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Research Article

Morphometric Study and Immunolocalization of Androgen Receptors in Epididymis During Postnatal Development in D'Man Lamb Reared Under Arid Environment in Algeria

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Abstract

The aim of this study was to examine the morphometry and the immunolocalization of androgen receptors in the epididymis of D'Man lamb during postnatal development. The epididymis was collected at the slaughtering lamb, aged of 2 to 5 month. The weight of the epididymis increased with a significant difference at 3 months. The tubular diameter of the corpus and the cauda epididymal tubules increased respectively from 2 till 4 months, and from 2 till 5 months. The luminal diameters of the caput epididymal tubules increased significantly at 3 months. The luminal diameters of the corpus and cauda epididymal tubules increased significantly from 3 and 4 months respectively. The epithelial height of the caput and cauda epididymal tubules increased significatively at 5 months. The epithelial height of the corpus epididymal tubules increased significantly at 3 months. The androgen receptor immunostaining was localized in nuclei and cytoplasm of epithelial cells, smooth muscle cells and in the cytoplasm of interstitial cells of the epididymis at each age. In conclusion, both the morphometric changes and androgen receptors immunolocalization during the postnatal development of epididymis indicated the necessity of androgens for postnatal differentiation and maintaining the structure of the epididymis.

Keywords: *Epididymis, Morphometry, Androgen receptor, Immunohistochemistry, Postnatal development, D'Man lamb*

Cezayir'in Kurak İkliminde Yetiştirilen D'Man Kuzularinda Postnatal Gelişim Süresince Epididimiste Androjen Reseptörlerinin İmmunolokalizasyonu ve Morfometrik Bir Çalışma

Özet

Bu çalışmanın amacı D'Man kuzularında postnatal gelişim süresince epididimiste androjen reseptörlerinin immunolokalizasyonunu ve morfometrisini araştırmaktır. 2 ile 5 ay arasında yaşları değişen kuzulardan kesim sonrasında epididimisler toplandı. Epididymis ağırlığı 3 aylık olanlarda anlamlı oranda farklı bulundu. Corpus ve cauda epididimal tüplerin tubular çapları sırasıyla 2'den 4 aylığa ve 2'den 5 aylığa kadar olanlarda artma gösterdi. Caput epididimal tüplerin luminal çapları 3 aylıklarda anlamlı oranda arttı. Corpus ve cauda epididimal tüplerin luminal çapları sırasıyla 3 ve 4 aylıklarda anlamlı oranda arttı. Caput ve cauda epididimal tüplerin epitel yükseklikleri 5 aylıklarda anlamlı oranda artış gösterdi. Corpus epididimal tüplerin epitel yükseklikleri 3 aylıklarda anlamlı oranda artış gösterdi. Androjen reseptör immunboyanması tüm yaş gruplarında epididimisin epitel hücrelerinin çekirdek ve sitoplazmalarında, düz kas hücrelerinde ve intersitisyel hücrelerinin sitoplazmasında lokalize oldu. Sonuç olarak; morfometrik değişimler ve androjen reseptör immunolokalizasyonu epididimisin postnatal gelişimi ve oluşumu süresince androjenlerin gerekli olduğunu göstermiştir.

Anahtar sözcükler: *Epididimis, Morfometri, Androjen reseptör, İmmunohistokimya, Postnatal gelişim, D'Man kuzu*

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INTRODUCTION

Sheep is an important part of the Algerian agricultural economy. D'Man and other second breeds (Hamra, Barbarine, Sidahou, Tazegzawt) represent less than 1% of the Algerian sheep population. D'Man breed acquires its importance from its exceptional reproductive performances and its high adaptation to the oasian environment [1]. The productivity was consistently higher due to generally high fertility and prolificacy [2] with an early onset of puberty at 3 months [3].

The morphohistological change is essential to gain a comprehensive knowledge on the reproductive physiology of epididymis [4,5]. Indeed, epididymal functions can be divided into several general categories: concentration of sperm; functional maturation; storage in a quiescent state until ejaculation, removal of degenerating sperm, provision of appropriate conditions for survival, transport by the myoid cells, protection and maintenance of the blood epididymal barrier [6]. In most species, the epididymis is divided into the caput, corpus and cauda regions [4,5,7]. The activity of these regions is regulated by endocrine, lumicrine, and paracrine factors, the relative importance of which remaining a topic of investigation [8]. The presence of androgen receptors (ARs) during spermatogenesis was investigated in rodent models in which testosterone levels were chemically deleted, or in models with transgenic disruption of ARs [9]. The use of these models made it possible to identify the steps of spermatogenesis requiring ARs, specifically the maintenance of spermatogonia number, integrity of blood-testis barrier, completion of meiosis, adhesion of spermatids and spermiation. Together these studies detailed the essential nature of androgens in the promotion of male fertility [10]. Luminal factors from the testis, in addition to androgens, are important for both the epididymal development [11] and maintenance of adult tissues [12]. The presence of steroids and their receptors, specifically ARs, which are responsible for maintaining epididymal structure and functions throughout the postnatal development [13] has not yet been shown in the epididymis of D'Man lambs. In the prepubertal period several factors can cause epididymal obstruction, such as iatrogenicities due to inguinal herniotomies, inflammatory, tumoral, cystic and similar causes [14]. The effect of these conditions on the testes and epididymal ARs distribution are of major importance. In this study, the objective was to characterize the morphological normal changes and immunolocalization of ARs of the epididymis in 2 to 5 months old D'Man lamb.

MATERIAL and METHODS

Twelve lambs aged from 2 to 5 months reared at El Meniaa experimental station in Algeria (30° 34' N., 02° 52' E.) have been used for this study. For each month, three lambs were weighed and immediately slaughtered; the epididymis was separated from testis and weighed. From each regions of epididymis (caput, corpus and cauda), a sample was fixed in 10% formaldehyde in phosphate buffered saline, dehydrated in a graded series of ethanol, clarified in xylene and embedded in paraffin. The sections were hydrated and stained with Masson's trichroma in order to study general histology. The diameters of tubules and lumen tubules of the epididymis were measured on 10 cross-sections per animal. The height of epithelial cells was measured from the basement to the apical membrane in cross-sections of 10 tubules using a computer program of light microscope Nikon Eclipse E 400 connected to a Nikon DXM 1200 digital camera.

Androgen Receptor Immunohistochemistry

The immunohistochemical studies of ARs were performed using the avidin-biotin complex method (ABC), with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Paraffin sections (3 µm thick) were deparaffinized, hydrated through a graded ethanol series (100%, 95% and 70%), and washed in PBS. Immunohistochemistry was performed on deparaffinized adjacent sections with heat -induced antigen retrieval in citrate buffer (pH 6.0) using *water bath* set at 94°C, as described in the prospectus for the kit "Vector Antigen Unmasking Solutions" (Vector Laboratories, CA, H3300). This step was followed with endogenous peroxidase blocking 3% H_2O_2 in PBS for 5 min at room temperature. All washes between antibody or reagent incubations were rinsed 5 min 2 times at room temperature in PBS, and all the incubations were carried out in a wet chamber. Tissue sections were first submitted to the appropriate serum in order to block the non-specific binding sites. After that, sections processed for ARs labeling were incubated with normal horse serum at room temperature for 5 min, and then with both avidin and biotin sites subsequently blocked (Vector Laboratories, CA, SP-2001). All sections were incubated overnight at 4°C with the primary antibody: a rabbit polyclonal antibody (C-19) raised against a peptide within the C-terminal domain of the human AR (sc-815, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was diluted at 1 : 200 in PBS. This antibody being currently used to detect the presence of androgen receptors in several mammals, it was used to detect androgen receptors in D'man lamb epididymis. Bound antibodies were visualized by incubating the sections with biotinylated secondary antibody (Vectastain Elite ABC kit-Vector Laboratories, CA, #PK-6200) for 30 min. Labeling of ARs was performed with 3,3'-diaminobenzidine-tetra-hydrochloride chromogenic substrate (SK-4100, DAB substrate kit for peroxidase; Vector Laboratories) and monitored microscopically. Sections were counterstained with hematoxylin (Hematoxylin QS, H-3404; Vector Laboratories, Burlingame, CA, USA). Those sections were dehydrated and mounted. Sections incubated with normal horse serum instead of primary

antibody were used as negative controls. Images were captured using a light microscope (Nikon Eclipse E 400 connected to a Nikon DXM 1200 digital camera).

The results of immunohistochemical staining, evaluated by semiquantitative methods, were given for the epithelial cells (principal, basal and apical cells), the interstitial stromal cells and the peritubular smooth muscle cells of each epididymis compartment. The staining intensity was evaluated at four different levels: ++ / strong, + / moderate, – / negative, +/– / variable.

Statistical Analysis

Results were expressed as Mean ± SEM after verification of their homogeneity. The CV observed was below 20% . Analysis of variance was performed using the One-way ANOVA. Each parameter was analyzed by pairwise comparison using the Tukey HSD test. All analysis was performed using XLSTAT version 2016. The correlation between the average epididymis weight and age was analyzed using a Pearson's Linear Correlation test. $P<0.05$ was considered as significant.

RESULTS

Epididymis Weight

The weight of the paired epididymis related to the age presented in *Fig 1*, increased ($P<0.001$) from 2 to 5 months. At 2 month, the epididymis weight was 2.8±0.3 g, and increased quickly to 10.7±1.4 g at 5 month with a significant increase ($P<0.05$) observed at 3 months. The average epididymal weight was significantly affected by the age ($P<0.001$) and a positive correlation ($r=0.76$, $P<0.05$) was observed between the average epididymis weight and age *(Fig. 1)*.

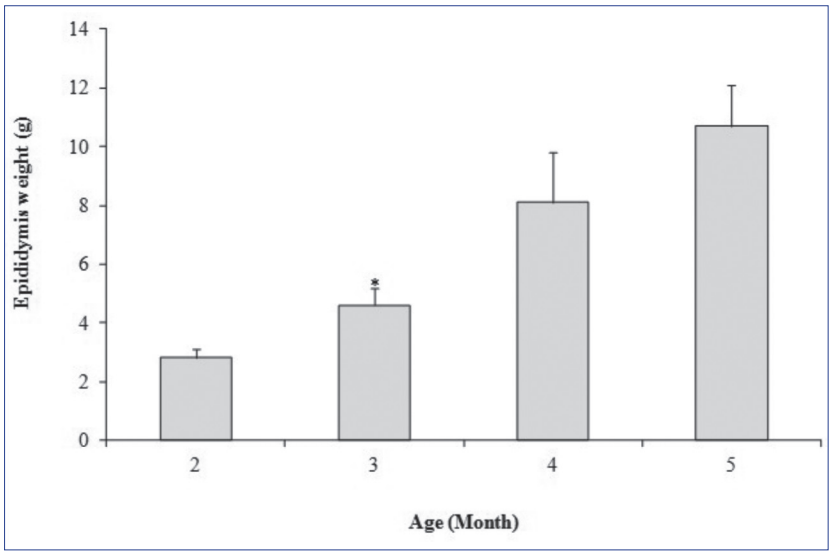

Fig 1. Age-related changes in the epididymis weight (g) in D'Man lamb

Morphometry Development of the Epididymis

The evaluation of the effects of the age on the tubular diameter, luminal diameter, and epithelial height of the epididymis is shown in *Fig. 2*. The measures of tubular and luminal diameters, and the epithelial height showed some regional differences in the three regions of the epididymis *(Fig. 2)*.

The tubular diameter of the caput epididymal tubules presented a high increase ($P<0.001$) from 2 (389±9.7 µm) to 3 (542±11.3 µm) months, then it stabilized at 5 months (550±13.7 µm). In 2 months-old animals, the epididymal tubular diameter was 279±9.9 and 294±9.9 µm, respectively for the corpus and cauda epididymis, increased to 490±7.5 and 467±11.8 µm respectively in 5 months-old animals. The tubular diameter of the corpus and the cauda epididymal tubules increased ($P<0.001$) gradually, respectively between 2 to 4, and between 2 to 5 months *(Fig. 2a)*. During the growth period, there were age effects ($P<0.001$) on the tubular and luminal diameters of the epididymal tubules *(Fig. 2a)*.

The luminal diameters of the caput epididymal tubules increased continuously with the age, with a significant ($P<0.001$) increasing in 3 months-old animals (412±12.8 µm) *(Fig. 2b)*. The epididymal luminal diameters measuring 180±3.7 and 221±7.9 µm, respectively for the corpus and cauda epididymis in 2 months-old animals, increased to 366±8.3 µm and 373±12.8 µm in 5 months-old animals. The luminal diameters of the corpus and caudal epididymal tubules increased significantly ($P<0.001$) from 3 and 4 months respectively *(Fig. 2b)*. During the postnatal growth, there were age effects ($P<0.001$) on luminal diameters of the epididymal tubules *(Fig. 2b)*.

The epithelial height of the caput and caudal epididymal tubules remained unchanged between 2 till 4 months, then it increased significatively ($P<0.001$) in 5 months-old animals *(Fig. 2c)*. The epithelial height of the corpus epididymal tubules increased significatively ($P<0.001$) in 3 months-old animals *(Fig. 2c)*. During the postnatal growth, age effected ($P<0.001$) the epithelial height of epididymal tubules *(Fig. 2c)*.

Immunolocalization of Androgen Receptor During Epididymis Development

ARs immunostaining was observed in all the segments of lamb epididymis *(Fig. 3)*. ARs were localized in the principal cells of the caput, corpus and cauda. The cytoplasm was slightly positive by comparison with nuclei in all the regions. In the epithelium, the ARs immuno-expression was observed in the basal and

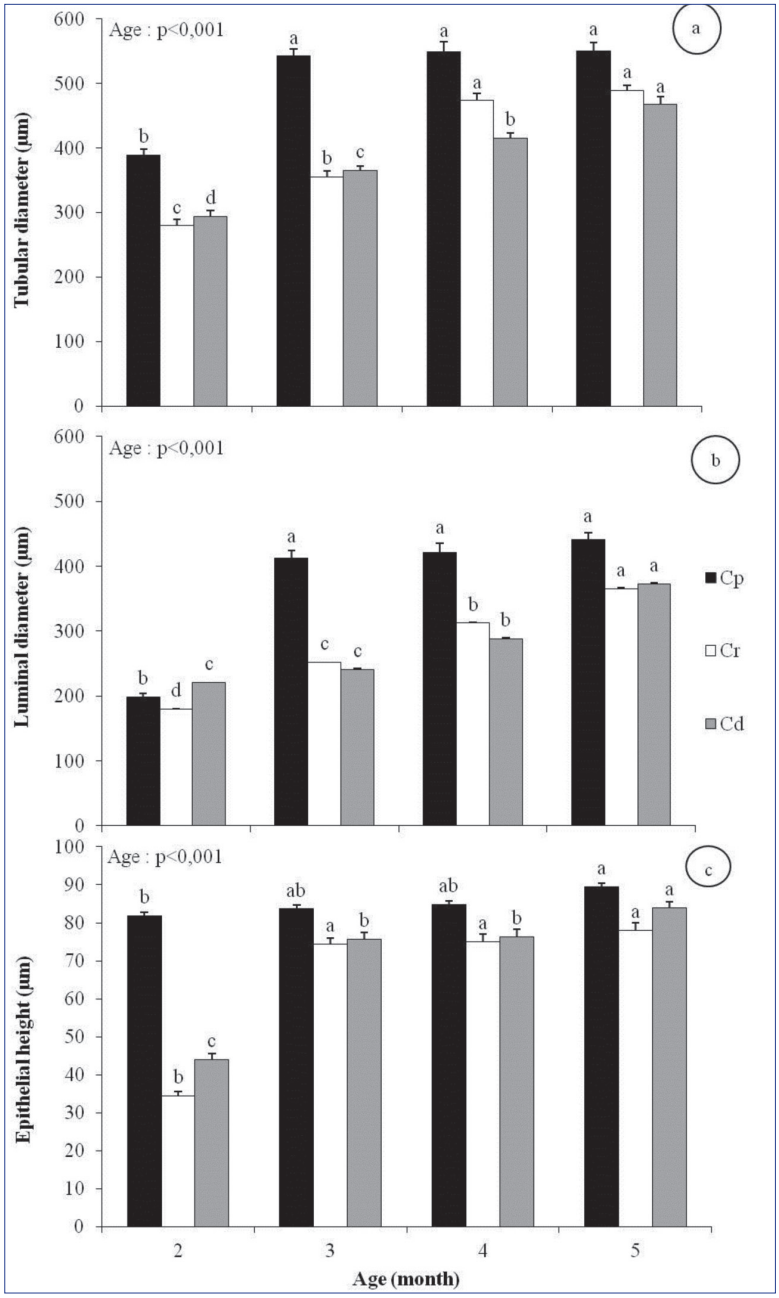

Fig 2. Effect of age on the tubular diameter (a), luminal diameter (b), and epithelium height (c) in epididymis. Bars represent means + SEM (n = 3). Cp: caput, Cr: corpus, Cd: cauda

apical cytoplasm of non-ciliated cells and in apical cytoplasm of ciliated cells.

In the corpus and cauda epididymis, the same pattern of ARs repartition was observed. However, a decreasing intensity of immuno-expression was observed, with the lowest intensity in principal cells of corpus, and cauda epididymis *(Table 1)*. The peritubular connective tissue was negative. Intermittent immunostainings for ARs were also seen in smooth muscle and connective tissue. Sperm in the lumen appeared positive for ARs immunostaining. Nuclei of cells belonging to connective tissue and smooth muscle cells were also positive. Additionally, nuclei of interstitial cell and sperm were positive for antibodies directed against ARs. Luminal sperm in corpus and cauda epididymis showed positive immunostaining for ARs in the cytoplasmic droplet.

No immunoreaction was observed in the caput, the corpus and the cauda epididymidis incubated without any primary antibody. ARs staining in the epithelial cells appeared to be stronger than in the peritubular smooth muscle cells. In the epithelial cells, staining intensity was stronger in the principal cells than in basal and apical cells. The staining intensity of AR positive cells changed depending on the age of animal. The AR immunostaining intensity increased between 2 till 5 months for all the different regions of epididymis.

DISCUSSION

After this study, the epididymis weight increased quickly during the period comprised between 2 and 5 months. The same increase pattern in testis weight was observed in lambs in postnatal development [3]. Several authors reported epididymis weight was increasing with age [15,16].

Table 1. *Average staining intensity of ARs in the different region of epididymis (caput, corpus and cauda) at 2 and 5 months of age during postnatal development in D'Man lamb*

Epithelial Cells	2 Months			5 Months		
	Caput	Corpus	Cauda	Caput	Corpus	Cauda
Principal cells	+	+	+	++	++	++
Basal cells	-	-	-	+/-	+	+/-
Apical cells	+	+	+	++	++	++
Interstitial stromal cells	-	+/-	+	+	+/-	+/-
Peritubular smooth muscle cells	-	-	+/-	+/-	+	-

** Symbols are as follows: ++ strong, + moderate, – negative, +/– variable*

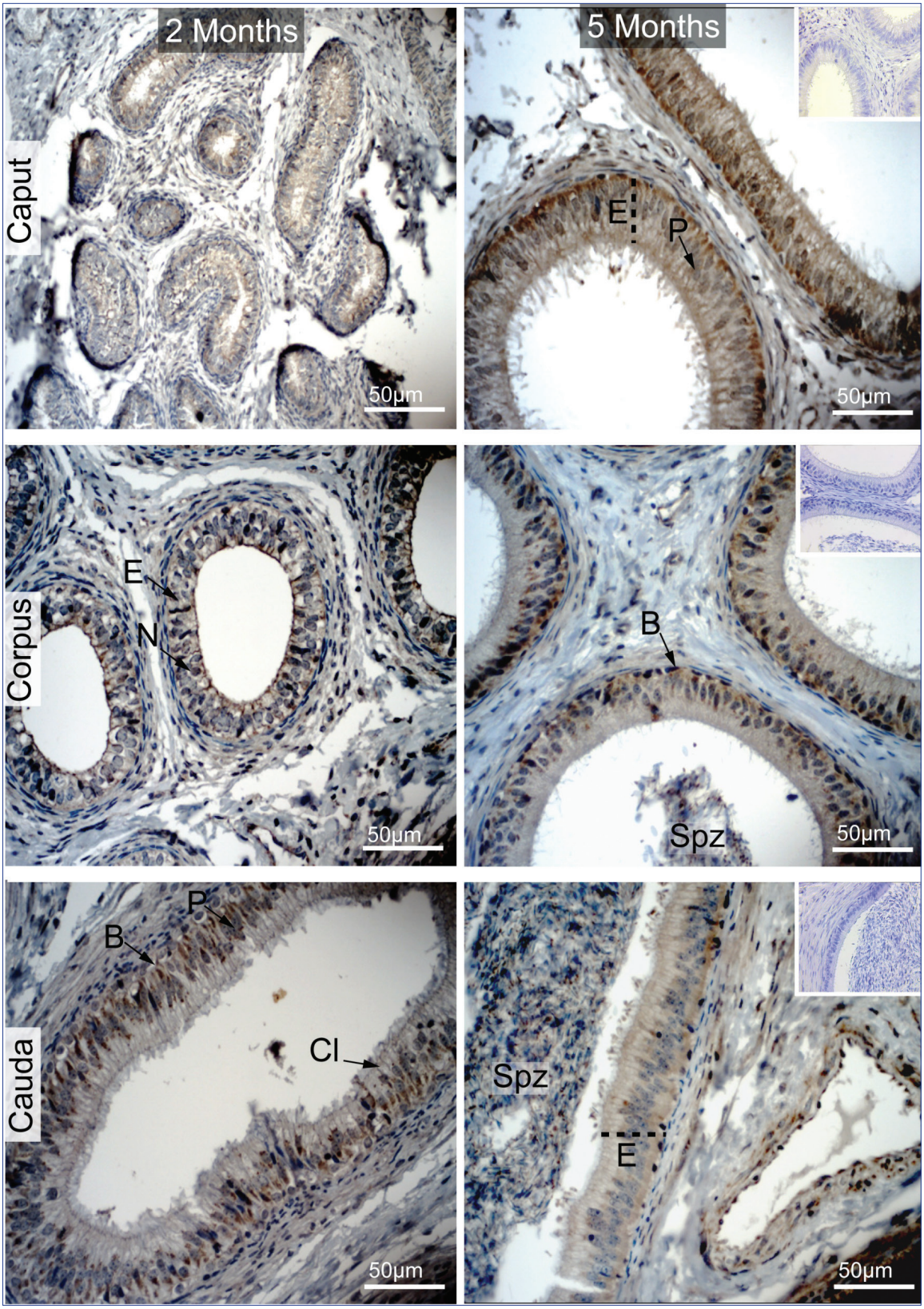

Fig 3. Immunolocalization of androgen receptor in the epididymis of D'Man lamb

AR immunostaining is observed in the nucleus and cytoplasm of epididymal epithelial cells in the caput, corpus and cauda in 2 and 5 months-old D'Man lambs. The images shown here are representative of the results of immunostaining observed in three animals for each age group. No immunostaining was observed in negative controls (inset). E: epithelial cell; P: principal cell; B: basal cell; N: narrow cell; Cl: clear cell; Spz: spermatozoa

At each age, the tubular diameter decreased from the caput to cauda in D'Man lamb. Noviana et al.[17] reported in Kacang goats and local sheep that the diameter in the corpus was smaller than the caput and cauda regions'ones, due to the narrowed and elongated anatomical structure of the corpus epididymis. The tubular diameter increased significantly with the age in the cauda, because spermatozoa were stocked in this epididymis area.

The luminal diameter of the caput epididymal tubules presented a high significant increase in 3 months-old animals. The epididymal luminal diameters decreased from caput then increased in cauda. For the domesticated adult African great cane rat *(Thryonomys swinderianus)* [18] and in age-related study in the rat [19], the epididymal luminal diameters increased progressively from caput to cauda. While the cauda epididymis acted as a sperm reservoir, both the caput and corpus were responsible for sperm maturation [20].

The height of the epithelium of each region increased, as the age increased. The epithelial height of caput was more developed than in corpus and cauda epididymis. In One-Humped Camel *(Camelus dromedaries)* [21], in rat [19], the highest epithelium was seen in the caput and decreased gradually toward the cauda; wards the epididymal duct might mechanically facilitate the passage of the sperms toward the terminal segment [21].

The development of a fully differentiated epithelium is dependent on androgens and also requires the influence of luminal factors from the testis [22]. The epithelial cells of epididymis are able to synthesize some steroid hormones because the cytoplasm has accumulated lipid droplets and contains the active enzymes of steroidogenesis, capable to moderate the *in vitro* synthesis of androgens [23]. Androgens play a crucial role in the proliferation, differentiation and function of the epididymis [24]. Immunocytochemistry identified the epithelium of the epididymis such as a site of ARs expression, while the connective tissue stroma and the blood vessels lacked specific signals throughout the organ. Androgens are also implicated in the regulation of epididymal blood flow [25]. The ARs immunostaining in D'Man lamb epididymis was observed in both the nuclei and cytoplasm of ciliated and non-ciliated epithelial cells, in addition to the peritubular and some stroma cells. The epididymal localization of the ARs was reported for various species [26,27]. However, the presence of ARs epididymis is well documented in adult ram [26-28] than during postnatal development of lambs. Additionally, sperm in the lumen appeared positive for ARs. The heterogeneous signal distribution for ARs expression along the ram epididymis did not change depending on age as reported in the rat [29]. During postnatal development, the luminal secretion of androgens is essential for the maintenance of epithelial cell identity [30], and for the normal development and function of the stromal cells [31]. Gur and Timurkaan [14] reported the progressive degenerative alterations occurred in the seminiferous tubules after prepubertal epididymal ligation. These degenerative changes included increase at the seminiferous tubule diameter and basal membrane thickness, decrease at the germinal epithelium thickness, depletion of spermatids and presence of multinucleated spermatids [32]. Both the regionalized differentiation of the epididymis and the variation in the luminal fluid composition take place under the control of androgens [33].

The morphometric changes and immunolocalization of androgen receptors during the postnatal development of epididymis indicated the necessity of androgens for postnatal differentiation and maintaining the structure of the epididymis.

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Research Article

The Antioxidant Activity, Vitamin C Contents, Physical, Chemical and Sensory Properties of Ice Cream Supplemented with Cornelian Cherry *(Cornus mas* L.) Paste

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Abstract

The aim of this research was to investigate the effect of cornelian cherry *(Cornus mas* L.) paste (CP) on the quality properties of ice cream. CP was added to an ice cream mix at four concentrations (0, 5, 10 and 15%, w/w) for ice cream production. The increment of CP level caused the increased of vitamin C content, a values and overrun values, whereas it decreased the viscosity of samples compared to Control ice cream sample. The results indicated that lyophilised water extracts of CP (LWECP) contain remarkable phenolic compounds. The findings showed that there is a positive correlation between the total phenolics and flavonoid contents in LWECP and ice cream samples antioxidant activity. CP has shown to be an effective source of natural antioxidants. CP may be used as a source of natural colour and flavour agent in ice cream manufacture. CP enhanced vitamin C amounts of ice cream, and improve sensory properties. In the literature surveys of Science Direct, Web of Science, Google Scholar and Scopus databases an ice cream study using cornelian cherry fruit was not found.

Keywords: *Ice cream, Cornus mas* L., *Cornelian cherry, Antioxidant activity, Sensory properties*

Kızılcık *(Cornus mas* L.) Ezmesi İlaveli Dondurmanın Antioksidan Aktivitesi, C Vitamini İçeriği, Fiziksel, Kimyasal ve Duyusal Özellikleri

Özet

Bu araştırmanın amacı, kızılcık ezmesinin (KE) dondurmanın kalite özelliklerine etkisini incelemektir. Dondurma üretimi için, dört farklı seviyede (%0, 5, 10 ve 15, w/w) KE dondurma miksine ilave edilmiştir. KE seviyesinin artmasıyla, Kontrol dondurma örneğine kıyasla, C vitamini, a değeri ve hacim artışı değerleri artmış, görünür viskozite değerleri azalmıştır. Sonuçlar, KE'nin liyofilize su ekstraktlarının (KELSE) dikkate değer fenolik bileşik içerdiğini göstermiştir. Bulgularımız, KELSE ve dondurma örneklerinin antioksidan aktivitesinde toplam fenolik madde ile flavonoid içeriği arasında pozitif bir korelasyon bulunduğunu göstermiştir. KE, doğal antioksidanların etkili bir kaynağı olduğunu göstermiştir. KE dondurma üretiminde doğal renk ve lezzet verici madde olarak uygun bir kaynak olarak kullanılabilir. KE, C vitamini miktarlarını arttırmış, duyusal özellikleri geliştirmiştir. Science Direct, Web of Science, Google Scholar ve Scopus kaynaklarında yapılan literatür taramasında, kızılcık meyvesi kullanılarak yapılan bir dondurma çalışmasına rastlanmamıştır.

Anahtar sözcükler: *Dondurma, Cornus mas* L., *Kızılcık, Antioksidan aktivite, Duyusal özellikler*

INTRODUCTION

Ice cream is a popular and nutritious dairy product which is consumed at all seasons. Its quality depends on mix formulation and processing. Actually, the highest quality of ice cream manufacture depends on excellent quality of ingredients and a mix which is formulated and balanced the proper function of components, freezing and hardening process [1]. As a result of consumer's trend ice cream technology rapidly has been developed by different taste demands [2-8]. Many researches have been focus on this field which shows that additives affects nutritional value, functional and sensory properties. In recent years, the interest of consumers has increased, the fruit rich in antioxidant compounds. Among these fruits, cornelian cherry fruits gained lately an increasing

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importance [9]. Cornelian cherry fruits are rich source in terms of their nutrients for humans such as vitamin C, organic acid, tannin, dietary fiber and some minerals [10,11] and have antioxidant effect [9,12-14]. This fruit contain significant amounts of polyphenols [9]. Antioxidant compounds have been widely used as food additives to protect food against oxidative degradation, especially caused by lipids [15]. Cornelian cherry tastes sweet-sour and slightly astringent. Fruits are not only consumed fresh but also used to produce jam, marmalade, pestil, compotes and several types of soft drinks in food area. It is known that significant amounts of anthocyanins in cornelian cherry can be used as natural food colorants [16]. Due to many beneficial effects of cornelian cherry, this study aims to examine usage of cornelian cherry fruits in ice cream production. For this reason, there are several analyses are planning to carry out physicochemical and colour analyses, vitamin C amounts, antioxidant properties and sensory characteristics of ice cream samples with cornelian cherry paste (CP). In the literature surveys of Science Direct, Web of Science, Google Scholar and Scopus databases an ice cream study using cornelian cherry fruit was not found.

MATERIAL and METHODS

Materials

Cornelian cherry fruits were collected from Tortum/Erzurum, Turkey in September, 2013. All samples were sorted in terms of shape, colour, size, ripening stage, physical damage and then transported to the laboratory. After removal of kernels, fruits were broken into small pieces with a blender (Waring, 7011HS). Sugar, salep and emulsifier (mono- and di-glycerides) were obtained from local market. Skim milk powder was supplied by Pınar Dairy Products Co. (İzmir, Turkey). The cream and cows' milk were obtained from the Dairy Factory of Food Engineering Department, Atatürk University (Erzurum, Turkey).

Methods

Preparation of Mixes and Ice Cream Samples

Ice cream samples were produced in the Dairy Factory of Food Engineering Department, Atatürk University (Erzurum, Turkey). The mix samples were prepared at four different compositions of 0%, 5%, 10%, 15% CP, respectively. The formulation with 0% CP was accepted as Control. The fat content of milk was adjusted to 6% with cream which had 38% w/v fat content. The milk was divided into four equal parts of 4.0 L. After that 18% sugar, 0.7% stabilizer (salep), 4.8% skim milk powder and 0.2% emulsifier (mono- and di-glycerides) were added to all mixes. The mixes were stirred consistently and pasteurized at 85°C for 25 s. After cooling to 4°C and they were remained at constant temperature for 24 h. Fresh CP was added to the aged ice cream mixes at three different concentrations of 5%, 10% and 15%, respectively. They were frozen in ice cream machinery (Ugur Cooling Machineries Co., Nazilli, Turkey) and hardened at -22±1°C for one day. Ice cream samples stored at -18±1°C.

Physical and Chemical Analysis

Colour analysis were carried out with a colorimeter (Minolta, Model CR-200; Minolta Camera Co., Osaka, Japan). Measurements were done according to Chunthaworn *et al*.[17]. Colour saturation (C) and Hue angle (H°) values were calculated according to the formula by Mendoza *et al*.[18]. For the overrun (OR) analysis was used a standard 100-mL cup. OR values were calculated using the following formula [19].

Overrun (%) = (weight of the ice cream mix – weight of same volume of the ice cream sample)/(weight of same volume of the ice cream sample) × 100

First dripping and complete melting analysis of the samples were carried out at room temperature (20°C) according to the method of Güven and Karaca [20]. The viscosity of ice cream samples was determined with a digital viscometer (Brookfield Engineering Laboratories, Model DV-II, Stoughton, MA, USA) according to Çakmakçı *et al*.[7]. pH, total solids, protein and ash of ice cream samples were done according to AOAC [21], while fat was determined according to Gürsel and Karacabey [22]. Ascorbic acid amount of fresh CP and ice cream samples were determined according to Çakmakçı *et al*.[7].

The overall experimental procedures were duplicated.

Antioxidant Methods

Antioxidant activity of liyophilised water extract of CP (LWECP) was determined by various *in vitro* methods. To prepare LWECP, 100 g of seedless fruit was grounded into a fine powder in a mill and added to 250 mL distilled water. This mixture was stirred by a magnetic stirrer for 1 day at 25°C. Then, the extract was filtered through filter paper (Whatman No.1). The filtrates were frozen and lyophilised in a lyophiliser (Labconco, Freezone 1 L) at 5 mmHg at -50°C. Following measurements and evaluations were done according to Apak *et al*.[23] and Çakmakçı *et al*.[7]. FRAP assay is based on the reduction of Fe^{3+} – TPTZ complex under acidic conditions [24]. $DPPH^{\cdot}$ scavenging activity assay was used in order to determine the DPPH free radical scavenging activity of LWECP. $DMPD^{\cdot+}$ radical scavenging activity of sample was measured by the N,N´-dimetil-*p*-fenilendiamin dihidroklorür (DMPD) method proposed by Fogliano *et al*.[25]. Total phenolic and flavonoid compounds in the CP and ice cream samples were done according to Slinkard and Singleton [26]. The concentration of total phenolic and flavonoid compounds in CP was determined as µg of standard compounds equivalent.

Sensory Analysis

The sensory characteristics of the ice creams were evaluated according to the modified version of hedonic scale suggested by Bodyfelt *et al*.[27]. Samples were tested by fifty consumer panellists. All sensory properties were graded from 1 to 9 (1: poor, 9: excellent) on point scales. Coded ice cream samples were stored at -18°C for 2 days before analysis. The samples (~50 g) were placed on white colour plates. Each panel member evaluated the ice cream samples in terms of their six sensory attributes including colour & appearance, texture, flavour, sweetness and overall acceptability.

Statistical Analysis

The experimental design consisted of a completely randomised design in a factorial arrangement: four treatments (ice cream samples: Control (without CP), +5% CP, +10% CP and +15% CP, as seen *Table 1*), and two replicates. Statistical analysis were performed with using SPSS 17.0 (SPSS Inc., Chicago, U.S.A) software. Statistical comparisons between samples were carried out using Duncan's Multiple Range method. Differences were considered as significant at $P<0.05$.

RESULTS

Physical Properties

The colour values of ice cream samples are shown in *Table 1*. The average of the overrun values achived in the ice cream samples are summarised in *Table 2*. The highest overrun and the lowest viscosity values were observed bythe 15% CP added ice cream sample *(Fig. 1)*. The first and complete melting behaviours of the samples are illustrated in *Fig. 2*. The lowest first dripping and complete melting times (840 and 3870 s, respectively) were determined in Control sample while the highest (1290 and 4620 s, respectively) were 15% CP added ice cream sample.

Chemical Properties

Dry matter (%), protein (%), ash (%), pH and vitamin C (mg/100 g) contents of CP were found, respectively, as follows: 14.83; 0.66; 0.54; 3.93; 48.8. Our ascorbic acid result is in accordance with this reported by Tural and Koca [28]. Our dry matter and ash results are lower reported by Ayar *et al*.[29].

The chemical composition of ice creams can be seen from *Table 2*. Control sample had the highest dry matter, protein, fat, ash and pH values. There were no significant difference ($P>0.05$) in the ash and fat contents for ice cream samples. As can be seen from *Table 2*, pH values of samples were found between 5.3 and 6.7. Vitamin C content of the CP which is the material of ice creams in this study had an average of 0.488±0.42 mg/g. It is clearly shown that in *Table 2*, increasing level of CP which was added in to the ice cream samples, was significantly increased the vitamin C amount ($P<0.05$).

Antioxidant Properties

Table 3 indicates the reducing activity of CP and the standards (BHA, BHT, α-tocopherol and trolox) using different antioxidant activity methods. The human diet contains plants which include a variety of phenolic and flavonoid compounds. Additionally, it was reported that these molecules are with respect to antioxidant capacity.

Table 1. *Comparison of colour parameters for ice cream samples and CP*

Samples	L*	a*	b*	H°	C*
Control	90.02±0.18e	-2.77±.0.09a	9.66±0.02e	106.00±0.71e	10.05±0.04d
+5% CP	83.03±0.08d	2.50±0.01b	6.34±0.11d	70.93±0.17d	6.82±0.04a
+10% CP	79.12±0.37c	5.62±0.13c	4.49±0.05c	39.85±0.64c	7.20±0.12b
+15% CP	74.89±0.16b	9.08±0.10d	3.25±0.07b	16.95±0.17b	9.64±0.09c
CP	29.50±0.20a	34.69±0.07e	2.43±0.28a	4.01±0.05a	34.72±0.02e

Means ± standard deviation; Values followed by different letters in the same column are significantly different (P<0.05); ***CP:*** *Cornelian cherry paste*

Table 2. *Effect of the addition of CP on the gross chemical composition of ice cream samples*

Ice Cream Samples	Total Solids (%)	Protein* (%)	Fat* (%)	Ash* (%)	pH	Vitamin C (mg/100 g dry matter)	Overrun (%)
Control	41.4±0.11c	12.0±0.67a	13.0±0.34a	2.6±0.07a	6.7±0.07d	< 6.0a	28.4±3.39a
+5% CP	38.0±0.09b	11.9±0.60a	12.9±0.40a	2.7±0.08a	5.9±0.04c	11.8±0.72b	39.6 ±2.83b
+10% CP	37.7±0.08a	11.6±0.40a	12.8±0.40a	2.7±0.11a	5.5±0.02b	17.5±0.61c	41.2±2.55c
+15% CP	37.7±0.19a	11.6±0.72a	12.6±0.36a	2.8±0.09a	5.3±0.07a	18.3±0.50c	42.3±2.97c

** in dry matter; Mean values followed by different letters in the same column are significantly different (P<0.05);* ***CP:*** *Cornelian cherry paste*

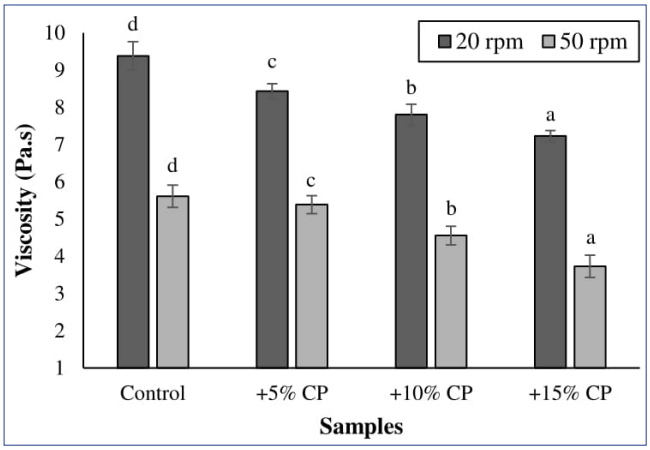

Fig 1. Viscosity values of ice cream mixes
Different letters above the bars indicate significant differences by Duncan multiple comparison test (P<0.05)

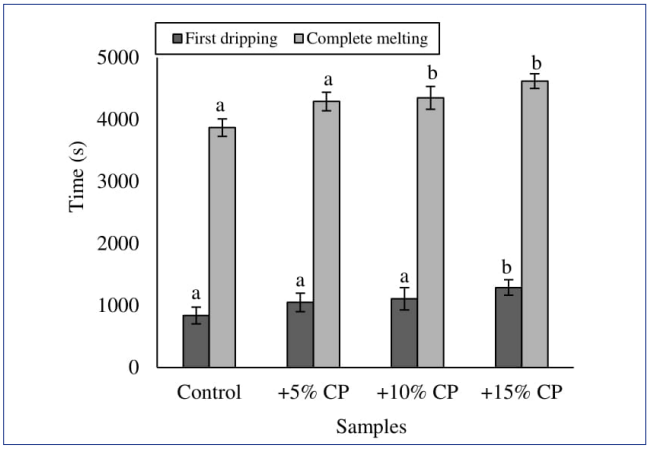

Fig 2. First dripping and complete melting times of ice cream samples
Different letters above the bars indicate significant differences between samples (P<0.05) (Duncan multiple comparison test)

Table 3. *Ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reductive potential and FRAP assay, $DPPH^{\cdot}$ scavenging assay, $DMPD^{\cdot+}$ scavenging assay of CP and reference antioxidants at the same concentrations (20 µg/L)*

Antioxidants	Fe^{3+}-Fe^{2+} Reducing Assay	Cuprac Assay	FRAP Assay	$DPPH^{\cdot}$ Scavenging Assay	$DMPD^{\cdot+}$ Scavenging Assay
BHA	1.86	0.45	2.16	0.08	0.61
BHT	1.95	0.46	2.14	0.49	0.64
α-Tocopherol	0.78	0.33	1.32	0.04	0.55
Trolox	0.31	0.31	0.38	1.18	0.68
CP	0.19	0.07	0.52	1.28	0.76

CP: *Cornelian cherry paste*

In this study, total phenolic and total flavonoid content of CP and ice cream samples are shown in *Table 4*. It was observed that, 89.5 µg of gallic acid equivalent of phenols and 67.20 µg of quercetin equivalent of flavonoids were found in 1 mg of CP.

Sensory Properties

The sensory characteristics of ice cream samples are shown in *Table 5*. The addition of the CP as ingredient significantly affected the sensory analysis results of the ice cream samples.

DISCUSSION

Colour is an important factor for consumers due to the fact that colour of food is closely associated with freshness, ripeness, desirability and food safety. *L** and *b** values decreased significantly (P<0.05) with increase in CP concentration. Black and blue character-istics increase when CP concentration increase in the ice cream samples. A disparate trend was observed in *a** values. The highest *a** value was determined at 15% CP added ice cream sample. Morever, Yüksel *et al.*[8] reported that similar result was observed in terms of *a* values of ice cream with terebinth coffee. The highest *C** value was achieved with Control while the lowest was 15% CP added sample. Data also showed that *H°* value progressively decreased by CP addition in increasing concentrations. The hue angle value of Control sample represents a colour in the yellow/green region due to between 90° and 180°. This region changed towards the red/yellow region with CP addition. The phenolic substance addition changes the colour properties of ice creams compared to Control sample [30].

Overrun is an important parameter for evaluating an ice cream product. This situation relates to a rising in the volume of ice cream during processing [31]. The highest overrun and the lowest viscosity values were observed by the 15% CP added ice cream sample. The lowest overrun was determined Control sample. There are opposite results about overrun and viscosity values at literature. Researchers generally studied with dried fruits or concentrated fruit products in these studies. For example, one of the study of Hwang *et al.*[32] about grape wine lee (GWL) paste explain that addition of GWL in ice cream product with increasing concentration caused decreasing of overrun and increasing in viscosity values. In this study, CP which has high water content is the material of this experiment. Therefore, the general increment in overrun and decrement in viscosity values

Table 4. *Total phenolic and flavonoid contents of CP and ice cream samples*

Samples	Total Phenolic Content (µg GAE/mg)	Flavonoid Content (µg QE/mg)
CP	89.5±1.45e	67.20±1.10e
Control	18.1±2.20a	13.6±2.10a
+5% CP	47.06±2.13b	38.65±1.30b
+10% CP	53.50±3.10c	49.23±2.50c
+15% CP	68.75±1.20d	53.47±2.00d

Mean values followed by different letters in the same column are significantly different (P<0.05); ***CP:*** *Cornelian cherry paste*

Table 5. *Sensorial properties of ice cream samples (1: poor, 9: excellent)*

Properties	Samples			
	Control	+5% CP	+10% CP	+15% CP
Colour and appearance	7.2±1.04a	7.4±0.93b	7.8±0.82c	7.9±0.48c
Texture	7.2±0.57a	7.4±1.14b	7.9±0.54c	8.0±0.64d
Flavour	6.8±1.01a	7.5±0.83c	7.4±0.25b	7.4±0.45b
Sweetness	7.4±0.82c	7.4±0.78c	7.2±0.48b	6.9±0.25a
General acceptability	7.2±1.00a	7.6±0.56b	7.6±0.59b	7.7±0.86b

Mean values followed by different letters in the same column are significantly different (P<0.05); ***CP:*** *Cornelian cherry paste*

with CP addition were considered as normal. *Fig. 1* illustrates the viscosity values of samples which are measured at 20 and 50 rpm. Addition of CP with a concentration of 5%, 10% and 15% caused a significant decrement (P<0.05) in the apparent viscosity when it was compared to Control sample.

The lowest first dripping and complete melting times were determined in Control sample while the highest one was 15% CP added ice cream sample. At the same time 15% CP added sample had the highest overrun value, as previously reported. Similarly, Sakurai *et al*.[33] and Sofjan [34], reported that ice creams with lower overruns had faster melting rates.

The chemical composition of ice cream samples can be seen in *Table 2*. Control sample had the highest total solids, protein, fat, ash and pH values. There were no significant difference (P>0.05) in the ash and fat contents for ice cream samples. As can be seen from *Table 2*, pH values of samples were in the range of 5.3 to 6.7. All of ice cream samples added to CP had statistically (P<0.05) lower pH values when it compared to Control sample.

The cornelian cherry fruit contains high amount of ascorbic acid between 0.164 and 0.786 mg/g as reported by researchers [10,11,35]. In this aspect, ascorbic acid content is significantly higher when it compared to other fruits which contain high ascorbic acid such as oranges (0.31 mg/g), strawberries (0.46 mg/g) and kiwis (0.29-0.80 mg/g) [13]. Vitamin C analysis is essential for fruity ice creams which have high vitamin C contents. The added of CP with increasing level (5%, 10% and 15%) dramatically increased the vitamin C amount of ice cream samples (P<0.05). The highest vitamin C value was achieved by 15% CP added sample (18.3 mg/100 g) while the lowest was in Control sample (<6.0 mg/100 g).

Antioxidants may be used to help the human body in reducing oxidative damage by free radicals and active oxygen [36]. The antioxidants use to protect quality of lipids and fatty foods. Therefore, antioxidants play much more important role in the food industry [15,37]. The antioxidant activity of CP and the standards (BHA, BHT, α-Tocopherol and Trolox) with using different antioxidant method are presented in *Table 3*. For measuring reductive activity of CP, the Fe^{3+}-Fe^{2+} transformation was investigated in the existence of CP. CP has a approximate effect trolox rather than other standarts in all metods. In addition, cupric ion (Cu^{2+}) reducing power of CP and standards decreased in order of CP < trolox < α-Tocopherol < BHA < BHT. When the FRAP (Ferric reducing antioxidant power) assay of CP and the same amount of the standard compounds was examined, CP has a higher activity than Trolox.

$DPPH^{\cdot}$ scavenging activity of CP and standards increased in the following order: α-Tocopherol> BHA> BHT> Trolox> CP. According to results obtained from $DMPD^{\cdot+}$ radical scavenging assay *(Table 3)*, DMPD scavenging ability of CP has similar to the all standards.

Flavonoids are the most common group of polyphenolic compounds in the human nutrition and they are commonly found in fruits and vegetables [6,12]. Known quantities of gallic acid and quercetin were used as the standart for calibration curves for analysis of total

phenolic and flavonoid compounds. Our datas showed that phenolic and flavonoid contents of ice cream samples increased with the addition of CP when compared with control group. It was observed that, 89.5 µg of gallic acid equivalent of phenols and 67.20 µg of quercetin equivalent of flavonoids were found in 1 mg of CP. Our results are higher than the results reported by Moldovan *et al*.[9]. As seen in *Table 4* total phenolic and total flavonoid content of ice cream samples in the range of 18.1-68.75 µg GAE/mg and 13.6-53.47 µg QE/mg, respectively. These findings clearly demonstrate that there is a positive correlation between the total phenolics and flavonoid contents in the samples and antioxidant activity.

The addition of the CP as ingredient significantly affected the sensory analysis of the ice cream samples *(Table 5)*. Samples have different colour and appearence score range between 7.2 and 7.9. The highest score was determined in sample +15% CP. CP gave reddish colour to ice cream that was desirable for the panellists. The samples containing CP showed relatively high scores in terms of organoleptic characteristics such as colour and appearance, texture, flavour compared to the Control group. The highest value of colour is +15% CP, although the lower values of colour are +10% CP, +5% CP and Control samples, respectively. Morever, the addition of CP positively affected colour, texture, flavour. The highest flavour score is determined in +5% CP sample. The highest sweetness score was determined in Control sample and following order +5% CP, +10% CP and +15% CP. Therefore, the addition of CP decreased sweetness of ice cream samples. The general acceptability scores were higher in all CP samples compared to Control group. Ayar *et al*.[29] have determined that yogurt made with CP is more appreciated than yoğurt samples made with *Diocypros kaki*, *Diocypros lotus* and rosehip (*Rosa rugosa*). Also, Celik *et al*.[38] stated that CP can be successfully used in fruit yogurt production.

The addition of CP significantly affected the physico-chemical, colour, sensory and antioxidant properties of ice cream. CP has a sweet-sour, slightly astringent taste and pleasing colour. Thus, ice cream samples with the fruit were preferred by the panellists. CP can be used as easily accessible source of natural antioxidants and a potential food additive in food industry. CP enhanced the amounts of vitamin C and improved sensory properties in ice cream. Generally, the best properties were determined in +10% CP concentration compared with Control group. CP may be used as a source of natural colour and flavour agent in ice cream manufacture. In general evaluation of the research results, this fruit which is less known can be as a potential functional food or value ingredients in our digestive system.

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Research Article

Surgical Treatment Results of Young and Adult Cats with Pectus Excavatum

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Abstract

Pectus excavatum (PE) is an uncommon, congenital thoracic wall deformity that has been previously documented in a variety of species. Most of the time, the preferred treatment modality is surgical. External and internal splints may also be used according to the age of the patient and pliability of the sternum. In this study, the authors have aimed to share the clinical and radiographic results obtained in the treatment of PE by traditional external splintage, internal splintage and sternal turnover techniques in cats. For this purpose, PE was treated using the sternal turnover technique in 8 patients, internal splint in 1 patient and external splint in 9 patients. Ages of the patients varied between 2-24 months. The external splint technique was preferred to treat cats aged younger than 12 months. Frontosagittal indices (FSI), vertebral indices (VI) and clinical severity scores (CSS) were determined before and after surgical interventions. Mean FSI, VI and CSS values were 2.1, 10.0 and 0.8 and 1.8, 11.3 and 0.4 in external splint and sternal turnover groups, respectively. At the end of the study, FSI and VI values did not reach the reference interval, however, CSS values were improved in both the external splint and sternal turnover groups. In conclusion, results of the sternal turnover technique, used for the first time in feline patients with PE, were satisfactory even though complete recovery in FSI and VI values could not be achieved. Also, in the opinion of the researcher, results of using sternal turnover technique in feline PE patients younger than 12 months of age should be further investigated.

Keywords: *Pectus Excavatum, External Splint, Sternal Turnover, Cat*

Pektus Ekskavatum Hastası Genç ve Yetişkin Kedilerde Cerrahi Tedavi Sonuçları

Özet

Pektus ekskavatum (PE) farklı türlerde görüldüğü önceki yıllarda rapor edilmiş bir kongenital toraks duvarı deformitesidir. Tedavisinde çoğu zaman cerrahi yöntemler tercih edilir ve hastanın yaşı ve sternumun esnekliği göz önüne alınarak eksternal veya internal splintlerden faydalanılır. Bu çalışmada PE 'nin geleneksel eksternal ve internal splint uygulaması ve sternal döndürme teknikleri ile tedavisi ile elde edilen klinik ve radyografik bulguların paylaşılması amaçlanmıştır. Bu maksatla çalışmamızda PE, 8 hastada sternal döndürme, 1 hastada internal splint, 9 hastada ise eksternal splint kullanılarak tedavi edilmiştir. Hastaların yaşı 2 ila 24 ay arasında değişmekteydi. Oniki aylıktan küçük kedilerin tedavisinde eksternal splint tekniği tercih edildi. Frontosagital indeks (FSI), vertebral indeks (VI) ve klinik önem skorları cerrahi girişim öncesi ve sonrasında tespit edildi. Ortalama FSI, VI ve klinik önem skorları, eksternal splint ve sternal döndürme uygulanan gruplar için sırasıyla 2.1, 10.0 ve 0.8 ve 1.8, 11.3 ve 0.4 olarak hesaplandı. Çalışmanın sonucunda eksternal splint ve sternal döndürme grubunda FSI ve VI değerleri normal referans aralığa getirilemedi ancak klinik önem skorları iyileştirilebildi. Sonuç olarak kedilerde PE 'un tedavisinde ilk kez kullanılan sternal döndürme tekniği ile elde edilen sonuçlar her ne kadar FSI ve VI indeks değerleri normal sınırlara çekilemese de tatminkâr bulundu. Ayrıca araştırıcıların görüşüne göre, sternal döndürme tekniğinin 12 aylıktan küçük kedilerde PE 'un tedavisi için kullanılmasının ayrıca araştırılması gerekmektedir.

Anahtar sözcükler: *Pektus Ekskavatum, Eksternal Splint, Sternal Döndürme, Kedi*

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INTRODUCTION

Pectus excavatum (PE) is an uncommon congenital abnormality of the chest wall and it is characterised by a concave deformity of the caudal sternum and consecutive narrowing of the entire thorax [1,2]. This anomaly has been reported in humans, cats, dogs, cows, sheep, ruffled lemurs and sea otters. While the aetiology of PE is poorly understood, possible reasons have been reported as; shortening of the central diaphragmatic tendon, abnormal diaphragmatic musculature or abnormal intrauterine pressure, especially in the congenital form [3]. PE is inherited in humans, and the predisposition of Burmese kittens and brachycephalic dogs suggests a possible heritable basis in small animals [1].

The major clinical symptoms in PE are growth retardation, exercise intolerance, cyanosis, tachypnea and vomiting [4]. Cardiac displacement, murmurs and cardiomegaly may occur in humans and small animals [5,6]. A diagnosis of PE can be made by palpation and radiography. The deformity may be evaluated objectively by measuring the fronto-sagittal and vertebral indices on thorax radiographs [7].

Animals with solely a flat chest may contour to near normal configuration, medical management could be prefered [2]. Surgical treatment of PE is indicated when cardiopulmonary signs are severe. In very young patients, when the tissues are still pliable, external splinting may be used [4,6,8]. Older animals usually require surgery on the hard or soft tissues of the thoracic wall to allow correction of the defects [9-13].

In this study, the authors evaluated the surgical treatment results of both young and adult cats with PE using external splint and sternal turnover technique.

MATERIAL and METHODS

Patient Selection and Preoperative Evaluation

All cats were brought with the complaint of respiratory distress. Cats with concave deformity of the caudal sternum determined during clinical examination were included in the study. Depending on respiratory rate and the severity of exercise intolerance and dyspnoea, clinical severity score (CSS) was rated between 0-5 during clinical examination. No clinical signs during examination was scored 0 and prolongesd periods of dyspnea with evidence of significant extrathoracic disease was scored as 5, according to previous study [14]. Radiographic examination of the thorax and blood tests were carried out routinely.

Frontosagittal (FSI) and vertebral indices (VI) were used to assess the severity of PE in all patients. FSI is the ratio of thoracic width at T10 level in dorsoventral radiography and the distance from the centre of the ventral surface of T10 or the vertebra overlying the deformity to the nearest point on the sternum in laterolateral radiography. VI is the ratio of the distance from the centre of the dorsal surface of the vertebral body overlying the deformity to the near point of the sternum and the dorsoventral diameter of the centrum of the same vertebra. FSI between 0.7-1.3 and VI between 12.6-18.8 were considered normal [14]. FSI >2 and VI <9 were considered as surgical candidates.

Surgery

Butarphanol (Butomidor®, Interhas) 0.4 mg/kg SC was used for preanesthethesia. Also we took precaution to avoid postoperative respiratory distress by the use of butarphanol at the same time. Anesthesia was induced with 2-4 mg/kg IV propofol (Pofol®, Sandoz) and maintained with isoflurane in oxygen. Non-rebreathing anesthesia circuit was used. Patients were positioned in dorsal recumbency and the ventral thorax was aseptically prepared for surgery. Perioperative fluid therapy consisted of Lactated Ringer's solution (10 mL/kg/h). Three different surgical treatment method were used depending on the pliablility of the hard and soft tissues. Ceftriaxone (Novosef® Zentiva) 50 mg/kg IV was administered 30 min before the operation.

External Splint Application

Preoperatively, a U-shaped, two sided splint made of PVC material was prepared by making holes at 1 cm intervals to include the sternum. The patient was positioned in dorsal recumbency. Non-absorbable monofilament no:1 suture material (Propilen®, Dogsan) was passed under the internal surface of the sternum starting from the caudal xiphoidea. Suture ends were left long and tagged with mosquito haemostats. All stay sutures were passed through the holes on the splint using an 18-gauge needle and then tied securely. The edges of the splint were padded. A bandage was applied lightly to cover the splint. The splint was removed three weeks post-surgery. After removal of the splint, normal thoracic depth was observed in physical and radiographic examinations.

Internal Splint Application

A medial ventral surgical approach to the sternum was used. Sternebrae were liberated from the superficial, deep pectoral and rectus thoracis muscle attachments by periosteal elevation [13]. Non-absorbable no:1 mono-filament sutures were passed under the sternebrae and costae and then were passed through the holes on the special design T-shaped plate (316-L steel) and finally tied securely. The skin was closed routinely *(Fig. 1)*.

Sternal Turnover Technique

The cat was positioned in dorsal recumbency, and both the ventral aspect of the thorax and the abdomen were prepared aseptically for surgery *(Fig. 2-A)*. A midline skin incision was made extending from the manubrium of the sternum to 2 cm cranial to the umbilicus *(Fig. 2-B)*.

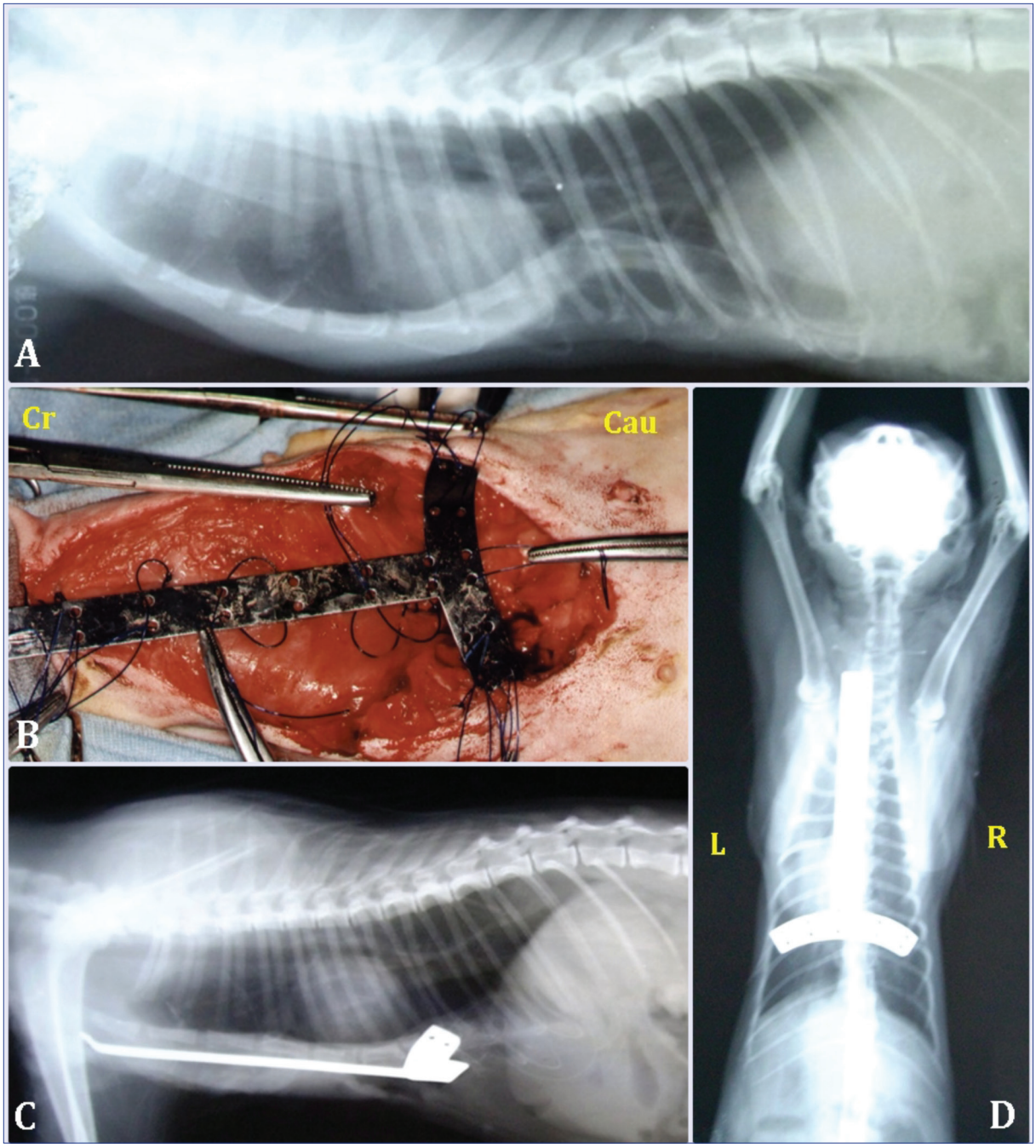

Fig 1. Internal splint application, case no 2. Preoperative lateral radiographic view, **A**; Placement of internal splint to ventral thorax by circumcostal and circumsternal sutures, **B**; Postoperative lateral radigraphic view, **C**; Postoperative dorsoventral radiographic view, **D**; *Cr*, cranial; *Cau*, caudal; *L*, left; *R*, right

Following this, the sternum was transected just above the point where it begins its posterior displacement. Costal cartilages and medial ends of the intercostal muscles were then resected en bloc *(Fig. 2-C,D)* [15]. The sternum was freed from its dorsal attachments, turned over and attached to its cranial part using Kirschner wires *(Fig. 2-E,F,G,H)*. Next, the lateral and caudal parts of the sternum were attached to muscles and other soft tissue structures using no:0 monofilament absorbable suture material (Tekmon®, Dogsan) in a simple interrupted fashion *(Fig. 2-I)*. Skin and subcutaneous tissues were closed using 2/0 monofilament non-absorbable suture material (Propilen®, Dogsan) *(Fig. 2-J)*. Laterolateral and dorsoventral thorax radiographs were taken and evaluated in the pre- and post-operative periods and also following removal of the Kirschner wire *(Fig. 3)*.

Postoperative Management and Evaluation

Patients were placed in an oxygen tent and respiratory rate, mucous membrane colour and existence of dyspnoea was

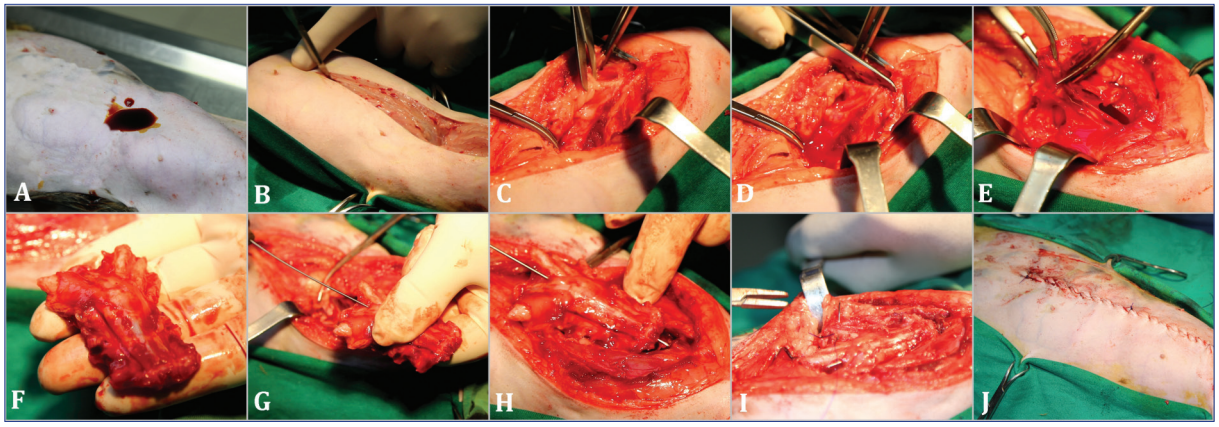

Fig 2. Operation stages of the sternal turnover technique. Patients were positioned in dorsal recumbency and ventral thorax was aseptically prepared, **A**; Median skin incision was made from manubrium to the caudal of xyphoid process, **B**; Costal cartilages and the medial ends of the intercostal muscles were then resected en bloc, **C**; Sternum was transected just above the point where it begins its posterior displacement, **D**; Sternum was freed from its dorsal attachments, **E-F**; Sternum was turned over 180° and ø 1.5 kirshner wire was passed through the centre of the sternebrae, **G-H**; Next, sternum was attached to its cranial part by kirchner wire and lateral and caudal parts of sternum were attached to muscles and other soft tissue structures using no:0 monofilament absorbable suture material, **I**; Subcutaneous tissues and skin were closed routinely, **J**

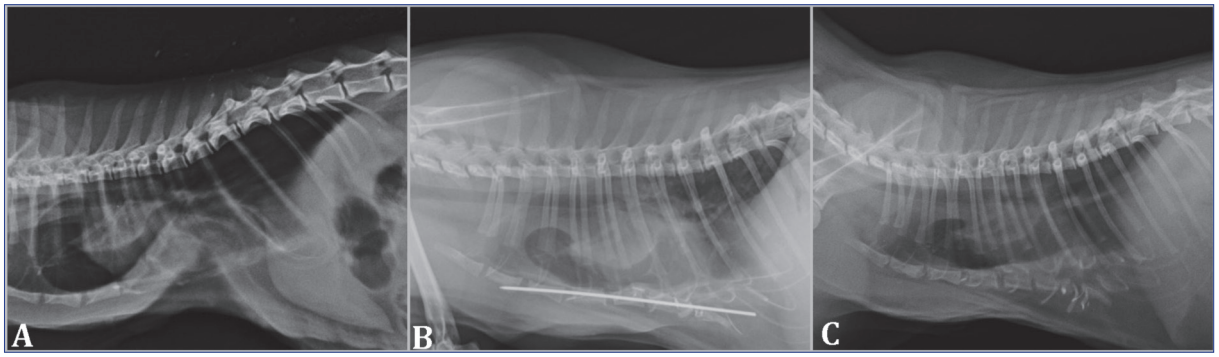

Fig 3. Preoperative and postoperative control radiographs of a cat (Case no: 6) in the sternal turnover group. Preoperative view of the sternal concavity, **A**; Changes in the sternal concavity immediately after operation, **B**; Lateral thorax radiography of the same cat 2 months after the operation, immediately after removal of the kirschner wire, **C**

observed at regular intervals. Administration of antibiotic and analgesic medications (Ceftriaxone and butarphanol) were continued for 7 days. External splints were removed 20 days after the operation and control radiographs were taken. In patients treated using the sternal turnover technique, Kirschner wires were removed 1.5-2 months postoperatively. FSI and VI values were recalculated in external splint and sternal turnover groups on radiographs taken 20 days and 1.5-2 months postoperatively, respectively. CSS was reevaluated and recorded at the same time.

RESULTS

The material of this study comprised a total of 18 cross bred cats (10 male and 8 female). Their ages varied between 2 and 24 months (average 11.1 months). During this study PE was diagnosed in 2 littermates from the same mother. Case no 16 and 17 were brothers and external splint application was carried out. Clinical signs included palpable abnormality of the sternum, as well as exercise intolerance and dyspnea in all of the patients. CSS was evaluated in both the preoperative and postoperative period. Patient were split into groups according to their age at the time of admission. While the external splint technique was used in cats younger than 12 months old, the sternal turnover technique was used in cats older than 12 months of age.

PE was treated using the sternal turnover technique in 8 patients, internal splint in 1 patient and external splint in 9 patients. One of the patients (case no: 7) treated with an external splint died in the acute postoperative period due to respiratory distress. Also another patient in the external splint group (case no: 4) was presented to the clinic with a complaint of severe dyspnea one week after removal of the external splint. Control radiography revealed that the concave deformity of the sternum had worsened after splint removal. The external splint was reapplied. The internal splint application in one patient (case no: 2) failed. A reaction occurred between the soft

Table 1. *Signalment, preoperative and postoperative FSI and VI indices and CSS, surgical techniques used and complications including information belonging to the patients*

Case No	Age (month)	Breed	Sex	Technique	Preop CSS	Postop CSS	Preop FSI	Postop FSI	Preop VI	Postop VI	Complications
1	6	Cross bred	Female	External Splint	3	1	3.2	2.1	5.3	7.5	
2	12	Cross bred	Male	Internal Splint	3	3	2.1	N/A	8.2	N/A	Seroma formation and soft tissue reaction. Splint was removed
3	18	Cross bred	Male	Sternal Turnover	4	1	3.3	2.1	3.2	11.7	
4	2	Cross bred	Female	External Splint	3	2	3.1	2.4	4.4	7.1	Respiratory distress, external splint was removed and reaplicate 1 month later
5	7	Cross bred	Male	External Splint	4	0	3.0	1.2	7.1	11.9	
6	14	Cross bred	Female	Sternal Turnover	3	0	2.6	1.4	7.5	12.4	
7	4	Cross bred	Male	External Splint	4	N/A	3.1	N/A	7.3	N/A	Died
8	19	Cross bred	Male	SternalTurnover	3	0	2.6	1.9	7.9	11.1	
9	9	Crossbred	Female	External Splint	3	1	2.6	1.9	7.9	12.2	
10	8	Cross bred	Male	External Splint	3	0	3.1	2.5	6.1	9.1	
11	16	Cross bred	Female	Sternal Turnover	3	0	2.5	1.7	7.7	10.3	
12	14	Cross bred	Male	Sternal Turnover	2	0	4.5	1.4	5.7	12.9	
13	19	Cross bred	Female	Sternal Turnover	2	0	2.2	1.5	6.9	10.8	
14	20	Cross bred	Male	Sternal Turnover	3	1	3.4	1.7	7.1	10.6	
15	24	Cross bred	Female	Sternal Turnover	3	1	3.5	2.2	7.4	10.9	
16	3	Cross bred	Male	External Splint	3	1	3.1	1.8	6.8	12.2	
17	3	Cross bred	Male	External Splint	3	1	3.8	2.4	6.4	8.8	
18	3	Cross bred	Female	External Splint	4	1	3.2	2.4	8.1	11.2	Relapse of respiratory signs and rising the concave deformity of thorax after removing the external splint- reoperated

FSI: frontosagittal indices; ***VI:*** vertebral indices; ***CSS:*** clinical severity score; ***N/A:*** not applicable

Table 2. *Evaluation of the postoperative mean FSI, VI and SCC values of the patients in sternal turnover and external splint groups*

Groups	Mean FSI	*sd*	Mean VI	*sd*	Mean CSS	*sd*
Sternal Turnover Group (n=8)	1.8	*0.3*	11.3	*0.8*	0.4	*0.5*
External Splint Group (n=8)	2.1	*0.3*	10.0	*1.5*	0.8	*0.4*

FSI: *frontosagittal indice;* ***VI:*** *vertebral indice;* ***CS:*** *clinical severity score;* ***sd****= standart deviation*

tissue and the plate and the material had to be removed *(Table 1)*.

When the external splint and sternal turnover groups were evaluated separately, the improvement in FSI and VI values in the postoperative period were different. Mean FSI and VI values were 2.1 and 10.0 in the external splint group and these values did not reach the reference interval. On the other hand, general clinical condition and CSS (mean 0.8) values were satisfactory in the external splint group when compared to the preoperative period. FSI and VI values were close to the reference interval in the sternal turnover group (FSI, mean 1.8; VI, mean 11.3) at 1.5-2 months postoperatively and general condition and CSS (mean 0.4) values had improved as much as the external splint group *(Table 2)*.

Postoperative skin abrasions, suture abscesses, and dermatitis-like complications were rarely observed and any additive evaluation was not required.

DISCUSSION

Pectus excavatum, also known as funnel chest or trichterbrust, is a skeletal abnormality of the sternum characterised by dorsal deviation of the sternebrae and resultant dorsoventral compression of the thorax [3]. Aetiology of the congenital form is poorly understood. Theories such as; shortening of the central diaphragmatic tendon, abnormal diaphragmatic musculature or abnormal intrauterine pressure have been put forward [6]. Predisposition in Burmese cats suggests that this developmental defect

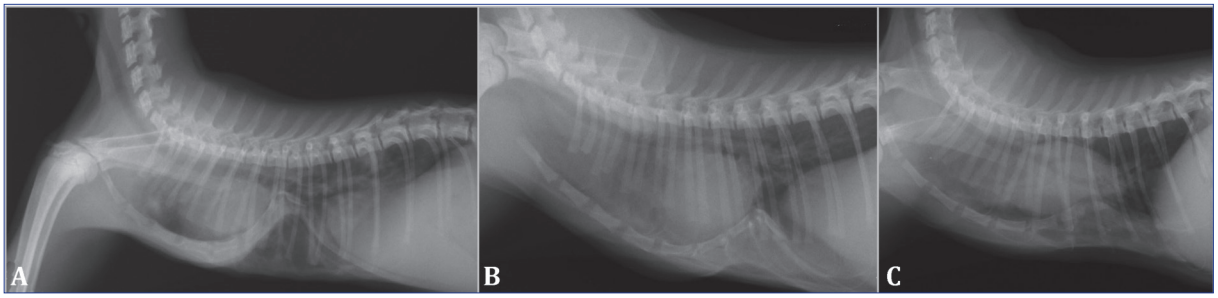

Fig 4. Case no: 4. Preoperative radiography, **A**; One week after removal of external splint, **B**; One month after second external splint application, **C**

may be heritable in some cases [16]. In this study, PE was determined in 2 littermates. Although no genetic tests have been performed, it has been suggested that PE may be heritable in such cases.

PE has been reported in Bengal, Main Coon, Burmese, domestic shorthair and domestic longhair cats [4,8,14,16,17]. Domestic shorthair and domestic longhair cats are cats of mixed ancestry and thus not belonging to any particular recognised cat breed [18], in other words cross bred. In the present study, all of the cats were cross bred. When the authors analyzed previous studies, PE was commonly seen in cross bred cats. The breed distribution of cats in this study is compatible with previous cases and studies.

External splint application to the ventral aspect of the thorax is the most common technique used to correct PE defects in small animals. However, the external splint technique can be used in young animals due to the pliability of the costal cartilage and sternum, and the thorax can be reshaped by applying traction to the sternum using sutures [2]. Although some studies suggest that external splintage should be used before 4 months of age [4], it is also known that this technique can be used in older animals and FSI and VI could be improved [8]. In this study, the external splint technique was used in cats older than 4 months old (oldest 9 months-old). Postoperative mean FSI and VI values did not reach the normal reference ranges but respiratory and cardiopulmonary signs had regressed. Body weight was increased and exercise intolerance and high respiratory rates were decreased in most of the patients in the postoperative period. Mean CSS in this group was 0.8. One patient (case no:18) was brought to the clinic 1 month after removal of the external splint, with complaints of a relapse of the respiratory signs and an increase in the concave deformity of the sternum. In this case, the external splint was reapplied and removed 3 weeks later as usual *(Fig. 4)*. It is known that, the ensuing costal mineralisation provides sufficient rigidity to maintain the costal arch and sternum in their final correct shape after removal of the external coaptive device [13]. Ongoing contraction of the central diaphramatic tendon, especially in ages 2 to 4 months may be the reason of the rising concavity after removal of the external splint in this case. According to the authors' observations, external splint was considered to be better tolerated in 5 to 9 month-old cats in terms of postoperative adaptation and respiratory signs regressed more rapidly. On the other hand, the reanimation period was longer and respiratory distress-like complications were more common in cats younger than 4 months of age in the external splint group in this study.

