that maize contained high B_3 fractions, because of high zein protein content (prolamins). DIP_{1X} values were highest in WM-2 and lowest in maize. The result of DIP_{1X} values were similar to Fox et al.[8] in maize (65%), wheat (85%), barley (80%), rye (86%), and wheat middling (86%).

The Estimation of Effective Protein Degradability (EPD)

The all EPD and DIP values are lined up starting from the highest to the lowest as WM-2, rye, WM-1, wheat, barley, maize, similarly. This result disagree with Bach et al.[31] that some mathematical models may not be appropriate for all types of feedstuffs and the feedstuffs could be ranged in a different order. However, we tested same type of feedstuffs. Results indicated that all determination coefficients were significantly high for the all equations ($R^2 \geq 0.96$) to predict EPD values. These regression relations did not improve when the different rumen outflow rates were used to estimate EPD. In Shannak et al.[1], Zhoa and Cao [16] ($n = 30$, $R^2=0.90$, $P<0.0001$) and Westreicher-Kristen et al.[17] similar to our findings, they reported that *in situ* rumen undegradable protein (1-EPD) obtained from nylon bag method may be the reliable and accurately predicted from CP fractions based on CNCPS. Zhoa and Cao [14] indicated that the regression equations could be used as a possible alternative, when rumen cannulated sheep or cattle are not available in some laboratories.

The present study showed that i*n situ* effective protein degradability (EPD) can be reliably and accurately predicted from CP fractions and DIP values in cereals and wheat middling based on CNCPS. In Turkey, more studies about feedstuffs based on type are needed to increase the reliability of the regression equation, which is used to estimate the crude protein degradability.

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RESEARCH ARTICLE

Investigation on Serum Hormonal Parameters (Ghrelin, Corticosterone, Insulin, T_3 and T_4) in Chronic Mild Stress Rat Model of Depression [1]

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Summary

The aim of present study was to investigate the effects of depression induced with chronic mild stress (CMS) protocol on serum hormones (ghrelin, corticosterone, insulin, T_3 and T_4) which is in interaction with stress or depressive disorders. Forty-five 40-45 d old laboratory rats were assigned to two groups; control (n: 10) and CMS (subjected to CMS procedure, n: 30). Rats in the control group were reared in single cages without any environmental stress. Rats in CMS were entered the CMS procedure. This protocol consisted of mild unpredictable stressors (intermittent illumination, stroboscopic light, grouping, food or water deprivation, exposure to an empty water bottle, solid cage, cage tilting, etc.). After CMS protocol sucrose preference (SP) test was used for the identification of depressed animals. Rats with lower than 65% SP were defined as depressed animal. Blood serum was taken from two groups (control and CMS) for the determination of blood hormonal variables by Elisa kits. Analyzed data showed a significant elevation in ghrelin, corticosterone, and insulin levels for CMS group (depressed animals) in comparison with control group, as elevation was very significant for corticosterone. There was not any significant change for T_3, whereas decreased T_4 was observed for CMS groups when compared with the control. It was concluded that chronic mild stress induced depression can cause ghrelin, corticosteroid, and insulin increases, and T_4 decreases. Mentioned effects of CMS induced depression on hormonal indices (with exception to thyroid hormones), can be similar with effects of major depression.

Keywords: *Chronic mild stress, Depression, Glucocorticoids, Insulin*

Depresyonun Kronik Hafif Stres Sıçan Modelinde Serum Hormonal Parametrelerin (Ghrelin, Kortikosteron, İnsülin, T_3 ve T_4) Araştırılması

Özet

Bu çalışmada, depresyon etkilerinin kronik hafif stres (CMS) işlemi ile stres ve depresif bozukluklarla etkileşim halinde olan serum hormonları (ghrelin, kortikosteron, insülin, T_3 ve T_4) üzerine etkilerinin araştırılması amaçlanmıştır. Kırk beş adet 40-45 günlük laboratuvar sıçanları kontrol (n: 10) ve CMS (CMS işlemine tabi, n: 30) olmak üzere iki gruba ayrıldı. Kontrol grubundaki sıçanlar herhangi bir çevresel stres olmadan tek bir kafeste yetiştirildi. CMS grubu sıçanlar CMS işlemine dahil edildi. Bu protokol hafif öngörülemeyen stres oluşturucularının (kesintili aydınlatma, stroboskopik ışık, gruplama, yiyecek veya su yoksunluğu, boş bir su şişesine, katı kafese, kafes eğimine maruziyet, vb) oluşmaktadır. CMS işleminden sonra sükroz tercih (SP) testi depresif hayvanların tanımlanması için kullanıldı. %65 SP'den daha düşük sıçanlar depresif hayvan olarak tanımlandı. Kan serumu ELISA kitleri ile kan hormonsal değişkenlerin tespiti için iki grutan (kontrol ve CMS) alındı. Analiz edilen sonuçlar kontrol grubu ile karşılaştırıldığında çok önemli kortikosteron artışıyla beraber CMS grupta (depresif hayvanlar) ghrelin, kortikosteron ve insülin seviyelerindeki önemli artışı gösterdi. CMS gruplarında kontrol grubu ile karşılaştırıldığında düşmüş T_4'e rağmen T_3 için önemli bir değişiklik gözlemlenmedi. Kronik hafif stres kaynaklı depresyonun ghrelin, kortikosteroid ve insülin artışına ve T_4 azalmasına sebep olabileceği sonucuna varıldı. Hormonsal endeks üzerindeki (tiroit hormonları hariç) CMS kaynaklı depresyonun adı geçen etkileri majör depresyon etkileri ile benzer olabilir.

Anahtar sözcükler: *Kronik hafif stres, Depresyon, Glukokortikoidler, İnsülin*

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INTRODUCTION

Depression is one of the common psychological disorders in human society, and it is much more prevalent in industrialized societies. Research undertaken in this regard has largely prescribed anti-depressant medicines or medicinal plants as well as different levels of effective hormones being studied. However, studies with adverse effects of depression on endocrine parameters are very limited. Also, these studies have focused mainly on major depression cases [1-3] not chronic or depression resulting from mild environmental stresses. So, studies on endocrine indices of various models of depression are necessary [4].

Among trusty models of depression, chronic mild stress (CMS) model of depression in rodents has been proposed to model some of the environmental factors that contribute to the induction of depressive disorders in humans [5-7]. In the present protocol (CMS), sequential exposure to a variety of mild stressors causes behavioral deficits in different paradigms that measure sensitivity to rewards. Thus, CMS suppresses the consumption of and preference to palatable sweet solution such as sucrose or saccharin [8], and the rewarding properties of food pellets, sweet solutions and amphetamine, as assessed by the place preference conditioning procedure [9]. Effects of these stresses (CMS) on animal hormonal profile are not reported by any comprehensive study. Albeit sporadic investigations were published in relation to effects on chronic-mild stress on glucocorticoids [10,11] and thyroid hormones [12], however, these experiments with exception to Kioukia *et al*.[12], are not conducted in general case of CMS models. So, the aim of present study was to investigate the effects of CMS on serum hormones which are in interaction with stress or depressive disorders.

MATERIAL and METHODS

Animals

Forty-five 40-45 d old Male Wistar rats were kept into the laboratory animal room for 1 week pre-experimental adaptation period. Animals were weighted and assigned as two groups; control (n: 10) and CMS (subjected to CMS procedure, n: 30). Animals in the control group were reared in single cages without any environmental stresses. Animal in CMS were entered into the CMS procedure *(Table 1)*. With exception to limitations of CMS procedure, the food and water were available ad libitum for all animals. Other environmental conditions included light/dark cycle (12 h: 12 h), light intensity, and ventilation were the same for both groups. Experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985). The protocol was arranged in according to certificate of Tabriz University of medical sciences ethical committee (Reg. no. 5.4.411- April 10, 2011) for present project.

CMS Procedure

CMS has been used to achieve depressive-like symptoms in Wistar rats [7,8]. It was designed to maximize the randomness of the stressors. The protocol was carried out for 4 weeks as described in *Table 1*.

This protocol consisted of mild unpredictable stressors which are: intermittent illumination, stroboscopic light (300 flashes/min), grouping, food or water deprivation, exposure to an empty water bottle immediately following a period of water deprivation, solid cage (300 ml water spilled into bedding) and 45° cage tilting. Grouping indicates housing a rat in pairs with different partners while an individual rat alternately becomes a resident or an intruder. Details of the CMS procedure are presented in *Table 1*.

Sucrose Preference Test

Sucrose preference (SP) test is a measure to evaluate anhedonic effect of CMS [8] and efficiency of protocol to induce depression. In this test, animals were trained to consume a 1% sucrose solution following 18 h of food/water deprivation at week three. Sucrose intake measure with weight losses of sucrose contained bottle at the end

Table 1. *Time and length (h) of stressors used in the CMS procedure*
Tablo 1. *CMS işleminde kullanılan stres oluşturucuların zamanı ve uzunluğu (saat)*

Stressor	Timing						
	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Intermitted lighting (light/dark)	00:00-14:00 (1 time/2 h)	-	-	-	-	-	-
Strobe light	-	-	00:00-12:00	-	-	-	-
Cage tilt 45°	-	13:00-23:00		-	-	13:00-23:00	00:00-12:00
Solid cages	-	-	12:00-24:00	-	-	-	13:00-23:00
Feed/Water deprivation	-	00:00-10:00	-	10:00-24:00	-	00:00-12:00	-
Empty water bottle	-	10:00-12:00	-	-	-	-	-
Paired housing	-	-	-	-	10:00- 24:00	-	-

of the test for 24 h during a no-stress period. Rats with lower than 65% SP (net sucrose consumption/ [sucrose consumption+ water consumption] × 100 %) were defined as depressed animal [13-15].

Blood Sampling and Analysis

Healthy animals (control) and depressed animals recognized by SP test were subjected to blood sampling for identification of effects of CMD on serum hormonal variables. Blood samples taken from orbital sinus were centrifuged at 1.200 x g for 7 min at 18°C, and serum was prepared for determination of blood hormonal variables (ghrelin, corticosterone, insulin, T_3 and T_4) with an auto-analyzer (Alcyon 300; Abbott Park, IL, US) and Elisa commercial kits.

Present experiment was arranged with two treatments and four replicates for each. Data were analyzed with SAS (SAS Inst. Inc., Cary, NC, US) and the differences between treatments were assessed by unpaired t-test, and $P<0.05$ was considered to be significant.

RESULTS

The hormonal measures of serum are presented in *Table 2*. There is a significant elevation in ghrelin, corticosterone, and insulin levels for CMS group (depressed animals) in comparison with control group, as elevation was very significant ($P<0.01$) for corticosterone *(Table 2)*. There was not any significant change for T_3, whereas decreased T_4 was observed for CMS groups when compared with control ($P<0.05$).

DISCUSSION

Nowadays, after 13 years from discovery of ghrelin in rat [16], it has been identified that ghrelin has considerable role in mental health, chronic or acute stress [17]. Chuang and Zigman [17] have stated that serum ghrelin can decrease following chronic stress and negative energy balance. In present study *(Table 2)*, ghrelin was in greater level in depressed group (CMS) when compared with control ($P<0.05$). According to Chuang and Zigman [17] report, the regulatory potential of ghrelin may be the reason for ghrelin elevation in onset of stress for moderating metabolic damages of stress or stress induced depression. Also, ghrelin can moderate depressive symptoms.

In previous studies, ghrelin elevation was observed in acute stress [18] water privation condition [19] or tail cutting-stress [20]. Ochi *et al.*[21] have shown that stress can elevate levels of different forms of ghrelin include pre-proghrelin, ghrelin mRNA, acyl-ghrelin and des-acyl-ghrelin. The present finding about elevation of acyl-ghrelin in stress-induced depression condition is in agreement with Ochi *et al.*[21]. In their study, the number of ghrelin- Ip cells (ghrelin-producer cells) has increased following 5 days solid-cage stress (part of CMS protocol). In Lutter *et al.*[18], animals under social stress have greater acyl-ghrelin concentration witch ghrelin level rise to peak point on day-10 of stress protocol. Whereas ghrelin level returned to normal after protocols finished. In this regard, Rouach *et al.*[22] reported that individuals with psychological stress have greater levels of plasma ghrelin. Another evidence for regulatory role of ghrelin in stressful condition is that ghrelin elevation occures along with epinephrine raises induced with stress [23]. Monteleone *et al.*[24] reported considerable increase in salivary ghrelin following social stress protocol. Investigations on major depression cases show constant level for ghrelin [2]. Findings of present study for ghrelin levels of depressed (CMS) animals *(Table 2)* are in agreement with Chuang and Zigman [17], Asakawa *et al.*[19], Kristenssson *et al.*[20] and Monteleone *et al.*[24] reports witch indicate that ghrelin can increase in chronic psychological or environmental stress, and various protocols for depression induce.

It is suggested that exposure to various stressors in CMS protocol is a main cause of ghrelin elevation. Ghrelin has regulatory role for elimination of depressive effects on animal mental or metabolic health. In other word, ghrelin elevation may be an efficient defense mechanism to avoiding depression related damages in CMS animals or in chronic depression cases. But in major depression this mechanism (increase in ghrelin level) may not be efficient [2].

In major depression, hypothalamic- pituitary-adrenal (HPA) axis is hyperactive and releases greater amount of

Table 2. *The hormonal variables of serum in rats subjected to CMS procedure*
Tablo 2. *CMS işlemi uygulanan sıçanlarda serum hormonsal değişkenleri*

Group	Variable				
	Ghrelin pg/ml	Corticosterone ng/l	Insulin mU/l	T_3 ng/ml	T_4 µg/ml
Control	250.38 [b]	160.62 [b]	6.25 [b]	0.40	2.00 [a]
CMS (depressed)	304.75 [a]	190.50 [a]	8.50 [a]	0.55	1.65 [b]
P value	0.0223	0.0042	0.0312	0.2782	0.0259
SEM*	12.582	4.717	0.568	0.088	0.084

** Standard error of the mean; - Different letters (a or b) shows significant difference between means*

glucocorticoide [25]. Abdul Aziz *et al.*[11] reported that pregnant CMS models have greater amniotic corticosterone, and the level can rise significantly at d 13 of gestation. Control group (pregnant non-CMS) had normal level of corticosterone at d 13 of gestation, and they have an increase in corticosterone rate at d 18. Also, in a study [26], infants from mothers with exposure to stress during pregnancy had greater level of plasma cortisol. It was reported that variety in kind and number of chronic stresses can activate hippocampal receptors and releases great amount of corticosterone [27]. In present study which was conducted with similar protocol with Raudkivi *et al.*[27], the various chronic stresses can affect corticosterone level *(Table 2)*. It seems that continuous exposing to variable chronic stresses without dietary energy intake (feed deprivation) for long time of CMS protocol cause corticosterone raises in CMS group.

Depressed individuals are susceptible to insulin-related disorders such as hyperinsulinemia or type 2 diabetes [28]. An epidemiological study showed that insulin- resistance indices are common in depressed people or individuals with mental potent for depression [29].

Less consumption of glucose sources (such as sucrose) in depressed models may be a potential factor for tribulation in insulin release, insulin sensitivity, and depression treatment process. In this regard, Ramasubbu [30] reported correlation between insulin-resistance disorder and depression and this correlation was independent from age, weight, nutritional status, plasma GH or glucagon level and cortisol circadian rhythm. In Pan *et al.*[29] study, it was observed that hypercortisolemia is a booster factor for insulin-resistance in depressed models. In according to Castillo-Quan *et al.*[31] suggestion, depression has insulin-resistance disorder similar with type-2 diabetes. So, it can be cause of hypercortisolemia. In present study, there is a significant increase in insulin and corticosterone level for CMS group *(Table 2)*. Whereof corticosterone and insulin is a major role in metabolic equilibrium of body, any change in these hormones can cause serious metabolic disorder [31]. It can be suggested that the stressors in CMS protocol can cause less dietary glucose intake. Also presents protocol can cause hyperinsulinemia witch is a symptom of depression.

Commonly, T_3 level is not affected by depression, whereas in some studies on depression cases it is reduced due to incidence of depression [32,33]. It is indicated that decreases in T_3 in major depression cases may be because of secondary effect of depression such as starvation, swoon and anti-depressant drugs [34]. But T_4 level can be change due to depression or environmental stresses [35]. In an idea [36], loss of serotonin was announced as main cause of change in thyroid activity. Findings of present study about thyroid hormones (T_3 and T_4) *(Table 2)* are in according to Kirkegaard [32] who reported decreases in T_4 level following depression induction. In present study, T_3 level remained unchanged following CMS protocol, which was unlike to Baumgartner *et al.*[33] reports in major depression (decreased T_3). In Olivares *et al.*[35] study, animals had decreased T_4 level and transient hypothyroidism following social stress protocol. Findings of present study *(Table 2)* conducted with CMS protocol was in agreement with Olivares *et al.*[35]. It seems that T_4 decreases in CMS animals *(Table 2)* were in related to serotonin losses, minor energy intake and subsequent declines in basal metabolism.

It was concluded that chronic mild stress protocol and induced depression can cause ghrelin, corticosteroid, and insulin increases, and T_4 decreases. Mentioned effects of CMS induced depression on hormonal indices (with exception to thyroid hormones), can be similar to the effects of major depression. It seems that T_4 decreases in CMS animals are in relation to serotonin losses, minor energy intake (because of continuous stresses) and subsequent declines in basal metabolism. Further studies on CMS effect on other hormones are necessary to completing hormonal profile of CMS protocol.

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RESEARCH ARTICLE

Comparative Production of Rapid Slide Agglutination Test (RSAT) Antigen Used in Serological Diagnosis of *Brucella canis* in Different Culture Media in the Fermenter [1]

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Summary

In this study, rapid slide agglutination test (RSAT) antigens were produced by using homologous (*Brucella canis* RM6/66 and a wild *B. canis*) and heterologous brucella strains (*B. abortus* 45/20 and *B. melitensis* B115) for a quick, practical and economic diagnosis of *B. canis* infection in dogs and humans. All the test strains were grown in three different culture media, namely, trypton liquid media (TLM), brucella broth media (BBM) and brain heart infusion broth media (BHIBM) to compare their optimal growths. The best growth rate was obtained by *B. canis* RM6/66 strain. *B. melitensis* B 115 showed the poorest growth in all test media. The culture media that supported the best growth rate in all tested strains was TLM. RSAT antigens from each test strains were produced without any stringy formation and standardised successfully. It was thought that *B. canis* RM6/66 and/or wild *B. canis* might be suitable candidate in commercial RSAT antigen production according to the test results supporting the fact that using homotypic strain increases the diagnostic sensitivity. As a conclusion, it was decided that this antigen will be able to used extensively as a part of routine clinical examination in dogs in Turkey. Furthermore, it was considered that to include RSAT in humans as a part of routine brucellosis diagnosis could help to evaluate the disease more accurately.

Keywords: *Brucella canis, RSAT, Antigen production, Rough Brucella strains*

Brucella canis'in Serolojik Tanısında Kullanılan Çabuk Lam Aglütinasyon Test (ÇLAT) Antijeninin Fermentörde Farklı Besiyerlerinde Karşılaştırmalı Olarak Üretimi

Özet

Bu çalışmada *Brucella canis* infeksiyonunun çabuk, pratik ve ekonomik teşhisi için homolog ve heterolog suşlar ile hazırlanmış çabuk lam aglütinasyon test (ÇLAT) antijenleri üretildi. Antijen üretiminde homolog suşlar (*B. canis* RM6/66 suşu ve saha *B. canis* suşu) ve heterolog suşlar (*B. abortus* 45/20 ve *B. melitensis* B115) kullanıldı. Bu amaçla çalışmada kullanılacak test suşları, tripton sıvı besi yeri (TSB), brusella broth besiyeri (BBB) ve brain heart infüzyon broth besiyeri (BHIB) olmak üzere 3 ayrı besi yerinde optimal üremeyi değerlendirmek üzere karşılaştırmalı olarak üretildiler. Test besiyerlerinde en iyi üremeyi *B. canis* RM6/66 suşu ve en zayıf üremeyi *B. melitensis* B 115 suşu gösterdi. Suşların en iyi üredikleri besi yerleri TSB oldu. Sonuçta tüm test suşlarından hazırlanan ÇLAT antijenleri başarılı bir şekilde üretilip standardize edildiler. Test sonuçlarına göre *B. canis* RM6/66 ve/veya saha *B. canis* suşlarının ticari ÇLAT antijeni üretimi için seçilebileceği düşünüldü. Sonuç olarak üretilen ÇLAT antijeninin Türkiye'de köpeklerde, yaygın olarak rutin klinik muayenenin bir parçası olarak hastalığın serolojik teşhisinde kullanılabileceği ve bu testin insanlardaki klasik bruselloz tanısına dahil edilmesinin, hastalığın insanlardaki durumunun daha sağlıklı olarak değerlendirilmesine olanak sağlayacağı sonucuna varıldı.

Anahtar sözcükler: *Brucella canis, ÇLAT, Antijen üretimi, Rough Brucella suşları*

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INTRODUCTION

Canine brucellosis is an important cause of canine abortion and infertility worldwide. Although the disease is reported from many countries of the world, the exact epidemiologic data regarding to its prevelance in many parts of the world are missing [1-3]. Because of the signs of the disease are relatively mild, diagnosis of the disease is usually based on bacterial isolation which is laborious and lengthy and its sensitivity might be decreased by the intermittent bacteremia [4]. Therefore, serological tests are most commonly used for routine clinical diagnosis. The most widely used serological tests are rapid slide agglutination test, tube agglutination test with and without 2-mercaptoethanol (2ME-RSAT and RSAT; 2ME-TAT and TAT, respectively) and agar gel immunodiffusion test (AGID) [3,5,6].

Tube agglutination test and 2ME-TAT have some technical disadvantages that limit their widespread usage in the field: Inability to detect low level antibody titers in chronically infected and bacteremic dogs, and prozone phenomena. Besides, these tests take at least 48 hours to interpret the results. AGID test has been used but it is a complex test, fails to detect early stages of infection and sometimes precipitin lines are difficult to interpret [3,7]. RSAT is very sensitive, practical and easily interpreted screening test [8-12]. The only disadvantage of RSAT is that many false positive reactions are observed because of the heterospecific reactions between surface antigen of *B. canis* and naturally rough organisms, nonsmooth *Brucella* species, and a variety of other bacterial species [13,14]. Therefore all the RSAT positive samples should be confirmed by more specific tests to alleviate false positives [11].

Production of suitable diagnostic antigen requires considerable attention because of *B. canis* shows great tendency to become ropy and form stringy sediments after long incubation period and in pH below 6.8. These sediments are tried to obviate by adjusting pH of growth media and resuspending buffer to pH level above 7.4 [9,15].

The disease was reported serologically in Turkey and seroprevelance rates in dogs that ranged from 6.3% to 12.7% have been reported in different regions in Turkey [16-18]. There are extremely few data regarding to human infection [19-21]. The impact on public health might be underestimated because of lack of standardized antigens, unawareness of medics to use these specific antigens instead of those for detecting smooth lipo-polysaccharide (sLPS) and poor reporting system in many parts of the world [22,23].

In this study, we present data on the production of four different RSAT antigens prepared by different rough *Brucella* strains and their growth rates in different culture media. The aim of this study was to produce appropriate RSAT antigen that can be used serological diagnosis of canine brucellosis. This antigen also will be able to used in serological diagnosis of brucellosis caused by *B. ovis* infection in sheep and goats and human brucellosis caused by rough species.

MATERIAL and METHODS

Reference Bacterial Strains and Sera

Brucella canis RM6/66 ATCC 23365 strain, *B. canis* positive serum prepared in rabbit (BC4) and negative dog serum, standardized RSAT antigen were kindly provided from Animal Health Veterinary Laboratory Agency (AHVLA), UK. *B.canis* positive serum obtained from experimentally infected specific pathogen free (SPF) beagle by oro-nasal route was kindly supplied from Dr. Carmicheal (Cornell University). Other test strains of *B. abortus* 45/20, *B. melitensis* B115 and *B. canis* wild isolate (10-PBC-87) and other reference materials used in classical biotyping were obtained from culture and reference materials collection from National Reference Laboratory for Brucellosis in Pendik, Istanbul, TURKEY.

Culture Media and Solutions

All the liquid and solid culture media and Tris-maleate buffer (TMB 0.4 M), PBS (0.15 M) and Rose Bengal dye solution (2%) were prepared to the methods described by Alton et al.[24]. *Brucella* Broth Medium (BBM, Becton Dickinson) and Brain Heart Infusion broth media (BHIBM, Himedia) were prepared according to manufacturer's instructions.

Bacteriological Studies

All test strains were identified according to the classical biotyping procedures described in OIE Manuel [25].

Preparation of Seed Cultures of Test Strains

Freeze-dried master culture of test strains were re-hydrated in sterile PBS (pH 6.4) and inoculated on serum dextrose agar slants. The slants were incubated at 37°C for 3 days.

Fermentation

Bioreactor (Biostat A Plus CC, Sartorius, Germany) with a working volume of 1 L was used to produce each test strain for a bulk production. Each of the test media was sterilized by filter and aseptically transferred to previously sterilized bioreactor. The cells from the each seed culture were inoculated to be 5% into bioreactor. The pH of the media was adjusted to 7.4±0.2. Temperature was fixed at 36±1°C. The aeration was maintained by addition sterile compressed air at 4-8 liter/min and agitation speed controlled at 300-600 rpm and the production process was started and after seed inoculation, samples were collected at 24 h interval up to 96 h to determine the viability count,

pH, dissolved oxygen, mucoid appearence, sticky sediment formation, purity and colonial morphology. All viability counts were performed in three separate occasions by three different persons and the results were expressed as the mean value of the viable counts.

Preparation of Stock Antigens for Slide Agglutination Test

Antigens prepared from each test strains were produced as described with some modifications [9,10]. Briefly, all the test strains cultured in bioreactors were harvested 96 hours later of their incubation and inactivated by heat at 60°C for 2 h. Inactivated cultures were checked for mucoidness, purity and viability. Then all test cultures were centrifuged at 3500 g for 30 min. After centrifugation, the supernatant fluid was decanted and resulted pellet washed twice in PBS (0.15 M, pH 7.4). The washed cells were resuspended to approximately 10% packed cell volume (PCV) in PBS and labeled as "stock RSAT antigen" for future use of standardized RSAT antigens production. The stock cell suspensions then were stained by the stock solution of 2% Rose Bengal (Sigma) dye previously filtered through Whatman no 1 filter paper. Stained suspensions were agitated at slow speed with magnetic stirrer overnight at 4°C to avoid foaming and then centrifuged at 3500 g for 30 min to sediment stained bacteria. Stained pellets were suspended with vigorous shaking in 0.4 M TMB buffer (pH 9.0) using a magnetic stirrer and filtered to exclude possible large bacterial clumps. The suspensions were diluted to PCV of 6% and filtered through sterile glass wool. Finally, sodium azide was added to each suspension for a final concentration of 0.1%.

Standardisation of RSAT Antigens

The prepared four separate RSAT antigens were standardised by using positive BC4 serum dilutions of 1/10, 1/40 and 1/80 made in TMB buffer. Equal amount of serum and each test antigens and standard antigen were mixed on a glass plate with a wooden stick and the plate was rocked gently for 4 min. Positive and negative control sera were included in each test. If agglutination appears within 15 sec, it was recorded as a "++" reaction and any agglutination occured later was scored as a "+" reaction. No agglutination was recorded as negative. Each test antigens was evaluated as standardised when it produced positive agglutination at a 1/10 and 1/40 dilution of BC4 serum and no agglutination at a 1/80 dilution of control and negative serum. Each test was carried out in three separate trials. Final products were checked for sterility.

Each test RSAT antigens were also tested with serially diluted *B. canis* positive serum from experimentally infected SPF beagle. This serum (AS 1048) which was kindly supplied from Dr. Carmicheal was obtained from a beagle experimentaly infected with *B. canis* by oro nasal route 9 months post infection and had TAT test titer of 1/500. The degree of agglutination was scored from +1 to +4 of agglutination reaction.

Statistical Analysis

For statistical comparisons of the test culture media and test strains, one-way analysis of variance (ANOVA) was done (with 95% confidence level) using SPSS (IBM, SPSS Inc.).

RESULTS

All test strains were identified and results were consisted with described reference strain characteristics [25]. Test results were shown in *Table 1*. All test strains were lysed by R/C phage while none of them was lysed by Tbilisi phage *(Fig. 1)*.

Table 1. *Cultural characteristics of reference and test strains*
Tablo 1. *Referans ve test suşlarının kültürel özellikleri*

Cultural Characteristics	*B. canis RM/66*	*B. abortus 45/20*	*B. melitensis B115*	*B. canis 10-PBC-87*
Agglutination with acriflavine	+	+	+	+
CO_2 requirement	-	-	-	-
H_2S production	-	+	-	-
Growth on thionine 20 µg/ml	+	-	+	+
Growth on basic fuchsin 20 µg/ml	-	+	+	+
Agglutination with monospecific A serum	-	-	-	-
Agglutination with monospecific M serum	-	-	-	-
Agglutination with monospecific R serum	+	+	+	+
Serum requirement	-	-	-	-
Urease production	+	+	+	+
Oxidase production	+	+	+	+
Lysis by Tbilisi phage	-	-	-	-
Lysis by R/C phage	+	+	+	+

Fig 1. Lysis evaluation of test strains by R/C and Tbilisi phage
Şekil 1. Test suşlarının R/C ve Tbilisi fajı ile lizis değerlendirmesi

All four strains were grown in the three different culture media, namely, TLM, BHIBM and BBM in a bioreactor. Samples were taken daily intervals for a viability count. Best growth (CFU/ml) was obtained after 72 h of incubation for each test strain and in each test culture media *(Table 2)*. Each test culture was terminated after 96 h of incubation and viability counts for each test strain in test media were summarized in *Table 3*. During growth of the test strains, bacterial cultures did not become stringy and no sticky sediments formed. But bacterial growth in BHIBM produced more foam compared to others and to compensate the bioreactor pumped antifoam solution into the reactor at more frequent intervals. The maximum viable count expressed as CFU per ml for all test culture media was obtained by *B. canis* RM6/66, *B. canis* 10-PBC-87, *B. abortus* 45/20 and *B. melitensis* B 115 strains, respectively. The culture medium yielded the best growth for each test strain was TLM, BBM, and BHIBM, respectively.

There was no significant difference (P>0.05) in growth between *B. canis* RM6/66 and *B. canis* 10-PBC-87 in different test media at 95% confidence level but the viability counts of these strains were significantly different (P<0.05) from the rest of the strains. The viability counts were significantly higher (P<0.05) in TLM than those of BBM and BHIB. The number of bacteria found after 48 h of incubation were not significantly different (P>0.05) than those of 72 h of incubation.

RSAT antigens prepared from each test strains were standardized against reference antigens and known positive and negative sera. All of them agglutinated 1/10 and 1/40 serum dilution of BC4 serum while there was no agglutination occurred with 1/80 dilution of the control serum. No bacterial and fungal contamination was detected in any RSAT antigens *(Table 4)*.

