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Research Article

Biological Variation of Oxidative Stress Biomarkers and Lactic Dehydrogenase in Mice

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Abstract

This is the first report, we aimed to determine the biological variations, analytical quality specifications of oxidative stress biomarkers such as glucose-6-phosphate dehydrogenase (G6PD), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), reduced glutathione (GSH), malondialdehyde (MDA), and also lactic dehydrogenase (LDH) in mice. In the our study, *Mus musculus* albino mice, forty-one adult (female 20, male 21), weighing between 23 and 40 g were enrolled for biological variation study during 30-day period. Samples were stored and then tested at the same time. Results were assessed in duplicate and coefficients of variation for each analyte which were isolated to distinguish variation such as within-, and between-individual variations. From these results, an index of individuality were determined for each analyte. The analytical, within-individual, between-individual variations were assessed in apparently healthy mice and were found to be; 1.19%, 22.51% and 7.38% for G6PD, 14.09%, 23.03% and 29.42% for CAT, 6.18%, 11.12% and 21.14% for SOD, 9.13%, 12.00% and 12.58% for GST, 4.12%, 23.40% and 11.20% for GSH, 2.11%, 16.45% and 11.27% for LDH, 4.75%, 9.03% and 31.55% for MDA, respectively. As a result, while population-based reference intervals for G6PD, GSH and LDH were appropriate, Subject-based reference intervals for SOD and MDA were more appropriate. CAT and GST had intermediate individuality so population-based reference intervals should be used with caution.

Keywords: *Biological variation, lactic dehydrogenase, mice, oxidative stress biomarkers*

Farelerde Oksidatif Stres Biyobelirteçlerinin ve Laktik Dehidrogenazın Biyolojik Varyasyonu

Özet

Bu çalışma, glukoz-6-fosfat dehidrogenaz (G6PD), katalaz (CAT), süperoksit dismutaz (SOD), glutatyon S-transferaz (GST), indirgenmiş glutatyon (GSH), malondialdehit (MDA) gibi oksidatif stres biyobelirteçlerinin ve laktik dehidrogenaz (LDH)'ın biyolojik varyasyonlarını, analitik kalite spesifikasyonlarını ve referans aralıklarını saptamak amacıyla yapılan ilk araştırmadır. Bu çalışmada, 41 erişkin, 23-40 g ağırlığındaki *Mus musculus* albino fare (20 dişi, 21 erkek), 30 gün boyunca biyolojik varyasyon ve referans aralıkları çalışmaları için kullanıldı. Sağlıklı görünen farelerde analitik, kişisel ve kişiler-arası varyasyonlar değerlendirildi ve sırasıyla G6PD: %1.19 %22.51 ve %7.38; CAT: %14.09, %23.03 ve %29.42; SOD: %18, %11.12 ve %21.14; GST: %9.13, %12.00 ve %12.58; GSH: %4.12, %23.40 ve %11.20; LDH: %2.11, %16.45 ve %11.27; MDA: %4.75, %9.03 ve %31.55 olarak bulundu. Bu bulgular G6PD, GSH ve LDH'ın referans aralıkları, toplum tabanlı referans aralıklara uygun iken SOD ve MDA'nın kişisel-tabanlı referans aralıklara daha uygun olduğunu gösterdi. CAT ve GST'nin toplum tabanlı referans aralıklarda dikkatli kullanılması gerektiği bulundu.

Anahtar sözcükler: *Biyolojik varyasyon, Laktik dehidrogenaz, Oksidatif stres biyobelirteçleri, Fare*

INTRODUCTION

Reactive oxygen species (ROS) are produced during normal metabolism and the biological effects of ROS are controlled *in vivo* by a variety of oxidative stress biomarkers such as glucose-6-phosphate dehydrogenase (G6PD), catalase (CAT), superoxide dismutase (SOD), and also glutathione-S-transferase (GST). They are inducible enzymes which react with activated oxygen species, both in the cytosol and in subcellular organelles, and function in decreasing the damaging effects of these molecules [1,2]. In addition to these enzymatic components of the oxidant defence system there are also non-enzymatic "radical scavengers" such as alpha-tocopherol, reduced glutathione

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(GSH) and ascorbic acid. Lipid peroxidation is a normal phenomenon that occurs continuously at low levels in all living cells. These peroxidation reactions are in part toxic to cells and cell membranes; however, they are normally controlled by countervailing biologic mechanisms [1]. Lactic dehydrogenase (LDH), a glycolytic enzyme, is present in various tissues and neoplasms of the mammalian body in multiple forms. Doherty et al.[3] reported that there was a relation between neoplasia and LDH activity.

Laboratory mouse is an animal most commonly used in mammalian biological studies and in the human disease modeling. In spite of numerous experiments conducted on laboratory mice, their biochemical phenotype still remains not fully discovered. Only limited data on the clinical biochemistry of mice are available from studies of various authors [4]. Currently, the biochemistry results of laboratory animal are assessed in relation to population-based reference intervals [5]. However, it is considered more appropriate to use 'subject-based' reference values to assess analytes that have a high degree of between-individual variation because many unhealthy individuals may have values that significantly differ from their regular analyte determination, but fall within population-based reference intervals [6,7].

Determination of 'subject-based' reference values requires knowledge of inherent physiological variation of analytes which is referred to as biological variation. Suitability of an analyte to be assessed in relation to population- or subject-based reference intervals is determined by that analyte's index of individuality (II), a ratio of within-individual biological variation (CV_I) to between-individual (CV_G) biological variation. Although biological variation data have been generated for several blood components in various veterinary animals (dog, cat and horse), there are no biological variation data for mice blood component published in the peer-reviewed literature. Therefore, in the present study, we aimed to determine biological variation data of erythrocyte oxidative stress biomarkers and LDH in mice. These data will be used to set analytical quality specifications, as well as to select those biomarkers most appropriate, for consideration in a larger observational study investigating on oxidative stress biomarkers and LDH in mice.

MATERIAL and METHODS

All chemicals used in the levels of oxidative stress biomarkers and LDH activity assays were analytical grade and were from the Sigma Chemical Company (St. Louis, MO, USA).

Animals

Mus musculus albino mice, forty-one adult (female 20, male 21), weighing between 25 and 35 g were obtained from the Experimental Research Center of the University of Sutcu Imam. They were fed with a standard laboratory diet and tap water. Illumination was 12 h light/dark cycle and room temperature was 22-24°C. They were enrolled for biological variation study during 30 days. This study was approved by the local ethics board in Kahramanmaras Sutcu Imam University Faculty of Medicine, Experimental Animals Laboratory (KSU Ethics Committee Number: 2000-18).

The Blood Samples

The blood samples were taken into EDTA tubes from tail vein from each individual (between 08:00 and 09:00 a.m.) on the zero, 1st, 3rd, 5th, 7th, 15th and 30th days. All the blood samples were centrifuged and the plasma was removed. The erythrocytes were then washed three times in 0.9% saline solution. All the oxidative stress biomarkers and LDH in erythrocyte were measured as spectrophotometric. Furthermore, erythrocyte samples previously collected from a group of eight healthy mice as described in this study and stored in 250 µL aliquots directly at -80°C, were used as internal control material for the oxidative stress biomarkers (G6PD, CAT, SOD and MDA) and LDH. All analyses were carried out in duplicate, in randomized order on separate tubes of EDTA, during the same day, using one batch of reagents and one calibration of the analyzers.

Determination of Oxidative Stress Biomarkers and LDH

Assay of G6PD Activity: G6PD activity was determined at 37°C in the erythrocyte according to Beutler [8]. The reaction mixture contained 1M Tris-HCl pH 8.0, 6 mM G6P Na, 2 mM NADP, 0.1 M $MgCl_2$ and hemolysate in a total volume of 3.0 mL. One unit of enzyme activity was the amount of enzyme catalyzing the reduction of 1mM of NADP per minute. G6PD activity was expressed as U/g Hb.

Assay of CAT Activity: CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 230 nm by the method of Beutler [8]. Assay medium consisted 1 M Tris HCl, 5 mM Na_2EDTA buffer solution (pH 8.0), 10 mM H_2O_2 and haemolysate in a final volume of 1.0 mL. CAT activity was expressed as U/g Hb.

Assay of SOD Activity: SOD activity was measured according to the method described by Fridovich [9]. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with p-iodonitro-tetrazolium violet (INT) to form a red formazan dye which was measured at 505 nm. Assay medium consisted of the 0.01 M phosphate buffer, CAPS (3-cyclohexilamino-1-propanesulfonicacid) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated NaOH) with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM INT) and 80 U/L xanthine oxidase. SOD activity was expressed as U/g Hb.

Assay of GST Activity: GST activity was measured by the method described by Mannervik and Guthenberg [10]. 1mM 1-chloro-2.4-dinitrobenzene (CDNB) was used as

the substrate. The reaction mixture contained phosphate buffer (pH 6.5), 20 mM GSH and 20 mM CDNB. Increase in optical density was followed at 340 nm and GST activity was expressed as U/g Hb.

Assay of LDH Activity: In the assay the oxidation of NADH was followed spectrophotometrically at 340 nm by the method of Beutler [8]. Assay medium consisted of the 1M Tris-HCl and 5mM EDTA solution with pH 8.0, 2mM NADH, hemolysate and 10 mM sodium pyruvate in a final volume of 1 mL. LDH activity was expressed as U/g Hb.

Measurement of GSH: GSH levels were determined by measuring a highly coloured yellow anion formed by the reduction of DTNB [5,5′-Dithiobis (2-nitrobenzoic acid)] with nonprotein sulfhydryl compounds of erythrocytes by the method of Beutler [8]. The optical density of yellow anion was measured at 412 nm within the first 10 minutes of colour development. Entire procedure was carried out at room temperature. The level of GSH was calculated as µmol/g Hb.

Measurement of MDA: Malondialdehyde (MDA) levels, as an indicator of lipid peroxidation. It was measured in erythrocyte according to procedure of Ohkawa et al.[11]. The reaction mixture contained 0.1 mL of sample, 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% aqueous solution of TBA. The mixture pH was adjusted to 3.5 and volume was finally made up to 4.0 mL with distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, *v/v*) were added. The mixture was shaken vigorously. After centrifugation at 4.000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm.

The hemoglobin concentration of the hemolysate was measured in digital Spectronic-20 spectrophotometer by the method of Beutler [8].

Statistical Analysis

Seven blood samples were taken from each individual on the zero, 1st, 3rd, 5th, 7th, 15 and 30th days. General statistical analyses were carried out with SPSS 15.0 for Windows. Outliers were determined according to Fraser and Harris. We looked for outliers in the sets of duplicate results. When one outlying variance (an unexpected large difference in the duplicates) was detected, both data points were rejected [12]. As a result of these steps the assay results of 4 subjects were identified as outliers for GST and were excluded from the estimations. After exclusion of any outliers, the data of 37 individuals for oxidative stress biomarkers and LDH were evaluated to estimate components of biological variation. Each sample from one individual was assayed in duplicate and, the average value of the individual's duplicate measurements was used for the statistical procedures. Analytical variance (SD_A^2) was calculated from the difference between each pair of duplicates according to the formula [12,13]:

$SD_A^2 = (\Sigma d^2/2n)$ and $CV_A = (SD_A/\text{Mean}) \times 100$ as a percentage.

CV_I was calculated from the total within subject variance (CV_{TI}) minus the analytical variance according to the formula described by Fraser and Harris as $CV_I = (CV_{TI}^2 - CV_A^2)^{1/2}$. CV_{TI} includes both analytical and biological variation. To determine between-individual variance (SD_G^2), the total variance (SD_T^2) was calculated by use of all of the individual data sets and transformed to relative SD (CV_T) by use of the overall mean. CV_G was calculated by means of the following formula as $CV_G = (CV_T^2 - CV_I^2 - CV_A^2)^{1/2}$. Index individuality (II) was calculated as CV_I/CV_G [12-15]. The reference change value (RCV) or critical differences, which is the difference required for two serial measurements of the oxidative stress biomarkers to have significantly changed at P<0.05, was calculated as $RCV = 2^{1/2} \times 1.96 (CV_I^2 + CV_G^2)^{1/2} = 2.77(CV_{TI})$. The objective analytical performance standards for imprecision $CV_{max} = 0.5CV_I$, inaccuracy $B_{max} = 0.25(CV_I^2 + CV_G^2)^{1/2}$ and total error $TE = [1.65(CV_{max}) + B_{max}]$ were also calculated [16].

Linear regression analysis was used to look for significant trends in values for the levels of oxidative stress biomarkers and LDH and to investigate the time dependence of the within-individual variations. Also, comparisons according to days were performed using the Wilcoxon test. A P<0.05 was considered to be statistically significant.

RESULTS

Of the 2009 data points of 41 subjects, 4 subjects were classified as outliers, but as the duplicate results were similar, these results were considered to be physiological variation and maintained. Also shown in *Table 1* are the overall means, ranges of each oxidative stress biomarker and LDH activity. There was no significant differences for all parameters between sexes (P>0.05). Therefore only the total results for all parameters are presented.

Table 2 shows, the day-to-day changes as within-subject CVs for all analytes in mice. There were no significant differences between values of each day and initial day by Wilcoxon test. The criterion for comparing variables with the initial day was chosen at P<0.05.