In adult cats, the sternum is less pliable and the external splint technique may not be sufficient enough to correct sternal abnormality. Because of this, instead of external splints, the plate and Kirschner wire were both used to correct sternal deformity in cats older than 4 months [12,13]. In this study, the researchers used a special design T-shaped plate to correct sternal deformity in a 12 month-old cat as an internal splint but the results were not satisfactory. In this case, the plate was placed on the sternum and attempts to stabilise were made using circumsternal and circumcostal sutures. In this case, poor stabilization of the plate resulted in soft tissue reaction and seroma formation under the skin. The plate was removed and no other treatment options were applied since consent was not given by the owner.

Sternal turnover technique was first described in 1965 for the treatment of PE in human patients [10]. Satisfactory results using this technique were obtained in 97% of the 199 patients in the 15-year experience. Despite the excellent results and the appeal of such a direct method of repair, experience with sternal turnover has been limited even in human medicine until the 1980s [11]. In this study, the researchers preferred to use this technique to treat PE in cats older than 12 months. When preoperative and postoperative FSI and VI values were examined, it was determined that FSI and VI values close to normal chest conformation could be reached in the sternal turnover group. In addition, postoperative CSS values were between 0-1 in all of the patients in the postoperative period. Although sternal turnover has some disadvantages, such as a long operation time and more invasive surgical technique when compared to other internal splint methods, results were satisfactory. On the other hand, sternal turnover technique may give better results, if this

technique is used in cats younger than 12 months of age, since the cats continue to grow.

In conclusion, the results achieved by the authors suggest that, sternal turnover technique may be used successfully to correct sternal deformity in PE in cats older than 12 months of age. Also, results of using the sternal turnover technique in PE patient cats younger than 12 months of age should be further investigated.

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Research Article

Using Cell-free Matrix Derived from Ostrich Plantar Ligament for Repair of Articular Cartilage Defect of Rabbit

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Abstract

Articular cartilage lesions are most common injury of the knee for which several repair methods have been described. We described *in-vivo* potential of collagen type I scaffold extracted fromknee of ostrich. Full thickness defects were created bilaterally in the weight bearing area of femoral condyles of the both femurs of 7 white New zealand adult male rabbits weighting 1800±100 g. The six left medial condyles as experimental group I were filled by using cell-free tissue and six right medial condyles of the same rabbit were treated by cell-tissue as experimental group II and the right medial condyle defect of one rabbit as control group was left without treatment. Histopathology data demonstrated significant difference between three groups with respect to the cartilage repair indicators as the morphology of the cells, color matrix and cartilage thickness ($P<0.05$). Furthermore, sub-chondral bone formation showed a significant difference and relationship between two experimental groups and control group ($P<0.05$). The use of cell-free ligamentum plantar matrix showed markedly significant improvement in the processes of joint cartilage repair compared control group ($P<0.05$). Results support the use of cell-free ligamentum plantar can be useful and promising bio-engineering procedure as a scaffold for the treatment of the articular cartilage surface.

Keywords: *Cell-free matrix, Ostrich plantar ligament, Repair, Articular cartilage defect, Rabbit*

Tavşanlarda Artikular Kartilaj Defekt Onarımında Devekuşu Plantar Ligament Kökenli Hücre İçermeyen Matriks Doku Kullanımı

Özet

Artikular kartilaj lezyonları dizin en sıklıkla gözlenen yaralanması olup birkaç onarım metodu tanımlanmıştır. Bu çalışmada devekuşunun dizinden ekstrakte edilen kollajen tip I doku iskelesinin *in vivo* potansiyeli tanımlanmıştır. Ağırlıkları 1800±100 g olan 7 adet Yeni Zelanda ergin erkek tavşanın her iki femurunda femoral kondulusların ağırlık taşıyan bölgelerinde bilateral olarak tüm kat hasarı oluşturuldu. Altı sol medial kondulus hücre içermeyen doku ile doldurulurken (Grup I) aynı tavşanların altı sağ medial kondulus hasarı hücre içeren doku ile tedavi edildi (Grup II) ve bir tavşanın sağ medial kondulus hasarı tedavi uygulanmayarak kontrol grubu olarak bırakıldı. Histopatoloji bulguları kartilaj onarım belirteçleri olarak hücre morfolojisi, renk matriks ve kartilaj kalınlığı bakımlarından her üç grup arasında anlamlı derecede fark olduğunu gösterdi ($P<0.05$). Ayrıca, sub-kondral kemik oluşumu iki deney grubu ile kontrol grubu arasında anlamlı derecede fark olduğunu göstermiştir ($P<0.05$). Hücre içermeyen ligamentum plantar matriks kullanımının kontrol grubu ile karşılaştırıldığında eklem kartilaj onarımında belirgin derecede iyileşme oluşturduğu gözlemlendi ($P<0.05$). Elde edilen sonuçlar hücre içermeyen ligamentum plantar kullanımının artikular kartilaj yüzey onarımında bir doku iskelesi olarak ümit verici biyomühendislik prosedüründe yararlı olabileceğini göstermiştir.

Anahtar sözcükler: *Hücre içermeyen matriks, Devekuşu plantar ligament, Onarım, Artiküler kartilaj hasarı, Tavşan*

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INTRODUCTION

Cartilage defects have been indicated to being the most common injury of the knee joint caused by trauma, inflammation and/or biomechanical dysfunction. Chondral and osteochondral lesions related to injury or other pathologic conditions are mainly associated with the progression of osteoarthritis, leading to joint destruction. The articular cartilage defects are one of the main challenges in orthopedic surgery and may progress symptomically to severe osteoarthritis in the stifle joint [1,2].

Several approaches have been described for cartilage repair such as bone marrow stimulation via micro-fracture or drilling, arthroscopic resurfacing, autologous chondrocyte implantation (ACI), and osteochondral trans-plantation. The repair of articular cartilage remained as a challenge for many years particularly because of cartilage's hypocellularity and insufficient nutrient supply. Furthermore, other factors responsible for delayed healing include the inability of bone marrow stem cells or resident chondroprogenitor cells for hyaline cartilage formation played role [2-10].

ACI has been demonstrated to be promising when implanting culture-expanded chondrocytes alone in clinical trials [7,8]. The disadvantages of autologous or allogeneic osteochondral transplants are shown to be the limited amount of grafts and donor site morbidity and also the incongruence of the surface. The implantation of autologous chondrocytes has been used for the treatment of full-thickness cartilage lesions. It is worth noting that this method is not effective for treatment of many types of lesions such as deep defects involving the subchondral bone [9,10].

Different scaffolds with osteochondral regenerative potential have been provided in order to overcome problems of joint treatment, and promising findings were achieved using these scaffolds [11,12]. In recent years, matrix-induced autologous chondrocyte implantation has successfully been used in animal models [13] and even in clinical trials [14]. However, mentioned technique is time-consuming and implicates the risk of potential donor-site morbidity because of chondrocyte harvesting [15,16]. Collagen-based materials have been already used for cartilage repair in rabbits with satisfying findings [17,18].

Various matrices were applied in orthopedic applications including polylactic acid based matrices, fibrin glue, hyaluronic acid, alginate, polyglycolic acid, and collagen [19-23]. It has been indicted that collagen-based matrices may be satisfying in this respect because of their biocompatibility, biodegradability and mechanical integrity.

Cell-free collagen type I scaffold has been reported to be correlated with high-quality tissue repair in defects up to 12 mm in diameter [6,22]. Moreover, a new technique using a cell-free collagen type I matrix in human have been suggested promising findings.

This study was aimed to investigate the effectiveness of a cell-free scaffold derived from ostrich type I collagen in healing of loading aspect of cartilage defect in rabbit.

MATERIAL and METHODS

This study was carried out on rabbit as an animal model after approval by ethical committee of the Iranian laboratory animal ethic frameworks under the reference code IAEC-205-P.

Tissue Restoration

A total of five pairs of ostrich's limb with an average age of 11 months were collected from a slaughterhouse in Iran. Metatarsophalangeal fibrocartilage tissue of limbs were used for tissue engineering and creating extracellular scaffold in order to replace a cartilaginous defect in rabbit femoral condyle. Histological examination on this tissue revealed fibrocartilage tissue rich in collagen fibers.

Briefly, the tissue samples were harvested and placed in the plantar surface of the metatarsophalangeal joint of 3rd and 4th digits. The tissues were transferred to the laboratory of Faculty of Specialized Veterinary Sciences, Science and Research Branch of Islamic Azad University. After washing with sterile sodium chloride solution and 10% povidone iodine, specimens were fixed with 10% neutral buffered formalin for histological evaluation and stored in PBS (Phosphate buffered saline) solution for decellularization process.

For preparing a cell- free tissue, a combination of physical and chemical methods were applied. The protocol used in this study was a modification of the method described previously by Tischer et al.[24]. An equal approximately 5 mm thick specimens were placed in deionized water for 48 h. Then sonicator was used as a mechanical stage of decellularization process. It was followed by ionic detergent (Sodium dodecyl sulfate (SDS) 2%) in a vacuum for 10 days. This process continued by placing them in deionized water and 70% ethanol for 24 h separately. Finally, they were washed and put in PBS.

In order to confirm decellularization process, the tissues were sent to laboratory for DAPI (4,6-diamino-2-phenylindole) staining. To determine types of bacteria in the fibrocartilage tissue and select effective post-operative antibiotics, bacterial cultures were performed on Blood-agar and Mac-conkey medias. A sample was also cultured on Muller-Hinton agar and antibiotic discs including Ciprofloxacin, Oxytetracycline, Chloramphenicol, Cloxacillin, Ampicillin, Gentamicin, Penicillin, Lincomycin, Enrofloxacin and sultrim were used to test bacterial sensitivity. The cultures were incubated in 37°C for 48 h.

To inhibit bacterial growth in the acellular tissue, an antibiotic cocktail containing 100 mg streptomycin, 80 mg of gentamicin and 100 units of penicillin was added to PBS solution. The *in-vivo* study was performed on seven white Newzealand adult male rabbits with average weight of 1800±100 g. In order to respect ethics, we involved less animals in the study. Therefore left and right limbs of each rabbit were prepared for surgery. The rabbits were randomly divided into three groups (two experimental and one control). The left limbs of six rabbits designed as experimental group I and were implanted with acellular scaffold, the right limbs of same rabbits were designed as experimental group II and were implanted with unprocessed scaffold. Both limbs of another rabbit were used as control group with no treatments. In order to achieve environmental adaptation, all animals were kept for a week before initiating the study under appropriate temperature, humidity and diet. Animals were kept NPO (Nil Per Os) for 6 hours and were prevented from drinking water for 2 h before surgery. They were anesthetized with intramuscular injection of acepromazine (1 mg/kg) and ketamine hydrochloride (40 mg/kg) cocktail. After aseptic preparation of stifle joint, animals were positioned in dorsal recumbency. Arthrotomy of each stifle was performed via lateral approach and medial femoral condyle was exposed *(Fig. 1-A)*.

A 4 mm osteochondral defect was made on the weight bearing surface of medial femoral condyle with orthopedic drill. Cell-free and cellular plantar ligament were trimmed to a diameter equal to the defect size and implanted with press fit technique in left and right femoral medial condyle defects in experimental group I and II, respectively *(Fig. 1-B)*. Both condylar defects in control group was left untreated. The joint was irrigated with diluted Gentamicin. Afterward, the joint capsule was sutured with 4-0 polyglactin910 with simple interrupted suture pattern. Subcutaneous tissue was sutured with the same suture material with continuous suture pattern. The skin was sutured with nylon suture material with simple interrupted suture pattern in all rabbits.

In first 48 h, ice pack was placed over the surgical site twice a day for 15 min. For the next 72 h, warm compress was placed on the surgical site every 12 h for 15 min. All the animals received a daily dose of Enrofloxacin (5 mg/kg) and Flunixin meglumine (1 mg/kg) two times a day for 3 days. Clinical assessment was also done. Both groups were evaluated for walking pattern, pain, joint effusion and range of motion (ROM) and infection. The animals were euthanized with intravenous injection of high dose thiopental sodium 12 weeks after surgery and specimens were collected and fixed in 10% Buffered formalin. Decalcification process was performed with EDTA 5.5% solution. The specimens were embeded in paraffin, cut with 5 micrometer microtome and stained with

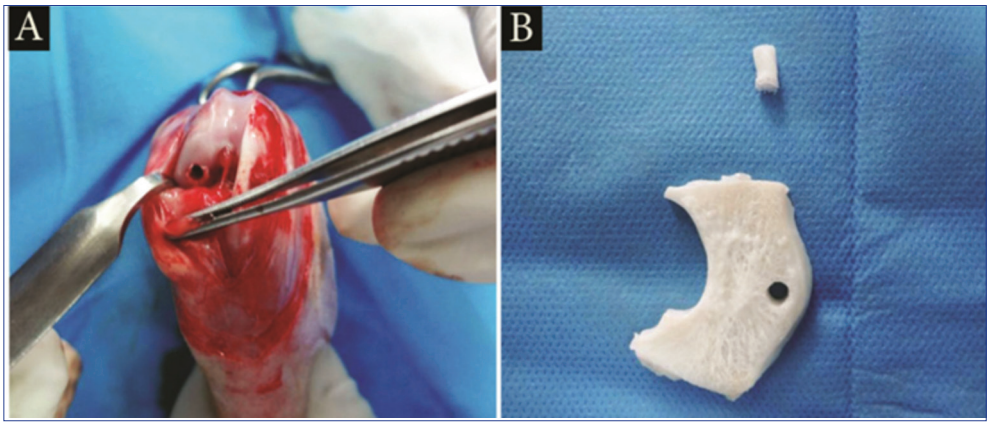

Fig 1. A- Osteochondral defect in the femoral medial condyle, **B-** Preparing blocks from decellularized Plantar ligament to press-fit into the defect

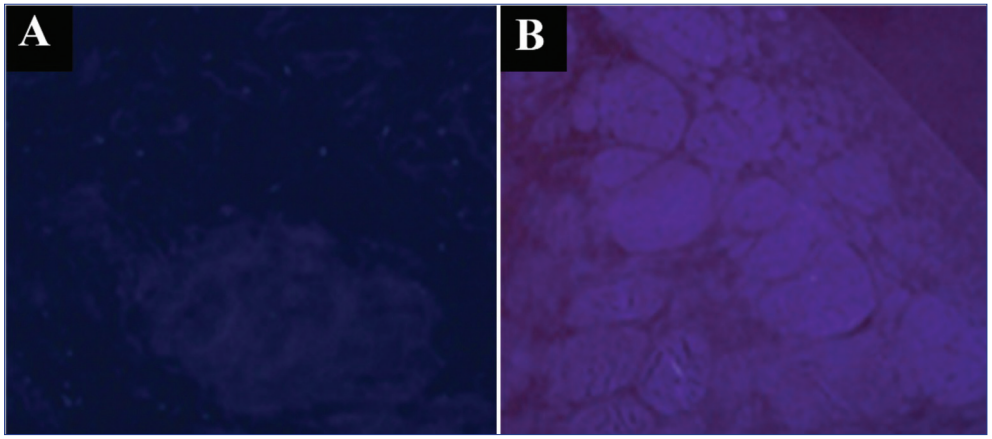

Fig 2. Morphology of Plantar ligament before **(A)** (×10) and after **(B)** (×40) Decellularization process with DAPI staining

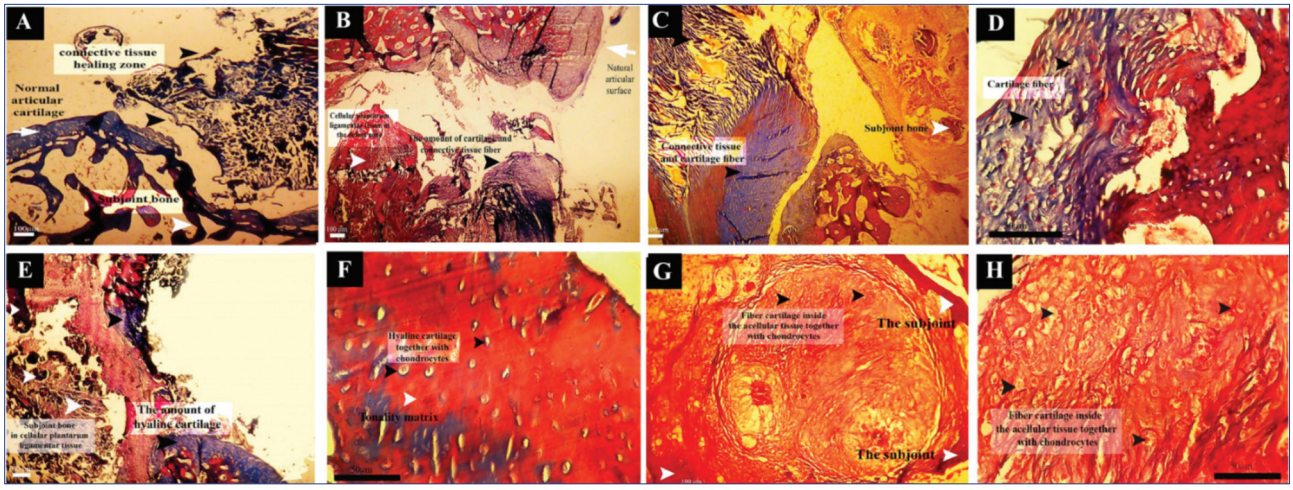

Fig 3. Histopathological samples stained with Trichrome staining. **A-** Control group; **B,C,D-** Un-processed (cellular) group; **E,F,G,H-** Acellular group

Table 1. *Evaluation and scoring of histologic factors of experimental groups*

Parameters	**Parameters**	**Score**	**Experimental Groups**													
			1		**2**		**3**		**4**		**5**		**6**		**7**	
			R	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**
The morphology of the cell	Hyaline cartilage	4	1	2	1	2	2	2	1	2	1	2	1	3	0	0
	Mostly hyaline cartilage	3														
	Fiber cartilage and transparent	2														
	Mostly cartilage fiber	1														
	Mostly without cartilage	0														
Color matrix	Like natural areas	4	1	2	1	2	0	3	2	3	1	2	2	3	0	0
	Slightly dimmer	3														
	Decreased	2														
	Highly decreased	1														
	Without color	0														
Regular levels	Smooth	2	0	1	0	1	1	0	1	0	0	0	1	1	0	0
	Slightly irregular	1														
	Irregular	0														
Thickness of cartilage	100%	4	1	2	1	2	0	3	1	1	0	2	1	3	0	0
	75%	3														
	50%	2														
	25%	1														
	0%	0														
Connecting two levels	Both integrated edge	2	2	2	1	2	0	2	2	2	1	2	2	2	1	1
	An integrated edge	1														
	Both non-integrated edge	0														
% [The formation of new bone under the cartilage under the original trade mark; (indicating normal bone repair following a similar cartilage)]	90-100	3	1	2	1	2	0	1	1	0	0	1	1	2	0	0
	75-89	2														
	25-74	1														
	Less than 25	0														
Total			6	11	5	11	1	11	8	8	3	9	8	14	1	1

hematoxylin-eosin (H&E) and Trichrome.abbits.staining for histopathological evaluation.

Statistical analysis was performed using SPSS 16 software package. Difference between groups was analyzed using the Mann-whitney test and $P<0.05$ was considered statistically significant.

RESULTS

DAPI staining demonstrated that almost 95% of the cells were removed and the native structure of tissue was preserved without any destruction as well *(Fig. 2-A,B)*.

In clinical assessment, no signs of pain was observed in about 4-5 days after surgery. The quality of walking was reported favorably in all animals. Weight bearing on operated foot was seen immediately after recovery. All rabbits were able to walk freely in their cages and natural ROM of stifle joint was seen. Macroscopically, xenografts were completely in their place in both experimental groups and tissue necrosis or transplant rejection was not grossly observed. Pathologic changes were observed only on one of the femoral condyle of rabbits in the control group.

H&E and Trichrome stained slides *(Fig. 3)* were evaluated microscopically. The results are shown in the following table *(Table 1)*. Based on four indices including cellularity, the predominant cell type, the morphological matrix and collagen formation. We scored specimens using some components of the International Cartilage Repair Society Visual Assessment Scale and a total score was obtained [25]. Histopathology data demonstrated a significant difference between acellular and cellular groups with respect to the cartilage repair indices, such as the morphology of the cells, color matrix and cartilage thickness ($P<0.05$). Furthermore, a significant difference was present between experimental groups I and II in respect to subchondral bone formation ($P<0.05$). Moreover, there were significant correlations among the 2 experimental and control groups ($P<0.05$). Cell-free plantar ligament matrix showed markedly significant improvement in articular cartilage repair compared to control group ($P<0.05$). As the matter of fact, a similar pattern to normal tissue was seen in terms of the type of cartilage formation (hyaline cartilage).

DISCUSSION

Hyaline cartilage as an avascular, alymphatic and aneural tissue is susceptible to trauma because of the current life style and activity of human societies [26]. Over 151 million people suffer from osteoarthritis worldwide, indicating a massive clinical and socioeconomic load [26]. Several strategies are being studied for restoring of articular cartilage defects and the scaffold-based cartilage treatments have been addressed as a fascinating treatment option. Investigations are mainly focusing on simplifying the surgical methods and adopting more effective agents to stimulate tissue regeneration.

The most important aspect of our study was using a new biomaterial derived from ostrich plantar ligament as an acellular scaffold which can facilitate repair of the articular cartilage defects of up to 4 mm in diameter in femoral condyle surface of rabbits.

Several studies focused on evaluating cartilage repair using different animal models including sheep, horse and goat [13,27-29]. A specialized characteristic of rodents and lagomorphs is that they possess auto-intrinsic repair of cartilage which is not present in larger animal and human [30].

Scaffold-based cartilage treatments have been reported to be effective in the improvement of cartilage repair processes [31,32]. The preclinical and clinical researches of scaffold therapy indicated that it had superior results in cartilage healing [4]. The advantage of Cell-free matrices is avoiding of cell manipulation and its regulatory obstacles with good clinical findings [33]. Additionally a cell-free one step procedure is time saving and also cost effective. Current studies showed that new generation of biomaterials are able to exploit the intrinsic tissue regeneration potential.

Many kinds of matrices have been used in reconstruction of bony and cartilaginous defects including polylactic acid, fibrin glue, hyaluronic acid, alginate, polyglycolic acid, and collagen matrices [19-23]. It has been suggested that collagen-based matrices may be promising sources due to their biocompatibility, biodegradability and mechanical integrity.

It is clear that transplanting unprocessed tissues is associated with immune reactions. Therefore, tissue-engineering methods such as decellularization are used to diminish the immune response and elimination of post-operative suppressive therapies. Therefore, the main goal of our study was to investigate the bio-behavior of a xenogenic decellularized scaffold originating from Ostrich in the repair of rabbit stifle osteochondral defects. Metatarsophalangeal fibrocartilage tissue of 11-months-old ostrich was used for tissue engineering and creating alternative extracellular matrix in osteochondral defects. Histological examination conducted on this tissue revealed a fibrocartilage ground substance rich in collagen fibers. Other studies assessed microstructure, mechanical properties and collagen content of ostrich plantar ligament highlighting that it has a good mechanical strength and it may be appropriate for application in tissue engineering. Since it is considered as a slaughterhouse waste, it is very cost-effective and has resolved the problem of expensive tissue procurement. On the other hand, as ostrich still is not the main source of humanitarian needs in the world, common and serious zoonosis disease has not been

reported, so risk of disease transmission is low.

In literature it has been documented that collagen has well biocompatibility and a number of advantages for use in tissue repair. Greater cellular interaction because of the presence of ligands in cell adhesion, ease of integration *in vivo* without any resultant adverse response, and great capacity to be co-polymerized with other biological materials to increase their bio-functionality are some of the aforementioned advantages. Schneider et al.[34], indicated that implantation of a cell-free collagen type-I gel consisted of 4.8 mg/mL rat tail collagen was associated with a high-quality tissue repair in the Goettinger minipig articular defect which can be considered equal to cell-based procedure one year postoperatively. They showed the high chondrogenic potential of collagen gel, which may be useful for *overcoming inherent disadvantages* of using in conventional cartilage tissue engineering techniques [34]. Another study evaluated a cell-free collagen type 1 matrix *in vitro* and also on nude-mouse as an animal model and their results revealed that this cell-free scaffold offered a suitable environment that leads to chondrocyte colonization and subsequent transformation of the former matrix into repair tissue [35]. Many studies have been evaluated different cell-seeded collagen matrices in cartilage repair because of their high biocompatibility [36-39]. In addition collagen gels increase chondrocyte proliferation and proteoglycan synthesis *in vitro* [40].

Ostrich acellular plantar ligament as a source of collagen in this study had milky appearance after processing and maintained its 3D structure and viscoelasticity following decellularization procedure and sterilization. Furthermore, immune response similar to what is present in porcine decellularized matrix was not observed in our decellurized ostrich collagen matrix.

The presence of fibroblasts and fibrocytes in decellularized matrix was the most interesting finding in this. Decellularization has opened new insights in trans-plantation of xenogenic tissues, that cost-effectiveness and the availability of extracellualr matrix are two significant advantages of them. The specific tissue after decellularization process is resilient and stiff, crucial to proper functioning of the fibrocartilage tissue. In Microscopic overview of tissue, presence of inflammatory mononuclear cells was reported only in experimental group II and a single rabbit of experimental group I with joint effusion.

In conclusion, the extracellular matrix of ostrich plantar ligament rich in collagen type I and proteoglycans is a complex 3D structure that as an acellular scaffold could be a potential bioactive bed in mimicking the natural tissue behavior. It can promote cell proliferation, differentiation and enhance the healing of osteochondral defects in rabbit stifle and therefore it might be a very valid option in regenerative medicine in future.

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Research Article

Effect of Parenteral Administration of Vitamin B to Goats on Performance, Lice (Phthiraptera) Infestations and Cellular Immunity [1]

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Abstract

This study was carried out on 20 of 8-10 months old Anatolian black goats which were healthy or *Linognathus africanus* infested (n=10 each). Each goat was housed individually all along the study. Five of ten louse-infested goats were administered vitamin B complex (Benefor enj) intramuscularly at a dose of 40mL/goat.Remaining goats (n=5) were not given vitamin B complex. The other goats that have not been infested with L. africanus were grouped exactly the same as above. All the animals (n=20) were first sensitized and then measured for hypersensitivity reaction in terms of cellular immune response. Infested goats given vitamin B complex were shown a decrease in *L. africanus* infestation. The other 5 number of infested goats that not given vitamin B complex were detected with an increase in the louse infestation. At the end of the trial, the higher live weight was recorded in healthy goats than those not administered vitamin B complex.

Keywords: *Linognathus africanus, Vitamin B, Performance, Cellular immunity, Goat*

Keçilerde Parenteral Verilen B Vitamininin Performans, Bit (Phthiraptera) Enfestasyonları ve Hücresel İmmünite Üzerine Etkisi

Özet

Bu araştırma 20 adet 8-10 aylık, *Linognathus africanus* ile enfeste (n=10) ve sağlıklı (n=10) erkek kıl keçilerinde yapıldı. Her bir keçi çalışma süresince bireysel bölmelerdeayrı ayrı beslendi. Bitli olan 10 adet keçinin 5 tanesinevitamin B kompleksi (Benefor enj) 40mL/keçi dozunda kas içi yolla uygulandı. Diğer 5 adet bitli hayvana vitamin B kompleksi uygulanmadı. Bitsiz 10 adet keçi ise aynı dozda vitamin B komleksi uygulanan ve uygulanmayan olmak üzere iki gruba ayrıldı. Tüm hayvanlar (n=20) önce duyarlılaştırıldı ve ardından aşırı duyarlılık reaksiyonu ile hücresel immün cevap yönünden değerlendirildi. Çalışma sonunda, bitli olup vitamin Bkompleksi uygulanan 5 adet keçide *L. africanus* enfestasyonunda önemli derecede azalma görülürken, vitamin B kompleksi uygulanmayan diğer 5 adet keçide ise bit enfestasyonunda artış gözlendi. Deneme sonunda en yüksek canlı ağırlık bit enfestasyonu olmayan ve B vitamini verilmeyen gruptaki keçilerden elde edildi.

Anahtar sözcükler: *Linognathus africanus, B Vitamini, Performans, Hücresel immünite, Keçi*

INTRODUCTION

Infestation of louse is called *pediculosis* or *phthirapteriasis* [1]. Louse infestation is the most common in winter and early spring. The rate of infestation is higher in animals with poor body structure than animals with good body composition [2-9]. Lice cause significant decline in productivity and major economic losses worldwide [1,10-13]. It has been reported that inTurkey there are 109 lice species belonging to a large number of genus that morphologically diagnosed in birds and mammals up to day. Among these mammalian lice, 20 lice species belonging to 8 genera in the Anoplura, 8 lice species belonging to 3 genera in Ischnocera and 2 lice species belonging to 2 genera in Amblycera were identified [14].

Konya is a pioneering region of national goat breeding with the number of 260.577 goats [15]. Huge economic

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loses in the yields of meat, milk and wool are caused by lice infestations in goats every year. Lice are known to cause weakening in animals by diminishing body conditions of animals. This causes great damage not only to the country's economy but also to its animal health. Millions of dollars of drugs are used every year depending on *pediculosis* throughout the World [12,16,17].

Despite not enough, studies have been conducted on lice infestations in various regions of the country [18-20]. In a study from the Region Van, a positive lose rate of 57.60% was found in examined goats. Infestations caused by the species of *Bovicola caprae*, *B. crassipes*, *B. limbata*, *L. africanus*, *L. stenopsis* were reported as being 52.51%, 7.56%, 6.39%, 17.81%, 15.71%, respectively. Additionally, it was emphasized that the infestation was more common in winter months with the rate of 40.16%. By some other studies carried out in Turkey,the rate of lice infestations in goats was found to vary between 57.60% and 82.10% [2,21,22]. The lice on sheep can cause itching because of intense irritation, secondary bristle and wool lossby destroying nerve endigs in the skin. However, small ruminants carrying few lice do not show clinical signs. This parasite also causes restlessness, weak condition, low efficiency and leads to friction and thickening of the skin caused by the itching [16,17]. Blood-sucking lice may penetrate through the mouth organelles and bleed. Serum leaked from wound cause the wool to become dirty. These wounds attract flies and predispose the infested goats to myiasis [23,24].

The purpose of this research is to measure relationship between the lice infestation, cellular immune response and performance in goats whichvitamin B complex were administered parenterally and thus indirectly determine the effect of vitamin B complex on host resistance to infestation.

MATERIAL and METHODS

The ethic committee document related to this study was taken from the Ethics Committee of the Experimental Animal Production and Research Center of the Veterinary Faculty of Selcuk University with the number of Decision 2016/16.

This research was carried out between 21 January and 21 March 2016. The research material consisted of 20 goats obtained from a private flock in Konya region and from which lice collected. All the goats from the same breed (hair goat) and gentle (male) were placed to individual compartments one by one in sheep unit of Prof. Dr. Hümeyra Özgen Research and Application Farm, Faculty of Veterinary Medicine, Selcuk University.

Firstly, animals were weighed in the experiment, then were divided into two groups in which each group has 10 goats. Infested animals were put into the first group. The first group were further divided into two groups, consisting of one given the vitamin B complex via parenteral route (n=5), and the other (n=5) not given vitamin B complex. The same trial procedure was also applied to the second group including 10 animals.

Parasitological Examination

Flotation, Sedimentation and Barmen-Wetzeltechniques were applied to all animals before starting to trial. Positive animals for the presence of other parasites were only included in the study provided that treatment completed.

At first, 10 goats infested with lice were macroscopically examined. Later, the hairs on the back, neck, tail and foot parts were collected by scraping into a plastic cuvette with an appropiate comb. White-barrel-shaped eggs were identified by appearing on the bristles of dark hairy goats. Identification of lice, nymphs and ovaries was performed by macroscopic and microscopic examinations [16]. In addition, the hair in the form of pinches was taken from these areas by using scissors. These bristles were put in nylon bags separately and the protocol numbers were noted.

Afterwards all samples were brought to the laboratory of the Parasitology, Veterinary Faculty of Selcuk University. Lice and eggs fallen into a white plastic cuvette were collected and taken into bottles containing 70% alcohol. The lice were fixed on slides with Canadian Balsam followed by a treatmentin 10% KOH to make them transparent.Lice were identified by examining in binocular light microscopein accordance with the relevant literature. Data for performance, condition and parasite presence were evaluated in goat groups with and without vitamin B complex.

Vitamin B complex usedin this study includes100 mg Thiamin HCl (Vit. B), 5 mg Riboflavin (Vit. B2), 10 mg Pridoxin HCl (Vit. B6), 100 mcg Cyanocobalamin (Vit B]2), 100 mg Niasin and 10 mg D-Panthenol per 1 mL. Five goats in first group infested with lice and other 5 in second group without infestation were injected with vitamin B complex intramuscularly (dose 5 mL/goat/week; 8 times, totally 40 ml/goat) and performance values were evaluated.

Determination of Nutrition Performance

At the beginning of the study,all the goats, taken to the experimentwere weighed usingan electronic weighing machine on an empty stomachin the morning. During the experiment, weights were measured four times for two months, once an every two weeks. The live weights as well as live weight gains were determined.

Individual feeding program was applied in the trial. Every goat was nourished with 4% of its weight concentrated feed. Roughage was given to the animals as *ad libitum*.

Immunologicalexamination

For the immune measurements, all the goats were first sensitized and then measurements for delayed type allergy

reactionwere performed. For this purpose,a method were used with modifications [25]. On the 50th day of the experiment, one side of necks of all the goats were shaved 15 cm x 15 cm in dimensions and topical application of 0.2 mL 90% (in alcohol) DMSO (Dimethylsulfoxide; Sigma D2650) was applied to the center of this field. Three minutes later, the same area was again topically applied with 0.2 mL of 5% DNCB (1-Chloro-2,4-dinitrobenzene; Sigma-Aldrich-237329) (wt/vol solvent alcohol). This sensitization was repeated in the same manner and to the same spots after 2 days (the 52nd day of the experiment).

The skin thicknesses of the two points (10 cm apart from each other) in sensitized area were measuredat 57th day of trial. 0.1 mL as control to first spot (4:1acetone:oil) of 0.1% acetone (Sigma Aldrich 320110) + olive oil (Sigma C8392) and 5% DNCB as test to second spot were injected. Sensitivity determination was recorded by measuring skin thickness at 24 h.

Statistical Analysis

Anderson Darling normality test was first applied to analyze distribution of the samples then Paired sample t test was used. P values of weight gain and skin thickness for all groups less than 0.05 (P<0,05) were considered as statistically significant.

RESULTS

By the parasitological examination of the lice from infested group, all the lice collected from all parts of the body were found to be *L. africanus*, a blood-sucking louse species *(Fig.1, Fig. 2)*. In the second subgroup of infested group and the goats from which the vitamin B complex has not been applied to, lice infestation was observed to increase at the end of the study. At the beginning of the work, the average number of lice counted was 25 in the 10 cm^2 area, while at the end it was 50 from the same area.

Significant losses of wool amounts and levels of irritations were observed due to severe infestations in goats without vitamin B complex of the infested group.

Skin thicknesses were recorded before and after sensitization as well as following testing by using digital calipers *(Fig. 3)*.

Based on groups, degrees of hypersensitivity reactions and values measured afterwards are shown in *Table 1*. When skin thickness of control site compared with

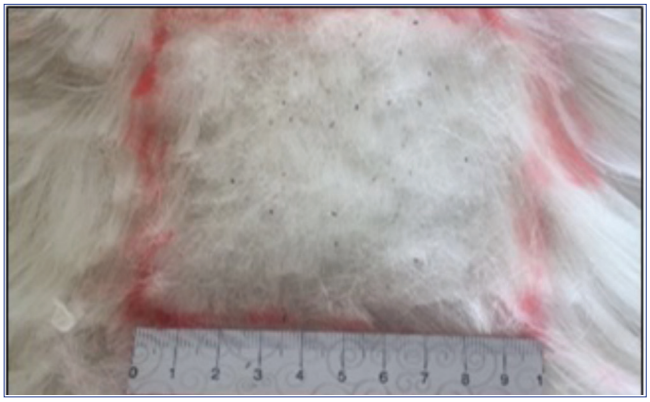

Fig 1. *Linognathus africanus*

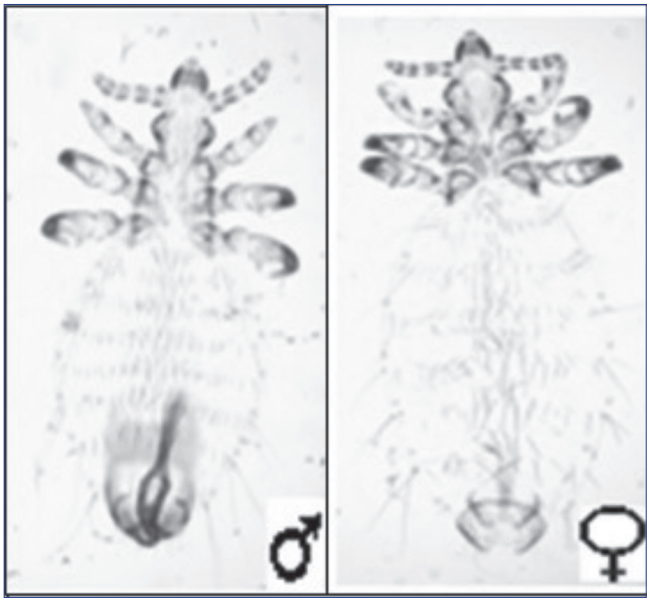

Fig 2. *Linognathus africanus*, original, microscopic appearence

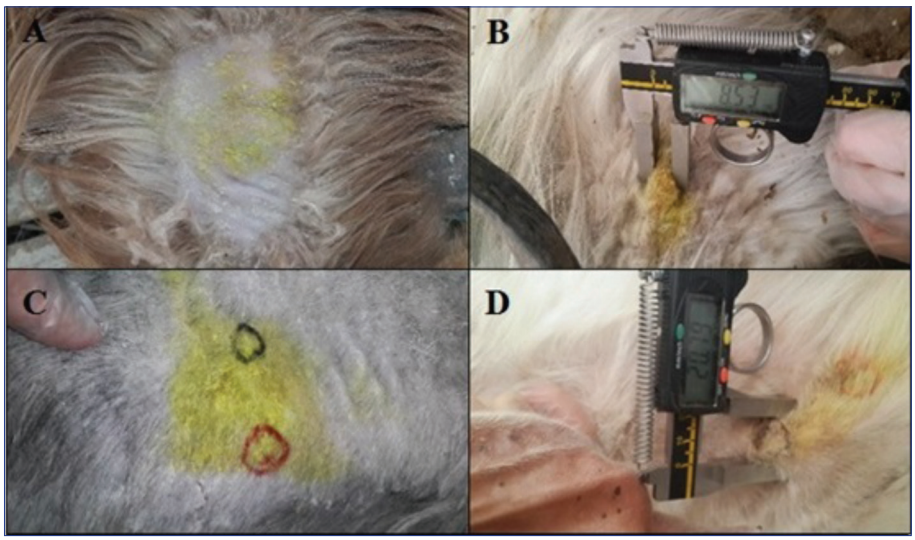

Fig 3. Normal, sensitized and test skin thickness measurements, A- Shaved skin, skin ready for sensitization, B- Sensitized skin, C- Control and test injections, D- Test reading at 24th h

Tablo 1. *Effect of vitamin B application on hypersensitivity reaction in healthy and lice-infested goats*

Skin Thickness (ST)		Groups*			
		1	2	3	4
Before sensitization ST		3.89±0.63	4.13±0.45	3.49±0.36	3.78±0.75
Before ID application ST		6.71±0.77	8.55±1.09	7.62±2.78	8.31±2.36
24 h after ID application ST	Control ST	9.72±1.80	9.89±1.79	7.55±2.02	7.85±1.58
	Test ST	17.98±1.93	19.51±2.26	19.89±1.96	21.43±4.19
	Test ST-Control ST	8.26±2.41	9.62±1.94	12.34±1.77	13.58±4.02

** **1:** Lice infested, vitamin B complex applied, **2:** Lice infested, vitamin B complex unapplied, **3:** Non-lice infestation, vitamin B complex applied, **4:** Non-lice infestation, vitamin B complex unapplied*

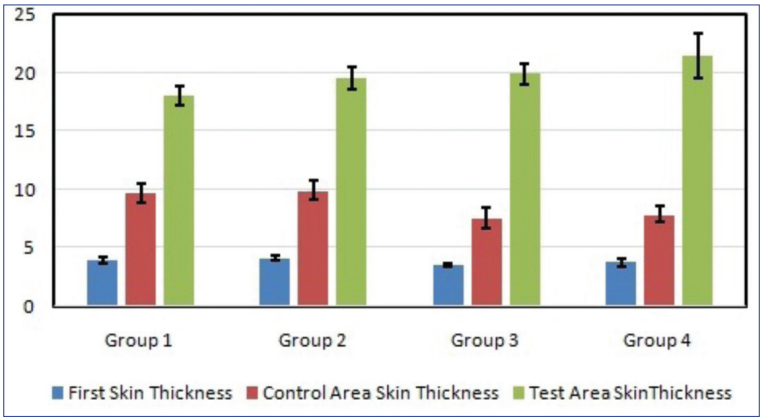

Fig 4. Statistically comparison of each group skin thickness

Fig 5. Statistically comparison of each group at the beginning and end of the study

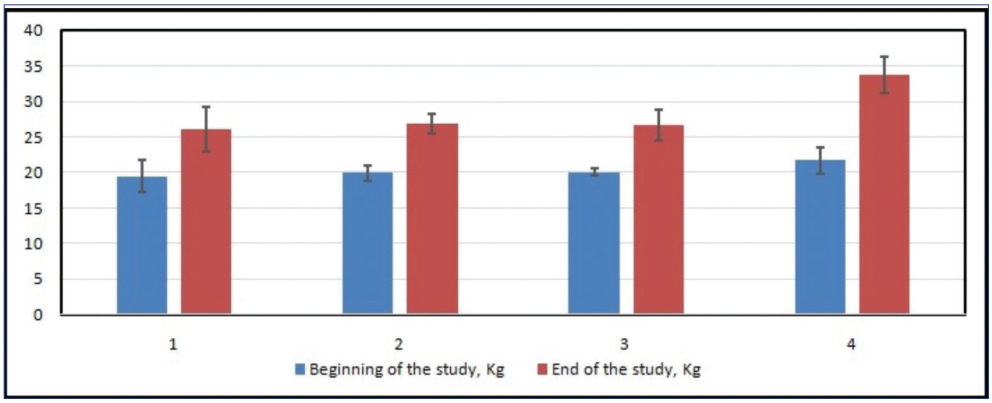

Table 2. *The live weight averages obtained at the end of the study*

Days	Lousy		Not Lousy	
	1 (Vitamined, Kg)	2 (Without Vitamin, Kg)	3 (Vitamined, Kg)	4 (Without Vitamin, Kg)
26.01.2016	19.50	19.92	20.02	21.68
09.02.2016	21.62	20.14	22.22	24.20
23.02.2016	23.48	22.80	23.66	28.44
12.03.2016	24.16	24.38	24.70	29.26
23.03.2016	26.08	26.86	26.72	33.72
Average	22.97	22.82	23.46	27.46

those of first (for sensitization) or second (for trial) injections all determined to be statistically significant (P<0.05). However, no differences were seen between the groups with or without louse infestations (P>0.05). This is also the same case for the groups of vitamine supplemantation (P>0.05) *(Fig. 4)*.

The measurements of nutrition performances are presented in *Table 2* and *Fig. 5*.

DISCUSSION

Single-sex goats were used and no gender comparisons were made in this study, sincetype of goat genderhas been reported as not one of the determining factors for spreading of lice [2,9,22,26,27].

L. africanus toxins cause paralysis in hair roots and lead to alopecia resulting in development of suppurative lesions by disruping skin integrity. However, no dermatitis occurrence was not detected in this study.

Two sides of neck are the body regions recommended for the measurement of cellular immunity using allergic skin tests [28]. In addition, as the primary region where the lice are localized on the host and in cases where the number of lice is low, both sides are suggested as suitable sides. In this trial, these regions were preferred for intra-dermal injection.

The delayed type of hypersensitivity reactions are used to assess cellular immune response. IVth type of hypersensitivity reactions is an *in vivo* test and has found clinical use in animals [29-31]. It has long been known that the vitamin B complex is one of the major factors in the development of resistance to ectoparasites in animals [32,33]. Various studies have been made to explain this effect of vitamin B [34,35]. Atpresent study, the development of the hypersensitivity reaction induced by DNCB was examined in both healthy and *L. africanus* infected goats. The adaptation of the method to the goats was clinically determined to develop a hypersensitivity reaction in the goats. Of vitamin B complex applied goats, the skin thickness of *L. africanus* infested goats (17.98±1.93 mm) was found to be lower than that measured by healthy goats (19.89±1.96 mm) (P>0.05).

A similarity was the case for the groups which did not receive vitamin B complex (19.51±2.26, 21.43±4.19 mm) (P>0.05) *(Fig. 4)*. These findings may led to the conclusion that *L. africanus* infestation had an effect on suppressing the development of cellular immune response. On the other hand, with the planning of new trials with more goats and different types of lice infestations in the future, it would then be possible to determine statistically meaningful conclusions.

It is known that various toxins of blood-sucking lice cause local paralysis and alopeciaby reaching the hair roots [16]. Although the *L. africanus* lice are not deeply established on surface skin, it is suggested that migration of cells which affect the skin and subcutaneous tissuescontributing to the formation of cellular granulomas (primarily T cells) to the region was inhibited by lice.Lice may cause this effect either locally via their secretions on wool to reach the skin or by a systemic wayfollowing absorption of secretions leading to T cell differentiation or even by both ways.

At the beginning of the trial, live weights were determined as 19.50, 19.92, 20.02 and 21.68 kg in the groups. At the end of the experiment, the average live weights obtained from the same groups were as 22.97, 22.82, 23.46 and 27.46 kg, respectively. At this time of the study, the highest live weight was obtained from a group which no lice infestation occurred and no vitamin applied (P<0.05). In this particular group, the increase in live weight can be due to the absence of lice-infestation and injection stress.

In the first group of the infested animals applied with vitamin B complex, there was a decrease in the number of lice at the end of the experiment while the lice had already been removed in the goat number 3. This was inter preted as the possibility that the application of the vitamin B complex would likely remove the stress factors in the goat and reduce the intensity of the lice infestation.

It was concluded that the DNCB hypersensitivity reaction can be used when cellular immunity measurementis needed in *L. africanus*infested goats, but this should be interpreted with caution and the recommendation of applying alternative tests or methods should be taken into account. In addition, when the vitamin B complex was administered in dose of 40 mL/goat by parenteral route (IM) in *L. africanus* infested goats, cellular immune system of the host and development of lice are negatively influenced. Vitamin B complex in goat breedingis concluded to be recomended parenterally at the dose used in this study.

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Research Article

Milk Production, Body Condition Score and Metabolic Parameters at the Peak of Lactation as Risk Factors for Chronic Lameness in Dairy Cows

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Abstract

The objective of this case-control study was to examine the milk production, body condition score and metabolic profiles at the peak of lactation as risk factors for chronic lameness present in cows during the first six months of lactation. A total of 100 Holstein-Friesian cows were enrolled in the study, out of which 30 were classified as lame (a locomotion score (LS) >3 according to 4 of 5 monthly measurements) and 70 exhibited no signs of clinical lameness (LS ≤3). The cows with milk production above 30.9 kg/day showed a higher risk for chronic lameness (OR=1.9, a 95% confidence interval (CI)=1.2-4.5), and the risk peaked at a milk production of 39.1 kg/day (OR=4.8, CI=2.1-8.8). A suboptimal BCS <2.5 or >3 at the peak of lactation increased the probability of lameness in the exposed group of cows (OR=4.9, CI=2.2-8). The cows were exposed to higher risk factors for chronic lameness under the following circumstances: BHBA>0.8 mmol/L (OR=3.5, CI=1.2-9.9), LDH>1900 IU/L (OR=2.3, CI=1.4-5.9), and triglycerides>0.22 mmol/L (OR=2.2, CI=1.5-2.9). The interaction between two risk factors showed a higher OR for developing chronic lameness in comparison with a single-factor exposure: BCS × BHBA (OR=22, CI=1.2-1000), BCS×LDH (OR=33, CI=1.8-1400), milk production × BHBA (OR=18.24, CI=2.1-433) and milk production×LDH (OR=14.2, CI=1.5-327). Lameness cows exposed to risk factor showed un-significant lower concentration of urea, ALP and higher concentration of cholesterol and triglycerides probably due to energy and protein malnutrition. Glucose concentrations were similar in healthy and lameness cows. Same mean concentration of glucose was maintained with decrease of LDH activity in healthy cows, but with increase LDH in lame cows, probably due to high glycolysis. Metabolic adaptation in pick of lactation and its relation with lameness need further research.

Keywords: *Cows, Lameness, BCS, Milk production, Metabolic parameters*

Sütçü İneklerin Laktasyon Pikinde Kronik Topallığın Risk Faktörleri Olarak Süt Üretimi, Vücut Kondisyon Skoru ve Metabolik Parametreler

Özet

Bu olgu-kontrol çalışmasının amacı ineklerde laktasyonun pik yaptığı ilk altı ayı süresince kronik topallığın risk faktörleri olarak süt üretimi, vücut kondisyon skoru ve metabolik parametrelerin incelenmesidir. Çalışmada toplam 100 adet Holstein-Friesian inek kullanıldı. Bu ineklerin 30'u topal (5 aylık ölçümlerin 4'üne belirlenen lokomosyon skoru (LS) >3'e göre) olarak sınıflandırılırken 70'i klinik olarak topallık göstermemekteydi (LS ≤3). 30.9 kg/gün'den daha fazla süt üretimine sahip olan inekler topallık için daha yüksek risk gösterirken (OR=1.9, 95% güvenlik aralığı (CI)=1.2-4.5) süt üretimi 39.1 kg/gün'de risk pik yaptı (OR=4.8, CI=2.1-8.8). Laktasyon pikinde suboptimal BCS <2.5 veya >3 maruz kalan ineklerde topallık olasılığını artırdı (OR=4.9, CI=2.2-8). İnekler belirtilen şartlarda kronik topallık için daha fazla risk faktörü gösterdiler: BHBA>0.8 mmol/L (OR=3.5, CI=1.2-9.9), LDH>1900 IU/L (OR=2.3, CI=1.4-5.9) ve trigliseridler>0.22 mmol/L (OR=2.2, CI=1.5-2.9). İki risk faktörünün etkileşmesi tek faktöre maruz kalanlarla karşılaştırıldığında kronik topallığın gelişmesi için daha yüksek OR gösterdi BCS × BHBA (OR=22, CI=1.2-1000), BCS×LDH (OR=33, CI=1.8-1400), süt üretimi × BHBA (OR=18.24, CI=2.1-433) ve süt üretimi×LDH (OR=14.2, CI=1.5-327). Risk faktörlerine maruz kalan topal inekler muhtemelen enerji ve protein dengesizliğine bağlı olarak anlamsız derece daha düşük üre konsantrasyonuna, ALP ve daha yüksek kolesterol ve trigiliserid konsantrasyonuna sahipti. Topal ve sağlıklı ineklerde glikoz konsantrasyonu benzerlik göstermekteydi. Sağlıklı ineklerde düşük LDH aktivitesi ile birlikte benzer ortalama glikoz konsantrasyonu sağlanırken topal ineklerde artmış LDH mevcuttu. Bu durum muhtemelen yüksek glikolizise bağlıydı. Laktasyonun pik döneminde metabolik adaptasyon ve topallık ile ilişkisi daha fazla çalışmaya ihtiyaç olduğunu göstermektedir.

Anahtar sözcükler: *İnek, Topallık, BCS, Süt üretimi, Metabolik parametreler*

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INTRODUCTION

After infertility and mastitis, lameness is the third most common reason for involuntary culling of dairy cows [1]. Lameness is a painful condition which adversely affects longevity, fertility and milk production. Moreover, lameness has been classified as the most representative animal-based indicator of compromised welfare in dairy cattle [2-5]. The etiology of lameness is complex and multifactorial. The factors influencing lameness include the following: housing conditions, social interactions/influence, stages of lactation, pregnancy or calving and high yielding [6-9].

In a cross-sectional study, Bicalho et al.[10] found a greater risk of lameness and claw horn disruption lesions developing in cows with lower body condition scores (BCS) and lower digital cushion thickness (DCT). Cows with a low BCS ≤2.5 (on a scale from 0 to 5) are more likely to be treated for lameness in 0 to 2 or >2 to 4 months following such a score [11]. This result supports the hypothesis that a low BCS is correlated with reduced digital cushion thickness, which can be associated with claw horn disruption lesions [12]. Our previous study showed that BCS assessment is a suitably strong predictor of lameness in fat cows, but in normal or thin cows, lameness prediction required the combination of both BCS and ultrasound measurement of subcutaneous fat deposit [13].

Zhang et al.[14] demonstrated higher values of inflammatory cytokines, beta-hydroxybutyrate (BHBA) and nonesterified fatty acid (NEFA) in the transition period prior to a clinical diagnosis of lameness in early lactation. Some other metabolic diseases, which become clinically manifest later in lactation (e.g. laminitis), can be traced back to metabolic insults that occurred during early lactation [15]. Days in milk (DIM) and lameness are significantly interrelated, thus lameness is most common in the first 100 DIM [16]. A relation between the metabolic status, milk production, a BCS and lameness in later lactation has not been argued in the literature. The purpose of the present study is to examine the milk production, body condition scores and metabolic profiles, as well as their mutual interaction, at the peak of lactation as risk factors for chronic lameness developing during the subsequent lactation period.

MATERIAL and METHODS

Animals

A total of 100 Holstein-Friesian cows were enrolled in a case-control study from the group with a high prevalence of lameness. The cows were milked twice a day and fed a total mixed ration, according to NRC recommendations [17]: Hay 4.5 kg; Concentrate mixture 9.95 kg; Straw 0.30 kg; Sugar beet 0.7 kg; Haylage, 7.0 kg; Sylage 20 kg; Provided DM 21.5 kg; Crude protein 3440 g; Digestible crude protein 2250 g; Percentage of protein 16.5 kg/SM; Total energy 144; NEL 6.65(MJ/kg DM); CF 17.2%; ADF 21.7%; NDF 36%; Ca 0.85%; P 0.50%; NaCl 0.30%.

Locomotion Score

The locomotion score was assessed using a 1-5 lameness scoring system (LS) as argued by Sprecher et al.[18]. This system uses a 1-5 numerical scale where a score of 1 denotes sound locomotion, 2 and 3 indicates clinically unimportant changes in gait, and a score of 4 to 5 describes different severities of clinical lameness. In the present study, the cows were described as lame (a locomotion score ≥4) or non-lame (a locomotion score ≤3). Lameness was indicated by a LS >3 from the 2nd to 6th month of lactation according at least to 4 of 5 monthly measurements.

Milk Production

The average milk yield in the previous lactation was 7794±1210 kg/305 days. All the cows were supervised daily by a veterinarian. Milk production was recorded by a farm software program. In this research, the peak of milk production was used as a risk factor. Cows reach the peak of milk production between the sixth and eighth week of lactation.

Body Condition Score

A 1-5 BCS system [19], which incorporates a numerical scale where thin animals receive lower scores and fat animals higher scores, was employed in the study. All the cows were scored at the end of a dry period and on a monthly basis during the first six months of lactation.

Metabolic Profile

Blood samples were obtained from the *v. jugularis*, using sodium heparin vacutainers (Becton, Dickinson & Co, GB), during the second month of lactation, when cows achieve peak milk production. The samples were stored on ice (max. 2 h) until centrifugation (2000 g. for 20 min. at 4°C), after which plasma was harvested. The plasma samples were stored at -20°C until analysis. Concentration of plasma level of glucose, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total cholesterol, triglycerides, urea were measured by colorimetric reaction (Accent 200, P.Z. Cormay, S.A.) and blood level of BHBA was measured by ketone meter (FreeStyle Optium - Abbot Germany).

Statistics and Design

The milk production, LS and BCS were recorded at the peak of lactation, and blood samples were taken for the measurement of metabolic parameters. In order to classify the cows, locomotion scores were recorded throughout the first six months of lactation. At the end of month 6 of lactation, the cows were divided into two groups, lame and non-lame cows, depending on the LS observed during lactation. A linear regression line for the LS from months 1 to 6 of lactation will be displayed for each cow. The data

are presented in 2x2 tables: lame cows, non-lame cows, cows with parameter values lower than the cut-off value, and cows with parameter values higher than the cut-off value. An odds ratio (OR) was calculated for each value of milk production, BCS and metabolic parameters in order to find the optimal cut-off point for those continuous parameters in binary outcomes. An OR was calculated was calculated between groups with normal BCS (2.5-3.0) and suboptimal BCS (BCS <2.5 or >3). Thereafter, the cows were divided into two groups: lame and healthy cows. Each group was divided into two groups: group with risk factor (significant parameters above the risk cut-off value) and group without risk factor. The difference between the mean values of measured parameters was confirmed using a t-test. Regression between glucose and LDH was examined in order to detect influence of glycolysis to maintaining glucose level in blood. For statistical purposes, the Analyse-it program (v3.90.70.) for Microsoft Excel was used.

RESULTS

A total of 30 cows were classified as lame (a locomotion score (LS) >3 according to 4 of 5 monthly measurements), whereas 70 exhibited no signs of clinical lameness (LS ≤3). The regression lines for the LS from months 2 to 6 of lactation are shown in *Fig. 1* with a clear distinction between lame and non-lame cows.

The cows with milk production above 30.9 kg/day showed a higher risk for chronic lameness (OR=1.9, a 95% confidence interval (CI)=1.2-4.5), and the risk peaked at a milk production of 39.1 kg/day (OR=4.8, CI=2.1-8.8). A suboptimal BCS <2.5 or >3 at the peak of lactation increased the probability of lameness in the exposed group of cows (OR=4.9, CI=2.2-8.0). The cows were exposed to higher risk factors for chronic lameness under the following circumstances: BHBA >0.8 mmol/L (OR=3.5, CI=1.2-9.9), LDH>1900 IU/L (OR=2.3, CI=1.4-5.9), and triglycerides >0.22 mmol/L (OR=2.2, CI=1.5-2.9). The ALP, cholesterol and urea concentrations showed significant ORs at higher values, i.e. the highest ORs at the most extreme parameter values. The glucose concentrations did not indicate the cut-off value with significant ORs (all the CIs contain 1). The maximum odds ratios (maxOR) for BHBA and LDH concentrations were obtained using the 97.5th percentile cut-off value: BHBA >0.9 mmol (maxOR=20) and LDH >2300 IU/L (maxOR=12.6). These cut-off values are only to be used for risk assessment on account of a very small number of animals with such parameter values in the blood. The interaction between two risk factors showed a higher OR for developing chronic lameness in comparison with a single-factor exposure: BCS×BHBA (OR=22, CI=1.2-1000), BCS×LDH (OR=33, CI=1.8-1400), milk production×BHBA (OR=18.24, CI=2.1-433), and milk production×LDH (OR=14.2, CI=1.5-327) *(Fig. 2)*.

When the cows are classified in the two categories (lame cows and cows with no signs of lameness), we conclude that the body condition, milk production, LDH and BHB are the most sensitive indicators of lameness, because their values are significantly changed in lame cows compared to the healthy cows and in healthy cows exposed to risk factors compared to the entirely negative control (healthy cows with no risk factors). On the other hand, there is a deviation in cholesterol, triglycerides, urea and ALP in a group of cows which is under a risk factor with lameness compared to the negative control *(Table 1)*.

The glucose level was not statistically significantly different in the examined groups of cows. LDH activity and glucose concentration exhibit polynomial quadratic relation. In the lame cows, LDH activity increases from the lowest values up and peaks at the mean values of the glucose, and there on the LDH activity decreases and this relationship is statistically significant ($LDH=-634.5 \times GLU^2 + 4391.7 \times GLU - 5609.1$; $R=0.365$; $P<0.05$). However, in healthy cows LDH and glucose demonstrate an inverse relation compared to the lame cows, so that the lowest LDH activity is reached at the mean values of the glucose, and its activity increases with increasing and decreasing values of glucose, but this relationship was not statistically significant ($LDH=196.74 \times GLU^2 - 1351.7 \times GLU + 4019.1$; $R= 0.19$; NS) *(Fig. 3)*.

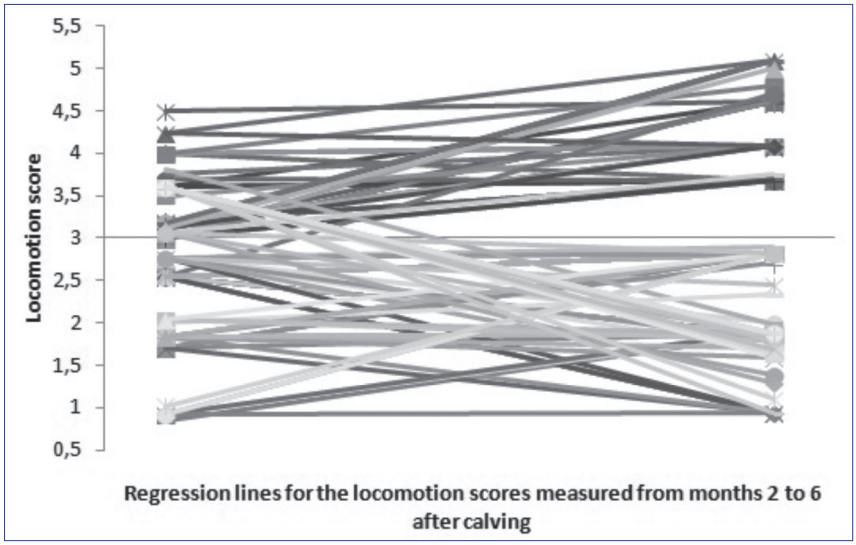

Fig 1. Lameness in dairy cows from months 2 to 6 of lactation

DISCUSSION

Milk production is an important risk factor for lameness. High milk production can cause lameness in

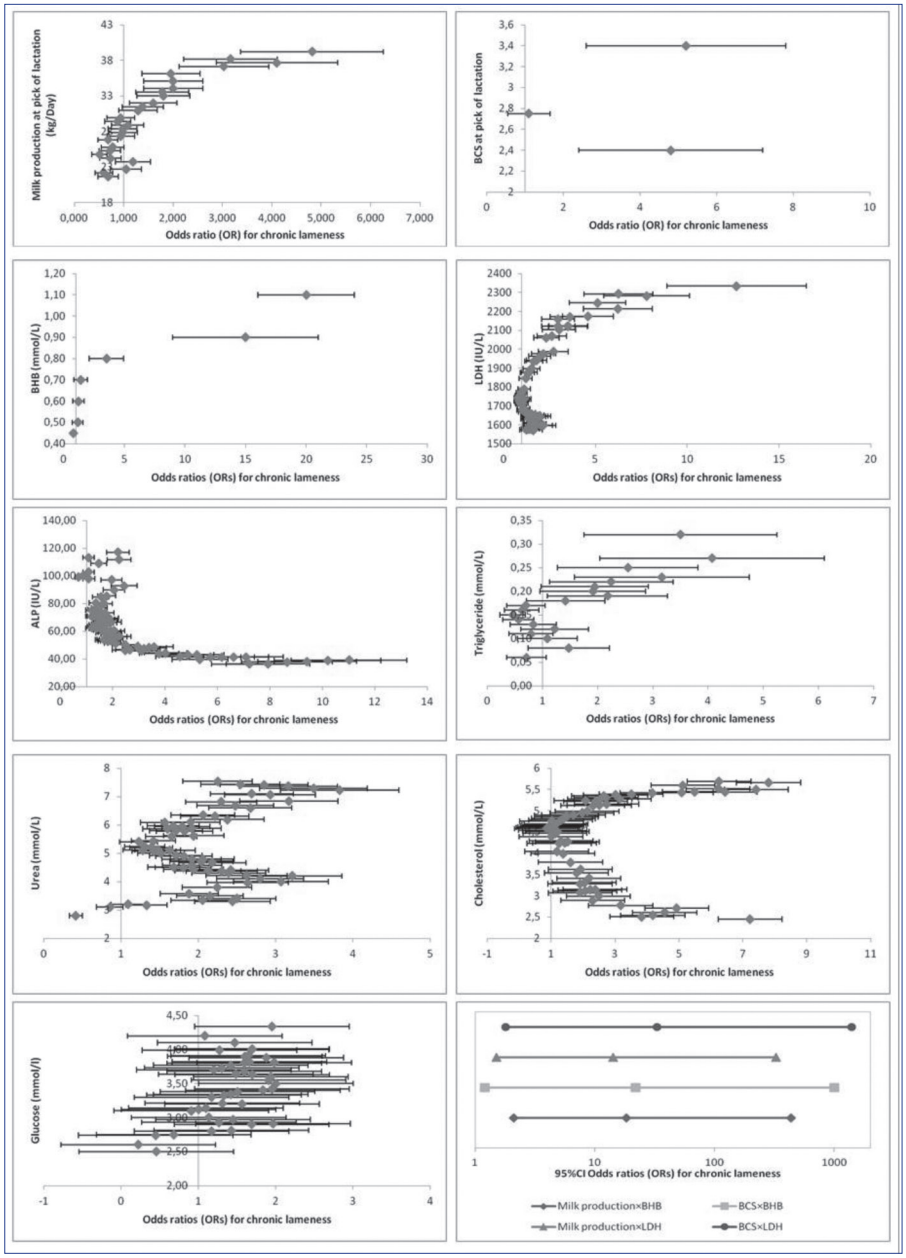

Fig 2. Odds ratios (ORs) for chronic lameness in the function of milk production, BCS, metabolic parameters in the blood and their combination

cows [20]. Bicalho et al.[9] showed that lame cows produced 3.02±0.23 kg more milk prior to lameness in comparison with the control group. Using body fat reserves for milk production entails mobilizing fat from many tissues, including the digital cushion [8]. Other studies have also revealed a connection between a lower BCS and lameness [6,10,12,21], as well as a connection between a higher BCS and a higher LS [22]. In a prospective longitudinal study, Randall et al.[23] found that a low BCS predisposes cows to lameness and that maintaining a BCS ≥2.5 is optimal for reducing the risk of a lameness event.

High concentrations of BHBA and LDH in cows at the peak of lactation indicate high odds of chronic lameness. According to Reist et al.[24] there is a strong correlation between the BHBA concentration and the energy balance. Zhang et al.[14] found higher concentrations of NEFA and BHBA in the serum within the first week of diagnosis and at four weeks postpartum. The concentration of BHBA in the aforementioned study was about 0.85 mmol/L within the first week of diagnosis, which is 0.05 mmol/L higher than our cut-off value. Cows with a higher concentration of BHBA in early lactation are at great risk for developing other diseases such as abomasal displacement and ketosis [15,25]. Oetzel [26] categorizes hyperketonemia (due to high milk production) as type 1 ketosis which occurs in cows 3 to 6 weeks post-calving on account of the highest milk energy outflow at this time. These cows usually do not have calving difficulties and a negative energy balance in early lactation, but underfeeding (in relation to the milk energy output) leads to the negative energy balance with a lipid mobilization and higher ketosis. Cows with high locomotion scores have a lower dry matter intake [27]. Type 1 ketosis and a decreased dry matter intake in lame cows could account for higher odds of lameness when cows are exposed to two risk factors (high milk production or a low BCS with a high BHBA concentration) in comparison with a single-factor exposure. Consequently, a low energy balance at the peak of lactation can predict the development of lameness during a longer period of lactation.

A high LDH concentration is an important risk factor for lameness. A high LDH concentration could be a consequence of a higher BHBA concentration affecting the liver. Furthermore, cows stand longer during lameness with consequently higher muscle loads. High muscle loads lead to high lactate production, which is reduced by LDH in muscle tissues. LDH is not an organ-specific enzyme as it is found in large concentrations in the muscles, heart, kidneys and liver, and is released during acute inflammations of those organs. Moreover, the activities of LDH in the blood are closely correlated with the degree of fatty infiltration of the liver [28]. The LDH cut-off value in

Table 1. *Metabolic profile, body condition scores and milk production in lame cows with different numbers of risk factors, and in healthy cows*

Parameter	Lame Cows with Risk Factor	Lame Cows without Risk Factor	Healthy Cows with Risk Factor	Healthy Cows without Risk Factor
Glucose (mmol/L)	3.52±0.51^{a}	3.6±0.42^{a}	3.5±0.48^{a}	3.41±0.42^{a}
LDH (IU/L)	2150±224.8^{A}	1812±192.3^{B}	1725±101.5^{B}	1657.82±231.7^{B}
ALP (IU/L)	49.1±18.3^{a}	61.3±11.5^{a}	51.4±18.1^{a}	60.33±21.2^{a}
Urea (mmol/L)	4.49±1.12^{A}	7.2±0.91^{B}	4.5±0.95^{B}	6,51±1.7^{A}
Cholesterol (mmol/L)	4.68±1.62^{a}	4.51±1.5^{a}	4.11±1.3^{a}	4.01±0.98^{a}
Triglycerides (mmol/L)	0.21±0.09^{a}	0.19±0.08^{b}	0.17±0.09^{c}	0.18±0.1^{d}
BHBA (mmol/L)	1.2±0.11^{A}	1.1±0.09^{A}	0.96±0.1^{B}	0.61±0.11^{B}
Suboptimal BCS (%)	90^{A}	0	5^{B}	5^{B}
Milk production (L)	33.34±5.35^{A}	30.5±2.9^{B}	29.87±3.1^{B}	26.90±3,14^{B}

Different superscript means significant difference at level: ^{a,b,c} P<0.05; ^{A,B,C} P<0.01

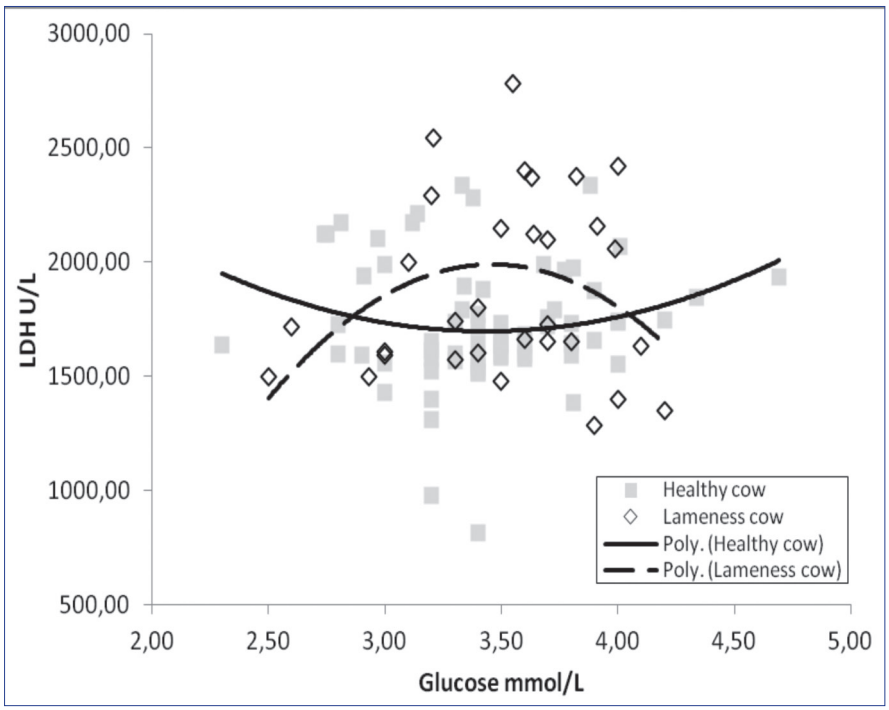

Fig 3. Regression lines between glucose concentration and LDH in healthy and lame cows

this research is approximate to the mean value of LDH in healthy cows during early lactation [29].

A high risk for lameness occurs when the concentrations of cholesterol and urea are either at high or low levels. The cholesterol concentration indirectly reveals the ability of the liver to produce VLDLs (very low density lipoproteins) and decreased cholesterol and triglycerides are sign of fatty liver [30,31]. Gross et al.[32] showed that change in lipid metabolic parameters during starvation is depends on lactation stage. During early lactation we have decrease of cholesterol and triglyceride concentration, but in later period concentration of this parameters increase during feed restriction. Yeruham et al.[33] found lower concentrations of cholesterol and urea, and higher blood activities of ALP and LDH in heifers associated with an excessive carbohydrate intake. Decrease concentration of urea could be in relation with malnutrition in proteins and lower ALP could be sign in low protein intake or hypophosphatemia.

LDH and BHBA are metabolites which together with milk production and body condition score help in the assessment of risk for the development of lameness. Lame cows have either prolonged time standing or prolonged time lying when compared to healthy cows [34]. These changes can lead to changes in glucose metabolism in muscle and the result is an increase in the production of lactate. The later was confirmed by Zhang et al.[14] who found elevated plasma lactate in lame cows. On the other hand, lactate is always the end product of cell glycolysis and is accompanied by high activity of LDH [35]. Lactate is then transported to the liver through the bloodstream where in the process of gluconeo-genesis it is again transformed into glucose that flows into the muscles and other tissues, a process known as the Cori cycle. However, in dairy cows the udder is the preferred site to use glucose, so it is possible that the produced glucose goes to the udder where it is used directly. As a result, the muscle tissue continues to utilize glycolysis that further increases the lactate. In support of this speculation speaks the fact that the relation of LDH and glucose is significantly different and shows the inverse relationship in healthy and lame cow.

In cows that have BHBA values higher than 0.8 mmol/L, the

risk for lameness increases. Elevated levels of BHBA indicate negative energy balance and the use of fat for energy. Although the changes in the BHBA value is small compared to the healthy controls, it is known that BHBA increases in cows in late lactation as a result of underfeeding, but not as much as in early lactation [36]. Also, in the late lactation there is no rapid decline of glucose as in early lactation [36], which renders glucose not a significant risk factor.

In conclusion, this study demonstrated that high milk production, a low or high BCS, a high BHBA concentration and a high LDH concentration are important risk factors for developing chronic lameness. Furthermore, it was demonstrated that the interaction between milk production, a BCS and metabolic parameters (high BHBA and LDH concentrations) poses a higher risk for developing chronic lameness in dairy cows in comparison with the exposure to a single risk factor. Metabolic adaptation in pick of lactation and its relation with lameness need further research.

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Research Article

Effect of Supplementary Liquid Colostrum on Growth Performance, Carcass Yield, Ceruloplasmin, Sialic Acid and Some Antioxidant Levels in Quails

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Abstract

This study was conducted to determine the effects of supplementary liquid-colostrum (LiqC) on growth performance, carcass yield, ceruloplasmin, sialic acid, and antioxidant levels in growing quails. In this study, a total of 90 ten-days-old mixed-sexed Japanese quail chicks were used. Quails were divided randomly into 3 groups. Chicks were fed one of three diets: basal diet or basal diet supplemented with 2% or 4% liquid colostrum. Birds were exposed to a 14L:10D illumination cycle for 32 (days of 10-42) days. When the effects of dietary liquid colostrum supplementation on performance were examined, values of final body weight, live weight gain, cumulative feed intake, feed efficiency, cold carcass weight and cold carcass yield in quails were higher in the trial groups compared to control group ($P<0.05$); but organ weights were not affected ($P>0.05$). Levels of liver malondialdehyde (MDA), ceruloplasmin, and sialic acid were lower, but levels of superoxide dismutase (SOD) were higher in trial groups ($P<0.05$), and there was no effect on total antioxidant status (TAS) levels ($P>0.05$). Serum MDA levels were lower and SOD levels were higher in liquid colostrum supplemented groups ($P<0.05$), although a numerical increase was found in TAS levels, no statistically important difference was found in trial groups. In conclusion, the oxidative, transport and slaughter stresses can be attenuated by liquid colostrum supplementation at 4% of diets in quail.