RSAT antigens were also tested by various serum dilutions of positive dog antiserum (AS 1048). Agglutination degree was scored from +1 (minimum degree of agglutination) to +4 (maximum degree of agglutination) reaction. The weakest reaction was observed in the

Table 2. *Viability counts (CFU/ml) in test media after 72 hours of incubation in the bioreactor*
Tablo 2. *Biyoreaktörde 72 saatlik inkübasyon sonrasında test besiyerlerindeki canlılık sayımları (KOB/ml)*

Test Strain	Test Culture Media		
	TLM	BHIBM	BBM
B. canis RM6/66	42.5X10^9	32.5 X10^9	35.8 X10^9
*B. canis*10-PBC-87	31.3 X10^9	24.9 X10^9	30.3 X10^9
B. abortus 45/20	18.9 X10^9	12.7 X10^9	15.9 X10^9
B. melitensis B115	11.9 X10^9	8.9 X10^9	10.3 X10^9

Table 3. *Viability counts (CFU/ml) in test media after 96 hours of incubation in the bioreactor*
Tablo 3. *Biyoreaktörde 96 saatlik inkübasyon sonrasında test besiyerlerindeki canlılık sayımları (KOB/ml)*

Test Strain	Test Culture Media		
	TLM	BHIBM	BBM
B. canis RM6/66	39.1X10^9	30.5 X10^9	31.2 X10^9
*B. canis*10-PBC-87	22.3 X10^9	18.3 X10^9	20.3 X10^9
B. abortus 45/20	17.6 X10^9	10.6 X10^9	12.6 X10^9
B. melitensis B115	9.1 X10^9	7.1 X10^9	8.8 X10^9

Table 4. *Results of standardization of RSAT antigens*
Tablo 4. *ÇLAT antijenlerinin standardizasyon sonuçları*

Test Strains of RSAT Antigens	BC4 Positive Serum Dilutions				Sterility
	1/10	1/40	1/80	Negative Serum	
B. canis RM6/66	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
B. canis PBC-10-87	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
B. abortus 45/20	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
B. melitensis B115	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile
Standard antigen	++, ++,++	+, +,+	-, -,-	-, -,-	Sterile

Table 5. *Results of agglutination reactions of RSAT antigens with positive dog serum dilutions*
Tablo 5. *ÇLAT antijenlerinin pozitif köpek serumunun dilüsyonları ile aglütinasyon sonuçları*

Test Strains of RSAT Antigens	Positive Dog Serum (AS 1048) Dilutions					
	1/5	1/10	1/15	1/20	1/25	1/30
B. melitensis B115	++ ++	++ ++	+ +	- -	- -	- -
B. canis PBC-10-87	++++ ++++	+++ +++	++ ++	++ +	+ -	- -
B. abortus 45/20	++++ ++++	+++ +++	++ ++	+ +	- -	- -
B. canis RM6/66	++++ ++++	+++ +++	++ ++	+ +	- -	- -
Standard antigen	++++ ++++	+++ +++	++ ++	+ +	- -	- -

agglutination caused by *B. melitensis* B115. The sensitivity of RSAT antigen prepared from *B. canis* PBC-10-87 seemed to be highest since only this antigen gave positive reaction with the 1/25 dilution of positive dog serum. Results were shown in *Table 5*.

DISCUSSION

Serological diagnosis of the canine brucellosis constitutes a vital first stage in control and eradication of the disease. In this context, a sensitive, practical and easily interpreted screening serological test that allows to diagnose early stage of infection is highly required for routine diagnosis [3,4]. The production of RSAT antigens that employs M+ virulent strains requires a great care because of the tendency of the agent to become stringy and to form sticky sediments especially for longer incubation period. These M+ strains are always mucoid in nature and autoagglutinates in relatively acidic pH, which is something that is feared to be happened in agglutination test antigens, and might be responsible for lack of complete agglutinations [4,10,13]. Because of the difficulties reported in antigen production using *B. canis*, some heterologous strains were also chosen in antigen production to evaluate their potential usage in this test.

Today, commercial RSAT kits for *B. canis* employ *B. ovis* as antigen. However, being more fastidious and having strict requirements for CO_2 and serum for growth [24], *B. ovis* might not be the best alternative for RSAT antigens production. It was reported that the usage of *B. canis* instead of *B. ovis* in RSAT production reduced the false positives from 50% to 12% and they concluded that the specificity of RSAT might be increased using homologous strains in antigen production [15]. There are some other researchers who supported the same conclusions [12,26,27].

The growth rate of *B. canis* RM6/66 and *B. canis* 10-PBC-87 was found as similar statistically in all tested media ($P>0.05$). But heterologous strains grew poorly compared to the *B. canis* strains ($P<0.05$). The highest viability rate was obtained by using TLM, BBM, and BHIBM, respectively. TLM is the medium of choice for growth of brucella vaccine strains (*B. melitensis* Rev.1 and *B. abortus* S19) [25] and we have been using this medium for vaccine production in our laboratory. BHIBM supported the poorest growth rate among others. This might be due to dilution of growth media by antifoam solution pumped into bioreactor because of foamy growth of test strains in this medium. Since the aim of this study was to produce cost effective reagent for commercial bulk production in fermenter, we decided to determine the growth media that stimulated the highest number of bacteria and test strain that grew optimally in this test media for antigen production. The viability counts made from the samples taken after 48 h of incubation were not significantly different ($P>0.05$) than those of 72 h of incubation. But it was significantly different from those observed in 96 h of incubation. The commercial biological production should not only consider the highest yield but also consider to reach same results by using minimum sources. In this context, to harvest cultures after 48 h of incubation instead of 96 h of incubation will significantly decrease the operational costs by bioreactor and will serve to more economical production.

Longer incubation periods in growth media especially in those of having pH below 6.8 were reported to cause sticky sediments in culture media [24]. In our study, no such sediments were observed. Stained pellet was suspended with vigorous shaking in 0.4 M TMB buffer (pH 9.0). It was reported that nonspecific agglutination was reduced by increasing the molarity of TMB to 0.2 M or greater [10]. The disease was reported serologically in Turkey in both dogs [16-18] and in humans [19-21]. But there are no enough data to clearly identify the disease situation in Turkey. The lack of commercially available standardized antigens that show no cross reactivity between antibodies to *B. canis* and smooth *brucella* species in serologic diagnosis of the disease might be responsible for few studies conducted with relatively low number of serum samples.

Human infections are probably more common than indicated in published reports because routine brucellosis diagnosis does not include serological test with *B. canis* antigen. Therefore, infection with this species or any rough species of brucella might be undetected [23,28,29]. Therefore, a RSAT that employs a suitable rough strain should be a part of a routine serologic brucellosis scheme in humans in order to evaluate the disease situation more precisely.

It has been reported that *B. canis* strain has the advantage of making a satisfactory antigen by replacing AGID as a screeining test for diagnosis of *B. ovis* infection which causes a genital disease in sheep [26].

According to the results of the study, *B. canis* RM6/66 and *B. canis* PBC-10-87 strains might be used in commercial large scale antigen production by using TLM. However, future studies that determine sensitivity and specificity of each test antigen comparatively in the field with large number of serum samples in carnivores, sheep and goats and in humans would be necessary to decide the best antigen for serologic diagnosis of infection caused by rough *brucella* strains.

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RESEARCH ARTICLE

Kuzu İzole Pulmoner Arterlerinde Gelişen Hipoksik Pulmoner Vazokonstriksiyonda G_i ve G_s Proteinlerinin Rolü [1]

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Özet

Hipoksik pulmoner vazokonstriksiyon akciğerlerde ventilasyon perfüzyon dengesini sağlayan fizyolojik uyum mekanizmasıdır. Hipoksemi ile seyreden progressif akciğer hastalıklarının patogenezinde önemli rol oynamakla birlikte sağlıklı bireylerde de görülmektedir. Yoğun çalışmalara rağmen, hipoksik pulmoner vazokonstriksiyonun mekanizması henüz bulunamamıştır. G proteinleri vasküler endotel ve düz kas hücrelerinde reseptör aracılıklı sinyal iletimine aracılık ederek vasküler tonusun düzenlenmesinde önemli rol oynayan membrana bağlı protein ailesidir. Biz bu çalışmada, kuzu izole pulmoner arterlerinde izlenen hipoksik vazokonstriksiyonun oluş mekanizmasında G_i ve G_s proteinlerinin rolünü saptamayı amaçladık. Pulmoner arterler yeni kesilmiş kuzuların sol akciğer alt loblarından izole edildi. Arterler Krebs-Henseleit solüsyonu ile dolu olan izole organ banyosuna asıldı ve izometrik kontraksiyonlar bilgisayarlı poligrafi sistemi ile bağlantılı olan izometrik transdüsır yoluyla devamlı olarak kaydedildi. Solüsyon %75 N_2 - %20 O_2 - %5 CO_2 (Normoksik) ve %95 N_2 - %5 CO_2 (Hipoksik) karışımı içeren gazlar ile havalandırıldı. Organ banyosundaki parsiyel oksijen konsantrasyonu oksijen elektrodu kullanılarak sürekli olarak ölçüldü. G_i ve G_s proteinlerin rolünü araştırmak için kolera ve pertusis toksini kullanıldı. Çalışmada, dinlenme gerimindeki geniş çaplı pulmoner arterlerde hipoksiye bağlı bir gerim artışı izlenmedi ancak hipoksi 3 μM 5-HT ile prekontrakte edilmiş dokularda kontraksiyona (1.7±0.5 mN/mm², n=10) sebep oldu. Hipoksik vazokonstriksiyon 2 μg/ml kolera toksini ile inkübasyon sonrası inhibe (2.6±0.4 mN/mm²'den 1.0±0.4 mN/mm²'ye kadar, n=6) ve 2 μg/ml pertusis toksini ile inkübasyon sonrası potansiyalize (0.6±0.4 mN/mm²'den 1.7±0.3 mN/mm²'ye kadar, n=6) oldu. Bu sonuçlar G_i ve G_s proteinleri ile ilişkili sinyal iletiminin geniş çaplı kuzu izole pulmoner arterinde gelişen hipoksik vazokonstriksiyonda önemli bir mekanizma olabileceğini gösterdi.

Anahtar sözcükler: *Hipoksi, Pulmoner vazokonstriksiyon, Kolera toksini, Pertusis toksini, G proteinleri, Kuzu*

The Role of G_i and G_s Proteins in Hypoxic Vasoconstriction of Lamb Isolated Pulmonary Artery Rings

Summary

Hypoxic pulmonary vasoconstriction (HPV) is an intrapulmonary adaptive mechanism that matches alveolar ventilation to perfusion. However during prolonged alveolar hypoxia HPV occurs with many pulmonary diseases. Despite intensive studies, cellular mechanisms of HPV are still not well defined. G proteins are a family of membrane-associated proteins believed to be involved in the transduction of various signals including the regulation of vascular tonus. In this study, we aimed to determine the contribution of G_i and G_s proteins in hypoxic vasoconstriction of lamb isolated pulmonary artery rings. Pulmonary arteries were isolated from left lower lobe of freshly slaughtered lamb. Arteries suspended in an organ bath filled with Krebs-Henseleit solution and isometric contraction recorded continuously via an isometric transducer connected to a computerised polygraphy system. The solution aerated with 75% N_2 - 20% O_2 - 5% CO_2 (normoxic) and 95% N_2 - 5% CO_2 (hypoxic) pO_2 of bathing medium was measured continuosly using an oxygen electrode. Pertussis toxin and cholera toxin were used to investigate the role of G_i and G_s proteins. In the present study, we observed that hypoxia had no effect on resting force in large artery rings, but it caused a further contraction (1.7±0.5 mN/mm², n=10) in 3 μM 5-HT precontracted pulmonary arteries rings. Hypoxic vasoconstriction was inhibited by preincubation with 2 μg/ml cholera toxins (from 2.6±0.4 mN/mm², to 1.0±0.4 mN/mm², n=6) and potentiated by preincubation with pertussis toxins (2 μg/ml) (from 0.6 ± 0.4 mN/mm², to 1.7±0.3 mN/mm², n=6). These results indicate that signal transduction mediated by G_i and G_s proteins may be an important mechanism in the hypoxic vasoconstriction in lamb isolated large pulmonary arteries.

Keywords: *Hypoxia, Pulmonary vasoconstriction, Cholera toxins, Pertussis toxins G-proteins, Lamb*

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GİRİŞ

Hipoksik pulmoner vazokonstriksiyon (HPV) hipoksiye karşı pulmoner arterlerde ortaya çıkan fizyolojik adaptasyon mekanizmasıdır. HPV ile kötü ventilasyonlu akciğer bölgelerindeki kan akımı ventilasyonu normal olan bölgelere doğru yönlendirilir ve arteriyel hipoksemi minimale indirilmeye çalışılır. Ancak HPV'nin sürekli olması ile pulmoner arterlerde direnç artar ve pulmoner hipertansiyon (PHT) ortaya çıkar [1].

HPV'nin mekanizması son yarım yüzyıldır birçok araştırmaya konu olmuştur. Hipoksik vazokonstriksiyon balık, amfibi, kuş ve memeli gibi birçok türde gösterilmiştir. Bu deneysel çalışmalarda hipoksi süresinin uzunluğu, canlı türü ve üzerinde çalışılan arterlerin büyüklüğü ile farklılık gösteren çeşitli mekanizmalar öne sürülmüştür [2]. Çoğu araştırmada HPV'nin pulmoner vasküler endotelden salınan çeşitli mediatörlere bağlı geliştiği ya da hipoksinin pulmoner vasküler düz kas üzerinde yaptığı doğrudan etki ile ortaya çıktığı ileri sürülmüştür [1]. Ancak, sonraki çalışmalarda pulmoner vasküler düz kas hücrelerindeki oksijene duyarlı iyon kanallarının ve lokal nöral kontrol mekanizmalarının da önemli rolü olduğu ortaya konmuştur [3].

Ökaryot canlıların hücrelerinde izlenen reseptör aracılıklı sinyal iletiminde çeşitli proteinler rol oynamaktadır. Guanin nükleotit bağlayıcı proteinler (G proteinleri) reseptör aracılıklı sinyal iletiminde rol oynayan ve bu yolla hücre fonksiyonlarını etkileyen membrana bağlı protein grubudur. Bu proteinler yoluyla iyon kanal aktivitesi, veziküler transport ve nörotransmitter salınımı gibi birçok hücre fonksiyonu değiştirilebilmektedir [4]. G proteinleri vasküler endotel ve düz kas hücrelerinde reseptör aracılıklı sinyal iletimine aracılık ederek vasküler tonusun düzenlenmesinde önemli rol oynamaktadırlar [5]. Ancak literatürde pulmoner vasküler yapılarda gelişen HPV'nin oluşum mekanizmasında G proteinlerinin rolünü inceleyen yeterli sayıda çalışma bulunmamaktadır.

G proteinleri kolera toksini (CTX) ve pertusis toksini (PTX) gibi çeşitli bakteriyel toksinlere maruz bırakıldığında yapısal değişimlere uğramaktadır [6]. CTX G_i proteininin α subünitindeki arjinin rezidüsünü etkileyerek G_i proteininde kalıcı aktivasyona, PTX ise G_s proteininin α subünitindeki sistein rezidüsünü etkileyerek G_s proteininde inaktivasyona yol açmaktadır [7]. Bu yapısal değişimler ile G protein fonksiyonları etkilenmekte, pulmoner vasküler endotel ve düz kas fonksiyonları değişmekte ve beraberinde HPV'nin şiddetinde farklılıklar ortaya çıkmaktadır [5].

Sunulan çalışmada, CTX ve PTX kullanarak kuzu izole pulmoner arterlerinde izlenen hipoksik vazokonstriksiyonda G_i ve G_s proteinlerinin rolünün saptanması amaçlanmıştır.

MATERYAL ve METOT

Deneyler Düzce Belediyesi mezbahasında günlük olarak kesilen kuzuların akciğerlerinden izole edilen pulmoner arterlerde gerçekleştirildi. Çalışma 4-9 aylık kuzularda gerçekleştirildi. Deneyler sırasında yaklaşık 90 kuzu akciğeri kullanılarak çalışma 6 aylık bir dönemde tamamlandı. Kullanılan bütün kimyasal maddeler Sigma Chemical Co' dan (St. Louis USA) sağlandı ve kimyasal maddeler distile suda çözüldü.

Pulmoner Arterlerin Disseksiyonu ve İzole Organ Banyosuna Asılması

Pulmoner arterler günlük olarak kesilen kuzuların sol akciğer alt lobunun ilk dalından izole edildi. İzole edilen arterlerin çapları yaklaşık 0.3-0.4 cm, uzunlukları 0.3-0.5 cm idi. Arterler halka şeklinde kesildi, yağ ve bağ dokudan temizlendi. İzolasyondan sonra arter dokuları Krebs-Heinseleit solüsyonu içeren bir petri içine alındı. İzole edilen arterler izole organ banyosuna çengeller yardımıyla asıldı. Deneylere pulmoner arterlerde önceden belirlenen 5 g optimum dinlenme gerimiyle devam edildi. İzole organ banyoları 10 ml Krebs-Heinseleit çözeltisi içermektedir ve %75 N_2, %20 O_2 ve %5 CO_2 (normoksik koşullar) karışımı içeren gaz ile havalandırılmıştır. Hipoksik koşullar ise %95 N_2 ve %5 CO_2 gaz karışımı ile sağlanmıştır.

İzometrik Kasılmaların Saptanması ve Hesaplanması

Pulmoner arter dokularındaki kasılma değerleri sürekli olarak Biopac Student Lab (BSL) bilgisayar programından izlendi ve kaydedildi. Kasılma değerleri milinewton (mN) cinsinden ölçüldü. Ancak her dokunun kesit alanı dolayısıyla kasılma gücü farklı olacağı için hesaplamalarımızda BSL programından elde edilen kasılma değerleri değil formülle hesaplanan izometrik kasılma değerleri kullanıldı. İzometrik kasılmalar aşağıdaki formüle uygun olarak BSL sisteminden elde edilen kasılma değerinin arterin kesit alanına (mm^2) bölünmesi ile saptandı [8,9].

$$\text{İzometrik kasılma } (mN/mm^2) = \frac{\text{Kasılma değeri (mN)}}{A}$$

Bu formülü hesaplamak için aşağıdaki formüle göre hesaplanan A değeri kullanıldı.

$$A = \frac{W}{h \times \beta}$$

A: Arterin enine kesit alanı

β: Arter halkasının yoğunluğu (Koyun karotit arterinde 1.05 mg/mm^3olduğu bildirilmiştir [10])

W: Filtre kağıdında kurutulmuş arterin ağırlığı (mg)

h: Optimum dinlenme geriminde damarın iki çengel arasındaki uzunluğu (mm)

Prekontraktil Agonistin Saptanması

Pulmoner arterlerde hipoksinin etkisi damarın ön gerimine bağlı olarak değişmektedir. Bu nedenle hipoksiye bağlı gelişen vazokonstriksiyonu daha kolay tespit etmek için prekontraktil bir madde kullanımı yararlı olmaktadır [11]. Yapılan deneysel çalışmalarda pulmoner arterlerde histamin, noradrenalin, ET-1, sodyum florid ve 5-HT gibi maddeler prekontraktil agonist olarak kullanılmıştır. Çalışmamızda ise 5-HT kasılma süresi ve büyüklüğü ile uygun profil göstermesi nedeni ile prekontraktil agonist olarak seçildi. Deneylerimizde kullanılacak olan 5-HT'nin EC_{50} değeri 3 μM olarak belirlendi.

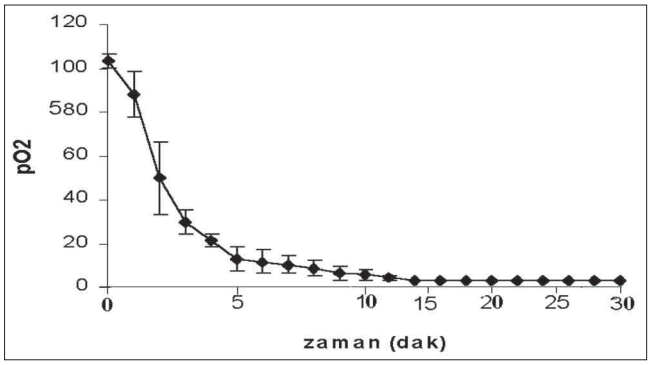
Deney Protokolü

İzole edilen damarlar optimum dinlenme geriminde 1 saat dengelenmeye bırakıldı. Daha sonra izole edilmiş pulmoner arterlerin deneye uygun ve kasılan bir doku olup olmadığının tesbiti için iki kez ardarda 40 μM KCl uygulaması yapıldı. Ardarda benzer kasılma yanıtı alındıktan sonra deneylere geçildi.

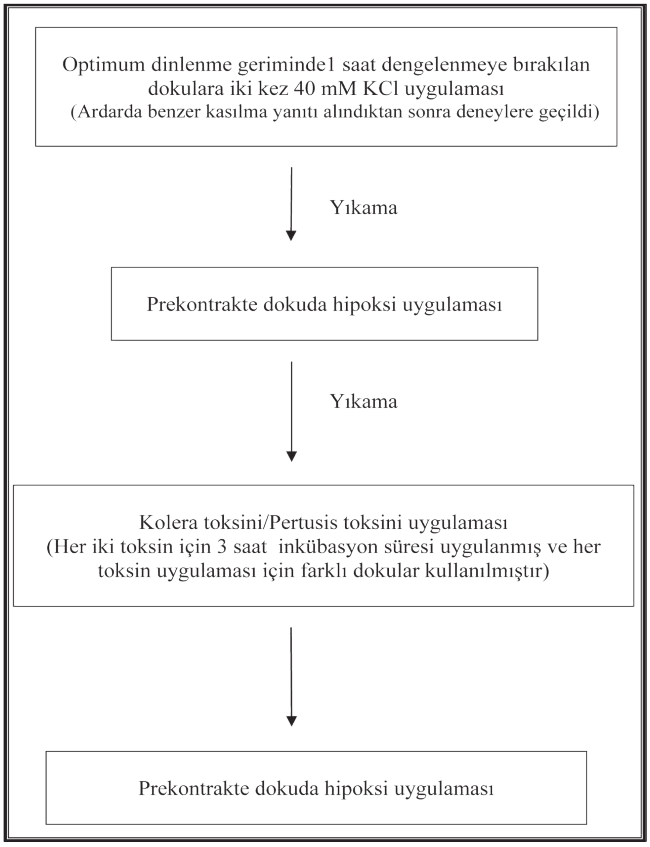
Deneylerimizde öncelikle kuzu izole pulmoner arterlerinde normoksi ve hipoksiye bağlı gerim değişiklikleri hem optimum dinlenme geriminde hem de 3 μM 5-HT ile prekontrakte dokularda araştırıldı. Her iki doku önce 30 dak. süre ile normoksik gaz karışımı ile (%75 N_2, %20 O_2 ve %5 CO_2) daha sonra 30 dak. boyunca hipoksik gaz karışımı ile (%5 CO_2-% 95 N_2) havalandırıldı. Normoksi ve hipoksi uygulaması sırasında dakikalık gerim değişiklikleri kaydedildi ve ortalamaları hesaplandı. Elde edilen sonuçlar ortalama±standart sapma olarak hesaplandı ve iki ortalama arasındaki farkın anlamlılığının saptanması için student's t testi kullanıldı. P<0.05 değerler anlamlı kabul edildi.

Deneyler süresince oksijenmetre ile ortamın oksijen konsantrasyonu ölçüldü. Hipoksi uygulaması sonucu ortamın PaO_2 değerinin zamana karşı değişimi *Şekil 1*'de gösterildi.

Deneylerimizde CTX ve PTX toksininin HPV gelişmiş kuzu izole pulmoner arterleri üzerindeki etkisinin gösterilmesi için *Şekil 2*'deki protokol uygulandı. Bu protokole göre kuzu izole pulmoner arterleri öncelikle 3 μM 5-HT ile



Şekil 1. 30 dakikalık hipoksi uygulaması boyunca değişen pO_2 değerleri
Fig 1. Changes in pO_2 values during 30 min hypoxia



Şekil 2. Deney protokolü
Fig 2. Experimental protocol

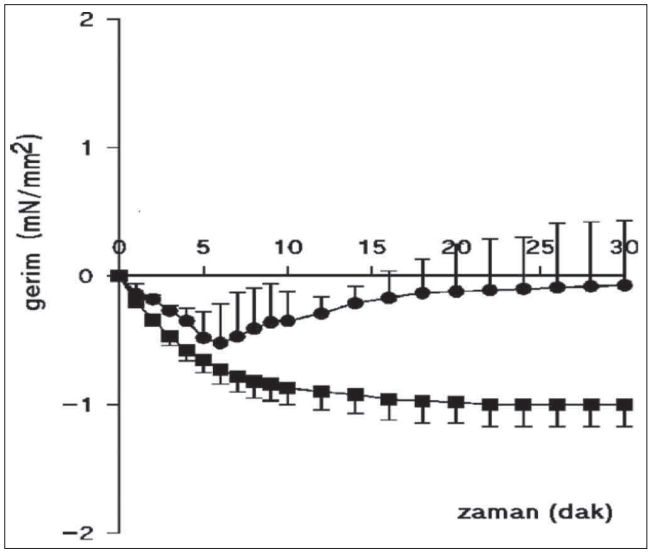
prekontrakte edildi ve bu dokulara 30 dak. süre ile hipoksi uygulandı. Bu işlemlerden sonra normoksik koşullara geri dönüldü ve yıkama işlemi yapıldı. Daha sonra dokular kolera ve pertusis toksini ile 3 saat inkübasyona bırakıldı. CTX ya da PTX 2 μM/ml konsantrasyonlarda kullanıldı [12,13]. İnkübasyon süresi bitiminde dokular 3 μM 5-HT ile tekrar prekontrakte edildi ve dokulara 30 dak. hipoksi uygulaması yeniden yapıldı (5-HT ile prekontrakte edilen dokular, ortalama 5 dak. süren optimum prekontraksiyon sağlanıncaya kadar yani kasılma eğrisi plato çizene kadar normoksik gaz ile havalandırılmıştır ve daha sonra hipoksi uygulamasına geçilmiştir). Normoksik koşullarda prekontraksiyon sonrası elde edilen maksimum kasılma değerlerinin ortalamaları ile prekontrakte dokuda hipoksi uygulaması sonrası elde edilen maksimum kasılma değerlerinin ortalamaları alınarak, bu ortalamalar arasındaki fark hesaplandı. Bu işlem her iki toksin verilmeden önce ve verildikten sonra ayrı ayrı yapıldı. Bulunan her iki farkın (Toksin verilmesinin öncesi ve sonrası) arasındaki anlamlılığının saptanması için student's t testi kullanıldı. P<0.05 değerleri anlamlı kabul edildi. Tüm hesaplamalar instat programı kullanılarak yapıldı.

BULGULAR

Kuzu İzole Pulmoner Arterlerinde Hipoksinin Etkisi

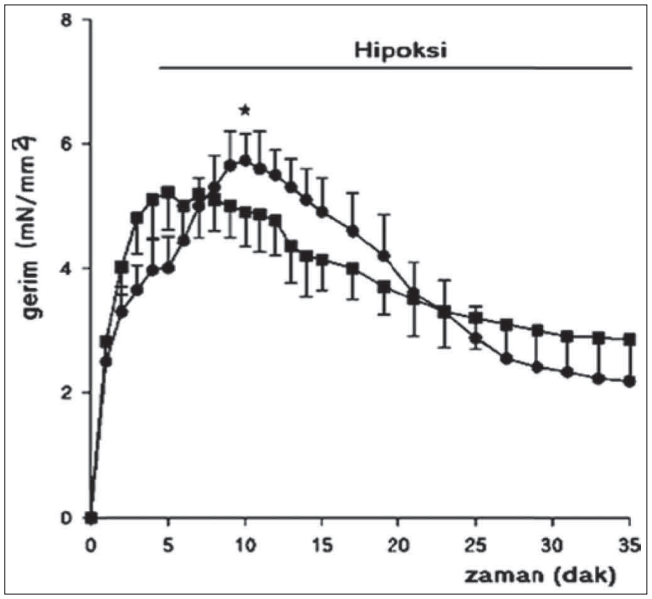
Dinlenme Gerimindeki Kuzu İzole Pulmoner Arterlerinde

Hipoksinin Etkisi: Dinlenme gerimindeki geniş çaplı izole pulmoner arterlerde hipoksiye bağlı bir gerim artışı belirlenememiştir *(Şekil 3)*. Bu arterlerde hipoksi boyunca -0.25±0.12 N/mm^2 (n=10) gerim kaybı olurken, normoksi uygulaması sonunda -1.03±0.17 N/mm^2 (n=10) gerim kaybı gerçekleşmiştir. Bu sonuçlar istatistiksel olarak anlamlı bulunmamıştır.



Şekil 3. Dinlenme gerimindeki izole pulmoner arterlerde hipoksi uygulaması; g Normoksi boyunca izole pulmoner arterlerde oluşan gerim değişikliği (n=10), nHipoksi boyunca izole pulmoner arterlerde oluşan gerim değişikliği (n=10)

Fig 3. Hypoxia in resting force on isolated pulmonary arteries; g Changes of force in isolated pulmonary arteries during normoxia (n=10), n Changes of force in isolated pulmonary arteries during hypoxia (n=10)



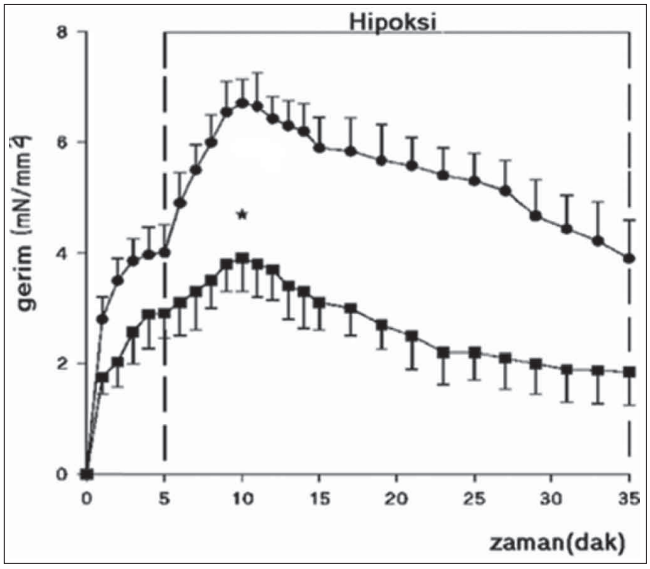
Şekil 4. 3 mM 5-HT ile prekontrakte edilmiş izole pulmoner arterlerde hipoksinin etkisi; g Normoksi boyunca 5-HT'nin oluşturduğu kasılma (n=10), n 5-HT ile prekontraksiyon sonrası 30 dak. hipoksi uygulaması ile oluşan HPV (n=10)

Fig 4. The effect of hypoxia on isolated pulmonary arteries precontracted by 3mM 5-HT: g 5-HT inuced contraction during normoxia (n=10), n Changes of force in isolated pulmonary arteries during hypoxia (n=10). HPV during 30 min hypoxia after precontracted by 3 mM 5-HT

Kuzu Prekontrakte Pulmoner Arterlerinde Hipoksinin Etkisi: 3 μM 5-HT ile prekontrakte edilen geniş çaplı kuzu izole pulmoner arterlerinde prekontraksiyonun platoya ulaştığı anda başlatılan ile hipoksi uygulaması (30 dak.) kontraksiyona neden olmuştur (1.7±0.5 mN/mm^2, n=10). Normoksi esnasında ise bu kontraksiyon 0.10±0.4 mN/mm^2 (n=10) olarak gerçekleşmiştir *(Şekil 4)*.

Geniş Çaplı Kuzu İzole Pulmoner Arterlerinde Kolera ve Pertusis Toksininin HPV Üzerine Etkisi

Kolera Toksininin HPV Üzerine Etkisi: Prekontrakte kuzu izole pulmoner arterlerinde oluşan kontraksiyon (2.6±0.4 mN/mm^2, n=6) 2 mg/ml kolera toksini varlığında inhibe olmuştur (1.0±0.4 mN/mm^2, n=6) *(Şekil 5)*.