Table 3 shows, CV_I, CV_G, II and categories of II, which are derived from the data on biological variation. Because there were no significant differences in the CV_I between the sexes (*F*-test) for the analytes investigated in this study, only the totals were presented. The CV_I values of G6PD, LDH and GSH were larger than CV_G values ($CV_I > CV_G$). However, CV_I values of CAT, SOD and MDA were smaller than CV_G values ($CV_I < CV_G$). CV_I and CV_G values of GST analyte were found as similar to each other. Furthermore, While G6PD, LDH and GSH showed high index of individuality (II>1.4), SOD and MDA showed low index of individuality (II<0.6). CAT and GST showed intermediate individuality

Table 1. *The levels of oxidative stress biomarkers and LDH in mice.* ***Tablo 1.*** *Farelerde oksidatif stres biyobelirteçlerinin ve LDH'ın düzeyleri*						
Parameters	**Mean**	**SD**	**Median**	**Min-Max**	**95% Confidence Interval**	***Values Reference Pool**
G6PD	19.31	4.92	18.00	10.40-30.50	9.47-29.15	17.00
CAT	2.31	1.04	2.30	0.80-4.20	0.23-4.39	2.03
SOD	1620.80	409.40	1520	1000-2500	802.00-2439.60	1632.42
GST	8054.31	1403.65	8255	5000-10300	5247.12-10861.52	7245.03
LDH	277.60	44.53	285	187-390	188.60-366.60	254.01
GSH	4.17	0.88	3.95	3.00-6.30	2.41-5.93	3.87
MDA	680.70	217.49	710	350-1020	245.72-1115.68	584.02

*The activities of G6PDH, CAT, SOD, GST and LDH were expressed as U/g Hb; The levels of GSH and MDA were expressed as μmol/g Hb and nmol/g Hb, respectively; * Values reference pool were given as mean, which consisted of eight blood samples*

Table 2. *The within-individual variations in oxidative stress biomarkers and LDH for 30 days duration* ***Tablo 2.*** *Oksidatif stres biyobelirteçlerinin ve LDH'ın 30 günlük sürede ki birey-içi varyasyonları*								
Days	**Body wg**	**G6PD**	**CAT**	**SOD**	**GST**	**LDH**	**GSH**	**MDA**
Zero-day	31.8±4.4 (13.9)	19.3±4.6 (23.8)	2.2±1.0 (45.4)	1555±364 (23.3)	7702±1404 (18.2)	279±46 (16.3)	4.3±0.8 (18.6)	681±218 (31.9)
First day	32.1±4.4 (13.8) [1]p:0.776	18.8±5.4 (28.7) [1]p:0.776	2.3±1.1 (47.8) [1]p:0.776	1641±394 (23.9) [1]p:0.293	8202±1553 (18.9) [1]p:0.118	300±69 (23.0) [1]p:0.079	4.0±1.7 (42.5) [1]p:0.690	695±231 (33.2) [1]p:0.826
Third day	32.4±4.5 (13.9) [2]p:0.660	18.9±5.3 (28.0) [2]p:0.820	2.3±0.9 (39.1) [2]p:0.753	1614.3±485 (30.0) [2]p:0.433	7779±1785 (22.9) [2]p:0.551	280±52 (18.5) [2]p:0.315	4.0±1.5 (37.5) [2]p:0.432	674±229 (34.0) [2]p:0.421
Fifth day	33.0±4.4 (13.4) [3]p:0.967	17.6±3.5 (19.8) [3]p:0.410	2.2±0.8 (36.3) [3]p:0.875	1563±432 (27.6) [3]p:0.887	8190±1557 (19.0) [3]p:0.321	284±45 (19.2) [3]p:0.182	4.4±0.8 (18.1) [3]p:0.330	683±231 (33.8) [3]p:0.593
Seventh day	33.2±4.6 (13.9) [4]p:0.835	18.7±4.3 (22.9) [4]p:0.732	2.6±0.9 (34.6) [4]p:0.735	1730±434 (25.1) [4]p:0.240	7860±1718 (21.8) [4]p:0.513	278±53 (19.2) [4]p:0.561	3.8±1.4 (36.8) [4]p:0.095	676±214 (31.6) [4]p:0.417
Fifteenth day	33.8±4.4 (13.0) [5]p:0.505	16.4±3.4 (20.7) [5]p:0.235	2.3±0.1 (4.34) [5]p:0.318	1621±398 (24.5) [5]p:0.397	7869±1573 (19.9) [5]p:0.495	276±59 (21.6) [5]p:0.535	4.3±0.7 (16.2) [5]p:0.197	683±219 (32.0) [5]p:0.557
Thirtieth day	34.0±4.6 (13.7) [6]p:0.337	18.8±3.3 (17.5) [6]p:0.734	2.5±0.8 (32.0) [6]p:0.826	1666±342 (20.5) [6]p:0.253	8200±1576 (19.2) [6]p:0.096	306±55 (18.0) [6]p:0.087	4.5±1.1 (24.4) [6]p:0.123	759±286 (37.6) [6]p:0.176

The activities of G6PDH, CAT, SOD, GST and LDH expressed as U/g Hb. The levels of GSH and MDA were given as μmol/g Hb and nmol/g Hb, respectively; [1,2,3,4,5,6]*Comparison of the values with initial value was done by Wilcoxon test (P<0.05).*

Table 3. *Components of biological variation for oxidative stress biomarkers and LDH in mice* ***Tablo 3.*** *Farelerde oksidatif stres biyobelirteçlerinin ve LDH'ın biyolojik varyasyon komponentleri*					
Variables	[a]**CV_A (%)**	[a]**CV_I (%)**	[b]**CV_G (%)**	[d]**RCV (%)**	[e]**II (Category[f])**
G6PD (U/g Hb)	1.19	22.51	7.38	62.43	3.05 (High)
CAT (U/g Hb)	14.09	23.03	29.42	74.78	0.78 (intermediate)
SOD (U/g Hb)	6.18	11.12	21.14	35.23	0.52 (Low)
GST (U/g Hb)	9.13	12.00	12.58	41.76	0.95 (intermediate)
LDH (U/g Hb)	2.11	16.45	11.27	45.93	1.45 (intermediate)
GSH (μmol/g Hb)	4.12	23.40	11.20	65.81	2.08 (High)
MDA (nmol/g Hb)	4.75	9.03	31.55	28.26	0.28 (Low)

All samples analyzed in duplicate; [a]*CV_I, within-subject coefficient of variation;* [b]*V_G, between-subject coefficient of variation;* [d]*RCV, reference change value;* [e]*II, index of individuality;* [f]*Categories of II=High (>1.4), Low (<0.6), intermediate (0.6-1.4)*

(II, between 0.6 and 1.4). Furthermore, the observed CV_A of G6PD, LDH and GSH were lower than CVmax except for the CAT, SOD, GST and MDA ($CV_A<CV_{max}$) as shown in *Table 4*. The CV_A values which achieved desired performance of 0.5CV_I are marked with a superscript.

The regression analysis showed no trends for the changes in the levels of oxidative stress biomarkers and LDH activity during 30 days in mice.

***Table 4.** Method imprecision and quality specifications for oxidative stress biomarkers and LDH measurements in mice*

***Tablo 4.** Farelerde oksidatif stres biyobelirteçleri ve LDH ölçümleri için metot belirsizliği ve kalite özellikleri*

Parameters	CV_A[a] %	CV_{max}[b], %	B_{max}[c], %	TE_{max}[d], %
G6PD (U/g Hb)	1.19*	11.25	5.92	24.48
CAT (U/g Hb)	14.09	11.51	9.34	28.33
SOD (U/g Hb)	6.18	5.56	5.97	15.14
GST (U/g Hb)	9.13	6.00	4.34	14.24
LDH (U/g Hb)	2.11*	8.25	4.98	18.59
GSH (µmol/g Hb)	4.12*	5.60	6.48	15.72
MDA (nmol/g Hb)	4.75*	15.77	8.20	34.22

*All samples analyzed in duplicate; [a]CV_A, analytical coefficient of variation; CV_{max}[b], objective analytical performance standard for imprecision; B_{max}[c], objective analytical performance for inaccuracy; TE_{max}[d], objective analytical performance standard for total error; *Desirable performance ($CV_A < CV_{max}$)*

DISCUSSION

To our knowledge, this is the first study to carry out the determination and application of data on biological variations of oxidative stress biomarkers and LDH in erythrocytes of mice. Limited studies have been conducted to address these methodological issues [17,18] and there is still controversy over which oxidative stress biomarkers and LDH to use. It has been suggested that oxidative stress biomarkers and LDH should be utilized for research purposes until more sensitive and specific assays are developed [19,20]. The current consensus in laboratory medicine is that quality specifications should be based on the parameters of biological variation [15,21]. In this study we generated data of biological variations for oxidative stress biomarkers and LDH that are commonly measured in the laboratory when assessing oxidative stress in mice. In the present study, we found no significant difference in the within-subject CVs of all analytes in mice, so these analytes can be used in deriving criteria for decision making. The CV_G values for CAT, SOD and MDA analytes in our study were found as generally larger than CV_I values. We thought that these analytes displaying small CV_I also allow more precise knowledge of the homeostatic set point and leave less margin for ambiguity in recognizing the patient's status. However, the average CV_G values of G6PD, GST, GSH and LDH analytes was smaller than CV_I values, probably because of the lack of homogeneity in the CV_I and perhaps due to problems of stability. The lack of homogeneity in the data of these analyte may cause erroneous calculations of biological variation and false interpretations of results, as such calculations involve an analysis of means and may incorporate individuals with CV_I values much larger than CV_G values.

For detection of disease, subject-based reference intervals are more sensitive than population-based reference intervals when CV_I values are less than CV_G values, because many individuals will have values that differ from their usual values, but which fall within population-based reference intervals [15,21,22]. For any individual, the homeostatic health setting for a given analyte may be near the limits or toward the center of a population-based reference interval, but maintains its relative position within the reference interval [20]. When CV_I is markedly lower than CV_G, any change must be considerable to be detected using a population-based reference intervals, especially if the individual's health setting is near the population's mean. To determine how much lower CV_I must be in order for subject-based reference intervals to be of use, the "individuality" of the analyte, namely the index of individuality, can be calculated. Using the criteria described by Fraser and Harris [14], and recently reviewed in the veterinary literature [15], an II <0.6 indicates that subject-based reference values are more appropriate to use; when the II >1.4, population-based reference intervals are more appropriate, and when between 0.6 and 1.4, population-based ranges should be used with caution [15]. In our study, SOD and MDA had low index of individuality and therefore subject-based reference intervals are more appropriate; G6PD, LDH and GSH had high index of individuality, indicating that population-based reference intervals are appropriate. CAT and GST had intermediate index of individuality so population-based reference intervals should be assessed in relation to subject based reference intervals. To our knowledge, there is no study on this subject, therefore we did not compare our results. We only compared our results with human blood for genetically close to each other. Covas et al.[23] reported that II for SOD and glutathione peroxidase in human blood was 0.45 (low individuality), suggesting that it has little value as a diagnostic or screening tool. The components of variation for MDA in human plasma reported in the literature [18,24] are similar to our values. The RCV is an important clinical tool for the assessment of changes in patient animal being monitored in pathologicalsituations [24,25]. In the present study, the RCVs were high for G6PD, CAT, LDH, GST and GSH due to their higher within-subject component of variation. Furthermore, RCV can be of diagnostic use in cases where the population-based reference range may be an insensitive interpretation criterion (i.e. low index of individuality). In such cases the RCV can be used to detect small, but statistically significant changes within the reference range between serial measurements in the same individual. For a change between serial measurements exceeding the RCV, and thus the limits of biological variation, could identify animal "at risk of disease" so that further diagnostic workup or clinical intervention can be instituted at an earlier stage of disease.

Every test result is subject to a number of sources of biological variation causing measurements in the same individual to change with time. Knowledge of these temporal changes is useful information when establishing whether the performance of a test is appropriate for

interpreting results and making a diagnosis. Desired analytical imprecision and within-subject biological variation are particularly important in this regard. The influence of both these parameters on the precision of single test results and on the number of samples required to make clinical decisions can be easily calculated using simple formulae. Furthermore the effect of performing replicate analyses of the same sample versus taking multiple samples can also be investigated. In the present study, we have determined desired analytical imprecision on the biological variation of oxidative stress biomarkers and LDH assays and furthermore assessed the utility of reference intervals for interpretation of results. The CVmax should be perceived as a desired minimum requirement for the level of imprecision [26]. However studies in humans have shown, that the desired analytical variation (CVmax) is difficult to achieve when working with oxidative stress biomarkers and LDH assays and often laboratories fail to accomplish this analytical goal [18]. Of these analytes, G6PD, LDH and GSH achieved a CV_A lower than desired [27]. However, CAT, SOD, GST and MDA achieved a CV_A higher than desired CV_A for this assay. The results indicated that for CAT, SOD, GST and MDA analysis in duplicate could be necessary, which would theoretically lower the CV_A and bring analytical variation below the desired level. There is no apparent analytical error, which can explain the observed high analytical variation of the MDA (34.0%), CAT (28.3%) and G6PD (24.4%) assays examined in this study. Internal controls of these tests using the pooled erythrocyte analyzed with each batch of samples was within a well defined control range, which indicates that there may be some pre-analytical variation causing the aberrant results of the samples from the eight mice examined. Oxidative stress biomarkers and LDH tests in laboratory medicine can be measured with a wide variety and combination of reagents and instruments, which can influence the results of the analysis. All seven measured erythrocyte oxidative stress biomarkers and LDH parameters met the objective analytical performance standard for imprecision except for the G6PD, LDH, GSH and MDA for which analysis in duplicate could also be necessary. An approach the estimate of within-subject variation would be confounded by between batch analytical variations. Thus an estimate of between-batch analytical variation must be obtained from assays of quality control materials, which are currently not available for these markers and LDH assays. Though oxidative stress biomarkers and LDH performed on erythrocyte samples might be as diagnostically relevant or informative of the patient animal. Therefore, it is hoped that these biological variation data may serve to apply this approach in the analysis of oxidative stress biomarkers and LDH in mice.

This study represents the first assessment of biological variation for oxidative stress and LDH in blood of mouse within the peer-reviewed literature. The results of this study showed that SOD and MDA had low index of individuality (<0.6), which may make the use of population-based reference ranges alone an insensitive interpretation criterion. However, G6PD, GSH and LDH had high index of individuality (>1.4), which may make the use of conventional population-based reference ranges is a sensitive interpretation criterion of these measurements. Another our results indicated that CAT and GST had intermediate index of individuality (between 0.6-1.4), population-based reference of these analytes values may be used, but with caution. Analytical quality specifications were derived from biological variation data, and imprecision goals can be reasonably achieved with current methods. Only three (G6PD, LDH and GSH) of the six traditionally used assays examined in this study achieved the analytical goal of $CV_A < CV_{max}$, indicating that the analytical variation of the four other assays (CAT, SOD, GST and MDA) was too high and that measures need to be instituted to address this. Here, we suggest that biological variations and quality specifications for analytical performance of a method should not be veterinary diagnostic ignored in assessing oxidative stress biomarkers and LDH obtained through the same method in the same laboratory or from laboratories applying different methods. There is no information regarding biological variation studies in mice so this paper may provide strong evidence for serial sampling in small animal practice.

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Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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Research Article

Effect of α-Lipoic Acid Supplementation to Extender on Quality of Frozen-Thawed Bull Semen [1] [2]

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Abstract

This study was conducted to investigate the possible protective effect of α-lipoic acid (ALA) supplementation to extender on damages in the quality of bull semen elicited by freeze-thawing process-induced oxidative stres. Ejaculates were collected via an artificial vagina from the bulls at once a week. Then they were split into five aliquots and extended with the Tris base extender containing different doses of ALA, except control. The extended samples were equilibrated slowly to 4°C for 4 h and then frozen using a digital freezing machine. Frozen straws were thawed to analyse progressive motility and sperm motility characteristics as well as plasma membrane integrity. Biochemical assays were performed in a spectrophotometer using commercial kits. Treatment of extender with ALA groups caused a significant decrease in percentages of post-thaw sperm CASA, progressive motilities and sperm motility characteristics such as VAP, VSL and VCL except ALA0.5. Besides ALA0.5 showed lower percentages of acrosome and total abnormalities in comparison to the control. In conclusion, findings generated here showed that ALA0.5 supplementation in Tris based semen extender was of great beneficial effect on frozen-thawed bull semen in terms of morphology and plasma membrane integrity.

Keywords: *Bull sperm, Cryopreservation, α-lipoic acid, Oxidative stress*

Sulandırıcıya Katılan α-Lipoik Asidin Dondurulmuş-Çözdürülmüş Boğa Spermasının Kalitesi Üzerine Etkisi

Özet

Bu çalışma dondurma-çözündürme işleminin neden olduğu oksidadif stresden dolayı boğa spermasında meydane gelen hasarlar üzerine sulandırıcıya katılan α-lipoik asidin (ALA) muhtemel koruyucu etkisini araştırmak amacı ile yapıldı. Ejakülatler haftada bir kez olmak üzere suni vajen yardımı ile toplandı, sonrasında beş eşit parçaya ayrıldı ve kontrol grubu dışında diğerleri farklı dozlarda ALA içeren Tris bazlı sperma sulandırıcısı ile sulandırıldı. Sulandırılan spermalar 4°C'de 4 saat süre ile ekülibre edildi ve otomatik sperma dondurma cihazı kullanılarak donduruldu. Dondurulan spermalar çözündürülerek plazma membran bütünlüğünün yanı sıra spermanın ileri yönlü hareketi ve sperma hareket özellikleri değerlendirildi. Biyokimyasal analizler ticari kit kullanılarak spektrofotometre de yapıldı. ALA0.5 grubu dışında ALA ilave edilmiş sulandırıcı ile sulandırılan gruplarda dondurma çözündürme sonrası CASA ve ileri yönlü spermatozoa hareketi ile VAP, VSL, VCL (spermatozoonun tüm hareketlerinin ortalaması, spermatozonun ilk harekete başladığı ve hareketini sonlandırdığı yer arasındaki en kısa mesafe, gerçek eğrisel yolda kat ettiği mesafe) gibi spermatozoa hareket özellikleri üzerine olumsuz etki gösterdi. Bunun yanında, kontrol grubu ile karşılaştırıldığında ALA0.5 akrozom ve total anormalitenin daha düşük oranlarda olmasını sağladı. Sonuç olarak, bu çalışmada elde edilen bulgular Tris bazlı sperma sulandırıcısına ilave edilen ALA0.5'in morfoloji ve plazma membran bütünlüğü üzerine olumlu etkilerinin olduğunu gösterdi.