Keywords: *Colostrums, Growth performance, Ceruloplasmin, Sialic acid, Quail*

Bıldırcınlarda Sıvı Kolostrumun Büyüme Performansı, Karkas Verimi, Seruloplazmin, Sialik Asit ve Bazı Antioksidan Düzeyleri Üzerine Etkisi

Özet

Bu çalışma, bıldırcınlarda sıvı kolostrumun büyüme performansı, karkas verimi, seruloplazmin, sialik asit ve bazı antioksidan düzeyleri üzerine etkisini belirlemek amacıyla yapıldı. Çalışmada toplam 90 adet 10 günlük yaşta karışık cinsiyette Japon bıldırcını civcivleri kullanıldı. Bıldırcınlar rastgele 3 gruba ayrıldı. Bıldırcınlara bazal diyet; bazal diyete %2 ve %4 oranında sıvı kolostrum ilave edilen üç farklı yem verildi. Hayvanlar 32 gün boyunca 14 saat aydınlık:10 saat karanlık olacak şekilde bir aydınlatma programına tabi tutuldu. Bıldırcın rasyonlarına ilave edilen sıvı kolostrumun performans üzerine etkileri incelendiğinde; deneme sonu ağırlığı, canlı ağırlık artışı, yem tüketimi, yemden yararlanma oranı, soğuk karkas ağırlığı ve soğuk karkas randımanının deneme gruplarında kontrol grubuna göre daha yüksek olduğu ($P<0.05$), ancak organ ağırlıkları üzerine herhangi bir etkisinin olmadığı görüldü ($P>0.05$). Deneme gruplarında karaciğer malondialdehit (MDA), seruloplazmin ve sialik asit düzeylerinin daha düşük olmasına rağmen; süperoksit dismutaz (SOD) düzeylerinin daha yüksek olduğu görüldü ($P<0.05$). Ancak TAS düzeyine herhangi bir etkisinin olmadığı tespit edildi ($P>0.05$). Serum MDA, SOD ve TAS düzeylerine etkisi incelendiğinde MDA düzeylerinin deneme gruplarında daha düşük, SOD düzeyinin ise daha yüksek olduğu görüldü ($P<0.05$). Deneme grupları TAS düzeylerinde sayısal bir artış olmasına rağmen istatistiksel olarak önemli bir fark bulunmadı. Sonuç olarak, bıldırcın rasyonlarına %4 düzeyinde katılan sıvı kolostrum ile oksidatif, taşıma ve kesim kaynaklı stresler azaltılabilir.

Anahtar sözcükler: *Kolostrum, Büyüme performansı, Seruloplazmin, Sialik asit, Bıldırcın*

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INTRODUCTION

Colostrum is a liquid lactated by the mammary glands of female mammals for the first few days after birth. Colostrum contains not only nutrients such as protein, carbohydrate, fat, vitamins and minerals, but also various bio-active components such as growth and antimicrobial factors [1,2].

Two of the most important components contained in colostrum are immune and growth factors [3,4]. Immune factors are the substances that reduce the effects of micro-organisms causing diseases, protect from diseases and help the body [5]. Growth factors contain components that increase healing effects by building and aiding recovery of bones, muscles, fibers and cartilage, stimulating fat metabolism, sustaining blood glucose level balance and helping to regulate brain chemicals controlling state of mind [6].

Sialic acid is one of the most important structures in biological membranes and is available within the structures of glycolipids, polysaccharides, glycoprotein and muco-protein [7]. It is commonly available in animal tissues and bacteria. Serum total sialic acid increases considerably in infectious, tumoral, and metabolic diseases [8]. Cerulo-plasmin, which is an acute phase protein, functions as an antioxidant within the organism. Free copper and iron ions are powerful catalysts of free radical damage. Cerulo-plasmin by binding copper, prevents free copper ions from catalyzing oxidative damage. Ceruloplasmin levels rise following infection, trauma, or inflammation [9]. In recent years, colostrum has been used as a good protein source to support the development of the musculoskeletal system and healthy and powerful immune system, to shorten the time of regeneration after workouts, to shorten recovery period after injuries, and as a stress reducing factor [10]. Additionally, research about colostrum's effects on performance and the immune system are limited, except for studies performed on ruminants; there has not been sufficient research done on poultry. This study was conducted to determine the effects of liquid cow colostrum on growth performance, some blood parameters, and antioxidant levels in quail.

MATERIAL and METHODS

Animals, Treatments and Management

The ethical committee approval of Dicle University (DÜHADEK: 2010-44) was taken in order to conduct this study. Ten-Days-old (47±0.5 grams) mixed-sexed Japanese quails *(Coturnix coturnix japonica,* 90), obtained from the poultry unit of the Veterinary Faculty of Dicle University. The birds were distributed randomly to one of three groups, and each of the experimental groups was replicated in six cages (60x120x30), each containing five birds. Quails were housed in wire cages at 37.5°C on the first days in a temperature-controlled room. The room temperature was then gradually decreased to 22°C by the end of the third week and was then kept constant. Birds were exposed to a 14L:10D illumination cycle for 32 (days of 10-42) days. Feeds as isocaloric and isonitrogenic were prepared (LiqC was added into diets at expense of corn). Feed and fresh water were offered *ad libitum* throughout the experiment.

Sample and Data Collection

Quails were divided into 3 groups randomly. No additions were made to the basal diet of the first group. 2% LiqC was added to the second group's diet (Per kilogram of basal diet was added 100 mL LiqC), and 4% to the third group (Per kilogram of basal diet was added 200 ml LiqC). Colostrum dry matter, protein, fat and lactose was calculated as 20.4%, 12%, 6.3% and 3.2%, respectively (Funke Gerber, Laktostar, Milk Analyzer, Berlin, Germany). Nutrient and chemical components of the diets of trial groups are given in *Table 1*.

Table 1. *Ingredient and nutrient composition of the basal diet*[a]

Ingredient	Starter phase (days 10-21) (%)	Grower phase (days 22-42) (%)
Corn	53	58.9
Soybean meal	36.6	32.2
Soy oil	6	5
Limestone	1.7	1.3
Dicalcium phosphate	1.6	1.6
Sodium chloride	0.4	0.4
Vitamin-mineral premix[b]	0.5	0.5
DL-Methionine	0.2	0.1
Chemical analyses, dry matter basis		
Crude protein	22.7	20.6
Crude fat	6.73	6.15
Crude fiber	3.85	3.87
Calcium	1.0	0.9
Phosphorus	0.75	0.71
Calculated compositions[c]		
Metabolizable energy, MJ/kg	12.91	12.91
Lysine	1.18	1.05
Methionine + cystine	0.9	0.63

[a] LiqC was added into diets at expense of corn; [b] Vitamin premix provides the following per kg: all-trans-retinyl acetate, 1.8 mg; cholecalciferol, 0.025 mg; all-a-tocopherol acetate, 1.25 mg; menadione (menadione sodium bisulphate), 1.1 mg; riboflavin, 4.4 mg; thiamine, 1.1 mg; pyridoxine, 2.2 mg; niacin, 35 mg; Ca-pantothenate, 10 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.55 mg; d-biotin, 0.1 mg. Mineral premix provides the following per kg: Mn (from MnO), 40 mg; Fe (from FeSO4), 12.5 mg; Zn (from ZnO), 25 mg; Cu (from $CuSO_4$), 3.5 mg; I (from KI), 0.3 mg; Se (from NaSe), 0.15 mg; choline chloride, 175 mg; [c] Calculated value according to tabular values listed for the feed ingredients [17]

Feed consumption and body weight gain of quails were recorded weekly. The weight gain and feed utilization efficiency of the birds were calculated weekly. At the end of the study, 30 quails from each group were slaughtered to determine carcass yield. Mix sexed 12 quails (one male and one female quails per replicate) were used to determine serum and liver MDA, TAS, ceruloplasmin, and sialic acid levels. The carcasses were obtained after the feathers, feet, and visceral organs were removed. The carcasses were kept at 4°C for 18 h, and cold carcass yields were calculated. Blood samples were collected into biochemical tubes to determine the level of the blood serum. The tubes containing the blood samples were centrifuged at 4°C at 3.000 rev/min for 10 min. Serum and liver samples were stored at -20°C until analysis.

Laboratory Analyses

Tissue samples were immediately weighed and washed with 0.9% NaCl solution, homogenised (2.000 rpm/min for 1 min, 1:10 w/v) using a stirrer (Stuart SHM 1, UK) in 1.15% KCl solution in an ice bath. Then homogenate was centrifuged at 5000×g for 60 min at 4°C. The resultant supernatant was used at the analyses. Protein analysis in homogenate and supernatant was performed according to the Lowry method [11].

SOD Analysis in serum was performed to the Sun et al.[12]. MDA levels in the homogenate and serum were determined by using the single heating method of Yoshioka et al.[13] based on thiobarbituric acid (TBA) reactivity. Total antioxidant capacity (TAS) of the supernatant or serum was determined using an automated measurement method with a commercially available kit developed by Rel (Total Antioxidant Status Assay kit, Rel Assay Diagnostics, Turkey). The antioxidative effect of the sample against the potent-free radical reactions initiated by the reduced hydroxyl radical is measured using this method. The results were expressed as millimoles of Trolox equivalent per mg tissue protein in supernatant or millimoles of Trolox equivalent/L in serum. Serum and supernatant total sialic acid levels were measured spectroscopically using Warren's [14] thio-barbituric acid method (Shimadzu UV 1800, Japon). Serum and supernatant ceruloplasmin levels were determined by measurement of p-phenylenediamine oxidase activity defined by Sunderman and Nomoto [15].

The nutritional composition of the diets was determined according to the Association of Official Analytical Chemists [16]. Energy and amino acid (lysine and Methionine + cystine) contents were calculated from tabular values listed for the feedstuffs [17].

Statistical Analysis

Performance variables [feed intake, weight gain, and feed efficiency], and serum MDA, SOD, and TAS levels were analyzed by one-way ANOVA using the PROC MIXED procedure (SAS, 2002). The linear model to test the effects of dietary LiqC supplementation on response variables was as follows: yij = μ + b0 + Ri + ej, where y = response variable; μ = population mean; b0 = covariate, measurements obtained at the end of the pretest period; R = LiqC supplementation; and e = residual error being N (σ, μ; 0, 1). The model also included orthogonal and polynomial contrast to determine changes in response variables as supplemental LiqC level was increased [18]. Mean differences of interaction effects were compared to Duncan test. Statistical significance was considered at P<0.05.

RESULTS

The effects of the LiqC added to the quail diet on growth performance are given in *Table 2*. When the data were examined, it was found that quail in the experimental groups increases final body weight, body weight gain, cumulative feed intake, cold carcass weight, and cold carcass yield of the quails, and decrease in feed efficiency as supplemental LiqC increased from 0% to 4% (P<0.05).

When the effect of the LiqC added to the feed mix on organ weight was investigated, it was observed that it has no effect *(Table 3)*.

The effects of the LiqC added to the quail feed mix on liver MDA, SOD, TAS, ceruloplasmin, and sialic acid levels are

Table 2. *Effects of liquid colostrum (LiqC) supplementation to quail diets on growth performance[a]*

Variable[c]	LiqC, %			SEM	Statistical Significance, *P*>Fb		
	0	2	4		S	L	Q
Final body weight, g[d]	181.52[b]	187.20[a]	191.09[a]	1.901	0.001	0.0001	0.651
Live weight gain, g	133.53[c]	139.79[b]	146.23[a]	0.913	0.0001	0.0001	0.957
Cumulative feed intake, g	635.25[c]	649.08[b]	658.33[a]	1.880	0.0001	0.0001	0.416
Feed efficiency[e]	4.76[a]	4.64[a]	4.50[b]	0.038	0.002	0.001	0.815
Cold carcass weight, g	119.71[c]	125.03[b]	129.35[a]	1.081	0.0001	0.0001	0.675
Cold carcass yield, %	65.65[b]	67.72[ab]	69.80[a]	0.984	0.030	0.009	0.995

*[a] Data are the least square means from 10-42 days animal experimentation; [b]Statistical contrast: **S** = LiqC supplementation effect (quail supplemented with LiqC vs. quail not supplemented with LiqC); **L** = Linear effect of increasing dietary LiqC; **Q** = Quadratic effect of increasing dietary LiqC; [c]Different letters within the same rows indicate differences among groups (P<0.05); [d] **n** = 30 quails per group; [e]Feed efficiency = feed consumed, **g:** weight gained, g*

Table 3. *Effects of liquid colostrum (LiqC) supplementation to quail diets on organ weights*

Variable[b]	LiqC, %			SEM	Statistical Significance, *P* > F[a]		
	0	2	4		S	L	Q
Liver, g	2.97	3.26	3.33	0.185	0.446	0.234	0.671
Heart, g	1.73	1.72	1.70	0.038	0.950	0.758	0.941
Spleen, g	0.11	0.12	0.11	0.011	0.822	0.734	0.602

*[a] Statistical contrast: **S** = LiqC supplementation effect (quail supplemented with LiqC vs. quail not supplemented with LiqC); **L** = Linear effect of increasing dietary LiqC; **Q** = Quadratic effect of increasing dietary LiqC; [b] **n** = 12 quails per group*

Table 4. *Effects of liquid colostrum (LiqC) supplementation to quail diets on liver MDA, SOD, TAS, ceruloplasmin and sialic acid levels[a]*

Variable[c, d]	LiqC, %			SEM	Statistical Significance, P > F[b]		
	0	2	4		S	L	Q
MDA[e]	14.53[a]	13.42[ab]	12.18[b]	0.434	0.006	0.002	0.907
SOD[f]	24.41[b]	27.82[ab]	29.63[a]	1.657	0.018	0.006	0.582
TAS[g]	1.40	1.48	1.63	0.096	0.274	0.119	0.765
Ceruloplasmin[h]	27.06[a]	23.26[b]	21.70[b]	1.017	0.012	0.004	0.431
Sialic acid[i]	184.08[a]	90.04[b]	81.00[b]	9.632	0.0001	0.0001	0.014

*[a] Data are the least square means from 10-42 days animal experimentation; [b] Statistical contrast: **S** = LiqC supplementation effect (quail supplemented with LiqC vs. quail not supplemented with LiqC); **L** = Linear effect of increasing dietary LiqC; **Q** = Quadratic effect of increasing dietary LiqC; [c,d] Different letters within the same rows indicate differences among groups (P<0.05), n = 12 quails per group (six male and six female); [e] **MDA** = µmol/ mg protein; [f] **SOD** = % inhibisyon/mg protein; [g] **TAS** = mmol trolox equiv./mg protein; [h] Ceruloplasmin = g/mg protein; [i]* Sialic acid = µg/mg protein

Table 5. *Effects of liquid colostrum (LiqC) supplementation to quail diets on serum MDA, SOD and TAS levels[a]*

Variable[c]	LiqC, %			SEM	Statistical Significance, P > F[b]		
	0	2	4		S	L	Q
MDA[e]	12.75[a]	11.14[b]	9.96[c]	0.606	0.0001	0.0001	0.629
SOD[f]	19.80[b]	26.97[a]	31.30[a]	1.586	0.001	0.0001	0.522
TAS[g]	2.31	2.49	2.56	0.276	0.644	0.374	0.799

*[a] Data are the least square means from 10-42 days animal experimentation; [b] Statistical contrast: **S** = LiqC supplementation effect (quail supplemented with LiqC vs. quail not supplemented with LiqC); **L** = Linear effect of increasing dietary LiqC; **Q** = Quadratic effect of increasing dietary LiqC; [c,d] Different letters within the same rows indicate differences among groups (P<0.05), **n** = 12 quails per group (six male and six female); [e] **MDA** = µmol/mg protein; [f] **SOD** = % inhibisyon/mg protein; [g] **TAS** = mmol trolox equiv./mg protein*

given in *Table 4*. When the table was examined, it was seen that the MDA, ceruloplasmin, and sialic acid levels are lower and SOD level is higher in the trial groups when compared with the control group (P<0.05). On the other hand, it was detected that it has no effect on the TAS level (P>0.05).

When the effect of the LiqC on serum MDA, SOD and TAS levels was analyzed *(Table 5)*, it was seen that MDA levels are lower and SOD level is higher in the trial group (P<0.05). However, it was determined that although there is a numerical increase in TAS levels, there is not a statistically significant difference.

DISCUSSION

This study was designed to determine the effect of the LiqC on growing performance, sialic acid, ceruloplasmin, and some antioxidant levels in quails. In a previous research, we found that the addition of powdered colostrum increased final body weight, body weight gain, cumulative feed intake, cold carcass weight, cold carcass yield, and decreased feed efficiency in quails [19]. Similarly, in the present study we observed that LiqC supplementation increased body weight gain, and decreased feed efficiency in quails with increasing dietary LiqC supplementation for 42 days of the experimental period. King et al.[20] reported that dietary spray-dried colostrum improved feed conversion ratio at day 14, and in another study conducted in broiler, it was declared that colostrum added to the feed mix increased body weight gain on the 13th day of the growth [21]. Additionally, in studies made on humans, it has been reported by many researchers that cow colostrum increases muscle growth, accelerates muscle-skeleton regeneration, and enhances power and strength [10,22]. The findings of many researchers mentioned above show similarity with our findings. This can be explained by the following: colostrum is a nutritious liquid which is rich

in essential and nonessential amino acids, fatty acids, minerals and vitamins, contains growth factors for cell and tissue growth [2] and helps the growth of intestinal system [23]. Lipid peroxidation occurring through free oxygen radicals is a significant cause of injury to cell membranes. It causes excessive accumulation of Ca^{2+} in cells by affecting membrane permeability [24]. Cell membrane dysfunction results in cell swelling and cell death. Malondialdehyde is an end result of lipid peroxidation and is used to show oxidative injury level. Plasma MDA and tissue MDA levels are measured as an indicator of free radicals [25]. Both tissue and serum MDA levels' being lower in the trial groups when compared with the control groups is an indicator for oxidative damage being less in this study.

Superoxide dismutase is an essential enzyme that is produced as endogen and for each cell constituting the organism. The first defense against free radicals within this organism is made with superoxide dismutase (SOD) enzyme. It protects the organism from harmful effects of oxidants by transforming superoxide radical, which causes cell injury, to less harmful hydrogen peroxide and molecular oxygen [26]. In a study made on elderly people, it was reported that addition of cow colostrum to their diets caused an increase in the level of serum SOD. In a study made on mice, Mahenderan et al.[27] reported that the level of SOD in the group fed with colostrum was higher when compared with the control group. The results of our study show similarity with the studies reporting that tissue and serum SOD level is higher in trial groups when compared with the control groups. Bovine colostrum has significant amounts of enzymatic and non-enzymatic antioxidants. Lactoperoxidase, catalase, superoxide dismutase, and glutathione peroxidase are the important enzymatic antioxidants present in bovine colostrum. The high levels of these oxidants in colostrum may cause an increase in SOD levels in trial groups [28].

Antioxidants function as a cell protection against destructive side effects of oxidative stress. Oxidative and antioxidative status evaluation can be done with the measurement of TAS and MDA levels [29]. In this study, the TAS levels in the trial groups showed no difference statistically. However, when compared with the control group, there was a numerical increase in the trial groups. This increase is caused by the fact that antioxidant substance level is high in the nutritious component of colostrum.

Acute phase proteins (AFP) synthesized by the liver as a reaction to the acute phase response may have different functions and characteristics [30]. While these proteins are present at a insignificant level in healthy animals, they increase during inflammation and have an indicator role. During the acute phase response, the synthesis and release of certain plasma proteins such as ceruloplasmin, sialic acid increase [31]. Ceruloplasmin is an α2 globulin composed of a single polypeptide chain. The transfer of copper, lipid peroxidation, oxidation of toxic ferrous iron to non-toxic ferric form and prevention of free radical generation are among the duties of ceruloplasmin [32]. In a study made on dogs, it was reported that ceruloplasmin concentration increased with infection and tissue damage [33]. With the present study, ceruloplasmin concentration was found higher in control groups when compared with the trial groups. There may be two reasons for this increase. First, it may be caused by the presence of a subclinical course of disease or a noneffective inflammatory in control groups. Second, ceruloplasmin level may have increased depending on the stress level during the transport and bird slaughter. Since no disease symptoms were detected clinically in any of the groups of birds subjected to the study, the second factor is thought to be in effect.

Sialic acid concentration increases rapidly in situations such as tissue damage, inflammation, infection, and transport [34]. During the study, sialic acid level was found higher in control groups when compared with the trial groups. As in ceruloplasmin, the increase of sialic acid in the control group may have resulted from the stress increase occurring during the transfer and slaughter of the birds.

It was observed that addition of LiqC to the feed mix increased slaughter weight, body weight gain, feed intake, carcass weight, and carcass yield but had no effect on organ weight. It was observed that it decreased MDA, ceruloplasmin and sialic acid levels, increased SOD levels, and had no effect on TAS levels. Additionally, ceruloplasmin and sialic acid levels' being low in the groups to whose feed mix was added colostrum may be a significant indicator of the animals' welfare. During transport and slaughter, the birds become stressed, and this can reduce the quality of meat. Addition of colostrum to the feed mix can minimize the oxidative, transport, and slaughter stresses that may occur.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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Research Article

Growth Performance, Carcass and Viscera Yields, Blood Constituents and Thyroid Hormone Concentrations of Chronic Heat Stressed Broilers Fed Diets Supplemented with Cumin Seeds *(Cuminum cyminum* L.)

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Abstract

This study was conducted to determine the effect of dietary supplementation with cumin (*Cuminum cyminum* L.) seeds on growth performances, relative weights of carcass and viscera, haematological and biochemical parameters, and thyroid hormones concentrations of broiler chickens subjected to natural fluctuation of Algerian summer ambient temperatures. A total of 440 28-day old chickens were divided into 2 groups (5 replicates of 44 birds) with similar body weight (971±48 g): a "Control" group fed with a standard diet and a "Cumin" group receiving a basal diet supplemented with 0.2% of cumin. As a result of this study, dietary cumin supplementation did not significantly modify the growth rate and final body weights of heat-exposed chickens but it slightly improved feed conversion ratio (-7%, P=0.1). Carcass traits, viscera (liver, heart, gizzard, spleen, bursa and thymus) intestine morphology and abdominal fat of heat stressed birds did not reveal any changes by cumin inclusion compared to the control ones. Also, thyroid hormones (T3 and T4) concentrations were not significantly influenced by dietary cumin (P>0.05). However, heat-exposed chickens supplemented with cumin exhibited a significant (P<0.01) lower values of plasma glucose, cholesterol, triglycerides and total proteins and higher calcium concentrations than those of control group. Cumin dietary may be a successful means to enhancing diet conversion and reducing glaecimic, lipidaemic and calcaemic disorders in chronically-heat exposed chickens.

Keywords: *Blood parameters, Broilers, Carcass, Chronic heat stress, Climate, Cuminum cyminum, Performance, Thyroid hormones*

Kimyon *(Cuminum cyminum* L.) Çekirdeği İle Beslenen ve Kronik Isı Stresine Maruz Bırakılan Broiler Tavuklarda Büyüme Performansı, Karkas ve Visseral Organ Ağırlıkları, Kan Bileşenleri ve Tiroit Hormon Konsantrasyonları

Özet

Bu çalışma, Cezayir'de yaz çevre ısısının doğal dalgalanmasına maruz kalan broiler tavuklarda yeme kimyon (*Cuminum cyminum* L.) çekirdeği ilavesinin büyüme performansına, karkas ve vissera ağırlığı oranına, kan ve biyokimyasal parametrelere ve tiroit hormon konsantrasyonuna etkilerini belirlemek amacıyla yapıldı. Toplam 440 adet 28 günlük tavuklar vücut ağırlıkları (971±48 g) benzer olmak üzere 2 gruba (44 tavuk, 5 tekrar olarak) ayrıldı. "Kontrol grubu" standart diyet ile beslenirken, "Kimyon grubuna" % 0.2 oranında kimyon ilave edilmiş bazal diyet verildi. Elde edilen sonuçlar ısı stresine maruz kalan broiler tavuklarda diyetsel kimyon ilavesinin büyüme oranına ve nihai vücut ağırlığına önemli bir etki yapmadığını fakat az oranda yem konversiyon oranına (% -7, P=0.1) etki ettiğini gösterdi. karkas özellikleri, visseral organ (karaciğer, kalp, taşlık, dalak, bursa ve timüs), bağırsak morfolojisi ve abdominal yağ miktarı ısı stresine maruz kalan kontrol grubu ile karşılaştırıldığında kimyon ilave edilen grupta bir farklılık göstermedi. Tiroit hormonları (T3 ve T4) konsantrasyonları diyetsel kimyon ile anlamlı derecede farklılık göstermedi (P>0.05). Ancak, kimyon verilen ve ısı stresine maruz bırakılan tavuklarda kontrol grubu ile karşılaştırıldığında anlamlı derecede olmak üzere (P<0.01) düşük plazma glikoz, kolesterol, trigliseritler ve total proteinler seviyeleri ile yüksek kalsiyum konsantrasyonu belirlendi. Diyete kimyon ilavesinin kronik ısı stresine maruz kalan tavuklarda diyet konversiyonunu artırmak ve glasimik, lipidemik ve kalsemik bozuklukları düzeltmek amacıyla kullanılabileceği kanısına varılmıştır.

Anahtar sözcükler: *Kan parametreleri, Broiler, Karkas, Kronik Isı Stresi, İklim, Cuminum cyminum, Performans, Tiroit hormonları*

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INTRODUCTION

In summer season, chronic heat stress continues to be a great problematic for poultry production. The prolonged periods of high ambient temperatures compromise productivity and cause large economic losses [1]. Heat exposure decreases broilers growth rate, which is generally due to the reduced feed intake and generate many physiological, hormonal and molecular changes [2]. Also, it has been established that chronic heat stress affect development of several internal organs such as digestive (proventriculus, gizzard, intestine) and lymphoid organs (thymus, spleen, and bursa of fabricius), which could compromise the efficacy of nutrient digestibility and absorbability as well as the immune responses of birds [2,3].

To alleviate the negative effects of high environment temperature on poultry production, scientific investigations have considerably increased in the last decades [4]. Several nutritional approaches using safe and natural feed additives have been suggested. In this respect, many studies have shown that antioxidant nutrients supplementation in their synthetic (vitamins, minerals) or natural (herbs, spices) form can be used to improve productivity [5], enhance nutrient availability and prevent against detrimental effect of heat stress [6,7].

Cuminum cyminum is a small annual and herbaceous plant belonging to Apiaceae (Umbelliferae) family, which is cultivated in Arabia, India, China and in the countries bordering the Mediterranean Sea [8]. *Cuminum cyminum* seeds have shown excellent antioxidant, anti-inflammatory and antimicrobial activities which are generally attributed to their major components such as cuminaldehyde, terpinenes, polyphenols and flavoids [9]. Little is known about the effect of cumin *(Cuminum cyminum L.)* on zootechnical performances and physiological responses of broilers reared under high environmental temperatures. The few existing studies have focused on the effects of dietary *Cuminum cyminum L.* inclusion on growth performance of broilers under standard thermoneutral conditions [10,11]. Al-Kassy [11], found that dietary supplementation with 0.5 or 1% of cumin seed improved body weight gain and feed conversion ratio of broilers reared under thermoneutral conditions, with decreasing hematological values such as haemoglobin, red blood cells and packed cell volume. Under heat stress conditions, cumin dietary feed supplementation at level of 2 g/kg has been shown to ameliorate weight gain, enhance carcass, liver, pancreas and proventriculus percentage and increase haematocrit values and blood haemoglobin concentration of slow-growing chicks [12]. To our knowledge, this study is the only published trial on the effect of *Cuminum cyminum* dietary inclusion on broiler chickens subjected to heat stress. The influence of cumin on growth, performance, metabolism and thyroid hormones of commercial broilers under hot climate remains to be explored. In this context, the aim of this study is to assess the efficacy of dietary supplementation with cumin seeds (2 g/kg) to improve growth performances, carcass characteristics, lymphoid organs weights, and some haematological, biochemical and hormonal parameters of commercial broiler chickens reared in hot conditions (natural fluctuation o f A lgerian summer ambient temperatures).

MATERIAL and METHODS

Birds and Experimental Design

This research was approved by the scientific c ouncil o f the Superior National Veterinary School of Algiers, Algeria. A total of 500 one-day-old of unsexed ISA HUBBARD broilers chicks obtained from a local hatchery were used in this experiment. From 1 to 28 days of age, chicks were given a standard starter diet (2.800 kcal ME/kg, 20% crude protein) from 1 to 10 d of age and a grower diet (2.900 kcal ME/kg, 19% crude protein) from 11 to 28 d of old. All animals were reared under standard breeding conditions. At 28 days old (experimental period), 440 chicks were selected on the basis of their body weight and equally divided into 2 groups, with similar average body weight (971±48 g) with 5 replicate pens of 44 birds each. Control chickens were fed a basal diet (grower diet from 28 to 42 d old and a finisher diet from 43 to 49 d old) as shown in *Table 1*. In cumin group, chickens received the same basal diet supplemented with 0.2% of cumin *(Cuminum cyminum L.)* in form of fine powder. During all the experimental period, all chicks were given free access to diet and water and were kept under similar rearing conditions and were exposed to the natural fluctuations of the summer ambient temperature *(Fig. 1)*.

Growth Performance

Body weights, feed intake and feed conversion ratios (g of feed/g of gain) for each group were recorded at 42 days and 49 days of old.

Carcass Traits

At the age of 49 d, ten birds per group were weighed, slaughtered, and then their feathers were removed. After eviscerating, carcasses, abdominal fat and the removed internal organs (liver, heart, gizzard, spleen, thymus, bursa, and intestine) were weighed and then expressed as percentage of live body weight. Also, the total length of intestine was measured and the intestine density was calculated.

Laboratory Analysis

At the end of experimental period (49 d), ten birds having a similar body weight to the average of their group were selected from each group and used for determination of the blood parameters. The birds were fasted for approximately 12 h before blood collection.

Table 1. *Composition of the basal diet of the experiment*

Ingredient (g/100 g)	Starter (d1-10)	Grower (d11-42)	Finisher (d43-49)
Maize	60.90	64.80	68.60
Wheat bran	5.90	5.00	6.00
Soybean meal (46%CP)	29.20	26.40	21.80
Limestone	0.57	1.20	1.30
Dicalcium phosphate	2.40	1.60	1.30
Methionine	0.03	-	-
Vitamin-mineral premix S-G[a]	1.00	1.00	-
Vitamin-mineral premixF[b]	-	-	1.00

[a] Each kilogram of starter and grower diet provided: 850.000 IU of vitamin A; 170.000 IU of vitamin D_3; 1.350 IU of vitamin E; 199 mg of vitamin K_3, 100 mg of vitamin B_1; 450 mg of vitamine B_2; 150 mg of vitamine B_6; 1 mg of vitamine B_{12}; 1.5 mg of biotin; 600 mg of pantothenic acid; 1.000 mg of niacin; 40 mg of folic acid; 34.800 of choline chloride: 100.000 mg ; 738.8 mg of crude ash; 142.7 mg of calcium; 5.1 mg of magnesium; 130.5 mg of sodium; 1.2 mg of sulfur; 15.5 mg of insoluble ash; 3.600 mg of iron (Fe carbonate); 7.488 mg of zinc; 2250 mg of copper (sulfate); 75.052 mg of manganese (oxyd); 121 mg of iodine (iodate); 40 mg of cobalt (carbonate); 25 mg of selenium (selenite); 120.000 mg of methionin; 10.000 mg of BHA-ethoxyquine; 85 mg of molybdenum; 6.000 mg of narasin; [b] Each kilogram of finicher provided: 1.000.000 IU of vitamin A; 240.000 IU of vitamin D_3; 2.500 IU of vitamin E; 200 mg of vitamin K_3, 180 mg of vitamin B_1; 600 mg of vitamine B_2; 290 mg of vitamine B_6; 1 mg of vitamine B_{12}; 1.5 mg of biotin; 900 mg of pantothenic acid; 2.900 mg of niacin; 26 mg of folic acid; 49.998 mg of choline chloride: 100.000 mg; 766 mg of crude ash; 204.3 mg of calcium; 5.1 mg of magnesium; 130.5 mg of sodium; 1.2 mg of sulfur; 15.5 mg of insoluble ash; 3.600 mg of iron (Fe carbonate); 7.488 mg of zinc; 2.250 mg of copper (sulfate); 75.052 mg of manganese (oxyd); 121 mg of iodine (iodate); 40 mg of cobalt (carbonate); 25 mg of selenium(selenite); 120.000 mg of methionin; 10.000 mg of BHA-ethoxyquine; 85 mg of molybdenum

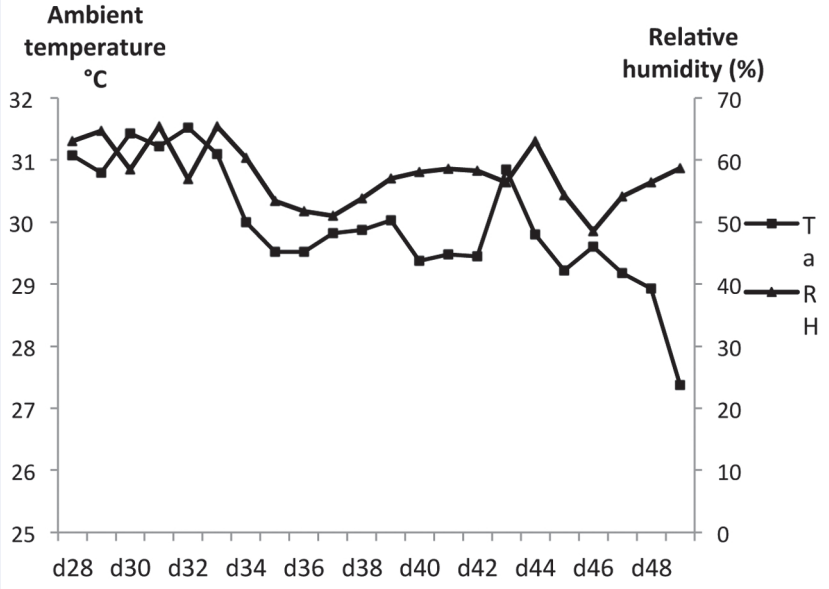

Fig 1. Evolution of daily mean ambient temperature and relative humidity during the experimental period (28-49d ays). Ta, ambient temperature; RH, relative humidity

Haematology Analysis

At slaughtering, blood samples were collected into EDTA tubes. The measurement of haematocrit percentage, haemoglobin concentration and total red blood cells (RBC) were performed on the same day using automated hematology analyzers as described by Post et al.[13].

Biochemical Analysis

Blood samples were collected from the slaughtered birds in heparinized tubes and then centrifuged at 3.000 rpm for 10 min. The blood plasma obtained were collected and stored at -20°C until analysis. Plasma glucose, total protein, total cholesterol, triglycerides and calcium were determined by using a spectrophotometer (LKB Novastec) and available commercial kits (SPINREACT, SA, Espagne) at various wavelengths as follows: Glucose concentration was determined as mg/dL by using GOD POD method [14] at 505 nm; total protein concentration was analyzed by the Biuret Method (colorimetric test) [15] at 540 nm; plasma cholesterol concentration was determined as mg/dL by using the CHOD PAP [16] method at 505 nm; plasma triglyceride concentration was determined as mg/dL by using the GPO-PAP enzymatic method [17] at 505 nm; plasma calcium concentration was determined as mg/dL by using the o-Cresophtalein v/v complex method [18] at 570 nm.

Thyroid Hormone Analysis

Blood samples were collected in heparinized tubes, centrifuged at 3.000 rpm for 10 min. The plasma samples were collected and kept at -20°C until analysis. The total plasma triiodothyronine (T3) and thyroxin (T4) concentrations were measured by radioimmunoassay (RIA) [19].

Statistic Analysis

Data are expressed as means ± standard error and subjected to one factor variance analysis (ANOVA1)

performed with the STATVIEW software [20]. Differences were considered statistically significant at P<0.05.

RESULTS

Growth Performance

The effects of dietary supplementation of cumin seed at 0.2% on body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of chronically heat stressed broilers are presented in *Table 2*. In the present study, body weights of cumin supplemented chickens were similar to those of control animals both at the age of 42 d (1886±68 g *vs.* 1799±59 g; P>0.05) and 49 d (2302±62 g *vs.* 2264±69 g; P>0.05) (data not presented in *Table 2*). When considering overall weight gain of heat-stressed broilers, we found that cumin inclusion in diet did not affect this parameter despite a lower weight gain during finishing period (-11%, P=0.03). In heat-exposed chickens, feed intake recorded from 28 to 42 d of age was significantly (P<0.05) decreased by the dietary inclusion of cumin (-20% comparing to control) while it was similar in the two groups from 43 to 49 d of age. In the growing period (28 to 42 d of old), dietary cumin supplementation resulted in a significant improvement of feed conversion ratio (FCR) while in the finishing period (42 to 49 d of age), this parameter was significantly increased comparing to control animals. Throughout the experiment (28 to 49 d of age), the inclusion of 0.2% of cumin to diet seemed slightly enhanced FCR (-7%, P=0.1).

Carcass and Internal Organs Traits

The effects of cumin seeds inclusion on carcass and viscera weights and their relative weights are shown in *Table 3*. Supplementing diet with 0.2% of cumin did not significantly (P>0.05) affect weights and relative weights of carcass, gizzard, liver, heart, abdominal fat per 100 g of body weight of broiler reared under chronic heat stress. Intestine weight, length and density of cumin supplemented birds did not differ significantly from those of control. Also, results showed that none of lymphoid organ (thymus, bursa, and spleen) weights and their relative weights were significantly influenced by dietary cumin supplementation.

Table 2. *Effect of dietary cumin supplementation on body weight gain, feed intake and feed to gain ratio of broiler chickens reared under high temperature summer from 28 to 49d of age*

Variables	Control Group	Cumin Group	P-value
Feed intake (g)			
28-42 days	1012±53	810±48	0.03
43-49 days	1082±68	1146±26	0.41
28-49 days	2094±120	1956±65	0.35
Body weight gain (g)			
28-42 days	845±35	898±19	0.23
43-49 days	465±12	416±13	0.03
28-49 days	1310±43	1314±19	0.93
Feed to gain ratio			
28-42 days	1.20±0.03	0.90±0.05	<0.01
43-49 days	2.32±0.10	2.76±0.11	0.02
28-49 days	1.60±0.04	1.49±0.04	0.11

Table 3. *Effect of dietary cumin supplementation on carcass and internal organs weights and as a percentage of body weight (BW) of broiler chickens reared under high temperature summer*

Variables	Control Group	Cumin Group	P-value
Carcass (g) Carcass (%BW)	1600.13±48.33 69.40±9.00	1616.53±47.27 70.02±7.00	0.81 0.46
Liver (g) Liver (%BW)	45.23±2.54 2.00±1.00	44.29±2.29 1.90±1.00	0.76 0.85
Heart (g) Heart (%BW)	10.15±0.62 $2.00\pm22\times10^{-4}$	9.36±0.44 $2.00\pm17\times10^{-4}$	0.31 0.26
Gizzard (g) Gizzard (%BW)	31.94±1.27 1.40±0.01	32.85±0.83 1.40±0.00	0.55 0.46
Abdominal fat (g) Abdominal fat (%BW)	29.00±1.42 1.30±0.01	28.66±2.48 1.20±0.01	0.68 0.72
Intestine weight(g) Intestine weight/%BW	101.53±4.68 4.37±0.12	95.89±3.74 4.07±0.13	0.35 0.10
Intestine length (cm) Intestine density (g/cm)	239.70±4.21 0.42±0.02	241.30±4.18 0.40±0.01	0.79 0.25
Spleen weight (g) Spleen (%BW)	2.96±0.22 0.13±0.10	3.07±0,16 0.13±0.09	0,68 0.82
Bursa (g) Bursa (%BW)	1.53±0.31 0.07±0.01	1.20±0.15 0.05±0.01	0.35 0.28
Thymus (g) Thymus (%BW)	7.26±0.46 0.36±0.04	8.57±0.07 0.39±0.03	0.27 0.38

Table 4. *Effect of dietary cumin supplementation on haematological and biochemical parameters and plasma thyroid hormones concentration of broiler chickens reared under high temperature summer*

Variables	Control Group	Cumin Group	P-value
Glucose (mg/dL)	236.38±9.08	212.13±3.63	0.019
Total proteins (g/dL)	3.27±0.10	2.75±0.07	0.0002
Triglycerides (mg/dL)	94.19±11.91	43.95±2.93	0.0003
Cholesterol (mg/dL)	107.33±4.71	70.29±4.27	<0.0001
Calcium (mg/dL)	4.71±0.38	10.51±0.48	<0.0001
Haematocrit (%)	26.40±0.57	25.19±0.44	0.103
RBC ($x10^6/\mu L$)	2.54±0.06	2.44±0.05	0.190
Haemoglobin (g/dL)	8.66±0.23	8.13±0.29	0.166
T3 (pmol)	2.38±0.23	2.70±0.35	0.457
T4 (pmol)	6.13±3.88	6.73±2.91	0.701
T3/T4	0.86±0.29	0.48±0.09	0.222

Biochemistry, Haematology and Thyroid Hormones Concentrations

The effects of dietary cumin supplementation on blood biochemistry, haematological parameters and thyroid

hormones (T3; T4) concentrations of chronically heat stressed broiler chickens are presented in *Table 4*. A significant decrease of plasma glucose (-10 %, P<0.05), triglyceride (-53%, P<0.001), cholesterol (-34%, P<0.0001) and total proteins (-16%, P<0.001) concentrations were recorded in the cumin supplemented groups compared to the control ones, while, a significant (P<0.0001) increase in plasma calcium concentration by this diet additive was observed.

Haematological parameters in term of haematocrit percentage, red blood cell count and haemoglobin concentration were not affected by supplementing diet with 0.2% of cumin. However, a slight tendency to a decrease of these blood contents by cumin diet was observed.

There were no significant (P>0.05) effects of cumin seeds supplementation on plasma T3 and T4 concentrations and on T3:T4 ratio. However, numerical results revealed slight increases for T3 (+13%; P>0.05) and T4 (+10%; P>0.05).

DISCUSSION

In the present study, the overall body weight gain of heat stressed chickens did not differ significantly by cumin inclusion. These results disagree with those obtained in fast-grower chicks reared under thermoneutral conditions [11], and those reported in slow-growing chicks subjected to heat stress [12]. The improvement of weight gain induced by cumin supplementation of local Egyptian strains [12] could be explained by the rusticity and thus a lower sensitivity of these breeds to heat stress. It can also be justified by the length and magnitude of the applied heat challenge which was 4 h per day during 3 successive days per weeks while in our conditions, birds were daily exposed to the high temperature of the summer period. In this study, the lower feed consumption occurred at the beginning of the dietary cumin supplementation could be related to a period of adaptation of chickens to the smell and the taste of the diet. However, the overall feed intake (d 28-49) of cumin supplemented birds was similar to those of control group. In slow-growing chickens subjected to heat stress, dietary cumin supplementation at the level of 0.2% did not induce a significant variation of feed consumption [12]. Similarly, in commercial broiler chickens maintained at thermoneutral conditions, adding cumin to diet (increasing doses of 0.5 to 1.5%) did not significantly alter feed intake [11]. Throughout the experiment (28 to 49 d of age), the inclusion of 0.2% of cumin to diet numerically enhanced FCR (-7%) *(Table 2)* as it has slightly reduced feed intake without affecting the growth rate. The positive effect of cumin on FCR is in agreement with previous results [11,12] and could reveal a better feed efficiency probably related to the beneficial properties of this spice to enhance digestive enzymatic activities, and thus, nutrients digestibility and absorbability [21]. At high ambient temperatures, birds reduce their feed intake and consequently less nutrients were provided to the internal organs, which compromise their developments [2]. In the present study, diet supplemented with 0.2% of cumin seeds did not ameliorate carcass and the selected internal organs weights measured at 49 d of age. In literature, inconsistent results have been reported on the effects of natural [22] and synthetic [6] diet additives used in avian diet on broiler carcass traits and organs yields. In contrast to our results, Ali et al.[12] stated that the negative effect of heat stress on carcass yield was reduced by adding 0.2% of *Cuminum cyminum* during 12 weeks (21-84 d). Also, they recorded a significant improvement for liver, pancreas and proventriculus percentage. However, similar to our results, these researchers have reported no significant effect of cumin feeding on relative weights of heart, gizzard and abdominal fat. It is very likely that the responses of broilers to this feed additive may be influenced by heat stress challenge and also by duration and period of cumin inclusion. In addition, the lack of positive impact on the carcass yield in the present study is probably associated with the absence of differences in body life weight at slaughtering age between the two groups of birds. Indeed, some researchers have reported a strong relationship between live body weight and carcass yield of broiler chickens [23]. Several studies have shown that heat stress can affect intestinal maturation [24]. Marchini et al.[3] have stated a decrease in intestinal length and a reduction in the intestinal absorption capacity of broiler chickens reared under hot climate. In the current study, cumin supplementation did not affect intestine morphometry of birds reared under high ambient temperatures *(Table 4)*. To our knowledge; there were no available data on the effect of cumin on intestine morphometric characteristics when broilers were heat challenged. The most reports on digestive effects of cumin seeds on broiler chickens were carried out under thermoneutral conditions. It has been reported that *Cuminum cyminum* seed improve the absorption of nutrients and reduce time of transit in intestine [21]. Recently, Sharifi et al.[25] failed to detect any improvement in gastrointestinal tract by adding 1.5% of *Cuminum cyminum* in diet of broilers reared under standard conditions. In our study, the lack of enhancement of the intestinal morphology was may be due to the inclusion period of cumin in diet (28 to 49 d) which coincides with the slow development period of intestine. Thus, some researchers have reported that the rapid rate growth of small intestine related to the body weight of broilers occurs earlier at the beginning of the starter phase (1 to 6-10 d of age), and after 10 d post-hatch, this rate begins to slow, determining the end of the rapid development phase [26]. Also, Teixeira et al.[27] have reported that the total length of intestine was influenced by weight gain of broiler but, in the current study, this last parameter remains unchanged by the cumin supplementation. It was well documented that heat stress can perturb the immune system and thus, affect the defense mechanisms of poultry [2,28] Reduced relative weights of both primary

and secondary lymphoid organs, such as thymus, bursa of Fabricius and spleen have been found under heat stress [28]. Also, it has been reported that changes in dietary ingredients, greatly influences the development of this above-mentioned organs [29]. Our results showed that none of lymphoid organ weights and their relative weights (thymus, bursa, and spleen) were significantly influenced by dietary cumin supplementation *(Table 3)*. In this respect, no effect of cumin supplementation on the relative thymus weight was observed in slow-growing chickens subjected to heat stress [12], whereas, this feed additive increased significantly the relative bursal weight of these chickens strain [12]. Also, in thermoneutral conditions, Alimohamadi et al.[30] failed to obtain any enhancement of immune responses expressed by measuring lymphoid organ weights when birds were fed a diet supplemented with cumin at the level of 0.4%. At higher level of inclusion (0.8%), these authors have recorded higher relative weight of bursa of Fabricius and thymus. In another study, cumin seed was shown to have the potential to stimulate the cellular immunity and increase spleen and thymus weights of normal and immune-suppressed swiss albino mice [31]. These authors demonstrated that the immunomodulatory activity recorded by this spice can be attributed to its flavonoid glycoside compound.

Several metabolic disturbances such as carbohydrates, fat, protein and calcic metabolism were observed when broiler chickens were exposed to heat stress [28]. Also, researchers have reported a decrease in haematological values of heat challenged birds [32]. Our results demonstrated that feed cumin addition greatly influence the plasma nutrients concentration of broiler reared under summer high ambient temperatures *(Table 4)*. The observed anti-hyperglycaemic, antihypercholesterolaemic and antihypertriglyceridaemic properties of cumin were expected, the fact that, some investigations on humans, rabbits and rats have revealed such beneficial effects [33,34]. Also, the contribution of the antioxidants in the form of herbs, spice or synthetic substances to improve the negative effects of heat stress on term of blood metabolites has been shown [28,35]. In termoneutral conditions, Golian et al.[10] and Alimohamadi et al.[30] have not observed any changes of plasma glucose when broilers were respectively fed diet supplemented with 0.2, 0.4, 0.6, 0.8 and 1% of cumin seed and with 0.4 and 0.8% of cumin essential oil. However, other researchers [33] have shown that glycaemia in diabetic or normal rats and normal rabbits may be lowered by cumin inclusion. This hypoglycaemic response was attributed to the enhancement of insulin sensitivity by protecting pancreatic beta cells integrity, improvement of serum insulin content [33] and/or to the regulation of glucose homeostasis by increasing peripheral utilization of glucose, increasing hepatic glycogen synthesis or decreasing the glycogenolysis. Furthermore, similarly to our results, the hypolipidaemic and hypocholesteroleamic properties of *Cuminum cyminum* in form of seeds, essential oil and/or extracts components have also been reported previously in human, rabbits, turkeys and rats [36,37]. In broiler, Al Kassi [11] have reported the effectiveness of adding 1 and 1.5% of cumin seeds to commercial strain chickens diet and maintained at thermoneutral conditions in reducing plasma cholesterol and triglycerides. In another study, the inclusion of 0.8 g of cumin essential oil per 1 kg of broiler diet have not change triglycerides level, whereas, cholesterol concentration was significantly reduced [38]. In contrast to our results, slow-growing chicks subjected to heat stress, have showed no significant effects of dietary cumin seeds supplementation on plasma cholesterol concentration [12]. Also, an insignificant difference in blood triglycerides and cholesterol levels has been reported in broilers fed diets supplemented with cumin seeds at 0.4 and 0.8% compared with no supplemented birds reared under standards conditions [30]. Several mechanisms by which cumin can lower blood cholesterol and triglycerides concentrations were suggested. Some published researches have reported that the active compound (flavones) of *Cuminum cyminum* can activate the antioxidant enzymes (superoxide dismutase and catalase) [39] which can reduce the rate-limiting enzyme for cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase: HMG-CoA reductase) [40]. Also, presence of sterol in *Cuminum cyminum* seeds can increase bile acids production and lipase activity [41]. The triglycerides lowering effect induced by cumin was associated with the unsaturated fatty acids of this spice [42]. Birds'plasma protein profile can be modulated by heat stress [32]. In the present study, total plasma proteins concentration decreased significantly in the blood of cumin supplemented birds reared under hot summer temperatures *(Table 4)*. This result was inconsistent with those reported by Ali et al.[12] who showed no significant variation of plasma total protein of slow-growing chickens subjected to heat stress. Also, under thermoneutral conditions, plasma total proteins concentration was not affected by dietary cumin inclusion [30,38]. It is well established that during heat stress, immune responses require specific nutrients needs and the reduction in nutrients availability due to reduce feed intake result in redistribution of body resources to detriment of growth requirements. However, the plasma total protein results lead us to suppose that cumin supplemented broilers expressed less stress, since as we have previously mentioned and discussed, cumin feeding have reduced feed intake without affecting growth, which may mean that proteins were rather used for growth than for immune responses. In this respect, Ali et al.[43] have shown that the addition of cumin seed at 0.2% to a low energy diet of broilers has significantly decreased plasma total protein and reduce numerically globulin values, but did not influence growth performances. Thus, these authors have concluded that this spice reduces immune cost for a more efficient use of proteins in growth and supposed that cumin can protect proteins from free radical injuries. In the present study, calcium concentration showed a significant

increase by diet cumin supplementation *(Table 4)*. This result agrees that reported for broiler chickens [44] and for Turkey poults [45] reared under standard environmental conditions. In the best of our Knowledge there is no study available concerning the effect of *Cuminum cyminum* seed on calcium profile of broilers under heat stress. In this study, the plasmatic concentration increase of calcium, lead us to suggest an enhancement in absorption and digestibility of this mineral element by cumin feeding. Also, Pradeep et al.[46] have reported that cumin seeds are good source of minerals. In same trend of our results, no significant variation was observed on blood haematology by dietary cumin of broiler chickens reared under hot [12] and thermoneutral [30] conditions. However, the deleterious effect of cumin on haematopoiesis by decreasing haemoglobin concentration, red blood cells number and percentage of haematocrit was reported when high level of *Cuminum cyminum* 1.5% [11] was included in broiler diet.

A rise in ambient temperature was generally accompanied with decreased in the level of triiodothyronine (T3) and tetraiodothyronine (T4) hormones in response to a reduction on thyroid activity in order to reduce internal heat production of broilers [47]. In our study, there were no significant effects of *Cuminum cyminum* seeds supplementation on plasma T3 and T4 concentrations and on T3:T4 ratio *(Table 4)*. Up to now no studies have reported the effects of *Cuminum cyminum* seeds on broiler blood thyroid hormones whether in thermoneutral or heat stress conditions. Also, limited numbers of animal studies on the effects of some feeding spice products and medicinal herbs acting as an antioxidant on thyroid hormones were reported and discrepant results were obtained [48,49].

The supplementation of broiler chicken's diet with *Cuminum cyminum* seeds at a level of 0.2% enhanced broiler feed efficacy during the summer and concomitantly, alleviated the negative effects of chronic heat stress on glycaemia, lipidaemia and calcaemia of broilers. However, this feed additive did not affect blood haematology, carcass and viscera yields, and thyroid hormones concentrations. The mechanisms of action of this medicinal spice need more clarification.

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Research Article

Caecal Bacterial Populations and Growth of Broiler Chickens Fed Diets with Different Particle Sizes and Forms

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Abstract

The main objective of the present study was to evaluate the effects of feed particle size, form and also pellet quality on intestinal pH, population of beneficial bacteria and pathogenic bacteria and performance of broiler chickens during growth period. Chicks were fed the same starter ration, but at day 11 of age, chickens were randomly assigned to eight treatments and four replicates and fed dietary treatments until day 28 of age as grower diet. Dietary treatments were as: pellet diets with (1.8 and 2.2 mm) or without dust, graded diets (0.78 and 1.27 mm) and mash diets (2 and 3 mm diameter), poor quality pellet and high quality pellet with die diameter of 1.8 and 2.2 mm. The lowest pH of duodenal and ileal contents was for pellet 2.2 mm and mash 3 mm, respectively (P<0.05). In mash diet, chicks fed the particle size of 3 mm had 7.3% greater the relative weight of gizzard than those fed particle size of 2 mm. Daily feed intake and daily weight gain of chicks were the highest in pellet diets and the lowest in graded diet 1.27 mm. The worse feed conversion ratio was for chicks fed mash 3 mm and graded diet 1.27 mm (P<0.05). There were no differences (P>0.05) among treatments for bacterial populations, except *Escherichia coli* population. The highest *Escherichia coli* population was found in high quality pellet 1.8 mm and poor quality pellet 1.8 mm and lowest one was found in mash 2 and 3 mm (P<0.05). It was concluded that both feed form and particle size may influence on the gizzard weight and the pH of duodenal and cecal contents and *Escherichia coli* population in the cecal of broiler chicks. High quality pellet 2.2 mm resulted in better feed conversion ratio, although the *Escherichia coli* population was the highest in the cecal content of these chickens.

Keywords: *Broiler chicken, Bacterial population, Feed form, Particle size, Performance*

Değişik Boy ve Formda Diet İle Beslenen Broiler Tavuklarda Büyüme ve Sekum Bakteri Popülasyonu

Özet

Bu çalışmanın amacı broiler tavuklarda büyüme periyodu süresince verilen yemin partikül büyüklüğü, formu ve aynı zamanda pelet kalitesinin bağırsak pH'sı, yararlı ve zararlı bakteri popülasyonları ve performans üzerine etkilerini araştırmaktır. Civcivler aynı başlangıç yemi ile beslendikten sonra 11. günde rastgele olarak dörder tekrar olmak üzere sekiz uygulama grubuna ayrıldı ve 28. güne kadar farklı büyüme yemleri ile beslendi. Yem uygulamaları şu şekilde gerçekleştirildi: tozlu veya tozsuz pelet diyet (1.8 ve 2.2 mm), dereceli diyet (0.78 ve 1.27 mm) ve lapa diyet (2 ve 3 mm çapında), 1.8 ve 2.2 mm çapında düşük kaliteli pelet ve yüksek kaliteli pelet. En düşük duodenum ve ileum içeriklerin pH değerleri sırasıyla 2.2 mm pelet ve lapa 3 mm için bulundu (P<0.05). Lapa diyette 3 mm partikül boyutu ile beslenen civcivler partikül büyüklüğü 2 mm olan ile beslenenlerle karşılaştırıldığında %7.3 daha fazla taşlık ağırlığına sahipti. Civcivlerin günlük yem tüketimi ve günlük ağırlık kazanımı en yüksek olarak pelet diyette gözlemlenirken en düşük 1.27 mm dereceli diyette tespit edildi. En kötü yem dönüşüm oranı lapa 3 mm ve 1.27 mm dereceli diyetle beslenenlerde gözlemlendi (P<0.05). *Escherichia coli* hariç bakteriyel popülasyonlar açısından uygulamalar arasında fark bulunmadı (P>0.05). En yüksek *Escherichia coli* popülasyonu yüksek kaliteli 1.8 mm pelet ve düşük kaliteli 1.8 mm pelet ile tespit edilirken en düşük değer lapa 2 ve 3 mm ile bulundu (P<0.05). Yem formu ve partikül boyutunun taşlık ağırlığı, duodenum ve sekum içerikleri ve sekum *Escherichia coli* popülasyonu üzerine etki yapabileceği sonucuna varıldı. Yüksek kaliteli 2.2 mm pellet daha iyi yem dönüşüm oranına neden olurken sekumdaki *Escherichia coli* popülasyonu bu grupta en yüksek olarak tespit edildi.

Anahtar sözcükler: *Broiler tavuk, Bakteriyel popülasyon, Yem formu, Partikül boyutu, Performans*

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INTRODUCTION

Particle size and form of feed and feed form quality with an important role in broiler nutrition has long remained the basic paradigm of feed manufacturers. From a bird's view, a coarse feed particle without dust is instinctive preference. From a feed manufacturing perspective, smaller feed particle size is preference to improve efficiency and uniformity of mixing, reduce ingredient segregation, and improve performance [1,2]. From a nutritional perspective, smaller feed particle size could increase the relative surface area, improve digestibility, and reduce selective feeding by the bird. From an alternative nutritional perspective, larger particle size could promote gizzard development and activity, longer ingredient retention in the gizzard, more uniformity of particles when pass to the small intestine, and consequently improve digestibility [1,3]. There is a discrepancy like particle size for feed form among different perspectives.

During recent two decades, many papers published concerning the effects of feed particle size and form on performance, nutrient availability, gizzard development, gut morphometry, gizzard or stomach Salmonella population in poultry or pigs, but their effects on population of beneficial and common pathogenic bacteria in broilers has been limited studied. It has been demonstrated that pigs fed finely ground compared with those fed coarsely ground feed had a higher incidence of *Salmonella* [4]. Furthermore, pelleted feed increases the risk of *Salmonella* infection in pigs compared with mash feed [5,6]. In poultry, it was demonstrated that enhance in gizzard activity by large particle size could increase digesta retention time [7] and consequently changed the colon pathogenic bacteria [8].

The main objective of the present study was to evaluate the effects of feed particle size, form and also pellet quality on intestinal pH, population of beneficial bacteria (*Bifidobacterium* and *Lactobacillus)* and pathogenic bacteria *(Clostridium perfringens* and *Escherichia coli)* and performance of broiler chickens during growth period.

MATERIAL and METHODS

Chicks used in this study received human care based on criteria outlined in the Guide for the Care and Use of Laboratory Animals [9] published by the National Institutes of Health in the United State, and the experimental protocol was approved by the Research Committee of Islamic Azad University, Science and Research Branch (19.02.2015; 190215).

A total of 384 chicks 1-day-old male broiler chicks (Ross 308) were obtained from a local hatchery. Chicks were divided in floor pens (100 cm × 90 cm) covered with new wood shavings and raised under environmentally controlled conditions and lighting program based on Ross 308 broiler guides. The chicks had *ad libitum* access to feed and water. Feeders were shaken once per day to prevent variation in feed intake. Chicks were individually weighed, and assigned to pens (12 birds per pen) so that the average weight per pen was similar. Broiler diets were formulated and manufactured according to suggested requirements of Ross 308 broilers.

Chicks were fed the same pre-starter and starter rations, but at day 11 of age, chickens were randomly assigned to eight treatments and four replicates and fed dietary treatments until day 28 of age as grower diet period *(Table 1)*. Dietary treatment were as: mash diet with dust screened at 1.8 and 2.2 mm; mash diet without dust screened to have particles with diameter 0.78 and 1.27 mm; poor quality pellet (75% crumbles and 25% dust) with die diameter of 1.8 and 2.2 mm and high quality pellet with die diameter of 1.8 and 2.2 mm. Corn grain and soybean meal were ground with a hammer mill equipped with two screens and blended in mixer to produce the mash diets. For pelleting, grounded mixed particles were conditioned at 85°C for 45 s and pelleted with a ring die pellet set. In the initial and day 28 of the experiment, feed intake and live body weight of chickens were recorded, and then feed conversion ratio was calculated with considering the weight of dead chickens.

At day 25 of age, two chicks per replicate were randomly chosen, weighed, and euthanized by cervical dislocation. Immediately, the gastrointestinal tract was eviscerated and the pH of different parts of small intestine was quickly recorded by inserting the pH meter directly into the digesta. The gizzards were opened, the contents were removed, weighed and expressed as a percentage of body weight. Also, caecal contents of these chicks (two chicks per each replicate) were collected, pooled and a sample was used to assay beneficiary bacteria (lactic acid bacteria and Bifidobacteria) and pathogen bacteria *(E. coli.* and *Clostridium perfringens)*. The populations of bacteria were estimated as the log 10 of colony forming units (CFU) per gram of content.

One gram of each sample was tenfold serially diluted (10^{-1} to 10^{-9}) in 0.9% sterile bacteriological peptone diluents. *Bifidobacteria* and *Lactobacillus* were cultured on MRS agar in anaerobic and aerobic condition, respectively. Anaerobic cultures jar was used for creating anaerobic conditions. *Clostridium perfringens* was cultured on SPS agar in anaerobic cultures jar. *E. coli* and other C*oliforms* were counted by EMB agar (*Eosin methylene blue)* and *Mac Conkey agar.* All commercial media (Merck, Dusseldorf, Germany) were rehydrated in distilled water. The agar media were sterilized by autoclaving at 121°C for 15 min. All the cultured samples were incubated at 37°C after 48 h, the plates containing 25 to 250 colonies were enumerated and recorded as CFU/g of sample. The number of replicate samples was three, and the experimental program was repeated twice.

Table 1. *Ingredient composition and nutritional values of the experimental diets fed during three phases*

Ingredients (%)	Pre-starter (days 1-7 of age)	Starter (days 8-21 of age)	Grower (days 22-28 of age)
Corn	55.20	59.10	62.00
Soybean meal	38.00	34.44	30.884
Soybean oil	2.200	2.350	3.350
Calcitic limestone	0.950	0.900	0.850
Dicalcium phosphate	1.950	1.800	1.670
Salt	0.266	0.250	0.240
Sodium bicarbonate	0.334	0.360	0.355
DL-methionine	0.255	0.175	0.175
L-lysine	0.345	0.225	0.220
Vit and Min Supp.[1]	0.500	0.400	0.400
Total	100	100	100
Chemical composition			
Metabolizable energy (kcal/kg)	2945	3000	3120
Crude protein (%)	22.05	20.75	19.35
Calcium (%)	0.95	0.90	0.87
Available phosphorus (%)	0.47	0.45	0.42
Methionine (%)	0.58	0.48	0.47
Sulfur amino acids (%)	0.93	0.82	0.80
Lysine (%)	1.47	1.28	1.20
Sodium (%)	0.22	0.21	0.20
Chlorine (%)	0.20	0.20	0.19
Linoleic acid (%)	2.45	2.60	3.15
Potassium (%)	0.85	0.81	0.76

[1] *Vitamins and minerals supplement (per kg feed): pantothenic acid 12.8 mg, folic acid 1.25 mg, B.H.T 2.5 mg, biotin 0.125 mg, choline chloride 800.0 mg, niacin 38 mg, Vit-A 13.000 IU, Vit-D_3 2.500 IU, Vit-E 30.0 mg, Vit-K 2.5 mg, Vit-B_1 2.5 mg, Vit-B_{12} 25mg, Vit-B_2 5.5mg, Vit-B_6 5.0 mg, Copper 12.5 mg, Iron 62.65 mg, Iodin 0.025 mg, Manganese 68.0 mg, Selenium 0.23 mg , Zinc 69.0 mg*

At first, the normality of data distribution was checked using the Kolmogorov-Smirnov test. The Statistical analysis was done with ANOVA of SAS using GLM procedure for Windows version 14.1 [10]. Means were separated by Tukey test at P≤0.05.

RESULTS

As shown in *Table 2*, significant differences exist among treatments for pH of duodenum and cecum contents, but there was no significant effects of feed forms or feed particle size on pH of jejunum and ileum contents. The lowest pH of duodenum and ileum contents was for high quality pellet 2.2 mm and mash 3 mm, respectively.

The effects of dietary treatments on the relative weight of gizzard and performance of broilers on day 28 of age are presented in *Table 3*. The relative weight of gizzard was influenced by the form of diet. Chicks in mash 3 mm had higher gizzard weight than other treatments. Chickens fed pellet had the lowest gizzard weight. Chicks fed graded

Table 2. *The effect of mash form and particle size on the pH of different section of broilers intestine*

Treatments	Duodenum	Jejunum	Ileum	Cecum
Mash 2 mm	6.12^{a}	6.59	7.11	5.97^{ab}
Mash 3 mm	6.13^{a}	6.52	7.09	5.93^{b}
Good quality pellet 1.8 mm	5.55^{bc}	6.13	6.75	6.18^{a}
Good quality Pellet 2.2 mm	5.41^{c}	6.10	6.72	6.20^{a}
Poor quality Pellet 1.8 mm	6.03^{ab}	6.31	6.93	6.10^{ab}
Poor quality pellet 2.2 mm	6.12^{a}	6.28	6.75	6.17^{ab}
Graded 0.78 mm	6.14^{a}	6.75	7.10	6.08^{ab}
Graded 1.27 mm	6.17^{a}	6.54	7.15	6.15^{ab}
SEM	0.112	0.147	0.124	0.048
P value	0.005	0.0605	0.0876	0.006

Means within a column with different superscripts are significantly different (P<0.05)

diet had higher gizzard weight than poor quality pellet, but lower than those fed mash diet. Regardless of diet form, the gizzard weight of chicks fed different particle size was not significant, but significant difference appeared between mash 1.8 and 2.2 mm. In mash diet, chicks fed the particle size of 3 mm had 12.5% greater relative weight of gizzard than those fed particle size of 2 mm. Daily feed intake and daily weight gain of chicks were the highest in pellet diets and the lowest in graded diet 1.27 mm. The worse feed conversion ratio was for chicks fed mash 3 mm and graded diet 1.27 mm.

The effects of dietary treatments on bacterial populations on day 25 of age are presented in *Table 4*. There were no significant differences among treatments for bacterial populations, except *Escherichia coli* population. The highest *Escherichia coli* population was found in high quality pellet 1.8 mm and poor quality pellet 1.8 mm and lowest one was found in mash diets 2 and 3 mm. Both mash diets have the highest counts of Lactobacillus and Bifidobacterium.

DISCUSSION

In this study, pH of duodenum was the highest in chicks fed mash and graded diets and the lowest in good quality pellet 2.2 mm. As stated by Huang et al.[11] mash diets are retained for longer period in the gizzard of broilers as compared with pelleted diet. Hence, mash and graded diets require further grinding and gizzard muscular activity. The higher activity of gizzard stimulates the proventriculus and increases in the secretion of hydrochloric acid. Thus, the pH of the duodenum in broilers fed mash, graded and poor quality pellet diets is lower compared with those fed pelleted diets as this result is in agreement with the finding of Engberg et al.[12]. Our finding is in contrast to the report of Dahlke et al.[13] who concluded that proventriculus pH and intestinal pH were not affected by different particle sizes.

In the jejunum and ileum of chicks, pancreatic and bile secretion influence on pH of digesta and no difference observed among treatments. The bacterial activity could

Table 3. *The effect of mash form and particle size on the relative weight of gizzard and performance parameters of broilers during grower period*

Treatments	Relative Gizzard Weight (g)	Body Weight Gain (g/day)	Feed Intake (g/day)	Feed Conversion Ratio
Mash 2 mm	1.89^{b}	31.6^{bcd}	41.5^{ab}	1.313^{ab}
Mash 3 mm	2.04^{a}	29.3^{d}	38.7^{bc}	1.320^{a}
Good quality pellet 1.8 mm	1.19^{d}	36.1^{a}	43.1^{a}	1.192^{cd}
Good quality Pellet 2.2 mm	1.22^{d}	36.0^{a}	42.7^{a}	1.187^{d}
Poor quality Pellet 1.8 mm	1.36^{c}	33.0^{b}	41.4^{ab}	1.256^{abcd}
Poor quality pellet 2.2 mm	1.42^{c}	32.7^{bc}	41.5^{ab}	1.269^{abc}
Graded 0.78 mm	1.77^{b}	30.0^{cd}	37.0^{cd}	1.235^{abc}
Graded 1.27 mm	1.82^{b}	26.1^{e}	34.5^{d}	1.323^{a}
SEM	0.028	0.93	0.83	0.024
P value	0.001	0.006	0.005	0.008

Means within a column with different superscripts are significantly different (P<0.05)

Table 4. *The effect of mash form and particle size on the bacteria population (log_{10} cfu/g) in the cecum of broilers during grower period*

Parameter	Lactobacilus	Bifidobacterium	Colostridium	*Escherichia coli*	Coliforms
Mash 2 mm	9.176	8.824	3.853	8.068^{c}	8.927
Mash 3 mm	9.246	8.728	2.606	8.281^{bc}	8.629
Good quality pellet 1.8 mm	8.920	8.042	2.201	10.091^{a}	9.493
Good quality Pellet 2.2 mm	8.795	7.938	2.634	9.704^{a}	9.215
Poor quality Pellet 1.8 mm	9.182	8.188	3.460	10.108^{a}	9.303
Poor quality pellet 2.2 mm	9.188	8.033	2.068	9.553^{ab}	9.508
Graded 0.78 mm	9.477	8.367	2.514	8.904^{abc}	8.609
Graded 1.27 mm	9.437	8.289	1.515	9.283^{abc}	9.545
SEM	0.0655	0.0858	0.3059	0.1701	0.1163
P value	0.081	0.116	0.195	0.008	0.109

Means within a column with different superscripts are significantly different (P<0.05)

change the pH of the cecum content, as in *Table 1* was seen, mash diet with coarse particle size caused a decrease and good quality pellet diet caused an increase in the pH of cecum contents. The availability of nutrient from mash diet in the cecum resulted in a non-significant increase in the lactobacillus population and consequently declined the pH of content. In the present study, the form and particle size of diet influenced on the gizzard weight. Chicks fed good and poor quality pellet diets had lower gizzard weight as compared with mash and graded diets. In line with our finding, several authors [12,14] reported lower gizzard weights in broiler chickens fed the pelleted diet. Lower gizzard weight was also attributed to low coarse particles in pelleted diets [3]. Regardless the diet form, gizzard weight numerically increased as particle size increased in the present study. Mash diet with coarse particle size needs higher muscular activity for grinding, and more activity causes an increase in the weight and size of gizzard. Of course, dietary form had higher impact than dietary particle size on the relative weight of gizzard. Our result is in agreement with finding of Nir et al.[14] who reported that broilers were fed diet with particles in coarse and medium sizes, the gizzard weight increased as compared with the chicks fed fine particles. The gizzard has an important and unique role in the gastrointestinal tract of broilers, as it acts a mechanical grinder for reduction of dietary particle size [15] which effectively resulted in gastrointestinal motility [16]. The gizzard weight has a positive broiler growth response, as Parsons et al.[17] reported an increase in mash feed particle size and coarser diet, chicks had the largest gizzard weight and the highest weight gain. In this study, differences for gizzard weight among particle size in each feed form was not significant, but Amereh et al.[1] reported significant differences. In the study of Parsons et al.[17], the chickens fed the coarse corn mash diets had greater absolute and relative gizzard weights as compared with the chickens fed the fine corn mash diets.

One of strategy for development of gizzard is manipulation of diet as mentioned previously by Nir et al.[14] and Engberg et al.[8] Graded feed in this study was a mash diet with the uniform particle size and compare with pellet diets resulted in greater gizzard weight, but its effect was lower than mash diet. The existence of large uniform particles in graded diet resulted in longer retention of feed in the gizzard [7], the development and activity and consequently the weight of gizzard [1,3]. As seen in *Table 2* and *Table 4*, a higher relative weight of gizzard resulted in a decrease in the population of *E. coli* that support the finding of Bjerrum et al.[18], thus may be reduce the risk of enteric diseases as speculated by Engberg et al.[8] and Bjerrum et al.[18].

Broilers fed pellet diet had the highest feed intake and body weight gain but had the lower feed conversion ratio. The result of this study is inconsistent with finding of Amerah et al.[1] who reported birds fed pellet diets showed lower feed conversion ratio than those fed mash diets. In contrast to feed intake of chicks in this study, Parsons et al.[17] reported no difference in feed intake when birds were fed mash versus pellet diets. In agreement with our study, Lemme et al.[19] indicated that pellet diets of good quality had the highest weight gain than poor-quality pellet feed. In the present study, chickens fed mash diet had lower feed intake and feed conversion ratio. The differences may be attributed to the particle size in mash and pellet diets, the passage rate of digesta and the particles distribution. Amerah et al.[1] demonstrated that large particles in mash diets promoted broiler performance and pelleted diets had controversial results on performance.

An increase in the beneficial bacteria population and a decrease in the pathogenic bacteria population or both is a favorite strategy for broiler industry. In this study, the dietary form had higher impact on the bacterial population than particle size. Diets with larger particle sizes have an indirect effect on reducing enteric disease problems in broilers. These diets may alter the microbial populations of the gastrointestinal tract. The gizzard development stimulated through increase in grinding activity, which leads to an improvement in gut motility [20]. Gizzard activity also increases the secretion of hydrochloric acid, which ultimately reduces the pH of intestinal contents. This event, in combination with the increased retention time of feed in the gizzard, has an antimicrobial effect. When pathogenic bacteria such as *Escherichia coli* entered to the gastrointestinal tract, they inactivated by the highly acidic media of intestine [14,20]. Therefore, the gizzard acts as a barrier in reducing the entrance of pathogenic bacteria to the distal intestinal tract [8].

In this study, lactobacilli populations were numerically higher in mash diets than pellet diets. The finding of this study was inconsistent with the report of Engberg et al.[12]. An increase in lactobacilli populations is usually considered to be beneficial to the host, because they can prevent colonization of diarrhea-causing pathogens such as *E. coli* [8,21,22]. Engberg et al.[8] compared coarse or finely ground mash or pelleted feed and reported that there was an increase in lactobacilli populations in the ceca and rectum when birds were given coarse mash diets, with the lowest counts of lactic acid bacteria in birds given finely ground pellets. There was no additional study concerning the effect of feed form and particle size on bacterial population in the intestine of broiler chickens.

Both feed form and particle size influence on *Escherichia coli* population in the cecal of broiler chicks. *Escherichia coli* population was the highest in the cecal content of chickens fed high quality pellet diets. This finding is in agreement with study of Engberg et al.[8], who observed that broilers fed pelleted diets presented higher coliform and enterococci counts in the ileum and lower lactobacilli and *Clostridium perfringens* numbers in the cecca and rectum compared with mash diet.

The findings of this study suggest that both feed form and particle size may influence on the gizzard weight and the pH of duodenal and cecal contents and *Escherichia coli* population in the cecal of broiler chicks. Based on the results of this study, high quality pellet resulted in better feed conversion ratio, although the *Escherichia coli* population was the highest in the cecal content of chickens. The use of good quality pellet with fine particles could significantly improve the broiler performance and health.

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Research Article

Effects of Birth Type on Growth, Fattening Performance and Carcass Characteristics in Honamlı Male Kids [1] [2]

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Abstract

The aim of this study is to determine fattening performance, slaughter and carcass characteristics of single and twin male Honamlı kids raised under semi- intensive conditions. In the study, 90 day-olds (weaned at the age of 75 days and the 15-day period of adaptation to feeding), 10 single and 10 twin Honamlı male kids were fattened for 56 days and all kids were sent for slaughter at the end of the fattening period. Final average live weights were found to be 34.4 kg and 30.4 kg for single and twin kids. Average daily live weight gain during fattening was 203 g for single kids and 231 g for twin kids. Hot carcass weights obtained after slaughter were 14.47 kg and 12.43 kg; dressing percentages calculated based on empty body weight were determined to be 52.46% and 51.11% for single and twin kids, respectively (P>0.05). Cold carcass weights were determined to be 14.19 kg and 11.17 kg for single and twin kids, respectively; cold carcass yields calculated based on empty body weight were determined to be 51.53% and 50.04% for single and twin kids. The surface areas of M. longissimus dorsi (MLD) were found to be 13.39 cm2 and 12.43 cm2, respectively. Withers height, rump height, body length, chest girth, and nose length of single kids were relatively higher compared to twin kids at the end of the fattening period.