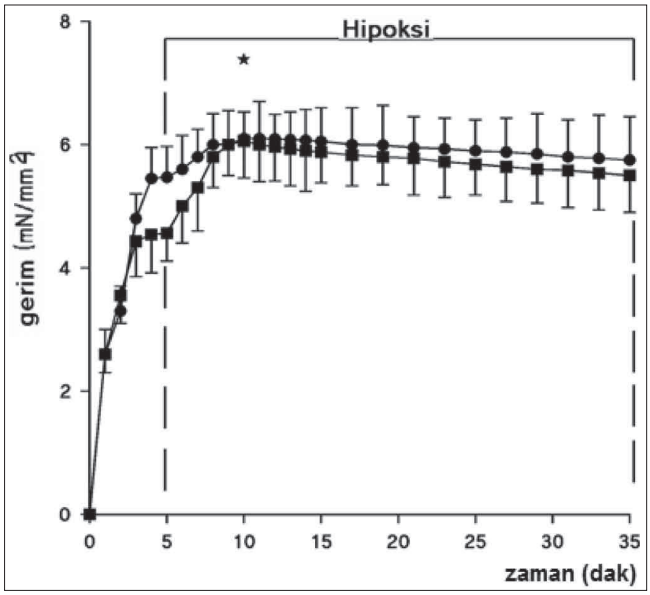
Şekil 5. 3 mM 5-HT ile prekontrakte edilmiş izole pulmoner arterlerde kolera toksininin HPV üzerine etkisi; n 5-HT ile prekontrakte izole pulmoner arterlerde hipoksi uygulaması (n=6), g 2 mg/ml kolera toksini varlığında 5-HT ile prekontrakte izole pulmoner arterlerde hipoksi uygulaması (n=6)

Fig 5. The effect of cholera toxin on HPV in isolated pulmonary arteries precontracted by 3 mM 5-HT; g Hypoxia application to isolated pulmonary arteries precontracted by 5-HT (n=6), n Hypoxia application to isolated pulmonary arteries precontracted by 5-HT in the presence of 2 mg/ml cholera toxin (n=6)

Pertussis Toksininin HPV Üzerine Etkisi: Prekontrakte pulmoner arterlerde hipoksi uygulamasına bağlı gelişen kasılma yanıtı, pertusis toksini (2 mg/ml) ile inkübe edildikten sonra potansiyalize olmuştur (sırasıyla 0.6±0.4 mN/mm^2, 1.7±0.3 mN/mm^2, n=6) *(Şekil 6)*.

TARTIŞMA ve SONUÇ

Sunulan bu araştırmada izole pulmoner arterlerde HPV oluşum mekanizmasında G_i ve G_s proteinlerinin rolünü araştırmak amaçlanmıştır. Bu amaçla önce kuzu pulmoner



Şekil 6. 3 mM 5-HT ile prekontrakte edilmiş izole pulmoner arterlerde pertusis toksininin HPV üzerine etkisi; n 5-HT ile prekontrakte izole pulmoner arterlerde hipoksi uygulaması (n=6), g 2 mg/ml pertusis toksini varlığında 5-HT ile prekontrakte izole pulmoner arterlerde hipoksi uygulaması (n=6)

Fig 6. The effect of pertusis toxin on HPV in isolated pulmonary arteries precontracted by 3 mM 5-HT; g Hypoxia application to isolated pulmonary arteries precontracted by 5-HT (n=6), n Hypoxia application to isolated pulmonary arteries precontracted by 5-HT in the presence of 2 mg/ml pertusis toxin (n=6)

arterlerinde hipoksinin etkisi belirlenmiştir. Ardından prekontrakte pulmoner arterlerde kolera toksinine bağlı olarak HPV'nin inhibe olduğu ama pertusis toksini varlığında hipoksiye bağlı kasılmanın arttığı belirlenmiştir.

Araştırmamızda geniş çaplı pulmoner arterler kullanılmıştır ve çapları yaklaşık 3-4 mm'dir. Oysa literatürde HPV'nin asıl bölgesinin çoğunlukla dar çaplı pulmoner arterler olduğuna vurgu yapılmaktadır [14,15]. Bununla birlikte pulmoner arterlerin hipoksi ya da çeşitli vazokonstriktör mediyatörlere kasılma cevabının bölgesel farklılık gösterdiği de bilinmektedir [16]. Bu açıdan literatürdeki çalışmaları değerlendirdiğimizde HPV'nin sunulan bu çalışmada olduğu gibi geniş çaplı pulmoner arterlerde de hatta pulmoner venlerde de oluşabildiği dikkati çekmektedir [17,18]. İzole preparatlardaki çalışmalar açısından aradaki fark dar çaplı pulmoner arterlerde genellikle prekontraksiyon gerekmeksizin hipoksiye kasılma yanıtının alınabilmesidir [19]. Diğer taraftan dinlenme gerimindeki koyun izole pulmoner arterlerinde yapılan bir çalışmada burada sunulan araştırmaya benzer şekilde geniş çaplı pulmoner arterlerde hipoksinin prekontraksiyon olmaksızın kasılmaya neden olmadığı da gösterilmiştir [17]. Bizim bulgularımız da bu sonucu desteklemektedir. Bu konuda başka bulgular da vardır. Örneğin, 20 dak. hipoksiye maruz bırakılmış dar (0.38-0.68 mm) ve geniş çaplı (2.2-4.5 mm) insan pulmoner arterlerinde yapılan başka bir çalışmada dinlenme gerimiinde benzer bir hipoksik vazokonstriksiyon gözlenmiştir [20]. Pulmoner arterler içerisindeki düz kas hücreleri değişik tiptedir. Hücrelerin büyüklüğü, biçimi, organel içeriği ve kontraktil proteinleri farklıdır [21]. Rat aortik hücre kültürlerinde yapılan bir çalışmada epiteloid ve iğsi olmak üzere ilk defa iki farklı hücre yapısı ortaya konulmuştur [22]. Epiteloid hücreler vazokonstriktör cevaptan sorumludur ve K_{ca} kanallarından zengindir. K_{ca} kanalları hücre içi Ca^{+2} düzeyini artırmaktadır. ET-1 gibi vazokonstriktör ajanlar iğsi hücreleri depolarize, epiteloid hücreleri ise hiperpolarize ederek hücre içi kalsiyum düzeylerini artırmaktadır. Öküz pulmoner arterlerinde yapılan diğer bir çalışmada ise immunolojik, morfolojik ve sitoskeletal protein dağılımı açısından 4 farklı hücre fenotipi tanımlamıştır [21]. Bu çalışmalar bize hipoksiye karşı pulmoner damar yatağında neden farklı yanıtların ortaya çıktığını açıklamaktadır.

Pulmoner arterlerde hipoksinin etkisi damarın ön gerimine bağlı olarak değişmektedir. Bu nedenle hipoksiye bağlı gelişen vazokonstriksiyonu daha kolay tespit etmek için prekontraktil bir madde kullanımı yararlı olmaktadır. Rodman ve ark.[11] izole rat pulmoner arterleri üzerinde yaptıkları çalışmada norepinefrin, angiotensin II, fenilefrin, U46619 gibi prekontraktil maddelerin kullanılması ile kontrol grubuna göre hipoksik vazokonstriksiyonun daha şiddetli olduğunu göstermişlerdir. Aynı çalışmada HPV'nin çeşitli türlerde sadece prekontraktil ajan kullanımı sonrası ortaya çıktığı ileri sürülmüştür. Çalışmamızda 5-HT kasılma süresi ve büyüklüğü ile uygun profil göstermesi nedeni ile prekontraktil agonist olarak seçilmiştir. 3 μM 5-HT ile prekontrakte edilen geniş çaplı kuzu izole pulmoner arterlerinde 30 dak. süre ile hipoksi uygulaması kontraksiyona neden olmuştur. Bu sonuç geniş çaplı kuzu izole pulmoner arterlerinde de hipoksik vazokonstriksiyonun meydana geldiğini ve HPV'nin ortaya çıkarılması için en azından kuzu pulmoner arterlerinde prekontraktil agoniste ihtiyaç olduğunu göstermektedir. Prekontrakte agonist kullanımı HPV'nin gelişiminde asıl rolü oynamamakta sadece HPV'nin şiddetinin artırmaktadır [23].

G proteinleri reseptör aracılı sinyallere aracılık ederler ve böylece uyarana bağlı olarak hücrenin cevabının oluşumunu sağlarlar. Düz kasın kontraksiyonunda G proteinlerinin rolünü anlamaya yönelik çok sayıda çalışma bulunmaktadır. Örneğin, ratlarda yapılan bir çalışmada kan basıncının regülasyonunda G_s proteinleri ile ilişkili α_1 adrenoreseptörler sorumlu tutulmuştur [24]. İnsan koroner arterleri üzerinde yapılan diğer bir çalışmada adenozin reseptörü içeren koroner arterlerde G_s proteini aracılığı ile vazodilatasyon meydana geldiği gösterilmiştir [25]. Düz kas kasılmasında önemli rolü olan K^+ kanallarına bağlı asetilkolin, histamin gibi bazı ligand reseptörleri ile G proteini ilişkilidir [26]. G_s proteini yine K_{Ca} üzerinde doğrudan etki göstererek düz kasları gevşetmektedir. Bununla beraber G_s proteini ile ilişkili olarak PGE_2, prostasiklin ve vazoaktif intestinal peptid düz kas reseptörleri saptanmıştır [27]. Bu çalışmalar G_s proteinleri ile vasküler yapılar arasındaki ilişkiyi ortaya koymaktadır. Bununla birlikte HPV'de bu proteinlerin doğrudan rolü üzerine bir araştırma bulunmaktadır [28].

Bu araştırmada bizim çalışmamızda olduğu gibi CTX kullanılmış ve koyun izole pulmoner arterlerinde HPV yanıtı üzerine etkisi araştırılmıştır. CTX G_s proteininde bulunan α subünitini etkileyerek bu proteinde kalıcı aktivasyona yol açmaktadır [7]. Sonuçta G_s proteininin ADP ribosilasyonunu katalize ederek adenilil siklazı aktive etmektedir [29]. Nitekim G proteinlerini aktive eden NaF varlığında da HPV inhibe olmaktadır [28]. Sonuç olarak araştırmamız kuzu izole pulmoner arterlerinde de adenilil siklaz aktivasyonunun HPV'yi inhibe ettiğini doğrulamaktadır.

Araştırmamızda HPV'nin oluşum mekanizmalarında G_s protein ile beraber G_i proteininin rolü de incelenmiştir. Bunun için G_i proteininin α subünitindeki sistein rezidüsünün ribozilasyonunu katalizleyen ve G_i proteininde inaktivasyona yol açan PTX kullanılmıştır. Deneylerimizde prekontrakte dokularda izlenen hipoksik vazokonstriksiyon yanıtının PTX (2 mg/ml) ile 3 saat inkübe edildikten sonra potansiyalize olduğu belirlenmiştir. Bu sonuç HPV oluşum mekanizmasında G_i proteinin rolü olabileceğini ortaya koymuştur. Bugüne kadar hipoksiye bağlı pulmoner vazokonstriksiyon yanıtında G_i proteinlerinin rolünü değerlendiren yeterli sayıda araştırma yoktur. Sweeny ve ark. nın yaptıkları bir çalışmada 5-HT ile prekontrakte edilmiş ve hipoksiye maruz bırakılmış inek pulmoner arterlerinde G_i proteininin aracılık ettiği bir mekanizma ile vasküler düz kas kasılmasını etkileyen cAMP düzeylerinin azaldığı gösterilmiştir [30]. Bu araştırma sonucu bizim bulgularımızı desteklemektedir. Diğer tarftan CTX ile yaptığımız deneylerde elde ettiğimiz sonuçlarla da uyumludur. PTX duyarlı G_i proteini pulmoner dolaşım dışında birçok vasküler yatak endoteli ve düz kas hücreleri üzerinde saptanmıştır [31]. Bu proteinler düz kas kasılmasında önemli rolleri olan iyon kanallarının aktivitesini değiştirmekte ve çeşitli mediyatörlerin vasküler yapılar üzerinde oluşturdukları etkilere aracılık etmektedir. Albert ve ark.[32] G_i proteininin birden fazla potasyum kanalı için spesifik G proteini olduğunu ve hücre içine potasyum akımı sağladığını ileri sürmüşlerdir. Diğer bir çalışmada PTX sensitif G_i proteinleri yoluyla Ca^{+2} ve K^+ kanallarında stimulasyon meydana geldiği vurgulanmıştır [25]. Rat ventrikül myositlerinde ise ventriküler K_{ATP} kanallarının G_i proteini yoluyla aktive edildiği gösterilmiştir [33]. İzole edilmiş domuz koroner arterinde NaF gibi G protein aktivatörleri ile EDRF-NO salıverilmesi artmakta ve endotel bağımlı vazodilatasyon sağlanmaktadır. PTX yoluyla bu aktivasyon engellenmektedir [34]. Köpek koroner arter endotelinde yapılan bir çalışmada serotonin resepörleri ile ilişkili PTX sensitif G proteinleri gösterilmiştir. Yine kronik hipoksemiye maruz bırakılmış neonatal kuzu kardiyak miyositlerinde β adrenoseptörlerde azalma ve G_i proteinleri ile ilişkili M_2 muskarinik reseptörlerin duyarlılığında artma saptanmıştır [35]. Akut hipoksiye maruz bırakılmış inek pulmoner arter adventisyasındaki fibroblastlarda G_i proteinlerinin aktive olduğu ortaya konmuştur [36]. Yapılan bu araştırmalar bizim çalışmamızda olduğu gibi hipoksinin vasküler yapılar üzerindeki etkilerine G_i proteini aracılıklı mekanizmaların da katılabileceğini göstermektedir.

Sonuç olarak kuzu izole pulmoner arterlerinde yaptığımız bu çalışma HPV'nin mekanizmasında G_s proteinlerinin rolünü bir kez daha doğrulamaktadır. Diğer taraftan G_i proteinlerinin rolü olabileceğini de ilk kez göstermektedir. Bu mekanizmaların ayrıntılarının açıklanabilmesi için başka çalışmalara gereksinim olduğu açıktır. HPV'nin mekanizmasının aydınlanması hipoksemik akciğer hastalıklarının tedavisine yeni yaklaşımlar sağlayacaktır.

Teşekkür

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RESEARCH ARTICLE

Zavot Irkı Sığırlarda Koroner Arterler ve Kalp Kası Köprüleri [1]

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Özet

Bu çalışma, Zavot ırkı sığırların kalbindeki koroner sirkülasyonu ve koroner arterler üzerinde bulunan kalp kası köprüsünün makroskobik ve mikroskobik özelliklerini belirlemek amacı ile yapıldı. Çalışmada 10 adet ergin Zavot ırkı sığır kalbi kullanıldı. Kalbin koroner arterlerini ortaya çıkarmak için a. coronaria sinistra ve a. coronaria dextra'dan renklendirilmiş latex enjekte edildi. Kalbin arteriyel vaskularizasyonunun aorta ascendens'ten orijin alan a. coronaria sinistra ve a. coronaria dextra tarafından sağlandığı tespit edildi. A. coronaria sinistra'nın çapının a. coronaria dextra'dan daha büyük, dallarının ise daha fazla olduğu belirlendi. Sağ ve sol koroner arter arasındaki anastomozların r. circumflexus sinister ile r. circumflexus dexter, r. interventricularis paraconalis ile a. coronaria dextra'nın ventriküler dalları ve r. coni arteriosi'ler arasında olduğu saptandı. Septum interventriculare'nin r. interventricularis paraconalis ile r. interventricularis subsinuosus'dan orijin alan rr. septales tarafından beslendiği gözlendi. Kalp kası köprüleri genişlikleri 6.39 mm ile 30.88 mm ve kalınlıkları 1.34 mm ile 6.25 mm olarak tespit edildi. R. marginis ventricularis sinistri üzerinde kalp kası köprüsüne rastlanılmadı. Kalp kası köprülerindeki kas liflerinin normal kalp kası liflerine benzer şekilde merkezi konumlu tek çekirdeğe ve enine bantlaşmalara sahip olduğu, bağlantı bölgelerinde interkalat disklerin bulunduğu görüldü. Ayrıca köprü içerisinde bulunan artere eşlik eden vena da tespit edildi.

Anahtar sözcükler: *Koroner arter, Kalp kası köprüsü, Zavot ırkı sığır*

The Coronary Arteries and Myocardial Bridges in Zavot-Breed Cattle

Summary

This study was conducted to determine of the coronary circulation and macroscopic and microscopic properties of myocardial bridge on coronary arteries in Zavot-breed cattle's heart. In this study, 10 hearts, adult Zavot breed cattle, were used. Colored latex was injected into a. coronaria sinistra and a. coronaria dextra to visualize coronary arteries of hearts. The arterial vascularization of the heart was determined to be supplied by a. coronaria sinistra and a. coronaria dextra which originated from the aorta ascendens. Diameter of the a. coronaria sinistra was larger than a. coronaria dextra and its branches had more. It was ascertained that anastomoses among the left and right coronary arteries were found between r. circumflexus sinister and r. circumflexus dexter, left and right r. coni arteriosi and ventricular branches of r. interventricularis paraconalis and a. coronaria dextra. It was investigated that septum interventriculare was supplied by rr. septales originated from r. interventricularis paraconalis and r. interventricularis subsinuosus. The thickness and width of myocardial bridges were determined as 6.39 mm to 30.88 mm and 1.34 mm to 6.25 mm, respectively. Myocardial bridge was not found above r. marginis ventricularis sinistri. It was seen that the muscle fibers in myocardial bridge had single central core and transverse-bands similar to the regular fibers and intercalated discs were also found in connection regions. It was determined that vein was accompanied with to artery in the bridge.

Keywords: *Coronary artery, Myocardial bridge, Zavot-Breed Cattle*

GİRİŞ

Ülkemizin iklim ve yer şekilleri özelliklerinin bölgesel olarak değişmesi hayvan yetiştiriciliğinde farklı kültür ırklarının ortaya çıkmasına neden olmuştur. Kış mevsiminin uzun ve soğuk geçtiği Kars, Erzurum ve Ardahan yörele-

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rinde bu iklim şartlarına uyum sağlayabilen Zavot ırkı sığır yetiştiriciliği yapılmaktadır. Bu ırk Simental, İsviçre esmeri ve bölgenin yerli ırklarının bir melezi olarak ortaya çıkmıştır [1,2].

Dolaşım sisteminin merkezi olan kalbin arteriyel vaskularizasyonu, a. coronaria dextra ve a. coronaria sinistra tarafından sağlanır. Bu arterlerden a. coronaria dextra valvula semilunaris dextra düzeyinde, a. coronaria sinistra ise valvula semilunaris sinistra düzeyinde aorta ascendens'den orijin alır [3-6].

Kalp kası köprüsü, koroner arterin subepikardiyal olarak seyreden bir dalının miyokard iplikleriyle sarılması sonucu oluşan anatomik bir yapıdır. Kalp kası köprüsü ateroskleroz, anjina pektoris, miyokardiyal iskemi, trombozis, miyokardiyal infarktüs ve ventriküler fibrilasyon gibi farklı kalp rahatsızlıklarına neden olabilir [7-10]. Kalp kası köprüsü genellikle facies atrialis'te, sulcus interventricularis subsinuosus üzerinde, facies auricularis de ise sulcus interventricularis paraconalis üzerinde yer alır [11,12].

Koroner arterler ile ilgili insan, kanatlı, laboratuar hayvanları ve evcil memeli hayvanlarda çok sayıda çalışma yapılmıştır [13-20]. Fakat Zavot ırkı sığırlarda koroner arterler ve kalp kası köprüleri hakkında bir araştırma bulunmamaktadır. Dolayısıyla yapılan bu çalışmada Zavot ırkı sığırlarda koroner arterler hakkındaki bilgi eksikliğinin giderilmesi ve mevcut bilgilere katkı sağlanması amaçlanmıştır.

MATERYAL VE METOT

Çalışmada materyal olarak Kars Belediyesi Mezbahasından cinsiyet ve yaş farkına bakılmaksızın elde edilen erişkin 10 adet Zavot ırkı sığır kalbi kullanıldı. Koroner arterler %0.9'luk tuzlu su ile yıkandıktan sonra kırmızı kumaş boyası (Artdeco) ile renklendirilmiş latex (ZPK-582-G Educational-Scientific Products Ltd. West Sussex, UK) enjekte edildi [24]. Latex enjekte edilen kalpler oda sıcaklığında bir gün bekletildi. Daha sonra bir hafta süre ile %10'luk formaldehit solüsyonu içerisine bırakıldı. Diseksiyon işlemlerini takiben kalpler incelenerek görüntülendi. Kalp kası köprülerinin genişlik ve kalınlık ölçümlerinde elektronik kumpas (BTS, U.K.) ile araştırma mikroskobu (Olympus BX51) kullanıldı. Histolojik incelemeler için alınan kalp dokusu örnekleri %10'luk formol solüsyonunda tespit edildikten sonra rutin histolojik yıkama ve takip aşamalarından geçirilerek parafinde bloklandı. Parafin bloklardan alınan 4 µm'lik kesitlere dokuların histolojik görünümlerini incelemek için üçlü boyama yapıldı [25]. Hazırlanan preparatlar araştırma mikroskobunda (Olympus BX51) incelenerek fotoğraflandı. Çalışmanın terminolojisinde Nomina Anatomica Veterinaria [26] esas alındı.

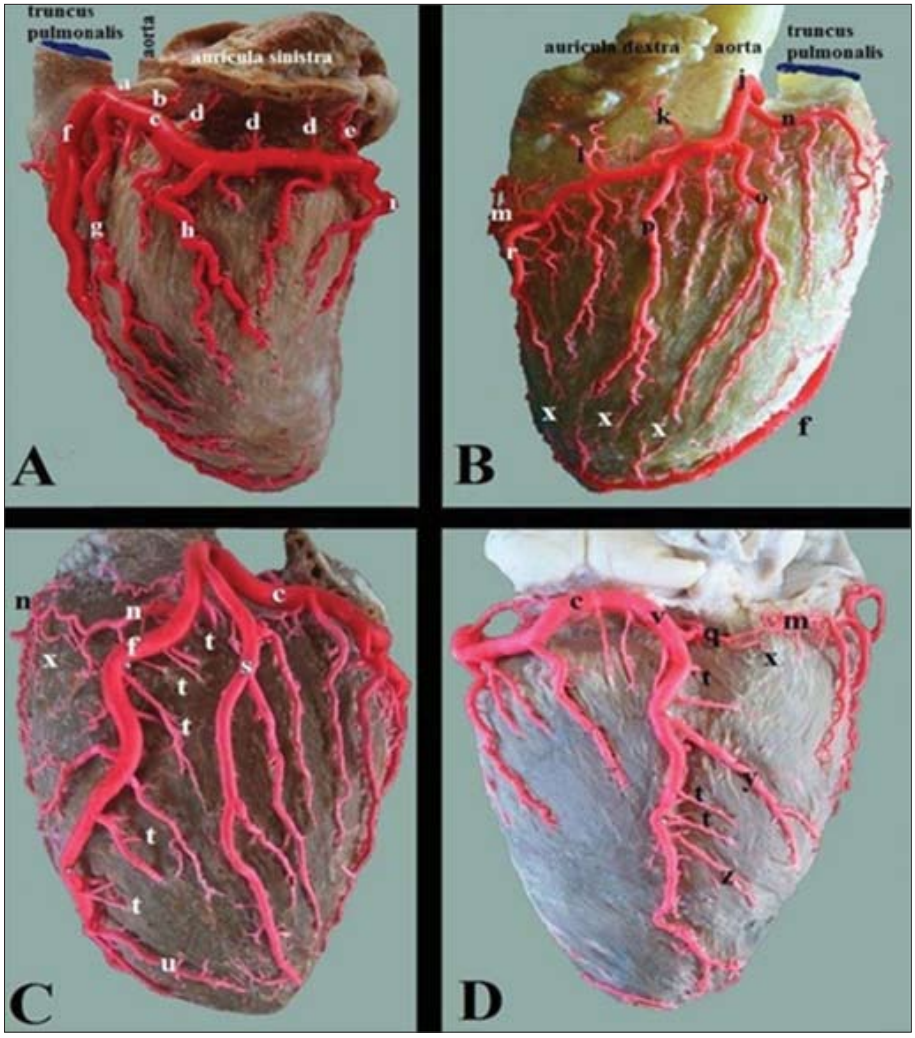
BULGULAR

A. coronaria sinistra'nın aorta ascendens'in başlangıç kısmında bulunan valvula seminularis sinistra'nın hemen üzerinden ortalama 8.93 mm çap ile tüm kalplerde tek bir dal halinde orijin aldığı gözlendi *(Şekil 1, a)*. A. coronaria sinistra'nın 10-15 mm sonra truncus pulmonalis ile auricula sinistra arasından geçerek, ortalama 6.68 mm çapında r. circumflexus sinister ve 6.87 mm çapındaki r. interventricularis paraconalis olmak üzere iki dala ayrıldığı belirlendi *(Şekil 1, c, f)*.

R. circumflexus sinister orijininden ortalama 17.9 mm sonra tüm kalplerde ilk olarak ortalama 3.11 mm çapındaki r. proximalis ventriculi sinistri'yi verdiği saptandı *(Şekil 1, g)*. R. proximalis ventriculi sinistri'nin 2 kalpte kalbin apex cordis'ine kadar uzandığı, diğer 8 kalpte ise ventriculus sinister duvarının orta seviyesinde sonlandığı gözlendi. R. circumflexus sinister'in dorsal duvarından ortalama 2.42 mm çapında r. proximalis atrii sinistri'nin orijin aldığı belirlendi *(Şekil 1, b)*.

Çalışmada r. proximalis atrii sinistri'nin üç dala ayrıldığı ve bu dallardan birinin auricula sinistra'nın medial yüzü ile aorta arasından geçerek atrium dextrum'un yakınlarına kadar uzandığı, diğer dalın ise caudal yönlü bir seyir izleyerek atrium sinistrum'un ön 1/3'ü civarında sonlandığı tespit edildi. R. circumflexus sinister'in r. proximalis atrii sinistri'den hemen sonra sayıları 3-5 arasında değişen ortalama 0.94 mm çapındaki r. intermedius atrii sinistri'leri verdiği belirlendi *(Şekil 1, d)*. R. intermedius atrii sinistri'lerin tüm kalplerde r. circumflexus sinister'in dorsal duvarından orijin aldığı ve atrium sinistrum üzerine dağıldığı saptandı. İlk r. intermedius atrii sinistri'nin, r. proximalis atrii sinistri'nin son dalı ile anastomoz yaptığı gözlendi. R. circumflexus sinister'in ventral duvarından ilk iki r. intermedius atrii sinistri'nin arasından ortalama 3.65 mm çapında r. marginis ventricularis sinistri'nin orijin aldığı tespit edildi *(Şekil 1, h)*. R. marginis ventricularis sinistri'nin orijininden sonra ventral bir seyir izlediği ve ventriculus sinister duvarının ortalarında iki dala ayrıldığı, bu dallardan birinin r. proximalis ventriculi sinistri'den gelen dalla anastomoz yaptığı tespit edildi. R. circumflexus sinister'in dorsal duvarından ortalama 1.87 mm çapında son atrial dal olan r. distalis atrii sinistri'nin orijin aldığı belirlendi *(Şekil 1, e)*. Bu dalın orijininden sonra dorsal bir seyir izlediği ve atrium sinistrum üzerine dağıldı tespit edildi. R. distalis atrii sinistri'den orijin alan bir dal ile r. ventriculi dextri'den ayrılan ince dalların anastomoz yaptığı belirlendi. R. distalis atrii sinistri'nin orijin aldığı yerin hemen karşısından ortalama 3.49 mm çapında r. distalis ventriculi sinistri'nin orijin aldığı gözlendi *(Şekil 1, ı)*. Bu dalın orijininden sonra ventral bir seyir izleyerek ventriculus sinister duvarının üst 1/3'ünde bir kaç dala ayrıldıktan sonra kas içerisinde dağıldığı belirlendi.

R. circumflexus sinister'in ortalama 4.53 mm çapında ve ventral seyirli r. interventricularis subsinuosus'u verdiği tespit edildi *(Şekil 1, v)*. R. interventricularis subsinuosus'un ventral seyrinin hemen başında septum interatriale ve ventriculus dexter duvarının üst kısmına dağılan ortalama 1.94 mm çapındaki r. ventriculi dextri'yi verdiği gözlendi



Şekil 1. A, C ve **D-** a. coronaria sinistra ve dalları, **B-** a. coronaria dextra ve dalları

Fig 1. A, C and **D-** The left coroner artery and its branches, **B-** The rigth coroner artery and its branches; **a)** a. coronaria sinistra, **b)** r. proximalis atrii sinistri, **c)** r. circumflexus sinister, **d)** r. intermedius atrii sinistri, **e)** r. distalis atrii sinistri, **f)** r. interventricularis paraconalis, **g)** r. proximalis ventriculi sinistri, **h)** r. marginis ventricularis sinistri, **ı)** r. distalis ventriculi sinistri, **j)** a. coronaria dextra, **k)** r. proximalis atrii dextri, **l)** r. intermedius atrii dextri, **m)** r. circumflexus dexter, **n)** r. coni arteriosi, **o)** r. proximalis ventriculi dextri, **p)** r. marginis ventricularis dextri, **r)** r. distalis ventriculi dextri, **s)** r. collateralis sinister proximalis, **t)** rr. septales, **u)** r. collateralis sinister distalis, **v)** r. interventricularis subsinuosus, **y)** r. collateralis dexter proximalis, **z)** r. collateralis dexter distalis, **q)** r. ventriculi dextri, **x)** anastomoz

(Şekil 1, q). Bu dalla birlikte r. circumflexus sinister'den çıkan ince dalların r. circumflexus dexter ile anastomoz yaptığı tespit edildi. Ayrıca r. interventricularis subsinuosus'tan ventriculus dexter duvarının üst 1/3 seviyesinde dağılan ortalama çapı 1.85 mm olan r. collateralis dexter proximalis' in ayrıldığı gözlendi *(Şekil 1, y)*.

