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Research Article

Effect of Dietary Orange Peel Essential Oil and Thermotolerance on Histo-morphometry and Serotonin-immunoreactive Endocrine Cell Numbers in the Small Intestines of Heat Stressed Japanese Quails

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Abstract

This study was conducted in order to measure the effects of early period thermal conditioning, feed restriction, supplementation of orange peel essential oil (OEO) into ration or combinations of them on small intestinal morphology and density of serotonin-immunoreactive (IR) endocrine cells (ECs) found in small intestines. 168 7-day-old Japanese quails were divided into six groups of 24-h fasting or thermal conditioning and their subgroups with and without supplementation of OEO (300 ppm) into ration. We determined that fasting and thermal conditioning increased villus height for duodenum in control groups and for jejunum in OEO groups. In addition, we detected that while fasting and thermal conditioning increased villus height/crypt depth (VH/CD) ratio in duodenum and jejunum, these applications did not affect this ratio in ileum. We found that supplementation of OEO into ration increased the number of serotonin-IR ECs in crypts of small intestine. We revealed that early period thermal conditioning increased the number of serotonin-IR ECs in duodenum, jejunum, and ileum especially in groups in which OEO was supplemented into ration. These results indicated that applications of early period thermal conditioning and feed restriction in quails may generally prevent adverse effects, caused by heat stress, on intestinal morphology.

Keywords: *Thermotolerance, Fasting, Orange peel essential oil, Intestinal morphology, Immunohistochemistry, Serotonin*

Yeme Eklenen Portakal Kabuğu Esansiyel Yağının ve Termotoleransın Sıcaklık Stresi Uygulanan Japon Bıldırcınlarında İnce Bağırsak Histo-morfometrisine ve Serotonin-immunoreaktif Endokrin Hücre Sayısına Etkisi

Özet

Bu araştırma erken dönem termal koşullandırmanın, yem kısıtlamasının, rasyona portakal kabuğu esansiyel yağı (PEY) ilavesinin veya bunlarının kombinasyonlarının ince bağırsak morfolojisi ve ince bağırsaklardaki serotonin-immunoreaktif (IR) endokrin hücrelerin yoğunlukları üzerine olan etkilerinin ölçülmesi için yapıldı. 168 adet 7 günlük yaşta Japon bıldırcınları, 24 saatlik açlık veya sıcaklık stresi uygulaması ve bunların rasyona PEY (300 ppm) eklenen veya eklenmeyen alt grupları olacak şekilde altı gruba ayrıldı. Yem kısıtlaması ve termal koşullandırmanın duodenum için kontrol gruplarında, jejunum için ise PEY gruplarında villus yüksekliğini artırdığı saptandı. Ayrıca yem kısıtlaması ve termal koşullandırmanın duodenumda ve jejunumda villus yüksekliği/kript derinliği (VY/KD) oranını artırırken, bu uygulamaların ileumda bu oranı etkilemediği belirlendi. Rasyona PEY ilavesinin tüm ince bağırsak kriptlerindeki serotonin-IR endokrin hücre sayısını arttırtığı tespit edildi. Erken dönem termal koşullandırmanın özellikle rasyona PEY eklenen gruplarda duodenum, jejunum ve ileumdaki serotonin-IR endokrin hücre sayısını arttırdığı gösterildi. Bu bulgular, bıldırcınlarda erken dönem termal koşullandırma ve yem kısıtlaması uygulamalarının sıcaklık stresinin neden olduğu intestinal morfoloji üzerindeki olumsuz etkilerini genel olarak önleyebileceğini göstermektedir.

Anahtar sözcükler: *Termotolerans, Açlık, Portakal kabuğu esansiyel yağı, İntestinal morfoloji, İmmunohistokimya, Serotonin*

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INTRODUCTION

During the past 20 years, animal production has increased particularly in tropical and subtropical regions all over the world [1]. Production of farm animals in tropical and subtropical regions is affected by several factors. Climate of these regions is known as one of primary factors restricting the production efficiency [2].

Heat stress is the major problem to be taken into consideration for production of poultry animals and significantly affects animal health and productivity [3]. Because poultry animals have no sweat gland, they increase their respiratory rate up to 140-170 to stabilize their body temperature at high ambient temperatures, and consequently respiratory alkalosis occur. Depending on these changes in bloodstream, decreases are observed in feed consumption, body weight gain, and feed conversion ratio. For example, in case that ambient temperature exceeds 30°C, feed consumption decreases at the rate of 1-6% per each increase of 1°C [4].

Epithelium of small intestine consists of continuously renewable clusters of cells. Stem cells are located in crypt region, they form enterocytes by migrating towards villi, and progress to the end points of villi [5,6]. Heat stress also causes some changes specific to gastrointestinal tissue like decrease in intestinal epithelial integrity [7].

Lots of literature information is found concerning possible techniques to eliminate adverse effects caused by heat stress in broiler chicks. One of the practical approaches is that high ambient temperature is applied at early periods of life; promising results were obtained by this way. Thermal conditioning applied at the early age period causes a decrease in weight gain during the first week of life; on the other hand, it leads to faster growing when compared to unconditioned chicks at the following process and enable them to reach higher body weight at marketing age [8-10]. The main purpose of thermal conditioning is to facilitate adaptation of chicks to the ambient in unexpected acute temperature attacks at older ages by activating temperature regulation mechanisms which have not developed yet at the early period of life [8].

One of the methods frequently applied in order to reduce adverse effects of high ambient temperature on performances of poultry animals is dietary changes. Several studies reveal that vitamin A, vitamin E, vitamin C, organic acid, prebiotic, probiotic supplementation to feeding rations of broiler chicks prevent the effects caused by temperature [11-14]. Orange oil (essence), is an essential oil produced by cells in peel of orange fruit *(Citrus sinensis fruit)*. Compared to numerous essential oils, orange oil is extracted as a by-product via centrifugation in production of orange juice and produced as cold pressed oil. A great majority of its components consists of d-limonene (more than 90%) and a very little part of them is β-myrcene (2-2.1%) [15]. In some studies conducted recently, orange peel essential oil (OEO) was used as antiparasitic [16], antifungal [17], antioxidant, antimicrobial, and growth regulator [18] agent.

Japanese quails *(Coturnix coturnix japonica)*, are produced as a source of meat and egg in several countries of the world. They are preferred more as an animal model in biological studies compared to other domesticated poultry animals because they reach sexual maturity in early period and have high egg laying rates, low feed and area requirements, and the most importantly they display high durability against environmental conditions [19].

In previous years, several studies [13,14,18] in which various herbal nutritional supplements were used were conducted in order to decrease numerous adverse effects caused by heat stress, including their effects on morphological structures of small intestines. Upon the deficiency we observed as a result of literature reviews; in this study, it was aimed to measure the effects of early period thermal conditioning, fasting, supplementation of OEO or combinations of them on small intestine morphology and density of serotonin-immunoreactive (IR) endocrine cells (ECs) found in small intestines.

MATERIAL and METHODS

Animal, Feeding and Experimental Design

This study was approved by Fırat University Committee of Animal Welfare and Use. Experimental procedure and protocol of the study was found appropriate by the committee (FUHADEK) (FUHADEK, Approval Number: 04.04.2013/55). 168 7-day-old Japanese quail chicks used for this study were weighed and divided randomly. With a totally random design, 4 replicate clusters (30x80 cm) were formed by dividing them into 6 groups including 7 chicks in each cage. Then, fasting and thermal conditioning among early period stress factors were applied to 4 groups for 24 h. While two of these 4 groups were exposed to fasting, other two were applied thermal conditioning (36±1°C and 70-80%, relative humidity). Accordingly, the groups were formed as follows: 1) The group without stress and supplementation (Negative Control) (NC), 2) Fasting group (F), 3) The thermal conditioning group (TC), 4) The group with supplementation of OEO and without stress (Positive control) (PC+OEO), 5) The group that was subjected to fasting and supplemented with OEO (F+OEO), 6) The group that was applied thermal conditioning and supplemented with OEO (TC+OEO). The temperature stress of 33±1°C was applied to all groups for 6 h on 42th day at the end of application.

Following the early period stress factors applied, conventional production and enterprise procedures were carried out until 42th day (for 35 days). The quails were exposed to continuous light, fed and given water *ad libitum*. The diet was calculated in such a way to meet feed

requirements of Japanese quails (24% HP and 2900 kcal/kg ME for beginning and growth periods) in accordance with recommendation of National Research Council [20]. 1 kg zeolite was supplemented as carrier for each 100 kg basal diet. In essential oil groups, 30 g OEO was mixed with 970 g zeolite in order to provide an essential oil concentration of 300 ppm and added into basal diet. Substances found in diet and composition of the diet were given in *Table 1*. The concentration of the volatile component of OEO was shown in *Table 2*.

Chemical composition of food contents (dry matter, crude protein, ash, and ether extract) was analyzed in accordance with AOAC procedures [21] and the amount of crude cellulose was determined according to methods of Crampton and Maynard [22].

Table 1. *Ingredients and chemical composition of standard diet (g/kg)*

Feed Ingredients	Starting and Growing	Calculated Analysis	
Maize	564.3	Crude protein	236.0
Soybean meal	315.0	ME, MJ/kg	12.7
Vegetable oil	30.0	Ether extract	46.5
Fish meal	58.0	Crude cellulose	25.5
Dicalcium phosphate	8.0	Crude ash	63.5
Calcium carbonate	8.0	Calcium	8.1
Salt	2.5	Available Phosphorus	3.6
DL-Methionine	0.5	Methionine + Cystine	8.4
L-Lysine	0.2	Lysine	13.9
Vitamin-Mineral Premix*	2.5		
Zeolite**	10.0		
Total	1000.0		

** Provided per kg of diet: retinol, 2.64 mg; cholecalciferol, 0.04 mg; dl-a-tocopherol-acetate, 11 mg; riboflavin, 9.0 mg; pantothenic acid, 11.0 mg; vitamin B_{12}, 0.013 mg; niacin, 26 mg; choline, 900 mg; vitamin K, 1.5 mg; folic acid, 1.5 mg; biotin, 0.25 mg; iron, 30 mg; zinc, 40 mg; manganese, 60 mg; copper, 8 mg; selenium, 0.2 mg; ** No added orange peel essential oil groups (10 g zeolit); 300 ppm orange peel essential oil added groups (3 g orange oil + 7 g zeolite)*

Table 2. *The concentration of the volatile components in orange peel essential oil (%)*

Analysis	Result*
Limonene	92.3%
Beta Myrcene	3.3%
Alpha Pinen	1.4%
Linalool	0.9%
Sabinen	0.6%
Delta 3 Caren	0.2%
Octanal	0.2%
Undefined	1.1%

** obtained by GC-MS analysis*

Morphometric Analyses

At the end of experiment, 10 quails from each experimental groups that had body weights near to group averages were decapitated by cutting jugular veins. Tissue parts were taken from segments of small intestine through abdominal dissection and divided into 3 segments; duodenum, jejunum, and ileum. 2-cm long tissue parts taken from middle points of each small intestine segments (duodenum, jejunum, and ileum) were washed with cold sterile saline solution. Then, they were fixed with formalin containing 10% neutral buffer. Fixed tissues were washed respectively, dehydrated through series of increasing alcohol concentrations, clearing through series of xylol, and embedded within paraffin blocks. 5-7 µm-thick cross sections taken by using a microtome were placed on glass slides covered with normal and poly-L-lysine. Cross sections placed on normal glass slides were processed according to conventional hematoxylin and eosin staining method [23]. Finally, they were examined by using light microscope (Olympus CX31, Olympus USA) equipped with digital imaging system (Olympus DP20, Olympus USA).

In all morphometric examinations, villus heights, villus bottom widths, villus edge widths and crypt depths related to villi were measured from 7 consecutive villus-crypt complex. Villus heights [AB] was measured form joining point of villi and crypts to top points of villus. Crypt depths [CD] was measured from the middle point of two neighboring villi to base of crypts. While villus edge widths [EF] was measured from just below top point of villus, villus bottom widths [GH] was measured from just above the joining point of villi and crypts *(Fig. 1)*. By using the measured values, ratios of villus height/crypt depth (VH/CD) were also calculated [24].

Immunohistochemical Analysis

Deparaffinization of sections placed on glass slides covered with poly-L-lysine was performed. They were passed through series of alcohol in decreasing concentration beginning from absolute alcohol. The samples were boiled in citrate buffer (10 mM citric acid, pH 6.0) for 20 min in order to enhance antigenity of the ECs. Immunohistochemical analysis was carried out according to protocol of Histostain®-Plus 3rd Gen IHC Detection Kit (Invitrogen: Cat. No. 85-9673). They were incubated with rabbit-anti serotonin (Zymed Lab., 18.0077) antibody at 4°C for over-night in order to determine presence of serotonin-IR ECs in duodenum, jejunum, and ileum of the quails. Immuno-staining was ensured to occur by treating sections with DAB chromogen found in kit for 5 min at the end of protocol. Mayer's hematoxylin was used as counterstain and sections were covered with coverslips by entellan. Sections were examined with Olympus CX31 microscope and photographs were taken. The IR ECs on each section were counted at 10x40 times magnification. The mean numbers of IR ECs in each sample obtained

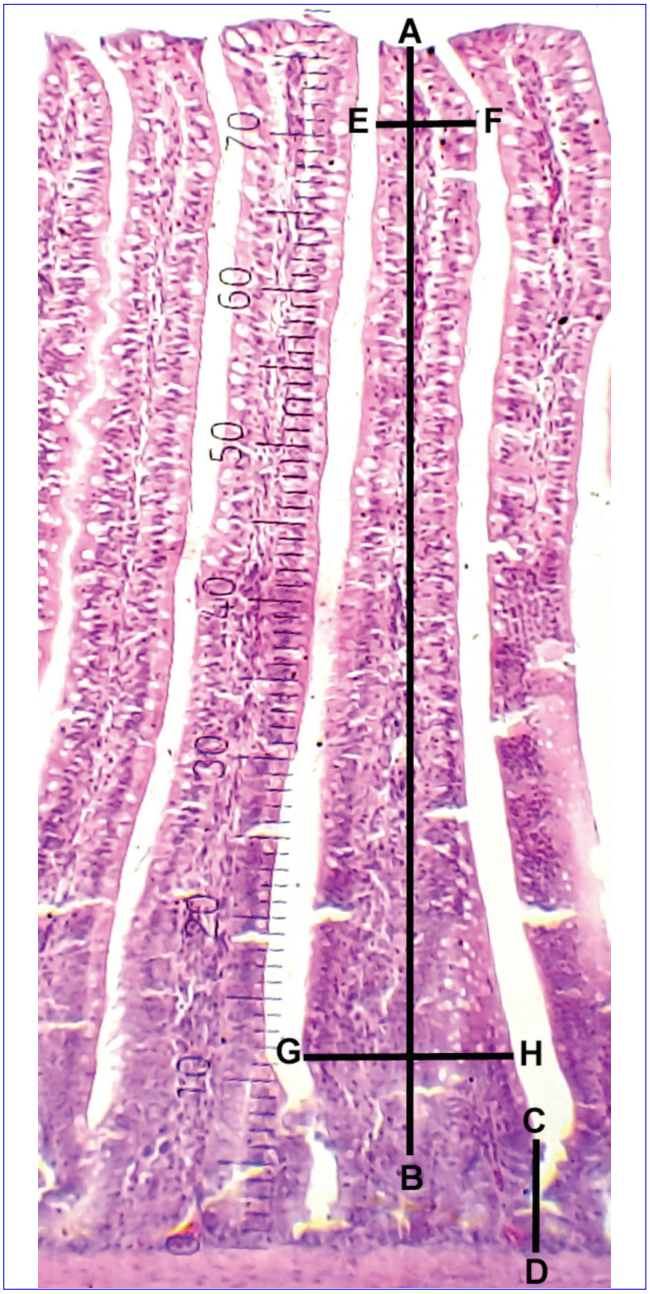

Fig 1. Section of the jejunum (thermal conditioned and OEO supplemented group), haematoxylin-eosin stained, showing the measured parameters. Villus height [AB], crypt depth [CD], villus edge widths [EF] and villus bottom widths [GH], (x 200)

from small intestines were determined by counting the IR ECs in 10 randomly selected microscopic fields with 40x magnification (quantification of IR ECs number/ microscopic field).

Statistical Analysis

The data were subjected two-way anova analysis by using GLM (General Linear Model) procedure. Then, significant differences were subjected to Duncan's multiple range test [25]. The results were accepted as significant if *P* values were < 0.05.

RESULTS

Morphometric Results

Morphometric changes caused by feed restriction, early period thermal conditioning, and OEO supplementation into ration in small intestines of quails were showed in *Table 3*.

It was determined in the present study that feed restriction and early period thermal conditioning significantly increased the villus heights of duodenum in control feeding group compared to negative control group ($P<0.001$). On the other hand, supplementation of OEO into ration significantly decreased villus heights of duodenum compared to control group ($P<0.001$). The longest villus height of duodenum in groups with OEO supplemented into ration was found in feed restriction group ($P<0.001$). Early period thermal conditioning was also determined to have a reducing effect on duodenum crypt depth in groups with control feeding ($P<0.05$). It was observed that there was an interaction between application and OEO in terms of duodenum crypt depth. Feed restriction and early period thermal conditioning increased duodenum VH/CD ratio significantly ($P<0.001$). OEO supplemented into ration decreased VH/CD ratio significantly ($P<0.001$). A difference was not found between feed restriction and early period thermal conditioning in terms of duodenum VH/CD ratio ($P>0.05$). Interaction between OEO supplemented into ration and applications was found to be significant.

It was determined that feed restriction and early period thermal conditioning increased significantly jejunum villus height in groups with OEO supplemented into ration ($P<0.001$). OEO supplemented into ration also increased villus edge width ($P<0.05$) and crypt depth ($P<0.01$) of jejunum. While feed restriction and early period thermal conditioning significantly increased VH/CD ratio of jejunum ($P<0.01$), OEO supplemented into ration did not affect this ratio ($P>0.05$).

Ileum villus height was found to increase in groups with OEO supplemented into ration ($P<0.01$). The highest ileum villus height in both control feeding and OEO supplemented groups was observed in early period thermal conditioning application ($P<0.001$). It was determined that OEO supplemented into ration decreased villus bottom width ($P<0.01$) and edge width ($P<0.05$) in ileum. The effect of applications on ileum VH/CD ratio was not detected ($P>0.05$).

Immunohistochemical Results

The effect of feed restriction, early period thermal conditioning, and OEO supplemented into ration on serotonin-IR ECs number found in small intestines *(Fig. 2)* of quails was showed in *Table 4*.

It was observed that early period thermal conditioning increased the number of cells in jejunum in control feeding groups (P<0.001), but decreased the number of cells in ileum (P<0.05). Early period thermal conditioning increased the number of serotonin-IR ECs in crypts of duodenum (P<0.001), jejunum (P<0.001), and ileum (P<0.05) in groups with OEO supplemented into ration. OEO supplemented into ration increased the serotonin-IR ECs number in crypts of duodenum (P<0.001), jejunum (P<0.001), and ileum (P<0.05).

Table 3. *Effects of orange peel essential oil supplementation and thermotolerance on histo-morphometric features of small intestines, (μm) Mean±S.E.M., (n=60)*

Histological Parameters	Control			Orange Peel Essential Oil, 300 ppm			P		
	NC	F	TC	PC	F	TC	O	T	O x T
Duodenum									
Villus height	825.71±21.61^{B}	999.95±23.58^{A}	939.04±30.89^{A}	659.53±27.02^{b}	807.47±21.87^{a}	699.42±23.21^{b}	***	***	NS
Villus bottom width	158.85±9.44	152.96±10.06	173.80±13.23	154.63±7.03	160.29±10.56	161.31±12.02	NS	NS	NS
Villus edge width	99.71±5.76	106.88±5.29	112.14±4.46	103.42±4.21	105.30±6.11	102.32±5.26	NS	NS	NS
Crypt depth	147.1±11.56^{A}	105.80±4.46^{AB}	102.14±3.17^{B}	127.49±6.35	122.70±4.22	116.09±5.83	NS	*	*
Villus height/Crypt depth	5.61±0.56^{B}	9.45±0.69^{A}	9.19±0.59^{A}	5.17±0.39^{b}	6.58±0.30^{a}	6.02±0.43^{a}	***	***	*
Jejunum									
Villus height	522.25±18.34	563.94±28.70	541.12±17.98	453.50±16.09^{b}	575.84±17.31^{a}	575.38±16.53^{a}	NS	***	NS
Villus bottom width	107.26±7.56	189.61±7.29	84.16±5.10	107.75±5.64	111.51±5.44	107.32±4.64	NS	NS	NS
Villus edge width	77.49±4.24	73.15±4.16	71.54±2.62	74.74±3.28	82.42±4.22	82.11±3.42	**	NS	NS
Crypt depth	83.80±6.23	86.70±5.80	78.03±3.43	95.39±5.37	83.06±3.17	86.07±4.48	*	NS	NS
Villus height/Crypt depth	6.23±0.59	6.50±0.91	6.93±0.84	4.75±0.36^{b}	6.93±0.35^{a}	6.68±0.38^{a}	NS	**	NS
Ileum									
Villus height	343.04±9.76^{B}	355.91±16.32^{AB}	409.69±14.57^{A}	375.28±12.72^{b}	410.61±12.41^{ab}	436.96±18.10^{a}	**	***	NS
Villus bottom width	98.99±4.56	98.95±6.20	89.12±5.26	86.39±3.31	83.22±4.04	81.28±4.77	**	NS	NS
Villus edge width	76.86±3.77	75.15±4.52	73.92±3.33	71.41±2.95	59.73±2.21	71.6±2.76	*	NS	NS
Crypt depth	50.49±2.69	56.08±3.49	65.67±3.53	64.52±2.61	62.21±2.39	59.54±3.80	NS	NS	NS
Villus height/Crypt depth	6.79±0.41	6.34±0.74	6.23±0.46	5.81±0.53	6.60±0.44	7.33±0.46	NS	NS	NS

NC: *Negative control,* ***F:*** *Fasted, TC: Thermal conditioned,* ***PC:*** *Positive control,* ***O:*** *Oil,* ***T:*** *Treatment,* ***NS:*** *Not significant, * P<0.05, ** P<0.01, *** P<0.001; -The differences between the mean values with different superscripts (A, B: P<0.05) in the same row within the no added groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests. -The differences between the mean values with different superscripts (a, b: P<0.05) in the same row within the orange peel essential oil groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests*

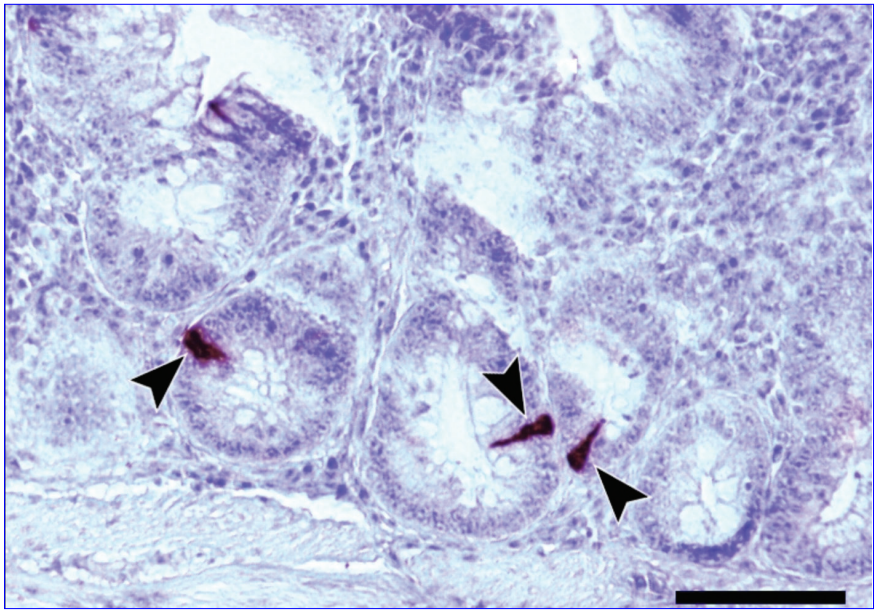

Fig 2. Serotonin-immunoreactive endocrine cells *(arrowheads)* showed in the crypt epithelium of the duodenum (negative control group)

Table 4. *Effects of orange peel essential oil supplementation and thermotolerance on serotonin-releasing endocrine cell numbers of small intestines, (μm) Mean±S.E.M. (n=60)*

Parts of Small Intestine	Control			Orange Peel Essential Oil, 300 ppm			P		
	NC	F	TC	PC	F	TC	O	T	O x T
Duodenum	4.22±0.32	2.96±0.28	3.70±0.31	4.16±0.40^{b}	4.00±0.28^{b}	7.24±0.38^{a}	***	***	***
Jejunum	1.32±0.15^{B}	0.74±0.14^{B}	3.10±0.46^{A}	2.20±0.49^{b}	1.82±0.23^{b}	3.00±0.31^{a}	*	***	NS
Ileum	3.16±0.46^{A}	2.10±0.28^{AB}	1.80±0.24^{B}	2.40±0.28^{b}	2.28±0.18^{b}	4.13±0.51^{a}	*	*	***

NC: *Negative control,* ***F:*** *Fasted,* ***TC:*** *Thermal conditioned,* ***PC:*** *Positive control,* ***O:*** *Oil,* ***T:*** *Treatment,* ***NS:*** *Not significant, * P<0.05, ** P<0.01, *** P<0.001; -The differences between the mean values with different superscripts (A, B: P<0.05) in the same row within the no added groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests. -The differences between the mean values with different superscripts (a, b: P<0.05) in the same row within the orange peel essential oil groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests*

DISCUSSION

Intestinal villi are projections that protrude from mucosa of small intestine towards lumen in order to extend the surfaces of digestion and absorption [26,27]. All epithelial cells in villi form by mitotic division of stem cells found in base parts of intestinal crypts and they can reach up to top of villus within a few days by migrating throughout the surface of villus. Such a cellular migration can be altered by the intestinal function [26]. Intestinal crypts are glands found in lamina propia and consist of different cell types including hormone secreting ECs [27]. Intestinal mucosa can activate rapid morphological and functional adaptation mechanisms in response to environmental and nutritional changes [28]. A great majority of intestinal morphology studies conducted in recent years indicate that heat stress decreases villus heights of duodenum [29-32], jejunum [30,32], and ileum [30,32,33], and villus width of duodenum and jejunum [31] in poultry animals. Decreases observed in villus heights and villus widths may be associated with feed intake reduced depending on heat stress [29]. Differently, Burkholder et al.[7] reported that heat stress of 24 h did not change villus height and VH/CD ratio, but decreased crypt depth. On the other hand, Santos et al.[32] determined that heat stress increased villus width in duodenum, decreased VH/CD ratio in duodenum and ileum, and increased crypt depth in jejunum. Sandıkçı et al.[29] showed that heat stress also did not have any effect on villus width of duodenum. Differences between our results and previous studies may be related to starting age and application period of heat stress.

In numerous studies, it was reported that early period thermal conditioning adjusted the body weight gain decreasing due to heat stress [8,34], increased the decreasing slaughter body weight [34,35], caused decreasing plasma T_3 concentration [8,36], decreased the high body temperature [8,37], and decreased the mortality at high temperature applied before slaughtering [8]. When literature information were examined in details, a limited number of study were found about the effects of thermal conditioning and feed restriction on morphology of small intestine. El-Badry et al.[38] showed that early period thermal conditioning increased villus height of jejunum but did not change the crypt depth when compared to control group. Uni et al.[36] determined that early thermal conditioning narrowed villi in jejunum just 24 h after the application and increased the distance between neighboring villi, and accordingly decreased villus volume significantly. However, they reported a rapid increase was observed in villus volume and the highest villus volume was observed 48-72 h after thermal conditioning. The present study reveals that early period thermal conditioning increased the villus height of duodenum and ileum, and did not change the villus height of jejunum in groups with control feeding compared to negative control group. Early period thermal conditioning and feed restriction increased duodenum VH/CD ratio significantly. Early period thermal conditioning increased significantly villus height of jejunum and ileum in groups with OEO supplemented into ration. On the other hand, thermal conditioning had a reducing effect on duodenum crypt depth in groups with control feeding *(Table 3)*. When the results were compared, it was observed that while there was agreement in terms of villus height, a difference was present in terms of crypt depth. This difference may be arising from the age at which thermal conditioning was applied or difference in degree of temperature applied.

In a study conducted on broiler chicks, it was reported that body weight was suppressed at first depending on feed restriction, it was balanced with control group as a result of rapid compensatory growth observed in following process, plasma T_3 concentration was suppressed, mortality occurring due to being exposed to temperature on 42th day decreased [10]. Numerous light microscopy studies also reported that fasting applications caused a decrease in intestinal villus heights. However, the decrease in villus height observed based on fasting was stated to recover depending on nutrition 1 day after re-feeding [26,39]. Yamauchi et al.[41] determined that 24-h feed restriction at early age period decreased significantly villus heights of duodenum and gradually those of jejunum, but a significant decrease was not observed in ileum. In the same study, they reported that a distinct increase was seen for all intestinal villus heights just 24 h after re-feeding started following the feed restriction. They observed that recovery in villus heights occurred rapidly in duodenum but slowly

in ileum. Slow recovery of ileum villus height may indicate that ileum could be less effective in absorptive function. In accordance with the results in these studies, we determined that feed restriction increased villus heights of duodenum significantly in control feeding groups compared to negative control group and significantly villus heights of jejunum in groups with OEO supplemented into ration, but did not cause any change in ileum. In previous studies, feed restriction was reported to decrease significantly mitotic division in intestinal cells. Transition to re-feeding also increases mitotic division, thus activates cell renewal and makes it to reach control values. According to these researchers, the change in villus height occurred as a result of the increase in number of epithelial cells depending on increased mitosis in cells [40,41].

In a recent study conducted on broilers fed under high ambient temperature; it was reported that extracts of orange and lemon peels supplemented into ration did not affect villus height, villus depth, crypt width, and VH/CD ratio [42]. On the other hand, in the present study, we determined that OEO supplemented into ration decreased villus heights of duodenum compared to control group and increased villus heights of ileum. We also determined that OEO supplemented into ration increased villus end width and crypt depth of jejunum, but decreased both villus edge width and bottom width in ileum. We observed there was interaction between application and OEO in terms of duodenum crypt depth. While supplementation of OEO into ration decreased VH/CD ratio in duodenum, VH/CD ratio did not change in jejunum. Differences between results of these two studies may be associated with the amount of herbal supplement added into feed, different starting age, and different time of supplementation. It was reported in another study that a mixture of oregano, cinnamon, and pepper essential oils (200 mg/kg) increased villus height [43].

Serotonin (5-hydroxytryptamine, 5-HT), is a monoamine neurotransmitter. It is released from serotonergic neurons of central nervous system and enterochromaffin cells of gastrointestinal tissue. Approximately 95% of peripheral serotonin is released and stored by enterochromaffin cells found in crypts in gastrointestinal tissue. In addition to the other functions of serotonin, it also has functions related to cardiovascular and gastrointestinal tract smooth muscles, and cell growth and differentiation. Serotonin activates neural reflexes and plays a vital role in intestinal secretion, sensitivity, and peristaltic movements [44]. In rats, it was stated that while feed restriction of 24 and 48 h caused a decrease in brain serotonin level, it led to an increase in gastrointestinal serotonin level [45]. When we reviewed the literature, we did not encounter any study concerning the effects of early period thermal conditioning and supplementation of OEO into ration on serotonin-IR ECs in intestinal tract. In the present study, it was determined that thermal conditioning increased the number of serotonin-IR ECs in jejunum, but decreased in ileum in groups with control feeding. It was found that supplementation of OEO into ration increased the number of serotonin-IR ECs in all crypts of small intestine. Early period thermal conditioning increased the number of serotonin-IR ECs in duodenum, jejunum, and ileum especially in groups with OEO supplemented into ration. Şimşek et al.[46] reported that prebiotics/probiotics and/or organic acids and antibiotics supplemented into feeds of quails caused a significant decrease in the number of serotonin-IR ECs in all intestinal segments compared to control group. The difference between results is likely resulted from difference of supplements supplemented into ration.

Consequently, in this study, we examined the effects of thermal conditioning, feed restriction, supplementation of OEO into ration or combinations of them on morphology of small intestine and the number of serotonin-IR ECs in small intestine. We determined that feed restriction and thermal conditioning increased villus height in control groups for duodenum and in OEO groups for jejunum. We also found that while feed restriction and thermal conditioning increased VH/CD ration in duodenum and jejunum, these applications did not affect this ratio in ileum. Early period thermal conditioning, particularly supplementation of OEO into ration increased the number of serotonin-IR ECs in all crypts of small intestine. We showed that supplementation of OEO into ration increased the number of serotonin-IR ECs in duodenum, jejunum, and ileum in groups with OEO supplemented into ration. In addition, the effects of early period thermal conditioning, feed restriction, and herbal extract essential oils especially on serotonin-IR ECs should be supported by new results in future further studies.

REFERENCES

1. FAO Statistics: http://faostat.fao.org/, 2010.

2. Renaudeau D, Collin A, Yahav S, de Basilio V, Gourdine JL, Collier RJ: Adaptation to hot climate and strategies to alleviate heat stress in livestock production. *Animal*, 6, 707-728, 2012. DOI: 10.1017/S1751731111002448

3. Humphrey T: Are happy chickens safer chickens? Poultry welfare and disease susceptibility. *Br J Poultry Sci*, 47, 379-391, 2006. DOI: 10.1080/00071660600829084

4. Deyhim F, Teeter RG: Sodium and potassium chloride drinking water supplementation effects on acid-base balance and plasma corticosterone in broiler reared in thermoneutral and heat-distressed environments. *Poultry Sci*, 70, 2551-2553, 1991. DOI: 10.3382/ps.0702551

5. Potten CS, Loeffler M: A comparative model of the crypts of the small intestine of the mouse provides insight into the mechanisms of cell migration and the proliferation hierarchy. *J Theor Biol*, 127, 381-391, 1987. DOI: 10.1016/S0022-5193(87)80136-4

6. Johnson LR, McCormack SA: Regulation of gastrointestinal mucosal growth. **In,** Johnson LR (Ed): Physiology of the Gastrointestinal Tract. 611-633, Raven Press, New York, NY, 1994.

7. Burkholder KM, Thompson KL, Einstein ME, Applegate TJ, Patterson JA: Influence of stressors on normal intestinal microbiota, intestinal morphology and susceptibility to *Salmonella entiritidis* colonization in broilers. *Poultry Sci*, 87, 1734-1741, 2008. DOI: 10.3382/ps.2008-00107

8. Yahav S, Hurwitz S: Induction of thermotolerance in male broiler chickens by temperature conditioning at an early age. *Poultry Sci,* 75, 402-406, 1996. DOI: 10.3382/ps.0750402

9. Yahav S, Shamai A, Haberfeld A, Horev G, Hurwitz S, Einat M: Induction of thermotolerance in chickens by temperature conditioning: heat shock protein expression. **In,** Blatteis CM (Ed): An Update in Thermoregulation from Cellular Functions to Clinical Relevance. 628-636, New York Academy of Science, New York, 1997.

10. Yahav S, Plavnik I: Effect of early-age thermal conditioning and food restriction on performance and thermotolerance of male broiler chickens. *Br Poultry Sci,* 40, 120-126, 1999. DOI: 10.1080/00071669987944

11. Lin H, Jiao HC, Buyse J, Decuypere E: Strategies for preventing heat stress in poultry. *World Poultry Sci J*, 62, 71-86, 2006. DOI: 10.1079/WPS200585

12. Solis de los Santos F, Donoghue AM, Farnell MB, Huff GR, Huff WE, Donoghue DJ: Gastrointestinal maturation is accelerated in turkey poults supplemented with a mannan-oligosaccharide yeast extract (alphamune). *Poultry Sci*, 86, 921-930, 2007. DOI: 10.1093/ps/86.5.921

13. Awad WA, Ghareeb K, Abdel-Raheem S, Böhm J: Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. *Poultry Sci*, 88, 49-55, 2009. DOI: 10.3382/ps.2008-00244

14. El-Moniary MMA, Hemid AA, El-Wardany I, Gehad AE, Gouda A: The effect of early age heat conditioning and some feeding programs for heat-stressed broiler chicks on: 1-Productive performance. *World J Agric Sci,* 6, 689-695, 2010.

15. Sun J: D-limonene: Safety and clinical applications. *Altern Med Rev,* 12, 259-264, 2007.

16. Abdelqader A, Qarallah B, Al-Ramamneh D, Daş G: Anthelmintic effects of citrus peels ethanolic extracts against Ascaridiagalli. *Vet Parasitol*, 188, 78-84, 2012. DOI: 10.1016/j.vetpar.2012.03.003

17. Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Rezaee MB, Jaimand K, Alinezhad S, Saberi R, Yoshinari T: Chemical composition and antiaflatoxigenic activity of *Carumcarvi* L., *Thymus vulgaris* and *Citrus aurantifolia* essential oils. *Food Control*, 20, 1018-1024, 2009. DOI: 10.1016/j.foodcont.2008.12.007

18. Hong JC, Steiner T, Aufy A, Lien TF: Effects of supplemental essential oil on growth performance, lipid metabolites and immunity, intestinal characteristics, microbiota and carcass traits in broilers. *Livest Sci*, 144, 253-262, 2012. DOI: 10.1016/j.livsci.2011.12.008

19. Baumgartner J: Japanese quail production, breeding and genetics. *World Poultry Sci J*, 50, 227-235, 1994.

20. National Research Council: Nutrient Requirements of Poultry, 9th edn., Washington, DC, National Academic Press, 1994.

21. AOAC: Official Methods of Analysis Association of AOAC International. 17th edn., AOAC International Maryland, 2000.

22. Crampton EW, Maynard LA: The Relation of cellulose and lignin content to nutritive value of animal feeds. *J Nutr,* 15, 383-395, 1983.

23. Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd edn., 32-33, Blackiston Division, McGraw-Hill Book Company, 1968.

24. Song J, Jiao LF, Xiao K, Luan ZS, Hu CH, Shi B, Zhan XA: Cello-oligosaccharide ameliorates heat stress-inducedimpairment of intestinal microflora, morphology and barrierintegrity in broilers. *Anim Feed Sci Tech,* 185, 175-181, 2013. DOI: 10.1016/j.anifeedsci.2013.08.001

25. SPSS, Inc: SPSS for Windows Release 11.5 Standard Version, Copyright SPSS Inc., Chicago, 2002.

26. Yamauchi K: Review on chicken intestinal villus histological alterations related with intestinal function. *J Poultry Sci*, 39, 229-242, 2002. DOI: 10.2141/jpsa.39.229

27. Yörük M: Sindirim sistemi II: Sindirim kanalı. **In,** Özer A (Ed): Veteriner Özel Histoloji. 161-183, Nobel Yayın Dağıtım, Ankara, 2008.

28. Garriga C, Hunter RR, Amat C, Planas JM, Mitchell MA, Moreto M: Heat stress increases apical glucose transport in the chicken jejunum. *Am J Physiol Regul Integr Comp Physiol*, 290, 195-201, 2006. DOI: 10.1152/ajpregu.00393.2005

29. Sandıkçı M, Eren U, Onol AG, Kum S: The effect of heat stress and the use of *Saccharomyces cerevisiae* or (and) bacitracin zinc against heat stress on the intestinal mucosa in quails. *Revue Med Vet*, 155, 552-556, 2004.

30. Karslı MA, Dönmez HH: Sıcaklık stresi oluşturulan broylerlerde rasyona ilave edilen bitki ekstraktının büyüme performansı ve ince bağırsak villusları üzerine etkisi. *Atatürk Üniv Vet Bil Derg,* 2, 143-148, 2007.

31. Ashraf S, Zaneb H, Yousaf MS, Ijaz A, Sohail MU, Muti S, Usman MM, Ijaz S, Rehman H: Effect of dietary supplementation of prebiotics and probiotics on intestinal microarchitecture in broilers reared under cyclic heat stress. *J Anim Physiol Anim Nutr,* 97, 68-73, 2013. DOI: 10.1111/jpn.12041

32. Santos RR, Awati A, Roubos-Van den Hil PJ, Tersteeg-Zijderveld MHG, Koolmees MA, Fink-Gremmels J: Quantitative histo-morphometric analysis of heat-stress-related damage in the small intestines of broiler chickens. *Avian Pathol*, 44, 19-22, 2015. DOI: 10.1080/03079457.2014.988122

33. Deng W, Dong XF, Tong JM, Zhang Q: The probiotic *Bacillus licheniformis* ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poultry Sci*, 91, 575-582, 2012. DOI: 10.3382/ps.2010-01293

34. Oral Toplu HD, Nazlıgül A, Karaarslan S, Kaya M, Yagın O: Effects of heat conditioning and dietary ascorbic acid supplementation on growth performance, carcass and meat quality characteristics in heat-stressed broilers. *Ankara Üniv Vet Fak Derg*, 61, 295-302, 2014.

35. Çiftçi M, Şimşek ÜG, Azman MA, Çerçi İH, Tonbak F: The effects of dietary rosemary *(Rosmarinus officinalis L.)* oil supplementation on performance, carcass traits and some blood parameters of Japanese quail under heat stressed condition. *Kafkas Univ Vet Fak Derg*, 19, 595-599, 2013. DOI: 10.9775/kvfd.2012.8474

36. Uni Z, Gal-Garber O, Geyra A, Sklan D, Yahav S: Changes in growth and function of chick small intestine epithelium due to early thermal conditioning. *Poultry Sci*, 80, 438-445, 2001. DOI: 10.1093/ps/80.4.438

37. Teeter R, Smith M, Wiernusz C: Research note: Broilers acclimation to heat distress and feed intake effects on body temperature in birds exposed to thermoneutral and high ambient temperature. *Poultry Sci*, 71, 1101-1104, 1992. DOI: 10.3382/ps.0711101

38. El-Badry ASO, El-Kholy KH, Mahrousa MH: Effect of early-age acclimation on development of small intestine and growth performance in Muscovy ducks during summer season. *4th World Poultry Conference*, Sharm El- Sheikh, Egypt, 27-30 March, 2007.

39. Yamauchi K, Tarachai P: Changes in intestinal villi, cell area and intracellular autophagic vacuoles related to intestinal function in chickens. *Br Poultry Sci,* 41, 416-423, 2000. DOI: 10.1080/00071660050194902

40. Yamauchi K, Buwjoom T, Koge K, Ebashi T: Histological intestinal recovery in chickens refed dietary sugar cane extract. *Poultry Sci*, 85, 645-651, 2006. DOI: 10.1093/ps/85.4.645

41. Yamauchi K, Kamisoyama H, Isshiki Y: Effects of fasting and refeeding on structures of the intestinal villi and epithelial cells in White Leghorn hens. *Br Poultry Sci,* 37, 909-921, 1996. DOI: 10.1080/00071669608417922

42. Akbarian A, Golian A, Gilani A, Kermanshahi H, Zhaleh S, Akhavan A, De Smet S, Michiels J: Effect of feeding citrus peel extracts on growth performance, serum components, and intestinal morphology of broilers exposed to high ambient temperature during the finisher phase. *Livestock Sci*, 157, 490-497, 2013. DOI: 10.1016/j.livsci.2013.08.010

43. Garcia V, Catala-Gregori P, Hernandez F, Megias MD, Madrid J: Effect of formic acid and plant extracts on growth, nutrient digestibility, intestine mucosa morphology, and meat yield of broilers. *J Appl Poult Res*, 16, 555-562, 2007. DOI: 10.3382/japr.2006-00116

44. Jonnakuty C, Gragnoli C: What do we know about serotonin? *J Cell Physiol*, 217, 301-306, 2008. DOI: 10.1002/jcp.21533

45. Bubenik GA, Ball RO, Pang SF: The effect of food deprivation on brain and gastrointestinal tissue levels of tryptophan, serotonin, 5-hydroxyindoleacetic acid, and melatonin. *J Pineal Res*, 12, 7-16, 1992. DOI: 10.1111/j.1600-079X.1992.tb00020.x

46. Şimşek N, Can I, Karadeniz A, Kara A, Gümüş R: Effects of dietary various supplementations on the mucin- and serotonin- releasing cell numbers in small intestine of quails. *Revue Méd Vét*, 163, 328-334, 2012.

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Research Article

Effects of Different Doses of Boric Acid Injected into Chicken Egg on Bursa of Fabricius and Spleen: A Histological and Stereological Study [1]

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Abstract

The aim of the study was to investigate the effects of different doses of boric acid injected into chicken eggs on bursa of Fabricius and spleen. The study material consisted of three treatment and one control group. On the fourth day of incubation, boric acid dissolved in 0.9% NaCl were injected into eggs at 1.000, 1.500 and 2.000 ppm doses and control group received only 0.9% NaCl injection. Chicks hatched were raised until ten weeks of age (n=10). Bursa of Fabricius and spleen were evaluated by histological, immunohistochemical and stereological methods. Weight of bursa of Fabricius, relative weight of bursa of Fabricius ($P<0.01$), volume of bursa of Fabricius, cortical thickness, follicle area and area of follicular medulla significantly reduced ($P<0.001$) by injection of 1.500 ppm boric acid compared to control group. The number of white blood cells also decreased in 1.000 ppm and 1.500 ppm groups. An increase ($P<0.001$) was recorded in hemoglobin ratio in 2.000 ppm group when compared with the other treatment groups and control group. There was an increase in 1500 ppm group in terms of spleen weight ($P<0.001$), relative spleen weight ($P<0.01$) and plasma cell count ($P<0.05$) and likewise an increase was noted in 1.000 and 2.000 ppm groups with respect to follicle count and number of apoptotic cells in comparison to control group. In conclusion, low doses of boric acid induced the bursal involution and implicitly increased plasma cell count in the spleen.

Keywords: *Boric acid, Bursa of Fabricius, Spleen, Apoptosis, Chicken*

Yumurtaya Enjekte Edilen Farklı Dozlarda Borik Asitin Bursa Fabricius ve Dalak Üzerine Etkileri: Histolojik ve Stereolojik Çalışma

Özet

Çalışmamızda farklı dozlarda yumurtaya enjekte edilen borik asitin, bursa Fabricius ve dalak üzerine olan etkileri belirlendi. Kuluçkanın 4. gününde %0.9 NaCl içinde çözülmüş 1.000, 1.500 ve 2.000 ppm dozlarında borik asit enjekte edilen deney grupları ve sadece %0.9 NaCl enjekte edilen kontrol grubu kullanıldı. Yumurta çıkımından sonra dişi civcivler 10. haftaya kadar büyütüldü (n=10). Bursa Fabricius ve dalaklar histolojik, immunohistokimyasal ve stereolojik tekniklerle değerlendirildi. 1.500 ppm borik asit uygulanan deney grubunda bursa Fabricius ağırlığı, bursa Fabricius relativ ağırlığı ($P<0.01$), bursa Fabricius hacmi, korteks kalınlığı, follikül alanı ve follikül medulla alanı ($P<0.001$) kontrol grubuna göre istatistiksel önemde düşüş görüldü. Akyuvarlarda 1.000 ppm ve 1.500 ppm grubunda kontrol grubuna göre düşüş tespit edildi. Hemoglobin oranının ise 2.000 ppm ($P<0.001$) dozundaki grupta kontrol ve diğer deney grupları göre bir artış olduğu belirlendi. Dalak ağırlığı ($P<0.001$), relativ dalak ağırlığı ($P<0.01$) ve plazma hücre sayısı ($P<0.05$) bakımından 1.500 ppm deney grubunda kontrol grubuna göre, follikül sayısı ve apoptotik hücre sayısı bakından ise 1.000 ve 2.000 ppm grubunda kontrol grubuna göre bir artış tespit edildi. Bu değerlendirmeler sonucunda borik asidin düşük dozlarda bursa Fabricius'un involasyonuna ve dolaylı olarak dalaktaki plazma hücre sayısında artışa sebep olduğunu belirlendi.

Anahtar sözcükler: *Borik asit, Bursa Fabricius, Dalak, Apoptozis, Tavuk*

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INTRODUCTION

Boron is a widely distributed element which exists in various forms and structures in nature [1]. It is obtained mainly from arid, volcanic and hydrothermal regions of Turkey and USA combined with oxygen (borates) [2]. Boron salts that are most commonly found in nature are known as boric acid and borax [3]. These are widely distributed in soil, water and foods at ppm levels [3,4]. This element was also reported to be essential for humans and animals [5]. Boric acid is a highly exposed simple inorganic compound due to its widespread commercial use [6]. Borax and boric acid are used in several industrial fields, for instance, in the manufacture of glassware and ceramic products, disinfectants, dust repellers, wooden wares and fabrics as flame retarding materials, emery products as well as in rocket fuels as additives [4].

It is beneficial for bone structure and functions and plays role in inflammation process as well as in regulation of the physiologic functions [1,7,8]. It was shown that boron did not exhibit toxic effects on the reproduction system of rats and was found to be effective on skeletal system at high doses [9]. In another study, boron was found to decrease spleen weight and besides, it caused arthritis in rats [10]. Bai and Hunt [11], reported that boron increased the level of antibodies. It has recently been shown that boron affected the immune response against injuries and infections and it particularly had an impact on inflammatory cell population [12]. Experimentally, boron was injected into fertilized turkey eggs and tibial length, body weight and bone ash content of hatching chicks were evaluated. On the basis of histological analysis, increase in bone mineralization and thus enhanced embryonic development were observed [13].

Bursa of Fabricius which is an organ found only in avian species is located between cloaca and sacrum. It is a primary lymphoid organ which functions in the differentiation of B-lymphocytes in birds [14,15]. The alterations in the development of bursa of Fabricius were shown to have adverse effect on antibody production [14,16]. Anlage of bursa of Fabricius arises by the 4-5th days of embryonic development and starts to regress between the following 10-12th weeks of hatching under the influence of steroid hormones [17,18].

This study was conducted in an attempt to determine how chicken was affected by the extensity of boron in the environment. For this purpose, various doses of boron were injected into the chicken eggs during embryonic development and the effects of this element on bursa of Fabricius which is the primary lymphoid organ, in association with the spleen as secondary lymphoid organ in chicken, were investigated.

MATERIAL and METHODS

Animals and Experimental Design

Fertilized eggs were obtained from a local manufacturer (Has Tavuk, Turkey). The eggs were incubated at 37.5ºC with a relative humidity of 60% and turned every three hour. A total of 120 fertilized eggs (Süper Nick) were allocated into 4 groups (3 experimental and one control). Different doses (1.000 ppm, 1.500 ppm and 2.000 ppm, respectively) of boric acid dissolved in 0.9% NaCl were injected into eggs on the 4th day of incubation, when anlage of bursa of Fabricius starts to develop. The control group received only 0.9% NaCl injection (100 µl per each egg). The injections were performed via insulin injector with disposable needles (0.6X15 mm) through a tiny hole drilled into the blind spot of the egg and the substances were injected right into the egg yolk. Then the holes were covered by paraffin and the eggs were placed back in the incubator.

Weights of females were recorded after hatching. The chicks were raised on the floor with wood shaving. Room temperature was maintained at 33ºC between days 0 and 3 and then gradually reduced by 3ºC/week. The chicks were fed on commercial diet *(ad libitum)*. Chicks and experimental conduct were in accordance with the Guidelines for Animal Experiments by the Ethical Committee of Istanbul University (Approval number: 2013/16).

Relative Weights of Bursa of Fabricius and Spleen

At 10 weeks of age, 10 birds in each group were euthanized with xylazine (1.0 mg/kg) and ketamine HCl (20 mg/kg). The body weights were recorded prior to sacrification and then necropsy was performed. Bursa of Fabricius and the spleen were harvested from each chick and weighed after dissecting the connective tissues around the organs. Relative weights of bursa of Fabricius and the spleen were calculated by the following formula:

Relative weight = organ weight (g)/body weight (g) [19].

Preparation of Samples for Histological Methods

Bursa of Fabricius and spleen were fixed in modified Davidson's solution for 24 h and then the organs were cut into 0.5-cm-thick pieces. Paraffin blocks were prepared from each piece and sectioned at 5 µm thickness and then stained with Masson's trichrome stain. Histopathological changes, apoptotic index, follicle count, follicle area, thickness of follicular cortex, area of follicular medulla, height of epithelium and plasma cell count in the spleen were stereologically evaluated.

A light microscope (Leica DM4000 B), a digital camera (MBF Bioscience) and a Stereo Investigator program compatible with this microscope were used for stereological evaluations.

The distribution of progesterone receptors (PR) was evaluated in order to determine the estrogenic activity of boric acid in the bursa of Fabricius.

Stereological Analyses

Total volume of bursa of Fabricius was calculated in accordance with *Cavelier's* principle. It is known that the volume of the regular shaped objects can be estimated by the following formula:

V = t x a;

Where (t) is the height and (a) is the base area of the object.

Slices sectioned in order to calculate the volume of bursa of Fabricius were cut at 5µm-thickness providing that whole surface area of each section has appeared on the slide. A point counting grid was used for the surface area estimation of each slice and the volume of the relevant slice was obtained by multiplying surface area by slice width. Total volume of bursa of Fabricius was calculated by adding up the volumes of all slices [20].

Follicle Count, Follicle Area, Area of Follicular Medulla, Thickness of Cortex and Height of Epithelium

For this purpose, we chose an area fraction approach with an area of an unbiased counting frame of 1.000 x 1.000 μm^2 (1 mm^2) and meander sampling of each sectioned area was done in a 4.000 x 4.000 μm^2 (4 mm^2) step size in a systemic-random manner.

Mean follicle count in an area of 1 mm^2 was determined by dividing the value (follicle count) obtained from each animal by the number of counting steps. A two-dimensional isotropic uniform nucleator was used to measure follicle area, area of follicular medulla, height of epithelium and thickness of cortex on the follicle aligned with the right top corner of the counting frame at every counting step [21].

Plasma Cell Count of Spleen

Sections of 5 µm thickness were prepared and stained with methyl green-pyronin [8]. Six animals from each group were subjected to evaluation. An unbiased counting was achieved in a systematic random manner with a counting frame area of 40 x 40 (1.600 μm^2) and a meander sampling was performed with a step size of 500 x 500 µm (250.000 μm^2). Mean plasma cell count in an area of 1 mm^2 was estimated by dividing the value (plasma cell count) obtained from each animal by the number of counting steps.

Evaluation of Blood Parameters

Blood samples were collected from the animals by heart punctuation into EDTA tubes under anesthesia. Hematocrit value and hemoglobin level were assessed by microhematocrit and spectrophotometric methods, respectively. Additionally, blood smears were prepared, air-dried, fixed in methyl alcohol, stained with Wright stain and finally immersed in Sorensen buffer and kept at room temperature to be evaluated by light microscopy under 100x magnification. One hundred leukocytes were counted on each slide and the ratio of leukocyte subtypes was estimated.

The level of IgY (Cusabio, CSB-E09872Ch) was assessed by ELISA.

Immunohistochemical Staining Method

- Progesterone Receptor (PgR): Sections of bursa of Fabricius were mounted on slides coated with poly-l-lysine (Sigma, St. Louis, MO, USA). Three sections per animal (n = 10 animals per group) were analyzed. Intensity of immuno-labelling was assessed by examination of 10 representative high-power fields (objective 40x) [22].

- Determination of Fragmented DNA in situ: Apoptotic Index (AI) is used as a measure of the extent of apoptosis. To present the apoptotic cells, the fracture in DNA were labelled using Terminal deoxynucleotidyltransferase (TdT)-mediated nick end-labelling (TUNEL) technique in paraffin sections, following the procedure of applied kit (Apop Tag® Peroxidase In Situ Apoptosis Detection Kit, EMD Millipore). AI was detected in each section by light microscopy. For this purpose, in each case, TUNEL-positive cells and total cells were counted in 10 random areas, under 40x magnification [23].

- Apoptotic Index (AI): Percentage values of apoptotic and non-apoptotic cells were calculated. Ten different microscopic fields were determined on the sections obtained from different portions of bursa of Fabricius of each animal by systematic random sampling (Area: 50 x 75 μm^2). AI was calculated by the formula: 100x (mean number of TUNEL positive cells in 10 random fields)/(mean number of total cells in 10 random fields).

AI= 100 x (positive cell number/total cell number) [23].

Statistical Analyses

Statistical analyses were performed with Duncan's One-Way ANOVA Method by using Windows SPSS (Statistical Package for the Social Science Version 10.0).

RESULTS

Histological and Stereological Evaluation in the Bursa of Fabricius

Histologically, no difference was noted in the cortical and medullary portions of bursa of Fabricius in the control and experimental groups in accordance with the above mentioned properties except for the dose of 1.500 ppm which caused marked extracellular vacuolization particularly in the medullary regions of bursal follicles in this group *(Fig. 1)*.

Although follicle area and area of follicular medulla

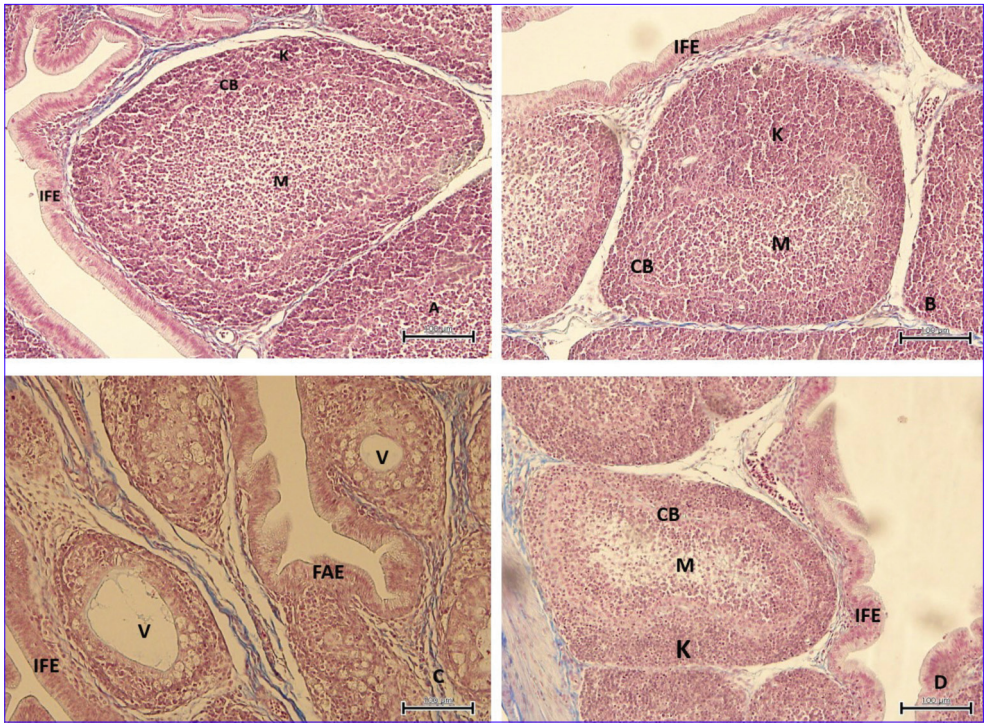

Fig 1. Histological Appearence of bursa of Fabricius in control and experimental groups, A: Control, B: 1.000 ppm, C: 1.500 ppm, D: 2000 ppm, K: Cortex, M: Medulla, CB: Corticomedullary border, IFE: Interfollicular epithelium, FAE: Follicle associated epithelium, V: Vacuolization, H&E, Bar: 100 µm

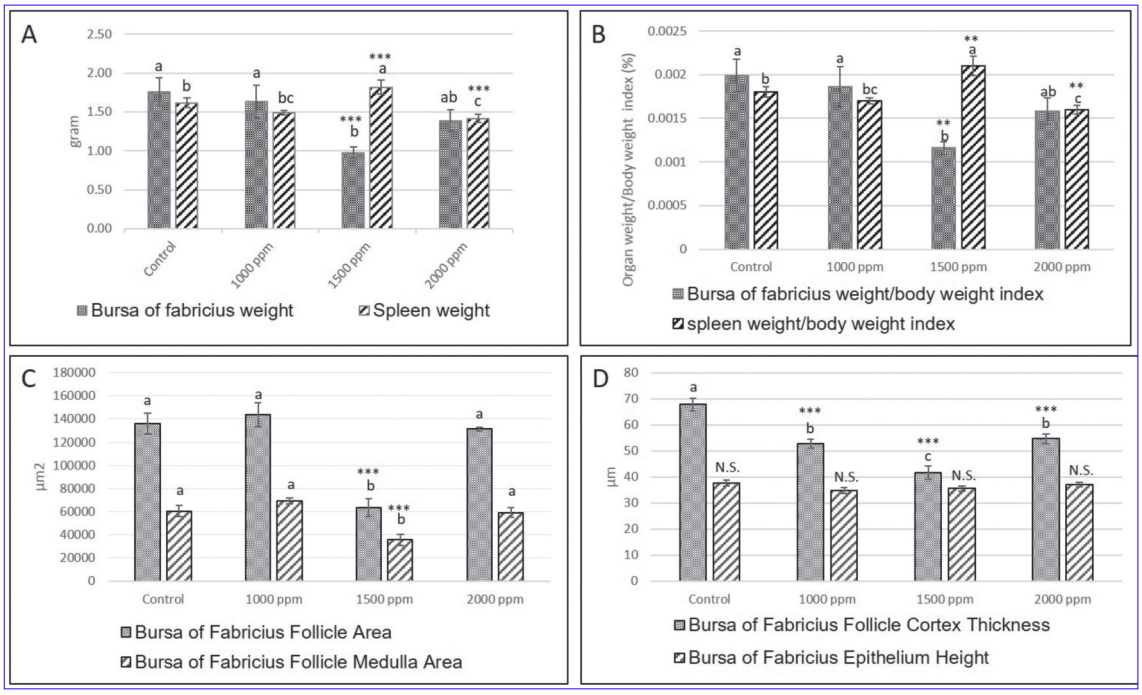

Fig 2. Bursa of Fabricius and spleen weights in control and experimental groups (A) bursa of Fabricius and spleen weight indice in control and experimental groups (B) Follicle area and area of follicle medulla of bursa of Fabricius in control and experimental groups (C) Follicle cortex and epithelium thickness values of bursa of Fabricius in control and experimental groups (D) Differences between values that doesn't share a common letter in each parameter at the graphics are statistically important (** P<0.01, *** P<0.001)

in 1.000 ppm boric acid group increased in comparison with the other groups, area of follicular medulla in groups of higher doses (1.500 and 2.000 ppm) decreased. The reduction in 1.500 ppm group was statistically significant at P<0.001 level *(Fig. 2C)*. Follicle count in 1.000 ppm (P<0.001) and 2.000 ppm groups increased when compared with control group while it decreased in 1.500 ppm group *(Fig. 3)*. Height of epithelium decreased in 1.000 and 1.500 ppm groups in comparison to control group and 2.000 ppm group (NS). A reduction was noted in the thickness of

Table 1. *Body weight, Bursa of fabricius volume, plasma cell number and blood parameter values in groups. (N.S. = Non Significant, * P<0.05, *** P<0.001)*

Parameters	Control	1000 ppm	1500 ppm	2000 ppm	Significant
Body weight (g)	873.32±19.51	874.11±9.07	834.94±19.77	874.13±12.30	NS
Bursa F volume (cm^3)	1.437±0.214^{a}	1.265±0.179^{ab}	0.627±0.063^{c}	0.883±0.094^{bc}	***
Plasma cell number	184.83±11.53^{a}	337.43±54.61^{b}	340.83±52.49^{b}	188.83±13.13^{a}	*
HCT%	31.93±0.573	30.87 ± 0.568	30.87±2.532	31.60±0.400	N.S.
HGB (g/dL)	10.1933±0.3798^{b}	9.9187±0.22132^{b}	10.2349±0.33414^{b}	11.736±0.28311^{a}	***
WBC	15.80±1.628^{a}	10.60±1.447^{b}	12.53±1.191^{ab}	15.40±1.137^{ab}	*
IgY (µg/mL)	1582.805±177.1964	1841.575±267.4685	1377.623±84.35289	1562.658±161.3952	N.S.

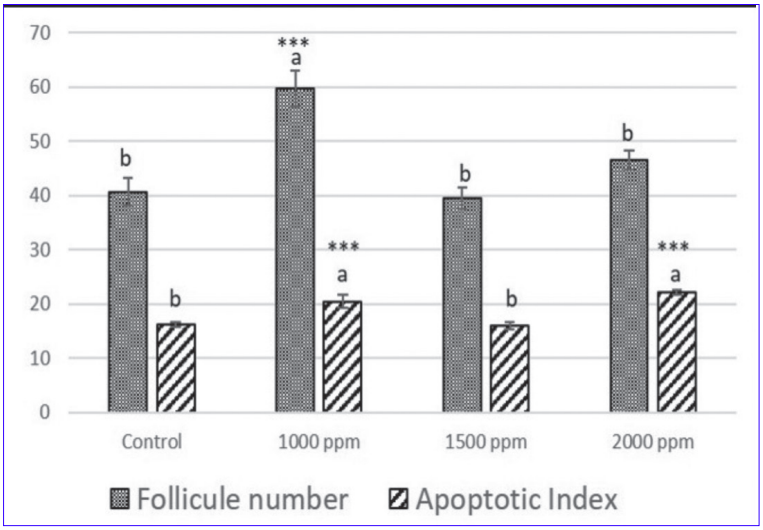

Fig 3. Follicle numbers and apoptotic index values of bursa of Fabricius in control and experimental groups. Differences between values that does not share a common letter in each parameter at the graphics are statistically important (*** P<0.001)

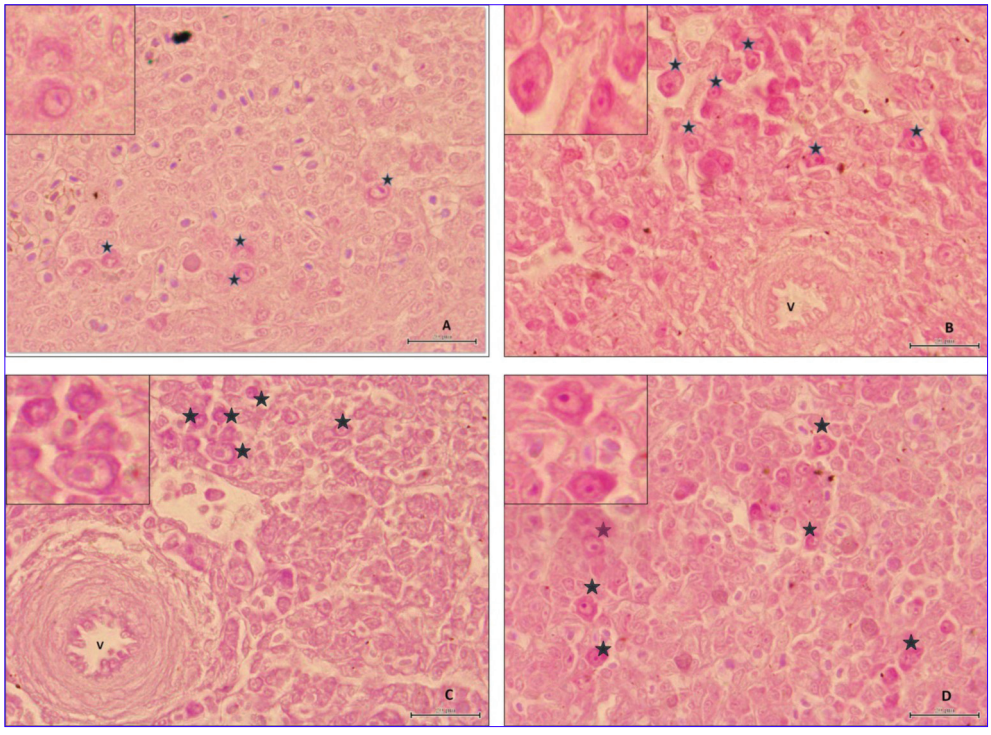

Fig 4. Plazma cell distribution and density in spleen; A: Control, B: 1000 ppm, C: 1500 ppm, D: 2000 ppm, V: vessel, *Asteriks:* Plazma cell, Methyl green-pyronin, Bar: 20 µm

follicular cortex in all experimental groups when compared with control group. A significant difference was obtained between 1.500 ppm and the other groups at P<0.001 level *(Fig. 2D)*.

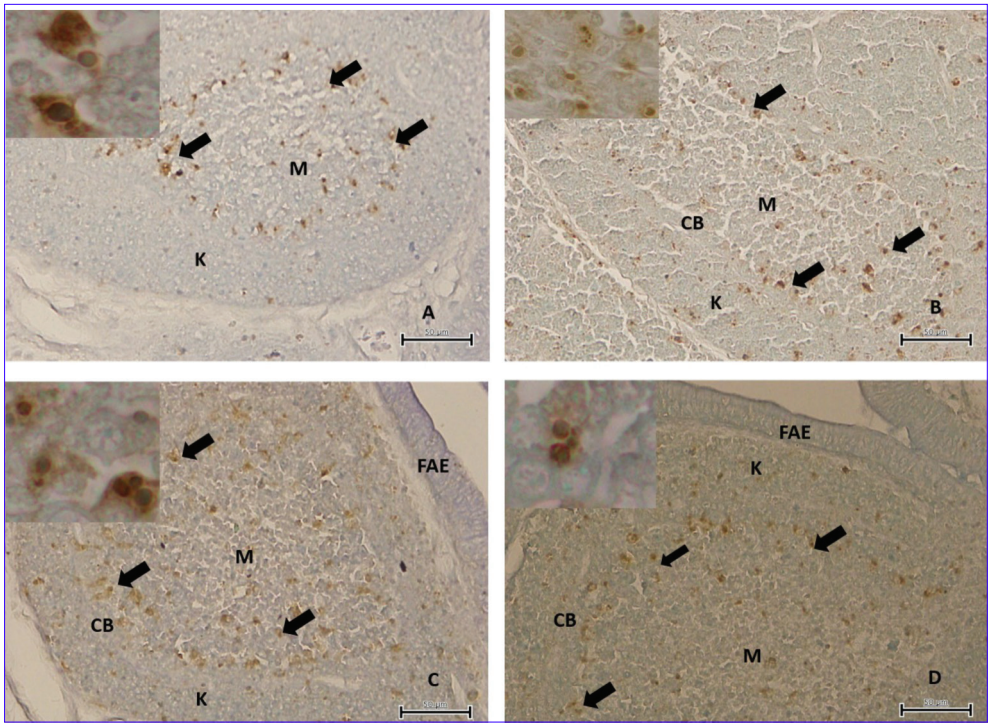

Fig 5. Apoptotic cell distribution and density in Bursa of Fabricius; A: Control, B: 1.000 ppm, C: 1.500 ppm, D: 2.000 ppm, K. Cortex, M: Medulla, CB: Corticomedullary border, FAE: Follicle associated epithelium, *Arrow:* Apoptotic cell, TUNEL, Bar: 50 µm

Body Weight, Weight of Bursa of Fabricius, Volume of Bursa of Fabricius and Relative Weight of Bursa of Fabricius

Live body weights increased by the doses of 1.000 and 2.000 ppm boric acid in comparison to control group. Although a reduction was recorded in 1500 group, the difference was not statistically significant *(Table 1)*. Bursal weights decreased in the treatment groups in comparison to control group however, the decrease was statistically significant (P<0.01) only in 1.500 ppm group *(Fig. 2A)*.

A marked reduction was recorded in 1.500 ppm group in terms of relative body weight and this reduction was statistically significant (P<0.01) when compared with 1.000 ppm group and control group *(Fig. 2B)*. Moreover, a marked decrease in the volume of bursa of Fabricius was noted in treatment groups in comparison to control group and the difference was statistically significant (P<0.001) between 1.500 ppm and 1.000 ppm and the control group *(Table 1)*.

Spleen Weight, Relative Spleen Weight and Plasma Cell Count

A reduction was recorded in the treatment groups in terms of spleen weight and relative spleen weight in comparison to control group. The difference with respect to spleen weight was statistically significant in 1.500 ppm group at P<0.001 level and likewise a statistically significant reduction (P<0.01) was noted also for relative spleen weight in the same group *(Fig. 2A, 2B)*.

Plasma cell count increased by the injection of different doses of boric acid in the treatment group when compared with control group. The differences between 1.000 ppm and 1.500 ppm groups and 2.000 ppm and control group were statistically significant at P<0.05 level *(Table 1)*. Plasma cells were most abundant in the vicinity of the blood vessels *(Fig. 4)*.

Changes in Apoptotic Index in the Bursa of Fabricius

In our study, no evaluation was carried out in the embryonic development stage. However, an increase was noted in apoptotic cell count by injection of 1.000 ppm and 2.000 ppm boric acid on 10 weeks of age in comparison to 1.500 ppm group and control group (P<0.001) *(Fig. 3)*. Apoptotic cells were mostly detected in corticomedullary regions *(Fig. 5)*.

Changes in Blood Parameters

The number of white blood cells reduced by injection of 1.000 ppm (statistically significant at P<0.05 level) and 1.500 ppm boric acid compared to 2.000 ppm and control groups.

Although hematocrit percentage values decreased in 1.000 ppm and 1.500 ppm groups, no statistically significant change was noted between the treatment groups and the control. Hemoglobin (g/dl) levels increased in control group and in 2.000 ppm group (statistically significant at P<0.001 level). On the other hand, serum IgY levels increased in 1.000 ppm while decreased in 1.500 ppm groups compared to control group (NS) *(Table 1)*.

Progesterone Receptor Expression

In our study, progesterone receptor was not expressed in the bursa of Fabricius during 10 weeks of experimental period.

DISCUSSION

In a study in which the effects of Boron on body weight were investigated, body weight of the chicks which were given 100 mg/L in drinking water for 2 weeks were reduced but those that received the same amount for six weeks gained weight. However, boron at the doses of 200 and 400 mg/L decreased their body weights [24]. Hunt [7] reported that different doses of boron with vit D caused an increase in the body weight of the chicks. King et al.[13], reported that different doses of boron injected into the egg had no effect on the body weight.

Jin et al.[24] indicated that 200 mg/L boron increased the weight of bursa of fabricius in six-week-old chicks but boron at the doses of 100 and 400 mg/L decreased the same parameter in two and four-week-old chicks.

Spleen weight was lower in the chickens which received 200 mg/ L boron in drinking water for two and six weeks when compared with the control group [24]. In a study carried out with rats boron increased weight and relative weight of the spleen [10]. The difference was considered to be associated with the difference in species.

When the results we obtained were compared with other studies, boron similarly, caused a reduction in the size and weight of bursa of fabricius in the chickens. Likewise, spleen weight increased at the dose of 1.500 ppm but decreased with other doses. Divergent results obtained for quails depend on the type of the species. Bursa of fabricius is permanent during life time in quails unlike other avian species [25].

Other criterion regarding the development of bursa of fabricius is apoptosis. Apoptosis is an important control mechanism in organogenesis [26]. Cell dead through apoptosis is linked with immune suppression [27]. Biochemical and morphological manifestations of apoptosis primarily occur at the 18th day of incubation and after hatching it occurs with sexual maturation [28]. Motyka and Reynolds [29], reported that apoptosis occurred at 4, 7 and 10 weeks of age.

On the basis of our results that the number of apoptotic cells was lower in 1.500 ppm treatment group, in which lowest values were recorded for bursal weight and volume, than those of 1.000 and 2.000 ppm groups, was considered to be associated with putative initiation of apoptosis at an early phase.

T-lymphocytes differentiate in timus and B-lymphocytes differentiate in the bursa of fabrius which is an organ found only in avian species [30]. Calander et al.[31], indicated that spleen is the organ where plasma cells proliferate and memory B cells are located. Kurtoglu et al.[8], reported that 25 mg/kg boron statistically increased plasma cell count. In a study carried out with rats antibody levels were increased by boron [11].

Therefore plasma cell count was performed only in the spleen. On the basis of data obtained, plasma cell count increased in 1.500 ppm group, in which involution was prominent. This showed that certain doses of boron led to early development of bursa of fabricius in chickens.

Likewise, Fairbrother et al.[32], evaluated the effects of boron, arsenic and selenium on immune function, development and maturation of avocet chicks. For this purpose, they investigated white blood cell count (WHC) of the chicks younger than 5 weeks of age that hatched from the eggs collected from different environments containing different levels of boron, arsenic and selenium under laboratory conditions and found no statistically significant difference. Kurtoğlu et al.[8], reported that different doses of boron (5 and 25 mg/kg) taken with food in combination with vit D_3 slightly increased WBC in 45-day-old chicks but the increase was not statistically significant.

In our study, WBC in 1.000 ppm ($P<0.05$) and 1.500 ppm groups were decreased in comparison to 2.000 ppm and the control groups. The difference in our findings which contrasted with those of Fairbrother et al.[32] and Kurtoğlu et al.[8] was considered to be associated with age and species.

In parallel with the findings obtained for plasma cell count, WBC was lower in the groups with higher plasma cell count. This was associated with the early occurrence of humoral immunity because it is known that white blood cells rather take place in cellular response. Likewise, Ig-y levels were higher in the groups of low doses.

Involution period of the bursa of Fabricius varies among different species. Period for bursal regression may not be determined only by age at least in some species but by the beginning of egg production.

Involution of the bursa starts at approximately 8 weeks of age in both sexes of white leghorns. In reality, scattered, atrophic or cystic follicles occur at 20 weeks of age and they become definitive by 24th week and the involution process is completed by 26th week and finally cicatrized bursal remnants were found at 28 weeks of age [15]. Vacuolization was present in the bursal medullary regions in the chickens that received 400 mg/L boron in drinking water for 6 weeks [24] and in antiandrogen injected quails [25] which was compatible with our experimental design.

Many studies showed that boron affected the mechanism of steroid hormone activity and particularly supported the hypothesis, stating that boron was essential for hydroxylation step in steroid synthesis [25,33]. Besides, boron was reported to regulate glucose, fat and protein metabolism by its enzymatic properties [33,34]. Jin et al.[24], administered 200 mg/dL of boron to the broilers in drinking water Follicle area and follicle count decreased in the chicks which were fed on this diet between 2 to 4 weeks of age and in 2-week-old chicks when compared with the control group but no difference was noted at 6 weeks of

age. In the same study, boron at 400 mg/dL level reduced the area of follicular nodules and follicle count in 2-6 week old and 4-6 week old chicks, respectively.

It has been reported in previous studies regarding the hormonal mechanisms of bursal involution that bursa of fabricius of the quails which received estrogen were smaller than those of the control group; however estradiol administration during embryonic period increased their sizes unlike the situation in adult quails [16]. In our previous study we have seen that Bisphenol A and Diethylstilbestrol caused involution of bursa of fabricius in chickens [35].

It was reported that progesterone receptor expression was not detectable in the chicken bursa until 10 weeks of age, however the expression started to appear between 12 to 15 weeks and expression status of progesterone receptor increased by maturation [36,37]. In our study, we found no evidence of progesterone receptor expression during a period of 10 weeks. Occurrence of bursal involution despite the lack of progesterone receptor expression in this period was associated with the influence of extrinsic boron administration.

Taking into account the changes in the thickness of follicular cortex, area of follicular medulla, follicle area, follicle count and height of epithelium, boron had an impact on the involution of bursa of Fabricius particularly at the dose of 1.500 ppm, as indicated by above mentioned studies. It may be associated with its effectiveness on the metabolism of steroid hormones as well as that on fat, glucose and protein metabolism by regulating the enzymatic activity.

When results obtained were compared with the parameters of previous studies the majority of data were compatible and ultimately boron treatment at low doses had positive impact on the development of bursa of fabricius which functions as the principle organ of avian immune system but it had no effect at high doses. In this study, alternatively, we consider that application of stereological techniques pioneering unbiased evaluations and presentation of new parameters will contribute to future researches.

REFERENCES

1. Naghii MR, Samman S: The role of boron in nutrition and metabolism. *Prog Food Nutr Sci*, 17, 331-349, 1993.

2. Woods WG: An introduction to boron - History, sources, uUses, and chemistry. *Environ Health Persp*, 102, 5-11, 1994.

3. Howe PD: A review of boron effects in the environment. *Biol Trace Elem Res*, 66, 153-166, 1998. DOI: 10.1007/BF02783135

4. Robbins WA, Xun L, Jia J, Kennedy N, Elashoff DA, Ping L: Chronic boron exposure and human semen parameters. *Reprod Toxicol*, 29, 184-190, 2010. DOI: 10.1016/j.reprotox.2009.11.003

5. Nielsen FH: Boron in human and animal nutrition. *Plant Soil*, 193, 199-208, 1997. DOI: 10.1023/A:1004276311956

6. Ku WW, Chapin, RE, Moseman RF, Brink RE, Pierce KD, Adams KY: Tissue disposition of boron in male Fischer rats. *Toxicol Appl Pharmacol*, 111,145-151, 1991. DOI: 10.1016/0041-008X(91)90143-3

7. Hunt CD: The biochemical effects of physiological amounts of dietary boron in animal nutrition models. *Environ Health Persp*, 102, 35-43, 1994.

8. Kurtoğlu F, Kurtoğlu V, Celik I, Kececi T, Nizamlioglu M: Effects of dietary boron supplementation on some biochemical parameters, peripheral blood lymphocytes, splenic plasma cells and bone characteristics of broiler chicks given diets with adequate or inadequate cholecalciferol (vitamin D-3) content. *Brit Poult Sci*, 46, 87-96, 2005. DOI: 10.1080/00071660400024001

9. Chapin RE, Ku WW, Kenney MA, McCoy H: The effects of dietary boric acid on bone strength in rats. *Biol Trace Elem Res*, 66, 395-399, 1998. DOI: 10.1007/BF02783150

10. Hu QQ, Li SH, Qiao EM, Tang ZT, Jin EH, Jin GM, Gu YF: Effects of boron on structure and antioxidative activities of spleen in rats. *Biol Trace Elem Res*, 158, 73-80, 2014. DOI: 10.1007/s12011-014-9899-5

11. Bai Y, Hunt CD: Dietary boron alleviates adjuvant-induced arthritis (AIA) in rats. *FASEB J*, 9 (3): A576-A576, 1995.

12. Samman S, Naghii MR, Wall PML, Verus AP: The nutritional and metabolic effects of boron in humans and animals. *Biol Trace Elem Res*, 66, 227-235, 1998. DOI: 10.1007/BF02783140

13. King N, Odom TW, Sampson HW, Pardue SL: *In ovo* administration of boron alters bone mineralization of the chicken embryo. *Biol Trace Elem Res*, 30, 47-58, 1991. DOI: 10.1007/BF02990341

14. Pedernera EA, Romano M, Besedovsky HO, Aguilar MC: The bursa of Fabricius is required for normal endocrine development in chicken. *Gen Comp Endocrinol*, 42, 413-419, 1980. DOI: 10.1016/0016-6480(80)90172-0

15. Ciriaco E, Pinera PP, Diaz-Esnal B, Laura R: Age-related changes in the avian primary lymphoid organs (thymus and bursa of Fabricius. *Microsc Res Tech*, 62, 482-487, 2003. DOI: 10.1002/jemt.10416

16. Quinn MJ, Jr McKernan M, Lavoie ET, Ottinger MA: Effects of estradiol on the development of the bursa of Fabricius in Japanese quail. *J Exp Zool A Ecol Genet Physiol*, 311, 91-95, 2009. DOI: 10.1002/jez.504

17. Quinn MJ, Jr Lavoie ET, Ottinger MA: Reproductive toxicity of trenbolone acetate in embryonically exposed Japanese quail. *Chemosphere*, 66, 1191-1196, 2007. DOI: 10.1016/j.chemosphere.2006.07.085

18. Ratcliffe MJ: The ontogeny and cloning of B cells in the bursa of Fabricius. *Immunol Today*, 6, 223-227, 1985. DOI: 10.1016/0167-5699(85)90039-8

19. Chen K, Fang J, Peng X, Cui H, Chen J, Wang F, Chen Z, Zuo Z, Deng J, Lai W, Zhou Y: Effect of selenium supplementation on aflatoxin B(1)-induced histopathological lesions and apoptosis in bursa of Fabricius in broilers. *Food Chem Toxicol*, 74, 91-97, 2014. DOI: 10.1016/j.fct.2014.09.003

20. Sahin B, Emirzeoglu M, Uzun A, Incesu L, Bek Y, Bilgic S, Kaplan S: Unbiased estimation of the liver volume by the Cavalieri principle using magnetic resonance images. *Eur J Radiol*, 47, 164-170, 2003. DOI: 10.1016/S0720-048X(02)00152-3

21. Piskin A, Kaplan S, Aktas A, Ayyildiz M, Raimondo S, Alic T, Bozkurt HH, Geuna S: Platelet gel does not improve peripheral nerve regeneration: An electrophysiological, stereological, and electron microscopic study. *Microsurg*, 29, 144-153, 2009. DOI: 10.1002/micr.20599

22. Gonzolez-Moran MG: *İmmunohistochemical* localization of progesterone receptor isoforms and estrogen receptor alpha in the chicken oviduct magnum during development. *Acta Histochem*, 117, 681-687, 2015. DOI: 10.1016/j.acthis.2015.10.003

23. Yildirim F, Sonmez K, Ozyogurtcu H, Sennazli G, Gurel A, Gunduz MC, Sontas BH: Evaluation of Bcl-2, Bcl-X-L and Bax expression and apoptotic index in canine mammary tumours. *Kafkas Univ Vet Fak Derg*, 20, 513-520, 2014. DOI: 10.9775/kvfd.2013.10450

24. Jin EH, Gu YF, Wang J, Jin GM, Li SH: Effect of supplementation of drinking water with different levels of boron on performance and immune organ parameters of broilers. *Ital J Anim Sci*, 13, 205-214, 2014. DOI: 10.4081/ijas.2014.3152

25. Quinn MJ, Summitt CL, Ottinger MA: Effects of androgen disruption by DDE on the development and functioning of the immune system in

Japanese quail. *Immunopharmacol Immunotoxicol*, 28, 535-544, 2006. DOI: 10.1080/08923970600927850

26. Ghobrial IM, Witzig TE, Adjei AA: Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin*, 55, 178-194, 2005. DOI: 10.3322/canjclin.55.3.178

27. Sun EW, Shi YF: Apoptosis: The quiet death silences the immune system. *Pharmacol Ther*, 92, 135-145, 2001. DOI: 10.1016/S0163-7258(01)00164-4

28. Reynaud CA, Bertocci B, Dahan A, Weill JC: Formation of the chicken B-cell repertoire: Ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion. *Adv Immunol*, 57, 353-378, 1994. DOI: 10.1016/S0065-2776(08)60676-8

29. Motyka B, Reynolds JD: Apoptosis is associated with the extensive B-cell death in the sheep ileal peyers patch and the chicken bursa of Fabricius - A possible role in B-cell selection. *Eur J Immunol*, 21, 1951-1958, 1991. DOI: 10.1002/eji.1830210825

30. Sharma JM: The Structure and function of avian immune system. *Acta Vet Hung*, 45, 229-238, 1997.

31. Calander D, Stanberg J, Larsson A: Chicken Antibodies. *Ups J Med Sci*, 104, 179-189, 1999. DOI: 10.3109/03009739909178961

32. Fairbrother A, Fix M, O'Hara T, Ribic CA: Impairment of growth and immune function of avocet chicks from sites with elevated selenium, arsenic, and boron. *J Wildl Dis*, 30, 222-233, 1994. DOI: 10.7589/0090-3558-30.2.222

33. Devirian TA, Volpe SL: The physiological effects of dietary boron. *Crit Rev Food Sci Nutr*, 43, 219-231, 2003. DOI: 10.1080/10408690390826491

34. Li SH, Li WC, Zhou JX, Jin GM, Gu YF, Chen HI, Zhao J, Huang J: Effects of drinking boron on the growth and the biochemical indexes and antioxidant ability in serum of rats. *Chin J Vet Sci*, 3, 486-492, 2013. DOI: 10.4081/ijas.2014.3152

35. Yiğit F, Aktas A, Daglioglu S: Effects of bisphenol A and diethylstilbestriol on the involution of bursa Fabrisius in the hens. *J Fac Med Istanbul Univ*, 39, 168-174, 2013.

36. Pasanen S, Ylikomi T, Palojoki E, Syvala, H., Pelto-Huikko, M, Tuohimaa P: Progesterone receptor in chicken bursa of Fabricius and thymus: Evidence for expression in B-lymphocytes. *Mol Cell Endocrinol*, 141, 119-128, 1998. DOI: 10.1016/S0303-7207(98)00086-0

37. Ylikomi TJ, Isola JJ, Gasc JM, Tuohimaa PJ: Sexual maturation-associated and estrogen-induced progesterone receptor expression in the bursa of Fabricius. *J Immunol*, 138, 3174-3178, 1987.

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Research Article

Kinetic Modeling of Quality Aspects of Fermented Sausage (Sucuk) During Storage

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Abstract

Quality characteristics of traditional fermented sausage (sucuk) including pH, free fatty acid (FFA), thiobarbituric acid reactive substances (TBARS), residual nitrite contents, colour values (L*, a*, b*, hue angle (Hue), browning index (BI), chroma and total colour difference (ΔE*)), total mesophilic aerobic bacteria (TMAB), lactic acid bacteria (LAB), total *Enterobacteriacea* (TE) and *Staphylococcus* and *Micrococcus* (SM) counts were investigated during storage period, and kinetic model estimation for the changes in these quality parameters were performed. After 9 days of fermentation period, the sucuk samples were stored for 90 days under controlled conditions. Analyses were done at the beginning, 30th, 60th and 90th days of the storage period. Zero, first and second order kinetic model equations represented successfully the changes in chemical and colour properties. A linear (first order) kinetic model equation demonstrated the kinetics of microbial changes well in the sucuk samples during storage period.

Keywords: *Traditional fermented sausage, Sucuk, Storage, Kinetic model, Quality*

Fermente Sucuğun Depolama Sırasında Kalite Özelliklerindeki Değişimin Kinetik Modellemesi

Özet

Geleneksel fermente sucuğun pH, serbest yağ asidi değeri (FFA), tiyobarbütirik asit reaktif maddeler (TBARS), kalıntı nitrit içeriği, renk değerleri (Hunter L*, a*, b*, hue açısı (Hue), esmerleşme indeksi (BI), kroma ve toplam renk farkı (ΔE*)), toplam mesofilik aerobik bakteri (TMAB), laktik asit bakterisi (LAB), toplam *Enterobacteriacea* (TE) ile *Staphylococcus* ve *Micrococcus* (SM) sayısı değerlerindeki değişimler depolama süresince incelenmiş ve bu özelliklerdeki değişimlerin kinetik modellemesi gerçekleştirilmiştir. Sucuklar 9 günlük fermantasyonun ardından kontrollü koşullarda 90 gün boyunca depolanmıştır. Analizler depolamanın 0, 30, 60 ve 90. günlerinde yapılmıştır. Sucukların depolama sırasındaki kimyasal özellikleri ve renk değerlerindeki değişimleri 0, 1 ve 2. dereceden kinetik modeller başarılı bir şekilde ifade ederken mikrobiyal özelliklerindeki değişimleri doğrusal (birinci dereceden) kinetik modelin iyi bir şekilde temsil etiği saptanmıştır.

Anahtar sözcükler: *Geleneksel fermente sucuk, Depolalama, Kinetik model, Kalite*

INTRODUCTION

Fermented sausage production is a method to preserve meat since ancient times. Contrary, today the production of fermented sausages is carried out for their desired sensory attributes by the consumers. Sucuk, is a widely consumed traditional fermented sausage in Turkey and culturally neighboring countries in Asia, Europe and North Africa. There are numerous documents showing that ancient Turks made and consumed sausages thousands of years ago. It has been documented that sucuk was an easy to carry, easy to prepare and a palatable meat product in Manas saga, a thirteen hundred years old Kyrgyz legend [1].

Literally, sucuk is produced from a mixture of meat and fat. This mixture may include cattle, sheep and/or water buffalo meat, beef fat and sheep tail fat, salt, sugar, garlic and some spices and seasonings. After mixing it is filled into a casing and then subjected to fermentation at certain conditions resulting in a semi dry or dried meat product [2,3].

Fermentation is the main stage of the curing process of sausages, since at this phase the main physical, chemical

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and microbiological changes start to occur. These transformations also continue in storage and are influenced by attributes of the raw material and the process conditions, which determine the sensory properties, shelf life and safety of the final product. These changes are mainly; changes in initial microflora, acidification, pH decrease, reduction of nitrates into nitrites than the nitric oxide, production of nitrosomyoglobin, solubilisation and jellification of myofibrillar and sarcoplasmic proteins, proteolytic, lipolytic and oxidative phenomena and dehydration [4-7]. Due to these continuous changes, the quality parameters of the last product show wide variations.

Kinetic modeling can be used to predict changes in physical, chemical or microbiological quality parameters of food products during processing and storage [8]. Numerous studies exist about kinetic modeling of quality changes in foods. Kinetic modeling of quality parameters of sucuk during fermentation was reported previously [9]. However, changes in quality parameters in sucuk like other food products continue during storage period after production processes. Due to these changes the product may become inconsumable. Kinetic modelling is a good way of demonstrating these changes in quality parameters during production (like fermentation) and storage for food products (like sucuk). Therefore, the aim of this study was to make a kinetic modeling of the changes in physical, chemical or microbiological quality parameters of the traditional fermented sausage "sucuk" during 90 days of storage period.

MATERIAL and METHODS

Three batches of the sucuk were produced under industrial conditions in a meat processing plant situated in İzmir, Turkey. Sucuk production was done according to method reported previously [9]. After fermentation the sucuk samples were vacuum packed in polyethylene bags and stored at 4°C during the storage period.

The study was carried out in three replicates. Percent moisture, salt, ash, fat, protein, contents of the samples were analyzed at the beginning of the storage. pH, free fatty acid, thiobarbituric acid reactive substances, residual nitrite content, instrumental colour and total mesophilic aerobic bacterial (TMAB), total *Enterobacteriacea* (TE), *Staphylococcus* and *Micrococcus* (SM) and lactic acid bacteria (LAB) counts were analyzed at the beginning, 30th, 60th and 90th days of storage. Determination of the chemical, color and microbial parameters were carried out according to the methods reported previously [9].

Kinetic Modeling

Zero-order (Eqn. 1), first order (Eqn. 2) and second order (Eqn. 3) kinetic models were used to describe the chemical and physical changes during the storage of the sucuk samples [8];

$$c = c_0 - kt \quad (1)$$

$$c = c_0 \exp(-kt) \quad (2)$$

$$\frac{1}{c} = \frac{1}{c_0} + kt \quad (3)$$

where, c is the quality parameter; c_0 is the value of this quality parameter at the beginning of the storage period, t is the storage time (day) and k is the rate constant (day^{-1}).

The linear (first order) kinetic model was used to demonstrate the changes in microbial quality parameters of the samples during the storage;

$$\frac{dN}{dt} = -k'N \quad (4)$$

after integration, it is,

$$\ln\left(\frac{N}{N_0}\right) = -k't \quad (5)$$

Base-10 logarithms of population sizes are used generally to present microbial changes [10,11];

$$\log\left(\frac{N}{N_0}\right) = -kt \quad (6)$$

where, N_0 and N are the initial number and number of survivors after a time t (day) of microorganisms and spores (cfu/g), respectively, k' is the rate constant (day^{-1}) and $k = k'/\ln 10$ (day^{-1}).

RESULTS

Percent moisture, salt, ash, fat and protein contents of the samples at the beginning of the storage were found as 35.29, 3.33, 4.57, 31.65 and 17.38%, respectively.

Estimated kinetic parameters of pH, FFA, TBARS and residual nitrite during storage are presented in *Table 1*. The positive (+) sign of the k shows a decrease while the negative (-) sign shows an increase in quality parameter during the storage. Comparison of the experimental values with the modeling results is given in *Fig. 1*. Only the models having highest R^2 values are presented in the graphs. During the 90 days of storage in refrigerator conditions the pH value of sucuk decreased from 5.05 to 4.76 *(Fig. 1-A)*.

The FFA amount of the sucuk samples showed a significant increment during the storage. The FFA amount increased from 3.40 mg KOH/g fat to 5.15 mg KOH/g fat

Table 1. *Estimated model constants for pH, FFA, TBARS and residual nitrite*

Quality Parameter	Zero Order Model			First Order Model			Second Order Model		
	c_0	k (day^{-1})	R^2	c_0	k (day^{-1})	R^2	c_0	k (day^{-1})	R^2
pH	4.9960	0.0030	0.8005	4.9953	0.0006	0.8049	4.9950	0.0001	0.8093
FFA (mg KOH/g fat)	3.3390	-0.0196	0.9929	3.3814	-0.0047	0.9985	3.4106	-0.0011	0.9974
TBARS (mg malonaldehyde/kg)	0.3985	-0.0016	0.9711	0.3995	-0.0034	0.9567	0.4001	-0.0075	0.9395
Residual Nitrite (ppm)	5.2430	0.0084	0.5228	5.1049	0.0014	0.4765	4.9579	0.0002	0.4408

FFA: *Free Fatty Acid,* ***TBARS:*** *Thiobarbituric Acid Reactive Substances*

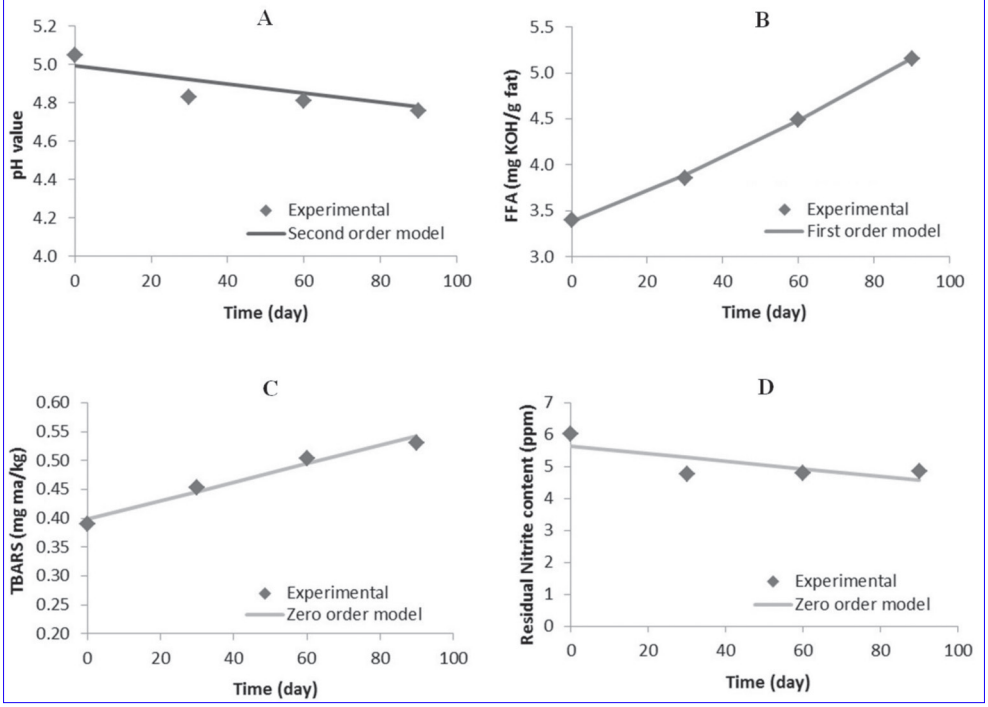

Fig 1. Changes in pH **(A)**, FFA **(B)**, TBARS **(C)** and residual nitrite **(D)** values during storage

FFA: Free Fatty Acid, TBARS: Thiobarbituric Acid Reactive Substances

Table 2. *Estimated model constants for L*, a*, b*, Hue, BI, chroma and ΔE**

Quality Parameter	Zero Order Model			First Order Model			Second Order Model		
	c_0	k (day^{-1})	R^2	c_0	k (day^{-1})	R^2	c_0	k (day^{-1})	R^2
L*	45.447	0.0428	0.8829	45.4449	0.0010	0.8883	45.4545	0.00002	0.8935
a*	15.386	0.0231	0.9872	15.3990	0.0016	0.9900	15.4083	0.0001	0.9923
b*	12.008	0.0167	0.9583	12.0131	0.0015	0.9630	12.0192	0.0001	0.9672
Hue	0.6625	-6.0E-05	0.4607	0.6625	-0.0001	0.4600	0.6625	-0.0001	0.4592
BI	54.494	0.0319	0.9146	54.5054	0.0006	0.9140	54.6448	0.00001	0.9135
Chroma	19.517	0.0285	0.9787	19.5309	0.001.6	0.9824	19.5313	0.00009	0.9856
ΔE*	7.1663	-0.0417	0.9409	7.1807	-0.0048	0.9183	7.1839	-0.0006	0.8916

Hue: *Hue Angle,* ***BI:*** *Browning Index,* ***ΔE*:*** *Total Colour Difference*

during 90 days of storage *(Fig. 1-B)*. The increase in FFA amount during storage was well represented by all of the three kinetic models with negative k values *(Table 1)*.

The TBARS value rose from 0.390 to 0.530 mg malonaldehyde/kg during the storage *(Fig. 1-C)*. The increase in TBARS amount during storage was best demonstrated by zero order kinetic model with negative k value *(Table 1)*.