Anahtar sözcükler: *Boğa sperması, Dondurarak koruma, α-lipoik asit, Oksidatif stress*

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INTRODUCTION

Cryopreservation is known to have a harmful effect on sperm motility [1,2]. The processes of cooling, freezing and thawing cause osmotic and chemical stresses on the sperm membrane that decreases sperm viability and fertilizing ability. This cold shock and freezing damages are associated with increased reactive oxygen species (ROS) and oxidative stress [3]. ROS stimulates a loss of sperm function associated with peroxidative damage to the mitochondria and plasma membrane, when produced in excessive amounts. Further, spermatozoa are more susceptible to peroxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity [4]. Depletion of antioxidant defences or rise in free radical production can affect antioxidant balance and cause oxidative stress leading to cell death [5,6]. Enzymatic and non-enzymatic antioxidants play an important role in scavenging free radicals [7]. Lipoic acid is a disulfide compound that is found naturally in mitochondria as coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [8]. There is general agreement about the antioxidant properties of α-lipoic acid (ALA). It scavenges hydroxyl radicals [9] and singlet oxygen [10]. Besides ALA crosses biological membranes easily and suppresses free radicals, because of its small size and high lipophilicity [11]. Exogenous supplementation with this substance has been reported to increase unbound lipoic acid levels, which can act as a potent antioxidant and reduce oxidative stress both in vitro and in vivo [12]. Because of this antioxidant attributes, a number of experimental and clinical studies have been carried [8]. However, few studies were made on the efficacy of ALA suplementation during freze process of semen. It is hypothesized that in order to protect sperm against cryopreservation-induced oxidative stress, a successful antioxidant treatment should be implemented. The combination of extender with ALA may be appropriate approach to reduce the side effects of oxidative stress. Therefore, this study was conducted to investigate the possible protective effect of ALA supplementation to extender at various amounts on damages in the quality of bull semen elicited by freze-thawing process-induced oxidative stress.

MATERIAL and METHODS

Animals and Semen Collection

In this study, three Holstein bulls with clinically healthy, fertility proven and 2-3 years old which they were raised and maintained at the Livestock Central Research Institute (Ankara, Turkey), were used for semen collection. Ejaculates were collected via an artificial vagina from the bulls at once a week. The ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than $1.0x10^9$ spermatozoa/ml were used in this study. The ejaculates were pooled in order to increase semen volume for making replication and to eliminate variability among the samples. This study was replicated eight times for each group. The semen sample was immersed in a water bath at 34°C until their evaluation; each ejaculate was evaluated to determine percentages of CASA progressive and total motilities as well as concentration. The experimental procedures were approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine (number and date: 2006/172 and September 27, 2006).

Semen Processing

The volume was read from the graded collection tube soon after collection concentration was determined using Accucell photometer (IMV Technologie, L'Aigle, France). A Tris-based extender (T) (Tris 30.7 g, citric acid 16.4 g, fructose 12.6 g, egg yolk 20% (v/v), glycerol 6% (v/v), 1.000 ml distilled water, pH 6.8) was used as the base extender. Pooled ejaculates were split into five aliquots and diluted to a final concentration of $18x10^6$/ml spermatozoa with the base extender containing different doses ALA (Sigma-Aldrich Chemical Co., USA); 0.5 mM, (ALA0.5); 1 mM (ALA1); 2 mM (ALA2), 4 mM (ALA4) and no additive (control), respectively. Then all aliquouts were slowly cooled to 4°C equilibrated for 4 h. Diluted semen samples were loaded into 0.25 ml French straws after equilibration and frozen in a programmable digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen at -196°C.

Assessment of Sperm Quality

Progressive and total motilities were evaluated using a computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA). In addition CASA was also used to analyse sperm motility characteristics. CASA was set up as pre-adjusted for bovine sperm analysis. A semen sample was diluted 1:4 in lactate ringer and then diluted semen sample was put onto a pre-warmed chamber slide (20 mm; Leja 4; Leja Products BV, The Netherlands), and sperm motility characteristics were determined with a 10x objective at 37°C. The following motility values were recorded: motility (%), progressive motility (%), average path velocity, µm/s (VAP), straight linear velocity, µm/s (VSL), curvilinear velocity, µm/s (VCL), amplitude of lateral head displacement, µm/s (ALH), beat cross frequency, Hz (BCF). A minimum of 10 microscopic fields were analysed which include at least 300 cells, for each assessment. The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoa membranes. HOS test was performed by incubating 30 µl of semen with 300 µl of a 100 mOsm hypo-osmotic solution at 37°C for 60 min After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide and then was examined under a phase-contrast microscopy (400x, Olympus BX43, Tokyo, Japan) [13]. Two hundred spermatozoa were counted for their swelling,

which is characterized by coiled tail, meaning intact plasma membrane. For the evaluation of sperm abnormalities, two drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution [14]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000x, Olympus BX43, Tokyo, Japan) under oil immersion.

Biochemical Assays

The levels of lipid peroxidase (LPO) were assessed with the commercial LPO-586TM Oxis research kit, Glutathione peroxidase (GPx) levels with GPx-340TM Oxis research kit, superoxide dismutase with Sigma-Aldrich Fluka FL 19160 kit, Catalase (CAT) with OxisresearchTM Catalase-520TM kit, GSH with Oxis researh-420TM kit and antioxidant capacity with Sigma-Aldrich Antioxidant assay CS 0790 kit. The assesment of sperm biochemical assays was investigated using spectrophotometric analysis by the method of previously described by Tasdemir et al.[15].

Statistical Analysis

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

RESULTS

As seen in *Table 1*, treatment of extender with ALA groups caused a significant decrease in percentages of post-thaw sperm CASA, progressive motilities and sperm motility characteristics such as VAP, VSL and VCL as compared to the control except ALA0.5 ($P<0.05$). ALA0.5 and ALA1 exhibited the greatest values for membrane integrity than that of the other groups ($P<0.01$). Besides ALA0.5 showed lower percentages of acrosome and total abnormalities in comparison to the control ($P<0.05$, $P<0.01$) *(Table 1)*.

As seen in *Table 2*, there were no significance differences in GPx and LPO activity among treatment groups ($P>0.05$). Conversely GSH, CAT and total antioxidant activity were affected by supplementation of ALA, notably group ALA1 yielded the greatest results in comparison to the other groups ($P<0.05$).

Table 1. *Mean (±SE) sperm values in frozen thawed bull semen*

Tablo 1. *Boğa spermasının dondurma çözündürme sonrası ortalama spermatolojik değerleri*

Analysis	Control	Lipoic acid (0.5 mM)	Lipoic acid (1 mM)	Lipoic acid (2 mM)	Lipoic acid (4 mM)	P
CASA motility (%)	39.50 ± 3.33^{a}	43.25 ± 1.87^{a}	24.63 ± 3.06^{bc}	29.63 ± 4.75^{b}	18.13 ± 2.39^{c}	*
Progressive motility (%)	20.63 ± 1.65^{a}	23.63 ± 2.67^{a}	8.75 ± 1.70^{b}	11.25 ± 1.95^{b}	7.63 ± 1.49^{b}	*
VAP (µm/s)	90.88 ± 0.93^{a}	91.00 ± 1.96^{a}	75.75 ± 4.17^{b}	78.38 ± 4.26^{b}	64.25 ± 2.96^{c}	*
VSL (µm/s)	73.13 ± 0.93^{a}	70.00 ± 1.69^{a}	60.50 ± 2.99^{b}	61.00 ± 3.05^{b}	48.75 ± 2.43^{c}	*
VCL (µm/s)	148.25 ± 1.83^{ab}	156.63 ± 3.36^{a}	127.38 ± 6.81^{bc}	135.13 ± 7.72^{b}	111.25 ± 5.45^{d}	**
ALH (µm/s)	6.50 ± 0.14^{bc}	7.75 ± 0.31^{a}	6.25 ± 0.25^{bc}	6.75 ± 0.41^{ab}	6.63 ± 0.50^{bc}	*
BCF (Hz)	14.50 ± 0.71^{abc}	13.63 ± 0.57^{abc}	15.75 ± 0.80^{a}	12.38 ± 0.63^{c}	14.50 ± 1.41^{abc}	*
HOS T (%)	36.63 ± 0.84^{b}	42.75 ± 0.41^{a}	41.50 ± 0.42^{a}	37.00 ± 0.82^{b}	33.63 ± 0.37^{c}	**
Acrosome abnormalities (%)	5.75 ± 0.49^{ab}	4.38 ± 0.53^{c}	4.63 ± 0.32^{bc}	4.63 ± 0.57^{bc}	5.00 ± 0.39^{bc}	*
Total abnormalities (%)	14.25 ± 0.62^{b}	10.50 ± 0.98^{c}	11.88 ± 0.99^{bc}	13.88 ± 0.74^{b}	18.25 ± 0.96^{a}	**

^{a,b,c} *Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05)*

Table 2. *Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioxidant activities in frozen thawed bull semen*

Tablo 2. *Boğa spermasında dondurma çözündürme sonrası ortalama glutatyon peroksidaz (GPx), lipit peroksidaz (LPO), redükte glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Analysis	Control	Lipoic acid (0.5 mM)	Lipoic acid (1 mM)	Lipoic acid (2 mM)	Lipoic acid (4 mM)	P
GPx (mU/ml-10^{9} cell/ml)	15.00 ± 0.38	15.88 ± 0.79	14.88 ± 0.13	15.00 ± 0.27	14.88 ± 0.13	-
LPO (µm/ml-10^{9} cell/ml)	0.63 ± 0.32	0.25 ± 0.25	0.13 ± 0.13	0.38 ± 0.26	0.38 ± 0.18	-
GSH (µm/ml-10^{9} cell/ml)	18.25 ± 1.05^{c}	27.38 ± 8.86^{bc}	54.13 ± 11.71^{a}	30.00 ± 8.94^{b}	32.13 ± 9.50^{b}	*
CAT (µm/ml-10^{9} cell/ml)	11.63 ± 2.76^{b}	$6.25 \pm 2.13b^{c}$	24.00 ± 5.25^{a}	8.25 ± 2.34^{bc}	17.13 ± 4.08^{ab}	*
Total antioksidant activities (mmol/trilox/ml-10^{9} cell/ml)	10.25 ± 1.74^{bc}	6.63 ± 1.41^{bc}	18.50 ± 3.52^{a}	8.13 ± 1.54^{bc}	13.88 ± 2.75^{ab}	*

^{a,b,c} *Different superscripts within the same row demonstrate significant differences (* P<0.05)*

DISCUSSION

Oxidative stress is a condition associated with an increased rate of cellular damage induced by ROS [16]. Unsaturated fatty acid is high level take part in phospholipid of cell membrane and the supplementation to the extender for sperm freezing is promoted the sperm cryopreservation efficiency [17]. ALA and its reduced form have been referred to as a universal antioxidant that functions in both membrane and aqueous environment [18]. It increases in semen quality and minimized to the oxidative stress [19] and puts down a variety of ROS [7]. These facts indicate ALA thus to has a protective action against the impairment of mitochondrial function, oxidative damage and sperm motility [20]. Current study showed that addition of ALA did not improve the CASA and progressive motilities as well as sperm motility characteristics with respect to the control. Earlier reports have shown when ROS increased during cryopreservation in bovine sperm, it impairs sperm function [21]. The results related to CASA and progressive motilities are in consistent with our previous findings Tasdemir et al.[15] and Büyükleblebici et al.[22] which demonstrated that supplementing various antioxidants to freeze the spermatozoa did not have any marked effects on the progressive and CASA motilities. This study also similar to recent studies in the bull [23], boar [24], rabbit [25] and stallion [26] concluded that were no improvement of sperm motility during cryopreservation when their diet was supplemented with fatty acids. However, current findings disagree with Japanese Black bull study, the addition of linoleic acid albumin to the extender marked improved motility parameter after freeze thawing [27]. In point of post thaw sperm motility patterns (VAP, VSL, VCL, ALH and BCF), current findings contrary to those reported by Soares et al.[21] who demonstrated that no improvement dietary supplements of oleic and linoleic acids were deleterious to the quality of ram sperm. Besides, VAP, VSL and VCL were lower than previous reports in which supplemented with the different doses sugars [28] as well as different doses curcumin and dithioerythritol [29]. Based on our results, after all we can hypothesize that these discrepancies between the studies may be due to the testing form.

Phospholipids, which are cell membrane components, have ensured membrane fluidity, in the event they have protected bull spermatozoa from cold shock [30]. It shows that fatty acids are important compounds to prolong the viability of spermatozoa [31]. Similar to our findings, Bucak et al.[29] showed that bull sperm samples in which 0.5 mM curcumin had been added, caused a decrease on the total abnormality (20.40±2.36%) and an increase membrane functional integrity (54.40±2.09%), but their membrane integrity and total abnormality results were greater than those obtained in our study in which supplemented with ALA0.5. In a dog breed sperm freezing study, Michael et al.[32] reported that after freezing and thawing, supplementing N-acetyl cysteine, taurine and catalase, to freeze the spermatozoa showed marked effects on the sperm morphology and plasma membrane integrity. It was indicated the ALA had a protective action against to oxidative damage and abnormal sperm motility [20]. Otherwise, in contrast with our findings, it has been proposed the adding antioxidants to freeze the spermatozoa do not have any marked effects on the acrosome and total abnormalities [27].

Thiols are the major components of cellular antioxidant system, which play an important role in detoxification of xenobiotic compounds and in the antioxidation of ROS and free radicals [33]. Consistent to the present investigation, ALA is also reported to accelerate the glutathione pool by reduction of oxidized glutathione [8]. Consistent to the present investigation Selvakumar et al.[19] who demonstrated a significant advance treatment with ALA increased in semen quality and minimized to the oxidative stress. Besides, Bucak et al.[34] reported antioxidants can improve sperm morphology and functional membrane integrity without influencing ROS formation in Angora goat. Otherwise, the addition of inositol do not improve sperm and biochemical parameters [35]. ALA0.5 showed a clear effect on sperm morphology and the plasma membrane integrity. However, this healing effect was not supported by antioxidant activities. This suggests that ALA0.5 might be due to the direct effect on the sperm morphology and membrane integrity or might be the antioxidant properties of egg yolk proteins, which are capable of preventing the oxidation of polyunsaturated fatty acids following freeze-thawing. Besides, it has dentrimental effect excess of 0.5 mM.

In conclusion, findings generated here showed that ALA0.5 supplementation in Tris based semen extender was of great beneficial effect on frozen-thawed bull semen in terms of morphology and plasma membrane integrity.