Keywords: *Honamlı male kids, Type of birth, Fattening, Slaughter, Carcass*

Honamlı Erkek Oğlaklarında Büyüme, Besi Performansı ve Karkas Özelliklerine Doğum Tipinin Etkisi

Özet

Çalışmanın amacı, yarı entansif koşullarda yetiştirilen Honamlı tek ve ikiz erkek oğlaklarının besi performansı ile kesim ve karkas özelliklerinin belirlenmesidir. Çalışmada 90 günlük yaşta (75. günde sütten kesim yaşı ve sonrasında 15 gün yeme alıştırma dönemi) 10'ar baş Honamlı ırkı erkek tek ve ikiz oğlak 56 gün süreyle besiye alınmış ve besi sonunda tüm oğlaklar kesime sevk edilmiştir. Besi süresi sonunda ise canlı ağırlıklar tek ve ikiz oğlaklar için 34.4 kg ve 30.4 kg olarak tespit edilmiştir. Besi süresince ortalama günlük canlı ağırlık artışı tek doğan oğlaklarda 203 g, ikizlerde ise 231 g olarak gerçekleşmiştir. Kesim sonrasında elde edilen sıcak karkas ağırlıkları da sırasıyla 14.47 kg ve 12.43 kg; boş vücut ağırlığına göre hesaplanan sıcak karkas randımanları tek ve ikiz oğlaklar için sırasıyla %52.46 ve %51.11 olarak tespit edilmiştir. Soğuk karkas ağırlıkları tek ve ikiz oğlaklar için sırasıyla 14.19 kg ve 11.17 kg; boş vücut ağırlığına göre hesaplanan soğuk karkas randımanları ise yine tek ikiz oğlaklar için sırasıyla %51.53 ve %50.04 olarak belirlenmiştir (P>0.05). M. longissimus dorsi (MLD) kesit alanı ise sırasıyla 13.39 cm2 ve 12.43 cm2 olarak tespit edilmiştir. Besi sonunda tek doğan oğlakların cidago yüksekliği, sağrı yüksekliği, vücut uzunluğu, göğüs çevresi ile burun uzunlukları ikiz doğan oğlaklara göre nispeten daha yüksek bulunmuştur.

Anahtar sözcükler: *Honamlı erkek oğlak, Doğum tipi, Besi, Kesim, Karkas*

INTRODUCTION

Honamlı goats are reared in the Mediterranean region throughout the Taurus mountains, especially west part of the region is the popular place for this breed. Nomad, semi-nomad and settled breeders keep the Honamlı for the kid production. Rapid growth rate and bigger body parts are the characteristics of the breed. Honamlı goat

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was officially registered by the Turkish Ministry of Food, Agriculture, and Livestock as an original goat breed of Turkey in the year of 2015.

When it comes to meat yields of farm animals, generally not only the amount of carcass obtained from the animals is considered but also the amount of edible meat on carcasses comes to forefront economically. While the amount and quality of carcass are crucial in terms of meat production, particularly determination of fattening performances is also important economically and for revealing yield capability of animals. Fattening performance is affected by several factors such as species, breed, age, gender, and feeding style [1]. The quality of carcass is one of the critical factors for determining meat yield in animals [2]. Meat production from animals is fundamentally based on revealing carcass characteristics of these animals reaching the maturity for slaughtering by intensively-feeding methods applied by focusing on growth traits of offspring. In addition, the quality which allows meat to be utilized commercially is also significant [3]. This is also valid for goats as well as for all farm animals. In addition to different grazing preference of goats and high adaptation for current environmental conditions, traditional habits and socio-economic structures of communities could be asserted to play a role in preference of consumption of goat meat [4]. Due to similar raising conditions, numerous studies have been conducted to reveal differences of goat meat compared to sheep meat. Goat meat containing lesser water than sheep meat and thus having a less tender structure has been found to be really flavorful and preferably by consumers [5]. There has been an obvious increase in consumption of goat meat all over the world in last 20 years [6]. The goal for raising sheep and goats for meat is to reach as much offspring as mother can care for weaning period. This is because lamb and kid meat is the most important outcome of this system. In this situation, multiple births provides the advantage but lambs and kids develop late. Sale of kids is leading in terms of incomes provided for goat breeding [7]. It is very important for kids raised for meat yield be fed sufficiently during the suckling period. By this way, the meat of kids has a more tender structure as a result of rapid adaptation of goats to forage consumption and environmental factors and their rapid development [8]. Survival rate of born kids, therefore, ensuring low mortality levels in the flock require to perform regularly management-feeding, health controls applied to kids [9].

As a tradation, twin kids are not very wellcome among the extansive and semi-entansive goat breeders in the region. Even they want to rear twin kids as single and sparate one of them just afeter bird. In the raelity twin kids can produce more calibro at the end of weaning and they can find time to compensate their low birth weight until slaughter time. Twin birth rate of the investigated flocks were 63.8% and 61.6% in the years of 2014 and 2015 respectively [10]. It is important to show the capability of twin born kids for the slaughter characteristics, in order to persuade the breeders to keep their twin kids in the flock. In this study, the effect of birth type (single-twin) on growth and carcass characteristics of Honamlı kids was examined in order to evaluate this concept.

MATERIAL and METHODS

The age of 90 days (weaned at the age of 75 days and the 15-day period of adaptation to feeding), 10 single and 10 twin Honamlı male kids were fattened for 56 days in order to determine growth, fattening, and carcass characteristics of Honamlı kids. Concentrated feed containing 15% raw protein and 2800 Kcal ME per 1 kg was given ad-libitum for the kids during the stated fattening period in addition to grazing in the pasture. Live weights of the kids were measured every week during fattening period. Some morphological body measurements (withers height, rump height, body length, chest girth, and nose length) of the kids were also taken at the beginning, middle, and end of fattening period. After fattening period, the kids were sent for slaughter. Live weights of the kids were determined before slaughter. Head, feet, skin, and internal organs were resected and weighed after bleeding process and the hot carcass was obtained. Hot carcass yield was also calculated based on empty body weight as well as live weight before slaughter. Cold carcass yields and chilling loss occurring in the course of holding time were calculated from these measurements with respect to pre-slaughter live weight and empty body weight of carcasses that were kept in cold storage at +4ºC for 24 h. Then, some measurements (carcass length, leg length, buttock and chest girth) were taken from hung carcasses in such a way to compatible with the reports by Fisher and De Boer [11] and Caneque et al.[12].

Following carcass measurement, chilled carcasses were split into left and right halves along the vertebral column. When measuring weights of carcass parts, left half of carcass was separated into a total of 5 parts including shoulder, neck, flank, ribs, and long leg according to the method reported by Colomer-Rocher et al.[13]. Weights and propertions of every mentioned pieces were recorded. The region between 12-13th costa was utilized for determining surface area of M. Longissimus dorsi (MLD). The surface area of MLD was drawn onto tracing paper as was indicated by Akbaş and Saatcı [14] and transferred into computer environment. Then, surface area of MLD was determined by using Autocad drawing program [15]. Thickness of back fat was calculated by measuring by using digital caliper on the same surface.

Project has been approved by Mehmet Akif Ersoy University Local Ethical Committee on Animal Experiments (6.9.2012, meeting number: 1, resolution number: 6).

The aim of the statistical analyses was to compare the twin

and single groups at the same age to see the differences between two birt type. In this condition, the only reached carcass parameters are important in terms of production aspect. Therefore, two-sample T test was applied by using Minitab [16] statistical software in order to compare slaughter and carcass characteristics of the contemporary kids.

RESULTS

Table 1 shows initial and final average live weights and mean scores of fattening performances at different periods of fattening for 10 single and 10 twin born Honamlı male kids fattened for 56 days in the study. When *Table 1* was examined, mean initial live weights of single and twin kids approximately at 90 days of age were 23 kg and 17.53 kg, respectively. Final live weights were determined to be 34.4 kg and 30.4 kg for single and twin kids. Average daily live weight gain during fattening was 203 g for single born kids and 231 g for twins. Compared to single born kids, twin kids were observed to reach higher values in terms of live weight gain between 0-28th and 28-56th days of fattening. Daily concentrated feed consumption of single and twin kids during fattening increased after 28 days of fattening and was detected to be 500 g and 460 g between 0-56th days, respectively *(Table 1)*.

Table 2 shows some morphological body measurements of Honamlı kids at different periods of fattening since the initiation of fattening. Body measurements of single born kids were relatively greater than twin born kids, only the differences indicated for body length at the beginning of fattening process were statistically significant (P<0.05).

Table 3, Table 4, and *Table 5* showed slaughter and carcass

Table 1. *Birth weight, fattening performance and average daily feed intake (concentre) of Honamlı male kids at different periods (X±Sx)*

Traits		Single	Twin	P
No. of observations		10	10	
Birth weight (kg		4.8±0.23	4.1±0.21	0.05
Initial Age (90 days)		89.2±2.4	90.9±1.22	0.53
Initial live weight (kg)		23.0±5.22	17.53±4.14	0.01*
Final live weight (kg)		34.4±2.00	30.4±1.50	0.13
Average Daily Gain (g)	0-28 days	231.0±9.14	275±18.06	0.04*
	28-56 days	176.0±16.00	188±17.10	0.63
	0-56 days	203.0±12.01	231±8.12	0.07
Average Daily Feed (concentre) Intake (g)	0-28 days	486	441	
	28-56 days	513	479	
	0-56 days	500	460	

Table 2. *Some morphological body measurements of Honamlı kids at different periods (X±Sx)*

Traits		Single	Twin	P
Withers Height (cm)	90th day	62.2±1.1	59.1±1.2	0.06
	120th day	68.0±0.9	66.3±0.9	0.23
	146th day	71.6±1.0	69.1±1.0	0.10
Rump Height (cm)	90th day	63.1±0.9	60.0±1.2	0.06
	120th day	68.4±1.0	66.7±1.1	0.26
	146th day	71.7±0.9	69.3±0.8	0.07
Body Length (cm)	90th day	59.3±1.6	53.5±1.4	0.01*
	120th day	63.1±1.3	60.6±1.2	0.17
	146th day	70.0±1.0	69.3±1.3	0.67
Chest Girth (cm)	90th day	61.4±1.3	56.4±1.4	0.02
	120th day	66.4±1.2	63.2±1.3	0.09
	146th day	70.9±1.2	69.0±1.4	0.32
Nose Length (cm)	90th day	19.5±0.3	19.7±0.4	0.70
	120th day	21.6±0.4	21.1±0.4	0.39
	146th day	22.7±0.4	22.4±0.4	0.64

Table 3. *Some slaughter and carcass characteristics of Honamlı male kids (X±Sx)*

Traits	Single	Twin	P
Slaughter weight (kg)	33.29±1.12	28.92±1.43	0.025*
Empty body weight (kg)	27.52±1.08	24.18±0.92	0.041*
Hot carcass weight (kg)	14.47±0.71	12.43±0.62	0.044*
Dressing percentage-1^{DP1}, %	43.37±0.43	42.83±0.83	0.575
Dressing percentage-1^{DP2}, %	52.46±0.41	51.11±0.80	0.159
Head weight (g)	1998.02±27.54	1847.23±38.43	0.109
Four feet weight (g)	1134.41±26.33	960.21±12.23	0.012*
Skin weight (g)	3247.32±21.54	2473.11±27.40	0.006**
Lungs and trachea weight (g)	531.13±20.45	516.23±23.50	0.750
Heart weight (g)	136.05±4.20	115.08±3.75	0.070
Liver weight (g)	698.03±11.05	650.27±15.10	0.128
Spleen weight (g)	65.12±0.85	60.00±0.29	0.538
Full stomach weight (g)	5670.35±59.01	4635.73±44.04	0.018*
Full intestine weight (g)	3282.40±25.72	2989.58±32.10	0.074
Empty stomach weight (g)	1258.09±28.75	1152.43±27.25	0.226
Empty intestine weight (g)	1923.06±22.20	1740.02±42.03	0.076
Internal fat weight (g)	132.61±3.55	60.53±4.98	0.004**

DP1: *Dressing percentage based on slaughter weight;* ***DP2:*** *Dressing percentage based on empty body weight*

Table 4. *Some cold carcass characteristics of Honamlı male kids (X±Sx)*

Traits	Single	Twin	P
Cold carcass weight (kg)	14.19±0.70	11.17±0.83	0.033*
Chilling loss (%)	1.95±0.08	2.10±0.07	0.244
Dressing percentage-1^{DP1}, %	42.60±0.46	41.93±0.82	0.490
Dressing percentage-1^{DP2}, %	51.53±0.48	50.04±0.80	0.133
Right half of carcass weight (kg)	6.68±0.35	5.80±0.34	0.088
Left half of carcass weight (kg)	7.39±0.35	6.21±0.29	0.019*
Shoulder weight (g)	1545.03±93	1327±67	0.074
Flank weight (g)	445.13±25	388.09±24	0.114
Neck weight (g)	756.05±43	556.09±54	0.021*
Ribs weight (g)	2276.23±48	1855.06±61	0.006**
Sirloin weight(g)	1850.33±56	1453.41±44	0.009**
Loin weight (g)	426.11±28	402.21±17	0.470
Long leg weight (g)	2328.29±112	2074.36±104	0.131
Back fat thickness (mm)	0.73±0.05	0.66±0.07	0.380
M. Longissimus dorsi area (cm^2)	13.39±0.47	12.43±0.66	0.353
Carcass Measurements			
Carcass length (cm)	74.35±1.20	70.50±1.10	0.034*
Leg length (cm)	29.30±0.39	29.05±0.61	0.735
Buttock girth (cm)	53.70±0.93	53.26±0.91	0.738
Chest girth (cm)	71.00±1.01	65.26±1.13	0.005**

DP1: *Dressing percentage based on slaughter weight;* ***DP2:*** *Dressing percentage based on empty body weight*

Table 5. *Percentages of the valuable parts and noncarcass components in Honamlı male kids (X±Sx)*

Traits	Single	Twin	P
[1]Percentages (%) of carcass parts			
Shoulder	21.33±0.34	21.05±0.30	0.534
Flank	9.26±0.12	9.00±0.21	0.479
Neck	10.20±0.14	10.03±0.27	0.430
Ribs	27.97±0.65	26.92±0.83	0.354
Sirloin	19.35±0.18	18.52±0.30	0.086
Loin	8.61±0.08	8.39±0.07	0.681
Long leg	31.22±0.42	31.05±0.53	0.418
[1]Percentages (%) of noncarcass parts			
Head	7.65±0.10	7.29±0.06	0.010*
Four Feet	4.13±0.12	4.05±0.07	0.385
Skin	11.78±0.31	10.26±0.24	0.044*
Lungs and Trachea	2.08±0.06	2.14±0.08	0.381
Heart	0.49±0.02	0.47±0.04	0.536
Liver	2.60±0.05	2.72±0.14	0.076
Spleen	0.24±0.02	0.23±0.03	0.795
Internal fat	0.46±0.06	0.24±0.03	0.005**

[1] Percentage based on cold carcass weight

characteristics according to birth type (single and twin) of Honamlı kids. As is seen from relevant tables, live weights of single and twin Honamlı kids before slaughter were found to be 33.29 kg and 28.92 kg, respectively. Hot carcass weights obtained after slaughter were 14.47 kg and 12.43 kg; hot carcass yields calculated based on empty body weight were 52.46% and 51.11% for single and twin kids, respectively. Cold carcass weights were 14.19 kg and 11.17 kg and cold carcass yields calculated based on empty body weight were 51.53% and 50.04% for single and twin kids, respectively. The surface area of M. longissimus dorsi (MLD) was determined to be 13.39 cm^2 and 12.43 cm^2, respectively. As is seen, the highest mean scores in terms of mentioned values were obtained from single born kids and the differences between the groups were not found to be statistically significant for indicated yield values (P>0.05).

As measurements on carcasses of single and twin Honamlı kids were evaluated *(Table 4)*; carcass length, leg length, buttock girth and chest girth values were observed to be 74.35 cm, 29.30 cm, 53.70 cm, and 71 cm for single born kids; respectively. The same values were determined to be 70.50 cm, 29.05 cm, 53.26 cm, and 65.26 cm respectively for twin born kids. However, statistically significant differences were found between single and twin kids only in terms of carcass length and chest girth (P<0.05). There was no statistically significant difference between single and twin kids in terms of proportional values of carcass parts (shoulder, flank, neck, rib, and long leg) (P>0.05).

DISCUSSION

Fattening performances, slaughter and carcass characteristics of single and twin Honamlı kids were examined comparatively. Kids with the same gender (male) were weaned at similar days of age and started fattened with the same forage and concentrated feed. High twinning rates seen in Honamlı goats have brought along to determine if or not birth type had an effect on fattening performance as well as revealing fattening performances of kids. In the study, a statistically significant difference was found between initial average live weights (23 kg and 17.53 kg) of single and twin kids at the same age (P<0.05); this was considered to be associated with birth weights of kids and the amount of milk they sucked.

Even though average daily live weight gain during fattening was relatively higher in twin born kids compared to single born kids and the relevant difference was found to be statistically significant only in the first 28 days of fattening period (P<0.05). Daily live weight gain in the study was found to be relatively higher than the value (197 g) reported by Aktaş et al.[17] for Honamlı kids during 60 days of fattening and considerably higher than values reported by Koşum et al.[18] for various breeds during 56 days of fattening period.

In the study, daily feed consumption of single male kids in the first half of fattening (28 days) was lower compared to the second period. This could be associated with low capacity of digestive system and feed adaptation period

in kids. Additionally, single born kids were observed to consume more daily concentrated feed (500 g) compared to twin kids.

Some morphological body measurements of kids were also determined at various period of fattening in order to reflect their growth performances. Single kids were observed to have higher values compared to twin kids; the reported values (except for chest girth) were found to be relatively higher than those reported by Gök et al.[19] for Honamlı kids and lower (nose length) than reports by Elmaz et al.[20]. Body measurements of kids at various growth periods were found to be higher than different values reported for kids by Alade et al.[21].

When calculating slaughter and carcass characteristics in the study, empty body weight was also considered in addition to pre-slaughter live weight as it was done by numerous researchers [22,23]. It was observed that when single and twin born kids were taken into account in terms of hot carcass yields calculated based on initial live weight, hot carcass yields were between 42.83% - 43.37%, they were 51.11% - 52.46% based on empty body weight and the difference between yields of single and twin kids was statistically insignificant (P>0.05). Similarly, reported that there was no statistically significant difference between single and twin kids in terms of yield values [24]. Hot carcass measurements calculated based on empty body weight were compatible with those reported by Dhanda et al.[25] and Daskiran et al.[26]; higher than those reported by Kor [27] and Pena et al.[28]; and lower than reports by Kebede et al.[23] and Koyuncu et al.[29].

Cold carcass yield values (between 41.93% - 42.60% according to slaughter weight, 50.04% - 51.53% according to empty body weight) determined in the study were compatible with values noted by Daskiran et al.[26], and lower than those reported by Kor [27], Pena et al.[28], Bonvillani et al.[30] and Santos et al.[31]. In addition, cold carcass yield values revealed in the study were observed to be higher than values reported by Gökdal [32].

No statistically significant difference was determined between single and twin kids for thickness of back fat which is important in terms of revealing fat level of carcasses (P>0.05); and twin kids were observed to have relatively lower body fat thickness compared to single kids. Values determined in the study (0.66 mm and 0.73 mm) were found to be higher than values detected by Akbaş and Saatcı [14] for Honamlı kids raised under extensive conditions. Additionally, these were observed to be higher than values reported by Koşum et al.[18], for kids from different breeds; and lower than values reported by Koyuncu et al.[29].

Values (13.39 cm^2 and 12.43 cm^2) determined for surface area of M. longissimus dorsi (MLD) which gives information about the amount of meat on carcass were relatively lower than those reported in the study conducted by Akbaş and Saatcı [14] on the same breed**;** and higher than values reported by Aktaş et al.[17], for Honamlı kids with higher slaughter weight. The current condition is thought to result from genotype of Honamlı breed.

When carcass measurements in the study were examined generally, single Honamlı male kids were observed to have higher values compared to twins. While they were higher than values reported by Kor [27] for kids of Hair and Akkeçi sent for slaughter with about 25 kg of carcass weight, generally had similar quality with reports by Şimşek and Bayraktar [22] for kids of Hair and Saanen X Hair hybrid with a weight of about 35 kg sent for slaughter.

In the study, there was no statistical significance between single and twin male kids in terms of ratios of important carcass parts served for consumption (P>0.05). In addition, ratios of shoulder (21.05% and 21.33%), rib (26.92% and 27.97%) and long leg (31.05% and 31.22%) determined in the study were relatively lower than the reports by Akbaş and Saatcı [14] and Aktaş et al.[17], for long leg ratio in the same breed with similar weight sent for slaughter. Furthermore, values found in the study were lower than values stated by Koşum et al.[18], Daskiran et al.[26], Pena et al.[28] and Bonvillani et al.[30]; and compatible with the values reported by Kor et al.[33] and Atay et al.[34].

A statistically significant difference was observed between single and twin male kids only for head and skin from non-carcass part ratios calculated in the study. This situation is considered to be associated with the differences in initial live weights of kids in both groups. Values of head percentage calculated in the study were generally compatible with values stated by several researchers [22,34], for different breeds. Also, skin percentage determined in the study were found to be higher than the reports by Atay et al.[34] and Koyuncu et al.[29], for Hair goats with various slaughter weights.

With this study, limited fattening and carcass data of Honamlı goats were used. This is important for determining characteristics of the breed. Multiple births which comes forefront in some flocks is an issue required to focus on Honamlı goat breed. These have also been tried to be answered with this study, as well. Having 11.17 kg cold carcass weight from each twin kids can be considered total 22.34 kg carcass and this amont dramatically much more from the carcass (14.19 kg) of single born kids.

No any statistical differences were found in the percentages of carcass parts between single and twins. This is a reflection that if twin kids are fed until at the end of the weaning they can be used a good product for the mentioned breeding sytem.

Live weight gain determined particularly during the last part of fattening period indicated that fattening could

be prolonged and thus heavier carcass and meat yield could be obtained. Also, back fat thickness which was found to be relatively low pointed out that carcass weight could be increased without gaining much fat. In fattening process applied to Honamlı which is a quickly developing breed, birth weight and compensation of weight losses during suckling period in twin kids could be solved by prolongation of fattening period. Trying different fattening periods for this issue namely determination of optimum fattening duration will clearly reveal characteristic of the breed.

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Research Article

Türkiye'de Veteriner Hekim Odalarının Çalışmalarına Yönelik Bir İnceleme: Kars Bölgesi Veteriner Hekimler Odası Örneği [1]

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Özet

Türk Veteriner Hekimleri Birliği, 6343 sayılı *Veteriner Hekimliği Mesleğinin İcrasına, Türk Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair Kanun* kapsamında, 1954 yılında kurulmuştur. Aynı yıl içerisinde, Birliği, ülkenin çeşitli illerinde temsil eden ilk veteriner hekim odaları da oluşturulmuştur. Bu odalardan Erzurum Bölgesi Veteriner Hekimleri Odası, uzun yıllar, Kars'ta çalışan veteriner hekimleri de çatısı altında toplamıştır. Ancak izleyen süreçte, Kars'taki veteriner hekimlerin meslek odası açılabilmesi için yeterli sayı olan 30'u geçmesi ile birlikte, 1989 yılında Kars Bölgesi Veteriner Hekimler Odası (Kars VHO) kurulmuştur. Bu çalışma, Kars VHO'nun veteriner hekimliği ile hayvancılığa yönelik faaliyetlerine ışık tutmak amacıyla gerçekleştirilmiştir. Çalışmanın ana materyalini, Kars VHO ve Kafkas Üniversitesi Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı Arşivlerinden sağlanan ilk elden kaynaklar oluşturmuştur. Bu kaynaklar, belge analizi uygulanarak değerlendirilmiştir. Araştırma sırasında, Kars VHO'nun, kuruluşundan itibaren geçen zaman içerisinde, üyelerinin ve bölge halkının bilinçlendirilmesi amacıyla çok sayıda toplantı düzenlediği; ayrıca Oda tarafından, Bölgede görev yapan veteriner hekimlerin mesleki becerilerini geliştirebilmelerine olanak tanıyacak çeşitli meslek içi eğitim programlarının da hayata geçirildiği görülmüştür. Çalışma ile Kars VHO'nun kuruluş amaçlarıyla örtüşen bir gelişim sergilediği, üyelerine, mesleğe ve hayvan yetiştiricilerine yönelik önemli işlevlerinin bulunduğu; bununla birlikte denetim-yaptırım etkinliklerinin artırılmasının ve Odaya ait derginin yayın hayatına devam ettirilmesinin gerekli olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Kars Bölgesi Veteriner Hekimleri Odası, Türk Veteriner Hekimleri Birliği, Veteriner hekim odaları, Veteriner hekimliği tarihi*

A Study on the Activities of Veterinary Chambers in Turkey: The Veterinary Chamber of the Kars Region as an Example

Abstract

The Turkish Veterinary Medical Association was founded in 1954, pursuant to *Law 6343 on the Execution of the Veterinary Medical Profession and the Establishment and Tasks of the Turkish Veterinary Medical Association and Veterinary Chambers*. The first veterinary chambers to represent the Association in the various provinces of Turkey, were established the same year. Of these chambers, the Veterinary Chamber of the Erzurum Region served veterinarians working within the borders of the Kars province for many years. In the course of time, when the number of veterinarians working in Kars reached a level sufficient (at least 30) to establish a separate professional chamber in this province in 1989, the Veterinary Chamber of the Kars Region (Kars VC) was established. This study was aimed to provide an insight on the activities of the Kars VC on veterinary services and livestock production. Primary resources were obtained from the archives of the Kars VC and the Department of the History of Veterinary Medicine and Deontology of the Faculty of Veterinary Medicine, Kafkas University, constituted the basic material of this study. These resources were assessed by means of the document analysis method. In this study it was determined that, since its establishment in 1989, the Kars VC has organized multiple meetings to raise awareness among its members and the local people of the region. Furthermore, the Chamber was ascertained to have carried out vocational trainings for the veterinary practitioners working in the region with a view to improve their professional skills. In this respect, it is concluded that the Kars VC has been successfully accomplished its original targeted development and has had a significant role in serving both the veterinary profession and its members and local animal breeders. However, in our opinion, the Chamber must increase its effectiveness on sanctions control framework and continue to publish its ceased journal.

Keywords: *Veterinary Chamber of the Kars Region, The Turkish Veterinary Medical Association, Veterinary chambers, History of veterinary medicine*

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GİRİŞ

Türkiye'de, veteriner hekimliği alanındaki ilk kamu kuruluşu niteliğinde sivil toplum örgütü olan Türk Veteriner Hekimleri Birliği (TVHB) [1], *Veteriner Hekimliği Mesleğinin İcrasına, Türk Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair* 6343 sayılı Yasa[1] ile 1954 yılında kurulmuştur. TVHB'nin kuruluş amaçları, veteriner hekimler arasında mesleki deontoloji ve dayanışmayı korumak, veteriner hekimliğin kamu ve kişi yararına uygulanıp geliştirilmesini sağlamak ve meslek mensuplarının hakları ile yararlarını gözetmektir. TVHB, işlevlerini Merkez Konseyi (MK), Yüksek Haysiyet Divanı (YHD), Büyük Kongre, Denetleme Kurulu ve veteriner hekim odaları isimli organları aracılığıyla yürütmektedir. Veteriner hekim odaları, Birliğin veteriner hekimlerle iletişimini ve üyelerinin mesleklerini mevzuat hükümleri çerçevesinde sürdürmelerini sağlamakla yükümlüdürler. Odalar, en az 30 veteriner hekimin görev yaptığı illerde ya da birkaç ilin birleştirilmesiyle oluşturulan bölgelerde kurulurlar[2].

TVHB'nin hukuksal dayanağını oluşturan 6343 sayılı Yasa'nın geçici 5. maddesinden yetki alan Türk Veteriner Hekimleri Derneği[3] Merkez Kurulu, Birliğe işlerlik kazandırmak üzere, 1954 yılında ilk veteriner hekim odalarını[4] kurmuştur [2,3]. Bunlardan Erzurum Bölgesi Veteriner Hekimler Odası, uzun yıllar, Kars ilinde görev yapan veteriner hekimleri de çatısı altında toplamıştır. Ancak geçen zaman içerisinde, Kars'ta çalışan veteriner hekimlerin, ayrı bir oda açabilmek için yeterli sayıya ulaşması, bu veteriner hekimlerde, Kars Odasının kurulması istemini doğurmuştur[5].

Türkiye'de yer alan tüm veteriner hekim odalarının bağlı bulunduğu TVHB'nin kuruluşu, gelişimi ve bazı etkinlikleri, Melikoğlu ve Kızıltepe [1] tarafından gerçekleştirilen bir araştırma ile ortaya konmuştur. Ancak 6 Temmuz 2017 itibariyle sayısı 56'ya ulaşan [3] bu odaların tarihine ilişkin olarak, yalnızca Samsun Bölgesi Veteriner Hekimler Odası hakkında bir bilimsel yayına [4] rastlanmıştır. Bu çalışma, Kars Bölgesi Veteriner Hekimler Odasının tarihsel gelişimi ile veteriner hekimliği ve hayvancılığa yönelik işlevlerini ortaya koymak ve gelecekte yapılacak araştırmalara veri sağlamak amacıyla gerçekleştirilmiştir.

MATERYAL ve METOT

Çalışmada, Kars VHO ve Kafkas Üniversitesi Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji Anabilim Dalı Arşivlerinden sağlanan ilk elden kaynaklar ile Odada görev almış bazı veteriner hekimler ve TVHB MK temsilcileriyle yapılan görüşmelerden elde edilen veriler kullanılmıştır. Konuya ilişkin yayınlardan da yararlanılmıştır. Oda karar defterlerinde yer alan etkinliklerin gerçekleştirilip gerçekleştirilmedikleri, çeşitli yayınlar, Kars VHO Arşivinde yer alan özgün belgeler ve ilgili dönemin oda başkanı ile yapılan görüşmeler aracılığıyla netleştirilmiştir. Belge analiziyle ve görüşmelerin değerlendirilmesi yoluyla sağlanan veriler, kronolojik sıra ile yazıya aktarılmıştır. İlk elden belgelerin künyeleri ve açıklayıcı ek bilgiler dipnotlarda gösterilmiştir.

BULGULAR

Kars Bölgesi Veteriner Hekimler Odasının Kuruluşu

Kars'ta veteriner hekim odası açmak için, İlde yer alan çeşitli kuruluşlarda görevli 37 veteriner hekim, 6343 sayılı Yasanın 16. Maddesi ve bu Yasayı değiştiren 2993 sayılı Kanunun[6] 3. maddesini dayanak göstererek, 5 Aralık 1988 tarihinde TVHB MK'ne başvuruda bulunmuştur[7]. MK tarafından 10 Aralık 1988'de yapılan toplantıda, "Kars Bölgesi Veteriner Hekimler Odası" adı altında yeni bir odanın kurulmasına ve Odanın Geçici Yönetim Kurulunun, Kafkas Üniversitesi Veteriner Fakültesi Cerrahi AD Öğretim Üyesi Doç. Dr. Seçkin Gündüz *(Şekil 1)*, Serbest Veteriner Hekim Bahattin Özdemir, Kars Tarım İl Müdürlüğünde görevli veteriner hekimlerden Hüseyin Bolluk, Nermin Güneş ve Melahat Türker'den oluşturulmasına karar verilmiştir. Doç. Dr. Seçkin Gündüz başkanlığındaki Geçici Yönetim Kurulu, Odayı, kuruluş aşamalarından geçirerek ilk genel kurula taşımıştır[8].

Şekil 1. Kars VHO Kurucu Başkanı Doç. Dr. Seçkin Gündüz (KAÜ Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji AD Arşivi)

1- 18 Mart 1954 gün ve 8661 sayılı Resmi Gazete (RG).
2- 6343 sayılı Yasa'nın 16. Maddesi. Bak. Dipnot 1.
3- 1930'da kurulan bu Dernek, bugün "Veteriner Hekimleri Derneği" ismiyle çalışmalarını sürdürmektedir.
4- Ankara, Balıkesir, Bursa, Diyarbakır, Erzurum, Eskişehir, İstanbul, İzmir, Konya, Sakarya, Samsun ve Trabzon veteriner hekim odaları [3].
5- Kars'ta görevli bazı veteriner hekimlerin TVHB MK'ne verdikleri dilekçeler, 1988. Kars VHO Arşivi.
6- *9/3/1954 Tarih ve 6343 Sayılı Veteriner Hekimliği Mesleğinin İcrasına, Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair Kanunun Bazı Maddelerinin Değiştirilmesi ve Bu Kanuna Bazı Maddeler Eklenmesi Hakkında 1/6/1983 Tarihli 68 Sayılı Kanun Hükmünde Kararname ile Bu Kararnamenin Bazı Hükümleri ile 6343 Sayılı Kanunun Bazı Hükümlerinin Değiştirilmesine Dair 6/9/1983 Tarihli 86 Sayılı Kanun Hükmünde Kararnamenin Değiştirilerek Kabulü Hakkında Kanun.* 7 Nisan 1984 gün ve 18365 sayılı RG.
7- Bak. Dipnot 5.
8- Kars VHO Geçici Yönetim Kurulu Çalışma Raporu, 1988-1989. Kars VHO Arşivi.

Tablo 1. *Yıllara göre Kars VHO yönetim kurullarında görev alan veteriner hekimler*

Dönem	Yıllar	Veteriner Hekimler				
		Başkan	**Yazman**	**Sayman**	**Üye**	**Üye**
1	1989-92	Seçkin Gündüz	Hüseyin Bolluk	Gülsüm Takhizade	Akif Şenyürek	Nermin Güneş
2	1992-94	Seçkin Gündüz	Fuat Kutluay	Turgay Şeyda	İsa Özaydın	Kemal Irmak
3	1994-96	Şinasi Umur	Alkan Kamiloğlu	Mete Cihan	Salih Otlu	Abdullah Doğan
4	1996-97	Gürbüz Gökçe	Şahin Aslan	Fethi Polat	Erkan Sural	Mesut Karadurmuş
4[i]	1997-98	Şahin Aslan	Nesrin Acarer	Erkan Sural	Gürbüz Gökçe	Mesut Karadurmuş
5	1998-00	Alkan Kamiloğlu	A.HaydarKırmızıgül Erkan Sural[ii]	Kamil Genek M. Sadık Aras[ii]	M. Sadık Aras Erkan Sural[ii] Süleyman Tolun[ii]	Ejder Kurtbaş
6	2000-02	Alkan Kamiloğlu	Şahin Aslan	Nevzat Göksu	M. Sadık Aras	Erkan Sural
7	2002-04	Alkan Kamiloğlu	Şahin Aslan	Asuman Kızıltepe	Olcay Oktay Öztürkler	Birol Gül
8	2004-06	Çağlar Yarıcı	Birol Gül	İzzet Tolu	Yavuz Öztürkler	Şemistan Kızıltepe
9	2006-08	Yavuz Öztürkler	Engin Kılıç	Savaş Yıldız Doğan Akça[ii] Turgay Deprem[ii]	M. Sadık Aras	Tolgay Aktürk
10	2008-10	Yavuz Öztürkler	Engin Kılıç	Savaş Yıldız	M. Sadık Aras	Tolgay Aktürk
11	2010-12	Yavuz Öztürkler	Engin Kılıç	Recai Kulaksız	Tolgay Aktürk	M. Sadık Aras
12	2012-14	Vargın Boy	A. Serhat Uslu	Şemistan Kızıltepe	Ş. Çağlar Gülyiyen	Selçuk Özdamar
13	2014-16	Vargın Boy	Asuman Kızıltepe	Özkan Soylu	A. Serhat Uslu	Korkut Cabak
14	2016-18	Şemistan Kızıltepe	Gökhan Katok	Özgür Çelebi	Erdinç Koç	Tahir Gezer

[i]Fethi Polat'ın 1997'de Kars'tan ayrılması ile Yönetim Kurulu ve Kurulun görev dağılımı değişmiştir; [ii]Askerlik, şehirden taşınma gibi nedenlerle Kuruldan ayrılan üyelerin yerine atanan veteriner hekimler

18 Kasım 1989 tarihinde gerçekleştirilen 1. Olağan Genel Kurulda seçimi kazanan üyelerden Doç. Dr. Seçkin Gündüz, ilk dönem olan 1989-1992 yılları arasında Oda Başkanlığı görevini yürütmüştür. İlk Yönetim Kurulunda bulunan diğer veteriner hekimler ile Oda kurullarında 1.-14. Seçim Dönemleri arasında hizmet veren üyelerin isimlerine, *Tablo1* ve *Tablo 2*'de[9] yer verilmiştir.

Kars'a bağlı Ardahan ve Iğdır'ın 1992'de il olmasıyla birlikte, Kars VHO, bünyesinde üç ili barındıran bir meslek kuruluşu kimliğine sahip olmuştur. Zamanla, Odaya ait resmi yazışmalarda, "Kars-Ardahan-Iğdır Bölgesi Veteriner Hekimler Odası" ismi benimsenmeye başlanmıştır. Bununla birlikte, TVHB MK'ne göre, Odanın kayıtlı adının hala Kars Bölgesi Veteriner Hekimler Odası olduğu saptanmıştır[10].

Kuruluşunda 37 üyesi bulunan Kars VHO'nun, Şubat 2017 itibariyle Kars, Ardahan ve Iğdır illerinde görev yapan toplam 338 üyeye sahip olduğu belirlenmiştir[11].

Odanın Etkinlikleri (1989-Haziran 2017)

Odanın, kuruluşundan itibaren gerçekleştirdiği faaliyetlere bakıldığında, üye kayıt-nakil işlemleri ve bürokratik ziyaretlerde bulunmak yoluyla mesleğin tanıtımının sağlanması gibi çalışmaların yanı sıra, veteriner hekim olmayan kişilerin mesleği icra etmesi, yetkisiz ve ruhsatsız yerlerde ilaç satışı, bilinçsiz ilaç kullanımı, haksız rekabet ve diploma kiralama gibi mesleki sorunların çözümüne yönelik bazı girişimlerde bulunulduğu görülmektedir. Söz konusu sorunlar için, meslekten olmayan kişilere karşı, savcılığa suç duyurusunda bulunmak, üyelerine yönelik olarak da özel veteriner kliniklerinin denetimi, asgari ücret tarifesi hazırlama ve meslek içi eğitim ve bilgilendirme gibi çözüm yöntemlerine başvurulduğu anlaşılmaktadır[12]. Bununla birlikte, Oda Haysiyet Divanı kararlarına yönelik bir araştırma yapıldığında, yalnızca bir veteriner hekime yazılı ihtar cezası verildiği belirlenebilmiştir[13].

Oda Yönetim Kurulu, 1991 yılında, mesleki camianın talebi olan Hayvancılık Bakanlığının kurulmasını desteklemek üzere, dönemin koalisyon hükümetinin temsilcilerine resmi başvuruda bulunmuştur[14]. Yine Oda yetkilileri, Hayvancılık ve Su Ürünleri Müsteşarlığı kurulması hakkındaki yasa tasarısına destek vermek amacıyla, Cumhurbaşkanlığı, Meclis Başkanlığı, Başbakanlık ve ilgili bakanlıklar nezdinde bazı girişimler gerçekleştirmiş ve yöre milletvekilleri ile yerel basının dikkatini bu konuya çekmeye çalışmıştır[15].

Kars VHO, Kurban Bayramları öncesinde, halkı kesim

9- *Tablo 1* ve *Tablo 2* (Kars VHO Seçim Tutanakları ve Karar Defterleri, 1989-2017. Kars VHO Arşivi.)
10- Kars VHO Gelen ve Giden Evrak Dosyaları, 1989-2017. Kars VHO Arşivi; TVHB MK Başkanı Talat Gözet ile 12 Mayıs 2017, TVHB MK çalışanlarından Şaban Aydemir ile 20 Haziran 2017 tarihlerinde yapılan görüşmeler.
11- Kars VHO Üye Listesi, 2017. Kars VHO Arşivi.
12- Kars VHO Çalışma Raporları ve Karar Defterleri, 1989-2017. Kars VHO Arşivi.
13- Kars VHO Haysiyet Divanı Dosyası, 1989-2017. Kars VHO Arşivi.
14- Kars VHO 1. Dönem Çalışma Raporu, 1989-1992. Kars VHO Arşivi.
15- Kars VHO 3. Dönem Çalışma Raporu, 1994-1996. Kars VHO Arşivi.

Tablo 2. *Yıllara göre Kars VHO Onur ve Denetleme Kurulları ile Büyük Kongre Delegeleri*

Dönem	Onur Kurulları					Denetleme Kurulları		Büyük Kongre Delegeleri					
1	Necdet Leloğlu	Mustafa Oktay	Enver Beytut	Melahat Türker	Faruk Kurnaz	Turgay Şeyda	İbrahim Öz	Turgay Şeyda	Yüksel Ağca	-	-	-	-
2	Necdet Leloğlu	Erdoğan Finci	Mustafa Oktay	Nurettin Öztürk	İsmet Çelik	Salih Otlu	Erdal Eken	Fuat Kutluay	Kemal Irmak	Tayfur Bekyürek	Turgay Şeyda	-	-
3	Seçkin Gündüz	Necdet Leloğlu	Nurettin Öztürk	Erdoğan Finci	Selami Turan	Narin Liman Burhan Özba[i]	Mithat Şahin	Seçkin Gündüz	Ufuk Kamber	Turgay Şeyda	B. Cem Liman	Alkan Kamiloğlu	Burhan Özba
4	Erdoğan Finci	Nurettin Öztürk	Armağan Çolak	Burhan Özba	Hayati Çamaş	Salih Otlu	Fuat Aydın	Alkan Kamiloğlu	Savaş Öztürk	Mete Cihan	Şinasi Umur	Selami Turan	Ejder Kurtbaş
5	Şinasi Umur	İsmet Takçı	Nurettin Öztürk	Bülent Güven	Fuat Aydın	Fuat Aydın	Burhan Özba	Şahin Aslan	M. Sadık Aras	Şinasi Umur	Mete Cihan	Erkan Sural	Oktay Genç
6	Nurettin Öztürk	Çetinkaya Şendil	Bülent Güven	İsmet Takçı	İsa Özaydın	Burhan Özba	İsa Özaydın	Salih Otlu	Şahin Aslan	Turgut Kırmızıbayrak	Mete Cihan	M. Sadık Aras	İsa Özaydın
7	Kemal Yılmaz	İsa Özaydın	Vedat Baran	Burhan Özba	Abamüslüm Güven	Engin Kılıç	Doğan Akça	İsa Özaydın	Şahin Aslan	Mete Cihan	Nesrin Kaya	Yavuz Öztürkler	Abamüslüm Güven
8	İsa Özaydın	Abamüslüm Güven	A. Rıza Aksoy	Yunus Kılıç	Fatih Kılınç	Engin Kılıç	Doğan Akça	İsa Özaydın	Mete Cihan	Engin Kılıç	Mithat Şahin	Yunus Kılıç	Şahin Aslan
9	Nurhayat Gülmez	Abamüslüm Güven	İsa Özaydın	A. Rıza Aksoy	M. Özkan Arslan	Mithat Şahin	Celal Aydın	Şahin Aslan	Salih Otlu	Alkan Kamiloğlu	Mete Cihan	Tolgay Aktürk	S. Ali Bingöl
10	Nurhayat Gülmez	Şahin Aslan	Nurettin Öztürk	A. Rıza Aksoy	Abdullah Doğan	Mithat Şahin	Celal Aydın	M. Sadık Aras	Salih Otlu	Alkan Kamiloğlu	Mete Cihan	S. Ali Bingöl	Özcan Yıldırım
11	Nurhayat Gülmez	İsa Özaydın	Şahin Aslan	A. Rıza Aksoy	Abdullah Doğan	Mithat Şahin	Turgay Deprem	Salih Otlu	Mete Cihan	Alkan Kamiloğlu	Özgür Aksoy	Recai Altun	-
12	A. Rıza Aksoy	Burhan Özba	Sefa Akbulut	Çağlar Yarıcı	Abdurahman Gürbüz	Gökhan Katok	Sezgin Sağlam	Özcan Yıldırım	Yunus Baydar	Ejder Kurtbaş	M. Fatih Kılınç	Erdinç Koç	-
13	A. Rıza Aksoy	Gürbüz Gökçe	Çağlar Yarıcı	G. Taşkın Taşçı	Azer Tutar	Cumhur Saltan	Gündoğan Mazaklı	Gökhan Katok	Hasan Kılıç	Erdinç Koç	Ş. Çağlar Gülyiyen	Kemal Çakın	-
14	Abdurrahman Gürbüz	Gürbüz Gökçe	Salih Otlu	İsa Özaydın	Alkan Kamiloğlu	Ercan Ödül	Hasan Altun	AdemArancı	Sezgin Sağlam	Vargın Boy	Cumhur Saltan	Hasan Kılıç	-

[i]*Narin Liman'ın Kayseri Odasına nakli nedeniyle, kendisinden boşalan yere Burhan Özba getirilmiştir*

ve hijyen açısından bilgilendirmek üzere, medya aracılığıyla gerçekleştirdiği çalışmaların yanı sıra, Irak Savaşı gibi toplumsal konularda da basın açıklamalarında bulunmuştur[16] [5]. Ayrıca Oda başkanlarından Prof. Dr. Yavuz Öztürkler (2006-2012), yerel televizyon kanalı Serhat TV'de, 21 Şubat 2009 tarihinde başlayan, dört bölümlük "Hayvancılıkta Bilinçli miyiz?" adlı bir program hazırlayıp sunmuştur[17].

Geçmişte Kafkas Üniversitesi Veteriner Fakültesinde ve il merkezinde kiralanan çeşitli yerlerde hizmet vermiş olan Oda için, 27 Temmuz 2012 tarihinde bir büro satın alınmış ve aynı yıl içerisinde, Eski Belediye İşhanı 1. Kat 108 numarada bulunan bu yere taşınılmıştır[18].

Kars VHO, Bölgede görev yapan veteriner hekimlerin mesleki donanımlarını geliştirebilmelerine olanak tanıyacak birçok meslek içi eğitim programını hayata geçirmiştir *(Tablo 3*[19]*)*. Söz konusu kursların birçoğunda, dersleri vermek üzere Kafkas Üniversitesi Veteriner Fakültesi öğretim elemanları görevlendirilmiştir. Oda tarafından, gerek üyelerinin gerekse bölge halkının bilgilendirilmesi ve sorunlarının ortaya konularak çözüm yollarının araştırılması amacıyla, birçoğu Kafkas Üniversitesi Veteriner Fakültesi ile işbirliği içerisinde gerçekleştirilen, çok sayıda toplantı da düzenlenmiştir *(Tablo 4*[20]*, Şekil 2)*.

Kars VHO, ulusal ve uluslararası nitelikteki bazı bilimsel toplantılara da mali yönden destek vermiştir. Bu etkinlikler, 5. Ulusal Veteriner Cerrahi Kongresi (Kars-1996)[21], İneklerde Üreme ve Meme Sağlığı Paneli (Kars-2009)[22], 39. Uluslararası Dünya Veteriner Hekimliği Tarihçileri Birliği Kongresi ve 3. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu[23] (Antalya-2010)[24], 13. Ulusal Veteriner Cerrahi Kongresi (Uluslararası katılımlı) (Kars-2012)[25] ve 5. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu (Bursa-2016)[26]'dur. Bilimsel toplantıların desteklenmesinin yanı sıra, Odayı temsilen, Yönetim Kurulu üyelerinin, ulusal ve uluslararası düzeyde gerçekleştirilen bazı etkinliklere de katıldıkları belirlenmiştir. Bu toplantılar arasında, 8. Hayvancılık Kongresi (Ankara-2000)[27], Kars-Gence Hayvancılığı ve Bilimsel İşbirliği Sempozyumu (Kars-2001)[28], Gence-Kars Veteriner Hekimliği Sempozyumu (Gence/Azerbaycan-2003)[29] *(Şekil 3)*, Uluslararası Kuş Gribi Kongresi (Antalya-2007)[30], 3. Türk Veteriner Hekimliği Kurultayı (Ankara-2010)[31] yer almaktadır.

Tablo 3. *Kars VHO'nun gerçekleştirdiği eğitim çalışmalarından bazıları*

Eğitim Çalışmasının Konusu	Tarihi[i]
Sığırlarda Rekto-Vaginal Suni Tohumlama	4-8 Aralık 2006
	17-21 Eylül 2007
	19-23 Şubat 2007
	26-30 Ocak 2009
	29 Haziran-3 Temmuz 2009
	19-23 Temmuz 2010
	26-30 Temmuz 2010
	1-5 Kasım 2010
	11-15 Temmuz 2011
	18-22 Temmuz 2011
	25-29 Temmuz 2011
Akredite Veteriner Hekimlik	15 Mayıs 2007
	15-17 Haziran 2007
	10-12 Aralık 2007
	9-11 Ocak 2009
	4-6 Temmuz 2009
	16-18 Temmuz 2010
ISO 22000: 2005 Gıda Güvenliği Yönetim Sistemleri (HACCP)	10-12 Aralık 2007
	11-12 Temmuz 2009
	23-25 Temmuz 2010
	9-10 Temmuz 2011
	9 Ekim 2011
	14 Haziran 2012
Veteriner Temel Ultrasonografi ve Sığırlarda Jinekolojik Ultrasonografi	21-23 Mayıs 2007
	14-16 Haziran 2007
Sığırlarda Reprodüktif Ultrasonografi	20-22 Haziran 2009
Zorunlu Hizmet İçi Eğitim	24 Ocak 2012
	19-20 Haziran 2012
	21-22 Haziran 2012
	26-29 Haziran 2012
	14-15 Mart 2013
Proje Döngüsü[ii]	1-5 Temmuz 2013
Tarımsal Başarı İçin Etkili İletişim[iii]	1-5 Ağustos 2016

[i]Tabloda, yalnızca tarihleri belirlenebilen eğitim çalışmalarına yer verilmiştir; [ii]Persona TR isimli şirket ile birlikte gerçekleştirilmiştir; [iii]Serhat Kalkınma Ajansı, Konya ABİGEM AŞ ve Kalkınma Bakanlığı ile birlikte gerçekleştirilmiştir

16- Kars VHO 7. Dönem Çalışma Raporu, 2002-2004. Kars VHO Arşivi.
17- Kars VHO, 10. Dönem Çalışma Raporu, 2008-2010. Kars VHO Arşivi.
18- 30 Temmuz 2012 gün ve 125 sayılı Yönetim Kurulu Kararı (YKK). Kars VHO Arşivi.
19- Kars VHO Karar Defterleri ve Eğitim Dosyaları, 2006-2016. Kars VHO Arşivi.
20- Tablonun oluşturulmasında, tablo başlığında atıfta bulunulan kaynaklara ek olarak şu bilgi ve belgelerden yararlanılmıştır: 13 Nisan 1998 gün ve 2 sayılı YKK, 20 Nisan 1999 gün ve 3 sayılı YKK, 29 Eylül 2000 gün ve 6 sayılı YKK, Kars VHO 6. Dönem Faaliyet Raporu (2000-2002), 8 Şubat 2004 ve 15 sayılı YKK, Kars VHO 7. Dönem Çalışma Raporu (2002-2004), Kars VHO 8. Dönem Faaliyet Raporu (2004-2006), 30 Ekim 2008 gün ve 37 sayılı YKK, 30 Ekim 2010 gün ve 82 sayılı YKK. Kars VHO Arşivi; Etkinlik Davetiyesi (2010), Etkinlik Davetiyesi (2011), KAÜ Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji AD Arşivi; Prof. Dr. Yavuz Öztürkler ile 20 Nisan 2016 tarihinde yapılan görüşme; Prof. Dr. Şahin Aslan ile 16 Şubat 2017 tarihinde yapılan telefon görüşmesi.
21- 2 Ağustos 1996 gün ve 96 sayılı YKK. Kars VHO Arşivi.
22- 27 Nisan 2009 gün ve 48 sayılı YKK. Kars VHO Arşivi.
23- Orijinal adı *XXXIXth International Congress of the World Association for the History of Veterinary Mecidine & IIIrd National Congress of the History of Veterinary Medicine & Professional Ethics*'tir.
24- 6 Eylül 2010 gün ve 77 sayılı YKK. Kars VHO Arşivi.
25- 15 Haziran 2012 gün ve 120 sayılı YKK. Kars VHO Arşivi.
26- 21Mayıs 2016 gün ve 35 sayılı YKK. Kars VHO Arşivi.
27- 12 Nisan 2000 gün ve 2 sayılı YKK. Kars VHO Arşivi.
28- 23 Eylül 2001gün ve 10 sayılı YKK. Kars VHO Arşivi.
29- 11 Nisan 2003 gün ve 10 sayılı YKK. Kars VHO Arşivi.
30- 6 Haziran 2007 gün ve 12 sayılı YKK. Kars VHO Arşivi.
31- 10 Mart 2010 gün ve 62 sayılı YKK. Kars VHO Arşivi.

Tablo 4. *Kars VHO'nun düzenlediği toplantılardan bazıları* [5-15]

Toplantının Adı	Toplantının Niteliği	Tarih
Kars Bölgesi Hayvancılığının Sorunları ve Çözüm Önerileri	Sempozyum	30 Haziran 1998
Veteriner Hekimlikte Etik	Konferans: Prof. Dr. Ferruh Dinçer	14 Mayıs 1999
Türkiye'de Veteriner Hekimliğin Konumu ve Geleceği	Konferans: Prof. Dr. Hazım Gökçen	23 Ekim 2000
Klinik Veteriner Hekimlikte Son Gelişmeler	Panel: Prof. Dr. İsa Özaydın, Doç. Dr. Mitat Şahin, Doç. Dr. Yavuz Öztürkler, Dr. Kutlay Gürbulak	28 Nisan 2004
Günümüzde Veteriner Hekimlik	Panel: Prof. Dr. Abamüslüm Güven, Doç. Dr. Hakan Kocamış, Özel Veteriner Hekim M. Sadık Aras	30 Nisan 2005
Avrupa Birliği'nde Veteriner Hekimlik	Panel: Prof. Dr. Abamüslüm Güven, Prof. Dr. Salih Otlu, Doç. Dr. Mustafa Saatcı, Doç. Dr. Yavuz Öztürkler, Doç. Dr. Savaş Öztürk,	29 Nisan 2006
Dünya Veteriner Hekimler Günü	Panel: Prof. Dr. Abdullah Doğan, Prof. Dr. Şahin Aslan, Doç. Dr. Doç. Dr. Mustafa Saatcı, Hakan Kocamış, Doç. Dr. Engin Kılıç	Nisan 2007
Veteriner Hekimlerin Sorunları	Panel: Prof. Dr. Abdullah Doğan, Prof. Dr. Şahin Aslan, Doç. Dr. Yavuz Öztürkler, Özel Veteriner Hekim M. Sadık Aras	Nisan 2008
Türkiye'de Veteriner Hekimliği Eğitimi	Panel: Prof. Dr. Abdullah Doğan, Prof. Dr. İsa Özaydın, Prof. Dr. Şahin Aslan, Doç. Dr. Yavuz Öztürkler	19 Kasım 2008
Zoonotik Hastalıklar	Panel: Prof. Dr. Yavuz Öztürkler, Prof. Dr. Zati Vatansever, Yrd. Doç. Dr. Yakup Yıldırım	23 Nisan 2009
Serbest Veteriner Hekimliğinde Deontolojik-Etik Sorunlar	Konferans: Dr. Asuman Kızıltepe	29 Nisan 2010
Kurban Bayramı ve Halk Sağlığı	Panel: Prof. Dr. Yavuz Öztürkler, Doç. Dr. Atilla Akça	Kasım 2010
Süt Sığırcılığında Kritik Dönemler ve Veteriner Hekimlik Eğitimi	Konferans: Prof. Dr. Veysi Aslan	28 Nisan 2011
Veteriner Hekim Yönüyle Mehmet Akif Ersoy	Konferans: Dr. Asuman Kızıltepe	28 Nisan 2011
Hayvansal Gıdalarda Kimyasal Kalıntı Problemi ve Antibiyotik/ Antelmintik Direnç	Panel: Prof. Dr. Sezai Kaya, Prof. Dr. Ender Yarsan	14 Mayıs 2012
Gıda Güvenliği ve Halk Sağlığı	Panel: Prof. Dr. Mustafa Atasever, Doç. Dr. Nebahat Bilge, Kars VHO Başkanı Veteriner Hekim Vargın Boy	30 Nisan 2013
Veteriner Fakültesi Kariyer Günleri	Panel: Prof. Dr. İsa Özaydın, EGE VET Genel Müdürü Tahir Yavuz, Kars VHO Başkanı Vargın Boy, Resmi Veteriner Hekim Şemistan Kızıltepe, Özel Veteriner Hekim Örsan Karaçöp	15 Mayıs 2015

Şekil 2. Klinik Veteriner Hekimlikte Son Gelişmeler Paneli (2004) (KAÜ Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji AD Arşivi)

Şekil 3. Oda başkanlarından Yrd. Doç. Dr. Alkan Kamiloğlu, Azerbaycan Kend Teserrüfatı Akademiyası Rektörü Prof. Dr. İ. Memmedtağı Ceferov'a Kars VHO adına plaket verirken (2003) (KAÜ Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji AD Arşivi)

Oda, çeşitli dönemlerde, hayvan yetiştiricilerinin hayvancılığa ilişkin konularda bilgilendirilmesine yönelik bir dizi projeyi de hayata geçirmiştir. 1000 Köyde 1000 Seminer Uygulaması kapsamında, 2006-2008 yılları arasında ilçe merkezlerinde muhtarlara, 2008-2010 yılları arasında ise köylerde yetiştiricilere yönelik olarak, hayvancılıkla ilgili seminerler verilmiştir[32]. 2013 yılı içerisinde yürütülen, Şarbon ve Brusella Hastalıkları ile Mücadele ve Hayvan Yetiştiricilerinin Bilinçlendirilmesi Projesi çerçevesinde ise tanıtıcı afiş ve broşürler hazırlanarak köylerde dağıtılmış; Kars İli ve ilçelerine bağlı çeşitli köylerde yaşayan 541

32- Kars VHO Çalışma Raporu, 2008-2010. Kars VHO Arşivi.

Şekil 4. Doç. Dr. Seçkin Gündüz, Kars Veteriner Fakültesinin ilk mezunlarından Dönem Birincisi Ayla Biçer'e Veteriner Hekimlik Rozeti takarken (1990) (KAÜ Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji AD Arşivi)

yetiştiriciye eğitimler verilmiş; bir bölümüne ise anketler uygulanarak, elde edilen veriler bir kitapçık halinde yayımlanmıştır [16].

Türkiye'de modern veteriner hekimlik öğretiminin yıldönümü nedeniyle, Oda ve Veteriner Fakültesinin ortaklaşa gerçekleştirdiği törenlerde, süt koşuları düzenlenerek, koşu sonucunda ilk üçe giren öğrencilere ve sonuncu öğrenciye süt hediye edilmiştir. Yine veteriner fakültesi öğrencilerinin mezuniyet törenlerinde etkin biçimde rol alınarak *(Şekil 4)*, meslektaş adaylarıyla Oda arasındaki iletişimin geliştirilmesine çalışılmıştır[33]. Oda tarafından, meslektaşların sosyal ortamda bir araya gelmesini sağlamak üzere, Veteriner Hekimler Günü baloları da düzenlenmiştir. Bu kutlamalara, mesleğin topluma tanıtımı amacıyla, veteriner hekim olmayan insanların da katılımı sağlanmıştır[34].

Yayın Organı

Odanın yayın organı olan "İsimsiz" adlı dergi, 2007 yılında yayın hayatına başlamıştır. İlk sayısında, "Neden Hayvancılık Kurultayı?", "Bölgemizde Brusella'dan Kurtulma Umudu", "Veteriner Hekimlik ve Toplum İlişkileri", "Meslek Hukuku Dersi" ve "Kuş Gribi Fay Hattı" başlıklı makaleler ile mesleğe ilişkin haberlere yer verilen dergi *(Şekil 5)* [17], ilk sayıdan sonra yayımlanamamıştır[35].

TARTIŞMA ve SONUÇ

TVHB, veteriner hekimler arasında mesleki deontoloji ve dayanışmayı korumak, veteriner hekimliğin kamu ve kişi yararına uygulanıp geliştirilmesini sağlamak ve meslek mensuplarının hakları ile yararlarını gözetmek üzere kurul-

Şekil 5. "**İsimsiz"** (Kars VHO Yayın Organı) (KAÜ Veteriner Fakültesi Veteriner Hekimliği Tarihi ve Deontoloji AD Arşivi)

muştur. Veteriner hekim odalarının yönetim kurullarına ise Birliğin veteriner hekimlerle iletişimini ve üyelerinin mesleklerini mevzuat hükümleri çerçevesinde sürdürmelerini sağlamak, üyelerinin mesleki ve bilimsel yönden gelişmelerini desteklemek, meslek onurunu ve veteriner hekimlerin hak ve yararlarını korumak gibi bazı sorumluluklar yüklenmiştir[36]. Bu çalışma ile Kars VHO'nun, kendisine yüklenen misyonu gerçekleştirmeye yönelik etkinlikler yürüttüğü belirlenmiştir. Oda etkinliklerinin değeri, genel içerisinde irdelenirse anlam kazanacaktır. Ancak diğer veteriner hekim odalarına ilişkin bilimsel çalışmaların noksanlığı, böyle bir değerlendirmeyi güçleştirmektedir. Öte yandan, çalışmanın, Kars VHO'nun faaliyetlerini gün ışığına çıkarması açısından, diğer veteriner hekim odalarının kendilerine vizyon oluştururken yararlanabilecekleri bir model ortaya koyduğu ve gelecekte benzer konularda yapılacak çalışmalar için bilgi sağlayabileceği ileri sürülebilir.

Sanal ve Melikoğlu Gölcü tarafından gerçekleştirilen araştırmada [4], Samsun-Sinop Veteriner Hekimleri Odasının çok sayıda eğitim faaliyeti yaptığı ve bazı mesleki ve bilimsel toplantıları desteklediği saptanmıştır. Benzer biçimde, bu çalışmada da Kars VHO'nun etkinlikleri arasında,

33- Kars VHO Çalışma Raporları ve Karar Defterleri, 1989-2016. Kars VHO Arşivi.
34- Kars VHO Çalışma Raporları ve Karar Defterleri, 1989-2017. Kars VHO Arşivi; Prof. Dr. İsa Özaydın ile 15 Mart 2016 tarihinde yapılan görüşme.
35- Kars VHO Arşivi (1989-2017).
36- *Veteriner Hekimliği Mesleğinin İcrasına, Türk Veteriner Hekimleri Birliği ile Odalarının Teşekkül Tarzına ve Göreceği İşlere Dair* 6343 sayılı Kanun'un 14. ve 23. maddeleri. (Bak. Dipnot 1)

veteriner hekimliği meslek içi eğitim kurslarının *(Tablo 3)* ve hayvan yetiştiricilerine yönelik eğitim projelerinin[37] [16] hayata geçirildiği belgelenmiştir. Meslek içi eğitim çalışmalarının, veteriner hekim odalarının, üyelerinin mesleki gelişimlerini destekleme göreviyle örtüştüğü ileri sürülebilir. Yetiştiricilere yönelik etkinlikler ise veteriner hekimliğin ilerlemesi için önce hayvancılığın geliştirilmesi hedeflenerek, hayvan yetiştiricilerinin bilgi seviyesinin daha yüksek düzeylere çekilmesi çabası olarak değerlendirilebilir. Nitekim, 2006-2012 yılları arasında Oda Yönetim Kurulu Başkanlığını yürüten Prof. Dr. Yavuz Öztürkler, bölgedeki hayvan üreticilerinin yeterince eğitimli olmamasının, birçok mesleki soruna yol açtığı, bu nedenle Odanın öncelikli amacının üreticilerin bilinçlendirilmesi olduğunun altını çizmiştir [18].

Çalışma sırasında, Kars VHO tarafından, bilimsel toplantıların düzenlenmesine *(Tablo 4)*, desteklenmesine ve bu gibi etkinliklerde Odanın temsil edilmesine ayrı bir önem verildiği görülmüştür. Oda faaliyetlerinde eğitim etkinliklerinin *(Tablo 3)* ve bilimsel toplantı odaklı çabaların ön plana çıkması, Kars'ta veteriner fakültesinin bulunması ve gerek fakülte yönetiminin gerekse burada görevli öğretim elemanlarının bu çalışmalara çeşitli katkılar sunması ile açıklanabilir.

Odanın eğitsel, mesleki ve bilimsel nitelikli etkinlikleri, gerek TVHB'nin kuruluş amaçları gerekse veteriner hekim odalarının görevleri ile ilişkilendirilebilir.

Çeşitli çalışmalarda [19-24], Türkiye'de görev yapan özel klinisyen veteriner hekimlerin deontolojik-etik ihlalleri ortaya konulmuştur. Kızıltepe [20], "Türkiye'de Klinik Veteriner Hekimliği Uygulamalarında Karşılaşılan Deontolojik-Etik Sorunlar Üzerine Bir Araştırma" adlı doktora tez çalışmasında, Türkiye'de "mesleğe saygı ve rekabet", "hasta ve hasta sahibiyle ilişkiler" ve "meslektaşlar arası ilişkiler" boyutlarındaki ihlallerin en fazla, Doğu ve Güneydoğu Anadolu bölgelerinde görüldüğünü bildirmiştir. Anılan çalışmada, TVHB YHD'nin, 1994-2009 zaman dilimine ait olan, özel klinisyenlikle ilgili kararları da incelenmiş ve bu kararlar ile veteriner hekimlere beş yazılı ihtar, dört para cezası ve dört meslekten geçici men cezası olmak üzere 13 adet ceza verildiği; 10 dosyanın ise iade veya iptali kararına gidildiği kaydedilmiştir. Tong ve ark.[25] ise İzmir Veteriner Hekimler Odası (İzmir VHO) Haysiyet Divanının aldığı kararlara yönelik olarak yaptıkları araştırmada, 1992-2013 yılları arasındaki Divan kararlarından 59'unun özel klinisyenlik, beşinin ise kamuda çalışan veteriner hekimler hakkında alındığını saptamışlardır. Aynı çalışmada, divanın yaptığı dosya incelemelerinin 33'ünün yazılı ihtar, üçünün para cezası ve üçünün de meslekten geçici men cezası şeklinde karara bağlandığı, 25'inde ise kusur bulunmadığı sonucuna varıldığı bildirilmiştir. YHD ve İzmir VHO Haysiyet Divanının verdiği cezalar ile karşılaştırıldığında, Kars VHO Haysiyet Divanı tarafından cezalandırılmış yalnızca bir üyenin tespit edilebilmesi, Odanın üye sayısının görece azlığı ve 1954 yılında yapılandırılmış bu örgütlerle karşılaştırıldığında yeni bir oda sayılabilecek Kars VHO'nun, henüz yeterince kurumsallaşamaması ile açıklanabilir. Bununla birlikte, deontolojik-etik ihlallerin en fazla görüldüğü bölgelerden birinde yer alan Kars VHO'nun, ihlallere yönelik yalnızca bir cezalandırma yapmış olması, Oda Haysiyet Divanının yeterince işletilemediğini düşündürmektedir. Odanın gerek mesleki etik alanıyla ilgili etkinliklere verdiği destek gerekse deontolojik-etik sorunlara yönelik girişimleri, bu sorunların çözümüne ilişkin iyi niyetli çabalar olarak değerlendirilebilir. Ancak deontolojik-etik ihlallerin önlenmesiyle ilgili denetim-yaptırım mekanizmasının önemli dişlilerinden olan odalara, bu konuda daha fazla sorumluluk düştüğü ortadadır.

Odanın "İsimsiz" adlı dergiyi çıkarmaya başlaması [17], üyelerle iletişimin sağlanması ve veteriner hekimler ile toplumun bilinçlendirilmesi adına, önemli bir girişim olarak değerlendirilebilir. Bununla birlikte, yayının sürekliliğinin sağlanamamasının büyük bir eksiklik olduğu ileri sürülebilir.

Sonuç olarak, Kars VHO'nun faaliyetlerinin, bir veteriner hekim odasından beklenen sorumlulukları yerine getirmeye yönelik olması, Odanın kuruluş amaçlarıyla örtüşen bir gelişim sergilediğine ve üyelerine, mesleğe ve -hayvan yetiştiriciliğinin yoğun olarak yapıldığı bir bölgede hizmet verdiği göz önünde bulundurulduğunda- ülke hayvancılığına yönelik önemli işlevlere sahip olduğuna işaret etmektedir. Ancak Odanın kurumsallaşması ve hizmetlerinin daha etkin bir biçimde gerçekleştirilebilmesi açısından, denetim-yaptırım konusundaki çalışmalarının artırılması ve Odaya ait derginin yayın hayatına devam ettirilmesi gerekmektedir. Veteriner hekim odalarının gelişiminin ve işlevlerinin daha iyi anlaşılabilmesi, bu mesleğe ilişkin diğer odalara ait verilerin gün ışığına çıkarılmasıyla mümkün olacaktır.

37- Bak. Dipnot 32.

Teşekkür

Önerileriyle çalışmamı zenginleştiren Prof. Dr. R. Tamay Başağaç Gül'e, Odanın geçmişine ait tanıklıklarından yararlanmamı sağlayan Prof. Dr. Seçkin Gündüz, Prof. Dr. Şahin Aslan, Prof. Dr. Yavuz Öztürkler, Prof. Dr. Alkan Kamiloğlu'na ve Odanın ismiyle ilgili yönlendirmeleri nedeniyle TVHB MK Başkanı Talat Gözet ile MK Çalışanı Şaban Aydemir'e teşekkür ederim.

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Research Article

Expression of ISG15 in Bone Marrow During Early Pregnancy in Ewes

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Abstract

Interferon-tau (IFNT) is the main signal for maternal recognition of pregnancy in ruminants. IFNT acts on the endometrium, corpus luteum, and liver through paracrine and endocrine style, which is involved in inhibiting the development of luteolytic mechanism and suppressing maternal immune rejection of the semi-allogeneic fetus. Bone marrow (BM) is a key component of the lymphatic system, supports the body's immune system through producing the lymphocytes. At present study, the BM was obtained from days 13, 16 and 25 of pregnant ewes, day 16 of non-pregnant ewes to study the expression of interferon stimulated gene 15 kDa protein (ISG15) mRNA and protein through a qRT-PCR assay, Western blot, and an immunohistochemistry analysis. Our results showed that the expression of ISG15 mRNA, proteins and conjugated proteins were up-regulated in the stroma of BM during early pregnancy, and the immunohistochemistry results confirmed that the ISG15 proteins were localized in the cytoplasm of different cells in the stroma of BM. In conclusion, IFNT derived from the conceptus induced up-regulated expression of ISG15 and conjugated proteins in the stroma of BM through an endocrine style, which were involved in regulating the maternal immune response during early pregnancy in ewes.

Keywords: *Ewes, Bone marrow, Pregnancy, Interferon stimulated gene 15 kDa protein*

Koyunların Erken Gebelik Döneminde Kemik İliğinde ISG15 Ekspresyonu

Özet

İnterferon-tau (IFNT) ruminantlarda gebeliğin maternal tanısındaki başlıca sinyaldir. IFNT endometrium, korpus luteum ve karaciğer üzerinde parakrin ve endokrin yollarla etki ederek luteolitik mekanizmanın gelişmesini inhibe eder ve semi-allojenik fetusun anne tarafından reddini baskılar. Kemik iliği lenfatik sistemin en önemli bileşenlerinden biri olup lenfositleri üretmek suretiyle vücudun bağışıklık sistemini desteklemektedir. Bu çalışmada, 13, 16 ve 25. günlerde gebe koyunlardan ve 16. günde gebe olmayan koyunlardan kemik iliği alınarak interferon tarafından uyarılan gen 15 kDa protein (ISG15) mRNA ve protein ekspresyonu qRT-PCR, Western blot ve immunohistokimyasal yöntemlerle araştırıldı. Elde edilen sonuçlar ISG15 mRNA, proteinler ile konjuge proteinlerin ekspresyonunun erken gebelik döneminde kemik iliğinde upregule edildiğini ve immunohistokimyasal olarak ISG15 proteinlerin kemik iliğinin stromasında farklı hücrelerin sitoplazmasında yer aldığını göstermiştir. Sonuç olarak, gebeliğe bağlı olarak üretilen IFNT; ISG15 ve konjuge proteinlerin ekspresyonunu kemik iliğinin stromasında endokrin yolla upregüle etmiş ve böylece koyunların erken gebelik döneminde maternal bağışıklık cevabın düzenlenmesinde rol almıştır.

Anahtar sözcükler: *Koyun, Kemik iliği, Gebelik; Interferon tarafından uyarılan gen 15 kDa protein*

INTRODUCTION

It has been documented that interferon-tau (IFNT) is the main signal for maternal recognition of pregnancy in ruminants. IFNT acts on the endometrium to inhibit the development of luteolytic mechanism, and prolongs the lifespan of the ovarian corpus luteum (CL) to produce progesterone (P4) continuously [1]. It is reported that many interferon-stimulated genes are up-regulated in the ovine uterus during early pregnancy, such as 2′,5′-oligoadenylate synthetase (OAS-1) [2], ubiquitin-like interferon-stimulated gene 15-kDa protein (ISG15) [3], Mx proteins [4], Stat-1, Stat-2, interferon regulatory factor 1 (IRF-1) and IRF-9 [5]. In addition, interferon-stimulated genes are also up-regulated in the ovine CL and blood cells during early pregnancy, including ISG15, OAS-1 [6], receptor transporter protein 4 (RTP4) [7]. It is through an endocrine style that IFNT secreted by the conceptus releases into the uterine vein, and extends luteal life span in ewes [8]. It is also reported that ISG15 and MX-1 mRNAs are detectable in the liver on day 18 of pregnancy in cattle, which is induced by IFNT [9].

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As an ubiquitin cross-reactive protein, ISG15 conjugates to the target proteins by its C-terminal LRLRGG motif. ISG15 modification (ISGylation) does not target proteins for degradation, but enhances the cellular response to interferon through enhancing Jak-1 and Stat-1 activities [10], which is different from ubiquitination. ISG15 plays a role in innate immunity, and is closely regulated by specific signaling pathways. During ruminant pregnancy, the maternal immune system must inhibit its immune rejection of the semi-allogeneic fetus to adapt the existence of the conceptus [11,12]. Bone marrow (BM) is a key component of the lymphatic system, supports the body's immune system through producing the lymphocytes. It is reported that there is a progressively diminished in the numbers of pre/pro B and immature B cells in the BM of pregnant mice [13]. However, it is unclear that the expression of ISG15 in the BM during early pregnancy in sheep. In this study, the BM from non-pregnant and early pregnant ewes were sampled to explore the expression of ISG15, which may be helpful to make out the immune suppression mechanism regulated by BM during early pregnancy in sheep.

MATERIAL and METHODS

Animals and Experimental Design

Small Tail Han ewes approximately 18 months of age with normal oestrous cycles were observed daily for oestrus using vasectomized rams, and were mated three times with intact rams at 12-h intervals after the detection of sexual receptivity. The experimental protocol was approved by the Hebei University of Engineering Animal Ethical Committee (HUEAE 2015-021), and humane animal care and handling procedures were followed throughout the experiment. The day of coition was counted as day 0 of pregnancy, and the ewes were randomly divided into four groups (n = 5 for each group), and ewes assigned to the non-pregnant group were not mated with intact ram. The BM was obtained from ewes on days 13, 16 and 25 of pregnancy, day 16 of non-pregnancy at the time of slaughter. Pregnancy was confirmed through observing the presence of conceptus in the uterus. The BM was sampled from the femur, and several sections of BMs (0.3 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in PBS buffer (pH 7.4). The remaining portions of BMs were frozen in liquid nitrogen for subsequent quantitative Real Time PCR (qRT-PCR) and protein analysis.

RNA Extraction and qRT-PCR Assay

The total RNA from the BM sample was extracted using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen, California, USA), and the cDNA was synthesized with FastQuant RT Kit (Tiangen Biotech Co., Ltd., Beijing). The SuperReal PreMix Plus Kit (Tiangen Biotech Co., Ltd., Beijing) was employed for qRT-PCR. The primer sequences of ISG15 (accession no. NM_001009735.1) was designed and synthesized by Shanghai Sangon Biotech Co., Ltd. *(Table 1)*, and GAPDH was based on Oliveira et al.[6] *(Table 1)*. The relative levels of mRNA expression were calculated using an internal control gene (GAPDH). The relative expression value was set as 1 for the group of day 16 non-pregnant BMs.

Table 1. *Primers used for qRT-PCR*

Gene	Primer	Sequence	Size (bp)
ISG15	Forward	CATCCTGGTGAGGAACGACAA	186
	Reverse	AAAGACAGCCAGAACTGGTCC	
GAPDH	Forward	GGGTCATCATCTCTGCACCT	176
	Reverse	GGTCATAAGTCCCTCCACGA	

Western Blot

The total proteins of the BMs were extracted by RIPA Lysis Buffer (Biosharp, BL504A). The protein concentration was measured using a BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing, PA115) with bovine serum albumin as the standard. An equal amount of total proteins (10 μg/lane) was separated using 12% SDS-PAGE, and the proteins were transferred to 0.22 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The PVDF membranes were blocked in 5% non-fat milk in Tris-buffered saline plus Tween 20 (TBST) at 4°C overnight. The mouse anti-ovine ISG15 monoclonal antibody was prepared as described by Austin et al.[14] with roISG15 instead of rboISG15. ISG15 and conjugated proteins were detected using the ISG15 monoclonal antibody (1:20000) at 37°C for 1 h. The secondary goat anti-mouse IgG-HRP was at a dilution 1:20000 (37°C for 1h). Pro-light HRP chemiluminescence detection reagent (Tiangen Biotech Co., Ltd, Beijing) was used to detect immunoreactive bands. Sample loading was monitored with the GAPDH antibody (Santa Cruz Biotechnology, Inc., sc-20357) at a dilution of 1:1000. Immunoreactive proteins greater than 30 kDa were deemed conjugates [15] and semi-quantified together. The intensity (INT) of blots were quantified using Quantity One V452 (Bio-Rad Laboratories), and the unit was INT/mm^2.