The residual nitrite content showed a decrement in storage and its amount in sucuk was lower than the added nitrite amount during the preparation of sucuk dough *(Fig. 1-D)*. The models tested were demonstrated the experimental data of the residual nitrite content with low

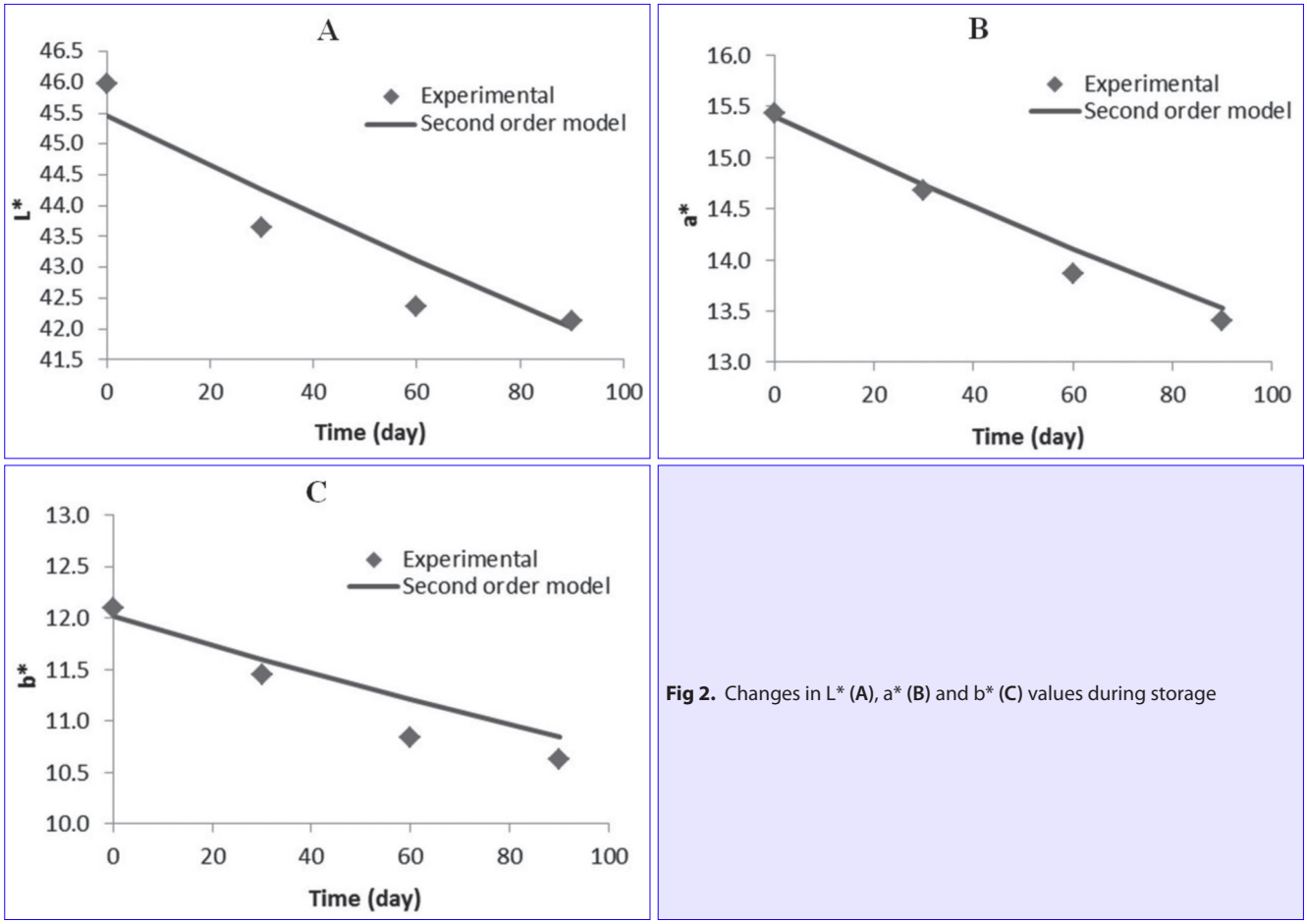

Fig 2. Changes in L* (**A**), a* (**B**) and b* (**C**) values during storage

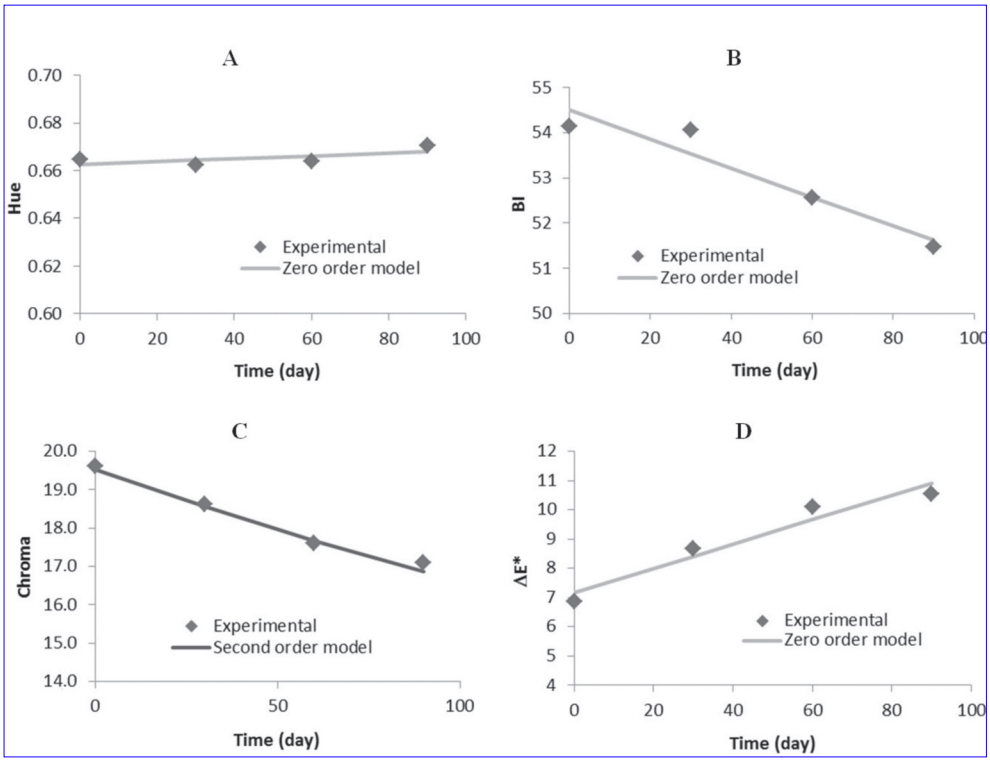

Fig 3. Changes in Hue (**A**), BI (**B**), chroma (**C**) and ΔE* (**D**) values during storage

Hue: Hue Angle, BI: Browning Index, ΔE*: Total Colour Difference

correlation. Amongst them the zero order model had the highest R^2 value of 0.5228 *(Table 1)*.

The estimated kinetic parameters of L*, a*, b*, hue angle (Hue), browning index (BI), chroma and total

Table 3. *Estimated model constants for microbial counts*

Microbial Counts	Log N_0 (cfu/g)	k (day^{-1})	R^2
TMAB	8.503	0.0022	0.5509
LAB	7.894	0.0047	0.7630
SM	6.303	0.0145	0.8208
TE	-	-	-

TMAB: *Total Mesophilic Aerobic Bacteria,* ***LAB:*** *Lactic Acid Bacteria,* ***SM:*** *Staphylococcus and Micrococcus,* ***TE:*** *Total Enterobacteriacea*

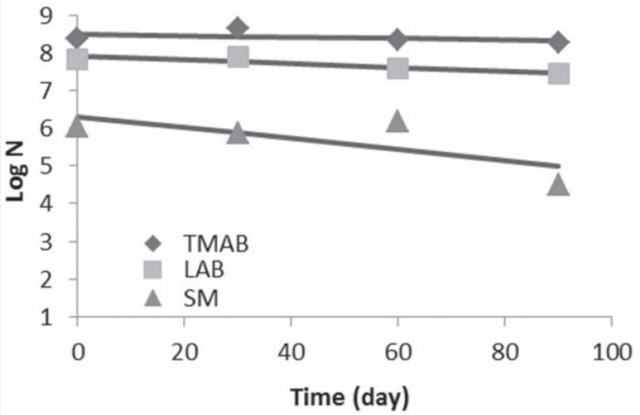

Fig 4. Changes in TMAB, LAB and SM values during storage
TMAB: Total Mesophilic Aerobic Bacteria, LAB: Lactic Acid Bacteria, SM: *Staphylococcus* and *Micrococcus*

colour difference (ΔE*) during the storage are given in *Table 2*. The L*, a* and b* values of the sucuk decreased during the storage *(Fig. 2)*. These changes in L*, a* and b* values during storage were best represented by a second order model *(Table 2, Fig. 2)*. The hue angle of the sucuk increased very slightly, the BI and Chroma values decreased significantly and the ΔE* values increased significantly during storage *(Fig. 3)*. The decrease in chroma value was represented by a second order model and changes in others were best demonstrated by zero order models *(Table 2, Fig. 3)*.

The estimated parameters of the first order kinetic model for microbial counts of the sucuk are given in *Table 3*. Statistically the model had moderate level R^2 for LAB and SM and low level R^2 for TMAB. However, graphical analysis of the experimental and the model values show that the errors between them are very low and the model equation demonstrated the kinetics of microbial changes in the sucuks well *(Fig. 4)*. The TMAB, LAB and SM counts decreased until the end of storage, however, this decrease in the TMAB and LAB is very slight which was caused the R^2 values to be lower.

DISCUSSION

Turkish Food Codex states that the fermented sucuks have a maximum moisture content of 40%, minimum protein content of 16% and a maximum fat content of 2.5 times of protein content [12]. At the beginning of the storage (or at the end of fermentation) properties of the sucuk samples were in these ranges.

According to Turkish Food Codex high quality ripened sucuk should have a pH at most 5.4 [12]. The sucuks were still in this range during the storage *(Fig. 1-A)*.

An increase in FFA content of sucuk during fermentation was also reported in a previous study [9]. This increase is faster than the increase in FFA content during storage, because the rate constants reported for fermentation period are greater than the rate constants obtained for storage period. The fast increase in the FFA amount during fermentation indicates that the lipolysis is carried out by auto-hydrolysis and enzymatic hydrolysis reactions; however the slow increase in the FFA content during storage could be attributed to the limitations of the enzymatic hydrolysis due to the lower water activity. Similar changes in the FFA amount of fermented sausages have been observed by several authors [13,14].

The TBARS value increases with a higher rate during fermentation of sucuk due to the more intense lipid oxidation during processing [9]. The increment in TBARS value continued with a slower rate during storage due to the decomposition of the formed TBARS to volatile compounds. Balance between the rate of formation and the rate of decomposition determines the amount of TBARS in a product [15,16].

The residual nitrite is the amount analytically detectable in the product and its value is considerably lower than the amount added, since it interacts with various constituents of the meat during manufacturing process [17]. The residual nitrite levels decreased significantly in both fermentation and storage periods; however the rate of decrease was higher in fermentation than that of storage [9]. Alteration of the redox potential to a reduced state in vacuum may increase the transformation of the nitrite ion to nitric oxide; due to this a reduction of residual nitrite in the sucuk was observed during the storage. The residual nitrite levels may decrease during storage and distribution, also during preparation and consumption [18]. The residual nitrite levels in traditional sucuks have been reported in the range of 4.00 - 11.25 mg/kg [19].

The L* value decreases in sucuk samples during fermentation stage, due to the moisture loss or drying [20,21] results shows that this decrease continued during storage. The a* value indicating the redness of the sucuk decreased during storage *(Fig. 2-B)*. The denaturation of myoglobin may cause this decrease in the redness value. These changes in the colour parameters during storage continued at slower rates than the changes in fermentation stage [9].

The TMAB and LAB counts decreased slightly until

the end of storage *(Fig. 4)*. This is a result of lactic acid production by LAB and decrease in pH [22,23].

The TE was uncounted during storage, therefore parameters of the models were unable to estimate *(Table 3)*. However, a decrease in TE count during fermentation was reported previously [9], as a result of the decrease in pH, dehydration [23], and suppression of *Enterobacteriacea* by fermentation microflora [24]. This shows that this decrease continued during storage and the TE became uncountable. Due to the lack of data, the model equation for storage period was not given for the TE counts.

Sucuk is a fermented sausage and its quality characteristics changes during storage period. The changes in pH, FFA, TBARS, residual nitrite contents, L*, a*, b*, Hue angle, BI, chroma and ΔE* values, TMAB, LAB, TE and SM of the sucuk were determined during 90 days of storage after 9 days of fermentation period. Increases or decreases in these parameters were observed during fermentation. Kinetic modelling of these changes was performed. Zero, first and second order kinetic model equations demonstrated successfully the changes in chemical and colour parameters, and linear kinetic model equation demonstrated well the changes in microbial parameters.

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REFERENCES

1. Gülensoy T: Manas Destanı. 366, Akçağ Yayınları, İstanbul, 2002.

2. Ertaş AH, Göğüş AK: Değişik oranlarda kuyruk yağı ve farklı starter kullanılmış olan sucuklar üzerine araştırmalar. *Doğa Bilim Dergisi, Veterinerlik ve Hayvancılık/Tarım ve Ormancılık,* 4, 48-53, 1980.

3. Gökalp HY, Ockerman HW: Turkish-type fermented sausage (soudjouk) manufactured by adding different starter culture and using different ripening temperatures. II. Growth of total, psychrophilic, proteolitic and lipolytic micro-organisms. *Fleischwirtschaft,* 65**,** 1235-1240, 1985.

4. Mottram DS, Edwards RA: The role of triglycerides and phospholipids in the aroma of cooked beef. *J Sci Food Agr*, 34, 517-522, 1983. DOI: 10.1002/jsfa.2740340513

5. Beriain MJ, Peña MP, Bello J: A study of the chemical components which characterize Spanish saucisson. *Food Chem,* 48, 31-37, 1993. DOI: 10.1016/0308-8146(93)90217-4

6. Lizaso G, Chasco J, Beriain J: Microbiological and biochemical changes during ripening of salchichon, a Spanish dry cured sausage. *Food Microbiol,* 16, 219-228, 1999. DOI: 10.1006/fmic.1998.0238

7. Ordōñez JA, Hierro EM, Bruna JM: Changes in the components of dry-fermented sausages during ripening. *Crit Rev Food Sci Nutr,* 39, 329-367, 1999. DOI: 10.1080/10408699991279204

8. Van Boekel MAJS: Statistical aspects of kinetic modeling for food science problems. *J Food Sci*, 61, 477-486, 1996. DOI: 10.1111/j.1365-2621.1996.tb13138.x

9. Ercoşkun H, Özkal SG: Kinetics of traditional Turkish sausage quality aspects during fermentation. *Food Control,* 22, 165-172, 2011. DOI: 10.1016/j.foodcont.2010.06.015

10. Peleg M: Microbial survival curves: Interpretation, mathematical modeling and utilization. *Comment Theor Biol,* 8, 357-387, 2003. DOI: 10.1080/08948550302436

11. Avşaroğlu MD, Buzrul S, Alpas H, Akçelik M, Bozoğlu F: Use of the Weibull model for lactococcal bacteriopage inactivation by high hydrostatic pressure. *Int J Food Microbiol,* 108, 78-83, 2006. DOI: 10.1016/j.ijfoodmicro.2005.10.027

12. Turkish Food Codex: Communiqué on fresh meat, prepared meat and prepared meat mixtures. The Official Gazette: 05.12.2012-28488, Communication no: 2012/74, 2012.

13. Hernandez P, Navarro JL, Toldra F: Lipolytic and oxidative changes in two Spanish pork loin products: Dry-cured loin and pickled -cured loin. *Meat Sci*, 51, 123-128, 1999. DOI: 10.1016/S0309-1740(98)00108-9

14. Molly K, Demeyer D, Civera T, Verplaetse A: Lipolysis in a Belgian sausage: Relative importance of endogenous and bacterial enzymes. *Meat Sci*, 43, 235-244, 1996. DOI: 10.1016/S0309-1740(96)00018-6

15. Newburg DS, Concon JM: Malonaldehyde concentrations in food are affected by cooking conditions. *J Food Sci*, 45, 1681-1683, 1980. DOI: 10.1111/j.1365-2621.1980.tb07588.x

16. Shahidi F, Hong C: Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *J Food Biochem*, 15, 97-105, 1991. DOI: 10.1111/j.1745-4514.1991.tb00147.x

17. Jiménez-Colmenero F, Carballo J, Cofrades S: Healthier meat and meat products: Their role as functional foods. *Meat Sci*, 59, 5-13, 2001. DOI: 10.1016/S0309-1740(01)00053-5

18. Cassens RG: Residual nitrite in cured meat: USA. *Food Technol*, 51, 53-55, 1997.

19. Üren A, Babayiğit D: Colour parameters of Turkish-type fermented sausage during fermentation and ripening. *Meat Sci*, 45, 539-549, 1997. DOI: 10.1016/S0309-1740(96)00129-5

20. Üren A, Babayiğit D: Determination of Turkish-type fermented sausage colour by a reflectance method. *Food Chem*, 57, 561-567, 1996. DOI: 10.1016/S0308-8146(96)00042-8

21. Kayaardı S, Gök V: Effect of replacing beef fat with olive oil on quality characteristics of Turkish soudjouk (sucuk). *Meat Sci,* 66, 249-257, 2004. DOI: 10.1016/S0309-1740(03)00098-6

22. Incze K: Raw fermented and air dried meat products. *Fleischwirtschaft,* 72, 58-64, 1992.

23. Lücke FK: Utilization of microbes to process and preserved meat. *Meat Sci.* 56, 105-155, 2000. DOI: 10.1016/S0309-1740(00)00029-2

24. Bauer F: Residues associated with meat production. **In,** Jensen WK, Devine C, Dikeman M (Eds): Encyclopedia of Meat Sciences. 1187-1192, Academic Press, NY, 2004.

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Research Article

Retrospective Study of Medial Patellar Luxation Surgery Using Combination of Four Techniques without Bone Reconstruction in Non-flattened Femoral Sulcus: 133 Cases in 10 Years' Period (2006-2015)

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Abstract

To assess signalment and outcome for dogs surgically treated for non-flattened femoral sulcus medial patellar luxation with combined soft tissue reconstruction techniques, cranial sartorius desmotomy, medial retinacular fascia release, patellar antirotational suture, and fascia lata overlapping, medical records from January 2006 to December 2015 were reviewed. Information obtained from medical records of cases in the study population included breed, age, sex, weight, type of patellar luxation (unilateral or bilateral), grade of patellar luxation, lameness score record, time of last follow-up, and time of luxation recurrent. The data were analyzed to determine factors influencing outcomes. Twenty-three dogs from 133 presented recurrent patellar luxation. A total of 12 from the 23 recurrent patellar luxation dogs were bilateral (52%) and 11 (48%) were unilateral. The time of recurrence after surgery varied from 1 month to 79 months. From the medical records as on the day of the study, it was found that the longest time that there was no recurrence in dogs was 10 years. It was found that, among the many factors, only grade 4 patellar luxation was a risk factor of considerable recurrence rate (OR=12.44, P=0.02). These results suggest that the combined four soft tissue reconstruction techniques demonstrated excellent outcome in the treatment of medial patellar luxation (with non-flattened femoral sulcus) in small breed dogs. At the same time, it can be stated that bone reconstruction technique is not always necessary.

Keywords: *Patellar luxation, Dog, Surgery, Recurrence, Soft tissue*

Düzleşmemiş Femoral Sulkusda Kemik Rekonstruksiyonu Yapılmaksızın Uygulanan Dört Tekniğin Kombine Kullanıldığı Medial Patella Lukzasyon Operasyonu Hakkında Retrospektif Çalışma: 10 Yıllık Periyotta 133 Olgu (2006-2015)

Özet

Düzleşmemiş femoral sulkus medial patella lukzasyonu kombine yumuşak doku rekonstrüksiyon teknikleri olan, kranial sartorius desmotomi, medial retinakular fasyanın serbestleştirilmesi, patella antirotasyonal dikiş ve fasia lata üstüste getirme ile tedavi edilen köpeklerin durumlarını ve elde edilen sonuçları değerlendirmek amacıyla, Ocak 2006 ile Aralık 2015 tarihleri arasındaki hasta raporları gözden geçirildi. Çalışmadaki olguların raporlarından elde edilen bilgiler ırk, yaş, cinsiyet, ağırlık, patella lukzasyon tipi (unilateral veya bilateral), patella lukzasyon derecesi, topallık skoru, son takip zamanı ve lukzasyon tekrarlama zamanı bilgilerini içerdi. Sonuçları etkileyen faktörleri belirlemek amacıyla veriler analiz edildi. Toplam 133 köpekten 23'ünde tekrarlayan patella lukzasyonu bulunmaktaydı. Bu olguların 12'si (%52) bilateral ve 11'i (%48) unilateral karakterdeydi. Operasyon sonrası tekrarlama zamanı 1 ay ile 79 ay arasında değişti. Çalışmanın yapıldığı zaman itibariyle tekrarlama görülmeyen en uzun zaman 10 yıl olarak belirlendi. Birçok faktör arasından sadece 4. derece patella lukzasyonu tekrarlama oranı (OR=12.44, P=0.02) için risk faktörü oluşturmaktaydı. Bu sonuçlar kombine olarak dört yumuşak doku rekonstrüksiyon tekniğinin küçük cüsseli köpeklerde medial patella lukzasyon (düzleşmemiş femoral sulkuslu) tedavisinde mükemmel sonuç gösterdiğini belirtmektedir. Ayrıca, kemik rekonstrüksiyon tekniğinin her zaman gerekli olmadığı söylenebilir.

Anahtar sözcükler: *Patella lukzasyonu, Köpek, Cerrahi, Nüks, Yumuşak doku*

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INTRODUCTION

As well documented, in small animal orthopedics, patellar luxation is one of the most common joint diseases in dogs, particularly small breed dogs [1-4]. Consequence of this disease being left untreated is that it can cause many complications and disorders such as cranial cruciate rupture [1,5], cartilage erosion [2,6], and osteoarthritis [7,8]. Veterinarians around the world have proposed many techniques for correcting patella luxation [9-14]. These techniques can be categorized into two main types: soft tissue reconstruction and bone reconstruction. So far, no studies have reported the standard procedure to be used for the correction of patellar luxation in different patient conditions. Bone reconstruction technique is effective in treating medial patellar luxation and lowest recurrent rate [5]. But this technique is highly invasive and traumatic to the animal, and the procedure needs well-trained surgeons and special instruments. Moreover, bone reconstruction technique has shown higher post-surgery complications such as articular cartilage damage, tibial tuberosity avulsion, and trochlear wedge displacement [15-17].

The objective of the study reported here was to evaluate the results of a series of cases in which treatment of medial patellar luxation (non-flattened femoral sulcus) is carried out without the bone reconstruction procedure. A combination of four soft tissue reconstruction techniques, including cranial sartorius desmotomy, medial retinacular fascia release, patellar antirotational suture, and fascia lata overlapping (imbrication), was applied in all the cases. We sought to assess the signalment and the outcomes, and evaluate whether osteoarthritis progressed during the 6 months' period following surgery. Our hypothesis was that the combination of the four procedures was enough to prevent the recurrence of the disease. We also hypothesized that there would be no progression of osteoarthritis detected in follow-up radiographs obtained 6 months after surgery. The advantage of this study over previous studies is that while the previous studies selected the difference surgical procedure to study the outcomes, this study included the same surgical procedure with variations of the grade of patellar luxation.

MATERIAL and METHODS

Animal Inclusion/Exclusion Criteria

Medical records for all patellar luxation surgery procedures performed at animal hospitals/clinics in Chiang Mai Province, Thailand, from January 2006 to December 2015 were reviewed. Information obtained from the medical records of cases included in the study population consisted of breed, age, sex, weight, type of patellar luxation (unilateral or bilateral), grade of patellar luxation, lameness score record, time of last follow-up, and time of luxation recurrent. One hundred and thirty-three dogs presented with medial patellar luxation were treated and included in this study, and the selection was done according to the following inclusion and exclusion criteria.

Inclusion criteria for dogs included the following: dogs weighing below 10 kg diagnosed of medial patellar luxation. They were treated using a combination of four techniques including cranial sartorius desmotomy, medial retinacular fascia release, patellar antirotational suture, and fascia lata overlapping. All the dogs were operated upon by the same veterinarian. The dogs did not present any history of illness or injury that may have involved some abnormality of the musculoskeletal system such as fractures, severe hip osteoarthritis, severe hind limb deformity, cranial cruciate ligament rupture or meniscus tear. Moreover, in the case of bilateral patellar luxation, the dogs' nerves underwent surgery for the contralateral leg. Exclusion criteria included the following: the dog was operated by other veterinarians or other techniques such as tibia tuberosity transposition or trochlear groove deeping technique were performed on the dogs; such dogs were excluded from this study. Dogs weighing more than 10 kg; and dogs presenting osteo-arthritis, dysplasia, or other joint diseases at the hip joint (by radiography) at the time of the surgery were also excluded. Moreover, bilateral patellar luxation dogs whose both legs had undergone surgery were also excluded from this study.

Patellar Grading

The degrees of patellar luxation were classified into four grades, as determined by manipulation [1,18]. Grade I: The patella can be pushed out of the femoral groove when the stifle is fully extended, and the patella can return into the femoral groove immediately. Grade II: The patella moves out of the femoral groove for some time, but it can return to the normal position by itself. Grade III: The patella usually moves out of the femoral groove, and it can return to the normal position by manipulation only. Grade IV: The patella always moves out of the femoral groove and cannot return to the normal position.

Surgical Procedures

To eliminate potential errors from surgical techniques, all dogs underwent the same procedure for the correction of patellar luxation performed by the same surgeon. The surgery was performed with the animal under anesthesia, by induction with 4 mg/kg propofol (Propofol-®Lipuro 1%; B. Braun Melsungen AG, Germany) and maintenance with isoflurane (Terrell™; Piramal Critical Care, Orchard Park NY, USA) and oxygen. A prophylactic antibiotic (20 mg/kg cefazolin; Nida Pharma, Bangkok, Thailand) was given intravenously in two doses in a 24 h period during and after surgery, and an anti-inflammatory drug (4.4 mg/kg carprofen; Rimadyl®; Pfizer, Thailand) was given subcutaneously in one dose 30 min before anesthesia. Those medicines (antibiotic and anti-inflammatory drugs) were given orally for 7 days after surgery.

Four techniques that were combined in this study include cranial sartorius desmotomy, medial retinacular fascia release, patellar antirotational suture, and fascia lata overlapping [19,20]. Briefly, the joint was thoroughly explored on the lateral side for observing intraarticular structures such as the patella, femoral sulcus, cranial cruciate ligament and meniscus tear; then the joint capsule was closed. Cranial sartorius desmotomy and medial retinacular fascia release started with cutting through the medial retinaculum with a scalpel blade without cutting the joint capsule. The incision extended from the tibial plateau to the insertion of the cranial sartorius muscle. At this point, we slightly modified the traditional technique by completely cutting the part of the insertion of the cranial sartorius muscle. After that, patellar antirotational suture was performed using non-absorbable suture material. The suture was passed around the lateral fabella. The suture was then passed through the patellar tendon just proximal to the patella in a lateral-to-medial direction. Then, the suture was passed along the medial side of the patella in a proximal manner toward the distal direction. Finally, fascia lata overlapping was performed as the last procedure. Extensive fascia lata was trimmed and then the fascia lata overlapping was performed in a Mayo mattress suture pattern.

Assessment of Outcome of Surgery

The data and the methods used for the assessment of the outcome of the surgery included radiographic image scoring, clinical lameness evaluation, and recurrence of laxation after surgery.

Radiographic Scoring

The standard craniocaudal and mediolateral radiography of stifle was performed to evaluate the progression of osteoarthritis before surgery and 6 months after surgery. The parameter used was adapted from previous studies [12,21,22] by using osteophytosis at the margins of the stifle joint and subchondral bone sclerosis. Our study did not use other parameters such as joint effusion or soft tissue thickening because the quality of the images was not good (classical film, not digital film). The grading scores of the lesions were given subjectively from 0 to 4 (0=normal, 1=slight, 2=mild, 3=moderate, and 4=severe). All the radiographic images were evaluated at the same time without being aware of the name or the number of animals. The same observer repeated the assessment with a minimum interval of 2 weeks to obtain the estimate for the intra-rater agreement, and there were two observers carrying out the evaluation. The intra-rater agreement and the inter-rater agreement for each radiographic feature were assessed using the unweighted kappa (κ) test [22]. This radiography grade was report as the median (min-max) of the dogs.

Clinical Lameness Evaluation

Lameness scores from the medical records were categorized into four grades: 0=weight bearing, 1=partial weight bearing (temporary), 2=partial weight bearing (permanent), and 3=non weight bearing. The evaluations were done at day 0 (pre-surgery), day 3, day 7, day 14, and day 30 after surgery. This grade was reported as median (min-max) of the dogs' recovery after surgery.

Recurrence Rate

The number of dogs that had recurrence and the number of dogs that did not have recurrence were recorded and presented as numbers of dogs and percentages. The periods between the surgery date and the recurrence date were recorded as months. In the non-recurrence group, the dates were record from the surgery date to the end of December, 2015. The recurrence rate was compared between the grade of patellar luxation (1-4), and between the unilateral and the bilateral patellar luxation.

Statistical Analysis

General information regarding the dogs was reported as the number of dogs and the percentage. Some data were compared between the unilateral and the bilateral patellar luxation. The risk factors for the unilateral disease and the bilateral disease, and the recurrence of patella luxation were determined by odds ratio with 95% confidence intervals (CI) through univariate logistic regression. The Wald test was used to analyze significance, and the factors were considered as significant at P-value <0.05.

Lameness scores were compared between unilateral and bilateral patellar luxation on the same day and the comparison between the observations on the day after the surgery and the day pre-surgery was carried out by the Mann–Whitney U test. In the comparison between the grades of luxation, the bilateral patellar luxation group was excluded because most of the bilateral luxation had different grades of luxation. The correlation between the patellar luxation grade and the lameness score was determined by Spearman's rank correlation. The difference in the lameness or radiography score between unilateral and bilateral disease, as well as between grades of unilateral and bilateral patellar luxation was examined by Mann-Whitney U test. The radiographic score was done by two experts, and the agreement of the obtained results was statistically tested by kappa statistics [23]. The recurrence probability of the time period (months) after the surgery between patellar luxation with grade 3 and that with grade 4 was analyzed by using the Bayesian logistic regression model using JAG in R with rjags package.

RESULT

Statistical Summary

One hundred and thirty-three dogs met the inclusion criteria of this study *(Table 1)*. The most common breeds were Pomeranian (39%), Chihuahua (17%), Poodle (16%),

Yorkshire (11%), Miniature (7%), Shih Tzu (6%), and mixed (4%). Of the 133 dogs, 79 (60%) had unilateral disease and 53 (40%) had bilateral disease. The female dogs (57%) were more affected than the male dogs (43%), and 71% of the dogs were not sterile yet. The median age of the dogs that underwent surgery was 25 months, and the ages of the dogs ranged between 6 months and 10 years. The median weight of the dogs was 3.3 kg, and the weights of the dogs ranged between 0.8 kg and 9.2 kg.

Table 2 shows the comparison between dogs with unilateral and bilateral patellar luxation; most cases in this study had unilateral patellar luxation. More than half of the dogs in both the groups were neutered dog, while the average age, weight, lameness score, and recurrent period were not different between the two groups (P>0.05). Many factors were studied, along with the luxation type (unilateral or bilateral), and no risk factor of the luxation type was found *(Table 3)*. Besides, we found that the correlation between patellar luxation and lameness score (pre-surgery) was mild, with Spearman's rank correlation = 0.2820 and P-value=0.0148, but there was no significant difference in the lameness scores between the unilateral disease and the bilateral disease *(Table 2)*.

Lameness Score

Pre-surgery data found that the lameness score between unilateral patellar luxation and bilateral patellar luxation did not have a significant difference. Almost all the dogs had the lameness score significantly high on day 3 after surgery, and the score decreased at day 7 to day 30 *(Table 4)*. Before the surgery, it was found that the lameness score of grade 4 of the unilateral group was significantly high. At day 30 after the surgery, the lameness scores in almost all of the dogs were lower than the pre-surgery scores, and were significantly lower in grade 2 and grade 3. The lameness scores were compared between the four grades of unilateral patellar luxation, and significant difference was found between the grades at day 0, day 7, day 14, and day 30, with the highest score in grade 4.

Radiographic Progress of Stifle Osteoarthritis

The kappa test score of pre-surgery radiography was 0.90 and the score of radiography conducted 6 months after surgery was 0.97. Only 86 dogs had radiographic images that fit our criteria (preoperative and 6 months after operation) *(Fig. 1)*.

The radiographic images of 59 dogs (68%) from 86 did not change in 6 months after the surgery. In this group, it was found that 80% (47/59), 7% (4/59), 12% (7/59), and 3% (1/59) of the dogs had scores of 0, 1, 2, and 3, respectively. The remaining 27 dogs (32%) from the 86 presented changes in the radiographic scores: 30% (8/27), 4% (1/27), 63% (17/27), and 4% (1/27) of the dogs had changed scores, from 0 to 1, 0 to 2, 1 to 2, and 2 to 3. The radiographic scores were compared between the scores pre-surgery and the scores 6 months after surgery *(Table 5)*, and it was found that there was significant increase in the score in grade 4 of unilateral as well as bilateral patellar luxation.

Table 1. *Information concerning total 133 dogs in this study*

Breed	Total	Patellar Luxation		Age (month)	Sex		Weight (kg)	Neutered	
		Unilateral	Bilateral		Male	Female		Yes	No
Pomeranian	52	28	24	34±23	24	28	3.8±1.9	15	37
Chihuahua	22	13	8	25±19	7	15	2.7±1.5	6	16
Poodle	21	8	13	40±26	11	10	4.2±1.6	7	14
Yorkshire	15	12	3	32±22	6	9	2.8±1.1	3	12
Miniature	9	6	3	26±13	3	6	4.8±1.4	2	7
Shih Tzu	8	7	1	37±23	3	5	5.5±1.4	3	5
Mixed	6	5	1	29±20	3	3	5.6±2.8	2	4
Total	133	79	54	33±22	57	76	3.8±1.8	36	95

Table 2. *Comparison between unilateral and bilateral patellar luxation in dogs*

Patellar Luxation	N (m/f)	Nor./Ster.	Age# (month)	Weight# (kg)	Lameness Score*	Recurrent Rate	Recurrent Period# (month)
Unilateral	79 (33/46)	25/54	31.5±21.6	3.9±2.0	1 (0-3)	13.92% (11/79)	21.0±21.4 (1-79)
Bilateral	54 (23/31)	14/40	35.0±24.1	3.7±1.7	1 (0-3)	22.22% (12/54)	24.3±20.2 (2-79)
P-value	N.D.	N.D.	0.394	0.631	0.980	N.D.	0.699

N*=number,* ***N.D.****=not determined; Statistical analysis between the unilateral and the bilateral groups used the pair t-test; Symbol "#" indicates that the data are presented as mean±sd, symbol "*" indicates that the data are presented as median (min–max)*

Table 3. *Odds ratio (OR) with 95% confidence interval (CI) and P-value of bilateral vs unilateral, along with recurrence of patellar luxation after surgery*

Parameters	Bilateral vs Unilateral		Recurrence	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Breed				
Mixed (reference)	-	-	-	-
Shih Tzu	7 (0.50, 97.76)	0.15	3 (0.23, 39.61)	0.40
Yorkshire	4 (0.52, 30.76)	0.18	1.82 (0.16, 20.71)	0.63
Poodle	0.62 (0.10, 3.82)	0.60	0.83 (0.07, 9.86)	0.88
Pomeranian	1.17 (0.22, 6.33)	0.86	1.19 (0.12, 11.35)	0.88
Chihuahua	1.75 (0.28, 10.81)	0.55	0.5 (0.04, 6.68)	0.60
Miniature	2 (0.24, 16.61)	0.52	0.62 (0.03, 12.41)	0.76
Gender (female vs male)	0.95 (0.48, 1.91)	0.90	1.91 (0.73, 5.01)	0.19
Age (>12 months vs <12 months)	0.72 (0.27, 1.92)	0.52	1.92 (0.41, 8.95)	0.41
Weight (<5 kg vs >5 kg)	0.83 (0.35, 2.01)	0.68	1.44 (0.55, 3.74)	0.45
Side (left vs right)	-	-	1.64 (0.78, 3.47)	0.19
Bilateral vs Unilateral	-	-	1.69 (0.68, 4.15)	0.26
Grade				
1 (reference)	-	-	-	-
2	0.65 (0.25, 1.70)	0.38	0.39 (0.02, 6.65)	0.51
3	1.03 (0.41, 2.60)	0.95	2.33 (0.26, 20.66)	0.45
4	1.21 (0.44, 3.34)	0.72	12.44 (1.47, 105.50)	0.02
Sterilized vs Fertile	0.88 (0.38, 2.04)	0.76	1.44 (0.55, 3.74)	0.45

Table 4. *Median (min-max) of lameness scores in dogs that underwent surgery with four combined techniques*

Type and Grade of Luxation	Day 0	Day 3	Day 7	Day 14	Day 30
Bilateral patellar luxation	1 (0-3)	3 (2-3)*	2 (0-3)*	1 (0-3)	1 (0-3)*
Unilateral patellar luxation	1 (0-3)	3 (1-3)*	2 (0-3)*	1 (0-3)	1 (0-3)*
P-value	0.980	0.990	0.574	0.887	0.896
Categorized unilateral patellar luxation by grade					
Grade 1	0.5 (1-0)	2 (1-3)*	1 (0-2)*	0 (0-2)	0 (0-2)
Grade 2	1 (1-2)	2 (2-3)*	1.5 (1-2)	1 (0-2)	0 (0-1)*
Grade 3	1 (0-3)	2.5 (2-3)*	2 (1-3)*	1 (0-2)	1 (0-3)*
Grade 4	1.5 (0-3)	3 (2-3)*	2 (1-3)*	2 (1-3)	1 (0-2)
P-value (among grades within same day)	0.006	0.080	0.004	0.004	0.001

Superscript "" indicates p-value <0.05 when compared with the lameness score at pre-surgery (day 0) within the same type of luxation (unilateral or bilateral) or the same grade (grades 1-4)*

Recurrence of Disease After Surgery

In this study, it was found that 23 dogs (17%) from 133 presented recurrent patellar luxation. The numbers of no recurrence and recurrence in the dogs after surgery were found to be the highest in grade 4 (18:16), followed by grade 3 (43:7), but in grade 1 (14:0) and grade 2 (35:0), no case of recurrence was found *(Fig. 2)*. The percentages of recurrence and time in unilateral and bilateral patellar luxation are presented with a Kaplan-Meier curve *(Fig. 3)*, which shows that bilateral patellar luxation has higher change as regards recurrence of the disease. From 23 recurrent patellar luxation dogs, 12 were bilateral (52%), and the other 11 (48%) were unilateral. The recurrence time varied from 1 month to 79 months after surgery *(Table 6)*. From the medical records at the day of study, it was found that the longest duration of non-recurrence in dogs is 10 years (n=2; Poodle and Pomeranian with unilateral patellar luxation). Bilateral patellar luxation presented higher percentage of recurrence (22%) in comparison with unilateral patellar luxation (14%).

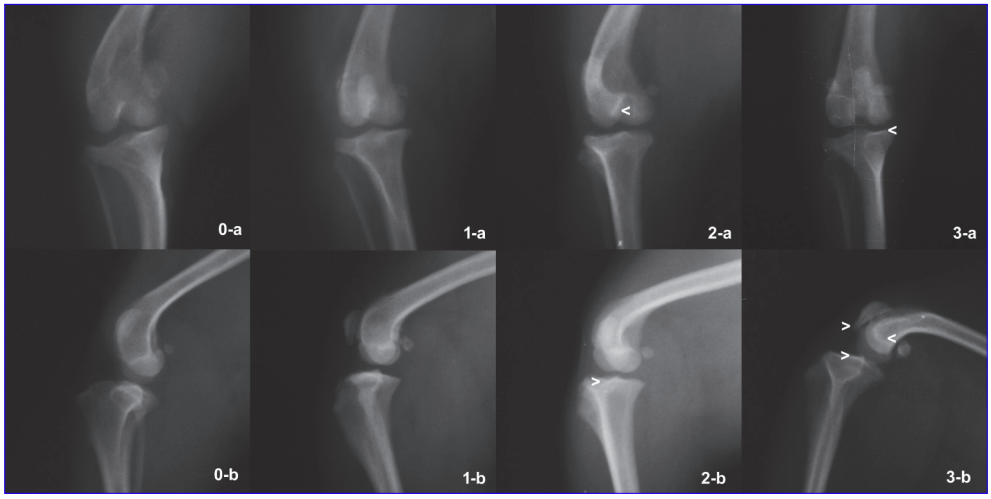


Fig 1. Representative craniocaudal (a) and mediolateral (b) radiography image of stifle in grades 0, 1, 2 and 3. The *arrow head* (<) indicates subchondral bone sclerosis

Table 5. *Median (min–max) of radiographic scores of dogs that underwent surgery with four combined techniques in comparison between pre-surgery and 6 months after surgery*

Type and Grade of Luxation	Pre-surgery	6 Months After Surgery	P-value
Bilateral patellar luxation	0 (0-2)	0 (0-2)	0.426
Unilateral patellar luxation	0 (0-3)	0 (0-3)	0.194
P-value	0.833	0.703	-
Categorized unilateral patellar luxation by grade			
Grade 1	0 (0-0)	0 (0-0)	1.000
Grade 2	0 (0-2)	0 (0-2)	0.406
Grade 3	0 (0-2)	0 (0-2)	0.366
Grade 4	1 (0-3)	2 (1-3)	0.009
P-value (among grades within same day)	0.000	0.000	-
Categorized bilateral patellar luxation by grade			
Grade 1	0 (0-0)	0 (0-0)	1.000
Grade 2	0 (0-0)	0 (0-0)	0.368
Grade 3	0 (0-2)	0 (0-2)	0.721
Grade 4	1 (0-2)	2 (0-2)	0.037
P-value (among grades within same day)	0.003	0.000	-

Table 6. *Comparison of numbers of dogs as regards non-recurrence and recurrence in unilateral and bilateral patellar luxation in different age groups*

Age at Surgery (month)	Unilateral Patellar Luxation		Bilateral Patellar Luxation	
	Non-recurrence	Recurrence	Non-recurrence	Recurrence
1-12	8 (89%)	1 (11%)	9 (90%)	1 (10%)
13-24	30 (86%)	4 (12%)	9 (90%)	1 (10%)
25-36	16 (89%)	2 (11%)	12 (12%)	1 (8%)
37-48	6 (86%)	1 (14%)	8 (64%)	4 (33%)
49-60	2 (100%)	0	1 (100%)	0
61-72	1 (100%)	0	0	5 (100%)
73-84	3 (100%)	0	0	0
85-96	1 (25%)	3 (75%)	0	0
97-108	0	0	0	0
109-120	0	0	2 (100%)	0
Total	67 (86%)*	11 (14%)#	41 (78%)*	12 (22%)#

The superscript symbols ("" and "#") represent high significance (P<0.05)*

The risk factor for recurrence of the disease was analyzed *(Table 1)*. Among the many factors, only grade 4 patellar luxation (OR=23.68, P=0.00), along with grade 3 patellar luxation (OR=7.2, P=0.06), was found to be a risk factor for recurrence. The median time of recurrence was observed approximately 21 months after the surgery, while the time period after surgery of grade 3 and grade 4 patellar luxation without recurrence was about 42 months of median *(Fig. 4)*. In addition, the time period after the surgery corresponding to recurrence among patellar luxation with grade 3 and grade 4 was considered. It was found that the risk of recurrence in patellar luxation with grade 3 and grade 4 decreased to 50% 18.7 months after the surgery (95%CI; 0.46 and 37.04 months) and that the recurrence chance was less than 2.5% after more than 37 months after the surgery *(Fig. 4)*.

DISCUSSION

In this study, it was demonstrated that combining four soft tissue techniques, namely cranial sartorius desmotomy, medial retinacular fascia release, patellar antirotational suture, and fascia lata overlapping for treating medial patellar luxation with not-fatted femoral sulcus in dogs of body weight lower than 10 kg yields excellence outcomes in grade 1 and grade 2.

As mentioned in most literature studies about patellar luxation treatment in dogs, in the cases of both medial and lateral patellar luxation, recurrence or reluxation is a major complication [5,12,13,15,16,24,25]. Our result found that

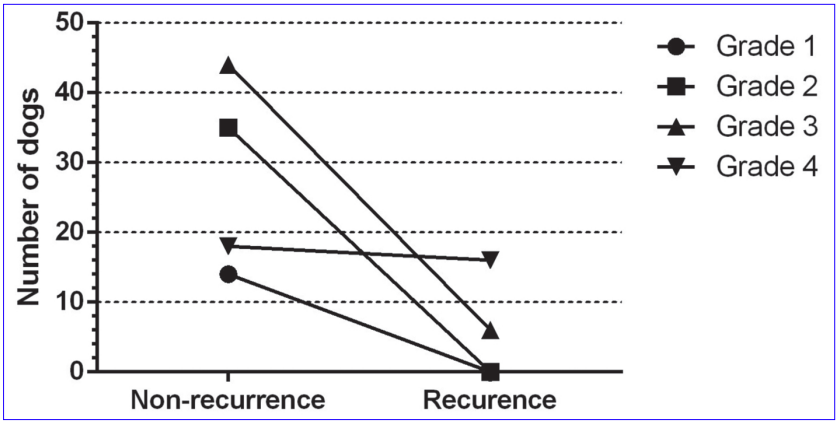
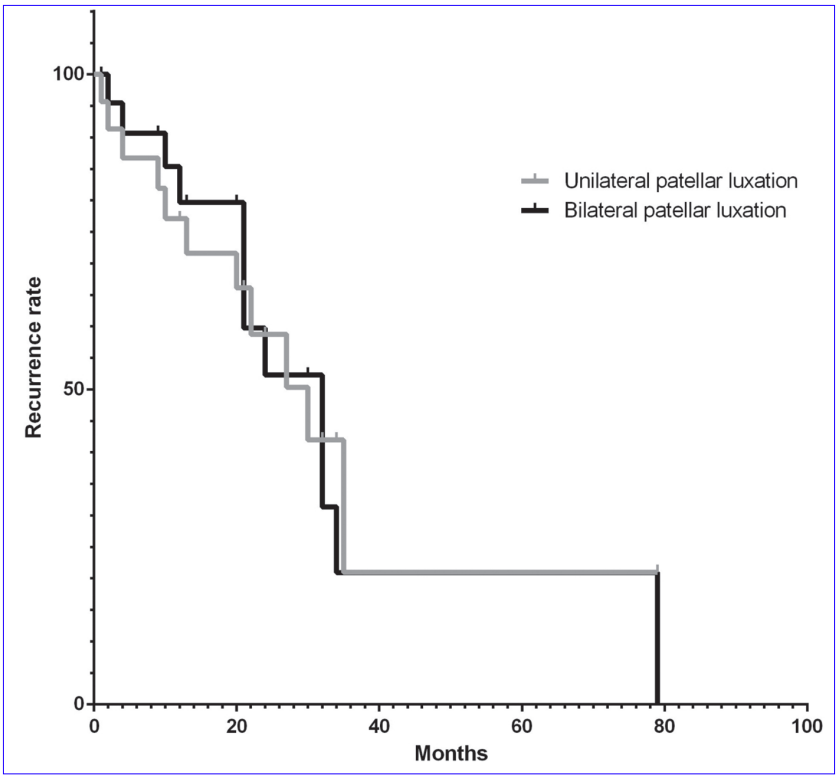


Fig 2. Comparison of the numbers of dogs that presented non-recurrence and recurrence in the four grades of patellar luxation

Fig 3. The Kaplan-Meier curve of time to patellar luxation recurrence of 12 bilateral luxation and 11 unilateral luxation dogs that underwent a combination of the procedures of cranial sartorius desmotomy, medial retinacular fascia release, patellar antirotational suture, and fascia lata overlap procedure. There were no significant differences between the two groups (P=0.8325)



the highest recurrence rate occurred in grade 4 (48%) and grade 3 (16%); additionally, there was no recurrence found in grade 1 and grade 2. This means that combining these four techniques may not be enough to prevent recurrence in particular grade 3 and grade 4 patellar luxation. The recurrent rate from this study is due to many factors, but we believe a major problem comes from tibia rotation in patellar grades 3 and 4, which affected the patellar tendon and quadriceps femoris muscle. Only 4 combined techniques were not strong enough to hold the patella in the femoral sulcus, even in cases of a non-flattened femoral sulcus. As mentioned in most literature, there are recommendations to do tibial translocation as treatment for grades 3-4 patella luxation for tibial tuberosity rotating over 30 degrees [19]. The overall recurrence percentage of the disease after surgery in our study is 17%, which is not such high in comparison with what other publications have reported as the recurrent rate found with variations in the rate from 7.6% up to 48%, depending on the grade of luxation and the surgical procedure [12,24,25]. Moreover, application of combinations of different procedures resulted in increased success rates [26]. One possibility that our overall recurrence rate was not high is because all the dogs included in this study had non-flattened femoral sulcus medial patellar luxation, while other publications did not exclude this condition.

In this study, by modifying a procedure from the literature on sartorius desmotomy, we performed completely cut insertion of the cranial sartorius muscle, as mentioned in the method. The cranial sartorius muscle is a lining between the crest of ilium and thoracolumbar fascia (origin) and the insertion was done at the patella. This muscle helps hip flexion and the extended stifle joint. From our

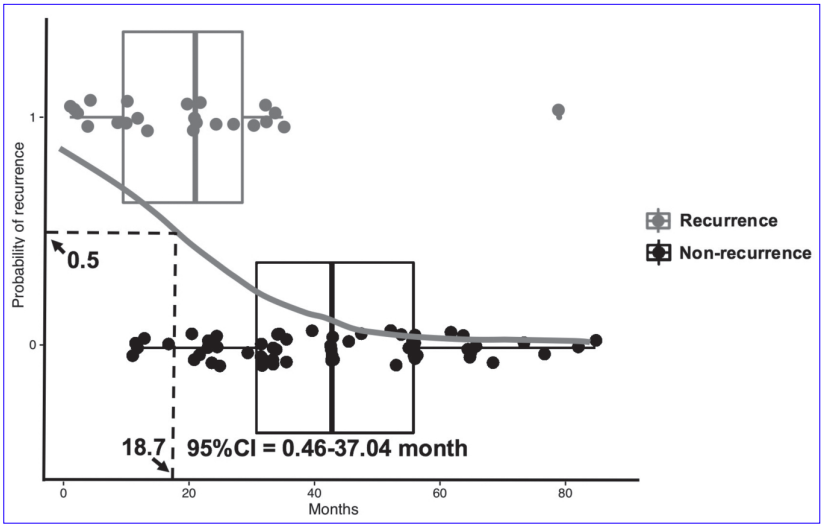


Fig 4. The recurrence and non-recurrence of patellar luxation of grade 3 and grade 4 in the time duration of months after the surgery are displayed in boxplot. The gray dots and the black dots represent the individual numbers of months after surgery with regard to recurrence and non-recurrence, respectively. The lines show the recurrence probability of the time period after the surgery among patellar luxation of grade 3 and grade 4. The red dashed line indicates that the time period after the surgery, 18.7 months (95% confidence interval (CI): 0.46 and 37.04 months), gives the chance of 0.5 for recurrence

observation, it was found that this muscle contracts more in medial patellar luxation dogs. However, so far, no study has reported this point, and we are studying the correlation between the cranial sartorius muscle and medial patellar luxation. From the lameness score evaluated following the surgery, in a month, it was not found that the dogs had any abnormality in walking. For this reason, we do believe that complete cranial sartorius desmotomy did not have any effect on gait. Our conclusion is supported by the findings of a previous study by Deban [27] on the activity of limb muscles in dogs at walk, trot, and gallop: It was reported that the cranial sartorius muscle shows significantly lower excitation in trotting. Previously, a study was carried out, in 2009, by Schilling [28] on the function of the extrinsic hind limb muscles in trotting dogs. It was demonstrated that the sartorius muscle had low activity during trotting at constant speed on flattened levels but increased activity during trotting uphill. The activity was not found to change when the dog trotted downhill.

Upon doing a comparison between the types of luxation (unilateral or bilateral), it can be observed that the recurrence rate in bilateral luxation (22%) is two times higher than the recurrence rate in unilateral patellar luxation (14%). A previous study reported that in unilateral patellar luxation, the dogs showed no effect in the opposite limb [29]. It is possible that the weight bearing ability of the hind limb after correction of one side of the affected limb in bilateral luxation is still abnormal and causes reluxation to occur easier than in unilateral luxation. On the other hand, unilateral luxation can balance the weight bearing, thus leading to lower recurrence rates. However, to confirm or prove our hypothesis, further experiments should be done by using faceplate analysis and gait analysis after correcting patellar luxation.

The radiography images of 32% of the dogs changed in 6 months after surgery, with significant changes in grade 4 patella luxation (both unilateral and bilateral). In patellar luxation grade 1 to grade 3, the score of most radiographic images was 0, and the score did not change within 6 months after surgery. This shows good prognosis that the patellar luxation joint would not develop osteoarthritis, in particular in low grades.

This study is not the first study to look at the outcome of patellar luxation treated without the bone reconstruction technique. Linney [12] reported the outcome of surgical treatment of medial patellar luxation without femoral trochlear groove deepening in 91 dogs. Eighteen of the 91 (19.8%) dogs had recurrence. So, taken together with our study, this demonstrates that the trochlear groove deepening procedure is not always necessary.

However, this study had many limitations. First, the outcome of this study can only be evaluated from radiographs (6 months post-surgery) and rates of recurrence. We cannot perform a CT-scan in the cases after surgery to study the morphology of the bone after surgery. Moreover, we cannot perform the gait or motion analysis after surgery as well as we cannot evaluate the lameness score for periods longer than 1 month after surgery. The lameness scores from the medical records are limited to scores of 1 month after surgery. Other limitation was that the duration between the occurrence of the disease and the treatment is unknown as the records do not carry that information; hence, delay in treatment might be a cause for increase in recurrence rate.

In conclusion, our study demonstrated the outcome of combining four soft tissue techniques for treating medial patellar luxation with non-flattened femoral sulcus in dogs. These techniques demonstrated excellent results for grade 1 and grade 2 medial patellar luxation with non-flattened femoral sulcus by showing fast recovery after surgery, prevention of progression of osteoarthritis, and no rates of recurrence. These techniques may be a good choice for patients with conditions similar to those in this study (grades 1 and 2 with non-flattened femoral sulcus)

before the surgeon makes the decision to perform bone reconstruction.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

Authors' Contribution

Nganvongpanit K. was a major contributor, who designed, conducted, and collected all the data used in this study. Buddhachat K. assisted in the statistical investigation and support of information for discussion. Boonsri B. and Sripratak T. assisted in data collection and Punyapornwithaya V. provided advice regarding statistical analysis. Nganvongpanit K. wrote the manuscript and Buddhachat K. assisted in the discussions and writing of the manuscript.

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REFERENCES

1. Nganvongpanit K, Yano T: Prevalence of and risk factors of patellar luxation in dogs in Chiang Mai, Thailand, during the years 2006-2011. *Thai J Vet Med*, 41, 449-454, 2011.

2. Boonsri B, Pradit W, Soontornvipart K, Yano T, Chomdej S, Ongchai S, Nganvongpanit K: Prevalence of cartilage erosion in canine patellar luxation and gene expression in affected joints. *Kafkas Univ Vet Fak Derg*, 22, 561-570, 2016. DOI: 10.9775/kvfd.2016.15036

3. Bellumori TP, Famula TR, Bannasch DL, Belanger JM, Oberbauer AM: Prevalence of inherited disorders among mixed-breed and purebred dogs: 27.254 cases (1995-2010). *J Am Vet Med Assoc*, 242, 1549-1555, 2013. DOI: 10.2460/javma.242.11.1549

4. Soontornvipart K, Wangdee C, Kalpravidh M, Brahmasa A, Sarikaputi M, Temwichitr J, Lavrijsen IC, Theyse LF, Leegwater PA, Hazewinkel HA: Incidence and genetic aspects of patellar luxation in Pomeranian dogs in Thailand. *Vet J* , 196, 122-125, 2013. DOI: 10.1016/j.tvjl.2012.07.027

5. Fattahian HR, Mohyeddin H, Molookpour H, Hoseinzadeh AR: Retrospective study of surgical treatment of various patellar luxations in dogs from 2004 to 2007. *IJVR*, 12, 56-60, 2011.

6. Daems R, Janssens LA, Béosier YM: Grossly apparent cartilage erosion of the patellar articular surface in dogs with congenital medial patellar luxation. *Vet Comp Orthop Traumatol*, 22, 222-224, 2009. DOI: 10.3415/VCOT-07-08-0076

7. Alam MR, Lee HB, Kim MS, Kim NS: Surgical model of osteoarthritis secondary to medial patellar luxation in dogs. *Vet Med*, 56, 123-130, 2011.

8. Alam MR, Ji JR, Kim MS, Kim NS: Biomarkers for identifying the early phases of osteoarthritis secondary to medial patellar luxation in dogs. *J Vet Sci*, 12, 273-280, 2011. DOI: 10.4142/jvs.2011.12.3.273

9. McKee WM, Cook JL: The stifle. **In,** Houlton JEF, Cook JL, Innes JF, Langley-Hobbs SJ (Eds): BSAVA Manual of Musculoskeletal Disorders. 350-395, Gloucester: British Samll Animal Veterinary Association, 2006.

10. Fitzpatrick CL, Krotscheck U, Thompson MS, Todhunter RJ, Zhang Z: Evaluation of tibial torsion in Yorkshire Terriers with and without medial patellar luxation. *Vet Surg*, 41, 966-972, 2012. DOI: 10.1111/j.1532-950X.2012.01041.x

11. Fujii K, Watanabe T, Kobayashi T, Hayashi K: Medial ridge elevation wedge trochleoplasty for medial patellar luxation: A clinical study in 5 dogs. *Vet Surg*, 42, 721-726, 2013. DOI: 10.1111/j.1532-950X.2013.12041.x

12. Linney WR, Hammer DL, Shott S: Surgical treatment of medial patellar luxation without femoral trochlear groove deepening procedures in dogs: 91 cases (1998-2009). *J Am Vet Med Assoc*, 238, 1168-1172, 2011. DOI: 10.2460/javma.238.9.1168

13. Pinna S, Venturini A, Tribuiani AM: Rotation of the femoral trochlea for treatment of medial patellar luxation in a dog. *J Small Anim Pract*, 49, 163-166, 2008. . DOI: 10.1111/j.1748-5827.2007.00532.x

14. Wangdee C, Hazewinkel HA, Temwichitr J, Theyse LF: Extended proximal trochleoplasty for the correction of bidirectional patellar luxation in seven Pomeranian dogs. *J Small Anim Pract*, 56, 130-133, 2015. DOI: 10.1111/jsap.12248

15. Stanke NJ, Stephenson N, Hayashi K: Retrospective risk factor assessment for complication following tibial tuberosity transposition in 137 canine stifles with medial patellar luxation. *Can Vet J*, 55, 349-356, 2014.

16. Cashmore RG, Havlicek M, Perkins NR, James DR, Fearnside SM, Marchevsky AM, Black AP: Major complications and risk factors associated with surgical correction of congenital medial patellar luxation in 124 dogs. *Vet Comp Orthop Traumatol*, 27, 263-270, 2014. DOI: 10.3415/VCOT-13-08-0100

17. Babić T, Kos J, Babić A, Budeč M, Stanin D, Vuković S, Popović M: Healing of the cartilage and the subchondral bone tissue after trochleoplasty in dogs. *Vet Arhiv*, 71, 173-186, 2001.

18. Putnam RW: Patellar luxation in the dog. Ontario, Canada: University of Guelph. 1968.

19. Tomlinson J, Constantinescu GM: Repair of medial patellar luxation. *Vet Med*, 89, 48-56, 1994.

20. Piermattei DL, Flo GL, DeCamp CE: The stifle joint. **In,** Piermattei DL, Flo GL, DeCamp CE (Eds): Handbook of Small Animal Orthopedics and Fracture Repair. 4th edn., 562-632, Saunders Elsevier, Missouri, 2006.

21. Chuang C, Ramaker MA, Kaur S, Csomos RA, Kroner KT, Bleedorn JA, Schaefer SL, Muir P: Radiographic risk factors for contralateral rupture in dogs with unilateral cranial cruciate ligament rupture. *PLoS One*, 25, 9, 2014. DOI: 10.1371/journal.pone.0106389

22. Innes JF, Costello M, Barr FJ, Rudorf H, Barr AR: Radiographic progression of osteoarthritis of the canine stifle joint: A prospective study. *Vet Radiol Ultrasound*, 45, 143-148, 2004. DOI: 10.1111/j.1740-8261.2004.04024.x

23. Viera AJ, Garrett JM: Understanding interobserver agreement: The kappa statistic. *Fam Med*, 37, 360-363, 2005.

24. Arthurs GI, Langley-Hobbs SJ: Complications associated with corrective surgery for patellar luxation in 109 dogs. *Vet Surg*, 6, 559-566, 2006. DOI: 10.1111/j.1532-950X.2006.00189.x

25. Willauer CC, Vasseur PB: Clinical results of surgical correction of medial luxation of the patella in dogs. *Vet Surg*, 16, 31-36, 1987. DOI: 10.1111/j.1532-950X.1987.tb00910.x

26. Wangdee C, Kalpravidh M: Tube realigment for patellar luxation repair in dogs. *Thai J Vet Med*, 38, 39-44, 2005. DOI: 10.1111/j.1532-950X.1987.tb00910.x

27. Deban SM, Schilling N, Carrier DR: Activity of extrinsic limb muscles in dogs at walk, trot and gallop. *J Exp Biol*, 215, 287-300, 2012. DOI: 10.1242/jeb.063230

28. Schilling N, Fischbein T, Yang EP, Carrier DR: Function of the extrinsic hindlimb muscles in trotting dogs. *J Exp Biol*, 212, 1036-1052, 2009. DOI: 10.1242/jeb.020255

29. Nganvongpa K, Klinhom S, Chaichit T: A comparative study of range of motion of forelimb and hind limb in walk pattern and trot pattern of Chihuahua dogs affected and non-affected with patellar luxation. *AJAVA*, 10, 247-259, 2015. DOI: 10.3923/ajava.2015.247.259

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Research Article

Effect of Dried Thyme Pulp *(Tymbra Spicata L. spicata)* on Fermantation Quality and *In Vitro* Organic Matter Digestibility of Meadow Grass and Alfalfa Silages

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Abstract

The aim of the study was to evaluate the possible effect of dried Thyme *(Thymbra spicata* L.) pulp (DTP) on first-cut meadow grass *(Poa trivialis* L.) and alfalfa *(Medicago sativa* L.) silage fermantation parameters and *in vitro* organic matter digestility. Experimental silage consisted of four replicates for each silage group (Grass, (G) and Alfalfa (L), respectively). Each silages groups were supplemented with DTP at 0, 0.5, 1.0, 3.0, and 5.0% level, respectively. The silos were stored for 60 d at room temperature (about 22°C). Results showed that silages added with DTP had higher amounts of NDF, acetic acid and propionic acid whlist lower amounts of lactic acid and NH_3N-TN compared to those of control (P<0.001). DTP significantly reduced microbial load of silage opened at 60 days without changing lactobacilli (P<0.001). It was concluded that the reducing in the load of undesirable microorganisms *(Enterobacter,* yeast and mould) without causing a decline in the number of lactobacilli could be provided significantly advantages in terms of improving the aerobic stability of the silages.

Keywords: *Thyme (Tymbra Spicata L. spicata) pulp, Phenolic compunds, Silage quality, Organic matter digestibility, Meadow grass, Alfalfa*

Kuru Kekik *(Tymbra Spicata L. spicata)* Posasının Çayır ve Yonca Silajının Fermantasyon Kalitesi ve *İn Vitro* Organik Madde Sindirilebilirliğine Etkisi

Özet

Bu çalışmanın amacı, birinci biçim çayır *(Poa trivialis* L.) ve yonca *(Medicago sativa* L.) silajlarına farklı oranlarda ilave edilen kuru kekik *(Thymbra spicata* L.) posasının (KKP) silaj fermantasyon kalitesi ve *in vitro* organik madde sindirilebilirliği üzerine etkilerini belirlemekti. Silaj grupları her biri dört tekerrür olacak şekilde hazırlandı. Her iki silaj grubuna sırasıyla %0, 0.5, 1.0, 3.0, ve 5.0 düzeyinde kuru kekik posası ilave edildi. Birer kg cam kavanoz silajları 60 gün oda ısında bekletildi. Araştırma sonuçları, KKP ilaveli silajlarda NDF, asetik asit ve propiyonik asit miktarları artarken, laktik asit ve toplam azot içerisindeki amonyak azotu miktarının azaldığını gösterdi (P<0.001). Deneme silajlarında organik maddenin sindirilme derecesinde bir değişiklik tespit edilmedi. Altmışıncı günde açılan silajlarda, KKP ilavesinin laktobasil sayısında bir değişiklik oluşturmadan istenmeyen mikroorganizma yükünü önemli derecede düşürdüğü belirlendi (P<0.001). KKP'nın silajların laktobasil sayısında herhangi bir düşüşe neden olmadan istenmeyen mikroorganizmalar (Enterobakter, maya ve küf) üzerindeki bu önleyici etkisinin silajların aerobik stabilizasyonunu iyileştirmesi bakımından önemli avantaj sağlayabileceği sonucuna varıldı.

Anahtar sözcükler: *Kekik (Tymbra Spicata L. spicata), Fenolik bileşikler, Silaj kalitesi, Organik madde sindirilebilirliği, Çayır silajı, Yonca*

INTRODUCTION

Silage quliaty and the resistance to aerobic deterioration are directly related to the fermentation types and the fermentation products by microbial activity during the ensiling. Consequently, phenolics- rich ingredients such as medicinal and aromatic plants or its essential oils derived from herbs have a selective antimicrobial properties and a

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potential in binding of proteins. Therefore, these products can regulate the silage microbiology and proteolysis in silages. On the other side, Turkey exports average 8000 tones dried thyme every year [1]. Additionally, 1000 tones of thyme are consumed as condiments/culinary herbs and herbal tea and also around 1500 tones of them are used in essential oil (EOs) production within Turkey [2]. EOs have received attention in recent years as potential 'natural' alternatives for replacing antibiotic growth promoters (AGPs) in animal diets due to their positive impact on growth performance, gut microbiota, animals products and welfare [3,4]. Only about 0.5-3% essential oil containing active biological compounds such as carvacrol, thymol, ocimene, and γ-terpinene is obtained from thyme *(Thymbra spicata)* by distillation [5]. On the other hand, less amount of essential oil but higher amounts of medicine-aromatic plant water and herbal pulp were obtained during the production of plant exctraction process. Medicine-aromatic plant water contains similar active ingredients to be less than the essential oil [4]. As a by-product, approximately same amount of proceed thyme remained after exctraction process and it is defined as organic waste. Most silages materials are ensilaged at a dry matter content between 20-50%. Within this dry matter range, many enzymes in the plant are still active at ensiling [6]. Also the wide variety of bacteria, yeasts and molds can all grow within this range. So it is a substantial challenge to bring all of this biological activity under control, but that is what a well-managed ensiling process does [6]. There are two key: the creation of an anaerobic environment and the fermentation of sugars by lactic acid bacteria to lactic acid and other products are the main two processes that preserve the raw silage materils in the silo. Thyme pulp is considered to have many biological-active compounds such as polyphenols, which might be act a silage fermentation inhibitor by changing silage microbiota population and their fermentations metabolites. Unfortunately, there are no data about its nutrient composition, active- biological compounds, availability of feed additive, and natural feed preservation as silage. Therefore, in the present study, the effects of different levels of thyme *(Thymbra spicata* L.) pulp on fermantation parameters, *in vitro* organic matter digestility, and microbial load of meadow grass *(Poa trivialis* L.) and alfalfa *(Medicago sativa* L.) silages were investigated.

MATERIAL and METHODS

Silage Preparation and Treatments

Thyme pulp *(Thymbra Spicata L. spicata)* was kept at room temperature (about 22°C) till reach to constant weight for drying process (dried thyme pulp, DTP) after water distilation process of fresh material. Meadow Grass *(Bromus inermis)* and first cut at the early-bloom stage of lucerne *(Medicago sativa* L.) of fresh material, was used as silage material. The chopped fresh silage materials were weighed, and DTP was mixed by hand and placed into 1.5 L. anaerobic glass jars (silos) by hand compressing to a final density of about 800g L^{-1} on a weight basis (w/w). Both of grass (G) and alfalfa (A) silages groups were supplemented with DTP at 0, 0.5, 1.0, 3.0, and 5.0% level (ten treatments and four replicates). The silos were stored at room temperature (about 22°C) for 60 days.

Analytic Procedures

Total phenolics (gallic acid, equivalent mg kg^{-1}) amount of DTP was determined according to Bae and Suh [7] and Cuendent et al.[8], respectively. Total phenol concentration was calculated from the calibration curve formed by gallic acid and total phenols were expressed as gallic acid equivalent mg kg^{-1}. Condensed tannin contents of Thyme pulp, meadow grass and alfalfa were estimated by butanol-HCl method as suggested by Makkar et al.[9].

Silage jars were opened after 60 d of ensiling. The pH values and dry-matter contents of the silages were immediately measured. Dry-matter contents of the silages were determined by drying 20 g of the ensiled forage at 105°C for 24 h in a forced-air oven and then weighing it. A total of 25 g of fresh silage was macerated with 100 mL distilled water with a high-speed blender. The macerated silage samples were filtered through two layers of cheesecloth and then the pH values of the filtrate were measured with a laboratory pH meter (Orion, Thermo Electron Corp., Kent, WA, USA). After pH determination, 10 mL filtrate was acidified with HCl and stored at -22°C for NH_3-N analysis. The NH_3-N content was analysed according to Broderick and Kang [10] by the Kjeldahl method. Volatile fatty acids (VFA) were determined by gas chromatography with Hewlett Packard-6890 equipment (Palo Alto, CA, USA). Lactic acid was determined by high-performance liquid chromatography (HPLC). Dry matter (DM), ash, and crude protein (CP) content of samples were analyzed by the AOAC methods [11]. Neutral detergent fibre (NDF) and acid detergent fiber (ADF) contents were analysed according to methods described by Van Soest et al.[12]. Four replicates were used in order to determination *in vitro* organic matter digestibilties (IVOMD, OM%) and metabolisable energy values (ME, Mcal kg^{-1} DM) of the silages samples. For this purpose, the methods described by Menke et al.[13] was used and calculated by using the equation reported by Menke et al.[14]. In order to measure *in vitro* methane production of silages, the silage samples were incubated in the rumen fluid in calibrated glass syringes following the procedures of Menke et al.[13]. Methane content was determined as a percentage of 24 h the total amount of gas formed [15]. For microbial enumeration four replicate used for each silage groups. For his purpose, LAB counts were determined using de Man, Rogosa, and Sharpe agar and enterobacteria counts were obtained using violet red bile agar. Yeasts and moulds were enumerated on spread plates of yeast extract and malt extract agar (pH: 3.5, obtained using sterilised lactic acid) [16].

Statistical Analysis

The number of microbial colony-forming units (CFUs) was expressed as logarithmic (log_{10}) transformation per gram of silage. All microbiological amounts from each silage sample were subjected to log transformation prior to statistical analysis. All data were analysed by one-factor ANOVA using the general linear model procedure of SAS [17]. Differences among means were determined by Duncan's multiple comparison tests at a significance level of P<0.001 [18].

RESULTS

Total phenols and condansed tannen amounts of DTP were estimated as 14.43 mg kg^{-1} equivalent gallic acid, and 5.73 g kg^{-1} DM, respectively. Condansed tannen amounts of meadow grass and alfalfa were also determinded as 5.48 and 11.14 g kg^{-1} DM. Nutritive values, metabolisable energy and *in vitro* organic matter digestibilities of dried thyme pulp, meadow grass and alfalfa were given in *Table 1*.

Table 1. *Nutrient composition, metabolisable energy value and in vitro organic matter digestibility of dried thyme pulp, meadow grass and alfalfa (in DM)*

Item	Raw Materials		
	DTP	Meadow Grass	Alfalfa
DM, %	92.90	32.99	21.98
CP, %	11.26	14.74	19.89
ADF, %	41.89	29.62	30.80
NDF, %	51.53	56.24	40.21
ME, Mcal/kg DM	1.88	1.94	2.51
IVOMD, %OM	50.97	51.66	63.98

DM: *Dry Matter,* ***CP:*** *Crude Protein,* ***ADF:*** *Acid Detergent Fiber,* ***NDF:*** *Neutral Detergent Fiber,* ***ME:*** *Metabolizable Energy,* ***IVOMD:*** *In Vitro Organic Matter Digestibility*

Compared with the control group (GS_{CTL}), increasing addition levels of DTP did not changed the DM, ADF, ME, IVOMD and CH_4 values of silages (P>0.001). Crude protein level was decreased while NDF level was increased in grass silages, especially in the addition of DTP at 5% level (P<0.001) *(Table 2)*.

When silage fermantation parameters and microbial counts of GS were investigated it was determined that adding of different levels of DTP were not changed the silage pH (P>0.001) *(Table 3)* but reduced NH_3-N/TN levels of GS especially adding level at over 3% (P<0.001). However, acetic and propionic acid levels of GS increased while lactic acid and butyric acid levels were decreased depending on the addition DTP levels (P<0.001). Increasing DTP level was shown a strong antimicrobial effect on both of aerobic (enterobacter and clostridia) and anaerobic (yeast and mould) microorganism of silages except to lactobacilli (P<0.001) *(Table 3)*. Generally, antimicrobial effect of DTP was more pronounced in the groups with dried thyme pulp at over 1%.

Effect of DTP on nutrients composition, IVOMD and ME value of alfalfa silages was presented in *Table 4*. Compared with the control group (AS_{CTL}), DM content of alfalfa silages increased by the addition of DTP at 3 and 5% levels (P<0.001) while ADF and NDF contents of those silages increased at all adding levels of DTP in alfalfa silages for 60 days (P<0.001). CP, ME, IVOMD and CH_4 values of alfalfa silages were found to be similar (P>0.001).

Compared with the control group (AS_{CTL}), the addition of DTP did not affect the silage pH (P>0.001) but reduced the NH_3-N/TN values of alfalfa silages, especially the groups with DTP at 3 and 5% levels (P<0.001) *(Table 5)*. A decrease of lactic acid levels and an increase of propionic acid levels were observed in alfalfa silages depending on the addition of TP at 1.0, 3.0 and 5.0% levels (P<0.001). All levels of DTP increased the acetic acid levels of alfalfa silages (P<0.001). Butyric acid was not detected in the alfalfa silages with DTP.

Table 2. *Effect of dried thyme pulp on nutrients composition, metabolisable energy (ME) , methane (CH_4) production and in vitro organic matter digestibility (IVOMD) of meadow grass silage*

Item	Treatments					SEM	P
	GS_{CTL}	G-DTP_1	G-DTP_2	G-DTP_3	G-DTP_4		
DM, %	32.39	31.85	32.20	32.19	33.02	0.263	NS
CP, %	14.01^{a}	13.14^{ab}	13.31^{ab}	12.85^{ab}	12.67^{b}	0.142	***
ADF, %	29.23	29.43	29.23	31.05	31.10	0.287	NS
NDF, %	48.27^{b}	48.56^{ab}	50.82^{ab}	51.61^{ab}	52.66^{a}	0.512	***
ME, Mcal/kg DM	1.91	1.90	1.91	1.90	1.95	0.046	NS
CH_4, %	9.11	8.71	10.54	10.63	10.20	0.234	NS
IVOMD, %OM	51.93	51.18	51.58	51.50	52.19	0.278	NS

^{a,b,c} The groups in the same line labeled different letters are statistically significant (P<0.001) ***GS_{CTL}:*** *Grass silage with not additive,* ***G-DTP_1:*** *Grass with dried thyme pulp at 0.5%,* ***G-DTP_2:*** *Grass with dried thyme pulp at 1.0%,* ***G-DTP_3:*** *Grass with dried thyme pulp at 3.0%,* ***G-DTP_4:*** *Grass with dried thyme pulp at 5.0%,* ***DM:*** *Dry Matter, CP: Crude Protein,* ***ADF:*** *Acid Detergent Fiber,* ***NDF:*** *Neutral Detergent Fiber,* ***ME:*** *Metabolizable Energy,* ***IVOMD:*** *In Vitro Organic Matter Digestibility,* ***CH_4:*** *Amount of methane in total amount of produced gas,* ***NS:*** *Non-significant, *** P<0.001*

Table 3. *Effect of dried thyme pulp on fermentation parameters and microbial load of meadow grass silage*

Item	Treatments					SEM	P
	GS_{CTL}	G-DTP_1	G-DTP_2	G-DTP_3	G-DTP_4		
pH	4.62	4.51	4.59	4.52	4.34	0.041	NS
NH_3-N/TN, %	9.32^{a}	7.89^{b}	7.27^{bc}	6.47^{c}	4.64^{d}	0.421	***
LA, g/kg DM	25.20^{a}	22.46^{a}	21.91^{a}	20.41^{ab}	16.97^{b}	0.771	***
AA, g/kg DM	7.54^{c}	13.98^{b}	14.61^{b}	15.73^{b}	21.11^{a}	1.184	***
PA, g/kg DM	0.84^{b}	1.14^{b}	2.14^{b}	10.29^{a}	13.82^{a}	1.486	***
BA, g/kg DM	5.29^{a}	3.96^{a}	3.74^{a}	4.52^{a}	0.35^{b}	0.470	***
Microbial counts, log cfu/g							
Enterobacter	120.00^{a}	46.67^{b}	8.67^{c}	7.67^{c}	6.67^{c}	11.86	***
Clostridia	373.33^{a}	300.00^{a}	300.00^{a}	123.33^{b}	116.67^{b}	29.06	***
Lactobacilli	6.03	6.10	6.20	6.24	6.28	0.283	NS
Yeast	600.0^{a}	466.67^{a}	40.00^{b}	16.67^{b}	12.30^{b}	71.05	***
Mould	1150^{a}	550^{b}	330^{c}	47^{d}	44^{d}	109.88	***

^{a,b,c} The groups in the same line labeled different letters are statistically significant (P<0.001) ***GS_{CTL}:*** *Grass silage with not additive,* ***G-DTP_1:*** *Grass with added at dried thyme pulp 0.5%,* ***G-DTP_2:*** *Grass with dried thyme pulp at 1.0%,* ***G-DTP_3:*** *Grass with dried thyme pulp at 3.0%,* ***G-DTP_4:*** *Grass with dried thyme pulp at 5.0%,* ***NH_3-N/TN:*** *Ammonia percentage in total nitrogen,* ***LA:*** *Lactic Acid,* ***AA:*** *Acetic Acid,* ***PA:*** *Propionic Acid,* ***BA:*** *Butyric Acid,* ***NS:*** *Non-significant,* ******** *P<0.001*

Table 4. *Effect of dried thyme pulp on nutrients composition, metabolisable energy (ME), methane (CH_4) production and in vitro organic matter digestibility (IVOMD) of alfalfa silages*

Item	Treatments					SEM	P
	AS_{CTL}	A-DTP_1	A-DTP_2	A-DTP_3	A-DTP_4		
DM, %	21.11^{b}	21.38^{b}	21.45^{b}	23.03^{a}	23.47^{a}	0.270	***
CP, %	18.77	18.50	17.67	17.60	17.45	0.162	NS
ADF, %	31.01^{b}	35.58^{a}	35.86^{a}	35.99^{a}	36.53^{a}	0.558	***
NDF, %	37.97^{b}	41.58^{a}	41.04^{a}	42.56^{a}	42.55^{a}	0.472	***
ME, Mcal/kg DM	2.48	2.50	2.42	2.40	2.39	0.081	NS
CH_4, %	11.82	11.44	11.54	11.37	11.03	0.087	NS
IVOMD, %OM	64.71	64.45	62.88	62.54	61.57	0.509	NS

^{a,b,c} The groups in the same line labeled different letters are statistically significant (P<0.001) ***AS_{CTL}:*** *Alfalfa silage with not additive,* ***A-DTP_1:*** *Alfalfa with dried thyme pulp at 0.5%,* ***A-DTP_2:*** *Alfalfa with dried thyme pulp at 1.0%,* ***A-DTP_3:*** *Alfalfa with dried thyme pulp at 3.0%,* ***A-DTP_4:*** *Alfalfa with dried thyme pulp at 5.0%,* ***DM:*** *Dry Matter,* ***CP:*** *Crude Protein,* ***ADF:*** *Acid Detergent Fiber,* ***NDF:*** *Neutral Detergent Fiber,* ***ME:*** *Metabolizable Energy,* ***IVOMD:*** *In Vitro Organic Matter Digestibility,* ***CH_4:*** *Amount of methane in total amount of produced gas,* ***NS:*** *Non-significant,* ******** *P<0.001*

Microbial load of alfalfa silages were lower than compared with the grass silage. In similar to grass silage, a superior antimicrobial activity was also determined in alfalfa silage (P<0.001). Compared with control silage (AS_{CTL}) lactobacilli counts were not changed in the alfalfa silage with DTP at 0.5, 1.0, 3.0% level (P>0.001) respectively, but increased in the alfalfa silage with DTP at 5.0% level compared to that of other groups (P<0.001).

DISCUSSION

In the present study, possibility use of DTP as a silage additive in grass and alfalfa silage was investigated. When considering the nutritional proterties of DTP, it can be said that DTP has the potential to be used as a silage additive *(Table 1)*. Also, DTP may have silage additive potantial due to its phenolic compounds which could reduce protein degradation and growth undesirable microorganisms (enterobacters, yeasts and moulds) in silages. Addition of DTP did not changed DM, ADF, IVOMD, ME and CH_4 level whlist the addition decreased CP level and increased NDF values of meadow grass silages (P<0.001) *(Table 2)*. There are limited data on silage quality and fermantation parameters of essential oil or purified compounds derived from essential oil, such as carvacrol, eugenol, linalool, cinnamic aldehyde and thymol. In a recent study where the effect of *oregano* and *cinnamon* essential oils at 400 mg/kg level on fermentation quality and aerobic stability of field pea silage were investigated it was reported that both additives reduced dry matter losses and prevented the cell wall materials of silages [19]. In the present study, DTP addition increased the ADF and NDF levels of both

Table 5. *Effect of dried Thyme pulp on fermantation parameters and microbial counts of alfalfa silages*

Item	Treatments					SEM	P
	AS_{CTL}	A-DTP_1	A-DTP_2	A-DTP_3	A-DTP_4		
pH	4.82	4.78	4.76	4.74	4.72	0.012	NS
NH_3-N/TN, %	20.21^a	19.65^a	19.10^a	17.37^b	16.12^b	0.415	***
LA, g/kg DM	59.85^a	50.37^a	35.58^b	30.45^b	25.98^b	3.492	***
AA, g/kg DM	15.24^d	17.44^d	24.22^c	36.30^b	42.69^a	2.876	***
PA, g/kg DM	1.31^c	1.69^{bc}	2.61^{ab}	2.87^{ab}	3.82^a	0.248	***
BA, g/kg DM	5.43^a	0.00^b	0.00^b	0.00^b	0.00^b	0.581	***
Microbial counts, log cfu/g							
Enterobacter	12.0^a	7.0^b	7.0^b	7.0^b	6.0^b	0.63	***
Clostridia	325^a	285^a	80^b	75^b	85^b	30.52	***
Lactobacilli	6.31^b	6.36^{ab}	6.41^{ab}	6.47^{ab}	6.58^a	0.271	***
Yeast	160.0^a	53.3^b	13.3^b	8.3^b	7,67^b	16.10	***
Mould	226^a	110^b	85^b	38^c	27^c	19.26	***

^{a,b,c} The groups in the same line labeled different letters are statistically significant (P<0.001) ***AS_{CTL}:*** *Alfalfa silage with not additive,* ***AS-DTP_1:*** *Alfalfa with added at dried thyme pulp 0.5%,* ***A-DTP_2:*** *Alfalfa with dried thyme pulp at 1.0%,* ***A-DTP_3:*** *Alfalfa with dried thyme pulp at 3.0%,* ***A-DTP_4:*** *Alfalfa with dried thyme pulp at 5.0%,* ***NH_3-N/TN:*** *Ammonia percentage in total nitrogen,* ***LA:*** *Lactic Acid,* ***AA:*** *Acetic Acid,* ***PA:*** *Propionic Acid,* ***BA:*** *Butyric Acid,* ***NS:*** *Non-significant, *** P<0.001*

silage samples (P<0.001). The possible mechanism of DTP on ADF and NDF values of silages could be attributed to the limited fermantation caused by the active antimicrobial compound (phenylpropanoids and hydrocarbons) of DTP. According to end-products of the expression/ metabolization of phenolic compounds by Lactic Acid Bacteria (LAB), some of them *(L. plantarum)* are activated while some others are inhibited *(Lactobacillus collinoides* and *L. brevis)* during the ensilage. Thus, a heterofermentatif process are shaped in silages [20]. These finding were in agreement with the results of Soycan Önenç et al.[19]. Therewithal, ADF (41.89%) and NDF (51.53%) values of DTP could be also contributed to those values of silage samples. A decreasing observed in DM content of alfalfa silage with DTP at 3 and 5% level compared with L_{CTL} are supported to this review. In both silage groups, the addition of DTP did not change silage pH but decreased NH_3-N/TN, lactic acid and butyric acid levels (P<0.001). Additionally, treatments increased acetic acid and propionic acid levels of both silages (P<0.001). This current silage fermantation profile indicated that interaction between the phenolic compound with lactic acid bacteria has established a heterofermentative fermentation. This fermentation type was especially pronounced in the silages with higher level of DTP (P<0.001).

These findings were in agreement with the results of the previous studies where the effects of silage inhibitors on silage quality were investigated [21-23]. In both silage groups, pH values was over 4.0 (pH: 4.34-4.62 for grass silages and 4.72-4.82 for alfalfa silages, respectively) and the addition of DTP into the silages did not change silage pH (P>0.001). As considering DM content of grass silages (31.85-33.02%), it was thought that this high level of DM caused to a prevention the decreasing of grass silage pH. For alfalfa silages, this results could be attributed to the high level of nitrogen and buffer capasity of alfalfa. Hence, previously studies reported that lower pH values was observed in the grass silages *(Perennium ryegrass)* with lower DM (26.4-28.0%) content [21] and the alfalfa silages with the addition of soluble carbohydrates (Honey Locust Pods at 20-100 g kg^{-1} fresh material) [24]. These results of previously studies are supported to this interpretation. On the other hand, high acetic acid level of silages could be also caused the current pH range, which is a weaker acid than lactic acid [24]. Additionally, purified compounds derived from essential oils such as carvacrol and tyhmol directly increased the silaj pH value [19]. Soycan-Önenç et al.[19] reported that the addition of *Origanum onites* essential at 400 mg kg^{-1} fresh pea forage, did not change the silage pH (4.40) while the addition of *cinnamon* essential at same level increased directly the silage pH (4.47) compared with the control (pH: 4.31) (P<0.001). This finding was in agreement with the results orserved from the current study. NH_3-N/TN values of both silages groups reduced by the addition of DTP, especially alfalfa silages with DTP at 3 and 5% level (P<0.001) *(Table 3* and *Table 5)*. A reducing of NH_3-N/TN values could be attributed to the limited fermantantion caused by the antimicrobial active compounds preventing the proteolysis such as polyphenols. Polyphenol compunds of DTP could be reduced nitrogen losses by binding proteins of material. Thus, in a previos study where the effects of honey locust pods (HLP) as carbohydrate source with rich polyphenols on the fermentation parameters and microbiological characteristics of alfalfa *(Medicago sativa* L.) silages were

investigated, it was reported that increasing additive level of honey locust pods significantly reduced silages NH_3-N levels due to its high polyphenols compunds [24]. In contrary to the report that phenolic compounds reduce the methanogenesis, in the present study, the addition of DTP did not change methane production in both of silage groups. This could be attributed to the metabolization of phenolic compund to end-products during ensilage, and inefficacy of these end-products on inhibiting of the methanogenesis [25]. In genarally, the addition of DTP suppressed the lactic acid levels of silages and contrary to expectation, LA levels of alfalfa silages were higher than that of the grass silage (P<0.001). As DM contents of silages were considered, DM contents of alfalfa silages were lower compared with the grass silages. At this DM content, alfalfa silages might have higher water soluble carbohydrate caused to increase LA production compared with the grass silages. Also, low amount of LA in grass silage could be attribited the meadow grass used in the study that partially mixed with the legumes [26]. Hence, lactobacilli counts of alfalfa silages were higher than that of grass silages *(Table 3* and *Table 5)*. There was a reducing or a tendency reducing in LA levels of silages. This reducing was pronounced in the alfalfa silages starting from the addition of DTP at 1% level (P<0.001). This finding was in agreement with the results of Soyvan-Önenç et al.[19]. On the other hand, this reduction in LA level could be attributed to the pH range which might be contributed to acetobacter activity. LA is transformed to acetic acid by acetobacters activity at certain pH values (around pH: 4.0-4.5) causing an increasing of silage pH [25]. LA levels of a quality silages are reported as 50-70 g kg^{-1} of silage DM [23].

In the current study, pH values and LA amounts of silage were lower than generally acceptation for a quality silage. But there are different opinions on this issue. Thus, Baytok et al.[22] reported that higher pH level and lower LA amount did not adversly effect silage fermentation and it nutritive values. On the other hand, the high levels of LA are reduced the microbial protein production in ruminants, of which fed mainly silage [26]. Thereby, the higher LA level might be desirable in terms of fermentation quality but not be possible to say same thing in terms of its effect on animal performance. Maximun protection of dry matter and cell wall components of silage are essential. The source of LA in silage is either cell wall components or water soluble carbohyrdrates of the plant material. AA level of silages is also important as much as LA level in this protection [25]. Furthermore, the source of AA is LA produced in silage that is transforming to AA by asetobacter in certain pH of silage. Therefore, it can be speculated that the ideal silage fermantation should provide the minumin level of LA that will be form a protective effect in sufficient quantity. In the present study, it was observed that the addition of DTP enabled the acetobacter fermentation and thus increased the AA level by reducing the pH drop (P<0.001) *(Table 3* and *Table 5)*. The high level of AA caused by the high buffering capacity in the silages with low dry matter than 25% was the expected fermantation pattern from alfalfa silages [29]. In additionally, the silage pH values (around pH: 4.3-4.8) might be stimulated production of acetic acid. Acetic acid bacteria are obligate aerobic and acid-tolerant bacteria. When the silage pH is around 4.0-4.5, LA are transformed to AA by acetic acid bacteria for maintaining their bacterial activities [25]. Similarly, it was determined that propionic acid levels were also increased in the silage samples with added DTP (P<0.001) *(Table 3* and *Table 5)*. This could be attributed to the silage pH which allows the activity of propionic acid bacteria [25]. Acetic acid is a fungicidal agent such as propionic acid and it inhibits the growth of yeast and mold fungi [30]. There is a positive correlation among silage anerobic stabilisation and silage organic acid level, and acetic acid obviously acts as an inhibitor of the growth of spoilage organisms [25,30]. Acetic acid shows a stronger antibacterial property compared with lactic acid, especially in certain pH values (4.0-4.5), and improves aerobic stability of silages after opening [25,31]. Furthermore, it was reported that silage should be promoted by acetic acid fermantation to improve aerobic stability and acetic acid is not a factor that directly reduces the feed intake of animals [25,31]. In a previos study where the effects of various compounds on the aerobic stability of silages were evaluated, it was determined that acetic acid has been proven to be the sole substance responsible for the increased aerobic stability, and acetic acid acts as an inhibitor of spoilage organisms. This results in the higher antimicrobial activity of acetic acid in surroundings where the pH values are low (around pH 4), since a greater proportion of the acetate is not dissociated [25]. The amount of acetic acid in silages necessary to successfully preserve the silages after the silos are opened can be quite high. The silage used in an experiment required a concentration of acetic acid of more than 50 g kg^{-1} for a stability of 100 h. and lactic acid exhibited clearly lower inhibition of the yeasts and mold tested [25]. Similar results were reported by Moon [32] who observed that for the same degree of inhibition, lactate concentrations had to be about two times higher than the concentrations of acetate. Therefore, the results in the current study reconfirm the importance of undissociated acids in spoilage inhibition. Clostridia and enterobacteria are considered undesirable microorganism for silage fermentation and quality as well as yeast and mold [33-35]. Microbial load of grass silages were higher than compared with the alfalfa silage *(Table 3* and *Table 5)*. This could be related to the initial contamination of fresh plant material with soil or manure [35,36]. Additionally, the addition of DTP showed a superior antimicrabial effect and significantly reduced microbial counts, especially yeast and mould *(Table 3* and *Table 5)* (P<0.001). Unfortunately, we could not determine the antimicrobial potential of DTP and aerobic stabilities of silage samples. Therefore, it could not be possible to reveal the relationship among these parameters in the concrete. But, it could be argued that both phenolic compounds [19,37] and organic acids of silage

(acetic acid and propionic acid, especially in around pH: 4.0-4.5) [25] acted synergistically, thus the silage anerobic stabilities were probably improved. As far as we aware, there is no data on demonstrating the effect of phenolic-rich ingredients (feed-stuff obtained after essential oil distilation process of herbs) on silage quality. The results revealed that the ensiling of meadow grass and alfalfa by addition of dried thyme pulp increased silage fermentative quality but did not affect organic matter digestibility. Dried thyme pulp, showed antimicrobial effect by increasing acetic and propionic acid levels of both silage samples. A reducing in the load of undesirable microorganisms (yeast and mold etc.) without causing a decline in the number of lactobacilli can be provided significantly advantages in terms of improving the aerobic stability of the silages. More research is required to determine how such phenolics-rich ingredients affects the silage aerobic stability.

REFERENCES

1. Anonymous: Report of Aegean Exporters' Assciations. Aegean Exporters' Associations. Izmir - Turkey, 2005.

2. Baser KHC: Aromatic biodiversity among the flowering plant taxa of Turkey. *Pure Appl Chem,* 74, 527-545, 2002. DOI: 10.1351/pac200274040527

3. Aksu T, Sarıpınar Aksu D, Kaya Durmuş A, Duran N, Önel SE, Canoğulları S: The response of Japanese quails to dietary *Thymbra spicata* L. essential oil. *Rom Biotech Lett,* 2016 *(In press)*.

4. Özkaya S, Erbaş S, Özkan O, Baydar H, Aksu T: The use of aromatic water of oregano *(Origanum onites* L.) as feed additives in calves diets. *VI. International Scientific Agricultural Symposium,* October 15-18, Agrosym, Jahorina, 2015.

5. Aksu T, Aksu MI, Önel SE, Yakan A, Kaya DA, Baylan M: Effect of thyme oil *(Thymbra spicata* L. var. *spicata)* on meat quality in Japanese quails. *Arhive fül Gelfkunde*, 1, 2014. DOI: 10.1399/eps.2013.6

6. Muck RE: Silage microbiology and its control through additives. *R Bras Zootec*, 39, 183-191, 2010. DOI: 10.1590/s1516-35982010001300021

7. Bae SH, Suh HJ: Antioxidant activities of five different mulberry cultivars in Korea. *LWT-Food Sci Technol,* 40, 955-962, 2007. DOI: 10.1016/j.lwt.2006.06.007

8. Cuendet M, Hostettmann K, Potterat O: Iridoid glucosides with free radical scavenging properties from fagraea bluemi. *Helvetica Chimica Acta,* 80, 1144-1152, 1997. DOI: 10.1002/hlca.19970800411

9. Makkar HPS, Blummel M, Becker K: Formation of complete between polyvinyl pyrolidones or polyethylene glycol and tannins and their implication in gas production and true digestibility in *in vitro* technique. *Brit J Nutr,* 73, 897-913, 1995. DOI: 10.1079/bjn19950095

10. Broderick GA, Kang JH: Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *J Dairy Sci,* 63, 64-75, 1980. DOI: 10.3168/jds.S0022-0302(80)82888-8

11. AOAC: Association of Official Analytical Chemists. Official Methods of Analysis. 15th edn.,1298-1302, Washington DC, 1990.

12. Van Soest PJ: Methods of dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci,* 74, 3583-3597, 1991. DOI: 10.3168/jds.S0022-0302(91)78551-2

13. Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Scheinder W: Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim Res Development,* 28, 7-55, 1988.

14. Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Scheinder W: The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas prodution when they are incubated with rumen liquor in vitro. *J Agric Sci*, 93, 217-222, 1979. DOI: 10.1017/S0021859600086305

15. Goel G, Harinder Makkar PS, Becker K: Effects of *Sesbania sesban* and *Carduus pycnocephalus* leaves and Fenugreek *(Trigonella foenum-graecum* L.) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. *Anim Feed Sci Techn,* 147, 72-89, 2008. DOI: 10.1016/j.anifeedsci.2007.09.010

16. Li Y, Nishino N: Effects of ensiling fermentation and aerobic deterioration on the bacterial community in italian ryegrass, guinea grass, and whole-crop maize silages stored at high moisture content. *Asian-Australas J Anim Sci,* 26, 1304-1312, 2013. DOI: 10.5713/ajas.2013.13175

17. SAS: SAS/STAT® 9.1 User's Guide., SAS Inc Cary, NC, 1989.

18. Steel RG, Torrie JH: Principles and procedures of statistics: A biometrical approach. 3rd edn., Mc Graw Hill Book Company, New York, 1980.

19. Soycan-Önenç S, Koc F, Coşkuntuna L, Özdüven ML, Gümüş T: The effect of oregano and cinnamon essential oils on fermentation quality and aerobic stability of field pea silages. *Asian Australas J Anim Sci*, 28, 1281-1287, 2015. DOI: 10.5713/ajas.15.0122

20. Stead D: The effect of hydroxycinnamic acids on the growth of wine-spoilage lactic acid bacteria. *J Appl Microbiol*, 75, 135-141, 1993. DOI: 10.1111/j.1365-2672.1993.tb02758.x

21. Baytok E, Muruz H: The effects of formic acid or formic acid plus molasses additives on the fermentation quality and DM and ADF degradabilities of grass silage. *Turk J Vet Anim Sci,* 27, 425-431, 2003.

22. Baytok E, Aksu T, Karslı MA, Muruz H: The effects of formic acid molasses, and inoculant as silage additives on corn silage composition and ruminal fermentation characteristics in sheep. *Turk J Vet Anim Sci*, 29, 469-479, 2005.

23. Lorenzo FB, O'Kiely P: Alternatives to formic acid as a grass silage additive under two contrasting ensilability conditions. *Irish J Agric Food Res,* 47, 135-149, 2008.

24. Canbolat Ö, Kalkan H, Filya İ: The use of honey locust pods as a silage additive for alfalfa forage. *Kafkas Univ Vet Fak Derg,* 19, 291-297, 2013. DOI: 10.9775/kvfd.2012.7710

25. Kayembe K, Basosila L, Mpiana PT, Sikulisimwa PC, Mbuyu K: Inhibitory effects of phenolic monomers on methanogenesis in anaerobic digestion. *Br Microbiol Res J*, 3, 32-41, 2013. DOI: 10.9734/BMRJ/2013/2291

26. Tjandraatmadja M, Norton BW, Mac Rae IC: Ensilage characteristics of three tropical grasses as influenced by stage of growth and addition of molasses. *World J Microbiol Biotechnol*, 10, 74-81, 1994. DOI: 10.1007/BF00357568

27. Danner H, Holzer M, Mayrhuber E, Braun R: Acetic acid increases stability of silage under aerobic conditions. *Applied Environ Micribiol,* 99, 562-567, 2003. DOI: 10.1128/AEM.69.1.562-567.2003

28. Aksu T, Baytok E, Karslı MA, Muruz H: The effects of formic acid molasses, and inoculant as silage additives on corn silage composition, organic matter digestibility and microbial protein synthesis in sheep. *Small Ruminant Res,* 1, 29-33, 2006. DOI: 10.1016/j.smallrumres.2004.12.013

29. Kung L, Shaver R: Interpretation and use of silage fermentation analysis reports. *Focus on Forage,* 3, 1-4, 2001.

30. Filya İ, Muck RE, Contreras-Govea FE: Inoculant effects on alfalfa silage: Fermentation profile products and nutritive value. *J Dairy Sci,* 90, 5108-5114, 2007. DOI: 10.3168/jds.2006-877

31. Beck T, Groß F, Beck R: Untersuchungen zur Kenntnis der Gärfuttermikroflora. II. Mitteilung: Die Milchsäurebakterienflora von Silagen unterschiedlicher Qualität aus Praxisbetrieben. *Wirtschafsteigene Futter*, 33, 34-43, 1987.