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Research Article

Prevalence of *Bartonella henselae* in Pet and Stray Cats from the Aspect of Public Health: A Research Sample in the Concept of One Medicine - One Health [1] [2]

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Abstract

Cat Scratch Disease (CSD) is an important zoonosis seen in cats and a public health problem in all over the world. In this study, prospective cross-sectional serologic survey and examination of local health authority records for CSD, the seroprevalence of antibodies against *Bartonella henselae* in pet and stray cats, and its public health aspect were investigated. Total antibodies to *B. henselae* were evaluated by indirect fluorescent antibody test (IFAT) in serum samples taken from 93 pet cats and 93 stray cats from the Selcuk University Veterinary Faculty Animal Hospital and Konya Municipality Stray Animal Shelter. Percentages of pet cats and stray cats seropositive for antibodies against *Bartonella henselae* (26.88% and 41.94%, respectively) were significantly higher than percentages of pet cats. Total seroprevalence of *Bartonella henselae* was found to be 34.41% in the study. A total of 438 CSD cases were identified in the Konya region according to the data received from local health authority records in the previous 1.5 years (2011-2012). Stray cats have higher seroprevalences of antibodies against *Bartonella henselae*, but this likely was related to greater exposure to vectors of these organisms. In conclusion, it was observed that CSD is an important risk for public health in Konya region. Therefore in order to decrease CSD prevalence in this region and prevent transmission of the disease to humans, information, treatment and prevention studies must be carried out within the One Health concept.

Keywords: *Cat scratch disease, Zoonosis, Public health, Indoor cat, Stray cat*

Halk Sağlığı Açısından Pet ve Başıboş Kedilerde *Bartonella henselae* Prevelansı: Tek Tıp - Tek Sağlık Konseptinde Örnek Bir Çalışma

Özet

Kedi tırmık hastalığı (Cat Scratch Disease, CSD), kedilerde görülen ve bütün dünyada halk sağlığı problemi olan önemli bir zoonozdur. Bu çalışmada, prospektif kesitsel tarama ile pet ve başıboş kedilerdeki *Bartonella henselae* antikorlarının seroprevalansı ve bölge sağlık kuruluşlarının CSD kayıtları üzerinden hastalığın halk sağlığı açısından durumu araştırıldı. Total *B. henselae* antikorları, Selçuk Üniversitesi Veteriner Fakültesi Hayvan Hastanesi ve Konya Büyükşehir Belediyesi Geçici Hayvan Bakımevi'nden 93 pet ve 93 başıboş kediden alınan serum örneklerinde indirekt flörosan antikor testi (IFAT) ile değerlendirildi. *B. henselae* seropozitifliği pet ve başıboş kediler (sırasıyla, %26.88 ve %41.94) arasında yapılan karşılaştırmada, başıboş kedilerde belirgin şekilde yüksekti. Çalışmada, *B. henselae*' nın total seroprevalansı %34.41 olarak bulundu. Bölge sağlık örgütlerinin 1.5 yıllık (2011-2012) kayıtlarına göre Konya bölgesinde toplamda 438 CSD vakası görüldüğü tespit edildi. Başıboş kedilerde *B. henselae* seroprevalansının yüksek olması, başıboş kedilerin vektörlerle yoğun temasta olmalarına yorumlandı. Sonuç olarak, Konya bölgesinde CSD'nin halk sağlığı açısından önemli bir risk olduğu gözlendi. Bu çerçevede bölgedeki CSD prevalansını azaltmak ve insanlara geçişini önlemek için tek sağlık konsepti içerisinde bilgilendirme, tedavi ve koruma çalışmaları yapılması gerektiği sonucuna varıldı.

Anahtar sözcükler: *Kedi tırmık hastalığı, Zoonoz, Halk sağlığı, Evcil kedi, Başıboş kedi*

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INTRODUCTION

One Health (also known as One Medicine) describes veterinarians and physicians working together to advance the health and well-being of both humans and animals. In a broader concept, it also includes collaboration with members of the public health community and, other health care professionals as well as biomedical research scientists. One of the primary goals of the One Health concept is to advance the understanding, prevention, and treatment of zoonotic disease [1]. CSD, known since the 1950s, is an important zoonosis, caused by *Bartonella henselae* [2-5]. Cats act as reservoirs in the transmission of *Bartonella henselae* to humans [2-4]. Spreading of *Bartonella henselae* from cats to humans is either directly by cat scratch and bite or indirectly by cat fleas and flea excrement [4-6]. Cat fleas harbour *Bartonella henselae* in their intestines, spread it in the environment via faeces and transmit the infection among cats. Settling of flea faeces between teeth during scratching or grooming with claws contaminated with flea faeces increase the possibility of transmitting the infection to humans through biting [4,7]. Ticks (Ixodesricinus) may act as a vector (trans-stadial transmission) in the transfer of *Bartonella henselae* among cats, humans, dogs and other mammal species [4]. In general, progressing asymptomatically in cats, CSD is a natural infection characterized by mild clinical symptoms in cat owners [8].

In humans, *Bartonella henselae* causes CSD [9], bacillary angiomatosis [6], bacteriaemia and extended fever [10], benign regional lymphadenopathy [11], and stomatitis [8]. Clinically, CSD progresses in typical and atypical forms. Its typical form is characterized by erythematous papules in the scratch or bite area and lymphadenitis in the nearest lymph node [11,12]. A painless erythematous papule or pustule with a diameter of 0.5-1 cm develops within 3-10 days in the scratched or bitten area. In 2-3 weeks, the papule or pustule usually heals without leaving a scar. Regional lymphadenitis follows more than 80% of the cases and 10% of these have a suppurative character. Within 1-7 weeks, the nearest lymph node enlarges, becomes sensitive and lymphadenitis develops. Lymphadenitis continues for 2-4 months or longer [11,13]. If the immune system of the host is sufficient it recovers on its own, however, if the immune system is compromised then generalized lymphadenopathy may develop. Potentially, this may lead to fatal disorders, particularly neuroretinitis, uveitis, endocarditis and neurological disorders in the atypical form [4]. Atypical manifestations may develop in 5% to 15% of humans with cat scratch disease; these may include Parinaud's oculoglandular syndrome, encephalitis, endocarditis, hemolytic anemia, hepatosplenomegaly, glomerulonephritis, pneumonia, relapsing bacteremia, and osteomyelitis [11].

The aim of this study is to serologically determine the prevalence of the important zoonosis *Bartonella henselae* in pet and stray cats in the Konya region and investigate its public health.

MATERIAL and METHODS

This study was approved by the Selcuk University Veterinary Faculty Local Ethics Committee (29.02.2012-2012/018).

Cats and Regional CSD Records

The animal material of this study consisted of 93 pet cats and 93 stray cats brought to the Selcuk University Veterinary Faculty Animal Hospital. In the context of this study, Local Health Authority records in the Konya region were examined and the number of patients visiting the 28 hospitals in the region with a complaint of cat bite/cat scratch was determined.

Sample Collection

Blood samples were collected from 93 stray cats and 93 pet cats by means of saphenous venipuncture. Samples in plain glass tubes were allowed to clot, and serum was obtained. Serum samples were frozen at –20°C until analyzed.

Testing Procedures

Presence of *Bartonella henselae* antibodies in the cat blood serum was established with a fluorescent microscope (Olympus BX50) using the IFAT (*Bartonella Henselae* IgG - IFA Vircell 200 test). Collected cat blood serum were defrosted at room temperature and diluted at a ratio of 1/64 with PBS prepared in the laboratory. 20 µl of the diluted serum was placed into wells in laboratory slides coated with antigens, the slides were placed into a laboratory incubator at 37°C with high humidity and incubated for 20 min. Following incubation, the slides were washed twice with PBS, 5 min apart, then washed with distilled water and left to dry. Into the wells on the dry slides, 20 µl cat conjugate diluted with 1/50 PBS was placed and the slides were incubated in an incubator with high humidity at 37°C for 20 min. After incubation the slides were washed and dried. VIRCELL mounting medium was put into the dry slide wells, covered with a cover slip and examined under a fluorescent microscope. Views were assessed in a darkened room under x 40 magnification with a fluorescent microscope. Observation of homogenous bacteria distribution giving out green-yellow fluorescence on a black background was considered to be positive *(Fig. 1)*.

Statistical Analyses

Results of seroprevalences of antibodies against *Bartonella henselae* were compared between stray and pet cats. Statistical analyses of data obtained within this study were carried out using the X^2 test, values of $P<0.05$ were considered significant [14].

Fig 1. IFAT test, *Bartonella* positive reaction notice that apple green flourescence

Şekil 1. IFAT testi, *Bartonella* pozitif reaksiyon, elma yeşili floresan görünüm

Table 1. *Seroprevalence of Bartonella henselae determined by IFAT in domestic and stray cats*
Tablo 1. *Sahipli ve sahipsiz kedilerde IFAT yöntemiyle tespit edilen Bartonella henselae seroprevalansı*

Cats	N	IFAT (+)	IFAT (-)	Seroprevalence (%)
Domestic	93	25	68	26.88
Stray	93	39*	54	41.94
Totally	186	64	122	34.41

* X^2 *value = 4.669, P<0.05*

RESULTS

Findings obtained in the light of the aims of the study carried out on pet and stray cats in the Konya region. In the scanning of 93 pet and 93 stray cats in the study region, 64 positive cats and 122 negative cats were identified. In the light of these findings, the seroprevalence of *Bartonella henselae* in the Konya region was found to be 34.41%. In the comparison regarding presence of *Bartonella henselae* in pet and stray cats, rate of positivity was found to be 26.88% in pet cats and 41.94% in stray cats *(Table 1)*. According to this, *Bartonella henselae* infection was seen to proceed at a significantly high level in stray cats.

In the context of this study, the number of patients admitted to the Infectious Diseases Department in a total of 28 hospitals in the Konya region, with a complaint of cat bite/cat scratch and pre-diagnosed with CSD, was seen to be 438 in the 1.5-year period between the dates this study was carried out (01/01/2011-30/06/2012).

DISCUSSION

Seroprevalence of antibodies against *Bartonella henselae* is ongoing in investigations carried out in many countries and its zoonotic potential is being evaluated. In France, in a study performed on 436 cats, bacteriaemia was identified in 72 cats and 179 cats were found to be seropositive regarding *Bartonella henselae* and/or *Bartonella clarridgeiae* [15]. It has been stated that, *Bartonella sp.* seroprevalence is higher in hot and humid climates [16]. In healthy pet cats, *Bartonella henselae* seroprevalence has been reported to be 17% in Thailand [17]; 9.6-19.6% in China [18]; 32% in Jordan [19]; 44.2% in Denmark [20]; and 54% in Indonesia/Jakarta [21]. In a pilot study carried out in healthy cats in Brasil, *Bartonella henselae* prevalence was found to be 47.5% and it was stressed that performing this study in the whole region to include larger populations of animals was very important for human and animal health [22]. In a study performed in the United States of America, where a total of 170 owned and stray cats, as well as cats from animal shelter were assessed, a *Bartonella henselae* seropositivity of 14.7% was determined and it was expressed that cat infections are an important source of zoonoses in humans [23]. In two separate studies carried out in the United Kingdom [24], and the United States [25], a relatively high seropositivity of 40.6% and 75% in pet cats and 41.8% and 93% in stray cats was reported, respectively. In the Czech Republic, while the total prevalence was 8% in a study including stray cats and cats in an animal shelter, bacteriaemia prevalence was determined to be 67% in stray cats and 5% in cats in the animal shelter [26].

While *Bartonella henselae* seroprevalence of 44.2% was determined, bacteremia prevalence was not statistically different between shelter/stray cats (13/49, 26.5%) and pet cats (8/44, 18.2%) in Denmark [20]. In Algeria, *Bartonella henselae* seroprevalence was found to be 17% in stray cats [27]. In a study carried out in Turkey, *Bartonella henselae* seroprevalence in cats in the Ankara region was found to be 18.8% [28]. In a study carried out in six different regions in Turkey, the total seroprevalent cat ratio was 28.9%. With varying seropositivity rates in Bursa (41.3%), Adana (33.9%), Burdur (32.3%), Aydin (27.5%), Kayseri (17.9%) and Istanbul (12.5%), it was stated that *Bartonella henselae* is a significant pathogen in Turkey [29]. In the present study, *Bartonella henselae* seroprevalence in owned cats and stray cats was determined to be 26.88% and 41.94%, respectively. Seropositivity rate in stray cats was found to be at a statistically significant high level (X^2 value = 4.669, $P<0.05$). The total seroprevalence level (34.41%) determined in this study is approximately within international (8-93%) and national (12.5-41.3) data range. These results clearly state that owned cats and stray cats are both reservoirs of *Bartonella henselae* infection. When hospital data examined in the context of this study is taken into account, it is seen that the number of patients pre-diagnosed with CSD (n: 438) in the investigation area is at a significantly high level. These results indicate that, in diseases with zoonotic potential such as this disease and similar ones, studies within the one medicine-one health concept must be carried out and preventative measures taken.

CSD is an important zoonosis seen in cats, which may spread to humans. In immune-competent patients, while *Bartonella henselae* leads to the acute infection known as CSD, it may cause widespread clinical diseases, such as bacillary angiomatosis, encephalopathy, peliosis hepatitis, splenitis, osteomyelitis and bacteriaemia in immune-compromised patients [2-4,11,30,31]. Treatment of Bartonellosis is carried out in the light of personnel experience, expert opinion and microbiological sensitivity data depending on the infection agent, clinical disease duration and immunological status of the patient [4,30,32]. Efforts to standardize antibiotic dose and duration treatment regimens, based upon both in vitro antibiotic susceptibility testing and patient outcome assessments are critically needed to effectively manage patients with neurobartonellosis and to elucidate the mechanisms by which chronic interplay between the host and bacteria ultimately leads to neurological manifestations [31]. In addition, antibiotics used in these treatments have been reported to be ineffective in the rate of recovery or success at any significant level [32]. In China, *Bartonella sp.* seroprevalence was found to be significantly high in people bitten by dogs and dog bite was reported to pose a risk with regards to *Bartonella* infection [33]. In a study carried out in Italy, contact with a cat was reported in 61 of 74 patients diagnosed with CSD and cat-related trauma in 49 patients. In a screening including 27 cats, some of which were owned by these patients and others not (domestic and stray), 9 of the 11 cats belonging to CSD patients and 2 of the 16 remaining cats were identified as being *Bartonella henselae* seropositive. In a general screening carried out in the region, *Bartonella henselae* seropositivity rate was found to be 23.1% [34]. These data indicate that preventative measures need to be taken for protection against and control of CSD. It is recommended that at-risk individuals should take certain precautions when coming into contact with cats; such as, adopting cats older than 1-year, avoiding adopting cats from animal shelters or crowded cat homes, health and flea control carried out by veterinarians, avoiding cat bite and scratches, cleaning bite and scratch wounds with soap and water and seeking medical advice, protection from flea infestations and other possible vectors and keeping cats indoors to prevent zoonotic risks [4]. In the present study, the number of patients with pre-diagnosed CSD in a total of 28 hospitals in the Konya region was seen to be 438 in the 1.5-year period. In the light of these studies for prevention of Bartonellosis, we believe that veterinary surgeons must collaborate with human medicine in their common field of studies within the frame of the one health concept. It will be advantageous to develop the one-health concept with studies such as; information given on subjects concerning human health despite being unrelated directly to pets and other animals, activating local and regional health units, encouraging the collaboration of veterinary surgeons and medical doctors in the management of immune-compromised people and pets, student exchange programs between veterinary and medical students and assessment of human- animal relationships in veterinary clinical procedures [1].