Immunohistochemistry Analysis

The fixed BMs were embedded in paraffin, and paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. The sections were quenched endogenous peroxidase activity with 3% H_2O_2, and reduced non-specific binding was completed with 5% normal goat serum in PBS buffer. Immunocytochemical localizations of ISG15 in the BMs were performed using the ISG15 monoclonal antibody (1:2000). The immunoreactive protein was detected using secondary goat anti-mouse IgG-HRP (1:1000). A DAB kit (Tiangen Biotech Co., Ltd., Beijing) was used to visualize the antibody binding sites in the tissue sections, and the nucleus was stained with hematoxylin. Finally, the images were captured on using a light micro-

scope (Nikon Eclipse E800, Japan) and a digital camera DP12, and the intensity of staining and density of stained cells were analyzed through the images.

Statistical Analyses

The relative expression values for the qRT-PCR assay were calculated using the $2^{-\Delta\Delta Ct}$ analysis method. Statistical analyses were performed using Statistical Analysis System Package version 9.1 for Windows (SAS Institute, Cary, NC, USA). *P* value < 0.05 was considered statistically significant difference.

RESULTS

Relative Expression Level of ISG15 mRNA in BMs

The expression levels of ISG15 mRNA were significantly higher at days 13, 16 and 25 in pregnant BMs comparing with that at day 16 in non-pregnant BMs ($P<0.05$), and there was a statistical difference between days 13 and 25 of pregnant BMs in the expression levels of ISG15 mRNA. Furthermore, the relative expression level of ISG15 mRNA was significantly higher at day 16 in pregnant BMs than that at days 13 and 25 in pregnant BMs in sheep *(Fig. 1)*.

Western Blot Analysis for the ISG15 Proteins in BMs

Western blot analysis showed that the ISG15 and conjugated proteins were expressed at days 13, 16 and 25 in pregnant BMs, and only the ISG15 conjugated proteins were expressed at day 16 in non-pregnant BMs. Western blot analysis revealed that the ISG15 and conjugated proteins up-regulated increasingly from day 13 to day 16 in pregnant BMs, and down-regulated from day 16 to day 25 in pregnant BMs in ewes *(Fig. 2)*.

The Immunohistochemistry Analysis for ISG15 in BMs

The immunohistochemistry does not distinguish between free ISG15 and conjugated proteins in the tissue sections, because the antibody recognizes the both forms of free ISG15 and conjugated proteins. In this study, pregnant ewes were adult, so the BMs from femurs were converted to yellow marrow. Yellow marrow is mainly made up of fat cells and little stroma, and stroma includes fibroblasts, macrophages, osteoblasts, osteoclasts, and endothelial cells. Immunohistochemistry analysis confirm that the ISG15 and conjugated proteins were expressed in the stroma from days 13, 16 and 25 in pregnant BMs, and day 16 in non-pregnant BMs. The staining intensity was the strongest in the stroma from day 16 of pregnant BMs, and the staining intensity of the stroma from day 16 in non-pregnant BMs was the lowest *(Fig. 3)*. Furthermore, there was a strong staining intensity located in the cytoplasm of different cells in the stroma from pregnant BMs, including macrophages.

DISCUSSION

It is necessary for the maternal immune system to suppress maternal immune rejection of the semi-allogeneic fetus during pregnancy, which is essential for the conceptus to develop normally in ruminants [11,12]. Many researchers reported that the IFNT exists its effect on blood cells [7,16-18], CL [6,19-21], and liver [9] in the ovine and bovine. It is through paracrine and endocrine style that IFNT secreted by the conceptus releases into the uterine vein, and extends the luteal life span during early pregnancy in sheep [8,22,23]. Therefore, it is possible that IFNT exerts its effect on the BM, which is implicated in regulating the maternal immune system through blood circulation during early pregnancy in sheep.

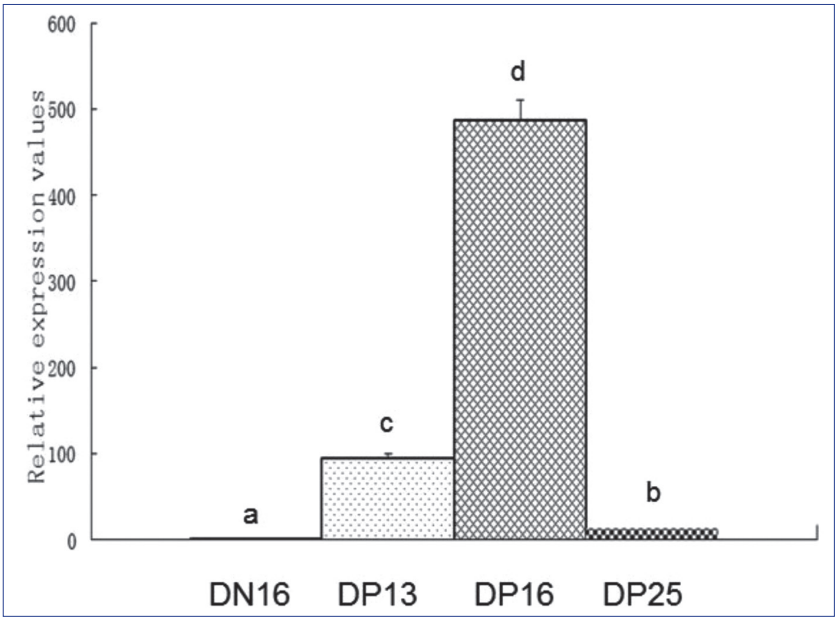

Fig 1. Relative expression value of ISG15 mRNA in day 16 non-pregnant, days 13, 16 and 25 pregnant bone marrow through quantitative real time PCR in sheep. Note: DN16 = Day 16 of non-pregnant bone marrow (BM); DP13 = Day 13 of pregnant BM; DP16 = Day 16 of pregnant BM; DP25 = Day 25 of pregnant BM. Significant difference was indicated by different letters within different column

Our results revealed that there were changed expression of ISG15 mRNA, ISG15 and conjugated proteins from day 13 to 25 in pregnant BMs *(Fig. 1* and *Fig. 2)*. These findings were similar to previous results that there were changed expressions of ISG15 in endometrium [3,24], blood cells [25,26], CL [6,27] during early pregnancy in sheep and cattle. It was indicated that the embryonic signal (IFNT) reached the blood cells, CL and BM to up-regulate ISG15 expression through blood or/and immune cells

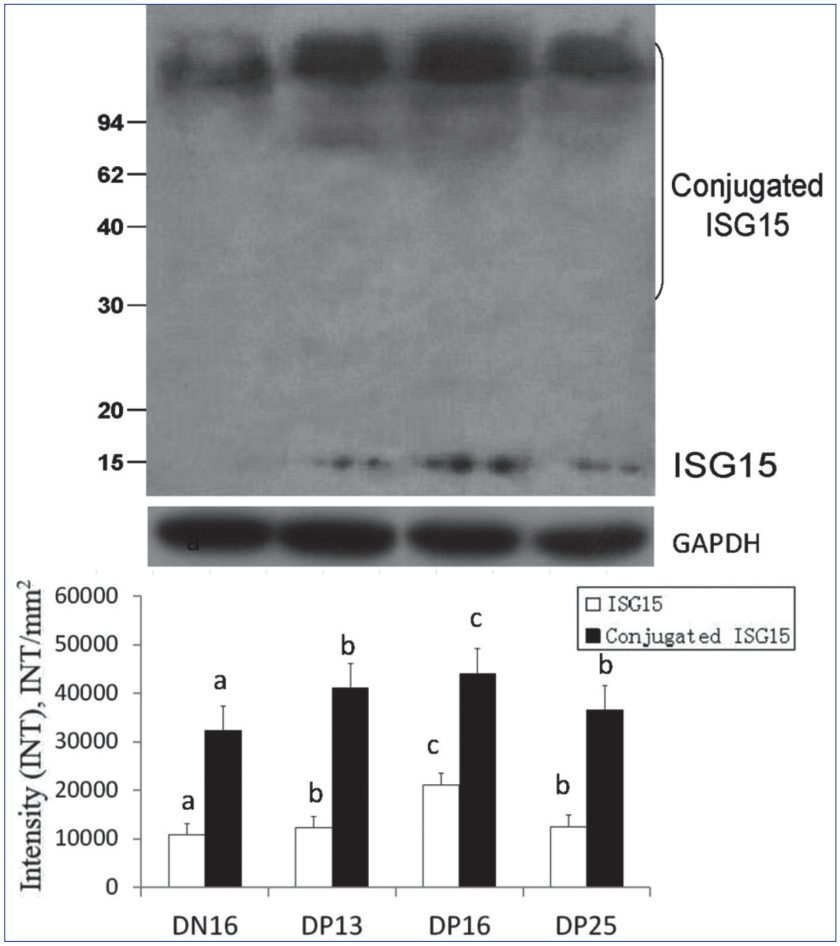

Fig 2. Expression of ISG15 and conjugated proteins in ovine bone marrow from day 16 of non-pregnancy, days 13, 16 and 25 of pregnancy. Note: DN16 = Bone marrow (BM) from day 16 of the estrous cycle; DP13 = BM from day 13 of pregnancy; DP16 = BM from day 16 of pregnancy; DP25 = BM from day 25 of pregnancy. Immunoreactive proteins greater than 30 kDa were deemed ISG15 conjugated proteins. Significant differences ($P<0.05$) are indicated by different superscript letters within same color column

during early pregnancy in sheep. However, there was no expression ISG15 protein in mammary gland during early pregnancy in cattle [27], and there was also no significant change in expression of ISG15 mRNA in cells from milk samples during early pregnancy in cows [28]. Furthermore, we also found that there was no expression ISG15 protein in lymphoid node and spleen during early pregnancy in ewes (our unpublished data), so IFNT exerted its effect selectively on different tissues and cells during early pregnancy in sheep. Therefore, we had no clear explanation that IFNT in the circulation selectively influenced expression of ISG15 by different immune organs during early pregnancy in sheep. It was probable that BM was in some part responsive to the circulating IFNT, or/and peripheral blood immune cells, which remained to be elucidated and verified.

As the central immune organ, BM produces lymphocytes to support the body's immune system. The immune cells mediate a cross-talk between the embryo and mother during early pregnancy in human [29]. There were significantly higher rate of preterm delivery and lower birthweight babies in female who conceived naturally after peripheral blood or BM transplantation than normal conception [30], which indicated that BM was of importance for normal conception. As an ubiquitin cross-reactive protein, ISG15 contains two domains with structural homology similar to ubiquitin [31]. Unlike ubiquitination, protein ISG15 modification (ISGylation) does not target proteins for degradation, but regulates the function of target proteins [32], and ISGylation plays a role in innate immunity [33]. Our results showed that there was an up-regulation of ISG15 and conjugated proteins in the BMs *(Fig. 2)*, which indicated that BM was implicated in regulating the maternal immune response during early pregnancy in ewes.

We also reported that there were changing expressions of Th1 and Th2 cytokines in bovine peripheral blood mononuclear cells (PBMC) during early pregnancy [34]. It is known that macrophages play a crucial role in initiating the immune response. Our present study showed that the positive signal for ISG15 was strongly localized to the cytoplasm of macrophages *(Fig. 3)*. It was suggested that IFNT derived from the conceptus entered the maternal blood circulation, which induced up-regulated expression of ISG15 and conjugated proteins in the stroma of BM. The immune cells from the stroma of BM up-regulated expression of ISG15 and conjugated proteins, and then the immune cells entered into the maternal blood circulation, which was implicated in regulating the maternal immune response. Therefore, it was suggested that up-regulated expression of ISG15 and conjugated proteins in the stroma of BM may be of importance in pregnancy maintenance in ewes.

In conclusion, the expression of ISG15 mRNA, proteins and conjugated proteins were up-regulated in the ovine BM during early pregnancy, which was suggested that IFNT derived from conceptus entered into the maternal blood circulation, induced up-regulated the expression of ISG15 and conjugated proteins in the stroma of BM. The immune cells from the stroma of BM were up-regulated expression of ISG15 and conjugated proteins, which were involved in regulating the maternal immune response through an endocrine style during early pregnancy in ewes.

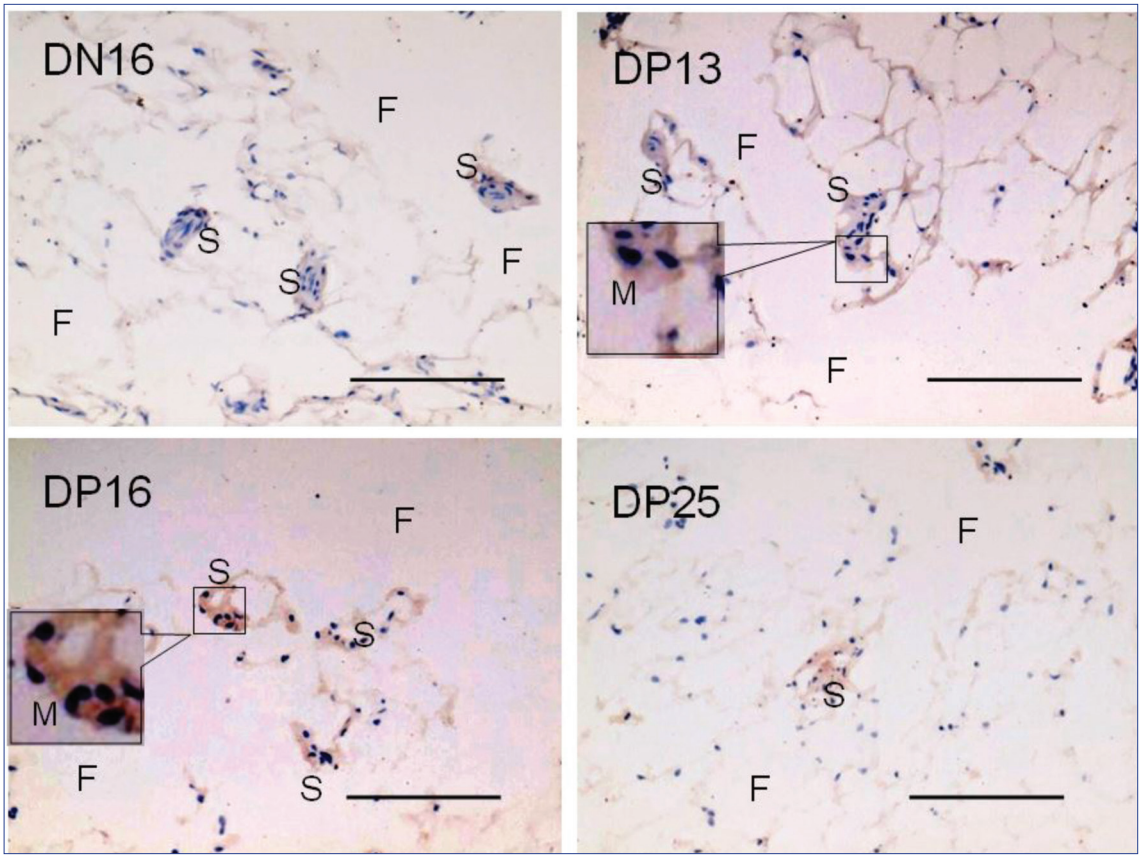

Fig 3. Representative immunohistochemical localization (200 × magnification) of ISG15 in ovine bone marrow collected on day 16 of the estrous cycle and days 13, 16 and 25 of pregnancy. The macrophages are conspicuous by their size and irregular nucleus. The immunohistochemistry does not distinguish between free ISG15 and conjugated proteins in the tissue sections. Note: DN16 = Bone marrow (BM) from day 16 of the estrous cycle; DP13 = BM from day 13 of pregnancy; DP16 = BM from day 16 of pregnancy; DP25 = BM from day 25 of pregnancy; S = Stroma; F = Fat; M = macrophages. Bar = 50 μm

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Research Article

Influence of Niacin Supplementation on the Metabolic Parameters and Lipolysis in Dairy Cows During Early Lactation

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Abstract

The objective of this study was to evaluate the effect of niacin on the metabolic parameters and lipolysis inhibition in dairy cows during early lactation. A total of 30 clinically healthy, multiparous Holstein-Friesian cows in late gestation were enrolled in the study (15 supplemented with niacin (120 g/d/cow) and 15 in the negative control group). Blood samples were taken weekly for 3 weeks after calving. The research results show that cows that received niacin indicated lower NEFA concentrations in all three weeks of the experiment. The NEFA concentrations did not change from weeks 0 to 2 after calving in the niacin group, whereas the NEFA concentrations in the control group were significantly increased ($P<0.05$). The administration of niacin exerted significant effects on the metabolic adaptations in cows during early lactation. Niacin significantly decreased BHB, MDA, total bilirubin, urea and phosphorus concentrations and liver enzyme activity (AST, ALP and GGT) and increased albumin, cholesterol, triglyceride and glucose concentrations. The administration of niacin significantly affected the correlation and regression between NEFA concentrations and other metabolic parameters, rendering the glucose, cholesterol, triglycerides, total bilirubin, AST, albumin, urea and phosphorus values less regressed against the NEFA values. In conclusion, niacin administration decreases lipolysis and metabolic adaptations proved to be less dependent on NEFA concentrations in niacin group compared to the control group.

Keywords: *Cow, Niacin, Lipolysis, Metabolic profile*

Erken Laktasyon Süresince Sütçü İneklerde Niasin İlavesinin Metabolik Parametreler ve Lipoliz Üzerine Etkisi

Özet

Bu çalışmanın amacı erken laktasyon süresince sütçü ineklerde niasinin metabolic parametreler ve lipolizi inhibe etme üzerine etkilerini araştırmaktır. Toplam 30 adet klinik olarak sağlıklı geç gebelik dönemindeki multiparous Holstein-Friesian inek (15'i 120 g/gün niasin takviyesi verilmiş ve 15'i negatif kontrol) çalışmada kullanıldı. Kan örnekleri doğumu takiben 3 hafta süresince haftalık olarak alındı. Çalışma sonuçları niasin alan ineklerde 3 hafta süresince NEFA konsantrasyonunun daha düşük olduğunu gösterdi. NEFA konsantrasyonu 0 ile doğumdan sonraki 2 hafta boyunca niasin grubunda değişmezken kontrol grubunda anlamlı oranda artma gösterdi ($P<0.05$). Niasin verilmesi erken laktasyon dönemindeki ineklerdeki metabolik adaptasyona anlamlı derecede etki etti. Niasin anlamlı oranda BHB, MDA, total bilirubin, üre ve fosfor konsantrasyonları ile karaciğer enzim aktivitesini (AST, ALP ve GGT) düşürürken albümin, kolesterol, trigliserid ve glikoz konsantrasyonlarını artırdı. Niasin verilmesi anlamlı oranlarda olmak üzere NEFA konsantrasyonları ile diğer metabolik parametreler arasındaki korelasyon ve regresyonu etkiledi. Glikoz, kolesterol, trigliserid, total bilirubin, AST, albümin, üre ve fosfor değerleri NEFA değerlerine göre daha az gerileme gösterdi. Sonuç olarak, niasin verilmesi lipolizi azaltırken metabolik adaptasyonun kontrol grubu ile karşılaştırıldığında niasin grubunda NEFA konsantrasyonuna daha az bağımlı olduğu gösterilmiştir.

Anahtar sözcükler: *İnek, Niasin, Lipoliz, Metabolik profil*

INTRODUCTION

During the transition period, energy requirements exceed energy intake, resulting in negative energy balances (NEB) and marked catabolism [1,2]. Increased plasma non-esterified fatty acids (NEFA) concentrations occur as a result of lipid mobilization. Consequently, the high influx of NEFA to the liver exceeds its oxidative capacity, which results in triacylglycerol (TG) accumulation in the liver and ketosis [3]. Lipolysis (NEFA) and ketogenesis (β-hydroxybutyrate- BHB)

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exert a significant influence on the metabolic profile in cows during the periparturient period [4]. Proper nutrition and management of cows during this period benefits their future health and milk production.

The term *niacin* is a generic descriptor for two compounds performing the biological action of the vitamin: nicotinic acid and nicotinamide [5]. Another physiological effect of niacin is the potential to suppress lipolysis when administered in pharmacological doses [6,7]. Nicotinic acid, under certain conditions, reduces the NEFA release from adipose tissue, whereas nicotinamide does not exert the same effect [8]. Reduced NEFA concentrations lead to a lowered TG accumulation and ketone body formation in the liver [9]. The antilipolytic effect of niacin is mediated by the activation of G protein-coupled GPR109A receptors, which exist in a functional form in cattle [10]. Consequently, the inhibition of adenyl cyclase activity occurs, reducing the intracellular levels of cyclic adenosine monophosphate (cAMP) and resulting in the suppression of lipolysis [11].

In this research, it has been hypothesized that antilpolytic effects of niacin is taken into consideration, niacin may show beneficial effect metabolic and oxidative status in dairy cows during early lactation.

The objective of this study was to evaluate the effect of niacin on the metabolic parameters and lipolysis inhibition in dairy cows during early lactation.

MATERIAL and METHODS

Animals, Treatments and Blood Collection

A total of 30 clinically healthy, Holstein-Friesian cows in second and third lactation were enrolled in the study. Cows were 3 to 4 years old. Their health status was evaluated on clinical examination and no signs of illness were found. None of the cows had history of abortion. Cows were divided into two groups, 15 cows in the experimental and 15 cows in the control group. The experimental group was treated with niacin for 14 days prior to the expected calving date and for 14 days after parturition. Niacin was not in a rumen-protected form such as nicotinic acids (Rovimix®Niacin, F. Hoffmann-La Roche AG, Switzerland) and was administered per os with food, 120 g/d per cow (in order to ensure about 12 g of niacin in small intestine). The control group was not treated with niacin. Cows were feed twice a day. The diet suited the energy necessary for cows in late pregnancy and early lactation. The cows in late pregnancy were fed with a diet consisting of 6 kg lucerne hay, 15 kg maize silage (30% DM) and 3 kg concentrate (18% crude proteins, CP). The cows in early lactation were fed with a diet consisting of 7 kg lucerne hay, 20 kg maize silage (30% DM) and 5 kg concentrate (18% CP).

Blood samples were collected by venipuncture from the coccygeal vein before morning feeding and were taken three times, on the day of calving and during the first and second week after parturition. Blood samples for biochemical analysis were collected in sterile 10-ml vacuum tubes with gel separator (BD Vacutainer® SST II Advance, BD Plymoth, UK) and appropriately marked. After centrifugation of the blood samples at 4.000x g for 10 min, the plasma was obtained. The samples were kept in a dry pace and protected from light until laboratory analysis.

Measurements of Metabolic Parameters

Metabolic parameters such as NEFA (colorimetric method based on acylation of ACoA), BHB (BHB dehydrogenase), triglyceride (GPO-PAP method), cholesterol (cholesterol esterase method), glucose (glucose oxidase test, GOT), total protein (Biuret method), albumin (BCG/BCP methods), aspartate aminotransferase (AST) (modified IFCC method), alkaline phosphatase (ALP) (DEA method), gamma-glutamyl transferase (GGT) (colorimetric method with final 5-amino-2-nitrobenzoat), total bilirubin (Modified Jendrassik and Vanadate Oxidation), urea (kinetic method), malondialdehyde (MDA) (method with thiobarbituric acid), calcium (CPC/AMP method) and phosphorus (UV method) concentrations were determined using colorimetric test kits (Randox, UK and Pointe Scientific, USA) and a semi-automatic biochemistry analyzer (Rayto RT/1904cv, China).

Calculation and Statistical Analyses

The effects of niacin administration on the metabolic parameter values were analyzed during the first three weeks after calving (week 0, 1 and 2). The two-way ANOVA analysis and LSD tests indicated differences in the mean values of metabolic parameters between cows that received niacin and cows that did not receive niacin (the control group) in all three weeks of the experiment.

The metabolic adaptations of cows, relative to the degree of lipolysis, were determined by a regression analysis and correlation between the metabolic parameters and NEFA concentrations (using the general linear formula $Y = bXi + a$) in all the samples obtained from the experimental and control groups after calving (N = 45, 15 samples x 3 weeks). The effects of niacin on the metabolic adaptations of cows in early lactation were determined by testing differences in the correlation and regression coefficients obtained for certain metabolic parameters and NEFA concentrations in the experimental and control groups. Statgraphics Centurion (USA) and Microsoft Excel (USA) were used for statistical calculations and analysis. $P<0.05$ level was accepted statistically significant.

RESULTS

The research results show that cows supplemented with niacin indicated lower NEFA concentrations in all three weeks of the experiment. The NEFA concentrations did

Table 1. *Effects of niacin administration on the metabolic parameter values in cows during early lactation*

Parameter	Group	Week Postpartum			Influence of Niacin (P)	Influence of Week (P)
		0	1	2		
NEFA mmol/L	Niacin	0.30±0.09	0.35±0.1	0.39±0.1	<0.01	NS
	Control	0.47±0.09	0.67±0.1	0.76±0.1		<0.01
BHB mmol/L	Niacin	0.49±0.11	0.68±0.12	0.79±0.12	<0.01	<0.05
	Control	0.62±0.12	0.86±0.13	1.03±0.11		<0.01
Glucose mmol/L	Niacin	3.08±0.3	2.73±0.29	2.51±0.3	<0.01	<0.05
	Control	2.53±0.29	2.11±0.25	1.97±0.2		<0.05
Triglycerides mmol/L	Niacin	0.12±0.008	0.12±0.009	0.10±0.01	<0.01	<0.01
	Control	0.11±0.007	0.097±0.008	0.087±0.01		<0.01
Cholesterol mmol/L	Niacin	2.53±0.15	2.41±0.16	2.02±0.13	<0.01	<0.05
	Control	2.05±0.12	1.95±0.17	1.64±0.14		<0.01
MDA µmol/L	Niacin	1.42±0.12	1.63±0.15	1.8±0.14	<0.01	<0.01
	Control	1.89±0.3	2.36±0.25	2.6±0.35		<0.05
T. bilirubin µmol/L	Niacin	6.31±1.2	7.82±0.95	9.18±1.2	<0.01	<0.01
	Control	8.35±1.1	10.85±1.1	13.1±0.99		<0.01
AST U/L	Niacin	83±9.5	101±10.5	107±9.9	<0.01	<0.01
	Control	93±10.1	121±12	130±10.8		<0.01
ALP U/L	Niacin	68±9	72±14	74±15	<0.01	NS
	Control	99±12	106±13	108±10		NS
GGT U/L	Niacin	15±2.5	17±2.1	18±2.6	<0.01	<0.05
	Control	18±2.2	21±2.3	22±2.4		<0.05
Total protein g/L	Niacin	77.1±4.1	72.5±4.3	71.1±4.1	NS	<0.05
	Control	78.9±4.2	74.1±4.4	72.7±4.1		<0.05
Albumin g/L	Niacin	41.7±3.5	38±3.2	37±3.5	<0.01	NS
	Control	34.6±3	31.5±3.7	30.7±3.3		NS
Urea mmol/L	Niacin	3.5±0.6	3.3±0.7	3.5±0.6	<0.01	NS
	Control	5.1±0.9	4.8±0.9	4.6±0.9		NS
Ca mmol/L	Niacin	2.25±0.25	2.16±0.24	2.13±0.22	NS	NS
	Control	2.1±0.23	2.07±0.25	2.04±0.24		NS
P mmol/L	Niacin	2.17±0.21	2.08±0.23	2.07±0.2	<0.05	NS
	Control	2.43±0.25	2.33±0.24	2.31±0.22		NS

not change from weeks 0 to 2 after calving in the group that received niacin, whereas the NEFA concentrations in the control group were significantly increased. The administration of niacin exerted significant effects on the metabolic adaptations in cows during early lactation. Cows supplemented with niacin were found to exhibit significantly lower BHB concentrations, higher cholesterol and triglyceride concentrations, lower MDA concentrations, higher glucose concentrations, decreased total bilirubin concentrations and liver enzyme activity (AST, ALP and GGT), higher albumin concentrations, and lower urea and phosphorus concentrations. The changes in metabolite values were also tested in all weeks of the experiment. The results are shown in *Table 1*.

In the niacin group, the metabolic adaptations proved to be less dependent on NEFA concentrations compared to the control group. With increased lipolysis, the administration of niacin significantly reduced the degree of ketogenesis *(Fig. 1)*. Increased NEFA concentrations greatly impeded the increase in MDA concentrations in cows that received niacin *(Fig. 2)*. In addition to ketogenesis and lipid peroxidation, the administration of niacin significantly affected the correlation between NEFA concentrations and other metabolic parameters, rendering the glucose, cholesterol, triglycerides, total bilirubin, AST, albumin, urea and phosphorus values less regressed against the NEFA values *(Table 2)*.

DISCUSSION

A typical metabolic profile of cows a week postpartum is characterized by reduced plasma concentrations of

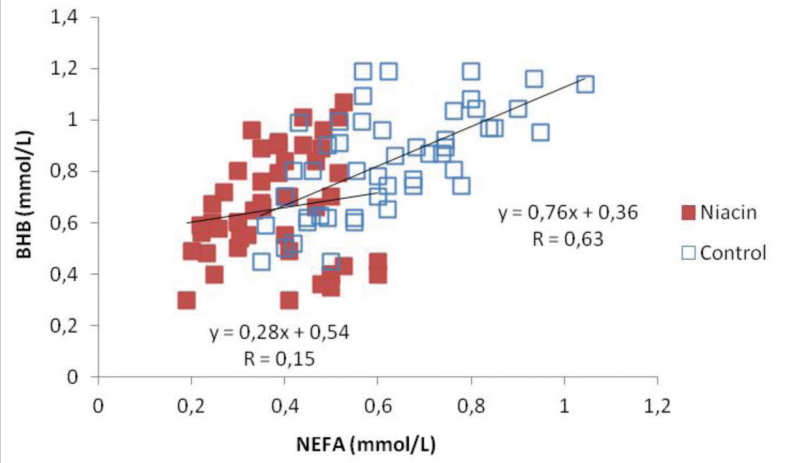

Fig 1. Regression and correlation between the NEFA and BHB concentrations in the group of cows supplemented with niacin and the control group

Fig 2. Regression and correlation between the NEFA and MDA concentrations in the group of cows supplemented with niacin and the control group

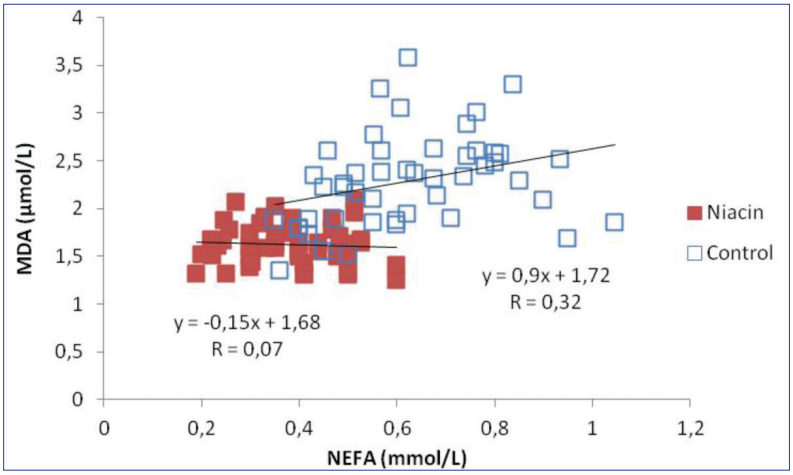

Table 2. *Effects of niacin administration on the regression and correlation between the metabolic parameters and NEFA concentrations*

Blood Parameters	Correlation with NEFA		P	Regression Against NEFA		P
	Niacin	Control		Niacin	Control	
BHB, mmol/L	0.15	0.63	<0.01	0.28	0.76	<0.01
Glucose, mmol/L	-0.17	-0.57	<0.01	-0.64	-1.16	<0.05
Triglycerides, mmol/L	-0.2	-0.72	<0.01	-0.02	-0.05	<0.05
Cholesterol, µmol/L	0.06	-0.69	<0.01	0.14	-0.87	<0.01
MDA, µmol/L	0.07	0.32	<0.01	-0.15	0.9	<0.01
T. bilirubin, µmol/L	0.06	0.61	<0.01	0.97	8.2	<0.01
AST, U/L	0.02	0.57	<0.01	3.3	63.1	<0.01
ALP, U/L	0.26	0.33	NS	15.5	20	NS
GGT, U/L	0.2	0.3	NS	6.37	5.38	NS
Total protein, g/L	0.05	0.4	<0.01	-2.4	-12.6	<0.01
Albumin, g/L	0.33	0.52	<0.05	17.7	-11	<0.01
Urea, mmol/L	~0	~0	NS	~0	~0	NS
Ca, mmol/L	0.06	0.07	NS	-0.15	-0.19	NS
P, mmol/L	~0	~0	NS	~0	~0	NS

glucose, protein, albumin, cholesterol and calcium, as well as high plasma concentrations of NEFA, BHB, bilirubin and liver enzymes [12,13]. Pharmacological doses of niacin have the ability to inhibit lipolysis, thereby reducing the hepatic uptake of NEFA, improve the metabolic profile of the transition cow and prevent energy-related metabolic

disorders associated with excessive mobilization of fat reserves [6,14]. Havlin et al.[15] concluded that effects of niacin in dairy cows dependent on dose level and feeding duration and low level of niacin feeding did not reduced blood serum NEFA concentration but it did result in a decreased prevalence of ketosis. In this study, cows that received niacin indicated decreased plasma NEFA and BHB concentrations in all three weeks of the experiment alongside a strong but lower correlation between the NEFA concentrations and metabolic parameters in comparison with the control group. Niacin was shown to reduce the dependence of the metabolic adaptations on the degree of lipid mobilization in cows during early lactation. Pires and Grummer [6] showed that a single abomasal infusion of niacin inhibits lipolysis and reduces plasma NEFA concentrations, followed by a rebound in plasma NEFA concentrations upon the termination of infusion and Rauls et al.[16] showed that niacin-supplemented cows had lower NEFA concentration in week 1 of lactation than non supplemented niacin cows but niacin had no effect if the cows are in late lactation. In our study, the niacin group did not indicate significant changes in the NEFA concentrations in all weeks of the experiment, which proved the antilipolytic effect of niacin and the importance of continuous niacin administration during the transition period. Serum levels of glucose, triglyceride, total cholesterol, total protein, albumin and urea are indicators of hepatic function [17,18], and a decrease in the plasma concentration of these metabolites may occur provided endogenous liver synthesis is reduced. In this study, the concentrations of triglyceride, cholesterol, glucose and albumin were higher in the niacin group, which emphasizes a better hepatic function. In human medicine the niacin is used as broad-spectrum lipid drug and has ability to reduce cholesterol concentration in plasma thereby inhibits diacylglycerol transferase, enzyme that is a key in hepatic synthesis of triglycerides. The niacin causes reduced catabolism rate of LDL (low-density lipoprotein) and VLDL (very low density lipoprotein) [19,20]. But metabolism in ruminates is different than metabolism of other mammals by having diminished capacity to form VLDL from cholesterol and to secrete VLDL from the liver [21,22]. There is negative correlation between cholesterol and NEFA [23] and the effect of niacin on triglyceride and cholesterol may be due to the action of niacin on NEFA. Further research is needed to established the direct effect of niacin on cholesterol in dairy cows.

When fat infiltrates the liver, a lesion appears in the hepatic tissues and the levels of enzymes indicating the liver injury (AST and GGT) are generally augmented [24]. Lower liver damage in the niacin group can be detected by a lower activity of liver enzymes (AST, ALP and GGT) and a lower concentration of total bilirubin. Plasma NEFA concentrations increase a few days before parturition and rise to higher levels in the week after calving [25,26]. In our study, cows supplemented with niacin were found to exhibit a reduced degree of ketogenesis upon increased lipolysis, which may be due to pharmacological doses of niacin administered. Niacin decreased the NEFA release from adipose tissue, leading to a reduced hepatic uptake of NEFAs and lowered BHB formation, i.e. an improved hepatic function. In the niacin group, less regression was recorded between the values of MDA and NEFA concentrations, which indicates that the administration of niacin may exert effects on lipid peroxidation. Moreover, Yuan et al.[9] speculate that niacin may have the antioxidant potential via inhibiting lipolysis, and thus decreasing the amount of fatty acid substrates available for lipid peroxidation.

Glucose concentrations change in early lactation in same manure in both of niacin and control group. Higher concentration of glucose in niacin group could be consequence of greater gluconeogenic activity, decrease removal of glucose or increased hepatic glucose production [7,27-29].

The administration of niacin affects the indicator values of carbohydrate metabolism, fats, proteins, as well as the functional status of hepatocytes and ions. Niacin decrease ketogenesis and oxidative stress in cows during early lactation.

In conclusion, niacin reduces the dependence of the metabolic adaptations on the degree of lipid mobilization in cows during early lactation.

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Research Article

Effect of *Laurocerasus officinalis* Roem. (Cherry Laurel) Fruit on Dimethoate Induced Hepatotoxicity in Rats [1]

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Abstract

Dimethoate is one of the most important organophosphate insecticides and may cause oxidative stress leading to production of free radicals and alterations in antioxidants. The aim of this study was to investigate the protective effects of *Laurocerasus officinalis* Roem. fruit extract containing antioxidant compounds on dimethoate-induced hepatotoxicity in rats. The rats were divided into six groups as follows: Control group; dimethoate-treated group; L. officinalis-treated group; the group of pre-treatment with *L. officinalis* prior to dimethoate; the group of pre-treatment with vitamin C before dimethoate; the group of post-treatment with *L. officinalis* after dimethoate. Aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin (TBil) as liver function tests and the oxidative stress parameters such as total oxidant status (TOS), total antioxidant status (TAS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) were measured. The DNA damage was determined with comet assay. The results indicated that dimethoate caused a significant increase in AST, ALT, TOS, MDA, DNA damage and an important decrease in TAS, SOD, CAT, GPx as compared to control group. However, administration of *L. officinalis* or vitamin C to dimethoate-exposed rats restored these biochemical and oxidative stress parameters to nearly normal levels. In conclusion, toxic effects of dimethoate on rat liver are mainly attributed to hepatic function enzymes, oxidative stress and DNA damage, while these effects were largely ameliorated by *L. officinalis* fruit extract.

Keywords: *Laurocerasus officinalis Roem, Cherry Laurel, Dimethoate, Hepatotoxicity, Rat*

Sıçanlarda Dimetoatla Oluşturulan Hepatotoksisite Üzerine Taflan *(Laurocerasus officinalis* Roem.) Meyvesinin Koruyucu Etkisi

Özet

Dimetoat en önemli organofosfatlı insektisitlerden biridir, serbest radikallerin üretimine ve antioksidanlarda değişimlere yol açarak oksidatif strese neden olur. Çalışmanın amacı, ratlarda antioksidan bileşikler içeren *Laurocerasus officinalis* Roem. meyve ekstresinin dimetoat kaynaklı karaciğer toksisitesindeki koruyucu etkilerini incelemektir. Ratlar aşağıdaki gibi altı gruba ayrıldı: Kontrol grubu; dimetoate ile muamele edilen grup; *L. officinalis* ile muamele edilen grup; dimetoat öncesi *L. officinalis* verilen grup; dimetoat öncesi vitamin C verilen grup; dimetoat sonrası *L. officinalis* ile muamele edilen grup. Karaciğer fonksiyon testleri olarak aspartat transaminaz (AST), alanin transaminaz (ALT) ve toplam bilirubin (TBil) ile toplam oksidan durum (TOS), toplam antioksidan durum (TAS), süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GPx) ve malondialdehit (MDA) gibi oksidatif stres parametreleri ölçüldü. DNA hasarı komet testi ile belirlendi. Sonuçlar, dimetoat'ın kontrol grubuna kıyasla AST, ALT, TOS, MDA, DNA hasarında belirgin bir artışa ve TAS, SOD, CAT, GPx'de önemli bir azalmaya neden olduğunu gösterdi. Bununla birlikte, dimetoat'a maruz bırakılan ratlara *L. officinalis* veya vitamin C'nin uygulanması, bu biyokimyasal ve oksidatif stres parametrelerini neredeyse normal seviyelere getirdi. Sonuç olarak, dimetoat'ın rat karaciğeri üzerindeki toksik etkileri esas olarak hepatik fonksiyon enzimleri, oksidatif stres ve DNA hasarına atfedilirken, bu etkiler *L. officinalis* meyve ekstresi tarafından büyük ölçüde iyileştirilmiştir.

Anahtar sözcükler: *Karayemiş, Taflan, Dimetoat, Hepatotoksisite, Sıçan*

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INTRODUCTION

Dimethoate (*O,O*-dimethyl *S*-*N*-methyl carbamoyl methyl phosphorodithioate) is one of the most significant and broad-use organophosphate insecticides on a great number of crops against various pests [1,2]. Producers, pesticide workers, and farm owners are the basic risk groups of dimethoate exposure [3]. Previous studies indicate that dimethoate leads to oxidative stress via production of free radicals and induction of lipid peroxidation [4-6]. Some studies have shown that dimethoate causes significant increase in lipid peroxidation by interacting with membrane lipids due to its lipophilic feature [7,8]. It has been noticed the dimethoate toxicity results in deleterious effects on various organs such as liver, brain, testes, pancreas of rats [9]. The liver is the target organ for chemicals and play a main role in xenobiotic metabolism. Therefore, evaluation of hepatotoxicity is an important process for the detection of toxic action of xenobiotics [10].

There is a growing interest in the importance of natural dietary antioxidant compounds as therapy to prevent damage in many health concerns related to oxidative stress [6,11]. Therefore, high fruit consumption may reduce the risk of some diseases such as hepatotoxicity, cancer, cardiovascular and coronary heart diseases, diabetes and atherosclerosis [12]. *Laurocerasus officinalis* Roem. (Cherry laurel) is grown as a native fruit in the coasts of the Black Sea region of Turkey and locally called "Taflan" or "Karayemiş" [13]. It was found that *L. officinalis* fruit is a rich source of antioxidant substances such as phenolics (chlorogenic acid, phenolic acids, anthocyanins, vanillic acid) and ascorbic acid [14-20]. Some studies indicated that *L. officinalis* fruit has radical scavenging action against superoxide and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals [15,19,21].

In previous studies, some pesticides have been shown to cause oxidative stress, DNA damage and cytotoxicity *in vitro* and *in vivo* test systems. Thus, the purpose of the research was to evaluate the hepatotoxic actions of dimethoate and the preventive efficacy of *L. officinalis* fruit on liver function markers, parameters of oxidative stress, and DNA damage in rats.

MATERIAL and METHODS

Chemicals

An emulsion form of dimethoate (Korumagor 40 EC, 40%, Koruma Agriculture, Turkey) was diluted in saline (0.9% NaCl) to get an effective dose (7 mg/kg body weight-bw) for rats. The percentage of the active content was used for the calculation of concentration of dimethoate. All other reagents were analytical grade and provided from Sigma Chemical Co. (St. Louis, MO, USA).

Plant Material and Preparation of Extract

L. officinalis fruits were collected from Akçaabat, Trabzon in Turkey and voucher specimen was deposited in the Herbarium of Pharmacy Faculty of Ankara University (AEF 26257). The fruits were washed with distilled water and dried at 40°C for 5 days. The fruits were macerated with methanol (MeOH) with magnetic stirrer for 8 h at room temperature and the extracts were filtered by Whatman No. 1 filter paper. This process was repeated twice with MeOH. All filtrates were dried at 40°C with a rotary evaporator and then lyophilized. The lyophilisate was kept in a freezer until usage.

Determination of Total Phenolic Content

Total phenolic content of fruit extract was identified by the method of Ahmed et al.[22]. 0.5 mL extract and 0.25 mL Folin-ciocalteu reagent and 0.5 mL sodium carbonate (20% w/v) were mixed and incubated for colour development at room temperature for 60 min. The absorbance was recorded at 765 nm with spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan). Total phenolic substance was defined as mg gallic acid equivalent (GAE)/g extract from the calibration curve. This experiment was repeated 3 times and the results were shown as mean±SD values.

DPPH Radical Scavenging Activity

Determination of radical scavenging effect of the fruit extract was assayed according to the method of Pourmorad et al.[23]. 50 µL of the extract solution at various concentrations were allowed to react with the 950 µL methanolic solution of DPPH (100 µM) in the dark for 10 min. Thereafter, the absorbance change was followed at 515 nm with a spectrophotometer. Measurements were repeated in triplicate. The known antioxidants, gallic acid, chlorogenic acid, and quercetin were applied as standards. The value of inhibitory concentration (IC_{50}) means the amount of sample, which is necessary to clearance 50% of DPPH free radicals.

Animals and Experimental Procedure

The experimental procedure approved by the Ethical Committee for Animal Research at Erciyes University (approval date:15.08.2012; no:12/82) was in accordance with the European Union Directive 2010/63/EU for care and use of laboratory animals. Sixty male Wistar albino rats (weighing 200-250 g) were housed under standard laboratory conditions (in polycarbonate cages, 12 h/12 h light/dark cycle, 22-24°C temperature and 55-60% relative humidity).

The rats were divided randomly into six groups consisting of ten animals each and were applied with oral gavage during sixty days as follow: Group I (controls) received only saline; group II (D) was exposed to dimethoate; group III (LOFE) was applied with only *L. officinalis* fruit extract; group IV (LOFE + D) received *L. officinalis* fruit extract 30 min prior to dimethoate; group V (Vit C + D) was given vitamin C 30 min before dimethoate; group VI (D + LOFE)

received dimethoate during the first month, the rats were exposed to dimethoate 30 min prior to *L. officinalis* fruit extract throughout the second month. The doses for dimethoate (7 mg/kg/day) [2], vitamin C (100 mg/kg/day) [5], and *L. officinalis* fruit extract (4 mg/kg/day) [24] were determined by using the results of other studies.

Sample Preparation

At the end of the experiment, blood was collected from anesthetized (xylazine/ketamine) rats and then centrifuged at 4400 rpm for 10 min at 4°C. Samples of plasma were kept at -20°C until analysis for the evaluation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBil) levels. Livers were removed and washed in ice-cold physiologic saline solution, blotted and divided into three parts. One part of liver was preserved in 10% formalin for histopathologic evaluation. For comet assay, a piece of the liver cut into thin slices were placed in phosphate buffered saline. Tissues were allowed to stir for dissociation at 500 rpm for 10 min to get the cell suspension [25]. For oxidative stress parameters, a homogenate from about 0.5 g of the remaining liver was obtained with a homogenizer (IKA Ultra-Turrax T10 basic model, Germany) in cold buffer solution of 140 mM KCl and 50 mM Tris-HCl (pH 7.6) for 2 min at 13000 rpm. Malondialdehyde (MDA) level was measured in the liver homogenate by using spectrophotometer. The homogenates were then centrifuged at 15000 rpm for 30 min at 4°C and clear supernatant was used for the assessment of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), total oxidant status (TOS) and total antioxidant status (TAS) levels. Protein content in tissue homogenate and supernatant was determined according to method of Lowry et al.[26].

Liver Function Tests

The levels of AST, ALT, and TBil in plasma were assayed spectrophotometrically using by commercial diagnostic kits (Biolabo Company, France)

Oxidative Stress Parameters

Activity of SOD was evaluated as defined by Eken et al.[27]. In brief, 840 µL of substrate solution (0.05 mmol/L xanthine sodium, 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride, 50 mmol/L 3-(cyclohexylaminol)-1-propanesulfonic acid, and 0.094 mmol/L ethylenediaminetetraacetic acid-EDTA) (pH 10.2) were mixed with 30 µL supernatant. After addition of 130 µL xanthine oxidase (120 U/L) to the mixture, the absorbance increase was recorded by spectrophotometer at 505 nm for 3 min. The activity of SOD was defined in U/mg protein.

Activity of GPx was identified by the method of Pleban et al.[28]. 30 µL supernatant and 970 µL of the reaction mixture (50 mmol/L tris(hydroxymethyl)aminomethane, 1 mmol/L EDTA-disodium salt, 2 mmol/L reduced glutathione, 4 mmol/L sodium azide, 0.2 mmol/L nicotinamide adenine dinucleotide, and 1000 U glutathione reductase) (pH 7.6) were mixed and incubated for 5 min at 37°C. When 8.8 mmol/L hydrogen peroxide (H_2O_2) was added, the decrement of absorbance was followed for 3 min by spectrophotometer at 340 nm. Activity of GPx was given as U/mg protein.

CAT activity was evaluated according to Aebi [29]. The rate of H_2O_2 decomposition in supernatant was recorded at 240 nm for 30 sec at 25°C. Activity of CAT was given as U/mg protein.

MDA level was detected for marker of lipid peroxidation by the method of Ohkawa et al.[30]. The MDA in the sample was reacted with thiobarbituric acid reactive substance and then the product was measured spectrophotometrically at 532 nm. Tetramethoxypropane was used as a standard. The result was expressed as nmol/mg protein.

Assays of TOS and TAS developed by Erel [31] were carried out by commercial kits (Rel Assay Diagnostic, Turkey) in supernatant. Oxidative stress index (OSI), which is a marker of the ratio of oxidative stress, was calculated from the percent ratio of TOS to TAS.

Determination of DNA Damage with Comet Assay

DNA damage was identified by comet technique as defined by Singh et al.[32] with some changes [33]. Firstly, microscope slides were pre-coated by using agarose with normal melting point (0.5%) in distilled water, drying at room temperature. Secondly, 10 µL of the cell suspension was mixed with 100 µL of agarose with low melting point (0.8%) in phosphate buffer saline at 37°C and dropped onto the first layer. Slides waited to concretion at 4°C in a humid container for 5 min were immersed in cold buffer solution (25 g sodium dodecyl sulfate in Tris Borate-EDTA) for 7 min at 4°C. The slides removed from the buffer solution was placed in the electrophoresis unit containing neutral electrophoresis solution (27.5 g boric acid, 54 g Tris, and 20 mL EDTA, pH 8.4) and left to unwind the DNA for 20 min. Electrophoresis was applied at 64 V for 2 min and was set to 250 mA. The neutralized slides were stained with 50 µL ethidium bromide. A fluorescent microscope (Olympus, BX51, Japan) was used for observations with a magnification of x400. Comet assay software project (CASP-1.2.2, Windows 2010) was applied for evaluation the images of 50 randomly selected nuclei. A tail of fragmented DNA that migrated from the cell head, causing a 'comet' pattern indicated damage. However, without a comet, whole cell heads were not regarded to be detriment.

Histopathologic Evaluation

The liver pieces fixed in formalin (10%) were embedded in paraffin. Thick paraffin sections about 5 µm were cut from each specimen and were stained with hematoxylin and

eosin dye. A microscope (Olympus BX-51, Japan) equipped with a high resolution of camera (Olympus DP-71, Japan) was used for the histologic assessment.

Statistical Analysis

Statistical package for the social sciences (SPSS version 18.0 for Windows, Chicago, IL, USA) was performed for the analysis of the data. The comparison of the values among the groups was performed by one-way ANOVA and followed by Tukey multiple comparisons test. The results were defined as mean±standard deviation (SD). P<0.05 value was considered significant.

RESULTS

Antioxidant Properties of L. officinalis Fruit Extract

The phenolic content of fruit extract was detected to be high (340±3.18 mg GAE/g extract) which may be responsible for antioxidant activity. As presented in *Table 1*, DPPH scavenging effect of the extract was referenced to known antioxidants (quercetin, gallic and chlorogenic acid). *L. officinalis* fruit extract was demonstrated to possess DPPH radical scavenging activity.

Hepatic Function Markers

Data represented in *Table 2* shows the liver function tests of control and experimental rats. Treatment with dimethoate alone caused a significant increase in AST (+43%) and ALT (+59%), and the level of TBil (+22%) compared to the control rats (P<0.05). On the other hand, pretreatment with *L. officinalis* fruit extract (-37%) or vitamin C (-37%) greatly reduced the activity of ALT in comparison to the group of dimethoate-applied alone (P<0.05). Pre- (-42%) and post-treatment (-27%) with *L. officinalis* fruit extract or pretreatment of vitamin C (-30%) significantly decreased the activity of AST compared with the group of dimethoate-treated alone (P<0.05). Pre- (-29%) and post-treatment (-28%) with *L. officinalis* fruit extract or vitamin C treatment (-33%) remarkably diminished the TBil level compared with the group of dimethoate-applied alone (P<0.05). *L. officinalis* fruit extract treatment without dimethoate did not show any important change in hepatic enzymes of AST and ALT and TBil level when compared to control rats (P>0.05).

Oxidative Stress Parameters

The results of *Table 3* indicate the MDA levels and the antioxidant enzyme activities of SOD, CAT, and GPx. Dimethoate treatment raised to a considerable increase in MDA levels, while an important decrease was seen in SOD, CAT, and GPx as compared to the control rats (P<0.05). In contrast, pre- and post-treatment with *L. officinalis* fruit extract or vitamin C greatly (P<0.05) diminished the elevated MDA level, whereas the SOD and GPx were prominently raised in comparison to the dimethoate-treated group (P<0.05). Pre-treatment with *L. officinalis* fruit extract or vitamin C treatment remarkably elevated the activities of CAT as compared to the dimethoate-applied group (P<0.05). Administration of *L. officinalis* fruit extract did not show any significant change in SOD, CAT, GPx and MDA when compared to controls (P>0.05).

TOS and TAS levels and the mean value of OSI were shown in *Fig. 1A, 1B,* and *1C*, respectively. An important rise in the values of TOS and OSI was observed in dimethoate administered rats, while a substantial decrement was detected in the TAS in comparison to the control rats (P<0.05). On the other hand, pre- and post-treatment with *L. officinalis* fruit extract or vitamin C treatment significantly (P<0.05) reduced the elevated TOS and OSI levels, whereas the TAS level was greatly increased in comparison to dimethoate-treated group (P<0.05). There was not seen any substantial change in the values of TOS, TAS, and OSI in the *L. officinalis* fruit extract-applied rats alone as compared to the controls (P>0.05).

DNA Damage

In this study, the tail DNA% in total comet DNA in liver cells of control and experimental rats was measured via comet assay as an indicator of DNA damage. The results are illustrated in *Fig. 2* and comet images of rat liver cells for each experimental group are shown in *Fig. 3*. An important increase by 94% in parameter of the tail DNA% was observed in rats receiving dimethoate alone compared with the control rats. Pre-treatment (-48%) and

Table 1. *Comparison of DPPH radical scavenging activity of L. officinalis fruit extract and those of standards*

Samples	Concentration (mg/mL)	Scavenging (%)	IC_{50} (mg/mL)
L. officinalis	50	83±0.94	17±0.09
Gallic acid	0.125	85±1.2	0.05±0.02
Quercetin	0.125	74±1.5	0.08±0.06
Chlorogenic acid	0.226	85±0.98	0.13±0.08

Each value in the table was obtained by calculating the average of three experiments±standard deviation

Table 2. *Toxic effect of dimethoate and the ameliorative potency of L. officinalis fruit extract on liver function tests*

Groups	AST (μKat/L)	ALT (μKat/L)	TBil (mg/dL)
Control	0.63±0.17	0.37±0.07	1.00±0.14
Dimethoate	0.90±0.16[a]	0.59±0.07[a]	1.22±0.21[a]
LOFE	0.55±0.20	0.43±0.11	0.93±0.12
LOFE + D	0.52±0.14[b]	0.37±0.10[b]	0.87±0.14[b]
Vit C + D	0.63±0.15[b]	0.37±0.14[b]	0.82±0.10[b]
D + LOFE	0.66±0.13[b]	0.57±0.15	0.88±0.21[b]

Values are expressed as mean±SD. ***LOFE:*** *L. officinalis fruit extract;* ***D:*** *dimethoate;* ***Vit C:*** *vitamin C;* ***AST:*** *aspartate aminotransferase;* ***ALT:*** *alanine aminotransferase;* ***TBil:*** *total bilirubin.* [a] *P<0.05 vs. control group;* [b] *P<0.05 vs. dimethoate-treated group*

Table 3. *Toxic effect of dimethoate and the ameliorative potency of L. officinalis fruit extract on oxidative stress parameters*

Groups	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Control	0.45±0.07	7.03±0.75	637.37±59.22	9.84±0.67
Dimethoate	0.97±0.06[a]	5.88±0.76[a]	496.24±54.99[a]	7.64±0.73[a]
LOFE	0.48±0.08	6.93±0.75	575.46±57.45	9.15±0.65
LOFE + D	0.57±0.08[b]	6.94±0.58[b]	579.36±31.09[b]	9.38±0.49[b]
Vit C + D	0.50±0.06[b]	6.99±0.63[b]	594.55±60.22[b]	9.65±0.59[b]
D + LOFE	0.55±0.09[b]	6.96±0.49[b]	514.67±50.29	9.42±0.65[b]

Values are expressed as mean±SD. ***LOFE:*** *L. officinalis fruit extract;* ***D:*** *Dimethoate;* ***Vit C:*** *vitamin C; MDA: malondialdehyde;* ***SOD:*** *superoxide dismutase;* ***CAT:*** *catalase;* ***GPx:*** *glutathione peroxidase.* [a]*P<0.05 vs. control group;* [b]*P<0.05 vs. dimethoate-treated group*

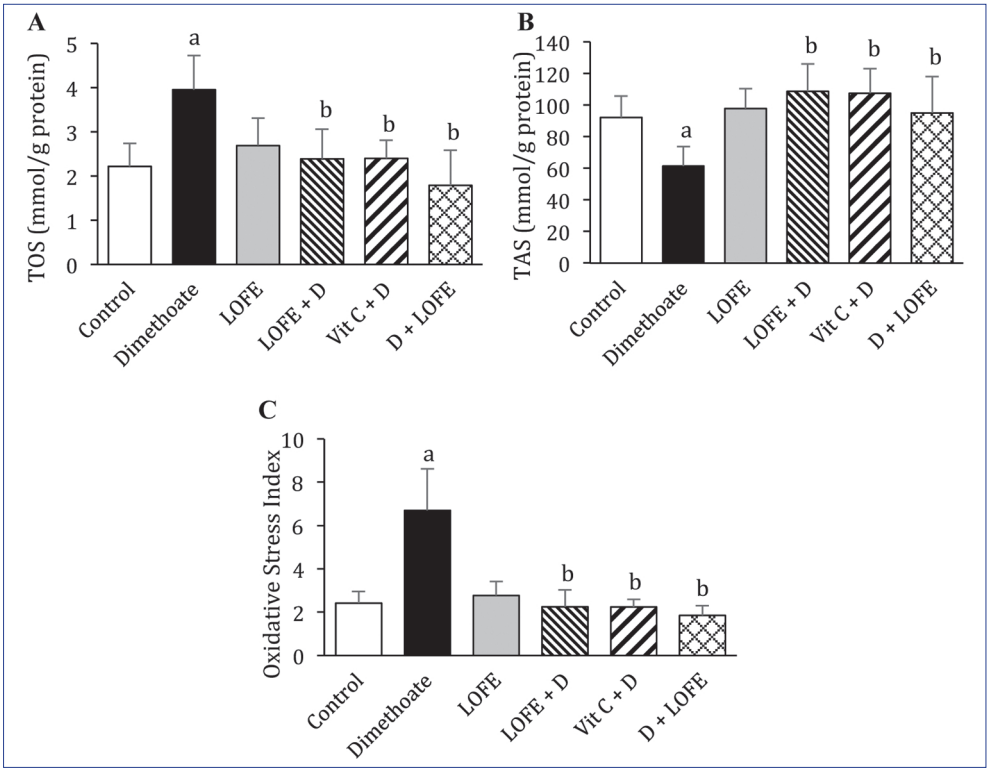
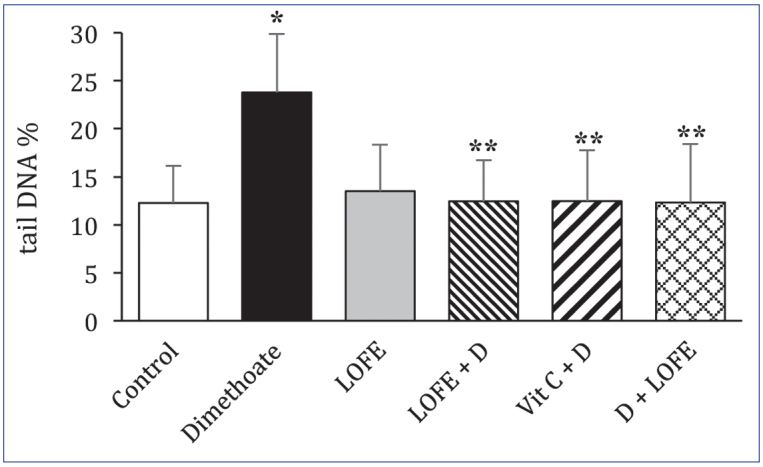


Fig 1. Toxic effect of dimethoate and the ameliorative potency of *L. officinalis* fruit extract on TOS (A), TAS (B), and OSI (C) parameters. LOFE: *L. officinalis* fruit extract; D: Dimethoate; Vit C: vitamin C; TOS: total oxidant status; TAS: total antioxidant status; OSI: oxidative stress index; Values are expressed as mean ± SD; [a]P<0.05 vs. control group; [b] P<0.05 vs. dimethoate-treated group

Fig 2. Toxic effect of dimethoate and the ameliorative potency of *L. officinalis* fruit extract on the tail DNA% with comet assay in hepatocytes of rats. LOFE: *L. officinalis* fruit extract; D: Dimethoate; Vit C: vitamin C; Values are expressed as mean ± SD. [*]P<0.05 vs. control group; [**]P<0.05 vs. dimethoate-treated group



post-treatment (-48%) with *L. officinalis* fruit extract or vitamin C administration (-48%) reduced DNA damage in rat hepatocytes as compared with dimethoate-applied alone group (P<0.05).

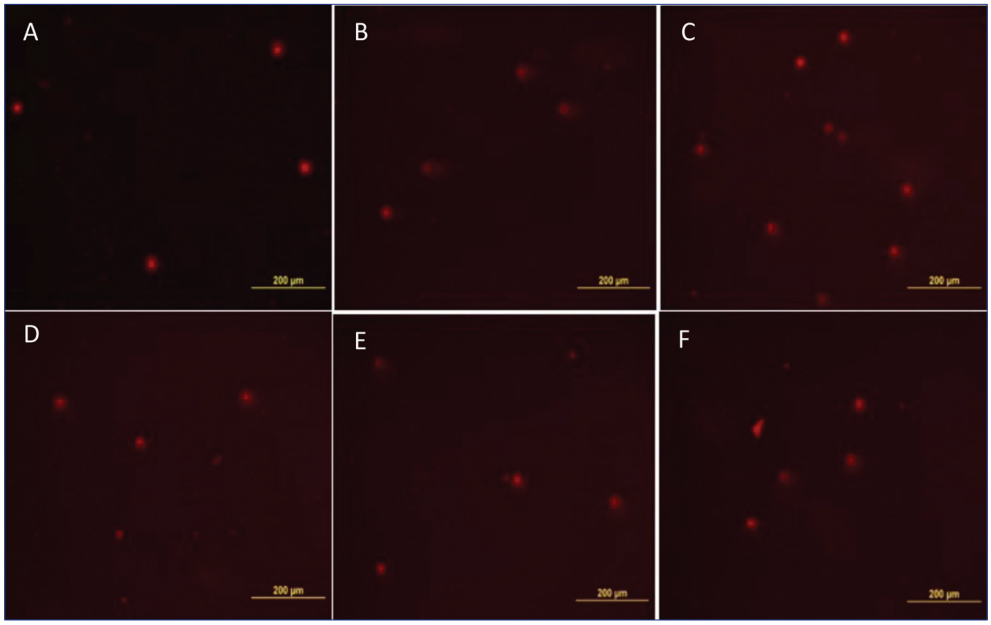


Fig 3. Comet images of rat liver cells for each experimental group (200× magnification). Control group (A), dimethoate-treated group (B), *L. officinalis* fruit extract-treated group (C), in group of pre-treatment with *L. officinalis* fruit extract before dimethoate (D), vitamin C-treated group (E), post-treatment with *L. officinalis* fruit extract after dimethoate (F)

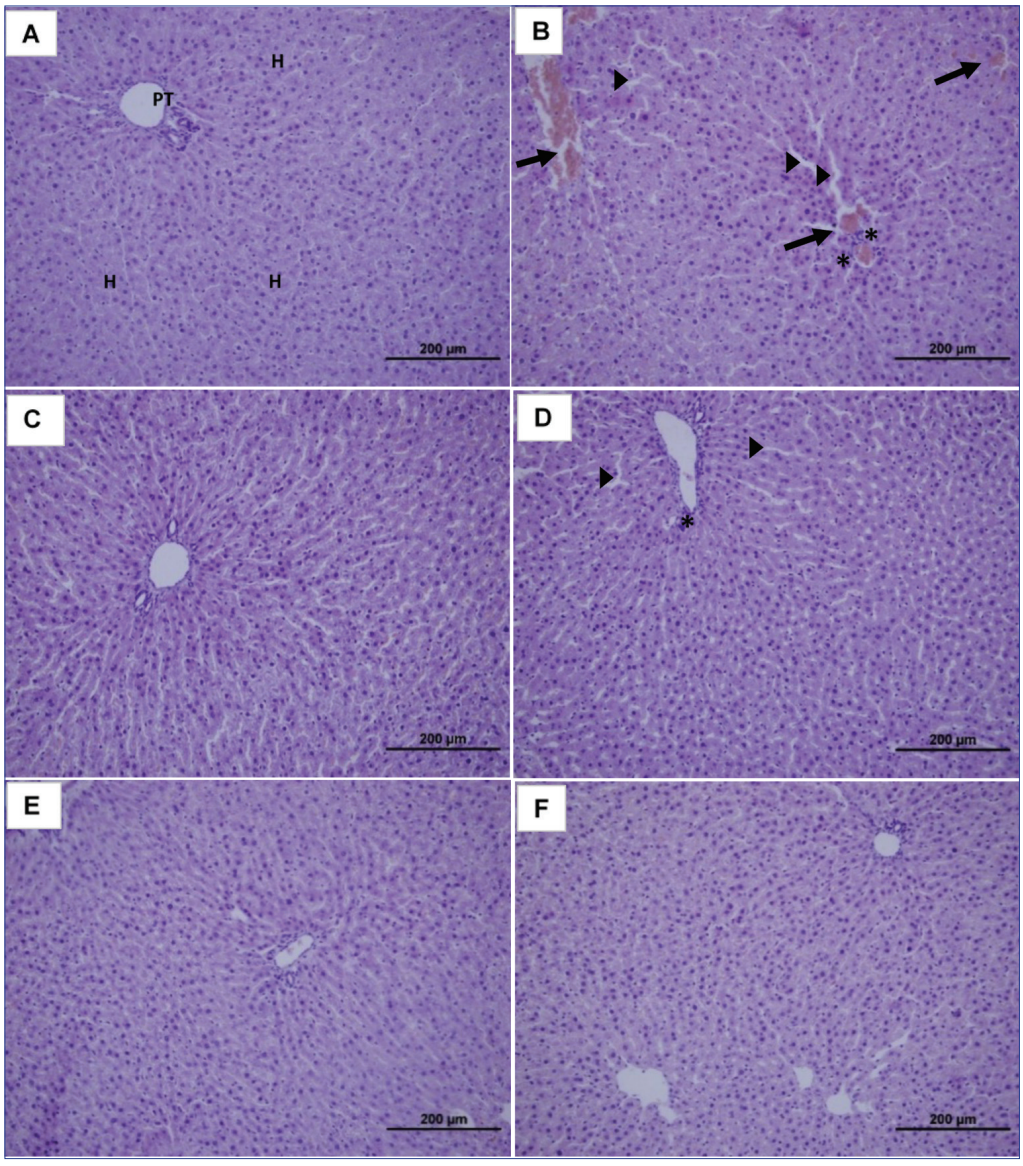


Fig 4. Photographs of sections of the liver from rat (H&E, x20). The liver of control rats had normal histological structure of hepatocytes (H), portal triad (PT), and blood sinusoid (A); Dimethoate-treated group showed dilated sinusoids *(arrow head),* congested blood vessels *(arrow),* lymphocytes infiltration (star) (B); *L. officinalis* fruit extract-treated group indicated normal architecture (C); The most of the hepatocytes exhibited normal histological morphology, although there was dilated sinusoids *(arrow head)* and lymphocytes infiltration *(star)* in group of pre-treatment with *L. officinalis* fruit extract before dimethoate (D); an improvement of liver morphology was observed in groups of vitamin C-treated (E) and post-treatment with *L. officinalis* fruit extract after dimethoate (F)

Histopathologic Evaluation

The liver section of control animals displayed a normal liver structure, including well-protected cytoplasm and nucleus of hepatocytes as shown in *Fig. 4A*. Dimethoate exposure exhibited severe histopathological changes in the liver sections compared with control rats as presented in *Fig. 4B*. The hepatocytes appeared large-sized cytoplasmic vacuolization. Moreover, dilated sinusoids, congested blood vessels, lymphocytes infiltration, and hepatocellular damage were observed. In addition, focal hepatocytes necrosis was also found. *L. officinalis* fruit extract-treated group did not indicate any pathological changes and the liver tissues appeared as the control group *(Fig. 4C)*. Liver sections in group of pre-treatment with *L. officinalis* fruit extract showed a reduction in the injury with little pathological alterations such as dilated sinusoids and lymphocytes infiltration when compared with only dimetoat-treated group *(Fig. 4D)*. In group of pre-treatment with vitamin C *(Fig. 4E)* and post-treatment with *L. officinalis* fruit extract *(Fig. 4F)* indicated an improvement of liver morphology.

DISCUSSION

Findings in the current study showed that the subchronic exposure to dimethoate caused hepatotoxicity in rats. Only administration of dimethoate alone caused a significant increase in AST and ALT, and TBil level compared to the control group. Conversely, pretreatment with *L. officinalis* fruit extract or vitamin C severely alleviated the activity of ALT compared with the group of dimethoate-treated alone. Pre- and post-treatment with *L. officinalis* fruit extract or pretreatment with vitamin C greatly decreased the activity of AST and TBil level in comparison with the group of dimethoate-treated alone. These findings were compatible with those obtained by other research. Saafi et al.[5] reported that dimethoate exposure produced liver toxicity in rats, increasing AST and ALT in comparison to controls. However, pre- and post-treatment with fruit extract of date palm *(Phoenix dactylifera* L.) significantly ameliorated these parameters in comparison with dimethoate-applied rats.

Oxidative stress mainly occurs through production of free radicals that consequently react with biological molecules, causing damage to membranes and tissues leading to lipid peroxidation [34,35]. It has been notified that the exposure to dimethoate induces oxidative stress in rats, as evidenced by enhanced lipid peroxidation, accompanied by concomitant decrement in the SOD, CAT, and GPx activities in tissues [34,36]. MDA is an oxidation product of polyunsaturated fatty acids and serves as an important biomarker of lipid peroxidation [37]. Increased MDA level indicates an enhanced lipid peroxidation, leading to tissue injury and failure of the antioxidant defense mechanism to prevent the excess production of free radicals [35]. Vitamin C is a well-known antioxidant that capable of scavenging free radicals such as superoxide, hydroxyl and singlet oxygen with its reversible properties of oxidation and reduction [4,24]. The enzymes of SOD, CAT, GPx act as the important antioxidant defense mechanism and provide protection against the deleterious effect of toxicants by means of their being free radical scavengers [38]. In the present research, MDA as a lipid peroxidation index, antioxidant enzymes of SOD, CAT, GPx were carried out to determine the oxidative damage in rat liver. Moreover, the values of TOS, TAS, and OSI were determined to more accurately assess oxidative stress. The current study results showed that subchronic subject to dimethoate caused a substantial increase in MDA and TOS levels and OSI value, while an important reduction was seen in SOD, CAT, and GPx enzymes and TAS level as compared with the control rats. Confirming the data in the current research, Sharma et al.[39] indicated that oral treatment with dimethoate at a dose of 30 mg/kg/day bw for 30 days markedly elevated the MDA level as compared with control rats. Sivapiriya et al.[36] observed that subacute exposure to dimethoate (18 mg/kg/day bw) for 14 days resulted in a significant rise in MDA, but a reduction in SOD, GPx, glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione in liver of experimental mice.

In the current study, pre- and post-treatment with *L. officinalis* fruit extract or vitamin C significantly diminished the elevated the levels of MDA, TOS and OSI, while the activities of SOD and GPx and the level of TAS were increased in comparison with the dimethoate-treated group. Pre-treatment with *L. officinalis* fruit extract enhanced the activities of CAT as compared to the dimethoate-applied group. Consequently, *L. officinalis* fruit extract provided protective effects against oxidative stress induced by dimethoate exposure. This may be reason that the antioxidant properties of *L. officinalis* fruit [15,19,20] and its hepatoprotective effect on rat liver toxicity induced by carbon tetrachloride [40] have been reported by the previous studies. In recent times, there is an attention for the significance of antioxidant compounds such as phenolic compounds present in the plants, which prevent toxic effects of free radicals because of their radical scavenger efficacy and have beneficial on human health [41]. We detected that *L. officinalis* fruit extract has activity of DPPH radical scavenging and antioxidant effect due to their high phenolic content. Our results were compatible with those presented by some studies. Saafi et al.[5] found that pre- and post-treatment with extract of date palm fruit alleviated the oxidative stress caused by orally exposure to dimethoate (20 mg/kg/day for 2 months), including improvement of antioxidant enzyme activities and histopathological alterations and the prevention of lipid peroxidation in liver of rats. Salim et al.[6] also reported that dimethoate administration (20 ppm) for 6 weeks produced a highly significant increase in the lipid peroxidation and an important decrease in antioxidant defense system in comparison to control rats. However, administration of

pomegranate seed due to its antioxidant content such as polyphenols, total flavonoids and phenols provided protection against dimethoate-induced oxidative stress. Similarly, Abu El-Saad and Elgerbed [34] demonstrated that dimethoate (21 mg/kg bw) treatment alone for 7 weeks produced a highly major rise in MDA level and a significant reduction in SOD, CAT, and GST compared with control rats. In contrast, treatment with vitamin E greatly reduced the MDA level and substantially elevated the SOD, CAT, and GST activities in comparison with dimethoate exposed group. Al-Awthan et al.[9] observed that the orally subject to dimethoate (7 mg/kg bw) for 28 days resulted in a significant increase of MDA, while major reductions in CAT, GST enzyme activities. On the other hand, co-treatment with vitamin C and vitamin E to dimethoate-applied guinea pigs restored all those parameters to nearly normal levels.

In this study, the tail DNA% in total comet DNA in liver cells of control and experimental rats was measured via comet assay as an indicator of DNA damage. Because the tail DNA% covers a widest range of damage and is linearly related to break frequency over most of this range [42]. An important increase in parameter of the tail DNA% was observed in rats receiving dimethoate alone compared with the controls. However, pre-treatment and post-treatment with *L. officinalis* fruit extract or vitamin C administration reduced DNA damage in rat hepatocytes as compared with dimethoate-treated alone group. This results were confirmed by the other studies. Similarly, Ayed-Boussema et al.[43] found that treatment with dimethoate (at doses ranging from 1 to 30 mg/kg bw) for 30 successive days produced DNA damage in liver of mice. In another study carried out by Abu El-Saad and Elgerbed [34], the increased DNA damage was found in dimethoate-applied rats compared to control group. Conversely, the combined treatment with vitamin E and N-acetylcysteine considerably decreased DNA damage in comparison to dimethoate group. Histopathological findings of the present research demonstrated that exposure to dimethoate exhibited severe changes in the liver including dilated sinusoids, congested blood vessels, lymphocytes infiltration, large-sized cytoplasmic vacuolization in hepatocytes, focal hepatocytes necrosis, and hepatocellular damage compared with control group rats. Our results are compatible with similar findings reported in the other studies [2,36,44]. Sharma et al.[39] found that dimethoate (6 mg/kg) administered group has portal inflammation in rats, and dimethoate (30 mg/kg) treatment caused variable portal inflammation, centrizonal congestion, and foci of necrosis, suggesting hepatocellular damage. In the present study, liver sections in group of pre-treatment with *L. officinalis* fruit extract indicated a reduction in the damage with little pathological changes such as dilated sinusoids and lymphocytes infiltration compared to only dimethoate group. In group of vitamin C-treated and post-treatment with *L. officinalis* fruit extract displayed an improvement of liver histology. It was also identified that treatment with fruit extract of the date palm showed improvement the hepatotoxicity induced by dimethoate in rats [5].