32. Moon NJ: Inhibition of the growth of acid tolerant yeasts by acetate, lactate and their synergistic mixtures. *J Appl Bacteriol,* 55, 453-460, 1983. DOI: 10.1111/j.1365-2672.1983.tb01685.x

33. Woolford MK: The Silage Fermentation. [Microbiological Series, No.14], 115-128. New York, Marcel Dekker Publisher, 1984.

34. Schlegel HG: General Microbiology. 6th edn., 224-229. Cambridge University Press, Cambridge, UK 1987.

35. McDonald P, Henderson AR, Heron SJE: The Biochemistry of Silage. 2nd edn., 186-198. Chalcombe Publications Marlow, UK, 1991.

36. Rammer C, Ostling C, Lingvall P, Lindgren S: Ensiling of manured crops - Effects on fermentation. *Grass Forage Sci*, 49, 343-351, 1994. DOI: 10.1111/j.1365-2494.1994.tb02009.x

37. Radwan IA, Abed AH, Abeer MR, Ibrahim MA, Abdallah AS: Effect of thyme, clove and cinnamon essential oils on *Candida albicans* and moulds isolated from different sources. *Am J Vet Sci*, 9, 303-314, 2014. DOI: 10.3844/ajavsp.2014.303.314

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Research Article

Clonal Heterogeneity and Efficacy of BOX and $(GTG)_5$ Fingerprinting Methods for Molecular Typing of *Escherichia coli* Isolated from Chickens in IRI

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Abstract

This study evaluates the clonal heterogeneity and efficacy of BOX-PCR and $(GTG)_5$-PCR for DNA-based typing of *Escherichia coli* strains isolated from feces of chickens in IRI. Fecal samples were collected from chicken husbandry followed by *E. coli* isolation through biochemical tests. Isolates were finger printed by BOX-A1 and $(GTG)_5$ primers. Dendrograms were generated based on 80% similarity and Shannon-Weaver index was calculated. One hundred and six *E. coli* isolates were obtained from chicken's fecal sample. By $(GTG)_5$ primer, of 106 isolates, two isolates were untypeable, while 104 isolates generated 100 unique, and 2 duplicate profiles. The dendrogram generated six clusters (G1-G6). With BOX-PCR, 106 *E. coli* isolates revealed 50 unique BOX profiles, in addition to 22 repetitive profiles, while 12 isolates were untypeable. Based on the bands and dendrogram, the 106 strains were grouped into six clusters (B1-B6). Shannon-Weaver index was 4.665 for $(GTG)_5$-PCR and 0.281 for BOX-PCR. $(GTG)_5$-PCR revealed complex clonal heterogeneity, more discriminatory power, less untypeable isolates, higher Shannon-Weaver index, and less isolates with the same profile in comparison to BOX-PCR. Although $(GTG)_5$-PCR proved to be a powerful typing method, it is recommended to combine two or more different typing methods for higher discriminatory power.

Keywords: *BOX-PCR, Chicken, Clonal heterogeneity, Escherichia coli, $(GTG)_5$-PCR, Molecular typing*

BOX ve $(GTG)_5$ Parmak izi Metotlarının İran'da Tavuklardan İzole Edilen *Escherichia coli*'nin Moleküler Tiplendirilmesinde Klonal Heterojenite ve Yeterliliği

Özet

Bu çalışma ile İran'da tavuk dışkılarından izole edilen *Escherichia coli* suşlarının DNA temelli tiplendirmesinde BOX-PCR ve $(GTG)_5$-PCR metotlarının klonal heterojenitesi ve yeterliliği araştırılmıştır. Biyokimyasal test ile *E. coli* tespit edilen kümeslerden dışkı örnekleri toplandı. İzolatların parmakizi BOX-A1 ve $(GTG)_5$ primerler kullanılarak alındı. Dendrogramlar %80 benzerlik baz alınarak üretildi ve Shannon-Weaver endeksi hesaplandı. Yüzsekiz *E. coli* izolatı tavuk dışkı örneklerinden elde edildi. $(GTG)_5$ primer ile yapılan analizde 106 izolatın 2'si tiplendirilemezken 104 izolattan 100 özgün ve 2 duplike profil üretildi. Dendrogram altı küme (G1-G6) oluşturdu. BOX-PCR ile 106 *E. coli* izolatından 22 tekrarlayan profile ile birlikte 50 özgün BOX profili oluşurken 12 izolat tiplendirilemedi. Oluşan bantlar ve dendrograma göre 106 suş 6 küme (G1-G6) içerisinde gruplandırıldı. Shannon-Weaver endeksi $(GTG)_5$-PCR için 4.665, BOX-PCR için ise 0.281 olarak belirlendi. $(GTG)_5$-PCR; BOX-PCR ile karşılaştırıldığında kompleks klonal heterojenite, daha fazla ayırt edici güç, daha az tiplendirilemeyen izolat, daha yüksek Shannon-Weaver endeksi ve daha az aynı profilli izolat elde edilmesini sağladı. $(GTG)_5$-PCR daha güçlü tiplendirme metodu olmasına rağmen iki veya daha fazla tiplendirme metodunun birlikte kullanılması daha yüksek ayırıcı güç için önerilmektedir.

Anahtar sözcükler: *BOX-PCR, Tavuk, Klonal heterojenite, Escherichia coli, $(GTG)_5$-PCR, Moleküler tiplendirme*

INTRODUCTION

Escherichia coli is a major member of the human and animal normal gut microflora. Although commensal *E. coli* strains are nonpathogen, pathogenic types of *E. coli*, including Enterotoxigenic (ETEC), Enterohemorrhagic (EHEC), Enteroaggregative (EAEC), Enteroinvasive (EIEC), and Enteropathogenic (EPEC) can cause intestinal diseases [1].

The ability to differentiate *E. coli* strains is critical for molecular typing, identifying bacteria at the strain level,

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studying bacterial population dynamics, and epidemiological surveillance of bacterial contamination; thus, it is necessary to apply rapid, reliable, and high-throughput typing methods [2]. Different phenotypic and biochemical characteristics have been previously used for the epidemiological investigations of *E. coli* [3]. However, the limitations of the phenotypically based typing methods (time consuming and lacking sufficient resolution amongst related strains), have led to the development of many DNA-based techniques. Therefore, a reliable genetic discriminatory method should be applied [4].

There are numerous methods to identify and characterize the diversity of bacteria, including; DNA banding pattern-based methods which classify bacteria according to the size of fragments generated by enzymatic digestion of genomic or plasmid DNA, DNA banding pattern-based methods which classify bacteria according to the size of fragments generated by PCR amplification, hybridization–based method, sequencing methods, detection of presence or absence of particular genes, and high Resolution Melting analysis [2]. All of the methods mentioned above are efficient typing methods because of revealing acceptable discriminatory power and reproducibility [5]. However, rep-PCR fingerprinting introduced by Versalovic *et al.*[6], is easy to set up, to use, to interpret, and inexpensive. Rep-PCR is a genotypic fingerprinting method that generates specific patterns by the amplification of repetitive elements present within bacterial genome [6]. Five rep-PCR methods are commonly used for genotyping of different bacterial strains including REP-PCR, ERIC-PCR, ERIC2 PCR, BOX-PCR and $(GTG)_5$-PCR among which BOX-PCR and $(GTG)_5$-PCR are of great interest [6]. Applying these methods will lead to the selective amplification of distinct genomic regions located between BOX and $(GTG)_5$ elements to produce specific banding profiles [7]. The use of BOX-PCR and $(GTG)_5$-PCR methods in the study of bacterial diversity has unveiled new insights in the composition of *E. coli* microbial communities and the number of data proving a considerable genomic diversity among *E. coli* strains is increasing steadily [8].

The ability to analyze *E. coli* populations by different methods not only can improve our understanding of the transport, viability and structure of *E. coli* populations but also can help us to develop strategies to identify bacterial pollution sources [9]. Thus, we conducted the current study to analyze clonal heterogeneity of *E. coli* isolated from chicken as well as to evaluate discriminatory power of BOX and $(GTG)_5$-PCR.

MATERIAL and METHODS

Ethics

For animal fecal samples, Permission was obtained from Alborz University of Veterinary Sciences, and Institutional Animal Care and Use Committee (IACUC) approved this study. To collect samples, written information about the study was given to the husbandry owner and Informed consent was obtained.

Sampling, Bacterial Isolates, and DNA Extraction

This cross-sectional study was performed from August 1, 2015 to August 20, 2015. Applying sterile cotton-tipped applicators (swabs), 106 enteric specimens from healthy chickens (aging from 5-7 weeks) were obtained from private chicken husbandry Qadir in Karaj city (suburb of Alborz province with geographic coordinate of 35.8840059, 50.9716793). Since fecal samples were collected from living animal, no animal was sacrificed. For sampling, following autoclaving swabs in the capped tubes, they were inserted into the cloaca and rectum of chickens in such a manner as to insure the collection of fecal material. The swabs and adhering fecal material were then placed in the tube and quickly shipped to laboratory. To isolate *E. coli*, fecal swabs were inoculated into lauryl sulphate tryptose (LST) broth (Merck KGaA) followed by *E. coli* (EC) broth (Merck KGaA) and incubated at 44.5°C, and then the broth cultures were streaked on Eosin Methylene Blue Agar (EMB) agar (Merck KGaA). Colonies showing metal sheen were considered as presumptive *E. coli* isolates and were subjected to IMV*i*C, tryptophanase and Beta-glucuronidase (Merck KGaA) tests for final confirmation [10]. Confirmed isolates were inoculated into sterile cryotube vials containing nutrient broth and were incubated overnight at 37°C. Sterile glycerol (Merck KGaA) was then added to each vial at a final concentration of 15% (vol/vol), and the vials were stored at -70°C. Following finishing sampling procedure, all frozen bacterial strains were revived in Brain Heart Infusion (BHI) broth under optimal growth condition and genomic DNA was extracted from the bacterial pellet applying *AccuPrep*® Genomic DNA Extraction Kit (Bioneer, South Korea). DNA was quantified and assessed for purity by spectrophotometry at 260 nm and 280 nm using a DR3900 Benchtop VIS Spectrophotometer (HACH, USA).

Molecular Fingerprinting

The primers used in this study were 5′-CTACGGCAA GGCGACGCTGACG-3′ (Bioneer, South Korea) for BOX-PCR and 5′-GTGGTGGTGGTGGTG-3′ (Bioneer, South Korea) for $(GTG)_5$-PCR. The final reaction mixture for both protocols, consisted of 12.5 µL, 2x CinnaGen PCR master kit containing Hotstart *Taq* DNA Polymerase (recombinant), PCR buffer, $MgCl_2$, dNTPs, in addition to 1 µL (approximately 100 ng) template DNA and 10 pmols of each primers sets to the final volume of 25 µL. Amplification was performed with Veriti® 96-Well Thermal Cycler (Applied Biosystems) as follows: Initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 20 s), annealing (30 s at 52°C for both protocols), extension (72°C for 1 min); and a final extension (72°C for 10 min). PCR products were resolved by horizontal electrophoresis in 1.5% (wt/vol) agarose (Bioneer, South Korea) and 1x Tris-

borate-EDTA (Merck KGaA) buffer. PCR products were evaluated by electrophoresis in 1% agarose gel (Merck KGaA) containing SYBR green (Thermo Scientific, Ukraine) and visualized by a Gel DOC™ XR+ (BIORAD) and analyzed by Image Lab™ 4.0 software.

Reproducibility Analysis of Rep-PCR

For reproducibility, five isolates were subcultured on tryptic soy agar (Merck KGaA) for 15 successive days (one subculturing per day). DNA was extracted after days 5, 10, and 15 of subculturing and used as template DNA for both $(GTG)_5$-PCR and BOX PCR methods (a total of 15 genomic DNA).

Computer-Assisted Image Analysis and Cluster Assignment

Gel images were normalized, and fingerprints were assigned to isolates, with Bio-Rad's Image Lab™ 4.0 software. The positions of fingerprints on gels were normalized using generuler 100 bp plus ladder (Thermo Scientific, Ukraine) as the external standard in the range of 100 bp to 3.000 bp. For cluster analyzing, the data were converted to a binary matrix, where the digits 1/0 represent the presence/absence of the corresponding DNA band. Using the PAUP software 4.0 beta windows, strains were assigned to different clusters by calculating the similarity coefficients with the curve-based Pearson similarity coefficient. Similarity trees were generated using the unweighted-pair group method using average linkages. Clusters were initially assigned using the PAUP software on the basis of 80% similarity.

For both protocols, the Shannon-Weaver index for the diversity of isolates was calculated using the formula $H=C/N*(N*log\ N-zn_i*log\ n_i)$, as described before [11].

RESULTS

A total of 106 *E. coli* isolates were obtained from chicken fecal samples. Genomic DNA was successfully extracted from all isolates and analyzed spectrophotometrically, confirming the purity and quantity of the DNA extracted.

Molecular typing of *E. coli* isolates by $(GTG)_5$-PCR generated 26 different bands ranging in size from 555 bp to 3.3 kb, while no common band was observed in all isolates. The majority of the isolates revealed complex banding patterns, while the most prevalent band (present in 73 isolates) was approximately 2.9 kb in size, and the least prevalent (present in 8 isolates) was approximately 1.87 kb in size. Among 106 isolates, two isolates were untypeable (generating no bands), while 104 isolates generated 100 unique (singletons) and 2 duplicate $(GTG)_5$-profiles *(Fig. 1)*. The dendrogram generated six clusters (G1-G6) for the 106 strains tested. G2 contained 40 isolates followed by G3 (28 isolates), G5 (19 isolates), G2 (14 isolates), G4 (3 isolates), and G6 (2 isolates) *(Fig. 2)*.

With the BOX-PCR method, profiles of *E. coli* strains revealed 10 amplified bands ranging from 0.7 to 3.5 kb, with various intensities. The most prevalent band which was present in 53 isolates was a band of approximately 2.9 kb in size, while the least prevalent band present in 8 isolates was approximately 1.87 kb. No common band was observed in all isolates. Because of low number of bands, the isolates did not reveal complex banding patterns. Visual comparison of the BOX-PCR banding results of 106 *E. coli* isolates revealed 50 unique BOX-PCR profiles in addition to 22 repetitive profiles, while 12 isolates showed no band. Based on the bands and dendrogram generation, isolates were grouped into six clusters (B1-B6). B3 contained 50 isolates followed by B2 (24 isolates), B4 (18 isolates), B5 (9 isolates), B6 (4 isolates), and B1 (1 isolates) *(Fig. 3)*.

The degree of diversity calculated for the 106 isolates, using the Shannon-Weaver index, was 4.665 for $(GTG)_5$-PCR and 0.281 for BOX-PCR.

Following reproducibility testing, all isolates (days 5, 10, and 15 of subculturing) gave repeatedly the same

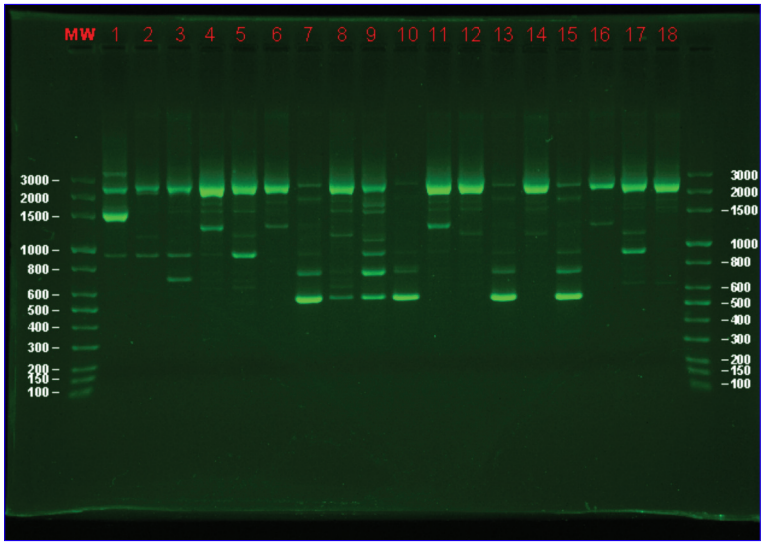

Fig 1. $(GTG)_5$-PCR fingerprinting patterns of the isolates. Lane M, generuler 100 bp plus ladder (Thermo Scientific, Ukraine). Lanes 1-18, $(GTG)_5$ profiles of the isolates

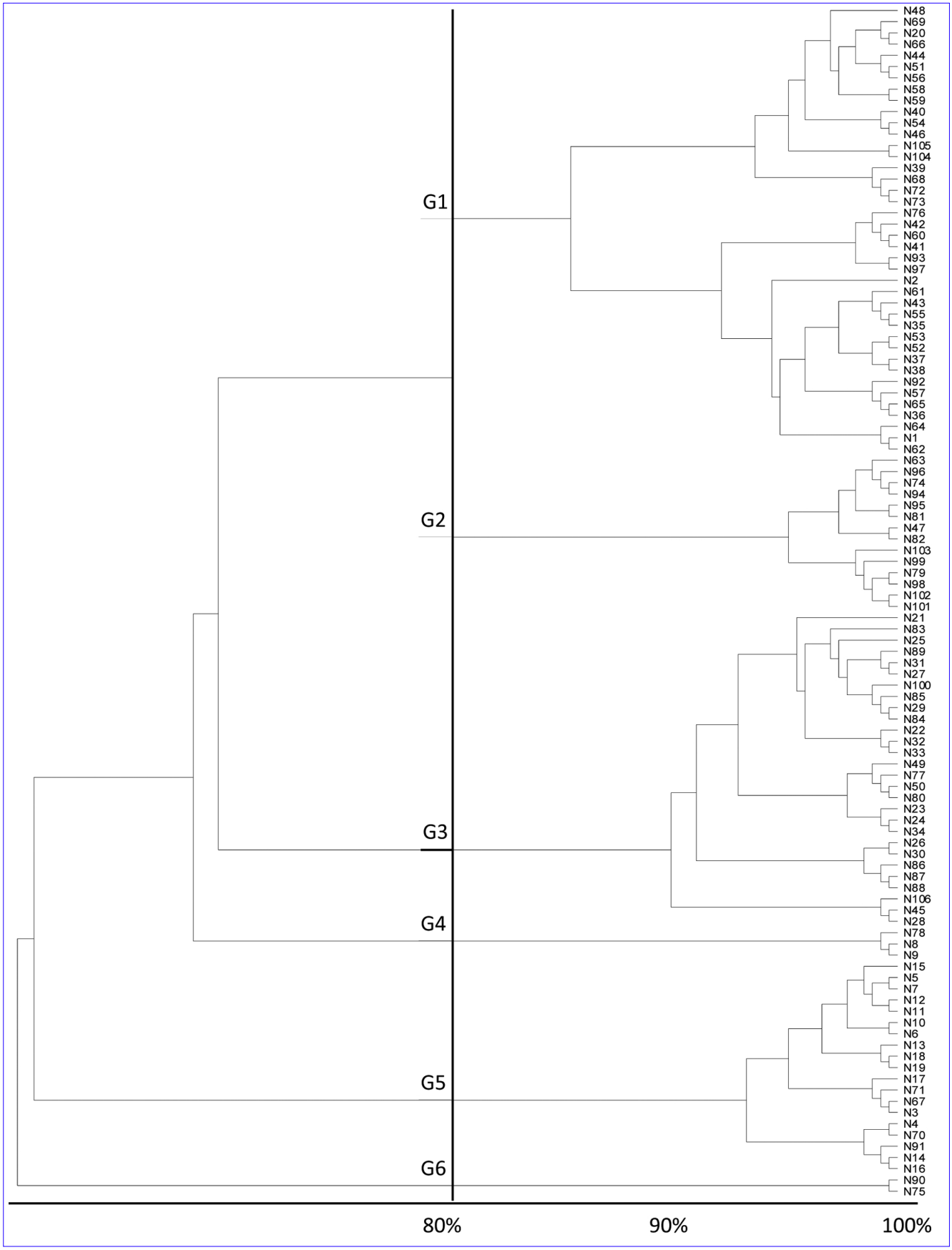

Fig 2. Dendrogram and cluster analysis of $(GTG)_5$-PCR fingerprints using the UPGMA clustering at a coefficient of 80% similarity. The bottom bar indicates the percent of similarity

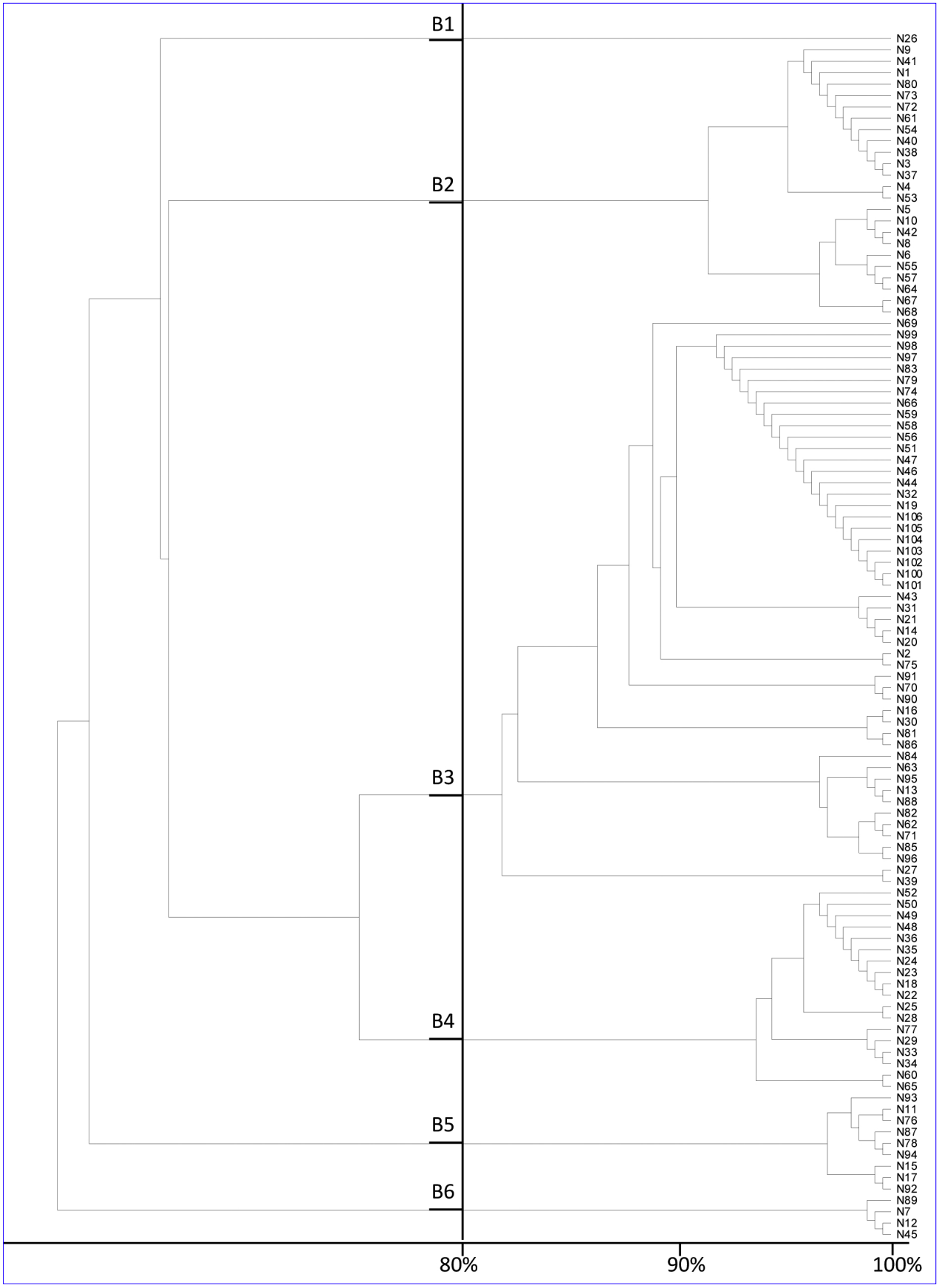

Fig 3. Dendrogram and cluster analysis of BOX-PCR fingerprints using the UPGMA clustering at a coefficient of 80% similarity. The bottom bar indicates the percent of similarity

band patterns without any difference due to missing or producing new bands. However, slight differences in intensity of some bands occurred.

DISCUSSION

Typing methods are efficient tools for the epidemiological study of bacteria. For a long time *E. coli* has been characterized by biotyping, phage typing, and serotyping with O-, H- and K- antigens [3]. From a biochemical and serological points of view, *E. coli* isolates have been demonstrated to be identical, but molecular studies have supported the existence of genetic variability among the isolates. Therefore, in recent years, traditional methods have been succeeded by molecular ones, among which pulse field gel electrophoresis is known to be the most efficient and gold standard of typing. PCR-based typing methods are other molecular tools which can be alternatives to PFGE, because they are fast and easy to setup and the results gained are to some extent comparable to PFGE [12]. However, the *E. coli* species has a comparatively clonal population structure, which can make the distinction of different isolates more difficult [13].

In this study we evaluated two PCR-based typing techniques, $(GTG)_5$ and BOX, which are frequently applied as molecular tools for fingerprinting of the different genus of the bacteria. The results obtained in this study revealed high clonal heterogeneity of the isolates. Furthermore, it was revealed that 98.5% of the chicken isolates were typeable by $(GTG)_5$-PCR, while just 53% of the isolates were typeable by BOX-PCR, this may confirm the robustness of $(GTG)_5$-PCR in comparison to BOX-PCR and consequently the lower applicability of BOX-PCR for typing compared with $(GTG)_5$-PCR. The lower Shannon-Weaver index with BOXA1 primer, the higher number of the untypeable isolates and the higher number of isolates with the same BOX fingerprints, would approve this claim. $(GTG)_5$-PCR is not only applicable for *E. coli*, but also is an efficient tool for fingerprinting other genus. For example, Pavel Svec *et al.* [14]. described that $(GTG)_5$-PCR is a reliable and fast method for species identification of Enterococci because it was able to discriminate Enterococci strains from known and unknown sources.

To evaluate the discriminatory power of REP-PCR, ERIC-PCR, ERIC2-PCR, BOX-PCR and $(GTG)_5$-PCR for fingerprinting of *E. coli* isolates, Mohapatra *et al.*[15] conducted a survey which based on discriminant function analysis, they introduced $(GTG)_5$-PCR followed by BOX-PCR as the most robust molecular tools for differentiation of *E. coli* isolates, which confirms the higher accuracy of $(GTG)_5$-PCR in comparison to BOX-PCR.

Luc De Vuyst *et al.*[16], evaluated $(GTG)_5$-PCR with acetic acid bacteria and validated this technique with DNA:DNA hybridization data. They claimed that exclusive patterns were obtained for most strains, suggesting that the technique can also be used for characterization below species level or typing of acetic acid bacteria strains.

In another survey, Mohapatra *et al*.[17] applied $(GTG)_5$-PCR to assess the ability of this typing method as a microbial source tracking tool. Following fingerprinting of 573 *E. coli* isolates from poultry and free living birds, Mohapatra *et al*.[17] indicated that $(GTG)_5$-PCR can be considered to be a complementary molecular tool for the fast determination of *E. coli* isolates identity and tracking the sources of fecal pollution.

Following $(GTG)_5$ fingerprinting of lactobacilli, Dirk Gevers *et al.*[18] found that $(GTG)_5$-PCR is a promising genotypic tool for fast and reliable speciation and typing of lactobacilli and other lactic acid bacteria. However, the result gained by Ma *et al.*[19] is not consistent with ours, because in a survey to differentiate between human, livestock, and poultry sources of fecal pollution, although the higher number of bands in $(GTG)_5$-PCR fingerprints could be observed, the discriminatory efficacy of BOX-PCR was superior to $(GTG)_5$-PCR. In another survey conducted by Dombek *et al*.[20] to differentiate *E. coli* isolates from human and animal sources of fecal pollution, BOX and REP primers were evaluated for DNA fingerprinting of *E. coli* strains. Using Jaccard similarity coefficients, Dombek *et al*.[20] managed to almost completely separate the human isolates from the nonhuman isolates.

To assess discriminatory power and suitability of BOX-PCR for bacterial source tracking, Carlos *et al.*[21], analyzed *E. coli* from different sources by BOX-PCR technique and a correct classification rate of 84% was achieved for strains from human and animal sources.

Based on our experiments, reproducibility results indicated that both $(GTG)_5$-PCR and BOX-PCR methods are of considerable repeatability and consistency. Following reproducibility testing, all isolates (days 5, 10, and 15 of subculturing) gave repeatedly the same band patterns without any difference due to missing or producing new bands. However, slight differences in intensity of some bands occurred. Identical results were gained by Pavel Sˇvec *et al.*[14] and Gevers *et al.*[18] who tested reproducibility of the $(GTG)_5$-PCR fingerprinting for lactobacilli and Enterococcus. Although they occasionally found minor quantitative variations in band intensity, none of the strains tested in their study showed qualitative differences in the fingerprint patterns.

Similarly, a good reproducibility of rep-PCR fingerprinting was proved by Kang and Dunne [22]. They demonstrated high stability of fingerprints obtained from DNA isolated from 24, 48 and 72 h old bacterial cultures and from 5, 10 and 15 successive subcultured strains. Furthermore, Abby Yang *et al.*[23] reported that different concentration of template DNA, presence or absence of

bovine serum albumin, different annealing temperature and the growth phase of the culture template may not have notable effect on the BOX- fingerprints of *E. coli* of either gull or duck origins. However, Rasschaert *et al.*[24] reported that reproducibility of $(GTG)_5$-PCR was poor between different PCR runs but high within the same PCR run. Of course it is noteworthy to indicate that for improving the reproducibility, we used Hotstart *Taq* DNA polymerase to eliminate any unspecific bands.

Regarding complex clonal heterogeneity of the isolates from chicken or any other sources, it is controversial to introduce the best molecular typing method, however, our results revealed that $(GTG)_5$-PCR method is more discriminative for typing of *E. coli* in comparison to BOX-PCR. Considering advantages and disadvantages of all typing methods, we can come to this conclusion that these approaches are complementary tools and combination of two or more different typing methods may lead to higher discrimination power rather than each of them when used individually.

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Ethical Considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Conflicts of Interest

The authors declare that they have no competing interests.

REFERENCES

1. Akter S, Islam M, Afreen KS, Azmuda N, Khan SI, Birkeland NK: Prevalence and distribution of different diarrhoeagenic *Escherichia coli* virulotypes in major water bodies in Bangladesh. *Epidemiol Infect*, 1-10, 2013. DOI: 10.1017/S0950268813000320

2. Wenjun L, Raoult D, Fournier E: Bacterial strain typing in the genomic era. *FEMS Microbiol Rev*, 33, 892-916, 2009. DOI: 10.1111/j.1574-6976.2009.00182.x

3. Fratamico P, Baranzoni M, DebRoy C, Feng P: Advances in molecular serotyping and subtyping of *Escherichia coli*. *Front Microbiol*, 7, 2016. DOI: 10.3389/fmicb.2016.00644

4. Olive M, Bean P: Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol*, 37, 1661-1669, 1999.

5. Singh A, Goering R, Simjee S, Foley S, Zervos MJ: Application of molecular techniques to the study of hospital infection. *Clin Microbiol Rev*, 19, 512-530, 2006. DOI: 10.1128/CMR.00025-05

6. Versalovic J, Schneider M, Bruijn F, Lupski J: Genomic fingerprinting of bacteria using repetitive sequence-based Polymerase Chain Reaction. *Methods Cell Biol*, 5, 25-40, 1994.

7. Kowalchuk GA, Bruijn FJ, Head IM, Akkermans AD, van Elsas JD: Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. *Molecular Microbial Ecology Manual*, 2513-2545, 2004. DOI: 10.1007/978-1-4020-2177-0_306

8. Baldy-Chudzik K, Stosik M: Specific genomic fingerprints of *Escherichia coli* strains with repetitive sequences and PCR as an effective tool for monitoring freshwater environments. *Pol J Environ Stud*, 14, 551-557, 2005.

9. Lyautey E, Lu Z, Lapen DR, Wilkes G, Scott A, Berkers T, Edge TA, Topp E: Distribution and diversity of *Escherichia coli* populations in the South Nation River Drainage Basin, Eastern Ontario, Canada. *Appl Environ Microbiol*, 76, 1486-1496, 2010. DOI: 10.1128/AEM.02288-09

10. Roohollah K, Naser H, Mehrouz D: Efficacy evaluation of four different culture and PCR-based methods of *Escherichia coli* detection in water samples. *Adv Environ Biol*, 7, 2689-2694, 2013.

11. Gustavo L, Borges d, Vechia VD, Cor G: Characterisation and genetic diversity via REP-PCR of *Escherichia coli* isolates from polluted waters in southern Brazil. *FEMS Microbiol Rev*, 45, 173-180, 2003. DOI: 10.1016/S0168-6496(03)00147-8

12. McLellan SL, Daniels AD, Salmore AK: Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA fingerprinting. *Appl Environ Microbiol*, 69, 2587-2594, 2003. DOI: 10.1128/AEM.69.5.2587-2594.2003

13. Jonasa D, Spitzmüllera B, Weistb K, Rüdenb H, Daschnera FD: Comparison of PCR-based methods for typing *Escherichia coli*. *Clin Microbiol Infect*, 9, 823-831, 2003. DOI: 10.1046/j.1469-0691.2003.00661.x

14. Švec P, Vancanneyt M, Seman M, Snauwaert C, Lefebvre K, Sedláček I, Swings J: Evaluation of (GTG)5-PCR for identification of *Enterococcus* spp. *FEMS Microbiol Lett*, 247, 59-63, 2005. DOI: 10.1016/j.femsle.2005.04.030

15. Mohapatra B, Mazumder A: Comparative efficacy of five different rep-PCR methods to discriminate *Escherichia coli* populations in aquatic environments. *Water Sci Technol*, 58, 537-547, 2008. DOI: 10.2166/wst.2008.424

16. Vuyst LD, Camu N, Winter TD, Vandemeulebroecke K, Perre V, Vancanneyt M, Cleenwerck I: Validation of the (GTG)5-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans. *Int J Food Microbiol*, 125, 79-90, 2008. DOI: 10.1016/j.ijfoodmicro.2007.02.030

17. Mohapatra BR, Broersma K, Mazumder A: Comparison of five rep-PCR genomic fingerprinting methods for differentiation of fecal *Escherichia coli* from humans, poultry and wild birds. *FEMS Microbiol Lett*, 277, 98-106, 2007. DOI: 10.1111/j.1574-6968.2007.00948.x

18. Gevers D, Huys G, Swings J: Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. *FEMS Microbiol Lett*, 205, 31-36, 2001. DOI: 10.1111/j.1574-6968.2001.tb10921.x

19. Ma HJ, Fu LL, Li JR: Differentiation of fecal *Escherichia coli* from human, livestock, and poultry sources by rep-PCR DNA fingerprinting on the shellfish culture area of East China Sea. *Curr Microbiol*, 62, 1423-1430, 2011. DOI: 10.1007/s00284-011-9870-z

20. Dombek PE, Johnson LK, Zimmerley ST, Sadowsky MJ: Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl Environ Microbiol*, 66, 2572-2577, 2000. DOI: 10.1128/AEM.66.6.2572-2577.2000

21. Carlos C, Alexandrino F, Stoppe NC, Sato MI, Ottoboni LM: Use of Escherichia coli BOX-PCR fingerprints to identify sources of fecal contamination of water bodies in the State of São Paulo, Brazil. *J Environ Manage*, 93, 38-43, 2012. DOI: 10.1016/j.jenvman.2011.08.012

22. Kang HP, Dunne M: Stability of repetitive-sequence PCR patterns with respect to culture age and subculture frequency. *J Clin Microbiol,* 41, 2694-2696, 2003. DOI: 10.1128/JCM.41.6.2694-2696.2003

23. Yang A, Yen C: PCR optimization of BOX-A1R PCR for microbial source tracking of *Escherichia coli* in waterways. *J Exp Microbiol Immunol*, 16, 85-89, 2012.

24. Rasschaert G, Houf K, Imberechts H, Grijspeerdt K, De Zutter, Heyndrickx M: Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. *J Clin Microbiol*, 43, 3615-3623, 2005. DOI: 10.1128/JCM.43.8.3615-3623.2005

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Research Article

Serum Leptin and Ghrelin Levels and Their Relationship with Serum Cortisol, Thyroid Hormones, Lipids, Homocysteine and Folic Acid in Dogs with Compulsive Tail Chasing

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Abstract

The aim of this study was to investigate serum leptin and ghrelin levels and their relations with circulating cortisol, thyroid hormones, lipids, homocysteine (Hcy) and folic acid in dogs with compulsive tail chasing (CTC). The material of this study consists of fifteen dogs with CTC and 15 healthy controls of various weights, breeds, ages of both sexes were enrolled in the study. CTC was diagnosed on the basis of the dog's behavioral history, clinical signs, and results of other medical assessments. None of the dogs were considered to have concurrent medical disease that would account for CTC. Dogs with CTC had a higher leptin (8.3±0.9 ng/mL vs 1.7±0.2 ng/mL, P<0.001) and lower ghrelin levels (74±7 pg/mL vs 144±41 pg/mL, P<0.05) than those of healthy controls. Serum cortisol, lipids (cholesterol, phospholipids and NEFA) and Hcy levels increased (P<0.05), whereas serum folic acid decreased (P<0.001) in dogs with CTC as compared with controls. Serum ghrelin correlated negatively with cholesterol (P<0.05), but serum leptin correlated positively with cholesterol, fT4, and phospholipids (P<0.05). These results suggest that serum leptin and ghrelin levels may bring up a new perspective on our understanding of the pathophysiological mechanisms associated with CTC. Serum levels of both hormones may be associated with serum levels of lipids and free T4.

Keywords: *Leptin, Ghrelin, Thyroid hormones, Lipids, Tail chasing, Dog*

Kompulsif Kuyruk Isıran Köpeklerin Serum Leptin ve Ghrelin Seviyeleri ve Serum Kortizol, Tiroid Hormonları, Lipidler, Homosistein ve Folik Asit İle İlişkileri

Özet

Bu çalışmanın amacı, kompulsif kuyruk ısıran köpeklerde serum leptin ve ghrelin seviyeleri ve sirküle eden kortizol, tiroid hormonları, lipidler, homosistein (Hcy) ve folik asit arasındaki ilişkiyi araştırmaktır. Çeşitli ağırlık, ırk, yaş ve her iki cinsiyetten 15 kuyruk ısıran ve kontrol grubu olarak 15 sağlıklı köpek çalışmaya dahil edildi. Kuyruk ısırma tanısı, davranış anamnez formu, klinik bulgular ve diğer medikal değerlendirmelerin sonuçlarına göre konuldu. Kompulsif kuyruk ısıran köpeklerin hiçbirinde eşlik eden başka bir medikal hastalık bulunmamaktaydı. Kompulsif kuyruk ısıran köpekler, kontrol grubundaki köpeklere göre yüksek leptin (8.3±0.9 ng/mL ve 1.7±0.2 ng/mL, P<0.001) ve düşük ghrelin (74±7 pg/mL ve 144±41 pg/mL, P<0.05) seviyesine sahipti. Kontrol grubu ile karşılaştırıldığında kompulsif kuyruk ısıran köpeklerin serum kortizol, lipid (kolesterol, fosfolipidler ve NEFA) ve Hcy seviyesi artmış (P<0.05), bunun aksine serum folik asid seviyesi (P<0.001) azalmıştır. Serum ghrelin seviyesi, kolesterol (P<0.05) ile negatif korelasyon gösterirken, serum leptin seviyesi, kolesterol, fT4 ve fosfolipidler (P<0.05) ile pozitif korelasyon göstermekteydi. Sonuçlar değerlendirildiğinde, serum leptin ve ghrelin seviyelerindeki değişikliklerin kompulsif kuyruk ısırmanın patofizyolojik mekanizmasını anlamak için yeni bir perspektif getireceği düşünülmektedir. Her iki hormon da serum lipid seviyeleri ve serbest T4 düzeyi ile ilişkili olabilir.

Anahtar sözcükler: *Leptin, Ghrelin, Tiroid hormonları, Lipidler, Kompulsif kuyruk ısırma, Köpek*

INTRODUCTION

Obsessive compulsive disorder (OCD) is a neuropsychiatric disorder in humans and animals. Canine compulsive disorder includes excessive tail chasing, light/shadow chasing and flank sucking [1-3]. Clinical and neurobiological similarities between dogs and humans with OCD were reported [3,4]. Thus, canine compulsive behaviors

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such as compulsive tail chasing (CTC) have been suggested as a promising model for human OCD [3].

Human and canine OCD have been associated with a biochemical disturbance at the level of neurotransmitter systems [1] and activation of hypothalamic-pituitary-adrenal (HPA) system [5,6]. Compulsive disorders are also known to be stress responsive and compulsive symptoms increase at times of stress [6,7]. Serum cortisol level as an indicator of activated HPA system is used to describe the presence of stress in humans [8], dogs [9], and cats [10].

Leptin and ghrelin are two hormones related with energy balance. Leptin, an anorexigenic hormone, is a mediator of long-term regulation of energy balance, suppressing food intake and thereby resulting in weight loss. Ghrelin, an orexigenic hormone, is playing a role in meal initiation [11]. Recently, leptin and ghrelin hormones have also been correlated in the pathophysiology of stress [12]. Leptin inhibits and ghrelin facilitates neuroendocrine stress responses in rats [12]. Hypercortisolemia increases serum levels of leptin and decreases serum levels of ghrelin in dogs. As serum levels of leptin and ghrelin are affected by cortisol [13], it is possible to hypothesize that these hormones might play a role in the regulation of stress response in dogs with OCD.

Current literature shows that some psychiatric disorders in humans might be related to the serum levels of folate and homocysteine (Hcy) [14-16], lipids [17,18] and thyroid function [19]. Thyroid status is considered as an important determinant of the serum level of total Hcy [20] and lipids (cholesterol, lipoproteins, etc.) in humans [17,18,21,22]. There is also one study indicating a relationship between serum level of lipid (serum cholesterol elevations) and OCD in dogs [23]. Based on the accumulated evidence, we hypothesized that circulating leptin, ghrelin, folate, Hcy, lipids and thyroid hormones might have a role in dogs with OCD. Thus, in this study, to understand of pathophysiological mechanism of OCD, we investigated serum leptin and ghrelin levels and their relations with circulating thyroid hormones, folate, Hcy, lipids, and cortisol in dogs with CTC.

MATERIAL and METHODS

Dogs

This study was performed on 15 healthy dogs and 15 dogs with CTC that were referred to the Small Animal Clinics Internal Medicine Department, Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey in January-July 2016. Dogs with CTC were 10-35 kg (mean 27.2 kg±1.5 kg), and of various breeds (3 Anatolian shepherd dogs, 3 German shepherd dogs, 3 Golden retrievers, 3 mixed breeds, 1 Terriers, 1 Doberman, 1 Labrador retriever), sex (11 males, 4 females) and age (8 months-9 years, mean 3.3±0.7 years) were evaluated. Dogs (n=15), which were referred for vaccination purposes, were also enrolled in the study as control after the consent of the owners and on the basis of normal physical examination results and complete blood count. Control dogs were of various weights (23.5±1.5 kg), mixed breeds of either sex (10 males, 5 females), and ranged from 12-96 months (34±2.5 months) in age. No significant differences were observed between the two groups regarding the aforementioned parameters. The body condition score for each dog was evaluated by using a 5-point scale (1: thin, 2: underweight, 3: ideal, 4: overweight and 5: obese) [24]. All experiments conducted by us in this study were performed in line with ethical approval from the ethical committee of University (16/1-5).

Diagnostic Procedures

A behavioural diagnosis was made for each dog on the basis of the dog's behavioural history, clinical signs, and results of other medical assessments, as published in the previous studies [23,25]. Dogs were assessed for seizure disorder, opioid-mediated stereotypy, local vasculitis or neuritis, anal sac diseases and pruritus. None of the dogs were considered to have concurrent medical disease (such as dermatological disease, vector–borne diseases, and renal diseases) that would account for CTC. Behavioral history included age at onset, frequency and duration of bouts since onset, general history, and current or previous medical conditions. All owners reported that their dogs commonly whined, barked, or growled during tail chasing. In this study, affected dogs had to have tail chasing bouts for a minimum of 60 s/bout at least 3 times/d during the previous two months in order to be included into the study.

Sample Collection and Measurements

Venous blood samples were collected, after a fasting period of 12-16 h, from cephalic veins into vacutainer tubes with or without EDTA (Becton Dickinson, Temse**,** Belgium) for complete blood count (Cell Dyne 3500R, Abbott, Germany) and serum biochemistry panel (Aeroset, Abbott), respectively. All dogs were screened for common vector-borne diseases (anaplasmosis, borrelliosis, dirofilariosis, ehrlichiosis, and leishmaniosis) by speed tests (Bionote, Anigen, South Korea), and dogs sero-positive for vector-borne pathogens were excluded from the study.

Serum leptin and ghrelin levels were measured by radioimmunoassay (RIA) using a commercially available kit (Multispecies leptin RIA kit and active ghrelin RIA kit, Linco Research, St. Charles, MO). Validity and reliability of these RIA kits for measuring serum leptin and ghrelin levels in dog were determined in our previous studies [13,26]. Since active ghrelin is too unstable to be measured in stored samples [27] and acidification of plasma prevents rapid desacylation of ghrelin [28], 1 N hydrogen chloride was added to serum samples before freezing[13]. Serum cortisol was measured by a solid-phase chemiluminescent enzyme immunoassay system (Immulite 2000, BioDPC, Los

Angees, CA) as reported earlier [26].

Serum samples were tested for total thyroxin (T_4), free T_4 (fT_4), triiodothyronine (T_3), and free T_3 (fT_3) concentrations. Hormone analysis was performed by RIA techniques (Advia Centaur™) as reported earlier [29].

Serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), very low-density lipoprotein cholesterol (VLDL-C) and triglycerides levels were measured by an automated clinical chemistry analyzer (Architect *ci*8200; Abbott GmbH Co KG, Wiesbaden, Germany) using commercially available assay kits (Abbott GmbH Co KG). Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the formula:

LDL-C(in milligrams per deciliter) = total cholesterol - (HDL – C + triglyceride/5).

Serum non-esterified fatty acid (NEFA) concentration was measured using a commercially available enzymatic colorimetric assay kit (Wako Chemicals, Neuss, Germany) [30]. Serum phospholipids were measured by an enzymatic colorimetric method using a commercially available assay kit (Wako Chemicals), which have been reported as choline-containing phospholipids (in milligrams per deciliter) [30].

Statistical Analysis

Data were analysed statistically by two-group comparison student t test (SigmaStat GmbH, Erkrath) and expressed as mean±SEM. Pearson's correlation analysis was used to determine a relationship between the mean levels of serum leptin, ghrelin, cortisol, Hcy, folate, lipids, and thyroid hormones. A *P* value of <0.05 was considered significant.

RESULTS

Routine clinical and hematological findings were within reference limits in all healthy dogs (data not shown). Dogs with CTC had a higher mean body condition score (4.2±0.6; P<0.001) than controls (3.2±0.4).

Dogs with CTC had a higher leptin (8.3±0.9 ng/mL *vs* 1.7±0.2 ng/mL, P<0.001) and lower ghrelin levels (74±7 pg/mL *vs* 144±41 pg/mL, P<0.05) than those of healthy controls *(Table 1)*.

Serum cortisol, lipids (cholesterol, phospholipids and NEFA) and Hcy levels increased (P<0.05), whereas serum folic acid decreased (P<0.001) in dogs with CTC as compared with controls. There were no statistically significant differences on serum thyroid hormones (except fT3) between the groups studied. Serum fT3 level in dogs with CTC was higher (P<0.05) than that of controls *(Table 2)*.

Serum ghrelin correlated negatively with cholesterol (P<0.05) and serum leptin correlated positively with cholesterol, fT_4, and phospholipids (P<0.05). There was a negative correlation (P<0.05) between changes in serum ghrelin and leptin levels in healthy controls.

Table 1. *Serum levels of leptin, ghrelin, lipids, thyroid hormones, homocysteine and folic acid in healthy dogs and dogs with compulsive tail chasing (CTC)*

Parameters	CTC Mean±SEM	Healthy Controls Mean±SEM	P Value
Leptin ng/mL	8.36±0.90	1.70±0.26	=<0.001
Ghrelin pg/mL	74.94±7.29	144.11±41.41	= 0.029
T. Cholesterol mg/dL	203.42±16.03	140.40±10.85	NS
HDL mg/dL	73.00±11.09	89.88±7.26	NS
LDL mg/dL	128.11±11.99	32.44±3.25	=<0.001
VLDL mg/dL	15.94±1.87	12.50±1.28	NS
Tg mg/dL	79.63±9.42	58.75±5.28	NS
Phospholipid mg/dL	338.16±17.05	276.11±14.60	=0.027
Nefa mg/dL	1.74±0.17	0.85±0.15	=0.002
TT_4 µg/dL	1.79±0.09	1.91±0.14	NS
fT_4 ng/dL	0.72±0.10	0.48±0.02	=0.072
TT_3 ng/mL	0.44±0.03	0.34±0.03	NS
fT_3 pg/mL	1.87±0.15	1.34±0.10	=0.03
Hcy µmol/L	11.52±0.84	6.29±0.23	=<0.001
Folic acide ng/mL	3.25±0.32	6.41±0.56	=<0.001

DISCUSSION

This study showed that serum leptin levels were higher and ghrelin levels were lower in CTC dogs when compared with the healthy counterparts [26]. Increased serum leptin and decreased ghrelin levels and their correlation with circulating thyroid hormones and lipids in dogs with CTC provide further information to understand the pathophysiology of OCD, and to develop new treatment strategies for these patients.

In the present study, CTC, one of the most common forms of OCD in dogs, was diagnosed as reported earlier [23,25]. Serum leptin concentrations (1.7±0.2 ng/mL), measured by RIA in healthy dogs, were slightly lower than those of healthy dogs in the previous studies: 2.5±0.1 ng/mL [13], 2.4±0.1 ng/mL [26], and 2.3±0.5 ng/mL [31]. This difference may be explained by diurnal rhythms of circulating leptin as well as using different measurement methods (RIA or ELISA) and kits (multi-species or canine specific leptin) [31]. In this study, no noticeable influence of age, gender, and breed on serum leptin levels was observed as reported by Ishioka et al.[32].

In this study, dogs with CTC had higher leptin (8.3±0.9 ng/mL) and lower ghrelin levels (74±7 pg/mL) than those of healthy controls, indicating a possible association between leptin and ghrelin systems and psychogenic disorders

Table 2. *Serum levels of leptin and ghreline and their relations with serum lipids, thyroid hormones*

Parameters	T. Cholesterol	HDL	LDL	fT4	Phospholipids
Leptin	r: 0.600 P<0.05	NS	NS	r: 0.634 P<0.05	r: 0.643 P<0.05
Ghrelin	r: -0.477 P<0.05	r: -0.512 P<0.05	r: - 0.462 P<0.05	NS	NS

as well as a good inverse correlation between them. The results of serum leptin in this study showed similarity to those of a human study [33], in which serum leptin level was slightly higher (but not statistically significant) in the OCD group than in the healthy control group. High body condition score, observed in dogs with CTC, may enhance the serum leptin concentration by increasing leptin secretion from adipose tissue [24,32,34].

Since active form of ghrelin as compared with total ghrelin is essential in particular for biological [28] and endocrine activities [35], and thus, is physiologically more crucial in terms of OCD [36], active ghrelin measurements were chosen in the present study. Observed serum ghrelin level (144±41 pg/mL) in healthy dogs was in good accordance with the levels of 172±17 pg/mL and 117±42 pg/mL reported for healthy beagle dogs [37] and humans [38], respectively. As compared to healthy controls, serum ghrelin levels in dogs with CTC were found lower in this study, whereas Atmaca et al.[39] and Emül et al.[33] reported a trend of higher ghrelin levels in patients with OCD. These differences of serum ghrelin between the studies might have resulted from the presence of depressive disorders in patients with OCD [33,39].

In this study, elevated levels of serum cortisol, as compared to control dogs, were thought to be associated with the HPA axis activation in dogs with CTC, in concordance with the results in patients with OCD [6]. In the previous studies, in addition to serum cortisol elevation, hyperactivity of HPA axis, the main mammalian system of stress response [6], was confirmed by increased corticotropin-releasing hormone levels in cerebrospinal fluid [40] and increased urinary cortisol levels in patients with OCD [41]. Elevated serum cortisol has been accepted as a physiological marker of stress and behavior abnormalities, particularly for dogs in animal shelter, as well [42].

Our observations on serum lipid profile confirmed and expanded the findings of the previous studies [23,43] reporting that dogs with CTC had significantly higher total cholesterol and LDL-cholesterol compared with control dogs, by demonstrating the increasing serum phospholipids and NEFA in dogs studied. The findings of this study were very similar to those of a previous study [23]; VLDL-cholesterol and triglyceride levels did not differ significantly between the groups. Similar to studies of dogs, elevated cholesterol [44], LDL- and VLDL-cholesterols and triglyceride levels were observed in human OCD patients compared with the control subjects [18]. In agreement with earlier studies in dogs [23,43] and humans [17,18], our observations confirm that serum lipid profile might be changed in dogs suffering from CTC.

On the other hand, a significant increase in serum phospholipid and NEFA brings up a new perspective which may help explain, at least in part, pathophysiological mechanisms associated with OCDs, such as CTC in dogs. Because phosphatidylcholine, as a primary phospholipid, is found at higher levels in myelin, cell membranes and brain parenchyma [45], it may have a crucial role for development of neuropsychiatric disorders in dogs as in humans [45]. In addition, excessive serum levels of NEFA, as observed in the present study, were reported to enhance oxidative stress [46] leading to several neuropsychiatric diseases including OCD [47]. Brain tissue has a high percentage of phospholipids that can easily be peroxidized. Recently, markers of oxidative stress and free radical induced injury to the brain tissues in OCD patients were reported, as well.

In this study, of thyroid hormones, only serum fT3 levels was found to be significantly higher in dogs with CTC than in controls, in accordance with euthyroid syndrome, most probably due to increased tissue metabolic demands in response to CTC as a non-thyroidal illness in dogs studied. One previous study [48] showed that basal values of thyroid hormones and thyroid stimulating hormone were normal in patients with OCD, and another study [19] reported that higher rates of panic disorder, OCD, and major depressive disorder were observed in thyroid patients than in the general population. Aizenberg et al.[49] underlined that dysregulation of the hypothalamic-pituitary-thyroid axis in OCD patients. Our results did not confirm the results arising from human studies indicating that altered levels of thyroid hormones might be associated with pathophysiology or maintenance of OCD.

Since serum Hcy has been considered as a sensitive marker for folate deficiency and they have important roles in carbon transfer metabolism (methylation) [16,39], in this study serum Hcy and folate levels were evaluated together. Our results of serum Hcy (6.2±0.2 µmol/L) and folate levels (6.4±0.5 ng/mL) in healthy dogs were in good accordance with those of the previous studies in dogs (5.1-10.9 µmol/L and 4.2-7.5 µg/L, respectively) [50] and humans (8.1±2.2 µmol/L and 7.5±1.9 ng/mL, respectively) [16]. In this study, serum Hcy levels increased (11±0.8 µmol/L), and serum folic acid decreased (3.2±0.3 ng/mL) in sick dogs, indicating the presence of hyperhomocysteinemia and serum folate deficiency in dogs with CTC. These results were very similar to the human studies on OCD [16,39]. It is well known that elevated serum Hcy and decreased folate

are associated with poor cognitive function and some psychiatric disorders. These results can be explained by the importance for the production of serotonin as well as for other monoamine neurotransmitters and catecholamines. Observations on the antidepressant effects of folate supplementation may support the importance of these nutrients in psychopathology [16].

In the present study, based on the correlation studies, it may be speculated that serum leptin and ghrelin might have a role to regulate circulating lipids (total cholesterol, HDL-C, LDL-C and phospholipids) in dogs with CTC. Serum leptin correlated positively only with serum fT_3 levels amongst thyroid hormones measured in this study. Similarly, in a previous study [51], circulating thyroid hormones were reported not to play a major role in the regulation of leptin synthesis and secretion. In contrast to previous studies reporting an inverse correlation between serum leptin and cortisol [39,52], in this study, there was no relationship between the two variables in dogs.

The data presented here should be interpreted with caution owing to some limitations. First, a relatively small sample size might not be representative of the dogs with CTC. However, more comprehensive and detailed studies are needed to determine the exact role of leptin and ghrelin, as well as the interactions between them in dogs with CTC.

In conclusion, our study results suggest that serum leptin and ghrelin levels bring up a new perspective which may contribute to clarify pathophysiological mechanisms associated with CTC. Increased serum Hcy and decreased serum folate levels should be taken into consideration at diagnostic and therapeutic approaches in dogs with CTC. Increased serum levels of fT_3 should be interpreted with caution during the diagnostic work-up in dogs suffering from CTC, due to euthyroid sick syndrome.

REFERENCES

1. Hewson CJ, Luescher UA: Compulsive disorders in dogs. **In,** Voith VL, Borchelet PL (Eds): Readings in Companion Animal Behavior. 153-158, Veterinary Learning Systems. Trenton, NJ, 1996.

2. Hewson CJ, Luescher UA, Ball RO: The use of chance corrected agreement to diagnose canine compulsive disorder: An approach to behavioral diagnosis in the absence of a 'gold standard'. *Can J Vet Res*, 63, 201-206, 1999.

3. Tiira K, Hakosalo O, Kareinen L, Thomas A, Hielm-Björkman A, Escriou C, Arnold P, Lohi H: Environmental effects on compulsive tail chasing in dogs. *PLoS One,* 7 (7): e41684, 2012. DOI: 10.1371/journal.pone.0041684

4. Goldberger E, Rapoport JL: Canine acral lick dermatitis: Response to the antiobsessional drug clomipramine. *J Am Vet Med Assoc*, 27, 179-182, 1990.

5. De Kloet E, Joels M, Holsboer F: Stress and the brain: From adaptation to disease. *Nat Rev Neurosci,* 6, 463-475, 2005. DOI: 10.1038/nm1683

6. Kluge M, Schüssler P, Künzel HE, Dresler M, Yassouridis A, Steiger A: Increased nocturnal secretion of ACTH and cortisol in obsessive-compulsive disorder. *J Psyciatr Res,* 4, 928-933, 2007. DOI: 10.1016/j.jpsychires.2006.08.005

7. Findley DB, Leckman JF, Katsovich L, Haiqun L, Zhang H, Grantz H, Otka J, Lombroso PJ, King RA: Development of the Yale Children's Global Stress Index (YCGSI) and its application in children and adolescents with Tourette's syndrome and obsessive-compulsive disorder. *J Am Acad Child Adolesc Psychiatry,* 42, 450-457, 2003. DOI: 10.1097/01.CHI.0000046816.95464.EF

8. Baig A, Siddiqui I, Naqvi H, Sabir S, Jabbar J, Shahid M: Correlation of serum cortisol levels and stress among medical doctors working in emergency departments. *J Col Physicians Surg Pak*, 16, 576-80, 2006.

9. Perego R, Proverbio D, Spada E: Increases in heart rate and serum cortisol concentrations in healthy dogs are positively correlated with an indoor waiting-room environment. *Vet Clin Pathol*, 43, 67-71, 2014. DOI: 10.1111/vcp.12118

10. Nibblett BM, Ketzis JK, Grigg EK: Comparison of stress experienced by cats examined in a clinic versus a home setting. *App Anim Beh Sci*, 173, 68-75, 2015. DOI: 10.1016/j.applanim.2014.10.005

11. Klok MD, Jakobsdottir S, Drent ML: The role of leptin and ghrelin in the regulation of food intake and body weight in humans: A review. *Obes Rev*, 8, 21-34, 2007. DOI: 10.1111/j.1467-789X.2006.00270.x

12. Kawakami A, Okada N, Rokkaku K, Honda K, Ishibashi S, Onaka T: Leptin inhibits and ghrelin augments hypothalamic noradrenaline release after stress. *Stress,* 11, 363-369, 2008. DOI: 10.1080/10253890701820257

13. Yilmaz Z, Ilcol YO, Golcu E: Serum leptin and ghrelin levels in response to methylprednisolone injection in healthy dogs. *Res Vet Sci*, 82, 187-194, 2007. DOI: 10.1016/j.rvsc.2006.07.005

14. Sumi-Ichinose C, Urano F, Kuroda R, Ohye T, Kojima M, Tazawa M, Shiraishi H, Hagino Y, Nagatsu Y, Nomura T, Ichinose H: Catecholamines and serotonin are differently regulated by tetrahydrobiopterin. A study from 6-pyruvoyltetrahydropterin synthase knockout mice. *J Biol Chem,* 276, 41150-41160, 2001. DOI: 10.1074/jbc.M102237200

15. Blom HJ, Smulders Y: Overview of homocysteine and folate metabolism with special references to cardiovascular disease and neural tube defects. *J Inherited Metab Dis,* 34, 75-81, 2011. DOI: 10.1007/s10545-010-9177-4

16. Turksoy N, Bilici R, Yalciner A, Özdemir YO, Ornek I, Tufan AE, Kara A: Vitamin B_{12}, folate, and homocysteine levels in patients with obsessive-compulsive disorder. *Neuropsychiat Dis Treat,* 10, 1671-1675, 2014. DOI: 10.2147/NDT.S67668

17. Peter H, Tabrizian S, Hand I: Serum cholesterol in patients with obsessive compulsive disorder during treatment with behavior therapy and SSRI or placebo. *Int J Psychiatr Med,* 30, 27-39, 2000. DOI: 10.2190/APWF-N1XU-Y7A0-TCBW

18. Agargun, MY, Dulger H, Inci R, Kara H, Ozer EA, Sekeroglu MR, Besiroglu L: Serum lipid concentrations in obsessive-compulsive disorder patients with and without panic attacks. *Can J Psychiatry*, 49, 776-778, 2004.

19. Placidi GP, Boldrini M, Patronelli A, Fioere E, Chiovato L, Perugi G, Marazatti D: Prevalence of psychiatric disorders in thyroid diseased patients. *Neuropsychobiology,* 38, 222-225, 1998. DOI: 10.1159/000026545

20. Catargi B, Parrot-Roulaud F, Cochet C, Ducassou D, Roger P, Tabarin A: Homocysteine, hypothyroidism, and effect of thyroid hormone replacement. *Thyroid*, 9, 1163-1166, 1999. DOI: 10.1089/thy.1999.9.1163

21. Iqbal A, Jorde R, Figenschau Y: Serum lipid levels in relation to serum thyroid-stimulating hormone and the effect of thyroxine treatment on serum lipid levels in subjects with subclinical hypothyroidism: The Tromsø study. *J Int Med*, 260, 53-61, 2006. DOI: 10.1111/j.1365-2796.2006.01652.x

22. Rizos CV, Elisaf MS, Liberopoulos EN: Effects of thyroid dysfunction on lipid profile. *Open Cardiovasc Med J,* 5, 76-84, 2011. DOI: 10.2174/1874192401105010076

23. Yalcin E, Ilcol YO, Batmaz H: Serum lipid concentrations in dogs with tail chasing. *J Small Anim Pract*, 50, 133-135, 2009. DOI: 10.1111/j.1748-5827.2008.00704.x

24. Usui, S, Yasuda H, Koketsu Y: Lipoprotein cholesterol and triglyceride concentrations associated with dog body condition score: Effect of recommended fasting duration on sample concentrations in Japanese private clinics. *J Vet Med Sci*, 77, 1063-1069, 2015. DOI: 10.1292/jvms.15-0032

25. Overall KL, Dunham AE: Clinical features and outcome in dogs and cats with obsessive-compulsive disorder: 126 cases (1989-2000). *J Am Vet*

Med Assoc, 221, 1445-1452, 2002. DOI: 10.2460/javma.2002.221.1445

26. Yilmaz Z, Ozarda Ilcol Y, Ulus IH: Endotoxin increases plasma leptin and ghrelin levels in dogs. *Crit Care Med*, 36, 828-833, 2008. DOI: 10.1097/01.CCM.0B013E3181611F5AA

27. Hosoda H, Kojima M, Matsuo H, Kangawa K: Ghrelin and des-acyl ghrelin: Two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochemical Biophys Res Commun*, 279, 909-913, 2000. DOI: 10.1006/bbrc.2000.4039

28. Nakai Y, Hosoda H, Nin K, Ooya C, Hayashi H, Akamizu T, Kangawa G: Plasma levels of active form of ghrelin during oral glucose tolerance test in patients with anorexia nervosa. *Eur J Endocrinol*, 149, R1-R3, 2003. DOI: 10.1530/eje.0.149R001

29. Yilmaz Z, Batmaz H: Short term effects of endotoxin on thyroid hormone concentrations in dogs. *Indian Vet J*, 79, 780-781, 2002.

30. Ilcol YO, Yilmaz Z, Cansev M, Ulus IH: Choline or CDP-choline alters serum lipid responses to endotoxin in dogs and rats: Involvement of the peripheral nicotinic acetylcholine receptors. *Shock*, 32, 286-294, 2009. DOI: 10.1097/SHK.0b013e3181971b02

31. Ishioka K, Hatai H, Komabayashi K, Soliman MM, Shibata H, Honjoh T, Kimura K, Saito M: Diurnal variations of serum leptin in dogs: Effects of fasting and re-feeding. *Vet J,* 169, 85-90, 2005. DOI: 10.1016/j.tvjl.2004.01.003

32. Ishioka K, Hosoya K, Kitagawa H, Shibata H, Kimura K, Saito M: Plasma leptin concentration in dogs: Effects of body condition score, age, gender and breeds. *Res Vet Sci*, 82, 11-15, 2007. DOI: 10.1016/j.rvsc.2006.06.002

33. Emul HM, Serteser M, Kurt E, Ozbulut O, Guler O, Gecici O: Ghrelin and leptin levels in patients with obsessive-compulsive disorder. *Prog Neuropsychopharmacol Biol Psychiatry*, 31, 1270-1274, 2007. DOI: 10.1016/j.pnpbp.2007.05.007

34. Park HJ, Lee SE, Oh JH, Seo KW, Song KH: Leptin, adiponectin and serotonin levels in lean and obese dogs. *BMC Vet Res*, 13, 113, 2014. DOI: 10.1186/1746-6148-10-113

35. De Ambrogi M, Volpe S, Tamanini C: Ghrelin: Central and peripheral effects of a novel peptidyl hormone. *Med Sci Monit*, 9, 217-224, 2003.

36. Aydin S, Karatas F, Geckil H: Simultaneous quantification of acylated and desacylated ghrelin in biological fluids. *Biomed Chromatogr,* 22, 1354-1359, 2008. DOI: 10.1002/bmc.1065

37. McKnight LL, Eyre R, Gooding MA, Davenport GM, Shoveller AK: Dietary mannoheptulose increases fasting serum glucagon like peptide-1 and post-prandial serum ghrelin concentrations in adult beagle dogs. *Animals,* 16, 442-454, 2015. DOI: 10.3390/ani5020365

38. Macedo DM, Diez-Garcia RW: Sweet craving and ghrelin and leptin levels in women during stress. *Appetite,* 80, 264-270, 2014. DOI: 10.1016/j.appet.2014.05.031

39. Atmaca M, Tezcan E., Kuloglu M, Kırtas Ö, Ustundag B: Serum folate and homocysteine levels in patients with obsessive-compulsive disorder. *Psychiatry Clin Neurosci,* 59, 616-20, 2005. DOI: 10.1111/j.1440-1819.2005.01425.x

40. Altemus M, Cizza G, Gold PW: Chronic fluoxetine treatment reduces hypothalamic vasopressin secretion *in vitro. Brain Res,* 593, 311-313, 1992. DOI: 10.1016/0006-8993(92)91326-A

41. Gehris TL, Kathol RG, Black DW, Noyes RJ: Urinary free cortisol levels in obsessive-compulsive disorder. *Psychiatry Res*, 32, 151-158, 1990. DOI: 10.1016/0165-1781(90)90081-F

42. Hennessy MB, Davis HN, Williams MT, Mellott C, Douglas CW: Plasma cortisol levels of dogs at a county animal shelter. *Physiol Behav*, 62, 485-490, 1997. DOI: 10.1016/S0031-9384(97)80328-9

43. Dodman NH, Knowles KE, Shuster I, Moon-Fanelli AA, Tidwell AS, Keen CL: Behavioural changes associated with suspected complex partial seizures in bull terriers. *J Am Vet Med Assoc*, 208, 688-691, 1996.

44. Peter H, Hand I, Hohagen F, Koenig A, Mindermann O, Oeder F, Wittich M: Serum cholesterol level comparison: Control subjects, anxiety disorder patients, and obsessive-compulsive disorder patients. *Can J Psychiatry,* 47, 557-561, 2002.

45. Maddock RJ, Buonocore MH: MR spectroscopic studies of the brain in psychiatric disorders. *Curr Top Behav Neurosci*, 11, 199-251, 2012. DOI: 10.1007/7854_2011_197

46. Barazzoni R, Zanetti M, Gortan Cappellari G, Semolic A, Boschelle M, Codarin E, Pirulli A, Cattin L, Guarnieri G: Fatty acids acutely enhance insulin-induced oxidative stress and cause insulin resistance by increasing mitochondrial reactive oxygen species (ROS) generation and nuclear factor-κB inhibitor (IκB)-nuclear factor-κB (NFκB) activation in rat muscle, in the absence of mitochondrial dysfunction. *Diabetologia*, 55, 773-782, 2012. DOI: 10.1007/s00125-011-2396-x

47. Chakraborty S, Singh OM, Dasgupta Mandal N, Nath Das E: Correlation between lipid peroxidation-induced TBARS level and disease severity in obsessive-compulsive disorder. *Prog in Neuro-Psychopharm*, 33, 363-366, 2009. DOI: 10.1016/j.pnpbp.2009.01.001

48. Hantouche E, Piketty ML, Poirier MF, Brochier T, Olie JP: Obsessive-compulsive disorder and the study of thyroid function. *Encephale*, 17, 493-496, 1991.

49. Aizenberg D, Hermesh H, Gilad I, Munitz H, Tyano S, Laron Z, Weizman A: TRH stimulation test in obsessive-compulsive patients. *Psychiatry Res,* 38, 21-26, 1991. DOI: 10.1016/0165-1781(91)90049-U

50. Patterson BE, Barr JW, Fosgate GT, Berghoff N, Steiner JM, Suchodolski JS, Black DM: Homocysteine in dogs with systemic inflammatory response syndrome. *J Small Anim Pract*, 54, 620-624, 2013. DOI: 10.1111/jsap.12144

51. Corbetta S, Englaro P, Giambona S, Persani L, Blum WF, Beck-Peccoz P: Lack of effects of circulating thyroid hormone levels on serum leptin concentrations. *Eur J Endocrinol,* 137, 659-663, 1997. DOI: 10.1530/eje.0.1370659

52. Liao SC, Lee MB, Huang TS: The counterbalance between leptin and cortisol may be associated with comorbid depression and anxiety. *Psychiatry Clin Neurosci*, 60, 120, 2006. DOI: 10.1111/j.1440-1819.2006.01472.x

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Research Article

Genomic Analysis of Endogenous Retrovirus Elements in Chinese Flocks of Economic Importance [1]

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Abstract

Since the status of endogenous retrovirus elements in Chinese chickens is currently unknown, the embryonated eggs were analyzed from 10 different chicken breeds throughout China. In this study, endogenous retrovirus elements were analyzed including: EAV, *ev, ev*/J, and ART-CH in the embryos of economically important chicken flocks in China. These results indicated that compared with the E51 and EAV-0, the EAV genomic sequence in the tested chicken breeds was more closely related to EAV-0. The ART-CH elements in Chinese chickens were not significantly different from the prototype ART-CH clones, 5 and 14. Although the nucleotide acid sequences of ev and ev/J in the tested chicken breeds was similar to other known ev and ev/J sequences, they belonged to different branches in the phylogenetic tree (except for Lohmann Brown layers and White Leghorns). In addition, the intact ev/J sequences indicated that these chickens were somewhat different from each other. The results reported here demonstrate that endogenous ALVs are widely distributed throughout a number of Chinese chicken breeds. The endogenous viral genomes present in the Chinese chicken breeds are genetically distinct from other endogenous viruses circulating within chicken populations.

Keywords: *Avian leukosis virus, Endogenous retrovirus, Chicken, Genetic divergence, Prevalence*

Çin'de Ekonomik Öneme Sahip Tavukçuluk İşletmelerinde Endojen Retrovirüs Elemanlarının Genomik Analizi

Özet

Çin tavuklarında endojen retrovirüs elemanlarının durumu henüz bilinmediğinden, Çin genelindeki 10 farklı tavuk ırkına ait embriyolu yumurta analiz edildi. Sunulan çalışmada, Çin'de ekonomik açıdan önemli tavuk sürülerinin embriyolarındaki EAV, *ev, ev*/J ve ART-CH'yi kapsayan endojen retrovirüs elemanları analiz edildi. Bu sonuçlar, test edilen tavuk ırklarındaki EAV genomik dizisinin E51 ve EAV-0 ile karşılaştırıldığında EAV-0 ile daha yakın ilişkili olduğunu gösterdi. Çin tavuklarındaki ART-CH elemanları, prototip ART-CH klonları 5 ve 14'ten önemli düzeyde farklı değildi. Test edilen tavuk ırklarındaki ev ve ev/J nükleotid asit dizileri, diğer bilinen ev ve ev/J dizilerine benzer idiyse de, filogenetik ağacın farklı dallarına aitti (Lohmann Brown yumurtacıları ve Beyaz Leghorn'lar hariç). Ek olarak, sağlam ev/J sekansları, bu tavukların birbirinden biraz farklı olduğunu gösterdi. Bildirilen sonuçlar, endojen ALV'lerin bir kaç Çin tavuk ırkı arasında yaygın şekilde dağıldığını göstermektedir. Çin tavuk ırklarında mevcut endojen viral genomlar tavuk popülasyonları içinde dolaşan diğer endojen virüslerden genetik olarak farklıdır.

Anahtar sözcükler: *Kuş leukosis virüsü, Endojen retrovirüs, Tavuk, Genetik farklılık, Prevalans*

INTRODUCTION

Avian leukosis viruses (ALV) were α-retrovirus that can be classified into 10 subgroups (A-J) based on characteristics, including host range, cross-neutralization, and envelope interference [1]. ALVs can be further divided into either exogenous or endogenous viruses based on the mechanism of transmission [2]. Exogenous viruses (subgroups A to D and J) can be spread vertically from the hen to the embryo through the egg, or horizontally from chicken to chicken [3]. ALV subgroups A, B, and J are common throughout the poultry industry; however,

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C and D are less common [2-5]. Among the endogenous viruses, four new ALV subgroups (F to I) were discovered and isolated from wild birds (e.g., pheasant and quail) [6]. ALV subgroup E (termed *ev* loci) is ubiquitous, exhibits low pathogenicity in chickens, and is the best studied endogenous virus to date [7].

Four major families of endogenous retrovirus elements: (i) Endogenous Avian Retrovirus (EAV), (ii) *ev* loci (Now termed ALV-E), (iii) avian retrotransposon from the chicken genome (ART-CH), and (iv) chicken repeat 1 (CR1) were identified in chickens [8]. The *ev* loci have been studied and characterized extensively [9]. The *ev* are inserted into the germline of normal chickens and are subsequently transmitted via Mendelian inheritance [10]. Some of the *ev* are closely related to the ALV exogenous viruses, with the exception of ALV-J [8]. The EAV consist of several different types of proviruses, including EAV-0, EAV-E51 and EAV-HP, meanwhile E51 was older than EAV-0 [11-13]. Members of the EAV family cannot form an infectious viral particle, but the RT (Reverse Transcriptase) remains functional and has been found in several human live vaccines, such as measles and mumps vaccines as chicken cells are used as a preparation method [14]. Additionally, ART-CH elements consisting of functional LTRs (Long Terminal Repeat) and short regions homologous to the ALV *gag*, *pol*, and *env* sequences can be found in around 50 genomic copies in the chicken genome [15,16]. The *gag*-related sequences are located within ART-CH elements, the longest of which is the coding sequences for p10, the matrix proteins (MA), and the capsid (CA) [16]. However, the sequences encoding the nucleocapsid (NC) and protease (PR) are not included [16]. The *ev*/J elements (Also termed EAV-HP) are novel sequences contained within the chicken genome that are highly similar to the HPRS-103 *env* gene of the ALV-J subgroup [8]. The ALV-J *env* was believed to have originated from the *ev*/J as it shares 95% identity to *ev*/J [17,18]. The CR1 element was a short, interspersed repetitive DNA section that belonged to the non-long terminal repeated (LTR) retrotransposons obtained by RT sequences. The majority of these sections contain between 7.000 to 20.000 repeats and are likely conserved ancient sequences, preceding aves evolution, but are not functional [3].

The emergence of ALV-J is thought to have derived from a recombination event between exogenous ALVs and the endogenous retrovirus *ev*/J [18-20]. The *ev*/J *env* gene is considered to be the source of the *env* gene found in all ALV-J viruses. The enhanced susceptibility of cancerous growths arising from the ALV-J virus varies among the various chicken genetic lines, but layers have a lower tendency to form tumors [6,10]. Therefore, it is extremely important to investigate the status of infections with avian endogenous retrovirus in Chinese chickens to elucidate the relationship between the prevalence exogenous and endogenous ALVs. This research will extend our insight into the characteristics of endogenous retrovirus elements in Chinese chicken flocks of economic importance. In addition, this information will aid in the development and implementation of measures that will help reduce exogenous and endogenous ALV infection prevalence in Chinese poultry. This study investigated the status of endogenous retrovirus elements in embryonated egg from seven breeds of indigenous Chinese chickens and three kinds of adventitious chickens raised widely throughout China using virus-specific PCR assays.

MATERIAL and METHODS

Samples

Fertile eggs were examined from ten different chicken breeds: 1) Shouguang chicken, 2) Beijing fatty chicken, 3) Langshan chicken, 4) Taihe chicken, 5) Pudong chicken, 6) Suqin chicken, and 7) Green eggshell chicken (All these embryos were from preserving species field in China) which are the important indigenous locks of economic importance in China, as well as 8) Lohmann Brown layer, 9) White Leghorn, and 10) Ross Brown layer. The adventitious chicken embryos were purchased from the chicken breeding companies in China. The DNA was extracted from the chicken embryo fibroblasts (CEFs) from 10-d-old embryonated chicken eggs obtained as described elsewhere [9], using with mincing and trypsin treatment.

DNA Extraction

DNA extraction was performed using a Genomic DNA Extraction Kit (Shanghai Generay Biotech. Co. Ltd., Shanghai, China). The genomic DNA of the CEFs of the tested chickens was resuspended in 50 µL of DNase-free water and was stored at -80°C.

Polymerase Chain Reaction (PCR)

The PCR assays used for detecting and sequencing the endogenous retrovirus elements in the CEFs were performed using the specific primer sets for each endogenous ALV *(Table 1)*. The reaction volume was 25 µL, which consisted of 1 µL of the template, 2.5 µL of 10 × buffer (Mg^{2+} free), 2 µL of $MgCl_2$ (25 mM), 2 µL of dNTP mixture (2.5 mM each), 1 µL of the forward primer (25 pmol), 1 µL of the reverse primer (25 pmol), 0.25 µL of Lataq polymerase (Takara Biotechnology Dalian Co. Ltd., Dalian, China), and the appropriate volume of DNase-free distilled water. The PCR procedure was as follows: preheat for 3 min at 94°C, 33 cycles of 40 s at 94°C, 40 s at the required temperature for each primer pair *(Table 1)*, extension at 72°C (according to the size of the fragments, 1 kb/min), and a final extension at 72°C for 10 min. 1.0% agarose gel electrophoresis was used to evaluate the PCR products.

Sequencing of DNA Products

Endogenous retrovirus genetic diversity was evaluated via nucleotide (nt) and amino acid (aa) sequences derived from a single 10-day-old embryonated egg from each

chicken breed. The *env* gene was used to design the primer pairs for the sequencing the *ev* and *ev*/J genomes due to the hyper variation of the sequences for nt and aa. The primer pair for detecting the intact *ev*/J genome sequence and that designed to allow for the specific amplification of proviruses containing complete *pol* genes were both set. The primer pair used to sequence the EAV genome was obtained from an area between the transmembrane (TM)-coding domain of the *env* and the long terminal repeats. This is because a large deletion occurred in the location of the EAV-0 *env* surface -coding domain. The sequencing primer pair for the ART-CHs was derived from the ART-CH *gag*-related sequences since the ART-CH internal regions were completely defective.

Table 1. *Oligonucleotide primers*

Family	Target Gene	Sequence 5′→3′	Annealing Temp. (°C)	Products Size (bp)	References
EAV	TM and LTR	F:gatgtgaggatgtcgaagg R:acaaagcatggaagacaga	46	241	[2,22]
ART-CH	*gag*- related region	F:ctcaaggtggctcatttaac R:acaaagcatggaagacaga	46	657	[2]
ev loci	*env*	F:ggatgaggtgactaagaaag R:tttgactgtctgcacatctc	48.5	881	[2,18]
		F:caatccttttctttaacagcg R:taacggaccaacaggctagt	46.5	713	[2]
ev/J	*env*	F:acaccattggtggcgcgtgtc R:cccgtcacatcgcgttc	48.5	1480	[9]
	Intact gene	F:ttcgtgattggaggaaacacttg R:gttacacttggcacacaaaggtggcataac	60	3900	[9]
	pol	F:ttcgtgattggaggaaacacttg R:cacgtttcctggttgttg	50	568	[15]

F: *forward primer,* ***R:*** *reverse primer*

Table 2. *GenBank accession numbers of the Chinese and the reference ALVs used in the phylogenetic analysis*

Subgroup/ Family	Name	Accession Number	Subgroup / Family	Name	Accession Number
A	RSA	M37980	*ev*-J	Line N chicken	NC_005947
B	RAV-2	M14902		Line 21 chicken	AJ238125
C	Prague C	V01197		Red jungle fowl	AJ238121
D	Schmidt-Ruppin D	D10652		Grey jungle fowl	AJ238122
E/ART-CH	clone 5	L25261		*ev*/J-BF	KU504577
	clone 14	L25262		*ev*/J-GS	KU504578
	ART-CH-BF	KR188978		*ev*/J-LB	KU504579
	ART-CH-GS	KR188979		*ev*/J-LS	KU504580
	ART-CH-LB	KR188980		*ev*/J-PD	KU504581
	ART-CH-LS	KR188981		*ev*/J-RB	KU504582
	ART-CH-PD	KR188982		*ev*/J-SG	KU504583
	ART-CH-RB	KR188983		*ev*/J-SQ	KU504584
	ART-CH-SG	KR188984		*ev*/J-TH	KU504585
	ART-CH-TH	KR188985		*ev*/J-WH	KU504586
	ART-CH-WL	KR188986	E/*ev* loci	*ev*-1	AY013303
	ART-CH-SQ	KR188987		*ev*-3	AY013304
E/EAV	EAV-0	X59844		*ev*-6	AY013305
	E51	M95189		*ev*-BF	KR188998
	EAV-BF	KR188988		*ev*-GS	KR188999
	EAV-GS	KR188989		ev-LB	KR189000
	EAV-LB	KR188990		*ev*-LS	KR189001
	EAV-LS	KR188991		*ev*-PD	KR189002
	EAV-PD	KR188992		*ev*-RB	KR189003
	EAV-RB	KR188993		*ev*-SG	KR189004
	EAV-SG	KR188994		*ev*-SQ	KR189005
	EAV-SQ	KR188995		*ev*-TH	KR189006
	EAV-TH	KR188996		*ev*-WL	KR189007
	EAV-WL	KR188997	J	HPRS-103	Z46390

The PCR products were purified using a Gel Purification kit (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China) according to the manufacturer's instructions and then cloned into a pMD-19T simple vector (Takara Biotechnology Dalian Co. Ltd., Dalian, China). The ligation products were transformed into DH5α and tested by bacterial fluid PCR (Beijing Dingguo Changsheng Biotechnology Co. Ltd.), then the positive samples were sequenced by Sanger method (Tsingke Biological Technology Co. Ltd. Wuhan, China). Sequences of the EAV, *ev*, *ev*/J, and ART-CH were retrieved from the GenBank database *(Table 2)*. Aligning and phylogenetic analyzes were performed using DNAStar (DNAStar, Inc., Madison, WI).

RESULTS

EAV, *ev*, *ev*/J, and ART-CH are the known endogenous retrovirus elements. The PCR assays that utilized specific primers for each endogenous retrovirus revealed that all of the retrovirus elements are present in the CEFs in all of the chicken breeds that were examined. An nt sequence comparison of the TM genome and the EAV LTR detected in embryonated eggs from all of the tested chickens showed a 95.9% - 100% sequence identity and between a 96.3% - 99.6% identity with EAV-0. However, E51 showed only 73.0% - 76.3% identity to the EAVs from the 10 chickens investigated in this study. The deduced aa sequences of the EAV detected in all the chickens showed 92.5% - 100% identity to each other, 93.8% - 98.8% identity to EAV-0, and 50.0% - 53.8% identity to E51. These results showed that the tested chicken EAVs are closely related to EAV-0, but distantly related to E51. The phylogenetic data derived from the total number of nt substitutions also found similar results *(Fig. 1)*.

To investigate the ART-CH prevalence and its genetic diversity in Chinese, as well as other chicken breeds, this study investigated the variability in the ART-CH, nt sequence since it does not encode aa as a result of large multiple nt deletions. Paired comparisons of the *gag*-related nt sequences revealed that the ART-CHs detected in the tested chickens had a sequence identity of 91.8% - 98.3% to each other but a higher sequence identity to prototype strains, the ART-CH clones 5 (93.5% - 99.2%) and 14 (92.1% - 99.1%). From the total number of nt substitutions and deletions, the *gag*-related sequence phylogenetic tree was analyzed using the prototype ART-CH elements, clones 5 and 14. It was found that the *gag*-related nt ART-CHs sequences showed that PD, TH, and BF were in a different branch of the phylogenetic tree compared with others. Moreover, LB and RB is closely related to the prototype ART-CHs clones 5 and 14 *(Fig. 2)*.

The nt sequence of the *ev* genomes (a part of the *env* gene) paired comparisons of the nt sequence substitutions denoted the maximum sequence divergence between the *ev* loci in the CEF of Ross Brown, and prototype *ev*-6, even for small substitutions (1.4%). Such nt substitutions only resulted in 4.2% of the aa alterations. In general, the nt and aa sequences for a region of the *env* gene from the *ev* genomes revealed a high level of identity. The nt sequence

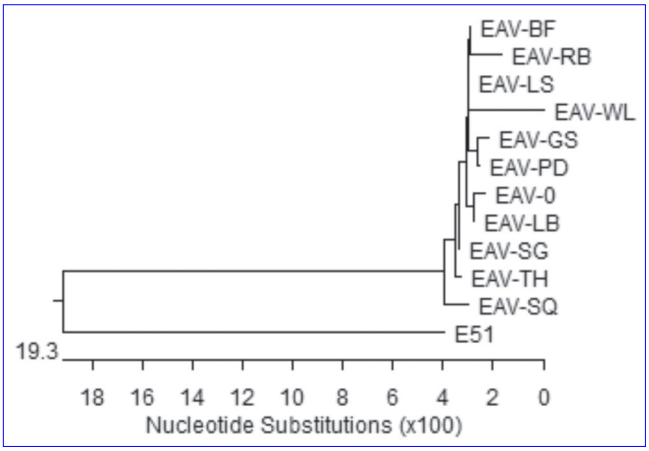

Fig 1. Phylogenetic tree of the nucleotide sequences from the endogenous EAV genomes made using MegAlign (DNAStar)
EAV-BF: Beijing fatty chicken; EAV-RB: Ross Brown layers; EAV-LS: Langshan chicken; EAV-WL: White Leghorn; EAV-GS: Green eggshell chicken; EAV-PD: Pudong chicken; EAV-LB: Lohmann Brown layers; EAV-SG: Shouguang chicken; EAV-TH: Taihe chicken; EAV-SQ: Suqin chicken; and EAV-0 and E51: Prototype of EAV

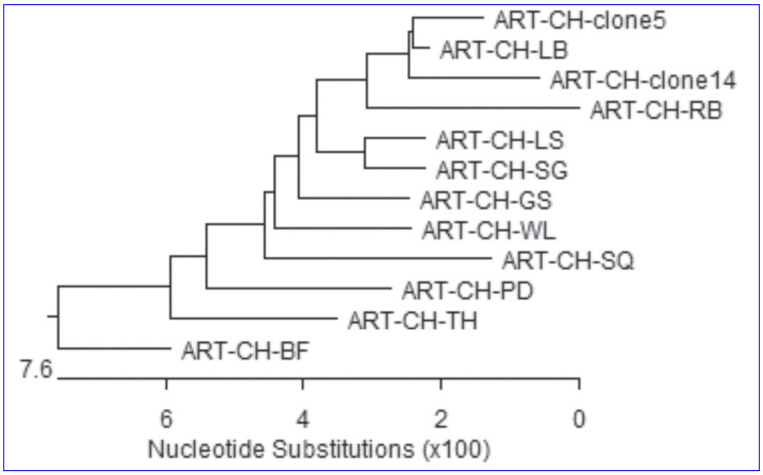

Fig 2. Phylogenetic tree of the nucleotide sequences from the endogenous ART-CH element made using MegAlign (DNAStar)
ART-CH-BF: Beijing fatty chicken; ART-CH-RB: Ross Brown layers; ART-CH-LS: Langshan chicken; ART-CH-WL: White Leghorn; ART-CH-GS: Green eggshell chicken; ART-CH-PD: Pudong chicken; ART-CH-LB: Lohmann Brown layers; ART-CH-SG: Shouguang chicken; ART-CH-TH: Taihe chicken; ART-CH-SQ: Suqin chicken; and ARTCH-clones 5 and 14: Prototype of ART-CH

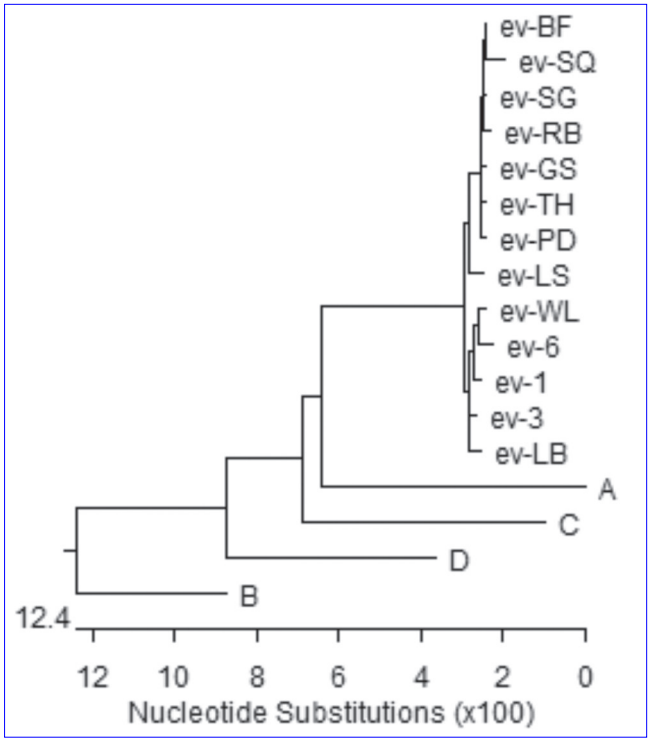

Fig 3. Phylogenetic tree of the nucleotide sequences from the endogenous ev genomes made using MegAlign (DNAStar)
ev-BF: Beijing fatty chicken; *ev*-RB: Ross Brown layers; *ev*-LS: Langshan chicken; *ev*-WL: White Leghorn; *ev*-GS: Green eggshell chicken; *ev*-PD: Pudong chicken; *ev*-LB: Lohmann Brown layers; *ev*-SG: Shouguang chicken; *ev*-TH: Taihe chicken; *ev*-SQ: Suqin chicken; and A, B, C and D: subgroup A, B, C and D ALSVs

between *ev* genomes and ALV-A was the most closely related one, while that with ALV-B was the most distant *(Fig. 3)*.

A section of the nt and aa sequences of the *env* gene from the *ev*/J genome demonstrated high sequence identity (nt; 95.9% - 99.1% and aa; 96% - 99.8%) among all of the tested chickens, as well as the other known sequences of *ev*/J. Moreover, all of the tested chickens contained the *ev*/J genomes, and the other known *ev*/J revealed a 92.8% - 98.1% nt and 96.1% - 98.2% aa identity to ALV-J. Phylogenetically, all of the compared *ev*/J genomes are randomly distributed, and the genetic distance of the *ev*/J genomes and ALV-J was relatively close *(Fig. 4)*.

Results of the PCR assay for detecting the complete *ev*/J genome were quite different between samples. Products of approximately 3.9 kb were amplified with the genome DNA template from all of the tested chickens. However, additional products of approximately 2.1 kb were amplified with Shouguang chickens, Beijing fatty chickens, Langshan chickens, and three adventitious breeds *(Fig. 5)*.

The 568 bp product was amplified with all the genomic DNA templates from all of the tested chickens in the test for detection of the *pol* gene in *ev*/J, with the exception of Taihe and Pudong chickens. These results illustrated that the complete *ev*/J genome differed between chicken breeds and none of the *ev*/J *pol* gene was intact in the tested chickens *(Fig. 6)*.

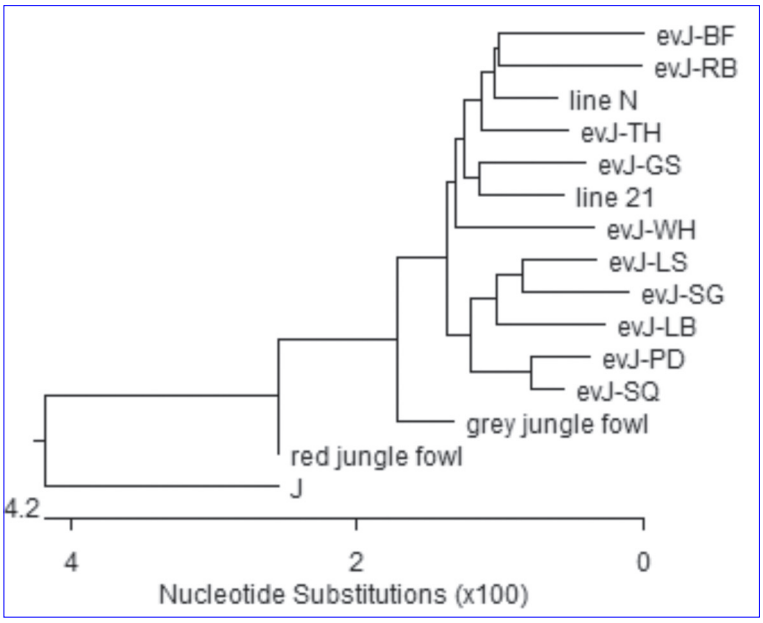

Fig 4. Phylogenetic tree of the nucleotide sequences from the endogenous ev/J genomes made using MegAlign (DNAStar).
ev/J-BF: Beijing fatty chicken; *ev*/J-RB: Ross Brown layers; *ev*/J-LS: Langshan chicken; *ev*/J-WH: White Leghorn; *ev*/J-GS: Green eggshell chicken; *ev*/J-PD: Pudong chicken; *ev*/J-LB: Lohmann Brown layers; *ev*/J-SG: Shouguang chicken; *ev*/J-TH: Taihe chicken; *ev*/J-SQ: Suqin chicken; and J: subgroup J ALSVs. Green jungle fowl, red jungle fowl, line 21 and line N: *ev*/J

phylogenetic tree from the *env* gene showed that the *ev* genomes of the tested chickens were highly related. However, the *ev* genomes, including prototype *ev*-1, *ev*-3, and *ev*-6 were separately clustered with the exception of the Lohmann Brown layers and White Leghorns. Besides, among the exogenous viruses, the genetic distance

DISCUSSION

Sequence analysis of the location between the TM and the LTR of the tested EAVs indicated that there is a low sequence identity to E51 and a high sequence identity with the other EAVs, including EAV-0 and the other tested

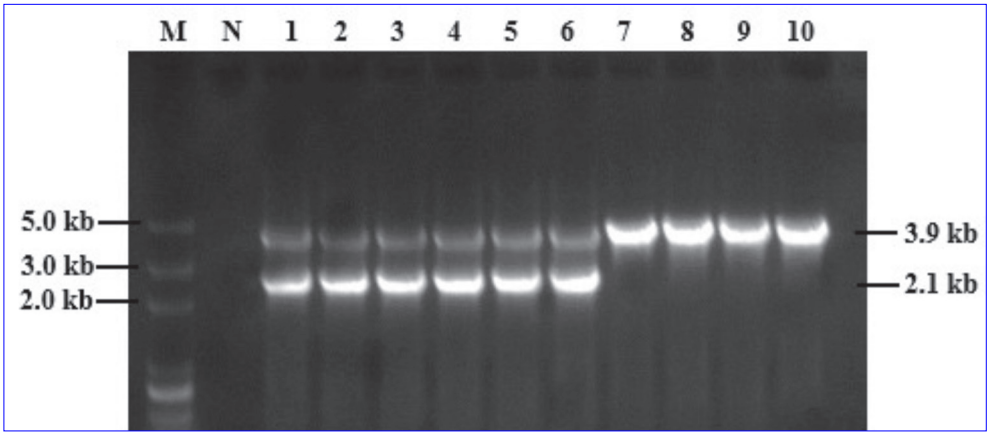

Fig 5. Reaction products of intact endogenous *ev*/J genomes subjected to electrophoresis on a 1% agarose gel.
Lane 1: Beijing fatty chicken; lane 2: Langshan chicken; lane 3: Lohmann brown layers; lane 4: Ross brown layers; lane 5: Shouguang chicken; lane 6: White Leghorns; lane 7: Green eggshell chicken; lane 8: Pudong chicken; lane 9: Suqin chicken; lane 10: Taihe chicken; N: Negative control (no template control); and M: DNA marker

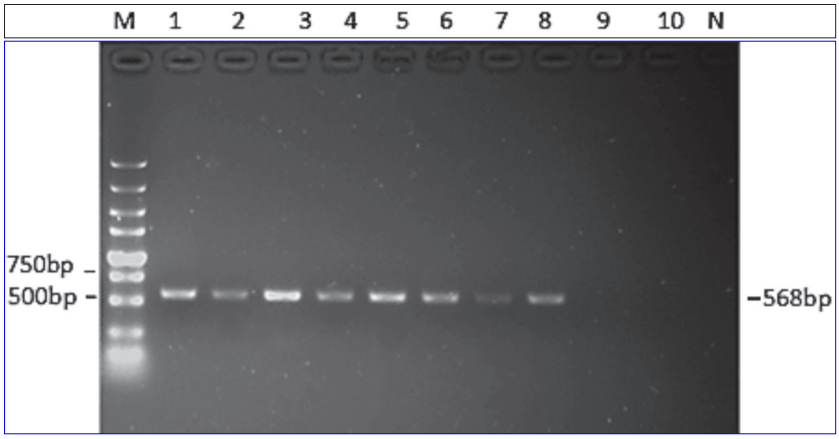

Fig 6. Reaction products of the selective amplification of *ev*/J proviruses with intact pol genes subjected to electrophoresis on a 1% agarose gel
Lane 1: Beijing fatty chicken; lane 2: Langshan chicken; lane 3: Green eggshell chicken; lane 4: Lohmann Brown layers; lane 5: Ross Brown layers; lane 6: Shouguang chicken; lane 7: Suqin chicken; lane 8: White Leghorn; lane 9: Pudong chicken; lane 10: Taihe chicken; N: Negative control (no template control); and M: DNA Marker

EAVs. The EAV is present in all Gallus species, consistent with a germline infection occurring before speciation. In addition, analysis of the EAV family revealed that the Gallus species genomes are heterogeneous. This is likely the result of prolonged evolutionary pressure compared to other endogenous retroviruses [21]. Correspondingly, different EAV family members might have infected the Gallus species throughout its evolutionary history. For example, EAV-0 may be younger than E51. Our results demonstrated all of the tested EAV underwent a pathway similar to EAV-0, but different from E51.

The evolutionary relationship between the various ART-CH and the other endogenous retroviruses remains unclear. A comparison of a section of the *gag*-related gene sequences observed in the ART-CH of the tested chickens with that of the other known ART-CH implies that they are distinct from each other (91.8% - 98.3% sequence identity). However, they are closely related to the ART-CH clones 14 (92.1% - 99.1% sequence identity) and 5 (93.5% - 99.2% sequence identity). The phylogenetic tree developed in this study also supports this claim.