In conclusion, data from the present study illustrates that *Bartonella henselae* infection is an important zoonosis and a public health problem in the Konya region as well as all over the world. In this context, veterinary and medical health workers, particularly at-risk people, must be informed on the subject of CSD and a common working ground established.

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Research Article

Immunopathologic Evaluation of Estrogen Receptor, Progesterone Receptor, and Ki-67 Antibody Expressions in Canine Mammary Tumours [1]

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Abstract

This study evaluates the expressions of Estrogen Receptor (ER), Progesterone Receptor (PR) and Ki-67 antibodies in canine mammary tumours and their prognostic values in regard to their histomorphologic subtypes. For this purpose, 60 tissue samples suspected as canine mammary tumour were examined and classified according to World Health Organisation's (WHO) criteria. Samples were labelled with anti ER, PR and Ki-67 using immunohistochemistry while ER, and PR values were showed significantly higher in benign tumours (P<0.001, P<0.001, respectively), contraversely Ki-67 value was significantly greater in malignant tumours (P<0.001). The statistical difference between malignant tumour types according to ER, PR and Ki-67 expressions were significant (P<0.001, P<0.01, P<0.001, respectively). Among malignant tumours, solid carcinomas and spindle cell carcinomas had the highest Ki-67 and lowest ER and PR expressions, complex adenocarcinomas and carsinosarcomas had the lowest Ki-67 and highest ER and PR expressions. It is concluded that presentation of immunohistochemical expression of differences between ER, PR and Ki-67 in canine mammary tumours, will provide contribution to the evaluation of tumours and determination of treatment processes in veterinary clinical pathology.

Keywords: *Canine mammary tumour, Estrogen receptor, Progesterone receptor, Ki-67 antibody, Immunohistochemistry*

Köpek Meme Tümörlerinde Östrojen Reseptör, Progesteron Reseptör ve Ki-67 Antikor İşaretlenmelerinin İmmunopatolojik Olarak Araştırılması

Özet

Bu çalışmada, köpek meme tümörlerinde Östrojen Reseptör (ER), Progesterone Reseptör (PR) ve Ki-67 antikorlarının işaretlenmeleri ve bunların histomorfolojik alt tiplerine göre prognostic önemi değerlendirilmiştir. Bu amaçla, köpek meme tümörü şüpheli 60 doku örneği incelenip, Dünya Sağlık Örgütü'nün (WHO) kriterlerine göre sınıflandırılmıştır. Örnekler, anti ER, PR ve Ki-67 ile immunohistokimyasal yöntem kullanılarak işaretlenmiştir. ER ve PR değerleri iyi huylu tümörlerde belirgin olarak yüksek iken, tam tersine kötü huylu tümörlerde Ki-67 değerleri daha yüksekti (P<0.001). Kötü huylu tümör tipleri arasında da ER, PR ve Ki-67 işaretlenmeleri bakımından istatistiksel farklılıklar belirgindi (sırasıyla; P<0.001, P<0.01, P<0.001). Kötü huylu meme tümörlerinden solid ve spindle hücreli karsinomalarda en yüksek Ki-67 ve en düşük ER ve PR işaretlenmeleri saptanırken, kompleks adenokarsinomalar ve karsinosarkomalar ise, en düşük Ki-67 ve en yüksek ER ve PR işaretlenmelerine sahipti. Köpek meme tümörlerinde ER, PR ve Ki-67 işaretlenmelerindeki farklılıkların immunohistokimyasal olarak gösterilmesi, veteriner klinik patolojide tümörlerin değerlendirilmesine ve tedavi süreçlerinin belirlenmesine katkılar sağlayabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Köpek meme tümörü, Östrojen reseptör, Progesteron reseptör Ki-67 antikor, İmmunohistokimya*

INTRODUCTION

Among all canine neoplasias mammary gland tissue tumours take the second place after skin cancers [1]. In dogs, the developmental risk of malignant mammary tumours throughout life time ranges between 2-20% and this value is 2-5 times greater than the benign mammary tumours [2].

The prognosis of canine mammary tumours, their histopathology and proliferative activity have been intensively studied [3-6]. Intracellular steroid-hormone receptor proteins, primarily Estrogen Receptor (ER) and Progesterone Receptor (PR) have been studied both as indicators of prognosis and as guides to hormone and endocrine therapy [7-9]. Estrogen and progesterone hormones are known to play an

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important role in the normal physiology of the mammary gland but these hormones have also been implicated in tumour development [10-13]. The Ki-67 antigen is a nuclear protein expressed in all active phases (G1, G2, S) of the cell cycle except GO, thus it demonstrates the proliferative activity of cells [14-16].

In the presented study about canine mammary tumours the association between histomorphological tumour types, their steroid hormone expression values and proliferation activity was aimed to be determined. For this purpose, we meant to evaluate ER, PR and Ki-67 expressions in 60 tumour suspected canine mammary tissues by immunohistochemistry (IHC). Demonstration of ER, PR and Ki-67 expression in canine mammary tumours, and the histomorphological differencies between benign and malignant mammary tumours are valuable data in veterinary clinical pathology for prognosis. These findings are supportive for the prediction of the possible response of the patients to the hormonal therapy and therefore will be useful in enlightenment of the further studies to be carried out in this field.

MATERIAL and METHODS

Animals and Tissue Samples

Routine mastectomy materials from 60 female dogs admitted to Department of Obstetrics and Gynecology aging between 4-15 years were investigated. The collected tissue samples were fixed in 10% buffered formalin, processed by routine methods, embedded in paraffin wax, sectioned at 4-µm and stained with haematoxylin and eosin (H.E.) and labelled with immunohistochemistry.

Histologic Examination

The tumours were examined histologically and classified independently by two pathologists according to the criteria of the World Health Organization [17]. Tumour malignancy was graded histologically with the method of Nottingham modified by Elston and Ellis [18]. The histologic grade for each case was derived from the assessment of 1- tubule formation, 2- nuclear pleomorphism, 3- mitotic figures, and each feature being scored 1 to 3 points. The scores were then added together to obtain an overall tumour grade as follows: 3-5 points: well differentiated cells (grade 1); 6-7 points: moderately differentiated cells (grade 2); 8-9 points: poorly differentiated cells (grade 3). Tumours were classified histologically according to their most aggressive components.

Immunohistochemical Assay

Tissue sections from paraffin blocks collected into poly-L-lysine-coated slides. They were put through deparaffinization, antigen retrival was performed using citrate buffer solution (pH 6.0, 10 nM), and endogen peroxidase and then protein blocking procedures were applied. Slides were incubated with the primary Ab ER (Zymed®, UK, Lot.No. 18-0174Z), PR (Clone 10A9; Immunotech, France, Cat.No. 1546), Ki-67(Clone MIB-1; Dako, Denmark, Code No. M 7240) overnight 4°C with dilutions of 1:50, 1:30, 1:100 respectively. Then they were treated with commercially seconder antibody kit (2nd Generation LAB-SA Detection System, Histostain®-Plus Kits Zymed®, Carlsbad, Cat. No. 85 9843) and marked with DAB chromogen. Section for Ki67 were marked with AEC chrromogen. Finally, the sections were counterstained with Mayer's haematoxylin. The negative control sections were incubated with PBS instead of the primer antibody.

Immunohistochemical Ki-67, ER, PR Assay Scoring System

In the study positively stained cells in neoplastic and non-neoplastic epithelial mammary gland sections were counted and scored. For each antibody, the sections were examined under light microscope (Olympus BX50) first at low power (40X) to identify 10 areas with high numbers of positive cells and a total of 2.000 cells were counted within these 10 random high-power (400X) fields and their arithmetical mean was calculated. Ki-67, ER and PR were scored on the basis of their IHC staining intensity in positive control tissues sections (canine small intestine for Ki-67 and uterus for ER and PR). Each high-power magnification scored as;- (none or less than 5% positive nuclei), + (between 5-19% positive nuclei), ++ (between 20-59% positive nuclei), and +++ (60% or more positive nuclei).

Statistical Analysis

The tumours were grouped according to their histopathological features. Kruskal- Wallis and Mann-Whitney tests were used to compare the ER, PR and Ki-67 expressions of different tumour types. Besides, to assess the effects of giving birth or spaying on benign and malignant tumours Chi-Square test was performed.

RESULTS

Clinic and Pathologic Findings

The mean ages of the 60 female dogs at the time of tumour removal was 9.2 years. No significant difference between the ages of dogs with benign and malignant tumours was determined ($P>0.05$). The results were summarised in *Table 1*.

Of 60 dogs in the study, 24 of them been were spayed, the others were intact female dogs and among them only 27 of them gave birth previously. When the results from these dogs were compared, while there was no significant difference according to status of giving birth ($P>0.05$), a difference was determined according to their status of spaying ($P<0.05$) *(Table 1)*.

Table1.*Comparison of ER, PR and Ki-67 expressions, grade of malignancy in malignant mammary and benign tumours*
Tablo1.*Kötü ve iyi huylu meme tümörlerinde ER, PR ve Ki-67 işaretlenmeleri ve malignite derecelerinin karşılaştırılması*

Histopathologic Diagnosis		**N**	**Age**	**Birth**		**Spayed**		**Grade of malignancy**			**ER**				**PR**				**Ki-67**			
				No	**Yes**	**No**	**Yes**	**I**	**II**	**III**	**-**	**+**	**++**	**+++**	**-**	**+**	**++**	**+++**	**-**	**+**	**++**	**+++**
Benign Mammary Tumour	Simple adenoma	7 (53.8%)	7.9 (6-10)	3	4	2	5	Not Graded			-	-	5	2	-	-	6	1	7	-	-	-
	Basaloid adenoma	1 (7.7%)	11	-	1	1	-				-	-	-	1	-	-	1	-	1	-	-	-
	Fibroadenoma	2 (15.4%)	7.5 (5-10)	1	1	-	2				-	-	-	2	-	-	-	2	2	-	-	-
	Benign mixed tumour	3 (23.1%)	8.3 (6-11)	2	1	1	2				-	-	-	3	-	-	3	-	3	-	-	-
BENIGN TOTAL		13 (21.7%)	8.1 (5-11)	6 (46.2%)	7 (53.8%)	4 (30.8%)	9 (69.2%)	-			-	-	5	8	-	-	10	3	13	-	-	-
Malignant Mammary Tumour	Simple tubulopapillary carcinoma	11 (23.4%)	9.8 (7-12)	7	4	7	4	8	2	1	2	5	3	1	4	-	5	2	3	6	1	1
	Simple solid carcinoma	8 (17%)	12.1 (10-15)	4	4	7	1	2	2	4	3	3	2	-	5	3	-	-	1	1	3	3
	Spindle cell carcinoma	3 (6.4%)	9.6 (8-11)	-	3	2	1	2	-	1	1	2	-	-	2	1	-	-	-	2	-	1
	Complex carcinoma	12 (25.5%)	7.5 (4-10)	7	5	9	3	12	-	-	-	1	6	5	1	6	4	1	10	2	-	-
	Osteosarcoma	6 (12.8%)	9.5 (6-12)	5	1	3	3	Not Graded			6	-	-	-	6	-	-	-	4	2	-	-
	Carcinosarcoma	7 (14.9%)	9.3 (6-11)	4	3	4	3				-	3	4	-	-	4	3	-	6	1	-	-
MALIGN TOTAL		47 (78.3%)	9.5 (4-15)	27 (57.4%)	20 (42.6%)	32 (68.1%)	15 (31.9%)	24			4	6	12	14	15	6	18	14	12	3	24	14
TOTAL		60 (100%)	9.2 (4-15)	33	27	36	24	24	4	6	12	14	20	14	18	14	22	6	37	14	6	5

Time-to-onset of metastasis and overall survival times were not included in analysis because this information was incomplete in medical records examined.

Of the 60 tumours, 13 (21.7%) were histologically benign and 47 (78.3%) were malignant. The ratio of benign to malignant tumours was 1:3.6. 10 different histologic types of mammary tumours were represented. In the benign mammary tumours group; simple adenoma (N:7), benign mixed tumour (N:3), fibroadenoma (N:2), basaloid adenoma (N:1) were determined, and in the malignant mammary tumours group; complex carcinoma (N:12), simple tubulopapillary carcinoma (N:11), simple solid carcinoma (N:8), carcinomsarcomas (N:7), osteosarcomas (N:6), and spindle cell carcinomas (N:3) were determined.

From those malignant tumours (except carcinosarcomas and osteosarcomas) 24 were classified as grade I (WDC), 4 were classified as grade II (MDC), 6 were classified as grade III (PDC) by use of histologic grading.

Immunostaining

In neoplastic mammary tissues, ER and PR expressions was observed in the nucleus of normal and tumoural epithelial cells, but stromal cells did not have positive staining results for ER and PR. Characteristics of PR expressions in positive control tissues were similar to those for ER expression. Ki-67(MIB 1) positive reaction was expressed as a granular nuclear staining often with prominent nucleolar positivity only in neoplastic epithelial cells.

Homogeneous ++ and +++ ER and PR positive staining in ductal and alveolar epithelial cells were observed in all benign tumours and normal mammary glands. Ki-67 positive staining was not observed in any of these samples.

ER, and PR values were showed significantly higher in benign than in malignant tumours (P<0.001, P<0.001, respectively), but conversely Ki-67 value was significantly greater in malignant tumours than in benign tumours (P<0.001). The ER, PR and Ki-67 expressions in benign and malignant mammary tumours are given in *Table 1*.

ER, PR and Ki-67 expressions of malignant mammary tumours are given in *Fig. 1*. The statistical difference between malignant tumour types according to ER, PR and Ki-67 expressions were significant (P<0.001, P<0.01, P<0.001, respectively). Among malignant mammary tumours 35 were ER (+), 12 were ER (-) and 29 were PR (+), 18 were PR (-). All of the complex adenocarcinoma and carcinosarcomas were ER (+), PR (+) and they represented the highest values *(Fig. 2* and *Fig. 3)*. Simple solid and spindle cell carcinomas had low values of ER (+) and PR (+) expressions *(Table 1)*.

Among malignant mammary tumours 23 were Ki-67 (+), 24 were Ki-67 (-). Most of the simple solid carcinoma, spindle cell carcinoma and simple tubulopapiller carcinomas were Ki67 (+) and they had high expression values *(Fig. 4)*. Carcinosarcoma, complex carcinoma and osteosarcomas had low values of Ki-67 (+) expressions.

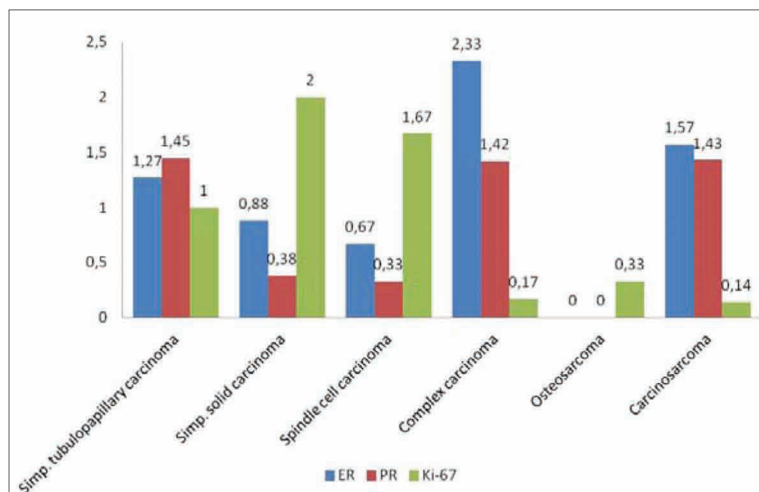
According to ER, PR and Ki-67 expression values statistically significant differences were observed between tumoural grades of malignant mammary tumours (P<0.01, P<0.01, P<0.001, respectively).