According to the findings of this study, dimethoate caused a significant oxidative damage in rat liver as evidenced by increase in lipid peroxidation and DNA damage, alteration in antioxidant status, depletion in the activities of CAT, SOD and GPx, and histopathological changes. *L. officinalis* fruit extract or vitamin C, on the other hand, was observed to ameliorate these disturbances induced by dimethoate. Thus, it can be suggested that the supplementation of a natural antioxidant such as vitamin C or *L. officinalis* fruit extract may act as a protective agent against dimethoate-induced hepatotoxicity. However, further studies are required to propose the potential therapeutic use of *L. officinalis* fruit extract in preventing the liver from xenobiotic-induced oxidative damage. In addition, further work should unravel the detailed role played by the individual components present in the *L. officinalis* fruit extract.

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Research Article

Effectiveness of Hesperidin on Methotrexate-Induced Testicular Toxicity in Rats [1]

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Abstract

The aim of this study was to investigate the effect of hesperidin on male reproductive system in rats to which methotrexate (MTX) was administered. In the study, 28 male Wistar albino rats at the age of 8 weeks and had 250-300 g of live weight were used. Four experimental groups were formed; Group 1 (n=7): The control group, only feed and water were given. Group 2 (n=7): MTX group, a single dose of 20 mg/kg of i.p. MTX was administered. Group 3 (n=7): Hesperidin group, 200 mg/kg of hesperidin was administered by gavage for 7 days. Group 4: MTX + hesperidin group (n=7): Following administration of a single dose of 20 mg/kg i.p. MTX , 200 mg/kg of Hesperidin was administered by oral gavage for 7 days. At the end of the experiment, rats were decapitated and biochemical, histopathological and spermatological parameters were examined. It was observed that in the MTX group, sperm motility and density, the enzymes CAT, GPx and SOD and GSH level decreased, TNF-alpha and IL-1 Beta, as well as MDA, levels were increased, regular structure of spermatogenic cells was impaired, and seminiferous tubules became necrotic and degenerative. It was determined that spermatological parameters improved and, necrotic and degenerative changes diminished by the administration of MTX+hesperidin. These outcomes indicated that hesperidin had a protective effect on destructive effects of MTX in rat testiclesv.

Keywords: *Male rat, Hesperidin, Methotrexate, Sperm, Reproductive system*

Ratlarda Metoraksat Kaynaklı Testiküler Toksisite Üzerine Hesperidinin Etkisi

Özet

Bu çalışmanın amacı, metoraksat (MTX) uygulanan ratlarda hesperidinin erkek üreme sistemi üzerindeki etkisini araştırmaktı. Çalışmada 8 haftalık yaşta, 250-300 g canlı ağırlığa sahip 28 erkek Wistar albino cinsi rat kullanıldı. Dört deney grubu oluşturuldu; Grup 1 (n=7): kontrol grubu, sadece yem ve su verildi. Grup 2 (n=7): MTX grubu, 20 mg/kg MTX i.p. tek doz uygulandı. Grup 3 (n=7): Hesperidin grubu, 200 mg/kg hesperidin 7 gün süre ile ağızdan sonda yardımıyla verildi. Grup 4 (n=7): MTX + hesperidin grubu, 20 mg/kg MTX i.p tek doz uygulamasını takiben 200 mg/kg hesperidin 7 gün süre ile ağızdan sonda yardımıyla verildi. Deney sonunda ratlar dekapite edilerek biyokimyasal, histopatolojik ve spermatolojik parametreler incelendi. MTX grubunda sperm motilitesi ve yoğunluğunun, CAT, GPx ve SOD enzimlerinin ve GSH düzeyinin düştüğü, TNF-alpha ve IL-1 Beta ile MDA düzeyinin arttığı, spermatojenik hücrelerin düzenli yapısının bozulduğu, seminifer tubullerin nekrotik ve dejeneratif bir hal aldığı gözlendi. MTX + hesperidin uygulaması ile spermatolojik parametrelerin düzeldiği, nekrotik ve dejeneratif değişikliklerin azaldığı tespit edildi. Bu sonuçlar hesperidinin rat testislerinde MTX tarafından oluşan yıkıcı etkiler üzerinde koruyucu etkiye sahip olduğunu gösterdi.

Anahtar sözcükler: *Erkek rat, Hesperidin, Metotreksat, Spermatozoon, Üreme sistemi*

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INTRODUCTION

Chemotherapeutics are well-known to have an acute toxic effect which influences many organs. One of these effects is testicular toxicity presenting with azoospermia and infertility in males [1]. Methotrexate causes defective oogenesis and spermatogenesis [2].

Presence of omega 3 polyunsaturated fatty acids (PUFAs) within the membrane of testicle makes this organ more susceptible to oxidative stress [3,4]. There are various antioxidant mechanisms in testicle which neutralize harmful effects of reactive oxygen species. However, MTX application causes an increase in formation of free radicals due to loss of effectiveness of antioxidant mechanisms, alterations in proinflammatory cytokine system and activation of phagocytic cells [5,6]. Activation of immune cells via proinflammatory cytokines leads to overproduction of ROS, resulting in overproduction of ROS, which leads to an increase in LP levels, just like MDA. Overproduced ROS interacts with structure and ratio of PUFA2 and leads to loss of fluidity in biological membranes [4,6].

It has been reported in previous studies that MTX leads to reductions in weights of reproductive organs, as well as leads to severe degradation of seminiferous epithelium, reduction of diameter of seminiferous tubules, reduction in size of spermatogenic cells and dilations in interstitial region [7,8].

It has been reported that MTX-induced organ toxicity can be as a result of oxidative stress [9]. Therefore, compounds with antioxidant properties may protect testicular tissue from harmful effects of the oxidative stress caused by MTX [10].

The antioxidant known as Hesperidin is a bioflavonoid antioxidant used in traditional Chinese medicine [11]. In many studies, it was demonstrated that Hesperidin reduced toxic effects [12-14].

HP has radical clearance and anti-inflammatory effects [15,16]. It has been reported that Hesperidin significantly reduces lipid peroxidation in testicular tissue, leads to positive effects on sperm parameters and biochemical parameters, as well as provides improvements in epididymal functions [14,17].

Although protective properties of some antioxidants in rats exposed to MTX have been investigated, protective property of Hesperidin has not been investigated yet. Therefore, in this study, effect of Hesperidin on male reproductive system is investigated at biochemical, histopathological and spermatological levels.

MATERIAL and METHODS

Chemicals

MTX (Ebewe Pharma® Unterach-Austria) was bought from a pharmacy. Hesperidin (Sigma-Aldrich St. Louis, MO, USA) was provided from a medical market. Rat TNF alpha ELISA kit and Rat IL-Beta ELISA Kit were procured from Cayma and other chemicals, however, were procured from Abcam and Merc.

Animals and Experimental Procedure

The study was approved by Yuzuncu Yıl University Local Ethics Committee On Animal Experiments (Approval number: 2015/28). The study was performed on 28 healthy male Wistar albino rats (8 week-old, with 250-300 g of body weight) which were provided from Fırat University Faculty of Medicine Experimental Research Center (Elazig, Turkey). Animals were adjusted to experimental conditions for a 1-week period before starting dosage. The animals were housed in standard laboratory conditions (24±3°C temperature, 40-60% humidity and 12 h light/12 h darkness). They were fed with commercial pelleted feed (Bayramoglu Food, Erzurum/Turkey) and fresh drinking water was provided *ad libitum*.

The experimental administrations were done in Experimental Animal Research Center of Yuzuncu Yil University. Rats were divided into 4 experimental groups, with 7 rats in each. Dosages of administered drugs were regulated in accordance with previous studies [10,14,18].

Group 1: Healthy control group (n=7): Only feed and water were given.

Group 2: MTX group (n=7): A single dose of 20 mg/kg of MTX was administered I.P. Because, it was revealed in previous studies that this dose (20 mg/kg) causes tissue toxicity [19]. MTX was dissolved in physiological saline.

Group 3: Hesperidin group (n=7): 200 mg/kg of hesperidin was administered by oral gavage for 7 days [14]. Hesperidin was dissolved in physiological saline.

Group 4: MTX + hesperidin group (n=7): Following administration of a single dose of 20 mg/kg MTX I.P., 200 mg/kg of Hesperidin was administered by oral gavage for 7 days.

Twenty-four hours after the last administration, the rats were decapitated under mild anaesthesia with sevoflurane. One of the testicular tissues was taken for biochemical analyses and kept under -80°C, and cauda epididymis of the other testicle was taken for spermatological examinations and the related testicle was fixed in Bouin's solution [20].

Collection of Samples and Evaluation of Spermatological Features

Testicles were removed. In order to obtain sperm from cauda epididymis, the method which was previously described by Sonmez et al.[21] was used. Briefly, epididymis is minced within 1.0 mL of physiological saline (0.9% w/v NaCl) by using an anatomical scissor in a petri dish. In order to provide transition of all spermatozoa into the fluid, it was left for incubation for 15 min. The obtained fluid was used as a sperm sample.

Sperm density was determined by using a slight modification of the method described by Sonmez et al.[21]. Sperm sample was poured into an eppendorf tube via an automated pipette with 10 µL of volume and 990 µl of eosine solution was added. Approximately 10 µL of diluted sperm suspension was transferred to counting chambers of a thoma slide (HHH Germany) and 5 min were awaited. They were counted at 200x magnification under phase contrast microscopy and calculated by using the formula used for calculation of sperm density.

Percentage of sperm motility was evaluated at 200x magnification by using a light microscope with a heating stage (ECLIPSE E 400 Nikon JAPAN), as it was stated by Sonmez et al.[21]. Average of three consecutive evaluations was used as the final motility score.

In order to evaluate abnormal sperm cells, the method which was reported in the study conducted by Turk et al.[22] was used. Briefly, one drop of sperm sample was mixed with the same amount of eosine-nigrosine staining (1.67% eosine, 10% nigrosine, and 0.1 M sodium citrate) and thin smear was prepared. 300 sperm cells were examined at 400x magnification under light microscope.

Biochemical Evaluations of Testicular Tissues

The testicular tissue which was triturated in porcelain mortar by using liquid nitrogen was diluted with 1.15% KCl and homogenized within ice with IKA-T-18 BASIC model ultra turrax. Firstly, obtained homogenate was centrifuged at 3500 rpm for 15 min and then the supernatant portion was separated for determination of protein, malondialdehyde, glutathione, superoxide dismutase and catalase.

The testicular tissue which was weighed according to dilution rate was diluted with 1.15% KCl and homogenized within ice by IKA T-18 BASIC ultra-turrax homogenisator. After the homogenate was centrifuged at 11.000 rpm for 20 min, the supernatant portion was separated for determination of glutathione peroxidase.

Determination of MDA was performed with thiobarbituric acid reaction method, which was reported by Placer et al.[23]. Measurement of GSH level was performed in accordance with the method reported by Sedlak and Lindsay [24]. SOD activity was performed with the method reported by Sun et al.[25]. SOD activity is measured via absorbance at 560 nm demonstrated by farmasone within the formed coloured solution. GPx activity was measured with the method of Matkovics et al.[26]. CAT activity in the testicular tissue was measured with method of Aebi [27]. Testicular cytokine production was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit in accordance with the instructions of the producer. Testis tumour necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) kits were provided from Sunred biological technology (Shangai, China). Protein amount was determined by using the method described by Lowry et al.[28].

Histopathological Evaluations

Obtained testicular tissues were fixed in Bouin's solution for 24-48 h. Tissues taken into paraffin blocks were passed through routine alcohol-xylol series. Slices which were sectioned 5 µm in thickness were stained with hematoxylin-eosin and examined under light microscope. For histopathological evaluation, Johnsen's testicular biopsy score was used [20].

Score	Definition
1	No cells
2	No germ cells, sertoli cells present
3	Only spermatogonia present
4	Few spermatocytes
5	Many spermatocytes
6	Few early spermatids
7	Many undifferentiated early spermatids
8	Few late spermatids
9	Many late spermatids
10	Complete spermatogenesis

Immunohistochemical Evaluations

Following deparaffinization, the slices were treated with 400 watts antigen retrieval solution (pH 6.0) in microwave oven for 15 min. To prevent endogenous peroxidase activity, it was awaited within 3% H_2O_2 for 10 min Slices washed with PBS were incubated for 15 min under room temperature with 8-OhDG primary antibody (cat no. Sc-66036, dilution ratio 1/200, Santa Cruz Biotechnology USA) and anti-NFKB1 antibody (cat no. ab7971, dilution ratio 1/200, Abcam, USA). Slices washed with PBS were continued to be stained with expose mouse and rabbit specific HRP/DAB detection IHC kit. 3,3' diaminobenzidine (DAP) was used as a chromogen. Slices which were passed through alcohol xylol series following counterstaining with hematoxylin were examined under light microscope. Immunopositivity was evaluated as none (0), mild (1), moderate (2) and severe (3).

Statistical Analysis

All statistical data were evaluated by using SPSS program (SPSS for windows, version 20.0). Spermatological and biochemical data were evaluated with Post hoc Tukey-HSD test and One-way variance analysis (ANOVA) in order to determine the differences between the groups. For histopathological and immunohistochemical findings, non-parametric Kruskal-Wallis and Mann-Whitney-U tests were used. $P<0.05$ value was accepted to be significant.

RESULTS

Spermatological Parameters

Sperm density, motility and abnormal sperm rate are given in *Table 1*. MTX group exhibited a significant decrease in sperm density and motility and a significant increase in

abnormal sperm rate, compared to the control and other groups. In addition to this, it was determined that MTX with Hesperidin treatment reduced the increased abnormal sperm rate and increased the reduced sperm motility and sperm density, compared to the only MTX group *(Table 1*, P<0.05).

Biochemical Results

Biochemical results of the testicular tissue are given in *Table 2*. It was determined that in the testicular tissue, MDA level increased in MTX group compared to the control group and MTX + hesperidin administration lowered this level to a near value in the control group (P<0.05). It was also determined that GSH level reduced in MTX group and MTX + hesperidin administration increased this lowered level (P<0.05).

It was determined that CAT, GPx and SOD activities were lower in the MTX-treated group compared to the control group, MTX + hesperidin administration led to an increase in enzyme activities and strengthened antioxidant defence system (P<0.05).

When the testicular tissue was examined in terms of TNF-alpha and IL-1 Beta levels among cytokines, it was determined that the levels were significantly lower in the group for which hesperidin was administered alone, compared to the control group. With MTX administration, increase in TNF-alpha and IL-1 Beta levels occurred, and addition of hesperidin treatment to MTX treatment was observed to decrease TNF-alpha and IL-1 Beta levels (P<0.05).

Histopathological Findings

Structure of seminiferous tubules was observed to be normal in the control and hesperidin groups *(Fig. 1A-1B)*. It was recognized that in the group for which MTX was administered, regular structure of spermatogenic cells was impaired, and seminiferous tubules became necrotic and degenerative, with development of sporadic flaking *(Fig. 1C)*. It was determined that in the group for which MTX was administered together with hesperidin, observed necrotic and degenerative changes were reduced compared to the group for which MTX was administered alone *(Fig. 1D, Table 3*, P<0.05).

Table 1. *Sperm motility, density and abnormal sperm rate in rats after administration of methotrexate and hesperidin. The different letters (a,b,c) in the same column indicate differences between the groups (P<0.05)*

Groups	Motility Rate (%)	Density ($x10^6$)	Abnormal Sperm Rate (%)		
			Head	Tail	Total
Control n=7	70.71±1.30^a	70.42±1.91^a	3.42±0.29^a	6.14±0.79^a	9.57±0.99^a
Hesperidin n=7	76.42±0.92^a	71.85±1.73^a	3.14±0.59^a	5.71±0.64^a	8.85±0.98^a
Methotrexate n=7	36.42±1.42^b	33.85±1.56^b	13.57±0.78^b	18.28±1.86^b	32.28±1.28^b
Methotrexate+ hesperidin n=7	45.71±2.02^c	44.14±0.98^c	9.42±0.71^c	10.71±0.80^c	20.28±1.50^c
Significance	(P<0.05)	(P<0.05)	(P<0.05)	(P<0.05)	(P<0.05)

Table 2. *MDA, GSH, GPx, SOD, CAT, TNFa and IL-1 Beta levels in testicular tissues of rats for which methotrexate and hesperidin were administered. The different letters (a,b,c,d) in the same column indicate differences between the groups (P<0.05)*

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	GPx (U/g protein)	SOD (U/g protein)	CAT (katal/g protein)	TNFa (ng/g tissue)	IL-1Beta (pg/g tissue)
Control (n=7)	73.47±1.28^c	4.47±0.03^b	8.57±0.16^b	18.96±0.35^b	6.02±0.07^b	3961±36.78^c	14078±58.84^c
Hesperidin (n=7)	68.14±0.64^d	4.94±0.04^a	9.57±0.14^a	21.54±0.32^a	6.81±0.09^a	3644±27.64^d	12892±74.29^d
Methotrexate (n=7)	103.42±2.83^a	3.59±0.04^d	6.63±0.17^d	14.50±0.22^d	4.22±0.08^d	5313±68.38^a	21153±292.78^a
Methotrexate+ hesperidin (n=7)	83.34±1.09^b	4.26±0.84^c	7.42±0.13^c	17.13±0.15^c	5.28±0.10^c	4081±40.84^b	17319±176.41^b
Significance	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05

Table 3. *Histopathological changes in testicular tissues of rats for which methotrexate and hesperidin were administered. The different letters (a, b, c, d) in the same column indicate differences between the groups (P<0.05)*

Groups	Johnsen's Testicular Biopsy Score
Control	9.42±0.20^a
Hesperidin	9.57±0.20^b
Methotrexate	7.14±0.26^c
Methotrexate + hesperidin	5.42±0.29^d
Significance	(P<0.05)

Table 4. *8-OhDG and NFKB1 expressions levels in testicular tissues of rats for which methotrexate and hesperidin were administered. The different letters (a,b,c,d) in the same column indicate differences between the groups (P<0.05)*

Groups	8-OhDG	NFKB1
Control	0.28±0.18^a	0.57±0.20^a
Hesperidin	0.57±0.20^b	0.85±0.14^b
Methotrexate	2.85±0.40^c	2.42±0.20^c
Methotrexate + hesperidin	1.85±0.26^d	1.57±0.20^d
Significance	(P<0.05)	(P<0.05)

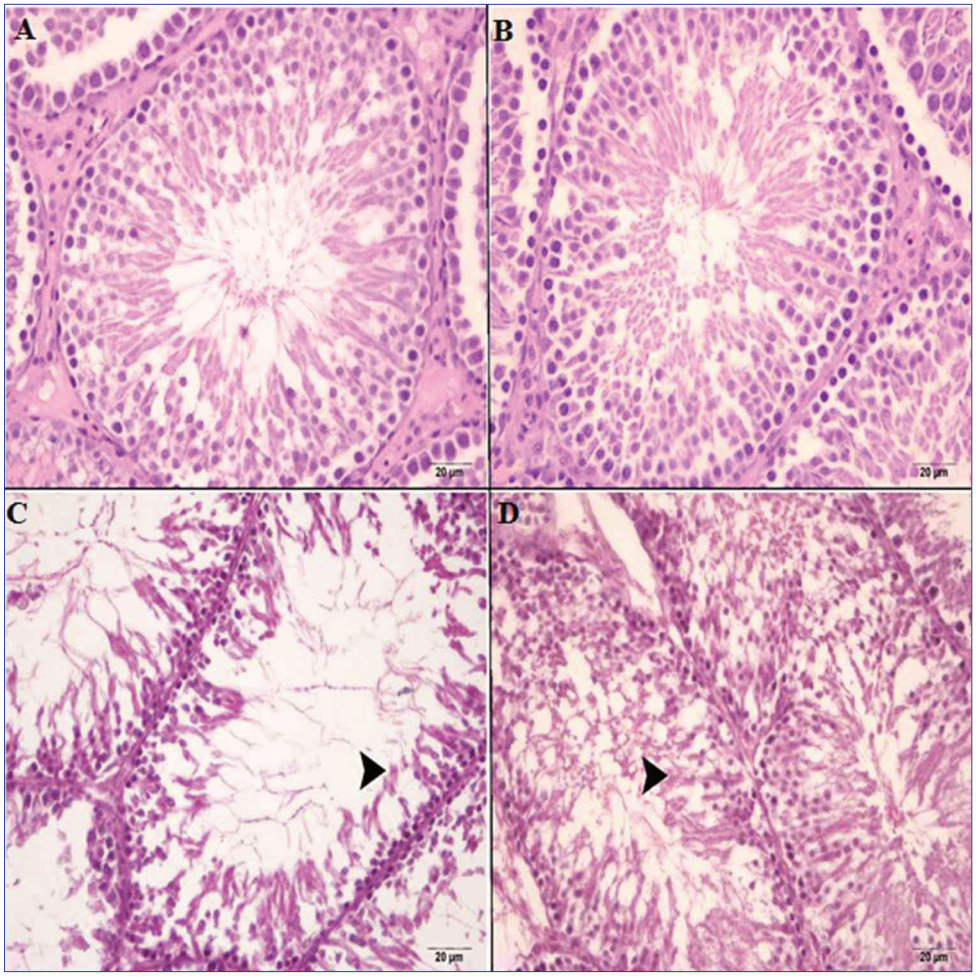

Fig 1. Structure of seminiferous tubules was observed to be normal in the control and hesperidin groups (A-B). Intense necrotic and degenerative alterations *(arrow head)* of seminiferous tubules are seen in the MTX alone group (C). Slightly necrotic and degenerative alterations *(arrow head)* are seen in the MTX + hesperidin group (D); H-E; (magnification 20×.)

Immunohistochemical Findings

8-OhDG expression was determined to be extremely low in control and hesperidin groups *(Fig. 2A-2B)*. It was determined that in the group for which MTX was administered alone, 8-OhDG was expressed strongly in seminiferous tubules but 8-OhDG expression level decreased in the group for which MTX was administered together with hesperidin *(Fig. 2C-2D, Table 4*, P<0.05). NFKB1 expression, however, was at extremely low level in control and hesperidin groups *(Fig. 3A-3B)*. Whereas it was observed that NFKB1 was expressed strongly in spermatids in the group for which MTX was administered alone, expression level was observed to begin decreasing in the group for which MTX was administered together with hesperidin *(Fig. 3C-3D, Table 4*, P<0.05)

DISCUSSION

Testicles are the most important target organs for oxidative stress due to their high content of polyunsaturated membrane lipids [29]. Increased oxidative stress in testicles damages to spermatological parameters [30].

In the current study, the decrease in sperm count and sperm motility and the increase in abnormal sperm rate determined following MTX administration confirmed previous studies [7,18,31]. The increase in abnormal sperm rate and reduction in sperm density and motility are associated with the increased lipid peroxidation. This situation can be explained by that MTX damages cell membrane integrity by disturbing lipids and proteins within the sperm membrane. It was determined in previous studies that like MTX, Cisplatin causes a marked decrease in sperm density and motility [32-34].

Hesperidin administration in addition to MTX decreased effects of MTX on sperm parameters and increased sperm count and motility. Protective effects of hesperidin are probably may be referred to its obvious antioxidant potential which was observed in this study.

Our biochemical findings indicated that MTX increased MDA level which is among the most important oxidant parameters in the testicular tissue, compared to the control group. This finding is consistent with some reports indicating that MTX stimulates oxidative stress by increasing MDA levels [8,10,35]. Also, it revealed that MTX treatment significantly decreased endogenous antioxidant enzyme activities such as SOD, CAT and GPx and GSH level, which are commonly used for monitoring oxidant/antioxidant status. In the study, it was determined that Gpx, CAT and SOD enzyme

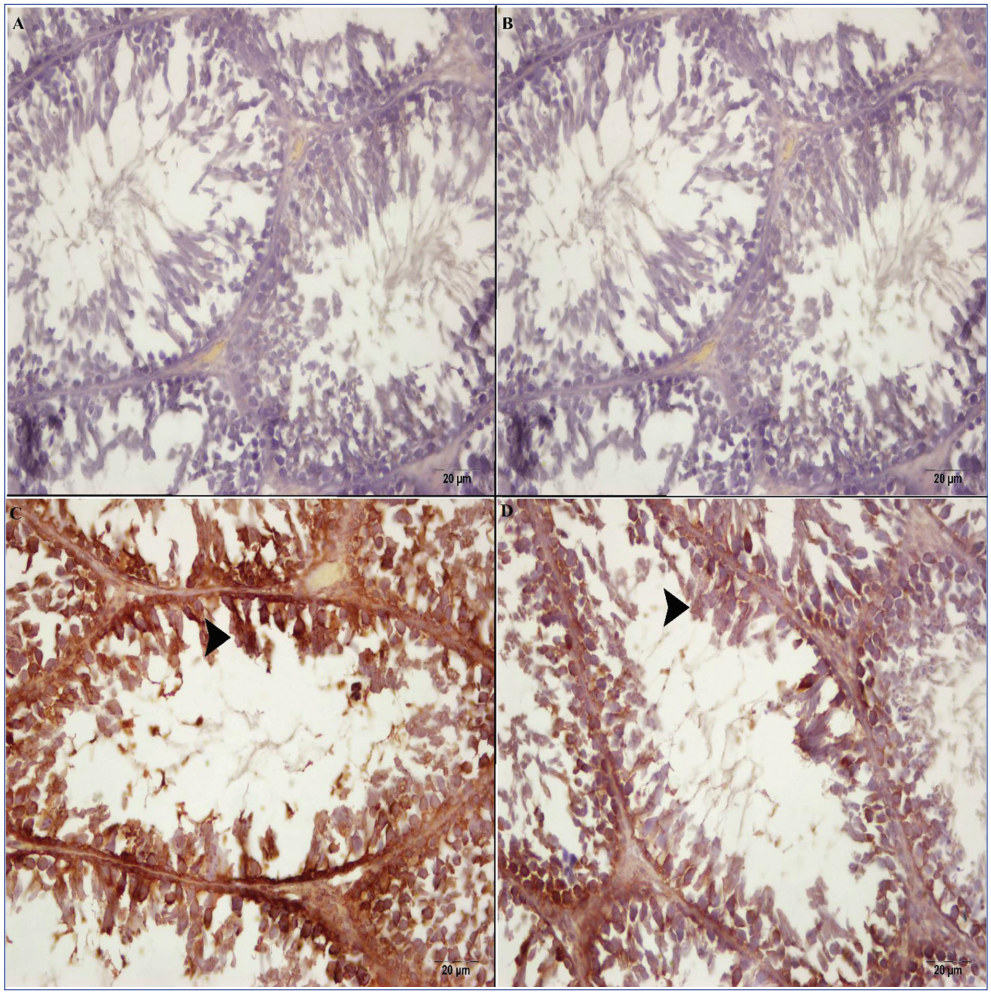

Fig 2. 8-OhDG expression was determined to be extremely low in control and hesperidin groups (A-B). In the MTX alone group, intensive 8-OhDG expression *(arrow head)* is detected (C). Slightly expression of 8-OhDG *(arrow head)* is seen in thye MTX + hesperidin group (D); IHC; (Magnification 20×.)

activities, as well as GSH level, were significantly increased and lipid peroxidation induced by MTX was significantly reduced with hesperidin administration. These results are consistent with those of previous studies [14,17,36].

TNF-alpha exists in seminiferous tubules and is strongly up-regulated under both pathological and physiological conditions [37,38]. IL-1 Beta is produced by macrophages. This cytokine is known as an important mediator of various cellular functions, including reproduction, differentiation and apoptosis, and of the inflammatory response [39]. The increase in TNF-alpha and IL-1 Beta levels which was observed after MTX administration suggested that MTX led to an inflammatory reaction. Reduction of this increase in TNF-alpha and IL-1 Beta levels by administration of hesperidin in addition to MTX suggests that hesperidin has anti-inflammatory effects. It was reported in a previous study that MTX led to an increase in TNF-alpha level [40]. It is consistent with this study that Cisplatin, Doxorubicin and Sodium nitrite increase TNF-alpha and IL-1 Beta levels among pro-inflammatory cytokines in testicular tissue [40-42].

In histopathological evaluation, severe necrotic and degenerative changes were determined in MTX group. In MTX + hesperidin group, however, necrotic and degenerative changes were determined to be milder. Therefore, possible protective effect of hesperidin was evaluated immunohistochemically with 8-OhDG in regard to DNA damage and with NfKB in regard to inflammatory reaction. 8-OhDG is a form of free radicals and a biomarker used in oxidative stress [43]. In various studies it was expressed that severity of DNA damage in testicular destruction due to oxidative stress was determined with 8-OhDG [44]. In the present study, whereas 8-OhDG was strongly expressed in MTX group, reduction in 8-OhDG expression in the group for which MTX was administered together with hesperidin suggested that DNA damage was diminished and, hence, hesperidin had a protective property. NfKB, however, is a molecule which is activated when oxidative stress is developed [45,46]. It was reported that level of NfKB increased with testicular intoxication [47,48]. Present study, NfKB was determined to be severe in MTX group and moderate in MTX + hesperidin group. This situation was expressed as that hesperidin reduced inflammatory reaction.

This study demonstrated that administration of a single dose of 20 mg/kg of MTX increased lipid peroxidation levels in testicles of Wistar albino rats and, thus, caused oxidative stress. Additionally, it also demonstrated that

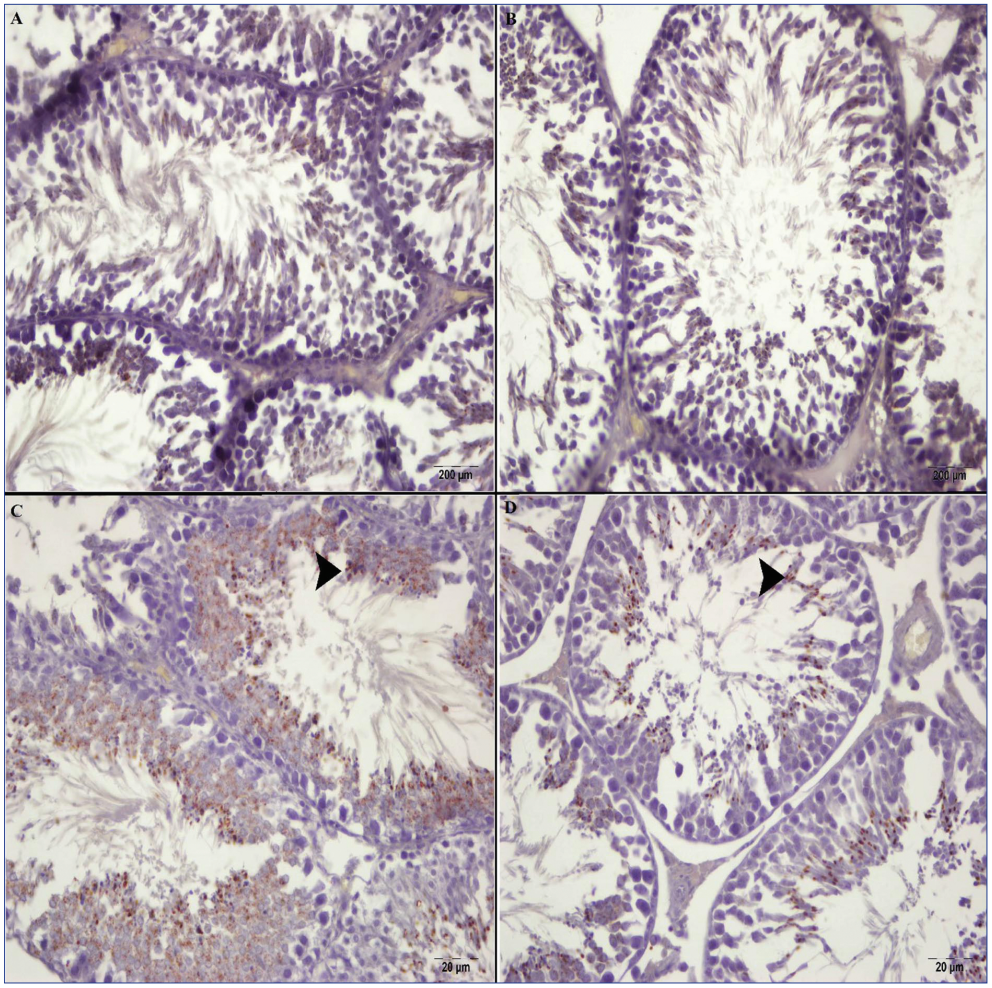

Fig 3. NFKB1 expression was at extremely low level in control and hesperidin groups (A-B). In the MTX alone group, intensive NFKB1 expression *(arrow head)* is detected (C). Slightly expression of NFKB1 *(arrow head)* is seen in the MTX + hesperidin group (D); IHC; (Magnification 20×.)

it had toxic effects including histopathological changes and spermatological damage. Treatment of MTX together with hesperidin was determined to significantly prevent toxicity of MTX on reproductive system. When results of all groups are taken into consideration, we suggest that hesperidin has a regenerative effect on testicular tissue and sperm parameters.

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Research Article

A Study on Observation of Respiratory Ultrasound Plethysmography in Donkeys

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Abstract

The respiratory efforts of the thorax and abdominal muscles of donkeys have not adequately been quantified. According to our knowledge, there is no objective, non-invasive monitoring system of lung mechanics in donkeys. This study is the first to describe the nature of thoracoabdominal asynchrony in donkeys. From 22 June 2016 to 19 January 2017 in turn all donkeys (n=18) owned by the Schlosshof GmbH were used for this study. The aim of this study was to analyse the differences of thoracic and abdominal excursions during breathing by using Respiratory Ultrasound Plethysmography (RUP). Synchronisation, rhythm and relative contribution of the thoracic and abdominal muscles were analysed. The final goal was to contribute to a reference data base for diagnostic purposes and find out if RUP could be a simple diagnostic technique for use in the field. The RUP system in its current form is too sensitive to signal noise and generated data are difficult to quantify. Nevertheless, using an alternative algorithm the respiratory strategy of healthy and coughing donkeys appeared different.

Keywords: *Donkey pulmonary function, Respiratory ultrasound plethysmography, Thoracic abdominal asynchrony*

Eşeklerde Solunum Ultrason Pletismografisinin Gözlemlenmesi Üzerine Bir Çalışma

Özet

Eşeklerin göğüs ve karın bölgelerinin respiratorik eforu yeterince tanımlanmamıştır. Bildiğimiz kadarıyla, eşeklerde akciğer mekaniğinin izlenmesi için objektif, invaziv olmayan bir sistem yoktur. Bu çalışma, eşeklerde torakoabdominal asenkroninin doğasını tanımlayan ilk çalışmadır. Bu amaç için, 22 Haziran 2016'dan 19 Ocak 2017'ye kadar, Schlosshof GmbH'ya ait tüm eşekler (n = 18) kullanılmıştır. Bu çalışmada, solunum sırasındaki göğüs ve karın bölgelerinin hareket farklılıklarının Solunum Ultrason Pletismografi (RUP) metodu ile analiz edilmesi amaçlandı. Bu amaçla göğüs ve karın bölgelerinin hareketlerinin senkronizasyon, ritim ve bağıl dağılımları analiz edildi. Çalışmanın nihai amacı, referans veri tabanına katkıda bulunmak ve sahada diyagnostik amaçlı basit bir RUP tekniği geliştirmekti, fakat, RUP metodunun mevcut haliyle sinyal gürültülerine aşırı duyarlı olduğu ve elde edilen verileri etkilediği gözlemlendi. Bununla birlikte, sağlıklı ve öksüren eşeklerin solunumları için alternatif algoritma stratejisi uygulanmasıyla da sonuçların farklı olduğu ortaya konuldu.

Anahtar sözcükler: *Eşeklerin solunum fonksiyonları, Respirasyon ultrason pletismografisi, Torasik-abdominal asenkroni*

INTRODUCTION

Domestic donkeys *(Equus africanus asinus)* descend from African wild ass *(Equus africanus)* populations. The domestication process started probably about 7000 years ago, which is 2000 years earlier than of the horse [1,2]. Donkeys were brought to Europe soon after domestication and were already widely distributed throughout the continent in the classical antiquity [3]. In Western Europe, donkeys played a role as working animal and for mule breeding; however, numbers of pure donkeys were modest over the course of time in Western Europe. On the contrary, in South Europe, the Mediterranean, Asia, Africa and America donkeys were or still are common working animals.

Pulmonary disorders are quite prevalent in working donkeys, but suitable diagnostic tools are absent or have not been validated. Of note is the apparently less developed cough reflex of donkeys compared with horses [4]. This blunts signs of pulmonary dysfunction and impedes rapid diagnosis.

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As the donkey is definitely not just a smaller, inferior type of horse [5], data from equine pulmonary function studies cannot be extrapolated to donkeys. In fact, a study by Delvaux et al.[6] showed that the breathing mechanics of donkeys compared more closely to bovines than horses. In particular, donkeys have increased respiratory frequency (13-31 breaths/min), increased total pulmonary resistance and a reduced dynamic compliance in comparison with horses.

In a recent UK survey of post mortem findings in 1444 aged donkeys [7] pulmonary fibrosis was found in more than 35% of all cases, making this the most important lethal respiratory cause. Earlier detection of this disease may reduce animal suffering.

The conventional pulmonary function test (PFT) of horses [8], is based on the measurement of the trans-thoracical pressure differences between in- and expiration measured in the oesophagus. Therefore, a balloon catheter must be inserted through a short naso-gastric tube via the ventral nasal passage into the thoracic part of the oesophagus. However, this procedure is not always well accepted by horses and thus sedation becomes mostly inevitable. Since sedatives affect the mechanics of breathing [9], blunting of measurements will occur. Moreover, PFT appeared not a very sensitive tool for detecting smaller changes in diseased airways [10]. Thus, alternative systems to measure changes in lung function parameters are being developed for horse since more than two decades, but most of them are still under study [11-15] or interest in the methods was lost.

Konno and Mead [16] described the lung as a model of two compartments (thorax and abdomen), whereby the movement of the abdomen predominantly reflects the activity of the diaphragm. Elastic bands around thorax or rib cage (RC) and abdomen (ABD) replace the classical body chamber of double-chamber plethysmography [16]. Induction loop incorporated into these bands function as sensors that detect and quantify movements of the thoracic and abdominal compartments at respiration. This method finds a broad application for the measurement of tidal volume, respiratory rate and monitoring of apnea, hypo- and hyperventilation in man [17]. Tidal volume measured by the pneumotachograph and by Respiratory Inductive Plethysmography (RIP) differed by only ±6% [18,19], indicating that RIP would provide accurate measures of ventilation. However, in a veterinary environment the RIP system was too sensitive for electric interference, therefore a different method, the Respiratory Ultrasonic Plethysmography (RUP), was developed to record changes in thoracic and abdominal changes in circumference [20].

The aim of the study was to record respiration-induced changes in the RUP signal in order to visualize and calculate normal respiratory patterns of donkeys.

MATERIAL and METHODS

Animals

Twelve healthy and six coughing sedentary pet donkeys aged between 3 and 17 years (see for the gender all other characteristics *Table 1)*, all owned by the Schlosshof GmbH were used for this study and allocated in two groups. Before the measurements started, donkeys were accustomed to the measuring procedure. Airway mechanics was measured with the RUP late in the morning (11.00 am) till mid after noon (3:00 pm). All donkeys were kept out on a grass paddocks and were not fed extra hay or concentrates during the experiments.

Experimental Design

The experimental protocol was approved by the Ethical Review Committee of the Veterinary University of Vienna Animal and by the Austrian Ministry of Education. Sciences and Culture licence number GZ: BMWFW-68.205/0097-WF/V/3b/2016. The study was observational and descriptive statistics were principally used.

Respiratory Ultrasound Plethysmography

The RUP system according to Schramel et al.[20] was used. Two ethanol filled rubber tubes, placed in the 11th intercostal space and behind the last rib, respectively, were used to measure alterations of abdominal and thoracic circumference during breathing, with 100 Hz

Table 1. *Donkeys used in the study*

Name	Gender	Age (year)	Chest Width (cm)	Cough	Weight (kg)
Donkey 1	Mare	8	170	1	200
Donkey 2	Mare	9	160	1	182
Donkey 3	Mare	5	155	0	167
Donkey 4	Mare	7	150	0	165
Donkey 5	Mare	5	170	1	180
Donkey 6	Mare	5	155	0	190
Donkey 7	Mare	17	170	1	170
Donkey 8	Mare	13	155	0	170
Donkey 9	Stallion	6	165	0	195
Donkey 10	Mare	6	165	1	190
Donkey 11	Stallion	8	155	0	180
Donkey 12	Mare	9	160	0	175
Donkey 13	Gelding	4	150	0	145
Donkey 14	Mare	3	150	0	146
Donkey 15	Mare	12	160	0	165
Donkey 16	Gelding	4	155	0	140
Donkey 17	Gelding	10	165	1	190
Donkey 18	Stallion	4	170	0	200
Mean		7.5	160		175

sample rate. Data were recorded by the software program BioSystem XA© Version 2.7.9. The data were analysed using Microsoft Office Excel 2016®. The system measures the stretching of compliant liquid - filled rubber tubes with an inner diameter of 5-8 mm that are fastened around the regions of interest. The elastic tubes act as ultrasonic waveguides between an ultrasonic transmitter and receiver at the respective ends. The distance between the tube endings is calculated from the time delay of ultrasonic pulses propagating in the liquid. The tubes are filled with 96% ethanol which has an acoustic velocity of 1207 m second^{-1} at 25°C The signal undergoes minimal attenuation even over distances in the magnitude of metres [20].

Special care must be taken not to introduce air bubbles into the fluid. A small electronic processing unit carried by the subject generates signals with a resolution of 0.3 mm and transmits them at sampling rates of 10 or 100 Hz via a Bluetooth link to a computer. Sensor length can be adapted individually within the range of 0.15-2.0 m. Additionally analogue output signals for the two channels use for thoracic and abdominal compartments are available for external data acquisition and comparison with other devices. Prior to measuring, the length of the tubes has to be adapted to the size of the subject to achieve a range of movement within 15% of the total length. The part of the perimeter between transmitter and receiver is completed by means of an adaptable metallic chain preferably placed over the spinal region. This method allows proper pre-tensioning of the waveguides with no slackening during end expiration. The electronics measure the running time of the ultrasonic waves in the ethanol and transfer the data to a laptop via Bluetooth® [20]. In all donkeys, RUP measurements were performed in standing position and during normal spontaneous breathing.

However, the placement of the abdominal band in the 16th and 18th intercostal space [21] gave technical problems due to sliding in the caudal direction *(Fig.1)*. For this reason, the system had to be adapted s uch that thoracic and abdominal bands were ventrally fixed with a 20 cm strap to each other *(Fig.1)*. This strap was fixed to 2 small rigid tubes through which the rubber measuring tubes were passing. This prevented sliding backwards, but may have affected measurements.

Data Handling and Statistical Analysis

Data and waveforms were analysed visually post hoc after processing into graphs with the Microsoft Office Excel 2016 statistical add-in. Thoracic-abdominal asynchrony (TAA) was planned to be measured by Pearson's correlation coefficient from 4 to 6 breath [22], but data deviated too much from the base line that further quantitative analysis with waveform Independent techniques on the unprocessed data was not performed. Using outlier analysis, the data base was cleaned from outliers such that visually reasonable Lissajous loops could be produced. Subsequently a semi-quantification was performed by calculation of the regression of the thoracic signal on the abdominal signal using IBM SSPS Statistic Version 23 software 2016. The arctan of the slope (tg θ) of the regression line of the data plots was subtracted from 45°, the slope of the perfect synchronisation of abdominal and thoracic excursions, for each individual donkey in order to establish the degree of asynchrony.

Another approach to analyse the 40.000 to 80.000 data points per animal was by selecting a region of 10000 data points per case. These sets were than normalized by their means and standard deviations. Normalised data were further analysed using cross correlation and by calculation of the regression line of xy-plots.

RESULTS

A proportion of the studied donkey occasionally coughed and since these animals were identified as having increased airway resistance, two groups were created for further analysis post hoc.

Many produced curves were too much affected by noise from moving legs, swiping tails or bending of the thorax to enable the detection of 4 to 6 uniform breaths for analysis as was severe base line drift. *Fig. 2* shows a suitable curve with a few normal breaths and occasional deep sighs of a quiet and cooperative donkey. The xy-plot of the thoracic and abdominal signals resulted in imperfect Lissajous loops *(Fig. 2)*, but the data cloud enabled calculation of the regression lines.

In this model, the healthy donkey group tended to have greater phase shift, with the thorax lagging behind the

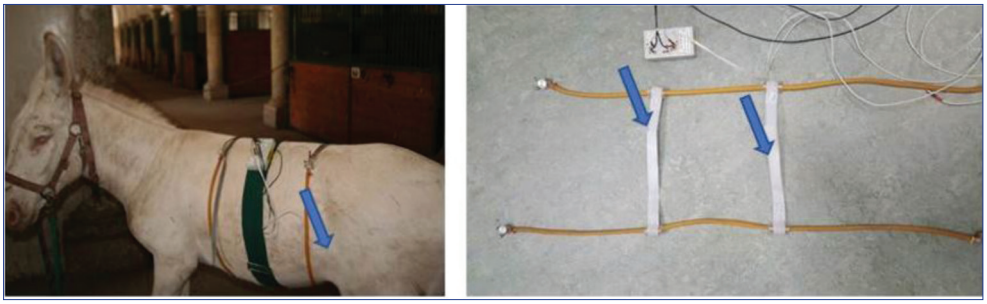

Fig 1. Sliding of the abdominal band towards the prepubic (flank) region causing decrease in circumferential movement. These tapes keep the tubes in a stable state

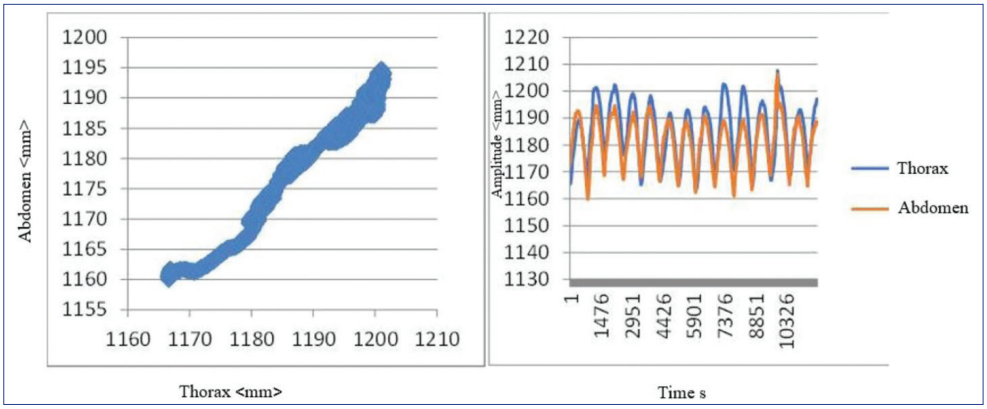

Fig 2. Thoracic (blue) and abdominal (red) signals of the RUP system (right) in a cooperative and quietly breathing donkey. Nearly perfect in-phase breathing is shown by the xy-plot (right). The calculated regression line was y=0.92 X + 158 and the level of agreement (r^2) was 0.86. The thoracic abdominal asynchrony in this animal was fast zero

abdomen movements than with the coughing animals but the differences were not statistically significant (P=0.96). So were the cross-correlation coefficient differences insignificant (P=0.55).

The second analytical approach, which is waveform independent, appeared more useful with large data sets in quickly finding phase shifts between thorax and abdomen excursions. On average, abdomen activity started before thorax activity in both groups as in the first model, but in the coughing donkeys, the lag between abdomen activity and thoracic activities was only 12 msec compared to the 23 msec of the healthy donkeys. However, the difference was not statistically significant (P=0.96).

DISCUSSION

The RUP system has been developed by Schramel [20,22] and validated as an alternative to the RIP system that appeared too sensitive to environmental electric noise. Yet, there are still conditions that decrease the accuracy of the device. Firstly, belts should not be placed too tight causing the actual cross sectional change of the chest or abdomen to be restricted and thus data will not reflect the patient's true breathing efforts. On the other hand, if belts are placed too loosely, the belts will slide to pre-pubic region in donkeys where there is less abdominal distension and hence weak signals are produced. In the horse the thoracic band is placed in the 11th intercostal space, which was also feasible with donkeys. Some authors prefer to position the thoracic band in the 6th intercostal space accepting small excursions of the thorax thereby offering larger excursion for specify [23].

Thoraco-abdominal asynchrony (TAA) is defined as the no coincident motion of thorax and abdomen and is considered as relevant clinically. TAA may be calculated using the Lissajous approach [24], but in case of much signal noise, the calculation of cross correlation coefficient $\rho_{XY}(\tau)$ has been shown to be a better approach [25]. In the case of sinusoidal signals, θ is independent of the choice of reference points for its calculation, but the respiratory signals may not be sinusoidal in shape [26]. Assessing start and stop of inspiration and expiration, merely from the RIP sum signal, is difficult. Especially in the case of severe asynchrony, the start of inhalation cannot be inferred from the RIP or RUP signals alone. In man, therefore an external source for respiratory timing is used [27]. This alone makes RUP unsuitable for fast and simple diagnostics in the field.

For the analysis of data in this study the $\rho_{XY}(\tau)$ approach appeared to produce the better results. The cross-correlation analysis indicated that in donkeys on average the thoracic activity lags behind the abdominal activity and in the coughing, donkeys this lag period on average seems smaller than in healthy animals suggesting that the thoracic activity attributes more early in the respiratory effort as has been shown to occur in horses with heaves [19].

It is still unclear how to interpret changes in TAA. The TAA pattern in infants with airway obstruction and/or lung restriction is such that the on-inspiration thorax lags behind the abdomen [27]. In contrast to diseased children, the healthy horses show the same pattern with none to a minimal phase shift, with the thorax lagging behind the abdomen during the onset of inspiration. On the other hand, horses with heaves have a phase shift that is characterized by the thorax leading in sequence before the abdomen. This pattern was unique to heaves. It was most pronounced in a group of horses with the abdominal paradox [19]. Furthermore, Haltmayer et al.[28] found that horses affected by a pulmonary disorder showed a lower degree of TAA than healthy horses, which agrees with our finding with the RUP in coughing donkeys. The RIP and possibly the RUP system too appear not that suitable for the diagnosis of pulmonary problems in a quick and simple way. So were Miller et al.[29] not able to show a significant correlation between the degree of change in resistive pulmonary load on foals and the degree to which phase angle was altered. Although RIP variables changed markedly with fixed upper airway resistive loading, the degree to which they changed was erratic and therefore not useful for grading these obstructions. Hoffman et al.[19] reported that RIP variables were insensitive measures of bronchoconstriction. RAO horses during exacerbation have severely increased θ with abdomen consistently

lagging behind the thorax, and a reduced contribution of the abdomen to ventilation [19]. This was pattern in contrast with the breathing strategy of healthy donkeys and most of the coughing animals.

Although it was possible to find a decrease in phase shift between the coughing and healthy donkey group, the RUP system is too sensitive for signal noise and therefore not specific enough to simply detect subtle changes in breathing pattern suggestive for pulmonary dysfunction in donkeys. Similarly, the RIP system also largely appeared unsuitable for diagnosing foal's respiratory disorders [29]. Therefore, measuring TAA in Donkeys with RIP instead of RUP likely would not have improved the outcome of the study.

Autor´s Declaration of Interest

The authors report no conflicts of interest.

Ethical Animal Research

The experimental protocol was approved by the Ethical Review Committee of the Veterinary University of Vienna and by the Austrian Ministry of Education, Sciences and Culture, licence number: (GZ): BMWFW-68.205/0097-WF/V/3b/2016

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Research Article

Class 1 Integrons and the Antibiotic Resistance Profile of *Salmonella* Infantis Strains from Broiler Chickens

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Abstract

Salmonella infections are one of the most important diseases and cause economic problems in poultry. The zoonotic feature of the agent and leading to food-borne infections are also important in public health issues. Increasing antibiotic resistance causes difficulties of controlling *Salmonella* infections, in recent years. Among non-typhoidal *Salmonella* serotypes the rate of *Salmonella* Infantis are increasing in Turkey. With this increase, it is important to know the antimicrobial resistance patterns of *Salmonella* Infantis strains as seen in other *Salmonella* serotypes. In this study, we aimed to investigate *Salmonella* Infantis strains which were isolated from feces of healthy broiler chickens for the presence of antibiotic resistance and frequency of Class 1 integrons that is responsible for the transfer of antibiotic resistance as plasmids, transposons. For this purpose a total of 150 S. Infantis strains which were isolated and identified according to the ISO 6579-2002 and Kauffmann-White serotyping scheme were used. Antimicrobial resistance of the strains was determined by the disc diffusion method following to the recommendations of CLSI 2011 standard protocol and also Class 1 integrons were investigated by PCR. According to the results, high rate of multi-drug antibiotic resistance (89.3%), high rate of sensitivity (100%) to cefotaxime, ciprofloxacin, gentamicin, ceftazidime were observed and also Class 1 integrons were determined in all isolates. In conclusion, the presence of Class 1 integron is all strains of *Salmonella* Infantis showed the potential importance of these strains as recipient for antibiotic resistance.

Keywords: *Antimicrobial resistance, Class 1 integron, Salmonella Infantis*

Broyler Tavuklardan Elde Edilen *Salmonella* Infantis Suşlarının Antibiyotik Direnç Profilleri ve Sınıf 1 İntegron Varlığı

Özet

Salmonella infeksiyonları kanatlı hayvanlarda görülen ve ekonomik kayıplara neden olan en önemli hastalıklardan biridir. Etkenin zoonotik karakteri ve gıda kaynaklı infeksiyonlara neden olması da halk sağlığı açısından önem göstermektedir. Son yıllarda artan antibiyotik dirençliliği *Salmonella* infeksiyonlarının kontrolünü güçleştirmektedir. Türkiye'de non-tifoidal *Salmonella* serotiplerinden *Salmonella* Infantis'in oranı artış göstermektedir. Bu artış ile birlikte, diğer *Salmonella* serotiplerinde yaygın olarak görülen antimikrobiyal dirençliliğin *Salmonella* Infantis suşlarında da bilinmesi önem taşımaktadır. Bu çalışmada, sağlıklı broyler tavukların dışkılarından izole edilen *Salmonella* Infantis suşlarında antibiyotik dirençliliği ve plasmidler, transpozonlar gibi antibiyotik dirençliliğinin taşınmasında rol oynayan sınıf 1 integronların sıklığının araştırılması amaçlandı. Bu amaçla ISO 6579-2002 ve Kauffmann-White serotiplendirme şemasına göre identifiye edilen 150 adet *Salmonella* Infantis suşu kullanıldı. Suşların antibiyotik dirençleri CLSI 2011 standart protokolüne göre disk difüzyon yöntemi ile belirlendi ve PZR ile sınıf 1 integronlar araştırıldı. Elde edilen sonuçlara göre yüksek oranda çoklu antibiyotik dirençliliği (%89.3), yüksek oranda (%100) sefotaksim, siprofloksasin, gentamisin, seftazidime karşı duyarlılık ve incelenen suşların tamamında sınıf 1 integron varlığı tespit edildi. Sonuç olarak, *Salmonella* Infantis suşlarının hepsinde sınıf 1 integronların varlığı, bu suşların potansiyel direnç alıcı haline gelmesi bakımından önem taşıdığını göstermektedir.

Anahtar sözcükler: *Antimikrobiyal direnç, Salmonella Infantis, Sınıf I integron*

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INTRODUCTION

Non-typhiodal *Salmonella* infection is one of the most significant health problem in poultry all over the world particularly in developing countries [1]. *Salmonella* is also the most important zoonotic pathogen in poultry [2] *Salmonella* may be transmitted to the raw chicken meat by improper evisceration of the intestine at the slaughter-house which leads to food-borne infections in humans. On the other hand, *Salmonella* isolates carrying the antibiotic resistant genes are also important for both human and animal health [3]. According to the European Food Safety Authority (EFSA), among *Salmonella* serovars *Salmonella* Infantis is the most commonly reported serovar in poultry flocks [4]. In Turkey, the prevalence of *S.* Infantis serovar from healthy broiler chicken flocks is 77.2% by percentage [5].

In Argentina, Australia, Brazil, Netherlands, Finland, Canada, Hungary, Japan, New Zealand and Russia this serovar has increasingly been reported and multidrug-resistant (MDR) *Salmonella* has been observed for more than 20 years [6,7]. Most of the antimicrobial resistance genes are integrated in mobile genetic elements such as plasmids, transposons and integrons [1,8,9]. Typically, Class 1 integrons have *intI1* gene which encodes integrase, a recombination specific site, a promoter and gene cassettes. They can be inserted into transposons/plasmids and carry antibiotic resistance genes from bacteria to bacteria. Class 1 integrons harbor more than 100 gene casettes some of which are encoding resistance to β-lactamases, rifampicin, trimethoprim, aminoglycosides, quinolones and chloramphenicol [10,11]. Class 1 integrons are the most frequent integron type classified in MDR *Salmonella* serotypes [12,13].

In recent years, several studies have been conducted to investigate integrons in *Salmonella* [8,9,12,13]. For example a study in Germany demonstrated that all *S.* Derby isolates obtained from pigs had Class 1 integrons. Another study in Thailand showed that 84 of 183 *Salmonella* strains (*S.* Anatum, *S.* Kedougou, *S.* Stanley, *S.* Weltevreden, *S.* Rissen, *S.* Baiboukoum) which were isolated from humans and pork had Class 1 integrons and 18 of the strains carried resistance gene cassettes [12,13]. Nevertheless, information of antimicrobial resistance related integrons of *S.* Infantis is limited in Turkey. Because of the relative prevalence of *S.* Infantis to other *Salmonella* serotypes in poultry production has increased in recent years, in this study multiple antibiotic resistance and the presence of Class 1 integrons related with antibiotic resistance was investigated in *S.* Infantis strains.

MATERIAL and METHODS

Salmonella Strains and Antimicrobial Susceptibility Tests

A total of 150 *S.* Infantis strains selected among 206 *S.* Infantis strains which were isolated from feces of healthy broiler chickens between 2012-2013 were used in this study. 150 strains were selected from four different regions (57 from Black Sea region, 39 from Central Anatolia, 30 from Aegean and 24 from Mediterranean region) of Turkey according to the capacity of flocks. Briefly, a 10% systematic sampling method was used for the flocks which were below 10.000 broiler chickens *(Table 1)*. The isolation method and serotyping was performed according to the ISO 6579-2002 and Kauffmann-White scheme, respectively. Antimicrobial resistance of the strains was detected by the disc diffusion method following to the recommendations of CLSI 2011 standard protocol. Discs were used as follows: ampicillin (AMP: 10 µg); cefotaxime (CTX: 30 µg); chloramphenicol (C: 30 µg); ciprofloxacin (CIP: 5 µg); gentamicin (GM: 10 µg); kanamycin (K: 30 µg); nalidixic acid (NA: 30 µg); streptomycin (S: 10 µg); tetracycline (TE: 30 µg); trimethoprim- sulfamethoxazole (SXT: 5 µg); sulfonamide (S3: 250 µg) and ceftazidime (CAZ: 30 µg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as positive control in all tests.

DNA Extraction

DNA was extracted from the isolates with a commercial kit (Genomic DNA Purification, Thermo Fisher Scientific, USA) following the manufacturer's recommended protocol. Then, all samples were kept in -20°C until the PCR assay was performed.

PCR Amplification of the intI1 Gene

Integrons were investigated by the detection of *intI1* gene [14]. PCR amplification was performed containing 0.2 µM of each primer (int1-F 5'-GCC TTG CTG TTC TTC TAC-3', int1-R 5'-GAT GCC TGC TTG TTC TAC-3'), 0.2 mM dNTPs (10 mM dNTP mix; Thermo Fisher Scientific, USA), 3 mM of $MgCl_2$ (Thermo Fisher Scientific, USA), 2.5 µL PCR reaction buffer, 2U of Taq DNA polymerase (Thermo Fisher Scientific; EP0402), and nuclease-free water to a final volume of 25 µl. In the reaction, 2 µL of DNA was used as template. The amplification was performed as follows: strand separation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Finally, there was a 7 min at 72°C for further strand extension. Ten microliters of the amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel (Promega Corporation, USA) with 4 µL of SafeView Classic (Applied Biological Materials, Canada) in Gel Electrophoresis Apparatus with 180V for 60 min. Positive (AVMA-SI14/2 *Salmonella* Infantis strain) and negative *(Leptospira* spp., *Listeria* spp., *Bordatella* spp., *Ornithobacterium rhinotracheale*, *Streptococcus* spp. and *Pseudomonas* spp.) strains were obtained from strain collection of Ankara University Faculty of Veterinary Medicine Department of Microbiology.

RESULTS

The isolates showing resistance to at least four and more antibiotic drug resistance were assessed as multiple-

Table 1. *Distribution of Salmonella Infantis strains according to the regions*

Regions/City	Number of Total Flocks	Number of Flocks ≤ 10.000	Number of Flocks with 10% Sampling	Number of Isolates
Black Sea/Bolu	378	70	7	57
Central Anatolia/Çorum	60	3	0.3*	7
Central Anatolia/Ankara	20	9	0.9*	19
Central Anatolia/Afyonkarahisar	70	2	0.2*	13
Aegean/İzmir	55	4	0.4*	30
Mediterranean/İçel	92	41	4	24

** The fractional results of 10% sampling calculations were accepted as one flock*

Table 2. *The antimicrobial resistance prevalence of Salmonella Infantis isolates*

Antimicrobials	Number of Isolates	Resistance Rates (%)
AMP	10	6.6
C	11	7.3
K	66	44
S	117	78
SXT	122	81.3
S3	139	92.6
TE	140	93.3
NA	142	94.6
CTX	0	0
CIP	0	0
GM	0	0
CAZ	0	0

Table 3. *Antimicrobial resistance patterns of 134 MDR Salmonella Infantis isolates*

Multiple Resistance Patterns	Number of Isolates (%)
K, NA, S, TE, SXT, S3	46 (30.6)
K, NA, TE, SXT, S3	6 (4)
K, NA, S, TET, S3	5 (3.3)
NA, S, TE, S3	8 (5.3)
NA, TE, SXT, S3	13 (8.6)
NA, S, TE, SXT, S3	43 (28.6)
AMP, C, NA, S, TE, SXT, S3	2 (1.3)
AMP, K, NA, S, TE, SXT, S3	1 (0.6)
AMP, C, NA, S, TE, S3	1 (0.6)
AMP, NA, S, TE, SXT, S3	1 (0.6)
AMP, C, K, NA, S, TE, SXT, S3	4 (2.6)
C, K, NA, S, TE, SXT, S3	3 (2)
C, NA, S, TE, SXT, S3	1 (0.6)

resistant strains. One hundred forty two (94.6%) isolates were resistant to nalidixic acid, 140 (93.3%) isolates were resistant to tetracycline, 139 (92.6%) isolates were resistant to sulfonamide and 122 (81.3%) isolates were resistant to trimethoprim- sulfamethoxazole *(Table 2)*. Also, all isolates were sensitive to cefotaxime, ciprofloxacin, gentamicin and ceftazidime (100%). Furthermore, 134 (89.3%) multi-drug resistance isolates were demonstrated *(Table 3)*.

All samples were investigated for the presence of *intI1* gene and thus the Class 1 integron *(intI1* gene) was detected in all isolates. Beside this, all multi-drug resistant isolates have been found to have Class 1 integron.

DISCUSSION

Increasing antibiotic resistance is an important public health problem and resistance continues to spread due to many factors. Antibiotics used for therapeutic and prophylactic purposes for broilers as stimulating growth in production. Some antibacterial drugs, such as ampicillin, tetracycline, chloramphenicol, enrofloxacin, neomycin, which are used in antibacterial treatment of *Salmonella* infections to become resistant by inhibiting the microflora of the digestive system and these bacteria play an important role in the transfer of resistance genes and cause serious infections that affect the food chain when spreading in nature [15-18]. For all these reasons, after January 2006, antibiotic feed additives are not allowed to be imported to Turkey by law [19].

In the present study, antibiotic susceptibility and presence of Class 1 integron of 150 *S.* Infantis strains that were isolated from healthy broiler chickens feces were detected. Nogrady et al.[20] also conclude that *S.* Infantis strains, which had multiple resistance spread through the poultry meat among humans and this supported that MDR has become a public health concern. According to the European Food Safety Authority (EFSA) report, it was emphasized that *S.* Infantis strains detected in poultry resistant to tetracycline, ampicillin and sulphonamides and 31% of MDR of these isolates [4]. Similar studies related to antibiotics that was used by poultry industry, performed by other researchers. Kudaka et al.[21] 99.2% of broiler isolates were identified as *S.* Infantis and antimicrobial resistance strains were detected as tetracycline, streptomycin and trimetoprim and especially sulphanamide. In our study, the resistance of nalidixic acid, tetracycline and sulfonamide against *S.* Infantis is 94.6%, 93.3%, 92.6% respectively. The high resistance rate of *S.* Infantis to quinolones was known in

Germany and Hungary, but in our study we had an opposite result of 100% sensitivity of ciprofloxacin [19]. Resistance to ciprofloxacin was reported to be 66.7% to 100%, especially in Germany, Slovakia, Bulgaria and Austria [4], while the study strains showed sensitivity to ciprofloxacin. Also high frequency of resistance to ciprofloxacin and nalidixic acid for *S.* Infantis was reported in several reports in Iran too [22,23]. Quinolones were used frequently in treatment regimens and also as a feed additives in poultry industry [24]. In recent years several studies have been reported about third-generation cephalosporin resistance patterns of *Salmonella* was isolated from domestic animals and human [25-27]. In parallel with this situation, Kameyama et al. emphasized that 3rd-generation cephalosporin-resistant *S.* Infantis associated with β-lactamases is increasing in worldwide [28]. Although cefotaxime and ceftazidime resistance were found in their research [28], our isolates were 100% sensitive.

Furthermore, in this study, it was observed that multiple antibiotic resistance was directly proportional to Class 1 integron presence. In our study *S.* Infantis strains were investigated for the presence of *intI1* gene and Class 1 integron was detected in all isolates. Previously, several laboratories mentioned that antibiotic resistance genes are chromosomally encoded and contain integrons. Integrons play an important role in the dissemination of antimicrobial resistance through horizontal transmission and it was known that prevalence of integron increases in *Salmonella* isolates [29,30]. Firoozeh et al.[8] showed that poultry MDR *Salmonella* isolates were carried Class 1 integron 91.4%, also Asgharpour et al.[1] reported that *S.* Infantis strains contain 36% Class 1 integron in Iran. Naghoni et al.[31] were detected Class 1 integron from *S. enterica* serovars and Kudaka et al.[21] was revealed that presence of Class 1 integron in *S.* Infantis isolated from broiler chickens as our study. Several other researches have been reported in many different countries about Class 1 integron but *S.* Infantis studies were limited [32-36]. This situation is similar in Turkey. To our knowledge there is only one study conducted to determine the association of antibiotic resistance with integrons of *S.* Infantis strains [37].

In our study, it was revealed that the antibiotic resistance rates were high in *S.* Infantis strains and a great number of these isolates have been found as MDR. Therefore, the more prudent usage of antibiotics and epidemiological studies are needed which can reveal the mechanism of spreading resistance and resistant strains must be included in prevention strategies.

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Research Article

The Prevalence and Associated Risk Factors of *Coa* Gene (Coagulase Positive *Staphylococcus aureus)* from Bovine Milk

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Abstract

The study describes prevalence of coagulase positive *Staphylococcus aureus,* a zoonotic pathogen, and rule out key risk factors associated with the spread of this pathogen. A total of 900 milk samples of animals (n = 450 cattle and n = 450 buffaloes) from three subdistricts of Faisalabad were collected and processed microbiologically for staphylococci identification while, PCR for coa gene identification. Surf field mastitis test was used to screen the samples for subclinical mastitis at milking. Chi-square test was used to assess association of risk factors with mastitis. The overall prevalence of subclinical mastitis was 55% comprising 54 and 56% in cattle and buffalo's milk, respectively. A significant difference ($P<0.05$) was seen in the prevalence of subclinical mastitis among the cattle from different cities. The prevalence of *coa* gene presented 39.33% from total samples. Highest prevalence was found in tehsil Samundari followed by Jaranwala and Faisalabad presenting 43%, 39%, and 36%, respectively. The quarter based prevalence of subclinical mastitis was found 32% while 5.58% of quarters were blind. Right side quarters and teats were more affected. The risk factors except body condition and parity presented significant association with mastitis. The study concluded that bacterial coa gene, was prevalent in milk, and determinants were strongly associated with higher prevalence.

Keywords: *Coagulase, Staphylococcus aureus, Coa gene, Risk Factors and subdistricts*

İnek Sütünde Koagulaz Pozitif Staphylococcus aureus *Coa* Geni Prevalansı ve İlgili Risk Faktörleri

Özet

Bu çalışma; zoonotik bir patojen olan koagülaz pozitif *Staphylococcus aureus*'un prevalansını ve bu patojenin yayılmasında rol oynayan risk fakörlerini tanımlamaktadır. Faisalabad'ın 3 ayrı bölgesindeki hayvanlardan toplanan toplam 900 adet süt örneğinde (n = 450 sığır ve n = 450 manda) *Staphylococci* identifikasyonu amacıyla mikrobiyolojik analiz ve coa geninin tespiti amacıyla PCR gerçekleştirildi. Subklinik mastitisin taranmasında sağım esnasında sörf mastitis testi uygulandı. Risk faktörlerinin mastitisle ilişkisini değerlendirmek amacıyla Ki-kare testi kullanıldı. Sığır ve manda sütlerinde subklinik mastitis prevalansı sırasıyla %54 ve %56'sı olarak hesaplanırken toplamda %55 olarak bulundu. Farklı şehirlerden örneklenen sığırlar arasında subklinik mastitis prevalansı yönünden anlamlı bir fark tespit edildi ($P<0.05$). *Coa* geninin prevalansı toplam örneklerde %39.33 olarak saptanmıştır. En yüksek prevalans oranı %43 olarak Samundari'de saptanırken bunu sırasıyla %39 ile Jaranwala ve %36 ile Faisalabad izlemektedir. Subklinik mastitisin meme loblarındaki prevalansı %32 olarak saptanırken meme loblarının %5.58'inde ise körelme belirlendi. Sağ taraftaki meme lobları ve meme başlarının daha fazla etkilendiği saptandı. Vücut kondisyonu ve doğum sayısı dışındaki risk faktörleri mastitis ile anlamlı ilişki içeriyordu. Çalışma, bakteriyel coa geninin sütte yaygınlığını ve göstergelerin yüksek prevalans ile kuvvetli bir ilişkisi olduğunu göstermektedir.

Anahtar sözcükler: *Koagülaz, Staphylococcus aureus, Coa geni, Risk faktörleri ve alt bölge*

INTRODUCTION

Milk quality and quantity deteriorates with bacterial invasion to milk producing glands that resultantly increases somatic cell count making milk unfit for consumers. This deterioration accounts for annual losses in dairy industry reaching to approximately 2 billion dollars in USA and 526 million dollars in India where subclinical mastitis gives

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its contribution to approximately 70% of these losses [1]. Among bacterial contaminants in bovine mastitis, *Staphylococcus aureus (S. aureus)* are overwhelming to control and are well-known to cause subclinical mastitis. The pathogen is a Gram positive organism with ability to survive in high salt and high temperature range appearing as an opportunistic pathogen and frequent colonizer of the epithelium causing severe diseases in humans and animals. The organism is of zoonotic importance that becomes more serious in its resistant form against antimicrobial agents [2,3]. The bovine *S. aureus* transferred to humans may lead to outbreak in humans and animal population [4].

The lower cure rate of bovine *S. aureus* mastitis, evading immune response of the host, and extensive use of beta-lactam antibiotics add to *S. aureus* nuisance [5,6] which influences the pathogenesis of *S. aureus* in mastitis. Coagulase positive *S. aureus* has ability to yield toxins such as; exfoliative, toxic shock syndrome toxin and panton-valentine leucodin. Subclinical mastitis has dynamic importance in its association with zoonotic impacts as milk serves as vehicle of pathogens transmission [7].

Intramammary infections caused by *S. aureus* damage the alveolar physiology; decrease in milk production, and impart harmful effects on milk composition [8,9]. *S. aureus* inflicted intramammary infections are long term and chronic as bacteria has the ability to hide itself in mammary epithelial cells and host phagocytes. Concurrent resistance of bacterial species to antimicrobials of different structural classes is increasing in multitude complicating therapeutic management of infections [10].

The traditionally infectious agents are identified on the basis of cultural examination that sometimes fails in case of non-cultivable strains thus creating ambiguity in identification. Polymerase Chain Reaction (PCR) works as an effective technique to diagnose coagulase positive *S. aureus* from human and animal origin [11,12]. Adding to this, scarce epidemiological studies regarding *S. aureus* prevalence in bovine milk may unleash zoonotic spread of pathogenic *S. aureus*.

Keeping in view the animal and human health hazard anchored with this pathogen, the current study was designed to estimate prevalence of *Coagulase* positive *S. aureus* from cattle and buffalo milk obtained from various private and public dairy farms of Pakistan and to rule out key risk factors associated with spread of this pathogen.

MATERIAL and METHODS

The district Faisalabad, 3rd largest city in Pakistan situated 184 meter higher to sea level with altitudes between 31°20′-31°33′ N and 73°13′-72°55′ [13]. The sole livelihood of people in and around the jurisdiction of district Faisalabad is agro based where lives of locals are directly linked with livestock rearing. Cattle and buffalo are kept as live banks by families in that mostly bovine serve as only income generator for house hold expenses. Three tehsils of district Faisalabad i.e. Faisalabad, Jaranwala and Samundari were selected as representative of bovine population of district. The bovine populations were approached using convenient technique of sampling as described by Thrusfield [14] keeping in view constraints of farm owner's willingness and access to the laboratory. The milk samples collected from bovine were divided into three categories; (1) 40-90 bovine, (2) 91-200 bovine and (3) >200 bovine in such a way that ten farms from each tehsil each with 30 milk samples from each farm (n=15 cattle, n=15 buffalo) totaling 900 milk samples (n=450 cattle, n=450 buffalo) were taken. Subclinical form of mastitis was assessed by Surf Field Mastitis Test (SFMT) proposed by Muhammad et al.[15].

Milk samples (n=900) were streaked on blood agar and later based on colony characters were sub-cultured on Mannitol Salt Agar. Gram staining and catalase test [16] were performed to differentiate organisms as Staphylococci. The Staphylococci were processed to DNA extraction using bacterial DNA extraction kit (GF-1 Bacterial DNA extraction kit, Vivantis Technologies Sdn. Bhd, Malaysia). The extracted DNA was quantified by Nano-Drop technique (NanoDrop 2000, Thermo-Scientifics, NanoDrop products, 3411 Silverside Rd, Bancroft Building, and Wilmington, DE 19810 USA).

The specification of PCR reaction included reaction mixture of 25 µL having, 12.5 µL of master mix (2x master mixes, Accuprime TM super mix11), 1.5 µL (10 pmol) from each primer, 8 µL distilled water, and 1.5 µL DNA sample. The primers, Coag 2 (CGA GAC CAA GAT TCA ACA AG), Coag 3 (AAA GAA AAC CAC TCA CAT CA) were used with product size of 970bp. The reaction of PCR was run in thermocycler (Eppendorf, Mastercycler®5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, and Germany) with following specification; initial denaturation at 94°C/10 min for once, and denaturation at 94°C/45 seconds, annealing at 54°C/1 min and extension at 72°C/2 min, respectively for 30 cycles each. Final extension at 72°C/10 min were given at the end for single time. The PCR product was run on 1.5% agarose gel stained with ethidium bromide [17] that was photographed under ultraviolet illuminator *(Fig. 1)*.

Risk factors assumed in dichotomous questionnaire to check their association with mastitis included information like specie, breed, age, diet, season, system of rearing, presence of ticks, body health, use of teat dips, feed and water, udder consistency and condition, animal physiological status either lactating or dry, parity, milking system, hygiene and milker's care during milking, use of antibiotics for general ailments, mastitis purpose, call for veterinary professionals, and call for veterinary consultancy.

Statistical Analysis

The data was subjected to statistical analysis using appropriate designs on SPSS version 22.0 computer program. Prevalence of mastitis was calculated as per formula described by Thrusfield [14] and chi-square test was used to statistically correlate the risk factors with prevalence at 5% probability.