In this study, the sequence analysis of an *env* gene section located between the *ev* of the tested chickens and other known *ev* genomes demonstrated a high degree of identity. Although phylogenetically their ev genomes cluster together, the prototypical *ev* genomes are clustered on a separate branch. The exception is the Lohmann Brown layers (in the same branch as *ev*-3) and the White Leghorns (in the same branch as *ev*-6), which indicates that the *ev* genomes discovered in the Chinese indigenous chickens had a differential evolutionary pathway from the prototype viruses, but that some adventitious breeds shared the same evolutionary pathway. Among the known exogenous ALVs, the *ev* genome (ALV-E) of tested chickens were closely related to ALV-A, which indicated that the ancestors of ALV-E and ALV-A were possibly similar with

each other because of the short genetic distance. Moreover, a sequences analysis of the *ev*/J genome *env* gene from the tested chickens exhibited both a high nt and aa identity in comparison to the other known endogenous genomes and exogenous ALV-J prototype strain HPRS-103. Phylogenetically, the tested *ev*/J genomes were distributed randomly among the other known *ev*/J genomes and the ALV-J prototype strain HPRS-103. However, *ev*/J genomes of the tested chickens and the prototype ALV-J strain were clustered in the same evolutionary branch indicating that they shared a common ancestor. It is well-known that the *ev*/J genomes are the material basis of the ALV-J *env* gene. However, there was no obvious nt substitution divergence observed between the *ev*/J and subgroup J ALV. Unlike gray jungle fowl, in which the *pol* gene was complete in the *ev*/J genome, none of the tested *ev*/J genomes exhibited a complete *pol* gene. Furthermore, the complete *ev*/J genome of the tested chickens was somewhat different from each other for obtaining either one or two fragments.

In this study, the primer pairs specific to the endogenous genomes of EAV, *ev*, *ev*/J, and ART-CH confirmed that these endogenous avian retrovirus elements are present in all seven types of indigenous Chinese chickens and three adventitious breeds via PCR. The results demonstrated that the endogenous retroviruses were closely related to each other in these ten chicken species, especially in indigenous Chinese chickens. Moreover, although none of the tested chickens had a complete *pol*, the genome of the *ev*/J was quite different between the Chinese indigenous chickens and the adventitious breeds.

The expression of particular endogenous proviruses may have an effect on the phenotype of the organism by influencing the susceptibility to related retroviruses, variations in the immune status, or creating genomic instability via recombination with sequences from other cells of retroviruses [22]. Additionally, the existence of different endogenous retroviruses in the various types of chicken breeds may have resulted in the broilers to have a greater probability of infection with ALV-J than the layers. Therefore, further study is required to elucidate whether other Chinese endogenous retroviruses contain similar traits as the endogenous viruses in Chinese indigenous chickens possessing these characteristics.

REFERENCES

1. Fadly AM: Isolation and identification of avian leukosis viruses. *Avian Pathol*, 29, 529-535, 2000. DOI: 10.1080/03079450020016760

2. Kim YJ, Park SI, Park SJ, Kim HH, Jung YW, Kwon JT, Jang BG, Kim HK, Cho KO: Molecular analysis of endogenous avian leukosis/sarcoma virus genomes in Korean chicken embryos. *J Vet Med Sci*, 70, 17-23, 2008. DOI: 10.1292/jvms.70.17

3. Sacco MA, Nair VK: Prototype endogenous avian retroviruses of the genus Gallus. *J Gen Virol*, 95, 2060-2070, 2014. DOI: 10.1099/vir.0.066852-0

4. Nikiforov MA, Gudkov AV: ART-CH: A VL30 in chickens. *J Virol,* 68, 846-853, 1994.

5. Ono M, Tsukamoto K, Tanimura N, Haritani M, Kimura KM, Suzuki G, Okuda Y, Sato S: An epizootic of subcutaneous tumors associated with subgroup A avian leukosis/sarcoma virus in young layer chickens. *Avian Dis*, 48, 940-946, 2004. DOI: 10.1637/7162-020204R

6. Venugopal K: Avian leukosis virus subgroup J: A rapidly evolving group of oncogenic retroviruses. *Res Vet Sci*, 67, 113-119, 1999. DOI: 10.1053/rvsc.1998.0283

7. Benson SJ, Ruis BL, Fadly AM, Conklin KF: The unique envelope gene of the subgroup J avian leukosis virus derives from *ev*/J proviruses, a novel family of avian endogenous viruses. *J Virol*, 72, 10157-10164, 1998.

8. Bai J, Payne LN, Skinner MA: HPRS-103 (exogenous avian leukosis virus, subgroup J) has an *env* gene related to those of endogenous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. *J Virol*, 69, 779-784, 1995.

9. Ruis BL, Benson SJ, Conklin KF: Genome structure and expression of the *ev*/J family of avian endogenous viruses. *J Virol*, 73, 5345-5355, 1999.

10. Grunder AA, Benkel BF, Chambers JR, Sabour MP, Gavora JS: Characterization of eight endogenous viral *(ev)* genes of meat chickens in semi-congenic lines. *Poul Sci*, 74, 1506-1514, 1995. DOI: 10.3382/ps.0741506

11. Venugopal K, Smith LM, Howes K, Payne LN: Antigenic variants of J subgroup avian leukosis virus: sequence analysis reveals multiple changes in the env gene. *J Gen Virol*, 79, 757-766, 1998. DOI: 10.1099/0022-1317-79-4-757

12. Borisenko L: Avian endogenous retroviruses. *Folia Biologica (Praha)*, 49, 177-182, 2003.

13. Adkins HB, Blacklow SC, Young JA: Two functionally distinct forms of a retroviral receptor explain the nonreciprocal receptor interference among subgroups B, D, and E avian leukosis viruses. *J Virol*, 75, 3520-3526, 2001. DOI: 10.1128/JVI.75.8.3520-3526.2001

14. Chernov MV, Tikhonenko AT, Zaĭtsevskaia TE, Denisova RA, Obukh IB: The genome of chickens free of endogenous avian leukosis-sarcoma proviruses contains sequences distantly related to Rous sarcoma virus. *Mol Gen Mikrobiol Virusol*, 3, 6-11, 1987.

15. Sacco MA, Howes K, Smith LP, Nair VK: Assessing the roles of endogenous retrovirus EAV-HP in avian leukosis virus subgroup J emergence and tolerance. *J Virol*, 78, 10525-10535, 2004. DOI: 10.1128/JVI.78.19.10525-10535.2004

16. Benkel B, Rutherford K: Endogenous avian leukosis viral loci in the Red Jungle Fowl genome assembly. *Poult Sci*, 93, 2988-2990, 2014. DOI: 10.3382/ps.2014-04309

17. Payne LN: Developments in avian leukosis research. *Leukemia,* 6, 150S-152S, 1992.

18. Smith L M, Toye AA, Howes K, Bumstead N, Payne LN, Venugopal K: Novel endogenous retroviral sequences in the chicken genome closely related to HPRS-103 (subgroup J) avian leukosis virus. *J Gen Virol*, 80, 261-268, 1999. DOI: 10.1099/0022-1317-80-1-261

19. Payne LN, Nair V: The long view: 40 years of avian leukosis research. *Avian Pathol*, 41, 11-19, 2012. DOI: 10.1080/03079457.2011.646237

20. Borisenko L, Rynditch AV: Complete nucleotide sequences of ALV-related endogenous retroviruses available from the draft chicken genome sequence. *Folia Biol (Praha)*, 50, 136-141, 2004.

21. Gudkov AV, Komarova EA, Nikiforov MA, Zaitsevskaya TE: ART-CH, a new chicken retrovirus like element. *J Virol*, 66, 1726-1736, 1992.

22. Tsang SX, Switzer WM, Shanmugam V, Johnson JA, Goldsmith C, Wright A, Fadly A, Thea D, Jaffe H, Folks TM, Heneine W: Evidence of avian leukosis virus subgroup E and endogenous avian virus in measles and mumps vaccines derived from chicken cells: Investigation of transmission to vaccine recipients. *J Virol*, 73, 5843-5851, 1999.

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Research Article

Morphologic and Morphometric Structure and Arterial Vascularization of Glandula Interdigitalis in Male Hemshin Sheep

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Abstract

The purpose of this study was to determine morphologic and morphometric structure and arterial vascularization of glandula interdigitalis in male Hemshin sheep. In the study, 40 feet (20 fore-feet, 20 hind-feet) were used as material. Latex was injected into arteries of fore- and hind-feet in order to determine vascularization of glandula interdigitalis. In the study, 6 measurements were taken from different points of glandula interdigitalis in order to determine its morphometric values. These values were statistically analyzed. Consequently, length of the gland was measured as 30.18±1.93 mm in fore-feet and 25.67±1.77 mm in hind-feet. The gland in fore-feet was determined to be considerably greater than the gland in hind-feet (P<0.05). It was found that the gland was vascularized by branches separated from arteriae digitales palmares communis II, III, and IV in fore-feet and arteria digitalis dorsalis communis III and arteriae digitales plantares propriae III and IV in hind-feet.

Keywords: *Arter, Interdigital gland, Morphology, Morphometry*

Erkek Hemşin Koyununda Glandula İnterdigitalis'in Morfolojik ve Morfometrik Yapısı ile Arterial Vaskularizasyonu

Özet

Araştırmada erkek Hemşin koyununda glandula interdigitalis'in morfolojik ve morfometrik yapısı ile arterial vaskularizasyonunun belirlenmesi amaçlandı. Materyal olarak 40 adet (20 ön, 20 arka) ayak kullanıldı. Çalışmada glandula interdigitalis'in vaskularizasyonunun belirlenmesi amacıyla ön ve arka ayak arterleri içerisine latex enjekte edildi. Glandula interdigitalis'in morfometrik değerlerini belirlemek için farklı noktalarından 6 adet ölçü alındı. Bu değerler istatistiksel analize tabi tutuldu. Sonuç olarak bezin uzunluğu ön ayakta 30.18±1.93 mm, arka ayakta 25.67±1.77 mm olarak ölçüldü. Ön ayaktaki bezin arka ayaktakine göre önemli oranda büyük olduğu tespit edildi (P<0.05). Bezin ön ayakta arteriae digitales palmares communis II, III ve IV' ten ayrılan dallar tarafından, arka ayakta ise arteria digitalis dorsalis communis III ve arteriae digitales plantares propriae III ve IV tarafından vaskularize edildiği saptandı.

Anahtar sözcükler: *Arter, Glandula interdigitalis, Morfoloji, Morfometri*

INTRODUCTION

Hemshin sheep breeding is performed in North-Eastern Anatolian Region of Turkey. It is reported that the number of Hemshin sheep has been decreased in recent years due to various reasons and the breed is under the danger of extinction [1,2].

Skin glands such as glandula (gl.) tarsalis, gl. interdigitalis and gl. infraorbitalis which are closely related to reproductive activity in ruminants, are localized in different parts of the body [3]. Gl. interdigitalis secreting apocrine and holocrine in primates is a dermal gland located between hooves [4]. Shape, size and location of the gland vary depending on species [4,5]. This gland was described as a hoof-skin organ by Raesfeld [6], a growing organ by Sivachelvan et al.[7], sinus interdigitalis by Badvek [8]. Density and composition of gl. interdigitalis's secretions are diverse [9-11]. Secretion of this gland in animals acts as pheromones [12]. In addition, it is also reported that the secretion of the gland has fungicidal and bactericidal effect and is protective against ultraviolet rays [4].

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In ruminants, arteriae (aa.) digitales palmares (or plantares) propriae axiales et abaxiales belonging to hooves III and IV are primarily responsible for the vascularization of digital area. Dorsal arteries of the feet (aa. digitales dorsales propriae III et IV), on the other hand, are less responsible for it [13]. Palmar and plantar digital arteries are separated from aa. digitales palmares communis II, III et IV and aa. digitales plantares communis II, III et IV in ruminants [14,15].

In literature reviews it is observed that there are a number of studies conducted morphology of gl. interdigitalis [4,9,16]. Hemshin sheep is a local breeds, in addition to there is not such a study on Hemshin sheep. Therefore, the purpose of this study was to determine morphologic and morphometric structure and arterial vascularization of gl. interdigitalis in male Hemshin sheep.

MATERIAL and METHODS

The healthy 10 male Hemshin sheep were used in this study. The Hemshin sheep were avarage of 42.70±5.07 kg, 8-9 months, and had not been castrated. Glandula interdigitalis in fore-feet and hind-feet of 10 male Hemshin sheep was assessed separately in the study. By considering that care and feeding conditions were the same, feet were provided from a slaughter house in Ardanuç district of Artvin in december. Approval required for conducting the study was received from Kafkas University Local Ethics Committee for Animal Trials (Date: 17.12.2015, Number: 119). While 5 of the sheep were used for morphological evaluations, the other 5 were used for determining the arterial vascularization of the gland.

For morphological evaluations, morphometric measurements were taken from 6 different points by dissecting gl. interdigitalis in feet separately. In addition, weight and volume of the gland were also calculated. Mean and standard deviation values of morphometric findings obtained from gl. interdigitalis in fore and hind feet were analyzed in SPSS (20.0 version) packaged software. The feet were randomly collected due to the slaughter-hause conditions, therefore differences between gl. inter-digitalis in fore and hind feet, on the other hand, were determined by using Independent Samples T test.

Latex stained with red colored rotring ink was injected to arteria (a.) mediana and a. saphena in order to examine arterial vascularization of gl. interdigitalis in feet of Hemshin sheep [17]. Arteries vascularizing the gland were revealed by dissecting after latex injected feet were kept in 10% formaldehyde for 24 hours.

For histological examinations, the interdigital gland samples of Hemshin sheep were kept in 10% formalin for fixation at room temperature, the routine procedure was applied and then they were embedded in paraffin. Serial sections with a thickness of 5 µm were cut from paraffin blocks. Mallory's modified triple staining (Triple) was used to show general structure of the interdigital gland. The sections were examined with light microscope (Olympus BX51, Japan).

RESULTS

Gl. interdigitalis was determined to consist of the parts corpus *(Fig. 1,2,3,4/c)*, excretory duct *(Fig. 1,2,3,4/b)* and orifice *(Fig. 1,2,3,4/a)*. In all the feet, it was determined that orifice of excretory duct and proximal end of corpus of the gland were localized between distal ends of phalanx proximalis and its excretory duct and corpus were between phalanx media. The gland was observed to have a pipe-shape and white color. It was observed that while distal end of gland's corpus in fore-feet leaned to ligamentum (lig.) interdigitale *(Fig. 1/d)*, gland's corpus in hind-feet did not touch to lig. interdigitale *(Fig. 2/d)*. While intensive hairs were determined in orifice of gland's excretory duct, a small amount of hairs was present in its lumen *(Fig. 3)*.

Table 1 shows morphometric results obtained from gl. interdigitalis in fore- and hind-feet. Accordingly, length of the gland was measured as 30.18±1.93 mm

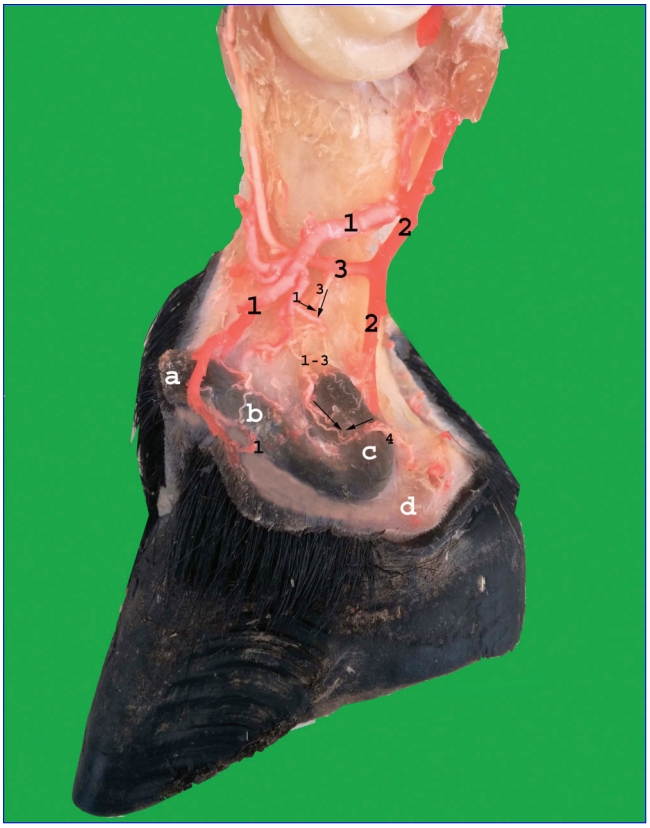


Fig 1. Interdigital region in fore hoof
1. Continuation of rami palmares phalangum proximalium in interdigital region, **2.** A. digitalis palmaris communis III, **3.** A. interdigitalis, **4.** A. digitalis palmaris propria, **a.** Orifice of excretory duct, **b.** Excretory duct, **c.** Corpus, **d.** Lig. interdigitale

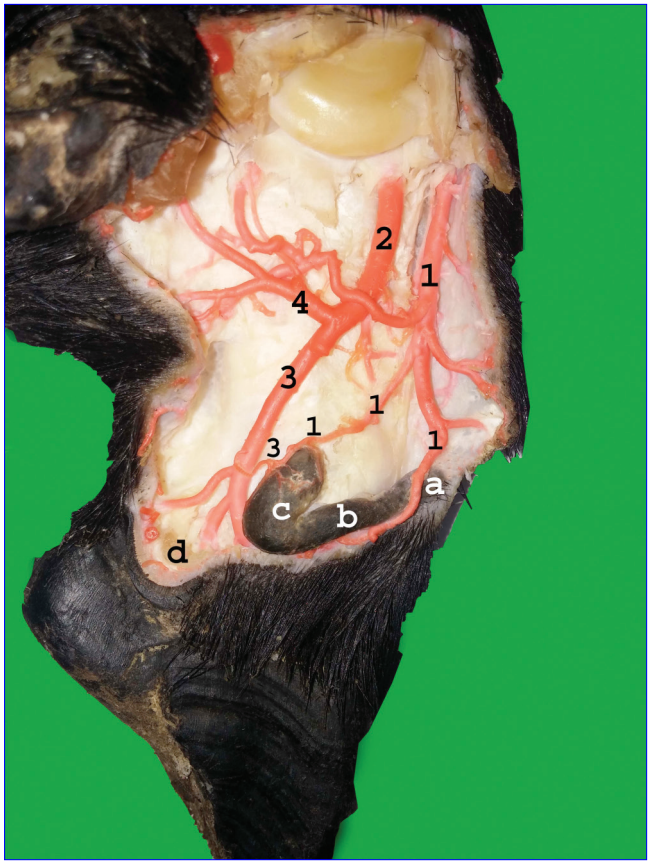


Fig 2. interdigital region in hind hoof
1. A. digitalis dorsalis communis III, **2.** A. metatarsea dorsalis III, **3.** Aa. digitales plantares propriae III et IV, **4.** A. digitalis plantaris communis III, **a.** Orifice of excretory duct, **b.** Excretory duct, **c.** Corpus, **d.** Lig. interdigitale

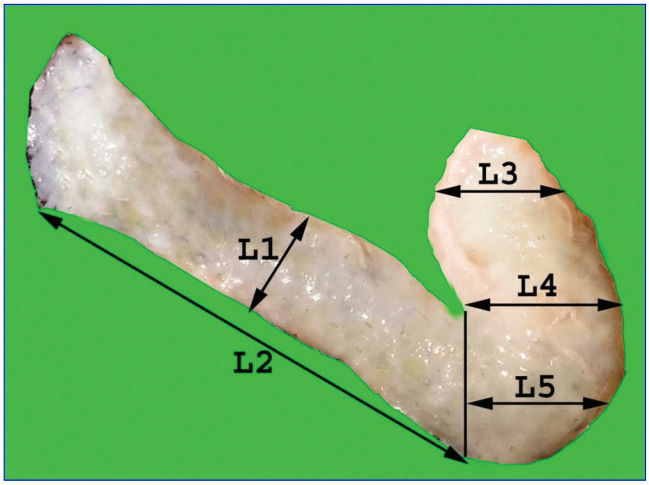


Fig 3. Morphometric measurements from gl. interdigitalis
L1; diameter of excretory duct, **L2;** length of excretory duct, **L3;** diameter of corpus's proximal end, **L4;** medium diameter of corpus, **L5;** diameter of corpus's distal end

palmares phalangum proximalium and a. interdigitalis *(Fig. 1/3)*, corpus was vascularized by a. digitalis palmaris communis III *(Fig. 1/2)* and a. digitalis palmaris propria *(Fig. 1/4)* which is one of the branches of aa. digitalis palmaris communis II et IV. Anastomosis developed rr. palmares phalangum

proximalium *(Fig. 1/1)*, a. interdigitalis and a. digitalis palmaris propria was observed on corpus of the gland. Rr. palmares phalangum proximalium in hooves of Hemshin sheep was determined to separate from a. digitalis palmaris communis II.

Table 1. *Mean length, weight, volume and standard deviation values of gl. interdigitalis found in fore- and hind-feet of male Hemshin sheep (*:P<0.05)*

Measurements	Gl. interdigitalis in fore-feet	Gl. interdigitalis in hind-feet
L1* (mm): Diameter of excretory duct	4.60±0.53	3.05± 0.47
L2* (mm): Length of excretory duct	20.90±1.95	18.95±1.50
L3* (mm): Diameter of corpus's proximal end	6.67±0.95	4.88±0.77
L4* (mm): Medium diameter of corpus	9.29±0.86	7.48±1.32
L5* (mm): Diameter of corpus's distal end	6.26±0.52	5.13±0.58
L6* (mm): Total length of the gland	30.18±1.93	25.67±1.77
W1* (gr): Weight of the gland	1.88±0.40	0.95±0.26
V1* (mL): Volume of the gland	2.00±0.39	1.00±0.28
** P<0.05*		

in fore-feet and 25.67±1.77 mm in hind-feet. The gland in fore-feet was larger than the gland in hind-feet in a statistically significant way (P<0.05).

Arterial Vascularization of Gl. Interdigitalis in Fore-Feet

The gland was determined to be vascularized by branches separated from aa. digitales palmares communis II, III et IV. It was found that while the gland's excretory duct was vascularized by continuation of rami (rr.)

Arterial Vascularization of Gl. Interdigitalis in Hind-Feet

It was determined that the gland's-excretory duct was vascularized by a. digitalis dorsalis communis III *(Fig. 2/1)*, corpus was vascularized by a. digitalis dorsalis communis III and aa. digitales plantares propriae III et IV *(Fig. 2/3)*. A. digitalis dorsalis communis III and aa. digitales plantares propriae III et IV developed anastomosis on corpus of the gland. A. metatarsea dorsalis III *(Fig. 2/2)* and a. digitalis plantaris communis III *(Fig. 2/4)* were observed to develop

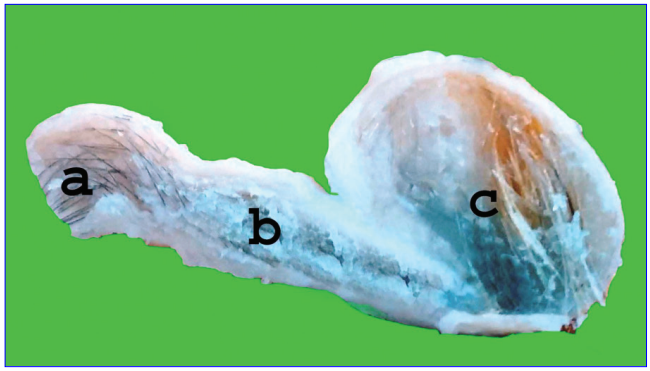


Fig 4. Cross section of gl. interdigitalis
a. Orifice of excretory duct, **b.** Excretory duct, **c.** Corpus

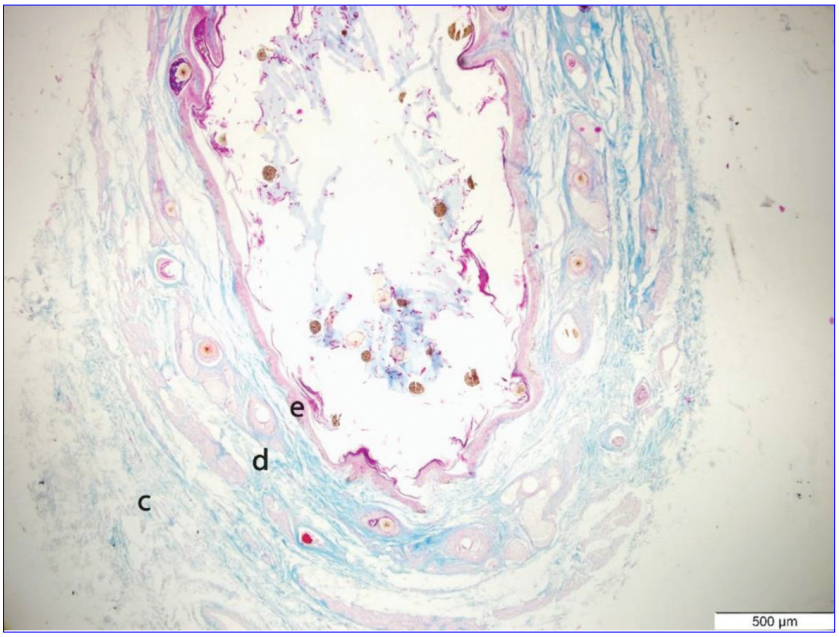
anastomosis in interdigital area at mid-level of phalanx proximalis.

The wall structure of the interdigital gland of the Hemshin sheep was composed of the epidermis, dermis and a capsule. The epidermis consisted of keratinized stratified squamous epithelium. Hair follicles, sebaceous glands, sweat-glands, muscle fibers, and apocrine glands were observed in the dermis *(Fig. 5,6)*.

DISCUSSION

Due to decrease in number of Hemshin sheep in recent years [2] slaughtering female Hemshin sheep in

Fig 5. Interdigital gland of Hemshin sheep. **e.** epidermis, **d.** dermis, **c.** capsule. Triple



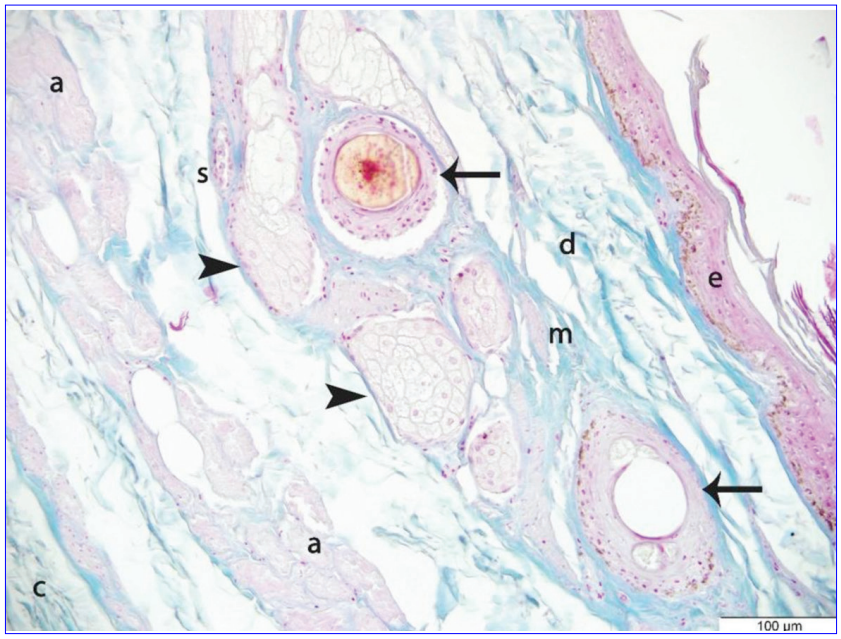


Fig 6. Interdigital gland of Hemshin sheep. **e.** epidermis, **d.** dermis, **c.** capsule, *arrows:* hair follicles, *arrow heads:* sebaceous glands, **s.** sweat gland, **m.** muscle fiber, **a.** apocrine glands. Triple

slaughterhouse has been limited. Therefore, differences of gl. interdigitalis in terms of sex could not be evaluated. Morphologic and morphometric differences of gl. interdigitalis in fore- and hind-feet of male Hemshin sheep were determined in the study.

It was previously reported that gl. interdigitalis was anatomically located between hooves of fore- and hint-feet in sheep, had a duct opening outside and had a shape pipe like dermal fold [18-21]. The gland was reported to be rudimentary in goats [5,22] and it was not found in fore-feet of male roebuck [23]. Avdic et al.[24] stated that the very proximal end of the gland in sheep had fundus, fundus was located on distal of phalanx proximalis, corpus and excretory duct were located between phalanx media; in Hemshin sheep, on the other hand, the very proximal end of the gland was observed to be orifice of excretory duct. The orifice of excretory duct was located at the proximal interphalangeal articulation in Baladi sheep [25]; in Hemshin sheep, it was located between distal ends of phalanx proximalis. In the line with the literature [4,24,26], it was determined that gland's fundus was at the level of distal of phalanx proximalis and corpus was at the level of phalanx media.

Some morphometric findings regarding the gland were found in literature reviews. Atoji et al.[9], stated that the total length of the gland was 25-40 mm in Japanese serow, Getty [27] reported that its total length was 25-30 mm in sheep. Length of the gland's excretory duct was reported as 23 mm by Çalışlar [18], 18-20 mm by Nickel et al.[20], 10 mm by Atoji et al.[9], 15 mm by Awaad et al.[25] and 16.74 mm by Süzer et al.[28]. Length of the gland's corpus was determined as 6.95 mm in female Kıvırcık sheep by Süzer et al.[28] and 9 mm in Akkaraman sheep by Çalışlar [18]. In Hemshin sheep, total length of the gland was determined as averagely 27.92 mm, length of its corpus as 8.39 mm and length of its duct as 19.92 mm.

Uğurlu [21] reported that there was no significant morphologic difference between sinus interdigitalis in fore- and hind-feet in sheep. It was determined that diameter of gl. interdigitalis' corpus in fore- and hind- feet of male Kıvırcık sheep was 4.29 mm and 5.14 mm, respectively; and length of its excretory duct was 25.03 mm and 27.23 mm, respectively [16]. These values in Hemshin sheep were found as 9.29 mm, 7.48 mm and 20.90 mm, 18.95 mm, respectively. Differently from the researchers [16,21], the gland in fore feet of Hemshin sheep was determined to be considerably larger compared to the hind feet as was reported in the literature [4,8,24] ($P<0.05$).

Aslan et al.[26] reported that arterial vascularization of the gland in Tuj sheep was provided by a. digitalis palmaris et dorsalis III, IV in fore-feet and by a. digitalis plantaris III, IV in the hind feet. It was determined in Hemshin sheep, on the other hand, arterial vascularization of the gland was provided by a. digitales palmares II, III et IV in fore-feet and by a. digitalis dorsalis communis III and aa. digitales plantares propriae III et IV in hind-feet.

The histological, histochemical, immunohistochemical and electronmicroscopic features of the interdigital gland in native sheep breeds were described [4,16,22,26,28,29]. The wall structure of the Hemshin sheep' interdigital gland was composed of epidermis, dermis, and a capsule and hair follicles, sebaceous glands, sweat glands, muscle fibers, and apocrine glands were observed in the interdigital glands of the Hemshin sheep like Kıvırcık [16] and Akkaraman [22] sheep. It was not run across the nerve plexus as described in Kıvırcık [16] sheep and lymph follicles as described in Tuj [26] sheep.

Consequently, length of the gland was measured as 30.18±1.93 mm in fore-feet and 25.67±1.77 mm in hind-feet. The gland in fore-feet was determined to be considerably greater than the gland in hind-feet ($P<0.05$). It was found that the gland was vascularized by branches separated from arteriae digitales palmares communis II, III, and IV in fore-feet and arteria digitalis dorsalis communis III and arteriae digitales plantares propriae III and IV in hind-feet.

REFERENCES

1. Anonymous: Tarım ve Köyişleri Bakanlığı Tarımsal Araştırmalar Genel Müdürlüğü Büyükbaş ve Küçükbaş Hayvancılık Araştırmaları Program Değerlendirme Toplantısı Ara Raporu. *TAGEM*, 1, 197-2001, 2009.

2. Anonymous: Artvin Provincial Directorate of Agriculture. Annual Data, *http://www.artvintarim.gov.tr/*, 2010.

3. Konig HE, Liebich HG: Veterinary Anatomy of Domestic Mammals, Textbook and Colour Atlas. 3rd edn., 609-615, Stuttgart, Germany, Schattauer Gmbh, 2007.

4. Abbasi M, Gharzi A, Mohammadzadeh S, Karami H: Morphology and histology of the interdigital gland in an Iranian native breed of sheep. *J Anim Vet Adv*, 8, 1157-1161, 2009.

5. Bahadır A, Yakışık M: Morphology of the interdigital sinus in native hair goat. *Uludağ Univ Vet Fak Derg*, 7, 87-92, 1988.

6. Raesfeld VF: Das Rehwild. Hamburg, Germany, Paul Parey Zeitschriftenverlag, 1978.

7. Sivachelvan MN, Yahaya A, Chibuzo GA: Developmental changes in the interdigital pouch of Yankasa sheep. *Small Ruminant Res*, 9, 303-312, 1992. DOI: 10.1016/0921-4488(92)90159-2

8. Bavdek S: Interdigitalni sinus ovaca. **In,** Koza in Njeni Derivati. VTOZD, Biotehniska Fakulteta Ljubljani, Slovenian, 1981.

9. Atoji Y, Suzuki Y, Sugimura M: Lectin histochemistry of the interdigital gland in Japanese serow *(Capricornis crispus)* in winter. *J Anat*, 161, 159-170, 1988.

10. Burger BV, Nell AE, Spies HSC, Le Roux M, Bigalke RC, Brand PAJ: Mammalian exocrine secretion. XII: Constituents of interdigital secretion of bontebok, Damaliscus dorcas dorcas, and blesbok, D-d. phillipsi. *J Chem Ecol*, 25, 2057-2084, 1999. DOI: 10.1023/A:1021036823079

11. Lamps LW, Smoller BR, Rasmussen LE, Slade BE, Fritsch G, Goodwin TE: Characterization of interdigital glands in the Asian elephant *(Elephas maximus). Res Vet Sci* 71, 197-200, 2001. DOI: 10.1053/rvsc.2001.0508

12. Parillo F, Diverio S: Glycocomposition of the aprocrine interdigital gland secretions in the fallow deer *(Dama dama). Res Vet Sci*, 86, 194-199, 2009. DOI: 10.1016/j.rvsc.2008.08.004

13. Schummer A, Wilkens H: Organs of the circulation. **In,** Schummer A,

Wilkens H, Vollmerhaus B, Habermehl KH (Eds): The Skin and the Cutaneus Organs of the Domestic Mammals. 533-534, Springer-Verlag Paul Parey, Berlin Hamburg, 1981. DOI: 10.1007/978-1-4899-7102-9

14. Dursun N: Veteriner Anatomi - II. 8. Baskı, 234-264, Medisan, Ankara, 2002.

15. International Committee on Veterinary Gross Anatomical Nomenclature: General Assembly of the World Association of Veterinary Anatomists. Nomina Anatomica Veterinaria. 5th edn., Gent, 2012.

16. Demiraslan Y, Akbulut Y, Deprem T, Karadağ Sarı E, Aslan K: Morphological and morphometrical characteristics of the interdigital gland in Kıvırcık sheep. *Turk J Vet Anim Sci,* 38, 485-489, 2014. DOI: 10.3906/vet-1403-21

17. Aycan K, Bilge A: Plastik enjeksiyon ve korozyon metodu ile vaskuler sistem anatomisinin araştırılması. *Erciyes Üniv Tıp Fak Derg,* 6, 545-552, 1984.

18. Çalışlar T: Sinus interdigitalis'in (sinus biflexe) morfolojik özellikleri. *Ankara Üniv Vet Fak Derg,* 18, 37-41, 1971. DOI: 10.1501/Vetfak_0000000436

19. Sisson S, Grossman JD: The Anatomy of the Domestic Animals. 1210-1211, WB, Saunders Company, Philadelphia, London, 1975.

20. Nickel R, Schummer A, Seiferle E: Lerhrbuch der Anatomie der Haustiere. Band III, 481-482, Verlag Paul Parey, 1984.

21. Uğurlu S: Koyunlarda sinus interdigitalis'lerin ışık mikroskopik yapısı üzerine incelemeler. *İstanbul Univ Vet Fak Derg,* 17, 1-7, 1991.

22. Karahan S, Yıldız D, Bolat D: Scanning electron microskopic features of the ovine interdigital sinus. *Acta Vet Hung,* 55, 417-424, 2007. DOI: 10.1556/AVet.55.2007.4.1

23. Janicki Z, Hraste A, Slavice A, Konjevic D, Marinovic Z, Stubican D: Morphohistological characteristics of interdigital gland in the roebuck *(Capreolus capreolus* L.). *Vet Arhiv,* 73, 27-37, 2003.

24. Avdic R, Katica A, Mlaco N, Softic A, Tandir F, Cengic B, Bejdic P, Jutahija V, Hadziomerovic N: Morphological characteristics of interdigital diverticulum (sinus cutaneous interdigitalis) of Dubska Pramenka. *Biotechnol Anim Husb,* 29, 441-448, 2013. DOI: 10.2298/BAH1303441A

25. Awaad AS, Tawfiek MG, Moawad UK, Abdel Razek AH, Abedellah BA: Morphohistological and surgical anatomy of the sinus interdigitalis in Egyptian native breeds of sheep. *Beni Suef Univ J Basic Applied Sci,* 4, 157-166, 2015. DOI: 10.1016/j.bjbas.2015.05.010

26. Aslan K, Kürtül İ, Nazlı M, Ateş S: Morphologic features of the interdigital sinus of the Tuj sheep. *Kafkas Univ Vet Fak Derg,* 16, 623-626, 2010. DOI: 10.9775/kvfd.2009.1307

27. Getty R: Sisson and Grossman's the Anatomy of the Domestic Animals. 5th edn., 1211, WB Saunders Company, USA, 1975.

28. Süzer B, Özgüden Akkoç CG, Arıcan İ, Yıldız H: Morphological and immunohistochemical features of interdigital sinus in Kıvırcık sheep. *Kafkas Univ Vet Fak Derg,* 22, 69-73, 2016. DOI: 10.9775/kvfd.2015.13902

29. Pourlis AF: Functional morphological characteristics of the interdigital sinus in the sheep. *Folia Morphol,* 69, 107-111, 2010.

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Research Article

The Angiogenic Effect of Testosterone Supplementation on Brain of Aged Mice [1]

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Abstract

The aim of this study was to determine, by histological and molecular techniques, the effects of testosterone hormone treatment on angiogenesis in brain of aged mice. A total of 30 mice were used in 3 study groups: Sham operation group (Control), gonadectomy group (G) and gonadectomy and testosterone supplementation group (GTS). The capillary number and the inner diameter of larger capillary vessels in brain were measured by light microscope. The levels of VEGF (vascular endothelial growth factor) mRNA in brain tissues were determined by RT-PCR. After gonadectomy operation, capillary densities of brain decreased in male ($P<0.05$) but did not changed in female. Testosterone supplementation to gonadectomized aged mice caused a mild increase on capillary number in female brain but did not affect in male. Gonadectomy caused a decrease in VEGF mRNA levels of brain in male and female mice. Interestingly, testosterone replacement caused an important decrease ($P<0.05$) in the expression of VEGF of brain in male compared to control group whereas resulted with no change in female. The present results showed that testosterone hormone has different angiogenic effect on brain in male and female old mice. The gonadectomy operation in old male mice has a negative effect on angiogenic events. However, testosterone replacement in male was not sufficient to convert this change and did not increase angiogenesis.

Keywords: *Angiogenesis, Testosterone, Old mice, Brain*

Yaşlı Farelerin Beyninde Testosteron Takviyesinin Anjiyogenik Etkisi

Özet

Bu çalışmanın amacı yaşlı farelerin beyninde testosteron hormon tedavisinin anjiyogenik etkisini moleküler ve histolojik olarak tespit etmektir. Toplam 30 fareden 3 çalışma grubu oluşturulmuştur. Bunlar; yalancı operasyon yapılan (kontrol) grup, gonadektomi yapılan (G) grup ve hem gonadektomi hem de testosteron takviyesi yapılan (GTS) gruptur. Beyin dokusunda kapillar sayısı ve geniş kapillarların iç çapı ışık mikroskobunda ölçülmüştür. Beyin dokusundaki VEGF (vasküler endoteliyal büyüme faktörü) mRNA seviyesi RT-PCR ile ölçülmüştür. Kısırlaştırmadan sonra erkeklerde beyindeki kapillar yoğunluk azalmış ($P<0.05$) dişilerde ise değişmemiştir. Kısırlaştırılmış hayvanlarda testosteron takviyesiyle dişilerde kapillar sayısı hafif bir artış gösterirken erkeklerde bir değişim görülmemiştir. Kısırlaştırmayla beyindeki VEGF mRNA seviyesinde hem dişi hem de erkek farelerde bir azalma saptanmıştır. Testosteron takviyesiyle erkeklerin beyindeki VEGF mRNA seviyesinde kontrol grubuyla karşılaştırıldığında önemli bir azalma ($P<0.05$) görülürken dişilerde bir değişim görülememiştir. Bu çalışmayla testosteronun erkek ve dişilerin beyninde anjigenik etkisinin farklı olduğu saptanmıştır. Yaşlı erkek farelerde kısırlaştırmanın anjiyogenik olaylarda negatif etkisinin olduğu belirlenmiştir. Ayrıca erkeklerde testosteron takviyesi bu değişiklikleri düzeltmek için yeterli olmamış ve anjiyogenezisi artırmadığı saptanmıştır.

Anahtar sözcükler: *Anjiyogenezis, Testosteron, Yaşlı fare, Beyin*

INTRODUCTION

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels. Angiogenesis, a normal process in growth and development, is also effective in tumor development. In absence of angiogenesis, cardiovascular and cerebrovascular diseases can occur. Angiogenic factors, found in a lot of organs and

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tissues, are made of proteins and stimulate angiogenesis. In these factors include vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [1]. There are many factors for increase of synthesis or stimulation of angiogenic agents. It is reported that especially the estrogen into many hormones effected to the factors [2]. It is known; the estrogen increased the vascularization in some organs (uterus, heart, brain etc.) by expression and releasing of angiogenic factors in female [3-5]. There are few and limited studies about the effect of testosterone on angiogenesis [6,7]. In addition to the factors mentioned above angiogenesis are also affected by the age. It is now known that the interruption and changes in angiogenesis by aging play as a major factor [8,9]. There is no literature about how aging and testosterone hormone affect the angiogenesis in mammalian brain. The aim of this study was to determine by histological and molecular techniques the effects of testosterone hormone treatment on angiogenesis in brain of aged mice.

MATERIAL and METHODS

A total of 15 female and 15 male Swiss albino mice are enrolled. The animals have been obtained from the Animal Experimental Unite of Veterinary Medicine Faculty in Adnan Menderes University, Turkey. Ethic committee approval was taken from Adnan Menderes University (with no: B.30. 2.ADÜ.0.00.00.00/050.04/2011/098). Mice were grown up 405 days (12 months + 10 days + 30 days) in cages under normal conditions (20-24°C and 50-60% humidity) and were fed ad libitum with available commercial pelleted feeds. Each 3 group consisted of 10 mice. Out of 10 mice of each group, 5 were male and 5 were female. Among the experimental groups, the first group of animals (control) exposed to the same stress sham operations at 12 months (only skin incision and closure) was performed. The second experimental group (G) had gonadectomy at the age of 12 months but did not have testosterone supplementation. The third experimental group had both gonadectomy at the age of 12 months and testosterone supplementation. Third group of animals (GTS) had testosterone supplementation for one month beginning after ten days of post-operative recovery period. For the animals in GTS group, 0.01 cc testosterone (250 mg/mL Sustanon 250®, Organon) was administered by subcutaneous injection as a single dose. Mice were anesthetized by intraperitoneal administration of (ip) Ketamine (90 mg/kg)/Xylazine (10 mg/kg). All animals were sacrificed and their blood and brain tissue were taken at the age of 405 days. Testosterone levels in bloods were measured using ELISA Kit (for Mouse Testosterone (T), USCN Life Science Inc.® Wuhan) in accordance with the manufacturer›s instructions. Blood was taken by exsanguinations from all animals after hormone supplementation period. Brains were dissected from the mice and cut in the middle of transversal plane. Half of the organs were collected for histological examination and the other half was devoted to molecular investigation. For histological examination, the tissue samples were kept in 10% buffered formalin buffer and were embedded in paraffin after appropriate tissue tracking. For each tissue from paraffin blocks of 5 µm three serial sections were taken with an interval of 30 µm. After applying the process deparaffinization, sections performed triple (Mason trichrome method) staining [10]. The number of capillaries and inner diameter of larger capillary vessels in brain tissues was measured. The slices were analyzed and photographed under a light microscope (Leica DMLB) that is equipped with a calibrated digital camera (Leica DC200 CD camera and Q-win Standard imaging analyses programme). Histomorphometric analyses were performed at a magnification of x 40-100. Both capillary number and inner diameter were measured and averaged results of 15 microscopic sites.

For molecular investigation, total RNA extraction from tissue samples (brain and hearth) was done using geneJET RNA Purification kit (Fermentas) according to the manufacturer's instructions. Reverse transcription using 2 µg of total RNA, was done with revertAid First Strand cDNA Synthesis kit (Fermentas) containing M-MuLV reverse transcriptase enzyme following manufacturer's instructions. The resulting cDNA was used for real time PCR amplification. For RNA extraction and PCR procedures of standardization and control the housekeeping transcript (GAPDH) was used. Genes were amplified using QuantiTect SYBR PCR Kit (ABM) as defined by Shidaifat et al.[6].

Statistical analysis was used in the SPSS 19.00 software package. Distributions of data were analyzed using Shapiro-Wilk test. Nonparametric distribution the data was checked using the Kruskal-Wallis test. Bonferroni- corrected Mann-Whitney U test was applied as post-hoc test. The presence of a correlation between the angiogenic events in brain tissue and the levels of testosterone was also tested by Spearsman's test. A *P*-value less than 0.05 were considered significant.

RESULTS

Testosterone Level

Blood testosterone levels were shown in *Table 1*. The highest level of testosterone was detected in testosterone supplemented female mice, followed by control male group. Among male mice after castration, testosterone levels were decreased 18.8 fold ($P<0.01$) and testosterone replacement increased hormone levels 13.1 fold among castrated mice ($P<0.01$). In female mice testosterone level was found to be decreased 2.8 fold in ovariectomized female. After hormone supplementation testosterone level increased 77.2 fold compared to ovariectomized female ($P<0.001$). In female mice after testosterone replacement the testosterone levels were increased in GTS group, in contrast to control ($P<0.05$) and G group ($P<0.001$) *(Table 1)*.

Capillary Number

The number of capillaries in brain was the highest in control group male animals. The least number of capillary was found in gonadectomized males group followed by GTS male group mice. After castration only 1.20 fold decrease was observed in the capillary densities of brain in both G ($P<0.05$) and GTS ($P<0.05$) group compared to control group. Hormone supplementation did not affect the capillary numbers in brain of castrated animals *(Table 2, Fig. 1)*.

In female brain, the maximum capillaries were found in GTS group animals, and least in control group animals. In female brain, ovariectomy did not affect capillary density but operation and hormone supplementation together caused 1.14 fold increases on capillary number in brain tissue *(Table 2, Fig. 1)*.

Vessel Diameter

Inner diameters of the larger capillary vessel of G groups were decreased in male and increased in female brain 1.26 and 1.03 folds, respectively, compared to control group. Testosterone supplementation did not affect brain vessel diameters in male and female mice *(Table 2)*.

Data of RT PCR

It was determined that the level of VEGF mRNA (ΔCT) in male brain was highest in the control group and lowest in GTS group animals. Amount of VEGF mRNA in brain tissue were 2.10 fold low in castrated animals compared to control group. In testosterone supplementation group expression level were decreased 8.62 fold and 4.75 fold compared to control ($P<0.05$) and G group, respectively. In female brain, VEGF mRNA levels decreased compared to control group 1.42 and 1.48 folds for G and GTS groups, respectively *(Table 2)*.

The presence of a correlation between the angiogenic events in brain tissue and the levels of testosterone was also tested by Spearsman's test. While there was a positive correlation ($r = 0.528$, $P<0.05$) between the capillary number in brain tissue and the testosterone levels of females, a significant negative correlation ($r = -0.536$, $P<0.05$) was found between inner diameter of large capillary in brain and the testosterone levels of females. This result showed that increased testosterone level in female blood caused elevated capillary number in brain tissue whereas a decrease the inner diameter of large capillary in female brain tissue.

Table 1. *Testosterone levels in male and female groups of mice. Control; sham operated group, G; gonadectomized group, GTS; gonadectomized and testosterone supplemented group*

Animal Groups (N)	Testosterone Level nmol/L (Mean ± SD)	
	Male	Female
Control (10)	1.517±1.173 [a]	0.140±0.101 [c]
G (10)	0.089±0.075 [a, b]	0.051±0.064 [d]
GTS (10)	1.051±0.478 [b]	3.862±2.838 [c, d]

[a, b] In male mice after castration testosterone levels were decreased ($P<0.01$) in G group of mice. Testosterone replacement increased hormone levels in GTS group of male mice ($P<0.01$), [c,d] In female mice after testosterone replacement the testosterone levels were increased in GTS group, to compared to control ($P<0.05$) and G group ($P<0.001$)

DISCUSSION

Few studies have been made regarding the effects of androgens on angiogenesis [6,7]. It was determined that androgens stimulate erythropoietin production via VEGF in cell culture and endothelial stem cells are regulated hormonally. In the reduced level of these hormones due to castration angiogenesis is also downward as it was shown previously [7]. The changes due to castration includes the decrease in endothelial function of vessel *(Arteria femoralis)* and deterioration in calcium channel activity as a result of decrease in testosterone level and the absence of androgen receptor in testicular feminized mice [11]. Castration in young male mice caused decrease in androgen receptors in the brain up to ten times [12]. Also using ischemic injury in male the effect of endogenous androgens in maintaining neovascularization was shown [7]. The results that castration decreased angiogenesis in male animals by lack of testosterone were led to investigations

Table 2. *The number of capillaries, inner diameters of larger capillary and VEGF mRNA (ΔCT) values of brain tissues in male and female groups of mice. Control; Sham operated group, G; Gonadectomized group, GTS; Gonadectomized and testosterone supplemented group.*

Gender	Animal Groups (N)	Capillary Number (Mean ± SD)	Inner Diameter of Larger Capillary (μm) (Mean ± SD)	VEGF mRNA (ΔCT) (Mean ± SD)
Male	Control (5)	3.865±0.226 [a, b]	8.527±1.508	7.375±0.564 [c]
	G (5)	3.226±0.193 [a]	6.778±0.448	3.510±2.268
	GTS (5)	3.222±0.234 [b]	6.686±0.774	-0.241±2.424 [c]
Female	Control (5)	3.316±0.400	7.160±0.410	6.205±0.624
	G (5)	3.384±0.237	7.418±0.758	4.372±2.500
	GTS (5)	3.770±0.167	6.916±0.675	4.186±1.940

[a, b] The number of capillary in male brain was decreased in both G ($P<0.05$) and GTS ($P<0.05$) group compared to control group; [c] The expression level of VEGF mRNA was decreased in the testosterone supplementation group ($P<0.05$) compared to control group

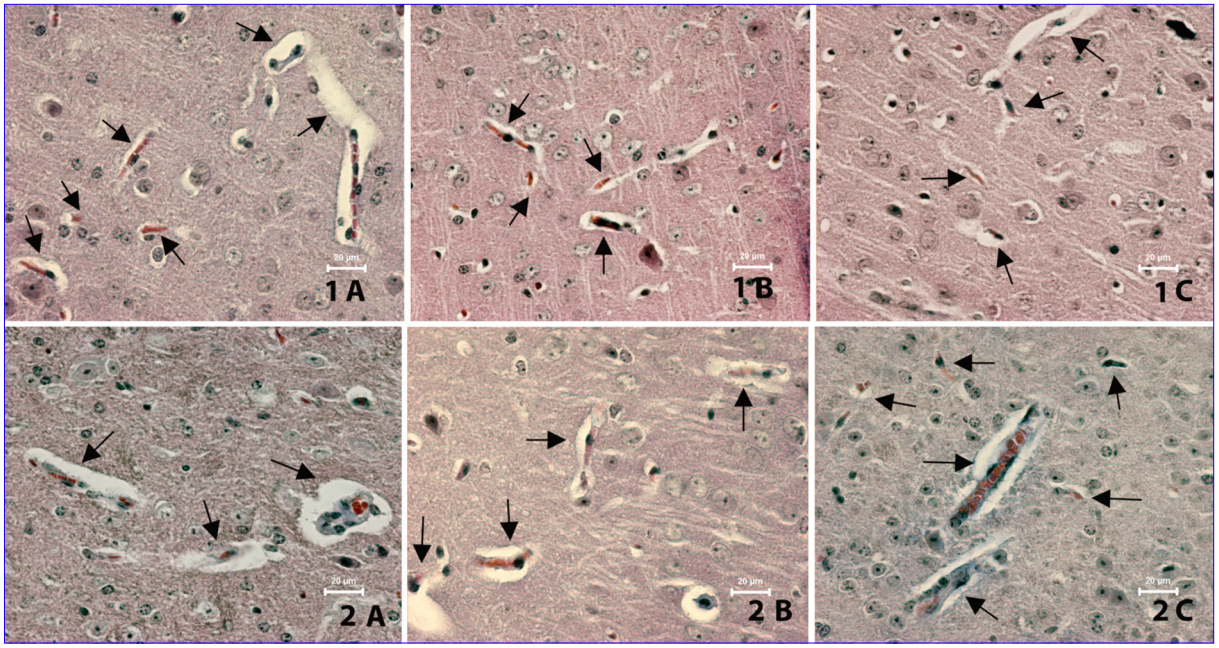

Fig 1. Capillary density in brain tissues from male (1A, 1B, 1C) and female (2A, 2B, 2C) aged mice. Sections performed triple (Mason trichrome method) staining (Crossman, 1937). A; sham operated group (Control), B; gonadectomized group (G), C; gonadectomized and testosterone supplemented group (GTS). Arrows show capillary. After gonadectomy operation, capillary densities of brain decreased in male ($P<0.05$) but did not changed in female. Testosterone supplementation to gonadectomized aged mice caused a slightly increase on capillary number in female brain but did not affect in male

on the effects of cancer tissue. Most of the studies have been made in prostate cancer. According to Hammarsten et al.[13], castration caused decreased vascular density in the normal tissue surrounding the tumor and consequently increased tumor hypoxia and apoptosis, and moderately decreased tumor growth in prostate. Also an *in vitro* study showed that testosterone affected development and function of early endothelial progenitor cells but there was no affect on late endothelial progenitor cells [14]. However in the present study, there was a reduction in capillary density and vessel diameter in brain tissue after castration. Hormone supplementation to castrated animals caused no effect on capillary number and vessel diameter in brain. Also in female brain tissue, with ovariectomy there was no change in capillary density and vessel diameters. With testosterone supplementation to ovariectomized animals an increase on capillary number in brain was observed. The disperancies among previous studies and our study results may be due to the age of the animals. That is why the aged animals were studied in present study to see the effects of the hormone replacement in elderly mice.

Previous studies showed that androgens stimulate erythropoietin production via VEGF in cell culture and endothelial stem cells. In the absence of these hormones by means of castration, angiogenesis is downward [7]. According to Sordello and colleagues testosterone caused an increase in transcription and biological activity of VEGF in human prostate cell culture [15]. *In vivo,* a transient increase in the weight of ventral lobes of the prostate gland and 7-fold increase in specific activity of VEGF belong to prostate in testosterone injected rats were seen. After castration a decrease is observed in both translation and transcription levels of VEGF of the prostate gland of the dog [6]. Also in cancer cells culture, an increase of transcription of VEGF is found after estrogens and androgens treatment [16]. However, Sieveking et al.[14] observed difference in the effect of testosterone hormone in male and female, an increase was observed in males in the angiogenesis depend on VEGF but no effect in females. In the present study, amount of VEGF mRNA in brain of both sex decreased due to gonadectomy. However, in contrary of the previous findings our results showed that testosterone supplementation caused an important ($P<0.05$) drawbacks in these values in male mice groups. However, no change was found in female groups.

Androgens have different effects of angiogenesis by gender. *In vitro* studies showed that the androgens stimulate angiogenic phenomena in male, but not in females. In addition, *in vivo* studies shown that the endogenous androgens regulated angiogenesis in males, but not in females [7]. Jesmin et al.[4], applied ovariectomie operation after 44 weeks to female rats and as a result observed that the vessel density, VEGF and receptors of VEGF levels decreased in brain (frontal cortex). Estrogen treatments caused a complete remission in these changes [4]. In the present study, the number of capillary vessels and diameter of vessels in the brain were decreased in gonadectomized male animals, but there was no change in females. In the brain, VEGF mRNA after gonadectomy is

reduced even more by hormone replacement in males, but there was no change in females.

In the old male mice, androgen deficiency caused a decrease in capillary number of brain tissue. Testosterone replacement possessed no increasing effect on angiogenesis in the male brain. In addition, the latter caused a decrease in the level of VEGF mRNA in male brain after gonadectomy and decrease was even more after hormone replacement. There was no change in the capillary density and vessel diameter in female brain.

Our results showed that the decreased testosterone levels in old mice had an important negative effect on VEGF mRNA levels. However, testosterone replacement in male was not sufficient to convert this change and did not increase angiogenesis. Interestingly, testosterone replacement caused an important decrease in the expression of VEGF in male brain although previous studies reported increased VEGF expression by testosterone supplementation. Further studies are necessary to show if this is a result of a feedback mechanism.

REFERENCES

1. Otrock ZK, Mahfouz RAR, Makarem JA, Shamseddine AI: Understanding the biology of angiogenesis: Review of the most important molecular mechanisms. *Blood Cells Mol Dis,* 39, 212-220, 2007. DOI: 10.1016/j.bcmd.2007.04.001

2. Castillo C, Cruzado M, Ariznavarreta C, Lahera V, Cachofeiro V, Loyzaga PG, Tresguerres JAF: Effects of ovariectomy and growth hormone administration on body composition and vascular function and structure in old female rats. *Biogerontology*, 6, 49-60, 2005. DOI: 10.1007/s10522-004-7383-x

3. Albrecht ED, Babischkin JS, Lidor Y, Anderson LD, Udoff LC, Pepe GJ: Effect of estrogen on angiogenesis in co-cultures of human endometrial cells and microvascular endothelial cells. *Hum Reprod,* 18, 2039-2047, 2003. DOI: 10.1093/humrep/deg415

4. Jesmin S, Hattori Y, Sakuma I, Liu MY, Mowa CN, Kitabatake A: Estrogen deprivation and replacement modulate cerebral capillary density with vascular expression of angiogenic molecules in middle-aged female rats. *J Cereb Blood Flow Metab,* 23, 181-189, 2003. DOI: 10.1097/01.WCB.0000043341.09081.37

5. Soares R, Balogh G, Guo S, Rtner FG, Russo J, Schmitt F: Evidence for the notch signaling pathway on the role of estrogen in angiogenesis. *Mol Endocrinol,* 18, 2333-2343, 2004. DOI: 10.1210/me.2003-0362

6. Shidaifat F, Gharaibeh M, Bani-Ismail Z: Effect of castration on extracellular matrix remodeling and angiogenesis of the prostate gland. *Endocr J,* 54, 521-529, 2007. DOI: 10.1507/endocrj.K07-009

7. Sieveking DP, Lim P, Chow RWY, Dunn LL, Bao S, McGrath KCY, Heather AK, Handelsman DJ, Celermajer DS, Ng MKC: A sex-specific role for androgens in angiogenesis. *J Exp Med,* 207, 345-352, 2010. DOI: 10.1084/jem.20091924

8. Reed MJ, Edelberg J M: Impaired angiogenesis in the aged. *Sci Aging Knowl Environ,* 2004 (7): pe7, 2004. DOI: 10.1126/sageke.2004.7.pe7

9. Reed MJ, Bradshaw AD, Shaw M, Sadoun E, Han N, Ferara N, Funk S, Puolakkainen P, Sage EH: Enhanced angiogenesis characteristic of sparc-null mice disappears with age. *J Cell Physiol,* 204, 800-807, 2005. DOI: 10.1002/jcp.20348

10. Crossman O: A modification of Mallory's connective tissue stain with a discussion of the principles involved. *Anat Rec*, 69, 31-38, 1937. DOI: 10.1002/ar.1090690105

11. Jones RD, Pugh PJ, Hall J, Channer KS, Jones TH: Altered circulating hormone levels, endothelial function and vascular reactivity in the testicular feminised mouse. *Eur J Endocrinol,* 148, 111-120, 2003. DOI: 10.1530/eje.0.1480111

12. Silva MA, Dorantes MR, Baig S, Montor JM: Effects of castration and hormone replacement on male sexual behavior and pattern of expression in the brain of sex-steroid receptors in BALB/c AnN mice. *Comp Biochem Physiol,* 147, 607-615, 2007. DOI: 10.1016/j.cbpa.2006.11.013

13. Hammarsten P, Halin S, Wikstöm P, Henriksson R, Rudolfsson SH, Bergh A: Inhibitory effects of castration in an orthotopic model of androgen-independent prostate cancer can be mimicked and enhanced by angiogenesis inhibition. *Clin Cancer Res*, 12, 7431-7436, 2006. DOI: 10.1158/1078-0432.CCR-06-1895

14. Fadini GP, Albiero M, Cignarella A, Bolego C, Pinna C, Boscaro E, Pagnin E, De Toni R, de Kreutzenberg S, Agostini C, Avogaro A: Effects of androgens on endothelial progenitor cells *in vitro* and *in vivo*. *Clin Sci,* 117, 355-364, 2009. DOI: 10.1042/CS20090077

15. Sordello S, Bertrand N, Plouet J: Vascular endothelial growth factor ıs up-regulated *in vitro* and *in vivo* by androgens. *Biochem Biophys Res Commun,* 251, 287-290, 1998. DOI: 10.1006/bbrc.1998.9328

16. Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Härkönen PL: Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. *Mol Cell Endocrinol,* 149, 29-40, 1999. DOI: 10.1016/S0303-7207(99)00003-9

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Research Article

Genetic Polymorphisms of Myogenin Gene and Their Associations with Growth Traits in the Chinese Tibetan Sheep

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Abstract

Myogenin gene encodes for skeletal muscle-specific transcription factors with highly conserved basic helix-loop-helix domain and play key role in growth and muscle development. In this study, the polymorphisms of myogenin gene were investigated to check whether they are associated with growth traits in Chinese-Tibetan sheep. In three sheep breeds with a total number of 632 individuals, three SNPs, including two in exon 1 (C109A and C183T) and one in intron 2 (A1403C) were found by DNA sequencing. Among those, C109A is a missense mutation (Gly37Arg). The single marker association analysis showed that the three mutations were significantly associated with growth traits (at P<0.01 or P<0.05). In conclusion, our results suggest that myogenin gene variation may be considered molecular markers for growth traits in Chinese-Tibetansheep.

Keywords: *Chinese Tibetan sheep, Myogenin gene, SNP, Growth traits*

Çin Tibet Koyununda Myogenin Geninin Genetik Polimorfizmi ve Büyüme Özellikleriyle Olan İlişkisi

Özet

Myogenin geni oldukça korunaklı olan temel heliks-ilmek-heliks domain yapısında olup iskelet kas-spesifik transkripsiyon faktörlerini kodlar ve büyüme ile kas gelişimde önemli rol oynar. Bu çalışmada, myogenin geninin polimorfizmi ve Çin-Tibet koyununda büyüme özellikleri ile ilgisinin olup olmadığı araştırıldı. Üç koyun ırkından oluşan toplam 632 hayvanda, DNA sekansı ile ikisi ekzon 1 (C109A ve C183T) ve biri intron 2 (A1403C)'de olmak üzere üç SNP bulundu. Bunlar arasında C109A missen mutasyonuydu (Gly37Arg). Tek markır asosiasyon analizi, üç mutasyonun anlamlı derecede büyüme özellikleri ile ilişkili olduğunu gösterdi (P<0.01 veya P<0.05). Sonuç olarak, elde edilen sonuçlar ışığında myogenin gen varyasyonunun Çin-Tibet koyununda büyüme özellikleri için moleküler belirteç olabileceği saptandı.

Anahtar sözcükler: *Çin-Tiber koyunu, Myogenin geni, SNP, Büyüme özellikleri*

INTRODUCTION

Myogenic regulatory factors *(MRFs)* belong to the family of conserved basic helix-loop-helix *(bHLH)* transcription factors [1], including *Mfy5, Mfy6,MyoD* and myogenin [2,3]. They are well known to control the determination of the myogenesis, from commitment and proliferation, through muscle fiber formation, to postnatal maturation and muscle function [3].

Myogenin gene plays a role during the terminal transformation of myoblasts into myofibres. Specifically, myogenin gene expression abrogates myoblast proliferation potential and regulates the differentiation of mono-nucleated myoblasts into multinucleated myofibers [4]. The expression of myogenin gene was continuous in all myogenic cell lines and associated with the number of muscle fibres during myogenesis [5]. Mice that lack myogenin shows no muscle fiber development, leading to striking phenotypes [6]. Additionally, the myogenin gene regulates the expression of muscle-specific genes, which encode several proteins that control the formation and apoptosis (or necrosis) of muscle fibers [7]. Taken together, these findings lend credence to the hypothesis that myogenin gene is considered excellent candidate gene for growth-related traits in livestock due to its potential roles in the development of skeletal muscles.

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At present, studies on mutation of myogenin gene associated with growth traits were mainly focused on swine, chicken, and cattle. Few studies were carried out on sheep. Therefore, the objective of this study was to detect ovine myogenin gene and to explore their possible association with growth traits in native Chinese breeds.

MATERIAL and METHODS

Experimental Population

A total of 632 ewes were collected from Chinese Tibetan sheep including Black Tibetan sheep (BT, N = 226), Gaoyuan Tibetan sheep (GT, N = 191), and Oula Tibetan sheep (OT, N = 215). All the sheep were in the artificial insemination system that were raised in provinces of Qinghai, Gansu, and Henan, respectively. Their growth traits (body weight, height, length and chest circumference were recorded at 3 years of age.

Genomic DNA Isolation and Genotyping

Genomic DNA was extracted from sheep blood (jugular vein samples) by the standard phenol-chloroform extraction procedure. DNA quantity and purity (A_{260}/A_{280} ratio) for each sample was assessed using a Nano-Drop™1000 Spectrometer (Thermo Scientific, Waltam, MA, USA), then stored at -20°C.

Primers to amplify of the ovine Myogenin gene were designed based on sequences in the NCBI database sequences (GenBank accession No. NC_019469.1) using Primer v5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). The information of the primers of Myogenin gene was shown in *Table 1*. A PCR was conducted in 20-μL reactions containing 50 ng DNA, 10 pM each primer, 0.20 mM dNTPs, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa, Shiga, Japan). The following PCR reaction conditions were used: 5 min at 95°C; 35 cycles of 30 s at 94°C, annealing for 35 s at optimum temperature, 40 s at 72°C, and final extension at 72°C for 10 min. Digested products were detected by electrophoresis on 1.0% agarose gels.

Thirty random DNA samples were mixed to from a single DNA pools, from which mutation of Myogenin gene were detected. Then, the PCR products were amplified from the 632 Chinese Tibetan sheep directly sequenced in both directions (Sangon, Shanghai, China).

Statistical Analysis

Genotype and allele frequencies, gene heterozygosity (*He*), effective allele numbers (*Ne*), polymorphism information content (*PIC*), and tests for deviation from Hardy-Weinberg equilibrium (*HWE*) were calculated by POPGENE v. 1.32 [8].

The association analysis between single marker and growth traits were analyzed by general linear model (GLM) procedure of SPSS 21 (IBM, Armonk, NY, USA).The following statistical linear model is used: $Y_{ij} = u + G_i + S_i + E_{ij}$, where Y_{ij} is the traits measured on each of the individual cattle, u is the overall population mean for the traits, $G_i$is the fixed effect associated with the genotype, S_i is the fixed effect with season, and E_{ij} was the standard error.

RESULTS

Identification of SNPs

Amplification and sequencing of Myogenin gene among three different sheep breeds revealed three variations, named C109A, C183T, and A1403C, respectively *(Fig. 1)*. Of these, C109A and C183T were identified in exon 1 and A1403C was found in intron 2. Sequence analysis showed that C109A was a missense mutation (Gly37Arg),

Table 1. *Genotype frequencies (%) of the myogenin for the SNPs in Chinese Tibetan sheep*

Site	Breed	Genotypic Frequency			Allele Frequency		χ^2 (HWE*)	H_E	N_E	PIC
g.109C>A		CC	CA	AA	C	A				
	BT	0.7611	0.1416	0.0973	0.8319	0.1681	55.1166	0.2406	0.2797	1.3884
	GT	0.5759	0.3194	0.1047	0.7356	0.2644	6.1169	0.3133	0.3890	1.6366
	OT	0.6279	0.2977	0.0744	0.7767	0.2233	4.3180	0.2867	0.3468	1.5310
g.183C>T		CC	CT	TT	C	T				
	BT	0.2080	0.3230	0.4690	0.3695	0.6305	21.2634	0.3574	0.4659	1.8724
	GT	0.2723	0.3037	0.4241	0.4241	0.5759	27.3398	0.3692	0.4885	1.9549
	OT	0.1953	0.2651	0.5395	0.3279	0.6721	34.1447	0.3436	0.4408	1.7882
g.1403A>C		AA	AC	CC	A	C				
	BT	0.0605	0.2186	0.7209	0.1698	0.8302	10.8372	0.2422	0.2819	1.3925
	GT	0.0628	0.3560	0.5812	0.2408	0.7592	0.1330	0.2988	0.3657	1.5765
	OT	0.0605	0.2186	0.7209	0.1698	0.8302	10.8372	0.2422	0.2819	1.3925

HWE, *Hardy-Weinberg equilibrium;* $\chi_{0.05}^2 = 5.991$, $\chi_{0.01}^2 = 9.210$

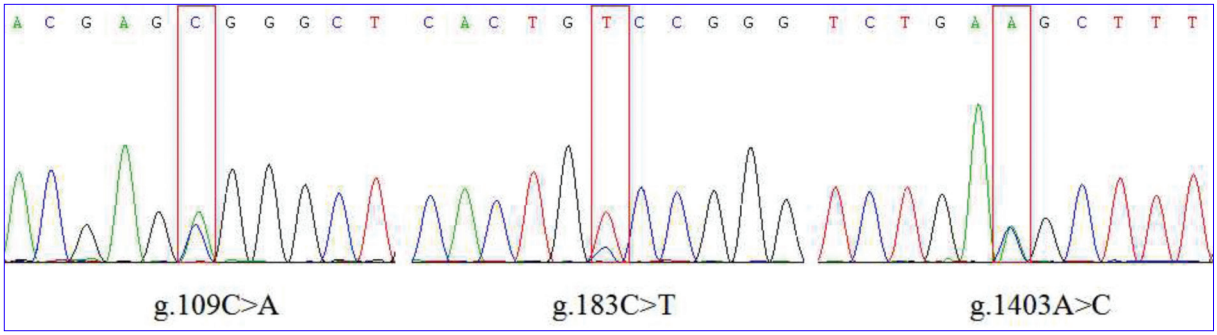

Fig 1. Sequencing map of the three identified SNPs in myogenin gene

Table 2. *Association of different genotypes of SNPs in myogenin with growth traits in BT sheep*

Site	Genotypes	Body Weight (cm)	Body Height (cm)	Body Length (cm)	Chest Circumference (cm)
g.109C>A	CC	39.81±3.05^{b}	59.87±5.38^{Bb}	63.68±5.36^{B}	87.84±8.14
	CA	41.78±4.52	63.07±6.28^{a}	68.15±3.25^{A}	87.38±8.61
	AA	42.99±3.77^{a}	65.91±5.42^{A}	68.77±3.70^{A}	89.42±7.33
g.183C>T	CC	38.88±3.69^{Bb}	62.77±5.69^{b}	65.45±3.68b	85.45±7.21^{B}
	CT	41.56±3.44a	63.19±6.39^{b}	68.33±3.72	87.08±7.55
	TT	43.52±4.85^{A}	66.22±6.17^{a}	68.62±3.58^{a}	90.36±8.19^{A}
g.1403A>C	AA	41.73±4.63	63.84±5.00	68.26±5.77	87.90±7.02
	AC	41.86±3.69	63.59±4.66	68.13±6.21	87.92±8.19
	CC	43.00±3.26	65.75±4.17	68.42±6.34	89.52±8.32

Means with different superscripted lower and upper case letters are significantly different at P<0.05 and P<0.01, respectively

while C183T resulted in a synonymous mutation (Cys61Cys). The detailed information about all SNPs are presented in *Table 1*.

Genotyping and Allele Frequencies

Genotyping was performed by DNA sequencing method. Allele frequencies of the SNP were investigated and performed by the χ^2 test in three different sheep breeds *(Table 1)*. The data shown here demonstrates that C (C109A), T (C183T) andC (A1403C) were the most prevalent alleles. By chi-square test, C109A in OT sheep and A1403C in GT sheep were in HWE ($\chi^2<\chi_{0.05}^2$). In this study, PIC values ranged from 0.2406 to 0.3692, according to the conventions for PIC classification (PIC <0.2500 is considered low polymorphism, 0.2500-0.5000 is intermediate polymorphism, and >0.5000 is high polymorphism), our data showed that C109A in BT sheep and A1403C in OT sheep has low genetic diversity, while others possessed an intermediate genetic diversity.

Effect of The Polymorphism Locus on Growth Traits in BT Sheep

Table 2 showed the effects of the SNPs on growth traits in GT sheep. At theC109A locus, individuals with genotype AA had higher values than those with CC for body height and body length (P<0.01). Additionally, the body weight of individuals with genotype AA was higher than those with genotype CC (P<0.05). At the C183T locus, individuals with genotype TT had higher values than those with CC on body weight and chest circumference (P<0.01), while genotype TT had higher mean values for body length and body height than those with genotype CC (P<0.05). As with similar the BT sheep, there were no significant correlation between A1403C and growth traits.

Effect of The Polymorphism Locus on Growth Traits in GT Sheep

Association results of single markers with four growth traits in the GT sheep population are shown in *Table 3*. At the C109A locus, individuals with genotype CC had higher values than those with AA for body weight (P<0.05). At the C183T locus, significant differences in body weight and height were observed between the CC and TT genotypes (P<0.05). Compared with TT, individuals with the CC genotype showed better performance for body length and chest circumference (P<0.01). At the A1403C locus, individuals with genotype AA had higher values than those with CC for body weight (P<0.05). In addition, the body length of individuals with genotype AA was higher than those with genotype CC (P<0.01).

Table 3. *Association of different genotypes of SNPs in myogenin with growth traits in GT sheep*

Site	Genotypes	Body Weight (cm)	Body Height (cm)	Body Length (cm)	Chest Circumference (cm)
g.109C>A	CC	47.79±3.36^{a}	66.54±5.89	70.35±5.02	92.41±7.95
	CA	46.05±3.22	65.38±5.64	69.33±5.11	91.33±7.77
	AA	43.49±4.49^{b}	66.37±6.54	68.92±3.90	91.22±8.21
g.183C>T	CC	47.98±3.69^{a}	69.94±7.25^{a}	74.41±4.29^{Aa}	95.43±8.33A^{a}
	CT	44.93±4.20^{b}	65.88±7.02	68.21±3.82^{b}	91.75±8.02b
	TT	43.15±4.65^{b}	63.88±6.32^{b}	66.82±3.24^{B}	88.75±6.58^{B}
g.1403A>C	AA	48.55±4.42^{a}	67.11±4.39	73.32±6.26^{A}	94.54±8.41
	AC	47.32±3.36^{a}	66.71±4.92	70.01±6.34	91.88±8.81
	CC	43.20±5.36^{b}	65.68±5.39	68.44±6.10^{B}	90.91±7.27

Means with different superscripted lower and upper case letters are significantly different at P<0.05 and P<0.01, respectively

Table 4. *Association of different genotypes of SNPs in myogenin with growth traits in OT sheep*

Site	Genotypes	Body Weight (cm)	Body Height (cm)	Body Length (cm)	Chest Circumference (cm)
g.109C>A	CC	59.01±3.69^{A}	74.21±7.32^{A}	76.01±4.24^{a}	93.83±8.25
	CA	56.96±4.35	72.50±6.21	73.82±3.59	90.72±8.46
	AA	53.61±5.11^{B}	67.10±6.03^{B}	72.29±4.22^{b}	90.37±7.62
g.183C>T	CC	59.42±4.28^{a}	73.64±7.21	75.88±3.52	93.83±7.56^{a}
	CT	56.36±4.19	72.95±6.50	74.00±3.89	91.40±7.31
	TT	55.17±4.89^{b}	71.28±6.24	73.98±5.21	89.94±8.26^{b}
g.1403A>C	AA	61.74±5.11^{a}	75.32±5.69^{a}	76.32±8.36	94.36±8.96
	AC	57.36±5.37^{b}	73.95±6.28	75.57±7.25	92.89±9.22
	CC	57.89±3.56^{b}	72.73±5.87^{b}	74.81±7.11	92.42±7.85

Means with different superscripted lower and upper case letters are significantly different at P<0.05 and P<0.01, respectively

Effect of The Polymorphism Locus on Growth Traits in OT Sheep

As shown in *Table 4*, the association analysis between each marker and the growth traits in OT sheep. At the C109A locus, individuals with genotype CC had increased body weight and height compared with the AA genotype (P<0.01). At the C183T locus, individuals with genotype CC had higher values than those with TT on body weight and chest circumference (P<0.05). At the A1403C locus, individuals with genotype AA had higher values than those with CC on body weight and body height (P<0.05).

DISCUSSION

Chinese Tibetan sheep were the first artificially bred sheep in the natural ecosystem of the Qinghai-Tibetan plateau and has adapted well to these conditions [9]. Undoubtedly, they are an important species of grazing livestock with great economic value and highly tolerant to unfavorable weather conditions, such as extreme cold, lower atmospheric oxygen and air pressure [10]. However, slower growth rate has hampered the commercialization of Tibetan sheep production. Therefore, this study aimed to identify potential polymorphisms of the ovine myogenin genes and to explore their relationships with growth traits in Chinese-Tibetan sheep.

In the present study, we detected the two SNPs (C109A and C183T) in exon and other one SNP (A1403C) mapping to intron to reveal their associations with growth traits in Chinese -Tibetan sheep. Specifically, At the C109A locus, individuals with genotype CC has significantly greater body weight in all breeds compared with genotype AA, meaning that C allele might be associated with an increase in body weight. At the C183T locus, individuals with genotype CC had significantlygreater body weight and chest circumference in all breeds compared with genotype TT, C allele appeared to be the beneficial genotype for body weight and chest circumference. At the A1403C locus, individuals with genotype AA had significantly greater body weight in all breeds compared with genotype CC, A allele appeared to be the beneficial genotype for body weight.

Recent evidence suggests that myogenin gene plays an essential role in skeletal muscle development and adult homeostasis [11]. Different myogenin gene function or timing of expression could have a major influence

on the number of muscle fibers that develop during myogenesis [12]. Growing observations indicate that genetic polymorphisms in myogenin gene are associated with growth traits in livestock. Xue et al.[13] identified a novel polymorphism (T314C) in the myogenin gene that was associated with growth traits in native Chinese breeds. Anton et al.[14] showed that one SNP in myogenin gene had a strong effect on growth rate in Hungarian large pig. One SNP (T36C in exon 3) detected in myogenin gene was associated with an alteration in body weight in Jinghai yellow chicken [15]. Combined with the results of our study, it was suggested that myogenin gene may mediate, directly or indirectly, growth traits in animals.

We noted that C183T was the synonymous mutation, and did not change the structure of the encoded proteins, but our results demonstrated that it was still associated with some of the growth traits. Growing evidence suggests that synonymous mutation could affect both the splice donor site or nearby regions and regulatory motifs [16-19]. In this study, we deduce that such associations may be the result of linkage disequilibrium between this SNP and other genes on the same chromosome that has a significant effect on the growth traits studied here. Importantly, further verifications are needed to understand the underlying mechanisms.

In summary, three mutations were identified in the ovine myogenin gene in this study. Substantial differences in allele frequencies were observed among three different breeds. The results presented here show significant associations between the myogenin gene SNPs with growth and in Chinese Tibetan sheep. The obtained results of this study will contribute to the understanding of the regulatory mechanisms of the myogenin gene, and may be used in molecular marker-assisted selection for excellent growth traits.

Conflicts of Interest

The authors declare no conflict of interest.

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REFERENCES

1. Massari ME, Murre C: Helix-loop-helix proteins: Regulators of transcription in eukaryoticorganisms. *Mol Cell Biol*, 20, 429-440, 2000. DOI: 10.1128/MCB.20.2.429-440.2000

2. Yan C, Xia X, He J, Ren Z, Xu D, Xiong YZ, Zuo B: MyoD is a novel activator of porcine FIT1 gene by Interacting with the canonical E-box element during myogenesis. *Int J Mol Sci*, 16, 25014-25030, 2015. DOI: 10.3390/ijms161025014

3. Chen SS, Hung HT, Chen TJ, Hung HS, Wang DC: Di-(2-ethylhexyl)-phthalate reduces MyoD and myogenin expression and inhibits myogenic differentiation in C2C12 cells. *J Toxicol Sci*, 38, 783-791, 2013. DOI: 10.2131/jts.38.783

4. Pas MF, Visscher AH: Genetic regulation of meat production by embryonic muscle formation - A review. *J Anim Breed Genet*, 111, 404-412,1994. DOI: 10.1111/j.1439-0388.1994.tb00477.x

5. Te Pas MF, Soumillion A, Harders FL, Verburg FJ, Van Den Bosch TJ , Galesloot P, Meuwissen TH: Influences of myogenin genotypes on birth weight, growth rate, carcass weight, back fat thickness, and lean weight of pigs. *J Anim Sci*, 77, 2352-2356,1999.

6. Shen H, Lv Y, Shen XQ, Xu JH, Lu H, Fu LC, Duan T: Implantation of muscle satellite cells overexpressing myogenin improves denervated muscle atrophy in rats. *Braz J Med Biol Res*, 49, e5124, 2016. DOI: 10.1590/1414-431X20155124

7. Malik A, Lee E J, Jan AT, Ahmad S, Cho KH, Kim J, Choi I: Network analysis for the identification of differentially expressed hub genes using myogenin knock-down muscle satellite cells. *PLoS One*, 10, e0133597,2015. DOI: 10.1371/journal.pone.0133597

8. Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX: POPGENE: The user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada, 1997.

9. Xin GS, Long RJ, Guo XS, Irvine J, Ding LM, Ding LL, Shang SH: Blood mineral status of grazing Tibetan sheep in Northeast of the Qinghai-Tibetan Plateau. *Livest Sci*, 136, 102-107, 2011. DOI: 10.1016/j.livsci.2010.08.007

10. Zhou JW, Mi JD, Titgemeyer EC, Guo XS, Ding LM, Wang HC, Qiu Q, Li ZP, Long RJ: A comparison of nitrogen utilization and urea metabolism between Tibetan and fine-wool sheep. *J Anim Sci*, 93, 3006-3017, 2015. DOI: 10.2527/jas.2014-8865

11. Lu J, Wei C, Zhang X, Xu L, Zhang S, Liu J, Cao J, Zhao F, Zhang L, Li B, Du L: The effect of myostatin silencing by lentiviral-mediated RNA interference on goat fetal fibroblasts. *Mol Biol Rep*, 40, 4101-4108, 2013. DOI: 10.1007/s11033-013-2494-6

12. Wyszynska-Koko J, Pierzchala M, Flisikowski K, Kamyczek M, Rózycki M, Kurył J: Polymorphisms in coding and regulatory regions of the porcine MYF6 and MYOG genes and expression of the MYF6 gene in m. longissimus dorsi versus productive traits in pigs. *J Appl Genet*, 47, 131-138, 2006. DOI: 10.1007/BF03194612

13. Xue M, Zan LS, Gao L, Wang HB: A novel polymorphism of the myogenin gene is associated with body measurement traits in native Chinese breeds. *Genet Mol Res*, 10, 2721-2728, 2011. DOI: 10.4238/2011.November.4.6

14. Anton I, Zsolnai A, Komlosi I, Kiraly A, Fésüs L: Effect of MYOG genotypes on growth rate and production traits in Hungarian large white pigs. *Acta Vet Hung,* 54, 393-397, 2006. DOI: 10.1556/AVet.54.2006.3.9

15. Genxi Z, Ying T, Tao Z, Jinyu W, Yongjuan W: Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. *Mol Biol Rep*, 41, 7331-7338, 2014. DOI: 10.1007/s11033-014-3619-2

16. Capon F, Allen MH, Ameen M, Burden AD, Tillman D, Barker JN, Trembath RC: A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups. *Hum Mol Genet*, 13, 2361-2368, 2004. DOI: 10.1093/hmg/ddh273

17. Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchynskyi O, Makarov SS, Maixner W, Diatchenko L: Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science*, 314, 1930-1933, 2006. DOI: 10.1126/science.1131262

18. Van Laere AS, Nguyen M, Braunschweig M, Nezer C, Collette C, Moreau L, Archibald AL, Haley C, Buys N, Tally M, Andersson G, Georges M, Andersson L: A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. *Nature*, 425, 832-836, 2003. DOI: 10.1038/nature02064

19. Krawczak M, Thomas NS, Hundrieser B, Mort M, Wittig M, Hampe J, Cooper DN: Single base-pair substitutions in exon-intron junctions of human genes: Nature, distribution, and consequences for mRNA splicing. *Hum Mutat*, 28, 150-158, 2007. DOI: 10.1002/humu.20400

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Research Article

Comparing Biochemical Properties of Pure and Adulterated Honeys Produced By Feeding Honeybees (*Apis mellifera* L.) Colonies with Different Levels of Industrial Commercial Sugars [1]

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Abstract

In the present study, 100 pure and adulterated honey samples produced by feeding colonies with different levels (5, 20 and 100 L/colony) of various commercial industrial sugar syrups such as High Fructose Corn Syrup 85 (HFCS-85), High Fructose Corn Syrup 55 (HFCS-55), Glucose Monohydrate Sugar (GMS), Bee Feeding Syrup (BFS) and Sucrose Syrup (SS) were evaluated in terms of sugar ingredients, physicochemical, mineral matter, vitamin and enzyme contents. Proline, electrical conductivity, free acidity, vitamin, mineral and enzyme content of honey were significantly affected by sugar origin and syrup levels. Fructose and glucose content of honey were not reliable criteria for distinguishing adulterated honey obtained by overfeeding honey bees (100 L/colony syrup) with sugar having balanced monosaccharide fraction. For pure blossom honey having EC value below 0.20 mS/cm should be evaluated as indirect adulterated with commercial industrial sugars originating from both C3 and C4 plants. In addition, proline content of pure blossom honey below 300 mg/kg might be an indicator for overfeeding honey bee colonies with HFCS-85 and SS. It is clear that the important biochemical and biological degradation occurred in honey when the colonies overfed with industrial sugars syrup during the main nectar flow. The values of biochemical properties found in the present study are important references to be used for revision of national and international standards.

Keywords: *Honey, Commercial sugars, Colony, Feeding, Adulterated, Biochemical properties*

Endüstriyel Ticari Şekerlerin Farklı Şerbet Seviyeleri İle Beslenen Bal Arısı (*Apis mellifera* L.) Kolonilerinden Üretilmiş Katkılı ve Saf Balların Biyokimyasal Özellikler Yönünden Karşılaştırılması

Özet

Bu çalışmada, endüstriyel mısırdan üretilen ticari High Fructose Corn Syrup 85 (HFCS-85), High Fructose Corn Syrup 55 (HFCS-55) ve arı yemi (BFS) şurupları ile glukoz monohydrate (GMS) ve sukroz (SS) şekerlerinin 5, 20 ve 100 litre/koloni şerbet seviyeleri ile üretilmiş hileli ve saf (PBH) 100 bal örneğinin biyokimyasal özellikleri değerlendirilmiştir. 100 bal örneği su, fruktoz, glukoz, F/G, F+G, Glukoz/Su, maltoz, laktoz, sukroz, kül, serbest asitlik, viskozite, hydroxymethyfurfural (HMF), diyastaz, invertaz, α-glukosidaz, glukoz oksidaz, proline, elektriki iletkenlik, Na, K, K/Na, vitamin B5 ve vitamin C yönünden karşılaştırılmıştır. Laktoz, maltoz ve glukosidaz enzimi dışındaki biyokimyasal özellikleri şeker çeşidi ve şerbet seviyesine göre önemli ($P<0,001$) farklılık göstermişlerdir. Arılar saf glukozmonohidrat şekerinden yararlanamamış fakat sukroz şekerini aşırı düzeyde severek tüketmişlerdir. Şeker içeriğinde monosakkarit yapıdaki fruktoz miktarı arttığında arı bunu izomerize edememiştir. Koloniye verilen şerbet miktarı arttıkça arılar daha fazla enzim üretememişlerdir. Şerbet miktarı arttıkça balın protein, enzim, vitamin, mineral madde ve şeker yapısı gibi çok önemli düzeyde biyokimyasal değişim ve biyolojik kayıp meydana gelmiştir. Prolin, asitlik, iletkenlik ve K/Na oranı aşırı besleme ile üretilen hileli balları tanımlamada önemli özelliklerdir. Biyokimyasal özelliklerin belirlenen değerleri mevcut ulusal ve uluslararası standartların düzenlenmesi için önemli referanslardır.

Anahtar sözcükler: *Bal, Endüstriyel Şeker, Koloni, Besleme, Hile, Biyokimyasal özellikler*

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INTRODUCTION

In fact, as a human food, honey should be pure, hygienic and unadulterated [1-4]. However, adulteration has been performed directly via the addition of commercial sugar syrups to the honey [5,6] or indirectly via overfeeding honey bee colonies with these commercial industrial sugars during the main nectar flow period [3,7,8]. These industrial sugars used for adulteration are produced from sugar beet and mainly maize starch by heat, enzyme or acid treatment producing a substance known as High Fructose Corn Syrup (HFCS). HFCS is found in the market under different names according to the fructose content (for instance HFCS-42, HFCS-55, HFCS 85 and HFCS-92). Fructose is preferred by food industry and adulterated honey producers due to having many advantages such as high sweetness degree, having many similar features with honey such as late crystallization, low pH, high osmotic pressure, being liquid and cheap [9]. Thus consumption of fructose corn syrup increased markedly up to a share approximately 40% in the sweetener. On the other hand, almost all fructose syrup (90%) has been produced from GMO corn. In Turkey each year 900 thousand tons starch-based sugar is produced by five domestic and foreign production firms [10]. However it is well known that industrial fructose usage causes serious health problems in living organism. It is reported that fructose is taken into the body without being controlled by insulin, which creates dependency, causes cell aging, impairs spatial memory, metabolic syndrome, obesity and many pathologic illness [11-13].

Fraudulent and adulterated honey production is a problem all over the world. These types of honey are produced by feeding honey bee colonies with industrial sugars ranging from 50 to 300 litres per colony during main nectar flowing period. It is unfair and not ethical to sell these types of honey as a pure honey in the market [1,3,14,15].

Many researchers reported that the use of excessive sugar with the intent of producing greater yields [5] adversely affected sugar content [3,5,7,9,10,16,17], mineral matter content and proline content of the honey [3-5,18]. In addition, sugar origin (C_3 or C_4) used for bee feeding affected the honey carbon isotope ratio and C_4% [5,8,12]. Many findings on honey adulteration belong to studies where adulterations were performed by the addition of sugar or syrups directly to the honey [19-23]. Namely, sugar-added honey does not undergo any changes due to the fact that samples are not processed biologically by the bee. Furthermore, in these studies, many honey samples subjected to biochemical analysis were taken from the market, meaning that their production conditions were not known exactly. In a similar way, the production conditions of the honey characterised as natural or pure in many of these studies were not clearly known. Also such indirect adulteration is extremely difficult to detect [24]. Above all, we do not know how to distinguish adulterated honeys taken from the colonies that were overfed with industrial commercial sugars from pure honeys. The standards should be revised by including new criteria for identification of indirectly adulterated honey [1,3,15]. We strongly believe that there is a need to assess different types and levels of industrial commercial sugars in feeding honey bee colonies on the field to address all of the questions discussed above.

We investigated the changes occurring in the biochemical properties of those honeys coming from honey bee colonies fed with different levels of various commercial sugars including HFCS-85), HFCS-55, glucose monohydrate (GMS), bee feeding syrup (BFS), sucrose syrups (SS) and Pure Blossom Honey (PBS) produced under the same environmental conditions. The aims of the study were: (1) to determine changes in the wide range of biochemical properties of pure and adulterated honeys, (2) to characterised these kind of honey biochemically and find which biochemical properties can be used for the identification of adulterated honeys and (3) to make a contribution to the current methods and standards.

MATERIAL and METHODS

Honey Bee Colonies

Honey samples were taken from colonies in the Apicultural Research and Application Unit of the Ondokuzmayis University, Samsun, Turkey. Colonies were retained in the vicinity of Gulacar Valley, near Gumushane province (40.274°N, 39.29°E), during the nectar flow period. The Gulacar valley is rich in nectar-producing plant species [8].

Sugar Sources

Types, origins, compositions, forms and proportions of the industrial sugars used in the study are summarised in *Table 1*.

Maintenance and Preparation of The Colony

The colonies with two- aged queen bees of the same genetic origin of Caucasian honey bee subspecies (Apis mellifera caucasica G.) were used in the study. All of the environmental factors such as frames covered with adult bees, frames covered with brood, foundation comb, drugs, transport and control procedures were standardised. Standard bee-feeding methods were applied in the early spring [9,25]. The shaking method was applied to the colonies [25].

Syrup Levels and Preparation

Sugars and syrup levels were used for the first time as a bee food supplement during the main nectar flow period in this study. Syrups were prepared daily, mixed often, left for one day and were finally given to the colonies. Levels of 5, 20 and 100 L/colony of HFCS-85, HFCS-55, BFS and SS were used in the study. Syrup was given to the experimental colonies at different intervals (twice for the 5, eight times

Table 1. *Types, origins, compositions, forms, proportions and supplied company names of the industrial sugars used in the experiment*

Sugar Sources	Origin of Sugar	Form	Composition	Usage Proportion
HFCS.85	Corn *(Zea mays)*	Liquid	84.9% fructose, 12.8% dextrose	1:3 (water : sugar, w:w)
HFCS.55	Corn *(Zea mays)*	Liquid	55.6% fructose, 39.6% dextrose	1:3 (water : sugar, w:w)
GMS	Wheat *(Triticum vulgare)*	Powder	99.0% glucose	70% water + 30% sugar
SS	Beet sugar *(Beta vulgaris)*	Crystalline	99.5% sucrose	1:1.5 (water : sugar, w:w)
BFS	Beet sugar *(Beta vulgaris)*	Liquid Pasteurised	30-36% sucrose, 27-30% glucose, 37-40% fructose	1:3 (water : BFS, w:w)

for the 20 and forty times for the 100 L/colony). Before the application of new syrup, the amount of unconsumed syrup (g/colony) was recorded on each colony's card.

Honey Harvest and Honey Sample Preparation

The honey produced in this study was regarded as a polyfloral honey. Honey was harvested by centrifugation and filtered through a 0.2-mm sieve into lactin. Honey samples were taken from six colonies which were chosen randomly from all of the available groups, and a total of 100 (five industrial sugars * three syrup levels * six repetitions = 90 adulterated samples + 10 pure honey or control) samples were used.

Analytical Methods

The following compositional properties were determined for pure blossom honey and adulterated honey samples: moisture (%), ash (%), free acidity (meq kg^{-1}), viscosity (cP), hydroxymethylfurfural (HMF, mg kg^{-1}), diastase number (DS, Schade scale value), invertase (u kg^{-1}), α-glucosidase (u kg^{-1}), proline (mg 100 g^{-1}), electrical conductivity (mS cm^{-1}), Na (mg 100 g^{-1}), K (mg 100 g^{-1}), fructose (g 100 g^{-1}), glucose (g 100 g^{-1}), sucrose (g 100 g^{-1}), maltose (g 100 g^{-1}), lactose (g 100 g^{-1}), vitamin B_5 (mg 100 g^{-1}) and vitamin C (mg 100 g^{-1}).

Moisture was measured at 20°C by Abbe refractometer (Digital refractometer Atoga, Germany) by refractive methods [19]. Fructose, glucose, maltose, and sucrose (g 100 g^{-1}) were identified and determined by high performance liquid chromatography (HPLC) according to AOAC [26] and DIN 10758 [27]. Hydroxymethylfurfural (HMF) was determined spectro-photometrically as outlined by the Harmonisation methods of International Honey Commission (IHC) [14]. The diastatic activity was based on starch hydrolysis [26] (method 958.09) at 300/time to a value of absorbance of 0.235 at 660 nm. A weighed sample was ignited in a muffle furnace at 550°C to a constant weight for ash determination (method 923.03) [26]. Potassium and sodium were determined using the Atomic Absorbance Spectrophotometer (AAS) according to [26] (AOAC, 1998 method 985.35). Proline was determined spectrophotometrically using ninhydrin in methyl cellosolve, and absorbance was read at 512 nm. A standard curve using pure proline was constructed according to [26] (AOAC, 1998 method 979.20). After calibrating the conductimeter, the electrical conductivity of each honey solution at 20% dry matter was measured at 20°C by the Harmonised Methods of the IHC [14]. Free acidity was determined photometrically by [26] (AOAC, 1998 method 962.19), and vitamin C and vitamin B_5 were determined by the R-Biopharm Vitafast Panthotenic Acid, Microbiological microtitre Plate Test to quantitate amounts present.

Statistical Analysis

The study was carried out according to the Randomised Factorial Plot Design and a Pairwise Permutation Test was used for comparison. Homogeneity of variance was performed using the Levene Variance Homogeneity Test and One-Sample Kolmogorov-Smirnov Test [28]. The variance analysis was performed by using the SAS packet programme and Duncan test was used for the comparison of averages [29].

RESULTS

There were statistically significant differences ($P<0.001$) in all of the investigated biochemical properties except for lactose and maltose, which were not found in any of the honey samples *(Table 2, 3, 4)*. In addition, interaction between the sugar type and sugar level was significant ($P<0.001$).

Water

The mean water content of honey samples are presented in *Table 2*. The water values ranged between 15.72±0.10% and 19.20±0.03%.

Sugar Ingredients

Sugar content of the honey samples were evaluated by using fructose, glucose and sucrose content, Fructose/Glucose ratio (F/G), sum of fructose and glucose (F+G), and glucose and water ratio (G/W) *(Table 2)*. All of these parameters were significantly affected by sugar types and syrup levels ($P<0.000$).