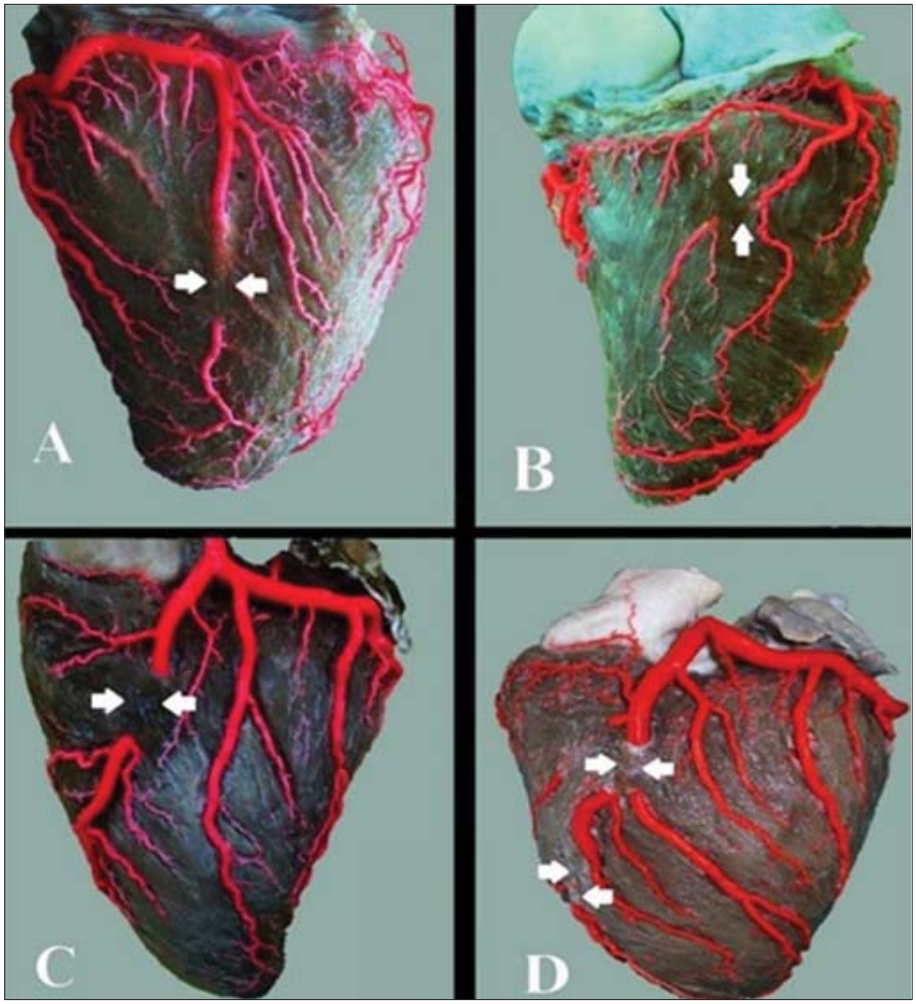
İncelenen 10 kalbin 6'sında r. interventricularis subsinuosus'un r. ventriculi dextri ve r. collateralis dexter proximalis'i verdikten sonra farklı büyüklük ve genişlikte kalp kası köprüleri tespit edildi *(Şekil 2/A)*. Kalp kası köprülerinin altında ya da hemen bitiminde, ventriculus dexter duvarının alt 1/3'nü besleyen ve ortalama 1.72 mm çapında r. collateralis dexter distalis'in r. interventricularis subsinuosus'dan orijin aldığı belirlendi *(Şekil 1, z)*. R. interventricularis subsinuosus'un ventral duvarından ayrılan farklı çaplarda ortalama 12 adet rr. septales ile birlikte, r. collateralis dexter proximalis ve r. colateralis dexter distalis'den gelen ince dalların septum interventriculare'nin beslenmesine katkı sağladığı gözlendi *(Şekil 1, t)*.

R. interventricularis paraconalis'in orijininden ortalama 22 mm sonra ilk olarak ortalama 3.20 mm çapındaki r. collateralis sinister proximalis'i ve hemen sonrasında 2.17 mm çapındaki r. coni arteriosi'yi verdiği gözlendi *(Şekil 1, s, n)*. Bu iki dalın kalp kası köprüsü görülen 8 kalpte kalp kası köprülerinden önce r. interventricularis paraconalis'den orijin aldığı belirlendi.

R. collateralis sinister proximalis'in orijininden sonra ventriculus sinister duvarının üst 1/3'üne ince dallar vererek dağıldığı ve bu dalların r. proximalis ventriculi sinistri'nin ince dalları ile anastomoz yaptığı tespit edildi.

R. coni arteriosi'nin tek dal halinde orijin aldığı ve daha sonra iki dala ayrıldığı belirlendi. Bu dallardan birisinin a. coronaria dextra'dan orijin alan r. coni arteriosi ile anastomoz yaptığı, diğer dalın ise conus arteriosus ve sinus trunci pulmonalis üzerinde dağıldığı gözlendi. Ayrıca r. coni arteriosi'ye paralel olarak seyreden 1-2 adet ince dalın r. interventricularis paraconalis'den orijin aldığı belirlendi.

R. collateralis sinister distalis'in r. interventricularis paraconalis'den ortalama 2.95 mm çap ile orijin aldığı ve ventriculus sinister duvarının alt 1/3'üne ve apex cordis'e kadar giden ince dallar verdiği gözlendi *(Şekil 1, u)*. R. interventricularis paraconalis'den orijin alan r. collateralis sinister proximalis'in r. coni arteriosi ve r. collateralis



Şekil 2. A- r. interventricularis subsinuosus üzerindeki köprü, **B-** a. coronaria dextra üzerindeki köprü, **C-** r. interventricularis paraconalis üzerindeki köprü, **D-** r. interventricularis paraconalis üzerindeki çift köprü

Fig 2. A- Myocardial bridge on r. interventricularis subsinuosus, **B-** Myocardial bridge on a. coronaria dextra, **C-** Myocardial bridge on r. interventricularis paraconalis, **D-** Double myocardial bridges above r. interventricularis paraconalis

sinister distalis'in orijin yerlerinden hemen sonra ince dallar vermek suretiyle septum interventriculare'nin beslenmesine katkı sağladığı tespit edildi. Bu dallarla birlikte r. interventricularis paraconalis'in ventral yüzünden orijin alan farklı çaplardaki ortalama 15 adet rr. septales'in septum interventriculare'nin beslenmesine katkı sağladığı belirlendi *(Şekil 1, t)*.

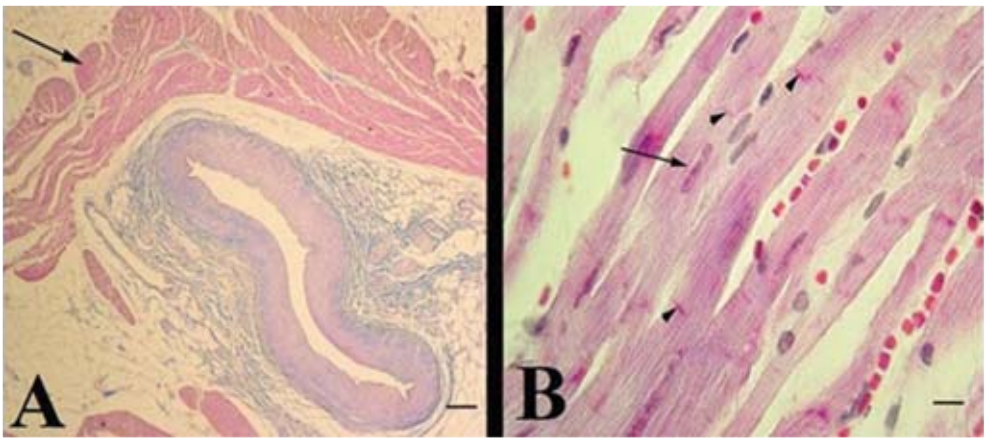
İncelenen 10 kalbin 8'inde sulcus interventricularis paraconalis içerisinde seyreden r. interventricularis paraconalis'in üzerinde farklı kalınlık ve genişlikte kalp kası köprüleri tespit edildi *(Tablo 1)*. Kalp kası köprülerinin oluğun genellikle üst 1/3'üne yakın bir yerde yoğunlaştığı belirlendi. Kalp kası köprüsü görülen kalplerin birinde iki adet kalp kası köprüsüne rastlandı *(Şekil 2/ D)*.

A. coronaria dextra'nın aorta ascendens'in başlangıç kısmında bulunan valvula seminularis dextra'nın hemen üzerinden ortalama 4.66 mm çap ile tüm kalplerde tek bir dal halinde orijin aldığı gözlendi *(Şekil 1, j)*. İncelenen 10 kalbin 2 sinde ventriculus dexter'in yaklaşık olarak ortalarında a. coronaria dextra'nın üzerinde farklı kalınlık ve genişlikte kalp kası köprüsü tespit edildi *(Tablo 1)*. A. coronaria dextra'nın orijininden yaklaşık 10-20 mm sonra ortalama 2.88 mm çapında r. coni arteriosi'yi verdiği belirlendi *(Şekil 1, n)*. İncelenen kalplerin 2'sinde ise r. coni arteriosi'nin tam karşısından ortalama 1.92 mm çapında r. proximalis atrii dextri'nin orijin aldığı tespit edildi. R. coni arteriosi'nin orijininden sonra 2-3 dala ayrıldığı ve conus arteriosis üzerinde dağıldığı belirlendi. R. coni arteriosi'nin uç dallarının ise r. interventricularis paraconalis'den orijin alan aynı isimli dal ile anastomoz yaparak sonlandığı gözlendi.

A. coronaria dextra'dan ayrılan ilk atrial dalın ortalama 1.92 mm çapındaki r. proximalis atrii dextri olduğu belirledi *(Şekil 1, k)*. R. proximalis atrii dextri'nin tek kök halinde a. coronaria dextra'dan orijin aldıktan sonra 2-3 dala ayrıldığı gözlendi. Bu dalların ilk ikisinin atrium dextrum üzerinde dağıldığı, son dalın ise r. intermedius atrii dextri ile anastomoz yaptığı tespit edildi. R. intermedius atrii dextri'nin ortlama 1.1 mm çapında olduğu ve a. coronaria dextra'dan orijin aldığı belirlendi *(Şekil 1, l)*. Kalplerin 2'sinde ise r. intermedius atrii sinistri'nin olmadığı görüldü. R. intermedius atrii dextri'nin orijininden sonra atrium dextrum'un serbest kenarlarına giden 2 adet dal ile r. proximalis atrii dextri ile anastomoz yapan 1 adet dal olmak üzere toplam 3 adet ince dala ayrıldığı belirlendi. A. coronaria dextra'nın bahsi geçen dalları verdikten sonra ortalama çapı 1.95 mm olan r. circumflexus dexter ile ortalama çapı 2.03 mm olan r. distalis ventriculi dextri'ye

Tablo 1. *Koroner arterler üzerindeki kalp kası köprülerinin genişlikleri ve kalınlıkları*
Table 1. *The widths and thickness of myocardial bridges on coronary arteries*

Kalpler	R. interventricularis paraconalis üzerindeki köprünün genişlik/kalınlık	R. interventricularis subsinuosus üzerindeki köprünün genişlik/kalınlık	A. coronaria dextra üzerindeki köprünün genişlik/kalınlık
1. Kalp	21.25 mm/3.12 mm	*	16.80 mm/3.50 mm
2. Kalp	17.68 mm/3 mm	29.90 mm/2.50 mm	*
3. Kalp	16.65 mm/2.87 mm	6.39 mm/4 mm	*
5. Kalp	17.36 mm/3.55 mm	8.12 mm/1.85 mm	*
5. Kalp	14.70 mm/4.25 mm	11.45 mm/2.74 mm	*
6. Kalp	*	*	*
7. Kalp	11.43 mm/2.50 mm	19.50 mm/3.22 mm	23.53 mm/2.42 mm
8. Kalp	15.76 mm/3.75 mm	*	*
9. Kalp	16.32/19.95 mm (iki köprü) 6.25/5.60 mm	30.88 mm/1.34 mm	*
10. Kalp	*	*	*



Şekil 3. A- Kalp kası köprüsü *(ok), Bar:* 250 µm, **B-** Kalp kası köprüsünün histolojik görünümü. *Ok:* Kalp kası lifine ait nükleus, *Ok başları:* İnterkalat diskler, *Bar:* 10 µm

Fig 3. A- Myocardial bridge *(arrow) Bar:* 250 µm, **B-** Histologic appearance of the bridge of the heart muscle. *Arrow:* Nucleus of the heart muscle fiber grups, *Arrow heads:* intercalated discs, *Bar:* 10 µm

ayrıldığı gözlendi *(Şekil 1, m, r)*. R. circumflexus dexter'den orijin alan çok sayıda ince dalın atrium dextrum'un serbest kenarlarına dağıldığı tespit edildi. R. circumflexus dexter'in son dallarının r. ventriculi dextri ve r. circumflexus sinister'den orijin alan ince dallarla anastomoz yaptığı görüldü *(Şekil 1/D, x)*. R. circumflexus dexter'den ortalama 1.14 mm çap ile orijin alan r. distalis atrii dextri'nin atrium dextrum üzerinde dağıldığı belirlendi.

A. coronaria dextra'dan ventriculus dexter için ilk olarak ortalama 1.96 mm çapındaki r. proximalis ventriculi dextri' nin orijin aldığı belirlendi *(Şekil 1, o)*. R. proximalis ventriculi dextri'nin 2-3 dala ayrıldığı ve bu dalların ventriculus dexter duvarının beslenmesine katkı sağladığı tespit edildi. R. proximalis ventriculi dextri'nin r. interventricularis paraconalis'den gelen dallarla anastomoz yaparak sonlandığı gözlendi *(Şekil 1/B, x)*. Ventriculus dexter için a. coronaria dextra'dan orijin alan diğer bir dalında ortalama 1.97 mm çapındaki r. marginis ventricularis dextri olduğu belirlendi *(Şekil 1, p)*. R. marginis ventricularis dextri'nin ventriculus dexter için ince dallar verdikten sonra r. interventricularis paraconalis'den gelen dallarla anastomoz yaparak sonlandığı gözlendi *(Şekil 1/B, x)*.

Histolojik araştırmalar için alınan doku örneklerinin mikroskobik incelemelerinde kalp kası köprülerindeki kas liflerinin merkezi konumlu tek çekirdeğe ve enine bantlaşmalara sahip olduğu, bağlantı bölgelerinde interkalat disklerin bulunduğu görüldü *(Şekil 3/B)*. Kas liflerinde kollateral bağ denilen dallanmalara ve endomizyumda bol miktarda kapiller damara rastlandı. Tüm bu özellikler göz önüne alındığında, kalp kası köprülerinin histolojik yapısının normal kalp kası özelliğinde olduğu, kalp kasına ait genel özelliklerden farklı herhangi bir yapı göstermediği tespit edildi.

Yapılan histolojik incelemelerde köprü içerisinde bulunan artere eşlik eden venaların bulunduğu gözlendi *(Şekil 3/A)*. Periarteriyel alanda bol miktarda yağ hücresi, diğer bağdoku ve sinir dokuya ait yapılar belirlendi.

TARTIŞMA ve SONUÇ

Literatür [3-6] bilgilerine uygun olarak Zavot ırkı sığırlarda kalbin arteriyel vaskularizasyonunu aorta ascendens'den orijin alan a. coronaria sinistra ve a. coronaria dextra'nın sağladığı belirlendi. Tecirlioğlu ve ark.[3], ile Karadağ ve Soygüder'in [5] bildirdiği gibi a. coronaria sinistra'nın, çok sayıda atrial ve ventriküler dalı olan r. circumflexus sinister'e,

r. interventricularis paraconalis'e ve r. interventricularis subsinuosus'a orijinlik etmesinden dolayı a. coronaria dextra'dan daha kuvvetli olduğu tespit edildi.

Literatürde [18,20,27-29] r. interventricularis subsinuosus'un a. coronaria dextra'dan orijin aldığını bildirilmiştir. Fakat Zavot ırkı sığırlarda r. interventricularis subsinuosus'un a. coronaria sinistra'nın sulcus interventricularis subsinuosus içerisinde devamı eden bir kolu olduğu belirlendi.

Özgel ve ark.[18], Yuan ve ark.[28], Tıpırdamaz'ın [30] çalışmalarındaki bilgilere paralel olarak araştırmamızda r. proximalis atrii sinistri'nin r. circumflexus sinister'den orijin aldığı tespit edildi. Ancak Doğruer ve Özmen [15] çalışmalarında 9 kalpte, Tecirlioğlu ve ark.[3], nadir olarak r. proximalis atrii sinistri'nin a. coronaria sinistra'dan orijin alabileceğini belirtmişlerdir.

Septum interventriculare'nin beslenmesini literatür [3,13,15,18] bilgilerine uygun olarak r. interventricularis paraconalis ve r. interventricularis subsinuosus'dan orijin alan rr. septales'in sağladığı belirlendi.

Özgel ve ark.[18], Taha ve Abel-Magied [27], Yuan ve ark.'nın [28] araştırmalarında r. circumflexus sinister ile r. circumflexus dexter arasında bir anastomozun olmadığını belirtmişlerdir. Fakat çalışmamızda r. circumflexus sinister ile r. circumflexus dexter arasında bir anastomozun olduğu tespit edildi.

Van Nie ve Vincent [31], hayvan türlerine göre farklı olmak üzere köprü genişliklerinin dar (<5 mm), orta (6-15 mm) ve geniş (<15 mm) olmak üzere üç ayrı tip olabileceğini belirtmişlerdir. Yapılan bu çalışmada ise dar tipte köprü bulunmamasına rağmen orta tipte 6 adet (%35.30), geniş tipte 11 adet (%64.70) olmak üzere toplam 17 adet kalp kası köprüsü tespit edildi.

Erden ve ark.[12], koroner arterlerin interventriküler dallarının seyri ve kalp kas köprülerinin şekillenişi arasındaki ilişkiye göre kalp kası köprülerini üç grup altında incelemişler ve çalışmalarında kalplerden birinin sağ yüzünde (%5.88) ve beşinin sol yüzünde (%29.41) olmak üzere, toplam altı kalpte (%35.29) kalp kası köprüsü oluşumunu tespit etmişlerdir. Çalışmamızda ise kalplerin 2'sinin sağ yüzünde (%12.5) ve 8'sinin sol yüzünde (%87.5) olmak üzere toplam 8 kalpte (%80) kalp kası köprüsü tespit edildi.

Shinjo ve ark.[32], %75, Severino ve Bombonato [33], %94, Santos ve ark.[34], %100 oranında kalp kası köprüsünden söz ederken, çalışmamızda ise %80 oranında kalp kası köprüsü belirlendi.

Bezerra ve ark.[35], a. coronaria dextra üzerinde, Yuan ve ark.[28] da r. interventricularis subsinuosus üzerinde kalp kası köprüsü varlığından söz etmez iken, araştırmamız da a. coronaria dextra'da 2 adet, r. interventricularis subsinuosus'da 6 adet kalp kası köprüsü tespit edildi. Fakat sulcus intermedius içerisinde seyreden r. marginis ventricularis sinistri üzerinde kalp kası köprüne rastlanılmadı.

Shinjo ve ark.[32], sığırlarda myokardial köprülerin morfolojisini ve bu yapıların koroner atheroskleroz ile ilişkisini araştırdıkları çalışmasında, koroner arterler üzerinde bulunan kalp kası köprülerindeki kas liflerinin morfolojik olarak kalp kası tellerine ait karakteristik özellikler gösterdiğini bildirmişlerdir. Bizim yaptığımız incelemelerde de kalp kası köprülerinin histolojik yapısının normal kalp kası özelliğinde olduğu, kalp kasına ait genel özelliklerden farklı herhangi bir yapı göstermediği belirlendi.

Çalışmada kalp kası köprüsü içerisindeki periarteriyel alanda bol miktarda yağ hücresi, diğer bağ doku unsurları ve sinir dokuya ait yapılar tespit edildi. Bulgularımıza benzer olarak Iuchi ve ark.[36], myokardial köprülerle ilgili yaptıkları çalışmalarında köprü içindeki periarteriyel alanda çoğunlukla yağ dokusunun, sinir liflerinin ve küçük damarların bulunduğunu belirtmişlerdir.

Dursun ve ark.[37], ramus interventricularis paraconalis ve ramus interventricularis subsinuosus üzerindeki kas köprülerinin içerdiği artere eşlik eden vena sayısını esas aldıkları araştırmada, beş sığır kalbinde söz konusu artere eşlik eden venanın bulunmadığı, üç sığır kalbinde de iki adet venanın bulunduğu köprü tipleri belirlemişlerdir. Yaptığımız mikroskobik incelemelerimizde ise tüm örneklerde köprü içindeki artere eşlik eden venaların bulunduğu gözlendi.

Kalp kası köprüsünün bulunup bulunmaması üzerine yapılan bazı araştırmalarda cinsiyet ve yaş faktörünün etkisinin olmadığı belirtilmiştir [11,21-23]. Çalışmada da literatür verileri dikkate alınarak cinsiyet ve yaş faktörü farkı gözetmeksizin Zavot ırkı sığır kalbi kullanılmıştır.

Sonuç olarak bu çalışmada yüksek rakım ve soğuk iklim şartlarında yaşayan Zavot ırkı sığırların koroner arterlerinin dağılımı ve koroner arterler üzerinde bulunan kalp kası köprülerinin morfolojik özellikleri ortaya konuldu.

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RESEARCH ARTICLE

The Effect of Post-Weaning Steer Diets Supplemented With Field Pea, Flaxseed and a Field Pea-Flaxseed Combination on Feedlot Finishing Performance, Carcass Quality and Immune Response [1]

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Summary

This study objective was to compare the effect of feeding field pea, flaxseed and field pea-flaxseed combination on steer performance and immune response during the 50-d post-weaning period (PWP). Subsequently, the effect on feedlot finishing performance, immune response and carcass quality were determined. Crossbred Angus x Hereford x Gelbvieh steers (castrated male calves, age=7.4 month, n=173) were used in the 3 year replicated study. The four pelleted 50-d PWP diets (PWD) were: 1) Control (C), 2) 12.5% Flaxseed (FLX), 3) 20.0% Field Pea (P), and 4) 20.0% Field Pea + 12.5% Flaxseed (PFLX). In the PWP, average daily weight gain (ADG) was increased (P<0.05) for FLX and PFLX when compared with C and P, but feed cost/kg of gain for FLX and PFLX was decreased (P<0.05). In the feedlot period, initial weight, slaughter weight, fattening period, weight gain, ADG, average daily feed intake and feed conversion ratio was not significantly different among the diets (P>0.10). For carcasses, PWD did not affect hot carcass weight, marbling score, percent US Department of Agriculture quality grade (P>0.05); however, FLX treatment reduced rib-eye area (REA), while P treatment increased REA (P<0.05). FLX and PFLX treatments did not increase serum neutralization titer level and did not reduce morbidity (P=0.96) and health care cost (P>0.10). Overall, Flaxseed improved 50-d PWP performance, but PWDs had no carryover effect on feedlot finishing period net return.

Keywords: *Beef cattle steer, Carcass quality, Field pea, Flaxseed, Immune response*

Sütten Kesilmiş Dana Rasyonlarına İlave Edilen Keten Tohumu, Yemlik Bezelye ve Keten Tohumu-Yemlik Bezelye Kombinasyonunun Besi Sonu Performansı, Karkas Kalitesi ve Bağışıklık Sistemi Üzerine Etkisi

Özet

Bu çalışmanın amacı, 50 gün boyunca sütten kesim sonrası periyot (PWP)'ta rasyona ilave edilen keten tohumu, yemlik bezelye ve keten tohumu-yemlik bezelye kombinasyonun danaların besi performansı ve bağışıklık sistemi üzerine etkisini karşılaştırmaktır. Bunu takiben, besi sonu performansı, karkas kalitesi ve bağışılık sistemi üzerine etkisini belirlemektir. Üç yıl tekrarlanan bu çalışmada melez Angus x Hereford x Gelbvieh danaları (kısırlaştırılmış erkek dana, yaş=7.4 ay, n=173) kullanılmıştır. 50 günlük PWP rasyonları (PWD); 1) Kontrol (C), 2) %12.5 Keten tohumu (FLX), 3) %20.0 yemlik bezelye (P) ve 4) %20 yemlik bezelye + %12.5 keten tohumu (PFLX) olarak dört grupta peletlenmiştir. PWP'da FLX ve PFLX gruplarında günlük canlı ağırlık artışı (ADG) daha yüksek (P<0.05) ve birim ağırlık artışı için yem maliyeti daha düşüktür (P<0.05). C ve P gruplarında ADG (P=0.004) daha düşüktür. Besi döneminde; başlangıç ağırlığı, kesim ağırlığı, besi süresi, canlı ağırlık artışı, ADG, ortalama günlük yem tüketimi, yemden yararlanma oranında farklılık görülmemiştir (P>0.10). Karkas ölçümlerinde, PWD'leri sıcak karkas ağırlığını, kas içi yağ dağılımını, ve USA Tarim Bakanlığı kalite derece yüzdesini etkilememiştir (P>0.05); bununla birlikte, P grubunda sırt kası alanı (REA) artarken, FLX grubunda REA azalmıştır (P<0.05). FLX ve PFLX gruplarında serum nötralizasyon titre seviyesi artmamış ve morbidite oranı (P=0.96) ve tedavi maliyetleri (P>0.10) önemli ölçüde düşmemiştir. Genel olarak, keten tohumu 50-d PWP performansını artırmıştır, ancak PWD'nin daha sonraki besi bitirme peryodunda net kâr üzerine herhangi bir etkisi olmamıştır.

Anahtar sözcükler: *Besi danası, Karkas kalitesi, Yemlik bezelye, Keten tohumu, Bağışıklık sistemi*

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INTRODUCTION

Field pea and flaxseed are protein and energy dense feedstuffs for growing and finishing cattle. Field pea contains 20-27% crude protein (CP), 88-90% total digestible nutrients (TDN), 7-8% acid detergent fiber (ADF), and 1.40 Mcal NEg/kg for cattle. Field pea protein and starch components are highly rumen degradable and can replace corn and barley in beef cattle diets. Feeding field peas has resulted in better ADG and improved dry matter intake (DMI) in post - weaning calf diets [1,2].

Flaxseed contains 20-23% CP, 35-37% fat, 105-110% TDN, 7-8% ADF, and 1.97 Mcal NEg/ kg for cattle. Feeding up to 8% flaxseed to feedlot heifers has been associated with significant increases in gain and gain efficiency, but did not affect DMI [3]. Flaxseed is associated with enhancing immune resistance due to anti-inflammatory and immune aiding properties that may reduce morbidity and mortality among weaned, highly stressed, feeder calves during the PWP [4-6]. Kansas State University researchers compared the value of flaxseed to tallow, which is commonly fed in USA feedlots, and concluded that the addition of 10-15% flaxseed during the first 5 to 6 weeks after weaning would result in greater feed intake, growth, feed efficiency, and may reduce the incidence of bovine respiratory disease (BRD) [7-9]. In another experiment, the authors concluded that calves fed flaxseed during the stressful 5-6 week period following weaning illicit a stronger immune response, and may require less antibiotic therapy [10].

The purpose of this research was to compare 50-d PWD supplements formulated with P, FLX, and a PFLX combination to determine the effect on PWP steer performance and immune response, and to determine the carryover effect on feedlot finishing performance, carcass quality, immune response, and net return. We also hypothesized that 50-d PWD formulated with FLX and PFLX would have a positive effect on immune response, morbidity, and treatment cost.

MATERIAL and METHODS

This research project was conducted in accordance with guidelines approved by The North Dakota State University Institutional Animal Care and Use Committee (Protocol Approval number A401).

During a 3-year period, three annually replicated treatment groups (n=173) of 7.4 month old crossbred steers (Angus x Hereford x Gelbvieh) with an average weight of 293±0.51kg were weaned and randomly assigned to one of four pelleted PWD treatments. The treatments were: 1) C - no flaxseed and no field pea, 2) FLX - 12.5% flaxseed, 3) P - 20% field pea and, 4) PFLX - 20% field pea and 12.5% flaxseed. The steers were fed an alfalfa-brome grass hay (*Medicago sativa* and *Bromus inermis,*10.0% CP) and the experimental receiving diets were formulated according to National Research Council specifications *(Table 1)* [11]. Each treatment consisted of four pen replicates with four steers per pen. Steers were weaned the first week of November each year and fed for an average 50-d at the Dickinson Research Extension Center. The pelleted supplements were top-dressed over chopped alfalfa-brome grass hay (5.1 cm screen) and, as hay was removed, the amount of daily supplement was increased. The steers consumed an average 8.9, 9.6, 8.8, and 9.4 Mcal of ME for gain per day in the C, FLX, P, and PFLX, respectively.

Table 1. *PWD ingredient composition and nutrient analysis (Dry Matter)*
Tablo 1. *Deneme rasyonlarının içeriği ve besin madde düzeyleri (%KM'de)*

Ingredients	**C**[a]	**FLX**[a]	**P**[a]	**PFLX**[a]
Flaxseed, %	0.0	12.5	0.0	12.5
Field Pea, %	0.0	0.0	20.0	20.0
Corn, %	15.0	15.0	15.0	10.0
Soybean Hulls, %	21.5	28.703	30.703	34.203
Wheat Midds, %	24.953	11.75	10.0	12.0
Barley Malt Sprouts, %	20.0	15.0	10.0	5.0
Distillers Dried Grain With Solubles, %	12.25	10.75	8.0	0.0
Other, % [b]	6.297	6.297	6.297	6.297
Total, %	100.00	100.00	100.00	100.00
Analysis				
CP, % [c]	15.54	15.54	15.53	15.56
ADF, % [c]	16.03	17.68	18.03	18.79
NEg, Mcal/kg	1.13	1.27	1.16	1.27

[a] ***C:*** *No Flaxseed and no Field pea,* ***FLX:*** *12.5% Flaxseed,* ***P:*** *20% Field pea,* ***PFLX:*** *20% Field pea+12.5% Flaxseed;* [b]*Molasses, 5.0%; Salt, 0.50%; Calcium, 0.55%; Dicalcium Phos., 0.10%; TM Premix, 0.075%; Vitamin A & D Premix, 0.025%; Decoquinate, 0.027%; Monensin Sodium, 36.31 g/kg;* [c] ***CP:*** *Crude protein,* ***ADF:*** *Acid detergent fiber*

To evaluate the effect of flaxseed on health status, serum humoral antibody level and BRD incidence were monitored. Three weeks before weaning, the steers were vaccinated against economically important bacterial and viral diseases (Bovi-Shield Gold 5®) [12] and a booster vaccination for clostridial myonecrosis diseases and pneumonic pasteurellosis at weaning (One Shot Ultra 7®) [12]. Blood samples were collected from steers 3 weeks before weaning, at weaning and 30 and 90 days post-weaning. Serum humoral antibody levels for bovine virus diarrhea (BVD) Types I and II and infectious bovine rhinotracheitis (IBR) virus were determined, and morbidity and treatment cost were recorded. Virus serum neutralization was conducted using the procedure described by Leannette and Schmidt [13]. For IBR, neutralizations are run from 4 to 256 and for BVD viruses, dilutions are run from 4 to 4,096. Values < 4 were considered negative.

At the end of the 50-d PWP, the steers were shipped 1090 km to the Decatur County Feedlot, Oberlin, Kansas, USA, for feedlot finishing. Dietary energy concentration at the feedlot was increased incrementally until the steers were consuming 109.3 Mcal/kg of NEg per day. The Decatur County Feedlot is a commercial feedlot that uses the ACCU-TRAC electronic cattle management system to determine slaughter time [14]. Slaughter time prediction is determined based on the animal weight and ultrasound carcass measurements for fat depth (FD), REA, and percent of intramuscular fat (IMF) [14]. These measurements are collected at the start of the feedlot feeding period and after 80 d of feeding. Growth rate and fat deposition data collected are then used in the computer database to predict a future slaughter time when each steer is estimated to have attained a predetermined backfat depth of 12.0 mm. The steers were slaughtered at the Cargill Meat Solutions meat packing plant in Ft. Morgan, Colorado, USA. Carcass measurements were collected by Diamond Livestock Services and US Department of Agriculture quality grade (USDA QG) determinations were made by US Department of Agriculture meat graders.

Data was analyzed using analysis procedures of SAS [15]. Receiving, finishing, carcass trait and closeout data were analyzed as a randomized complete block design using PROC GLM, and USDA QG was analyzed using Chi-square procedures in PROC GENMOD. Antibody serum neutralization was analyzed using PROC MIXED procedures. In the models, diet served as the fixed effect and block, and year were random effects. Pen served as the experimental unit. Differences between treatment groups were considered significant at P≤0.05 and a trend at P≤0.10.