DISCUSSION

In the canine mammary tumour, age is clinically an important risk factor. It is a known fact that the frequency of mammary tumour development is directly proportional with age [19-21]. In the present study, average age at the time of surgery was 9.2 years (ranging from 4 to 15 years) which was similar to ages reported in previous studies [22,23]. In the presented study the benign mammary tumours were generally observed in dogs aged ≤7 years old and malignant mammary tumours were generally observed in dog aged between 10-11 years old. The difference, between benign and malignant mammary tumours was

Fig 1. ER, PR and Ki-67 levels of malignant mammary tumours

Şekil 1. Malign meme tümörlerinde ER, PR ve Ki-67 seviyeleri



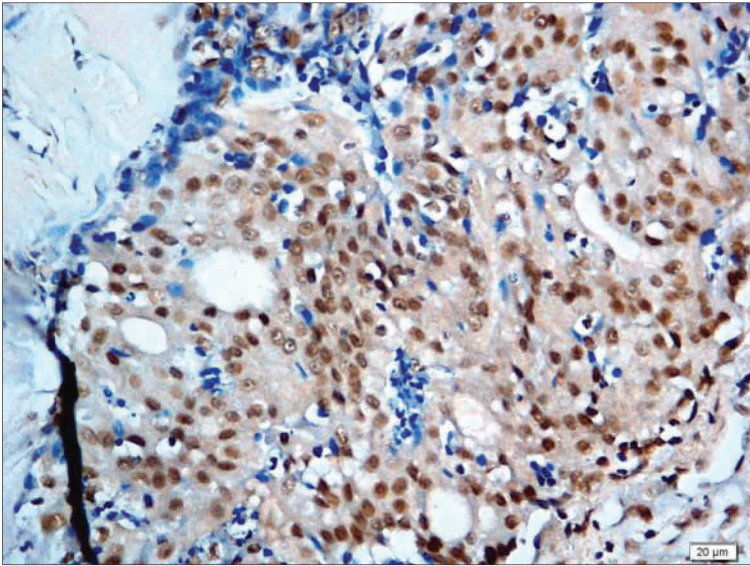


Fig 2. Complex carcinoma, diffuse, nuclear staining of tumor cells with IHC-ER. Streptavidin-biotin-peroxidase

Şekil 2. Kompleks karsinoma, IHC-ER ile tümör epitel hücrelerinde yaygın çekirdek boyanması, Streptavidin-biotin-peroxidase

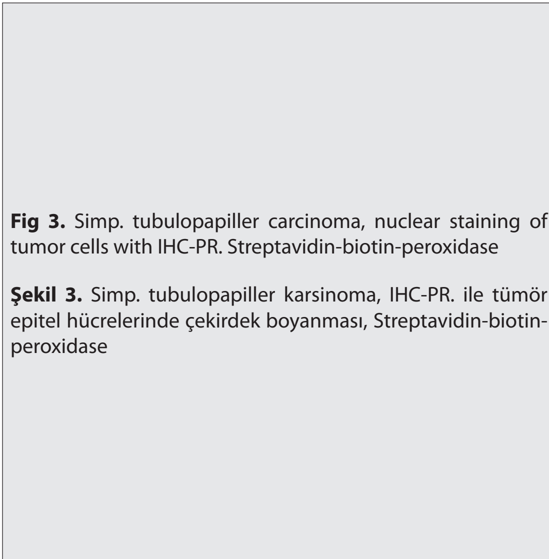
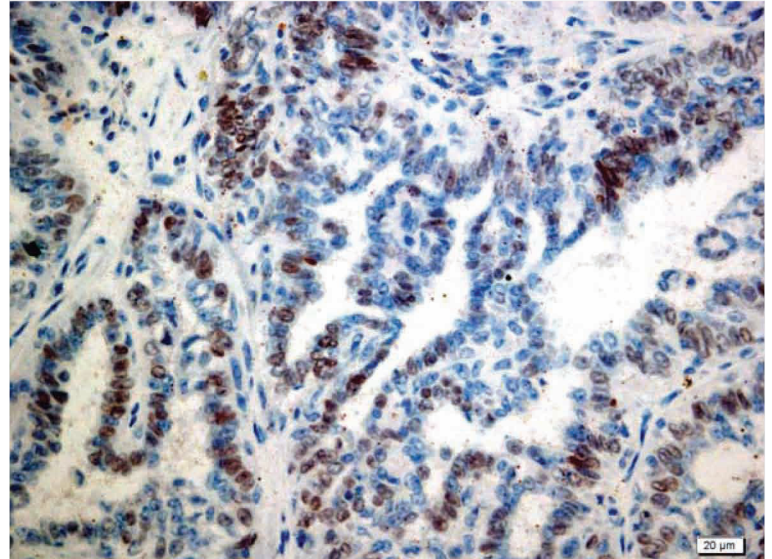


Fig 3. Simp. tubulopapiller carcinoma, nuclear staining of tumor cells with IHC-PR. Streptavidin-biotin-peroxidase

Şekil 3. Simp. tubulopapiller karsinoma, IHC-PR. ile tümör epitel hücrelerinde çekirdek boyanması, Streptavidin-biotin-peroxidase



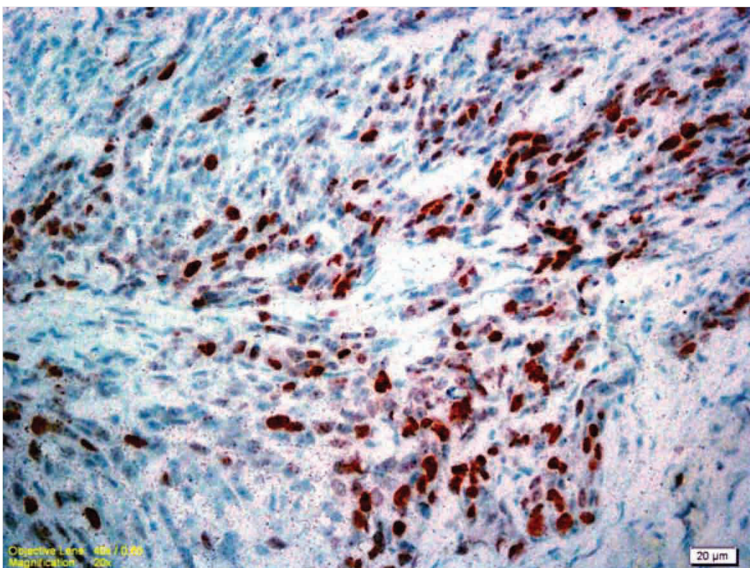


Fig 4. Spindle cell carcinoma, Diffuse, nuclear staining of tumor cells with IHC-Ki-67. Streptavidin-biotin-peroxidase

Şekil 4. Spindle hücreli karsinoma, IHC-Ki-67 ile tümör epitel hücrelerinde yaygın çekirdek boyanması, Streptavidin-biotin-peroxidase

not significant (P>0.05). Also, it was observed that the dogs diagnosed as simple solid carcinoma had highest mean age values (≥11) than other dogs. But conversely to our findings, Chang SC et al.[22] announced that age was not a significant prognostic factor in his research. Philibert et al.[24] reported that in dogs' age was not an important risk factor as in humans.

There was a statistical difference between benign and malignant mammary tumours according to the status of spaying (P<0.05), but there was no statistical difference according to the status of giving birth (P>0.05). Although spaying did not have statistically significant effect on ER and PR expression (P>0.05), it had a significant impact on the proliferative capacity (Ki-67) of the neoplastic cells (P<0.01). By these results we can conclude that spaying is a more preservative factor than giving birth for the formation of malignant mammary tumours. Parallel to our findings previous researchers [22,25,26] verify that in bitches ovariectomy offered a protective effect against the development of this condition and this effect is most pronounced if this operation is performed early in life, before 2.5 years of age.

In the presented study from 60 tumours 47 of them were malignant (78.3%) and 13 of them were benign (21.7%). This finding is compatible with previous reports about the prevalence of canine mammary tumours [20,27]. Tumours are recognised late because of dogs' long hair or late operative intervention. Even though those tumours are recognised in early stages, depending to the time period spent they generally expected to have malign transformation to take form because in dogs the prevalence of malignant mammary tumours are 3-4 times greater than benign tumours.

The clinical and histological characteristics of canine mammary tumours often do not provide sufficient predictive information for the clinician to know how the disease will behave in a given individual and how to implement therapy based on the predicted behaviour. Analysis of potentially important prognostic factors has been the focus of many studies [20,22,23,28,29]. Both ER and PR expression values and tumour cell proliferation (Ki-67) value are accepted to be important parameters for the evaluation of biologic behaviour and demonstrating the malignity potentials of mammary tumours [30]. Variable values detected in ER and PR levels of the mammary gland tumours are accepted to be an important evidence of the possible relation of the tumours with the hormonal factors [31]. In this study, 50 of 60 (83%) canine mammary tumours were positive for ER and/or PR, and 23 of 60 (38%) canine mammary tumours were positive for Ki-67. Statistically significant differences were detected in the expression of ER, PR and Ki-67 between benign and malignant mammary tumours. While ER and PR expression levels were high in benign mammary tumours conversely Ki-67 levels were only high in malignant tumours. This significant differences between benign and malign mammary tumours demonstrated that both hormone receptors and Ki-67 receptors had independent prognostic value. De Las Mulas et al.[4], Geraldes et al.[28], Rutteman et al.[32], Donnay et al.[33] and Nieto et al.[34] reported compatible results to present study.

In this study, it is determined that, among diagnosed malignant mammary tumours, tumours with dominant epithelial morphology such as simple solid carcinomas, spindle cell carcinomas and simple tubulopapiller carcinoma and tumours with dominant mesenchymal morphology such as carcinosarcomas, and complex carcinomas there were statistically significant differences between steroid hormone receptor expressions and tumour cell proliferation capacities just like their cellular morphologic characteristics differences. In other studies [13,30,35,36], parallel to our findings it was reported that solid carcinomas of the malignant mammary tumours have highest Ki-67 and lowest ER expressions. De Las Mulas et al.[4] determined that complex carcinoma and carcinosarcomas have higher ER and PR expression values than simple histologic subtypes of tumours. On the contrary, Yang et al.[37] stated that simple tubulopapillary carcinomas have higher Ki-67 expression than solid carcinoma and complex carcinoma had the highest Ki-67 expression values.

In the presented study, there was no significant difference between tumours with different grades according to their steroid hormone expressions, but there was significantly important difference according to their Ki-67 expression values.

Rutteman et al.[32] reported that ER, PR scores and Ki-67 values of both benign and malignant mammary tumours showed a non-significant inverse correlation. ER and PR expression decreased significantly with increased values of Ki-67, they reported that these decreases in hormone receptors depend to the increased cellular transformation and the loss of stimulant effects of hormones on epithelial tumour cells. According to the data we established in the presented study we share this reasoning.

Different immunohistochemical investigations contribute new possibilities to the veterinary clinic pathology, which are still ill-defined to demonstrate the differences and for the evaluation and treatment of these tumours even with the golden criteria of histomorphologic structures of canine mammary tumours.

In the presented study about canine mammary tumours an association between histomorphological tumour types, their steroid hormone expression values and proliferation activity was determined. We think that the presentation of these values contribute hugely to the determination of clinical and biologic behaviour of tumours and their malignancy potentials and indirectly interests the course of disease and determination of treatment.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Research Article

Determination of Helicobacter Infections with ^{14}C Urea Breath Test (^{14}C UBT) and Polymerase Chain Reaction (PCR) in Dogs and Treatment [1] [2] [3]

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[3] *This study is summarized from the PhD Thesis of Güvenç GOKALP*

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Abstract

It was aimed to determine that if ^{14}C UBT (^{14}C Urea Breath Test) application as a "Gold Standard" in the human practice for diagnosing Helicobacter infections could also be used in an easy, practical, and a reliable method in the veterinary practice. Three groups were allocated in the study. Dogs (n=41) were detected as clinically healthy in their physical examinations were grouped as healthy (Group I). Dogs (n=32) had clinical symptoms of vomiting after feeding, anorexia, epigastric pain during abdominal palpation were grouped as diseased and these diseased dogs (Group II) that were treated grouped as treatment (n=32) (Group III). Helicobacter staining, Polymerase Chain Reaction (PCR) and ^{14}C UBT were compared for diagnosis of Helicobacteriosis before and after therapy. Based on the results of the present study ^{14}C UBT was found 2 times more reliable when compared to PCR and 65 times more reliable than Helicobacter staining technique. Sensitivity for ^{14}C UBT, PCR and Helicobacter staining technique was found as 96.55%, 93.75%, and 59.38%, respectively. Moreover specificity of the ^{14}C UBT, PCR and Helicobacter staining technique was detected as 97.73%, 97.56% and 92.68%, respectively. Consequently, it was thought that ^{14}C UBT test could be used as a reliable method in veterinary practice.

Keywords: *Dog, Helicobacter spp, Diff-quick staining, PCR, ^{14}C Urea breath test*

Köpeklerde Helikobakter Enfeksiyonlarının ^{14}C Üre Nefes Testi (^{14}C UNT) ve Polimeraz Zincir Reaksiyonu (PZR) ile Belirlenmesi ve Tedavisi

Özet

Çalışmamızda; insan hekimliği pratiğinde Helikobakter enfeksiyonlarının teşhisinde "Altın Standart" olarak tabir edilen ve başarıyla uygulanan ^{14}C UNT (^{14}C Üre Nefes Testi)'nin Veteriner Hekimlik pratiğinde de kolay, pratik ve güvenilir bir yöntem olarak uygulanabileceğini belirlemeyi amaçladık. Çalışmamız klinik muayenesinde sağlıklı olan köpekler (n=41) (Grup I), anoreksi, yemeği takiben kusma ve epigastric ağrı şikâyeti ile kliniğimize gelen helikobakter enfeksiyonlu köpekler (n=32) (Grup II) ve tedavi edilen bu helikobakter enfeksiyonlu köpekler (n=32) (Grup III) olmak üzere 3 grup oluşturuldu. Helikobakteriyosizin karşılaştırmalı tanısı için, Helikobakter boyama, Polimeraz Zincir Reaksiyonu (PZR) ve ^{14}C Üre Nefes Testi (^{14}C UBT) tedavi öncesi ve tedavi sonrası köpeklere yapıldı. Çalışma sonucunda, ^{14}C UNT'nin PZR'a göre 2 kat ve Helikobakter boyama yöntemine göre 65 kat daha güvenli bir tanı yöntemi olduğu bulundu. Sensitivite; ^{14}C UNT'de %96.55, PZR'de %93.75 ve Helikobakter boyamada %59.38 bulunurken, spesifite sırasıyla; %97.73, %97.56 ve %92.68 olarak bulundu. Sonuç olarak, ^{14}C UBT'nin Veteriner Hekimlik pratiğinde güvenli bir metod olarak kullanılabileceğini düşünmekteyiz.

Anahtar sözcükler: *Köpek, Helicobacter spp., Diff- quick boyama , PZR , ^{14}C Üre nefes testi*

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INTRODUCTION

Helicobacter pylori was firstly described as a cause of b type human gastritis in 1982 in West Australia by Warren and Marshall [1]. Later it is named as *Campylobacter pyloridis* and its etiologic relations with gastritis were confirmed by applications of Coch Postulates [2].