Physicochemical Properties

There were significant differences ($P<0.000$) among treatments in Hydroxymethylfurfural (HMF) content of the honey *(Table 2)*. The HMF content ranged from 2.63±0.53 mg/kg to 10.93±0.74 mg/kg. Viscosity, electrical conductivity

Table 2. *Means (X) and standard errors (±Sx) related to water, fructose, glucose, sucrose (g/100 g), F/G (ratio), F+G, glucose/water (ratio) and HMF (mg/100 g) contents of pure and adulterated honeys*

Sugar Sources	SL	Water (Moister)	Fructose	Glucose	Sucrose	F/G	F + G	Glucose/Water	HMF
HFCS-85	5	17.42±0.07 h	39.05±0.83 cd	29.72±0.80 ef	0±0 b	1.32±0.01 cd	68.77±1.62 b-e	1.707±0.049 bcd	2.63±0.53 c
	20	19.20±0.03 a	44.02±0.59 b	24.00±0.39 g	0±0 b	1.84±0.02 b	68.02±0.95 b-e	1.250±0.020 f	6.27±1.16 b
	100	16.77±0.05 k	57.07±1.11 a	17.08±0.53 h	0±0 b	3.35±0.07 a	74.15±1.52 a	1.019±0.032 g	10.68±1.68 a
HFCS-55	5	18.60±0.00 c	37.40±0.96 def	29.87±0.83 def	0±0 b	1.25±0.01 def	67.27±1.79 cde	1.606±0.044 cde	5.43±1.64 bc
	20	17.07±0.05 l	37.78±0.49 c-f	29.35±0.61 ef	0±0 b	1.29±0.01 cd	67.13±1.09 cde	1.719±0.035 bc	6.27±1.19 b
	100	18.40±0.00 d	37.20±0.23 def	27.85±0.27 f	0±0 b	1.34±0.01 c	65.05±0.46 e	1.514±0.015 e	10.93±0.74 a
GMS	5	19.17±0.02 a	37.45±1.35 def	29.60±1.41 ef	0±0 b	1.27±0.02 cde	67.05±2.73 cde	1.545±0.075 e	2.80±0.59 bc
	20	19.20±0.00 a	40.08±0.47 c	33.43±0.45 ab	0±0 b	1.20±0.01 fg	73.52±0.86 a	1.741±0.015 bc	2.93±0.39 bc
	100	19.03±0.05 b	36.42±0.30 ef	34.23±0.32 a	0±0 b	1.06±0.00 h	70.65±0.62 a-d	1.798±0.015 b	3.73±0.68 bc
BFS	5	18.02±0.02 fg	39.95±0.57 c	31.77±0.49 bcd	0±0 b	1.26±0.01 def	71.72±1.02 ab	1.763±0.027 bc	4.78±1.13 bc
	20	18.98±0.04 b	39.20±0.67 cd	32.22±0.63 bc	0±0 b	1.22±0.02 efg	71.42±1.20 abc	1.697±0.033 bcd	5.08±1.02 bc
	100	18.12±0.02 ef	38.57±0.79 cde	32.37±0.81 ab	0±0 b	1.19±0.01 fg	70.93±1.57 abc	1.787±0.045 b	4.50±1.19 bc
SS	5	18.55±0.07 c	36.98±0.35 def	28.97±0.3 ef	0±0 b	1.28±0.01 cde	65.95±0.63 e	1.542±0.015 de	3.67±1.34 bc
	20	18.20±0.06 e	38.95±1.07 cd	30.27±0.79 cde	0±0 b	1.29±0.00 cd	69.22±1.86 b-e	1.663±0.045 cde	4.67±0.85 bc
	100	15.72±0.10 m	36.07±0.43 f	30.40±0.41 cde	3.05±0.08	1.19±0.02 fg	66.47±0.50 de	1.934±0.023 a	4.68±0.43 bc
PBH	-	17.93±0.03 g	35.49±0.68 f	30.35±0.31 cde	0±0 b	1.17±0.01 g	65.84±0.97 e	1.693±0.017 bcd	3.71±0.93 bc
Significance		<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000
Codex Standard		<20%	Sum of both >60 g/100 g		<5 g/100 g		>60 g/100 g		<40 mg/kg
European Union		<20%	Sum of both >60 g/100 g		<5 g/100 g		>60 g/100 g		<40 mg/kg

♦*=For each syrup level n=6,* ***HFCS****= High Fructose Corn Syrup,* ***GMS****=Glucose Monohydrate Sugar,* ***BFS****=Bee Feeding Syrup,* ***SS****=Sucrose Sugar,* ***PBH****=Pure Blossom Honey (n=10),* ***SL****=Syrup levels (L/colony), * Values within rows with different letters differ significantly (P<0.001)*

(EC) and free acidity of the honeys showed significant differences (P<0.000) depending on sugar types and syrup levels *(Table 3)*. EC and free acidity values of the honey were within the range reported by Codex Alimentarius [2] and EU Council [30], Turkish Food Codex, Honey Notification [31].

Mineral Matter Content

There were significant differences among treatments in terms of these parameters (P<0.001). The Na content of honey decreased rapidly in the 100 L/colony of HFCS-85 and HFCS-55, it was increased in the 100 L/colony of BFS and SS *(Table 3)*. Potassium content of honey decreased with increasing syrup level of all sugar types except for GMS.

Proline Content

The proline content of pure and adulterated honey ranged from 772.83±17.56 to 249.33±6.27 mg/kg. Except for GMS, the proline content of honey decreased considerably with increasing syrup level of all sugar types. The decrease in proline was more than 2.5-3.0-fold. The highest proline was found in the pure honey, whereas there was a considerable decline in the honey taken from the 100 L/colony of HFCS, BFS and SS *(Table 4)*.

Vitamin and Enzyme Content

Sugar types and sugar levels had significant effect (P<0.001) on vitamin (vitamin C and B_5) and enzyme content of honey samples. There were great decline in vitamins content of honeys taken from the 100 L/colony of all sugar types except for GMS. In the present study diastase number did not change with sugar types originating from C_3 or C_4 plants. Enzyme invertase content of honeys decreased with increasing syrup levels of all sugar types. There was no consistency in the enzyme α-glucosidase content of honeys. It increased with increasing sugar levels of HFCS-85 and BFS; whereas it decreased when the syrup levels of SS and GMS were increased *(Table 4)*. In addition we found significant positive and negative correlations between investigated biochemical characteristics *(Table 5)*.

DISCUSSON

The water values ranged between 15.72±0.10% and 19.20±0.03%, indicating that all values were below the standard value (20-21%) reported by Codex Alimentarius [2], the International Honey Commission [17], the EU Council [30] and the Turkish Honey Codex [31] for all pure and adulterated honeys. This might be resulted from the fact that all honeys were taken from colonies when completely ripening [14].

Table 3. *Means (X) and standard errors (±Sx) related to viscosity (cP), free acidity (meq/kg) electrical conductivity (mS/cm), ash (mg/100 g), Na (mg/100 g), K (mg/100 g) and K/Na (ratio) contents of pure and adulterated honeys*

Sugar Sources	SL	Viscosity	Free Acidity	Electrical Conductivity	Ash	Na	K	K/Na
HFCS-85	5	12405.5±340.81 d	16.2±0.02 b	0.211±0.001 bcd	0.121±0.011 a	0.783±0.019 ab	17.15±0.10 cde	21.97±0.47 de
	20	5111.17±52.1 h	14.7±0.02 d	0.201±0.000 de	0.113±0.01 ab	0.792±0.021 ab	16.88±0.10 cde	19.69±1.98 ef
	100	14605.42±280.05 c	9.2±0.02 g	0.117±0.001 h	0.056±0.007 g	0.585±0.093 c-f	6.81±0.10 f	12.87±1.55 gh
HFCS-55	5	7005.58±96.57 g	17.3±0.02 a	0.216±0.001 abc	0.088±0.006def	0.730±0.076 a-d	16.52±0.19 de	23.96±2.40 de
	20	15650.08±609.63 b	16.0±0.03 b	0.203±0.001 cde	0.098±0.007b-e	0.809±0.140 a	20.9±2.32 ab	26.67±3.94 cd
	100	7611.08±390.73 g	11.0±0.00 f	0.138±0.000 fg	0.055±0.004g	0.465±0.016 f	7.6±0.09 f	16.35±0.19 fg
GMS	5	5527.67±135.97 h	16.2±0.02 b	0.211±0.001 a-d	0.083±0.004ef	0.724±0.078 a-d	22.49±2.57 a	30.87±0.74 bc
	20	5394.42±172.62 h	15.8±0.02 bc	0.211±0.003 a-d	0.097±0.011 b-e	0.558±0.049 def	17.13±0.17 cde	32.76±2.24 ab
	100	11361.08±588.55 e	16.3±0.02 b	0.224±0.001 a	0.124±0.002 a	0.610±0.066 c-f	21.81±0.86 a	37.16±2.41 a
BFS	5	9600±456.83 f	17.0±0.00 a	0.215±0.001 abc	0.105±0.006 a-d	0.598±0.011 c-f	18.16±0.11 cd	30.41±0.59 bc
	20	6038.83±204.56 h	17.0±0.00 a	0.216±0.001 abc	0.091±0.007 cde	0.528±0.016 ef	17.4±0.08 cde	33.12±0.96 ab
	100	9116.67±373.24 f	11.5±0.02 e	0.146±0.001 f	0.082±0.004 ef	0.622±0.038 b-f	9.28±0.08 f	15.15±0.79 fg
SS	5	8955.5±158.3 f	16.9±0.01 a	0.221±0.001 ab	0.090±0.006 c-f	0.635±0.034 b-f	19.25±0.23 bc	30.86±1.53 bc
	20	8888.83±174.34 f	15.5±0.02 c	0.195±0.000 e	0.082±0.005 ef	0.699±0.019 a-e	15.05±0.13 e	21.60±0.46 de
	100	33111.0±359.39 a	8.0±0.00 h	0.130±0.040 g	0.070±0.005 fg	0.746±0.016 abc	7.34±0.05 f	9.86±0.18 h
PBH	-	10773.2±318.81 e	16.8±0.02 a	0.213±0.001 a-d	0.109±0.004 abc	0.603±0.020 c-f	18.11±0.18 cd	30.75±1.59 bc
Significance		<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000
Codex Standard			<50 mEq/kg	<0.8 mS/cm				
European Union			<50 mEq/kg	<0.8 mS/cm				

♦*=For each syrup level n=6,* ***HFCS****=High Fructose Corn Syrup,* ***GMS****=Glucose Monohydrate Sugar,* ***BFS****=Bee Feeding Syrup,* ***SS****=Sucrose Sugar,* ***PBH****=Pure Blossom Honey (n=10),* ***SL****=Syrup levels (L/colony), *Values within rows with different letters differ significantly (P<0.001)*

In the 20 and 100 L/colony levels of HFCS-85, honey bees stored a high amount of fructose in honey as fructose. This might be resulted from high fructose content of the HFCS-85 compared to the other sugars *(Table 1)*. This result was compatible with the result of Ruiz-Matute et al.[7] who used HFCS-42, HFCS-55, HFCS-75, HFCS+SS and SS sugars for feeding the honey bees. In our study, the honeys taken from the 20 and 100 L/colony of HFCS-85 evaluated as fraudulent due to the fact that they showed significant deviations from standard values [2,30]. The result also indicated that as being in the BFS, if the monosaccharide fraction of sugar is arranged in a balanced way (for instance 40-45 g/100 g fructose and 30-35 g/100 g glucose), honey bees can use them efficiently. This may explain why USA beekeepers use HFCS-42 syrup to feed their colonies [9].

Honeys taken from the 20 and 100 L/colony of HFCS-85 and honey taken from the 100 L/colony of GMS were accepted as fraudulent because of the lower and higher glucose content, respectively, when compared to the standards [2,30]. These differences might be attributed to the glucose proportion of the used sugars; namely the HFCS-85 has no glucose, GMS has the highest glucose content (99.0%). It can be inferred from these results that if fructose content of used sugar is high, honey bees could not isomerise it efficiently to acceptable level of glucose in the honey. Of the 100 honey samples, only 12 samples were identified as fraudulent when the fructose contents of the honeys were taken into consideration. On the other hand, 18 samples were classified as fraudulent when the glucose contents of honeys were taken into account. Therefore it is obvious that fructose and glucose content are not reliable parameters in order to identify indirect adulteration [3,32]. Sucrose was only found in the honey taken from the 100 L/colony of SS (3.05±0.08 g/100 g). PBH had no sucrose. Ozcan et al.[33] and Ruiz-Matute et al.[7] also found no sucrose in control honey. The source of sucrose in the pure honey, where sucrose was not used as a supplemental food in this group, is nectar [5,32-34].

The F/G values determined for the 20 and 100 L/colony of HFCS-85 were higher than the values (F/G 0.95 for *Brassica* honey to 1.61 for *Robinia* honey) reported by Piazza and Oddo [35] and Oddo et al.[36] for the 19 unifloral honey types generated from different plant sources. In their study total range of the F/G ratio of 6719 honey samples produced in 21 countries of the European geographical area was 1.03. Whereas, in our study difference in the F/G ratio of honey between the 20 and 100 L/colony of HFCS-85 was 1.51. These two groups were evaluated as fraudulent due to the fact that they showed important deviation from the standard. The sum of fructose and glucose (F+G) values corresponded with the value (>60 g/100 g) given by Codex Alimentarius [2]. Therefore, there was no adulterated

Table 4. *Means (X) and standard errors (±Sx) related to proline (mg/100 g), vitamin C (mg/100 g), vitamin B_5 (mg/100 g), diastase (u/kg), Invertase (u/kg) and α-glucosidase (u/kg) of pure and adulterated honeys*

Sugar Sources	SL	Proline	Vit C	Vit B_5	Diastase	Invertase	α-glucosidase
HFCS-85	5♦	709.33±7.57 bc	2.54±0.01 ef	0.105±0.001 a	8.33±0.00 a	66.23±0.10 b	22.88±0.63 c
	20	530.00±3.85 f	1.89±0.02 g	0.077±0.001 g	7.70± 0.00 b	58.15±0.63 fgh	27.43±0.72 b
	100	279.17±2.83 h	0.20±0.01 k	0.062±0.003 i	7.70±0.00 b	57.65±0.19 ghi	27.64±0.87 b
HFCS-55	5	707.50±16.12 bc	2.76±0.06 cd	0.096±0.002 b	7.14±0.00 c	71.08±0.56 a	30.78±0.57 a
	20	618.17±13.73 e	3.70±0.08 a	0.084±0.001 ef	7.14±0.00 c	70.20±0.33 a	31.23±0.50 a
	100	348.67±6.42 g	0.34±0.02 k	0.059±0.001 i	7.70±0.00 b	62.57±0.35 c	26.94±0.92 b
GMS	5	673.17±9.25 cd	3.44±0.07 b	0.081±0.003 fg	7.70±0.00 b	62.65±0.35 c	28.02±0.47 b
	20	646.33±26.25 de	2.45±0.15 f	0.090±0.001 cd	8.33± 0.00 a	55.94±0.75 I	23.25±0.62 c
	100	719.67±10.73 b	3.65±0.16 a	0.097±0.001 b	8.33±0.00 a	57.33±1.76 ghi	22.62±0.78 c
BFS	5	702.33±18.37 bc	1.57±0.07 h	0.091±0.002 bcd	8.33±0.00 a	56.73±0.37 hi	24.08±0.35 c
	20	709.00±10.39 bc	2.72±0.08 de	0.092±0.001 bc	7.70±0.00 b	62.15±0.33 cd	27.86±0.78 b
	100	372.83±3.61 g	0.26±0.01 k	0.069±0.001 h	7.70±0.00 b	61.27±0.24 cde	26.67±0.98 b
SS	5	772.83±17.56 a	2.83±0.04 cd	0.092±0.001 bc	7.70±0.00 b	61.38±0.74 cde	27.70±0.74 b
	20	704.50±9.67 bc	1.30±0.02 i	0.086±0.001 de	7.70±0.00 b	60.23±1.02 def	26.40±0.98 b
	100	249.33±6.27 h	0.19±0.01 k	0.050±0.001 k	7.70±0.00 b	58.53±0.83 fgh	22.38±0.59 c
PBH		768.20±13.06 a	2.94±0.02 c	0.094±0.003 bc	7.70±0.00 b	59.33±0.73 efg	27.36±1.24 b
P		<0.000	<0.000	<0.000	<0.000	<0.000	<0.000
Codex Standard		>180 mm/kg			>8 u/kg		
European Union					>8 u/kg		

♦=For each syrup level n=6, ***HFCS****=High Fructose Corn Syrup,* ***GMS****=Glucose Monohydrate Sugar,* ***BFS****=Bee Feeding Syrup,* ***SS****=Sucrose Sugar,* ***PBH****=Pure Blossom Honey (n=10),* ***SL****=Syrup levels (L/colony), *Values within rows with different letters differ significantly (P<0.001)*

Table 5. *Correlation coefficients in terms of some biochemical properties of pure and adulterated honeys*
Tablo 5. *Saf ve katkılı balların bazı biyokimyasal özellikleri arasındaki ilişkiyi ifade eden korelasyon katsayılarına (r) ilişkin değerler*

Characteristics	G	S	F/G	F+G	P	HMF	W	Ac	EC	K/Na	Vit B_5	Vit C	I	Dyz	Gls
F	-0.636**	-0.159	0.908**	0.629**	-0.416	0.399	-0.180	-0.394	-0.432	0.262	- 0.267**	-0.390**	-0.243*	0.026	0.056
G		0.055	-0.877**	0.199	0.450	-0.430	0.286	0.433	0.471	-0.396	0.444	0.341	0.332	0.283**	-0.299**
S			-0.101	-0.146	-0.503	-0.034	-0.644	-0.587**	-0.436	0.590	-0.513	-0.369	-0.138	-0.060	-0.311**
F/G				0.269	-0.486	0.461	-0.287	-0.477	-0.518	0.398	-0.365**	-0.410**	-0.195	-0.107	0.165
F+G					-0.075	0.073	0.060	-0.064	-0.074	-0.066	0.049	-0.134	-0.264**	-0.319**	-0.221*
P						-0.438	0.515	0.954**	0.914**	-0.770**	0.866	0.823	0.428	0.104	0.094
HMF							-0.162	-0.400	-0.457	0.346	-0.332**	-0.412**	0.044	-0.244*	-0.250*
W								0.602**	0.584**	-0.675**	0.432**	0.442**	-0.063	0.186	0.074
Ac									0.935**	-0.808**	0.854	0.835	0.429	0.103	0.169
EC										-0.781**	0.788	0.833	0.431	0.177	0.023
K/Na											0.652**	0.745**	-0.051	0.269**	-0.080
Vit B_5												0.713**	0.223*	0.249*	0.030
Vit C													0.320**	-0.014	0.174
I														-0.593**	0.521**
Dyz															-0.741**

G*=glucose,* ***S****=sucrose,* ***F****=fructose,* ***P****=proline,* ***Ac****=acidity,* ***EC****=electrical conductivity,* ***W****=water,* ***Dyz****=diastase,* ***I****=invertase,* ***Gls****=glucosidase,* ***HMF:*** *hydroxymethylfurfural, *, ** shows significance at P<0.05 and P<0.01 levels.*

honey when the sum of F+G was used as a criterion. These results indicated that the F+G was not a good parameter for identification of indirect adulteration [32]. Of the 100 honey samples, 12 honeys from the 20 and 100 L/colony of HFCS-85 were classified as fraudulent when the F/G ratio was taken into consideration. This result indicated that F/G ratio over 1.84 can be an indicator of overfeeding honey bees with HFCS-85.

The rate of G/W did not change with the source of the sugar (C_3 and C_4 plant). The highest changes occurred for different levels of HFCS-85. The rate of change was about 65-70%. The G/W ratio decreased with increasing levels of HFCS-85. Oddo et al.[36] reported that the G/W showed differences according to the plant sources of honey. The reason for the higher rate of G/W in the 100 L/colony of SS is that honey bees used it efficiently. While the fructose and glucose content of the given sucrose were equal (50%), these rates changed to 36.07±0.43% fructose and 30.35±0.41% glucose after being processed by the honey bees. Namely, even in the adulterated honey produced by feeding the honey bees with the 100 L/colony of sucrose syrup, the fructose and glucose levels of honeys were similar to those of pure honeys. In fact, we expected the highest G/W ratio for the GMS due to its high glucose content, but this expectation did not realise, because the water content of honey in the GMS was higher, as the honey bees did not ripening the GMS syrup properly. The G/W ratio is a criterion for crystallisation [34,35]. If the G/W ratio of honey is lower than 1.70, it does not crystallise and remains liquid; if this rate is over 2.10, the honey crystallises in a short time. In our study, all honeys crystallised, even the pure honey, with the exception of the 100 L/colony of HFCS-85. Although the G/W ratio of some of them were lower than 1.70, they crystallised *(Table 2)*.

HMF is a criterion that shows whether honey was stored under good conditions and also whether the honey was treated with heat or not [1,34,37]. The low HMF content of the honeys in the present study was attributed to the fact that they were newly harvested honeys (not stored for a long duration), not treated with heat, and stored under proper conditions before sending for analysis. These results supported by Visquert et al.[38] who found low HMF (<10 mg/kg) in all honey samples. Therefore, HMF should not be used for the identification of adulterated honey produced by overfeeding the honey bees with industrial commercial sugars.

In all sugar types except for GMS, electrical conductivity, free acidity and ash content of the honey decreased with increasing level of sugar syrups. In the highest syrup level (100 L/colony) of these sugars decrease in EC, free acidity and ash content was dramatic, which indicated that honey underwent important biochemical change. Significant correlations between EC and free acidity ($r=0.935$, $P<0.001$), proline ($r=0.914$, $P<0.001$), vitamin C ($r=0.833$, $P<0.001$) and B_5 ($r=0.788$, $P<0.001$) support this result *(Table 5)*. Free acidity shows great variation depending on the plant sources. Terrab et al.[39] reported the range of free acidity as 10.3-102 meq/kg for Moroccan unifloral honeys. Oddo et al.[36] gave the total range of free acidity as 49.1 meq/kg for 19 European unifloral honeys. Decrease in free acidity of honey produced by feeding honey bees with industrial commercial sugar syrup reported also by Ozcan et al.[33] and Guler et al.[3] According to our result, the free acidity of honey is an important criterion for the determination of adulteration made by HFCS and SS and it should be at least 15-16 meq/kg in pure blossom honey. Although EC is the most important trait for determination of the botanic or floral source and ash content of the honey [4,34,36,40], it is also important for the identification of adulterated honey with industrial sugars via colony feeding. Guler et al.[3] reported that EC is the second biochemical characters following proline for discriminating pure and adulterated honeys produced by sucrose feeding. According to the Codex Alimentarius [2] EC should not be more than 0.8 mS/cm. According to us for blossom honeys having EC value under 0.20 mS/cm should be evaluated as indirect adulterated with commercial sugars originating from both C_3 and C_4 plants. This supported by the results of Guler et al.[3] who found that while EC value of control and pure honey over 0.20 mS/cm (0.224 mS/cm for control and 0.230 mS/cm for pure honey) and it was below 0.20 mS/cm for sucrose adulterated honey (0.176 mS/cm).

The Na content of honey decreased rapidly in the 100 L/colony of HFCS-85 and HFCS-55, it increased in the 100 L/colony of BFS and SS. These differences might be attributed to origin of the sugar used. While the origin of the first two sugars (HFCS-85 and HFCS-55) is C_4 plant, the origin of SS is C_3 plant, of BFS is composed of C_3 and C_4 plant. The finding of high Na content of SS honey compared to pure blossom honey is incompatible with the result of Ozcan et al.[33] who reported slightly decrease in Na content for sucrose syrup honey and inverted sucrose syrup honey. Differences might be resulted from differences in given amount of sugar syrup to the bee colonies and differences in sugar composition. They heated sucrose syrup, added HCl solution for pH adjustment and added Na_2CO_3 for neutralization.

In the present study the K content of the honey in the 100 L/colony of SS (7.34±0.05 mg/100 g) was lower than the value reported by Guler et al.[3] for sucrose-adulterated honey with the 100 L/colony of sucrose. Potassium, an essential nutrient for nerve cell functioning in honeybees, is used for the definition of plant sources of honey or the identification of honeydew and blossom honey [32,34]. It is also an important tool for the identification of adulteration. According to our results, the K content of honey should be over 16 mg/100 g.

The K/Na ratio also decreased greatly with increasing syrup level of all sugar types except for GMS. The K/Na ratio of honey is one of the biochemical properties negatively

affected by adulteration following vitamin C and proline content of honey. Therefore, it is an important characteristic for the identification of adulterated honey produced by overfeeding honey bee colonies with industrial commercial sugar. We suggest that the K/Na rate of honey should be considered in standards. If the K/Na rate of blossom honey is less than 20, it should be considered adulterated. Thus, in the standards, the K/Na rate should be evaluated as >20.

Potassium and sodium content of honey shows great variability depending on plant sources. Fernandez-Torres et al.[41] found that K content of Spanish honeys ranged between 43.4-193.5 mg/100 g and Na content ranged between 1.17-21.8 mg/100 g. Conti et al.[42] reported that K content of Argentina honeys ranged between 281.3-13.4 mg/100 g and Na content ranged between 10.5-1.31 mg/100 g.

The proline content of honey decreased considerably with increasing syrup level of all sugar types except for GMS. Oddo et al.[36] and Piazza and Oddo [35] reported that differences in proline stemmed from plant sources. However, in the present study, differences in proline resulted from excessive sugar syrup usage rather than plant source; because the colonies were placed in the same apiary and we know that the HFCS originated from corn (C_4), the GMS from wheat (C_3), the BFS from mixture of C_3 and C_4 plants, the SS from sugar beet (C_3). Proline is accepted as an indicator of maturity level [34,36]. Therefore, we concluded that differences in proline did not stem from maturation or plant source but from excessive feeding of the colonies with industrial sugars. Previously, proline was reported by many authors as the most important biochemical component for monitoring adulteration [3,5,33,34,36]. In Codex Alimentarius [2], proline is considered a quality criterion and should be greater than 180 mg/kg. When Codex standards were taken into account, none of our honey samples were identified as adulterated. However, we know that the honey samples produced by feeding the colonies with 100 L/colony of HFCS, GMS, BFS and SS were exactly adulterated. Therefore, we suggest that the amount of proline given as a standard by Codex Alimentarius is too low to discriminate adulterated honey. For that reason, in the present study, the proline amount of PBH (768.2±13.06 mg/kg) should be an important quality criterion for the standards. In addition there were significant positive correlations ($P<0.001$) between proline and water, acidity, electrical conductivity, vitamin B_5, and vitamin C. Therefore, proline and EC parameters should be evaluated elaborately to rearrange their limits in order to use them as quality criteria for distinguishing pure and indirectly adulterated honeys with commercial sugars. For blossom honey proline content below 300 mg/kg might be an indicator for overfeeding honey bee colonies with HFCS-85 (C_4) and SS (C_3).

There were great decline in vitamins C and B_5 content of honeys taken from the 100 L/colony of all sugar types except for GMS, indicating a biological loss of adulterated honey. Significant correlations among these vitamins and proline, EC, acidity and the K/Na ratio support this result *(Table 5)*.

In the present study diastase number did not change with sugar types originating from C_3 or C_4 plants. The diastase number of most samples, including pure honey (PBH), was slightly lower than the standard value (>8 u/kg). The diastase number of honey changes with plant source, ranging from 4.6±2.8 to 39.3±7.9 u/kg [36], and is an indicator of samples that have not been subjected to heat treatment [34,43,44]. As a matter of fact, a statement from the researcher who performed the analyses confirmed this hypothesis.

Studies showed that there were enormous differences in enzyme activity depending on the botanical origin, even though the enzymes are usually added by the bees [24]. Lichtenberg-Kraag [45] found that the correlation between the concentration of sucrose and invertase activity is highly significant in the process of honey ripening. In the present study we found negative correlation between invertase and fructose and sucrose *(Table 5)*. It is known that honey bees use invertase to invert sucrose of the nectar into fructose and glucose [5,34]. Enzyme invertase content of honeys decreased with increasing syrup levels of all sugar types. A greater reduction in the invertase enzyme levels of honey from HFCS sugars might have resulted from the fact that they contain more fructose than other monosaccharide sugars used in the study. Ruiz-Matute et al.[7] reported that when the fructose proportion of the sugar increased, honey bees encounter difficulty to izomerize the sugar into fructose (39-40%) and glucose (28-30%) to a level that found in natural honey.

We found no consistency in the enzyme α-glucosidase content of honeys. It increased with increasing syrup levels of HFCS-85 and BFS. Whereas there was a decline in the enzyme α-glucosidase content of honeys when the syrup levels of SS and GMS were (both C_3 plants) increased. Differences might be attributed to differences in the origin of the commercial sugars and proportion of fructose, glucose and sucrose in the used commercial sugars.

In conclusion, honey bees utilised commercial sugar syrups depending on the sugar origin (C_3 and C_4 plants), purity levels and amount provided to the colonies. It is not possible to define the adulterated honey by using its sugar constituents such as fructose, glucose, fructose/glucose and sucrose. Whereas it is possible to determine or monitor adulteration in honey when the monosaccharide fructose rate of industrial sugars syrup is increased (>50 g/100 g), as honey bees do not sufficiently or efficiently utilize these types of sugars. When glucose and fructose proportions were balanced or close to each other (fructose 40-45 and glucose 30-35 g/100 g), they were used efficiently by honey bees to the honey. Important biological

degradation occurred in some properties of honey especially in proline, electrical conductivity, free acidity, vitamin, mineral and enzyme contents when the colonies were fed with commercial sugars (20 L/colony and over) in the main nectar flow period. So it is unfair and not ethical to produce and sell these types of honey as a pure honey in the market.

REFERENCES

1. Anklam E: A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem,* 63, 549-562, 1998. DOI: 10.1016/S0308-8146(98)00057-0

2. Codex Alimentarius Commission: Codex Standard for honey, CODEX STAN 12-1981; Food and Agriculture Organization of the United Nations and the World Health Organization. Rev.1, Rev. 2, Rome, Italy, 2001.

3. Guler A, Bakan A, Nisbet C, Yavuz O: Determination of important biochemical properties of honey to discriminate pure and adulterated honey with sucrose syrup. *Food Chem,* 105, 1119-1125, 2007. DOI: 10.1016/j.foodchem.2007.02.024

4. Ruoff K, Luginbühl W, Kilchenmann V, Bosset JO, Ohe KVD, Ohe WVD, Amado R: Authentication of the botanical origin of honey using profiles of classical measurands and discriminant analysis. *Apidologie,* 38, 438-452, 2007. DOI: 10.1021/jf060838r

5. White JWJR: Methods for determining carbohydrates, hydroxymethyl-furfural and proline in honey: Collaborative study. *J Assoc Off Anal Chem,* 62, 509-514, 1979.

6. Ruiz-Matute AI, Soria AC, Martinez-Castro I, Sanz ML: A new methodology based on GC-MC to detect honey adulteration with commercial syrups. *J Agric Food Chem,* 55, 7264-7269, 2007. DOI: 10.1021/jf070559j

7. Ruiz-Matute AI, Weiss M, Sammatora D, Finely J, Sanz ML: Carbohydrate composition of high-fructose corn syrups (HFCS) used for bee feeding: Effect on honey composition. *J Agric Food Chem,* 58, 7317-7322, 2010. DOI: 10.1021/jf100758x

8. Guler A, Kocaokutgen H, Garipoglu AV, Onder H, Ekinci D, Biyik S: Detection of adulterated honey produced by honeybee *(Apis mellifera* L.) colonies fed with different levels of commercial industrial sugar (C_3 and C_4 plants) syrups by the carbon isotope ratio analysis. *Food Chem,* 155, 155-160, 2014. DOI: 10.1016/j.foodchem.2014.01.033

9. Sammataro D, Weiss M: Comparison of productivity of colonies of honey bee, *Apis mellifera*, supplemented with sucrose or high fructose corn syrup. *J Insect Sci,* 13, 1-13, 2013. DOI: 10.1673/031.013.1901

10. Leblebici MJ, Leblebici F: Şeker ve Tatlandırıcılar. *Sugar Company*, NO: 1, 2011.

11. Farr SA, Yamada KA, Butterfield DA, Abdul HM, Xu L, Miller NE, Banks WA, Morley JE: Obesity and hypertriglyceridemia produce cognitive impairment. *Endocrinology*, 149, 2628-2636, 2008. DOI: 10.1210/en.2007-1722

12. Weiss M: Supplemental carbohydrates in apiculture: Effects upon honey bee (*Apis mellifera*) health and productivity. *MSc Thesis,* University of Arizona, Tuscon, 2009.

13. Ross AP, Bartness TJ, Mielke JG, Parent MB: A high fructose diet impairs spatial memory in male rats. *Neurobiol Learn Mem*, 92, 410-416, 2009. DOI: 10.1016/j.nlm.2009.05.007

14. Bogdanov S, Lullmann C, Martin P, Ohe WVD, Russmann H, Vorwohl G, Oddo PL, Sabatini AG, Marcazzan GL, Piro R, Flamini C, Morlot M, Lheretier J, Borneck R, Marioleas P, Tsigouri A, Kerkvliet J, Ortiz A, Ivanov T, D'Arcy B, Mossel B, Vit P: Honey quality and international regulatory standards: Review by the International Honey Commission. *Bee World,* 80, 61-69, 1999. DOI: 10.1080/0005772X.1999.11099428

15. Önder H, Güler A, Biyik S, Ekinci D, Garipoğlu AV, Kocaokutgen H: Origin estimation of honey samples by using constant and discriminative function coefficients of pure honey and honey produced by colonies feeding with different sugars. *Kafkas Univ Vet Fak Derg,* 22, 21-28, 2016. DOI: 10.9775/kvfd.2015.13586

16. Cotte JF, Casabianca H, Chardon S, Lheritier J, Grenier-Loustalo MF: Application of carbohydrate analysis to verify honey authenticity. *J Chromatog,* 1021, 145-155, 2003. DOI: 10.1016/j.chroma.2003.09.005

17. Bogdanov S, Lullmann C, Mossel BL, D'Arcy BR, Russmann H, Vorwohl G, Oddo L, Sabatini AG, Marcazzan GL, Piro R, Flamini C, Morlot M, Lheretier J, Borneck R, Marioleas P, Tsigour, A, Kerkvliet J, Ortiz A, Ivanov T, Vit P, Marti, P, von der Ohe W: Honey quality, methods of analysis and international regulatory standards: Review of the work of the International Honey Commission. *Mitt Lebensm Hyg,* 90, 108-125, 1999.

18. Sahinler N, Sahinler S, Gul A: Biochemical composition of honeys produced in Turkey. *J Apicultural Res,* 43, 53-56, 2004. DOI: 10.1080/00218839.2004.11101110

19. Eflein L, Raezke KP: Improved detection of adulteration by measuring differences between $^{13}C/^{12}C$ stable carbon isotope ratios of protein and sugar compound with a combination of elemental analyser-isotope ratio mass spectrometry and liquid chromatography-isotope ratio mass spectrometry ($\delta^{13}C$-EA/LC-IRMS). *Apidologie*, 39, 574-587, 2008. DOI: 10.1051/apido:2008042

20. Padovan GJ, De Jong D, Rodrigues LP, Marchini JS: Detection of adulteration of commercial honey samples by the 13C/12C isotopic ratio. *Food Chem,* 82, 633-636, 2003. DOI: 10.1016/S0308-8146(02)00504-6

21. Cabanero AI, Recio JL, Ruperez M: Liquid chromatography coupled to isotope ratio mass spectrometry: A new perspective on honey adulteration detection. *J Agric Food Chem,* 54, 9719-9727, 2006. DOI: 10.1021/jf062067x

22. Padovan GJ, Rodriques LP, Leme IA, Jong David D, Marchini JS: Presence of C_4 sugars in honey samples detected by the carbon isotope ratio measured by IRMS. *Eurasian J Analytical Chem,* 2, 134-141, 2007.

23. Tosun M: Detection of adulteration in honey samples added various sugar syrup with $^{13}C/^{12}C$ isotope ratio analysis method. *Food Chem,* 138, 1629-1632, 2013. DOI: 10.1016/j.foodchem.2012.11.068

24. Zábrodská B, Vorlová L: Adulteration of honey and available methods for detection: A review. *Acta Vet Brno,* 83, 85-102, 2014. DOI: 10.2754/avb201483S10S85

25. Guler A: The effects of the shook swarm technique on honey bee *(Apis mellifera L.)* colony productivity and honey quality. *J Apicultural Res,* 47, 27-34, 2008. DOI: 10.1080/00218839.2008.11101420

26. AOAC: Official Methods of Analysis. 16th edn., Chapter 44, 27-29, USA: Association of Official Analytical Chemist, Arlington, VA, 1998.

27. DIN 10758: Analysis of Honey - Determination of the content of saccharides fructose, glucose, saccharose, turanose and maltose-HPLC method, 1997.

28. Onder H: Using permutation tests to reduce type I and II errors for small ruminant research. *J Appl Anim Res,* 32, 69-72, 2007. DOI: 10.1080/09712119.2007.9706849

29. SAS Institute: SPSSx user's guide. McGraw-Hill; New York, USA, 806 pp, 2004.

30. Council Directive: 2001/110/EC of 20 December (2001) relating to honey. Official Journal of the European Communities, OJ L, 109: 29 pp, 2002.

31. Turkish Food Codex: Honey Codex. No.28366, Ministry of Food and Agriculture, 2012.

32. Basoglu FN, Sorkun K, Löker M, Dogan C, Werherilt H: Identification of pure and adulterated honeys by using physical, chemical and palynological criteria. *Gıda,* 21, 67-73, 1996.

33. Ozcan M, Arslan D, Ceylan DA: Effect of inverted sucrose on some properties of honey. *Food Chem,* 99, 24-29, 2006. DOI: 10.1016/j.foodchem.2005.07.009

34. Bogdanov S, Ruoff K, Oddo LP: Physico-chemical methods for the characterisation of unifloral honeys: A review. *Apidologie,* 35, 4-17, 2004. DOI: 10.1051/apido:2004047

35. Piazza MG, Oddo LP: Bibliographical review of the main European unifloral honeys. *Apidologie,* 35, 94-111 2004. DOI: 10.1051/apido:2004046

36. Oddo LP, Piro R, Bruneau E, Decklerck CG, Ivanov T, Piskulova J,

Flamini C, Lheritier J, Morlot M, Russmann H, Ohe WVD, Ohe KVD, Gotsiou P, Karabournioti S, Kefalas P, Katrali MP, Thrasyvoulou A, Tsigouri A, Marcazzan GL, Piana ML, Piazza MG, Sabatini AG, Kerkvliet J, Godinho J, Bentabol A, Valbuena AO, Bogdanov S, Ruoff K: Main European unifloral honeys: Descriptive sheets1. *Apidologie,* 35, 38-81, 2004. DOI: 10.1051/apido:2004049

37. Sahinler N: Effects of heating and storage on Hydroxylmethylfurfurol and diastase activity of different Turkish honeys. *J Apicultural Res*, 46, 34-39, 2007.

38. Visquert M, Vargas M, Escriche I: Effect of postharvest storage conditions on the colour and freshness parameters of raw honey. *Int J Food Sci Tecnol,* 49, 181-187, 2014. DOI: 10.1111/ijfs.12296

39. Terrab A, Diez MJ, Heredia FJ: Characterisation of Moroccan unifloral honeys by physicochemical characteristics. *Food Chem,* 79, 373-379, 2002. DOI: 10.1016/S0308-8146(02)00189-9

40. Devillers J, Morlot M, Pham-Deleque MH, Dore JC: Classification of monofloral honeys based on their quality control data. *Food Chem,* 86, 305-312, 2004. DOI: 10.1016/j.foodchem.2003.09.029

41. Fernandez-Torres R, Perez-Bernal JL, Bello-Lopez MA, Callejon-Mochon M, Jimenez-Sanchez JC, Guiraum-Perez A: Mineral content and botanical origin of Spanish honeys. *Talanta*, 65, 686-691, 2005. DOI: 10.1016/j.talanta.2004.07.030

42. Conti ME, Finoia MG, Fontana L, Mele G, Botre F, Lavicoli I: Characterization of Argentine honeys on the basis of their mineral content and some typical quality parameters. *Chem Centl J,* 8, 44, 2014. DOI: 10.1186/1752-153X-8-44

43. White JWJR, Winters K, Martin P, Rossmann A: SCIRA of honey: Validation of internal standard procedure for worldwide application. *AOAC*, 81, 610-619, 1998.

44. Ruoff K, Iglesias MT, Luginbühl W, Bosset JO, Bogdanov S, Amado R: Quantitative analysis of physical and chemical measurands in honey by mid-infrared spectroscopy. *Eur Food Res Technol*, 223, 22-29, 2006. DOI: 10.1007/s00217-005-0085-z

45. Lichtenberg-Kraag B: Evidence for correlation between invertase activity and sucrose content during the ripening process of honey. *J Apicultural Res,* 53, 364-373, 2014. DOI: 10.3896/IBRA.1.53.3.03

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Research Article

Pathomorphological and Immunohistochemical Studies of Tumours in the Urinary Bladders of Water Buffalo in Marmara, the Central and Western Black Sea Region of Turkey [1]

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Abstract

In this study, non-neoplastic and neoplastic lesions in the urinary bladders of water buffalo were determined in the Marmara, the central and western part of the Black Sea region of Turkey. In this context, water buffalo with lesions in the urinary bladder were obtained from the private and public slaughterhouses of several provinces. The lesions were evaluated pathologically and immunohistochemically. In addition, species of bracken ferns were identified in these provinces. Histopathologically, 39 of the collected tissue samples were diagnosed as neoplasia. These neoplasms were classified and graded according to The World Health Organization Classification of Tumours published in 2004. In this context, papilloma (16 cases), papillary urothelial neoplasm of low malignant potential (PUNLMP) (7 cases), low-grade papillary carcinoma (13 cases), high-grade papillar carcinoma (2 cases), and low-grade papillary carcinoma with hemangiosarcoma (1 case) were detected in samples diagnosed as neoplasia. In the collected bracken ferns, *Athyrium filix-foemina* (L.) Roth; *Dryopteris dilatata* (Hoffm.) Gary; *Dryopteris filix-mas* (L.) Schott; *Polystichum aculeatum* (L.) Roth; *Polystichum setiferum* (Forsk.) Woynar; *Polystichum woronowii* Fomin, *Polypodium vulgare* L. and *Pteridium aquilinum* (L.) Kuhn spp. were identified. As a result, it has been understood that the consumption of identified bracken ferns leads more frequently to epithelial neoplasms.

Keywords: *Bracken fern, Chronic enzootic haematuria, Neoplasia, Tumour, Pteridium spp, Water buffalo*

Türkiye'de Marmara, Orta ve Batı Karadeniz Bölgelerinde Bulunan Mandaların İdrar Keselerinde Rastlanılan Tümörlerin Patomorfolojik ve İmmunohistokimyasal Yönden İncelenmesi

Özet

Çalışmada, Marmara, Orta ve Batı Karadeniz Bölgelerinde bulunan mandaların idrar keselerinde saptanan neoplazik ve neoplazik olmayan bulgular değerlendirildi. Orta, Batı Karadeniz ve Marmara Bölgelerindeki bazı illerde bulunan özel ve kamu kurumu mezbahalarında kesilen mandaların idrar keselerinde rastlanılan lezyonlar patolojik ve immunohistokimyasal yönden incelendi. Ayrıca hastalığın bulunduğu bölgelerdeki hayvanların otladıkları yerlerde doğal olarak yetişen eğrelti otları toplanarak tür teşhisi yapıldı. Çalışma sonucunda toplanan idrar keselerinin histolojik incelenmesinde; otuzdokuz adet neoplazi görüldü. Bu neoplazilerin tanısı Dünya Sağlık Teşkilatı'nın 2004 yılındaki tümör klasifikasyonuna göre yapıldı. Histolojik incelemelerde; otuzdokuz adet neoplazik idrar keselerinin onaltı adeti papillom; yedi adeti düşük malignite potansiyelli papiller ürotelyal neoplazi, onüç adeti düşük dereceli papiller karsinom, iki tanesi yüksek dereceli papiller karsinom ve bir tane ise hemangiosarkom görülmüştür. Düşük dereceli papiller karsinom görülen idrar keselerinden bir tanesinde aynı zamanda hemangiosarkoma da rastlandı. Çalışma sonucunda toplanan eğrelti otlarında ise; *Athyrium filix-foemina* (L.) Roth; *Dryopteris dilatata* (Hoffm.) Gary; *Dryopteris filix-mas* (L.) Schott; *Polystichum aculeatum* (L.) Roth; *Polystichum setiferum* (Forsk.) Woynar; *Polystichum woronowii* Fomin; *Polypodium vulgare* L. ve *Pteridium aquilinum* (L.) Kuhn türleri saptandı. Sonuç olarak, manda idrar keselerinde türleri tayin edilen eğrelti otlarının tüketilmesiyle daha çok epitelyal neoplazilerin tetiklendiği anlaşılmıştır.

Anahtar sözcükler: *Eğrelti otu, Kronik enzootik hematüri, Manda, Neoplazi, Tümör, Pteridum spp.*

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INTRODUCTION

Enzootic haematuria syndrome has occurred in cattle and water buffalo after they have grazed for an extended period of time on the natural plant cover of an area that is infested with bracken fern. The bracken ferns contain toxic chemicals. The syndrome is primarily encountered in Brazil, China, Italy, Japan, Portugal, Turkey, and New Zealand [1-9]. Haematuria is a clinical finding of chronic enzootic haematuria appearing sporadically and attracting attention permanently. Anaemia and weight loss are also amongst the common clinical findings [2,8]. The long process of digestion triggers the syndrome in cattle and buffalo over the age of two [5,10,11]. It has been determined that different types of ferns, e.g. *Pteridium aquilinum, Pteridium esculentum, Cheilantes sieberi, Cheilantes farinosa, Christella dentata, Polystichum squarrosum, Dryopteris juxtaposita,* etc. are found in the areas where the syndrome is encountered [12-14]. In the flora of Turkey are found 16 families, 24 genuses, and 79 taxa (species and sub-species) of ferns. The ferns thrive in humid and shady areas, in forest underbrush, alongside streams, and in rock fissures. The ferns grow in every region of Turkey, especially in the Black Sea region [15,16].

Ferns which builded up the largest part of sporophytes live mostly on land and are autotrophs. However, they need water for insemination. They are standing out mostly with their green leave alike structures which are great sporophyte mostly pinnate leaves. Green leaved alike sporophytes are doing photosynthesis and carry bags producing spor and providing proliferation [17]. It has been documented that bracken ferns contain ptaquiloside and quercetin. It is also suggested that the ferns can have carcinogenic, mutagenic, and clastogenic effects [1,11]. Among them, bracken fern comprises predominantly major toxin of ptaquiloside [18]. This toxin cause errors in DNA synthesis (especially, H-ras expression) and activation of proto-oncogenes which are controlled under cell division mechanism [4,19,20]. Because disruption of signalling cascades in the cells, this situation creates any changes in morphological diversity and biological behaviour of the cells and consequently leads to unbalanced tissue microenvironment [21,22].

Cystitis, epithelial hyperplasia, and hydropic degeneration are mentioned as non-neoplastic findings of the syndrome. Neoplastic findings of mesenchymal origin (hemangioma, hemangiosarcoma, fibroma, and myxoma) and epithelial origin (papilloma and transitional cell carcinoma) are reported [2,5,6].

In this study, it was aimed to reveal neoplastic and non-neoplastic lesions in urinary bladder of water buffaloes and to show types of bracken fern, which found naturally in the habitat of Marmara, the Central and Western Black Sea Regions of Turkey ,and to proven the possible relationship between urinary bladder tumours and this herb.

MATERIAL and METHODS

The urinary bladders of water buffalo aged three through eight years and of both genders were collected from slaughterhouses in İstanbul, Adapazarı, Bolu, and Samsun in which chronic enzootic haematuria was previously detected.

Pathomorphological and Immunohistochemical Examinations

In total, 163 urinary bladders with lesions were evaluated. The bladders were fixed in 10% neutral formalin and evaluated macroscopically in the Department of Pathology, Faculty of Veterinary Medicine, at the University of Ankara. Portions of lesions were taken from the samples, subjected to routine tissue processing, and embedded in paraffin. Tissue sections were cut at a thickness of 5-6 µm and then stained with a specific haematoxylin and eosin.

For immunohistochemistry, the streptavidin- biotin-peroxidase complex (GBI SPlink HRP Broad Bulk Kit, D01-110) method was used. All sections were cut by microtome and adhered to positively charged slides All slides were deparaffinized, hydrated, and put into citrate buffer pH 6.0 for antigen retrieval. An 800 watt microwave pressure cooker was run for 20 min. The primary antibodies used for urothelium were Lifespan (LS-C40107/5F161) and mouse monoclonal anti-Uroplakin III antibody (1/10 dilution, 60 min at RT) and for endothelium Santa Cruz (SC-1506R) and rabbit polyclonal CD31 antibody (1/400 dilution, 60 min at RT). Then, biotinized secondary antibody and streptavidin-peroxidase were dropped onto the tissue sections. After this process, sections were visualised with 3-amino-9-ethylcarbazole (AEC, C01-12, GBI) chromogen. The background was colourised with Gill's (I) Hematoxylin. Slides were covered with aqueous mounting medium.

Drying Collected Plant Samples and Performing Species Identification

Samples of bracken fern were collected from pastured areas in which chronic enzootic haematuria cases had been previously detected during field studies.

RESULTS

Macroscopic Results

Diffuse and petechial hemorrhages were observed in some of the urinary bladders. Varying sizes of the white foci were seen on the surface of the bladder, some of which had the appearance of cauliflower *(Fig. 1)*. In one case, the urinary bladder was filled with blood. The bladder reached a size 6-7 times larger than normal. It had a very thin wall and a dark blackish color.

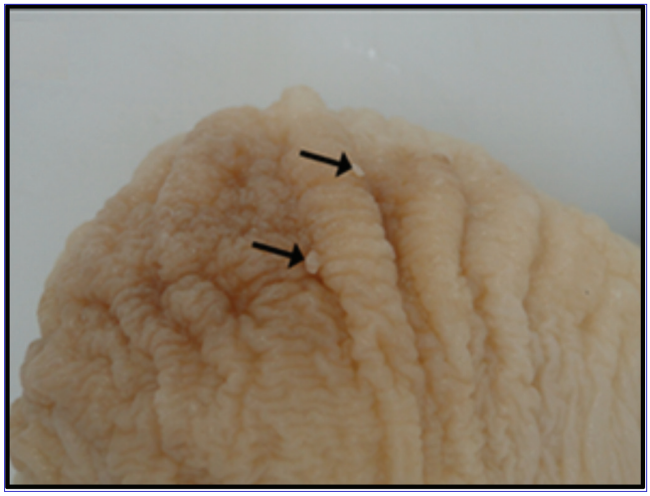

Fig 1. White papillomatous foci on the surface of the bladder *(arrows)*

In some cases, mononuclear cell infiltrations composed of lymphocytes and plasma cells were seen on the epithelium of the bladders.

In 39 cases, neoplasia was observed. These neoplasms were diagnosed according to The World Health Organization Classification of Tumours published in 2004. According to this classification, 43.8% of all tumours were papilloma; 17.9% were papillary urothelial neoplasms of low malignant potential (PUNLMP); 33.3% were low-grade papillary carcinoma; 5.1% were high-grade papillar carcinoma; and 2.5% were hemangiosarcoma. In one urinary bladder, low-grade papillary carcinoma and also hemangiosarcoma was observed *(Table 1)*.

An immunohistochemical technique was used to support the diagnosis of possible neoplasms. Immuno-

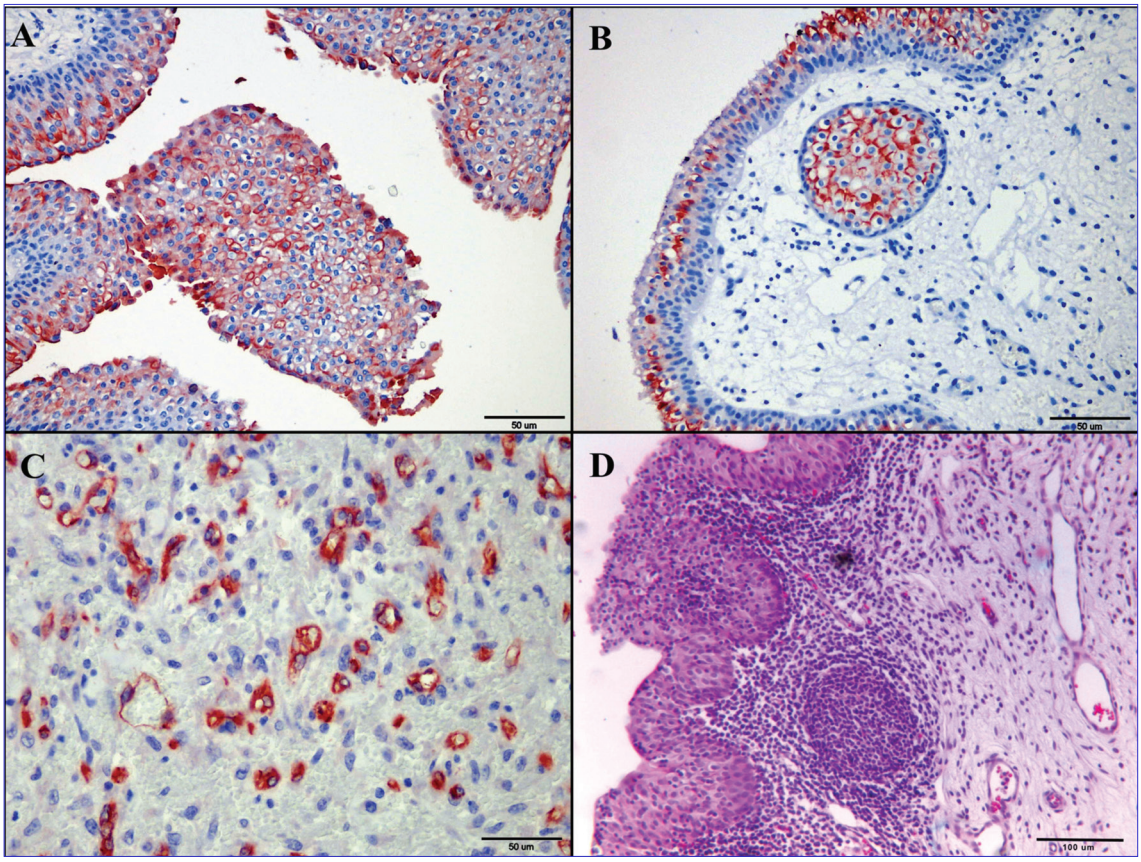

Fig 2. A- Positive staining with UPIII of Papilloma, **B**- Positive staining with UPIII of Brunn's nest, **C**- Positive staining with CD31 of hemangiosarcoma, **D**- Follicular cystitis

Histopathological and Immunohistochemical Results

In histopathology, chronic cystitis was observed in 67 urinary bladders. 74.6% of these bladders with cystitis had lesions like aggregate lymph follicle in propria mucosa. These were diagnosed as follicular cystitis. They also were associated with severe inflammation *(Table 1), (Fig. 2-D)*.

histochemically, positive staining by UPIII was observed at the cytoplasms of the umbrella and intermediate cells. However, the umbrella cells reacted more than the intermediate cells *(Fig. 2-A)*. The cells located in the center of the Brunn's nest stained stronger than the peripheral cells *(Fig. 2-B)*. In one case, positive staining by CD31 was observed at the cytoplasms of the tumour endothelial cells *(Fig. 2-C)*.

Table 1. *Histopathological findings of 67 urinary bladders with chronic enzootic hematuria*

Epithelium of the Urinary Bladder							
Desquamation	**Hydropic degeneration**	**Hyperplasia**	**Inflammatory cells on the epithelium**	**Haemorrhagia on the epithelium**	**Ulcer**	**Cystitis glandularis**	**Cystitis cystica**
5	58	81	4	1	7	14	2
Epithelium of the Urinary Bladder (Urothelial lesions)				**Propria mucosa of Urinary Bladder**			
Papilloma	**Papillary urothelial neoplasm of low malignant potential (PUNLMP)**	**Low grade papillary carcinoma**	**High grade papillar carcinoma**	**Inflammation**	**Lymph follicle**	**Hemorragia**	**Vascularisation**
16	7	13	2	65	49	44	64

Findings Regarding Species Determination

It was observed that the water buffalo did not eat the bracken ferns by design, but consumed them incidentally while grazing on grasslands. The identified species of fern were *Athyrium filix-foemina* (L.) Roth, *Dryopteris dilatata* (Hoffm.) Gary, *Dryopteris filix-mas* (L.) Schott, *Polystichum aculeatum* (L.) Roth, *Polystichum setiferum* (Forsk.) Woynar, *Polystichum woronowii* Fomin, *Polypodium vulgare* L., *Pteridium aquilinum* (L.) Kuhn.

DISCUSSION

Enzootic haematuria cases have been described in cattle that graze on fern-covered areas of Turkey [5,6]. However, there are limited studies regarding water buffalo with enzootic haematuria [5,23-26]. In this study, we determined the age of the water buffalo to be between three and eight years old, which refers to the possible age of the chronic enzootic haematuria syndrome. Pamukcu [6], Ozkul and Aydın [5] also studied this syndrome in cattle after a period of life that was an old age.

In our study, 76 cases had chronic cystitis. However, it was not the acute cystitis previously reported by Somvanshi et al.[25] and mentioned in their study. Of that study's subjects, 53.7% had epithelial hyperplasia (flat/papillary) and 32.8% had hydropic degeneration. 8.9% of the total subjects had lymphocyte and plasma cell infiltrations on the epithelium of the urinary bladder. In terms of the chronic cystitis, 74.6% of cases were diagnosed as follicular cystitis which was formed like aggregate lymph follicles in propria mucosa. It was commented that severe inflammation had developed. However, Aydın and Ozkul [27] worked on urinary bladders both of cattle and water buffalo which had non-neoplastic findings and determined only 20% lymphoid cell aggregation in propria mucosa of the urinary bladders. The recent study pointed out the significant aggregate lymph follicles in more subjects than the previous study. Also, 21 of the chronic cystitis cases had haemorrhages that were in accordance with other studies [6,26].

As a result of the histopathological examination of the urinary bladders, we observed 39 neoplasia that were classified according to The World Health Organization Classification of Tumours published in 2004 [28]. 43.8% of all tumours were papilloma; 17.9% were papillary urothelial neoplasm of low malignant potential (PUNLMP); 33.3% were low-grade papillary carcinoma; 5.1% were high-grade papillar carcinoma; and 2.5% were hemangiosarcoma.

In previous studies, mesenchymal tissue tumours were more frequently reported than epithelial tissue tumours in cattle [2,5,9,24]. However, we observed that epithelial tissue tumours occurred more frequently than mesenchymal tumours in water buffalo. Our data also correspond to others [6,24].

Immunohistochemically, examination of the tumours of water buffalo with chronic enzootic haematuria and pathologically diagnosed as urothelial epithelial cell tumours, just similar to the findings of Carvalho et al.[29]. We also observed that the umbrella cells were intensely stained with UPIII and, unlike the umbrella cells, the intermedial cells were weakly stained. In addition, the cytoplasms of the tumour endothelial cells of the urinary bladders with hemangiosarcoma were stained with CD31, and those results were also in accordance with Carvalho et al.[29].

Many varieties of bracken ferns grow around the world. The types detected in India, Athyrium, Dryopteris, Polystichum, and Pteridium, were also found in our working field [14,30]. Some types of bracken ferns, such as *P. aquilinum*, commonly grow across a wide area of Turkey and are the cause of chronic enzootic haematuria.

Water buffalo, unlike cattle which are bred intensively, require extensive breeding. As a result of their need to graze in large pasturelands, water buffalo consume many types of grasses in addition to bracken ferns, which can cause the syndrome called chronic enzootic haematuria. This study will be informative for all researchers and breeders.

REFERENCES

1. Borzacchiello G, Ambrossio V, Galati P, Poggiali F, Venuti A, Roperto F: The pagetoid variant of urothelial carcinoma in situ of urinary bladder. *Vet Pathol*, 38, 113-116, 2001. DOI: 10.1177/0300985812469632

2. Carvalho T, Pinto C, Peleteiro MC: Urinary bladder lesions in bovine

enzootic haematuria. *J Comp Path*, 134, 336-346, 2006. DOI: 10.1016/j.jcpa.2006.01.001

3. Dawra RK, Sharma OP: Enzootic bovine haematuria-past,present and future. *Vet Bull*, 71, 10, 2001.

4. Lioi MB, Barbieri R, Borzacchiello G, Dezzi S, Roperto S, Santoro A, Russo V, Roperto F: Chromosome aberrations in cattle with chronic enzootic haematuria. *J Comp Path*, 131, 233-236, 2004. DOI: 10.1016/j.jcpa.2004.01.011

5. Ozkul IA, Aydin Y: Tumours of the urinary bladder in cattle and water buffalo in the black sea region of Turkey. *Br Vet J*, 152, 473-475, 1996. DOI: 10.1016/S0007-1935(96)80041-8

6. Pamukcu AM: Tumors of the urinary bladder in cattle and water buffalo with enzootic bovine hematuria. *Zentralb Veterinarmed,* 4, 185-197, 1957. DOI: 10.1111/j.1439-0442.1957.tb00464.x

7. Peixoto PG, França TN, Barros CSL, Tokarnia CH: Histopathological aspects of bovine enzootic hematuria in Brazil. *Pesq Vet Bras*, 23, 65-81, 2003. DOI: 10.1016/j.jcpa.2010.11.002

8. Smith BL, Beatson NS: Bovine enzootic haematuria in New Zealand. *New Zealand Vet J*, 115-118, 1970. DOI: 10.1080/00480169.1970.33878

9. Xu LR: Bracken poisoning and enzootic haematuria in cattle in China. *Res Vet Sci*, 53, 1, 116-121, 1992. DOI: 10.1016/0034-5288(92)90095-J

10. Pamukcu AM: IV. Tumours of the urinary bladder. *Bull Wld Hlth Org*, 50, 43-52, 1974.

11. Peretti V, Ciotola F, Albarella S, Russo Di Meo GP, Iannuzzi L, Roperto F, Barbieri V: Chromosome fragility in cattle with chronic enzootic haematuria. *Mutagenesis*, 22, 317-320, 2007. DOI: 10.1093/mutage/gem019

12. Dawra RK, Kurade NP, Sharma OP: Carcinogenicity of the fern *Pteridium aquilinum* collected from enzootic bovine haematuria-free hilly area in India. *Curr Sci*, 83, 8,1005-1009, 2002.

13. Smith BL, Embling PP, Lauren DR, Agnew MP, Ross AD, Greentree PL: Carcinogen in rock fern *(Cheilanthes sieberi)* from New Zealand and Australia. *Australian Vet J*, 66, 5, 154-155, 1989. DOI: 10.1111/j.1751-0813.1989.tb09785.x

14. Somvanshi R, Lauren DR, Smith BL, Dawra RK, Sharma OP, Sharma VK, Singh AK, Gangwar NK: Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. *Curr Sci*, 91, 11, 1547-1552, 2006.

15. Davis PH: Flora of Turkey and The East Aegean Islands. Edinburgh University Press, Edinburgh, 31-62, 1965.

16. Davis, PH, Mill RR, Tan K: Flora of Turkey and The East Aegean Islands, Vol: 2-10, Edinburgh University Press, Edinburgh, 1965-1988.

17. Tutel B, Çirpici A: Sporlu Bitkiler Sistematiği Laboratuar Kılavuzu. İstanbul Üniversitesi Fen Fakültesi Basımevi, İstanbul, 1986.

18. Shahin M, Moore MR, Worrall S, Smith BL, Seawright AA, Prakash AS: H-ras activation is an early event in the ptaquiloside-induced carcinogenesis: Comparison of acute and chronic toxicity in rats. *Biochem Biophys Res Commun*, 250, 491-497, 1998. DOI: 10.1006/bbrc.1998.9341

19. Prakash AS, Pereira TN, Smith BL, Shaw G, Seawright AA: Mechanism of bracken fern carcinogenesis: Evidence for H-ras activation via initial adenine alkylation by ptaquiloside. *Nat Toxins*, 4, 221-227, 1996. DOI: 10.1016/S0022-5347(01)63876-X

20. Sardon D, de la Fuente I, Calonge E, Perez-Alenza MD, Castano M, Dunner S, Pena L: H-ras immunohistochemical expression and molecular analysis of urinary bladder lesions in grazing adult cattle exposed to bracken fern. *J Comp Path*, 132, 195-201, 2005. DOI: 10.1016/j.jcpa.2004.09.011

21. Friedl P, Wolf K: Tumour-cell invasion and migration: Diversity and escape mechanisms. *Nat Rev Cancer*, 3, 362-374, 2003. DOI: 10.1038/nrc1075

22. Kramer RH, Shen X, Zhou H: Tumor cell invasion and survival in head and neck cancer. *Cancer Metastasis Rev*, 24, 35-45, 2005. DOI: 10.1007/s10555-005-5046-2

23. Gupta PP, Singh B: Infiltrative transitional cell carcinoma of urinary bladder in a water buffalo. *Vet Pathol*, 12, 468-469, 1975.

24. Pamukcu AM, Bryan GT: Pathology of the bovine urinary bladder tumors. *Fırat Üniv Vet Fak Derg*, 3 (3) : 27-44, 1976.

25. Somvanshi R: Papillomatosis in buffaloes: A less-known disease. *Emerg Infect Dis*, 58, 327-332, 2011. DOI: 10.1111/j.1865-1682.2011.01211.x

26. Somvanshi R, Pathania S, Nagarajan N, Pangty K, Kumar P: Pathological study of non-neoplastic urinary bladder lesions in cattle and buffaloes: A preliminary report. *Trop Anim Health Prod*, 44, 4, 855-61, 2011. DOI: 10.1007/s11250-011-9978-y

27. Aydın Y, Ozkul IA: Non-neoplastic findings of the urinary bladder in cattle and water buffalo in the black sea region of Turkey. *Ankara Üniv Vet Fak Derg,* 42, 189-192, 1995.

28. Miyamoto H, Miller SJ, Fajardo DA, Lee TK, Netto GJ, Epstein JL: Non-invasive papillary urothelial neoplasms: The 2004 WHO/ISUP classification system. *Path Int*, 60, 1-8, 2010. DOI: 10.1111/j.1440-1827.2009.02477.x

29. Carvalho T, Naydan D, Nune T, Pinto C, Peleterio MC: Immuno-histochemical evaluation of vascular urinary bladder tumors from cows with enzootic hematuria. *Vet Pathol*, 46, 2, 211-221, 2009. DOI: 10.1354/vp.46-2-211

30. Pathania S, Kumar P, Singh S, Khatoon S, Rawat AKS, Punetha N, Jensen DJ, Lauren DR, Somvanshi R: Detection of ptaquiloside and quercetin in certain Indian ferns. *Curr Sci*, 102 (12): 1683-1691, 2012.

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Research Article

Age Related Histopathological and Immunohistochemical Changes in Horse Brains [1]

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Abstract

The aim of this study was to investigate age dependent pathological changes in horse brains. In the study, 10 mature horses aged between 10-16 years, 13 old horses aged between 17-21 years old and 7 young horses between 4-7 years old were examined macroscopically, histopathologically and immunohistohemically. As compared young horse brains; ventricular dilatation, satellitosis, neuronal vacuolization, status spongiosis, ventricular dilatation and ependymal undulation, calcium deposits and axonal swellings were seen in old horse brains. Immunohistochemical staining was obtained with Glial Fibrillary Acidic Protein (GFAP), 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase), neuron-specific enolase (NSE), β-amyloid protein (Aβ) antibodies to indicate age related changes in brains.

Keywords: *Brain, Histopathology, Immunohistochemistry, Old horse*

Atlarda Yaşla İlgili Beyinde Oluşan Histopatolojik ve İmmunohistokimyasal Değişiklikler

Özet

Bu çalışmanın amacı at beyinlerinde yaşa bağlı oluşan patolojik bulguların araştırılmasıdır. Sunulan çalışmada 10 adet genç at; yaşları 10-16 arasında, 13 adet yaşlı at; yaşları 17-21 arasında ve 7 adet genç at; yaşları 4-7 arasında (3-6 yaş arası) atlara ait beyinler makroskobik, histopatolojik ve immunohistokimyasal olarak incelendi. Genç atlara ait beyinlerle kıyaslandığında, yaşlı atlara ait beyinlerde makroskobik olarak ventriküler dilatasyon dikkat çekerken mikroskobik olarak satellitozis, nöronal vakuolasyon, status spongiozis, ventriküler genişleme ve ependimal ondülasyon, lipofuksin pigmenti, kalsiyum birikimleri ve aksonal şişmeler gözlendi. İmmunohistokimyasal boyamalarda yaşlı atların beyinlerinde Glial Fibrillary Acidic Protein (GFAP), ubiquitin, 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase), neuron-specific enolase (NSE), β-amyloid protein (Aβ), tirozin hidroksilaz antikorları ile pozitif boyanmalar gözlendi.

Anahtar sözcükler: *Beyin, Histopatoloji, Immunohistokimya, Yaşlı at*

INTRODUCTION

With the improvements in diet, management and care in horses can allow them to live as long as humans. The average life expectancy of horses is about 24 years. Although 20 years old horses are considered to be 'geriatric', many of them are still physically fit when they reach this age [1]. The brains of the aging dogs and cats show pathologic changes similar to those that occur in people with Alzheimer's disease. Equine dementia has not been well-described in the horse but age-related changes that occur in horse brains, were similar to other species [2].

Aging brains undergo many changes and these are described in detail in human beings [3-5] and in many animal species [6-10]. Neuroaxonal dystrophy, calcification and inflammation are the common changes in the aging brains, pathogenesis and functional consequences of

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these changes are not fully understood [11-14]. Lipofuscin and hemosiderin pigments, calcium deposits, satellitosis [11] neuroaxonal dystrophy, cerebrovascular disease [13,14] and Alzheimer type II cells were reported in previous studies [13] in the elderly horse brains. Furthermore, changes in the aging brain were demonstrated by some markers. In parallel with aging, astrocytes and glial changes demonstrate the neuroaxonal destruction and it can be showed with immunoperoxidase technique by using GFAP, CNPase and NSE. In some studies β-amyloid antibody is used to show amyloid accumulation in cerebral blood vessels, and it is the most common change of aging brain [15-19].

The aim of this study was to evaluate age-related pathological findings in horses in detail by routine and immunohistochemical staining methods; we also aimed to compare our findings with the previous studies on human beings and animal models.

MATERIAL and METHODS

The material of the study consists of 7 young horses aged between 4-7 years (3 female, 4 male) and 10 mature horses aged between 10-16 years (5 female, 5 male) and 13 old horses aged between 17-21 years old (7 female, 6 male) that were collected from the Jockey Clubs of Turkey, zoos and commoner, between the years of 2001-2014. Natural death due to senile/various diseases was observed in horses and they showed no signs of any neurological disease. The research project and animal housing conditions were approved by the Ethical Committee for Animal Studies (Approval 2010-3-11).

After death, necropsy was immediately done and brain samples were immediately removed and were fixed in 10% neutral buffered formalin and embedded in paraffin by routine methods. Brain sections that were taken from frontal, parietal, occipital, temporal lobes, pons, medulla oblongata, spinal cord and cerebellum, were cut 5 µm and stained with hematoxylin and eosin (H & E) [20]. Additionally tissues were de-waxed and rehydrated by routine methods for immunohistochemical staining. For immunostaining, room temperature was used and the streptavidin-biotin-peroxidase complex method by a commercial kit (Zymed, USA) was carried out. Antigen retrieval was heated in citrate buffer, pH 6.0, for 10 min in microwave oven at 800 W. Endogenous peroxidase activity in tissue sections was blocked by applying 0.3% hydrogen peroxide in 0.01 M PBS containing 10% methanol to block nonspecific binding, and then the sections were incubated with 5% normal goat serum (blocking reagent) prior to exposure to primary antisera in oven for 20 min at 40°C. Sections were incubated with the primary antibodies anti-GFAP antibody (Abcam, UK, ab7260) (1:500) for astrocytes, anti-CNPase antibody (Sigma-Aldrich, USA, C9743) (1:200) for oligodendrocytes, anti-NSE antibody (Sigma-Aldrich, USA, SAB4500768) (1:200) for neurons and anti-Aβ antibody (Sigma-Aldrich, USA, A8354) (1:300) for amyloid deposits; overnight at room temperature. The antibodies were diluated with PBS. After incubation with the rabbit anti-mouse biotinylated secondary antibody for 20 min, for color labelling the chromogen as aminoethyl carbazole (AEC) (Dako, Glostrup, Denmark) was administered for 5 min at room temperature. Finally, Mayer's hematoxylin was used for 1 min, washed under tap water and mounted with gelatinous glue. Following each incubation step, sections were washed thoroughly with PBS with the exception of the step after incubation with normal goat serum. All sections were examined by light microscopy (Olympus CX31). The findings were recorded with the support of the studies done previously and comparison was done. Finally, microphotographs were obtained by Olympus DP12. Previously known to be positive for all antibodies, tissue sections used served as the positive controls and for the negative controls, the primary antibody was omitted and replaced by PBS.

RESULTS

No gross changes were observed in the young horse brains, also microscopically all young animal brains showed normal histology structure and no staining was observed against antibodies.

Macroscopically in older horse brains only ventricular dilatation was noted (at ≥18 years old 5 cases, case nos: 19-23). Microscopically at some sections (Case Nos: 4, 11, 18) inflammatory cell infiltration, hemorrhage and hyperemia were observed and it was considered that they were not associated with aging. The main microscopic changes in all aged brains were gliosis, with neuronal degeneration and satellitosis (chromatolysis) *(Fig. 1)* and these changes were most seen in medial temporal lobe, amygdala and hippocampus. Additional main microscopic results

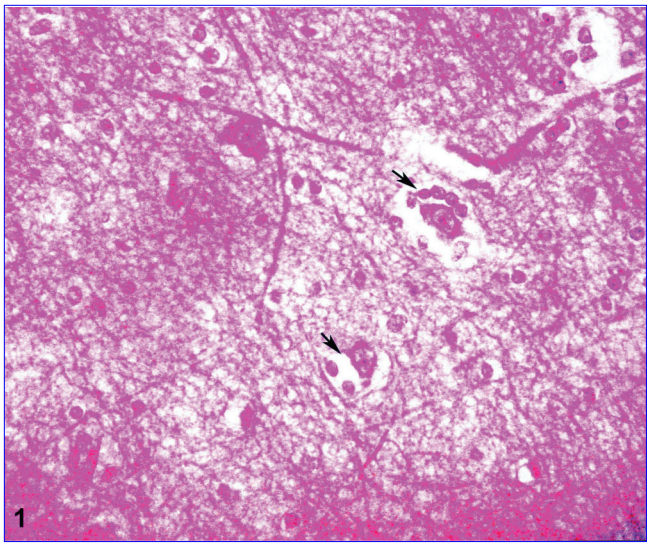

Fig 1. Satellitosis *(arrows)* and glia cell proliferation, H&E (Bar=12.5 µm)

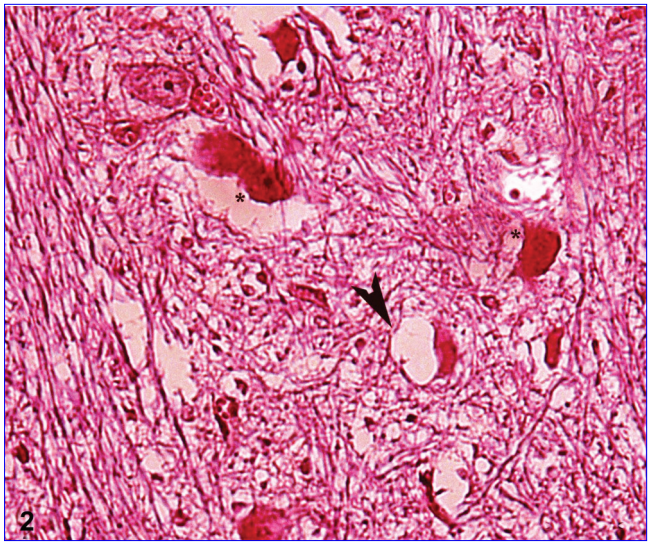

Fig 2. Neuronal vacuolation *(arrow head)* and perineural calcium deposits (*), H&E (Bar=12.5 µm)

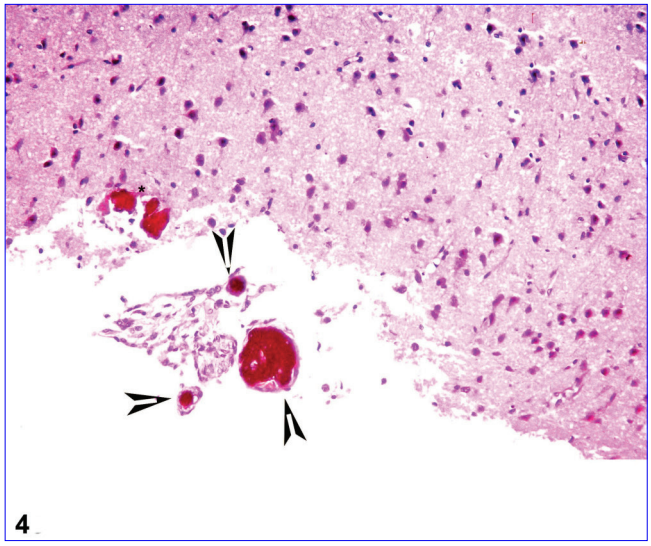

Fig 4. Ca^{++} deposits at subependymal region (*) and blood vessel lumens *(arrow heads),* H&E (Bar=125 µm)

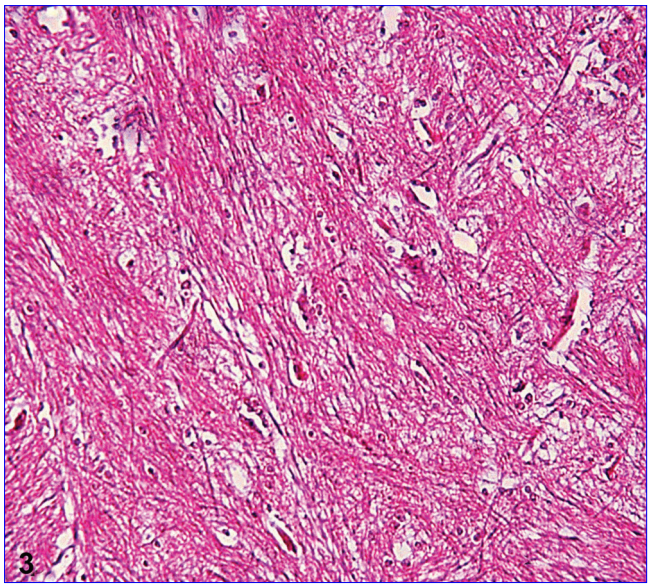

Fig 3. Status spongiosis at white matter, H&E (Bar=125 µm)

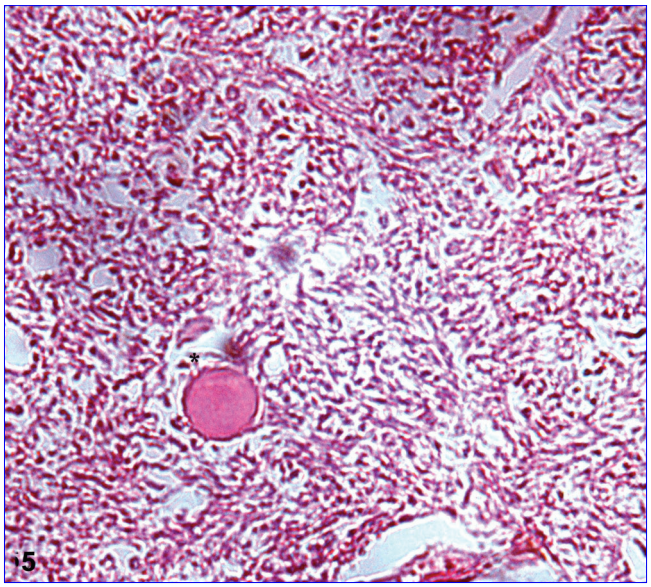

Fig 5. Axonal swelling (*), H&E (Bar=12.5 µm)

were neuronal vacuolation (one or more vacuoles with miscellaneous dimensions) (at ≥17 years old 12 cases) *(Fig. 2)* and remarkable white matter status spongiosis (at ≥16 years old 15 cases) *(Fig. 3)* observed in medulla oblongata and pons. Intraneuronal yellowish-brown lipofuscin pigment storage was commonly observed in cerebral cortex, thalamus and midbrain. Dark blue mineralization areas were detected in degenerated neurons with focal gliosis and these changes had no relationship with blood vessels. At five cases (Case Nos: 14, 18, 22, 23, 25) calcium deposits occurred free in subependymal parenchyma or totally filled the blood vessel walls *(Fig. 4)*. Focal axonal swelling (at cases 11, 14, 17- 20) *(Fig. 5)* with axonal calcification (Case Nos: 19, 21, 23) was observed at cerebral white matter and midbrain grey matter. At ≥18 years old aged brains (5 cases) ependymal convolution with inflammatory cell infiltration (mononuclear) was observed (Case Nos: 19-23). With aging, astrocytic glial cell proliferation increased and was demonstrated with GFAP antibody frequently in cerebral cortex, hippocampal area, thalamus, and cerebellar white matter *(Fig. 6)*. The increase of oligodendrocytes was detected by CNPase antibody *(Fig. 7)* in the white matter of cerebral and cerebellar cortex and like GFAP positive astrocytes with aging, the number of CNPase labelled oligodendrocytes was increased. NSE-positive immunoreaction was detected at degenerated neurons *(Fig. 8)* in basal nuclei, thalamus, hippocampal pyramidal neurons, cerebellar dentate nuclei and some nuclei of cerebral cortex. Amyloid deposits were observed at cerebral

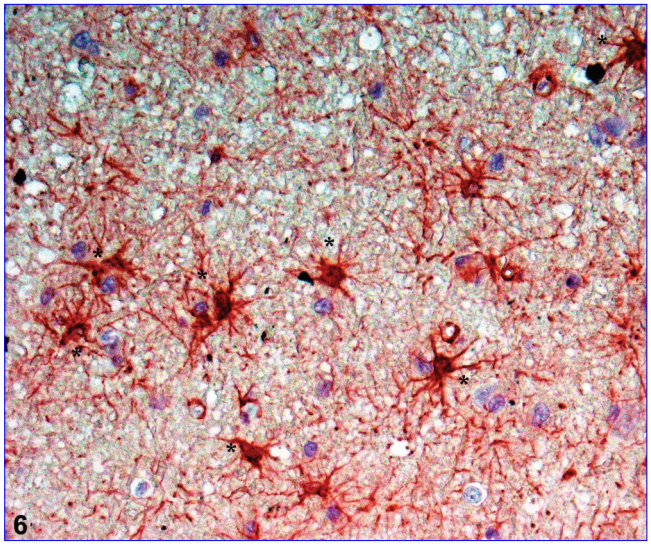

Fig 6. GFAP positive astrocytes (*), IHC (Bar=12.5 µm)

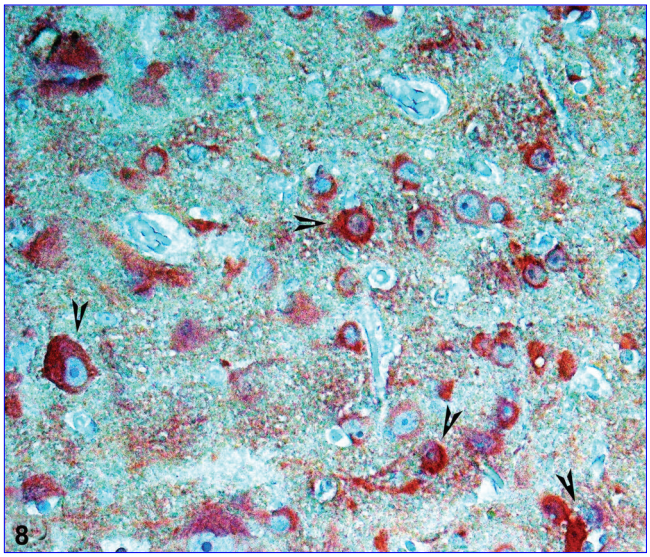

Fig 8. NSE positive neurons *(arrow heads)*, IHC (Bar=12.5 µm)

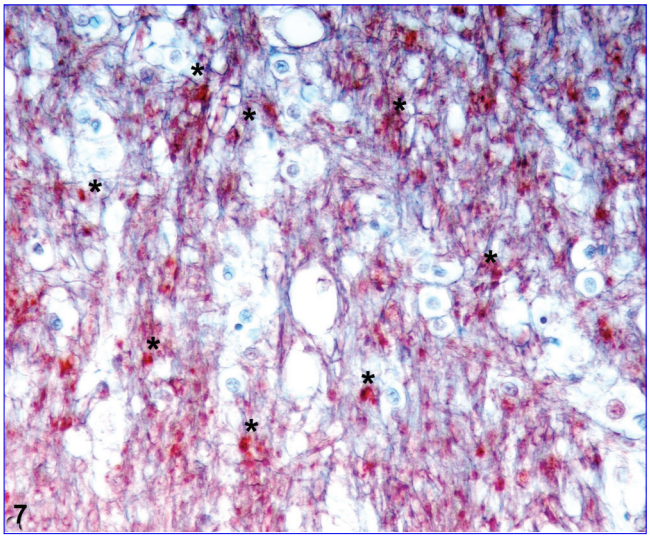

Fig 7. CNPase positive oligodendrocytes (*), IHC (Bar=125 µm)

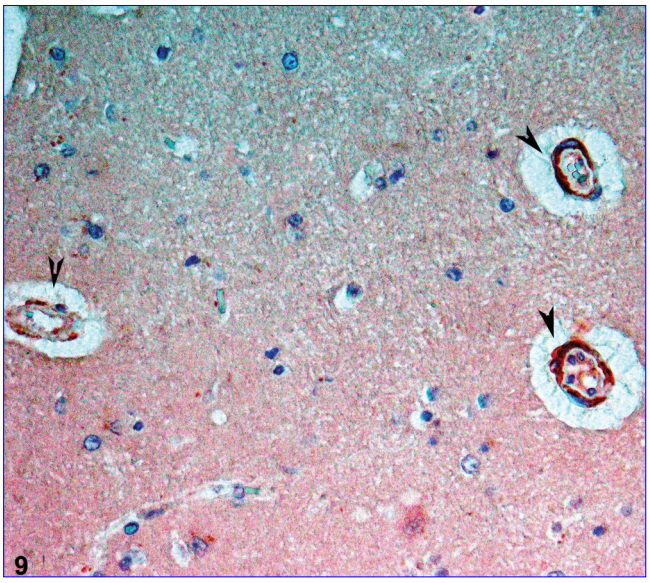

Fig 9. Aβ positive staining at cerebral vessel walls *(arrow heads)*, IHC (Bar=12.5 µm)

(Fig. 9) and meningeal blood vessels walls *(Fig. 10)*, and demonstrated by Aβ antibody. Amyloid was most often found in leptomeninges, neocortex, cerebellum and brain-stem vessels respectively. No senile plaque was observed. The main histopathological and immunohistochemical changes are summarized in *Table 1*.

DISCUSSION

Like in pet animals such as cats and dogs with correct maintenance, feeding, and good veterinary services, the lives of horses extend to the age 30's [21,22]. The average life span for a horse is considered to be 24 years [11].

It is considered that young horses' 1-year life span is equivalent to 3.5-year life span of people but the ratio varies with the aging of the horse, and it is said that 1-year life span is equivalent to 3-year life of people. Accordingly, it is accepted that about 10-year-old horse is equivalent to 35-year-old person while 20-year-old horse is equivalent to 60-year-old man. So, 20-year-old horse is considered as old [1,15].

Ventricular dilation (enlargement) is the most common macroscopic change of aging brain in people and it is shown by MR (cerebral magnetic resonance imaging scans) and tomography [3], but in animal species [21,22] this can be shown during the necropsy by macroscopic serial sections of the brain. In this study, macroscopically and microscopically ventricular dilatation and ependymal undulation with inflammation were noted; these results were previously reported in other animal species [8,10] but not in horses.

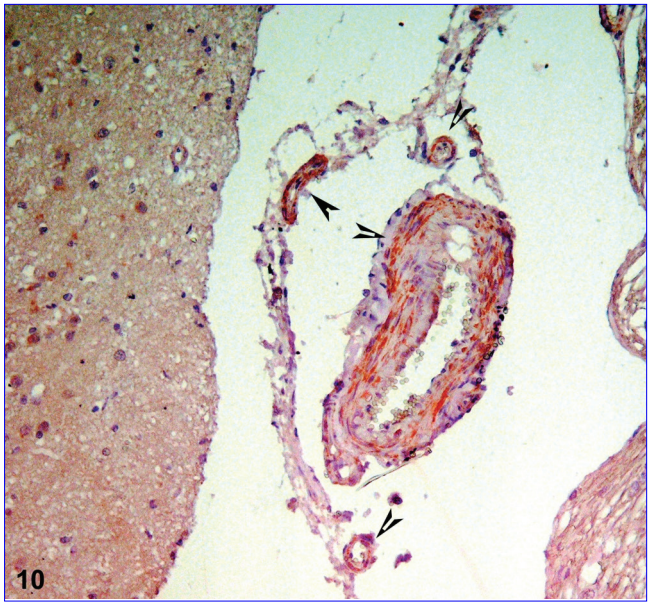

Fig 10. Aβ positive staining at meningeal blood vessel walls *(arrow heads)*, IHC (Bar=12.5 µm)

observed in all 10≥ years; we thought that these changes were related with aging.

Previous studies reported histopathological changes in aged animal brains like meningeal and choroid plexus fibrosis, hyalinization and fibrosis of the vessel walls [8,10] and cholesterol granulomas (cholesteatoma) that settled in the 4th ventriculus and causes hydrocephalus [11,13], but these findings were not shaped in this study. Neuronal lipofuscin pigmentation and mineralization areas (Ca^{++} deposits) with free accumulation of Ca deposits in the parenchyma were similar with previous studies [8,10,11,23,27]. Additionally to these, same mineralization occurred at axons.

It is known that with aging a certain neurodegeneration develops and during this astrogial activation, gliosis and disruption of myelin membranes occur [26,28,29]. In addition to routine H&E staining in the study, neurodegeneration was demonstrated by specific antibodies such as GFAP, CNPase and NSE. In this study, with aging (especially between the ages 18-23) response to neuronal degeneration, astroglial activation was increased and this leads to increased GFAP

Table 1. *Histopathological and immunohistochemical changes of the brain due to aging in horses.*

Lesion	Affected Areas	Horse Ages (Year)	Frequency
Gliosis	Medial temporal lobe, amiglada and hippocampus	12-21	19/23 (82.6%)
Neuronal degeneration	Medial temporal lobe, amiglada and hippocampus	13-21	18/23 (78.2%)
Satellitosis	Medial temporal lobe, amiglada and hippocampus	13-21	18/23 (78.2%)
Neuronal vacuolation	Medulla oblongata and pons	17-21	12/23 (52.1%)
White matter status spongiosis	Medulla oblongata and pons	16-21	15/23 (65.2%)
Lipofuscin pigment storage	Cerebral cortex, thalamus and midbrain	18-21	7/23 (30.4%)
Calcium deposits	Free in subependymal parenchyma/ blood vessel walls	17-21	5/23 (21.7%)
Axonal swelling	Cerebral white matter and midbrain grey matter	17-21	6/23 (26.1%)
Axonal calcification	Cerebral white matter and midbrain grey matter	18-21	3/23 (13.0%)
Ependymal convolution with inflammatory cell infiltration	Mesencephalic canal and lateral ventricles	18-21	5/23 (21.7%)
Astrocytes proliferation and GFAP staining (moderate to strong)	Cerebral cortex, hippocampal area, thalamus, and cerebellar white matter	10-21	20/23 (86.9%)
CNPase staining at increased ligodendrocytes (moderate to strong)	White matter of cerebral and cerebellar cortex	16-21	14/23 (60.9%)
NSE-positive immunoreaction at degenerated neurons (moderate to strong)	Basal nuclei, thalamus, hippocampal pyramidal neurons, cerebellar dentate nuclei, and some nuclei of cerebral cortex	17-21	11/23 (47.8%)
Amyloid deposits	Leptomeninges, neocortex, cerebellum and brainstem vessels	18-21	8/23 (34.8%)
Senile plaque	None	None	0/23 (0%)

Histopathological findings in the aging brains in horses were similar with earlier studies done with horses [11,13,14,23], other animal species [8,10,21,22,24] and people [4,25,26]. In the study, even though no sex or breed difference was found, significant difference was found with aging. Jahns et al.[13] reported that neuronal and white matter vacuolation and spheroids were not related with aging. However, in the study, neuronal degeneration, gliosis and satellitosis were expression. CNPase is a myelin-associated enzyme that makes up 4% of total CNS myelin protein, and is thought to undergo significant age-associated changes [30]. In the central nervous system, CNPase is thought to play a critical role in the events leading up to myelination and expressed exclusively by oligodendrocytes. CNP expression that is a key myelin protein is implicated in age-related changes in myelin and axons [30,31]. Here we again examine the extent

of CNP accumulation in brain white matter of aged horses and its relationship to CNP degradation and partitioning in myelin. NSE is considered as quite established and specific markers of the central nervous tissue damage [10,32-34]. In the study, positive anti-NSE staining results showed that aging brains undergo degenerative changes and especially neurons were mostly affected by this situation. It is known that amyloid is deposited in the walls of cerebral and meningeal blood vessels; it is the important disorder of the aging brain. Beta amyloid (Aβ) deposits are seen in aged individuals in many of the mammalian species that possess the same Aβ amino acid sequence as humans [35]. Lesions such as senile plaques, cerebral β-amyloid angiopathy appear with advancing age in the human brain but are not specific to the human brain [19]. We observed Aβ deposits in the cerebral and meningeal vessel walls but not in the brain cortex as senile plaques reported previously in other species [8,10,21,22].

As a result, we observed that aging had a significant effect on brain tissue. The histopathological and immunohistochemical findings encountered in the brain related to aging in horses are similar with earlier studies, other animal species and humans. Also with further molecular studies, pathogenesis of these lesions will be explained in detail.

REFERENCES

1. McDonnell S: Signs of equine dementia. http://www.thehorse.com/articles/29479/signs-of-equine-dementia, *Accessed:* 10.1.2016.

2. Geor R: Conditioning the older horse. http://www.thehorse.com/articles/10040/conditioning-the-older-horse, *Accessed:* 10.11.2015.

3. Breteler MM, Amerongen Van NM, Swieten Van JC, Claus JJ, Grobbee DE, Gijn Van J, Hofman A, Harskamp Van F: Cognitive correlates of ventricular enlargement and cerebral white matter lesions on magnetic resonance imaging. *Stroke*, 25, 1109-1115, 1994. DOI: 10.1161/01.STR.25.6.1109

4. Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribe E: Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Curr Alzheimer Res*, 2, 3-18, 2005. DOI: 10.2174/1567205052772713

5. Yamada S, Asano T, Enomoto M, Sakata M, Tanno M, Yamada H, Esaki Y, Mizutani T: Ventricular dilatation and brain atrophy of normal elderly individuals and patients with Alzheimer-type dementia: A retrospective longitudinal computed tomographic study of autopsy cases. *Neuropathol*, 18, 261-269, 1998. DOI: 10.1111/j.1440-1789.1998.tb00113.x

6. Gavier-Widen D, Wells GAH, Simmons MM, Wilesmith JWW, Ryan J: Histological observations on the brains of symptomless 7-year-old cattle. *J Comp Pathol*, 124, 52-59 2001. DOI: 10.1053/jcpa.2000.0428

7. Gunn-Moore DA, Mcvee J, Bradshaw JM, Pearson GR, Head E, Gunn-Moore FJ: Ageing changes in cat brains demonstrated by β-amyloid and AT8-immunoreactive phosporylated tau deposits. *J Feline Med Surg,* 8, 234-242, 2006. DOI: 10.1016/j.jfms.2006.01.003

8. Hazıroğlu R, Güvenç T, Tunca R, Özsoy ŞY, Özyıldız Z: Evaluation of the age related changes in dog brains. *Rev Med Vet,* 161, 72-78, 2010.

9. Kikuchi K: Über die Altersveränderungen am Gehirn des Pferdes. *Arch Tierheilkunde*, 58, 541-573, 1928.

10. Özsoy ŞY, Hazıroğlu R: Age-related changes in cat brains as demonstrated by histological and immunohistochemical techniques. *Rev Med Vet,* 161, 540-548, 2010.

11. Capucchio MT, Ma´rquez M, Pregel P, Foradada L, Bravo M, Mattutino G, Torre C, Schiffer D, Catalano D, Valenza F, Guarda F, Pumarola M: Parenchymal and vascular lesions in ageing equine brains: Histological and immunohistochemical studies. *J Comp Pat,* 142, 61-73, 2010. DOI: 10.1016/j.jcpa.2009.07.007

12. Head E, Moffat K, Das P, Sarsoza F, Poon WW, Landsberg G, Cotman CW, Murphy MP: β-Amyloid deposition and tau phosphorylation in clinically characterized aged cats. *Neurobiol Aging,* 26, 749-763, 2005. DOI: 10.1016/j.neurobiolaging.2004.06.015

13. Jahns H, Callanan JJ, Mcelroy MC, Sammin DJ, Bassett HF: Age related and non-related changes in 100 surveyed horse brains. *Vet Pathol,* 43, 740-750, 2006. DOI: 10.1354/vp.43-5-740

14. Yanai T, Masegi T, Ishikawa K, Sakai H, Iwasaki T, Moritoo Y, Goto N: Spontaneous vascular mineralization in the brain of horses. *J Vet Med Sci*, 58, 35-40, 1996.

15. Edward L, Johnson K, Jo D, Mark S: Caring for the older horse: Common problems and solutions. http://edis.ifas.ufl.edu/an253, *Accessed:* 10.1.2016.

16. Frankhauser R, Luginbuehl H, Mcgrath JT: Cerebrovascular disease in various animal species. *Ann N Y Acad Sci*, 127, 817-860, 1965. DOI: 10.1111/j.1749-6632.1965.tb49447.x

17. Sloane JA, Hinman JD, Lubonia M, Hollander W, Abraham CR: Age-dependent myelin degeneration and proteolysis of oligodendrocyte proteins is associated with the activation of calpain-1 in the rhesus monkey. *J Neurochem,* 84, 157-168, 2003. DOI: 10.1046/j.1471.4159.2003.01541.x

18. Summers BA, Cummings JF, deLahunta A: Principles of neuropathology. **In,** Summers BS, Cummings JF, deLahunta A (Eds): Veterinary Neuropathology. 40-50, Mosby, St Louis, MO, 1995.

19. Uchida K, Yoshino T, Yamaguchi R, Tateyama S, Kimoto Y, Nakayama H, Goto N: Senile plaques and other senile changes in the brain of an aged American black bear. *Vet Pathol*, 32, 412-414, 1995. DOI: 10.1177/030098589503200410

20. Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd edn., 32, McGraw-Hill Book Co, NewYork, 1968.

21. Borras D, Ferrer I. Pumarola M: Age related changes in the brain of the dog. *Vet Pathol*, 36, 202-211, 1999. DOI: 10.1354/vp.36-3-202

22. Brellou G, Papaioannou N, Lekkas S, Vlemmas I: Immunohistochemical investigation of amyloid β protein (Aβ) in the brain of aged cats. *Proceedings of the 22nd ESVP meeting*, Olsztyn, Poland, September, 57, 15-18, 2004.

23. Hurst EW: Calcification in the brains of Equidae and Bovidae. *Am J Pathol*, 10, 795-802, 1934.

24. Braak H, Braak E, Strothjohann M: Abnormally phosphorylated tau protein related to the formation of neurofibrillary tangles and neuropil threads in the cortex of sheep and goat. *Neuroscience Let,* 171, 1-4, 1994. DOI: 10.1016/0304-3940(94)90589-4

25. Davis S, Thomas A, Perry R, Oakley A, Kalaria RN, O'brien JT: Glial fibrillary acidic protein in late life major depressive disorder: An immunocytochemical study. *J Neurol Neurosurg Psychiatry*, 73, 556-560, 2002. DOI: 10.1136/jnnp.73.5.556

26. Lyck L, Dalmau I, Chemnitz J, Finsen B, Schrøder HD: Immunohistochemical markers for quantitative studies of neurons and glia in human neocortex. *J Histochem Cytochem,* 56, 201-221, 2008. DOI: 10.1369/jhc.7A7187.2007

27. Yanai T, Masegi T, Kawada M, Ishikawa K, Fukuda K: Spontaneous vascular mineralization in the brain of cows. *J Comp Pathol*, 111, 213-219, 1994. DOI: 10.1016/S0021-9975(05)80053-2

28. German DC, Liang CL, Song T, Yazdani U, Xie C, Dietschy JM: Neurodegeneration in the Niemann-Pick C mouse: Glial involvement. *Neurosci,* 109, 437-450, 2002. DOI: 10.1016/S0306-4522(01)00517-6

29. Hinman JD, Chen CD, Oh SY, Hollander W, Abraham CR: Age-dependent accumulation of ubiquitinated 2',3'-cyclic nucleotide 3'-phosphodiesterase in myelin lipid rafts. *Glia*, 56, 118-133, 2008. DOI: 10.1002/glia.20595

30. Sprinkle TJ, Lanclos KD, Lapp DF: Assignment of the human 2',3'-cyclic nucleotide 3'-phosphohydrolase gene to chromosome 17. *Genomics,* 13, 877-80, 1992. DOI: 10.1016/0888-7543(92)90174-Q

31. Kasama-Yoshida H, Tohyama Y, Kurihara T, Sakuma M, Kojima H, Tamai Y: A comparative study of 2',3'-cyclic-nucleotide 3'-phosphodiesterase in vertebrates: cDNA cloning and amino acid sequences for chicken and bullfrog enzymes. *J Neurochem*, 69, 1335-1342, 1997. DOI: 10.1046/j.1471-4159.1997.69041335.x

32. Hajduková L, Sobek O, Prchalová D, Bílková Z, Koudelková M, Lukášková J, Tuchová I: Biomarkers of brain damage: S100B and NSE concentrations in cerebrospinal fluid-A normative study. *Bio Med Res Int Art*, 2015 (2015), Article ID: 379071, 2015. DOI: 10.1155/2015/379071

33. Ozmen E, Ozsoy SY, Donmez N, Ozsoy B, Yumuşak N: The protective effect of L-carnitine against hippocampal damage due to experimental formaldehyde intoxication in rats. *Biotech Histochem,* 89, 336-341, 2014. DOI: 10.3109/10520295.2013.855818

34. Ozsoy SY, Ozsoy B, Ozyildiz Z, Aytekin I: Protective effect of L-carnitine on experimental lead toxicity in rats: A clinical, histopathological and immunohistochemical study. *Biotech Histochem,* 86, 436-443, 2011. DOI: 10.3109/10520295.2010.529825

35. Chambers K, Uchıda K, Harada T, Tsuboi M, Sato M, Kubo M, Kawaguchi H, Miyoshi N, Tsujimoto H, Nakayama HI: Neurofibrillary tangles and the deposition of a beta amyloid peptide with a novel n-terminal epitope in the brains of wild tsushima leopard cats. *James Plos One*, 7, 1-10, 2012. DOI: 10.1371/journal.pone.0046452

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Research Article

Effects of Supplementation with Rosemary *(Rosmarinus officinalis* L.) Volatile Oil on Growth Performance, Meat MDA Level and Selected Plasma Antioxidant Parameters in Quail Diets [1]

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Abstract

The current study was conducted to evaluate the effects of dietary supplementation with rosemary volatile oil on performance, meat quality and selected plasma antioxidant parameters of quails. A total of 192 1-day-old Pharaoh (*Coturnix coturnix Pharaoh*) quails, including both males and females, were divided into three groups containing 64 quails and treated as follows: (1) a control group with 0 mg volatile oil/kg of diet; (2) 200 mg/kg rosemary volatile oil plus-RVO1; and (3) 250 mg/kg rosemary volatile oil plus-RVO2. The diets were prepared fresh for each treatment. The experiment was carried out for 42 days. Dietary treatments did not have any significant effect on live weight gain, feed intake, feed conversion rate, hot and cold carcass yield. There were significant differences for the plasma MDA level ($P<0.01$) and meat MDA level ($P\leq0.001$) between the control and treatment groups. Plasma SOD activity not affected by addition of rosemary volatile oil but plasma GPx level was significantly ($P<0.01$) affected by dietary treatments. In conclusion, rosemary volatile oil may be a potential natural antioxidant for quails and used to retard lipid oxidation in animal diets to improve meat products quality and animal performance.