RESULTS

Post-Weaning Period

The effect of the PWD on steer performance during the 50-d PWP is summarized in *Table 2*. Control steers and steers that were fed P had similar gain (P=0.005) and ADG (P=0.004), which was less than steers that received either PFLX or FLX supplements (P=0.005). Steers that were fed P also tended to consume more feed per kg of gain resulting in a tendency for poorer feed to gain ratio (F:G; P=0.075). The P diet cost per kg of gain was similar to the C, and higher than steers fed either PFLX or FLX.

Table 2. *Effects of post weaning rations on growth performance.*
Tablo 2. *Sütten kesim sonrası rasyonlarının büyüme performansı üzerine etkileri*

Post Weaning Performance	C[a]	FLX[a]	P[a]	PFLX[a]	SEM[a]	P-Value
Growth performance						
Number of Steers [b]	43	43	44	43		
Initial Weight, kg	292.4	293.3	293.1	293.6	4.34	0.99
Final Weight (50-d), kg	363.6	371.5	362.9	371.9	4.76	0.38
Gain, kg [c]	71.2^{y}	78.2^{x}	69.8^{y}	78.0^{x}	1.99	0.005
ADG, kg [c, d]	1.42^{y}	1.56^{x}	1.40^{y}	1.56^{x}	0.040	0.004
Feed intake						
DMI, kg [d]	8.28	8.34	8.09	8.14	0.183	0.74
Hay/Day, kg	3.88	3.94	3.69	3.74	0.115	0.41
Supplement/Steer, kg	4.39	4.40	4.39	4.39	0.094	0.99
F:G, kg [d]	5.83	5.34	5.78	5.20	0.090	0.075
Feed cost						
Feed Cost/Steer, $	$41.44	$41.20	$39.97	$39.56	0.688	0.17
Feed Cost/kg Gain, $ [c]	$0.5820^{y}	$0.5269^{x}	$0.5726^{y}	$0.5072^{x}	0.0082	0.012

*[a] **C:** No Flaxseed and no Field pea, **FLX:**12.5% Flaxseed, **P:** 20% Field pea, **PFLX:** 20% Field pea+12.5% Flaxseed, **SEM:** Standard error of the mean; [b] One steer died of bloat in the C, FLX, and PFLX treatments; [c] Means in a row with unlike superscripts differ significantly (P<0.05); [d] **ADG:** Average daily gain, **DMI:** Dry matter intake, **F: G** Feed to gain ratio*

Compared to the C treatment diet, supplements that contained FLX were calculated to be 2.0 times higher in fat content and contained 6.2% greater net energy for gain. Average DMI did not differ between treatments (P=0.74). When flaxseed occurred alone in the supplement (FLX), or was blended with P (e.g. PFLX), there was a tendency for improved F:G (P=0.075) and feed cost per kg of gain was significantly lower (P=0.012) compared to either the C or P treatments. Feed cost per kg of gain was lower in treatments that included flaxseed (e.g. FLX and PFLX; P=0.012) and compared to feeding supplements containing P alone, feed cost per kg of gain was reduced 11.7%.

Feedlot Finishing Period

Upon completion of the 50-d PWP the carryover effect on finishing performance, carcass trait measurements, and finishing net return was evaluated and has been summarized in *Table 3*. Within the data set, treatment, year, and treatment x year interactions were analyzed. For feedlot finishing performance, carcass measurements, and net return, there were significant year effects identified. However, for feedlot finishing performance, there were no treatment or treatment x year interactions that were significant. There was no significant feedlot performance differences determined for slaughter weight, number of feedlot days, ADG, DMI, or F:G (P>0.10).

For carcass measurements, there were no treatment differences identified (P>0.10) for hot carcass weight (HCW), marbling score (MS) or percent USDA QG. When FLX had previously occurred alone in the 50-d PWD, finishing REA was smaller (P=0.044), FD tended to be greater (P=0.074), and US Department of Agriculture yield grade (USDA YG) also tended to be greater (P=0.083), suggesting that steers fed supplements during the 50-d PWP that contained FLX had an increased propensity for a greater fat to lean ratio that reduced carcass value (CV). Moreover, when FLX was blended with P in the PWD, there was a year-over-year tendency for USDA YG score to be less desirable. Carcass value was effected by yearly fluctuations in US fed cattle prices (P=0.0001) resulting in an ending numerical CV difference, but the observed numerical difference was not statistically significant for CV (P=0.862). Year-over-year interactions for USDA YG score (P=0.008) and MS (P=0.031) were identified; however, the subsequent and final effect

Table 3. *Effect of 50-d PWD on feedlot finishing performance, carcass parameters, expense and net return*
Tablo 3. *50 günlük PWD'nin besi sonu performansı karkas parametreleri, maliyet ve net kâr üzerine etkisi*

Feedlot Finishing Performance	C[a]	FLX[a]	P[a]	PFLX[a]	SEM[a]	P Value		
						TRT[a]	YR[a]	TRT x YR[a]
Feedlot performance								
Number of Steers	43	43	44	43				
Initial Weight, kg [b]	356.7	364.5	358.1	366.7	4.36	0.269	0.0001	0.943
Slaughter Weight, kg [b]	587.8	582.8	588.3	589.2	6.22	0.898	0.0001	0.481
Feedlot Days	147.3	137.0	143.6	141.3	3.91	0.291	0.0001	0.463
Gain, kg	231.1	218.3	230.2	222.5	5.94	0.355	0.0006	0.623
DMI, kg [d]	8.85	8.87	8.95	8.84	0.155	0.959	0.7790	0.456
ADG, kg [d]	1.568	1.593	1.603	1.575	0.032	0.834	0.0033	0.397
F:G, kg [d]	5.65	5.57	5.59	5.61	0.824	0.609	0.0001	0.888
Carcass parameters								
Carcass Number	43	43	44	43				
HCW, kg [d]	368.8	366.2	368.9	369.4	4.40	0.955	0.0001	0.284
REA, sq. cm [c,d]	87.03^{xy}	83.23^{y}	88.39^{x}	87.10^{xy}	1.330	0.044	0.0001	0.268
FD, mm [d]	11.25	13.08	11.79	11.63	0.518	0.074	0.0001	0.063
MS [d]	4.70	3.66	3.57	3.77	0.638	0.562	0.124	0.031
USDA YG Score [d]	2.43	2.69	2.39	2.60	0.940	0.083	0.057	0.008
USDA QG, % [d]	60.5	37.2	43.2	44.2		0.112	0.219	0.066
Expense and net return								
CV, $ [d]	1104.93	1088.67	1106.61	1108.44	18.24	0.862	0.0001	0.015
Calf & Feed Cost, $	1096.03	1086.30	1093.38	1095.39				
Net Return, $	8.90	2.37	13.23	13.05	17.85	0.943	0.0001	0.017

[a] ***C:*** *No Flaxseed and no Field pea,* ***FLX:*** *12.5% Flaxseed,* ***P:*** *20% Field pea,* ***PFLX:*** *20% Field pea+12.5% Flaxseed,* ***SEM:*** *Standard error of the mean,* ***TRT:*** *Treatment,* ***YR:*** *Year,* ***TRT x YR:*** *Treatment x year interaction;* [b] *Feedlot start weight age 9.3 months; slaughter age 14.0 months;* [c] *Means in a row with unlike superscripts differ (P<0.05);* [d] ***DMI:*** *Dry matter intake,* ***ADG:*** *Average daily gain,* ***F:G*** *Feed to gain ratio,* ***HCW:*** *Hot carcass weight,* ***REA:*** *Rib-eye area,* ***FD:*** *Fat depth,* ***MS:*** *Marbling score,* ***USDA YG:*** *US Department of Agriculture yield grade,* ***USDA QG:*** *US Department of Agriculture quality grade* ***CV:*** *Carcass value*

of PWD treatment on finishing net return did not differ (P=0.943).

Immune Response

The subsequent effect of treatments fed during the initial 50-d PWP on humoral antibody titer change is shown in *Fig. 1*, and morbidity and treatment cost during the critical first 60 days of the finishing period are depicted in *Fig. 2*. Pre-vaccination humoral antibody level, when vaccines were administered on pasture 3 weeks before weaning, was low and increased across treatments each time blood was drawn for serum recovery, but did not differ between dietary treatments for IBR (P=0.78), BVD Type I (P=0.11), and BVD Type II (P=0.90). The incidence of BRD during receiving and finishing was similar for all treatment groups and medical treatment cost did not differ (P=0.96).

DISCUSSION

Separating calves from their mothers at weaning (e.g. 7-8 months old) is stressful and limits feed intake immediately after weaning. For this study, steers were offered palatable alfalfa-brome grass mixed hay, which was readily consumed at the start. Then, during the 50-d PWP, hay was removed and the nutrient dense supplements were increased until the steers were consuming approximately 4.40 kg of supplement/steer/day.

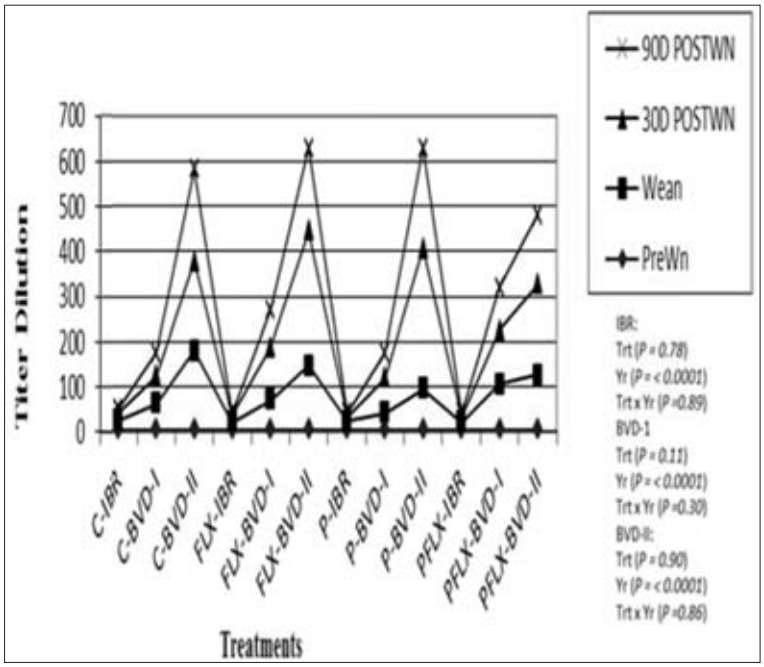

Fig 1. Effect of 50-d PWD treatments on antibody titer dilution

Şekil 1. 50 günlük PWD'nin grupların antikor titreleri üzerine etkisi

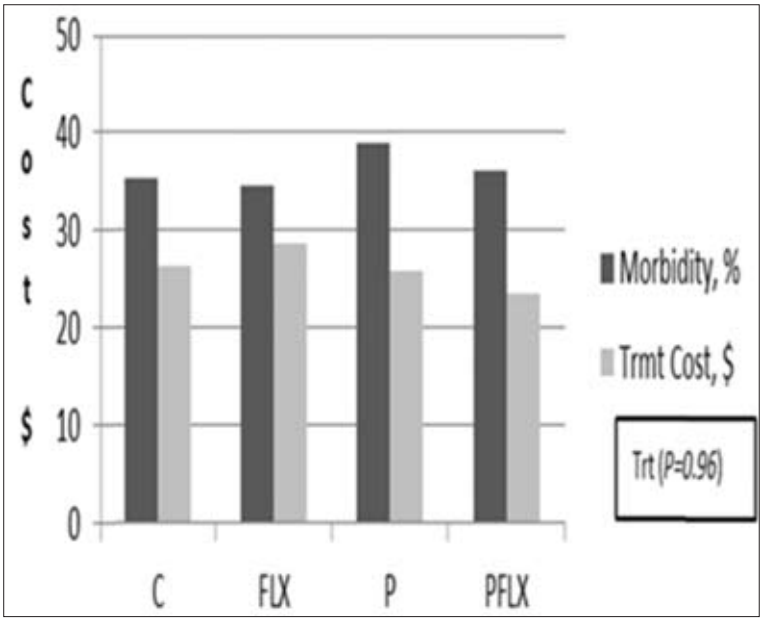

Fig 2. Effect of 50-d PWD treatments on morbidity and treatment cost

Şekil 2. 50 günlük PWD'nin grupların morbidite dilüsyonları ve tedavi giderleri üzerine etkisi

Field pea grain was included in the 50-d PWP supplements, since previous research with P has shown that DMI can be improved when P is included in both growing and finishing steer diets [1,2]. Including P alone or combined with FLX (e.g. PFLX) in the current study, resulted in DMI that did not differ from the other treatments (P=0.74). Steers that were fed the P supplement during the 50-d PWP performed as well as the steers that were fed the other PWD supplements. However, slower ADG from P and a subtle numerically lower DMI among the steers fed the P supplement combined to increase the feed to gain ratio. The resulting feed cost/kg of gain was higher for P compared to the FLX and PFLX treatments. At the end of the 50-d PWP, steers that were fed the P supplement had poorer performance, however, there was no death loss in the P treatment.

Comparing the C, P, and FLX treatments, formulations with FLX were calculated to contain twice as much fat. Based on previous research [6-10], we hypothesized that including FLX in the 50-d PWP diets would increase DMI and growth performance as well as improve health status resulting in greater immune response, less morbidity, and lower treatment cost. Including flaxseed did not result in greater immune response or improved health status, and did not result in greater DMI. However, growth performance was enhanced by FLX inclusion, which may have been due to the higher energy level from fat in the FLX treatments. Nonetheless, FLX tended to improve feed efficiency and lowered the feed cost/kg of gain making FLX inclusion a cost effective ingredient decision. Regardless of FLX inclusion success during the 50-d PWP, there was no compelling data to support a positive carryover effect from feeding FLX during the 50-d PWP on health status, finishing growth performance, carcass measurements or finishing net return. This is in sharp contrast to the results reported for highly stressed feeder cattle [7], but is in agreement with research that documented an improvement in PWD performance in one experiment without a positive reduction in mortality in a second experiment [8].

Reasons for the inability of the steers' immune system to demonstrate a difference in response to vaccine or the incidence of BRD are unknown. Data from this study indicates that flaxseed does not appear to influence serum neutralization titers in vaccinated cattle. The complexity of the immune system would preclude identifying any specific cause for this result, but different levels of stress can affect the occurrence of respiratory disease. Our study steers were not highly stressed compared to the highly stressed Kansas State University steers [7-10]. Steers in the current study were transported less than 16 km to the PWP study site. After completing the 50-d PWP, the steers were transported 7.5 h to the finishing feedlot. In the United States, calves that have just been weaned become highly stressed after spending nearly 30 hours or more in the marketing and transit process before feedlot arrival. This markedly increases a stress related inflammatory response. Moreover, development of BRD is dependent upon numerous factors such as pathogen dose, pathogen strain, presence of co-pathogens, and pathogen exposure pathway. Considering the myriad of possible variables, it is difficult to insure that when a group of animals are exposed to a certain pathogen load of infective agents that they will respond in the same way. Since the steers used in this study were exposed to a greatly reduced level of stress, flaxseed does not appear to increase a steers' ability to respond favorably to BRD.

We conclude from the results of this research and the results reported elsewhere [7-10] that moderately stressed feeder steers probably will not respond to FLX supplementation, whereas, highly stressed feeder steers are the most likely cattle to illicit a positive pro-anti-inflammatory response to FLX.

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RESEARCH ARTICLE

Comparision of DGAT1 K232A Polymorphism and its Effects on some Milk Quality Parameters in Holstein and Native Black Race Cattles [1]

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Summary

In this study, effects of DGAT1 K232 polymorphism and the allele frequency differences of "K" (alanine variant) and "L" (lysine variant) on some cattle milk qualities and some microbiological parameters which pose a risk on consumer health were investigated. For this purpose, 2 years old 50 cattles were used as material (25 Holstein race and 25 Native Black race). Blood samples were collected for determining the allele frequencies of DGAT1 gene. Then, during the experimental period (1 month) the milk of the cattles were collected once a week and the milk samples has been explored for some important foodborne pathogens (Total Aerobic Bacteria, Coliforms, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*) and milk quality parameters (pH, fat, density and acidity). According to the findings, it has been identified that DGAT1 gene frequency differences were significantly effective on some the parameters of milk qualities and the growth of coliforms, *Esherichia coli* and *Staphylococcus aureus*.

Keywords: *DGAT1 gene, Milk quality, Polymorphism, Cattle, Foodborne pathogens*

Holstein ve Yerli Kara Sığır Irklarında DGAT1 K232a Poliformizminin ve Bunların Bazı Süt Kalite Parametrelerine Etkisinin Araştırılması

Özet

Bu çalışmada, farklı ırk sığırlarda DGAT1 K232 polimorfizmi araştırılmış ve DGAT1 geninde bulunan "K" (alanin varyantı) ve "L" (lizin vasryantı) allel frekans farklılıklarının sığırların bazı süt kalite (süt verimi, sütte yağ oranı, pH vb.) parametrelerine ve tüketici sağlığı açısından risk teşkil eden önemli bazı gıda kaynaklı patojenlere etkisi incelenmiştir. Bu amaçla 2 yaşlı, 50 adet sığır (25 adet Holstein ırkı, 25 adet ise Yerlikara ırkı) materyal olarak kullanılmıştır. Sığırlardan kan örnekleri alınarak DGAT1 geni allel frekansları belirlenmiş ve daha sonraki dönemde ise haftada 1 kez olmak üzere 1 ay boyunca aynı sığırlardan süt örnekleri toplanmış ve toplanan süt örnekleri bazı önemli gıda kaynaklı mikrobiyolojik parametreler (toplam mezofilik erobik bakteri, koliformlar, *Escherichia coli, Listeria monocytogenes* ve *Staphylococcus aureus* olmak üzere) ve süt kalite parametreleri (pH, süt yağı, sütün yoğunluğu ve sütün asiditesi olmak üzere) açısından incelenmiştir. Elde edilen bulgulara göre DGAT1 geni allel frekans farklılıklarının, tüm süt kalite parametreleri ve mikrobiyolojik parametrelerden koliformlar, *Esherichia coli* ve *Staphylococcus aureus* üremelerinin üzerine istatistik açıdan belirgin derecede etkili olduğu tespit edilmiştir.

Anahtar sözcükler: *DGAT1 geni, Süt kalitesi, Polimorfizm, Sığır, Gıda kaynaklı patojenler*

INTRODUCTION

DGAT1 gene is determined as a 8.6 kb sized gene including 17 exon territories and its relation to milk productiveness in cattles is proven. According to rat trials, lack of double *DGAT1* gene results in the complete

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prevention of milk secretion due to the lack of triglyceride synthesis in mammary gland. The transformation of lysine amino acid in exon 8 region 232 of *DGAT1* gene to alanine results in two different haplotypes. The haplotype which encodes the lysine amino acid is the principal type, whereas the haplotype that encodes the alanine amino acid is the mutant one [1].

Diacylglycerol O-acyltransferase 1enzyme catalyses the last step of triglyceride synthesis in cellular triglyceride metabolism. The enzyme also carries important functions in adiposis tissue and intestinal fat absorption [1]. In European cattle races, it has been reported that the aforementioned gene may have some polymorphologic properties with the ability of lysine and alanine switching places/lysine replacing alanine in the 10433 and 10434 loci (positions) of the eighth exon (*DGAT1 K232A*) [2]. The milk can have higher rate of fat due to the mutation of "K" allele. Literature may also report that the "K" allele is responsible from the saturated fat acids in the milk [3]. The individual properties of the related loci of the cattle races can affect the *DGAT1 K232A* polymorphism and this may produce different effects on milk fat [4,5]. The utility of the cattles for milk and meat production, their adaptation to natural conditions and tolerance to diseases makes them economically advantageous [6,7]. Due to the mentioned reasons, the investigations of *K232A* polymorphism in cattle plays an important role in in milk industry and procurement of higher quality milk, thus consumer health.

MATERIAL and METHODS

Collection of Blood and Milk Samples

Fiftty cattles that are 2 years old have been used as test subjects of this study (with the acceptance number of 2012/16 by İstanbul University Local Ethical Committe of Animal Experiments). Blood samples have been collected from 25 Holstein race cattles which are thought to have *DGAT1* gene and high alele frequency and 25 indigeneous race cattles (native black), which are thought to have *DGAT1* gene but low allele frequency. Following this, the blood samples have been analyzed in molecular genetic methods and the cattles which have the *DGAT1* gene and which have high and low frequencies of "K" and "A" alleles have been positively identified. After that, the cattles which carry high allele frequency and low allele frequency have been grouped and milk enough for the microbiological and milk quality parameter analyses has been harvested from these test subjects every week for a month. During the study trial period, in order to maximize the homogenization, it has been provided that all the cattles are kept under the same conditions (hygienic conditions, feeding, age etc.).

Determination of DGAT1 Gene

Lysine (K) and the Alanine (A) alleles in the *DGAT1* gene have been determined, using PCR amplification *Cfr*I enzyme. In order to pick up the *K232A* polymorphism, PCR - SSCP (Polymerase Chain Reaction - Single Strand Conformation Polymorphism) procedure has been applied according to Ripoli's [4] method. PCR amplification has been applied in 26 µl total volume. 12.5 µl 2X PCR Master Mix, 0.5 µM (forward primer 5'-GCACCATCCTCTTCCTCAAG-3' and reverse primer 5'-GGAGCGCTTTCGGATG-3'), 50 ng DNA sample has been used for each primer. In PCR procedure, the reproduction operation is done as such: 15 min on 95°C, 1 min on 94°C 1 min 35 rotations and 1 min on 60°C and 1 min on 72°C and the last elongation 3 min on 72°C with *Cfr*I enzyme and 411bp alanine variant division to 203 and 208 bp. The DNA bands are visualised in 2% agaroz gel painted with etidium bromide.

Microbiological Analyses

TAB (total aerobic bacteria), coliforms, *E. coli (Escherichia coli), L. monocytogenes (Listeria monocytogenes)* and *S. aureus (Staphylococcus aureus)* were determined for each milk sample. Microbiological analyses were performed according to FDA/BAM [8].

TAB: TAB was enumerated in PCA (Plate Count Agar) after incubation at 30°C for 48 h.

Coliforms: Coliforms were enumerated by surface plating on VRBA (violet red bile agar). Plates were incubated at 37°C for 24 h.

E. coli: E. coli were examined by surface plating on TBX (Tryptone Bile X - glucurunide) Agar. Colonies on plates incubated at 44°C for 24 h were enumerated.

S. aureus: S. *aureus* was determined by surface plating on BPA (Baird Parker Agar) supplemented with egg yolk-tellurite emulsion. Spread plates were incubated at 35°C for 46-48 h. Colonies with typical S. *aureus* morphology were examined microscopically following Gram staining and tested for catalase and coagulase activity.

L. monocytogenes: 25 g sample has been put in 225 ml BLEB (Buffered Listeria Enrichment Broth Base), incubated for 4 h in 30°C and after that selective agents and 25 mg/L natamisin has been added in the mediums and incubated for 48 h in 30°C. During the 24 h of the incubation period, Oxford and Palcam agars has been used and they have incubated for 48 h in 35°C. By the end of the 48th h of the incubation, *Listeria monocytogenes/ivanovii* has been passaged to Chromogenic Listeria Agar Base. Yeast Extract added trypticase soy agar (TSA) passages have been made from the colonies with the *Listeria* spp. suspicion and cultures have been purified. The suspicious isolates have been identified according to gram staining, catalase, motion, dextroglucose, malt sugar, rhamnose, mannitol, xylose fermentation, esculin hydrolization and nitrate reduction properties.

Quality Parameter Analyses

pH: Digital pH meter (Hannah Instruments) was been used to determine the pH values of all milk samples.

Milk fat rate: Gerber method was used to determine the milk fat. 10 ml concentrated H_2SO_4, 11 ml milk and 1 ml isoamyl alcohol have been put in Gerber tubes. Then, the mixture has been centrifuged in Gerber centrifuge at 2.500 rpm for 5 min and the milk fat amount has been directly read from Gerber tube scala [9].

Milk density: Lactodensimeter was been used in order to determine the concentration of the milk samples.

Milk acidity: Acidity rate of the samples is determined according to % lactic acidity. For this procedure, N/10 NaOH was used. 25 ml milk has been poured in a beher glass and 1-2 drops of phenolphthalein have been instilled on it and titration has been performed with the help of a burette. After the permanent pink colour was achieved, titration has been stopped, the spent aount of NaOH has been determined and calculation has been done. The calculation formula is:

A X 10 = T X 0.009

A: NaOH amount spent/ml, T: Acidity level in Thörner scale [10].

RESULTS

The PCR analyses have shown that 25 Holstein race cattles (experimental group) had *DGAT1* gene and the "K" and "A" alleles of the gene have high allele frequencies (between 0.93 and 0.95). On the other hand, the 24 native black cattles (control group) have been determined in order to have the *DGAT1* gene, but the gene has been identified to have low allele frequency (between 0.35 and 0.60). The control and experimental groups have been compared with regard to, milk microbiological charges according to the TAB, coliforms, *E. coli, L. monocytogenes, S. aurues*, and milk quality parameters (pH, fat rate, concentration and acidity). Milk samples have been collected weekly for a month. According to the findings, no milk sample has been determined to have *L. monocytogenes,* therefore this microbiologic parameter has not been included in the assesment. *Table 1* shows the testing of goup differences of the milk from control group and the experimental group according to the microbiological parameters while *Table 2* indicates the testing of group differences of the milk from two according to the chosen quality parameters. *Fig. 1* shows the DGAT1 gene from the Holstein cattles' milk, treated with ethidium bromide 2% agaroz gel DNA band views.

DISCUSSION

The milk productivity of the cattle is under the influence of multigenes and it is economically important to increase the milk productivity of livestock through correct genetic improvement. This study has been done to investigate the effect of the *DGAT1* gene in cattles on the microbiological quality of the milk and milk quality parameters.

DGAT1 gene lysine variant ("K" allele) is related to the reduction in protein and milk productivity. Increase in fat productivity is related to the alanine variant ("A" allele) is effective on the increase in milk and protein productivity and the reduction in fat productivity [2]. In the studies where Kaupe et al.[11], investigated *DGAT1* locus *K232* amplification on 1748 samples from 38 different cattle races, it has been determined that *DGAT1* "A" allele

Table 1. *The testing of goup differences of the milk from control group and experimental group according to microbiological parameters (The parameters written in bold carry statistically meaningful differences between the groups, P<0.005)*

Tablo 1. *Kontrol ve deney gruplarının sütlerinin seçilen mikrobiyolojik parametreler açısından grup farklılıklarının sınanması (Koyu karakterle yazılmış olan parametreler istatistik açıdan P<0.005 olduğundan gruplar arası anlamlılığı ifade etmektedir)*

Microbiological parameters / Statistical values	Mean	Standard Deviation	Standard Error Mean	Sig. (2-tailed)
Total Mesophilic Aerobic Becteria Count	-.0500	.16018	-.3743	-.0500
Coliforms	-.0448	.50134	.01602	.001
E. coli	-.0140	.33786	.01069	.000
S. aureus	19.771	-.0295	.01953	.030

Table 2. *The testing of goup differences of the milk from control group and the experimental group according to the chosen quality parameters (The parameters written in bold carry statistically meaningful differences between the groups, P<0.005)*

Tablo 2. *Kontrol ve deney gruplarının sütlerinin seçilen bazı süt kalite parametreleri açısından grup farklılıklarının sınanması (Koyu karakterle yazılmış olan parametreler istatistik açıdan P<0.005 olduğundan gruplar arası anlamlılığı ifade etmektedir)*

Milk quality parameters / Statistical values	Mean	Standard Deviation	Standard Error Mean	Sig. (2-tailed)
pH	6.62	.0168	.01388	.040
Milk Fat Rate	37.495	.2622	.04271	.000
Milk Concentration Rate	25.785	.0249	.00876	.014
Milk Acidity Rate	23.199	.0297	.01193	.019

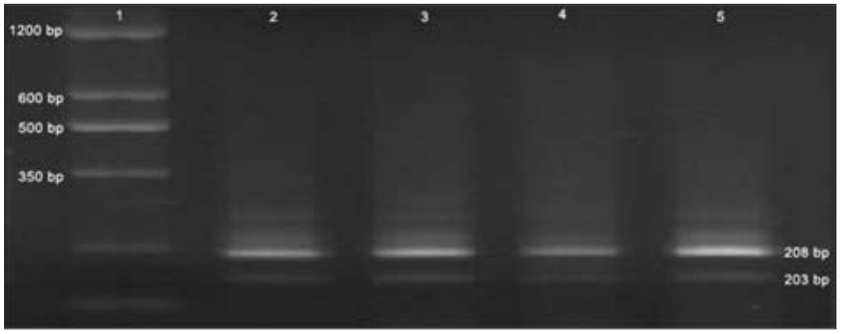

Fig 1. DGAT1 gene from the Holstein cattles, treated with ethidium bromide 2% agaroz gel DNA band views (partial sequence), (1: Marker, 2-5: The amplification products of the blood samples from the Holstein cattles)

Şekil 1. Holstein ırkı sığırlardan elde edilen DGAT1 geninin (parsiyel sekans olmak üzere) Ethidium bromide ile muamele edilmiş %2'lik agaroz jelde DNA bant görüntüleri, (1: Marker, 2-5: Holstein ırkı sığırların kan örneklerinden elde edilen amplifikasyon ürünleri)

frequency is high in meat types, whereas the related allele frequency is low in milk types. One of the few studies done in our country has shown that the *DGAT1* "K" allele and the *DGAT1* "A" allele frequencies are, respectively: Native Black 0.38-0.62 (N=73), Eastern Anatolian Red Cattle 0.25-0.75 (N=50), Western Anatolian Red Cattle 0.21-0.79 (N=48) and Grey Race 0.36-0.64 (N=49) and native races of Bos Indicus and Bostaurus centered in near east and African taurin-N'Dama cattle has higher *DGAT1* "K" allele frequencies [12]. Our findings are in line with Özdemir's reports and the *DGAT1* "K" and *DGAT1* "A" alleles gene frequencies of the native black cattles which have been reported as 0.35-0.60 (N=25). The obtained some milk quality parameters (milk fat rate, milk pH values, milk density and acidity) have shown that the "K" and "A" alleles of the *DGAT1* gene can directly affect the milk quality parameters. In addition to that, coliforms, *E. coli* and *S. aureus* is statistically and meaningfully less in the milk from the cattle with high allele frequency than in the milk from the cattle with low allele frequency.

Our findings show that *DGAT1* gene had a statistically meaningful effect on milk fat and milk concentration parameters. The study results indicate that the total fat in the milk of Holstein cattles which have a high allele frequency is higher than that of the milk of the native black race cattles which have a low allele frequency. According to this results, the native black race cattle's milk has higher density than the Holstein cattle's milk. Schennink et al.[3] have reported that the *DGAT1* gene is 50% effective in the quantity of the milk fat between the Holstein and Friesian cattle races. According to the results, milk fat in the milk from the cattle with high allele frequency is meaningfully higher than the milk from the cattle with the low allele frequency. Sun et al.[13] have pointed out that the "K" allele of the *DGAT1* gene increases the fat concentration of the milk but reduces the milk productivity. However, our findings in milk productivity parameter are not in parallel with Sun et al's findings. On the other hand, Mao et al.[1] have indicated that *DGAT1* gene is effective on fat concentration, total protein amount and total milk solid matter in cattles and have determined that "K" allele affects these parameters. The same researchers have also determined that the "A" allele has positive effects on milk productivity parameter. The findings of our study is similar to those of Mao et al.[1], about milk productivity. In our study, "K" and "A" alleles are determined in cattles with high allele frequency. According to our results, "A" allele is effective on milk productivity and it is thought to reduce/block the effect of "K" allele on milk productivity. The results show that the cattles with high allele frequency are "AK" haplotypes. This is relatively effective on the milk productivity of the cattles with low allele frequency but medical literature reports that "AA" haplotype cattles are more productive of milk than "AK" haplotype cattles whereas the milk of the cattles with the mentioned genomic profile includes less milk fat than the milk of the cattles of the "AK" haplotype [1].