The spiral shaped microorganisms in the dog stomach had been discovered a century before than the bacteria known as *H. pylori* in humans and later named as *Helicobacter* spp. It is believed that early identification of *Helicobacter* species in dogs related to their bigger morphological structures [3].

Among the gastric *Helicobacter* spp. species *H. heilmannii* group is detected in numerous mammalian species after *H. pylori*. They are presented most commonly in dog, cat, cheetah, pig, wild cats with multiple primate species and humans [4-6]. The clinical studies are obviously reported that spiral bacteria have been detected in the clinically healthy dogs' and cats' stomachs as much as their gastrointestinal diseased counterparts [6].

There are at least four spiral microorganisms- *H. felis* [5], *H. salomonis* [7], *H. bizzozeronii* [8,9]. *H. heilmannii* known that colonized in the dog and cat stomach.

In the last decade, O'Rourke et al.[10] succeeded in distinguishing the *H. felis, H. salomonis, H. bizzozeronii* ve *H. heilmannii* by sequence analyze of some of the urease gen complex. Later experimental studies are showed that *H. pylori* have similar disease procedures in dogs like humans [11].

The prevalence of *Helicobacter* spp. have been reported very high in the dogs and cats. It is reported in the healthy dogs as 67-100%, and 74-90% as in the infected dogs. On the other hand, in cats this prevalence is determined as 40-100% in healthy or infected cats [12].

However, it has not been clearly known how the *Helicobacter* spp. transmitted to people. It is propounded that *H. pylori* could be transmitted by oral-oral or fecal-oral way. Especially puppies in the lactation period have a very closer social life. Therefore, they could have exposed to *Helicobacter* spp. transmissions in their early lives [13].

Dogs and cats are propounded as reservoirs for transmission of *H. heilmannii* like organisms (HHLO) to people because of the progressed intimate life with humans and the companion animals [12].

Beyond the invasive approaches like endoscopy, rapid urease test, histology, culture, PCR (Polymerase Chain Reaction) that based on the phase-contrast microscopy of the stomach tissues, non-invasive approaches like serology, urea breath test, "*H. pylori* Stool Antigen test (HpSA)", stool, salvia or dental plaques are also could be used for detecting the microorganism with molecular techniques [14].

Urea breath is a non-invasive test that has a high sensitivity and specificity is applied with a radioactive method. Even it is a slightly expensive method; it could be used in both diagnosis and treatment [15].

Within the present study we tried to emphasize that ^{14}C UBT application in the human practice for diagnosing Helicobacter infections remains as a "Gold Standard" also could be used in an easy, practical, and a reliable method in the veterinary practice.

MATERIAL and METHODS

Dogs (n=41) belonging to Group I were clinically healthy and negative for *Helicobacter* spp by PCR and ^{14}C UBT. Inclusion criteria for Group II (before treatment) (n=32) were presence of clinical symptoms of vomiting after feeding, anorexia, epigastric pain during abdominal palpation and positivity of *Helicobacter* spp. by staining, PCR and ^{14}C UBT. All dogs in Group II were treated according to Aytuğ et al.[16] (amoxicillin, 20 mg/kg per orally, tid and with omeprazole, 0.75 mg/kg for one week). This group were classified later as Group III (after treatment) (n=32). On the other hand, care was taken that all groups had not received any antibiotics and active acid inhibitors one month before the procedure.

There were 3 German shepherds, 4 Golden Retrievers and 34 mongrels of which 31 were male and 10 were female in Group I. Group II were consisted of 22 Mongrels, 8 Golden Retrievers, 1 Doberman and 1 Anatolian Shepherd dog of which 15 dogs were male and 17 were female. Dogs in Group III were as in group II.

Complete blood count was performed in all groups. All blood samples were analysed by using an Abacus Vet Junior Hematology Analyzer (Diatron, Austria). All data were recorded into their individual protocols.

^{14}C - UBT test was applied to all groups. These animals, which were given the test, were starved for 6 h, free from antibiotics and active acid inhibitors for at least a month. At the same time, dogs of Group II were given ^{14}C- Urea Breath Test after therapy in order to detect the existence of Helicobacter *spp.* Before the test, the animals were made to swallow a ^{14}C- Urea capsule (HELICAP®)-with plenty of water- which requires no preparation, is easy to swallow, has no risk of spilling and is safe. After putting over the capsule, 2 mg/kg of 2% Xylazine hydrochloride was administered to the patient. There after this procedure breath collector was connected to the dry cartridge within 10 minutes. Suitable endotracheal tube was applied with sedation. The breath is collected with the attached system until the membrane's color of the cartridge changed to yellow from orange within 20 min (approximately this period continues 20 min). Then, dry cartridge system was scanned at an analyser (Heliprobe Analyser Nosterkibion System 2223- A™), and the results were received in 250

sec. The results evaluated as GRADE 0 meaning infection negative, GRADE 1 meaning suspicious; GRADE 2 meaning infected *(Table 1)*. GRADE 1 results were rechecked.

Gastroscopy was administrated to every dog that allocated in the study after applying the UBT test. Starting from the pharynx, in addition to the systematic examinations of oesophagus and antrum, the examination of the stomach was completed with the scrutinization along the angulus antrum and pyloric canal as a whole. Gastric smear and juice were collected during the examination of stomach with gastroscopy. The gastric smear was then put into the eppendorf tubes that filled 0.9% saline and samely gastric juice were collected into the eppendorf tubes and stored at -18°C until analysis. Every signalmen's and clinical data of the dogs were grouped and recorded in the individual protocols and all samples were numbered.

The smear applied on microscope slides were dried in the air and fixed with methanol, it was stained with diff-quick stains II and I and dried in the air for 2 min. Next, it was examined for S or spiral-shaped organisms by the light microscope with active x400 magnification. The results were recorded in the protocol [12].

The stomach contents and biopsy materials were used for DNA extraction. For this aim, commercially available DNA extraction kits (PureLink Genomic DNA Kits, Invitrogen, Canada) were used according to the manufacturers' instructions. The 100 µl of extracted DNA was kept frozen at -20°C until molecular tests were carried out. Genus-spesific PCR analysis was conducted as reported by Riley et al.[17] and the observation of a 375 bpm band was considered positive. Species-specific PCR was performed on DNA that belongs to the samples found out to be positive in species-specific PCR result. For that purpose, PCR was conducted separately for 16S rRNA gene [17,18], which is specific to *H. pylori*, for UreB gene [18,19], which is specific to *H. heilmannii,* and for 16S rRNA gene [19,20], which is specific to *H. felis* and *H. bizzozeronii*. The DNA bands of 295, 580, 577-596 and 636-656 bp were evaluated as positive for *H. pylori*, *H. heilmannii, H. felis* and *H. bizzozeronii*, respectively. All PCR analyses were conducted according to the methods and under the conditions stated in the related literature.

Data obtained from the study is the threshold character data obtained in existing (+) and non-existing (-) type. Hence, the most suitable mathematical modelling was investigated by initially determining the structure and the distribution of Binomial character data. In categorical data analysis, χ^2 test were used statistically [21]. The ethical committee approval numbered 2009-38 was taken from Ethical Committee for Experimental Animals of Ondokuz Mayıs University.

Table 1. *Evaluation of the Heliprobe Analyzer Results*
Tablo 1. *Heliprob Analizer ile Alınan Sonuçların Değerlendirilmesi*

Grading	Infection Status	d (cpm)
0	Infection negative	d ≤ 25 cpm
1	Suspicious	25 cpm < d < 50 cpm
2	Infection positive	d ≥ 50 cpm

RESULT

There were significant differences between WBC, RBC and RDWc of Group I, Group II and Group III (P≤0.001). Other parameters were not found to be statistically important *(Table 2)*.

The values obtained via the ^{14}C- UBT from all dogs are shown in *Table 3*. D value, which is the sum of ^{14}C isotope values gathered in d1 (lower ped) and d2 (upper ped), were detected to be 28.03±10.95cpm and 109±163cpm (P≤0.001), respectively in groups. A statistical comparison of d1 and d2 parameters between groups was not performed.

With the gastroscopic examination performed in accordance with the method, hyperaemia in gastric mucosa and oedema (erythematous) were detected in Group II. Gastric ulcer was detected in two cases.

According to the clinical examination data of the animals, of 32 dogs considered to have *Helicobacter* spp.-

Table 2. *The distribution and the significance level of the blood count values of animals included in the study*
Tablo 2. *Çalışmaya alınan hayvanların kan sayımı değerleri dağılımı ve önem derecesi*

Blood Count Parameters	Group I Healthy Group (n=41)	Group II Before Treatment (n=32)	Group III After Treatment (n=32)
WBC (10³ µl)	8.3±1.3ᵇ	15.34±6.67ᵃ	7.6±2.4 ᵇ
LYM (10³ µl)	2.42±1.64 ᵃ	3.93±1.77 ᵃ	3.42±1.36ᵃ
MONO (10³ µl)	0.44±0,27ᵃ	0.5±0.35 ᵃ	0.49±0.1 ᵃ
GRAN (10³ µl)	8.22±2.9ᵃ	8.2±3.6ᵃ	7.2±1.6 ᵃ
LYM (%)	20.78±8.66 ᵃ	20.92±13.82 ᵃ	20.41±7.1 ᵃ
MONO (%)	4.48±1.43ᵃ	3.93±2.19ᵃ	3.22±0.9 ᵃ
GRAN (%)	68.46±23.96 ᵃ	71.27±20.68 ᵃ	66.21±14.6 ᵃ
RBC (10⁶ µl)	6.08±1.17 ᵃ	5.13±1.27ᵇ	5.77±0.8 ᵇ
HGB (g/dl)	14.09±2.44 ᵃ	13.34±4.28 ᵃ	13.71±2.94ᵃ
HCT (%)	39.58±4.92 ᵃ	40.49±10.26 ᵃ	34±2.62 ᵃ
MCV (fl)	66.84±6.47 ᵃ	65.96±9.88 ᵃ	64.21±9.88 ᵃ
MCH (pg)	26.27±12.77 ᵃ	30.62±15.82 ᵃ	28.1±10 ᵃ
MCHC (g/dl)	36.31±8.82 ᵃ	36.21±8.21 ᵃ	36.9±9.2 ᵃ
RDWc (%)	19.93±4.7ᵇ	25.6±3.55ᵃ	21.2±2.4ᵇ
PLT (10³ µl)	414.52±166.24 ᵃ	405.24±191.26 ᵃ	366.3±117.2 ᵃ
PCT (%)	0.42±0.12 ᵃ	0.38±0.19 ᵃ	0.22±0.16 ᵃ
MPV (fl)	9.46±1.39 ᵃ	8.94±1.68ᵃ	8.44±1.2 ᵃ
PDWc (%)	38.6±4.7 ᵃ	39.06±4.72ᵃ	38.64±3.2 ᵃ

** Groups marked with a different letter are significant among themselves (P≤0.001)*

Table 3. *The distribution of d, d1 and d2 values obtained Via ^{14}C- UBT method used in the study*

Tablo 3. *Çalışmada uygulanan C^{14} UNT yöntemi ile elde edilen d, d1 ve d2 değer dağılımları*

^{14}C Isotope Values (cpm)	Group I Healthy Group (n=41)	Group II Before Treatment (n=32)	Group III After Treatment (n=32)
d1	15.03±7.54	108.7±92.27	21.2±10.2
d2	13±5.29	1±0.2	8.4±7.9
d	28.03±10.95 ^{b}	109±163 ^{a}	30.2±4.1 ^{b}

** Groups with different letters are significant among themselves (P≤0.001)*

induced gastritis, positivity was detected in 22 animals via diff-quick staining method.

According to PCR, taking the number of patients under consideration, of 32 dogs, while 29 of them had *Helicobacter* spp, 3 of them had *H. heillmanni* species. None of the other *Helicobacter* species were detected in our study.

DISCUSSION

S-structure microorganisms in the stomach of animals were first discovered at the end of the 19th century by Rappin. Later on, in 1893, Bizzozero discovered a type of bacterium that causes inhibition in gastric glands and canals. Salomon, on the other hand, discovered these types of microorganisms in the stomach of cats and rats [22].

In defining helicobacter infection, invasive intervention (endoscopy), which requires rapid urease test, histology, culture, tests based on Polymerase Chain Reaction (PCR) and examinations, such as phase contrast microscopy of the gastric tissue and non-invasive serology, ^{13}C and ^{14}C Urea Breath Tests (UBT), Helicobacter stool antigen test (HpSA) are used [14].

Among the invasive examination methods which require endoscopy are histological examination, bacteriological culture, PCR and rapid urease test. Both groups of diagnosis methods have advantages and disadvantages in terms of operation, the choice of patients, time needed to get the result, and costs [23].

Urea breath test, which is accepted as one of the non-invasive tests in the diagnosis of Helicobacter infections, provides an accurate diagnosis without performing endoscopy. It is also a test with high sensitivity and specificity which can be used both in the initial diagnosis of an active infection in unhealed patients and to follow up the effectiveness of the treatment in post-treatment period [24].

In our study, the average leucocyte value is 8.3±1.3x10^{3}/µl in the whole blood count analyses of samples taken from Group I, and the average leucocyte value in dogs with *Helicobacter* spp-induced gastritis is 15.34±6.67x10^{3}/µl (P≤0.001). While the average erythrocyte value in the whole blood count analysis of healthy dogs is around 6.08±1.17x10^{6}/µl, this average value in dogs with *Helicobacter* spp-induced gastritis has been found out to be 5.13±1.27x10^{6}/µl (P≤0.001). While this value ranges between the values 19.93±4.70 (%) in erythrocyte distribution range of Group I, it ranges between 25.6±3.55 (%) in dogs with *Helicobacter* spp-induced gastritis *(Table 2)*. In a study conducted on 34 cats and dogs, Jennifer et al.[25] established that there could be a reduction in the erythrocyte number of *Helicobacter* spp. types and in the erythrocyte distribution range due to Vitamin B_{12} and iron deficiency.

We think that the reason why 96.55% obtained in our study is higher than what other researchers obtained is that we transferred the other lung air to dry cartridges via endotracheal tube. However, in their study, Kubota et al.[26] used mask to prevent the contamination. On the other hand, because it is difficult to make an animal breathe directly and spontaneously into the kit used as suggested in the method performed on people, it might reduce the sensitivity of the study, transferring air in dry cartridges through endotracheal tubes increases the sensitivity of our research.

In two of the samples taken to detect Helicobacter species, the result of ^{14}C-UBT was positive, and the negative results of these samples were seen in PCR. It is thought that this situation results from the fact that samples taken through endoscopy for PCR were not taken from the prepiloric antrum region of the stomach [27]. In the same way, in spite of this false positivity obtained via ^{14}C- UBT, Zotta et al.[28] describe these false positive results may occur according to the urease activity of the *Streptococcus thermophilus* which exists in the gastric mucosa with *H. pylori*.

It has been established in our study that there is a high incidence of accuracy of ^{14}C-UBT in the diagnosis of *Helicobacter* infection *(Table 4, Table 5)*, and in line with our study, Raju et al.[29] stated in their study (n=64) that the positive result rate was higher in ^{14}C-UBT than in other diagnosis methods used in the diagnosis of *H. pylori* infection. In analyses performed in order to detect a healthy animal or to determine the existence of spiral bacteria activity, diagnosis with the ^{14}C-UBT method has been found out to be sensitive at a rate of 96.55%, while the sensitivity rate has been found out to be 93.75% in PCR method and 59.38% in *Helicobacter* staining method *(Table 4, Table 5)*.