Keywords: *Antioxidant, Performance, Quail, Rosemary, Volatile oil*

Bıldırcın Rasyonuna Biberiye Uçucu Yağ İlavesinin Büyüme Performansı, Et MDA Düzeyi ve Bazı Plazma Antioksidan Parametreleri Üzerine Etkisi

Özet

Bu çalışma, rasyona ilave edilen biberiye uçucu yağının, bıldırcınlarda performans, et kalitesi ve bazı plazma antioksidan parametreleri üzerine etkilerini değerlendirmek amacıyla yapıldı. Araştırmada toplam 1 günlük yaşta 192 adet Pharaoh (*Coturnix coturnix Pharaoh*) erkek ve dişi bıldırcınlar kullanıldı. Araştırmada her bir deneme grubunda 64 bıldırcın olmak üzere toplam 3 ana gruba bölündü ve deneme grupları sırasıyla: (1) rasyona 0 mg/kg biberiye uçucu yağı; (2) 200 mg/kg biberiye uçucu yağı ve (3) 250 mg/kg biberiye uçucu yağı ilave edildi. Rasyonlar her deneme grubu için taze olarak haftalık olarak hazırlandı. Deneme 42 gün boyunca yürütüldü. Araştırmada rasyona biberiye uçucu yağ ilavesinin canlı ağırlık artışı, yem tüketimi, yemden yararlanma oranı, sıcak ve soğuk karkas verimi üzerine önemli bir etkisi tespit edilmedi. Araştırmada plazma MDA ($P<0.01$) ve et MDA ($P\leq0.001$) düzeyinde kontrol ve deneme grupları arasında önemli farklılıklar belirlendi. Çalışmada plazma SOD aktivitesi biberiye uçucu yağ ilavesinden önemli düzeyde etkilenmemiş fakat plazma GPx oranını önemli ($P<0.01$) düzeyde etkilemiştir. Sonuç olarak, bıldırcın rasyonlarına ilave edilen biberiye uçucu yağının lipid oksidasyonu geciktirerek potansiyel bir doğal antioksidan katkısı olabileceği ve hayvan performansını iyileştirmek amacıyla kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Antioksidan, Performans, Bıldırcın, Biberiye, Uçucu yağ*

INTRODUCTION

Recently, aromatic plants and plant extract products have received attention also in their useful physiological functions and antioxidant activity. The oxidative deterioration of lipid and proteins is a major concern for food technologists due to the loss of quality associated with those processes. Lipid oxidation decreases nutritional

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and sensory properties of foods since it involves the loss of essential fatty acids and vitamins and generation of toxic compounds, causing additionally, flavour, texture and color deterioration [1]. Oxidative deterioration of lipids is an important factor limiting the shelf life of foods. Use of antioxidants can minimize the degree of lipid peroxidation [2]. To prevent or delay this autoxidation process, traditional antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), propyl gallate (PG) and tertiary butyl hydroquinone (TBHQ) have been used for more than five decades. However, these synthetic antioxidants (BHT, BHA and TBHQ) are known to have toxic and carcinogenic effects on human health. Therefore, we need to highly sensitive to lipid peroxidation and free radicals form research for alternative antioxidants. In particular, the extracts of many members of the *Labiatae (Lamiaceae)* family (oregano, marjoram, savory, sage, rosemary, thyme, and basil), which are antioxidative, have a high total phenol content. Among these, rosemary and sage have been widely used and most of their antioxidant components have been identified [3]. Dorman et al.[4] observed that, while these antioxidant characteristics are not completely concerned with the total phenolic contents, they do appear to be strongly conditional on rosmarinic acid, the major phenolic component present.

Rosemary is currently a widely used aromatic plant which has been recognized to have high antioxidant activity [5]. The objects associated with the antioxidant activity of rosemary are the phenolic diterpenes, such as carnasol, rosmanol, 7-methyl-epirosmanol, isorosmanol and carnosic acid, and the phenolic acids, such as rosmarinic and caffeic acids. Conversely, the major constituents of rosemary volatile oil are monoterpenes such as α-pinene, myrecene, 1.8-cineole and borneol. These components possess strong antibacterial, antifungal, antiviral and antimicrobial activities [6]. However, the properties of the volatile oils have been found to differ rely on extraction method used and different varieties of rosemary, grown in different area under different conditions may vary in the content of these phenolic compounds. So, the present study was aimed to determine the effect of rosemary volatile oil on quail performance, the susceptibility of raw breast meat lipid oxidation and selected plasma antioxidant parameters.

MATERIAL and METHODS

Animals, Diets and Experimental Design

A total of 192 1-day-old Pharaoh *(Coturnix coturnix Pharaoh)* quails, including both males and females, were divided into three groups containing 64 quails each group was randomly divided into four subgroups comprised of 16 quails each. Groups as follows: (1) a control group with 0 mg volatile oil/kg of diet; (2) 200 mg/kg rosemary volatile oil -RVO1; and (3) 250 mg/kg rosemary volatile oil -RVO2. Animals were obtained from the Uludag University Animal Health and Production, Research and Application Centre for quail breeding (Bursa, Turkey). In addition, this study was conducted under an approved protocol by Animal Care and Use Committee of University of Uludag (Approval number: 14.01.2010, 2010-01/108). The diet was fed to the quails in the form of mash and water *ad libitum* throughout the entire experimental period (42 d). Newly hatched chicks in all of the groups were reared under the same growing conditions in brooding cages (colony type) in an open-sided house with mechanical ventilation. The quails were transferred randomly at the fourth week of age from growing cages to laying cages (100 cm wide, 45 cm deep, 21 cm high in front, and 17 cm high in the rear, 112.5 cm^2 per quail) and housed there until the end of the study. All the chicks were brooded and reared at 28°C for the 1st wk, 27°C for the 2nd wk, 24°C for the 3rd wk, and 18-21°C from the 28th day until the quails reached 42 days of age. The quails received a basal diet (maize and soy bean based; 22.1% crude protein; 12.5 MJ/kg metabolisable energy) that was formulated to meet the NRC [7] requirements for nutrients including vitamins and minerals. The diet did not contain antibiotics, coccidiostats or growth promoters. The content of the basal diet for meat quails is presented in *Table 1*. Group feeding was used in all treatment groups.

Table 1. *Ingredients and chemical composition of the basal diet*

Ingredients for Meat Quail	**g/kg**
Corn	473.0
Soybean meal	360.0
Wheat	50.0
Corn gluten	50.0
Vegetable oil	30.0
$CaCO_3$	16.0
DCP	6.2
Salt	3.0
L-Lysine	3.5
D-L methionine	4.8
Vit-min premix[a]	3.5
Calculated nutrient concentration	
Metabolisable energy[b] MJ/kg	12.49
Crude protein %	22.12
Ether extract %	7.67
Ash %	5.64
Dry matter %	90.75
Starch %	33.88
Sugar %	55.98

[a] Provides (mg per kg diet): retinol, 2.4 mg; cholecalciferol, 0.075 mg; α-tocopherol acetate, 20 mg; thiamin, 3 mg; riboflavin, 3 mg; pyridoxal, 3.5 mg; cobalamin, 0.01 mg; niacin, 20 mg; panthotenic, 4 mg; folic acid, 1 mg; choline, 600mg; biotin, 0.03 mg; Mn, 80 mg; Fe, 60 mg; Zn, 60 mg; Cu, 5 mg; I, 1mg; Co, 0.2 mg; Se, 0.15 mg; [b] Metabolisable energy content of diets was estimated using the equation devised by Carpenter and Clegg [11]

The volatile oil (VO) dosages added to diet were chosen based on information from the literature and from the effective dosage determined by a previous study [8,9]. A 24-h constant lighting program was also maintained throughout the experimental period.

The nutritional composition of the diets was determined according to the Association of Official Agricultural Chemists [10]. The metabolisable energy (ME) levels of diets were estimated [11] using the following equation from Carpenter and Clegg:

ME, kcal/kg = 53 + 38 [(Crude Protein, %) + (2.25 x ether extract, %) + (1.1 x starch, %) + (1.05 x sugar, %)].

Performance Parameters of Quail Chicks

The quails were weighed individually at the beginning of the experimental period, after which the animals were weighed weekly to calculate live weight gain (LWG). Mortality was recorded when it occurred. Feed consumption was recorded weekly and expressed as g per quail per week. The feed conversion rate (FCR) was calculated as kg feed per kg body weight gain. At the end of the experimental period, the sex ratio was established in each group and 20 male quails from each group (5 male quails from each replicate) were randomly selected and weighed to determine the carcass yields (CY). The inert organs, heads and feet were removed after the carcasses were passed through a poultry defeathering machine. The chilled carcass weights were determined after incubation at 4°C for 18 h, and then the CY was calculated.

Determination of VO in Rosemary

The pure VO of rosemary (GC-MS tested, origin Mersin/Turkey, Semi Eterik Yağ Sanayi ve Dış Ticaret Ltd. Şti.) steam distillation extraction were obtained from a volatile oil company. Gas chromatography analysis was carried out on a Mass Spectrometry (HP 6890 Series Gas Chromatograph 5973 Mass Selective Detector System China - Agilent HP-Innowax capillary column) (60 x 0.25 µm x film thickness 0.25 µm). The temperature was programmed to rise from 60°C to 220°C at 4°C/min. The injection was performed at 250°C in split mode. Helium gas was used as a carrier at 1.3610 atm. The detection was performed by FID at 250°C, and the injection volume for all samples was 0.1 µL. Chromatograms were determined using mass spectrometer (MS) or MS/MS. The data was calculated using internal standards (Wiley GC/MS library). Volatile oil compositions of rosemary plant is shown in *Table 2*.

Table 2. *Volatile oil components of rosemary (Rosmarinus officinalis)*

Components	(%)
α-pinene	11.77
Camphene	5.21
β-pinene	1.29
Limonene	1.89
1,8-cineole (Eucalyptol)	51.63
Camphor	3.11
Borneol	4.16
Caryophyllene oxide	0.75
Bornyl acetate	2.56
Sabinene	0.54
β-myrcene	0.85
p-eymene	3.12
Linalool	0.63
β-caryophyllene	3.78
α-terpineol	2.73
Carvacrol	1.03

TBA Analysis of Meat Samples

Malondialdehyde (MDA) was measured as a secondary oxidation product according to the TBA method described by Tarladgis et al.[12] using spectrophotometry with some modifications. At the end of the experimental period, 30 breast-meat samples (10 samples from each group) were subjected to TBA analysis. The lipid oxidation value of breast-meat samples stored at +4°C was determined on days 3 and 7 post slaughter. A modification of the 2-thiobarbituric acid method was used, and the results are expressed as the amount of 2-thiobarbituric acid reactive substances (mg MDA). This method is based on the observation of a red colour that is created by the oxidation of unsaturated fatty acids with TBA after heating with MDA. For the analyses, a 10 g sample was homogenised with distilled water in a blender and transferred to a Kjeldahl flask where it was distilled to distillate aggregation by adding 2.5 mL 4 N HCl (Merck, Germany) and 1 mL Antifoam A. The reactant, 5 mL TBA (Merck, Germany), was added to 5 mL distillate and incubated in a boiling water bath for up to 30 min. The final solution and a blank were measured in a spectrophotometer at 538 nm. The obtained absorbance value was multiplied by 7.8. The final value was expressed as mg MDA per kg sample.

Blood Analyses

Blood samples (2 mL) were collected by venipuncture into an Etilen Diamin Tetra Aceticacid (EDTA) tube during slaughter time (42th day). Plasma was separated by centrifugation at 3.000 × *g* for 10 min. and placed in separate eppendorf tubes then stored at -80°C until analyses day. SOD activity was measured by using the assay kit (BioVision Research Products, Mountain View, USA, cat. no. K335-100). The sensitive SOD assay kit utilizes WST-1, which produces a water-soluble Formosan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidize activity and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined using a

colorimetric method. The results were expressed as the inhibition rate (%). GP_x activity was measured by using GP_x colorimetric assay kit (BioVision, cat. no. K762-100). All plasma measurements were read in the microplate spectrophotometer (Biotek Epoch, USA). Plasma malondialdehyde (MDA) level was measured the MDA method described by Ohkawa et al.[13] Method briefly, 100 µL plasma was added to 50 µL of sodium dodecyl sulfate (SDS 8.1%) and then vortexed, incubated for ten minutes at room temperature. 375 µL of acetic acid (pH 3.5, 20%) and 375 µL thiobarbituric acid (0.6%) was added and incubated in a boiling water bath for 60 min. The samples was left to cool to room temperature. 1.25 mL butanol:pyridine (15:1) was added, vortexed and centrifuged at 1000 rpm for five min. Finally, organic pink solution was measured in a spectrophotometer (Shimadzu UV 1601, Kyoto, Japan) at 532 nm. Results were defined nmol/mLfor plasma.

Statistical Analysis

All data were subjected to ANOVA using the ANOVA procedure of SPSS (SPSS, 2011). The data were first analysed as a completely randomized design as a random factor to examine the overall effect of treatments. Effect of rosemary was determined by the "contrast" option of the GLM procedure. When this effect was significant (i.e., $P<0.05$), orthogonal polynomial contrasts using contrast coefficients that were used to determine linear and quadratic responses to rosemary dosages. The significance of differences among treatments was perform using Dunnet's test.

RESULTS

The ingredients and chemical composition of the basal diet is presented in *Table 1*. Volatile oil compositions of rosemary plant is shown in *Table 2*. The 1-8 cineole (51.63%), α-pinene (11.77%), camphene (5.21%) and borneol (4.16%) were determined to be the main active components for rosemary volatile oil. The effects of dietary treatments on LWG, feed intake, FCR, hot and cold CY were shown in *Table 3*. Dietary treatments did not have any significant effect on LWG, feed intake, FCR, hot and cold CY. Lipid oxidation degree of meat samples (meat MDA level) stored for 3 and 7 day in refrigerator conditions and plasma

Table 3. *Effect of Rosemary volatile oil on live weight gain, feed intake, feed conversion rate, hot carcass yield and cold carcass yield*

Parameters	n	Period	Groups			P- values
			CG[1] mean±SD	RVO 1[2] mean±SD	RVO 2[3] mean±SD	
Live Weight Gain (g)	4	0-3 week	103.63±0.86	105.23±2.31	101.67±1.17	0.16
		4-6 week	98.64±0.98	93.47±2.07	94.07±0.73	1.00
		0-6 week	202.27±0.48	198.71±3.94	195.75±1.48	0.10
Feed Intake (g)	4	0-3 week	285.66±9.88	275.66±3.08	290.61±2.55	0.13
		4-6 week	671.84±16.57	672.68±21.28	671.31±35.32	0.97
		0-6 week	957.50±13.43	948.34±21.53	961.93±36.78	0.73
Feed Conversion rate (feed/gain)	4	0-3 week	2.85±0.037	2.62±0.044	2.75±0.010	0.099
		4-6 week	7.13±0.38	7.21±0.30	6.81±0.18	0.63
		0-6 week	4.91±0.18	4.77±0.15	4.73±0.074	0.67
Hot Carcass Yield (g)	20	End of trial	76.24±0.70	76.03±0.68	77.97±0.47	0.06
Cold Carcass Yield (g)	20	End of trial	74.66±0.76	74.84±0.59	76.38±0.49	0.07

[1] Control group; [2] Rosemary volatile oil (Group supplemented with 200 mg/kg rosemary volatile oil; [3] Rosemary volatile oil (Group supplemented with 250 mg/kg rosemary volatile oil)

Table 4. *Effect of Rosemary volatile oil on meat and plasma MDA level, plasma SOD activity and plasma GPx level*

Parameters	n	Period	Group			P-values
			CG[1] Mean±SD	RVO 1[2] Mean±SD	RVO 2[3] Mean±SD	
Meat MDA level (mg/kg meat)	10	A	0.11±0.01[a]	0.06±0.009[b]	0.05±0.007[b]	0.001
		B	0.86±0.01	0.73±0.017	0.64±0.11	0.14
Plasma MDA level (nmol/mL)	20	End of trial	0.24±0.25[a]	0.14±0.15[b]	0.20±0.10[a]	0.002
Plasma SOD(%)	20	End of trial	93.24±1.84	93.90±2.05	92.23±1.77	0.56
Plasma GPx(U/mL)	20	End of trial	0.59±0.04[b]	0.58±0.03[b]	0.74±0.03[a]	0.007

[1] Control group; [2] Rosemary volatile oil (Group supplemented with 200 mg/kg rosemary volatile oil; [3] Rosemary volatile oil (Group supplemented with 250 mg/kg rosemary volatile oil) A: storage of 3rd day at +4°C B: storage of 7th day at +4°C; MDA: Malondialdehyde SOD: Superoxide Dismutase GPx: Glutathione Peroxidase Letters; [a,b] in the same row indicate significant differences between different letters

MDA level, SOD activity and GPx levels are given in *Table 4*. Lipid oxidation, as measured by MDA formation, varied (P≤0.001) between the dietary treatments especially MDA value significant important at 3 day of storage period. Groups treated with rosemary volatile oil had the lowest MDA values. There were significant differences (P<0.01) for the plasma MDA level between the control and RVO 1 group. The lowest plasma MDA level was determined in the group including 200 mg/kg rosemary volatile oil (RVO 1). Plasma SOD activity was not affected by addition of rosemary volatile oil but plasma GPx level was significantly (P<0.01) affected by dietary treatment (RVO 2).

DISCUSSION

Volatile Oil Composition of the Rosemary Plant

In this study the main active components of rosemary volatile oil were determined 1.8-cineole (51.63%), α-pinene (11.77%) and camphene (5.21%). Ghazalah and Ali [15] stated that main active components were camphor (11-16%), pinene (15-20%) and cineole (30-35%). These findings agree with those obtained by Wolski et al.[16] and Porte et al.[17]. Debersac et al.[18] reported that the major component of dried leaves of Rosmarinus officinalis (L.) was monoterpene oxide 1-8 cineole (36.1%). Farag et al.[19] stated that these active compounds have high antioxidant activity due to the presence of phenolic groups in their structure. Plants belonging to the *Lamiaceae* family are very rich in polyphenolic compounds. Chemical composition of rosemary volatile oil can vary between regions and it depends mostly on climate, soil composition, plant organ, age, stage of vegetable cycle.

Growth Performance

The addition of rosemary volatile oil in quail diet did not impair LWG, feed intake, FCR and CY *(Table 3)*. Performance parameters are generally positively affected by the addition of aromatic herbs and their extracts into diets, and in this study, rosemary volatile oil did not have adverse effects on the performance parameters. However, few studies have shown negative effects on performance parameters when using mixtures of active compounds and volatile oils [20]. Al-Kassie [21] confirmed that adding 0.5 and 1 g/kg anise and rosemary oil, respectively, significantly improved live weight gain and the feed conversion value (P<0.05). Hernandez et al.[22] have demonstrated that adding 5.000 ppm of an herbal mixture of members of the *Labiatae* family such as rosemary can improve live weight gain for 42 d. These results may have derived from the positive effects of aromatic herbs and their volatile oils in the digestive system, where they can improve the activity of enzymes that help in the digestion of feed. Yesilbag et al.[9] determined that the increasing concentration of rosemary oil (140 mg/kg) caused a significant (P<0.05) increase in live weight, live weight gain and carcass yields during the growing and finishing periods. But feed intake and FCR were not significantly influenced by treatments. Yesilbag et al.[23] explained that the inclusion of rosemary VO at the level of 200 mg/kg to the laying quail diets improved FCR (P<0.01).

Meat MDA Level

The results of quail meat MDA levels are presented in *Table 4*. The effects of dietary treatment on TBA values of refrigerated (+4°C) raw breast meat during different storage times (3 and 7 d) are shown in *Table 4*. Thiobarbituric acid (TBA) analysis is an efficient way to measure antioxidant activity in meat products. This analysis is an indicator of MDA, a product of oxidation; thus, the MDA value increases during the storage period. At the end of this experiment, when the breast meat of the samples were tested, MDA value in the groups including rosemary volatile oil was significantly lower than the control group (P<0.001) especially MDA value significantly low in experiment groups at 3d of storage period. This result indicated that phenolic compounds from rosemary prevent thigh meat from oxidizing. Botsoglou et al.[24] determined that broilers that were given diets enriched in oregano essential oil (50, 100 mg/kg) increased the antioxidative stability of chicken tissue. As such, herbs contain several compounds that can extend the shelf life and improve the quality of meat products [25]. In addition, dietary administration of rosemary and sage essential oil extract to broilers resulted in a decrease in TBA levels from the Day 3 to Day 9 [5].

Biochemical Analyses

The results of quail plasma MDA, SOD and GPx values are presented in *Table 4*. Anti-oxidant enzymes are most effective when acting synergistically with one another or with other components of the anti-oxidant barrier of the organism when their activity remains balanced. It has been shown that nutrition plays a vital role in maintaining the pro-oxidant-antioxidant balance [26]. Antioxidant enzymes such as SOD and GPx are the first line defence antioxidants. In the present study, the GPx value in RVO 2 increased significantly but no statistically significant differences were found for SOD activity the control and the other experimental groups. The MDA level in plasma was reduced (P<0.01) by the inclusion of rosemary volatile oil at 200 mg/kg level. Lin et al.[27] reported that the intakes of herbs in chickens results in an increase in serum antioxidant enzyme activities and decrease in MDA level. Changes in these enzymes could be attributed to the presence of phenolic compounds in the rosemary plant [28]. The substances have strong antioxidant properties, which could protect organisms against oxidative stress. Yesilbag et al.[29] determined that the highest SOD activity value (527.48%) was found in group RVO1, which was fed 100 mg/kg rosemary oil. Overall, oil extract derived from herbal plants could be considered a potential growth promoter for poultry due to its digestive stimulant effect and antioxidant

effects. Additional research is needed to achieve a better understanding of the effects of these oils on plasma antioxidant parameters.

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REFERENCES

1. Morrissey PA, Sheehy PJ, Galvin K, Kerry JP, Buckley DJ: Lipid stability in meat and meat products. *Meat Sci,* 49, 73-86, 1998. DOI: 10.1016/S0309-1740(98)90039-0

2. Aruoma OI, Halliwell B, Aeschbach R, Loliger J: Antioxidant and pro-oxidant properties of active rosemary constituents: Carnosol and carnosic acid. *Xenobiotica*, 22, 257-268, 1992. DOI: 10.3109/00498259209046624

3. Chen H, Lin YC, Hsieh CL: Evaluation of antioxidant activity of aqueous extract of some selected nutraceutical herbs. *Food Chem*, 104, 1418-1424, 2007. DOI: 10.1016/j.foodchem.2007.02.004

4. Dorman HJD, Peltoketo A, Hiltunen R, Tikkanen MJ: Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chem,* 83, 255-262, 2003. DOI: 10.1016/S0308-8146(03)00088-8

5. Lopez-Bote CJ, Gray JI, Gomaa EA, Flegal CJ: Effect of dietary administration of oil extracts from rosemary and sage on lipid oxidation in broiler meat. *Br Poult Sci*, 39, 235-240 1998. DOI: 10.1080/00071669889187

6. Okoh OO, Sadimenko AP, Afoloyan AJ: Comparative evaluation of the antibacterial activities of the essential oils of *Rosmarinus officinalis* L. obtained by hydrodistillation and solvent free microwave extraction methods. *Food Chem*, 120, 308-312, 2010. DOI: 10.1016/j.foodchem.2009.09.084

7. NRC: National Research Council. Nutrient requirements of poultry. 9th rev. ed., National Academy Press, Washington, DC,1994.

8. Bulbul A, Bulbul T, Biricik H, Yesilbag D, Gezen SS: Effects of various levels of rosemary and oregano volatile oil mixture on oxidative stress parameters in quails. *Afr J Biotechnology*, 11, 1800-1805, 2012.

9. Yesilbag D, Gezen SS, Biricik H, Bulbul T: Effect of a rosemary and oregano volatile oil mixture on performance, lipid oxidation of meat and haematological parameters in Pharaoh quails. *Br Poult Sci*, 53, 89-97, 2012. DOI: 10.1080/00071668.2012.654763

10. AOAC: Official Methods of Analysis. Association of Official Analytical Chemists. 17th edn., Maryland, AOAC International, 2000.

11. Leeson S, Summers JD: Nutrition of the chicken. University Books, Guelph, Canada, 2001.

12. Tarladgis BG, Pearson AM, Dugan LR: Chemistry of the 2-thiobarbituric acid test for detemination of oxidative rancidity in foods. II. Formation of the TBA-malonaldehyde complex without acid-heat treatment. *J Sci Food Agr*, 15, 602-604, 1964.

13. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animals and tissues by thiobarbituric acid reaction. *Anal Biochem,* 95, 351-358, 1979. DOI: 10.1016/0003-2697(79)90738-3

14. SPSS: Statistical Package for the Social Sciences. Base system user's quide, version 7.5 SPSS Inc., Chicago, IL, USA, 2011.

15. Ghazalah AA, Ali AM: Rosemary leaves as a dietary supplement for growth in broiler chickens. *Int J Poult Sci*, 7, 234-239, 2008. DOI: 10.3923/ijps.2008.234.239

16. Wolski TA, Ludwiczuk A, Zwolan W, Maradarowicz M: GC/MS analysis the content and composition of essenttial oil in leaves and gallenic prepartions of rosemary *(Rosmarinus officinalis). Herba Polonica*, 46, 243-248, 2000.

17. Porte A, Godoy RLD, Lopes D, Koketsu M, Goncalves SL, Torquilho HS: Essential oil of Rosmarinus officinalis (rosemary) from Rio de Janeiro. Brazil. *J Essential Oil Res*, 12, 577-580, 2000. DOI: 10.1080/10412905.2000.9712163

18. Debersac P, Heydel JM, Amiot MJ, Goudonnet H, Artur Y, Suschetet M, Siess MH: Induction of cytochromeP 450 and/or detoxicatione enzymes by various extracts of rosemary: Description of specific patterns. *Food Chem Toxicol*, 39, 907-918, 2001. DOI: 10.1016/S0278-6915(01)00034-5

19. Farag RS, Daw ZY, Hewedi FM, El-Baroty GSA: Antimicrobial activity of some Egyptian spice essential oils. *J Food Protect*, 5, 665-667, 1989.

20. Lee KW, Everts H, Karpert HJ, Yeom KH, Beynen AC: Dietary carvacrol lowers body weight gain but improves feed conversion in female broiler chickens. *J App Poultry Res*, 12, 394-399, 2003. DOI: 10.1093/japr/12.4.394

21. Al-Kassie GAM: The effect of anise and rosemary on broiler performance. *Int J Poult Sci*, 7, 243-245, 2008.

22. Hernandez F, Madrid J, Garcia V, Orengo J, Megias MD: Influence of two plant extracts on broiler performance, digestibility and digestive organ size. *Poultry Sci*, 83, 169-174, 2004. DOI: 10.1093/ps/83.2.169

23. Yesilbag D, Biricik H, Gezen SS, Meral Y: Effects of dietary rosemary and oregano volatile oil mixture on quail performance, egg traits and egg oxidative stability. *Br Poult Sci*, 54, 231-237, 2013. DOI: 10.1080/00071668.2013.778389

24. Botsoglou NA, Florou-Paneri P, Christaki E, Fletouris DJ, Spais AB: Effect of dietary oregano essential oil on performance of chickens and on iron- induced lipid oxidation of breast, thigh and abdominal fat tissues. *Br Poult Sci*, 43, 223-230, 2002. DOI: 10.1080/00071660120121436

25. Liu HF, Booren AM, Gray JI, Crackel RL: Antioxidant efficacy of oleoresin rosemary and sodium tripolyphosphate in restructured pork steaks. *J Food Sci,* 57, 803-806, 1992. DOI: 10.1111/j.1365-2621.1992.tb14298.x

26. Cowey CB: The role of nutritional factors in the prevention of peroxidative damage to tissues. *Fish Physiol Biochem*, 2, 171-178, 1986. DOI: 10.1007/BF02264085

27. Lin CC, Wu SJ, Chang CH, Ng LT: Antioxidant activity of *Cinnamomum cassia*. *Phytother Res*, 44, 450-457, 2003. DOI: 10.1002/ptr.1190

28. Shan B, Cai YZ, Sun M, Corke H: Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J Agr Food Chem*, 53, 7749-7759, 2005. DOI: 10.1021/jf051513y

29. Yesilbag D, Eren M, Agel HE, Kovanlıkaya A, Balcı F: Effects of dietary rosemary, rosemary volatile oil and vitamin E on broiler performance, meat quality and eerum SOD activity. *Br Poult Sci,* 52, 472-482, 2011. DOI: 10.1080/00071668.2011.599026

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Research Article

Effects of Terebinth *(Pistacia terebinthus* L.) Fruit Oil Supplementation to Diets on Fattening Performance, Carcass Characteristics, Blood Parameters and Breast Meat Fatty Acid Composition in Japanese Quails *(Coturnix coturnix Japonica)* [1]

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Abstract

The objective of this study was to determine effects of terebinth fruit oil (TFO) supplementation to diet on growth performance, carcass characteristics, some blood parameters and composition of breast meat fatty acids in Japanese quails. Totally 240 unsexed daily Japanese quail chicks were assigned randomly to three treatment groups. Each group divided into 4 replicates, each containing 20 birds. A group was fed with basal starter diet for 1-21th days and grower diet for 22-42th days (Control). Treatment groups were also fed same diets additionally their ration added by 130 (Group A) or 260 mg/kg TFO (Group B) during the study. At 42 d of study, 20 quails (5 quails from each subgroup) from each group slaughtered for determination of carcass traits, blood parameters and fatty acid composition of breast meat. As a result of this study, final live weight of quails in Group B was found higher than Control and Group A ($P<0.05$). There were no differences on the average live weight gains of the birds among the groups. Average feed intake in Group A was found lower than Control and Group B on basis of whole study period ($P<0.05$). Average feed conversion ratio in Group A was found better than Control on basis of whole study period ($P<0.05$). Carcass weight in Group B was found higher than Group A ($P<0.05$). There were no differences on carcass yield and breast-, leg-, wing-, heart-, liver- and gizzard ratio to carcass weights of the birds in all groups. Serum total cholesterol, high density lipoprotein, aspartate amino transferase, alkaline phosphatase, total protein, albumin and globulin did not differ among the groups. There were no differences on composition of breast meat fatty acid profiles. In conclusion, due to supplementation of 260 mg/kg TFO enhanced live weight and carcass weight, and 130 mg/kg TFO decreased feed intake and improved feed conversion ratio, TFO could be used as a supplement at indicated doses for quail fattening rations.

Keywords: *Quail, Pistacia terebinthus fruit oil, Growth performance, Carcass, Blood parameters, Fatty acids*

Japon Bıldırcınlarının *(Coturnix coturnix Japonica)* Rasyonlarına Menengiç *(Pistacia terebinthus* L.) Meyvesi Yağı İlavesinin Besi Performansı, Karkas Karakteristikleri, Kan Parametreleri ve Göğüs Eti Yağ Asitleri Kompozisyonuna Etkileri

Özet

Bu çalışmanın amacı Japon bıldırcını rasyonlarına menengiç meyvesi yağı (MMY) ilavesinin büyüme performansı, karkas özellikleri, bazı kan parametreleri ve göğüs eti yağ asidi kompozisyonuna etkilerini belirlemekti. Toplam 240 karışık cinsiyetteki bıldırcın civcivi rastgele üç deneme grubuna ayrıldı. Her grup her birinde 20 civciv bulunan 4 alt gruba ayrıldı. Gruplardan biri başlangıç (1-21. günler) ve büyütme döneminde (22-42.günler) temel rasyonla beslendi (Kontrol). Deneme grupları deneme süresince temel rayona ilave olarak rasyonlarına 130 (Grup A), ya da 260 mg/kg MMY (Grup B) ilave edilerek beslendi. Araştırmanın 42. gününde her gruptan 20 adet (her alt gruptan 5 adet) bıldırcın karkas özellikleri, kan parametreleri ve göğüs eti yağ asidi kompozisyonunu belirlemek için kesildi. Çalışma sonucunda Grup B'nin deneme sonu canlı ağırlığı Kontrol ve Grup A'dan yüksek bulundu ($P<0.05$). Ortalama canlı ağırlık artışı bakımından gruplar arasında farklılık yoktu. Deneme geneli itibariyle Grup A'nın ortalama yem tüketimi Kontrol ve Grup B'den düşük bulundu ($P<0.05$). Deneme genelinde ortalama yemden yararlanma oranı Grup A'da Kontrol grubuna göre daha iyi bulundu ($P<0.05$). Grup B'nin karkas ağırlığı Grup A'dan daha yüksek bulundu ($P<0.05$). Gruplar arasında karkas oranı ile göğüs, but, kanat, kalp, karaciğer ve taşlık ağırlığının karkasa oranı bakımından farklılık yoktu. Serum total kolesterol, yüksek dansiteli lipoprotein, aspartat amino transferaz, alkalin fosfataz, total protein, albumin ve globulin miktarları gruplar arasında farklı değildi. Göğüs eti yağ asidi kompozisyonu bakımından gruplar arasında farklılık yoktu. Sonuç olarak; bıldırcın rasyonlarına 260 mg/kg MMY ilavesinin canlı ağırlığı ve karkas ağırlığını artırması, 130 mg/kg ilavesinin yem tüketimini azaltması ve yemden yararlanma oranını iyileştirmesinden dolayı her iki dozda da bıldırcın besi rasyonlarında kullanılabilir.

Anahtar sözcükler: *Bıldırcın, Menengiç meyvesi yağı, Büyüme performansı, Karkas, Kan parametreleri, Yağ asitleri*

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INTRODUCTION

Various subtherapeutic antibiotics have been used as feed additives since 1940's to enhance growth performance and to prevent disease in livestock, particularly in poultry. Usage of antibiotics as feed additives was banned in European Union in 2006 due to the antibiotic residue risk in animal products and the potential evolving of antibiotic resistant bacteria [1]. This banning has led to acceleration of research on alternative natural feed additives, such as probiotics, prebiotics and organic acids, in animal production [2]. Certain aromatic plants or essential oils (EO) derived from these plants have also increasingly been used for such purpose. Essential oils have antimicrobial [3], antioxidant [4] and digestive enzyme stimulant affect [5,6].

Pistacia terebinthus L. (terebinth or turpentine tree), is a small tree, widely distributed in the Mediterranean region and west Asia [7-10]. It commonly grows on dry rock slopes and hillsides areas, pine forest, and maquis vegetation in the Mediterranean-, Aegean-, Black sea-, and Southeastern Anatolia region of Turkey [11-13]. Fruits of terebinth (known as menengiç, çitlenbik, çıtlık) are very nutritious and have been used as an appetiser in Turkey. The fruits are consumed also as coffee and the oil extracted from the fruits is used as cooking oil as well as in making soap (known as bıttım sabunu) in Turkey [11]. In addition, it has anti-inflammatory, antipyretic, antiparasitic, expectorant, and spasmolytic effects [11]. Matured fruits of terebinth are small globular and dark greenish [14], it includes 35.26-47.52% ether extracts [15-17], 6.4% crude protein and 1.5% crude ash [17]. There has been 0.1% essential oils [17], and 0.06-0.73% volatile fatty acids in fruits of terebinth [7,18]. It also contains macro- (Ca, P, K, Na, Mg, S) and microelements (Fe, Al, Zn, Cu, Mn, Se, Co, Cr) [8,17] as well as tocopherols, tocotrienols and sterols [9,15]. Pharmacologically active substances in the mature fruit of terebinth are α-pinene, limonene, α-felandrene, terpinolene, p-cymen-8-ol and caryophyllene oxide [7,18]. It also contains phenolic and flavonoids such as; quercetin and α-tocopherol, which are antioxidant compounds [9].

There have been a lot of researches on usage of certain aromatic plants/herbs/spices by alone or essential oils (EO) derived from these plants or commercially produced EO as feed additives in the poultry feeding. According to our knowledge, there is no research on the usage of terebinth fruit oil (TFO) in poultry feeding. This study aimed to assess supplementation of TFO to diet on growth performance, carcass traits, some blood parameters and fatty acid composition of breast meat in quails.

MATERIAL and METHODS

The ethical committee approval of Kafkas University (KAÜ-HADYEK: 2015-078) was taken in order to conduct this study.

Birds, Experimental Design, Housing Environment and Diets

Totally 240 unsexed one day old Japanese quail chicks *(Coturnix coturnix japonica)* were divided into randomly to three dietary groups. Each group consisted of 4 subgroups, each subgroup included 20 birds. The experimental diets were an unsupplemented basal diet *(Table 1)*. Basal diets were supplemented with 0 (Control), 130 mg/kg (Group A), or 260 mg/kg TFO (Group B). The TFO was firstly mixed into the vegetable oil component of the ration, and then the oil mixture was added to the basal diets. The birds were housed in cages, in an environmentally controlled room. The environmental temperature in the room was maintained 31-33ºC and gradually reduced by 2-3ºC every week until 21-22ºC in the final week. Experimental diets were prepared according to NRC requirements for quails [19].

Table 1. *Ingredients and nutrient content of the basal diets used in the starter and grower period, (dry matter basis, %)*[1]

Item	Starter (d 1-21)	Grower (d 22-42)
Ingredients		
Corn	40.00	37.00
Wheat	6.15	20.25
Vegetable oil (sunflower)	5.00	4.30
Soybean meal, 44% CP	38.00	25.00
Sunflower meal, 32% CP	8.00	10.00
Dicalcium phosphate	0.65	1.32
Limestone	1.35	1.28
Vit./min. premix[2]	0.35	0.35
Salt	0.30	0.30
DL-Methionine	0.20	0.20
Calculated analysis		
Metabolizable energy, kcal/kg [3]	3000	3000
Crude protein	23.92	20.49
Calcium	0.80	0.90
Total phosphor	0.65	0.70
Lysine	1.42	1.11
Methionine + Cysteine	0.77	0.67
Linoleic acid	1.55	1.49
Analysed nutrient composition		
Dry matter	88.99	89.03
Crude protein	23.92	20.49
Ether extract	7.09	6.48
Crude fibre	4.35	4.10

[1] The basal diets were the same in the all groups. Treatment diets were supplemented with 130 or 260 mg/kg of terebinth fruit oil; [2] Supplied per kg diet: Vit A, 8400 IU; vit D_3, 4480 IU; vit E, 56 mg; vit K_3, 2.24 mg; vit B_1, 1.68 mg; vit B_2, 4.48 mg; niacin, 33.6 mg; cal.D-pantothenate, 10 mg; vit B_6, 2.8 mg; vit B_{12}, 9 µg; D-biotin, 0.112 mg; folic acid, 1.12 mg; vit C, 56 mg; manganese, 59 mg; iron, 47 mg; zinc, 47 mg; copper, 47 mg; cobalt, 0.112 mg; iodine, 0.56 mg; selenium, 0.100 mg; molybdenum, 0.582 mg; [3] Calculated based on NRC [19] data of feedstuffs nutrient tables

Experimental diets were in mash form and offered *ad libitum* during the both starter period (d 1-21) and grower period (d 22-42). Clean and fresh water was available throughout the study.

Terebinth Fruit Oil

The TFO used in this study obtained from local firm in Siirt city, TURKEY. Terebinth fruits were also obtained from the same firm for determination of crude nutrient analyses.

Making of Terebinth Oil

Terebinth fruit oil is made traditional method in Turkey, according to following way. The fruits are grind by a mill. They put into a large metal basin, and boiled water adds, to achieve the dough consistency. When the grinded fruit reaches the dough consistency, knead by manpower for 90-120 minutes. Then basins incline in half horizontally and waited for leakage of oil from the kneaded material. Accumulated oil in the bottom of the basins is taken.

Growth Performance, Carcass Traits, Blood Serum and Breast Meat Samples

Individual live weights of the birds and offered feed for each replicate pen was recorded at the beginning of the study and then at the end of each period. Feed conversion ratio (gain: feed) per pen were calculated at 21 d and 42 d of study. At 42 d of the study, 20 birds (5 birds from each replicate) similar body weight were selected from the each group, weighed and slaughtered for determination of the carcass traits and internal organ weights. In the slaughtering process blood samples were also taken. Blood samples were centrifuged at 3000 g for 10 min, and serum samples were stored -20°C for analyses. From the middle of 1 to 3 both side of the breast meat, meat samples were taken for fatty acid analyses and samples were stored at -20°C until analysis [20].

Analytical Procedures

Dry matter, crude protein, crude fat, crude fibre and crude ash in the diets were made according to AOAC methods [21]. Concentrations of blood serum total cholesterol, high density lipoprotein (HDL), aspartate amino transferase (AST), alkaline phosphatase (ALP), total protein, albumin and globulin were analysed by an auto analyser (Beckman Coulter AU5800, Beckman Coulter, Inc. USA), using the commercial kits belonging the same firm.

Extraction of the terebinth oil for determination of individual fatty acids, in line with Agilent Technologies Firm proposal, was made with the method described by Ichihara et al.[22]. Concentrations of individual fatty acids of terebinth oil were analysed using a gas chromatography (Agilent Technologies 7890A GC/5975C MS) equipped with an auto sampler according to methods described by Paquot [23]. One microlitre of the sample volume was injected using the splitless mode. Chromatographic separations were accomplished with an Optima delta-6 capillary column (0.25 mm i.d. x 60 m, film thickness 0.25 µm). Analysis was carried out using nitrogen as the carrier gas. The column temperature was arranged from 1 min in 50°C to 50°C to 300°C at 3°C/min for 5 min. The detector temperature was 250°C. The separated components were identified by retention time of matching standard fatty acid methyl esters. External standard method was used as standard. Quantitative determination was carried out based on peak area integration.

Statistical Analyses

All data were subjected to one-way ANOVA using the SPSS software package, version 19.00 (SPSS Inc., Chicago, IL, USA) [24]. When significant treatment effects were disclosed, Duncan's multiple range test was performed [25].

RESULTS

Crude nutrient contents of the fruit of terebinth used in this study for making TFO were analysed as follows; 94.36% dry matter, 38.91% crude fat, 10.07% crude protein and 3.26% crude ash, in dry matter basis. Individual fatty acid composition of the TFO used in this study is shown in *Table 2*. The highest fatty acid in the TFO was oleic acid (46.82%). The other fatty acids were linoleic- (23.07%), palmitic- (22.00%), stearic- (4.61%), palmitoleic- (2.14%), and linolenic acid (1.36%).

As shown in *Table 3*, average body weight of the birds in the Group B was significantly higher than those of the birds in the Control and Group A at the end of the starter period ($P<0.001$) and as well as the grower period ($P<0.05$). There were no statistical differences on the average weight gain of the birds in all groups both at starter- and grower periods. However, average weight gain of the birds in the Group B was higher than those of the birds in the Control and Group A in overall the study ($P<0.001$). There were no statistical differences on average daily feed intake of the birds in all groups both starter- and grower periods, but average daily feed intake of the birds in the Group A was lower than those of the birds in the Control and Group B in overall the study ($P<0.05$). There were no statistical differences on feed conversion ratio of the birds in all the groups both starter and grower periods, but feed conversion ratio of the birds in the Group A was lower than those of the birds in the Control group in overall the study ($P<0.05$).

Average slaughter body weights of the birds in the Group B were higher ($P<0.05$) than those of the birds in the Control and Group A *(Table 4)*. Average carcass weights of the birds in the Group B were higher than those of the birds in the Group A ($P<0.05$). There were no statistical differences on carcass ratio and breast-, leg-, wing-, heart-,

Table 2. *Fatty acid composition of terebinth fruit oil used in this study*

Amount	Fatty Acids					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
%	22.00	2.14	4.61	46.82	23.07	1.36

C16:0= *Palmitic acid,* ***C16:1***= *Palmitoleic acid,* ***C18:0*** = *Stearic acid,* ***C18:1*** = *Oleic acid,* ***C18:2*** = *Linoleic acid,* ***C18:3*** = *Linolenic acid*

Table 3. *Effects of 0 (Control), 130 (Group A) and 260 mg/kg (Group B) terebinth fruit oil supplementation to diet on performance of quails during the 42 d of study*

Parameters	Control	Group A	Group B	SEM	Sig.
Body weight, g					
1 d	8.6	8.6	8.5	0.05	NS
21 d	92.8^{b}	90.1^{b}	98.6^{a}	0.78	***
42 d	194.1^{b}	194.7^{b}	204.5^{a}	1.64	*
Daily weight gain, g/d					
1 to 21 d	3.98	3.89	4.13	0.06	NS
22 to 42 d	4.88	5.10	5.09	0.06	NS
1 to 42 d	4.43^{b}	4.49^{b}	4.61^{a}	0.04	***
Daily feed intake, g/d					
1 to 21 d	7.69	7.43	8.03	0.14	NS
22 to 42 d	19.25	18.27	18.99	0.24	NS
1 to 42 d	13.47^{a}	12.70^{b}	13.55^{a}	0.17	*
Feed conversion ratio, g/g					
1 to 21 d	1.93	1.91	1.95	0.02	NS
22 to 42 d	3.95	3.59	3.74	0.07	NS
1 to 42 d	3.04^{a}	2.83^{b}	2.94^{ab}	0.04	*

NS: *Non significant (P>0.05);* ^{ab}*Mean values in the same row with a common letter are significantly different. * P<0.05, ***P<0.001*

Table 4. *Effects of 0 (Control), 130 (Group A) and 260 mg/kg (Group B) terebinth fruit oil supplementation to diet on carcass yield traits of quails slaughtered at 42 d*

Parameters	Control n=20	Group A n=20	Group B n=20	SEM	Sig.
Body weight, g	194.2^{b}	194.8^{b}	204.1^{a}	1.69	*
Carcass weight, g	134.5^{ab}	131.3^{b}	137.7^{a}	1.06	*
Carcass ratio, %	69.33	67.51	67.56	0.46	NS
The relative organ ratio to the carcass weight, %					
Breast	32.11	32.37	32.61	0.40	NS
Leg	23.12	22.74	22.43	0.23	NS
Wing	10.62	10.18	10.91	0.15	NS
Heart	1.54	1.57	1.58	0.02	NS
Liver	3.35	3.93	4.13	0.15	NS
Gizzard	3.59	3.69	3.44	0.07	NS

^{ab}*Mean values in the same row with a common letter are significantly different. * P<0.05;* ***NS:*** *Non significant (P>0.05)*

Table 5. *Effects of 0 (Control), 130 (Group A) and 260 mg/kg (Group B) terebinth fruit oil supplementation to diet on serum parameters of quails slaughtered at 42 d*

Parameters	Control n= 20	Group A n= 20	Group B n= 20	SEM	Sig.
Total cholesterol, mg/dL	230.3	201.4	212.0	8.16	NS
HDL, mg/dL	135.7	115.0	83.0	10.04	NS
AST, IU/L	280.1	310.6	299.3	14.47	NS
ALP, IU/L	786.8	936.0	813.3	30.07	NS
Total protein, g/dL	2.81	2.77	3.22	0.10	NS
Albumin, g/dL	0.98	1.00	1.10	0.04	NS
Globulin, g/dL	1.83	1.77	2.13	0.07	NS

NS: *Non significant (P>0.05)*

Table 6. *Effects of 0 (Control), 130 (Group A) or 260 mg/kg (Group B) terebinth fruit oil supplementation to diet on fatty acid composition (%) of breast meat of quails slaughtered at 42 d*

Parameters	Control n=20	Group A n=20	Group B n=20	SEM	Sig.
C14:0	0.49	0.53	0.44	0.03	NS
C16:0	26.09	24.16	25.42	0.37	NS
C17:0	0.21	0.16	0.17	0.06	NS
C18:0	18.06	16.41	17.30	0.54	NS
Σ SFA	**44.80**	**41.26**	**43.28**	0.83	NS
C16:1	2.48	3.02	2.89	0.23	NS
C18:1	15.81	18.11	14.64	0.89	NS
Σ MUFA	**17.78**	**21.13**	**17.53**	1.09	NS
C18:2	27.76	27.47	28.41	0.32	NS
C18:3	2.20	2.18	1.82	0.35	NS
C20:4	6.49	6.93	8.00	0.35	NS
Σ PUFA	**36.45**	**36.58**	**38.23**	0.81	NS
Σ PUFA/Σ SFA	**0.82**	**0.90**	**0.88**	0.02	NS

C14:0 = *Myristic acid,* ***C16:0*** = *Palmitic acid,* ***C16:1*** = *Palmitoleic acid,* ***C17:0*** = *Heptadecanoic acid,* ***C18:0*** = *Stearic acid,* ***C18:1*** = *Oleic acid,* ***C18:2*** = *Linoleic acid,* ***C18:3*** = *Linolenic acid,* ***C20:4*** = *Arachidonic acid,* ***SFA*** = *Saturated fatty acids,* ***MUFA*** = *Monounsaturated fatty acids,* ***PUFA*** = *Polyunsaturated fatty acids;* ***NS:*** *Non significant*

liver- and gizzard ratio to carcass weight in all groups.

There were no statistical differences on concentrations of serum total cholesterol, HDL, AST, ALP, total protein, albumin and globulin in all groups *(Table 5)*.

As seen from the *Table 6*, there were no statistical differences on both individual fatty acids composition and total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) as well as the total PUFA:SFA ratio in all the groups. The mostly found fatty acids were palmitic- and stearic acids in the SFA, oleic acid in the MUFA, and linoleic- and arachidonic acids in the PUFA of breast meat of birds.

DISCUSSION

Analysed fatty acid composition results of TFO used in this study showed that oleic-, linoleic, palmitic-, stearic-, palmitoleic- and linolenic acids were major oil components *(Table 2)*. In many previous studies have reported 34.8 to 52.67% oleic-, 19.91 to 23.58% palmitic-, 17.30 to 20.95% linoleic- and 0.17 to 0.79% linolenic acids for TFO [8,15-17]. Our results in this study for the oleic- and palmitic acids are in accordance with above literature reports; but, the linoleic- and linolenic acids are higher.

In the present study, supplementation of 260 mg/kg TFO to diet induced a significant increase on live weight of the birds both at the end of the starter period and the grower period as compared to the birds in the Control and 130 mg/kg TFO supplemented group *(Table 3)*. Weight gain of the birds in the 260 mg/kg TFO supplemented group also was higher than those of the other groups in overall the study. The results on the live weight and weight gain of the birds indicated that supplementation of high level TFO (260 mg/kg) to diet improved live weight and weight gain, but not low level TFO (130 mg/kg). The improvement on the live weight and live weight gain in the high level TFO supplemented group may be related to the antibacterial agents, such as luteolin, luteolin-7-glucoside [10] and α-pinene [26], antioxidant compounds, such as quercetin and α-tocopherol [9], and high linoleic acid content of the TFO used in this study [8,16,17]. In other hand, Jang et al.[27] reported that a commercial blend of EO showed a marked increase in digestive enzyme activities of the pancreas and intestinal mucosa from broiler chickens, leading in a significant growth performance. Further, Hernandez et al.[5] indicated that two different plant extracts improved the digestibility of the feeds for broilers. Similar results were also reported by Lee et al.[28] and Lee et al.[29] which indicates that EO in diets encourages secretions of endogenous digestive enzymes, which then enhance nutrient digestion and gut passage rates in chickens. Live weights of the birds were similar in the Control and 130 mg/kg TFO supplemented groups. This situation may be related to the basal diets used in the present study, which had adequate and balanced nutrient content, high digestibility, and optimum environmental conditions during the research. It has been proposed that dietary EO supplementation as growth stimulators could not give positive effect when chickens are raised at optimal condition such as highly digestible diets and clean condition [30].

In this study, supplementation of 130 mg/kg TFO induced lower feed intake than those of the other groups in overall the study *(Table 3)*. However, Kamel [31] suggested that herbs, spices and various plant extracts have appetiser and digestion-stimulating properties. Supplementation of 130 mg/kg TFO also improved feed conversion ratio as compared to Control group in overall the study *(Table 3)*. Küçükyılmaz et al.[32] have conducted two experiments to determine the effects of supplementation EO, which contains certain active substances found also in the TFO (such as; terpinene, α-pinene, limonene, caryophyllene), on growth performance in broilers. In experiment I (within October - November period); they have not found differences on the live weight, feed intake, and feed conversion ratio among the groups. However, in experiment II (including April to May); they have determined higher live weight and improved feed conversion in the EO supplemented group than the Control group. Likewise, Khosravinia [33] has made a study in broilers on the usage of *Satureja khuzistanica* oils, which contains some essential oils available in the TFO. He found 0.5, 1.0, 1.5, 2.0 and 2.5 g/L oil supplementation via by drinking water negatively affected weight gain, feed intake and feed conversion of the birds as compared to Control. Alçiçek et al.[34] found that supplementation of 24 mg/kg EO did not affect growth performance of broilers, but 48 and 72 mg/kg EO supplementation improved live weight and feed intake.

Slaughter body weight of birds in the 260 mg/kg TFO supplemented group was higher than the birds when compared to other groups *(Table 4)*. The carcass weight of birds in the 260 mg/kg TFO supplemented group was higher than that of the birds in the 130 mg/kg TFO supplemented group. Higher slaughter body weight and carcass weight in the 260 mg/kg TFO supplemented group than those of the other groups originated from the higher final live weight of the birds at the end of the study in this group. There were no differences on the carcass ratio and, carcass parts and visceral organ ratio to carcass weight among the groups. Similar results were found by Küçükyılmaz et al.[32] in broilers. Khattak et al.[35] reported that 300, 400 and 500 g/ton EO supplementation to broiler diet increased carcass weight but not 100 and 200 mg/kg. They also determined that 300 and 400 mg/kg EO supplementation induced higher breast ratio as compared to other groups. Alçiçek et al.[2] found that 36 mg/kg EO supplementation did not affect carcass ratio, but 48 mg/kg supplementation increased. Similarly, 24 mg/kg EO supplementation to diet did not affect carcass ratio but 48 and 72 mg/kg increased [34]. Results on the carcass traits in this study and other studies indicated that oil or essential oil type and their doses could be alter some carcass traits of birds.

It was determined that 130 or 260 mg/kg TFO supplementation to diet did not affect blood serum parameters *(Table 5)*. Similar to present study, Lee et al.[28] found no differences on total cholesterol and HDL cholesterol concentrations in broilers fed with supplemented EO diets. Khattak et al.[35] reported that no significant effect on the total cholesterol concentration depending on the supplementation of EO, and they speculated that this situation could be associated either ineffective in inhibiting hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase or due to their fast degradation rate in the

liver of broilers. In other hand, the absence or presence of hypo-cholesterolaemic effects of components in animals depend on breed, gender, age and also on the composition of the diet.

In the present study, 130 or 260 mg/kg TFO supplementation to diet did not affect fatty acid composition of the breast meat in quails *(Table 6)*. Total SFA amounts in the breast meat of birds fed with 130 or 260 mg/kg TFO supplemented birds were 7.90 and 3.39 % lower than those of the birds in the Control group, respectively. The decrease in the total SFA amount in the both TFO supplemented group were originated from the decrease in the palmitic-, heptadecanoic- and stearic acids amount in these groups as compared to Control. The numerical decrease in the total SFA of breast meat of quail fed with both TFO supplemented group may be related to the numerical decrease in the serum total cholesterol concentrations of these birds *(Table 5)*. In contrast the total SFA, 130 or 260 mg/kg TFO supplementation to diets induced a 0.36 and 4.88 % increase in the total PUFA amount as compared to Control, respectively. The numerical increase in the total PUFA in the both TFO supplemented group were primarily related to increase in the arachidonic acid. The numerical decrease in total SFA but numerical increase in the total PUFA in both TFO supplemented groups have indicated that fatty acid consumption of carcass from these birds may be poses a lower risk of coronary heart disease. PUFA accepts a more vulnerable indicator to lipid peroxidation. Although there were no statistical differences ($P<0.062$), it is worth to state that, the PUFA: SFA ratio was numerically higher in the both TFO supplemented group than Control.

Finally, supplementation of 260 mg/kg TFO to diet increased body weight and weight gain of birds, as compared to the Control and 130 mg/kg TFO supplemented groups. Yet, supplementation of 130 mg/kg TFO decreased feed intake of the birds, as compared to the Control and 260 mg/kg TFO supplemented groups. Supplementation of 130 mg/kg TFO improved feed conversion ratio, as compared to the Control. Supplementation of 260 mg/kg TFO induced higher slaughter body weight as compared to other groups. Supplementation of TFO at both doses had no effect on the carcass ratio, carcass parts and visceral organ ratio, blood serum parameters and fatty acid composition of breast meat. These affirmative results have indicated that TFO may be used as an alternative supplement at these doses in quail diets. Additionally, first findings on usage of TFO as feed additives in quails were demonstrated with this study.

REFERENCES

1. European Union: Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition. *Official Journal of European Union Legislation,* 268, 29-43, 2003.

2. Alçiçek A, Bozkurt M, Çabuk M: The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. *S Afr J Anim Sci,* 34, 217-222, 2004.

3. Jang IS, Ko YH, Kang SY, Lee CY: Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Anim Feed Sci Tech,* 134, 304-315, 2007. DOI: 10.1016/j.anifeedsci.2006.06.009

4. Vichi S, Zitterl-Eglseer K, Jugi M, Fraz C: Determination of the presence of antioxidants deriving from sage and oregano extracts added to animal fat by means of assessment of the radical scavenging capacity by photochemiluminescence analyses. *Nahrung,* 45, 101-104, 2001. DOI: 10.1002/1521-3803(20010401)45:2<101::AID-FOOD101>3.0.CO;2-W)

5. Hernandez F, Madrid J, Garcia V, Orengo J, Megias MD: Influence of two plant extracts on broiler performance, digestibility, and digestive organ size. *Poultry Sci,* 83, 169-174, 2004. DOI: 10.1093/ps/83.2.169

6. Jamroz D, Wiliczkiewicz A, Wertelecki T, Orda J, Sukorupinska J: Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. *Br Poultry Sci,* 46, 485-493, 2005. DOI: 10.1080/00071660500191056

7. Couladis M, Özcan M, Tzakou O, Akgül A: Menengic (*Pistacia terebinthus L.*) ağacının değişik organlarında uçucu yağ bileşimi. *14. Bitkisel İlaç Hammaddeleri Toplantısı.* Bildiriler, 29-31 Mayıs 2002, Eskişehir, Türkiye.

8. Özcan M: Characteristics of fruit and oil of terebinth *(Pistacia terebinthus L.)* growing wild in Turkey. *J Sci Food and Agric,* 84, 517-520, 2004. DOI: 10.1002/jsfa.1632

9. Topçu G, Ay M, Bilici, Sarıkürkçü C, Öztürk M, Ulubelen A: A new flavone from antioxidant extracts of *Pistacia terebinthus*. *Food Chem,* 103, 816-822, 2007. DOI: 10.1016/j.foodchem.2006.09.028

10. Kavak DD, Altıok E, Bayraktar O, Ülkü S: Pistacia terebinthus extract: As a potential antioxidant, antimicrobial and possible β-glucuronidase inhibitor. *J Molec Catalysis B: Enzymatic,* 64, 167-171, 2010. DOI: 10.1016/j.molcatb.2010.01.029

11. Baytop T: Türkiye'de Bitkiler ile Tedavi. İstanbul Üniversitesi Yayınları, Yayın No: 3255, İstanbul, Türkiye, 1984.

12. Baytop T: Türkçe Bitki Adları Sözlüğü. Türk Dil Kurumu Yayınları. Yayın No: 578, Ankara, Türkiye, 1994.

13. Anonymous: Menengiç Nedir? Nerelerde Yetişir Faydaları Nelerdir? http://bilgiliyem.com/bitkiler/menegic-nedir-nerelerde-yetisir-faydalari-nelerdir/ *Accessed:* 06.10.2016.

14. Davis PH: Flora of Turkey and the East Aegean Islands. Vol. 2. Edinburg University Press, Edinburgh, Scotland, 1967.

15. Matthaus B, Özcan M: Quantitation of fatty acids, sterols, and tocopherols in turpentine *(Pistacia terebinthus Chia)* growing wild in Turkey. *J Agric Food Chem,* 54, 7667-7671, 2006. DOI: 10.1021/jf060990t

16. Geçgel U, Arıcı M: Çıtlık *(Pistacia terebinthus)* çeşitlerinin bazı fizikokimyasal özellikleri ile yağ asitleri kompozisyonunun belirlenmesi. *Türkiye 10. Gıda Kongresi,* 21-23 Mayıs, s.301-304, Erzurum, Türkiye, 2008.

17. Kızıl S, Türk M: Microelement contents and fatty acid compositions of *Rhus coriaria L.* and *Pistacia terebinthus L.* fruits spread commonly in the south eastern Anatolia region of Turkey. *Natural Product Res,* 24, 92-98, 2010. DOI: 10.1080/14786410903132555

18. Özcan MM, Tzakou O, Couladis M: Essential oil composition of the turpentine tree *(Pistacia terebinthus L.)* fruits growing wild in Turkey. *Food Chem,* 114, 282-285, 2009. DOI: 10.1016/j.foodchem.2008.08.094

19. NRC: National Research Council. Nutrient Requirements of Poultry 9th rev. edn., National Academy Press, Washington, DC, USA, 1977.

20. Şekeroğlu A, Diktaş M: Yavaş gelişen etlik piliçlerin karkas özelliklerine ve et kalitesine serbest yetiştirme sisteminin etkisi. *Kafkas Univ Vet Fak Derg,* 18, 1007-1013, 2012. DOI: 10.9775/kvfd.2012.6922

21. AOAC: Official Methods of Analysis of AOAC International. 2000. 2nd edn., AOAC International, Maryland, USA.

22. Ichihara KI, Shibahara A, Yamamoto K, Nakayama T: An improved method for rapid analysis of the fatty acids of glycerolipids. *Lipids,* 31, 535-539, 1996. DOI: 10.1007/BF02522648

23. Paquot C: Standard Methods for the Analyses of Oils, Fats and Derivates. 6th edn., Centre Nationale de la Recherche Scientifique, Thiais,

France, 1979.

24. SPSS: Statistical Package in Social Sciences for Windows. Statistical Innovation Inc., Chicago, USA, 2010.

25. Dawson B, Trapp RG: Basic and Clinical Biostatistics. Third edn., Lange Medical Books/McGraw-Hill Publishing Division, New York, 161-182, 2001.

26. Tzakou O, Pitarokili D, Chinou IB, Harvala C: Composition and antimicrobial activity of the essential oil of *Salvia ringens*. *Planta Med,* 67, 181-183, 2001. DOI: 10.1055/s-2001-10627

27. Jang Ko YH, Yang HY, Ha JS, Kim JY, Kim JY, Kang SY, Yoo DH, Nam DS, Kim DH, Lee CY: Influence of essential oil components on growth performance and the functional activity of the pancreas and small intestine in broiler chickens. *Asian-Australian J Anim Sci,* 17, 394-400, 2004.

28. Lee KW, Everts H, Kappert HJ, Frehner M, Losa R, Beynen AC: Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens. *Br Poultry Sci,* 44, 450-457, 2003. DOI: 10.1080/0007166031000085508

29. Lee KW, Everts H, Kappert HJ, Beynen AC: Growth performance of broiler chickens fed a carboxymethyl cellulose containing diet with supplemental carvacrol and/or cinnamaldehyde. *Int J Poultry Sci,* 3, 619-622, 2004. DOI: 10.3923/ijps.2004.619.622

30. Botsoglou NA, Florou-Paner P, Christaki E, Fletouris DJ, Spais AB: Effect of dietary oregano essential oil on performance of chickens and on iron-induced lipid oxidation of breast, thigh and abdominal fat tissues. *Br Poultry Sci,* 43, 223-230, 2002. DOI: 10.1080/00071660120121436

31. Kamel C: Tracing modes of action and roles of plant extracts in non-ruminants. **In,** Garnsworthy PC, Wiseman J (Eds): Recent Advances in Animal Nutrition. 135-150, Nottingham University Press, Nottingham, UK, 2001.

32. Küçükyılmaz K, Çatlı AU, Çınar M: The effect of dietary essential oil mixture supplementation on the broiler performance, carcass yield and some internal organ weight. *Kafkas Univ Vet Fak Derg,* 18, 291-296, 2012. DOI: 10.9775/kvfd.2011.5443

33. Khosravinia H: Productive performance, litter characteristics and carcass defects of the broiler chickens given drinking water supplemented with *Satureja khuzistanica* essential oils. *J Medicanal Plants Res,* 7, 1754-1760, 2013.

34. Alçiçek A, Bozkurt M, Çabuk M: The effect of an essential oil combination derived from selected herbs growing wild in Turkey on broiler performance. *S Afr J Anim Sci,* 33, 89-94, 2003.

35. Khattak F, Ronchi A, Castelli P, Sparks N: Effects of natural blend of essential oil on growth performance, blood biochemistry, cecal morphology, and carcass quality of broiler chickens. *Poultry Sci,* 93, 132-137, 2014. DOI: 10.3382/ps.2013-03387

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Research Article

In Vitro Toxicity of Some Pesticides on Goat and Dog Spermatozoa [1] [2]

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Abstract

In vitro toxic effects of selected pesticides (cypermethrin, flumethrin, propoxur, carbaryl, chlorpyrifos, metamidophos) were evaluated on dog and buck fresh spermatozoa using MTT (mitochondrial activity/viability), NeutralRed-NR (lysosomal activity/viability) and CASA (motility parameters/VCL, VSL, VAP, linearity, straightness, wobble) tests. For buck, the most toxic compounds were carbamates followed by organophosphate and pyrethroids. Carbamates, again induced the highest toxicity in dog sperm, followed by pyrethroids. In general, all pesticide treatments were found to increase the hyperactivity of buck spermatozoa except flumethrin; whereas a decrease was present for dog spermatozoa (except carbaryl). A decrease of VCL, VSL and VAP parameters was evident in buck ($P<0.05$), whereas no difference was found in dog sperm ($P>0.05$). Overall, flumethrin was found to induce less effect on motility parameters compared to cypermethrin. As a conclusion, the combination of MTT and CASA along with NR would provide more accurate data for the *in vitro* evaluation of chemicals on spermatozoa; where alternative testing strategies for the ReproTox tests (especially for cosmetic product safety assessment where animal testing is banned) are recommended nowadays with battery strategies and species specific differences would play a key role for understanding the defense mechanisms.

Keywords: *Pesticides, Buck spermatozoa, Dog spermatozoa, In vitro toxicity, MTT, CASA, Neutral red*

Bazı Pestisitlerin Teke ve Köpek Sperması Üzerine *İn Vitro* Toksisiteleri

Özet

Bu çalışmada, bazı pestisitlerin (sipermetrin, flumetrin, propoksur, karbaril, klorprifos, metamidofos) köpek ve teke taze spermaları üzerindeki etkileri, MTT (mitokondriyal etkinlik/canlılık), Nötral Kırmızı-NK (lizozomal etkinlik/canlılık) ve Bilgisayar Destekli Semen Analizi-CASA (motilite parametreleri, VCL, VSL, VAP, lineerlik, doğrusallık, yalpalama) kullanılarak *in vitro* olarak araştırıldı. Teke sperması üzerine canlılık ve motilite bakımından en toksik bileşiğin karbamatlar, ardından organofosfatlar ve piretroitler olduğu; köpek spermasında ise aynı şekilde karbamatların, ardından da piretroitlerin toksik etki gösterdiği tespit edildi. Genel olarak tüm pestisit uygulamalarının teke spermasında hiperaktiviteyi (flumetrin dışında) arttırdığı, köpek spermasında ise azalttığı tespit edildi (karbaril dışında). Tekede VCL, VSL ve VAP parametrelerinde azalma gözlenirken ($P<0.05$), köpek spermasında anlamlı bir fark gözlenmedi ($P>0.05$). Piretroitler arasında flumetrinin, motilite parametreleri üzerine etkisi sipermetrine göre daha az bulundu. Sonuç olarak üreme toksisite testlerinde (özellikle kozmetik ürünlerin toksisite testlerinde hayvan deneylerinin yasaklandığı göz önünde bulundurularak) alternatif testler arasında bulunan ve batarya testleri içerisinde yer alan *in vitro* sperm toksisite araştırmalarında; MTT ve CASA ile birlikte NK'nın birlikte kullanılmasının kimyasalların sperma üzerine etkilerinin değerlendirilmesinde daha etkili olacağı ve türe özgü farklılıkların kimyasalların sperma canlılık ve motilite parametrelerini farklı şekil ve düzeyde etkileyeceği, dolayısıyla spermada türe özgü ksenobiyotik moleküler savunma mekanizmalarının araştırılmasının gerekliliği gösterilmiştir.

Anahtar sözcükler: *Pestisitler, Teke sperması, Köpek sperması, İn vitro toksisite, MTT, CASA, Nötral kırmızı*

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INTRODUCTION

Male infertility due to impaired semen quality is related to a variety of possible causes including genetic abnormalities, lifestyle (smoking, alcohol, clothing etc), diseases *(Brucella, Chlamydia)*, condition *(varicocele, hematocele* and *torsion)*, environment and occupation (hormone disrupters-xenoestrogens, anti-androgens, toxic substances-lead etc.) [1]. The physiological mechanisms that ultimately lead to healthy sperm production is complex; where a disturbance can take place at different periods in a lifetime and differ for each species [2]. Along with the increased concern on endocrine disrupting compounds, mainly due to their effects on triggering morphological and functional abnormalities in reproduction systems, screening chemicals for male infertility raised an increasing interest. Since March of 2013, European cosmetic products cannot be tested on animal for reproductive toxicity (the 7th amendment to the European Union's Cosmetics Directive). Non-animal *in vitro* alternative tests for the assessment of the effects of chemicals on reproductive system have been developed and many of them are validated. However, these models are often not fully reflective of physiological processes and need to be complemented with additional tests to accurately illustrate the overall reproductive function. Innovative non animal testing strategies are sought from regulatory authorities for rapid and accurate detection strategies [3].

Sperm motility is considered as one of the most important parameters in evaluating the fertilizing ability of sperm in the human or other mammal species where motility parameters directly affect sperm penetration of mucus or oocyte vestments [4]. The conventional, manual method of sperm count and assessment of motility under the optical microscope was found to be fraught with inevitable subjective [5]. Computer-assisted sperm analysis (CASA) has been developed as a sensitive and reliable method in the quantification of deficiencies in sperm motility pattern and, together with assessment of sperm concentration and viability for the evaluation of the fertilizing ability of an ejaculate [4].

The aim of this study was to evaluate the *in vitro* effects of pesticides *(carbaryl, chlorpyrifos, cypermethrin, flumethrin, methamidophos* and *propoxur)* on dog and goat spermatozoa using MTT and Neutral Red tests for viability and CASA system for the motility. In particular, the combination of viability assays and comparative investigation of species specific differences for the evaluation of xenobiotic exposure on spermatozoa *in vitro* has never been performed. Even though the combination MTT and CASA analysis as an objective, simple, inexpensive and efficient method for the screening of xenobiotics in spermatozoa was introduced previously, species specific differences are expected to provide an important aspect for the evaluation of these chemicals.

MATERIAL and METHODS

Animal experiments were conducted according to ethical principles and this study was approved by the animal ethics committee in Ankara University (2015-21-230).

Semen samples were collected from Angora buck (4 years of age) under uniform feeding and housing conditions in Ankara University Faculty of Veterinary Medicine Research Farm, where they are proven to be free from any general or genital diseases. Ejaculate was collected with the aid of an electro ejaculator (Ruakura®, New Zealand). Animals were strained and topical lidocaine was used to reduce the pain caused by rectal smooth muscle spasm or *intra pelvic nerve* stimulation, then after probe was inserted into *rectum*. Ejaculate from buck were directly immersed in a water bath at 35.5°C until it could be assessed for total and progressive motility as well as sperm concentration. Then samples at 35.5°C were cooled down to 24°C for laboratory transfer.

Semen samples from the known healthy fertile German shepherd male dog (2 years of age and sired a litter previously) were collected by digital manipulation. The first and third fraction were collected in two separate pre-warmed plastic tubes. The volume of 1st fraction, 2nd fraction and 3rd fraction with a graduated collection tubes and pH were determined with stripes. Only the second sperm-rich fraction of ejaculate collected was used for the experimental design.

Semen was evaluated macroscopically for volume, color and pH; microscopically for motility, concentration, viability and morphology. Total semen volume was determined from the graded collection tube soon after collection and the concentration was determined using an Accucell photometer. Spermatozoa were primarily analyzed by CASA and only ejaculates with a minimum concentration of 3×10^9 spermatozoa/mL and 75% progressively motile cells were used for dilution to a final concentration of 3.6×10^6/mL spermatozoa. For the dilutions a Tris-based extender (30.7 g of Tris, 16.4 g of citric acid, 12.6 g of fructose and 1000 mL of distilled water at a pH of 6.8 with no cryoprotectant) was used.

For cytotoxicity studies, collected sperm in Tris buffer were transferred to 96-well plates at 3.6×10^6 sperm/mL per well (100 µL per well). Pesticides *(carbaryl, chlorpyrifos, cypermethrin, flumethrin, methamidophos* and *propoxur)* were dissolved in Tris buffer (only for *flumethrin* 500 µL DMSO and 500 µL Tris) at 1000 µg/µL concentration. Working solutions at log conc. first, then half conc. were prepared (166.67-0.0000167 µg/µL per well). Viability was measured using MTT and Neutral Red Assays; wherein MTT, metabolic activity of the cells are evaluated by the conversion of a yellow tetrazolium salt to purple formazan particles by mitochondrial succinate dehydrogenase of

intact mitochondria of living cells and in Neutral Red, viable cells incorporate and bind the supravital neutral red dye in viable lysosomes. MTT assay was performed according to our previous study on sperm [6] modified from Mosmann [7] and neutral red assay was performed according to Repetto et al.[8]. Viability assays were both then quantified using a micro plate reader (SpectraMax i3x-Multimode Detection Platform, Molecular Devices, Sunnyvale, CA, USA) at 540 nm. Cytotoxicity values, expressed as percentage, was calculated with regard to the untreated cell control (containing only the vehicle buffer), which was set to 100% viability and the dead cell control (containing Triton-X) which was set to 0% viability. A plot of % cytotoxicity versus sample concentrations was used to calculate the concentration which was then evaluated to calculate 50% cytotoxicity defining IC_{50} value.

For motility studies, collected sperm in Tris buffer were transferred to 96-well plates at 3.6×10^6 sperm/mL per well (100 µL per well). Pesticides were dissolved in Tris buffer (only for *flumethrin* 500 µL DMSO and 500 µL Tris) and prepared in the stock concentrations to be applied at IC_{50} doses in 20 µL IC_{50}, $IC_{50}/2$, $IC_{50}/4$ and $IC_{50}/8$ concentrations were applied for both sperms. The results were recorded at 0 and 4th hour following the exposure.

A 5 µL sperm suspension was placed on preheated siliconized slides and covered with 22 × 22 mm^2 coverslips to achieve a calculated depth of 20 psm. Sperm movement was recorded using a 100 frame/s camera (Basler, 782 × 582 resolution) attached to a microscope (600×, Nikon eclipse 50I, SCA, Barcelona, Spain) with a phase-contrast objective (10 × 10 magnification) and connected to a computerized motion analysis system, the Sperm Class Analyzer (SCA, Microptics®, Spain).

The sperm motility characteristics were determined using a 10× objective microscope lens at 37°C. The SCA acquisition parameters were set with 5 µm^2 <particle area <80 µm^2; progressivity >8% of STR; Circular <50% LIN; Vap points = 5; frame rate = 100/s; total captured images = 180 for dog and 10 µm^2 <particle area <80 µm^2; progressivity >75% of STR; circular <50% LIN; Vap points = 5; frame rate = 100/s; total captured images = 180 for goat. Each recorded field consisted of a mean of five replicates, each one analyzing from 100 to 300 sperm tracks and minimum average path at 50 µm/s and 40 µm/s; with >50% and >55% progressive motility were accepted for dog and goat sperm, respectively. For 5 µL of each sample, at least 200 up to 300 spermatozoa in 5 different areas were evaluated.

Measured data were plotted against the corresponding inhibition values using NCSS 2007, resulting in the inhibition curves as regression analysis, selected by the highest coefficient of determination (R^2); where IC_{50} (half maximal inhibitory concentration) values were calculated by interpolation of experimental data. Data were checked for parametric test assumptions by Kolmogrov-Smirnov test and Levene test to determine the homogeneity. Statistical significance between the pesticides and the animal species were determined by one-way ANOVA followed by posthoc analysis. A "P" value of <0.05 was defined as statistically significant.

RESULTS

IC_{50} values for *methamidophos, chlorpyrifos, carbaryl, propoxur, flumethrin* and *cypermethrine* using MTT assays are as follows in µg/mL; 104.62; 3.61; 23.98; 15.14; 67.89; 1.71 for dog sperm and 45.47; 11.36; 25.01; 1.47; 114.47; 1.58 for buck sperm. IC_{50} values was found to be lower in Neutral Red assay (NR) compared to MTT in general. IC_{50} values for the tested pesticides using NR (except *flumethrin*, where IC_{50} cannot be calculated) were as follows in µg/mL; 33.16; 3.80; 20.47; 58.53; ND; 0.79 for dog and 19.45; 2.51; 25.40; 2.42; ND; 0.39 for buck *(Table 1)*. IC_{50} values were found to be lower in buck ($P<0.05$) except *carbaryl* by Neutral Red and MTT assays and *chlorpyrifos* and *flumethrin* by MTT assay. For buck, the most toxic compounds were carbamates followed by organophosphate and pyrethroids. *Carbamates*, again induced the highest toxicity in dog sperm, followed by pyrethroids then organophosphates.

All pesticide treatments at $IC_{50}/2$ concentrations were found to increase the hyperactivity of buck spermatozoa except *flumethrin* (no change was present). On the contrary

Table 1. *IC_{50} values for the tested pesticides on dog and buck sperm*

Chemical Family	Main Mechanism of Action	Drug Name	IC_{50}			
			MTT		Neutral Red	
			Dog	Buck	Dog	Buck
Organophosphates	Acetylcholine esterase inhibitors (Irreversible)	*Methamidophos*	104.62	45.47	33.16	19.45
		Chlorpyrifos	3.61	11.36	3.80	2.51
Carbamates	Acetylcholine esterase inhibitors (Reversible)	*Carbaryl*	23.98	25.01	20.47	25.40
		Propoxur	15.14	1.47	58.53	2.42
Pyrethroids	Sodium channel modulators	*Flumethrin*	67.89	114.47	ND	ND
		Cypermethrin	1.71	1.58	0.79	0.39

Table 2. *Effects on the kinetic parameters (VCL, VSL and VAP) of pesticides on buck and dog sperm (percent control)*

% of Control±SD (Total = Slow + Medium + Rapid)			Curvilinear Velocity (VCL)		Straightline Velocity (VSL)		Average Path Velocity (VAP)	
			Buck	Dog	Buck	Dog	Buck	Dog
Organophosphates	*Methamidophos*	IC_{50}	117.10±26.56	89.01±6.18	85.24±12.72	92.73±8.38	101.84±29.13	97.57±6.38
		$IC_{50}/2$	111.60±21.63	97.07±7.79	105.62±22.69	104.39±47.78	112.63±22.86	120.08±21.35
		$IC_{50}/4$	137.04±26.56	103.14±10.42	130.89±28.13	79.03±20.62	134.80±27.36	84.95±5.05
		$IC_{50}/8$	152.70±29.59	71.41±9.65	148.83±31.98	58.48±6.59	151.84±30.82	68.00±9.56
	Chlorpyrifos	IC_{50}	40.52±7.46	39.43±3.95	48.42±0.99	33.58±1.07	42.37±1.65	35.01±2.11
		$IC_{50}/2$	103.08±11.67	68.06±3.49	57.71±0.86	61.50±3.58	86.66±22.60	62.27±3.66
		$IC_{50}/4$	74.99±8.49	95.51±4.32	70.26±1.05	80.78±15.82	64.06±1.92	83.76±8.54
		$IC_{50}/8$	49.46±5.60	59.12±3.32	42.73±0.64	51.19±14.21	37.63±1.13	55.64±13.23
Carbamates	*Carbaryl*	IC_{50}	103.81±17.53	99.36±6.90	92.88±2.69	130.41±11.69	96.71±8.58	112.39±7.35
		$IC_{50}/2$	131.65±14.12	75.81±6.79	75.02±1.42	114.68±15.40	141.94±7.45	83.18±17.03
		$IC_{50}/4$	82.34±8.83	72.04±6.35	64.77±1.23	146.46±11.86	66.79±3.50	88.62±4.20
		$IC_{50}/8$	188.42±20.21	70.46±5.80	193.10±3.66	138.96±17.01	188.08±9.87	88.47±10.35
	Propoxur	IC_{50}	40.92±9.66	75.00±5.21	35.85±15.48	78.22±7.07	37.32±6.47	87.42±5.72
		$IC_{50}/2$	50.58±6.62	80.39±5.43	53.50±8.99	100.94±38.97	60.00±14.43	95.69±15.24
		$IC_{50}/4$	57.51±7.53	62.38±4.40	58.81±9.89	107.42±22.86	60.83±4.49	86.94±26.90
		$IC_{50}/8$	105.02±13.75	71.52±4.08	136.18±22.89	101.50±12.67	126.87±9.36	93.43±25.99
Pyrethroids	*Cypermethrin*	IC_{50}	41.99±8.09	92.19±14.18	45.28±3.03	78.15±11.93	41.23±4.36	91.75±18.76
		$IC_{50}/2$	99.46±11.58	100.38±18.46	80.68±2.43	86.63±26.42	79.63±4.62	91.44±27.76
		$IC_{50}/4$	48.59±5.66	62.78±15.04	58.21±1.76	99.32±30.60	50.85±2.95	85.26±11.34
		$IC_{50}/8$	63.22±7.36	62.77±12.25	88.93±2.68	85.73±14.33	73.76±4.28	74.93±23.66
	Flumethrin	IC_{50}	37.34±2.46	107.64±10.78	31.16±9.92	97.79±3.11	34.32±5.68	103.00±6.21
		$IC_{50}/2$	67.06±3.60	81.54±11.41	84.35±16.23	89.85±15.25	74.54±7.51	85.26±14.75
		$IC_{50}/4$	52.77±2.84	86.19±12.76	70.11±13.49	124.32±10.36	52.21±5.26	104.42±8.54
		$IC_{50}/8$	59.85±3.22	95.90±15.87	83.25±16.02	103.99±28.81	67.67±6.81	113.95±11.25

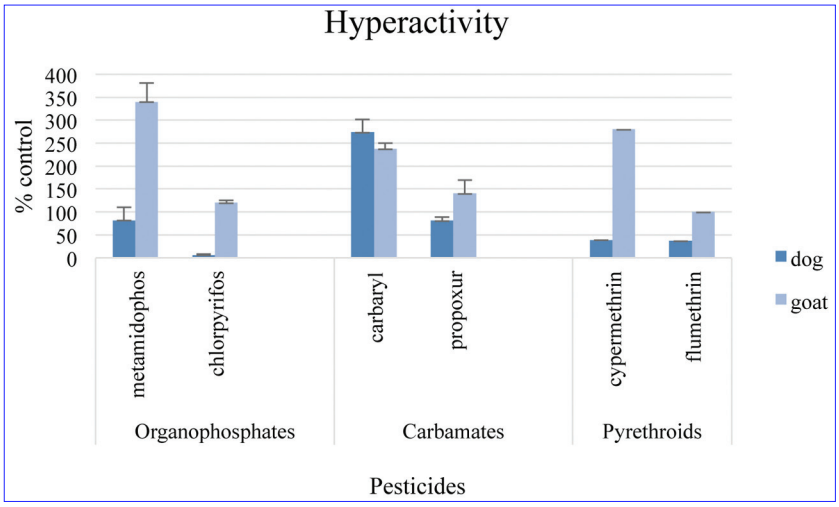

Fig 1. Effects on the hyperactivity of pesticides on buck and dog sperm (percent control)

a decrease was present for dog spermatozoa (except *carbaryl)*. For pyrethroids *(cypermethrin* and *flumethrin)* this decrease in hyperactivity in dog for both drugs were found as 39.09 and 37.27% compared to untreated control, whereas the most decrease was observed for *chlorpyrifos* treatment with a value of 6.37% *(Fig. 1)*. A decrease of VCL, VSL and VAP parameters was evident in buck (P<0.05), whereas no difference was found in dog sperm (P<0.05). Overall, *flumethrin* was found to induce less effect on motility parameters compared to *cypermethrin (Table 2)*.

Table 3. *Effects on the kinetic parameters (LIN, STR and WOB) of pesticides on buck and dog sperm (percent control)*

% of Control±SD (Total = Slow + Medium + Rapid)			Linearity		Straightness		Wobble	
			Buck	Dog	Buck	Dog	Buck	Dog
Organophosphates	*Methamidophos*	IC_{50}	72.79±1.43	104.37±4.95	83.70±1.43	95.26±4.65	86.97±2.14	110.01±0.87
		$IC_{50}/2$	94.64±5.69	107.75±22.51	93.78±5.69	87.14±7.96	100.92±3.51	124.15±2.33
		$IC_{50}/4$	94.85±5.70	76.76±7.29	96.73±5.70	93.25±14.59	97.97±3.62	82.65±4.15
		$IC_{50}/8$	96.92±5.83	82.05±25.21	97.44±5.83	86.21±24.73	99.56±3.65	95.56±8.53
	Chlorpyrifos	IC_{50}	119.49±1.42	85.32±12.83	114.29±1.04	96.13±5.46	104.56±3.65	89.11±8.06
		$IC_{50}/2$	55.99±0.79	90.53±48.99	66.60±0.57	99.00±10.63	84.07±3.64	91.82±15.94
		$IC_{50}/4$	94.27±8.33	84.73±17.51	109.77±0.95	96.67±20.21	85.82±3.72	88.01±20.21
		$IC_{50}/8$	87.07±1.23	86.75±6.41	114.29±0.99	92.22±16.64	76.24±3.30	94.45±11.45
Carbamates	*Carbaryl*	IC_{50}	89.48±17.53	130.41±6.19	96.04±2.69	115.36±5.63	93.16±1.25	113.51±0.90
		$IC_{50}/2$	56.98±14.12	114.68±29.93	52.85±1.42	104.57±11.56	107.82±1.34	110.12±2.13
		$IC_{50}/4$	78.78±8.83	146.46±18.49	97.20±1.23	119.12±27.08	81.31±6.01	123.45±5.67
		$IC_{50}/8$	102.61±20.21	138.96±10.83	103.18±3.66	110.72±28.95	99.94±1.24	126.02±15.38
	Propoxur	IC_{50}	87.62±2.85	104.50±4.96	96.08±18.57	89.69±4.37	91.20±14.93	116.98±0.93
		$IC_{50}/2$	105.77±2.41	125.80±26.31	89.17±13.34	105.74±9.09	118.61±4.92	119.46±2.38
		$IC_{50}/4$	102.26±2.33	172.52±19.14	96.68±16.45	123.85±22.14	105.77±4.39	139.87±7.18
		$IC_{50}/8$	129.67±2.96	142.18±11.48	107.34±19.61	108.89±17.29	120.80±5.01	131.10±20.27
Pyrethroids	*Cypermethrin*	IC_{50}	107.83±2.62	84.93±5.62	109.81±3.76	85.38±3.64	98.20±10.85	99.88±3.49
		$IC_{50}/2$	81.12±1.49	86.46±20.49	101.32±12.90	94.96±6.80	80.06±1.27	91.4±6.88
		$IC_{50}/4$	117.90±2.17	158.49±13.70	112.92±3.23	116.76±15.60	104.30±10.65	136.29±20.18
		$IC_{50}/8$	138.06±2.54	136.84±79.03	118.12±9.38	114.68±35.17	116.50±1.85	119.81±32.09
	Flumethrin	IC_{50}	82.83±10.06	91.02±13.69	89.40±17.44	95.16±5.41	92.26±3.98	96.03±8.68
		$IC_{50}/2$	126.88±5.95	110.39±3.73	115.27±10.63	105.63±11.23	110.88±3.85	104.94±19.63
		$IC_{50}/4$	133.74±6.27	144.51±38.84	136.32±12.57	119.34±26.35	98.64±5.42	121.58±21.40
		$IC_{50}/8$	140.60±6.59	108.64±17.80	125.31±11.56	91.48±27.39	112.74±8.91	119.25±13.20

For organophosphate drugs (chlorpyrifos and methamidophos), a decrease in VCL, VSL, VAP parameters for both drugs in buck was observed, whereas this decrease was present for only chlorpyrifos in dogs ($P<0.05$). Propoxur were found to decrease VCL, VSL and VAP values where no difference was found for carbaryl ($P>0.05$). Even though the minor changes, in general, the linearity, straightness and wobble values were found to have decreased in buck for all treatments except chlorpyrifos. Meanwhile this difference were insignificant in most of them and did not exert dose correlation *(Table 3)*.

DISCUSSION

The species specific differences in sperm morphology including spermatozoon membrane structural composition, ion channel distribution and oxidative damage are the key response elements for the toxic effects of xenobiotics on spermatozoa. Phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, compose the majority of lipid fraction of the sperm cell membranes, functioning as a natural barrier for chemical and physical stress defining the functional characteristics of the sperm [9]. Long-chain polyunsaturated fatty acids (PUFAs) in spermatozoa influence the membrane fluidity [10] can lead to reduced membrane fluidity and a functional defect in sperm-oocyte fusion and fertilization [11]. As PUFAs and docosahexaenoic acid (DHA) are expected to effect the sperm viability, motility and the ability of spermatozoa to survive cryogenic storage and restores the protection [9,10], species specific differences might play an important role in the xenobiotic transport to sperm; meanwhile the alteration in the PUFAs composition could be the common base of different degenerative processes [12]. The goat sperm plasma membrane was found to be particularly rich in ether lipids phosphatidylcholine and phosphatidylethanolamine [13] and yet dog semen also contained great amounts of PUFA [12]. This lipid structure of the spermatozoa membrane also effects the the absorption of the chemical into the cell which is also determined by partitioning between aqueous and lipid phases. The octanol/water partition coefficient (log K_{ow}), defining the ratio of the concentration of the chemical in octanol to the concentration of water, is important in the absorption

of drugs. Hydrophobic drugs with high octanol/water partition coefficient are preferentially distributed to the lipid bilayers of cells [14]. In the current study *cypermethrin* having the highest Log K_{ow}, (6.6) were found to exert the lowest IC_{50} in both viability assays; however, this relation (Log K_{ow} of the tested pesticides are 5.9 for *flumethrin*, -1.74 for *methamidophos*, 4.7 for *chlorpyrifos*, 1.45 for *propoxur* and 2.36 for carbaryl) was not present for the rest of the drugs.

The distribution of the ion channels in different species could also implicate the toxicity differences of the tested pesticides. Amiloride sensitive Na^+ channels contribute to the regulation of resting sperm membrane potential [15]; whereas voltage-dependent Na^+ channels are required for the regulation of mature sperm function with an important role in the initial capacitation steps for the hyperpolarization before the acrosome reaction [16]. After capacitation, the sperm cell hyperpolarizes with an increase in K^+ permeability and blocking of the epithelial sodium channel. This effect is similar to the mechanism of action of pyrethroids. In the current study, this *in vitro* effect might have induced a potency on the transmembrane ion channels; where an increase in the overall hyperactivity is evident. On the other hand, the sperm activates the oocyte by causing either a single or series of Ca^{2+} oscillations and T-type Ca^{2+} channels are the key components in male reproduction, such as in the acrosome reaction and sperm motility [17]. The influence on the voltage independent Ca^{2+} influx, especially from the extracellular environment such like the conditions in our experiment are utilized during fertilization and might initiate a false acrosome reaction making the cells more hyperactive.

Acrosome contains hydrolytic enzymes like acrosin, hyaluronidase and many other hydrolases and esterases including acetylcholine esterase and acetylcholine transferase [18]. Also several reports suggest that nACh receptors (α7 nicotinic acetylcholine receptor) are present in mammalian sperm which is involved in the zona pellucida-induced acrosome reaction along with increased intracellular calcium levels [19]. As well known, organophosphorus compounds (OPs) and *carbamates* bind to an active site of acetylcholinesterase (AChE) and inhibit the functionality of this enzyme. In the current study, OPs increased the hyperactivity in buck and decrease in dog; where *carbamates* increased hyperactivity in both species; which might also be attributed to the species specific differences in the nACh receptor expression.

Another key factor; free-radical induced oxidative damage to mitochondrial membrane lowers the production of ATP ultimately affecting the motility and it has deleterious effects on sperm plasma membrane and DNA damage [20]. In the study by Zalata et al.[21] *in vitro* effects of *cypermethrin* on human spermatozoa were investigated; where *cypermethrin* was demonstrated to preferentially get localized in the hydrophobic core of the membrane, increasing the lipid packing and decreasing the membrane fluidity. It was concluded that, *cypermethrin* produced oxidative stress by generating reactive oxygen species (ROS) and reducing the antioxidant defenses. Although high concentrations of ROS cause sperm pathologies resulting in a loss of sperm motility and viability, low concentrations play an important role in capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion [20]. This might explain current study findings; where *cypermethrin* was found to be highly toxic compared to other pesticides *in vitro*. In buck, the toxic effect of *cypermethrin* in the current study, was more evident with an increased hyperactivity and a possible loss of ATP and death compared to dog.

Since motility of the spermatozoon depends on the energy expense produced in mitochondria [21]; MTT results in the current study, indicating the mitochondrial function, with lower IC_{50} values reveal that pesticides altered the mitochondrial functions, with an over increase of hyperactivity leading to cell death. MTT method, is efficient in processing a large number of specimens and therefore may be a powerful tool for preliminary screening of toxic compounds in spermatozoa. Neutral red assay, for the lysosomal activity, could also be used as a supportive quantitative-colorimetric method for the viability assays along with MTT and not only for the microscopic analysis for the acrosome reaction [22]. As CASA variables unfolds the important indicators for the fertilization capability of spermatozoa such as motility; it is expected to provide as prognostic tool for *in vitro* toxicity assays along with the viability quantitative such as MTT and NR for the potential screening of xenobiotics *in vitro*. The combination of these protocols would provide cheap, repeatable results where many samples are screened in a very short time. Even though these *in vitro* models represent only a very simplified picture of reality which encompasses only a small part of the complex reproductive cycle and has its own limitations; it is required especially for the reproductive toxicity battery assays for the cosmetic products testing where *in vivo* studies are no longer allowed. Researchers should also include the species specific differences for their decisions using these simplified *in vitro* sperm toxicity assays.

REFERENCES

1. Comhaire F, Mahmoud A, Zalata A, Dhooge W: Factors affecting male fertility. **In,** De Vriese DR, Christophe AB (Eds): Male Fertility and Lipid Metabolism. 1-10. AOCS Publishing, Champaign, IL, 2003.

2. Knez K: Endocrine-disrupting chemicals and male reproductive health. *Reprod Biomed Online,* 26, 440-448, 2013. DOI: 10.1016/j.rbmo.2013.02.005

3. Lorenzetti S, Altieri I, Arabi S, Balduzzi D, Bechi N, Cordelli E, Galli C, Ietta F, Modina SC, Narciso L, Pacchierotti F, Villani P, Galli A, Lazzari G, Luciano AM, Paulesu L, Spanò M, Mantovani A: Innovative non-animal testing strategies for reproductive toxicology: The contribution of Italian partners within the EU project ReProTect. *Ann Ist Super Sanita,* 47, 429-444, 2011. DOI: 10.4415/ANN_11_04_16

4. Peiris LD, Moore HD: Evaluation of effects of 1,3-dinitrobenzene on sperm motility of hamster using computer assisted semen analysis

(CASA). *Asian J Androl*, 3 (2): 109-114, 2001.

5. Ohtani K, Yamazaki S, Kubota H, Miyagawa M, Saegusa J: Comparative investigation of several sperm analysis methods for evaluation of spermatotoxicity of industrial chemical: 2-bromopropane as an example. *Ind Health,* 42, 219-225, 2004. DOI: 10.2486/indhealth.42.219

6. Yurdakok B, Tekin K, Daskin A, Filazi A: Effects of polychlorinated biphenyls 28, 30 and 118 on bovine spermatozoa *in vitro. Reprod Dom Anim,* 50, 41-47, 2015. DOI: 10.1111/rda.12447

7. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods,* 65, 55-63, 1983. DOI: 10.1016/0022-1759(83)90303-4

8. Repetto G, del Peso A, Zurita JL: Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc,* 3, 1125-1131, 2008. DOI: 10.1038/nprot.2008.75

9. Mandal DK, Kumar M, Tyagi S: Effect of age on spermiogram of Holstein Friesian × Sahiwal crossbred bulls. *Animal,* 4, 595-603, 2010. DOI: 10.1017/S1751731109991273

10. Martinez-Soto JC, Landeras J, Gadea J: Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success. *Andrology*, 1, 365-375, 2013. DOI: 10.1111/j.2047-2927.2012.00040.x

11. Safarinejad MR: Effect of omega-3 polyunsaturated fatty acid supplementation on semen profile and enzymatic anti-oxidant capacity of seminal plasma in infertile men with idiopathic oligoasthenoterato-spermia: A double-blind, placebo-controlled, randomised study. *Andrologia* 43, 38-47, 2011. DOI: 10.1111/j.1439-0272.2009.01013.x

12. Gavazza M, Guttiérez AM, Marmunti M, Palacios A: Lipid peroxidase assays in canine fresh semen. *Rev Vet,* 20 (2): 97-102, 2009.

13. Rana AP, Majumder GC, Misra S, Ghosh A: Lipid changes of goat sperm plasma membrane during epididymal maturation. *Biochim Biophys Acta,* 1061, 185-196, 1991. DOI: 10.1016/0005-2736(91)90284-F

14. Avdeef A: Octanol-Water Partitioning. **In,** Avdeef A (Ed): Absorption and Drug Development: Solubility, Permeability, and Charge State, 2nd edn., 174-219, John Wiley & Sons, Inc., Hoboken, NJ, USA. 2012.

15. Hernández-González EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, López-González I, Demarco I, Wertheimer E, Darszon A, Visconti PE: Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyper-polarization in mouse sperm. *J Biol Chem,* 281, 5623-5633, 2006. DOI: 10.1074/jbc.M508172200

16. Pinto FM, Ravina CG, Fernández-Sánchez M, Gallardo-Castro M, Cejudo-Román A, Candenas L: Molecular and functional characterization of voltage-gated sodium channels in human sperm. *Reprod Biol Endocrinol,* 7, 71, 2009. DOI: 10.1186/1477-7827-7-71

17. Tao J, Zhang Y, Li S, Sun W, Soong TW: Tyrosine kinase-independent inhibition by genistein on spermatogenic T-type calcium channels attenuates mouse sperm motility and acrosome reaction. *Cell Calcium,* 45, 133-143, 2009. DOI: 10.1016/j.ceca.2008.07.004

18. Pesch S, Bergmann M: Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron,* 37, 597-612, 2006. DOI: 10.1016/j.micron.2006.02.006

19. Jaldety Y, Glick Y, Orr-Urtreger A, Ickowicz D, Gerber D, Breitbart H: Sperm epidermal growth factor receptor (EGFR) mediates α7 acetylcholine receptor (AChR) activation to promote fertilization. *J Biol Chem,* 287, 22328-22340, 2012. DOI: 10.1074/jbc.M111.292292

20. Agarwal A, Virk G, Ong C, du Plessis S: Effect of oxidative stress on male reproduction. *World J Mens Health,* 32, 1-17, 2014. DOI: 10.5534/wjmh.2014.32.1.1

21. Zalata A, Elhanbly S, Abdalla H, Serria MS, Aziz A, El-Dakrooy SA, El-Bakary AA, Mostafa T: *In vitro* study of cypermethrin on human spermatozoa and the possible protectiverole of vitamins C and E. *Andrologia,* 46, 1141-1147, 2014. DOI: 10.1111/and.12206

22. Jankovicova J, Simon M, Antalikova J: Methods for evaluation of an acrosome reaction of bovine spermatozoa. *Acta Fytotechn Zootechn,* 1, 118-119, 2006.