In our study, the effect of the *DGAT1* gene on some of the milk microbiological parameters has also been investigated. There is no study in the literature showing whether the *DGAT1* gene has any effects on the microbiological qualities of milk or not. According to our findings, *DGAT1* gene is effective on coliforms and *E. coli* parameters and in the study, it has been determined that coliforms and *E. coli* is statistically meaningfully less in the milk of the cattles with the high allele frequency than that of cattle with the low allele frequency. Most of the coliforms and especially *E. coli* ferment milk sugar and sucrose during growthand produce acid and gas in result [14]. The milk of the cattles that had high allele frequency is thought to have relatively negative effect on the growth of coliforms and *E. coli*.

The increase of the proportion of total fat concentration in the solid matter of the milk and the dispersive distribution of the fat globules in the milk is probably another parameter which reduces the milk sugar and sucrose usage of the mentioned pathogens. Another probable reason that *E. coli* and coliforms grow relatively less in the milk of the cattles with high allele frequency might be that the optimum growth pH values of these pathogens are near to being neutral. According to the results, the pH values of the milk of the cattles with high allele frequency is meaningfully lower than those of the milk of the cattles with the low allele frequency. The average pH values of the milk of the cattles with high allele frequency (according to the general measures during the study period) is 6.3 whereas the average pH values of the milk of the cattles with low allele frequency (according to the general measures during the study period) is 6.7. The general pH value of

each group is accepted normal [15], however the difference between the group values is statistically meaningful. Although *E. coli* can grow on low acidic values as 4.4 pH when the other conditions are suitable, together with fat concentration parameter, low pH values are thought to be effective on *E. coli* and coliforms microorganisms. In the studied cattle groups, another microorganism determined to have statistically meaningful difference is *S. aureus*. Like coliforms and *E. coli, S. aureus* also ferments the milk sugars but differs from those microorganisms as it produces acid without gas during fermenting foremost mannitol and other sugars [14]. According to the results of the study, like the *E. coli* and coliforms, *S. aureus* growth is meanningfully less in the milk of the cattles with high allele frequency than that of the cattles with low allele frequency. The probable reasons which slow down the *E. coli* and coliforms growth in the milk of the cattles with the *DGAT1* gene are thought to be valid for *S. aureus* as well. The findings do not indicate a meaningful difference between the groups with regard to total mesofilic aerobic bacteria. No *L. monocytogenes* has been found in the milk samples examined, therefore this microbiological parameter is not included in the assesments.

Studies have identified genetic variation in the composition of milk quality parameters such as milk fat, density and acidity [16,17]. As a result, PCR methods are thought to be effective in determining the "K" and "A" alleles of the *DGAT1* gene. In addition to that, the findings of our study indicate that the quality parameters (pH, milk fat amount, milk concentration and acidity) of the milk of cattle with high allele frequency (Holstein race) have a statistically meaningful difference from the quality parameters of the milk of the cattles with low allele frequency. Also, it was determined that the examined milk microbiological parameters *E. coli, S. aureus* and coliforms grow meaningfully less in the milk of cattle with high allele frequency than in the milk of the cattles with low allele frequency. The *DGAT1* gene and especially the "K" allele of the gene is reported to be very ancient in European cattle races and to be inherited from the ancestors [2]. Medical literature reports that the native races also have the allele frequency but almost all these races have a lower allele frequency than the pureblood European races [18]. The correct strategies of hybridization and artificial insemination among the native races are thought to increase the allele frequency of the native races and thus contribute to the development of milk quality parameters and this is thought to positively effect the supply of more qualified milk to the consumer.

Our study is the first to examine the effect of allele frequencies of the *DGAT1* gene on the milk microbiological charges and no study similar to our study has been encountered in the literature. To supply high quality milk to the consumer is very important but the supply of raw milk with minimum microbiological risk is also a very important factor for safe milk supply to the consumer. According to the results of our study, the milk of the cattle races with high allele frequency is considered to be important in safer supply of milk to the consumer and the protection of public health. Similar studies on the subject and to define the biochemical mechanisms of the "K" and "A" alleles of the *DGAT1* gene more clearly is thought to be very rewarding with regard to the supply of both high quality and safe milk to the consumer.

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RESEARCH ARTICLE

Distribution and Location of Endocrine Cells in the Pancreas of the Sparrowhawk, *Accipiter nisus*

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Summary

The distribution and location of endocrine cells were determined in the dorsal, ventral, and splenic lobes of sparrowhawk pancreas using immunohistochemical methods. In this study, pancreatic tissues were stained with anti-insulin, anti-glucagon, anti-somatostatin, anti-gastrin, and anti-serotonin primary antibodies using the streptavidin-biotin-peroxidase method. The results showed that the numbers of glucagon- (alpha cell), insulin- (beta cell), and somatostatin- (delta cell) releasing cells were high and located in the splenic lobe of the pancreas. These endocrine cells were grouped into alpha, beta, and mixed islets. Alpha islets were mainly composed of alpha and delta cells and also occasionally beta cells. Beta islets contained numerous beta cells and a few delta and alpha cells. Furthermore, in the exocrine tissue were showed as only one cell or 2-3 gastrin immunopositive cell groups, whereas serotonin immunopositive cells were not found anywhere in the exocrine and endocrine pancreas. In conclusion, the endocrine islet types, endocrine cell localizations and lobe numbers of pancreas in sparrowhawks are similar to predator bird species, but are determined different to domestic fowls.

Keywords: *Insulin, Glucagon, Somatostatin, Gastrin, Pancreas, Sparrowhawk*

Atmaca (*Accipiter nisus*) Pankreas Dokusu Endokrin Hücrelerin Dağılımı ve Lokalizasyonu

Özet

Bu çalışmada, atmaca pankreasının dorsal, ventral ve splenik loblarında bulunan endokrin hücrelerin dağılımı immunhistokimysal metotlarla belirlenmiştir. Pankreas dokusu anti-glukagon, anti-insulin, anti-gastrin ve anti-somatostatin primer antikorları kullanılarak streptavidin-biotin peroxidase metodu ile boyandı. Çalışmada, glukagon (alfa hücresi), insülin (beta hücresi) ve somatostatin (delta hücresi) salgılayan hücrelerin splenik lobda sayısının en fazla olduğu saptandı. Atmalarda pankreasın endokrin bölümlerinin alfa, beta ve miks adacıklar halinde gruplaştıkları saptandı. Beta adacıklarının çok sayıda beta hücresi, az sayıda delta ve alfa hücrelerini içerdiği belirlendi. Alfa adacıkları, genellikle alfa ve delta hücrelerinden nadiren de beta hücrelerinden oluşuyordu. Ayrıca, gastrin pozitif hücreler, sadece ekzokrin pankreasta 1 ya da 2-3 adet hücre grubu halinde bulunurken, seratonin pozitif hücrelere ise ekzokrin ve endokrin pankreasın herhangi bir bölümünde rastlanmadı. Sonuç olarak, atmaca pankreasının lop sayısı, endokrin adacık tipleri ve endokrin hücre lokalizasyonu açısından etçil kuşlarla benzerliği, evcil kuş türleriyle de farklılıkları belirlenmiştir.

Anahtar sözcükler: *İnsulin, Glukagon, Somatostatin, Gastrin, Pankreas, Atmaca*

INTRODUCTION

The pancreas consists of exocrine and endocrine (Langerhans islets) compartments, which both have different functions. Mammalians pancreases are comprised of head, body and tail regions [1,2], whereas pancreas of

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avian species consisted of three [3,4] or four lobes: dorsal ventral, third and splenic [4,5]. Also, the islets of Langerhans of avian species are different from those of mammals [6-8]. Avian islets of Langerhans are divided into alpha, beta, and mixed islets according to their cellular composition [6,7]. Each islet of Langerhans in mammalian pancreas consists of a few to several thousand cells [9,10], which are alpha (A, A2, α), beta (B, β), delta (D, δ), and pancreatic polypeptides (PP, F). Some authors have reported that the islets of Langerhans may also contain substance P, neuropeptide Y, serotonin, cholecystokinin-8, galanin [6], gastrin, and ghrelin-immunopositive cells in the some avian species [1]. In the mammalians, beta cells are located generally in the central of islets, and alpha cells located in the periphery of islets in humans, rats, and mice [1], however these cells are opposite position in the pancreatic islets of horses [2], monkey and kangaroo [11]. In the avian species, endocrine parts of pancreas are generally consisted of large diameter alpha islets, higher number of beta islets and a few or have not mixed islets [1,7,11]. Alpha islets consist of numerous alpha and delta cells, whereas beta islets are consist of numerous beta cells, mildly delta cells, rarely a few alpha cells [5,7].

Researchers have studied the anatomical, histological, and histopathological structures of the pancreas. The distributions of the different types of cells in the pancreas of avian species have been demonstrated by immunohistochemical methods in mynah [12], chickens [13], falcons [7], ducks [3], geese [14], and young Japanese quails [5]. To our knowledge, there is no study showing the histological distribution of endocrine cells in the pancreas of the sparrowhawk *(Accipiter nisus)*. The aim of this study was to determine the distribution of glucagon-, insulin-, somatostatin-, gastrin-, and serotonin-releasing cells in sparrowhawk pancreas by using immunohistochemical-staining techniques.

MATERIAL and METHODS

Nine sparrowhawks with injuries including shotgun wounds, broken legs, and wings or poisoning were recieved from the animal hospital at Atatürk University, Faculty of Veterinary Medicine, Erzurum, Turkey between 2011 and 2013. Their weights ranged from 150 g to 250 g. Sparrowhawks that could not recover from their injuries were euthanized using ether anesthesia, and pancreatic tissue was taken from the dorsal, ventral, and splenic lobe edges of the pancreas. The tissues were fixed in 10% neutral buffered formalin. The fixed materials were dehydrated in a graded series of ethanol and embedded in paraffin wax. Paraffin wax-embedded pancreases were sectioned in series at a thickness of 5-7 µm.

For examination of the histological structure of the tissue, the sections were stained with Crossman modified Mallory's triple stain [15]. Pancreatic endocrine cells were also detected by immunohistochemistry using the streptavidin-biotin-peroxidase method. The sections were deparaffinized in xylene and dehydrated in descending alcohols, and then antigen retrieval was performed by heating the slides in ethylene diamine tetra acetic acid (EDTA) buffer (pH:8.0). Endogenous peroxidase activity was blocked with 3% H_2O_2. Normal bovine serum was used to block nonspecific binding sites of antibodies, and then sections were incubated with primary antibodies (glucagon (Leica, 1/50 dilution), insulin (Cell Marque), somatostatin (Cell Marque), gastrin (Leica, 1/50 dilution), and serotonin (Dako, 1/50 dilution)) for one hour. Following this, they were incubated with biotinylated secondary antibody and then streptavidin horseradish peroxidase (Dako, Universal LSAB Kit, K0690) for 30 min each. To demonstrate the reactions, 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) was used. Nuclei were stained with Harris's haematoxylin, dehydrated through an ethanol series, and then cleared in xylene before being mounted using Entellan (Merck, German).

Immunohistochemical Evaluation Procedure

The binding of antibodies was evaluated using a high-power Nikon i50 light microscope (Nikon, Tokyo, Japan). The sections of all lobes were evaluated for the location of islets and the distribution of pancreatic endocrine cell types. For each animal, ten serial-sectioned pancreas tissue slides were analyzed for endocrine cell types. Evaluations and scoring of endocrine cell distribution or localization were insulin-positive, glucagon positive, somatostatin-positive, gastrin-positive, and serotonin-positive. The scores were derived semi-quantitatively using light microscopy on the preparations from each animal, and were reported as follows: none = –: not detected, +: rare, ++: a few, +++: moderate, ++++: numerous.

RESULTS

Sparrowhawk pancreas, which consisted of dorsal, ventral, and splenic lobes, was located in a horizontal position in the abdomen. Endocrine islets of different sizes and shapes were dispersed throughout the pancreatic tissue. In the immunohistochemical examination, glucagon- *(Fig. 1)*, insulin- *(Fig. 2)*, and somatostatin - *(Fig. 3)* immunopositive endocrine cells were detected in the pancreatic islets. Also, glucagon- *(Fig. 1)*, insulin- *(Fig. 2)*, and gastrin- *(Fig. 4)* immunopositive cells were found within the acini as both single and ductular areas, but serotonin-immunopositive cells were not detected in either the endocrine pancreas or the exocrine pancreas. The location and distribution of endocrine cells in the pancreas are presented in *Table 1*.

In the sparrowhawk pancreas, according to the distribution of endocrine cells, there were three types of islets: alpha, beta, and mixed islets. These islets were numerous in the splenic lobe compared to the other lobes.

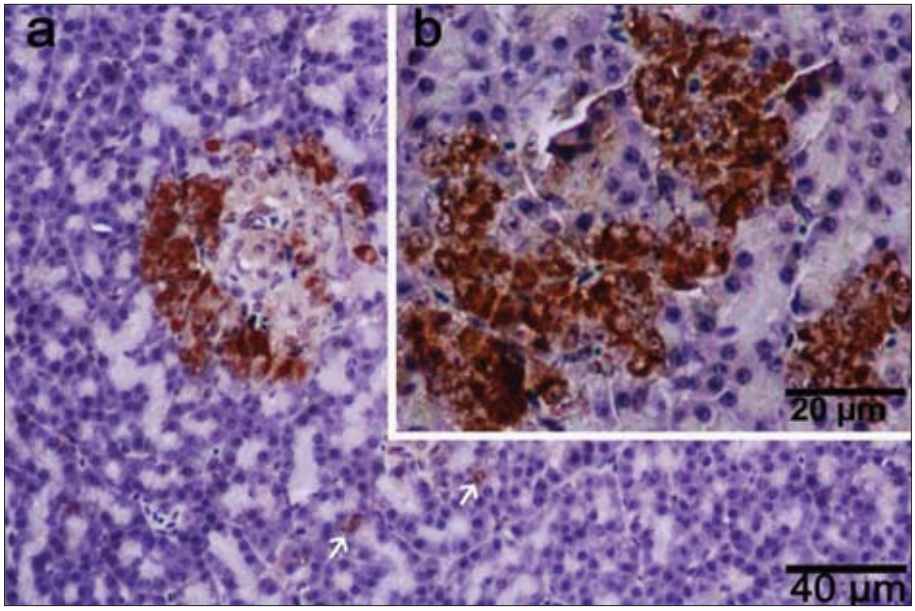
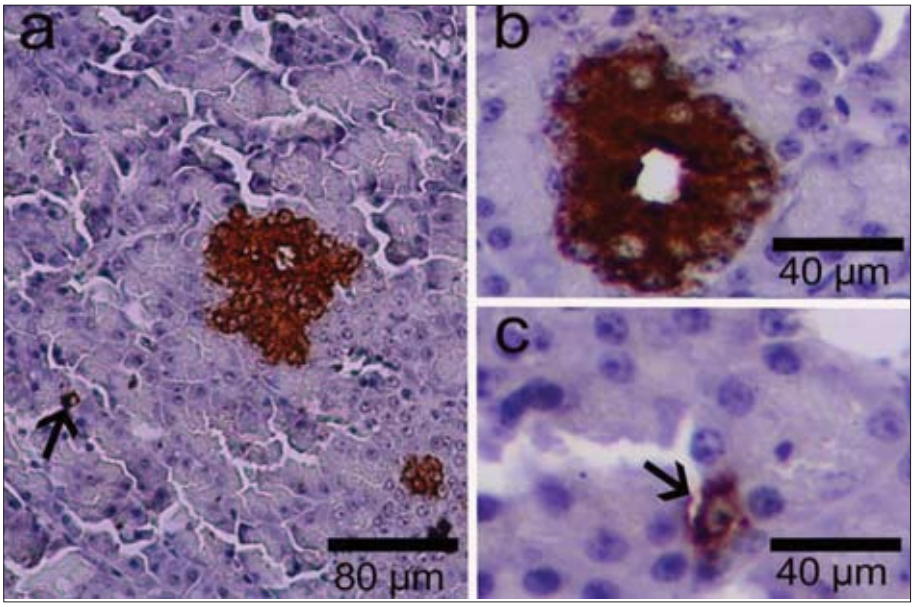


Fig 1. The distribution of the glucagon immunopositive cells within the mixed **(a)** and alpha **(b)** islets of pancreas; *arrows:* Some of glucagon immunopositive cells were located in the exocrine region of pancreas. Streptavidin-biotin peroxidase method

Şekil 1. Pankreatic miks **(a)** ve alfa **(b)** adalardaki glucagon immun-pozitif hücrelerin adacıklardaki dağılımı, *oklar;* pankreas ekzokrin bölümünde bazı immunopozitif glukagon hücrelerin dağılımı, Streptavidin-biotin peroxidase metodu

Fig 2. The distribution of the insulin immunopositive cells within the mixed **(a)** and beta **(b)** islet, and exocrine pancreas **(c)**, *arrows;* single located insulin immunopositive cells in the exocrine region of pancreas, Streptavidin-biotin peroxidase method

Şekil 2. Pankreatik miks **(a)** ve beta **(b)** adaları ve ekzokrin alan **(c)** immun-pozitif İnsülin immunopozitif hücrelerin dağılımı, *oklar;* ekzokrin pankreasda tek yerleşimli insulin pozitif hücreler, Streptavidin-biotin peroxidase metot



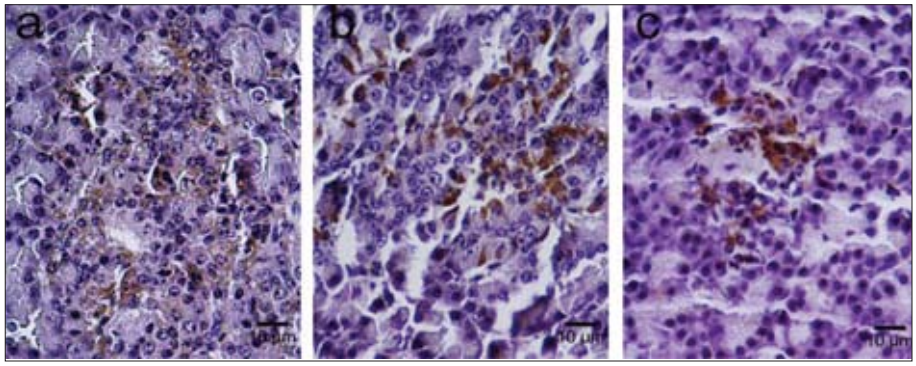


Fig 3. The distribution of the somatostatin immunopositive cells within the alpha **(a)**, beta **(b)** and mixed **(c)** islets in pancreas, Streptavidin-biotin peroxidase method

Şekil 3. Pankreatik alfa **(a)**, beta **(b)** ve miks **(c)** adalardaki somatostatin immun-pozitif hücrelerin adacıklardaki dağılımı, Streptavidin-biotin peroxidase metot

Alpha islets were found as generally large cell clusters and occasionally small islets, which principally contained alpha cells *(Fig. 1)*, a few delta cells, and occasionally beta cells. In addition, the somatostatin-immunopositive cells were observed in both the peripheral and central regions of the alpha islets *(Fig. 3)*. Beta islets were generally oval-shaped small endocrine islets. Beta islets were more numerous than alpha islets in all lobes of the sparrowhawk pancreas. Beta islets were comprised mainly of beta cells *(Fig. 2)* with a few delta cells located in the periphery of the islets.

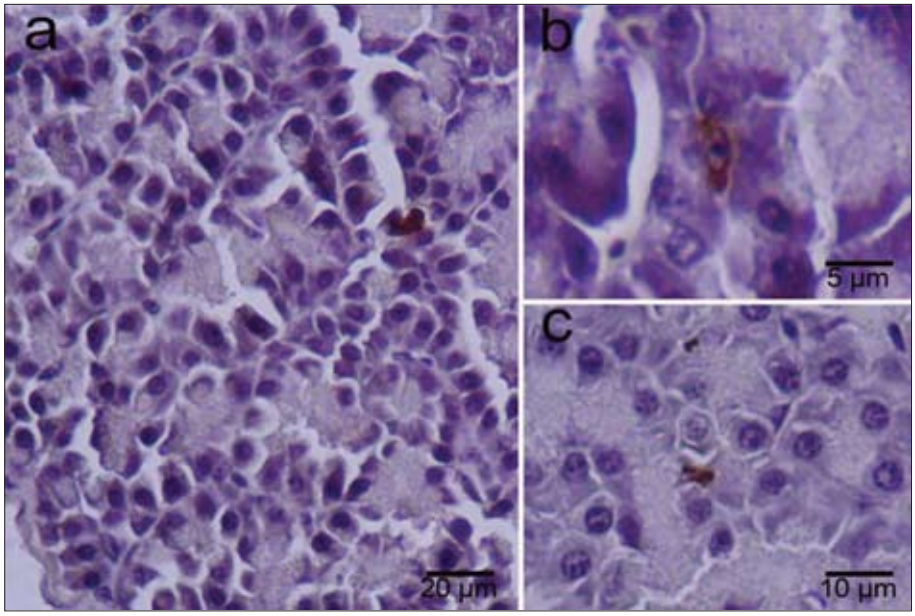


Fig 4. The distribution of the gastrin immunopositive cells within the exocrine region of the sparrowhawk pancreas; **a, b,** and **c;** gastrin immunopositive cells are generally located among the acini as a single in the exocrine region of pancreas, Streptavidin-biotin peroxidase method

Şekil 4. Atmaca pakreası ekzokrin alanında gastrin immun pozitif hücrelerin dağılımı, **a, b,** ve **c;** ekzokrin pankreas asinuslarına genellikle tek yerleşimli gastrin immun-pozitif hücreler, Streptavidin-biotin per-oxidase metot

Table 1. *Distribution and localization of endocrine cells in pancreas of the sparrowhawk*
Tablo 1. *Atmaca pankreası endoktrin hücrelerinin dağılımı ve lokalizasyonu*

Antibody	Alpha Islet		Beta Islet		Mixed Islet		Exocrine Areas
	Periphery	Central	Periphery	Central	Periphery	Central	
Glucagon	++++	+++	-/+	-	++++	++	+
Insulin	-/+	-/+	+	++++	+	+++	+
Somatostatin	++	+	++++	-	+++	++	-
Gastrin	-	-	-	-	-	-	+
Serotonin	-	-	-	-	-	-	-

Relative frequencies; –: not detected, +: rare, ++: a few, +++: moderate, ++++: numerous

Mixed islets generally consisted of central-positioned beta cells and peripheral-located alpha cells, or they were occasionally composed of side-by-side alpha and beta islets (*Fig. 1* and *Fig. 2*).

DISCUSSION

This study reveals, for the first time, the existence, location, and distribution of insulin-, glucagon-, somatostatin-, and gastrin-immunopositive endocrine cells in sparrowhawk pancreas using immunohistochemistry methods. The number of islets may vary greatly in size, with large islets being replaced near the arterioles and smaller islets being replaced in the deeper pancreatic parenchyma. Endocrine islets show different distributions, locations, and characteristics among avian species [1]. A high number of alpha-, beta-, and somatostatin-releasing cells, and a small number of gastrin-, serotonin-, substance P-, neuropeptide Y-, and cholecystokinin-8-releasing cells are located in the pancreatic endocrine islets of some avian species [3,5,6,12-14,16]. Therefore, the present study aimed to investigate the distribution and location of endocrine cells in the pancreas of the sparrowhawk.

The Endocrine region of the pancreas in avian species generally consists of large-diameter alpha islets, a higher number of beta islets [6,17], and a few or no mixed islets [5,14]. In many studies, the number of endocrine islets in both avian and mammalian species is higher in the splenic lobe (tail region) than the other lobes of the pancreas [14,18]. According to Rawdon and Larsson [4], small beta islets were found in the dorsal and ventral lobes of the pancreas; moreover, Gulmez et al.[14] and Simsek et al.[5] found predominantly alpha and beta islets in the splenic lobes of goose and quail pancreas, respectively. In the present study, in sparrowhawk pancreas, alpha, beta, and mixed islets were found in higher numbers in the splenic lobes than in the other lobes.

Glucagon

Pancreatic alpha cells secrete glucagon, which regulates glucose levels in the blood [19]. In this study, glucagon immunopositive cells were located numerously in the throughout of the alpha islets and a few in the periphery of the beta islets and rarely in the exocrine parenchyma. According to Tarakci et al.[8], the alpha cells of ostrich are located in the periphery of the islets. Our findings

revealed predominantly in the throughout of the alpha islets in the sparrowhawk pancreas, which is in accordance with previous studies [2,5,6,14]. Also, this study, single or 2-3 alpha cell clusters were observed in the pancreatic parenchyma. However, some authors are demonstrated that alpha cells found only in the endocrine islets of the falcon [7], goose [14] and quail [5]. These differences in locality might be related to paracrine interactions, which may affect the cell types in the different reproductive periods. Paracrine interactions have been proposed as another way of signaling to achieve glucose homeostasis [20]. The different locations of alpha cells in the sparrowhawk pancreas may be related to this mechanism.

Insulin

Each endocrine cell produces only one specific peptide hormone. Beta cells are responsible for the secretion and storage of insulin in response to decreased plasma glucose concentrations. Insulin regulates carbohydrate metabolism and has anti-apoptotic effects on pancreatic cells [21]. In common mammalian species, the beta cells are located in the center of the endocrine islets [1,11]. Conversely, in goose [14], falcons [7], quails [5], chickens [23], and ducks [1], beta cells are mainly located in the beta islets and are rarely observed in the alpha islets. In this study, insulin immunopositive cells were generally located in the beta and mixed islets, and similar to falcon's [7], single or 2-3 clustered beta cells were located in the alpha islets and exocrine pancreas.

Somatostatin

Somatostatin is a peptide hormone, which is found in neurons, the pancreas, the gut, and some other tissues. There are two forms of somatostatin in the body: SOM-14 and SOM-28, which inhibit both glucagon and insulin secretion [25]. In addition, they inhibit endocrine and exocrine secretions of the pancreas and have an effect on neurotransmission, the gastrointestinal system and biliary motility, intestinal absorption, and cell proliferation [16,26]. Previous studies have showed the existence of delta cells in the pancreases of geese [14], quails [5] and long-legged buzzards [6]. In these studies, somatostatin-releasing cells were found more frequently in alpha islets than in beta islets. Moreover, delta cells were not observed in the exocrine part of the pancreas. Similarly, Tarakci et al.[8] did not observe delta cells in the exocrine pancreas of the ostrich. Contrary to these results, delta cells are also observed in exocrine pancreas of quail and chicken [5,27].

Gastrin

Endocrine gastrin exists in part of the digestive tract, and gastrin secretions are affected by the regulation of stomach motility in the digestive system [28]. In some studies, gastrin stimulated pancreatic growth and differentiation by stimulation of epithelial growth factor (EGF) [29]. Gastrin or vasoactive intestinal peptide (VIP), is produced in some pancreatic endocrine neoplasms, although these are not found in normal mammalian endocrine islets. However, gastrin or the homologue of cholecystokinin is transiently expressed in the developing pancreas islets, and the highest gastrin expression is produced by the mammalian fetus pancreatic islets during development [30]. In the present study, immunopositive gastrin cells were found in the exocrine region. To date, no avian studies have detected anti-gastrin, and so the differences between avian and mammalian species are considered here. According to the literature, in developing mammalians, due to the effect of EGF, some ductular cells differentiated to gastrin-producing cells [30]. However, these gastrin secretions did not continue into adulthood, whereas the findings of this study showed that these gastrin-immunopositive cells are found in the adult sparrowhawk.

Serotonin

The existence of serotonin-releasing cells in the pancreas of mammals has been reported [31]. In this study, no serotonin immunopositive cells were found in either the exocrine or the endocrine regions of the sparrowhawk pancreas. On the contrary, some studies reported the existence of serotonin immunoreactive cells in the pancreases of ducks [3] and chickens [32]. Many immuno-histochemical studies have showed the similar morphological characteristics between different species, which may reflect the metabolic characteristics of some avian species.

In conclusion, the examination of the endocrine region of the sparrowhawk pancreas has showed that some differences exist among avian species. The endocrine region of the sparrowhawk pancreas consists of alpha, beta, and mixed islets. Although the insulin- and glucagon immunopositive cells were located in the endocrine and exocrine regions, the somatostatin-releasing cells were found as clusters and/or single cells in the endocrine region. And also, the gastrin immunopositive cells were detected as single cells in the exocrine pancreas, whereas serotonin immunopositive cells were not found in the all of the sparrowhawk pancreas.

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SHORT COMMUNICATION

Biochemical Profile of *Paenibacillus larvae* Repetitive Element Polymerase Chain Reaction (rep-PCR) Genotypes in Bulgaria [1]

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Summary

The aim of the present study was to determine the biochemical profile of *Paenibacillus larvae* repetitive element polymerase chain reaction (rep-PCR) genotypes in Bulgaria and to assess the link between genotype and phenotype. A total of 103 isolates (genotype AB, n=21; genotype ab, n=82) and a reference strain NBIMCC 8478 were analyzed using identification system BioLog Gen III and nitrate reducing ability. Genotypes AB and ab showed a particular metabolic fingerprint based on 71 carbon sources provided by BioLog system. Considering the nitrate reducing ability, mannitol and salicin utilization, the strains were distributed into biotypes I, III, IV and VIII. The majority of *Paenibacillus larvae* AB clustered into biotype III while ab were grouped mainly into biotype I. Biotypes I and IV were not found among the tested AB strains. This study showed the obvious link between rep-PCR genotypes AB/ab and biochemical phenotype that can be useful in epidemiologic situations to trace the source of infection and to control the disease.

Keywords: *Paenibacillus larvae, rep-PCR, genotype AB and ab, biochemical phenotype*

Bulgaristanda *Paenibacillus larvae* Repetitive Element Polymerase Chain Reaction (rep-PCR) Genotiplerinin Biyokimyasal Profili

Özet

Bu çalışmanın amacı Bulgaristan'da *Paenibacillus larvae* repetitive element polymerase chain reaction (rep-PCR) genotiplerini belirlemek ve genotip ile fenotip arasındaki ilişkiyi ortaya koymaktır. Toplam 103 izolat (genotip AB, n=21; genotip ab, n=82) ve referans suş olarak NBIMCC 8478 BioLog Gen III identifikasyon sistemi kullanılarak ve nitrat indirgeme kabiliyeti yönünden analiz edildi. Genotip AB ve ab, BioLog system ile 71 karbon temelli özel bir metabolik parmak izi gösterdi. Nitrat indirgeme kabiliyeti, mannitol ve salisin kullanımı göz önüne alınarak suşlar biyotip I, III, IV ve VIII olarak ayrıldı. *Paenibacillus larvae* AB'nin büyük bölümü biyotip III'de toplanırken ab çoğunlukla biyotip I'de gruplandı. Biyotip I ve IV test edilen AB suşları arasında tespit edilmedi. Bu çalışma rep-PCR genotipleri AB/ab ile biyokimyasal fenotipler arasındaki ilişki koyarak epidemiyolojik çalışmalarda enfeksiyon kaynağını tespit etme ve hastalığın kontrolünde faydalı olacaktır.