RESULTS

Subclinical form of mastitis is type in which there are apparently no abnormal signs except that screening test detects invisible abnormality in milk. The current study found 55% (495/900) subclinical mastitis in bovine from district Faisalabad with highest percentage noted in tehsil Samundari (67.67%) followed by Jaranwala (52.33%) and Faisalabad (45%) *(Table 1)*. There was a significant difference (P<0.05) of subclinical mastitis among different tehsils on overall bovine subclinical mastitis basis. However, subclinical mastitis in cattle from different tehsils indicated a non-significant difference (P<0.05) presenting 62.67%, 50.67%, and 48.67% of subclinical mastitis from tehsil Samundari, Jaranwala, and Faisalabad, respectively. Similar pattern was observed in case of buffalo milk subclinical mastitis from different tehsils of district Faisalabad. The comparison of specie showed a non-significant difference (P>0.05) of subclinical mastitis in cattle and buffalo from different cities of district Faisalabad with 48.67% versus 41.33%, 50.67% versus 54%, and 62.67% versus 72.67% of subclinical mastitis between cattle and buffalo in tehsil Faisalabad, Jaranwala, and Samundari was noted, respectively.

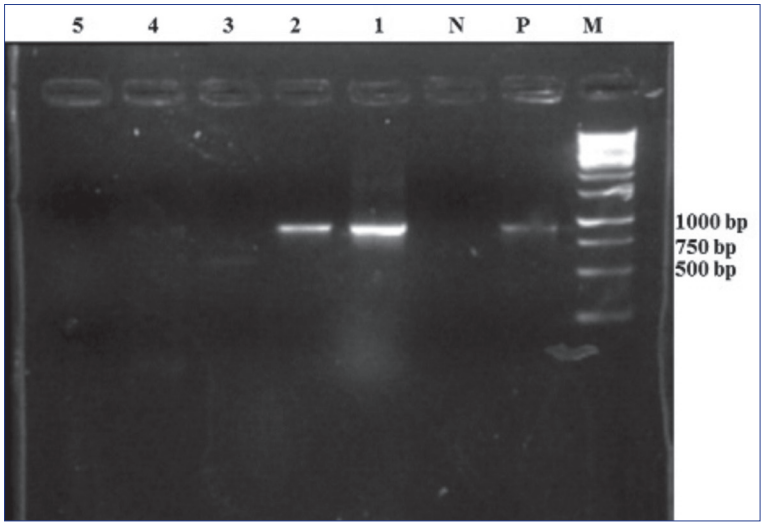

Fig 1. PCR results of *coa* gene, M = 1kb marker (product brand), P = positive control (coa gene, Momtaz et al.[18]), N = neative control, 1-2 positive samples at 907bp, 3-5 negative samples

Coa gene *(Staphylococcus aureus)* presented 39.33% in bovine from district Faisalabad from total collected milk samples *(Table 2)*. The comparison of *S. aureus* prevalence from different tehsils showed 43%, 39%, and 36% from tehsil Samundari, Jaranwala, and Faisalabad, respectively. Subclinical mastitis among different tehsils of district Faisalabad presented a non-significant difference (P>0.05) with 34%, 36%, and 40% of prevalence from tehsil Faisalabad, Jaranwala, and Samundari, respectively. Similar pattern was noted in case of buffalo milk subclinical mastitis from different tehsils of district Faisalabad.

The number of blocked quarters were found 5.58% (201/3600) with highest percentage of blocked quarter noted in case of front right (FR) followed by rear right (RR), front left (FL), and rear left (RL) with 6.89%, 6.56%, 4.67%, and 4.22%, respectively *(Table 3)*. The study found right side suffering higher mastitis compared to left side quarters with a non-significant difference (P>0.05). The quarter based prevalence was

Table 1. *Prevalence of subclinical mastitis in bovine from District Faisalabad*

Sampled Area	Cattle				Buffalo				Overall (Cattle and Buffalo)			
	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*
Faisalabad	150	73 (48.67)	39.98-56.02		150	62 (41.33)	33.42-9.24		300	135 (45)	39.36-50.64	
Jaranwala	150	76 (50.67)	41.97-58.03	0.022	150	81 (54)	46.00-2.00	0.001	300	157 (52.33)	46.67-58.00	0.001
Samundari	150	94 (62.67)	54.90-70.43		150	109 (72.67)	51.41-0.60		300	203 (67.67)	62.36-72.96	
Total	450	243 (54)	48.94-58.16		450	252 (56)	65.50-9.82		900	495 (55)	51.75-58.25	

C.I: *Indicates confidence interval set at 95%; P<0.05 indicate significant difference*
** Between specie comparison: cattle versus buffalo from Faisalabad P=0.246, Jaranwala, P=0.488, Samundari P=0.064, and Total district P=0.461*

Table 2. *Prevalence of coa gene (Staphylococcus aureus) from bovine milk of District Faisalabad*

Sampled Area	Cattle				Buffalo				Overall			
	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*
Faisalabad	150	51 (34)	26.40-41.61		150	57 (38)	30.21-45.80		300	108 (36)	30.56-41.44	
Jaranwala	150	54 (36)	28.29-43.71	0.547	150	63 (42)	34.07-49.92	0.373	300	117 (39)	33.47-44.53	0.212
Samundari	150	60 (40)	32.13-47.87		150	69 (46)	38.00-54.00		300	129 (43)	37.39-48.61	
Total	450	165 (36.67)	32.20-41.12		450	189 (42)	37.43-46.56		900	354 (39.33)	36.14-42.52	

C.I: *Indicates confidence interval set at 95%; P<0.05 indicates significant difference*
** Between specie comparison: cattle versus buffalo from Faisalabad P=0.470, Jaranwala P=0.287, Samundari P=0.294, and Total district P=0.101*

Table 3. *Prevalence of teat blockage and quarter based prevalence*

	Prevalence of Blocked Quarters				Prevalence on Quarter Basis			
Quarters	Total Quarter Examined	No. Blocked (%)	CI	P-value	Total Quarter Examined	No. Blocked (%)	CI	P-value
FR	900	62 (6.89)	5.23-8.55		838	290 (34.61)	31.38-37.83	
FL	900	42 (4.67)	3.29-6.05	0.028	858	252 (29.37)	26.32-32.42	0.001
RR	900	59 (6.56)	4.94-8.18		841	311 (36.98)	33.72-40.25	
RL	900	38 (4.22)	2.91-5.53		862	235 (27.26)	24.29-30.23	
Total	3600	201 (5.5)	4.83-6.33		3399	1088 (32.0)	30.35-33.48	

CI: *Indicates confidence interval set at 95%*

found 32% (1088/3399) from bovine. The right side quarters presented higher prevalence with 36.98% and 34.61% in rear right (RR) and front right (FR) compared to left side quarters with 29.37% and 27.26% in front left (FL) and rear left (RL), respectively. The difference of quarter based prevalence among different quarters showed significant difference (P<0.05).

The study found non-significant association of mastitis with age in case of cattle whereas age of buffalo was found significantly associated with subclinical mastitis *(Table 4)*. The breeds of cattle showed significant association with mastitis occurrence among them Frisian cattle had higher prevalence of subclinical mastitis (66.67%) than to Sahiwal (40%) and non-descript cattle (48.83%). However, breeds factor in case of buffalo subclinical mastitis did not have a significant (P>0.05) association with mastitis occurrence. Higher number of calving (>4 calving in life), lack of teat dipping, higher tick infestation, udder pathology, lactation status, higher milk yield, higher milking frequency, unhygienic milker's hands, use of beta-lactam drugs for general ailment, lack of veterinary professional services for disease treatment and prevention proved potential risk factors associated with this disease.

DISCUSSION

The prevalence of *S. aureus* mastitis in current study was calculated from milk samples irrespective of screening test. The findings of Farooq et al.[19] reporting 44% of from subclinical mastitis was in line with current study. Contrary to current study Ali et al.[20] stated 8.32% *S. aureus* prevalence from bovine milk. Highest Staphylococcal species in bovine milk had been reported by various studies [21,22]. Higher prevalence of *S. aureus* was also noted by Khan and Muhammad [23] presenting 45% on subclinical mastitis basis, respectively. The higher prevalence in current study might be due the significant association of assumed risk factors with mastitis. The discrepancies in *S. aureus* prevalence included its stay and survival in keratin of healthy animal's teat canal, and formation of biofilm that may give rise to lower than exact isolation of *S. aureus* during *in vitro* culturing. The prevalence of *S. aureus* varies specie to specie [24], breed, geographic zones, and farm management conditions. The surrounding of animal such as bedding and manure are true source of contagious microorganisms. These microbes may be present in soil as well as in air as environmental microorganisms. Milker's hands, towels and flies spread these pathogenic bacteria to clean udders during the

Table 4. *Bivariate analysis of risk factors associated with subclinical mastitis in cattle and buffalo from District Faisalabad*

Parameters	Levels	Cattle			Buffalo		
		Total Examined	Diseased (%)	*P*-value	Total Examined	Diseased (%)	*P*-value
Age	3-5 years	240	51.25	0.447	180	46.66	0.001
	>5 years	219	54.79		261	64.36	
Breed	* Sahiwal/**Nili-ravi	150	40.00	0.001	141	55.31	0.339
	* Frisian/**Kundi	180	66.67		129	53.48	
	*Scrub/** Scrub	129	48.83		171	61.40	
Rearing system	Open	270	61.11	0.001	273	63.73	0.001
	Confined	189	41.26		168	46.42	
No. of Calving	2-4	240	56.25	0.137	204	55.88	0.659
	>4	219	49.31		237	58.22	
Body health	Normal	240	53.75	0.748	216	55.55	0.659
	Thin	120	50.00		129	60.46	
	Emaciated	99	54.54		96	56.25	
Use of teat dips	Yes	153	29.41	0.001	144	41.66	0.001
	No	306	64.70		297	64.64	
Presence of ticks	Yes	285	60.00	0.001	267	67.41	0.001
	No	174	41.37		174	41.37	
Feed & Water	Well fed	390	56.15	0.001	198	45.45	0.001
	Underfed	69	34.78		243	66.66	
Udder consistency & condition	Normal	288	46.87	0.001	252	48.80	0.001
	Inflamed	51	76.47		66	68.18	
	Painful	120	57.50		123	68.29	
Lactation Status	Dry	198	42.42	0.001	171	42.10	0.001
	Lactating	261	65.43		270	66.66	
If Lactating then milk yield	<1	54	16.66	0.001	30	10.00	0.001
	2-5	216	55.55		114	44.73	
	5-10	114	57.89		177	64.40	
	>10	75	64.00		120	70.00	
No. of Milking if lactating	Once/day	54	16.66	0.001	30	10.00	0.001
	Twice/day	405	57.77		411	60.58	
Milker's hands hygiene during milking	Yes	156	30.76	0.001	144	41.66	0.001
	No	303	64.35		297	64.64	
Hygienic condition during milking	Yes	177	33.89	0.001	138	23.91	0.001
	No	282	64.89		303	72.27	
Generally used antibiotics	B-lactam group	390	56.15	0.001	381	61.41	0.001
	Others	69	34.78		60	30.00	
Antibiotics used in mastitis	B-lactam group	435	53.79	0.001	360	65.00	0.001
	Others	24	37.50		81	22.22	
Call for veterinary professional	Self-treatment	165	58.18	0.039	165	63.63	0.008
	Few day post self- treatment	183	54.09		186	56.45	
	Only emergency	30	30.00		21	28.57	
	Always on call	45	53.33		45	60.00	
	Always veterinarian	36	41.66		24	37.50	
Veterinary professional consultancy	Only veterinary assistant	231	68.83	0.001	240	70.00	0.001
	Only veterinarian	81	29.62		63	28.57	
	Both	147	40.81		138	47.82	

*P<0.05 indicate significant association, * Cattle breeds, ** Buffalo breed*

milking process and are responsible for most of the mastitis cases [25].

Subclinical mastitis serves as reservoir of infectious pathogens and together with other forms of mastitis piles up to 70% of economic losses [1]. The rate of subclinical cattle mastitis (54%) in current study was in line with findings of Singh and Baxi [26] who described 54% prevalence of subclinical mastitis in India while higher bovine subclinical mastitis was noted as 64% and 86.3% by Mureithi and Njuguna [27] and Abrahmsen et al.[28], respectively. Whereas, lesser to the current study was reported as 44% and 34.4% from Pakistan and Kenya [29,30] respectively.

The prevalence of subclinical mastitis in buffalo in present study is in agreement with findings of Getahun et al.[31] and Mustafa et al.[32] who reported 54.7% and 59.64% subclinical buffalo mastitis, respectively. Higher prevalence in buffalo might because of enriched nutrients in milk that favors comparatively higher growth of microbes. In addition to this pendulous udder and longer teats of buffaloes make it prone to mastitis. Contrary to the finding of current study lower prevalence was found to be 32.85% [33] and 23.18%. The discrepancies in prevalence are also linked with different climatic zones, management trends, and exposure to microbial environment.

The higher prevalence of subclinical mastitis in cross bred cattle was in line with findings of Sanotharan et al.[34] and Alemu et al.[35] who reported significant association ($P<0.05$) of crossbred animals with subclinical mastitis. The significant association ($P<0.05$) of breed with mastitis occurrence was also reported by Alebachew and Alemu [36] and Lakew et al.[37]. The difference in anatomical, genetic, production status may predispose occurrence of this disease [38]. Researchers have noticed native breed resistant to mastitis [39] and this could be due to genetic resistance and adaptation to the environment [40]. Higher prevalence of mastitis in late lactation was in agreement with results of Dego and Tareke [9]. However, Mungube et al.[41] reported higher prevalence of mastitis in early stage of lactation. The variations in the effect of stages of lactation among different studies could be related probably to disparities in age, parity and breed of the sampled animals as indicated by Getahun et al.[31].

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Conflict of Interest Statement

The authors declared that they have no conflict of interest.

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Research Article

Antitumorigenic Activity of the Herbal Mixture-AK27 on Ehrlich Ascites Carcinoma in Mice

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Abstract

The treatment of cancer is highly challenging and contains surgery, chemotherapy and radiotherapy alone or combinations which have various side effects on the patient health. This study aimed at observing the possible antineoplastic activity of AK27-herbal mixture, a combination of pistachio resin, rhus resin, pollen, *Nigella sativa* seed, pomegranate skin and olive oil, on experimentally induced Ehrlich ascites carcinoma (EAC) in a mouse model. EAC-bearing mice were evaluated by tumor cell count (viable and non-viable), median survival time, percentage increase in the life span and live body weight changes up to 30 days of EAC inoculation. Volume of EAC cells and viable cell count were found to be significantly decreased in AK27 treated groups when compared to EAC control group ($P<0.01$). The highest viable cell count (mean $15.5x10^7$/mL/mouse) and EAC volume (average 12 mL/mouse) was measured in the cancer group. Administration of AK27 mixture before tumor challenge prevented the EAC development whereas simultaneous administration or after tumor initiation, AK27 significantly reduced the number of viable EAC cells with respect to cancer control group. After the 4th day onwards until the 12th day significant differences were observed between groups in terms of live body weight ($P<0.001$). All animals in cancer control group died within 12 days as expected. Mean life span in AK27 treated groups were varied from 24 to 26 days with percentage increase in life span of from 100 to 150. The present study demonstrated that AK27-compound was exhibited promising antitumor efficacy in EAC bearing mice.

Keywords: *Ehrlich ascites carcinoma, Pistachio, Rhus, Nigella sativa, Pollen, Olive oil*

Farelerde AK27 Bitkisel Karışımının Ehrlich Ascites Karsinoma Üzerine Antitümorijenik Etkisi

Özet

Kanser sağaltımı oldukça zor ve hastanın sağlığı üzerine olumsuz etkileri olan cerrahi, kemoterapi ve radyoterapinin tek başına veya kombinasyonlarını kapsar. Bu çalışmanın amacı antep fıstığı reçinesi, sumak reçinesi, polen, çörek otu tohumu, nar kabuğu ve zeytinyağı kombinasyonundan oluşan AK27-bitkisel karışımın muhtemel antineoplaistik etkinliğini fare modelinde deneysel oluşturulan Ehrlich asites karsinomunda (EAK) göstermektir. EAK'lı fareler tümör hücre sayısı (canlı ve ölü), ortalama yaşam süresi, yaşam süresinde yüzde artış ve canlı ağırlık değişimi yönünden değerlendirildi. En yüksek canlı hücre sayısı (ortalama $15.5x10^7$/mL/fare) ve EAK hacmi (ortalama 12 mL/fare) kanser grubunda ölçüldü. AK27 karışımının tümör oluşturulmasından önce verilmesi EAK gelişimini engellerken aynı anda veya tümör oluştuktan sonra verilmesi kanser kontrol grubu ile karşılaştırıldığında canlı EAK hücre sayısını anlamlı düzeyde azalttığı gözlendi. Dördüncü günden itibaren 12. güne kadar canlı ağırlık artışı bakımında gruplararasında anlamlı farklar tespit edildi ($P<0.001$). Beklenildiği gibi kanser kontrol grubundaki hayvanların tümü 12. günde öldü. AK27 uygulanan gruplarda ortalama yaşam süresi 24 ile 26 gün arasında artarken yaşam süresinde yüzde artış 100 ile 150 arasında değişti. Bu çalışma AK27 bileşiminin EAK'lı farelerde dikkate değer antitümör etkisinin olduğunu gösterdi.

Anahtar sözcükler: *Ehrlich ascites karsinomu, Antep fıstığı, Sumak, Nigella sativa, Polen, Zeytin yağı*

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INTRODUCTION

Cancer is one of the most aggressive disease and closely associated to the causes of morbidity and mortality in humans with approximately 14 million new cases in 2012 [1]. By 2020, the number of new cases is expected to rise as high as 70%. It is the second leading cause of death globally, and was responsible for 8.8 million deaths (approximately 1 in 6 mortality) in 2015 [2].

Cancer is the disease of all vertebrated animals and can be defined as uncontrolled cellular growth, invasion and spreading of local cells from the primary site to other sites in the body to generate new colonies of cancer cells [3].

The nature of cancer can be evaluated by experimental tumor models especially transferable tumor cells from one individual to the other [4]. Under *in vivo* conditions transplanting tumor tissues subcutaneously from mouse to mouse was pioneered by Ehrlich and Apolant [5]. Then the liquid form of carcinoma "Ehrlich ascites carcinoma" (EAC) was deposited into the peritoneum of the mouse and achieved successful passage thus making next studies possible [6]. EAC is known as undifferentiated carcinoma, and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also has no tumor specific transplantation antigen [7].

The management of cancer is challanging and usually treated by one of or combinations of surgery, chemotherapy and/or radiotherapy. In metastasized cases, the treatment becomes more drastic and patients suffer various side effects during and/or after therapy. Several anti-tumor agents have cytotoxic effects and been designed to stop tumor growth [8]. Long term use of chemotherapeutics is closely associated with significant negative outcomes on patients' health status. Therefore several natural compounds have been investigated without any detectable side effects.

In folkloric and traditional medicine several plants, vegetables and herbs have been frequently used for the prevention or treatment of malignant masses as nutraceuticals and they served as the main source of cancer chemoprevention drug discovery and development [9-11].

Plant derived natural products (remedies) such as pistachio, rhus, *Nigella sativa* seed, olive oil, pommegrande skin have received significant attention due to their ignorable side effects on comsumers/patients. It was shown that pistachio or its resin possessed apoptotic activity through cytotoxic and apoptosis-inducing effects on human hepatoma cell line [12]. *Rhus coriaria* exhibits anticancer activities (suppressing angiogenesis, metastasis and tumor growth) by promoting cell cycle arrest and autophagic cell death of the breast cancer cells [13,14]. *N. sativa* has long been recognised as antitumorigenic effects especially on hard splenic masses [15]. It has been exclusively used for a wide range of tumors. Intraperitoneal administration of *N. sativa* extract dramatically restricted soft tissue sarcomas chemically induced in albino mice [16,17]. Several studies have shown that bee pollen has greater or lesser antimutagenic properties in certain types of cancer [18,19]. It has been also shown the growth of breast, prostate, colon and lung cancer cells in culture were successfully and selectively inhibited by pomegranate (*Punica granatum)* extracts [20]. Olive oil phenols as chemopreventive and therapeutic effects against cancer has been reviewed [21,22] and shown that olive oil prevented experimentally induced colon cancer in a mouse model [23].

We believe that due to many facets of cancer malignancy using single plant derived substance may have only limited effect however a combination of these matters may generate synergistic impact on the tumor cells. Thus, here, we evaluated possible effect of the combination (AK27 herbal mixture) of pistachio resin, rhus resin, pollen, *N. sativa* seed, pomegranate skin and olive oil on experimentally induced EAC in a mouse model.

MATERIAL and METHODS

Formulation of AK27 Mixture

A total of 100 g preparation of AK27 mixture contained followings and prepared by one of the author (AK). Resin of pistachio was collected from branches of pistachio trees approximately 5 year-old and 10 cm in diameter after pruning. Similarly rhus resin was obtained after pruning of the young branches (1-2 year-old) of the rhus plants. Freshly collected resins were melted at 50-55°C for 15 min and kept at room temperature overnight and then overlayed as a thin layer on a marble block to solidify. Thin layer of resins were removed from the marble and finely crushed into powder form. Ten g of each resin powder was mixed. Shadow-dried skin of unrippen pomegranates (local name: Delieksi) were crushed into fine particles. From this, 15 g was added to the formulation. *N. sativa* seeds and bee-pollen were purchased from a local herbalist and both were further triturated by a fine grinder. Twenty-five g of *n. sativa* seed and 20 g of pollen were added to the mixture. The formulation was filtered through a fine particular sized drain. Finally 20 g of natural olive oil (produced locally in a traditional manner) was added to the formulation and mixed until the end-product was viscous.

Animals and Handling

Handling animals carried out in accordance with the ethical guidelines for the care of laboratory animals of Afyon Kocatepe University, Turkey (Ethical approval no: 49533702-110, 14.06.2016). All effort made to control the experimental pain in conscious animals. A total of 84 eight-week-old, weighing 24-25 g male BALB-c mice were used and allocated into six groups each having 14 mice. Animals

were climatised and housed before the experiment for 10 days at the university's experimental animal research center and kept in separate polyacrylic cages without contacts between groups i.e. four animals per cage. All animals received 3 g standard pellet feed per day with *ad libitum* access to water. Room temperature was kept at 22-24ºC and humidity 50%±2. The light cycle was 12 h dark then 12 h light. The air in the room was cleaned by a bio-filter system (Airsopure S980, Airsopure International, USA).

EAC and Stock Animals

EAC cells were obtained from Department of Anatomy, Faculty of Medicine, Erciyes University, Kayseri, Turkey [24]. The tumor cells were maintained in our laboratory by serial intraperitoneal passage in male BALB-c mice for 7-10 days. EAC cells were tested for viability and contamination using trypan blue dye exclusion technique. Cell viability was usually found to be 95% or more. Tumor cell suspensions were prepared in phosphate buffered saline (PBS). Finally EAC cells were implanted into the peritoneal cavity of experimental groups' to establish the animal model for ascites carcinoma.

Experimental Study

Animals in group I (n=14) were given freshly prepared 2 g/mouse of AK27 mixture (0.2 g of pistachio resin, 0.2 g of rhus resin, 0.3 g pommegranate skin, 0.5 g of *N. sativa* seeds, 0.4 g of pollen, and 0.4 g of olive oil per mouse) orally for 7 days then $3x10^6$ EAC cells/mouse (0.2 mL/mouse) was administered intraperitoneally. AK27 mixture feeding was continued until the end of the experiment (to observe preventive effect of AK27 mixture). In group II (n=14) immediately after intraperitoneal administration of $3x10^6$ EAC cells/mouse, oral AK27 mixture (2 g/mouse) was given and continued until the end of the experiment (to observe effect of tumor initiation) whereas in grup III (n=14) after 5 days of single intraperitoneal administration of $3x10^6$ EAC cells/mouse, oral AK27 mixture (2 g/mouse) was given and continued until the end of the experiment (to observe effect on tumor development). Group IV (n=14) served as cancer control group where mice received intraperitoneal $3x10^6$ EAC cells/mouse and then daily oral 0.9% NaCl (0.2 mL/mouse) was administered. For the AK27 control, group V (n=14) mice had single injection of intraperitoneal normal saline solution (0.2 mL/mouse) followed by oral AK27 mixture (2 g/mouse) until the end of the experiment (AK27 mixture control). Group VI serves as sham control in which routine feeding was practiced. The experiment was terminated at the 30th day of the study.

EAC Cell Count

Six of the mice in each group were sacrificed at 10th day and abdominal ascites were removed to observe viability of the cells by microscopy. One ml of EAC ascites was added 1 mL of PBS making 2 mL total. A 100 microliter of diluted EAC was mixed with 100 microliter of trypan blue solution (Sigma, T8154, product of UK). Then approximately 50 microliter was subjected to cell count using a counting chamber (Thoma, Iso Lab, Germany). Five large squares of each counting area of the chamber was considered for cell count. The cells that did not stained by trypan blue were considered as viable and those stained were non-viable. All viable and nonviable cells were counted.

Volume of EAC

Ascites fluid were withdrawn from the abdomen of sacrificed mice and centrifuged at 3000 rpm for 10 min at 4°C. Subtracting the volume of the supernatant gave the volume of ascites fluid.

Mean Survival Time and Percentage Increase in Life Span

Mice that were not sacrificed (n=8 in each group) were observed for the mean survival time (MST) and percentage increased in life span (PILS) according to quotations given below [25]. The live body weight of animals were measured every other day up to 30 days.

$$MST = \frac{\text{Total survival time of each mouse in group}}{\text{Total number of mice in group}}$$

$$PILS = \frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \times 100$$

Statistics

Data were analysed using SSPS for windows. Differences between groups were evaluated by ANOVA and Duncan tests. Significant level was set at P<0.05.

RESULTS

Antitumor activity of AK27 compound against EAC-bearing mice was evaluated by tumor cell count (viable and non-viable), median survival time, percentage increase in the life span and live body weight changes. The findings of the study indicated that the AK27 compound produced significant antitumor effects on mice with EAC.

Live Body Weight Increase

When the live body weight (LBW) increases of the mice were considered, the differences between groups were not significant on days 0 and 2 (P>0.05). After the 4th day onwards until the 12th day significant differences were observed between groups (P<0.001). The highest mean LBW was noted in group IV (cancer control group) then followed by groups III and II. On day 4, no significant difference was seen in groups III and IV however the difference was significant between 6 and 12 days of the study *(Table 1)*.

After 14 days, the highest LBW increase was observed in group III (mean 28.36 g) followed by groups II (27.44 g) and I (27.11 g). The lowest LBW increase was in groups

V (26.19 g) and VI (25.39 g). AK27 significantly ($P<0.05$) maintained the body weight of mice in groups I, II and III toward normal with respect to EAC control animals (group IV) *(Fig. 1)*. Increase in the LBW was 44.9% for group IV however it was 8.6%, 11.4% and 15.1% for groups I, II and III, respectively. In control groups LBW was increased 6.1% and 5.3% *(Table 2)*.

Cell Count and EAC Volume

Volume of EAC cells and viable cell count were found to be significantly decreased in AK27 treated group when compared to EAC control group ($P<0.01$). No viable or non-viable CEA cells or abdominal fluid was recovered from groups I, V and VI however the highest viable cell

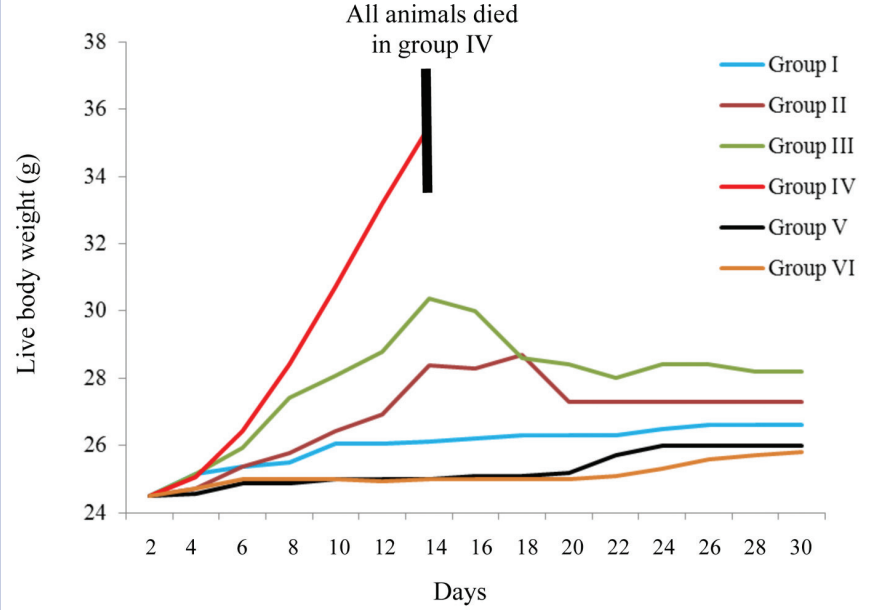

Fig 1. Representation of mean life span and body weight of normal and EAC bearing mice. Note: Animals in cancer control group (IV) were died at the 12th day of the experiment

Table 1. *Live body weight changes in normal and EAC bearing mice (first 12 days of the experiment)*

Groups	Days													
	0		2		4		6		8		10		12	
	Live body weight (g)													
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
I	24.50	0.68	25.14	0.53	25.36^{bc}	0.63	25.50^{cd}	0.65	26.07^{c}	0.47	26.07^{bc}	0.47	26.13^{d}	0.35
II	24.50	0.51	24.71	0.83	25.36^{bc}	0.74	25.79^{c}	0.89	26.43^{c}	0.85	26.93^{b}	1.27	28.38^{c}	2.62
III	24.50	0.52	25.14	1,29	25.93^{ab}	1.21	$27,43^{b}$	1,55	28.07^{b}	1.94	28.79^{b}	2.08	30.38^{b}	2.62
IV	24.50	0.52	25.07	0,83	26.43^{a}	1.16	28.43^{a}	1.60	$30,71^{a}$	2.13	33.21^{a}	2.67	35.50^{a}	2.78
V	24.50	0.52	24.57	0.51	24.86^{c}	0.36	24.86^{d}	0.36	25.00^{d}	0.00	25.00^{c}	0.00	25.00^{d}	0.00
VI	24.50	0.51	24.71	0.47	25.00^{c}	0.00	25.00^{cd}	0.00	25.00^{d}	0.00	24.93^{c}	0.27	25.00^{d}	0.00
Total	24.50	0.56	24.89	0.81	25.49	0.95	26.17	1.66	26.88	2.35	27.49	3.23	28.40	4.16
p	NS		NS		<0.001		<0.001		<0.001		<0.001		<0.001	

$^{a,b\,c,d}$ *Different letters respresent significant differecences between groups ($P<0.05$)*

Table 2. *Initial, final, average and increase in live body weight of mice*

Groups	Initial Body Weight (g)	Final Body Weight (g)	Average Body Weight (g)	Increase in Body Weight (%)
I	24.5	26.6	25.9	8,6
II	24.5	27.3	26.7	11.4
III	24.5	28.2	27.7	15.1
IV	24.5	35.5	28.1	44.9
V	24.5	26.0	25.6	6.1
VI	24.5	25.8	25.2	5.3

Table 3. *Viable and non-viable cell count and volume of EAC in mice*

Groups	Viable Cell Count (cell x 10^7/mL)			Non-viable Cel Count (cell x 10^7/mL)			EAC Volume (mL)
	Lowest	Highest	Mean	Lowest	Highest	Mean	
I	-	-	-	-	-	-	-
II	8.1	29.5	15.5	0.5	2	1.4	6
III	21.5	30.2	25.5	0.3	0.9	0.6	8
IV	30.1	44.3	33.1	0.2	0.5	0.4	12
V	-	-	-	-	-	-	-
VI	-	-	-	-	-	-	-

In groups I, V and VI no ascites fluid was obtained

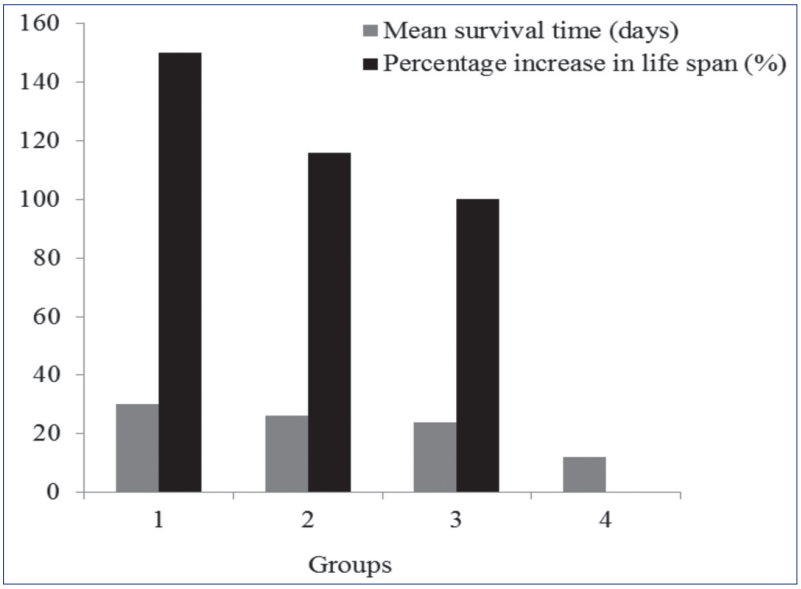

Fig 2. Chart showing mean survival time and percentage increase in life span in normal and tumor bearing mice

count (lowest 8.1x10^7/mL, highest 29.5x10^7/mL and mean 15.5x10^7/mL) and EAC volume (12 mL) was measured in group IV *(Table 3)*. Administration of AK27 before tumor challenge (group I) prevented the EAC development whereas simultaneous administration (group II) or after tumor initiation (group III), AK27 significantly reduced the number of viable CEA cells with respect to cancer control group (P<0.05) displaying negative effects on initiation and development of the tumor.

Longevity of Animals

All animals in group IV (cancer control) were died within 12 days as expected. No dead was observed in group I where AK27 was given 1 week before the EAC challenge. However in groups 2 and 3, two and three dead out of 8 animals were noted, respectively. In the cancer control group, the median survival time was 12 days whereas it significantly increased in AK27 treated groups to 30 days in group I, 26 days in group II and 24 days in group III with PILS of 150, 116 and 100, respectively *(Fig. 2)*.

Effect of AK27 on Normal Mice

After oral administration of AK27 compound at a dose of 2 g/day/mouse for 30 days, none of the mice exhibited either clinical side effects or abnormal behavioral responses (such as aggressiveness, inactiveness, loss of appetite, slow movement, dullness, dizziness, erection of hairs, and hypothermia; data not shown) in group V. Moreover repeated daily oral doses of 2 g/mouse for 30 days also did not have any effect on the live body weight of the mormal mice.

DISCUSSION

Nature was the main source of food, protection, clothing, transportation and remedies for humans since ancient times for survival in this planet [26]. This is also true today's modern world. Natural products are also considered as one of the major contributor that can be used for the design and development of potential chemotherapeutic agents [27]. A number of plant extracts have been used for major health problems for instance the management and combat of cancer and cancer related diseases in traditional medicine however, only a few of them have been scientifically explored [28].

The treatment of cancer is not promising in all cases. Several side effects on the patient's health is still significant

obstacle in modern medicine today. These effects may be short term or long term [29].

Using plant derived extracts remedy principles indicate cytotoxicity towards tumor cells [30] and antitumor activity in experimental animal models [31].

Pistachio consumption reduced cancer mortality and may protect prostate cancer, colorectal and colon cancers. *In vitro* studies and those studies carried out on animals suggested that the health properties of pistachios can be attributed partially to the content of the nut's dietary antioxidants activity [32]. Anti-cancer properties of *N. sativa* has been exclusively reviewed by Randhawa and Alghamdi [13]. The thymoquinone main constituent of the volatile oil of *n. sativa* seed enhanced the anti-cancer effect in rats and mice due to its antioxidant action [33] that interferes with DNA synthesis coupled with enhancement of detoxification processes and apoptosis and cell cycle [34]. Sumac is the generic name for genus Rhus that cover over 250 plant species and has a long historical background of use by indigenous people for medicinal and other uses including antimutagenic and antitumorigenic activities [35]. Anti-tumorigenic activities can be attributed to its promotion of cell cycle arrest [14]. Pollen possesses a wide range of primary and secondary metabolites that exhibit various properties and bioactivities i.e. anticarcinogenic. This effect is associated with the development by cytotoxic activity [19]. Elsewhere, the efficacy of pomegranate was evaluated and concluded that it has a very high antioxidant activity associated with anti-proliferative, anti-invasive and pro-apoptotic entities in various cancer cell lines and animal models [36] for example, pomegranate significantly suppresses TNFα-induced COX-2 protein expression and NF-κB binding suggesting anti-proliferative activity [37].

In our study the combination of aforementioned plants was formulated and successfully used for the prevention and treatment of CEA in the mouse model. The rationale behind combination approach may be explained that many tumors arise from a single malignant cell, by the time they are clinically detectable (1 cm^3 or 10^9 cells), they contain a heterogeneous population of cells. When tumor mass contains 10^6 cells, inherent drug resistance may develop [38]. This genetic instability may further be associated with a tumor that initially responds to treatment but later relapses due to resistance clones grow predominantly. Sometimes single agent therapy is not curative therefore induction regime should contain multiple drugs [39] and combination protocols must maximize cell kill and maintain acceptable toxicities, broaden the range of efficacy against a heterogeneous tumor population and prevent or slow the development of resistant tumor cells [40].

EAC is highly aggressive and causing mortality within 12 days in mice. Nascimento et al.[41] reported that the longevity of EAC bearing mice were 12 days maximum similar to the current study in which all animals in EAC treated group were died within 12 days.

To observe antineoplastic effect of agents on the EAC model the live body weight (LBW) of animals are compared between groups [42]. Our study showed that LBW in cancer group increased dramatically until death however it was not the case in groups I and II indicating EAC prevention effect. In group III, increase in LBW was not significant at 30th day of the tumor challenge mimicking anticancer activity.

It was suggested that studies directed to longevity after cancer cell inoculation should be monitored at least 30 days since the distribution of death occurrence with a 50% longevity of 14.3 days in mice models [43]. We observed the animals up to 30 days.

The prolongation of lifespan of animals is highly reliable and valuable criteria for judging the anticancer drugs effectiveness in experimental investigations [44]. Enhancements of life span by 25% are more over that of the control can be considered as effective antitumor response of the drug in question [45]. MST and PILS in study groups were significantly longer than control group in our study. This observation displays that increased lifespan of tumor bearing mice in experimental groups further corroborates the antitumor potential of AK27 mixture.

To conclude, the results of our study showed enhanced antitumor activity of AK27 compounds on experimentally induced EAC in mice. AK27 mixture formulation appeared to be stable at room temperature, safe to use and easy to administer by means of oral gavage. No side effects of AK27 was observed at a dose of 2g per mouse in this study. However, the maximum tolerated dose should be determined and also this formulation requires further evaluation to identify the possible antineoplastic action/mechanism (synergism or contribution) of the combinatorial fashion. Eventually, future phase studies in human cases should be considered.

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Short Communication

Soluble Expression, Protein Purification and Quality Control of Recombinant Porcine Interferon-α

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Abstract

Herein, we reported an *Escherichia coli*-based expression and purification method of recombinant porcine interferon alpha (rPoIFN-α). PoIFN-α coding sequence was cloned into pMD18-T vector and then subcloned into pET-32a (+) vector using standard recombinant DNA techniques and the resulting plasmid was transformed into BL21(DE3) competent cells. After induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG), rPoIFN-α was purified from the supernatant of the bacteria lysate using a simple two-step chromatography process consisting of a Ni^{2+} affinity chromatography and a DEAE anion exchange chromatography. rPoIFN-α was purified to >95% homogeneity with a yield of 48 mg/L of culture. It has isoelectic point of 6.09 and bacterial endotoxin was less than 1 EU/mg. N-terminal amino acid sequence and the peptide map digested by trypsin provided additional evidence for the authenticity of rPoIFN-α. The biological activity of rPoIFN-α was 1.1×10^6 IU/mL in HEp-2/ Vesicular Stomatitis Virus (VSV) titration system and its specific activity reached to 1.0×10^6 IU/mg. In conclusion, we obtained high-level expression of a soluble form of bioactive rPoIFN-α by using pET-32a (+) prokaryotic expression system.

Keywords: *Soluble expression, Protein purification, Quality control, Porcine interferon-α, Vesicular Stomatitis Virus (VSV)*

Rekombinant Domuz İnterferon-α'nın Çözünür Ekspresyonu, Protein Saflaştırması ve Kalite Kontrolü

Özet

Bu sunuda rekombinant domuz interferon alfa (rPoIFN-α)'nın *Escherichia coli*-temelli ekspresyonu ve saflaştırma metodu rapor edilmiştir. PoIFN-α kodlayan sekansı pMD18-T vektör içine klonlandı ve sonrasında standart rekombinant DNA teknikleri kullanılarak pET-32a (+) vektör içine subklonlandı ve elde edilen plazmid BL21(DE3) kompetan hücreler içine nakledildi. İzopropil-β-D-1-tiogalaktopiyranosid (IPTG) ile uyarmanın ardından rPoIFN-α, bakteri lizatının süpernatantından basit iki basamaklı kromatografi işlemi (Ni^{2+} affinite kromatografi ve DEAE anyon değişim kromatografi) kullanılarak saflaştırıldı. rPoIFN-α 48 mg/L kültür oluşumu ve >95% homojenite ile saflaştırıldı. Ürün 6.09 izoelektrik puanına sahip olup bakteriyal endotoksin 1 EU/mg'dan daha azdý. N-ucu amino asit sekansý ve tripsin ile oluþturulan peptit haritasý rPoIFN-α'nın özgünlüğü hakkında ilave kanýt saðladý. rPoIFN-α'nın biyolojik aktivitesi HEp-2/ Vesicular Stomatitis Virus (VSV) titrasyon sisteminde 1.1×10^6 IU/mL olarak tespit edilirken spesifik aktivitesi 1.0×10^6 IU/mg'a ulaştı. Sonuç olarak, pET-32a (+) prokaryotik ekspresyon sistemi kullanılarak biyoaktif rPoIFN-α'nın çözünür formunun yüksek derecede ekspresyonu sağlandı.

Anahtar sözcükler: *Çözünür ekspresyon, Protein saflaştırma, Kalite Kontrol, Domuz interferon-α, Vesicular Stomatitis Virus (VSV)*

INTRODUCTION

Among type I Interferons (IFNs), IFN-α plays important roles in inhibition of viral replication [1]. Previously, recombinant IFN-α has been successfully expressed in prokaryotes, eukaryotes and baculovirus [2-4]. However, the function of the *E. coli* expressed products was constrained by protein misfolding [3]. The protein expressed in *Pichia*

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was readily degradable. The baculovirus expression system does not sustain continuous high level expression.

In the present study, we represented the expression, purification, and quality control scheme for producing bioactive rPoIFN-α in large scale. It will facilitate the biological research and clinical application of porcine IFN-α.

MATERIAL and METHODS

Bacterial Strains, Reagents and Cell Lines

Molecular biology reagents were purchased from TaKaRa Biotech (TaKaRa, Dalian, China). The Ni^{2+} His-bind resin and DEAE -Sepharose Cl 6B column were obtained from GE Healthcare (Piscataway, NJ, USA). The mouse anti-PoIFN-α monoclonal antibody was purchased from Abcam (ab11408, Abcam, Cambridge, UK). The pET-32a (+) vector, *E. coli* DH5α, *E. coli* BL21 (DE3), and HEp-2 cell line were preserved in our laboratory.

Porcine IFN-α cDNA Cloning

Total RNA was extracted from peripheral white blood cells of a 6-month-old Bamei pig and was then reverse transcribed to cDNA. The primer sequences for RT-PCR of PoIFN-α (NCBI accessing number AY345969) were 5′-GGAATTCATGTGTGACCTGCCTCAG-3′ (forward) and 5′-CTCGAGTCACTCCTTCTTCCTGAGT-3′ (reverse) which included *EcoR*I and *Xho*I sites (underlined). The amplification length was 501 bp, and It did not include the signal peptide sequence. The RT-PCR product was cloned into pMD-18T vector and the resulted recombinant plasmid was further confirmed by PCR and DNA sequencing, The final product was named as pMD18T-PoIFN-α.

Expression Vector Construction

The inserted PoIFN-α gene in pMD18T-PoIFN-α was digested by *EcoR* I and *Xho* I, and was then ligated into the pET-32a (+) plasmid. The authenticity, orientation and reading frame of the recombinant plasmid pET-32a (+)-PoIFN-α was verified by DNA sequencing.

Expression of PoIFN-α Protein

The plasmid pET-32a (+)-PoIFN-α was transformed into competent *E. coli* BL21 (DE3). The bacteria were cultured in LB medium at 37°C to a density of OD_{600}=0.6. After 4 h induction by IPTG, the bacteria were collected and resuspended in lysis buffer for sonication The lysate was then centrifuged and the supernatant and pellet were collected separately [5].

Purification of rPoIFN-α Protein

The rPoIFN-α protein in the supernatant of cell lysate was purified with *Chelating Sepharose Fast Flow* Ni^{2+} *chromatography* (GE Healthcare, Piscataway, NJ, USA) *following the protocol from the manufacturer.* The chromatogram were shown in *Fig. 2A* and *Fig. 2B*.

Determination of Protein Concentration and Purity

The protein concentration was determined by the Bradford method [6]. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was used to determine the purity of the purified rPoIFN-α product. The integrity and specificity of the purified proteins were demonstrated by Western blot assay.

Mass Spectrometry Analysis

The purified protein was further analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). Mass analysis was performed using a Voyager DE-STR Biospectrometry™ Workstation (Applied Biosystems, Foster City, CA, USA).

Determination of rPoIFN-α Biological Activity

A cytopathic effect inhibition based IFN-α bioassay [7] was used to evaluate the ability of the recombinant protein to protect HEp-2 cells from *VSV* infection. Data were expressed as mean unit (U)/mL, where 1 unit of IFN-α activity was defined as the reciprocal of the dilution producing 50% inhibition of CPE. The titer of sample IFN, was determined by the Reed-Muench method as previously described [8].

Other Quality Control Measurement of rPoIFN-α

The peptide map, isoelectric point, endotoxin, ultraviolet spectroscopy, and N-terminal amino acid sequencing of rPoIFN-α were all determined according to the guidelines in Veterinary Pharmacopoeia of People's Republic of China (2010 edition) [9].

RESULTS

Soluble Expression of Recombinant Protein pET-32a (+)-PoIFN-α

The rPoIFN-α protein was over expressed as shown by a dominant band of 35.0 kDa in Coomassie blue stained PAGE gel *(Fig.1A)*. Besides, the over-expressed protein in the *E. coli* culture was found majorly in the supernatant, not in the pellet *(Fig.1B)*. By SDS-PAGE analysis, the expressed recombinant protein contituted to 32% of the total cellular protein, or 48 mg/L in *E. coli* culture.

Purification of pET-32a (+)-PoIFN-α Protein

In the supernatant of cell lysate, it was shown a single protein peak by Ni^{2+} affinity chromatography *(Fig. 2A)* and by DEAE anion exchange chromatography *(Fig. 2B)*. The result of purification by HPLC showed that there was a dominant protein peak with purity of 95.5% *(Fig.*

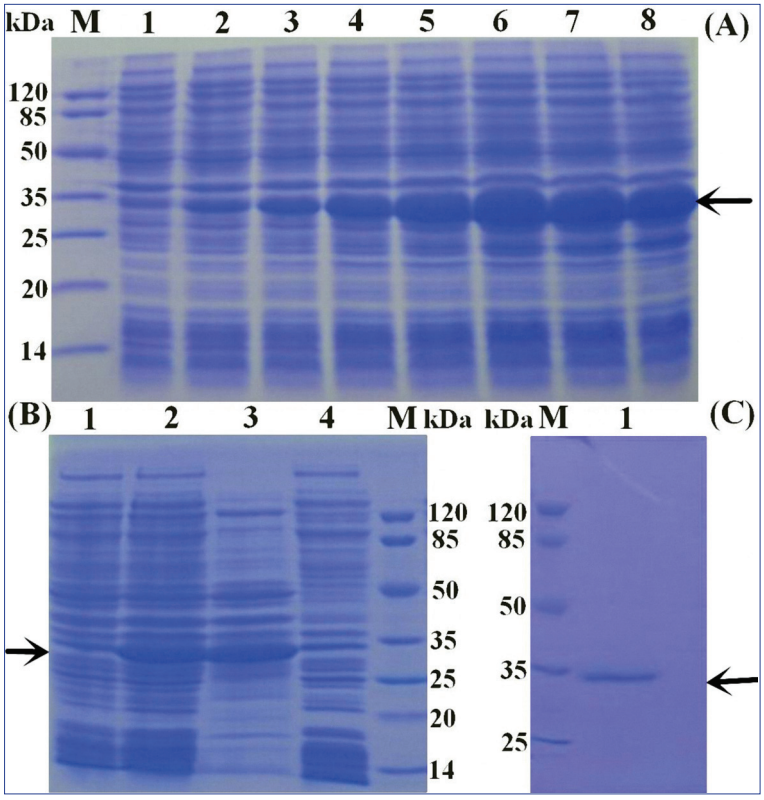

Fig 1. Analysis of rPoIFN-α protein by 12% SDS-PAGE

A: Lane M: molecular weight marker. Lane 1-8. Total cellular proteins from *E. coli* BL21(DE3) at 0h, 1h, 2h, 3h, 4h, 6h, 8h, 10h after IPTG induction, **B:** Lane 1. Total proteins before induction. Lane 2. Total proteins after induction. Lane 3 Supernatant of bacterial lysate after ultrasonication. Lane 4. Pellet of bacterial lysate after ultrasonication**.** Lane M: molecular weight marker, **C:** Lane M. Molecular weight marker. Lane 1. Purified rPoIFN-α after Ni^{2+} affinity chromatography and DEAE anion exchange chromatography

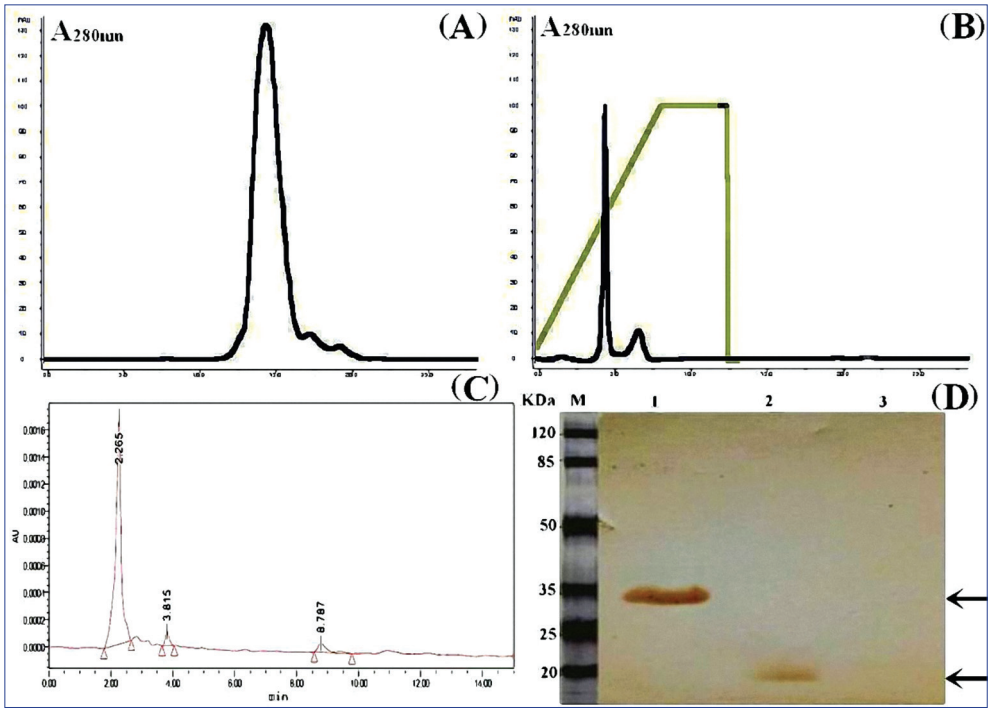

Fig 2. Protein characterization by affinity chromatography, anion exchange chromatography, HPLC and Western blot

A: Ni^{2+} affinity chromatography with absorbance at 280nm, **B:** DEAE anion exchange chromatography with absorbance at 280 nm, **C:** HPLC examination of purified proteins. The peak is the target protein which was detected at a wavelength of 280 nm, **D:** Western Blot analysis of purified proteins. Lane M. Molecular weight marker. Lane 1. Purified rPoIFN-α protein without enterokinase digestion. Lane 2. Purified rPoIFN-α with enterokinase digestion. Lane 3. Total cellular proteins before induction

2C). Western blot analysis with anti-PoIFN-α monoclonal antibody showed a non-enterokinase digested protein product at 35 kDa *(Fig. 2D)* and a enterokinase digested PoIFN-α protein at 19.3 kDa *(Fig. 2D)*, consistent with that in SDS-PAGE gel *(Fig. 1C)*.

The purification chart of rPoIFN-α from 300 mL of bacterial culture showed that the recombinant rPoIFN-α was purified to 4.9 fold by the two-step purification procedure and its specific activity reached to 1.0×10^6 IU/mg *(Table 1-A)*.

Bioactivity of Purified rPoIFN-α

The results showed that HEp-2 cells pretreated with 1 U of purified rPoIFN inhibited 50% of *VSV* infection

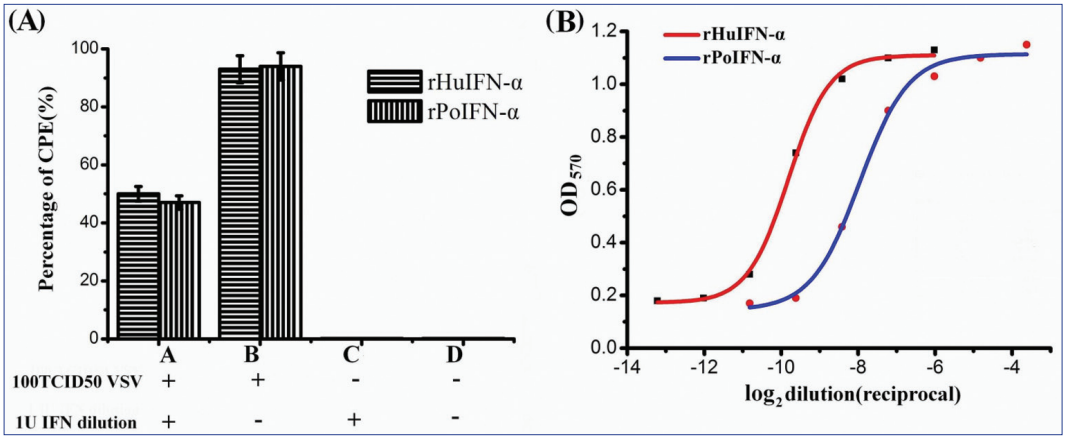

Fig 3. Antiviral activity of the rPoIFN-α in HEp-2/*VSV* titration system

A: 50% of CPE inhibition by 1 unit of rPoIFN-α and 1 unit of rHuIFN-α and the control groups for the titration of biological activity of IFN, **A:** about 50% CPE was observed in *VSV* infected cells pre-incubated with 1 unit of IFN-α, **B:** about 90% CPE was observed in *VSV* infected cells without IFN-α treatment, **C:** No CPE was observed in the cells pre-incubated with 1 unit of IFN-α without *VSV* infection, **D:** No CPE was observed in the cells which was treated with neither *VSV* infection nor IFN-α addition. **B:** The dose-response curve of interferon in HEp-2/*VSV* system. The figure shows that the titre of rHuIFN-α is slightly higher than that of rPoIFN-α in human cells

Table 1-A. *Purification chart of rPoIFN-α from 300 mL of bacterial culture**

Purification Step	Total Protein (mg)	Total Activity (IU)	Specific Activity (IU/mg)	Fold of Purification
Before purification (Cell Lysate)	77.9	1.6×10^7	2.0×10^5	1.0
After two-step purification	14.5	1.4×10^7	1.0×10^6	4.9

** Results were representative of three independent experiments*

Table 1-B. *Quality control of the bulk of rPoIFN-α*

Category	Method	Specification	Reference
Specific activity	HEp-2/*VSV*	≥1.0×10^6 IU/mg	
Purity	SDS-PAGE and HPLC	≥95.0%	Ref. [9], Appendix 36,41
Bacterial endotoxin	LAL(Limulus Amebocyte Lysate)	<1 EU/mg	Ref. [9], Appendix 130
Isoelectic point	Isoelectrofocusing	6.09(within 4.5~6.5)	Ref. [9], Appendix 41
UV maximum	UV scan	(278±3) nm	Ref. [9], Appendix 26
Peptide map	Tryptic digestion	Conformed to reference	Ref. [9], Appendix 107
N-terminal amino acid sequence	Edman degradation	CDLPQTHSLAHTRAL	Ref. [9], Appendix 32

(Fig. 3A). The antiviral activity of the final rPoIFN-α protein was determined as 1.1×10^6 IU/ml by the bio-activity assay. The inhibitory activity of rPoIFN-α on *VSV* replication in culture was dose dependent. The dose-response curve of interferon in HEp-2/*VSV* system was shown in *Fig. 3B*.

Study on Quality Control of rPoIFN-α

The primary structure of purified rPoIFN-α was confirmed by N-terminal sequencing and Mass Spectrometry analysis *(Table 1-B)*. Also, the recombinant molecules appeared to be homogenous by reversed-phase HPLC analysis and gel filtration *(Fig. 2C)* with no signs of aggregation (data not shown). The results of rPoIFN-α analysis of quality control are summarized in *Table 1-B*.

DISCUSSION

In the production of recombinant protein in heterologous expression systems, solubility is a key issue. Soluble recombinant proteins are usually properly folded, functional and they are much easier to be purified than aggregated proteins obtained from inclusion bodies.

The pET is one of the most powerful systems yet developed for the expression of the recombinant proteins in *E. coli*. The pET32 series were fused with the 109 amino acid Trx•Tag™ thioredoxin protein which is a solubilization tag that assists in the proper folding of the expressed peptides and keeps them from precipitating. This vector also contains cleavable His•Tag® and S•Tag™ sequences for detection and purification. Through the use of combination

of pET-32a (+) vector and BL21(DE3) host cell, the desired expression product can comprise more than 30% of the total cell proteins in a few hours after induction [5].

In summary, the present study demonstrated that a functional porcine IFN-α protein was expressed in *E. coli* in a soluble form. The recombinant protein was readily purified by a two-step chromatographic procedure. Its authenticity and bioactivity were verified by multiple tests of quality control. This protein could be further expected for mass production and clinical applications of rPoIFN-α.

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Short Communication

Pathological Examination of Deep Pectoral Myopathy in House Reared Broilers

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Abstract

Deep pectoral myopathy (DPM) is a disease characterized by focal necrosis, hemorrhages, and green discoloration in the pectoral muscle of broilers and turkeys. The lesions of the affected muscles are usually detected during dissection after slaughter. DPM causes significant economic losses in the poultry meat industry. The purpose of this study was to investigate the gross and microscopic findings in a house-reared broiler flock with DPM. In this study, the pathological findings of 12 house reared 100-120-day-old broilers with DPM were examined. All birds were clinically healthy but hemorrhages and green discoloration were detected on the pectoral muscle mass during dissection. Samples were collected from the lesioned muscles for a histopathological examination, which revealed necrosis, hyalinization, and hemorrhage. Inflammatory cell infiltration and atrophy of breast muscles was present in some cases. DPM was diagnosed based on gross characteristics and microscopic findings.

Keywords: *Deep Pectoral Myopathy (DPM), Pathology, Broiler*

Köy Koşullarında Beslenen Broylerlerde Saptanan Derin Pektoral Myopatide Patolojik İncelemeler

Özet

Derin Pektoral Myopati (DPM), broyler ve hindilerin pektoral kaslarında fokal nekroz, kanamalar ve yeşil renk değişikliği ile karakterize bir hastalıktır. Etkilenen kasların lezyonları kesimden sonra ve et parçalama sırasında fark edilir. DPM etlik kanatlı endüstrisinde önemli ekonomik kayıplara neden olabilen bir hastalıktır. Bu çalışmanın amacı köy koşullarında yetiştirilen bir broyler sürüsünde saptanan DPM olgusunun makro ve mikroskobik bulgularını incelemektir. Bu çalışmada, 12 adet köy koşullarında yetiştirilmiş, 100-120 günlük broylerlerde saptanan DPM'de patolojik bulgular incelenmiştir. Bütün tavuklar klinik olarak sağlıklı görünümde iken kesim sonrası et parçalama işlemi sırasında pektoral kaslarda kanama ve yeşil renk değişikliği fark edildiği bildirildi. Histopatolojik inceleme için lezyonlu kaslardan örnekler alındı. Mikroskobik incelemede pektoral kaslarda nekroz, hiyalinizasyon ve kanamalar izlendi. Bazı olgularda yangısal hücre infiltrasyonları ve göğüs kaslarında atrofi mevcuttu. Hastalığa karakteristik makro ve mikroskobik bulgulara göre DPM tanısı kondu.

Anahtar sözcükler: *Derin Pektoral Myopati (DPM), Patoloji, Broyler*

INTRODUCTION

Deep pectoral myopathy (DPM) or Oregon green muscle disease is a hidden and degenerative condition characterized by focal necrosis of the pectoral muscle in poultry. The disease involves the wing elevating muscle known as the deep pectoral muscle or *M. supracoracoidus*; hence, it is referred to as degenerative myopathy of the supracoracoideus [1-3]. The lesions often affect the muscle symmetrically and vary in color from hemorrhaged to a green discoloration. However, these symptoms are rarely detectable until the affected muscles are dissected. The disease appears as a surprise before preparation because of the unpleasant aspect. DPM is not a contagious disease, and the symptoms are not detectable in living animals [1,2,4].

Two forms of DPM reported; one results from normal muscular activity (spontaneous DPM), as it can be reproduced by repeated contraction of the pectoral muscles through electrical stimulation, and the other form occurs

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by inducing birds to flap their wings (induced DPM) [2]. The only affected pectoral muscle is the supracoracoid muscle. DPM appears to be the consequence of oxygen associated with wing flapping [1,2,4]. Blood circulation increases significantly during contraction of the pectoral muscle to supply oxygen and nutrients to the muscle. Insufficient blood supply to the myoglobin can cause ischemia and DPM [2].

DPM has caused important economical loses in the broiler industry [5]. The disease was first described in 1968 in turkeys, in 1975 in broiler breeder hens, and in 1980 in young broiler chickens [4,6,7]. DPM has been reported in North America and Europe [1-7]. There is no report about DPM in Turkey, and this is the first study about DPM in Turkey. The aim of this study was to examine naturally occurring DPM in broilers using pathological methods.

MATERIAL and METHODS

In this study, 12 broilers (nine males and three females) with DPM were used. The chickens originated from a flock consisting of 100 animals that a household reared and that were slaughtered by the owner. The broiler chickens were 100-120 days old and weighed 3.5-4.0 kg. The flock has been comprised of >60 birds but most had been previously slaughtered. These 12 birds were the last remaining animals in the flock and were slaughtered together. No DPM had been diagnosed previously in any slaughtered bird from the flock. Lesions of different severities were observed from hemorrhage to green discoloration during the necropsy.

The lesioned pectoral muscle samples were fixed in 10% buffered formalin for the histopathological examination. After routine processing of fixed samples, they were embedded in paraffin, sectioned to 5 µm with a Leica RM 2155 rotary microtome (Leica Microsystems, Wezlar, Germany), stained with hematoxylin-eosin (HE) and Masson's trichrome methods and examined microscopically.

RESULTS

All chickens were >3 months old and heavier than 3.5 kg. They were freely reared in the garden of the owner' home. No clinical sings were observed before slaughter. The owner stated that he also had laying poultry and slaughtered them occasionally but there were no DPM case in the layers of the same age or broilers slaughtered previously.

The carcasses of the broilers were normal on a gross examination. DPM was detected during dissection of the pectoral muscles. The lesions were localized to the middle and deep layers of the pectoral muscle. All affected pectoral muscles were greenish, pale, and swollen and covered by a fibrinous, sometimes hemorrhagic membrane *(Fig. 1)*. The cut surface of the green, dry, friable necrotic and hemorrhagic tissue was evident. The lesions were generally focal but some were diffuse in the pectoral muscle. Gross bilateral lesions were more common than unilateral lesions in the present study. Lesion severity was related to body weight, and lesions were more diffuse and severe in heavier chickens.

Fig 1. Gross appearance of the breast muscle from broilers with deep pectoral myopathy (DPM); pale yellow-green discoloration and necrosis *(arrows)* and hemorrhage *(arrow head)*

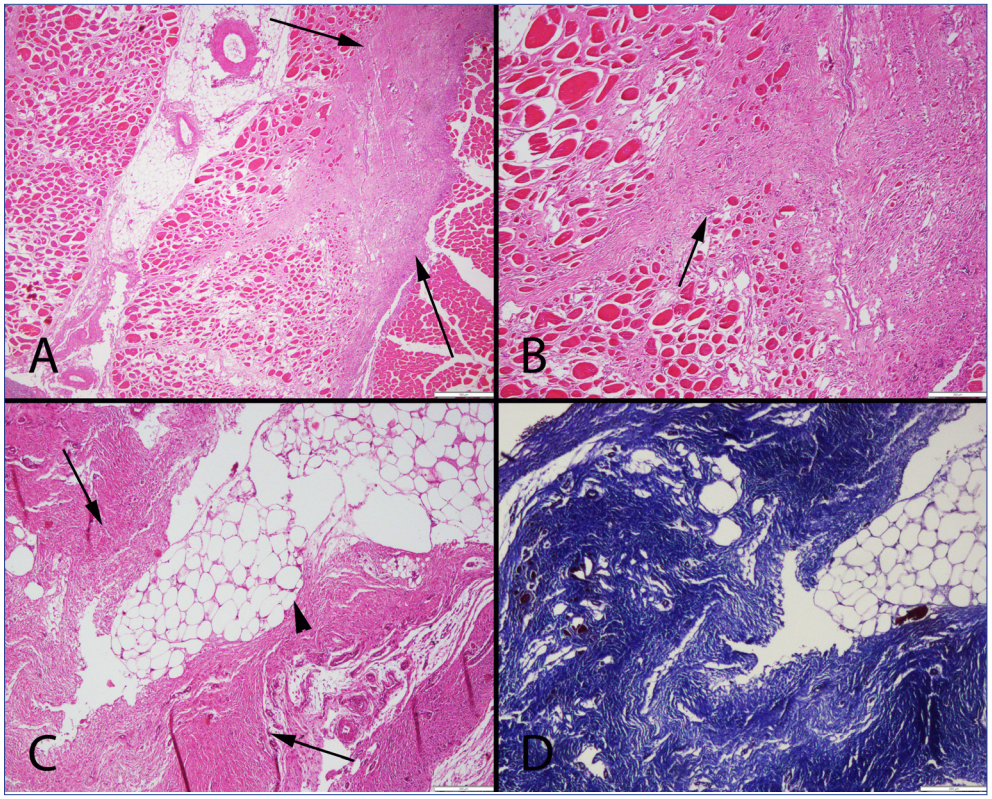

Fig 2. Microscopic appearance of the muscle with lesion, (A) numerous degenerated muscle cells and connective tissue *(arrows)*, (HE), Bar = 500 µm, (B) higher magnification of the lesioned area of the pectoral muscle, HE, Bar = 200 µm, (C) old and chronic lesion. Muscle fibers were replaced by fibro-adipose tissues, HE, Bar = 200 µm. (D) Fibrous tissue forming in the pectoral muscle, Masson's trichrome, Bar = 200 µm

The microscopic investigation revealed an acute inflammation characterized by heterophillic leucocyte and macrophage infiltration, edema, hyperemia, and hemorrhages in the affected area, particularly in early DPM lesions. Degenerative or necrotic changes and edema were commonly observed in these cases. Large necrotic areas, swollen, hyalinized and necrotic muscle fibers with hemorrhagic zones were observed in the chronic cases. Infiltration of inflammatory cells, mainly macrophages and lymphocytes, was seen around the lesions. In some cases, the breast muscles were atrophied *(Fig. 2)*. In addition, hyalinized areas and newly formed fibro-adipose tissue were observed in some cases.

The owner stated that it was impossible to detect DPM when the carcasses were inspected and that it appeared during muscle dissection. The unpleasant appearance of the meat was usually noticed during cooking in the kitchen.

DISCUSSION

In the present study, DPM was diagnosed in a house-reared broiler flock. The birds were heavy because of their long life expectancy (100-120 days) and prevalence was significantly higher in birds >110 days. This finding was in agreement with previous studies suggesting that DPM is more prevalent in heavy birds [1,2,8,9]. In addition, the typical chronic lesions were observed because of the older ages and heavier body weights of the chickens in the present study.

DPM is becoming more common problem in the broiler industry worldwide, especially in broilers grown to heavier body weights. Numerous studies have reported that rapid body weight gain in broilers may result in various forms of degenerative chronic diseases of muscle such as DPM [1,2,10,11]. In this study, broilers of numerous chicken breeds were reared together with this flock, and DPM was only observed in the broilers. This result supports the idea of a possible predisposition in broiler chickens.

According to some researchers [1,2], the condition appears to be more common in males than females. However, there is some disagreement on this matter [12]. The problem is also seen more frequently in free-range broilers [13]. In this study we observed a higher prevalence in male house-reared broilers.

The lesions can occur in acute or chronic forms. The supracoracoid muscle appears pale and swollen and is covered by a fibrinous or hemorrhagic area in the acute lesions. The necrotic tissue may be white to green color. The lesions are generally limited to the deep layer of the supracoracoid muscle. In chronic cases, the muscle is necrotic and green in color. The cut surface of a lesioned muscle is dry and friable [2,3]. All lesions in the present study were chronic and characteristic because of the older and heavier birds.

The histology of the affected muscle typically had large zones of swollen and necrotic muscle fibers. An inflammatory reaction, particularly macrophage infiltration, was seen in early stages of the lesions. Fibro-adipose tissue formations

were characteristic of older or chronic lesions and the affected parts of the deep pectoral muscle were replaced by adipose tissue. The breast mass was usually atrophied and thin. The main cause of these histopathological lesions is associated with circulatory failure [2]. A histopathological examination of the chickens in this study showed excessive fibrous and adipose tissue indicating the chronic form of the DMP.

The main cause of the disease is related to insufficient blood flow, oxygen, and nutrients for muscle contraction [13]. This finding also supports this hypothesis because the birds were heavy and moved freely. DPM is not a contagious disease and no bacteria can be isolated from affected muscle [8,14].

Classical knowledge indicates that the major cause of DPM is increased bird activity that results in wing flapping, increased body weight, and stress factors, such as excessive noise, that disturbs the birds. Owners should consider these factors to prevent the disease.

The present study demonstrated that DPM can cause meat loss in house-reared broiler chickens, particularly in heavier birds. The main lesions were located deep in the pectoral muscles, which generally had chronic lesions. Postmortem and microscopic examinations are needed to confirm the disease because no clinical findings or guidelines exist for this disease. This study also indicates that DPM may be a problem in the Turkish broiler industry.

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Case Report

Evaluation of Computed Tomography, Clinical and Surgical Findings of Two Cats with Paranasal Tumours

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Abstract

Feline paranasal tumours are relatively rare and require sufficient imaging for both diagnostic success and planning the treatment of cases. Radiography is inferior to computed tomography for diagnostic purposes near sinonasal cavities and tissues surrounding them, it is widely accepted that CT is the best imaging technique for tumours of this area. With the advent of 3D rendering software, it became possible to show borders of a lesion in a different manner. Such images are simpler to assess even to those with little to no experience with conventional CT. The preoperative CT images of two cats with paranasal area tumours were rendered in this study in hopes of providing better orientation to the surgeon. While the soft tissue details of 3D images were not adequate, they were useful in seeing outline of the tumours and determining the extent of bony destruction. The intraoperative findings of both cats confirmed that the 3D CT findings were useful in orientation and determination of bony defects, as the images were instrumental in determining the limits of the skull. However, the rendered images were inadequate to provide detail on soft tissue borders so conventional CT images were relied on to determine deep soft tissue borders in both cases.

Keywords: *Computed tomography, Paranasal tumour, Cat*

Paranazal Tümörü Olan İki Kedide Bilgisayarlı Tomografi, Klinik ve Cerrahi Bulguların Değerlendirilmesi

Özet

Kedilerde paranazal tümörler nispeten nadir gözlenir ve başarılı tanı ile sağaltımın planlanması için yeterli görüntüleme gerektirirler. Sinonazal kavitelerin ve bunları çevreleyen yumuşak dokuların görüntülenmesi için radyografi bilgisayarlı tomografiye (BT) göre daha başarısızdır, bu bölgenin tümörlerinin görüntülenmesi için en iyi yöntemin BT olduğu kabul edilmektedir. Üç boyutlu biçimlendirme yazılımlarının gelişimiyle lezyon sınırlarını farklı biçimde göstermek mümkün olmuştur. Bu görüntüler, uygulayıcının normal BT görüntülerini inceleme konusunda deneyimi kısıtlı olsa bile daha kolay değerlendirilebilmektedir. Bu çalışmada, operatöre daha iyi orientasyon sağlama amacıyla paranazal bölgede tümörü olan iki kedinin preoperatif BT görüntüleri biçimlendirilmiştir. Her iki kedinin intraoperatif bulguları, üç boyutlu BT ile elde edilen görüntülerin, kafatası sınırlarının belirlenmesini sağladığı için operasyona hazırlık ve kemik dokudaki defektlerin bulunması konusunda yararlı olduğunu gösterdi. Ancak, her iki olguda da, biçimlendirilmiş görüntüler, yumuşak doku sınırlarını belirlerken detay yetersiz kaldığı için, derin yumuşak doku sınırlarını değerlendirmek için, normal BT kesitleri kullanıldı.

Anahtar sözcükler: *Bilgisayarlı tomografi, Paranazal tümör, Kedi*

INTRODUCTION

Nasal and paranasal tumours are rarely seen in cats and the previously recorded incidences of these tumours are between 1% to 8.4% [1]. The most commonly encountered type of paranasal tumours in the cat are lymphomas [2]. It is a well known fact that most feline nasal and paranasal tumours are malignant [1]. Most aggressive tumours originating in the nasal cavity tend to expand and infiltrate into local tissues, obliterating nearby bone and soft tissue structures [3].

Radiography is not recommended for the diagnosis of paranasal tumors due to superimposition [4]. Computed tomography images are considered superior to radiography because they reduce the risk of misdiagnosis caused by

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superimposition of neighbouring structures and provide better anatomical detail [5,6]. Computed tomography can also allow detection of bone lysis and tumour extension to nearby tissues [5]. Those findings were previously used to differentiate tumours from nasal inflammation in humans and dogs [5]. Bone lysis seen as a CT finding is especially suggestive of neoplasia. Because of these properties CT has been used to determine the surgical approach to nasal and paranasal tumours [4].

This paper details the clinical, CT and surgical findings of 2 cats with paranasal sinus tumours.

CASE HISTORY

Both patients underwent complete physical and clinical examinations with bloodwork including complete hemogram and serum biochemistry. Craniocaudal, laterolateral and dorsoventral x-rays of the head and two way thorax x-rays of the thorax were taken. Rhinoscopy could not be attempted at the 1st case because the nostril was constricted and failed to yield clear images in the 2nd case.

Spiral CT scans (Xpress/GX model TSX 002a Toshiba, Toshigi-Ken) of the cats were taken in sternal recumbency under general anaesthesia. Image acquisition parameters were 120 kV, 250 mAs, with a slice thickness of 1mm. Window level was set for 200 HU and window width was set at 900 HU for the assessment both patients. A 64 bit image rendering software (Osirix®, Pixmeo SARL, Bernex - Switzerland) was used to convert conventional CT images.

Both cats were anaesthesized using the same protocol for surgery. The anaesthesia was induced with propofol (6 mg/kg, IV) before intubation and maintained with isoflurane in oxygen. Both cats received one dose of Cefazolin (20 mg/kg, IV) before surgery. Meloxicam was used for pain management (0.2 mg/kg, SC). Ringer's lactate solution was given during the operation (20 mL/kg/h). Amoxycillin clavulonate was administered for 7 days to the 1st case (20 mg/kg, PO, BID).

Case 1: A 3 year old, male, mix breed cat was presented to our clinic with an enlargement of the right eye and a small mass encompassing the right nasal and frontal area. The nasal tumour, which was approximately 3 cm in diameter grew to its final size in about 3 months' time *(Fig. 1)*.

The right submandibular lymph node was extremely large on palpation. Fine needle aspiration biopsies were collected from the tumour and submandibular lymph nodes and sent for pathological evaluation. Cytology results indicated lymphoma. The CT findings of the patient revealed a mass originating from just over the right nostril extending up to the frontal bone of the skull, with a mediocre invasion of the right orbit, pushing the right eye cranially and laterally. No bone lysis or septal deviation was seen in the nasal bone or skull, and there was some soft tissue contrast inside the right frontal sinus *(Fig. 2)*.