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Research Article

Identification of Risk Factors for Canine Transmissible Venereal Tumour (CTVT) in Owned Dogs in Pakistan

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Abstract

A matched case-control study was conducted to investigate the risk factors for canine transmissible venereal tumour (CTVT) in owned dogs of Lahore District, Pakistan. Ninety six laboratory-confirmed CTVT positive dogs were matched on the date of clinic visited with 96 control dogs. The univariate analysis was employed to assess the relationship between different population characteristics and the presence or absence of CTVT. Significant factors associated with CTVT that were obtained using univariate analysis were further included in multivariable logistic regression analysis.. Important identified risk factors were breeding strategies (OR=6.36), dog keeping purpose (OR=4.11), stray dog population (OR =3.35), dogs in neighborhood (OR=2.87), history of skin/genital diseases (OR=2.86) and high density population area (OR=2.54). Protective factors included chain/restrain the dog, use of separate cage, use of proper fencing around the kennel and knowing the difference between normal red discharge and clotted blood. With rigorous protective measures and controlling these risk factors, would decrease the spread of CTVT among the canine population in Pakistan. This is the first study performed regarding the quantification of risk factors for CTVT in Pakistan.

Keywords: *Canine, Pakistan, Risk factors, Case control, Transmissible venereal tumour*

Pakistan'da Sahipli Köpeklerde Köpek Bulaşıcı Veneral Tümör (CTVT) İçin Risk Faktörlerinin Belirlenmesi

Özet

Pakistan'ın Lahor Eyaletinde sahipli köpekler arasında köpek bulaşıcı veneral tümör (CTVT) hastalığının risk faktörlerini araştırmak amacıyla eşleştiriliş vaka kontrol çalışması yürütüldü. Doksan altı adet laboratuarda CTVT pozitif olduğu tespit edilen köpek kliniğe getirilen 96 adet kontrol köpek ile karşılaştırıldı. Değişik popülasyon özellikleri ile CTVT'nin bulunup bulunmadığı durumu arasındaki ilişkiliyi belirlemek amacıyla tek değişkenli analiz yöntemi uygulandı. Tek değişkenli analiz yöntemi kullanılarak CTVT ile ilgili olduğu saptanan anlamlı faktörler çok değişkenli lojistik regresyon analizine dahil edildi. Belirlenen önemli risk faktörleri olarak çiftleştirme stratejileri (OR=6.36), köpeği bulundurma sebebi (OR=4.11), sokak köpeği popülasyonu (OR =3.35), yakın çevredeki köpekler (OR=2.87), deri/genital hastalık geçmişi (OR=2.86) ve yüksek yoğunluk popülasyon alanı (OR=2.54) belirlendi. Koruyucu faktörler köpeği bağlama/kısıtlamayı, ayrı kafesin kullanılmasını, kulübe etrafında uygun çitin kullanılması ile normal kırmızı akıntı ile pıhtılı kan arasındaki farkın bilinmesini içermekteydi. Yoğun olarak koruyucu önlemlerin alınması ve risk faktörlerinin kontrol edilmesi Pakistan'da köpek popülasyonu içerisinde CTVT'nin yayılmasını azaltabilir. Bu çalışma Pakistan'da CTVT için risk oluşturan faktörlerinin belirlenmesinde yapılmış ilk çalışmadır.

Anahtar sözcükler: *Canine, Pakistan, Risk faktörleri, Vaka kontrol, Bulaşıcı veneral tümör*

INTRODUCTION

Canine Transmissible Venereal Tumour (CTVT) is one of the unique neoplasms that can be physically transmitted during coitus by direct contact such as licking, scraping, and biting [1,2]. Thus it represents the cells with high number of mutations that are developed into pathogen. Tumorous cells can be transmitted only across abraded

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mucosa with broken epithelium. Acting like an infectious pathogen, cells propagate and establish in the organism. These cells can also transmit from mother to the offspring during social interactions such as grooming and other maternal behaviour. It is a well-known tumour of domestic dogs [3,4]. Based on these, CTVT is seen as a major threat to the reproductive efficiency of canines [5].

CTVT is much prevalent in countries with tropical and sub-tropical settings due to loose import laws [6]. Due to similar settings and loose canine import laws, it is possibly a prevalent disease in Pakistan. Although considerable work regarding diagnosis and therapeutic management has been reported in Pakistan [7,8]. However, as compared to other countries, there is little work has been done in Pakistan regarding the risk factors associated with CTVT [6,9,10].

Previous studies have revealed that introduction and transmission of CTVT is associated with age, breed, sex, purpose of dog keeping [3,10], contact with stray dogs and high human population density areas [9]. These mentioned risk factors are important in identification of high-risk areas and kennels, which could be planned for interventions like treatment. Furthermore removal of these identified risk factors could be important part of disease control policies.

Based on such importance of breeding inefficiency and epidemiology of CTVT, the current study was planned. The main objective of this study was to investigate the risk factors associated with CTVT in owned dogs under local geographical conditions of Pakistan. These findings may also provide baseline for studying the spread of CTVT in similar geographical conditions.

MATERIAL and METHODS

A case control study was conducted in the Lahore District, Punjab, Pakistan;included the clinical cases between January 2014 and January 2015. Data related to cases and controls were collected from a total of 24 clinical practices in Lahore. From this data, 96 cases of CTVT and 96 controls (n=192) were required to achieve the study power of 80% for detection of 2.0 having odds ratio with 95% confidence interval [11]. A team of researchers visited the homes/kennels to collect the data related to risk factors. A structured questionnaire was used to get information from the dog owners.

Cases were included from the records of those pet dogs that exhibited the clinical signs (cauliflower like lesions on external genitalia, serosenguinus clotted bloody discharge, lethargy) of CTVT and were confirmed by (impression smear and aspiration needle) cytology with Leishman-Giemsa (LG) staining techniques followed by the microscopic examination [2]. In control, dogs visiting the clinics on the same day for other problems like vaccinations, routine check-ups, fever of unknown origin, ectoparasites, dyspnoea, wounds, and other infectious diseases were included. These dogs were considered as negative if they did not show any clinical signs [2,12,13]. Only those dogs were considered as cases which are kept as owned dogs and are not neutered. Those dogs were excluded from the study which were neutered and not owned.

A pre-designed questionnaire was used for the collection of data. The questionnaire contained several questions representing the different risk factors on the baseline parameters of the dogs including sex, age, type of dog (Purpose of dog keeping), breed and conditions related to management and breeding practices. Stray dog population was defined as the presence of free-roaming dogs with no owners in 1 km of the distance of the area. Proper fencing was considered as the presence of boundary walls around the house/kennel.Dogs in neighbourhood were defined as the presence of pet dogs in ten houses around the house/kennel. Usually bloody discharge during the proestrus phase and vaginal cytology are used to confirm the approaching oestrus. But considering only bloody discharge as the sole indicator heat can be easily confused with the clotted blood that is released during CTVT. That is why the knowledge related to breeding practices involving bloody discharge was also included as risk factors. These selected risk factors were the result of extensive review of previous studies and observations of local canine keeping practices [3,8-10]. The questionnaire and the variable boundaries were explained to the owners by the interviewer so that owner could answer those questions easily.

The location of all the cases was recorded with Global Positioning System (GPS, Garmin, Olathe, KS, USA) in WGS-84 datum. Chloropleth map was generated in Q-GIS 2.6.1 (Quantum-GIS Group). Geographical data of Lahore District consisting of boundaries and administrative town divisions were downloaded from the internet (http://www.diva-gis.org/datadownand http://lwmc.com.pk/company_profile.php).

To manage the data, a database was created in Microsoft Excel 2010. Data was analysed by using SPSS® (Version 16.0, Chicago, USA). Univariate analysis for dichotomous variables was applied to observe the association among different variables divided into 2 levels encoding Yes/High/Guard/Own = 1 and No/Low/Common/Pet = 2. Odds ratio (OR) was calculated for the assessment of risk factors. Logistic model regression was also applied to analyse the complete relation between the variables. Variables with P value <0.2 were selected in multivariable logistic regression model with forward method. Similar method was repeated until all remaining variables showed the P value <0.05 [14]. The two tailed P value < 0.05 was considered as statistically significant. The Hosmer and Lemeshow goodness-of-fit test was also employed in order to analyse the fit of the model to the data.

RESULTS

A total of 96 exposed dogs and 96 non-exposed dogs were visited and interviewed.Locations of exposed dogs were recorded and marked on the map in *Fig. 1*. Map depicts that cases were most frequentin urban areas/ towns of Lahore district compared to rural areas/towns.

Univariate analysis screened a total of 15 variables from which 12 variables (dog keeping purpose (guard/pet), dog in neighborhood, high density human population area, history of skin/genital diseases, mating partner, number of dogs per house, stray dog population, red vulvular discharge as only heat indicator, chain/restrain the dog, use of separate cage, use of proper fencing around the kennel and knowing the difference between normal red discharge and clotted blood were found associated with either case or control *(Table 1)*. The 3 intrinsic factors of dogs i.e. age, sex and breed were found having no association with CTVT. Of these 12 variables, 8 were found as potential risk factors i.e. associated with increasing the odds of exposure in cases as compared to controls. Among these, the type of breeding dog/mating partner is the most affecting variable with 170% increase followed by history of skin/genital diseases and dogs in neighbourhood affecting 110% increase in disease occurrence. But the Remaining 4 factors were determined as protective factors i.e. associated with decreasing the odds of exposure in cases to controls. These factors included chain/restrain the dog, use of separate cage, use of proper fencing around the kennel and knowing the difference between normal red discharge and clotted blood.

In first multivariable, all the variables except red blood discharge as sole indication of breeding and number of dogs per householdhad P-value <0.05 *(Table 2)*. Therefore a new multivariable model was produced without these two variables *(Table 3)*. No interactions were found in the final multivariable model under all the parameters with interaction coefficients found statistically non-significant. Confounding was not found among variables with difference between ORs lower than 0.012 The P-value for Hosmer and Lemeshow goodness-of-fit test was 0.6, which shows a good fit of theoretical model.

DISCUSSION

In Pakistan, limited information is available regarding canine diseases especially CTVT [6]. This study is the first document that explains the epidemiological aspects associated with the spread of this disease in Pakistan.

CTVT can be diagnosed with different methods but the commonly used methods are histopathology and cytology. During the study, cytology based approach was less costly and rapid as compared to 2-3 day long costly histopathology [2,12,13].

Current study implicates that there is no significant difference between genders, age groups with various breeds either imported or mongrels that can influence the disease burden. These results are similar to previous studies [9,10]. The possible cause of variable frequency among the groups is might be due to their key importance in dog breeding business and notion of obtaining higher sperms and ova quality [15,16].

This study entails that factors allowing contact between dogsare the main reasons that can spread this disease. This study involves several factors that directly relate to biosecurity of the kennel/homestead (i.e. chain/restrain the dog, use of separate cage, use of proper fencing around

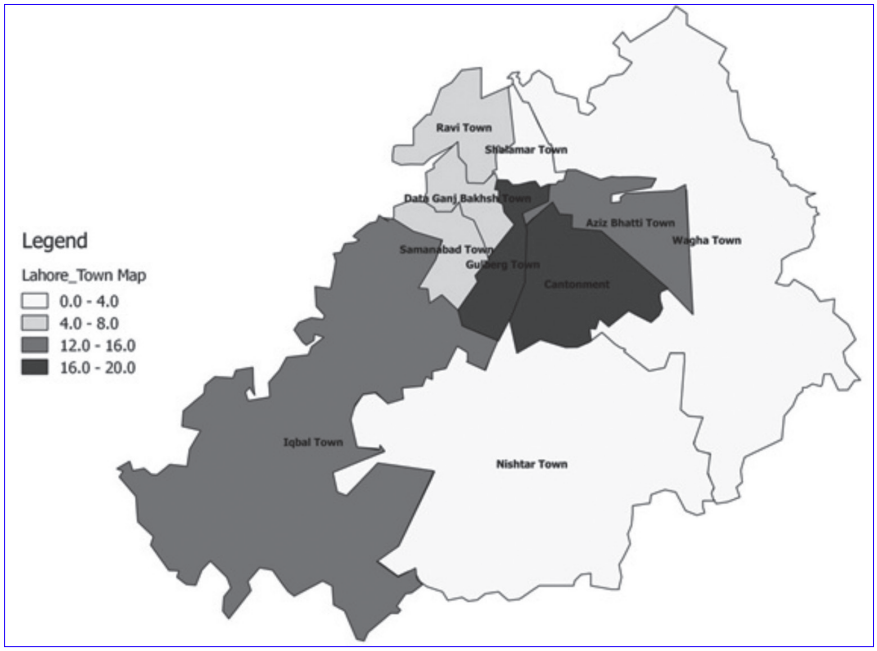

Fig 1. Range of CTVT cases in different towns of Lahore District

(Urban Towns = Ravi Town, Shalamar Town, Data GanBakhsh Town, Samanabad Town, Gulberg Town, Aziz Bhatti Town; Rural Towns = Wagha Town, Nishtar Town, Iqbal Town)

Table 1. *Univariate analysis of the factors included in the study*

S. No.	Risk Factors	Levels	Case	Controls	P Value	OR	CI 95%	
1	Age	< 5 Years	60	54	0.35	1.33	0.72	2.45
		> 5 Years	36	42				
2	Gender	Male	46	45	0.89	1.03	0.611	1.75
		Female	50	51				
3	Chain/Restrain the dog	Yes	38	55	0.019	0.51	0.291	0.9
		No	58	41				
4	Cage Type	Separate	35	53	0.0079	0.43	0.233	0.819
		Common	61	43				
5	Knowing the difference between normal discharge and clotted blood	Yes	33	47	0.04	0.56	0.31	1
		No	63	49				
6	Dog breed	Imported	47	45	0.87	1.08	0.5	1.8
		Mongrels	49	51				
7	Proper fence	Yes	28	41	0.032	0.48	0.24	0.95
		No	68	55				
8	Purpose of dog keeping	Guard	59	41	0.014	2.01	1.13	3.5
		Pet	37	55				
9	Dogs in neighbourhood	Yes	68	41	0.00015	3.25	1.7	6.2
		No	28	55				
10	Density of human population in area	High	68	38	0.00001	4.2	2.1	8.37
		Low	28	60				
11	History of skin/genital diseases	Yes	58	26	0.00001	4.2	2.1	8.37
		No	38	70				
12	Type of breeding dog	Common	69	30	0.0000013	5.4	2.6	11.08
		Own	27	66				
13	No of dogs per house	< 2 Dogs	58	36	0.0011	2.83	1.46	5.47
		> 2 Dogs	39	60				
14	Stray dog population in the area	Yes	71	49	0.0009	3.01	1.51	5.93
		No	25	47				
15	Red bloody discharge as sole indicator of heat	Yes	66	51	0.03	2.05	1.076	3.71
		No	30	45				

the kennel).These factors were found protective (OR <1.0) in this study and in previous study [17].

With compromised biosecurity, certain factors (i.e. stray dog population in the area, dogs in neighborhood, high density human population area and number of dogs per household) favour the introduction of infected dog and consequently the disease spread rapidly. All these factors were included in the multivariable models and they revealed high association with the occurrence of CTVT (OR >2.0). This multivariable model explained the guard dogs with common mating partner/practices and stray dog population in the area have more risk of getting the disease. Among all the variables, mating practices is the most important risk factor that can increase the risk of getting disease 3 times more. Although other variables, excluding above discussed variables, have less higher OR values but still they can pose a risk.

Stray population is considered as reservoir of CTVT infection [3]. In the final multivariable model, Stray population of the area showed the higher association with risk of contracting CTVT (OR =3.35, P<0.001). As mentioned in previous studies, failing the biosecurity measures introduces the infected stray dogs into kennels/ households that mightlead to spreading the infection [9]. Apart from the stray populations, infected dog in the kennels and nearest vicinity can be remained undiagnosed especially females due to less obvious clinical signs. Final multivariable modelrevealed that presence of dogs in neighborhood wasfound associated with the CTVT (OR=2.87). High density human population areas are

Table 2. *Mutivariable regression model including all the variables with P-value <0.25*

Potential Risk Factors	Levels	Regression Coefficient	Standard Error	OR	95% C.I.	P value
High density population area (N= 68)	High	0.91	0.385	2.503	1.176 - 5.327	0.017
	Low					
Dogs in neighborhood (N=68)	Yes	1.07	0.388	2.937	1.371 - 6.289	0.005
	No					
Stray dog population (N=71)	Yes	1.28	0.404	3.626	1.640 - 8.014	0.001
	No					
No of dogs per household (N=58)	< 2 Dogs	0.63	0.387	1.896	0.887 - 4.05	0.098
	> 2 Dogs					
Dog keeping type (N=59)	Guard	1.25	0.413	3.520	1.565 - 7.915	0.002
	Pet					
Mating partner (N=69)	Common	1.70	0.413	5.480	2.434 - 12.334	< 0.0000
	Own					
History of diseases (N=58)	Yes	1.04	0.390	2.838	1.319 - 6.105	0.007
	No					
Red bloody discharge as indication of heat (N=66)	Yes	0.67	0.392	1.967	0.911 - 4.25	0.084
	No					

Table 3. *Final multivariable regression model excluding the red discharge as indication of heat and number of dogs per household*

Potential Risk Factors	Levels	Regression Coefficient	Standard Error	OR	95 % C.I.	P value
High density population area	High	0.93	0.38	2.54	1.21-5.32	0.01
	Low					
Dogs in neighborhood	Yes	1.05	0.38	2.87	1.36-6.03	0.01
	No					
Stray dog population	Yes	1.21	0.40	3.35	1.54-7.25	0.00
	No					
Dog keeping type	Guard	1.41	0.40	4.11	1.87-9.05	0.00
	Pet					
Mating partner	Common	1.85	0.40	6.36	2.88-14.01	0.00
	Own					
History of diseases	Yes	1.05	0.38	2.86	1.35-6.05	0.01
	No					

The same no of cases were mentioned as in Table 2

also considered as risk for contracting CTVT (OR=2.54); might be because of more congesting living style and more interaction. Relationship of presence of dogs in neighborhood and high density human population areas with CTVT has been also discussed in previous studies [9,10].

Breeding and general management of kennel is considered to be playing very important role here in the spread of this disease. Factors such as purpose of dog keeping, red vulvar discharge as sole breeding indicator, knowing the difference between normal red discharge and clotted blood, and type of breeding partner were showing significant differences (P<0.001). Purpose of dog keeping was included in the final multivariable model that resulted in higher risk of contracting disease (OR=4.11). Guard dogs have also been considered as prone to risk of getting CTVT in previous studies [9]. Regardless of estrous cycle/breeding management, guard dogs are routinely released in night for security purposes which entails the risk of their mating with infected dog. While such practices are absent in pet dogs.

In canine breeding, red vulvar discharge in female dogs is considered as good indication of approaching oestrus but it should be further confirmed with the vaginal cytology [18]. Red discharges can also be produced in

different pathological conditions therefore should be confirmed [19]. This study implicates that owners considering red vulvar discharge as sole breeding indicator were posing their dogs to contract and spread this disease to other dogs (OR=1.96*)*. While knowing the difference between normal red discharge and clotted blood is considered as protective factor that can prevent the mating with infecting dog (OR=0.5*)*.

Another factor in the canine breeding practices is utilization of one of the two types of breeding dog i.e. common breeding dog and own breeding dog. Common breeding dog for several kennels/household is usually allowed to mate with any other dog on the account of money while own dog is not allowed to mate with any other dog outside the house and kennel. This study has revealed that due to utilization of common dog unchecked, this disease is usually spreading and persisting in Pakistan (OR=6.36*)*. In previous studies specifically related to CTVT [7,8], this factor has not been studied. This factor is commonly studied in all other reproductive disorders that spread through venereal transmission [20]. In addition to this, owners having less number of dogs are more exposed to this disease because they usually introduce the common breeding dog that exposes them to potential risk factor of getting this disease. These risk factors are newly devised to the risk factor studies related to CTVT. This is new addition to knowledge about epidemiological factors of this disease.

The basic objective of the study was the identification and quantification of potential risk factors associated with CTVT among owned dogs in Pakistan. This study has identified some new risk factors like utilizing common mating dogs and poor breeding practices along with similar risk factors that were already documented from previous studies. Guidelines and policies focusing the control of these risk factors could reduce the risk of CTVT infection in owned dogs in Pakistan and in other developing countries oftropics. Good management practices and strict biosecurity together can prevent the unchecked introduction of infected dogs. There should also be improvement in breeding practices and management. There are many examples of quarantines and biosecurity policies that include prohibits introduction of any dog into kennel either stray or owned, clinical examination before mating and confirmation of estrus.

Conflict of Interest Statement

The authors declared that they have no conflict of interest.

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REFERENCES

1. Murgia C, Pritchard JK, Kim SY, Fassati A, Weiss RA: Clonal origin and evolution of a transmissible cancer. *Cell*, 126, 477-487, 2006. DOI: 10.1016/j.cell.2006.05.051

2. Thangathurai R, Balasubramaniam GA, Dharmaceelan S, Balachandran P, Srinivasan P, Sivaseelan S, Manohar BM: Cytological diagnosis and its histological correlation in canine transmissible venereal tumour. *Veterinarski Arhiv*, 78, 369-376, 2008.

3. Das U, Das AK: Review of canine transmissible venereal sarcoma. *Vet Res Commun*, 24, 545-556, 2000. DOI: 10.1023/A:1006491918910

4. vonHoldt BM, Ostrander EA: The singular history of a Canine Transmissible Tumor. *Cell*, 126, 445-447, 2006. DOI: 10.1016/j.cell.2006.07.016

5. Ortega-Pacheco A, Segura-Correa JC, Jimenez-Coello M, Forsberg CL: Reproductive patterns and reproductive pathologies of stray bitches in the tropics. *Theriogenology*, 67, 382-390, 2007. DOI: 10.1016/j.theriogenology.2006.07.020

6. Strakova A, Murchison EP: The changing global distribution and prevalence of canine transmissible venereal tumour. *BMC Vet Res*, 10, 168, 2014. DOI: 10.1186/s12917-014-0168-9

7. Athar M, Suhail A, Muhammad G, Shakoor A, Azim F: Clinico-therapeutic Studies on canine transmissible venereal tumor. *Pak Vet J*, 21, 39-43, 2001.

8. Awan F, Ali MM, Ijaz M, Khan S: Comparison of different therapeutic protocols in the management of canine transmissible venereal tumour: Review of 30 cases. *Global Vet*, 12, 499-503, 2014.

9. Batamuzi EK, Kassuku AA, Agger JF: Risk factors associated with canine transmissible venereal tumour in Tanzania. *Prev Vet Med* 13, 13-17, 1992. DOI: 10.1016/0167-5877(92)90031-A

10. Kabuusu RM, Stroup DF, Fernandez C: Risk factors and characteristics of canine transmissible venereal tumours in Grenada, West Indies. *Vet Comp Oncol*, 8, 50-55, 2010. DOI: 10.1111/j.1476-5829.2009.00204.x

11. Schlesselman JJ: Case-Control Studies: Design, Conduct, Analysis. 354, Oxford University Press, Oxford, UK, 1982.

12. Florez MM, Pedraza F, Grandi F, Rocha NS: Cytologic subtypes of canine transmissible venereal tumor. *Vet Clin Pathol*, 41, 4-5, 2012. DOI: 10.1111/j.1939-165X.2012.00401.x

13. Igor U, Irena C, Ksenija I, Elena A, Goran N, Plamen T: Cytological diagnostic of Canine transmissible venereal tumor - Case report. *Mac Vet Rev*, 35, 91-96, 2012.

14. Lindahl E, Sattorov N, Boqvist S, Magnusson U: A study of knowledge, attitudes and practices relating to brucellosis among small-scale dairy farmers in an urban and peri-urban area of Tajikistan. *PLOS One*, 10, 1-10, 2015. DOI: 10.1371/journal.pone.0117318

15. Ortega-Pacheco A, Acevedo-Arcique M, Sauri-Arceo C, Bolio-González M, Gutiérrez-Blanco E: Prevalence of transmissible venereal tumor of stray dogs in Merida Yucatan Mexico. *Rev Biomed*, 14, 83-87, 2003.

16. Rogers KS, Walker MA, Dillon HB: Transmissible venereal tumor: A retrospective study of 29 cases. *J Am Anim Hosp Assoc*, 34, 463-470, 1998. DOI: 10.5326/15473317-34-6-463

17. Nak D, Nak Y, Cangul IT, Tuna B: A clinico-pathological study on the effect of vincristine on transmissible venereal tumour in dogs. *J Vet Med A Physiol Pathol Clin Med*, 52, 366-370, 2005. DOI: 10.1111/j.1439-0442.2005.00743.x

18. Feldman E, Nelson R: Canine and Feline Endocrinology and Reproduction. 3rd edn., 1044, Saunders, Philadelphia, 2003.

19. Jiang Z, Holyoak GR, Bartels KE, Ritchey JW, Xu G, Bunting CF, Slobodov G, Piao D: In vivo trans-rectal ultrasound-coupled optical tomography of a transmissible venereal tumor model in the canine pelvic canal. *J Biomed Opt*, 14, 1-3, 2009. DOI: 10.1117/1.3149852

20. Pearse AM, Swift K: Transmission of devil facial-tumour disease. *Nature*, 439, 549, 2006.

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Research Article

Evaluation of Analytical-Sensitivity and -Specificity of a Commercially Available One-Step Real-Time Polymerase Chain Reaction Assay Kit for the Detection of *Burkholderia mallei*

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Abstract

Burkholderia mallei (B. mallei) is the etiological agent of glanders, a highly infectious and zoonotic disease of solipeds. Prompt and accurate detection of *B. mallei* and diagnosis of glanders is important for both, humans and animals. The present study was designed to evaluate the analytical sensitivity and specificity of a commercially available one-step real-time (RT) PCR assay kit (Genekam Biotechnology AG, Germany) for the detection of *B. mallei*. Furthermore, the comparative evaluation of the analytical sensitivity of an already published fliP RT-PCR protocol and this kit was made using a real-time PCR (RT-PCR) platform: the Mx3000P TM (Stratagene®, Canada). Diagnostic parameters were assessed with a panel of 20 B. mallei, 20 *B. pseudomallei,* 15 *B. mallei* related and 10 clinically relevant non-Burkholderia species. Using the one-step RT-PCR on the Mx3000PTM platform, the limit of detection (LOD) was determined as 10 fg. Applying a modified fliP RT-PCR using the Mx3000P platform resulted in a LOD of 100 fg. Authors conclude that the one-step RT-PCR kit is specific for DNA of *B. mallei* strains. The one-step RT-PCR assay kit is a robust, rapid, reliable, specific and sensitive tool with a fast turnaround time for the specific identification and detection of *B. mallei* from culture material.

Keywords: *Burkholderia mallei, Analytical specificity, Analytical sensitivity, One-step RT-PCR kit*

Burkholderia mallei'nin Tespitinde Ticari Tek-Basamaklı Gerçek-Zamanlı Polimeraz Zincir Reaksiyon Kitinin Analitik Özgünlüğü ve Özgüllüğünün Değerlendirilmesi

Özet

Burkholderia mallei (B. mallei) ruamın etiyolojik ajanı olup, hastalık tek tırnaklıların oldukça enfeksiyöz ve bulaşıcı bir hastalığıdır. *B. mallei*'nin hızlı ve doğru tespiti ve ruamın diagnozu hem insanlar hem de hayvanlar için önemlidir. Bu çalışma, *B. mallei*'nin tespitinde ticari olarak satılan tek-basamaklı gerçek-zamanlı (RT) PCR kitinin (Genekam Biotechnology AG, Almanya) analitik özgünlüğü ve özgüllüğünün değerlendirilmesi amacıyla yapılmıştır. Daha önceden yayınlanmış olan fliP RT-PCR protokolü ile mevcut test edilen kitin özgünlüğü ve özgüllüğünün karşılaştırılması gerçek-zamanlı PCR, Mx3000P TM (Stratagene®, Kanada) ile yapıldı. Diagnostik parametreler 20 B. mallei, 20 *B. pseudomallei*, 15 *B. mallei* alakalı ve 10 klinik olarak ilgili *Burkholderia* olmayan türler ile değerlendirilirdi. Mx3000PTM platformunda tek basamaklı RT-PCR uygulayarak tespit etme limiti (LOD) 10 fg olarak belirlendi. Mx3000PTM platformu kullanılarak uygulanan fliP RT-PCR ile LOD 100 fg olarak belirlendi. Tek basamaklı RT-PCR'ın *B. mallei* suşları için spesifik olduğu sonucuna varıldı. Tek basamaklı RT-PCR kiti, kültürden *B. mallei*'nin spesifik olarak identifiye edilmesi ve belirlenmesinde kullanılabilecek hızlı geri dönüş zamanına sahip güçlü, hızlı, güvenilir, spesifik ve hassas bir üründür.

Anahtar sözcükler: *Burkholderia mallei, Analitik özgüllük, Analitik özgünlük, Tek basamaklı RT-PCR kiti*

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INTRODUCTION

Glanders is a highly infectious zoonotic disease of solipeds caused by Gram-negative pathogen *Burkholderia mallei (B. mallei)*. *B. mallei* is considered to be a clone of *B. pseudomallei* causes melioidosis both in animals and humans [1,2]. Main clinical signs of equine and human glanders are lymphangitis (farcy pipes), pneumonia and/or lymphadenitis (farcy buds) [3]. Glanders can be considered a re-emerging disease as the number of outbreaks in solipeds, zoo carnivores and dromedary is steadily increasing in the last two decades [2,4,5].

There is problem of accurate identification of *Burkholderia* spp. based on serology. False-positive and -negative serological results impose restrictions on the international trade of healthy animals and spread of glanders in disease free regions, respectively. However, combined use of serological and molecular methods increases the detection rate of glanders [2,6,7].

It is difficult to detect *B. mallei* directly from tissues and biological fluids of infected hosts as their low concentration. Cultivation and identification of *B. mallei* with conventional microbiological techniques is time-consuming and hazardous and should be performed under BSL (bio safety level)-3 conditions. However, isolation of *B. mallei* is the real proof of an infection.

Rapid and precise diagnosis of *B. mallei* is the mainstay for prompt treatment particularly in acute human glanders [6]. Similarly, rapid detection of equine glanders would be helpful for early countermeasures and quarantine time of suspicious cases of glanders can be shortened. Consequently, different polymerase chain reactions (PCR) and real-time PCRs (RT-PCR) with improved analytical-sensitivity and/or-specificity have been developed.

Many attempts have made to detect targets of the 16S rRNA, ISBma2transposase, 23S rRNA genes and the 16-23S rRNA spacer region for discrimination of *B. mallei* from *B. pseudomallei* [8-11]. In addition, RT-PCR assays have been developed that target the conserved type 3 secretion system (TTS1, *orf11* and *orf13)* encoded by both *B. mallei* and *B. pseudomallei* [12]. In a latter investigation, both *B. mallei* and *B. pseudomallei* were distinguished from *B. thailandensis* by amplification of *orf13* sequences, but the assay was not successful to differentiate *B. mallei* from *B. pseudomallei*. Recently, developed RT-PCR assay targeting the *B. pseudomallei* 16S rRNA, *fliC*, and the ribosomal subunit protein S21 *(rpsU)* genes could not differentiate *B. mallei* from *B. pseudomallei* [13]. Heterogenecity in the *fliC* gene from several *B. pseudomallei* isolates has been reported, which hindered the differentiation of *B. mallei* from *B. pseudomallei* [14]. It is demonstrated in another study that all *B. mallei* isolates contain specific *bimA* (intracellular motility A) gene with corresponding AT4 and AT5 primer pair would consider extremely useful for the identification of *B. mallei* and its differentiation from *B. pseudomallei* [15]. For rapid identification and screening of *B. mallei* and *B. pseudomallei*, a 5´ nuclease RT-PCR assay was developed and further improved for rapid, sensitive and specific identification and detection of *B. mallei* in clinical samples by targeting flagellin P (*fliP*) gene [16]. A Highly specific, rapid and validated RT-PCR assay "BurkDiff" which targets a single nucleotide polymorphism (SNP) and differentiates between *B. mallei* and *B. pseudomallei* has been developed [17]. The latest development is a commercially available one-step RT-PCR assay kit (Genekam Biotechnology AG, Germany) for the detection of *B. mallei*.

In the current study, analytical-sensitivity and -specificity of this kit for the diagnosis of glanders has been evaluated. The development of this kit is another step to reduce the turnaround time for the detection of *B. mallei*.

MATERIAL and METHODS

One-step RT-PCR kit has been manufactured by Genekam Biotechnology AG, Germany to detect glanders. This kit works on the principle of fluorogenic probes: 6-caboxyfluorescein (FAM/reporter) at the 5' end and black hole Quencher-1 (BHQ1/quencher) at the 3' end of the probe. Fluorescence indicates the presence of PCR amplified product. Regarding the manufacturers' instructions, this kit needs DNA which can be isolated from blood, serum, nasal swabs, infected lymph nodes, respiratory tract, bacterial culture, body fluid and other tissues of glanderous animals or humans. The RT-PCR kit (tube A, B, D1 and D2) was used according to manufacturers' instructions. Briefly, after thawing, 8 µl from tube 'A' and 10 µl from tube 'B' mixed together in a nuclease free PCR-tube or alternatively in an optical 96-well microtiter plate (q PCR 96-well plates, Micro Amp TM, Applied Biosystem). This mixture is now referred as a ready-to-go "mastermix" (18 µl) for one test sample. As this whole procedure of mixing was performed in one-step that's why it is called one-step RT-PCR. After addition of 2 µl of isolated template DNA the reaction mix (20 µl) was ready-to-go in a thermocycler. Similarly, D1 (positive control) and D2 (negative control) were used in a separate mastermix (18 µl) in each run. No internal amplification control is included in the kit to detect any amplicon contamination and/or amplification failure. In the current study, the performance of the one-step RT-PCR kit was compared in RT-PCR platform: Mx3000P™ real-time PCR system (Stratagene© La Jolla, Canada) software version 2.0 was used for amplification and detection. The real-time PCR reaction was performed in duplicate in this platform. Results were interpreted according to one-step RT-PCR assay kit manufacturers' instructions.

Furthermore, for the comparative evaluation of analytical sensitivity of aforementioned thermocycler, a real-time hot-start *fliP* PCR with little modification [18] was performed in a Mx3000P. In Mx3000P, the 25 µl

Table 1. *List of bacterial strains analysed in current study*

Species	Strain	Source	Geographic Origin	Year	+/- in one-step RT-PCR kit
B. mallei	235	un	un		+
	237	un	un		+
	242	un	un		+
	32	un	un		+
	M2				+
	34	un	un		+
	ATCC 23344^T	Human	China	1942	+
	Bogor	Horse	Indonesia		+
	Dubai 7	Horse	UAE	2004	+
	M1	un	un		+
	Mukteswar	Horse	India		+
	NCTC 10230	Horse	Hungry	1949	+
	NCTC 10247	Horse	Turkey	1960	+
	NCTC 10260	Human	Turkey	1949	+
	NCTC120-Lister	un	London/Lister	1920	+
	PRL-1	Donkey	Pakistan	2002	+
	PRL-3	Horse	Pakistan	2005	+
	PRL-4	Horse	Pakistan	2005	+
	Rotz 7 (SVP)	un	un		+
	Zagreb	Horse	ex-Yugoslavia		+
B. pseudomallei	09RR8920	Ring trail	Germany	2009	–
	AB2056	Human	Kenya	1980	–
	ATCC 23343^T	Human	un	<1957	–
	D4899/303	Environment	Venezuela		–
	EF15660	un	un		–
	Hainan 106	Environment	China		–
	Heckeshorn	Human	Germany	1999	–
	Holland	un	Holland		–
	NCTC 1688	Rat	Malaysia	1923	–
	NCTC 4845	Monkey	Singapore	1945	–
	PITT 225A	Human	Thailand	1986	–
	PITT 521	Human	Pakistan	1988	–
	PITT 5691	un	un		–
	SID 2889	Human	Bangladesh	1923	–
	SID 3477	Human	Thailand	1999	–
	SID 3511	Human	Bangladesh		–
	SID 3783	Human	Malaysia	1999	–
	SID 4075	Human	N Thailand	1999	–
	Soil 1977	Environment	Madagascar	1977	–
	UE10	Human	NE Thailand		–
***B. mallei* related strains**					
B. cenocepacia	DSM 16553^T	Human	NE Thailand		–
B. ambifaria	DSM 16087^T	Human	NE Thailand		–
B. cepacia	DSM 7288^T	Human	NE Thailand		–

Table 1. *List of bacterial strains analysed in current study (Continue)*

Species	Strain	Source	Geographic Origin	Year	+/- in one-step RT-PCR kit
B. dolosa	DSM 16088^T	Human	NE Thailand		–
B. fungorum	DSM 17061^T	Human	NE Thailand		–
B. gladioli	DSM 11318	Human	NE Thailand		–
B. glathei	DSM 50014^T	Human	NE Thailand		–
B. multivorans	DSM 13243^T	Human	NE Thailand		–
B. stabilis	DSM 16586^T	Human	NE Thailand		–
B. thailandensis	DSM 13276^T	Human	NE Thailand		–
B. vietnamiensis	DSM 11319^T	Human	NE Thailand		–
P. aeruginosa	ATCC 9027				–
P. alcaligenes	ATCC 14909				–
P. fluorescens	ATCC 13525				–
P. putida	ATCC 12633				–
Non-*Burkholderia* strains					
E. coli	DSMZ 30083				–
P. multocida	DSM 5281				–
R. equi	DSM 20307				–
Str. agalactiae	DSM 6784				–
Str. equi subsp. equi	ATCC 9528				–
Str. equi subsp. zooepidemicus	ATCC 700400				–
Str. equinus	DSM 20558				–
Str. parauberis	DSM 6631				–
T. equigenitalis	DSM 10668				–
Y. enterocolitica ssp. enterocolitica	ATCC 9610				–

B Burkholderia; E Escherichia; P Pasturella; Ps Pseudomonas; R Rhodococcus; Str Streptococcus; T Taylorella; un unknown history; Y Yersinia; + Positive; – Negative

reaction mixtures consisted of 9.75 µl of HPLC, 12.5 µl of 2x TaqMan™ Universal MasterMix (Applied Biosystem, Germany), 0.25 µl of each primer (Jena Bioscience, Germany) (10 µM/µl), 0.25 µl of the probes (Jena Bioscience, Germany) (10 µM/µl), and 2 µl of template DNA. Thermal profile conditions were as follows: 1 cycle at 50°C for 2 min (decontamination) followed by 95°C for 10 min (hotstart), initial amplification at 95°C for 25 s, followed by 50 cycles at 63°C for 1 min.

To determine analytical specificity (exclusivity and inclusivity) of one-step RT-PCR kit, a representative panel of genomic DNA from 20 *B. mallei*, 20 *B. pseudomallei,* 15 *B. mallei* related and 10 clinically relevant non-*Burkholderia* species *(Table 1)* were obtained from the Institute of Microbiology, Federal Armed Forces (Munich, Germany), the Department of Clinical Medicine and Surgery (University of Agriculture, Faisalabad, Pakistan), the German Collection of Microorganisms and Cultures (DSMZ, Braunschweig, Germany), and the strain collection of the National Reference Laboratory of Glanders at the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institute, Jena, Germany). *B. mallei* detection limit of one-step RT-PCR kit was determined with little modification [19]. Briefly, analysis was made of a dilution series of purified DNA of *B. mallei* ATCC 23344^T in PCR HPLC from 1 ng to 100 ag. This linear quantification was used to prepare the standard curve *(Fig. 1)*. The concentration of DNA preparations and purity were determined by using NanoDrop ND-1000 UV Vis spectrophotometer (NanoDrop Technologies, Wilmington, Germany). Dilution series and RT-PCR reactions were performed in duplicate separately in Mx3000P using one-step RT-PCR kit and modified *fliP* RT PCR protocol. For the assessment of analytical specificity, all DNA *(Table 1)* was used in a concentration of 100 pg/µl in HPLC in a volume of 2 µl per assay.

RESULTS

The linear range of the one step real-time PCR for *B. mallei* covered concentrations from 1 ng to 100 ag of bacterial DNA/µl *(Fig. 1, Fig. 2)*. During evaluation of

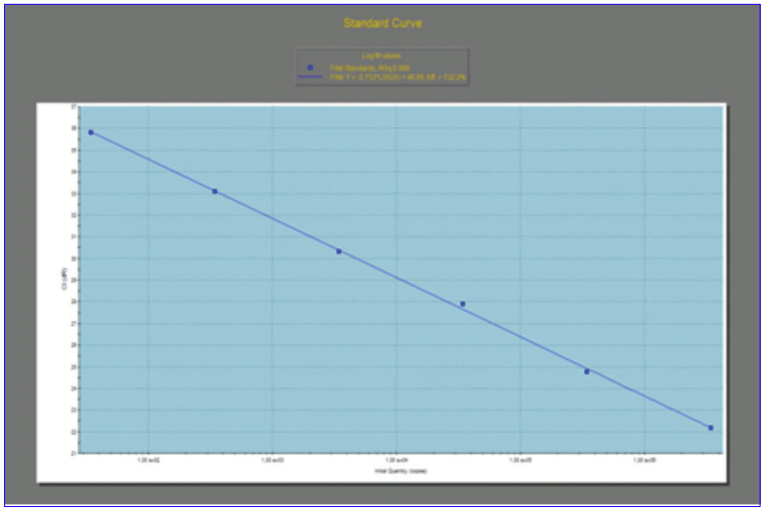
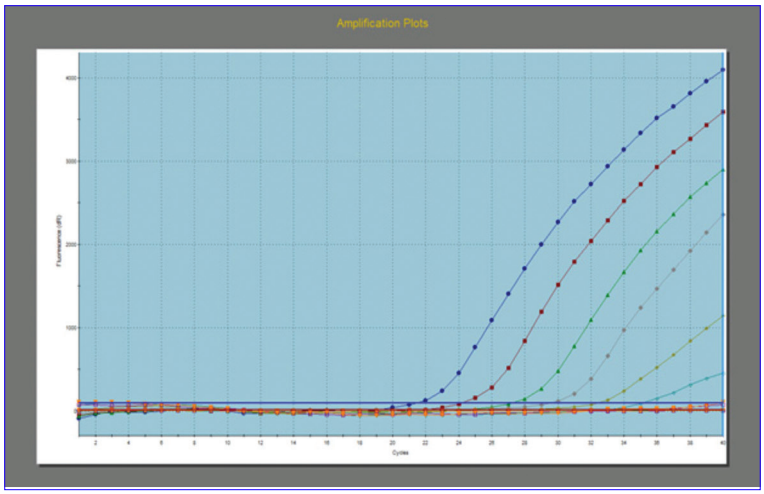


Fig 1. *Standard curve of dilution series containing 1 ng-100 ag/ µl DNA*

Fig 2. *Comparative evaluation of different RT-PCR assays at Mx3000P platform*



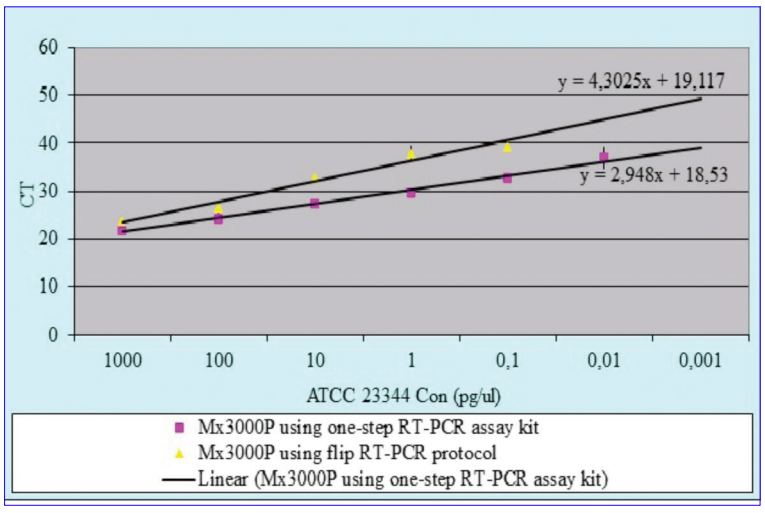


Fig 3. *Comparative evaluation of standard curve of different RT-PCR assays at Mx3000P platform*

analytical sensitivity of one-step RT-PCR with Mx3000P, lower limit of detection (LOD) for *B. mallei* ATCC 23344^T purified DNA was determined to be 10 fg, corresponding to 2 genome equivalents. However, *fliP* RT-PCR using Mx3000P platform determined LOD of 100 fg, 10 genome equivalents) *(Fig. 3)*.

DISCUSSION

It is difficult to rule out erroneous serodiagnostic results because use of crude *B. mallei* spot antigen [2]. Recently, ELISA has been developed based on purified *rBimA* (recombinant *Burkholderia* intracellular motility A) protein

as spot antigen. This protein showed no cross-reaction with sera from melioidosis patients or healthy humans [20]. This serodiagnostic test still needs to be validated and it yet has not been commercialised. Rapid and accurate diagnosis of *B. mallei* infection is still a major challenge for veterinary clinician. Glanders is often falsely diagnosed as melioidosis, strangles, equine influenza and epizootic lymphangitis. Glanders is still a major problem in many developing countries where BSL-3 laboratory facilities do not exist for the cultivation of *B. mallei* which itself is time taking process. Among direct methods, development of PCR assays and real-time PCR assays for specific identification and detection of *B. mallei* would reduce the time turn around for interpretation of final results but also overcome aforementioned shortcomings. Potential use of *B. mallei* as a bio-weapon stresses its prompt detection to start immediate prophylactic treatment. The main objective of current study is to evaluate analytical-sensitivity and -specificity of a commercially available one-step RT-PCR kit for the identification and detection of *B. mallei*.

A RT-PCR assay was developed using LightCycler 4.0 software to detect *B. mallei* and found LOD 1 pg or 424 genome equivalents [15]. However, another RT-PCR assay was developed targeting the *fliP* gene of *B. mallei* and used internal amplification control and found the detection limit of 60 fg of *B. mallei* DNA [21]. The aforementioned difference of LOD might be due to use pure genomic DNA *(Table 1)* in current study while in previous studies [16,21], RT-PCR assays used natural outbreak clinical samples and/ or *B. mallei* ATCC 23344^T challenged animal tissue samples for the specific identification and isolation of *B. mallei* due to which chance of extraneous analyte contamination cannot be ignored.

There are many factors including instrumentation, operator error, reagent choice, calibration, accuracy and acceptance limits of assay controls, water quality, pH, ionicity of buffers and diluents, durations, and error introduced by detection of closely related analytes that can influence on the analytical performance of an assay. One-step RT-PCR kit might have already overcome these shortcomings during its optimisation process, on the other hand its read-to-use availability for clinical laboratories minimise the chances of humans inherent errors.

While establishing exclusivity and inclusivity of one-step RT-PCR assay kit, all 20 *B. mallei* species DNA were accurately identified positive and 20 *B. pseudomallei*, 15 *B. mallei* related species and 10 clinically relevant non-*Burkholderia* species DNA tested negative *(Table 1)*. No cross-reactivity was detected with these 45 species DNA. Results of analytical specificity correlate with recent studies for the development of RT-PCR for the specific identification and detection of *B. mallei* [15,16,21].

The slop of a standard curve is used to estimate the PCR efficiency of a RT-PCR reaction. A RT-PCR standard curve is graphically represented as a semi-log regression line plot of C value versus input nucleic acid (pg) *(Fig. 2)*. A standard curve slope of 3.32 indicates a PCR reaction with 100% efficiency. One can draw the conclusion that Mx3000P using one-step RT-PCR kit relatively better in its amplification efficiency than Mx3000P using *fliP* based RT-PCR protocol for the detection of *B. mallei* DNA.

Commercially available one-step RT-PCR assay kit is robust, rapid, reliable, specific and sensitive tool with obvious less turnaround time for specific identification and detection of *B. mallei* within culture material. There is also need to validate one-step RT-PCR assay kit by screening a large panel of *B. mallei, B. pseudomallei* and *B. mallei* related and non-*Burkholderia* isolates. The application for clinical sample materials also needs to be evaluated. Generally, a positive PCR result in clinical samples is a confirming result in the diagnosis of glanders but a negative PCR result in any clinical sample cannot be used to exclude the disease.

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REFERENCES

1. Lau SKP, Sridhar S, Ho CC, Chow WC, Lee KC, Lam CW, Yuen KY, Woo PCY: Laboratory diagnosis of melioidosis: Past, present and future. *Exp Biol Med*, 240, 742-751, 2015. DOI: 10.1177/1535370215583801

2. Khan I, Wieler LH, Melzer F, Elschner MC, Muhammad G, Ali S, Sprague LD, Neubauer H, Saqib M: Glanders in animals: A review on epidemiology, clinical presentation, diagnosis and countermeasures. *Transbound Emerg Dis*, 60, 204-221, 2013. DOI: 10.1111/j.1865-1682.2012.01342.x

3. Meumann EM, Cheng AC, Ward L, Currie BJ: Clinical features and epidemiology of melioidosis pneumonia: Results from a 21-year study and review of the literature. *Clin Infect Dis*, 54, 362-369, 2012. DOI: 10.1093/cid/cir808

4. Khaki P, Mosavari N, Khajeh NS, Emam M, Ahouran M, Hashemi S, Mohammad TM, Jahanpeyma D, Nikkhah S: Glanders outbreak at Tehran Zoo, Iran. *Iran J Microbiol*, 4, 3-7. 2012.

5. Wernery U, Wernery R, Joseph M, Al-Salloom F, Johnson B, Kinne J, Jose S, Tappendorf B, Hornstra H, Scholz HC: Natural *Burkholderia mallei* infection in Dromedary, Bahrain. *Emerg Infect Dis*, 17, 1277-1279, 2011. DOI: 10.3201/eid1707.110222

6. Lowe CW, Satterfield BA, Nelson DB, Thiriot JD, Heder MJ, March JK, Drake DS, Lew CS, Bunnell AJ, Moore ES, O'Neill KL, Robison RA: A quadruplex Real-Time PCR Assay for the rapid detection and differentiation of the most relevant members of the *B. pseudomallei* complex: *B. mallei*, *B. pseudomallei*, and *B. thailandensis*. *PLoS ONE*, 11, e0164006, 2016. DOI: 10.1371/journal.pone.0164006

7. Kettle ANB, Wernery U: Glanders and the risk for its introduction through the international movement of horses. *Equine Vet J*, 48, 654-658, 2016. DOI: 10.1111/evj.12599

8. Janse I, Hamidjaja RA, Hendriks ACA, Rotterdam BJ: Multiplex qPCR

for reliable detection and differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei*. *BMC Infect Dis,* 13, 86, 2013. DOI: 10.1186/1471-2334-13-86

9. Antonov VA, Tkachenko GA, Altukhova VV, Savchenko SS, Zinchenko OV, Viktorov DV, Zamaraev VS, Ilyukhin VI, Alekseev VV: Molecular identification and typing of *Burkholderia pseudomallei* and *Burkholderia mallei*: When is enough enough. *Trans R Soc Trop Med Hyg*, 102, 134-139, 2008. DOI: 10.1016/S0035-9203(08)70030-0

10. Liguori AP, Warrington SD, Ginther JL, Pearson T, Bowers J, Glass MB, Mayo M, Wuthiekanun V, Engelthaler D, Peacock SJ, Currie BJ, Wagner DM, Keim P, Tuanyok A: Diversity of 16S-23S rDNA Internal Transcribed Spacer (ITS) reveals phylogenetic relationships in *Burkholderia pseudomallei* and its near-neighbors. *PLoS ONE*, 6, e29323, 2011. DOI: 10.1371/journal.pone.0029323

11. Gee JE, Sacchi CT, Glass MB, De BK, Weyant RS, Levett PN, Whitney AM, Hoffmaster AR, Popovic T: Use of 16S rRNA gene sequencing for rapid identification and differentiation of *Burkholderia pseudomallei* and *B. mallei*. *J Clin Microbiol*, 41, 4647-4654, 2003. DOI: 10.1128/JCM.41.10.4647-4654.2003

12. Thibault FM, Valade E, Vidal DR: Identification and discrimination of *Burkholderia pseudomallei*, *B. mallei*, and *B. thailandensis* by real-time PCR targeting type III secretion system genes. *J Clin Microbiol*, 42, 5871-5874, 2004. DOI: 10.1128/JCM.42.12.5871-5874.2004

13. Tomaso H, Pitt TL, Landt O, Al-Dahouk S, Scholz HC, Reisinger EC, Sprague LD, Rathmann I, Neubauer H: Rapid presumptive identification of *Burkholderia pseudomallei* with real-time PCR assays using fluorescent hybridization probes. *Mol Cell Probes*, 19, 9-20, 2005. DOI: 10.1016/j.mcp.2004.08.001

14. Sprague, LD, Zysk G, Hagen RM, Meyer H, Ellis J, Anuntagool N, Gauthier Y, Neubauer H: A possible pitfall in the identification of *Burkholderia mallei* using molecular identification systems based on the sequence of the flagellin fliC gene. *FEMS Immunol Med Microbiol*, 34, 231-236, 2002. DOI: 10.1111/j.1574-695X.2002.tb00629.x

15. Ulrich MP, Norwood DA, Christensen DR, Ulrich RL: Using real-time PCR to specifically detect *Burkholderia mallei*. *J Med Microbiol*, 55, 551-559, 2006. DOI: 10.1099/jmm.0.46350-0

16. Scholz HC, Joseph M, Tomaso H, Al-Dahouk S, Witte A, Kinne J, Hagen RM, Wernery R, Wernery U, Neubauer H: Detection of the reemerging agent *Burkholderia mallei* in a recent outbreak of glanders in the United Arab Emirates by a newly developed fliP-based polymerase chain reaction assay. *Diagn Microbiol Infect Dis*, 54, 241-247, 2006. DOI: 10.1016/j.diagmicrobio.2005.09.018

17. Bowers JR, Engelthaler DM, Ginther JL, Pearson T, Peacock SJ, Tuanyok A, Wagner DM, Currie BJ, Keim PS: BurkDiff: A real-time PCR allelic discrimination assay for *Burkholderia pseudomallei*and *B. mallei*. *PLoS One*, 5, e15413, 2010. DOI: 10.1371/journal.pone.0015413

18. Tomaso H, Scholz HC, Al-Dahouk S, Eickhoff M, Treu TM, Wernery R, Wernery U, Neubauer H: Development of a 5′-nuclease real-time PCR assay targeting *fliP* for the rapid identification of *Burkholderia mallei* in clinical samples. *Clin Chem*, 52, 307-310, 2006. DOI: 10.1373/clinchem.2005.059196

19. Thibault FM, Valade E: Vidal DR: Identification and discrimination of *Burkholderia pseudomallei*, *B. mallei*, and *B. thailandensis* by real-time PCR targeting type III secretion system genes. *J Clin Microbiol*, 42, 5871-5874, 2004. DOI: 10.1128/JCM.42.12.5871-5874.2004

20. Kumar S, Malik P, Verma SK, Pal V, Gautam V, Mukhopadhyay C, Rai GP: Use of a recombinant *Burkholderia* intracellular motility a protein for immunodiagnosis of glanders. *Clin Vaccine Immunol*, 18, 1456-1461, 2011. DOI: 10.1128/CVI.05185-11

21. Tomaso H, Scholz HC, Al-Dahouk S, Pitt TL, Treu TM, Neubauer H: Development of 5′ nuclease real-time PCR assays for the rapid identification of the *burkholderia mallei//burkholderia pseudomallei* complex. *Diagn Mol Pathol*, 13, 247-253, 2004.

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Research Article

Effect of Progestagen Application During Ovsynch Protocol on Pregnancy Rates of Lactating-Grazing Cows

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Abstract

The aim of this study was to test the effect of progesterone supplementation to Ovsynch protocol in lactating and grazing cows on pregnancy rate after fixed time artificial insemination (TAI). Animals, from a total of 158 dairy cows, were randomly assigned to two groups. In Grup 1 (n = 75) Ovsynch protocol was carried out (Group OV), and in Grup 2 (n = 83) Ovsynch + PRID protocol was carried out (Group OV + PRID). Conception rates following timed Artificial Insemination (TAI) were determined as 33.3% for Grup OV and 53.0% for Group OV+PRID. Differences between groups were statistically significant (P<0.05). When Body Condition Score (BCS) of cows considered, pregnancy rates of cows with 2.5 and upper BCS for Group OV and Group OV + PRID were determined as 36.8% and 59.2%, respectively. Differences between groups for BCS were statistically significant according to pregnancy rates (P<0.01). While premature heat rate was considered for TAI, 12.0 and 7.2 % were determined for Group OV and Group OV + PRID, respectively (P>0.05). The findings of the present study suggest that progesterone administration between first GnRH to PGF2α in Ovsynch protocol increases conception rates and numerically decreases premature heat rates, also BCS was a significant factor affecting conception rates in OV or OV + PRID protocols in cows. Moreover, usage of PRID with Ovsynch was more effective to increase conception rates in cows with optimum body condition (2.5-4 BCS) in Kars region.

Keywords: *Cow, Ovsynch, Premature estrus, PRID, TAI*

Ovsynch Protokolü Sırasında Progestagen Uygulamasının Laktasyonda-Otlayan İneklerin Gebelik Oranları Üzerine Etkisi

Özet

Bu çalışmanın amacı laktasyonda ve otlayan ineklerde Ovsynch protokolüne progesterone katkısının sabit zamanlı suni tohumlamada gebelik oranları üzerine etkisini test etmekti. Toplam 158 sütçü inek, rastgele iki gruba ayrıldı: Grup 1 (n = 75)'de bulunan ineklere Ovsynch protokolü uygulandı (Grup OV), Grup 2 (n = 83)'de bulunan ineklere ise Ovsynch + PRID protokolü uygulandı (Grup OV + PRID). Uygulama sonrası sabit zamanlı tohumlama ile gebelik oranları; Grup OV için %33.3, Grup OV + PRID için %53.0 olarak tespit edildi. Gebelik oranları açısından önemli derecede fark (P<0.05) oluştuğu belirlendi. İneklerin Vücut Kondüsyon Skoru (VKS)'na göre sınıflandırılarak incelendiğinde, VKS 2.5 ve üzerinde olan ineklerde gebelik oranı; Grup OV içerisinde %36.8, Grup OV+PRID grubunda ise %59.2 olarak tespit edildi. VKS'na göre gebelik oranları açısından ise önemli derecede (P<0.01) fark olduğu belirlendi. Sabit zamanlı suni tohumlamada, prematüre kızgınlık oranları açısından incelendiğinde, Grup OV için %12.0, Grup OV + PRID için %7.2 olarak saptandı, istatistiksel açıdan bir fark (P>0.05) olmadığı saptnadı. Çalışmadaki bulgular Ovsynch protokolünde, ilk GnRH ile PGF2α uygulaması arasında progesteron uygulamasının gebelik oranlarını yükselttiği ve prematüre kızgınlık oranlarını sayısal olarak aşağıya çekebileceği belirlenmiştir. Buna ek olarak hem Grup OV hem de Grup OV + PRID uygulamalarında VKS'nin gebelik oranlarını etkileyen önemli faktör olduğu tekrar görülmüştür. Ayrıca, bu çalışma ile Kars bölgesinde optimum VKS'ye sahip (2.5-4) ineklerde Ovsynch'le birlikte PRID uygulamasının gebelik oranlarını yükseltmede çok daha etkili olduğu ortaya konulmuştur.

Anahtar sözcükler: *İnek, Ovsynch, Prematüre östrus, PRID, TAI*

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INTRODUCTION

The application of Ovsynch protocol in cattle yields satisfactory conception rates since a report by Twagiramungu et al.[1], working with beef cattle in Canada. They suggested that pretreatment with GnRH, 6 days before the administration of prostaglandin, may eliminate the need for heat detection prior to breeding by AI. The first injection of GnRH was designed to induce ovulation and formation of a new or accessory corpus luteum and a new follicular wave. The corpora lutea was subsequently caused to regress by prostaglandin injection and ovulation occurred about a day after the second administration of GnRH. It is suggested that the protocol could eliminate the need for estrus detection. However, initiation of the protocol in late luteal phase (i.e. after day 13 of the estrous cycle) can induce pre-mature heat then ovulation before the timed insemination [2]. Therefore, reduced conception rates are to be expected when the protocol is initiated after day 13 of the estrous cycle [3]. In an attempt to increase conception rates, pre-synchronization with two luteolytic doses of PGF2a has been tested in Ovsynch by some researchers [3,4].

It has been shown that progesterone concentration during late luteal phase before insemination is positively linked with conception rates in cattle [5]. Adding progesterone to Ovsynch protocol to improve pregnancy rate has been employed with different degree of efficacy application of progesterone with Ovsynch protocol has been shown to improve conception rates. Moreover, duration of progesterone may be important factor affecting premature heat and pregnancy rate. In cyclic heifers that had a progesterone-releasing device (CIDR-B) in their vagina for 7 days and given PGF at CIDR-B removal, estradiol treatment 24-30 h later effectively synchronized estrus with acceptable pregnancy rate (52%) [6]. Similar pregnancy rates (65%) have been achieved after fixed-time AI with exogenous progesterone delivered by intra-vaginal CIDR-B devices in heifers assigned to an Ovsynch protocol [7]. Murugavel et al.[5] attained higher pregnancy rate with 9 days insertion of PRID + Ovsynch protocol (35.5%) compared Ovsynch (26.7%) and PRID (24.4%) protocols in cows. Moreover Ambrose et al.[8,9] determined that CIDR usage with Ovsynch may increase pregnancy rate and ovarian response to GnRH in heifers.

The main objective of this study was to evaluate the efficacy of the Ovsynch protocol with the inclusion of exogenous progesterone delivered by intra-vaginal PRID on pregnancy rate and also effects of BCS on the protocol's efficacy to increase synchronization and pregnancy rates in lactating and grazing cows in Kars region.

MATERIAL and METHODS

Animals

This study was conducted with lactating and grazing Brown-Swiss, Simental and crossbred cows from villages (N40.8055/E42.8917/Alt. approx. 2600 m; N40.2794/E42.9428/Alt. approx. 2200 m) in spring-summer period in Kars/Turkey. All cows (n = 158) were kept under similar management conditions in order to minimize environmental differences: animals were housed indoors, milked twice a day and fed with a total mixed ration ad libitum to meet the nutritional requirements of lactating cows (10-15 L per day) and also two times grass hay feeding. Only cows without a history of reproductive disorder and illness were included in the study. In addition, body condition scoring system from 1 = very thin to 5 = very fat was evaluated for each cows at the time of the first GnRH administration according to Ferguson et al.[10]. Only cows between 1.5 and 4 were included in the study (*Table 1*).

Synchronization Protocol, AI and Pregnancy Determination

One hundred fifty eight cows were randomly divided into two treatments: (a) group OV, standard Ovysnch protocol (n = 75) characterized by the administration of GnRH (gonadorelin diacetate tetrahydrate, 2 mL, 100 µg, i.m., Ovarelin®; CEVA-DİF İlaç A.Ş., İstanbul, Turkey) at days 0 and 56 h after PGF administration, PGF2α (dinoprost, 5 mL, 25 mg, i.m., Enzaprost®T, CEVA-DİF® İlaç A.Ş., İstanbul, Turkey) at day 7 and (b) group OV + PRID7, Ovsynch protocol integrated with the use of progesterone releasing intravaginal device (PRID®, 1.55 g, progesterone, without its estradiol capsule; CEVA-DİF® İlaç A.Ş., İstanbul, Turkey) inserted at the initial GnRH injection and removed at PGF2α administration for seven days (n = 83). PRIDs were used according to EU norms: all capsules (containing estradiol) on PRID were removed before insertion). All cows were inseminated 16-18 h after the second GnRH administration, with frozen-thawed semen with proven fertility sires. Cows detected in heat based on standing estrus were either artificially inseminated based on AM/PM rule or naturally bred by bull, and these cows were accepted as non-pregnant for TAI for analyses.

Pregnancy determinations were performed with trans-rectal B-mode ultrasonography (USG) between days 28-35 following TAI *(Fig. 1)*. Pregnancy diagnosis were performed in 158 cows; therefore, these cows were completed the experiment and used for further analyses.

Table 1. *Primary individual properties of cows in Ovsynch (OV) and Ovsynch + PRID (OV+PRID7) groups for pregnancy rates*

Groups	OV	OV+PRID7
Cows for TAI (n)	75	83
Body Condition Score, n (%)		
<2.5	37 (49.3%)	34 (41.0%)
≥2.5	38 (50.7%)	49 (59.0%)
Premature Estrus	9 (12.0%)	6 (7.2%)
Pregnancy	25 (33.3%)	44 (53%)

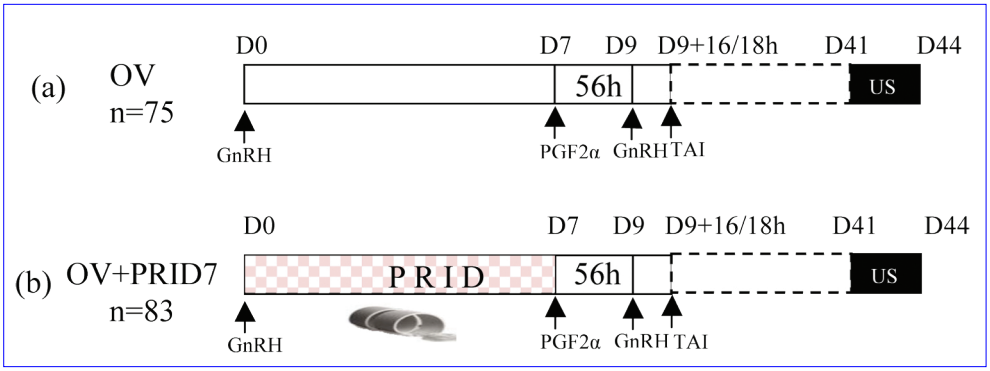

Fig 1. Diagram of treatments during current study. The protocols were **(a)** Ovsynch protocol (OV): 100 µg GnRH i.m. were administered on Day 0 (D0), 25 mg dinoprost (PGF2α) was injected i.m. on Day 7 (D7), second GnRH treatment was administered after 56 h (D9) from PGF2α, and timed artificial insemination n(TAI)s were carried out around 16-18 hours following second GnRH; **(b)** Application of progesterone releasing intravaginal device (PRID, 1.55 g) with Ovsynch (OV + PRID7): 100 µg GnRH i.m., and a PRID containing 1.55 g progesterone for 7 days were applied. At PRID removal 25 mg of dinoprost (PGF2α) was injected i.m. Cows received the second GnRH treatment at 56 h after PRID withdrawal and TAI at 16-18 h later. On 32-35 days (D41-44) after TAI for checking pregnancies, ultrasonographic (USG) examinations were performed following treatments

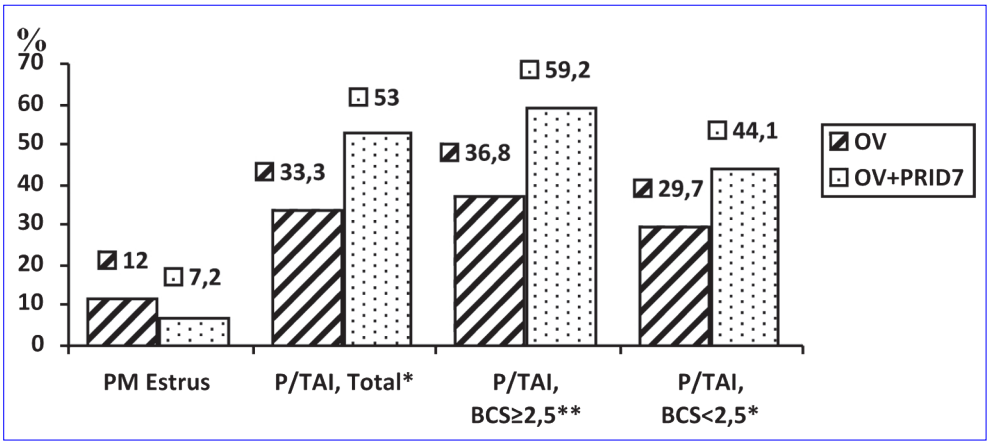

Fig 2. Effect of PRID treatment on Premature Heat **(PM Estrus),** Pregnancy per Timed Artificial Insemination (P/TAI) in whole animals (BCS 1.5-4; **P/TAI, Total**), BCS≥2.5 **(P/TAI, BCS≥2.5)** and BCS<2.5 **(P/TAI, BCS<2.5)** in Ovsynch Protocol. OV= Ovsynch Protocol, OV + PRID7 = Ovsynch Protocol with PRID, P/TAI=Pregnancy rate per timed artificial insemination, PM = Premature, BCS = Body Condition Score (5 point scale). * and ** determine that differences between OV and OV + PRID is statistically significant for parameters: * P<0.05 and ** P<0.01

Statistical Analysis

Binomial dependent variable were analyzed by stepwise selection method in logistic regression procedure (PROC LOGISTIC) of the SAS software (SAS Institute Inc., Cary, NC, USA). The effects of treatments, breed, body condition score (BCS; optimal vs poor), farm (location), replicate (year), parity (primiparous vs multiparous), and interactions of treatment by BCS and treatment by parity on the binominal dependent variables were considered in statistical models. In the final logistic regression models, P<0.05 was considered as a statistically significant. Results were reported as odds ratios along with 95% confidence intervals.

RESULTS

Fig. 2 shows overall reproductive performance for the two treatment groups. Premature heat showed a numerically decrease in OV + PRID7 group (7.2%), compared to the OV group (12.0%) (P>0.05). Pregnancy rate for TAI was higher in OV + PRID group (53.0%), as compared to the OV group (33.3%). Moreover, pregnancy rate for BCS≥2.5 was also higher in OV + PRID group (59.2%), as compared to the OV group (36.8%, P<0.01).

For premature heat expression rate, logistic regression stepwise selection procedure indicated that only main effect of BCS was significant (P<0.01). In this regard, premature heat expression rate did not differ between OV (12.0%; 9/75) and OV + PRID7 (7.2%; 6/83), although it was numerically higher in OV group. However, cows with optimal BCS were significantly (P<0.01) higher premature heat expression rate (14.3%; 14/98) than those with poor BCS (2.6%, 2/76) regardless of treatments.

For TAI pregnancy rate, logistic regression stepwise selection procedure indicated that pregnancy rates for cows in OV + PRID7 (53.0%; 44/83) were higher than those in OV group (33.3%; 25/75). However, logistic regression stepwise selection procedure indicated that only treatment by BCS interaction effect was significant (P<0.01) when interactions were included in the statistical model *(Fig. 2)*. In this regard, pregnancy rates were similar for cows in OV group with poor BCS (29.7%; 11/37) and optimal BCS (36.8%; 14/38). Whereas, pregnancy rates were higher in cows with optimal BCS (59.2%; 29/49) compared to those with poor BCS ((44.1%; 15/34) in OV + PRID7 group.

DISCUSSION

In current study, we found that simultaneous application of PRID with Ovsynch protocol increases pregnancy rates in cows. Especially, this beneficial effect of PRID was more evident in cows with optimum BCS (2.5-4) in our study. Moreover, usage of PRID with Ovsynch numerically decreased premature heat in cows.

Murugavel et al.[5] determined that PRID and PRID + Ovsynch combination provided higher pregnancy rates in cows with low progesterone levels while there was no effect on cows with higher progesterone levels. In other words, usage of PRID with Ovysnch more effectively treated cows in anestrus and may have induced ovulations in these animals. It is well known that anestrus is most common situation attributable to an anovulatory condition in early postpartum period [11]. Progesterone application is one of the treatment options in cows with ovarian cyst syndrome [12]. Similar condition may have occurred in our current study and progesterone supply with PRID in Ovsynch may have treated or beneficially affected to cows with anestrus and anovulatory syndrome.

It was determined that ovulation of an early-stage dominant follicle triggered with exogenous GnRH results in a reduced ovulatory follicle size and also fertility in dairy cattle. Moreover, low progesterone levels may result in the development of large persistent follicles, oocyte quality in such follicles is determined to be compromised resulting in increased embryonic loss found that diameter of follicle in first GnRH directly affects pregnancy rates [13-17]. They hypothesized that large CL producing high P4 are developed from large follicle at the time of first GnRH administration. Pre-ovulatory follicle developing in higher P4 condition could effectively response to second GnRH injection. Similar physiological condition, high P4 levels, may have been artificially created in application of PRID with Ovsynch in our study.

Although presynchronization treatment was not performed in current study, it was determined that PRID administration with Ovsynch increased pregnancy rates in TAI, compared with Ovsynch without any presynch. Similar results had been obtained by Colazo et al.[18]. They used not only Ovsynch but also presynch+ovsynch protocols with PRID administration. It is interesting (or predicted) that PRID did not have any effect on pregnancy/AI in Presynch + Ovsynch groups while PRID increased pregnancy rate in Ovsynch group and pregnancy rates in Ovsynch and presynch groups were similar while PRID application was administered with Ovsynch. So it may be said that PRID usage in Ovsynch increase pregnancy rate, although PRID usage may not be necessary in Ovsynch protocols used with Presynch. In field, PRID + Ovsynch application without Presynch or any other hormonal usage may have some advantages, reducing extra-expenses because of hormonal usage and time consumption. Nevertheless, PRID + Ovsynch or Presynch + Ovsynch applications may be chosen or decided according to expenses and time consuming in field.

It is well known that body condition score (BCS) of cows is an important factor determining success of artificial insemination. It is possible to base nutritional advice on target cow condition scores at critical points in the annual production cycle. It is determined that one critical target is the condition score at the time of mating. In autumn-calving herds the target condition score is set at 2.5 as cows expected to rebreed while mobilizing body reserves on a winter diet which is sufficient to prevent any loss of condition becoming prohibitively expensive. In comparison, the target condition score at mating in spring-calving cows is near 2; the high nutritive value of spring grass permits the cows to be in positive energy balance throughout the mating period [19]. In current study, BCS based artificial insemination had been carried out in cows with Ovsynch and Ovsynch + PRID and it was determined that cows with optimum body condition scores (2.5-4 BCS) had higher possibility to be pregnant after AI in Ovsynch + PRID group, compared with Ovsynch. Clearly, cows with optimum body condition score (BCS≥2.5 in current study) had higher pregnancy rates, compared those with poor conditions (BCS<2.5). However, PRID application increased pregnancy rates in cows with both poor and optimum body conditions. Moreover, supplementation of PRID to Ovsynch was more effective in cows with optimum BCS (≥2.5). It is well known that leptin is an important factor which determines success of reproduction in cattle. Leptin levels in blood are correlated with BCS in ruminants. Animals with low BCS have low leptin levels in blood [20,21].

Another important factor affecting success of timed artificial insemination programs is premature estrus/heat before planned time for AI. In ovulation synchronisation protocols (i.e. Ovsynch, Cosynch, Heatsynch etc.) some percentage of animals show premature estrus in estrus synchronization protocols. In current study, 7.2-12.0% of animals showed premature, undeserved estrus before second GnRH administration. The main reason of this unwanted condition is known that early or premature

increase of estrogens in blood and early ovulation of follicle following PGF2α injection before second GnRH administration. This condition causes uncontrolled, early ovulations. Premature estrus in TAI is undesired situation because these animals cause extra-time consumption and expenses if they are inseminated in unexpected/unplanned time. So a lot of studies have been carried out to prevent early-premature estrus and also ovulations before TAI in Ovsynch or Cosynch protocols with administration of progestagens [14,18,22-26]. In current study, usage of PRID with Ovsynch numerically prevented premature estrus in cows although there was not statistically significant difference. Nevertheless, premature estrus decreased more than 40% in PRID+Ovsynch compared with Ovsynch. In other words, approximately two-fold higher premature estrus had been detected in Ovysnch group. Sample size limitation of current study may have made findings not statistically significant. Colazo et al.[14] had found similar results and percentages in prevention of ovulations before TAI with PRID administration in Ovsynch. Based on our results and that of others, we suggest that it is possible to reduce by half the incidence of premature estrus or ovulation before TAI with addition of PRID to Ovsynch under field conditions.

Colazo and Ambrose [22] compared 5 days and 7 days duration of PRID in Ovsynch protocol. Moreover, they investigated effect of first GnRH injection with PRID application. In cows, it was determined that pregnancy rates in 7 days PRID application with Ovsynch was higher than in 5 days PRID even when no first injection of GnRH was given. First GnRH injection in PRID usage may be important to ovulate follicles with adequate size and develop a mature CL, which will be regressed with PGF2α. In this way, synchronization of ovulations may be more effectively controlled in PRID applications with Ovsynch.

Kars region has hard climate condition during winter and farmers cannot provide enough food and energy intake for their cows during winter period. During winter, cows have to be fed with grass and pasture oaths yielded during summer. Therefore, cows, which have not got enough food intakes, lose weight and BCS. Generally this condition causes “anestrus” at the end of winter in these animals because of low BCS (1.5-2). These animals show estrus again when pasture feeding start at the beginning of spring period. So in this region, treatment of anestrus cows with low BCS (<2.5) is important with usage of Ovsynch + PRID applications at the end of winter period to obtain satisfied pregnancy rates. There are a lot of studies [25,27-33] which have been carried out to deal with reproductive problems and to find good fixed time artificial insemination program using Ovsynch-based for cattle. However, it is certain that a lot of similar and endocrinologic studies are needed to gain good result from timed artificial insemination programs in cattle reared in hard climate condition and also Kars region.

In conclusion, it was determined that PRID application with Ovsynch protocol increased pregnancy rates and prevented premature heat in cows. Moreover, beneficial effect of PRID with Ovsynch is more clear in cows with optimal BCS in Kars region.

Conflict of Interests Statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement

The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals. All procedures on animals were carried out by veterinarians/researchers who are expert and certificated on cattle reproduction.

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REFERENCES

1. Twagiramungu H, Guilbault LA, Proulx J, Villeneuve P, Dufour JJ: Influence of an agonist of gonadotropin releasing hormone (Buserelin) on estrus synchronization and fertility in beef cows. *J Anim Sci,* 70, 1904-1910, 1992. DOI: 10.2527/1992.7061904x

2. Moreira F, Risco CA, Pires MFA, Ambrose DJ, Drost M, Thatcher WW: Use of bovine somatotropin in lactating dairy cows receiving timed artificial insemination. *J Dairy Sci,* 83, 1245-1255, 2000. DOI: 10.3168/JDS.S0022-0302(00)74990-3

3. Moreira F, de la Sota RL, Diaz T, Thatcher WW: Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. *J Anim Sci,* 78, 1568-1576, 2000. DOI: 10.2527/2000.7861568x

4. Thatcher WW, Moreira F, Santos JEP, Mattos RC, Lopes FL, Pancarci SM: Effects of hormonal treatments on reproductive performance and embryo production. *Theriogenology,* 55, 75-89, 2001. DOI: 10.1016/S0093-691X(00)00447-7

5. Murugavel K, Yaniz JL, Santolaria P, Lopez-Bejar M, Lopez-Gatius F: Luteal activity at the onset of a timed insemination protocol affects reproductive outcome in early postpartum dairy cows. *Theriogenology*, 60, 583-593, 2003. DOI: 10.1016/S0093-691X(03)00047-5

6. Lammoglia MA, Short RE, Bellows RE, Bellows SE, MacNeill MD, Hafs HD: Induced and synchronized estrus in cattle: Dose titration of estradiol benzoate in prepubertal heifers and postpartum cows after treatment with and intravaginal progesterone-realising insert and prostaglandin F2 alfa. *J Anim Sci*, 76, 1662-1670, 1998.

7. Martinez MF, Kastelic JP, Adams GP, Mapletoft RJ: The use of CIDR-B or melengestrol acetate with GnRH, LH or estradiol benzoate for fixed-time AI in beef heifers. *J Anim Sci*, 80, 1746-1751, 2002. DOI: 10.2527/2002.8071746x

8. Ambrose DJ, Kastelic JP, Rajamahendran R, Aali M, Dinn N: Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine response and pregnancy rates. *Theriogenology*, 64, 1457-1474, 2005. DOI: 10.1016/j.theriogenology.2005.03.010

9. Ambrose DJ, Emmanuel DGV, Colazo MG, Kastellic JP: Pregnancy rates to timed artificial insemination in Holstein heifers given prostagilandin F2α twenty-four hours before or concurrent with removal of an intravaginal progesterone-releasing insert. *J Dariy Sci*, 91, 2678-2683, 2008. DOI: 10.3168/jds.2007-0840

10. Ferguson JD, Galligan DT, Thomsen N: Principal descriptors of body condition score in Holstein cows. *J Dairy Sci*, 77, 2695–2703, 1994. DOI: 10.3168/jds.S0022-0302(94)77212-X

11. Wiltbank MC, Gümen A, Sartori R: Physiological classification of anovulatory conditions in cattle. *Theriogenology,* 57, 21–52, 2002. DOI: 10.1016/S0093-691X(01)00656-2

12. Crane MB, Bartolome J, Melendez P, de Vries A, Risco C, Archbald LF: Comparison of synchronization of ovulation with timed insemination and exogenous progesterone as therapeutic strategies for ovarian cysts in lactating dairy cows. *Theriogenology*, 65, 1563-1574, 2006. DOI: 10.1016/j.theriogenology.2005.09.003

13. Ahmad N, Schrick FN, Butcher RL, Inskeep EK: Effect of persistent follicles on early embryonic losses in beef cows. *Biol Reprod*, 52, 1129-1135, 1995. DOI: 10.1095/biolreprod52.5.1129

14. Colazo MG, Behrouzi A, Ambrose DJ, Mapletoft RJ: Diameter of the ovulatorty follicle at timed artificial insemination as a predictor of pregnancy status in lactating dairy cows subjected to GnRH-based protocols. *Theriogenology*, 84, 377-383, 2015. DOI: 10.1016/j.theriogenology.2015.03.034

15. Mihm M, Curran N, Hyttel P, Knight PG, Boland MP, Roche JF: Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. *J Reprod Fertil*, 116, 293-304, 1999. DOI: 10.1530/jrf.0.1160293

16. Savio JD, Thatcher WW, Morris GR, Entwistle K, Drost M, Mattiacci MR: Effects of induction of low plasma progesterone concentrations with a progesterone-releasing intravaginal device on follicular turnover and fertility in cattle. *J Reprod Fertil*, 98, 77-84, 1993. DOI: 10.1530/jrf.0.0980077

17. Stock AE, Fortune JE: Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. *Endocrinology*, 132, 1108–1114, 1993. DOI: 10.1210/endo.132.3.8440173

18. Colazo MG, Dourey A, Rajamahendran R, Ambros, DJ: Progesterone supplementation before timed AI increased ovulation synchrony and pregnancy per AI, and supplementation after timed AI reduced pregnancy losses in lactating dairy cows. *Theriogenology*, 79, 833-841, 2013. DOI: 10.1016/j.theriogenology.2012.12.011

19. Gordon I: Factors affecting fertility of the beef cow. **In,** Gordon I (Ed): Controlled Reproduction in Cattle and Buffaloes. 37, CAB International Publishing, New York, 1996.

20. Chilliard Y, Delavaud C, Bonnet M: Leptin expression in ruminants: Nutritional and physiological regulations in relation with energy metabolism. *Domest Anim Endocrinol*, 29, 3-22, 2005. DOI: 10.1016/j.domaniend.2005.02.026

21. Kacar C, Arı, U.C: Effects of leptin on energy metabolism and reproduction physiology in cows and sheep. *Kafkas UnivVet Fak Derg*, 13, 209-213, 2007. DOI: 10.9775/kvfd.2007.17-D

22. Colazo MG, Ambrose DJ: Effect of initial GnRH and duration of progesterone insert treatment on fertility of lactating dairy cows. *Reprod Dom Anim*, 50, 497-504, 2015. DOI: 10.1111/rda.12518

23. Mialot JP, Constant F, Dezaux P, Grimard B, Deletang F, Ponter AA: Estrus synchronization in beef cows: Comparison between GnRH+PGF2α+GnRH and PRID+PGF2α+eCG. *Theriogenology*, 60, 319-330, 2003. DOI: 10.1016/S0093-691X(02)01371-7

24. Palomares RA, Fishman HJ, Jones AL, Ferrer MS, Jenerette M, Vaughn A: Comparison of 4- versus 5-days Cosynch + controlled internal drug release (CIDR) + timed artificial insemination protocols in dairy heifers. *Theriogenology*, 84, 868-874, 2015. DOI: 10.1016/j.theriogenology.2015.05.021

25. Pancarcı SM, Lehimcioglu NC, Arı UC, Güngör O, Akbulut O: Efficacy of hCG and GnRH with respect to follicular size and presence of the corpus luteum in cosynch protocol integrated with Norgestomet in lactating cows. *Bull Vet Inst Pullawy*, 57, 61-64, 2013. DOI: 10.2478/bvip-2013-0011

26. Sakase M, Kawate N, Nakagawa C, Fukushima M, Noda M, Takeda K, Ueno S, Inaba T, Kida K, Tamada H, Sawada T: Preventive effects of CIDR-based protocols on premature ovulation before timed-AI Ovsynch in cycling beef cows. *Vet J*, 173, 691-693, 2007. DOI: 10.1016/j.tvjl.2005.12.012

27. Kacar C, Yıldız S, Pancarcı SM, Kaya M, Gungor O, Ari UC: Effect of administration of lesireline five days after artificial insemination on pregnancy rates and luteal function in cows. *Kafkas UnivVet Fak Derg,* 13, 139-142, 2007. DOI: 10.9775/kvfd.2007.20-A

28. Kacar C, Kamiloglu NN, Ucar O, Ari UC, Pancarcı SM, Kaya M, Gungor O: Effect of Ovsynch and Cosynch synchronization programmes with β-carotene + vitamin E administration upon the pregnancy rates in cows. *Kafkas Univ Vet Fak Derg*, 14, 45-50, 2008. DOI: 10.9775/kvfd.2008.02-A

29. Nak Y, Tuna B, Nak D, Karakaş, Şimşek G: The effect of Ovsynch, Ovsynch with Progestin and Progestin plus Double TAI on pregnancy rates in unobserved oestrus dairy cows and heifers. *Kafkas Univ Vet Fak Derg,* 17, 917-922, 2011. DOI: 10.9775/kvfd.2011.4704

30. Çevik M, Selçuk M, Doğan S: Comparison of pregnancy rates after timed artificial insemination in Ovsynch, Heatsynch and CIDR-based synchronization protocol in dairy cows. *Kafkas Univ Vet Fak Derg,* 16, 85-89, 2010. DOI: 10.9775/kvfd.2009.451

31. Yılmaz C, Yılmaz O, Ucar M: Effect of PGF2α and GnRH injections applied before Ovsynch on pregnancy rates in cows and heifers. *Kafkas Univ Vet Fak Derg,* 17, 641-644, 2011. DOI: 10.9775/kvfd.2011.4638

32. Uçar Ö, Özkanlar S, Kaya M, Özkanlar Y, Şenocak MG, Polat H: Ovsynch synchronization programme combined with vitamins and minerals in underfed cows: Biochemical, hormonal and reproductive traits. *Kafkas Univ Vet Fak Derg,* 17, 963-970, 2011. DOI: 10.9775/kvfd.2011.4863

33. Stevenson JS, Pursley JR, Garverick HA, Fricke PM, Kesler DJ, Ottobre JS, Wiltbank MC: Treatment of cycling and noncycling lactating dairy cows with progesterone during Ovsynch. *J Dairy Sci*, 89, 2567-2578, 2006. DOI: 10.3168/jds.S0022-0302(06)72333-5

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Research Article

Utilization of Cryopreserved Ruminal Liquor in *In Vitro* Gas Production Technique for Evaluating Nutritive Value of Some Feedstuffs [1]

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Abstract

In vitro gas production technique (IVGPT) is a routine method in nutritional sciences to determine energy content, organic matter (OM) digestibility, and fermentation kinetics of feedstuffs. After collecting from two ruminally cannulated Holstein heifers (350 kg), rumen liquors were used either fresh or cryopreserved form in the inoculums for IVGPT. Starch- (barley, wheat, and corn) and protein-rich (sunflower meal, cotton seed meal, and soybean meal) feedstuffs were evaluated for gas production kinetics, fermentation pattern, and energy content in 5 replicates. pH, NH_3-N concentration and volatile fatty acids (VFA) profile, gas production, and fermentation kinetics parameters were measured. Data were analyzed by 2-way ANOVA. Viable protozoa rate was found to be 70.8% in cryopreserved rumen liquor after thawing. Decrease in pH in thawed rumen liquor was less than fresh rumen liquor as the incubation period advanced. Utilization of frozen rumen liquor after thawing in IVGPT was associated with lower VFA and NH_3-N concentration, cumulative gas production, and metabolisable energy estimate for all feedstuffs. In conclusion, despite high correlation between in vitro data obtained from fresh and thawed rumen liquors to predict gas production, further experiments should cope with improving cryopreservation protocol for rumen liquors in order to optimize microbial activity for maintaining fermentation pattern.

Keywords: *Cryopreserved rumen liquor, Energy prediction, Fermentation, Gas production, In vitro gas test, Volatile fatty acid*

Bazı Yemlerin Besin Değerlerini Değerlendirmek için *In Vitro* Gaz Üretim Tekniğinde Dondurulmuş Rumen Sıvısının Kullanımı

Özet

In vitro gaz üretim tekniği (IVGPT), besleme çalışmalarında yem maddelerinin enerji içeriklerini, organik madde (OM) sindirilebilirliğini ve fermentasyon kinetiklerini belirlemede kullanılan rutin bir yöntemdir. İki adet rumen kanüllü Holstein düveden (350 kg) elde edilen rumen sıvıları taze veya dondurulmuş formda IVGPT için inoculum olarak kullanıldı. Nişasta (arpa, buğday ve mısır) ve protein (ayçiçeği küspesi, pamuk tohumu küspesi ve soya fasülyesi küspesi) bakımından zengin yem maddelerinin gaz üretim kinetikleri, fermentasyon profile ve enerji içerikleri 5 tekerrlü belirlendi. pH, NH_3-N konsantrasyonu, uçucu yağ aside (UYA) profili, gaz üretimi ve fermentasyon kinetik parametleri ölçüldü. Veriler 2-yönlü ANOVA ile analiz edildi. Dondurulduktan sonra çözdürülen rumen sıvısındaki canlı protozoa oranı %70.8 bulundu. Inkübasyon periyodu devam ederken çözünmüş rumen sıvısındaki pH düşüşü taze rumen sıvısındaki düşüşten daha azdı. IVGPT'de dondurulup çözdürülmüş rumen sıvısı kullanılmasıyla yem maddeleri için UYA düzeyleri, NH_3-N konsantrasyonu, kümülatif gaz üretimi ve tahmini metabolik enerji değeri düşük bulundu. Sonuçta, gaz üretim tahmini için taze rumen sıvısı ve dondurulup çözdürülmüş rumen sıvısı kullanılmasıyla elde edilen in vitro veriler arasında önemli bir benzerlik olmasına rağmen, optimum mikrobiyal aktiviteyi, dolayısıyla fermentasyonu sürecini sağlamak için gelecekte yapılacak çalışmalar rumen sıvılarının kriyoprezervasyon protokolünü geliştirmeye odaklanmalıdır.

Anahtar sözcükler: *Dondurulmuş rumen sıvısı, Enerji tahmini, Fermentasyon, Gaz üretimi, In vitro gaz testi, Uçucu yağ asitleri*

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INTRODUCTION

Nutrient content and digestibility are important feedstuff parameters to formulate cost-effective rations. Because of being less costly and laborious as well as reliable while producing a large number of data with a low variability in a short time in a controllable environment, *in vitro* gas production technique (IVGPT, also known as Hohenheimer Futter Test) has become common [1].

Many researchers have employed IVGPT to determine OM digestibility (OMD) [2,3], energy [4,5], fermentation kinetics [6], the adverse effects of anti-nutritional factors [7], and methane emission [8]. Nevertheless, there are inconsistencies among *in vitro* outcomes from different laboratories. It appears that availability of uniform and continuous rumen liquor supply is one of the major factors in yielding variable results [9].

Cryopreservation may offer some advantages for IVGPT, in terms of availability of constant rumen liquor from defined donors to eliminate variability in microbial inoculum source [10]. Controlling cell density, cryoprotectant concentration, equilibrium period, suspension temperature, and time to hold cells in suspension are shown to succeed in maintaining viability of rumen microorganisms [11]. However, studies coping with cryopreserved rumen liquor usage as a source of inoculum in IVGPT are limited.

This study was therefore conducted to evaluate fermentation characteristics and nutritive value of commonly used concentrate feedstuffs in ruminant nutrition by IVGPT employing fresh and cryopreserved rumen liquors in comparison with *in vivo* experimentation.

MATERIALS and METHODS

Animals and Management

The experimental protocol (#2008/028) was approved by the Selçuk University Ethic Committee on Animal Experimentation (Konya, Turkey). Two ruminally cannulated Holstein heifers weighing an average of 350 kg were served as rumen liquor donors. Heifers fed a ration consisting of 60% forage (13.43% CP and 2320 kcal/kg ME on DM basis) and 40% concentrate (17.32% CP and 2810 kcal/kg ME on a DM basis) twice daily at 08:00 and 17:00 h, delivering 5 kg 3rd cut alfalfa hay plus 3.5 kg concentrate per day, to meet nutrient requirement for maintenance and ~0.5 kg daily weight gain [12]. The ration contained 95.2% dry matter (DM), 15% crude protein (CP), 41.2% aNDF (neutral detergent fiber of organic matter with sodium sulphite and heat stable α-amylase), 26.7% acid-detergent fiber (ADF), 50.1% nitrogen-free extract (NFE), 3.1% ether extract (EE), and 9.8% crude ash (CA). Fresh water was available *ad libitum*.

Experimental Feedstuffs

Commercially available barley grain (BG), wheat grain (WG), corn grain (CG), sunflower seed meal (SFM), cotton-seed meal (CSM), and soybean meal (SBM) samples (n = 5) were ground to pass a 1-mm screen (Retsch, SM100 Comfort, Germany) and conserved for experimentations in plastic containers *(Table 1)*.

Rumen Liquor Collection and Cryopreservation Protocol

Before morning feeding, rumen liquors collected from different spots in both heifers were mixed and then poured into a prewarmed container. Liquor was filtered through a double-layer cheese cloth under CO_2 pump. A part liquor was separated for the cryopreservation protocol. Samples were put into 50 ml plastic containers and centrifuged at 4.640 *g* for 30 min (Allegra 64R, Beckman Coulter, Brea, CA) [13]. After removing supernatant, pellet was added with dimethyl sulfoxide (DMSO, 5%, vol/vol), a cryoprotectant and let stand at 25°C for 5 min (equilibration time). For the two-step cryopreservation protocol, treated aliquots (1 ml) were transferred into cryotubes for freezing in a computer-controlled freezer (Ice Cube 14S, Sy-Lab, Neupurkersdorf, Austria), from 25 to -2°C (extracellular ice nucleation temperature) at a rate of 7°C/min (the first step). Then, the suspension was continued to freeze from -2 to -30°C, at a rate of 1.4°C/min and kept at -30°C for 45 min (holding temperature, the second step) [14] before placing them into N tank (-196°C) (Taylor Wharton, Theodore, AL) until IVGPT experimentation. On the day of IVGPT, frozen rumen liquors were thawed at 39°C for 5 min in water bath. In the tests, thawed rumen liquors (1 ml) were added into incubation media (29 ml).

In vitro Gas Production Technique

For the *in vitro* gas production, the media were prepared using the Hohenheim Gas Test as outlined by Menke and Steingass [4]. Each of 0.2 g feedstuff samples, in triplicates, was incubated with the mixture (20 ml medium solution and 10 ml fresh rumen liquor; 29 ml medium solution + 1 ml cryopreserved rumen liquor after centrifugation) in a pyrex bottle (100 ml) a digital manometer (Keller Leo 1, Switzerland) was used for determination of gas production [15]. In each assay, blank bottle without a feed sample were run in triplicates.

Gas production was measured at 6, 12, 24, and 48 hrs post-incubation. Gas production kinetics parameters were calculated using NEWAY software (Version 5.0) as described by Ørskov and McDonald [16], which was as follows: $P = a + b*(1-e^{-c*t})$, where P = corrected gas production at time t relative to incubation (ml), a = gas production from soluble fraction (ml), b = gas production from insoluble but slowly fermentable fraction (ml), c = gas production rate from the fraction b (ml/h), and t = incubation time (h). The effective gas production (EGP) was calculated using the following

Table 1. *Nutrient contents (%) of the experimental feedstuffs **

Nutrient††	Feedstuffs†						P
	BG	WG	CG	SFM	CSM	SBM	
DM	91.2±0.3^{ab} (0.7)	91.4±0.3^{ab} (0.6)	89.2±0.4^{c} (1.1)	91.2±0.4^{ab} (0.9)	92.2±0.7^{a} (1.60)	90.8±0.4^{b} (0.9)	0.001
CP	13.7±0.5^{c} (8.3)	12.5±0.9^{c} (15.9)	7.8±0.5^{d} (13.6)	30.6±2.5^{b} (18.1)	29.0±2.3^{b} (18.0)	46.8±0.6^{a} (3.0)	0.0001
EE	2.44±1.09^{b} (11.1)	2.40±1.07^{b} (25.6)	4.16±1.86^{a} (12.1)	1.38±0.62^{b} (25.5)	4.30±1.92^{a} (67.7)	2.02±0.90^{b} (30.18)	0.006
CA	2.19±0.18^{b} (18.6)	1.46±0.33^{b} (50.8)	1.37±0.41^{b} (66.5)	6.61±0.27^{a} (9.1)	6.46±0.38^{a} (13.3)	6.78±0.14^{a} (4.5)	0.0001
CF	6.97±0.43^{bc} (13.7)	4.73±0.41^{c} (19.4)	4.89±0.15^{c} (7.0)	24.03±1.23^{a} (11.5)	22.78±1.31^{a} (12.8)	8.77±0.40^{b} (10.1)	0.0001
NFE	74.7±0.9^{b} (2.8)	78.9±1.6^{ab} (4.5)	80.3±2.4^{a} (6.8)	37.4±1.7^{c} (10.3)	37.4±1.5^{c} (9.0)	35.7±0.3^{c} (1.97)	0.0001
aNDF	21.5±0.4^{b} (4.3)	17.3±1.0^{bc} (13.4)	13.6±0.4^{cd} (6.6)	46.9±4.2^{a} (20.1)	51.1±1.9^{a} (8.2)	10.8±0.4^{d} (8.7)	0.0001
ADF	6.71±0.32^{b} (10.6)	4.08±0.25^{b} (13.9)	3.61±0.14^{b} (8.5)	32.3±2.7^{a} (18.8)	35.0±2.7^{a} (17.1)	6.77±0.47^{b} (15.7)	0.0001

** Data are LSM±SE (% CV), **n** = 5; Different superscripts within the same rows differ (P<0.05); †**BG** = barley grain; **WG** = wheat grain; **CG** = corn grain; **SFM** = sunflower seed meal; **CSM** = cottonseed meal; **SBM** = soybean meal; ††**DM** = dry matter; **CP** = crude protein; **EE** = ether extract; **CA** = crude ash; **CF** = crude fiber; **NFE** = nitrogen–free extract; **aNDF** = amylase-treated neutral detergent fiber; **ADF** = Acid detergent fiber*

formula: EGP, ml = a + b*c/(c + k), where k = ruminal flow rate (5%).

Using the cumulative gas production (CGP) as well as nutrients such as CP, EE, and CA, *in vitro* ME (IVME) values were calculated using following formula as defined by Menke and Steingass [4]:

IVME, Mcal/kg DM = [(1.06 + 0.157 x CGP + 0.084 x CP + 0.022 x EE - 0.0081 x CA) x 1000]/4.186.

In vitro NE_L ($IVNE_L$) value of feedstuffs was determined using the same variables as defined by Steingass [17] as follows: $IVNE_L$, kcal/kg DM) = [(1.64 + 0.0269 x CGP + 0.00078 x CGP^2 + 0.0051 x CP + 0.01325 x EE) x 1000] /4.186.

In vitro digestible OM (IVDOM) was calculated as defined by Öğretmen [18] as follows: IVDOM, % DM = 0.7602 x CGP + 0.6365 x CP + 22.53.

Measurements and Laboratory Analyses

Feedstuffs were subjected to wet chemistry for DM, CP, CF, EE, and CA [19] as well as for aNDF and ADF [20] using The Ankom^{200} Fiber Analyzer.