Anahtar sözcükler: *Paenibacillus larvae, rep-PCR, Genotip AB ve ab, Biyokimyasal fenotip*

INTRODUCTION

Paenibacillus larvae (P. larvae) is the etiologic agent of the most virulent bacterial disease of honey bees, American foulbrood (AFB) [1]. The bacterium is Gram-positive, rod-shaped, catalase negative and spore forming [2]. The infectious form of the agent are only spores which are difficult to be induced *in vitro* but are readily formed in infected larvae [3]. Therefore, spores are transmitted easily in the nest, between colonies and apiaries during the beekeeping practice leading to considerable beekeepers' losses worldwide [3]. American foulbrood is a notifiable disease in many countries including Bulgaria, where regulatory measures are observed to control the disease [4]. Incorporation of the research achievements on conventional and molecular characteristics of the agent into practice

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would be a valuable additional epidemiologic tool in AFB surveillance programs.

Biochemical properties of *P. larvae* have long been a subject of scientific interest. Carbolytic, proteolytic and lipolytic activities of the bacterium have been studied [5]. However, the researchers' reports have been somewhat in disagreement with each other. Jelinski [5] proposed a scheme to biotype *Bacillus larvae (B. larvae*, now *P. larvae)* based on nitrate reduction, acid production from mannitol and salicin. Hence, biotypes I-VIII were possible. These variable features might be of importance in distinguishing the biochemical types of the bacterium in disease outbreaks. With the development of molecular typing methods different genotypes of *P. larvae* have been recognized. The most exploited molecular techniques for typing *P. larvae* were the repetitive element polymerase chain reaction (rep-PCR) [6-9] and pulse-field gel electrophoresis (PFGE) [10,11]. There is still little information about the link between different genotypes established with rep-PCR and their phenotype [1,7].

Nothing is known about the biochemical profile of *P. larvae* genotypes in Bulgaria- that was the rationale to perform the present study. Hence, the aim of this work was to characterize genotypes of the agent determined in Bulgarian apiaries with the commercial identification system BioLog Gen III and by their nitrate reducing ability. The link between genotype and biochemical phenotype was also assessed.

MATERIAL and METHODS

Isolation, PCR Identification and rep-PCR Genotyping of P. larvae Isolates

A total of 103 *P. larvae* isolates and a reference strain NBIMCC 8478 were included in the study. Strains were isolated from brood combs with clinical symptoms of AFB originating from apiaries located in different regions of Bulgaria. Isolation and PCR identification of the isolates were done as previously described [12].

Genotyping of the isolates was based on rep-PCR protocol using BOX A1R and MBO REP1 primers developed by us [9].

Biochemical Profile of P. larvae rep-PCR Genotypes in Bulgaria

Biochemical profile of the isolates with known genotype was determined by the commercial identification (ID) system BioLog Gen III (Hayward, USA). Microplates were processed following the company's protocol. Briefly, protocol A was used to identify and characterize *P. larvae*. The isolates were cultured on trypticase soy agar (Fluka, India) supplemented with 5% defibrinated sheep blood and the inocula were prepared in a broth medium, procured by the manufacturer until achieving the cell density in the range of 90-98% T. The plates were filled with 100 µL of the inocula and incubated at 33ºC for 48-72 h aerobically. Then, the plates were analyzed using the computer system software OmniLog. After identification with the BioLog system, only the substrates providing a carbon source (n=71) were taken into consideration to characterize the strains. In addition strains were analyzed by their nitrate reducing ability and grouped into biotypes as described by Jelinski [5].

RESULTS

Isolated strains were identified by multiplex PCR protocol for detection of the fragments of 16S rRNA and metalloproteinase (Mlp) genes of *P. larvae*. Specific amplicon length of 973 bp (16S rRNA gene) and 273 bp (Mlp) was generated which confirmed the species affiliation.

Molecular typing with rep-PCR using BOX A1R and MBO REP1 primers resulted in two genotypes of the studied isolates. Most of them referred to genotype ab (n=82) followed by AB isolates (n=21). The reference strain NBIMCC 8478 belonged to ab genotype.

All tested isolates were confirmed as *P. larvae* by the BioLog Gen III system. The biochemical profile of *P. larvae* genotypes AB and ab determined with BioLog microplates is given in *Table 1*. Genotype AB and ab isolates were able to metabolize D-trehalose, N-acetyl-D-glucosamine and N-acetyl-β-D-mannosamine. All AB isolates used D-fructose and D-mannitol as carbon sources, while for ab isolates the results were 5% and 18%, respectively. Glucose was utilized by 100% of ab and 90% of *P. larvae* AB. All ab isolates used glycerol as a carbon source, positive reactions for the AB genotype were 10%. Zero percent of genotype AB metabolized D-turanose, L-alanine and L-lactic acid. None of the tested ab isolates used D-melibiose as for AB values were also low - 5%.

Considering the nitrate reducing ability, mannitol and salicin utilization, the strains were distributed into biotypes I, III, IV and VIII. Only the reference strain referred to biotype V. The majority of *P. larvae* AB clustered into biotype III (90%) while ab were grouped mainly into biotype I (62%). Biotypes I and IV were not found among the tested AB strains. Biotype VIII represented 10% of AB and 13% of ab isolates *(Fig. 1)*.

DISCUSSION

The present study has shown that the ID system BioLog GEN III can be successfully used for the correct identification of *P. larvae*. In addition to the probability values, BioLog provides information about the biochemical profile of analyzed isolates. Findings also revealed that the

Table 1. *Biochemical profile of P. larvae (genotype AB and ab) strains determined with BioLog Gen III microplates. Results are presented as percent positive reactions of using the respective carbon source*

Tablo 1. *P. larvae (genotip AB ve ab) suşlarının BioLog Gen III mikroplate ile belirlenen biyokimyasal profili. Sonuçlar ilgili karbon kaynağı kullanılarak yüzde pozitif reaksiyon olarak ifade edilmiştir*

Well	A4	A8	B3	B5	B6	B7	B8	C1	C2	C3	C9	D2	D5	E3	E9	G2	G4
Substrate	D-trehalose	D-Turanose	D-Melibiose	D-Salicin	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	N-Acetyl-D-Galactosamine	α-D-Glucose	D-Mannose	D-Fructose	Inosine	D-Mannitol	Glycerol	L-Alanine	L-Serine	Methyl Pyruvate	L-Lactic Acid
P. larvae genotype AB n = 21	100	0	5	10	100	100	43	90	52	100	33	100	10	0	10	29	0
P. larvae genotype ab n = 82	100	9	0	33	100	100	24	100	5	5	100	18	100	20	34	91	7

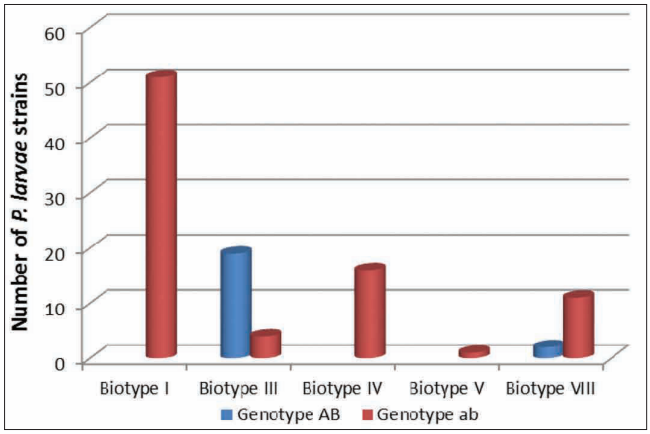

Fig 1. Distribution of Bulgarian *P. larvae* genotypes AB/ab into biotypes according to Jelinski [5]; Biotype I (reduction of nitrate to nitrite - ; acid production from mannitol - ; acid production from salicin -); Biotype III (reduction of nitrate to nitrite - ; acid production from mannitol + ; acid production from salicin -); Biotype IV (reduction of nitrate to nitrite - ; acid production from mannitol - ; acid production from salicin +); Biotype V (*P. larvae* NBIMCC 8478, reduction of nitrate to nitrite + ; acid production from mannitol - ; acid production from salicin +); Biotype VIII (reduction of nitrate to nitrite - ; acid production from mannitol + ; acid production from salicin +); + = positive reaction; - = negative reaction

Şekil 1. Bulgaristan *P. larvae* genotipleri AB/ab'nin Jelinski'nin bildirdiğine yönteme göre [5] biyotiplere dağılımı; Biyotip I (nitratı nitrite indirgeme - ; mannitolden asit üretimi - ; salisinden asit üretimi -); Biyotip III (nitratı nitrite indirgeme - ; mannitolden asit üretimi + ; salisinden asit üretimi -); Biyotip IV (nitratı nitrite indirgeme - ; mannitolden asit üretimi - ; salisinden asit üretimi +); Biyotip V (*P. larvae* NBIMCC 8478, nitratı nitrite indirgeme + ; mannitolden asit üretimi - ; salisinden asit üretimi +); Biyotip VIII (nitratı nitrite indirgeme - ; mannitolden asit üretimi + ; salisinden asit üretimi +); + = pozitif reaksiyon; - = negatif reaksiyon

system was a good alternative to PCR for detection of the agent, especially where the molecular techniques are not yet applicable. However, the ID results are obtained after 48-72 h incubation due to the slow growth and metabolic activity of *P. larvae*.

This is the first study concerning the biochemical profile of *P. larvae* rep-PCR genotypes in Bulgaria. A relatively small number of the BioLog Gen III substrates were utilized by both AB and ab genotypes included in the present work. Out of the 71 carbon sources, 17 were used. It was found that only three substrates were metabolized by 100% of isolates from genotypes AB and ab, namely D-trehalose, N-acetyl-D-glucosamine and N-acetyl-β-D-mannosamine. In a study of Neuendorf et al.[7] regarding the biochemical characterization of German *P. larvae* genotypes (AB, Ab, ab) using BioLog Gram positive identification test panel, the authors obtained similar results for D-trehalose (92% AB) and N-acetyl-D-glucosamine. With respect to N-acetyl-β-D-mannosamine the findings were not comparable. None of the German ab isolates and 19% of AB have used this substrate as carbon source. The same study exhibited the AB genotype as one with a most striking metabolic pattern, since it was the only able to utilize the carbohydrates D-fructose and D-psicose, and the only strains not capable to use glycerol. Also, the authors did not find any isolate which could use turanose or L-alanine. However, among tested isolates in our study we detected 5% of ab to metabolize D-fructose, 10% of AB able to use glycerol, 9% and 20% of ab to use turanose and L-alanine, respectively. Regarding some of the substrates incorporated in the test panel, similar to our results were reported by Carpana et al.[13] and Dobbelaere et al.[14] who evaluated the API 50CHB and BBL Crystal systems for identification and biochemical characterization of *B. larvae*. Jelinski [5] also observed consistent and variable biochemical properties of the studied 110 reference and field strains *B. larvae*. It has to be considered that in this three studies and in the past years' reports the genotypes of the bacterium were not known.

Alippi and Aguilar [15] observed no obvious linkage between the biochemical type and the genotype of the analyzed isolates. The results in this study are not comparable with those of Alippi and Aguilar [15] because of the different primers used for the rep-PCR molecular typing of the strains. An association between genotypes

and their biochemical phenotype was found by Neuendorf et al.[7] using the BioLog Gram positive panel with 95 carbon sources as mentioned above. Particular metabolic features of Bulgarian AB and ab isolates were also found based on the 71 carbon sources. Additionally, the strains were biotyped according to Jelinski [5] testing their nitrate reducing ability that was not included in the Biolog microplates layout. Thus the link between genotype and biotype became even more apparent. Biotypes I and IV correlated with the ab genotype. The majority of AB genotype belonged to biotype III versus 5% for ab strains. The reference strain belonged to biotype V. Interestingly, we found strains possessing characteristics for biotype VIII whereas according to Jelinski [5] no strains were known by that time. Pentikäinen et al.[11] also used the proposal of Jelinski [5] to biotype Finnish *P. larvae* strains. The authors found biotype V as the commonest followed by biotypes IV and I. Pentikäinen et al.[11] established a relationship between the genotype determined by pulse-field gel electrophoresis and the biotype. Obviously biotyping based on the work of Jelinski [5] combined with the genotype can be very useful to address the epidemiologic problems in AFB outbreaks.

The precise comparison between genotype and biochemical phenotype of the strains originating from different countries is impossible due to the different biochemical tests and ID systems or used genotyping methods and probably the geographically related metabolic features of the studied strains. However, this study has shown a clear connection between the rep-PCR AB/ab genotypes and the biochemical type. We suggest that biotyping using ID systems or the accepted biochemical scheme based on three substrates could add to the genotype specific characteristics and might improve the epidemiologic investigations with regard to tracing the source of infection and controlling the disease.

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CASE REPORT

Ultrasonographic Finding in Anterior Displacement of Abomasum in a Cow

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Summary

In this case report, the ultrasonographic appearance of the abomasum, changes in some biochemical and blood gases parameters in a cow with anterior abomasal displacement (ADA) were described. Hyperbasemia, hypokalemia, hypocalcemia and hyperlactatemia in the cow with anterior abomasal displacement was detected. Displaced abomasum was imaged approximately 10 cm cranial of the xyphoid process from the left and and right paramedian regions and from the ventral abdomen midline, immediately caudal to the reticulum. This aim of case report was to contribute to the litature data for anterior abomasal displacement that can be also rare in cows.

Keywords: *Cow, Anterior abomasal displacement, Ultrasonography, Diagnose*

Bir Sığırda Anterior Abomazum Deplasmanının Ultrasonografik Bulgusu

Özet

Bu vaka raporunda anterior abomazum deplasmanlı (ADA) bir sığırda kan gazları ve bazı biyokimyasal parametrelerdeki değişiklikle birlikte abomazumun ultrasonografik görüntüsü tanımlandı. Materyali bir yaşında holştein ırkı sığır oluşturdu. Anterior abomazum deplasmanlı sığırda hiperbazemi, hipokalemi, hipokalsemi ve hiperlaktemia belirlendi. Deplase olan abomazum sol ve sağ paramedian bölgesinden ve ventral abdomenin ortasından ksifoid prossesin yaklaşık 10 cm kraniyalinde, hemen retikulumun kaudalinde görüntülendi. Bu olgu sunumunun amacı sığırlarda nadir görülen abomazumun anterior deplasmanına ilişkin literatüre katkı sağlamaktır.

Anahtar sözcükler: *Sığır, Anterior abomazal deplasman, Ultrasonografi, Tanı*

INTRODUCTION

Abomasal displacement are the most importance problem of dairy cows due to cause serious economic loss [1,2]. Abomasal displacement occurs most frequently in high yielding cows during early lactation [3-5]. Left displacement of the abomasum in dairy cattle occurs when the cow's abomasum moves from its normal anatomic location and becomes entrapped between the rumen and left abdominal wall. Right displacement of the abomasum twists in 2 planes: on its longutidinal axis and on its mesenteric or omental axis when abomasum moves from its normal anatomic location and becomes entrapped between liver/intestine and right abdominal wall [6-8]. Otherwise, anterior displacement of abomasum in cattle very rare occurs and it's diagnose is difficult. Van de Watering et al.[9] first described anterior displacement of the abomasum in one cow. However, Radostits et al.[2] and Zadnik [8] reported anterior dis-placement of abomasum in cattle. It was reported that an ultrasonography a valuable techniques for evaluate of the size, position, and content of the abomasum [1,10].

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The ultrasonographic appearance of the abomasum, changes in some biochemical and blood gases parameters in a cow with ADA was described in this case report.

CASE HISTORY

A 1- year old male Holstein cow was described anterior abomasal displacement. The cow with ADA had clinically appetite, depressive, abdominal pain, decreased rumen motility, no defecation, increased of hearth and respiration rate. In auscultation and percussion of the ventral abdomen, tympanic resonance (a ping sound) was not taken. Splashing sound was also not heard on ventral abdominal wall.

After cow received rutin clinical examination, heparinised and K-EDTA venous blood samples were taken from the jugular vein. RBC and WBC counts were measured by automatic haemocell counter (MS4, CFE 279, France). Blood gas analysis and sodium, potassium, ionised calcium (ICa) and lactate measurement were performed by GEM Premier Plus 3000 automatic analyzer (Model 5700, 74351, USA). Serum calcium and glucose was measured with an automatic analyzer (BT 3000 plus, Biotecnical Inc, SPA, Via lizenca, 18 00155, Rome, Italy).

Hyperbasemia, hypokalemia, hypocalcemia and hyperlactatemia, and metabolic alkalosis in the cow with ADA were detected *(Table 1)*. Total WBC count was increased.

Ultrasonographic examination of the abomasum was performed to the ventral aspect of the thorax on both sides of the sternum and to the left and right lateral thorax up to the level of the elbow, and the area was examined from cranial to caudal (xyphoid process) using a real-time 3.5-5.0-MHz convex transducer [10,11].

In ultrasonographic examination, displaced abomasum was imaged approximately 10 cm cranial of the xyphoid process from the left and and right paramedian regions and from the ventral midline, immediately caudal of reticulum *(Fig. 2)*. The abomasal content were visible heteroform due to fluid ingesta. The visible ingest in the abomasum was seen hypoechogenic. The walls of abomasum and reticulum were appeared thin echogenic line. The abomasal folds were seen as echogenic structures within the content of the abomasum. Reticulum content was not well imaged due to gaseous composition *(Fig. 2)*. Abomasal content was taken from this area by ultrasound assisted paracentesis. pH of this content was 3.5. Also anterior abomasal displacement was confirmed by laparotomy. In a healthy cow, reticulum (3) and craniodorsal blind sac of the rumen (4) imaged from the left sternal region *(Fig. 1)*.

Table 1. *Blood gases, WBC, glucose, sodium, potassium, calcium, inorganic calcium and lactate values in the cow with anterior displacement of abomasum*

Tablo 1. *Anterior abomazum deplasmanlı bir sığırda kan gazları, lökosit, glikoz, sodyum, potasyum, kalsiyum, inorganik kalsiyum ve laktat değerleri*

Parameters	Values of Analysis	Reference Range
WBC (x10³/µl)	14.45	4.00-12.00
pH	7.54	7.35-7.50
pCO_2 (mmHg)	41	35-45
HCO_3 (mmol/L)	35.1	21-29
BE(W) (mmol/L)	12.6	4-12
pO_2 (mmHg)	37	35-45
O_2 saturation (%)	81	80-90
Sodium (mmol/L)	137	135-148
Potassium (mmol/L)	2.9	3.9-5.8
Glucose (mg/dl)	86	45-75
Lactate (mmol/L)	9.7	2-4
Calcium (mg/dl)	6.9	9.5-11.5
Inorganic calcium (mmol/L)	0.62	1.2-1.5

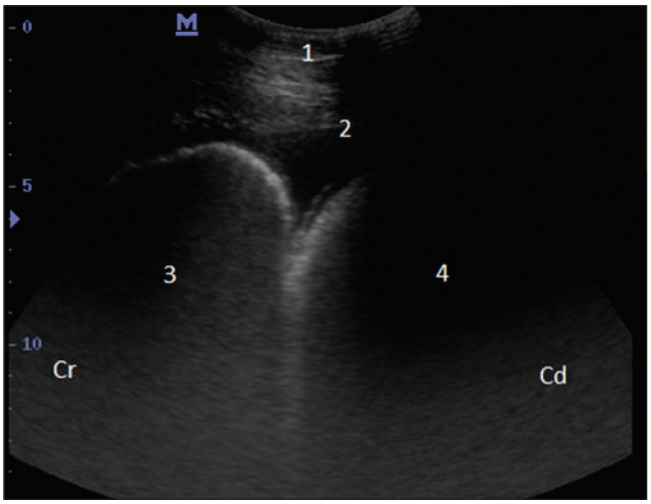

Fig1. Ultrasonogram of reticulum and craniodorsal blind sac of the rumen in a healthy cow imaged from the left sternal region. Ventral abdominal wall (1), diaphragm (2), reticulum (3), craniodorsal blind sac of the rumen (4), Cr: cranial, Cd: caudal

Şekil 1. Sağlıklı bir sığırda sol sternal bölgeden rumenin kraniodorsal kör kesesi ve retikulumun ultrasonogramı. Ventral abdominal duvar (1), diafram (2), reticulum (3), rumenin kraniodorsal kör kesesi (4), Cr: kranial, Cd: kaudal

DISCUSSION

The results of this case report indicate that the ADA could be easily diagnose by ultrasonograpic techniques. Displaced abomasum was imaged approximately 10 cm cranial of the xyphoid process from the left and and right paramedian regions and from the ventral midline, immediately caudal to the reticulum the displaced

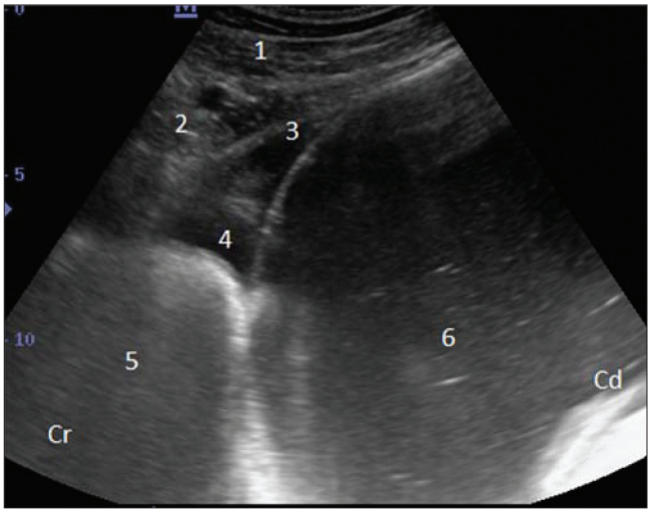

Fig 2. Ultrasonogram of reticulum and abomasum in a cow with Anterior abomasal displacement imaged from the left sternal region. Ventral abdominal wall (1), deposits of fibrin (2) diaphragm (3), mild asites (4), reticulum (5), abomasum (6) Cr: cranial, Cd: caudal

Şekil 2. Anterior abamazum deplasmanlı bir sığırda sol sternal bölgeden reticulum ve abomazumun ultrasonogramı. Ventral abdominal duvar (1), fibrin birikimi (2) diafram (3), sıvı birikimi (4), reticulum (5), abomazum (6), Cr: kranial, Cd: kaudal

abomasum could be easily distinguish from reticulum and craniodorsal blind sac of rumen by ultrasonography *(Fig. 1)*. Abomasal contents were seen as a hypoechogenic due to ingesta visible. However, the wall of the abomasum appeared as a narrow echogenic line. Parts of the abomasal folds were visible occasionally as echogenic structures within the abomasum *(Fig. 2)* [10,11].

In this case report, hyperbasemia, hypokalemia, hypocalcemia and hyperlactatemia, and metabolic alkalosis in the cow with ADA were detected *(Table 1)*. Decreased plasma concentration of K^+ attributable primarily to sequestration of gastric contents and anorexia and hyperbicarbonatemia and increased concentrations attributable to obstruction of abomasal outflow and the resultant accumulation of HCO_3^- in the extracellular fluid space [4,12]. The plasma lactate value in the cow with ADA was high. Increase of lactate in this cow may be related to decreased abomasal tissue perfusion [13]. Because ischemic necrosis was detected in abomasum by laparotomy. In additional there is fluid and fibrin deposits in abdominal cavity *(Fig. 2)*. The serum calcium and plasma inorganic calcium values in the cow with ADA were low *(Table 1)*. Hypocalcemia have been described for cases of abomasal displacement [12]. Increased WBC in the cow with ADA may be related to abdominal cavity inflammation.

As a conclusion, this case reported is the first description of the diagnose of the anterior displacement of abomasum by ultrasonography. In ultrasonographic examination, displaced abomasum was imaged approximately 10 cm cranial of the xyphoid process from the left and right paramedian regions and from the ventral midline, immediately caudal to the reticulum. Ultrasonography is a valuable tool in the diagnose of the anterior displacement of abomasum.

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REVIEW

Hayvansal Gıdalarda Bulunan *Listeria monocytogenes*'in Yüksek Hidrostatik Basınç Altında Yaşam Eğrilerinin Tanımlanması Üzerine Bir Derleme

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Özet

Son yirmi yılda, ısıl olmayan teknolojiler geliştirilmiş ve bu teknolojilerden yüksek hidrostatik basınç (YHB) gıda sanayinde şimdiden uygulama alanı bulmuştur. Çalışmalar göstermektedir ki YHB işlemi hayvansal gıdalarda bulunan *Listeria monocytogenes*'i başarı ile etkisizleştirebilmektedir. Uzun yıllardır gıdalardaki mikroorganizmaların yaşam eğrilerini tanımlamakta kullanılan doğrusal modelin hem ısıl hem de ısıl olmayan işlemler için bir kuraldan çok istisna olduğu halihazırda bilinmektedir. Sütte, peynirde, tavuk, hindi ve dana etinde bulunan ve YHB uygulanmış *L. monocytogenes*'in yaşam eğrilerinin dikkatlice incelenmesi göstermiştir ki hem doğrusal hem de doğrusal olmayan yaşam eğrilerini gözlemlemek mümkündür. Doğrusal olmayan yaşam eğrileri omuzlu, kuyruklu, iki evreli, dış bükey, iç bükey ve S biçiminde görülmektedir. Bu makalenin amacı YHB uygulanmış ve hayvansal gıdalarda bulunan *L. monocytogenes*'in etkisizleştirilmesini tanımlamak için önerilen matematiksel modelleri değerlendirmektir. Bu modellerin değişkenlerinin varsa biyolojik veya matematiksel anlamları da açıklanacak ve değişik şekillerdeki yaşam eğrileri için hangi model veya modellerin kullanılabileceği de ayrıca tartışılacaktır.

Anahtar sözcükler: *Yüksek hidrostatik basınç, Listeria monocytogenes, Matematiksel modeller*

A Review on Describing the Survival Curves of *Listeria monocytogenes* in Foods of Animal Origin under High Hydrostatic Pressure

Summary

In the last two decades, non-thermal technologies have been proposed and some of these technologies such as high hydrostatic pressure (HHP) have already found application in the food industry. Studies have shown that HHP treatment could successfully be used to inactivate *Listeria monocytogenes* in foods of animal origin. It is already known that linear model, that is being used for many years to describe the survival curves of microorganims in foods, is an exception rather than the rule for both thermal and non-thermal treatments. A careful inspection of survival curves of HHP-treated *L. monocytogenes* in milk, cheese, chicken, turkey and beef meat indicated that it is possible to observe both linear and non-linear survival curves. Non-linear curves are in the form of shoulder, tailing, biphasic, convex, concave and sigmoid. This manuscript aims to review the mathematical models proposed to decsribe the inactivation of HHP-treated *L. monocytogenes* in foods of animal origin. If it exists the biological or mathematical meanings of the parameters of these models will also be explained and further use of the models for different types of survival curves will also be discussed.

Keywords: *High hydrostatic pressure, Listeria monocytogenes, Mathematical models*

GİRİŞ

Günümüzde tüketiciler doğal veya az işlem görmüş ve katkı maddesi içermeyen gıdaları tercih etmektedirler [1]. Geleneksel ısıl işlemler gıdaların raf ömrünün uzamasını ve mikrobiyal açıdan güvenirliliğinin artmasını sağlarken, gıdalarda besin, vitamin ve renk kaybına veya değişimine neden olabilmektedir [2,3]. Gıdaların özelliklerinin geliştirilmesinde ve muhafazasında kullanılan katkı maddelerinin bir kısmının ise insan sağlığı üzerinde olumsuz etkileri bulunabilmektedir [4].

Gıda sanayinde son 50 yılda yaşanan gelişmeler ve günümüz tüketicilerinin talep ettikleri gıda ürünlerini

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ortaya çıkarma çabası yeni teknolojilerin araştırılmasına neden olmuştur. Bu teknolojilerin en başında geleni ve belki de en yaygın kullanım sahası olanı Yüksek Hidrostatik Basınç (YHB) uygulamasıdır [5].

YHB ambalajlanmış gıda maddelerini çevreleyen sıvıyı (genellikle su) sıkıştırma esasına dayalıdır ve gıda sanayinde uygulanan basınç değeri genellikle 4000 ile 6000 atmosfer [400 ile 600 MegaPaskal (MPa)] arasındadır. Günümüzde YHB birçok ülkede [Japonya, İspanya, Amerika Birleşik Devletleri (ABD), Fransa, Çek Cumhuriyeti v.s.] yasal olarak kullanılmakta olup YHB uygulanmış gıdalar marketlerdeki yerlerini almış bulunmaktadır [6,7]. Bu gıdaların yaklaşık üçte birini et ve et ürünleri oluşturmaktadır. YHB uygulanmış et ve et ürünlerini genellikle yenmeye hazır etler ve kurutulmuş-tütsülenmiş et ürünleri oluşturmaktadır [8,9].

L. monocytogenes gram pozitif, fakültatif ve intraselüler bir bakteridir [10]. *L. monocytogenes*'in çok yaygın dağılımı nedeniyle; üretim, işleme ve dağıtım aşamalarında gıdalar sıklıkla kontaminasyona maruz kalmaktadır. *L. monocytogenes* çiğ ve işlenmiş süt ve ürünleri, et ve ürünleri ile diğer bazı gıdalarda tespit edilmiştir [11]. ABD'de tüketime hazır kırmızı et ve kanatlı eti ürünlerinin *L. monocytogenes* açısından en riskli grubu oluşturduğu vurgulanmıştır. Türkiye'de ise tüketime hazır ürünlerde (kanatlı karkası, çiğ kanatlı eti gibi) *L. monocytogenes* için sıfır tolerans istenmektedir [12].

YHB uygulamasının en önemli özelliği ısıl işlemin gıdalarda meydana getirdiği tahribatı gerçekleştirmeden mikroorganizmaları etkisiz hale getirmesidir [13,14]. Uygulanan basınç seviyesi mikroorganizmaların morfolojisi, hücre duvarları ve hücre zarları, genetik mekanizmaları ve biyokimyasal tepkimeleri üzerinde önemli değişimlere yol açmaktadır [15-19]. YHB uygulamasıyla birlikte, hücre zarı geçirgenliği artmakta, hücre içi bileşenleri parçalanmakta, hücrede enerji üreten tepkimeler gerçekleşmemekte, hücre büyümesi için gerekli enzimler etkisizleşmekte ve büyüme için gerekli olan pH aralığı azalmaktadır [4,20-24].

Nicel gıda mikrobiyolojisi kavramı 1980'lerden günümüze gıda mikrobiyolojisinin en gözde konularından biri olmuştur. En basit ve yalın tanımıyla nicel mikrobiyoloji, mikroorganizmaların büyümesinin, hayatta kalmasının ve etkisizleştirilmesinin matematiksel modellerle tanımlanmasıdır [25-27]. Hangi ölümcül işlem (ısı, yüksek basınç, antimikrobiyal madde vs) söz konusu olursa olsun gıdalardaki mikroorganizmaların etkisizleştirme kinetiği genellikle doğrusal model kullanılarak tanımlanmaya çalışılır. Gıda sanayinde doğrusal model 90 yılı aşkın bir süredir başarıyla kullanılmaktadır. Konserve sanayindeki etkileyici güvenilirlik kayıtları bu modelin doğruluğunu tartışılmaz hale getirmiştir [28]. Bu model sürekli olarak eleştirilmesine rağmen bu eleştirilerin model üzerinde çok az etkisi olmuştur. Ancak, son yıllarda yapılan çalışmalar göstermektedir ki gıda sanayindeki bu başarı doğrusal modelin değil uygulanan ölümcül işlemin başarısıdır. Şöyle ki gıdalar olması gerekenden fazla işlenmekte ve bu da bir takım sıkıntılara yol açmaktadır [29,30].