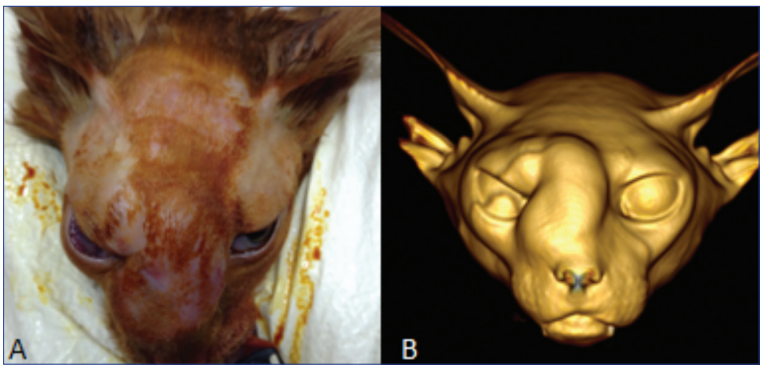

Fig 1. The preoperative (A) and 3D rendered CT image (B) of the 1st case

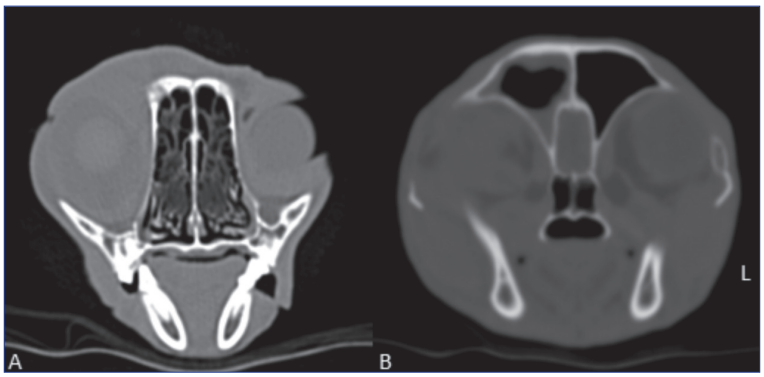

Fig 2. The mass over the right orbit and nasal bone with no invasion to the nasal cavity (A) and increase in soft tissue density on in the right frontal sinus (B)

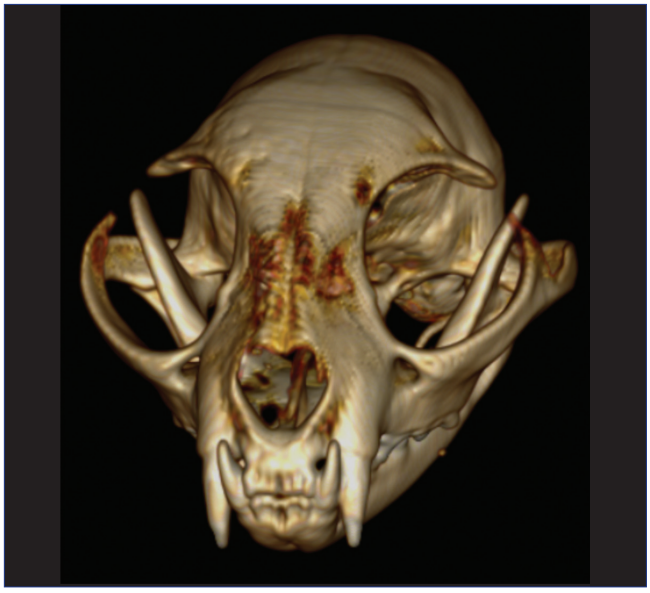

Fig 3. No bone lysis or deformation is seen on the 3D rendered image of the 1st case

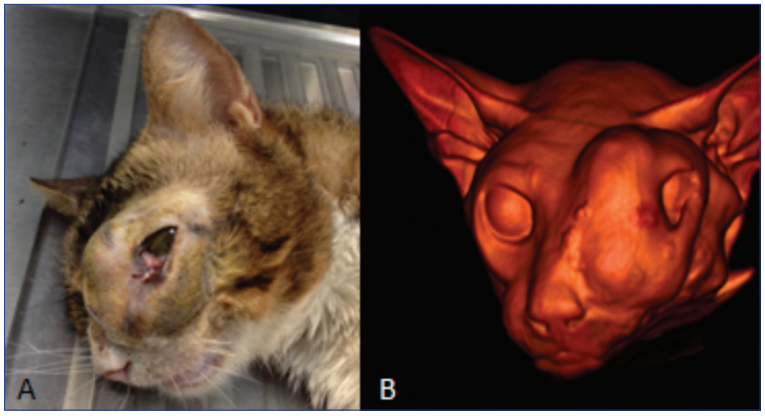

Fig 4. The clinical (A) and 3D rendered CT image (B) of the 2nd case

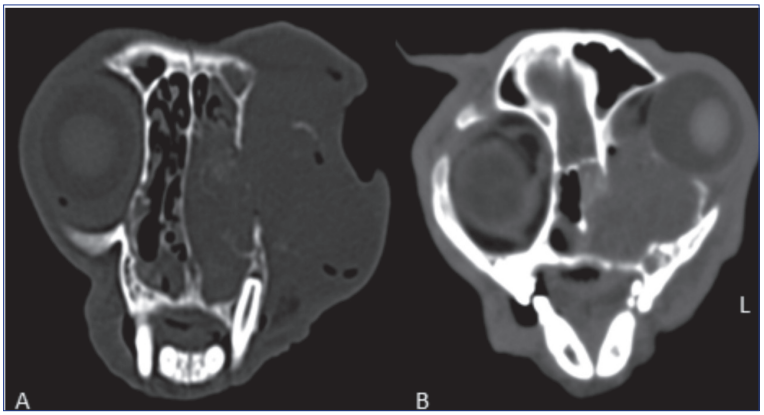

Fig 5. The mass completely filling and extending outward from the maxillar area, obliterating the bone and invading the nasal area completely and slightly deviating the nasal septum (A). The orbital part of the tumour is pushing the eye laterally, invading the nasal cavity and frontal sinus (B)

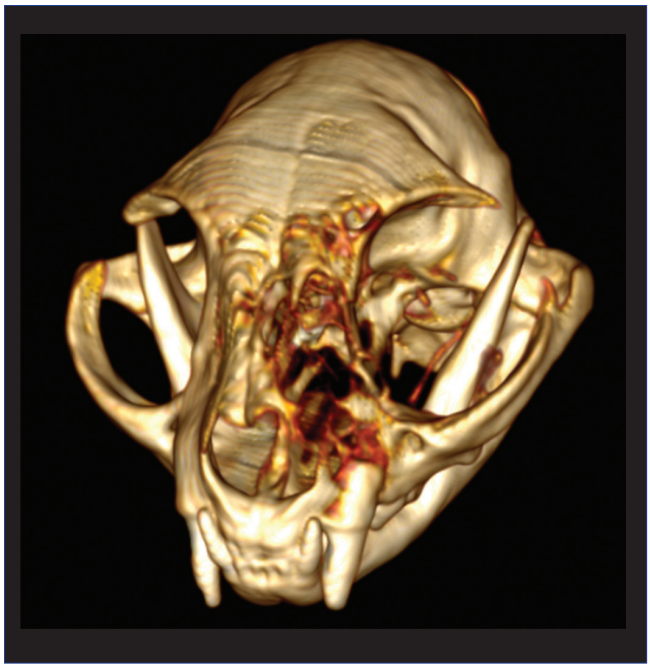

Fig 6. Extensive bone lysis on left nasal bone and orbital lamina

The patient's 3D CT rendering showed no lysis to the bones of the skull *(Fig. 3)*. The patient owner did not accept chemotherapy due to probable side effects so the cat was taken into surgery to improve his quality of life. The tumour had mild adhesions to nearby soft tissues and bones and could be easily excised. The exposed frontal and orbital regions of the skull were intact as was seen in 3D rendered images. A small sinusotomy defect was made to irrigate the right frontal sinus. The soft tissue contrast inside the sinus was confirmed to be mucous content during the operation. Pathology identified the mass as low grade lymphoblastic lymphoma. In the postoperative period the cat healed fine in 10 days and the stitches were removed. After five months, the tumour recurred in the same location and the cat was euthanized in accordance with the owner's wishes.

Case 2: A four year old, male, mix breed cat was presented to our clinic with a large tumoral mass on the left side of its head. The patient history was unclear as the cat was mostly free-roaming and the owner only saw it at the time of feedings. The cat had bloody-mucoid discharge from the left nostril. The left eye was enlarged and slightly exophthalmic and the conjunctive tissue surrounding it was oedematous and hyperaemic *(Fig. 4)*. The mass was solid and unmoving during palpation.

Fine needle aspiration and tru-cut biopsies were performed during short term propofol anaesthesia. Fine needle aspiration biopsies were obtained both from the mass and the closest (left) submandibular lymph node. Cytology results were inconclusive. Computed tomography images of the head were taken from the cat's head to determine the dimensions and invasion margins of the tumour. The cranial margin of the tumour originated from both nasal and maxillary regions, extending caudally to both the frontal area and the temporal boundary of the left eye. The tumour completely filled the left nasal cavity, deviating the nasal septum slightly to the right, the ventral wall of the frontal sinus was completely obliterated and the frontal sinus was mostly filled by the mass. Some fluid/soft tissue contrast was also seen inside the left sphenoid sinus. There was no lysis on the sinus wall neighbouring the brain. There was also some soft tissue contrast presence in the right frontal sinus, separate from the mass. The left eye deviated from the orbit, laterally and caudally, because of the mass, and there was bone lysis on the orbital lamina *(Fig. 5)*.

After 3D rendering was done to show the skull, the lysis of the bony structures could be clearly defined *(Fig. 6)*.

Surgery was chosen as the course of action in accordance with the patient owner's wishes after all possible

complications were explained. Amoxicillin Clavulonate was administered for 1 week before surgery, until the bloody discharge dissipated. The mass did not came off easily over the bone and orbit, necessitating aggressive resection. The void regions that could be seen in 3D rendered images were filled with tumour masses. In addition to successfully remove the tumour, a sinusotomy was performed in addition to removing some of the remaining bony coverage of the area and all of the exposed mass was excised. The left eye was also enuclateated. The tumour's margins were not very clear but all of the intra and periocular muscles were removed. The patient died 4 hours after the surgery due to respiratory collapse followed by cardiac failure. Histopathological examination identified the mass as rhabdomyosarcoma.

DISCUSSION

Two cases were presented to our hospital at a relatively advanced stage of their respective tumours so the clinical signs were rather obvious in differentiating the disease from rhinitis. Both had facial deformity, exophthalmus, epiphora, nasal discharge, and sneezing which are commonly known clinical symptoms of nasal tumours [7]. Paranasal tumours are more likely to be seen in older cats (8+ years), but reports of younger cases are present [1]. Both our patients were young adults, 3 and 4 years old respectively, which is common.

The recommended evaluation of paranasal tumour patients include bloodwork, serum biochemistry, urinalysis and orthogonal thorax and nasal radiographs [7]. Normal diagnostic procedure in nasal or paranasal tumour patients should also include rhinoscopy, biopsy, CT evaluation [4] and both cases in this report underwent these evaluations.

The most commonly encountered paranasal tumours in the cat are lymphomas, followed by squamous cell carcinomas [4,7]. Fine needle aspiration biopsies were collected from the closest lymph nodes of both cats in addition to other biopsies as suggested before [7]. When fine needle biopsy cytologies and tumour histopathology were evaluated in the 1st case, low grade lymphoblastic lymphoma was diagnosed. However the 2nd case was diagnosed with rhabdomyosarcoma, which originates from striated muscles [8]. This led us to believe the mass did not originate from the nasal cavity and invade the bulbar area but the other way round. Though retrobulbar tumours occur rarely in cats, some may cause bone lysis and invade sinonasal cavities [9].

The advantage of CT imaging over radiography is well known [4,6,10]. The addition of software that allows 3D rendering of images provide the clinician a better orientation in our opinion. In our opinion 3D CT images allow better visualisation of bony structures and make it easier to determine the margins of the operation. CT imaging provides "slices" of every possible location on the scanned locus, 3D software provides a better overview of the bony structures and simplifies explanation of lesions. In a recent study, the 3D CT views and prints were obtained and it was found to be successful in preoperative planning [11].

In our cases, the rendered images provided a better orientation in explaining and understanding the extent of deformation/obliteration on bones, mainly because there is little or no experience needed for reading 3D CT scans. In the cases reported here, the 3D rendered images were useful in showing the outside borders of lesions and determining the damage on bony structures. This helped estimate surgical approach beforehand. To determine the changes in deeper soft tissues we had to rely on cross-sectional CT images, 3D rendering could not provide a view of multiple layers of soft tissue. This may be because of poor rendering technique or limitations of the software used.

In conclusion, 3D rendering was a helpful tool to determine limits of invasive masses near sinonasal cavities in the aforementioned cases. But in our cases, only the outline of the head and skull provided useful images, rendering of soft tissues did not yield valuable information. Considering that, we think 3D rendering is a valuable technique when used in addition to conventional CT for imaging paranasal tumours.

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Case Report

Ovarian Tumour in a Bitch: Diagnosis, Surgery and Recovery

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Abstract

A 12 year old dog was submitted for a routine pregnancy examination. The information received from the owner was that the animal had been mated 45 days previously. During abdominal palpation, a hard, round mobile structure was detected. Extension of the tumour from the right ovary into the abdomen was determined by ultrasonography. Hemogram, blood biochemistry, estradiol and serum progesterone analyzes were performed before surgery. An ovariohysterectomy was performed and 1.6 L of ascites fluid aspirated. A tumour in the right ovary weighing 1.3 kg was detected. Histopathological examination revealed ovarian papillary cystadenoma. One month after the operation, the animal showed good general condition, however, five months after the operation, the state of health deteriorated. Euthanasia followed this, because the metastases were detected in the repeated laparotomy operation. As a result, Increased serum E2 and E2/P4 ratio (3.15), and sonographically detectable abdominal mass and ascites could be useful for the dectection of the ovarian tumour in bitch. In case of rapidly growing papillary adenomas, frequent post-operative controls should be recommendable.

Keywords: *Bitch, Ovarian tumour, Cystadenoma*

Bir Köpekte Ovaryum Tümörü: Teşhis, Cerrahi ve İyileşme

Özet

Oniki yaşında köpek gebelik kontrolü için getirildi. Alınan anamnezde köpeğin 45 gün önce çiftleştiği bilgisi verildi. Abdominal palpasyonda yuvarlak, sert kıvamda hareketli bir yapının olduğu hissedildi. Ultrasonografik muayenede sağ ovaryumla bağlantılı tümöral yapının varlığı saptandı. Cerrahi operasyon öncesi hemogram, kan biyokimya, estradiol ve serum progesteron analizleri yapıldı. Yapılan Ovariohysterektomi operasyonu ile abdomendeki asites aspire edildi (1.6 L). Sağ ovaryumdaki tümörün 1.3 kg ağırlığında olduğu tespit edildi. Histopatolojik kontroller sonucunda ovaryum papillar kist adenom tanısını konuldu. Ameliyattan bir ay sonra, hayvanın genel durumunun iyi olduğu, ancak ameliyattan beş ay sonra sağlık durumu kötüleştiği tespit edildi. Bu durum ardından tekrardan yapılan laparatomi operasyonunda metastazlar tespit edildiği için ötenazi uygulandı. Sonuç olarak, artmış serum E2 ve E2/P4 oranı (3.15) ile ultrasonografik olarak saptanabilen abdominal kitle ve asides varlığı ovaryum tümörünün tanısı bakımından önem taşımaktadır. Hızla büyüyen papiller adenomlar durumunda sık post-operatif kontroller yapılması önerilebilir.

Anahtar sözcükler: *Köpek, Ovaryum tümörü, Kist adenom*

INTRODUCTION

Ovarian tumours are rarely found in dogs. They constitute 0.5-1.2% of all tumours detected in dogs. Papillary adenoma and adenoma carcinoma constitute 40-50% of these phenomena. The most frequently occuring sex cord stromal tumours are granulosa cell tumours which comprise 50% of all ovarian neoplasms [1].

Clinical symptoms in dogs are: slow moving, lethargy and especially long-term enlargement of the abdomen [2]. They can cause symptoms like anoestrus, nymphomania, masculinization, hyperadrenocorticism, alopecia and occasionaly with mammary complex carcinoma but may also be asymptomatic [3,4].

In this case report, clinical, ultrasonographic, vaginoscopic, radiographic and laboratory findings along with post-operative and pathological results are presented. Additionally post-operative recovery and monitoring of

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a dog suffering from this type of tumour is described.

CASE HISTORY

Medical History and Clinical Findings

A 12 year old Labrador Retriever dog weighing 30 kg was brought to Near East University Animal Hospital with a history of abdominal distension and suspected pregnancy. In the medical history obtained from the owner, mating of the dog took place 45 days previously, soon afterwards, the dog calmed down, and her activity declined despite a normal appetite.

During clinical examination, abdominal extension was observed and a large structure with hard consistency was palpated on the right side of the abdomen. The mammary glands were not enlarged and no secretion was assessed. Body temperature was 38.6°C.

In the vagina, during vaginoscopical examination, hemorrhagic and petechial areas were seen. Furthermore, ulceration areas and local bleeding were observed towards the longitudinal folds of the vagina.

During ultrasonographic examination, the animal was found not to be pregnant, and the uterus showed physio-logical structure and dimension *(Fig. 1)*. In contrast, a structure with knotty-wavy-cauliflower like appearance, most probably related to the right ovary, filled the abdomen *(Fig. 2)*. Connection to other organs could not be excluded sonographically. The presence of anechogenic areas indicated fluid accumulation in the abdomen. A hyperechogenic structure and distinct boundaries of the intestine were determined free floating in the abdominal fluid.

No pathology was observed in 3-way chest radiography of the patient, however, peritoneal effusion and loss of details because of moderate accumulation of free fluid in the abdomen were assessed. Slight enlargement of

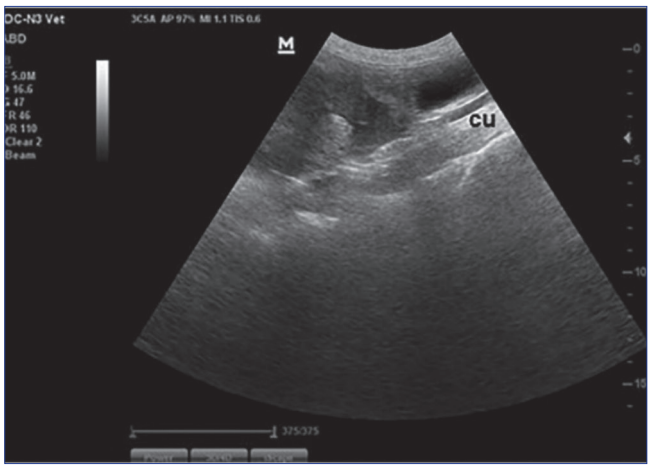

Fig 1. Wavy appearance of ovarian tumours in front of the cornu uteri (cu) and urinary bladder because of liquid accumulation

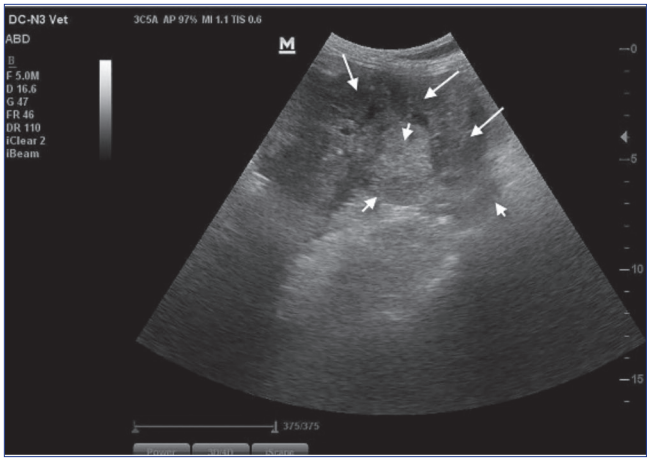

Fig 2. Round slightly wavy ovarian tumour in front of the urinary bladder and extended into the abdomen *(surrounded by arrows)*

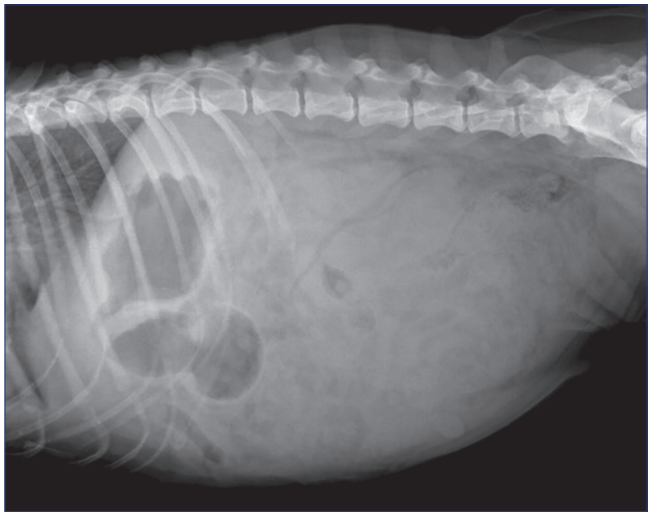

Fig 3. Radiography of the abdomen: Loss of details because of liquid accumulation

sternal lymph nodes were identified *(Fig. 3)*.

The haematological parameters were analyzed using an automatic analyzer (BC-2800Vet, Mindray, Shenzen, China), Serum biochemical analyses were measured by using commercial assay kits (Randox Laboratories Ltd., UK; Mindray Chemistry Reagents, Shenzen, China) and an automated blood chemistry analyzer (BS120, Mindray, Shenzen, China). Competitive ELISA test was used for serum progesterone (P4) and estradiol concentration measurement (Demeditec DE1651 Lot. 23K035, Kiel, Germany). Total blood analysis revealed an elevated number of white blood cells (WBC) ($36.60x10^9$) and granulocytes ($36.60x10^9$), albumin was low (1.61 g/dL), and haematocrit value was below normal limits. Estradiol and serum progesterone values were 119.06 pg/mL and 37.70 ng/mL, respectively (E2/P4 ratio: 3.15).

Operation

General anesthesia was induced with propofol. After

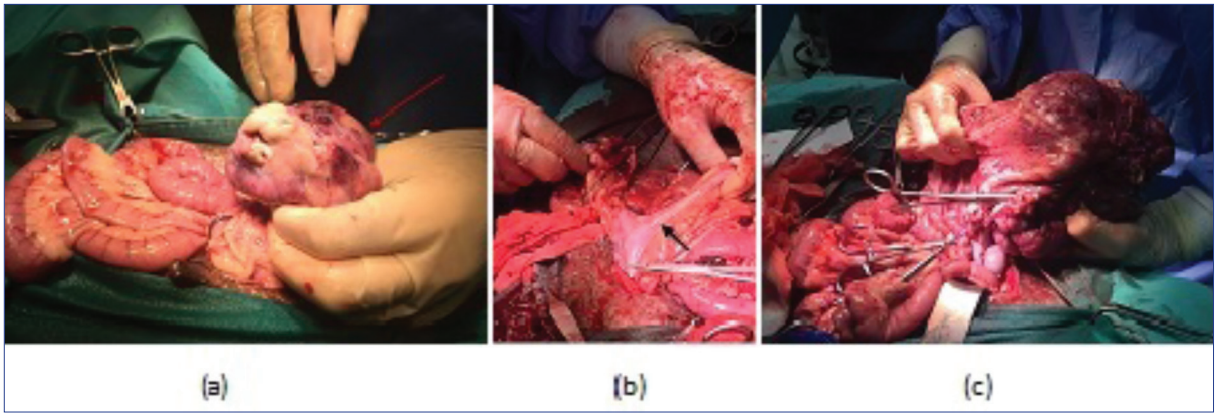

Fig 4. a- The tennis ball-sized tumour in the right ovary *(arrows),* **b-** No pathological changes or thickening in the uterus *(arrows),* **c-** Adhesions between mesometrium, mesovary and the ovary

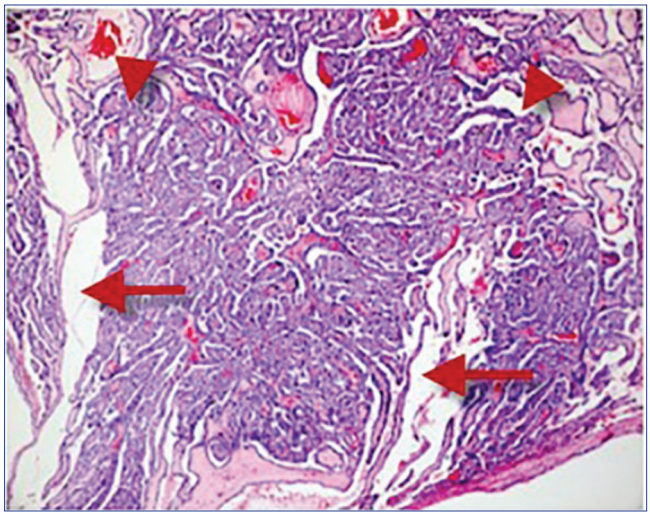

Fig 5. Cystic *(arrows)* and papillary structures *(arrowheads)* surrounded by a fibrovascular stroma. H&Ex100

intubation, anesthesia was maintained by means of inhalation of sevoflurane. The operation was performed by routine ovariohysterectomy method. First free abdominal fluid was aspirated (1.6 L). Then two tumours were detected, one which is a tennis ball-sized mass in the right ovary was removed by ligaturing *(Fig. 4a)*. The uterus did not show pathological changes *(Fig. 4b)*. Adhesions between mesometrium, mesovary and the ovary were determined *(Fig. 4c)*. The second tumour which completely removed from in the same ovary weighed 1.3 kg (20x16 cm).

Histopathology

Histopathological examination revealed ovarian papillary cystadenoma. The tumour was characterized by the presence of cysts and proliferation of papillae, both lined by single- or multi-layered cuboidal to columnar epithelial cells. The neoplastic epithelial cells with pale eosinophilic cytoplasm, distinct cell margins, round to ovoid nuclei, and prominent nucleoli were arranged mainly around the cystic and papillary structures, and surrounded by a fibrovascular stroma *(Fig. 5)*.

Patient Monitoring and Postoperative Findings

No problems were encountered during the post-operative week, the patient's general condition and appetite returned to normal. One month post-operative control of the blood profile, revealed an increased. Five months after the last clinical examination and blood testing, the owner brought his animal to the clinic, since the animals state of health had suddenly deteriorated. Blood analysis showed that BUN (8.27 mg/dL) and UREA (17.50 mg/dL) were decreased. The lymphocytes were (8.10%) decreased, and WBC (21.20x10^9/L), granulocyte (19.30x10^9/L-89.40%) and eosinophiles (1.70%) increased. In ultrasonographic examination, metastasis-like formations were detected in the spleen, kidney and other organs. Laparotomy was performed, and tumours were found in the spleen, kidney and all intestines. The animal was euthanized on request of the owner of the animal, however.

DISCUSSION

In bitch, mated 40-45 days ago, recent enlargement of the abdomen and since the general situation of the dog is normal, dogs are usually examined for pregnancy. Similarly, in some articles [2,5,6] it is mentioned that no deterioration was seen in the general situation of the patient and dogs were brought to controls only due to excess enlargement of the abdomen.

During the abdominal palpation, at the right side a bulk, rigid, round and large structure was assessed. In some reports it is stated that ovarian teratomas can be localized by abdominal palpation [2]. In the presented case, additional soft structures inside the large rigid mass prevented the definitive diagnosis of ovarian tumors.

In the presented case, sonographically round, heterogeneous, hypoechogenic and locally anechogenic regions

were detected on the ovary. If the diameter of the tumour is larger than 10cm, it is difficult to determine whether the tumor originates from the ovary or other organs [1]; however, similar ultrasonographical findings were described in cases of ovarian teratomas [1,2].

During laparotomy, increased amounts of peritoneal fluid were detected and 1.6 L were aspirated. In the right ovary, a mass with 20cm diameter was seen. The tumour had a weight of 1.3 kg. Tumours of the ovary have previously been described to reach diameters from 1.5 cm to 25 cm and up to 3.3 kg weight [1,7].

Clinical symptoms frequently comprise recurrant vaginal secretions, caused by ovarian tumour derived progesterone and estrogen, and leucocytosis [8]. In this case, no external vaginal secretion was detected during clinical controls. However vaginoscopically, blood and ulceration areas in the vagina were seen. Serum-estradiol was 3 times higher than P4, which is reflected in the appearance of the vagina. Unfortunately, no literature exists, whether P4 values being 3 times the E2 values have any diagnostic value in these cases, this warrants further investigation.

During pathological examination, irregular cystic, papillary areas and neoplastic cells were detected and histologically, a papillary cyst adenoma was diagnosed. The most frequently seen ovarian tumours are cyst adenomas [9]. During the operation, two tumours on the same ovary were detected; one had the size of a tennis ball and the other 20x16 cm in diameter. Previously tumours have been detected on both ovaries, but not several on one ovary [5]. Excessive growth of ovarian tumours covering the peritoneum, including ovary and bursa ovarica was reported in several cases [10]. In benign tumours, like papillary cyst adenomas, a grossly enlarged tumour incorporating the salpinx extends into the abdomen is characteristic.

The post-operative state of health of the dog was satisfying and improved continuously. Laboratory tests carried out one month later revealed that the blood parameters were normal. It is known that papillary adenocarcinomas metastasize into the kidney, mesenteric lymph nodes and lungs [10]. However, in this case, the diagnosis was papillary cyst adenoma which is supposed to be a benign tumour. In the presented case, 6 months after the operation, the general situation of the dog deteriorated. During laparotomy, adherence to other organs and metastases in peritoneum, spleen and mesenterium indicate bad prognosis; in these cases, if the general health status of the patient is bad, euthanasia should be recommended. In one study, 30 days after operative removal of a papillary adenocarcinoma, no metastases were assessed radiographically [9]. In our case, controls were made after 30 days, however, changes indicating metastases occurred thereafter. Controls in different intervals are recommended after the operation; in case of metastases, chemo-immunotherapy may prolong the animal's life [9].

In conclusion, any enlargement of the abdomen together with elevated estrogen might indicate an ovarian tumour. In our case, increased serum E2 and E2/P4 ratio (3.15), and sonographically detectable abdominal mass and ascites were additionally assessed. Some cases with a history of previous mating are typically introduced for pregnancy control. In case of rapidly growing papillary adenomas, frequent post-operative controls should be recommendable.

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Case Report

The Assessment of Diagnostic and Ultrasonographic Findings in a Bitch with True Vaginal Prolapse

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Abstract

A 4-year old crossbred Pointer, with observable vaginal prolapse was brought to our hospital at the 47th day of gestation. The prolapse reoccurred next day despite of the vulval suturing. During the examination of the prolapsed mass towards the cranium via palpation, it was observed that gestational sacs and urinary bladder were also in the prolapsed mass as well. Ultrasonographic imaging of the tissue revealed that puppies were presented into one-third section of the prolapsed vagina and there were no signs of vitality in the puppies. Although it is rare, vaginal prolapse might occur in association with simultaneous dilation of urinary bladder with a part of uterus in the third trimester of the gestation in dogs. In this case, the probability of true vaginal prolapse during gestation was restated and it was revealed that as an innovation ultrasound was a beneficial and guiding technique for the diagnosis of this case.

Keywords: *Bitch, Pregnancy, Ultrasonography, Vaginal prolapse*

Bir Dişi Köpekte Görülen Gerçek Vaginal Prolapsus Olgusunda Tanı ve Ultrasonografik Bulguların Değerlendirilmesi

Özet

Dört yaşlı, Pointer melezi bir köpeğin kontrolleri sonucunda gebeliğinin 47. gününde olduğu ve ilk muayenede vulva dikişi uygulandığı halde vaginal prolapsusun tekrardan şekillendiği tespit edildi. Prolabe olan kitlenin kaudalden kraniale doğru yapılan palpasyonunda, kitle içinde yavruya ait bölümler ve idrar kesesinin olduğu tespit edildi. Doğrudan prolabe olan kitleye yapılan ultrasonografik muayenede yavruların prolabe vaginanın ilk üçte birlik bölümünde bulunduğu ve ölü oldukları belirlendi. Köpeklerde çok ender de olsa, gebeliğin son üçte birlik döneminde prolapsus vagina olgularının uterusun bir bölümünü ve idrar kesesini de içine alabilecek şekilde gelişebildiği görülmüştür. Bu olguda köpeklerde oldukça az karşılaşılan gerçek vaginal prolapsus olgusunun gebelikte gelişebileceği bir kere daha gösterilmiş olmakla birlikte, yenilik olarak, benzer olguların tanısında olayın derecesi ve ciddiyetini belirlemede ultrasonografinin yararlı ve yol gösterici olarak kullanılabileceği gösterilmiştir.

Anahtar sözcükler: *Köpek, Gebelik, Ultrasonografi, Vaginal prolapsus*

INTRODUCTION

True vaginal prolapse might occur in dogs during pregnancy and is correlated with low levels of progesterone, together with elevated levels of estrogen [1]. True vaginal prolapse is the complete protrusion of vagina with cervix out of vulva and is commonly associated with dilatation of the urethral orificium and prolapse of other organs [2,3]. While vaginal hyperplasia or vaginal fold prolapse is observed mostly during the term close to estrus; true vaginal prolapse usually occurs antepartum in dogs [2,4]. No other organ observed on the vaginal prolapse besides protrusive oedamatous vagina. In spite of the vaginal mucosa, protrusion of urethral orificium is observed in Type III vaginal prolapses [5]. True vaginal prolapse cases are observed rarely in bitches. Whilst vaginal prolapse Type

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III cases were defined during pregnancy and especially on the last trimester of gestation. However; case reports presenting vaginal prolapse together with partial uterine prolapse are extremely rare and our purpose is to present this one.

CASE HISTORY

A 4-year old crossbred Pointer weighing 18 kg was referred to our hospital; it was assessed to be at the 47th days of the gestation. Despite of suturing in the first inspection vaginal prolapsus (VP) were determined.

One day previously a mass was detected and prolapsed mass is rejected and vulva suture was applied in any clinic. The vagina is protruded completely due to tenesmus and also green flux from orificium uteri externa were observed *(Fig. 1)*. Prolapsed vagina was approximately 17 cm in length and 9 cm wide. When the prolapsed mass were examined by external cranial palpation; various body parts of puppies determined to be inside this mass and also urinary bladder was observed to be protruded. Ultrasonography (USG) were performed directly on the tissue (Mindray®DC-N3Vet; 5.0 MHz; convex probe); puppies were revealed to be attached to the first third of the prolapsed vagina and showing no signs of vitality (no heartbeat or movement detected). The vagina was also shown to be interlocked with the cranial part of the uterus, with its boarders clearly defined *(Fig. 2)*. Ultrasonography

Fig 1. Appearance of vaginal prolapse

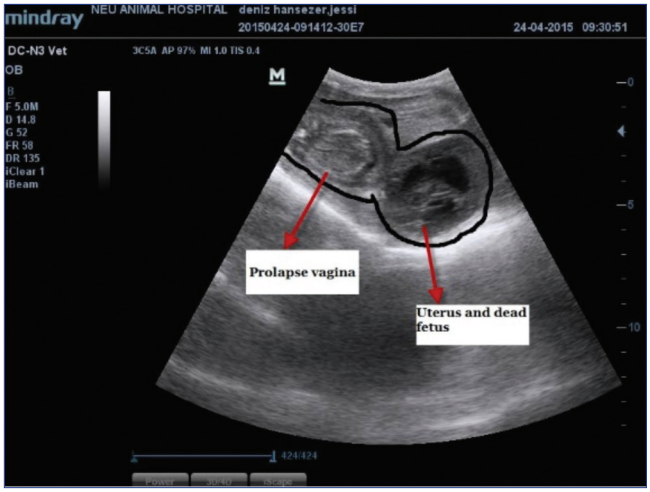

Fig 2. Ultrasonographic view of the prolapsed uterus and vagina

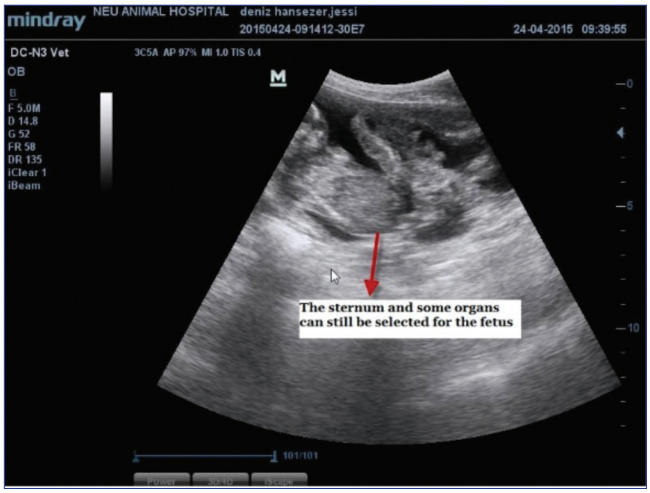

Fig 3. Abdominal ultrasonographic image of the fetus

examination of the abdomen presented osteoid sternum-like structures, but still no fetal movement or heartbeat were detected. Slow movement from caudal to cranial with the ultrasonography probe, revealed that the uterus continued behind the symphysis pelvis *(Fig. 3)*.

Following the consideration of blood tests (WBC 35.2x10^{9}/L, oestradiol 17-β measured value 2 days after the prolapsus is 23.5 pg/mL) and the request of the patient owner dead foetuses and prolapsed tissue were removed via "cesarean section". Prior to the surgery part of the mass were rejected to inside. The incision was made in line with linea alba. Uterus were removed without hysterotomy *(Fig. 4)*, and following the removal four dead puppies with approximately 8.0x3.2 cm dimensions were detected. Prolapsed uterus section have revealed enlargement of the cranial region and an increase in the levels of oedema *(Fig. 4)*. The prolapsed part of the vagina had shrunk completely within one day post ovariohysterectomy. 3 day after surgery, no VP was observed externally; in spite

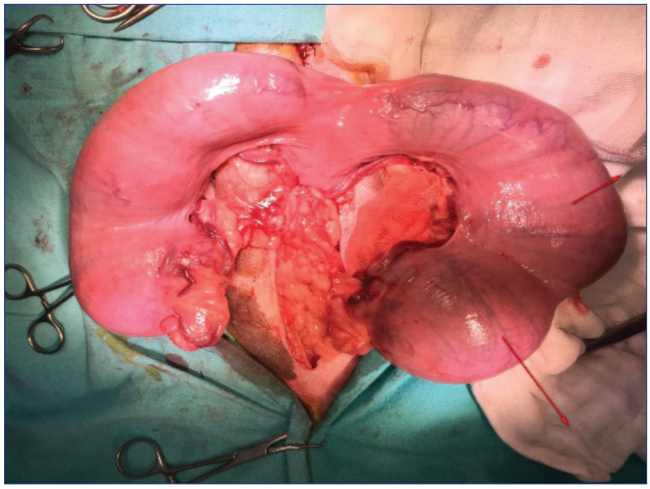

Fig 4. Oedema and color change in the right uterine corn which is a prolapsed *(arrows)*

of this the patient were transferred to the our hospital department of surgery for cystopexy and uteropexy procedures since the previously rejected urinary bladder was still partially prolapsed and could be easily seen externally. Urinary bladder was rejected during surgery and returned to its anatomical position were performed according to Mc Namara et al.[6]. After consultation, the bladder was reported to have regressed one day following the subsequent examinations revealed that the dog had fully recovered.

DISCUSSION

True vaginal prolapses are cases with protrusion of vaginal walls, urinary bladder, uterine tissue and/or distal part of colon. Cases accompanied with vaginal prolapse and uterine prolapse are extremely rare [6].

Even though exact etiology is not known, vaginal prolapses are more frequently encountered during terms where oestrogen levels are elevated such as cystic ovary [7], exogenous oestrogen application [8], granulosa cell tumors [9] and follicular phase [5] of sexual cycle. In addition, factors like relaxation of ligaments due to aging [5], abdominal pressure increase due to gestation and ascites, trauma [10] causes vaginal fold prolapse. It is more appropriate to define cases which are regressed at dioestrus and neither vaginal hyperplasia nor true prolapse as vaginal fold prolapses [11]. No neoplastic tissues were observed in this case. Vaginal prolapse cases are considered to be based on oestrogenic influences when rejected before arrival to the clinic and true vaginal prolapse is considered to be developed due to internal pressure which is caused by uterine contractions continued after vulva suture application. The potential for vaginal prolapse development is reported to be starting from estrus (or end of proestrus) until almost the end of gestation [12]. In this case, true vaginal prolapse is determined as developing at a time near the parturition. Concannon et al.[13] revealed that progesterone (P4) serum values are around 4.5±0.6 ng/mL nearly 120 h before postpartum. Having 5.6 ng/mL P4 values indicates that the cases occur closer to parturition. Other publications report that true vaginal prolapse occurs during a period near postpartum where P4 values start to decrease and oestrogen values begin to increase [4,14,15]. In the present case, it was easy to diagnose since puppies could be palpated on first one third caudal part of clinically prolapsed vagina and a second layer with palpation. Also, this shows that determination using ultrasonographic imaging diagnosis of partial uterine tissue engaged in vaginal prolapse at the caudal region of the mass is possible. Determination of additional foetus and other parts of uterus on the caudal parts when abdomen was palpated to the caudal of pecten pubis shows that part of the uterus remaining in cranial was prolapsed with vagina. Vaginal prolapses can be easily diagnosed with identification of protrusive cyclindiric part [16]. As understood from published cases that prolapsed uterus with vagina is revealed after operation [4]. This case reveals diagnosis of true vaginal prolapse with palpation and especially through the use of ultrasonographic imaging techniques. There are many publications regarding pathological and physiological changes of the uterus with ultrasonographic [17]; however, to our knowledge this is the first account regarding the determination of true vaginal prolapse using USG.

In previous reports in cases of true vaginal prolapse or vaginal fold prolapse upon determination of both live and dead puppies via ultrasonography; generally a ovariohysterectomy were performed [4,16]. In this case the reason for surgical intervention were the death of the foetuses and the failure of the repositioning of the tissue(s). While excessive tenesmus related to dystocia causes prolapse, stenosis related to dystocia on reproductive canal reported to be possibly prevents repositioning the organ [4].

As a result, it is concluded that, even though it is extremely rare in dogs, during the last third term of gestation, a part of uterus and bladder may be dilated accompanied with vaginal prolapse. In this report possibility of development of true vaginal prolapse in the pregnancy period were represented, and benefits and guidance of ultrasonographic method in the diagnosis of such cases are revealed.

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Review

Update on Canine Parvovirus: Molecular and Genomic Aspects, with Emphasis on Genetic Variants Affecting the Canine Host

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Abstract

Canine parvovirus (CPV), the etiology of hemorrhagic enteritis in dogs, was first isolated as CPV type 2 (CPV-2) almost 40 years ago, and was soon replaced by the emergence of new variant types. The major viral capsid proteins encoded by the VP2 gene are the sites where amino acids are often substituted, accounting for the unusual nature of this type of DNA virus. The alteration of specific residues has contributed to different antigenic variants which have affected the evolution of virus binding and host immunity to this virus. Sequence analysis of the VP2 gene and subsequent characterization have revealed three circulating CPV-2 strains, CPV-2a, CPV-2b, and CPV-2c, identified by mutations at amino acid residue 426. The latter strain displays increased pathogenicity in dogs and an extended host range. The present review article aimed at updating contemporary information on epidemiological studies and surveys from CPV field work. Moreover, we pointed out some sensitive and rapid diagnostic tools for detecting CPV in clinical samples, techniques which will be useful for health monitoring and management of CPV with currently available vaccines.

Keywords: *Canine Parvovirus, CPV type 2, Genetic Variation, VP2 Gene, Mutation, Dog*

Köpek Parvovirusu Üzerine Bir Güncelleme: Köpek Konakçıya Etki Eden Genetik Varyasyonların Moleküler ve Genomik Özellikleri

Özet

Köpeklerde hemorajik enteritin etiyolojik etkeni olan Canine parvovirus (CPV) neredeyse 40 yıl önce ilk olarak CPV tip 2 (CPV-2) olarak izole edildi ve hemen sonrasında ortaya çıkan yeni varyant tipler CPV tip 2'nin yerine geçti. VP2 geni tarafından kodlanan major viral kapsid proteinler aynı zamanda en sıklıkla amino asitlerin başka amino asitlerle değiştiği alanlar olup bu tip DNA virusların aykırı doğasının da sebebini oluşturmaktadır. Spesifik yapılardaki değişimler farklı antijenik varyantların oluşmasına katkıda bulunarak bu virüsün bağlanma ve virusa karşı konakçı bağışıklığının değişmesini etkilemiştir. VP2 geninin sekans analizi ve takibinde karakterizasyonu, 426. amino asitte mutasyon ile şekillenen CPV-2a, CPV-2b ve CPV-2c olmak üzere dolaşımda 3 farklı CPV-2 suşunun olduğunu göstermiştir. CPV-2c köpeklerde artmış patojenite ve daha geniş konakçı yelpazesi göstermektedir. Bu derlemede güncel epidemiyolojik çalışmalar ile CPV saha çalışmaları hakkındaki bilgilerin güncellenmesi amaçlanmıştır. Ayrıca, klinik örneklerde CPV'nin tanısında kullanılmak suretiyle sağlık taramasında faydalı olabilecek ve mevcut aşılarla CPV'nin kontrol altına alınmasında faydalı olabilecek bazı hassas ve hızlı tanı yöntemleri değerlendirilmiştir.

Anahtar sözcükler: *Canine Parvovirus, CPV tip 2, Genetik Varvasyon, VP2 Geni, Mutasyon, Köpek*

INTRODUCTION

Canine parvovirus (CPV) is a contagious, life-threatening viral disease in young dogs, with a wide host range in many mammalian families: Mustelidae (ferrets, minks, and badgers), Canidae (dogs, foxes, and wolves), Procyonidae (raccoons), and Felidae (cats, lions, tigers, and cheetahs) [1]. This viral disease is very common in unvaccinated dogs living in densely populated areas. The transmission of CPV is mediated by persons, animals, and fomites that come in contact with infected secretions or materials, such as feces, blood, food bowls, clothing, or bedding. The most

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clinically significant forms induced by CPV are hemorrhagic enteritis, or bloody diarrhea. The general clinical signs may present as anorexia, depression, vomiting, fever, and mucoid or watery diarrhea. In severe cases, dehydration and hypovolemic shock may occur. The mortality rate in puppies can reach more than 70%, whereas the rate in the adults is less than 1% [2]. Canine parvovirus replication occurs in host cell nuclei and requires rapidly dividing cells of fetuses, newborns, lymphoid tissue, and intestinal epithelium of animals. The CPVs spread easily and are highly stable in the environment, able to survive in harsh conditions for about six weeks [1,3].

Puppies without or inadequate titer of maternal-derived antibody (MDA) to this virus are prone to be infected [4]. In any circumstances, healthy dogs or infected dogs with hemagglutination inhibition (HI) titer of 320 or higher are suggested to be protected from virus replication [4]. With sufficient protective immunity, the feces of dogs challenged with CPV-2 remained undetectable of CPV DNA by real-time PCR if they had the HI titer level of 320 of MDA [4]. In case of a low HI titer, such as 160 and lower, active CPV can be demonstrated by utilizing a reliable, sensitive method such as real-time PCR to detect the presence of the viral genome [5-7].

CANINE PARVOVIRUS AND ITS GENOMIC ASPECTS

Canine parvovirus is a DNA virus and a member of the Parvoviridae family. This virus family consists of two subfamilies, *Parvovirinae* and *Densovirinae*. According to available information, Parvovirinae viruses are able to infect vertebrate hosts, while the latter subfamily can only infect insects. Currently, the *Parvovirinae* subfamily is comprised of eight genera, namely *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* and *Tetra parvovirus* [8]. The unique viruses in the genus *Parvovirus* are canine parvovirus (CPV) and feline panleukopenia virus (FPV), which are now well characterized [8].

Parvoviruses are non-enveloped viruses, single-stranded DNA approximately 25 nm in diameter. The parvovirus genome consists of approximately 5,323 nucleotides [9]. The full length of the viral genome contains two large open reading frames (ORFs). The first ORF is encoded for two nonstructural proteins (NS1 and NS2). The second ORF is built up of three structural proteins or capsid proteins (VP1, VP2, and VP3) through an alternative splicing of the same mRNAs [3]. The parvovirus capsid is icosahedral and consists mainly of 60 subunits of the polyproteins VP1 and VP2 [3,10]. VP3 is a product of VP2 from virus–host interactions when cleaved by proteolytic enzymes [9].

The global distribution of contemporary CPV is thought to be divergent from canine minute virus (CnMV) [9]. This virus, formerly known as canine parvovirus type 1 (CPV-1), has caused neonatal death in puppies [8]. It has been documented that CPV-1 emerged from feline parvovirus (FPV) and has been circulating worldwide since the 1970s [11]. A few years later, the first CPV-2 isolates were discovered [12]. CPV-2 causes severe hemorrhagic gastroenteritis in dogs, as well as myocarditis [11].

The evolution of the original CPV-2 was established in the mid-1980s [6]. Since that time, the original CPV-2 (simply called "CPV-2") has been completely replaced by alternative variants, the first two of which are known as CPV-2a and CPV-2b [6]. *This phenomenon suggests that CPV-2 has evolved a highly fit conformation* [13]. In 2000, a new CPV subtype, CPV-2c, was detected, and it is now confirmed to be co-circulating with the other presenting subtypes [6].

At present, the antigens or subtypes of CPVs can be systematically identified using certain amino acid residues positioned within the VP2 protein. The antigenicity of CPVs, which determines the host range, is associated with VP2 capsid proteins. There is an antigenicity difference frequency of CPV-2a/2b detection [5,14,15]. The introduction of the CPV-2c strain was reported in 2001 [16]. CPV-2c is more widespread in South America [17,18], with the exception of Brazil where all circulating strains were characterized as CPV-2a or -2b [19,20]; few CPV-2c strains have been detected in India [21,22].

The VP2 protein is a favored location for mutations. *This protein accounts for interactions with host transferrin receptor (TfR). Once alterations become permanent, the affinity to canine TfR could be significantly enhanced* [11,23]. The favorability of mitotically active tissues, such as actively dividing intestinal cells and myocardiocytes in canine puppies, leads to the pathogenesis of CPV infection because the transferin receptors are highly expressed in those cells [24].

CANINE TRANSFERRIN RECEPTOR (TFR), AND CPV RECEPTOR RECOGNITION

The adaptation of receptor binding to canine transferrin receptor (TfR) type-1 has resulted in the extension of the host range of this virus, which for the newer antigenic types now includes both dogs and cats [11,25,26]. *Canine parvovirus has evolved its ability to bind the TfR type-1 by naturally occurring mutation of capsid protein (VP2) which conferred small local changes* [27]. The binding of the canine TfR plays a critical role in the canine parvoviral infection [28]. The TfR-capsid interaction depicted asymmetrical docking conformation [29,30]. *It is postulated in vitro study that binding of viral capsid to canine TfR, required only a small number of TfR (one to five TfRs per capsid) in initiation of infection* [29-31].

The alteration of hydrogen bonds and amino acid sub-

stitution at position 300 of VP2 are likely to cause a great susceptibility of the host receptor in binding of the viral particles [27,32]. Adjacent to residue 300, replacement of Gly299 (G299) increased hydrogen bonds with aiding in the flexibility of capsid surface loop [27]. The single point mutation between two AA residues is unlikely to cause the major change in protein structure, but this phenomenon can enhance the thermodynamic properties or entropy of surrounding AAs [27,33] and further influence the interaction between viruses and TfR.

The CPV-2a, which has descended from CPV-2, has a broad host range of both domestic and wild carnivores [27]. The certain substitution of AAs on the exterior surface of VP2 (G299K/A300K) has demonstrated the efficient binding to the receptor and eventually allowed virus entry into both feline and canine cells [29]. Within the virus-binding region of canine TfR and closely related canids (e.g. coyotes, and gray wolves), the glycan molecules at glycosylation site has been discovered to influence the binding of the virus thus promote the infection of canine cells [29,34,35]. The presence of glycosylation site prevents binding and infection of FPV-like virus in dogs, but this event was later overcome by antigenic variants of CPV-2 [32,36-38], suggesting that a specific Gly300 residue has some potential to bind efficiently to canine TfR [32]. The changes of specific AAs of three-fold spike of new antigenic CPV-2a, -2b and -2c (e.g. AA # 87, 101, 297, 300, and 305) resulted in the cross-species viral transfer and adaptation to new hosts [28], while the differences of AA residue 426 dictate antigenic variants of CPV-2 [28]. The mutation at AA residue 300 (e.g. Trp300), and its neighboring AA residues 299 and 301, has rendered CPV non-infectious for a dog with an exception for other animal species (e.g. cat and fox) [32]. *It is important to note that AA position at 300 of VP2 proteins may be considered as a key determinant of CPV host tropism through TfR binding* [6] *and even more about the pandemic emergence of CPV* [24,27,32,39,40].

The study of glycosylation found at TfRs of some carnivorous animals demonstrated the variation in patterns in which highly suggesting that the presence of glycan of domestic dog TfR forces the susceptibility to CPV [32]. The binding of AA residues near the 3-fold spike of VP2, especially residues 299 to 301, with TfR required the change of residue 300 (A300G) of virus to gain access to dog host, whereas there were some evident dictated that the mutation of residue 299 (G299E) or residue 300 (A300D) causing reduced binding and infectivity of canine TfR [24,29,31,32,38,40].

AMINO ACID CHANGES AND CPV TYPE 2 VARIANTS

The emergence of new CPV-2 subtypes, specifically CPV-2c, has drawn attention to how well they fit to the canine host. *CPV-2c is thought to have a less severe clinical course and a lower mortality rate, as observed in dogs infected with the Glu-426 mutant (currently known as CPV-2c) compared with outbreaks caused by CPV-2a and CPV-2b* [16,41]. The alterations of amino acids (AA) in the VP2 protein at specific residues - asparagine to glutamic acid (N426E) and aspartic acid to glutamic acid (D426E) - as determined by the antigenicity of antibodies has resulted in different antigenic detection of monoclonal antibodies, as shown in many studies [6,11,42] *(Fig. 1)*. From the perspective of humoral immunity, the monoclonal antibodies (mAbs) A4E3 and C1D1 could recognize this novel antigenic determinant occurring within the major antigenic sites of VP2 in the CPV-2 virus [43]. The other site in VP2 where alterations are often detected is the amino acid residue at the 440 position. The threonine to alanine mutation (T440A) is of interest since it is located in close proximity to the Glu426 residue in the major antigenic site, or epitope A, found on the three-fold spike of the CPV capsid protein [6].

Indeed, progressive changes inside the capsid protein (particularly VP2) have been occurring throughout the past three decades, and these changes are continuing; however, the transformation seems rather small [11]. Changes at the 426 amino acid residue may account for the spread of all CPV-2 subtypes. *The new mutant VP2 structure may improve the biological properties of the virus, contributing to canine host adaptation, stabilization of the VP2 capsid structure, and enhanced antigenic escape from monoclonal antibodies* [44].

Many studies have identified the changes in amino acid residues located within the full-length gene encoding the main capsid protein VP2. Here, we list some of these changes in amino acids *(Table 1)*. It is currently unknown whether the various new mutants, such as S297A, D426E, or T265P, are also associated with altered receptor binding [11]. Analysis of the VP2 protein has shown that all CPV-2c strains and sequenced CPV-2a/2b strains retain the AA changes of the variants with respect to the original CPV-2 (M87L, I101T, A300G, D305Y, N375D) and display the S297A mutation typical of the recent CPV-2a/2b isolates [45]. The most relevant change was at T440A, which was encountered in one United States. type 2c strain (110/07-27), but also in two type 2a Italian strains (333/05 and

Table 1. *Frequent amino acid mutation sites found in full-length VP2 genes of canine parvovirus* [16,45-48] *compared with a reference strain (accession number M38245)*

87	101	267	297	300	305	324	375	389	418	426	440	555
M87L	I101T	F267Y	S297A	A300G	D305Y	Y324I	N375D	T389N	I418T	N426D N426E	T440A	V555I

80/08) [45]. This change was also present in some reference isolates, CPV-2a (northern India) and K022 (South Korea) and CPV-2b LCPV-V204 (Vietnam) [45]. In contrast, strain CPV-2b 311/04 (Italy) displayed a different change at the same position (T440N) and a further change in a nearby residue (D434V) [45].

The GH loop, situated between the βG and βH strands of the capsid surface (VP2) of the parvovirus, is formed by residues 267 to 498. This region contains sites with the most variability, influenced by its presentation on the capsid surface [10,45]. Aside from amino acids 297 and 440, changes detected in the GH loop of the VP2 protein of CPV-2c were R274K, F420L, N421Y, and V463I, of which the change at position 463 has been identified in a Korean CPV-2a isolate [49].

PREVALENCE OF CANINE PARVOVIRUS TYPE 2 VARIANTS

A new antigenic variation, carrying the AA substitution Asp426Glu (D426E) in the major antigenic site of the viral capsid protein VP2, was first reported in 2001 by a group of Italian virologists [16,41]. This newest variant, designated CPV-2c, has already been detected in other European countries, as well as in Asia, Africa, and the Americas. Five AA changes are present in the VP2 capsid protein, while the antigenic differences observed in CPV-2b are the consequence of only one AA substitution (Asn426Asp; N426D) located in the major antigenic site of the capsid (epitope A) *(Fig. 1)*. CPV-2, on the other hand, replicates poorly in feline cells *in vitro*; however, this finding was not in accordance with the results of an *in vivo* study of live cats [11]. CPV-2c displays a low genetic variability and shared amino acid changes already detected in recent CPV-2a/2b isolates [45].

The prevalence of CPV-2 subtypes has been intensively studied at only a few laboratories. The majority of reports were derived mainly from countries in Europe, America, and Asia, where there are suspected endemic areas. We have collected information from a public dataset, which is summarized in a phylogenetic tree *(Fig. 2)*. The phylogenetic clusters accounting for the geographical distribution were created using selected full-length amino acid sequences of the VP2 capsid protein (full 584 AAs) from various types of viruses (FPV, CPV-2, CPV-2a, CPV-2b, and CPV-2c) with the corresponding GenBank accessions *(Fig. 2)*.

CPV-2c has been identified by sequencing at the major antigenic variation within the VP2 capsid. CPV-2c is the dominant and most prevalent type of CPV-2 that has been spreading in Argentina [44,50,51], Ecuador [52], Uruguay [53], and Rio de Janeiro, Brazil [54]. In Colombia, the presence of the antigenic variants CPV-2a/2b with a possible new CPV-2a are currently circulating [55].

In the United States and Mexico, CPV-2 types have been documented as CPV-2, CPV-2b, as well as CPV-2c [1,6,53-55]. In Asia, CPV-2a and 2b are currently predominant in Japan [56,57], Taiwan [58,59], and South Korea [60,61]. In Vietnam, CPV-2c [42] is often used as a reference strain for the naturally occurring Vietnamese HNI-4-1 prototype [45]. In Thailand, dog populations are often crowded into urban and metropolitan areas such as Bangkok and Chiang Mai. It was previously reported that CPV-2, CPV-2a and CPV-2b were the pre-dominant types found in Bangkok and the vicinity [26].

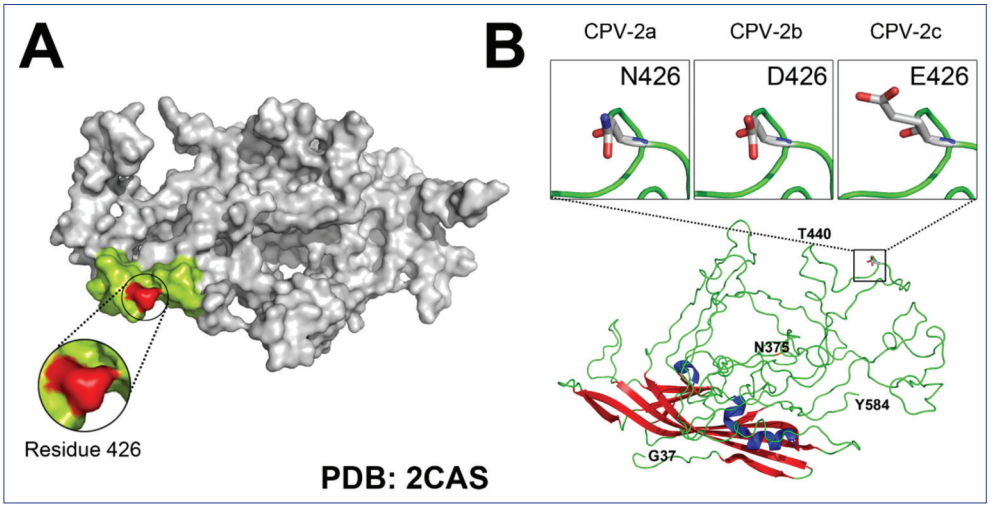

Fig 1. Amino acid variations in capsid protein (VP2) of canine parvoviruses (CPV). **(A)** Surface representation structures of VP2, with residue 426 highlighted in red and nearby amino acids shown in lime green. Enlarged views of the 426 position are provided. **(B)** Graphical representation of VP2 capsid protein depicts the β strands of the eight-stranded antiparallel β barrel (red), α helices (blue), and loops (green) in the structure. The N-terminal is labeled 'G37' and the C-terminal end is labeled 'Y584'. Magnified views of the original residue 426 from CPV-2a (N426), or substituted residues in CPV-2b (D426), and CPV-2c (E46), which account for CPV-2 variants, are shown with stick configurations. This graphic is derived from PDB accession number 2CAS

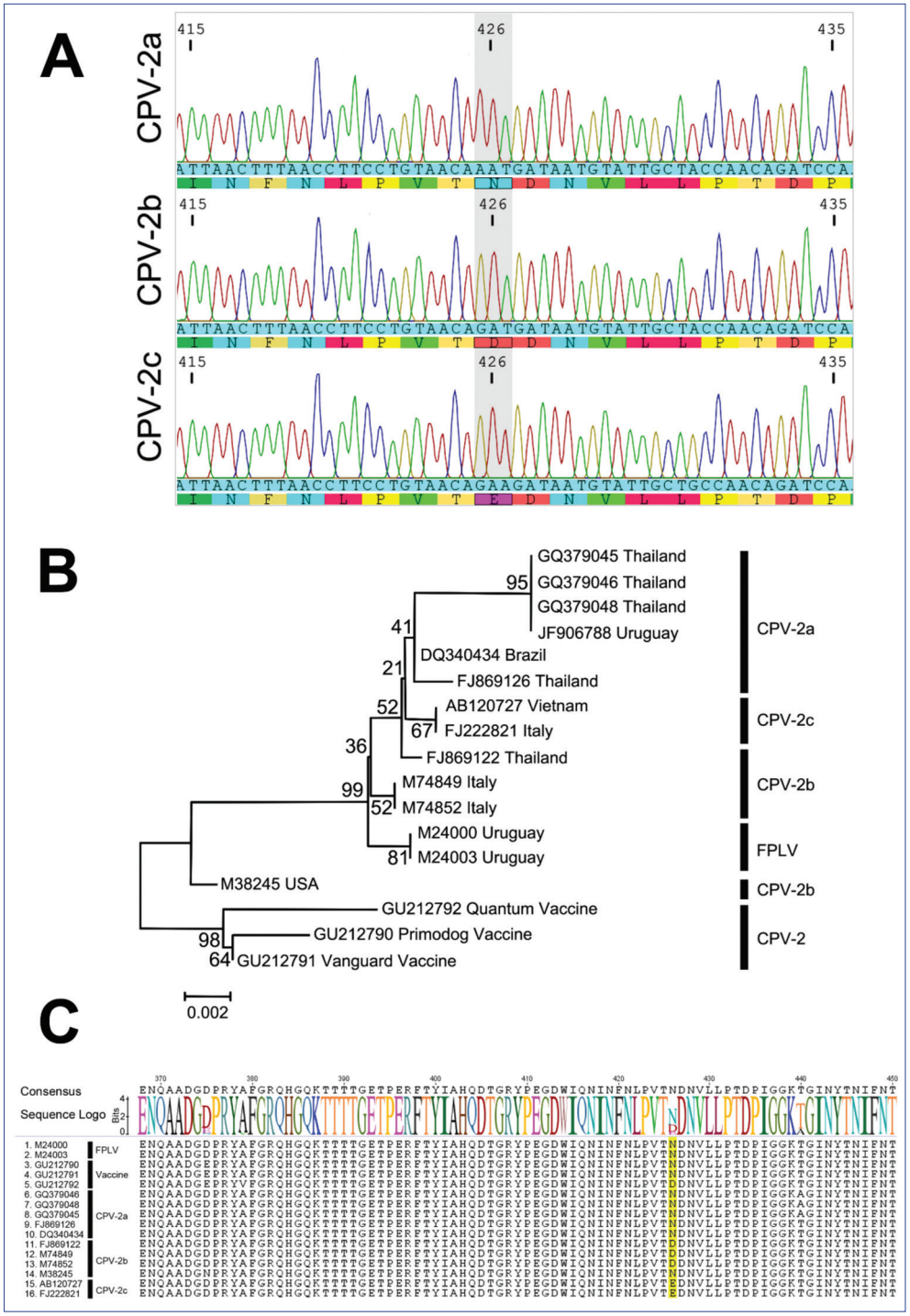

Fig 2. **(A)** Representative sequencing chromatograms of CPV-2a, -2b, and CPV-2c. The partial nucleotide sequences at residue 426 are labeled in order to compare the different amino acid substitution among the three strains. **(B)** Phylogenetic analysis based on selected GenBank complete VP2 amino acid sequences of FPLV and CPV viruses of various CPV-2, CPV-2a, CPV-2b, and CPV-2c strains. **(C)** Multiple amino acid sequence alignment of the complete *VP2* gene, with the amino acid at position 426 highlight. **(B)** and **(C)** are made from the same dataset

More recently, we discovered that mixed types of CPV-2 - CPV-2a, CPV-2b, and CPV-2c - circulate in combination and cause mucoid or bloody diarrhea in dogs residing in the Chiang Mai municipality (unpublished data). There has also been a recent CPV-2c epidemic in Vientiane, Laos [64] and Taiwan [65]. In China's capital city, Beijing, CPV-2a and 2b remain the dominant types of this virus [47,66-68], but more recently, the presence of CPV-2c has been confirmed in China [9,69-71]. In India and Iran, the dominant types in recent outbreaks were CPV-2a and -2b [21,72,73]. In 2016, CPV-2c was detected in northern and central India [74,75] and Iran [73].

In European countries, the co-circulation of CPV-2a, -2b, and -2c has been reported [76,77]. CPV-2c, causing gastroenteritis in dogs, has been detected in Spain [25], the United Kingdom (UK) [13], Italy [16,45], Germany, France [77] and Portugal [78,79]. In Albania [80], Hungary [81] and Turkey [82], CPV-2a and mutants are widely spread. In Australia, New Zealand, and Oceania, investigation of CPV in dogs has demonstrated that CPV-2a remains the predominant genetic variant, and has not been replaced by CPV-2b or CPV-2c, as in many other countries [83,84]. In Africa, infection of dogs with either CPV-2a or -2b was

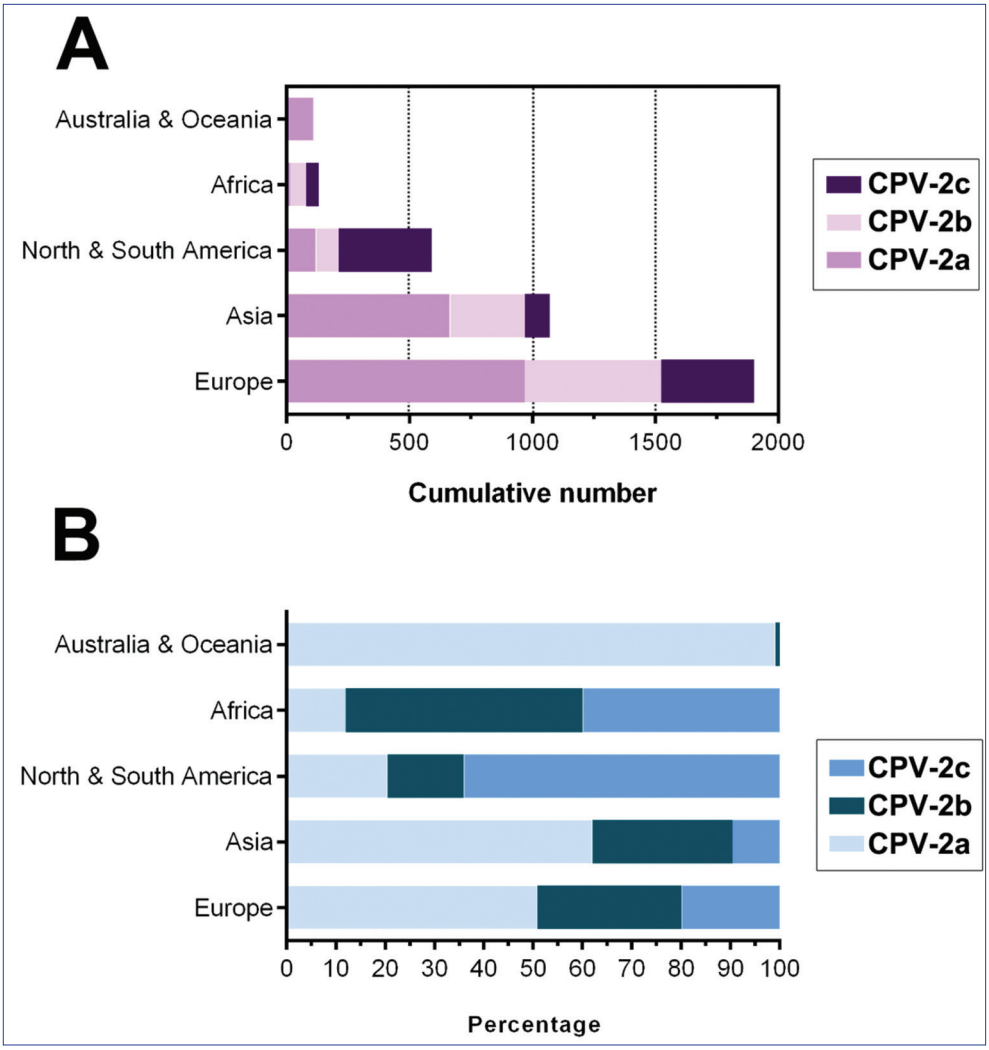

Fig 3. (A) Distribution of cumulative numbers of CPV case reports, and **(B)** the percentages of canine parvovirus variants, categorized by continent. Bar graphs are constructed from the available recent public data to mid-2016

reported in Nigeria, Mozambique, and South Africa [85,86]; however, the CPV-2c strain was found to be present in Morocco [87]. The numbers and percentages of detected CPV cases are summarized by region in *Fig. 3*.

DIAGNOSTIC TOOLS FOR CPV ANTIGEN AND ANTIBODY DETECTION

Canine parvovirus infection is the main viral etiology responsible for diarrhea in dogs. This disease may be differentially diagnosed by the time of clinical manifestation. Some viruses, such as morbillivirus, rotavirus, coronavirus, adenoviruses, reovirus, and norovirus, have contributed to causing diarrhea in dogs [2]. The virus causing gastroenteritis should be confirmed by laboratory diagnosis in order to be distinguished from bacterial enteritis. CPV infection causing gastroenteritis is usually a major cause of illness in the early life of dogs. The feces, intestinal contents, or tissues from affected dogs, or even EDTA blood samples at the time of viremia, have proven to be useful in diagnosis [25,43,88].

In recent years, many research groups have attempted to validate the use of various methods to detect the presence of CPV as a causative virus. Some are working on antibody-based tests for rapid detection of CPV antigens. Several studies have demonstrated that ELISA test kits are able to detect CPV antigens and have shown promising sensitivity and specificity toward new variants of CPV [89,90]. A recent study compared commercial antibody-based tests for rapid detection of CPV antigens with other detection methods, i.e. PCR and immunoelectron microscopy (IEM). The results revealed the high specificity and low sensitivity of the antigen-detection kits [91].

Molecular biology techniques, such as traditional polymerase chain reaction (PCR) and quantitative PCR (real-time PCR; qPCR) have been widely developed and used in the detection of CPV genetic materials from blood and fecal samples [7,41,43,48]. *Because of the sensitivity, specificity, and reproducibility of PCR and real-time PCR in detection of CPV DNA, this method might replace traditional methods such as virus isolation and antibody detection.* Real-time PCR technology, using SYBR Green or minor groove binding (MGB) TaqMan probes for PCR assays, has many

advantages over conventional PCR [6,7,92]. The quantification of virus load is one example of the many applications for exploiting qPCR. Real-time PCR can also be performed with a large throughput to achieve an inexpensive and time-saving method [41].

As documented in Desario et al.[43], monoclonal anti-bodies (mAbs) can be raised against CPV-2 types in order to determine the hemagglutination inhibition (HI) titers in different viral variants. Abs clones A4E3, B4A2, C1D1, and B4E1 were unequally recognized major epitopes in original CPV type 2, CPV-2a, CPV-2b, and CPV-2c (formerly known as Glu-426 mutant) [43]. The clones A4E3 and C1D1 demonstrated superior reactivity with nearly all CPV variants [43]. The change in CPV-2c at amino acid residue 426 has shown differences in antigenic determination by the monoclonal antibodies 21C3 and 19D7 [6,42].

CANINE PARVOVIRUS VACCINES: FUTURE PERSPECTIVES

The original CPV-2-based vaccines have been proven to provide secure immunization against CPV-2c in Italian isolates [1]. The antibodies produced in dogs vaccinated with the latest CPV-2b field strain have shown more promising reactivity than the traditional CPV-2 strain vaccines [59]. The observed antigenic contrasts may drive the selection of CPV strains by producing differential immunogenic pressures among canine populations, which raises concerns about immunization efficiency [44]. The CPV-2c variation displayed a one-of-a-kind antigenic example, since it was inadequately recognized by specific antibodies of dogs inoculated with CPV-2, CPV-2a, and CPV-2b strains [44]. Several studies have demonstrated that CPV-2 vaccines can be used to promote CPV-2 antibodies against CPV variants [93-96]. A new modified live CPV vaccine (CPV-MLV) recently launched in the marketplace is designed from the CPV-2b variant, or can be genetically engineered to simulate the new CPV-2c variant [44,93]. In the serum neutralization (SN) test, titers to the antigenic variants CPV-2a, CPV- 2b, and CPV-2c in immunized dogs were significantly lower than the homologous titers (raised to the original type) [11,44,95]. As previously observed by Pratelli et al.[95], the greatest antigenic differences were found in comparison with the original CPV-2, which is still largely utilized in vaccine manufacturing [44]. The SN immunologic method has been found to provide greater clarity and contrast than HI in cross-antigenic assessment of CPV-2 variability. The heterologous SN titers (versus CPV-2a and -2c) were significantly lower than the homologous SN titer (versus CPV-2b) [44]. After inoculation with the CPV-2c variant, lower SN titer was found in the sera of dogs and rabbits immunized with heterologous (CPV-2, -2a, and -2b) viruses. Moreover, these discoveries suggest the opportunity to develop modified live virus (MLV) vaccines from the CPV-2c strain [44]. Another study has also confirmed the notion that the current vaccine regimen, made from nucleotide sequences of CPV-2b, can provide antigenic cross-protection of dogs from the CPV-2c variant [97]. In the case of maternal antibodies, a specific titer might provide adequate resistance to disease caused by homologous CPV infection, but it may not fully protect puppies if they encounter a heterologous virus strain [11].

CONCLUSIONS

The continuous antigenic evolution of CPV-2 has caused the rapid displacement of older strains by a new antigenic variant strain, CPV-2c, which emerged in Italy in early 2000 [78] is spreading with high morbidity and mortality in the dog populations of Italy and neighboring countries. This progressive mutant is now replacing the antigenic variants CPV-2a and -2b [15]. Studies by authors from many countries, i.e., Italy, Portugal, Spain, France, United Kingdom, Belgium, Germany, Greece, Bulgaria, Tunisia, United States, Uruguay, Argentina, China, Taiwan, Vietnam, Thailand, Laos, and India, have demonstrated that the new variant 2c is a global threat for puppies. The substitution of CPV-2 by strains -2a and -2b, and then -2c, has been connected with expanded receptor-binding capacity to canine transferrin receptors. The mutation at Glu-426 confers the benefit of infectivity and, even more, influences clinical disease status. Continuing progress in vaccine development will determine whether the CPV vaccines currently in use will still provide full protection against the new variant or whether we should be prepared to replace those homologous vaccines with a novel technology, heterologous vaccines.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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Further considerations

• Journal policies detailed in this guide have been reviewed

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