On the day of using frozen rumen liquors after thawing at 39°C for 5 min, 3 cryotubes were subjected to viable protozoa enumeration by the same two individuals. Viability was determined immediately after mixing rumen liquors (15 µl) with 15 µl ml 0.5% trypan blue [21]. Enumeration was performed as described by Dehority [22].

pH was measured at 6, 12, 24 and 48 hrs relative to incubation using a digital pH meter (HI 8314, Hanna Instruments, Portugal). NH_3-N concentrations were determined using spectrophotometer (625 nm, UV Mini 1240, UV-VIS Spectrophotometer, Shimadzu, Japan) [23]. VFAs were determined using gas chromatography (Shimadzu, Model 15-A) with FID detector [24].

Statistical Analysis

Descriptive statistics of nutrient contents were determined using the Proc Univariate procedure [25]. Differences among feedstuffs were determined by one-way ANOVA using the Duncan Multiple Range Test option.

Two-way ANOVA with repeated measures option was employed to determine the effect of the rumen liquor form and feedstuff on pH, NH_3-N concentration, and gas production. The linear model was $y_{ijk} = \mu + RL_i + FS_j + (RL \times FS)_{ij} + T_k + (RL \times T)_{ik} + (FS \times T)_{jk} + (RL \times FS \times T)_{ijk} + e_{ijk,}$ where RL = i^{th} rumen liquor (fresh *vs*. cryopreserved), FS = j^{th} feedstuff, T = k^{th} incubation time, and e = residual error. For other variables measured at a single time point (*i.e.*, VFA, effective gas production, gas production kinetics parameters, IVME, $IVNE_L$, IVDOM), "time" parameter and its interaction terms were omitted from the linear model.

Finally, mathematical relationships between CGP and ME estimates as well as between ME and NE_L values obtained from inoculums prepared from fresh and thawed rumen liquors were established using the REG procedure. Statistical significance was declared at P<0.05.

RESULTS

Nutrient Content

Leguminous feeds (SBM > SFM = CSM) had greater CP level than gramineous feeds (BG > WG > CG) *(Table 1)*.

Protozoa Count and Viability

Rumen protozoa count in fresh rumen liquor was within normal range (1.5-5.3 x 10^5). In thawed rumen liquor the protozoa viability was 70.8% when protozoa count expressed as log basis.

Ruminal pH, NH_3-N and VFA Concentration

pH of media containing fresh ruminal liquor was lower than pH of media containing thawed ruminal liquor (6.90 *vs.* 7.01, P<0.0001; *Table 2)*. NH_3-N concentration was greater in media prepared from fresh ruminal liquor than in media prepared from thawed rumen liquor (14.4 *vs.* 12.9 mmol/l, P<0.0001; *Table 2)*. During incubation pH decreased slower and NH_3-N increased faster in media containing fresh rumen liquor than those containing thawed rumen liquor (P<0.0001 for both). As the incubation period progressed, media pH increased gradually with fermentation of protein rich-feedstuffs, whereas media pH decreased gradually with fermentation of starch rich-feedstuffs (P<0.0001). Increase in NH_3-N concentration was continuous when protein rich-feedstuffs were incubated, whereas there was a lag period in release of NH_3-N when starch rich-feedstuffs were incubated (P<0.0001), *(Table 2)*.

The acetate (51.77 vs. 54.72, P<0.0001) proportion was greater and the propionate portion was lower (20.00 vs. 24.29%, P<0.0001) when feedstuffs were incubated in fresh rumen liquor as compared to thawed rumen liquor *(Table 3)*. Cryopreservation caused 17.2 and 22.6% decreases in the Ac:Pr ratio (P<0.01) and total VFA concentration (P<0.0001), respectively *(Table 3)*.

Gas Production and Kinetics Parameters

The amount (40.32 vs. 39.10 ml, P<0.0001; *Table 2)* and rate *(Fig. 1A)* of gas production during the *in vitro* incubation with fresh rumen liquor were greater than thawed rumen liquor. Fermentation of starch-rich feedstuffs resulted in 1.73-fold greater gas production than protein rich-feedstuffs (P<0.0001; *Table 2)*. The rate of increase in gas production over time was greater for starch-rich feedstuffs than for protein-rich feedstuffs (P<0.0001; *Fig. 1B)*.

Cumulative gas production from media containing fresh and thawed rumen liquor within 24 h was highly correlated (r = 0.98) and fit to following model: *In vitro* CGP from fresh rumen liquor within 24 h (ml) = 11.74 + 0.77 x *In vitro* CGP from thawed rumen liquor within 24 h (ml) (R^2 = 0.97, Sy.x = 4.60%; P<0.0001; *Fig. 1C)*.

Cumulative gas production in media containing thawed rumen liquor fit considerably to predict ME value of feeds

Table 2. *The effect of the rumen liquor form on pH, NH_3-N concentration, in vitro gas production and kinetics parameters **

Treatments††	Response Variables†						
	pH³	NH_3-N³	CGP‡	EGP	a	b	c
Fresh rumen liquor							
BG	6.80±0.01	13.6±0.2	49.2±0.3	41.4±6.7	-10.44±1.04	86.6±10.8	0.077±0.003
WG	6.80±0.01	13.5±0.2	52.3±0.3	44.4±6.4	-9.84±1.01	93.8±10.4	0.069±0.003
CG	6.80±0.01	12.1±0.2	48.8±0.3	39.8±6.4	-19.45±1.04	100.8±10.8	0.073±0.003
SFM	7.05±0.01	15.4±0.2	26.7±0.3	23.0±5.8	-1.49±0.90	41.0±9.3	0.075±0.003
CSM	7.03±0.01	14.8±0.2	26.6±0.3	22.8±5.6	-1.71±0.88	47.6±9.1	0.055±0.003
SBM	6.94±0.01	16.8±0.2	38.3±0.3	32.8±6.4	-5.08±1.01	58.0±10.4	0.095±0.003
Group mean	6.90±0.004	14.4±0.1	40.3±0.1	34.0±0.2	-8.00±0.40	71.3±4.2	0.074±0.001
Thawed rumen liquor							
BG	6.92±0.01	12.0±0.2	48.9±0.3	40.0±6.4	-24.41±1.01	101.0±10.4	0.089±0.003
WG	6.87±0.01	11.8±0.2	55.0±0.3	43.8±6.4	-33.25±1.01	115.1±10.4	0.102±0.003
CG	6.92±0.01	10.8±0.2	47.8±0.3	38.4±6.4	-25.25±1.01	110.0±10.4	0.069±0.003
SFM	7.11±0.01	14.6±0.2	24.8±0.3	21.9±5.6	3.73±0.88	45.1±9.1	0.038±0.003
CSM	7.15±0.01	14.0±0.2	20.2±0.3	19.2±6.4	7.76±1.01	22.2±10.4	0.001±0.003
SBM	7.07±0.01	14.0±0.2	38.0±0.3	32.9±6.7	1.20±1.04	62.1±10.8	0.052±0.003
Group mean	7.01±0.004	12.9±0.1	39.1±0.1	32.7±0.2	-11.70±0.41	68.5±4.2	0.058±0.001
ANOVA (------ P > F ------)							
Rumen liquor (RL)	0.0001	0.0001	0.0001	0.0001	0.0001	0.63	0.0001
Feedstuff (FS)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
RLxFS	0.0001	0.0001	0.0001	0.04	0.0001	0.0001	0.0001

** Data are LSM±SE, **n** = 5; †**NH_3-N** = ammonia nitrogen (mmol/l); **CGP** = cumulative gas production within 24 h (ml); **EGP** = effective gas production (ml); **a** = gas production from soluble fraction (ml); **b** = gas production from insoluble but slowly fermentable fraction (ml). **c** = gas production rate from the fraction b (ml/h); ††**BG** = barley grain; **WG** = wheat grain; **CG** = corn grain; **SFM** = sunflower seed meal; **CSM** = cottonseed meal; **SBM** = soybean meal ‡P<0.0001 for the effects of incubation time (T). RLxT. FSxT. and RLxFSxT*

Table 3. *The effect of the rumen liquor form on volatile fatty acid proportion **

Treatments††	Response Variables†							
	Ac (%)	Pr (%)	Isobu (%)	Bu (%)	Isova (%)	Va (%)	Ac:Pr	Total VFA (mmol/L)
Fresh rumen liquor								
BG	53.71±1.40	19.99±0.78	3.14±0.49	17.55±0.64	2.91±0.30	2.69±0.20	2.80±0.38	96.2±5.3
WG	53.59±1.40	19.44±0.78	3.11±0.49	19.26±0.64	2.35±0.30	2.25±0.20	2.88±0.38	107.6±5.3
CG	53.42±1.25	19.77±0.69	2.75±0.43	19.50±0.57	2.85±0.27	1.70±0.18	2.80±0.34	100.4±4.7
SFM	55.69±1.10	20.36±0.61	4.89±0.38	10.59±0.51	5.34±0.24	3.14±0.16	2.81±0.30	87.9±4.2
CSM	60.20±1.07	18.81±0.59	4.06±0.37	9.88±0.49	4.32±0.23	2.72±0.15	3.30±0.29	84.4±4.0
SBM	51.73±1.15	21.64±0.64	4.22±0.40	12.73±0.52	5.86±0.25	3.81±0.16	2.88±0.31	123.5±4.3
Group mean	54.72±0.50	20.00±0.28	3.97±0.18	14.92±0.24	3.94±0.11	2.72±0.07	2.91±0.14	100.0±1.9
Thawed rumen liquor								
BG	49.30±1.27	25.56±0.70	2.12±0.44	18.40±0.58	2.06±0.28	2.56±0.18	1.98±0.34	88.7±4.8
WG	49.04±1.31	23.74±0.72	2.40±0.46	20.45±0.60	1.85±0.28	2.51±0.18	2.16±0.35	92.8±4.9
CG	51.04±1.43	24.62±0.79	2.19±0.50	18.68±0.66	1.55±0.31	1.92±0.20	2.11±0.38	87.9±5.4
SFM	52.14±1.23	24.65±0.68	5.81±0.43	9.90±0.56	4.82±0.27	2.68±0.17	2.75±0.33	65.8±4.6
CSM	55.27±1.19	24.260.66	5.47±0.42	9.46±0.55	3.28±0.26	2.26±0.17	2.35±0.32	44.4±4.5
SBM	53.80±1.38	22.92±0.76	3.68±0.48	11.03±0.63	4.95±0.30	3.61±0.20	3.10±0.37	84.6±5.2
Group mean	51.77±0.54	24.29±0.30	3.61±0.18	14.65±0.24	3.08±0.12	2.59±0.08	2.41±0.14	77.4±2.0
ANOVA (----- P > F -----)								
Rumen liquor (RL)	0.0001	0.0001	0.74	0.43	0.0001	0.22	0.01	0.0001
Feedstuff (FS)	0.0001	0.40	0.0001	0.0001	0.0001	0.0001	0.44	0.0001
RLxFS	0.06	0.03	0.02	0.15	0.68	0.17	0.41	0.001

** Data are LSM±SE.* ***n*** *= 5;* †*Ac = acetate;* ***Pr*** *= propionate;* ***Isobu*** *= isobutyrate.* ***Bu*** *= butyrate;* ***Isova*** *= isovalerate;* ***Va*** *= valerate;* ***VFA*** *= volatile fatty acid*
†† ***BG*** *= barley grain;* ***WG*** *= wheat grain;* ***CG*** *= corn grain;* ***SFM*** *= sunflower seed meal;* ***CSM*** *= cottonseed meal;* ***SBM*** *= soybean meal*

estimated using fresh rumen liquor *(Fig. 1D)*. Following models were developed to predict ME and NE_L values from fresh rumen liquor using CGP measured in 24 h from thawed rumen liquor and nutrient contents: *In Vitro* ME kcal/kg (fresh rumen liquor) = 1089 + 6.98 x CGP (24 h, ml, thawed rumen liquor) + 30.79 x CP (%) + 77.11 x EE (%) + 12.14 x NFE (%) + 5.08 x aNDF (%) – 77.11 x CA (%) - 16.70 x ADF (%) (R^2 = 0.97, Sy.x = 7.07%, P<0.0001). *In Vitro* NE_L kcal/kg (fresh rumen liquor) = 644 + 9.00 x CGP (24 h, ml, thawed rumen liquor) + 2.11 x NFE (%) + 13.20 x aNDF (%) – 24.93 x ADF (%) (R^2 = 0.97, Sy.x = 3.79%, P<0.0001).

Energy Estimation and Organic Matter Digestibility

The mean ME and IVOMD values for feeds incubated with thawed rumen liquor were lower than those for feeds incubated with fresh rumen liquor (2594 *vs*. 2643 kcal/kg; P<0.0001 and 74.1 *vs*. 75.9%, P<0.003; *Table 4)*. The rumen liquor form did not affect NE_L value of feeds.

DISCUSSION

In general, nutrient contents of tested feedstuffs are in agreement with those reported in the literature [26]. Variations in nutrient contents within and among feedstuffs are natural and are mainly due to hybrid, growth condition, soil composition, and climatological factors.

Protozoa count is consistent with literature and within the normal range [22]. Controlled freezing increases viability [27], such as until -30°C, 1°C/min freezing speed for anaerobic bacteria [28]. It is suggested that frozen anaerobic bacteria should be thawed at 37°C within 1 min [28]. Nevertheless, effective freezing protocol for rumen liquor has not been defined [29]. Survival response also seems to vary by the cryopreservation protocol. It was reported that viability of *Isotricha*, *Dasytricha*, *Epidinium*, *Polyplastron*, *Eudiplodinium*, and *Entodinium* were 100, 98, 85, 79, 63, and 60%, respectively [14]. Cryoprotectant (5% DMSO) usage and controlled-2 step freezing (holding phase of -30°C for 45 min at a rate of 1.4°C/min) application to assure extracellular ice enucleation temperature (-2°C) in this experiment may explain high protozoa survival rate.

Higher pH *(Table 2)*, lower NH_3-N concentration *(Table 2)*, and lower total VFA concentration *(Table 3)* in media containing thawed rumen liquor may result from decreased survival, perhaps activity of microorganisms, and hence inefficient fermentation [9]. Energy rich grains incubated within thawed liquor reached the lowest pH at 12 h when

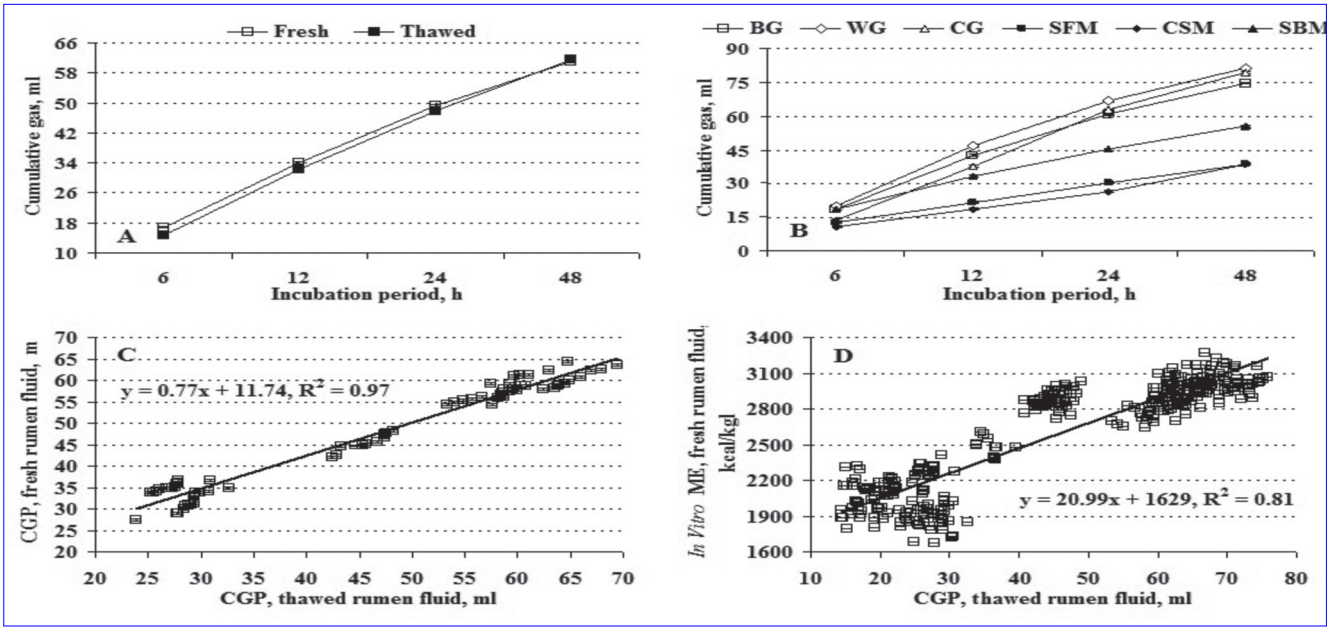

Fig 1. Comparison of fermentation products in inoculums prepared from fresh (--□--) and thawed (--■--) rumen liquor during the *in vitro* incubation Cumulative gas production in inoculums ($P<0.0001$, Panel A). Cumulative gas production from *in vitro* fermentation of barley grain (--□--), wheat grain (--◊--), corn grain (--Δ--), sunflower meal (--■--), cottonseed meal (--♦--), and soybean meal (--▲--) ($P < 0.0001$; Panel B). Regressing cumulative gas production (CGP) in inoculum prepared from thawed rumen liquor within 24-h incubation on CGP in inoculum prepared from thawed rumen liquor within 24-h incubation (Panel C) and *in vitro* ME value determined in inoculum prepared from thawed rumen liquor (Panel D)

Table 4. *Energy content and organic matter digestibility (OMD) determined in vitro gas technique employing fresh and frozen rumen liquors **

Treatments†	ME (kcal/kg)	NE_L (kcal/kg)	*In vitro* OMD (%)
Fresh rumen liquor			
BG	2882.5±23.9	1487.6±12.4	77.3±0.6
WG	3026.6±23.6	1608.4±12.3	79.6±0.6
CG	2950.3±23.6	1548.4±12.3	75.1±0.6
SFM	1989.3±21.1	827.8±11.0	65.8±0.5
CSM	2134.0±20.8	836.6±10.8	65.4±0.5
SBM	2874.5±23.6	1135.9±12.3	86.9±0.6
Group mean	2643±9.0	1241±5.0	75.9±0.2
Thawed rumen liquor			
BG	2960.4±23.6	1549.0±12.3	78.8±0.6
WG	3199.2±23.9	1759.7±12.4	83.3±0.6
CG	2971.4±23.9	1566.4±12.4	75.6±0.6
SFM	1872.7±20.8	768.9±10.8	63.5±0.5
CSM	1713.6±20.6	656.9±10.7	56.9±0.5
SBM	2849.8±24.4	1120.3±12.7	86.4±0.6
Group mean	2595±9.0	1247±5.0	74.1±0.2
ANOVA (----- P > F -----)			
Rumen liquor (RL)	0.0001	0.57	0.003
Feedstuff (FS)	0.0001	0.0001	0.0001
RLxFS	0.0001	0.0001	0.0001

Data are LSM±SE. **n = 5; †**BG** = barley grain; **WG** = wheat grain; **CG** = corn grain; **SFM** = sunflower seed meal; **CSM** = cottonseed meal; **SBM** = soybean meal*

gas production started to increase. Decrease in pH with SBM was more notable than CSM and SFM *(Table 2)*. High cellulose content of CSM and SFM might lead to slower decrease in pH. It was shown that freeze drying rumen liquor caused reduction in gas production, by 6-12% in concentrate feeds, 11-30% in hays, and 23-49% in straws, suggesting that freezing affects viability and/or activity of cellulolytic microorganisms [4].

Lower NH_3-N concentration in thawed rumen liquor than fresh rumen liquor is in agreement with a study by Luchini et al.[30]. Protozoa plays a role in protein degradation, as well; highly soluble protein fractions are degraded by bacteria, whereas poorly soluble protein fractions are degraded by protozoa [31]. Both decreased protozoa viability by 30% in thawed liquor and characteristics of CSM could result in its inefficient degradation.

In addition to survival and activity, freezing rumen liquor may alter bacterial cell wall, such as lipid composition [32] and porosity [13], which limits bacterial attachment, particularly to cell wall rich feedstuffs, such as CSM and SFM. This may lead to lower acetate and total VFA production *(Table 3)*. Responses of the propionate and acetate fractions [33] and the Ac:Pr ratio [30] to fermentation of starch- and protein-rich feedstuffs *(Table 3)* are in agreement with literature.

During incubation, degraded feed fraction is converted to gas, VFA, or incorporated into microbial mass [9]. Chaudhry and Mohamed [34] reported that CP and DM degradability of rapeseed meal and grass nuts were lower in thawed rumen liquor than in fresh rumen liquor. However, their degradations were highly correlated (r = 0.97), as in the

present experiment *(Fig. 1C)*. Degradation constants and effective degradabilities of feeds differed by the rumen liquor form.

Cryopreservation caused 3% reduction in gas production. In thawed rumen liquor the acetate proportion decreased by 4-fold compared to the propionate proportion. Relatively less reduced propionic acid could be due to low gas production from starch rich grains [35]. Moreover, gas loss occurs by 6-12% in *in vitro* experimentation employing thawed rumen liquor [4], which accounts for lower estimation of gas production. Rumen bacteria obtain their energy from fermentation of carbohydrate and protein. Energy from protein alone is not sufficient for bacterial growth, requiring readily available carbohydrates [36]. Indeed, the positive correlation between CGP and NFE content (r = 0.86, P<0.0001) in the present study and elsewhere [1] (r = 0.89) ascertains that bacterial growth is related to starch fermentation. Incubating starch-rich grains with thawed rumen liquor resulted in gas production at time 12 h was similar to those incubated with fresh rumen liquor, suggesting importance of starch content for bacterial activation. Pectin content of SBM stimulates growth of cellulolytic bacteria and their activities in early stage of *in vitro* incubation [37]. This could explain greater gas production from SBM than CSM and SFM in both fresh and frozen rumen liquor. Provision of soluble carbohydrates could increase efficiency of frozen rumen liquor. Moreover, even in fresh rumen liquor, gas production was shown to be negatively correlated with aNDF (r = -0.83) and ADF (r = -0.91) contents [24]. Similar relationship was determined in frozen rumen liquor (r = -0.84 for aNDF and r = -0.91 for ADF), suggesting that freezing adversely affect cellulolytic bacteria as reflected by 11-30% reduction in gas production [4].

In vitro incubation with thawed rumen liquor did not affect gas production from fraction "b", but reduced gas production per hr of incubation, fraction "c" and EGP. Hervas et al.[38] also reported these reductions in rumen liquors kept in ice for 24 hrs and those frozen in deep freezer. These were more notable in CSM and SFM, which had low fermentability and did not fit the model indicated by McDonald [39].

In this experiment, rumen liquor was subjected to condensation (centrifugation), 2-step controlled freezing, and keeping in liquid N. Freezing rumen liquor was associated with 2% underestimation of ME and NE_L values. Such a small percentage of underestimation could be due to low variability in CGP, a regression model component [40]. In a previous experiment, it was reported that ME level was 2820, 2892, and 2605 kcal/kg DM for WG, BG, and CG, respectively when IVGPT with fresh rumen liquor was employed [5]. ME values estimated from using fresh rumen liquor were lower than those reported by Getachew et al.[26], but similar to those reported by Seven et al.[41]. As compared with NRC [12], ME values were slightly lower in starch-rich grains, and markedly lower in protein-rich feedstuffs in the present experiment. Rumen liquor by feedstuff interaction revealed increases in estimate ME values for starch-rich grains and decreases in estimate ME values of protein-rich feedstuffs in thawed rumen liquor as compared to fresh rumen liquor.

In vitro OM digestibility data from fresh rumen liquors (75.1-79.6%) were lower than those reported by (78.5-87.3%) Umucalılar et al.[5], and similar to those reported by (78.2-81.3%) Şeker [2]. *In vitro* OM digestibility values had low variability in both rumen liquor forms. Limitations occurred for VFA formation and gas production in thawed rumen liquor appear to be valid for IVOMD data, as well.

Menke and Steingass [4] evaluated 700 feed samples using IVGPT and reported strong relationship between nutrient content and gas production. It was also shown that *in vivo* energy content and OMD were correlated with *in vitro* gas production measured using fresh rumen liquor [42]. However, many researchers [1,2] suggested that *in vitro* ME values were lower than their *in vivo* ME values, even in highly digestible feeds.

This experiment questioned feasibility of frozen rumen liquor usage in IVGPT through evaluating fermentation of commonly used concentrate feeds in ruminant nutrition. Despite achieving considerable viable protozoa count, usage of thawed rumen liquor increased pH and decreased NH_3-N concentration, the Ac:Pr ratio and total VFA concentration, gas production, and gas production rate as compared to usage of fresh rumen liquor. Moreover, ME and OMD values of feedstuffs were estimated to be lower in media containing thawed rumen liquor than in media containing fresh rumen liquor. However, CGP obtained using thawed rumen liquor was a good predictor of energy estimates obtained using fresh rumen liquor and digestibility. Also, response variables to fresh rumen liquor were highly correlated with response variables to thawed rumen liquor. The adverse effects of thawed rumen liquor usage were more notable on protein-rich feedstuff than starch-rich feedstuffs, particularly CSM. These may confirm detrimental effect of freezing on cellulolytic micro-organisms. It can be concluded that usage of frozen rumen liquor in IVGPT can be feasible if cryopreservation techniques are advanced to assure no change in microbial survival and activity.

REFERENCES

1. Getachew G, De Peters EJ, Robinson PH, Fadel JG: Use of an *in vitro* rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Anim Feed Sci Technol*, 123-124, 547-559, 2005. DOI: 10.1016/j.anifeedsci.2005.04.034

2. Şeker E: The determination of the energy values of some ruminant feeds by using digestibility trial and gas test. *Rev Med Vet*, 153, 323-328, 2002.

3. Umucalılar HD, Şeker E: Effects of sodium bicarbonate and magnesium oxide as buffers on *in vitro* digestibility of grains. *Vet Bilim Derg*, 16, 129-

135, 2000.

4. Menke KH, Steingass H: Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim Res Dev*, 28, 7-55, 1988.

5. Umucalılar HD, Coşkun B, Gülşen N: In situ rumen degradation and *in vitro* gas production of some selected grains from Turkey. *J Anim Physiol Anim Nutr,* 86, 288-297, 2002. DOI: 10.1046/j.1439-0396.2002.00381.x

6. Pell AN, Schofield P: Computerized monitoring of gas production to measure forage digestion *in vitro*. *J Dairy Sci*, 76, 1063-1073, 1993. DOI: 10.3168/jds.S0022-0302(93)77435-4

7. Makkar HPS: *In vitro* gas methods for evaluation of feeds containing phytochemicals. *Anim Feed Sci Technol*, 123-124, 291-302, 2005. DOI: 10.1016/j.anifeedsci.2005.06.003

8. Soliva CR, Hess HD: Measuring methane emission of ruminants by *in vitro* and in vivo techniques. **In,** Makkar HPS, Vercoe PE (Eds): Measuring Methane Production from Ruminants. 15-31, Springer Publishers, Netherlands, 2007.

9. Rymer C, Huntington JA, Williams BA, Givens DI: *İn vitro* cumulative gas production techniques: History, methodological considerations and challenges. *Anim Feed Sci Technol*, 123-124, 9-30, 2005. DOI: 10.1016/j.anifeedsci.2005.04.055

10. Thammavongs B, Poncet JM, Desmasures N, Gueguen M, Panoff JM: Resin straw as an alternative system to securely store frozen microorganisms. *J Microbiol Meth*, 57, 181- 186, 2004. DOI: 10.1016/j.mimet.2004.01.003

11. De La Fuente G, Perez-Quintana M, Cebrian JA, Fondevila M: Preliminary study on the effect of exposure to low temperature on the viability of both mixed and monocultures of rumen protozoa. *Acta Protozool*, 45, 225-230, 2006.

12. National Research Council (NRC): Nutrient Requirements of Dairy Cattle. Seventh Revised Edn, National Academy Press, Washington DC, 2001.

13. Hsu JT, Fahey GC: Effects of centrifugation speed and freezing on composition of ruminal bacterial samples collected from defaunated sheep. *J Dairy Sci*, 73, 149-152, 1990. DOI: 10.3168/jds.S0022-0302(90)78658-4

14. Nsabimina E, Kisidayova S, Macheboeuf D, Newbold CJ, Jouany JP: Two-Step freezing procedure for cryopreservation of rumen ciliates, an effective tool for creation of a frozen rumen protozoa bank. *APPl Environ Microbiol*, 69, 3826-3832, 2003. DOI: 10.1128/AEM.69.7.3826-3832.2003

15. Cone JW, Van Gelder AH: Influence of protein fermentation on gas production profiles. *Anim Feed Sci Technol*, 76, 251-264, 1999. DOI: 10.1016/S0377-8401(98)00222-3

16. Ørskov ER, Mc Donalds I: The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J Agr Sci Cambridge,* 92, 499-503, 1979. DOI: 10.1017/S0021859600063048

17. Steingass H: Bestimmung des energetischen futterwertes von wirtschaftseigenen futtermitteln aus der gasbildung bei der pansenfermentation *in vitro*. Dissertation, Univ. Hohenheim, 1983.

18. Öğretmen T: Geviş getirenlerin beslenmesinde kullanılan önemli bazı yemlerin NEL içeriklerinin *in vivo* ve *in vitro* yöntemleri ile saptanması. *PhD Dissertation,* Ege Üniversitesi, Turkey, 1991.

19. AOAC: Official Methods of Analysis, Vol. I, 15th edn., Association of Official Analytical Chemist. Arlington, VA, USA, 1990.

20. Goering HK, Van Soest PJ: Forage Fiber Analysis (Apparatus, Reagents and some Applications). US Dep. Agric. Handb. 379, 1970.

21. Miyake Y, Karanis P, Uga S: Cryopreservation of protozoan parasites. *Cryobiol*, 48, 1-7, 2004. DOI: 10.1016/j.cryobiol.2003.10.004

22. Dehority BA: Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *APPl Environ Microbiol*, 48, 182-185, 1984.

23. Weatherburn MW: Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem*, 39, 971-974, 1967. DOI: 10.1021/ac60252a045

24. Supelco: Analyzing fatty acids by packed column gas chromatography. Sigma-Aldrich Corp., Bulletin 856, Bellefonte, PA, 1998.

25. SAS Institute: *SAS* User's Guide. Statistics, Version 9th. Statistical Analysis System. SAS Inst., Inc., Cary, NC, 2002.

26. Getachew G, Robinson PH, De Peters EJ, Taylor SJ: Relationships between chemical composition, dry matter degradation and *in vitro* gas production of several ruminant feeds. *Anim Feed Sci Technol*, 111, 57-71, 2004. DOI: 10.1016/S0377-8401(03)00217-7

27. Brockbank KGM, Covault JC, Taylor MJ: Cryopreservation Guide. Bro-Lecs Cryoguide-0707, Thermo Fisher Scientific Inc. USA, 2007.

28. Malik KA: Cryopreservation of bacteria with special reference to anaerobes. World Federation of Culture Collection Technical Information Sheet No: 4, Germany, 1989.

29. Luchini ND, Broderick GA, Combs DK: Preservation of ruminal microorganisms for *in vitro* determination of ruminal protein degradation. *J Anim Sci*, 74, 1134-1143, 1996. DOI: 10.2527/1996.7451134x

30. Luchini ND, Broderick GA, Combs DK: *In vitro* determination of ruminal protein degradation using freeze-stored ruminal microorganisms. *J Anim Sci*, 74, 2488-2499, 1996. DOI: 10.2527/1996.74102488x

31. Jouany JP: Effect of rumen protozoa on nitrogen utilization by ruminants. *In* Conference: Altering Ruminal Nitrogen Metabolism to Improve Protein Utilization. 1335S-1346S, 1996.

32. Beal C, Fonseca F, Corrieu G: Resistance to freezing and frozen storage of *Streptococcus thermophilus* is related to membrane fatty acid composition. *J Dairy Sci*, 84, 2347-2356, 2001. DOI: 10.3168/jds.S0022-0302(01)74683-8

33. Prates A, De Oliveira JA, Abecia L, Fondevila M: Effects of preservation procedures of rumen inoculum on *in vitro* microbial diversity and fermentation. *Anim Feed Sci Technol*, 155, 186-193, 2010. DOI: 10.1016/j.anifeedsci. 2009.12.005

34. Chaudhry AS, Mohamed RAI: Fresh or frozen rumen contents from slaughtered cattle to estimate *in vitro* degradation of two contrasting feeds. *Czech J Anim Sci*, 57, 265-273, 2012.

35. Getachew G, Blummel M, Makkar HPS, Becker K: *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: A review. *Anim Feed Sci Technol*, 72, 261-281, 1998. DOI: 10.1016/S0377-8401(97)00189-2

36. Karslı MA, Russell JR: Effects of source and concentrations of nitrogen and carbohydrate on ruminal microbial protein synthesis. *Turk J Vet Anim Sci*, 26, 201-207, 2002.

37. Leedle JAZ, Hespell RB: Brief incubations of mixed ruminal bacteria: Effects of anaerobiosis and sources of nitrogen and carbon. *J Dairy Sci*, 66, 1003-1014, 1983. DOI: 10.3168/jds.S0022-0302(83)81895-5

38. Hervas G, Frutos P, Giraldez FJ, Mora MJ, Fernandez B, Mantecon AR: Effect of preservation on fermentative activity of rumen fluid inoculum for *in vitro* gas production techniques. *Anim Feed Sci Technol*, 123-124, 107-118, 2005. DOI: 10.1016/j.anifeedsci.2005.05.004

39. McDonald P: A revised model for the estimation of protein degradability in the rumen. *J Agr Sci Cambridge,* 96, 251-252, 1981. DOI: 10.1017/S0021859600032081

40. Mauricio RM, Mould FL, Dhanoa MS, Owen E, Channa KS, Thedorou MK: A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. *Anim Feed Sci Technol*, 79, 321-330, 1999. DOI: 10.1016/S0377-8401(99)00033-4

41. Seven PT, Çerçi İH, Azman MA: The different between methods and determining of metabolisable energy levels with enzyme and gas techniques in concentrate feeds. *FU Vet J Health Sci,* 21, 159-161, 2007.

42. Getachew G, Crovetteo GM, Fondevila M, Krishnamoorthy U, Singh B, Spangero M, Steingass H, Robinson PH, Kailas MM: Laboratory variation of 24 h *in vitro* gas production and estimated metabolizable energy values of ruminant feeds. *Anim Feed Sci Technol*, 102, 169-180, 2002. DOI: 10.1016/S0377-8401(02)00212-2

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Research Article

A Comparative Study on Detection of *Bartonella henselae* Infection by Culture Followed by PCR, Nested-PCR and IFA [1]

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Abstract

Cats are the main reservoirs of zoonotic *Bartonella henselae* which are the causative agents of Cat Scratch Disease (CSD). The aim of this study is to compare three diagnostic methods including culture followed by PCR from whole blood, nested-PCR from oral swab and whole blood, and IFA from serum samples. The diagnosis of *B. henselae* was compared with the bacteriological methods following conventional PCR and by two separate nested PCR from blood, and oral cavity swabs which were collected from 81 pet and stray cats in Istanbul, Turkey. Also the seroprevalence was determined by indirect fluorescent antibody (IFA) technique in the same animals. *Bartonella* spp. was determined in 26 (32%) of the blood samples by culture. Twenty of them were identified as *B. henselae* and 6 of them were *B. clarridgeiae* by following conventional PCR assay. Of 81 whole blood samples subjected to PCR, 29 (36%) were positive in the nested reaction. Of these, 20 were identified as *B.henselae* and 8 were *B. clarridgeiae.* However, one of the samples was found to be positive for both *B. henselae* and *B. clarridgeiae* DNA by the nested reaction. Of 81oral swab samples subjected to PCR, 25 (31%) were positive in the nested reaction. Of these, 19 were identified as *B. henselae* and 6 were *B. clarridgeiae. B.henselae* IgG antibody seroprevalence was detected as 67% (54/81). Using the combination of blood and oral samples by Nested-PCR simultaneously may increase the sensitivity of the test. Also, the combination of the blood culture with nested- PCR and serology is likely to give the most definitive information in the diagnosis of bartonellosis in cats.

Keywords: *Bartonella, Culture, Nested-PCR, IFA, Oral swab*

Bartonella henselae Infeksiyonunun Saptanmasında Kültür Sonrası PCR, Nested-PCR ve IFA Yöntemlerinin Karşılaştırılması

Özet

Kediler kedi tırmalama hastalığının etkeni olan zoonoz *Bartonella henselae* bakterisinin ana rezervuarıdır. Bu çalışmanın amacı tam kandan kültür sonrası PCR, tam kan ve oral svaptan nested-PCR ve serum örneklerinden IFA yöntemlerini içeren 3 farklı teşhis yöntemini karşılaştırmaktır. *B. henselae* tanısı için İstanbul, Türkiye'de yaşayan 81 ev ve sokak kedilerinden toplanan kan örneklerinden bakteriyolojik kültürü takiben yapılan konvansiyonel PCR, tam kan örnekleri ve ağız boşluğundan alınan svapların 2 farklı nested PCR'I ile karşılaştırıldı. Ayrıca aynı hayvanlarda indirect floresan antikor (IFA) tekniği ile seroprevalance belirlendi. Kültür sonucunda 26 (32%) kan örneğinde *Bartonella* spp saptandı. Konvansiyonel PCR testleri sonucunda bunların 20 adeti *B. henselae,* altı adeti *B. clarridgeiae* olarak identifiye edildi. Nested PCR sonucu, 81 tam kan örneğinin 29'u (36%) pozitifti. Bunların 20 adeti *B. henselae,* sekiz adeti *B. clarridgeiae* olarak identifiye edildi. Aynı zamanda, 1 örnekte hem *B. henselae* hem de *B. clarridgeiae* identifiye edildi. PCR'ı yapılan 81 oral svabın 25'i (31%) nested PCR ile pozitif bulundu. Bunların 19 adeti *B. henselae*, altı adeti *B. clarridgeiae* olarak identifiye edildi. *B.henselae*'ya karşı oluşmuş IgG antikorlarının varlığı incelenen serum örneklerinde seroprevalans %67 (54/81) olarak belirlendi. Sonuç olarak kan ve oral svap örneklerinden Nested-PCR kombinasyonunun kullanımı testlerin sensitivitesini arttırabilmektedir. Ayrıca kan kültürünün nested-PCR ve seroloji ile kombinasyonu kedilerde bartonellosis tanısında muhtemelen en kesin bilgiyi vermektedir.

Anahtar sözcükler: *Bartonella, Kültür, Nested-PCR, IFA, Oral svap*

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INTRODUCTION

Cat scratch disease (CSD) which can be found in many species including humans is a worldwide zoonosis. Agent of the disease, *Bartonella henselae* which is classified in the family Bartonellaceae, is an intraerythrocytic, slightly curved Gram negative bacteria [1]. The confirmatory diagnosis in cats cannot be determined based on clinical signs. Infected cats are usually asymptomatic, but can still present recurrent bacteraemia, which may last from months to years. Currently, the laboratory diagnosis of bartonellosis in cats is based on direct methods (bacterial isolation and PCR) and indirect methods (Serological tests: IFA, ELISA, Western Immunobloot) [2]. Because of their fastidious nature, standard biochemical methods are not convenient for identification [3] and cannot be used in differentiation of the species in the genus, therefore molecular methods are commonly used for this purpose [4]. The aim of this study was to compare three diagnostic methods including culture followed by PCR from whole blood, nested-PCR from oral swab and whole blood, and IFA from serum samples.

MATERIAL and METHODS

Sample Collection

The samples objected in the study were collected from 81 cats which stay or visit private clinics and streets of Istanbul, Turkey. Five of the cats have lesions on their gingiva while 76 of have no lesions.

Blood samples (3 mL) collected by aseptic procedure from the jugular vein of the cats, were placed in serum separator tubes and tubes with EDTA. In the laboratory, the blood with EDTA was divided into two parts. One part was used for blood culture immediately; the other part was stored at -80°C for nested PCR. The sera were stored at -20°C until analysed.

Dry cotton swab was rolled over the gums or oral lesions if exists, and swab specimens were collected into specimen transport media. They were stored in cooling boxes for transport to the laboratory. They were stored at -20°C until tested.

The present study was approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine, Approval no: 2012/182.

Bacterial Isolation

The samples in tubes with EDTA kept at -80°C were thawed at room temperature, vortexed and then centrifuged at 3800 rpm for 70 min. Upon pouring away the supernatant, the pellet was suspended in 125 µL Medium 199 Broth and finally mixed by vortex. 250 µL from the acquired suspensions were inoculated onto Heart Infusion Agar (HIA) supplemented with 5% defibrinated rabbit blood, and the suspension in the medium were diffused on the surface of the medium using their own viscosity. The plates were incubated at 35°C with 5% CO_2 in an incubator for 5 weeks. The presumptive identification of suspected gram negative bacteria was performed by conventional biochemical methods as a genus level [5].

From the isolates, DNAs were extracted by using DNeasy Tissue Kit (Qiagen, Valencia,CA, USA) according to the manufacturer's instructions. The PCR assay was performed as described by Jensen et al.[6]. PCR products were separated on a 3% agarose gel, stained with ethidium bromide and visualized under UV light. The fragments of 172 and 145 bp were evaluated as positive for *B. henselae* and *B. clarridgeiae*, respectively.

Nested-PCR Assay from Whole Blood and Oral Swab Samples

DNAs were extracted from whole blood by using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The oral swabs were immerged for 15 min in 400 mL of PBS and vortexed. Then, DNA was extracted by using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Primary reactions were performed in a 25 µL of volume as follows: 5 µL extracted DNA, 0.2 mM each dNTP, 0.5 mM each primer (P-bhenfa and P-henr1), 3 mM $MgCl_2$, 10X PCR buffer (50 mM KCL, 10 mM Tris-HCL pH 8.8), 0.5 U Taq DNA polymerase. DNA amplification was obtained with pre denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 48.2°C for 30 s and synthesis at 72°C for 30 s. A final extension step at 72°C for 5 min was included at the end of the cycles. The PCR mixtures were cooled at 4°C until using for nested reactions.

Nested reactions were performed in a 25 µL of volume as follows: 1 µL primary amplicon, 0.2 mM each dNTP, 0.5 mM each primer (N-bhenf1a and N-henr), 1.5 mM $MgCl_2$, 10X PCR buffer (50 mM KCL, 10 mM Tris-HCL pH 8.8), 0.5 U Taq DNA polymerase. DNA amplification was obtained with predenaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s and synthesis at 72°C for 30 s. A final extension step at 72°C for 5 min was included at the end of the cycles.

Positive controls, consisting of purified *B. henselae* and *B. clarridgeiae* DNAs, and a negative control (water blank) were included with each run. PCR amplification products were separated on 3% agarose gel, stained with ethidium bromide and visualized under UV light. Bands of 152 and 134 bp were evaluated as positive for *B.henselae* and *B.clarridgeiae*, respectively [7].

IFA

The presence of IgG antibodies against to *B. henselae*

was measured by indirect fluorescence assay (IFA) using commercial slides coated with *B. henselae* (Fuller Laboratories, California, USA). IFA was performed according to the instructions of the manufacturer. For detection of antibodies against to *B. henselae*, the serum samples were initially screened at 1: 64 dilutions in phosphate-buffered saline with goat anti-cat IgG marked with fluorescein isothiocyanate conjugate (Fuller Laboratories, California, USA). Positive and negative controls were run in each test. The intensity of the fluorescence was scored subjectively from 1 to 4, and a fluorescence score of 2 at a dilution of 1:64 was considered to be positive.

Statistical Analyses

The sensitivity, specificity, positive and negative predictive values and diagnostic values for IFA and nested PCR assays were determined for diagnostic effectiveness compared to the culture followed by PCR considered as a gold standard [8].

RESULTS

Culture

Bartonella spp. was determined in 26 (32%) of the blood samples by culture. Twenty of them were identified as *B. henselae* and 6 of them were *B. clarridgeiae* by following conventional PCR assay.

Nested-PCR from Whole Blood

Of 81 whole blood samples subjected to PCR, 29 (36%) were positive in the nested reaction. Of these, 20 were identified as *B. henselae* and 8 were *B. clarridgeiae*. However, one of the samples was found positive for both *B. henselae* and *B. clarridgeiae* DNA by the nested reaction.

Nested-PCR from Oral Swabs

Of 81oral swab samples subjected to PCR, 25 (31%) were positive in the nested reaction. Of these, 19 were identified as *B. henselae* and 6 were *B. clarridgeiae*.

IFA

B. henselae IgG antibody seroprevalence was detected as 67% (54/81).

Number of the positive cats are comparatively summarised according to sampling and test methods on *Table 1*.

Results of the cats with oral lesions are summarized on *Table 2*.

Statistical Analyses

In this study, the specificity and sensitivity of the nested-PCR assay from blood (81.8% and 73.1%, respectively) and oral swab samples (87.3% and 69.2%, respectively) were found relatively high. On the contrary, the sensitivity of the IFA test (80.8%) was found the highest of all when the specificity (40%) was the lowest.

The results are showed on *Table 3*.

Table 1. *Number of the positive cats according to sampling and test methods*

Culture	Nested-PCR from Whole Blood	Nested-PCR from oral samples	IFA	Number of Positives
+	-	-	-	1
-	+	-	-	1
-	-	+	-	0
-	-	-	+	27
+	+	-	-	2
+	-	+	-	1
+	-	-	+	2
-	+	+	-	4
-	+	-	+	3
-	-	+	+	1
+	+	+	-	1
+	+	-	+	3
+	-	+	+	3
-	+	+	+	2
+	+	+	+	13

+ : Bartonella spp positive, - : Bartonella spp negative

Table 2. *Results of the cats with oral lesions according to sampling and test methods*

No of the Cats with Gingivitis	Investigated Methods			
	Culture	Nested-PCR from Whole Blood	Nested-PCR from Oral Samples	IFA
1	-	+	_	+
2	-	-	-	+
3	+	-	-	+
4	+	+	+	-
5	-	-	-	+

+ : Bartonella spp positive, - : Bartonella spp negative

Table 3. *Diagnostic effectiveness for nested-PCR assays and IFA*

Diagnostic Effectiveness	Nested-PCR from Whole Blood	Nested-PCR from Oral Samples	IFA
Sensitivity	73.1%	69.2%	80.8%
Spesifity	81.8%	87.3%	40%
Positive predictivity	65.5%	72%	38.9%
Negative pedictivity	86.5%	85.7%	81.5%
Diagnostic value	79%	81.5%	53.1%

DISCUSSION

Bartonella organisms need special growth medium and they grow very slowly. Some researchers recommended that isolation of the bacterium was the gold standard and they indicated that the most successful method to detect *Bartonella* species from cat blood was culture and characterization of the isolate by PCR [4,9]. But, because of the high prevalence of infection in healthy cats in endemic areas, Pennisi et al.[10] determined that the positive culture was not corroboratory and other compatible diagnoses must be ruled out. Jensen et al.[6] presented a single-step PCR which was suited for the detection of *B. henselae* and *B. clarridgeiae* from culture and blood. However, Rampersad et al.[7] and Engvall et al.[11] reported that this method has questionable sensitivity and show less sensitivity than culture for the detection of *B. henselae* and *B. clarridgeiae* in blood. Therefore, Rampersad et al.[7] recommended enhancement methods such as a nested-PCR from blood.

Nasoiu et al.[2] indicated that sequencing and analysis of bacterial DNA by PCR was a sensitive test to amplify *Bartonella* spp. However, they pointed that because of the bacteria circulates only intermittently, PCR was not offered many advantages over culture. Bai et al.[12] explained the overall low success of culture with the observation of low concentrations of *Bartonella* bacteria in cat blood and they pointed that molecular approach does not provide evidence of viable bacteria in animal samples. In this study, the results of the nested-PCR from blood were 24.6% for *B. henselae* while 11.1% for *B. clarridgeiae*, and the results of the blood culture were 24.6% for *B. henselae* while 7.4% for *B. clarridgeiae*. Our detection rates by Nested-PCR and culture were quite similar. It has been considered that the detection limit of the bacteria in bacteriological and molecular methods might be varied. *Bartonella* species are very fastidious and the culture processes take longer time. In some samples, these bacteria might not survive and could not be cultured; despite of that, the DNA of the bacterium might be detected by the molecular methods. Therefore, nested-PCR from blood may be able to an opportunity for diagnosis of bacteraemia.

Pennisi et al.[13] indicated that the oral swab was an easier procedure than taking blood and testing both blood and oral samples may easily enhance the sensitivity of PCR testing, although their positive results of the nested-PCR from blood more than the rate of the nested-PCR from oral swabs. Furthermore, Kim et al.[14] reported that the results of nested-PCR from saliva (44.1%) were more than the results of nested-PCR from blood (41.8%). In our study, the results of nested-PCR from blood (36%) were higher than the results of nested-PCR from oral swabs (31%). These results have supported the results of Pennisi et al.[13]. Despite the sensitivity of nested-PCR from oral swabs was relatively high, positive results might not show the current infection. In the current study, there have been some cases that positive nested-PCR from oral swabs without positive results for culture and nested-PCR-from blood. It was been thought that, these might be developed due to ingestion of *Bartonella* bacteria in flea dirt when the cats acting grooming behaviour and the infection may be absent. Also, because of the bacteraemia can be intermittent [2], nested-PCR from blood can give negative results.

Quimby et al.[15] described for the first time for presence of microbial DNA from oral swabs collected from cats with and without gingivostomatitis (GS) and they reported that of the five *Bartonella* positive samples, only one was from a cat with GS. Namekata et al.[16] indicated that cats with oral lesions (bacteremic or not) had more frequently PCR positive oral swabs than cats without oral lesions. In our study, only one sample of the five cats with oral lesion was PCR positive. On the other hand, four (two of were culture positive while two of were blood PCR positive) of the five cats with oral lesion had bacteraemia while four cats were seropositive.

The positive results on the serological tests in cats only document exposure to infection [13]. Therefore, the serological test results are not be used to determine the *Bartonella* spp. infection status of individual cats [17]. Park et al.[18] emphasized that serology has only a retrospective value, and the cats might have been infected during some period of their life. Pennisi et al.[10] determined that IFAT is more useful for exclusion than for confirmation of the infection because of the low positive predictive value compared with the good negative predictive value. In our study, the diagnostic value of the IFA test was found that the positive predictive value was only 38.9% and the negative predictive value was 81.5%. These results were in parallel with the results of the authors indicated above.

Serologic tests results do not strictly correlate to PCR analysis and culture. Fabbi et al.[9] indicated the lack of the association between seropositivity and the level of bacteraemia. Lappin and Hawley [19] emphasized that *Bartonella* species serum antibody test results cannot be used to accurately predict bacteraemia in cats as some cats with *Bartonella* species DNA in blood were seronegative and some cats with *Bartonella* species IgG in serum were negative for *Bartonella* species DNA in blood. Beside these, Nasoiu et al.[2] indicated that compared to the bacterial isolation, which lasts between 4 and 6 weeks, the serological tests have the advantage that they are easier to use, and have duration of 1-2 days, while being economic. In this study, 28 cats with serum IgG to *B. henselae* were negative for *B. henselae* DNA in blood while 6 cats with *B.henselae* DNA in blood were seronegative. These results have supported the results of Lappin and Hawley [19] and Nasoiu et al.[2].

It is clear that the clinicians should focus on cats which are reservoir of the CSD and on preventing this zoonotic disease. Using the combination of blood and oral

samples by Nested-PCR simultaneously may be increased the sensitivity of the test. Also, the combination of the blood culture with nested- PCR and serology is likely to give the most definitive information in the diagnosis of bartonellosis in cats.

REFERENCES

1. Kordick DL, Breitschwerdt EB: Intraerythrocytic presence of *Bartonella henselae*. *J Clin Microbiol*, 33, 1655-1656, 1995.

2. Năsoiu L, Mitrea I, Ioniță M: Advanced diagnostic methods as tools to investigate the exposure to bartonella infections in cats. *Proc Rom Acad (Series B, Suppl. 1)*: 145-150, 2015.

3. Sander A, Buhler C, Pelz K, von Cramm E, Bredt W: Detection and identification of two *Bartonella henselae* variants in domestic cats in Germany. *J Clin Microbiol*, 35, 584-587, 1997.

4. Zanutto MdS, Mamizuka EM, Raiz-Junior R, Diogo CL, Okay TS, Hagiwara MK: Experimental infection and horizontal transmission of *Bartonella henselae* in domestic cats. *Rev Inst Med Trop São Paulo,* 43, 257-261, 2001. DOI: 10.1590/S0036-46652001000500004

5. Koehler JE, Quinn FD, Berger TG, LeBoit PE, Tappero JW: Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. *N Engl J Med*, 327, 1625-1631, 1992. DOI: 10.1056/NEJM199212033272303

6. Jensen WA, Fall MZ, Rooney J, Kordick DL, Breitschwerdt EB: Rapid identification and differentiation of *Bartonella* species using a single-step PCR assay. *J Clin Microbiol*, 38, 1717-1722, 2000.

7. Rampersad JN, Watkins JD, Samlal MS, Deonanan R, Ramsubeik S, Ammons DR: A nested-PCR with an internal amplification control for the detection and differentiation of *Bartonella henselae* and B. clarridgeiae: an examination of cats in Trinidad. *BMC Infect Dis*, 5, 63, 2005. DOI: 10.1186/1471-2334-5-63

8. Jekel JF, Katz DL, Elmore JG, Wild D: Epidemiology, biostatistics and preventive medicine. Elsevier Health Sciences, Philadelphia, 2007.

9. Fabbi M, De Giuli L, Tranquillo M, Bragoni R, Casiraghi M, Genchi C: Prevalence of *Bartonella henselae* in Italian stray cats: evaluation of serology to assess the risk of transmission of *Bartonella* to humans. *J Clin Microbiol*, 42, 264-268, 2004. DOI: 10.1128/JCM.42.1.264-268.2004

10. Pennisi MG, Marsilio F, Hartmann K, Lloret A, Addie D, Belak S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hosie MJ, Lutz H, Möstl K, Radford AD, Thiry E, Truyen U, Horzinek MC: *Bartonella* species infection in cats: ABCD guidelines on prevention and management. *J Feline Med Surg*, 15, 563-569, 2013. DOI: 10.1177/1098612X13489214

11. Engvall EO, Brandstrom B, Fermer C, Blomqvist G, Englund L: Prevalence of *Bartonella henselae* in young, healthy cats in Sweden. *Vet Rec*, 152, 366-369, 2003. DOI: 10.1136/vr.152.12.366

12. Bai Y, Rizzo MF, Alvarez D, Moran D, Peruski LF, Kosoy M: Coexistence of *Bartonella henselae* and B-clarridgeiae in populations of cats and their fleas in Guatemala. *J Vector Ecol*, 40, 327-332, 2015. DOI: 10.1111/jvec.12171

13. Pennisi MG, La Camera E, Giacobbe L, Orlandella BM, Lentini V, Zummo S, Fera MT: Molecular detection of *Bartonella henselae* and *Bartonella clarridgeiae* in clinical samples of pet cats from Southern Italy. *Res Vet Sci*, 88, 379-384, 2010. DOI: 10.1016/j.rvsc.2009.11.005

14. Kim YS, Seo KW, Lee JH, Choi EW, Lee HW, Hwang CY, Shin NS, Youn HJ, Youn HY: Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in cats and dogs in Korea. *J Vet Sci*, 10, 85-87, 2009. DOI: 10.4142/jvs.2009.10.1.85

15. Quimby JM, Elston T, Hawley J, Brewer M, Miller A, Lappin MR: Evaluation of the association of *Bartonella* species, feline herpesvirus 1, feline calicivirus, feline leukemia virus and feline immunodeficiency virus with chronic feline gingivostomatitis. *J Feline Med Surg*, 10, 66-72, 2008. DOI: 10.1016/j.jfms.2007.05.007

16. Namekata DY, Kasten RW, Boman DA, Straub MH, Siperstein-Cook L, Couvelaire K, Chomel BB: Oral shedding of Bartonella in cats: Correlation with bacteremia and seropositivity. *Vet Microbiol*, 146, 371-375, 2010. DOI: 10.1016/j.vetmic.2010.05.034

17. Ficociello J, Bradbury C, Morris A, Lappin MR: Detection of *Bartonella henselae* IgM in serum of experimentally infected and naturally exposed cats. *J Vet Intern Med*, 25, 1264-1269, 2011. DOI: 10.1111/j.1939-1676.2011.00820.x

18. Park HJ, Lee SE, Hong SH, Lee WJ, Seo KW, Song KH: Seroprevalence of *Toxoplasma gondii* and *Bartonella henselase* infection in stray cats of the Daejeon City, Korea. *Korean J Vet Res*, 54, 87-89, 2014. DOI: 10.14405/kjvr.2014.54.2.87

19. Lappin MR, Breitschwerdt E, Brewer M, Hawley J, Hegarty B, Radecki S: Prevalence of *Bartonella* species antibodies and *Bartonella* species DNA in the blood of cats with and without fever. *J Feline Med Surg*, 11, 141-148, 2009. DOI: 10.1016/j.jfms.2008.06.005

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Short Communication

Molecular Screening of LFS and PSSM-I Diseases in Arabian Horse Population in Turkey [1,2]

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Abstract

Polysaccharide Storage Myopathy (PSSM) and Lavender Foal Syndrome (LFS) are inherited diseases in horse. PSSM-I, a point mutation in *Glycogen Synthase-1* gen (*GYS1*) causes increased activation of the enzyme and abnormal polysaccharides formation. LFS caused by one nucleotide deletion in *Myosin-Va (MYO5A)* leads frame shift mutation and premature termination of the protein. In the study, a total of 239 blood samples, taken from Anadolu (n=70), Karacabey (n=70) and Sultansuyu (n=99) stud farms were screened. Diseases related mutations were not detected in the sampled Arabian horse population.

Keywords: *Arabian horses, LFS, PCR-RFLP, PSSM-I*

Türkiye Arap Atı Popülasyonunda LFS ve PSSM-I Hastalıklarının Moleküler Taraması

Özet

Atların "Polisakkarit Depolama Hastalığı" (Polisaccarite Storage Myopathy, PSSM) ve "Lavanta Renkli Tay Sendromu" (Lavander Foal Syndrome, LFS) aktarılan kalıtsal hastalıklardır. PSSM-I'de, *Glikojen Sentaz-1* (GYS1) geninde meydana gelen bir nokta mutasyonuna bağlı olarak enzim etkinliğinde artış şekillenmekte ve anormal polisakkaritler oluşmaktadır. Lavanta renkli tay hastalığında ise Miyozin-Va (*MYO5A*) geninde çerçeve kayması mutasyonuna neden olan bir bazlık delesyon sonucunda proteinde prematüre sonlanma şekillenmektedir. Sunulan araştırmada, Anadolu (n=70), Karacabey (n=70) ve Sultansuyu (n=99) Tarım İşletmeleri'nde yetiştirilen damızlık Arap atlarından temin edilmiş 239 adet kan örneği kullanılmıştır. İncelenen popülasyonda LFS ve PSSM hastalıklarıyla ilişkilendirilen mutasyonlara rastlanmamıştır.

Anahtar sözcükler: *Arap atı, LFS, PZR-RFLP, PSSM-I*

INTRODUCTION

Pleiotropic effects of pigmentation genes are well described in horses [1]. Melanocytes are the key cells in pigmentation. Melanocytes derived from the neural crest, which arise dorsal edge of the neural plate during embryogenesis. Neural crest derived melanocytes are found several parts of the body, such as skin, hair, eye layers and also several types of neurons and glia [2]. Hence, mutations in the genes that affect melanocyte development cause pleiotropy on neurologic functions, hearing, and sight. One of them causes Lavender Foal Syndrome (LFS) also known as Coat Color Dilution Lethal (CCDL) which only reported in Arabian horse foals [3-5] and is inherited in an autosomal recessive manner [3]. Brooks et al.[6] have determined the genetic mutation causing this syndrome by SNP-based whole genome association (WGA) study. According to this study, the mutation is a single base deletion of cytosine (c.4459delC) on *MYO5A* gene, located on ECA1 chromosome. This deletion causes frameshift resulting in a premature stop codon (p.Arg1487AlafsX13). Normally synthesized protein functions in organelle

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transport and membrane trafficking in brain and skin cells [7]. The protein also has a role in axonal and dendritic transport in neurons [8]. Thus newborn foals display diverse neurological abnormalities and have diluted coat colors like lavender, pewter and silver. Diluted coat color is the decisive symptom of LFS. LFS has no cure, and thus the foals with these conditions are euthanized after birth [6].

Abnormal polysaccharide and glycogen accumulation in striated muscles is called Polysaccharide Storage Myopathy (PSSM) and PSSM is distinct cause of Equine chronic exertional rhabdomyolysis. As a muscle disorder, rhabdomyolysis is disruption of striated muscles and causes of the disease could be associated with exercise (Exertional rhabdomyolysis) and unrelated to exercise [9]. Up to date there are two forms of PSSM in horses; PSSM-Type I (PSSM-I) and PSSM type II (PSSM-II). PSSM-I has a genetic base, which is a dominant mutation on *Glycogen Synthetize-1* (*GYS1)* gene. The mutation is a single base substitution (c.926G>A), causes an amino acid change (Arg309His). *GYS1* gene translates an enzyme called "glycogen synthase" [10]. The mutation increases the enzyme activity which results with production of amylase resistant polysaccharides. This type of PSSM is very common in several horses, especially in Quarter horses, Draft horses. PSSM-I is a rare genetic diseases for Arabian horses [10].

Restriction fragment length polymorphism method is based on whether a mutation forms specific enzyme cleavage site it has been widely used in diagnosis of mutations, polymorphisms which were previously associated with quantitative traits [11].

First aim of the presented study was to screen 30th exon of *MYO5A* gene and 6th exon of *GYS1* gene for the causative mutations for LFS and PSSM-I respectively by PCR-RFLP in the 239 Arabian horses, which bred in stud farms in Turkey. Second aim was to place the diagnosis of the diseases on routine laboratory work.

MATERIAL and METHODS

Animal Material

In the study, a total of 239 blood sample, taken from Anadolu (n=70), Karacabey (n=70) and Sultansuyu (n=99) stud farms of the Ministry of Agriculture was used for the further analyzes. The samples were collected in a previous study [12]. Also positive control samples carrying the mutations were sent by Dr. Brooks and Dr. McCue, for LSF and PSSM-I diseases respectively.

Total DNA Isolation

DNA extractions from samples were performed using the spin-column based commercial kits (DNeasy Blood& TissueKit, Qiagen GmbH, Hilden, Germany). DNAs were quantified by spectrophotometry at wavelengths of 260 nm and 280 nm. To determine degradation, Ethidium bromide (EtBr) stained 1% agarose gel electrophoresis were used.

PCR

PCR analysis for both regions was conducted in compliance with the method proposed by Brooks et al.[6] and McCue et al.[10]. The oligonucleotide sequences were shown in *Table 1*.

Electrophoresis of amplicons was conducted for 20 min at 120V in EtBr stained 2% agarose gel and visualized under UV light. The amplicons stored at -20°C until Restriction Fragment Length Polymorphism (RFLP) analysis.

RFLP Analysis

RFLP analysis is used to identify the mutations which cause differentiation in the enzyme cut sites. To detect the mutation causing LFS diseases, FauI (Smu) enzyme was used. This enzyme recognizes the patterns in the 5' strand "CCCGC(N)$_4$" and in the 3' strand "GGGCG(N)$_6$". The deletion mutation on the *MYO5A* gene causes a loss of enzyme cut region. To detect the mutation causing PSSM-I diseases, HpyCH4V enzyme was used. HpyCH4V recognizes the pattern TGCA, and cuts between G and C bases. The mutation on *GYS1* gene causes a gain of enzyme cut region. Thus the RFLP pattern in normal, heterozygote carrier and homozygous mutant individuals are different *(Table 2)*.

RFLP analysis was carried out with 2U of each enzyme on 10 µl PCR products. Incubations were performed as instructed (New England Biolabs Inc.). Electrophoresis of RFLP results were conducted 20 min in EtBr stained 2% agarose gel with 100V, then results were visualized with UV light (Gel Logic 2000, Kodak).

Sequence Analysis

Positive control samples (n=2) and randomly selected samples (n=10 for each gene), additionally 40 individuals presenting abnormal band pattern for RFLP analysis of *MYO5A* were sequenced for further analyses. PCR stage was repeated with the same conditions and same oligonucleotides were used for sequencing. Results were aligned to ENSECAG00000021742 and ENSECAG00000021428 for *MYO5A* and *GYS1* regions respectively by using BioEdit software [13].

Table 1. *Oligonucleotide sequences for GYS1 and MYO5A regions*

Oligonucleotide Name	Oligonucleotide Sequences 5'-3'	References
MYO5A_F	CAG GGC CTT TGA GAA CTT TG	[6]
MYO5A_R	CAG CCA TGA AAG ATG GGT TT	
GYS1_F	TGA AAC ATG GGA CCT TCT CC	[10]
GYS1_R	AGC TGT CCC CTC CCT TAG AC	

Table 2. *Expected patterns of MYO5A and GYS1 regions after restriction with FauI and HpyCH4V enzymes, respectively*

Enzyme	Homozygous Mutant	Homozygous Normal	Heterozygote Carrier	References
FauI	287bp, 482bp	96bp, 287bp, 396bp	96bp, 287bp, 396bp, 482bp	[6]
HpyCH4V	54bp, 80bp, 97bp	80bp, 152bp	54bp, 80bp, 97bp, 152bp	[10]

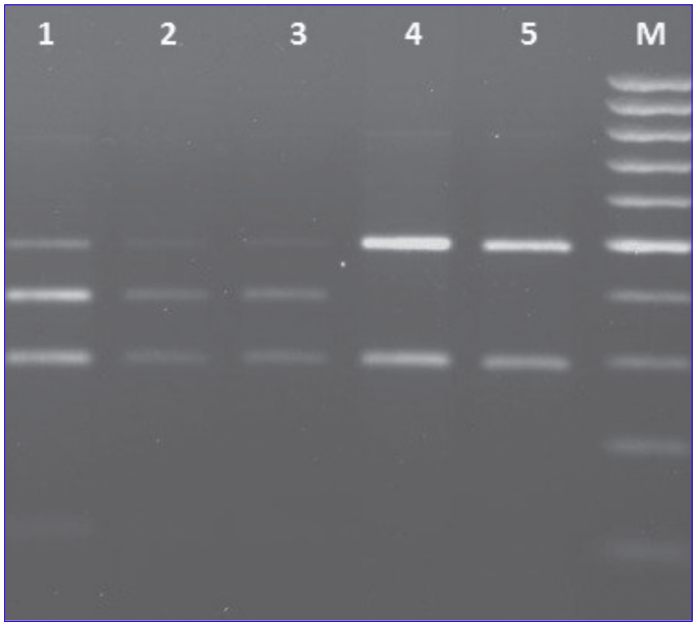
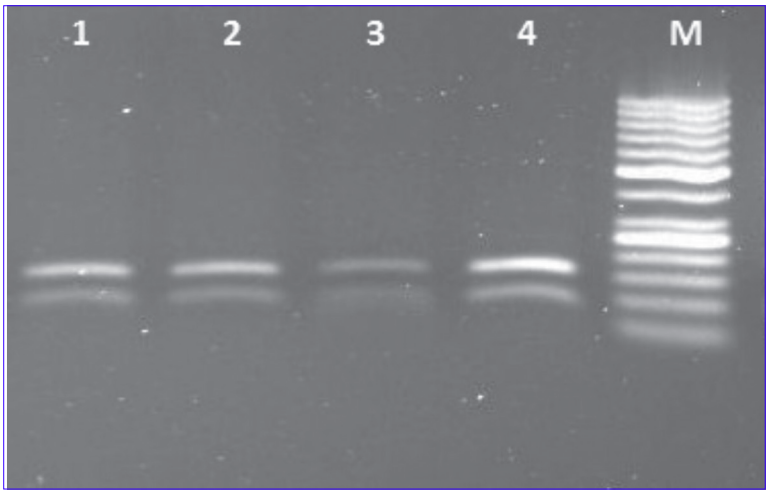


Fig 1. Observed band patterns of *MYO5A* region after restriction with FauI enzyme. Lane 1 heterozygous positive control sample. Lanes 2 and 3 are analysed samples from the present study. Lanes 4 and 5 are homozygous positive control samples. Lane M, molecular size marker (100 bp DNA ladder)

Fig 2. Observed band patterns of *GYS1* region after restriction with HpyCH4V enzyme. Lanes 1-3 are analysed samples from the present study. Lane 4 homozygous negative control samples. Lane M, molecular size marker (50 bp DNA ladder)



RESULTS

DNA concentrations results vary between 100-300 ng/µl. To optimize PCR protocol all DNA samples were diluted in to approximately 40 ng/µl concentration. By using PCR 769bp and 229 bp fragments were successfully amplified for *MYO5A* and *GYS1* genes, respectively. These amplicons were digested with FauI and HpyCH4v restriction enzymes to detect point mutations for each. Examples of gel images showing the digestion results of the amplified products cut by restriction enzyme were shown in *Fig. 1* and *Fig. 2*.

The sequences obtained from the samples showing abnormal band patterns were evaluated by comparison with the reference sequences and also mutation positive

controls samples for each gene regions. All of the analysed samples resulted with homozygous normal genotype.

DISCUSSION

LFS and PSSM-I diseases are genetic disorders. LFS is only observed in Arabian horses with the history of Egyptian originated or related Arabian horses [6]. The mutation causing LFS is recessive, thus heterozygous Arabian horses appear healthy. However when two carriers bred, the resulting offspring has 25% possibility of being affected with LFS. Brooks et al.[6] analyzed Egyptian Arabian Horses and the frequency of the carriers was found in 10.3% of the population consisting of 58 individuals. In the same study, another population, which does not have Egyptian origin, consisting 56 individuals the frequency of the disease determined to be 1.8% [6]. Another study conducted by Gabreski et al.[14] included the examination of 215 Arabian Horses, which has Egyptian blood line connection, the frequency of LFS found 1.62%. Tarr et al.[15] scanned Arabian horse population for severe combined immunodeficiency (SCID), LFS and cerebellar abiotrophy (CA) mutations, in 2004 and 2009. For LFS, the determined heterozygosity proportion was 13.3% (n=203) and 11.7% (n=197) in 2004 and 2009 respectively. In the presented study, we have examined 239 individuals from the Arabian horse population, the mutation causing LFS is not determined.

Abnormal activation of the enzymes in the glycogenolytic or glycolytic pathway cause different types of glycogen storage myopathies [9] and *GYS1* is one of them. *GYS1* mutation is determined in many horse populations including Belgian draught horses, Quarter Horses and Cob Normand horses [10]. In the study we analysed Arabian horses and only homozygous normal band patterns were observed for *GYS1*, 6th exon region as expected.

In this study we used PCR-RFLP method to scan two mutations in population conducted by 239 Arabian horses. Abnormal band patterns observed for *MYO5A* mutation in 40 of the studied samples. It has seen that none of the samples were carry the mutation or any other base change in the sequence. The unexpected band patterns were decided as "star activity" of Faul enzyme.

The study conducted by the authors of this publication are the most comprehensive LSF and PSSM-I mutation scan studies in the Arabian horses bred in Turkey.

REFERENCES

1. Bellone R: Pleiotropic effects of pigmentation genes in horses. *Anim Genet*, 41 (Suppl. 2): 100-110, 2010. DOI: 10.1111/j.1365-2052.2010.02116.x

2. Thomas AJ, Erickson CA: The making of a melanocyte: the specification of melanoblasts from the neural crest. *Pigment Cell Melanoma Res,* 21, 598-610, 2008. DOI: 10.1111/j.1755-148X.2008.00506.x

3. Bowling AT: Medical genetics. **In,** Bowling AT, Ruvinsky A (Eds): Horse Genetics. 105-106. CAB International, Wallingford, 1996.

4. Pascoe RR, Knottenbelt DC: Congenital/developmental diseases. **In,** Pascoe RR, Knottenbelt DC (Eds): Manual of Equine Dermatology. 148-149, WB Saunders Co, London, UK, 1999.

5. Fanelli HH: Coat color dilution lethal (lavender foal syndrome): A tetany syndrome of Arabian foals. *Equine Vet Educ* 17, 260-263, 2005.

6. Brooks SA, Gabreski N, Miller D, Brisbin A, Brown HE, Streeter, C, Antczak, DF: Whole-genome SNP association in the horse: Identification of a deletion in myosin Va Responsible for Lavender Foal Syndrome. *PLoS Genet* 6.4: e1000909, 2010. DOI: 10.1371/journal.pgen.1000909

7. Takagishi Y, Murata Y: Myosin Va mutation in rats is an animal model for the human hereditary neurological disease, Griscelli syndrome type 1. *Ann N Y Acad Sci,* 1086, 66-80, 2006. DOI: 10.1196/annals.1377.006

8. Reck-Peterson SL, Provance DWJr, Mooseker MS, Mercer JA: Class V myosins. *Biochim Biophys Acta,* 1496, 36-51, 2000. DOI: 10.1016/S0167-4889(00)00007-0

9. Valberg SJ: Exertional myopathies in horses. **In, Merk Manuel Online:** http://www.merckmanuals.com/vet/musculoskeletal_system/myopathies_in_horses/overview_of_myopathies_in_horses.html, *Accessed:* 25.05.2016.

10. McCue ME, Valberg SJ, Miller MB, Wade C, DiMauro S, Akman HO, Mickelson, JR: Glycogen synthase (GYS1) mutation causes a novel skeletal muscle glycogenosis. *Genomics,* 91, 458-466, 2008. DOI: 10.1016/j.ygeno.2008.01.011

11. Akyüz B, Ertuğrul O, Korkmaz Ağaoğlu Ö: Detection of bovine leukocyte adhesion deficiency (BLAD) allele in Holstein cows reared in Kayseri vicinity. *Kafkas Univ Vet Fak Derg,* 16, 519-521, 2010. DOI: 10.9775/kvfd.2009.1127

12. Cinar Kul B, Korkmaz Ağaoğlu O, Ertuğrul O, Durmaz M: Investigation of severe combined immunodeficiency (SCID) disease of Arabian horses raised at the state stud farms in Turkey. *Ankara Univ Vet Fak Derg,* 61, 59-63, 2014. DOI: 10.1501/Vetfak_0000002606

13. Hall T: BioEdit Sequence Alignment Editor. Ibis Therapeutics, Carlsbad, Ca, 1997.

14. Gabreski N, Brooks S, Miller D, Anczak D: Mapping of lavender foal syndrome using the EquineSNP50 Chip. *J Equine Vet Sci*, 29, 321-322, 2009. DOI: 10.1016/j.jevs.2009.04.029

15. Tarr CJ, Thompson PN, Guthrie AJ, Harper CK: The carrier prevalence of severe combined immunodeficiency, lavender foal syndrome and cerebellar abiotrophy in Arabian horses in South Africa. *Equine Vet J*, 46, 512-514, 2014. DOI: 10.1111/evj.12177

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Case Report

First Isolation of *Salmonella* Hessarek from *Sturnus vulgaris* in Turkey: A Case Report

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Abstract

In this paper, the first isolation of *Salmonella enterica subsp. enterica* serovar Hessarek (*S.* Hessarek) was reported from *Sturnus vulgaris* in Çorum, Turkey in 2010 and 2015. Eleven dead *Sturnus vulgaris* were necropsied, and *S.* Hessarek was isolated from seven of dead bird's tissue samples. The automated ribotyping was performed for epidemiologic typing of the all seven *S.* Hessarek isolates. The number and relationship of bands obtained by ribotyping were analyzed with a similarity index by using the Dice coefficient and unweighted pair group method with mathematical averaging (UPGMA) in GelCompar TM software. The six isolates from 2015 were evaluated as closely related. But, the other isolate apart from them was unrelated. In conclusion, we thought that there were two unrelated *S.* Hessarek outbreaks in a studied region.

Keywords: *Salmonella* Hessarek, *Sturnus vulgaris (Common Starling, European Starlings), Turkey, Wild birds*

Türkiye'de İlk Kez *Sturnus vulgaris*'ten *Salmonella* Hessarek İzolasyonu: Olgu Sunumu

Özet

Bu yazıda Çorum ilinde *Sturnus vulgaris*'ten 2010 ve 2015 yıllarında *Salmonella enterica subsp. enterica* serovar Hessarek (*S.* Hessarek)'in ilk defa izolasyonları bildirilmiştir. Onbir adet ölü *Sturnus vulgaris*'in nekropsisi yapıldı ve yedi ölü kuşun organ örneklerinden *S.* Hessarek izole edildi. *Salmonella* Hessarek izolatlarına epidemiyolojik tiplendirme için otomatize ribotiplendirme uygulandı. Ribotiplendirme ile elde edilen bantların sayı ve ilişkileri GelCompar TM jel analiz programı kullanılarak; küme analizi Dice benzerlik katsayısı ve UPGMA ilişki kuralı parametreleriyle değerlendirildi. 2015 yılında izole edilen altı izolat birbiriyle yakından ilişkili bulundu. Fakat 2010 yılındaki izolat diğerleri ile ilişkili bulunmadı. Sonuç olarak, çalışmanın yapıldığı bölgede iki farklı salgın olduğu düşünüldü.

Anahtar sözcükler: *Salmonella* Hessarek, *Sturnus vulgaris (Sığırcık, Avrupa sığırcığı), Türkiye, Vahşi kuşlar*

INTRODUCTION

Salmonella agents have a broad range of animal hosts, especially wild birds that are known as natural hosts for *Salmonella* throughout the world. The transmission of *Salmonella* agents from infected wild birds to the environment has a common risk for animals and humans [1,2].

Starlings, small wild birds, are among the most widespread birds in Europe and in the urban environment, and have been nominated to the list of the "100 World's Worst" invaders by the Invasive Species Specialist Group [3,4]. Starlings damage agriculture by consuming crops destined for human and livestock consumption [5-7]. In addition to the influence of animal and human health, these birds cause economic loss to agriculture; for example the economic loss in the U.S. was estimated at $800 million annually [8,9].

Salmonellosis can occur in starlings most commonly their omnivorous diet due to feed on the ground, food contaminated with fecal matter and live or feed in contaminated water [10,11]. Thus starlings can catch the disease and die or further excrete the disease through their feces to livestock, especially poultry and egg producers, and to humans [12,13].

Salmonella enterica subsp. enterica serovar Hessarek (*S.* Hessarek) is not a common serotype in Turkey. It was

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originally isolated from a Common Raven *(Corvus corax)* in Iran [14] and numerous occasions in outbreaks of septicemic salmonellosis in starlings [15]. It has also been reported that *S.* Hessarek caused an epidemic of salmonellosis amongst Blackbirds Turdus merula [16].

This paper describes two mortality events involving *Salmonella* Hessarek in *Sturnus vulgaris* in Turkey and epidemiological typing of the isolates.

CASE HISTORY

Case Definition

The area involved in both mortality events is a park called Hıfzı Veldet Velidedeoğlu Park (formerly called Yunus Emre Park) in the town of Çorum (40°33'18.,8"N, 34°58'5.17"E, Turkey), where eleven *Sturnus vulgaris* were found. Five of dead birds in 2010 and six dead birds in 2015 were found. Starlings fed from trash in the park and mortalities were detected in this place.

Necropsy

Necropsies were performed on all dead birds. Among all of the examined birds, the most consistent lesion was necrosis loci on the internal organs (spleen, liver, and heart).

Microbiological Examination

Tissue samples were inoculated on 5% blood agar and incubated aerobically at 37°C. The presumptive colonies of *Salmonella* were identified by biochemical tests: Triple Sugar Iron (Oxoid, CM0277), urea hydrolysis (Oxoid, CM0053B), H_2S, indole production, ONPG (β-galactosidase; Oxoid, DD0013), lysine decarboxylase (Oxoid, CM038) and Voges-Proskauer (Oxoid, CM0043) tests [17]. Then all presumptive *Salmonella* positive isolates were confirmed serologically with polyvalent and monovalent specific somatic and flagellar antisera (Statens Serum Instut, Denmark) and serotyping was performed based on the Kauffmann-White scheme [18]. A total of seven *Salmonella* spp. were isolated from only liver samples and these isolates were identified as *S.* Hessarek, named A, B, C, D, E, F (strains were isolated in 2015), and G (strain was isolated in 2010). But, there was no isolation from spleen and heart samples.

Molecular Typing

Ribotyping was performed as described in the user manual of RiboPrinter (Dupont Qualicon, Wilmington, DE, USA) [19]. The number and relationship of bands obtained by ribotyping were analyzed with a similarity index by using Dice coefficient with optimization and position tolerance set at 1-1.5%, respectively, and unweighted pair group method with mathematical averaging (UPGMA) in using Gelcompar II (version 6.5; Applied Maths, Saint-Martens-Latem, Belgium) software. According to the automated ribotyping, seven isolates separated into one cluster and one unique ribotype with a coefficient of similarity of 70%. The cluster included six isolates (A-F) that had been isolated in 2015. The unique ribotype, included one isolate (G) that had been isolated in 2010 *(Fig. 1, Fig 2)*. The isolate A and B were showed 100% similarity. Also, the isolates C to F were 100% similar. The similarity of these two groups was 95.2%.

DISCUSSION

In this study, *Salmonella* Hessarek was isolated from starlings for the first time in Turkey. *Salmonella* Hessarek was isolated from dead starlings at an interval of five years.

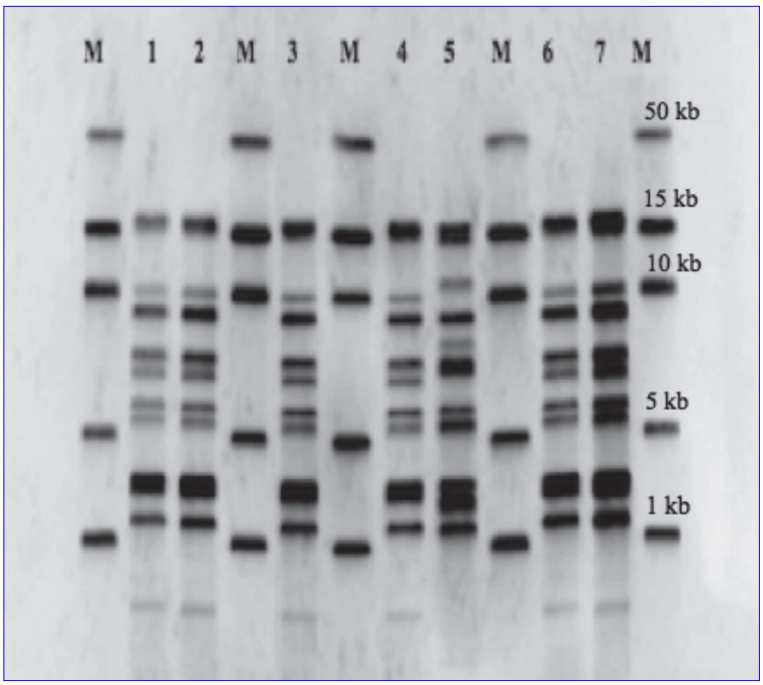

Fig 1. Banding patterns of S. Hessarek generated by PvuII. M: molecular size marker (kilobases). Lanes 1- 6: S. Hessarek isolates from 2015; Lane 7: S. Hessarek isolate from 2010

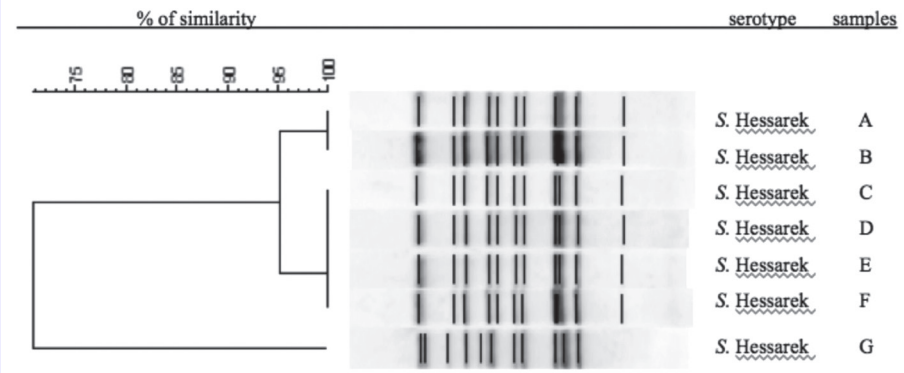

Fig 2. The dendrogram of S. Hessarek isolates that were analyzed by ribotyping

Similarity is high between two *S.* Hessarek isolates in 2010-2015. It is showed that similar strains could lead to birds in two cases and these strains could be persistent in this area. Therefore, it was revealed that migrating birds were exposed continuously to these strains. A similar study was performed in wild birds, Song Thrushes, in Spain [20]. *S.* Hessarek was isolated from wild birds in two cases and had high similarity between isolates with pulsed field gel electrophoresis (PFGE).

Salmonella Hessarek was isolated for the first time in Iran from a raven *(Corvus corax)* in 1953 [4]. However the first isolation of *S.* Hessarek from European Starlings in Isreal was notified that starlings as a reservoir for human [15]. Birds can acquire these pathogens from contaminated environments and spread it directly to humans or indirectly by contaminating commercial livestock vehicles [21]. In particular, the transmission route of Salmonella to humans occurs both by direct contact to contaminated fecal materials and by consumption of their contaminated meat [22].

In this study, there was no data about course of the disease in both cases. It was detected only in necrotic foci on internal organs, especially the liver, spleen, and heart. Other studies have reported that *Salmonella* Hessarek also can cause poor body condition, splenomegaly, moderate to severe pectoral muscle atrophy, hepatomegaly, fibrinous serositis, and hemorrhage in the proventriculus or intestine [15,20].

It has been stated that PFGE and RAPD were performed for diversity of Salmonella serovars, especially clonally related Salmonella isolates from the environment and feces or wildlife reservoirs that affected public health [13,23]. Ribotyping was an essential method for epidemiological typing in this study [24,25]. *Salmonella* Hessarek isolates were separated into seven ribopatterns and two clusters although they had similar profiles. This shows that in 2010-2015, isolates could have come from a different sources.

In conclusion, this knowledge may help to further identify potential epidemic *Salmonella* Hessarek in the wild, and control measures against the migration routes of wild birds avoid transmission of this infection both in humans and animals.

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REFERENCES

1. Chomel BB, Belotto A, Meslin FX: Wildlife, exotic pets, and emerging zoonoses. *Emerg Infect Dis*, 13, 6-11, 2007.

2. Kobayashi H, Kanzaki M, Shimizu Y, Nakajıma H, Khatun MM, Hata E, Kubo M: *Salmonella* isolates from cloacal swabs and footpads of wild birds in the immediate environment of Tokyo Bay. *J Vet Med Sci*, 69, 309-311, 2007. DOI: 10.1292/jvms.69.309

3. Jones FT: Understanding and control of European Starlings. *Avian Adv*, 9, 9-11, 2007.

4. Lowe S, Browne M, Boudjelas S, DePoorter M: 100 of the world's worst invasive alien species. A selection from the global invasive species database Auckland: IUCN-ISSG, 12 pp, 2004. http://www.issg.org/pdf/publications/worst_100/english_100_worst.pdf; *Accessed:* 04.03.2016.

5. Besser JF, Royal WC Jr, DeGrazio JW: Baiting starlings with DRC-1339 at a cattle feedlot. *J Wildl Manage*, 31, 48-51,1967. DOI: 10.2307/3798359

6. Carlson JC, Engeman RM, Hyatt DR, Gilliland RL, DeLiberto TJ, Clark L, Bodenchuk MJ, Linz GM: Efficacy of European starling control to reduce *Salmonella enterica* contamination in a concentrated animal feeding operation in the Texas panhandle. *BMC Vet Res*, 7, 1-10, 2011. DOI: 10.1186/1746-6148-7-9

7. Glahn JF, Mason JR, Woods DR: Dimethyl anthranilate as a bird repellent in livestock feed. *Wildl Soc Bull*, 17,313-320, 1989.

8. Hall AJ, Salto EK: Avian wildlife mortality events due to *Salmonellosis* in The United States, 1985-2004. *J Wildl Dis,* 44, 585-593, 2008. DOI: 10.7589/0090-3558-44.3.585

9. Pimentel D, Lach L, Zoniga R, Morrison D: Environmental and economic costs of nonindigenous species in the United States. *Biosci*, 50, 53-65, 2000. DOI: 10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2

10. Cizek A, Literak I, Hejlicek K, Treml F, Smola J: *Salmonella* contamination of the environment and its incidence in wild birds. *J Vet Med B*, 41, 320-327,1994. DOI: 10.1111/j.1439-0450.1994.tb00234.x

11. Tizard I: Salmonellosis in wild birds. *Semin Avian Exot Pet,* 13, 50-66, 2004. DOI: 10.1053/j.saep.2004.01.008

12. Benskin CMcWH, Wilson K, Jones K, Hartley IR: Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biol Rev*, 84, 349-373, 2009. DOI: 10.1111/j.1469-185X.2008.00076.x

13. Hernandez SM, Keel K, Sanchez S, Trees E, Gerner-Smidt P, Adams JK, Cheng Y, III AR, Martin G, Presotto A, Ruder MG, Brown J, Biehert DS, Cottrell W, Mauer JJ: Epidemiology of a *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain associated with a songbird outbreak. *Appl Environ Microbiol*, 78, 7290-7298, 2012. DOI: 10.1128/AEM.01408-12

14. Neel RL, Minor L, Kaweh M: Une nouvelle espece de salmonella

isolee chez un corbeau *(Corvus corax)*: Salmonella Hessarek. *Ann Inst Pasteur,* 85, 271-274, 1953.

15. Singer NY, Weismann YY, Marder U: Isolation of *Salmonella* Hessarek from starlings *(Sturnus vulgaris). Avian Dis,* 21, 117-119, 1977. DOI: 10.2307/1589370

16. Plant CW: Salmonellosis in wild birds feeding at sewage treatment works. *J Hyg Camb,* 81, 43-48,1978.

17. O.I.E. Manual: Fowl typhoid and pullorum disease. **In,** Manual of Standards for Diagnostic Tests and Vaccines. Chapter 2.3.11 pp.514-529, Office International des Epizooties, Paris, France, 2012.

18. Grimont PAD, Weill F: Antigenic Formulae of the *Salmonella* Serovars. 9th edn., Paris: WHO Collaborating Center for Reference and Research on Salmonella Institute Pasteur, 2007.

19. Anonymus: Riboprinter User Manual. Dupont Qualicon RiboPrinter System, Wilmington, USA, 2002 http: //www2.dupont.com/Qualicon/en_US/RiboPrinter_System/ *Accessed:* 07.03.2016

20. Velarde R, Porrero MC, Serrano E, Marco I, García M, Téllez S, Lucas Domínguez L, Aymí R, Lavín S: Septisemic Salmonellosis caused by *Salmonella Hessarek* in wintering and migrating *Song Thrushes (Turdus Philomelos)* in Spain. *J Wildl Dis*, 48, 113-121, 2012. DOI: 10.7589/0090-3558-48.1.113

21. Linz G M, Homan HJ, Gaulker S M, Penry LB, Bleier WJ: European Starlings: A review of an invasive species with far-reaching impacts. *Managing Vertebrate Invasive Species: Proceedings of an International Symposium*, USDA National Wildlife Research Center, Fort Collins, CO, 24 pp, 2007. http://digitalcommons.unl.edu/nwrcinvasive/24; *Accessed:* 07.03.2016

22. Pasquali F, Cesare AD, Braggio S, Manfreda G: Salmonella detection and aerobic colony count in deep-frozen carcasses of house sparrow *(Passer domesticus)* and starling *(Sturnus vulgaris)* intended for human consumption. *Italian J Food Safety*, 3, 117-119, 2014. DOI: 10.4081/ijfs.2014.1668

23. Farias LFP, Oliveira CJB, Medardus JJ, Molla BZ, Wolfe BA, Gebreyes WA: Phenotypic and genotypic characterization of *Salmonella enterica* in captive wildlife and exotic species in Ohio, USA. *Zoonoses Public Hlth*, 62, 438-444, 2015. DOI: 10.1111/zph.12170

24. Altwegg M, Hickman-Brenner FW, Farmer III JJ: Ribosomal RNA gene restriction patterns provide increased sensitivity for typing *Salmonella typhi* strains. *J Infect Dis,* 160, 145-149, 1989. DOI: 10.1093/infdis/160.1.145

25. Bailey JS, Fedorka-Cray PJ, Stern NJ, Craven SE, Cox NA, Cosby DE: Serotyping and ribotyping of Salmonella using restriction enzyme PvuII. *J Food Prot,* 65, 1005-1007, 2002.

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Letter to the Editor

Non-cardiogenic (Lone) Atrial Fibrillation in an Anatolian Shepherd Dog (Bir Anadolu Çoban Köpeğinde Non-Kardiyojenik [Yalnız] Atriyal Fibrilasyon)

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Dear Editor,

Herein, we are presenting a case of lone atrial fibrillation (AF) diagnosed by electrocardiography (ECG) in an Anatolian Shepherd Dog. Despite having some limitations, ECG is used as a conventional method to diagnose cardiac rhythm disorders in human and veterinary medicine. Combined with echocardiography and radiology, ECG gives speckle of data about the cardiac disease and/or the arrhythmia caused by metabolic disorders. Since several heart diseases such as dilated cardiomyopathy (DCM) and chronic valvular heart disease can cause AF, existence of AF without underlying cardiac disease is rare. There is only one case presentation on lone AF in dogs in literature [1].

A dog (Anatolian Shepherd Dog, 3-year-old, male, 63 kg) was presented to Animal Hospital (Dep. of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, Bursa) with a history of lethargy, exercise intolerance and fatigue. The dog was lying in lateral recumbency and tachypneuic (68 breahts/min). Cardiac auscultation revealed a gallop heart rhythm with strength decrease, and cardiac murmur was not existed. A weak femoral pulse and mental depression were seen on physical examination. All other parameters including complete blood cell count (VetScan HM5, Abaxis, UK), routine serum biochemistry profile (Comprehensive Profile, VetScan VS 2, Abaxis, UK) and urine analysis were within reference ranges, except a mild haemoconcentration with normal haemoglobin concentration (total protein 8.9 g/dL; reference range: 5.4-8.2 g/dL, haemoglobin 15.8 g/dL; reference range: 11.9-18.9 g/dL). Dog was negative for common vector borne diseases; *Dirofilaria immitis* antigen, *Borrelia burgdorferi* antibody, *Anaplasma phagocytophilum/Anaplasma platys* antibody and *Leishmania infantum* antibody (Anigen Rapid Leishmania Ab Test Kits, BioNote, Korea).

ECG examination showed a rapid ventricular rate with absence of the P waves, irregular R-R intervals, and atrial premature contractions *(Fig. 1)*. Thoracic radiography showed a mild bronchial pattern without evidence of cardiac pathology *(Fig. 2)*. There was no any cardiac

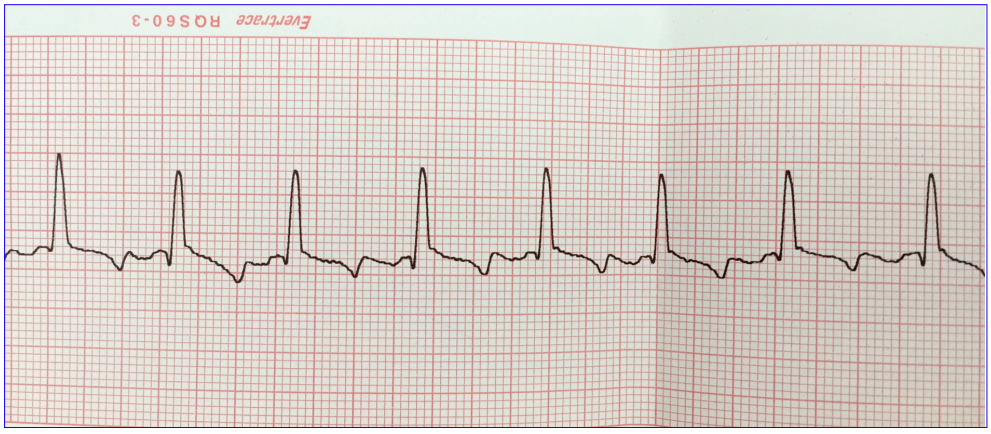

Fig 1. ECG paper of the patient shows a rapid ventricular rate (188 bpm) with absence of the P waves and irregular R-R intervals indicating presence of atrial fibrillation (50 mm/sec, 10mm/1 mV, derivation II)

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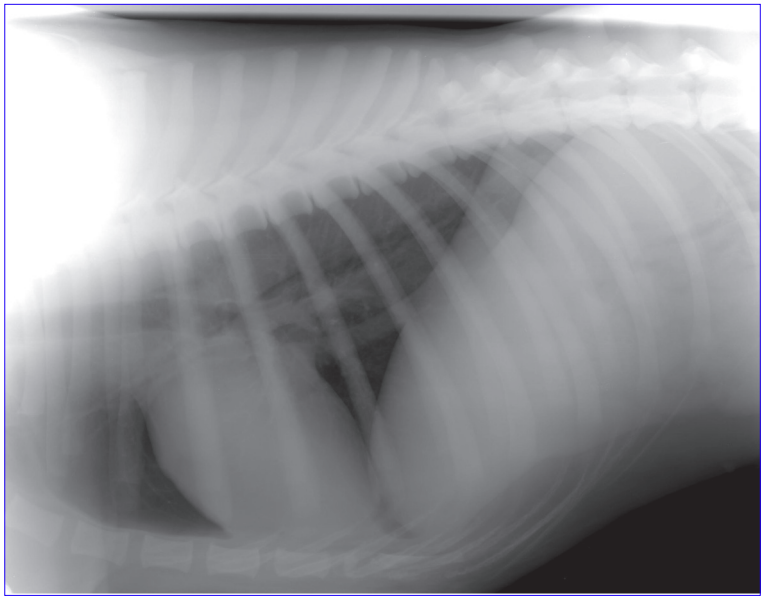

Fig 2. Lateral x-ray of thorax shows a mild bronchial pattern without evidence of cardiac pathology

pathology based on the echocardiographic examination, by using standard techniques such as two-dimensional echocardiography, M-mode, color flow imaging and spectral Doppler examinations (CarisPlus®, Esoate, Florence, Italy) with a 2.5-5 MHz phased-array transducer, as suggested [2]. These observations showed that dog was suffered from AF without cardiac pathology. The reason for observed AF in this patient might be due to haemoconcentration, as described in human and dogs [1].

According to literature [3,4], symptomatic treatments such as intravenous lactated Ringer solution combined with 5% dextrose (10 ml/kg/hr) were given to rehydrate the patient, prednisolone (1 mg/kg, q12h, PO), and amoxycillin clavulinate (20 mg/kg, q12h , PO) and digoxin (0.025 mg/kg, q24h, PO) was given in order to control AF in the patient.

In conclusion, ECG monitoring is clinically relevant in diagnosis of dogs with arrhythmias and also useful for monitoring treatment. AF is the most life threatening cardiac arrhythmia among canine and feline patients [3], although presence of AF does not always accompany with a cardiac pathology. A poor prognosis in canine DCM can be related to AF [4]. Also, uncontrolled arrhythmias may lead to DCM within days [5]. Clinician should be kept in mind that ECG examination is easy to apply and relatively cheap and gives ideas on already existed cardiological diseases or before their presence.

REFERENCES

1. Takemura N, Nakagawa K, Hirose H: Lone atrial fibrillation in a dog. *J Vet Med Sci.* 64 (11): 1057-1059, 2002.

2. Boon JA: Veterinary Echocardiography. 2nd edn., Wiley-Blackwell, USA, 2010.

3. Gelzer AR, Kraus MS: Management of atrial fibrillation. *Vet Clin North Am: Small Anim Pract*, 34, 1127-1144, 2004. DOI: 10.1016/j.cvsm.2004.05.001

4. Kocatürk M, Baykal AT, Türkseven Ş, Acıoğlu Ç, Agudelo CF, Yılmaz Z: Evaluation of serum and ascitic fluid proteomes in dogs with dilated cardiomyopathy. *Kafkas Univ Vet Fak Derg,* 22, 273-279, 2016. DOI: 10.9775/kvfd.2015.14429

5. Bounhoure JP, Boveda S, Albenque JP: Arrhythmia induced dilated cardiomyopathies. *Bull Acad Natl Med,* 190 (6): 1225-1235; Discussion: 1235-1236, 2006.

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup ve derleme türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve şekil ve tablo gibi görsel öğelerin metin içindeki yerleri belirtilmelidir.

Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.

Başvuru sırasında yazarlar yazıda yer alacak şekilleri online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.

3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- Makale Türleri

Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.

Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th edn., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir alan editörü ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

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1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, and review and on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text.

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Manuscripts consist of the title, abstract and keywords, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Abstract should contain 200±20 words.

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If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

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DOI number should be added to the end of the reference.

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