Doğrusal/doğrusal olmayan matematiksel modeller ve gıdalardaki mikroorganizmaların etkisizleştirilmesi için şunlar söylenebilir:

Ölümcül işlem uygulanan birçok mikroorganizmanın yaşam eğrileri açıkca doğrusal değildir. Dolayısıyla bu deneysel verileri bir doğruyla tanımlamaya çalışmak gerçek yaşam eğrisini gizlemek anlamına gelmektedir.

Mikroorganizmaların deneysel olarak etkisizleştirilmesi genellikle, uygulama olarak, 6-8 log azalmayla gösterilir. Bunun 4-6 log ötesini bir modelin doğru olduğu varsayımıyla (geleneksel modelde olduğu gibi) tahmin etmeye çalışmak gıda güvenliği açısından son derece tehlikelidir.

Günümüzde gıda sanayinde kullanılan ısıl işlemlerin gıdalarda aşırı-işlenmeye neden olması dolayısıyla gıdaların besinsel değerlerini ve toplam niteliklerini azaltması tüketicide bazı endişeler doğurmaktadır. Bu endişeleri gidermek aynı zamanda da gıdanın güvenilirliğini sağlayabilmek için doğrusal olmayan yaşam eğrilerini de tanımlayacak daha uygun modellere gerek duyulmaktadır.

Isıl olmayan işlemlere (özellikle YHB teknolojisi) artan bir talep vardır. Bu işlemlerin gıdalardaki güvenilirliğini sağlamak ve ısıl işlemlerle karşılaştırmak için, çoklukla gözlemlenen doğrusallıktan sapmalarla başa çıkabilecek yeni yordamlara ihtiyaç vardır.

Geleneksel (doğrusal) model ilk olarak ortaya çıktığında işlemleri hızlı ve etkin biçimde gerçekleştirecek bilgisayarlar ve yazılımlar henüz üretilmemişti. Günümüzde ise doğrusal olmayan modelleri kullanarak doğrusal olmayan uyumlama çözümlemelerini yapacak ve türevsel denklemleri çözecek araçlar mevcuttur. Dolayısıyla geçmişte doğru kabul edilen ve kullanılması için uzlaşılan bazı kavramların günümüzde de aynı şekilde geçerli olması için hiçbir neden yoktur.

Bu derlemenin amacı YHB uygulanmış ve hayvansal gıdalarda bulunan *Listeria monocytogenes*'in etkisizleştirilmesini tanımlamak için önerilen matematiksel modelleri değerlendirmektir. Bu modellerin değişkenlerinin varsa anlamları da açıklanacak ve değişik şekillerdeki yaşam eğrileri için hangi model veya modellerin kullanılabileceği de okuyucuya aktarılacaktır.

LİSTERİA MONOCYTOGENES'İN YAŞAM EĞRİLERİ

YHB uygulanmış *Listeria monocytogenes* için 6 farklı yaşam eğrisi gözlemlenmiştir:

1. Doğrusal

Genel ve gıda mikrobiyoloji kitaplarına göre, mikrobiyal

etkisizleştirme geleneksel olarak doğrusal modelle tanımlanmaktadır. Dolayısıyla basit bir hız sabiti *(k)* ya da bunun çarpımsal tersi (*D*-değeri) uygulanan YHB işlemine karşılık mikroorganizmaların gösterdiği direncin ölçütü olarak kullanılır [Denklem (1)] [28,31,32].

$$\log_{10} S(t) = -kt \qquad (1)$$

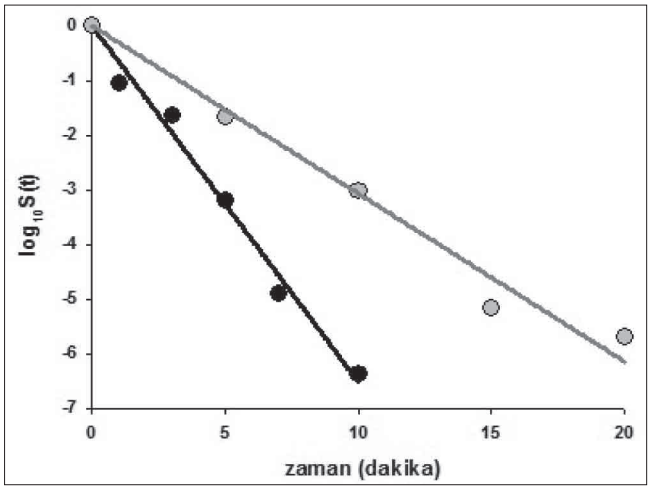
Burada $S(t)$ hayatta kalma oranı yani t zamanındaki mikroorganizma sayısının [$N(t)$] başlangıçtaki mikroorganizma sayısına (N_0) bölümüdür ($S(t) = N(t)/N_0$). Yukarıda da bahsedildiği üzere k hız sabiti olup birimi 1/zaman'dır (D değeri ise sabit bir basınç değerinde mikroorganizma sayısını %90 azaltmak için gereken süredir).

Şekil 1'de YHB uygulanmış sütte ve peynirde bulunan *L. monocytogenes*'in Denklem 1 ile tanımlanmaları gösterilmiştir. Şekilden de anlaşıldığı gibi doğrusal (geleneksel) model YHB uygulanmış süt ve peynirde bulunan *L. monocytogenes* yaşam eğrilerini başarı ile tanımlamaktadır.

2. Omuzlu

Yaşam eğrileri omuz şekline benzediği için bu şekilde isimlendirilirler. Bu tür yaşam eğrilerinde belli bir zamana kadar YHB işleminin etkisi yoktur. Diğer bir deyişle belli bir zamana kadar mikroorganizma sayısında bir değişiklik olmaz ya da çok az sayıda mikroorganizma ölür. Belli bir zamandan sonra ise doğrusal bir azalma [Denklem (1)] söz konusudur.

Bu tür verileri 2 ayrı model kullanarak tanımlamak mümkündür:



Şekil 1. Tam yağlı UHT sütte bulunan *Listeria monocytogenes* NCTC 10527'nin 550 MPa, 25°C'deki yaşam verilerine *(siyah daireler)* denklem (1)'in uygulanışı *(siyah çizgi)*. Özgün veriler Amina ve ark.'ndan [33] alınmıştır. Çiğ süt peynirinde bulunan *Listeria monocytogenes* Scott A'nın 300 MPa, 25°C'deki yaşam verilerine *(gri daireler)* denklem 1'in uygulanışı *(gri çizgi)*. Özgün veriler Shao ve ark.'ndan [34] alınmıştır

Fig 1. Survival data *(black circles)* of *Listeria monocytogenes* NCTC 10527 in UHT whole milk at 550 MPa, 25°C fitted with Eq.(1) *(black line)*. Original data are from Amina et al.[33] Survival data *(gray circles)* of *Listeria monocytogenes* Scott A in raw milk cheese at 300 MPa, 25°C fitted with Eq.(1) *(gray line)*. Original data are from Shao et al.[34]

Kesikli model ("Eğer" ifadesi kullanılarak) [35]

$$\text{Eğer } t \le t_i \quad \log_{10} S(t) = 0 \qquad (2)$$

$$\text{Eğer } t > t_i \quad \log_{10} S(t) = -k(t - t_i)$$

Burada t_i inaktivasyonun başladığı zaman olup k hız sabitidir (1/zaman). Görüldüğü gibi bu model doğrusal modelin aynısı olup doğrunun gözlemlenmesi t_i kadar gecikmeyle gerçekleşmektedir.

Sürekli model [36]

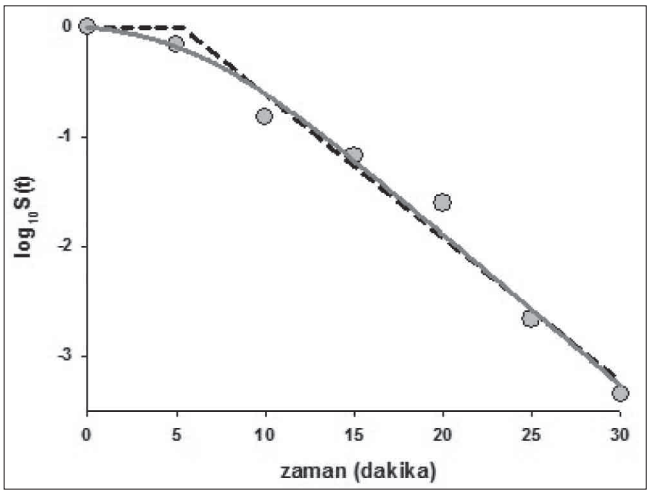
$$\log_{10} S(t) = -\frac{kt}{\ln 10}\left(\frac{e^{kS}}{1 + (e^{kS} - 1)e^{-kt}}\right) \qquad (3)$$

Burada k hız sabiti (1/zaman), S ise omuz kısmının sona erdiği zamandır.

Şekil 2'de YHB uygulanmış pişmiş tavuk kıymasında bulunan *L. monocytogenes*'in Denklem (2) ve (3) ile tanımlanması gösterilmiştir. Her iki modelin de iki değişkeni (kesikli modelde k ve t_i sürekli model de ise k ve S) bulunmaktadır ve *Şekil 2*'de görüldüğü gibi her iki model de veriye hemen hemen aynı uygunluğu göstermektedir. Dolayısıyla omuzlu yaşam eğrileri her iki model kullanılarak tanımlanabilir.

3. İki Evreli

İki evreli modeller bir anakitledeki mikroorganizmaların uygulanan YHB işlemine değişik hassasiyetler gösterdiği varsayımına dayanır. Mikroorganizmalar "basınca karşı hassas" ve "basınca karşı dayanıklı" olarak değerlendirilir. Hassas mikroorganizmalar kolayca ve önce ölür. Dayanıklı



Şekil 2. Pişmiş tavuk kıymasında bulunan *Listeria monocytogenes* Lm1'in 375 MPa, yaklaşık 18°C'deki yaşam verilerine *(gri daireler)* denklem (2)'nin *(kesikli siyah çizgi)* ve denklem (3)'ün *(gri çizgi)* uygulanışı. Özgün veriler Simpson ve Gilmour'dan [37] alınmıştır. Denklem (2) ve (3) verilere neredeyse aynı uyumu göstermiştir

Fig 2. Survival data *(gray circles)* of *Listeria monocytogenes* Lm1 in cooked chicken mince at 375 MPa, about 18°C fitted with Eq.(2) *(dashed black line)* and Eq.(3) *(gray line)*. Original data are from Simpson and Gilmour [37]. Eq.(3) and (4) produced almost the same fit to the data

olanları ise öldürmek daha zordur. Her iki evreyi de değişik hız sabitlerine sahip iki doğrusal modelle tanımlamak mümkündür.

Bu tür veriler de tıpkı omuzlu eğrilerde olduğu gibi kesikli ve sürekli 2 ayrı modelle tanımlanabilir:

A. Kesikli model ("Eğer" ifadesi kullanılarak) [38]

Eğer $t \le t_e$ $\log_{10} S(t) = -k_1 t$ (4)

Eğer $t > t_e$ $\log_{10} S(t) = -k_1 t_e - k_2(t - t_e)$

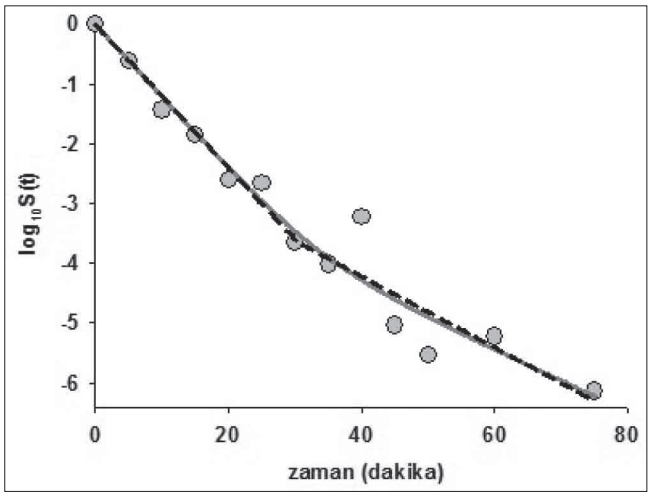
Burada t_e birinci evreden ikinci evreye geçiş zamanı olup k_1 birinci evrenin (hassas mikroorganizmaların) hız sabiti (1/zaman), k_2 ise ikinci evrenin (dayanıklı mikroorganizmaların) hız sabitidir (1/zaman). Görüldüğü gibi bu model iki farklı eğime dolayısıyla iki farklı hız sabitine sahip iki doğrusal modelin birleştirilmiş halidir.

B. Sürekli model [39]

$$\log_{10} S(t) = \log_{10}(fe^{-k_1 t} + (1-f)e^{-k_2 t}) \quad (5)$$

Burada f anakitledeki hassas üyelerin oranıdır [$(1-f)$ ise dayanıklı üyelerin oranıdır] k_1 tıpkı kesikli modelde olduğu gibi birinci evrenin (hassas mikroorganizmaların) hız sabiti (1/zaman), k_2 ise ikinci evrenin (dayanıklı mikroorganizmaların) hız sabitidir (1/zaman).

Şekil 3'te YHB uygulanmış sulu peynir altı suyu proteini süspansiyonunda bulunan *L. monocytogenes*'in Denklem (4) ve (5) ile tanımlanması gösterilmiştir. Her iki modelin de üç değişkeni (kesikli modelde k_1, k_2 ve t_e sürekli model de ise k_1, k_2 ve f) bulunmaktadır ve *Şekil 3*'te görüldüğü gibi her iki model de veriye tamamen aynı uygunluğu göstermektedir (modeller üst üste çakışmaktadır). Dolayısıyla iki evreli yaşam eğrileri her iki model kullanılarak tanımlanabilir.



Şekil 3. Sulu peynir altı suyu proteini süspansiyonunda bulunan *Listeria monocytogenes* OSY-8578'in 345 MPa, 20°C'deki yaşam verilerine *(gri daireler)* denklem (4)'ün *(kesikli siyah çizgi)* ve denklem (5)'in *(gri çizgi)* uygulanışı. Özgün veriler Doona ve ark.'ndan [40] alınmıştır. Denklem (4) ve (5) verilere neredeyse aynı uyumu göstermiştir

Fig 3. Survival data *(gray circles)* of *Listeria monocytogenes* OSY-8578 in aqueous whey protein suspension at 345 MPa, 20°C fitted with Eq.(4) *(dashed black line)* and Eq.(5) *(gray line)*. Original data are from Doona et al.[40] Eq.(4) and (5) produced almost the same fit to the data

4. Kuyruklu

Yaşam eğrileri kuyruk şekline benzediği için bu şekilde isimlendirilirler. YHB uygulanarak etkisizleştirilen mikroorganizmaların belli bir süre sonra (uygulanan işlem zamanı ne kadar artırılırsa artırılsın) inaktivasyon miktarında bir değişiklik olmaz [41].

$$\log_{10} S(t) = -\frac{at}{b+t} \quad (6)$$

Burada a azami inaktivasyon miktarı (veya hayatta kalma oranı), b ise inaktivasyon değerinin $a/2$ olduğu zamandır.

Şekil 4'te YHB uygulanmış pişmiş dana kıymasında bulunan bulunan *L. monocytogenes*'in Denklem (6) ile tanımlanması gösterilmiştir.

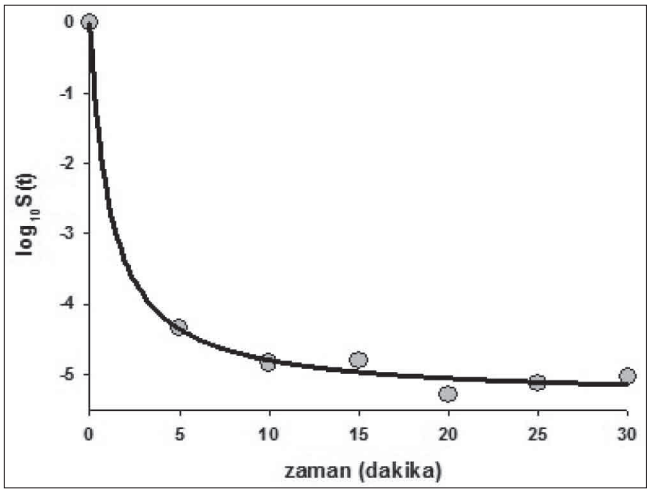
5. İç ve Dış Bükey

İç ve dış bükey yaşam eğrileri aynı modelle [Weibull modeli - Denklem (7)] tanımlanabilir [42,43].

$$\log_{10} S(t) = -bt^n \quad (7)$$

Burada b hız sabiti (1/zaman), n ise şekil değişkenidir; eğer birden büyükse ($n>1$) yaşam eğrisi dış bükey, birden küçükse ($n<1$) iç bükeydir. Şekil değişkenin bir olduğu durumlarda ($n = 1$) ise Weibull modeli [Denklem (7)] doğrusal modele [Denklem (1)] dönüşmektedir [44].

Şekil değişkeni (n) kullanılarak biyolojik bir bağlantı yapmak mümkündür: $n>1$ YHB işlemi ile etkisizleştirile-

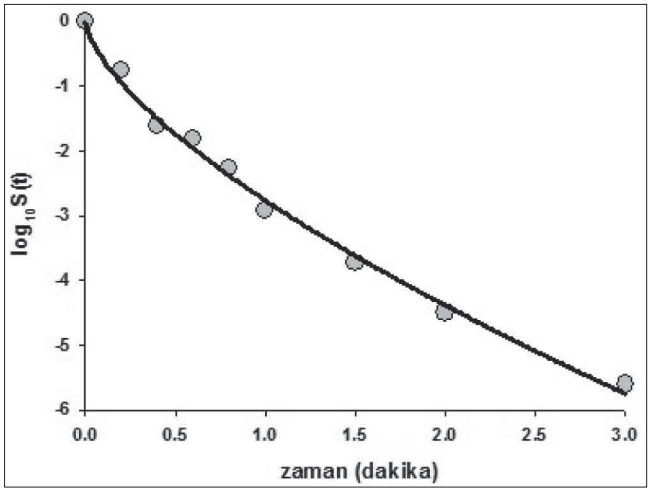


Şekil 4. Pişmiş dana kıymasında bulunan *Listeria monocytogenes* Lm1'in 375 MPa, 45°C'deki yaşam verilerine *(gri daireler)* denklem (6)'nın *(siyah çizgi)* uygulanışı. Özgün veriler Simpson ve Gilmour'dan [37] alınmıştır

Fig 4. Survival data *(gray circles)* of *Listeria monocytogenes* Lm1 in cooked beef mince at 375 MPa, 45°C fitted with Eq.(6) *(black line)*. Original data are from Simpson and Gilmour [37]

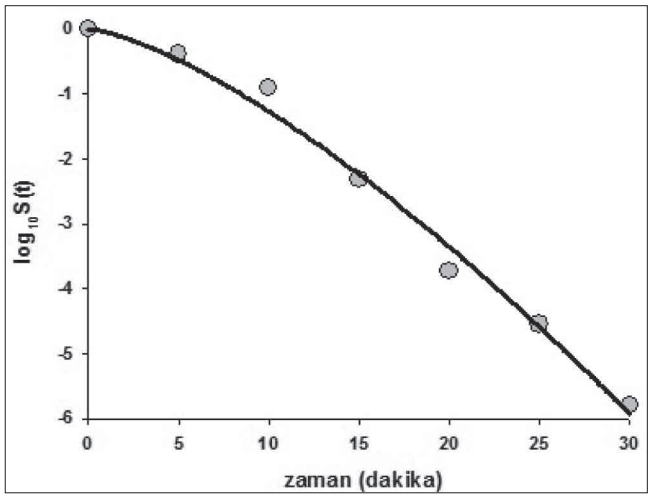
meyen hücrelerin zaman geçtikçe daha kolay hasara uğrayabileceklerini, $n<1$ ise ölümcül işleme karşı daha hassas olan organizmaların ölüp geriye kalan dayanıklı hücrelerin ölümcül işleme karşı uyum sağlayarak etkisizleştirmenin bu canlılar için daha zor olabileceğini ifade eder. $n = 1$ olduğu durumlarda bir anakitledeki bütün canlıların zamandan bağımsız eşit ölme olasılığı vardır (tıpkı doğrusal modelde olduğu gibi) [42].

Şekil 5'te YHB uygulanmış hindi göğüs etinde bulunan; *Şekil 6*'da ise YHB uygulanmış çiğ tavuk kıymasında bulunan *L. monocytogenes*'in Denklem (7) ile tanımlanması gösterilmiştir.



Şekil 5. Hindi göğüs etinde bulunan beş suşluk karışım *Listeria monocytogenes*'in (PSU1, PSU2, PSU9, PSU21 ve ATCC 19115) 400 MPa, 50°C'deki yaşam verilerine *(gri daireler)* denklem (7)'nin *(siyah çizgi)* uygulanışı. Özgün veriler Chen'den [45] alınmıştır

Fig 5. Survival data *(gray circles)* of five strain cocktail of *Listeria monocytogenes* (PSU1, PSU2, PSU9, PSU21 and ATCC 19115) in turkey breast meat at 400 MPa, 50°C fitted with Eq.(7) *(black line)*. Original data are from Chen [45]



Şekil 6. Çiğ tavuk kıymasında bulunan *Listeria monocytogenes* Lm1'in 375 MPa, yaklaşık 18°C'deki yaşam verilerine *(gri daireler)* denklem (7)'nin *(siyah çizgi)* uygulanışı. Özgün veriler Simpson ve Gilmour'dan [37] alınmıştır

Fig 6. Survival data *(gray circles)* of *Listeria monocytogenes* Lm1 in raw chicken mince at 375 MPa, about 18°C fitted with Eq.(7) *(black line)*. Original data are from Simpson and Gilmour [37]

6. "S" Biçiminde

Yaşam eğrileri "S" biçimindedir ve üç değişkenli iki farklı modelle tanımlanabilir:

A. İç bükey başlayıp dış bükey biten [46]

$$\log_{10} S(t) = -\frac{at}{(1+bt)(c-t)} \qquad (8)$$

Burada a ve b birimi 1/zaman, c ise birimi zaman olan değişkenlerdir.

Şekil 7'de YHB uygulanmış sütte bulunan *L. monocytogenes*'in Denklem (8) ile tanımlanması gösterilmiştir.

B. Dış bükey başlayıp iç bükey biten [48]

Bu tür eğrileri Gompertz denklemi [Denklem (9)] ile tanımlamak mümkündür.

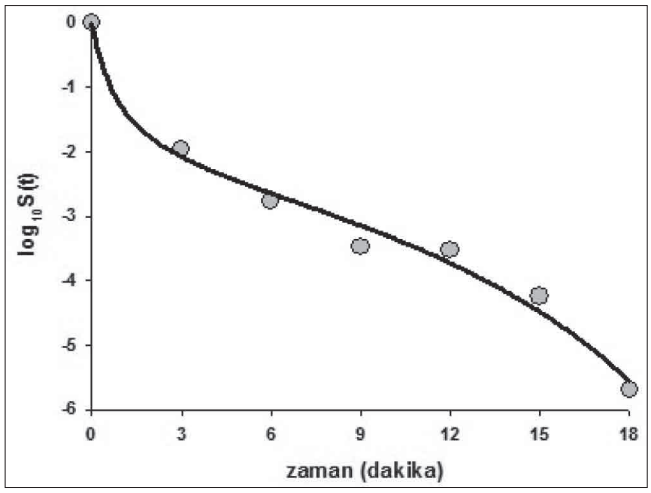
$$\log_{10}S(t) = A\exp[-\exp(kti)] - A\exp[-\exp(-k(t-ti))] \qquad (9)$$

Burada A azami inaktivasyon miktarı (veya hayatta kalma oranı), k hız sabiti, t_i ise inaktivasyonun en hızlı olduğu zamandır.

Şekil 8'de YHB uygulanmış sütte bulunan *L. monocytogenes*'in Denklem (9) ile tanımlanması gösterilmiştir.

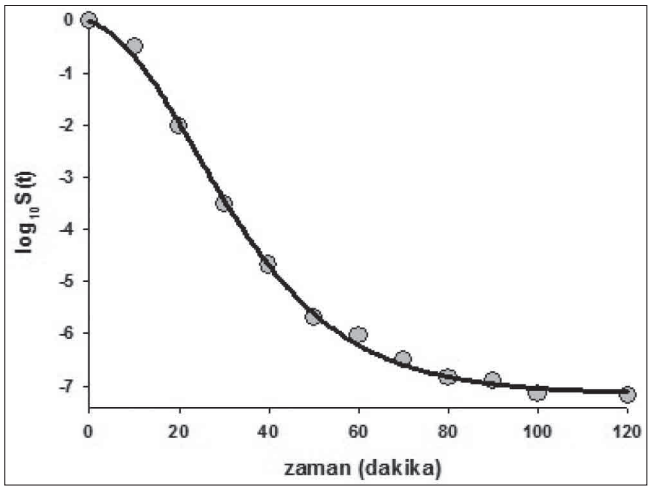
SONUÇ

Gıdalardaki mikroorganizmaları etkisizleştirmek için kullanılan ölümcül işlem ne olursa olsun (ısı, yüksek basınç, antimikrobiyal madde v.s.) veya hangi mikro-



Şekil 7. Tam yağlı UHT sütte bulunan *Listeria monocytogenes* ATCC 19115'in 400 MPa, 43°C'deki yaşam verilerine *(gri daireler)* denklem (8)'in *(siyah çizgi)* uygulanışı. Özgün veriler Mishra ve ark.'ndan [47] alınmıştır

Fig 7. Survival data *(gray circles)* of *Listeria monocytogenes* ATCC 19115 in UHT whole milk at 400 MPa, 43°C fitted with Eq.(8) *(black line)*. Original data are from Mishra et al.[47]



Şekil 8. Tam yağlı UHT sütte bulunan *Listeria monocytogenes* Scott A'nın 400 MPa, 22°C'deki yaşam verilerine *(gri daireler)* denklem (9)'un *(siyah çizgi)* uygulanışı. Özgün veriler Chen ve Hoover'dan alınmıştır [49]

Fig 8. Survival data *(gray circles)* of *Listeria monocytogenes* Scott A in UHT whole milk at 400 MPa, 22°C fitted with Eq.(9) *(black line)*. Original data are from Chen and Hoover [49]

organizma söz konusu olursa olsun (hastalık yapıcı bakteri, spor, bakteriyofaj v.s.) doğrusal olmayan yaşam eğrilerini gözlemlemek kaçınılmazdır. Bu çalışmada da YHB uygulanmış ve hayvansal gıdalarda bulunan *L. monocytogenes*'in 7 farklı yaşam eğrisinin 9 farklı model kullanarak tanımlanması gösterilmiştir. Bu modeller YHB dışında başka bir ölümcül işlem için veya *L. monocytogenes* dışında başka bir mikroorganizma için rahatlıkla kullanılabilir.

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ERRATUM

Kars Yöresinde Sığırlarda *Cryptosporidium parvum* Subtiplerinin Belirlenmesi (Determination of *Cryptosporidium parvum* Subtypes in Cattle in Kars Province of Turkey)

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Makale Kodu (Article Code): 2012/6565

Dergimizin 2012 Yılı, 18. Cilt, Supplement-A Sayısının A221-A226 Sayfalarında basılan makalenin A223. sayfasındaki *Tablo 1*'in son sütunu (Genbank No) sehven yanlış yazılmıştır. Bu sütun tablodan çıkarılmış olup, düzeltilmiş tablo aşağıda yeniden verilmiştir.

Publishing in the journal in Volume 18, Issue Supplement-A, Year 2012, between pages A221-A226, on the page A223 of the atricle, the final column of *Table 1* (Genbank Accession No) was inadvertently misspelled. This column has been removed from the table and the revised form of the table is given below.

Tablo 1. *Kars yöresindeki sığırlarda saptanan Cryptosporidium parvum subtiplerinin dağılımı*
Table 1. *Distribution of Cryptosporidium parvum subtypes in cattle in Kars province of Turkey*

Protokol No	Klinik Durum	Yaş	*C. parvum* Subtip Familyası	*C. parvum* Subtipi
CAG4	İshalli Buzağı	1 ay	IIa	IIaA15G2R1
AKB17	İshalli Buzağı	20 gün	IIa	IIaA15G2R1
SGK70	İshalli Buzağı	5 gün	IIa	IIaA15G2R1
KUM40	İshalli Buzağı	1 ay	IIa	IIaA15G2R1
BGD14	İshalli Buzağı	1 ay	IIa	IIaA15G2R1
ALC4	İshalli Buzağı	20 gün	IIa	IIaA15G2R1
ALC7	İshalli Buzağı	1.5 ay	IIa	IIaA15G2R1
AYD2	İshalli Buzağı	2 ay	IIa	IIaA16G3R1
KUM30	Sağlıklı Buzağı	3 ay	IIa	IIaA15G2R1
AKB30	Sağlıklı Buzağı	2 ay	IIa	IIaA15G2R1
AKB16	Sağlıklı İnek	>3 yaş	IIa	IIaA15G2R1
DIK16	Sağlıklı İnek	>3 yaş	IIa	IIaA16G3R1
KRK17	Sağlıklı İnek	>3 yaş	IId	IIdA15G1

Ayrıca, A224. sayfada;

Şekil 1'deki >gi|296067697|dbj|AB560741.1|,

Şekil 2'deki >gi|296067693|dbj|AB560739.1| ve

Şekil 3'teki >gi|296067695|dbj|AB560740.1|

Genbank No'ları sehven yanlış yazılmış olup, bu rakamlar makaleden çıkarılmış ve doğrusu aşağıda verilmiştir.

Şekil 1'deki doğrusu: Cryptosporidium parvum GP60 gene for 60 kDa glycoprotein, partial cds, isolate: Cp(C)198-IR

Şekil 2'deki doğrusu: Cryptosporidium parvum GP60 gene for 60 kDa glycoprotein, partial cds, isolate: Cp(C)63-IR

Şekil 3'teki doğrusu: Cryptosporidium parvum GP60 gene for 60 kDa glycoprotein, partial cds, isolate: Cp(C)112-IR

Yanlışlıklardan dolayı özür dileriz.

Furthermore, Genbank numbers on the page A224,

in *Figure 1* >gi|296067697|dbj|AB560741.1|,

in *Figure 2* >gi|296067693|dbj|AB560739.1| and

in *Figure 3* >gi|296067695|dbj|AB560740.1|

were inadvertently given incorrect. Theses numbers have been removed from the article and those have been given as below.

Figure 1, Cryptosporidium parvum GP60 gene for 60 kDa glycoprotein, partial cds, isolate: Cp(C)198-IR

Figure 2, Cryptosporidium parvum GP60 gene for 60 kDa glycoprotein, partial cds, isolate: Cp(C)63-IR

Figure 3, Cryptosporidium parvum GP60 gene for 60 kDa glycoprotein, partial cds, isolate: Cp(C)112-IR

We sincerely apologize for the mistakes.

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YAZIM KURALLARI

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Başvuru sırasında yazarlar yazıda yer alacak şekilleri online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.
3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır. Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.
Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.
Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
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Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.
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Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.
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Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

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