^{14}C-UBT, which is one of the leading non-invasive methods used in the diagnosis of *Helicobacter* spp. in carnivores, has been found out to be 96.55% in our urea breath test sensitivity rate study in the detection of the existence of spiral bacterium before the designation of species. Additionally, in the study conducted by Wong et al.[30] (n=68), this rate was found out to be around 94.5%, and around 95-97% in the study (n=85) by Vaira et al.[31].

Table 4. *Frequency (%), odd's ratio's, $\chi^2_{sd=1}$ and P value distributions of methods used in the study*
Tablo 4. *Çalışmada uygulanan yöntemlerin frekans (%), odd's ratio's , $\chi^2_{sd=1}$ ve P değer dağılımları*

Method	Frequency (%)		Odds Ratio's			$\chi^2_{sd=1}$	P Value
			Additional Risk	Relative Risk			
	Helicobacter (-)	Helicobacter (+)		Helicobacter (-)	Helicobacter (+)		
Clinical Signs	41 (60.27)	32 (39.73)	-	-	-	-	-
Diff-quick Stain	51 (69.86)	22 (30.14)	18.51	5.46±0.04	1.88±0.01	23.13	0.001
PCR	44 (60.27)	29 (39.73)	600	28.34±0.02	4.12±0.03	64.88	0.001
^{14}C UBT	42 (57.53)	31 (42.47)	1204	29.52±0.04	4.28±0.01	61.33	0.001

Table 5. *Frequency (n), sensitivity, specificity and Q value distributions of methods used in the study*
Tablo 5. *Çalışmada uygulanan yöntemlerin frekans (n), sensivite, spesivite ve Q değeri dağılımları*

Method	Frequency (n)		Sensitivity (%)	Specificity (%)	Q Value (1- Specifity) (%)
	Helicobacter (-)	Helicobacter (+)			
Clinical Signs	41	32	-	-	-
Diff-quick Stain	51	22	59.38	92.68	7.32
PCR	44	29	93.75	97.56	6.25
^{14}C UBT	42	31	96.55	97.73	3.45

When compared to each other, the urea test is 65 times more reliable than *Helicobacter* staining method, twice as reliable as PCR analysis method that it is a non-invasive method increases its suitability among other methods used for diagnosis *(Table 4, Table 5)*. Similarly, in the study of Kopanski et al.[32] it was reported that urea breath test as a non-invasive choice for detection of Helicobacter infections had higher sensitivity.

^{14}C UBT has superiority for the diagnosis of the existence of spiral bacterium in dogs, when compared with PCR analysis and Diff-quick test in our study. Cartridges that belong to ^{14}C Helicobacter Urea Breath dry cartridge system were anaesthetized and connected to the end of the intubation tube with a tape, and then dogs were made to breathe into this cartridge for 20 minutes. As a result of this, the existence of *Helicobacter* spp. was established as well as 96.55%, sensitivity rate and specificity at a rate of 97.73%.

Consequently, within the present study we tried to emphasize that ^{14}C UBT application in the human practice for diagnosing Helicobacter infections remains as a "Gold Standard" also could be used in an easy, practical, and a reliable method in the veterinary practice.

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Research Article

Chewing Lice (Phthiraptera: Amblycera, Ischnocera) on Several Species of Wild Birds around the Lake Van Basin, Van, Eastern Turkey

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Abstract

This study was performed to detect chewing lice on the wild birds in Eastern Turkey, between April 2013-September 2014. 108 injured birds brought to Wild Animal Protection Center of Yüzüncü Yıl University were examined for louse. The feathers of each bird specimens were inspected for louse, macroscopically. Collected lice samples on the birds were preserved in 70% ethyl alcohol and mounted on slides in Canada balsam after transparented in 10% KOH. Fifteen (14.95%) out of the 108 were found to be infested with at least one chewing louse species. Nineteen lice species in 15 genera were found on the infested birds. *Goniocotes megalocephalus* (Uchida, 1916) on the Helmeted Guineafowl *(Numida meleagris)*; *Actornithophilus piceus lari* (Packard, 1870) on the Armenian Gull *(Larus armenicus)*; *Kurodaia fulvofasciata* (Piaget, 1880) on the Long-legged Buzzard *(Buteo rufinus)*; *Laemobothrion* sp. on the Golden Eagle *(Aquila chrysaetos)*; *Trinoton anserinum* (Fabricius [J.C.], 1805) and *Holomenopon* sp. on the Greylag Goose (*Anser anser*) were recorded for the first time from Turkey in this study.

Keywords: *Chewing lice, Wild bird, Lake Van Basin, Van, Turkey*

Doğu Anadolu'da Van Gölü Havzası Çevresinde Bulunan Çeşitli Yabani Kuş Türlerinde Saptanan Çiğneyici Bit (Phthiraptera: Amblycera, Ischnocera) Türleri

Özet

Bu çalışma Doğu Anadolu'daki yabani kuşlardaki çiğneyici bit türlerini belirlemek amacıyla Nisan 2013-Eylül 2014 tarihleri arasında yapılmıştır. Yüzüncü Yıl Üniversitesi Yaban Hayvanlarını Koruma Merkezine getirilen 108 yaralı kuş bit yönünden incelenmiştir. Kuşların tüyleri bit yönünden makroskobik olarak muayene edilmiş, toplanan bitler %70'lik alkol içinde saklanmış, %10'luk KOH'de saydamlaştırıldıktan sonra Kanada balsamı ile lamlara yapıştırılmıştır. İncelenen 108 kuşun %14.95'i en az bir bit türü ile enfeste bulunmuş ve enfeste kuşlarda 15 cinse ait 19 bit türü saptanmıştır. Beç tavuğundan *(Numida meleagris) Goniocotes megalocephalus* (Uchida, 1916); Van Gölü Martısı *(Larus armenicus)*'ndan *Actornitophilus piceus lari* (Packard, 1870); Kızıl Şahin *(Buteo rufinus)*'den *Kurodaia fulvofasciata* (Piaget, 1880); Kaya Kartalı *(Aquila chrysaetos)*'ndan *Laemobothrion* sp.; Yaban Kazı (*Anser anser*)'ndan *Trinoton anserinum* (Fabricius [J.C.], 1805) ve *Holomenopon* sp., Türkiye'den ilk kez bu çalışma ile bildirilmiştir.

Anahtar sözcükler: *Çiğneyici bit, Yabani kuş, Van Gölü Havzası, Van, Türkiye*

INTRODUCTION

Chewing lice are small, wingless, dorso-ventrally flattened insects that parasitize birds and some mammals. Most of the lice species on birds feed on feathers, dead skin and skin products. Some species also feed on blood. Chewing lice normally cause small and subclinical infestation but when present in large numbers thay can cause severe irritation and serve as a vector of some bloodborne parasites including some species of filarial worms [1,2].

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The Van province (38° 28′ N 43° 20′ E) is part of the Eastern Region of Turkey. The city is located around the Lake Van, the largest lake of Turkey. There are approximately 500 bird species so far recorded in Turkey [3]. Lake Van Basin lies along flyways of many migratory birds and this closed basin is hosted 213 of the bird fauna found in Turkey [4]. In addition, Erçek lake that host about 179 bird species is located 20 km east of Van city [5]. Erçek Lake takes as important bird area according to national and international marshy grouping and accepted B group of marshy region [6].

There are approximately 4.000 lice species on birds in the worldwide [7]. Some studies had been performed to detect on chewing lice on the birds in Turkey, recently [8-22]. Up to this time, more than 100 chewing louse species were reported from birds in Turkey. A few studies were done on chewing lice on birds in Eastern Turkey. Unfortunately, there is only one study [15] on the chewing lice on the birds in Van province.

This study was carried out to detect chewing lice species on birds in Van province in Turkey.

MATERIAL and METHODS

The study was carried out between April 2013-September 2014 in Wild Animal Protection Center, Yüzüncü Yıl University, Van, Eastern Turkey. In this period, 108 birds specimens in 16 genera, 10 families belonging to 9 orders were examined for louse *(Table 1)*. The feathers of each bird were inspected carefully for louse, macroscpically. The lice collected on the birds were preserved in alcohol 70%, transparented in 10% KOH for a day, washed with distilled water, passed in alcohol series 70%, 80%, 90% and 99% in four consecutive days and mounted on slides in Canada balsam. They were examined under binoculer light microscope (Leica DM750) and identified to species using some literatures [7,23-30].

RESULTS

Fifteen out of the 108 (14.95%) were found to be infested with at least one chewing louse species and 19 lice species belonging to 15 genera were identified on infested birds *(Table 2)*.

Ischnocera

Anaticola anseris (Linnaeus, 1758): Studied material: 6 Adult, Van-Özalp. Host; Greylag Goose *(Anser anser)*.

Anaticola crassicornis (Scopoli, 1763): Studied material: 1 ♀, 1 ♂, December, 2013, Van-Özalp. Host; Mallard *(Anas platyrhynchos)*.

Table 1. *Orders, families, genera and species of the examined birds in the study*
Tablo 1. *Çalışmada incelenen kuşların takım, aile, cins ve türleri*

Orders	Families	Genera	Species	Numbers of the Examined Birds
Accipitriformes	Accipitridae	*Aquila*	*Aquila chrysaetos*	6
		Circus	*Circus pygargus*	3
			Circus aeruginosus	2
		Buteo	*Buteo buteo*	3
			Buteo rufinus	1
Falconiformes	Falconidae	*Falco*	*Falco naumanni*	7
Strigiformes	Strigidae	*Bubo*	*Bubo bubo*	5
		Asio	*Asio flammeus*	4
Galliformes	Numididae	*Numida*	*Numida meleagris*	10
	Phasianidae	*Meleagris*	*Meleagris gallopavo*	1
		Coturnix	*Coturnix coturnix*	10
		Alectoris	*Alectoris graeca*	5
Anseriformes	Anatidae	*Anas*	*Anas querquedula*	2
			Anas clypeata	2
			Anas platyrhynchos	2
		Anser	*Anser anser*	1
Ciconiiformes	Ciconiidae	*Ciconia*	*Ciconia ciconia*	2
Charadriiformes	Laridae	*Larus*	*Larus armenicus*	21
Passeriformes	Corvidae	*Corvus*	*Corvus corone*	10
Coraciiformes	Meropidae	*Merops*	*Merops apiaster*	11
Total				108

Table 2. *Bird species, infestation rate and detected louse species*
Tablo 2. *Kuş türleri, infestasyon oranları ve saptanan bit türleri*

Bird Species	Locality	Number of the Examined Bird	Number of the İnfested Bird	Lice Species
Eurasian Eagle Owl *(Bubo bubo)*	Bitlis	5	-	-
Helmeted Guineafowl *(Numida meleagris)*	Muradiye -Van	10	1	*Goniocotes megalocephalus*
Golden Eagle *(Aquila chrysaetos)*	Hakkari	6	1	*Laemobothrion* sp.
Montague's Harrier *(Circus pygargus)*	Muş, Karasu-Van	3	-	-
Marsh Harrier *(Circus aeruginosus)*	Van	2	1	*Degeeriella fusca* *Kurodaia fulvofasciata*
Lesser Kestrel *(Falco naumanni)*	Van	7	1	*Degeeriella rufa* *Laemobothrion tinnunculi*
Common Buzzard *(Buteo buteo)*	Van	3	-	-
Long-legged Buzzard *(Buteo rufinus)*	Van	1	1	*Kurodaia fulvofasciata*
Short-eared Owl *(Asio flammeus)*	Van	4	-	-
White Stork *(Ciconia ciconia)*	Bitlis	2	1	*Neophilopterus incompletus*
Armenian Gull *(Larus armenicus)*	Van	21	3	*Actornithophilus piceus lari* *Saemundssonia lari* *Quadraceps punctatus*
Greylag Goose *(Anser anser)*	Özalp-Van	1	1	*Anaticola anseris* *Trinoton anserinum* *Holomenopon* sp
Garganey *(Anas querquedula)*	Van	2	-	-
Northern Shoveller *(Anas clypeata)*	Van	2	-	-
Mallard *(Anas platyrhynchos)*	Özalp-Van	2	1	*Trinoton querquedulae* *Anaticola crassicornis* *Holomenopon* sp.
Turkey *(Meleagris gallopavo)*	Van	1	1	*Chelopistes meleagridis*
Carrion Crow *(Corvus corone)*	Van	10	-	-
European Bee-eater *(Merops apiaster)*	Erciş-Van	11	3	*Meropoecus meropis* *Meromenopon meropis* *Meropsiella apiastri*
Common Quail *(Coturnix coturnix)*	Erciş-Van	10	-	-
Rock Patridge *(Alectoris graeca)*	Van	5	-	-

Chelopistes meleagridis (Linnaeus, 1758): Studied material: 12 ♀, 4 ♂, August, 2014, Van. Host: Turkey *(Meleagris gallopavo)*.

Degeeriella fusca (Denny, 1842): Studied material: 1 ♀, July, 2014, Van. Host: Marsh Harrier *(Circus aeruginosus)*.

Degeeriella rufa (Burmeister, 1838): Studied material: 6 ♀, 2 ♂, July, 2014, Van. Host: Lesser Kestrel *(Falco naumanni)*.

Goniocotes megalocephalus (Uchida, 1916): Studied material: 1 ♀, July, 2014, Muradiye-Van *(Fig. 1)*. Host: Helmeted Guineafowl *(Numida meleagris)*. This species was recorded for the first time on Helmeted Guineafowl in Turkey.

Meropoecus meropis (Denny, 1842): Studied material: 1 ♀, 2 N, September, 2014, Erciş-Van; 12 Adult, September, 2014, Erciş-Van; 12 Adult, 2 N, September, 2014, Erciş-Van. Host: European Bee-eater *(Merops apiaster)*.

Meropsiella apiastri (Denny, 1842): Studied material: 2 ♀, 1 ♂, September, 2014, Erciş-Van. Host: European Bee-eater *(Merops apiaster)*.

Neophilopterus incompletus (Denny, 1842): Studied material: 2 ♀, 8 ♂, September, 2014, Van. Host: White Stork *(Ciconia ciconia)*.

Quadraceps punctatus (Burmeister, 1838): Studied material: 3 Adults, April, 2013, Van. Host: Armenian Gull *(Larus armenicus)*.

Saemundssonia lari (Fabricius [O], 1780): Studied material: 4 ♀, 5 ♂, Van; 1 ♂, 1 N, April, 2013, Van. Host: Armenian Gull *(Larus armenicus)*.

Amblycera

Actornitophilus piceus lari (Packard, 1870): Studied material: 2 ♀, 1 N, April, 2013, Van *(Fig. 2)*. Host: Armenian Gull *(Larus armenicus)*. This species was recorded for the first time on Armenian Gull *(Larus armenicus)* in Turkey and in the World.

Holomenopon sp.: Studied material: 1 ♀ 1 ♂, December, 2013, Özalp, Van. Host: Mallard *(Anas platyrhynchos)*; 1 ♀, December, 2013, Özalp, Van. Host: Greylag Goose *(Anser anser)*.

Remarks: The *Holomenopon* specimens collected from Mallard and Greylag goose are different species from each other.

Laemobothrion sp.: Studied material: 1 N, June, 2014, Hakkari. Host: Golden Eagle *(Aquila chrysaetos)*. It was recorded for the first time on Golden Eagle *(Aquila chrysaetos)* in Turkey.

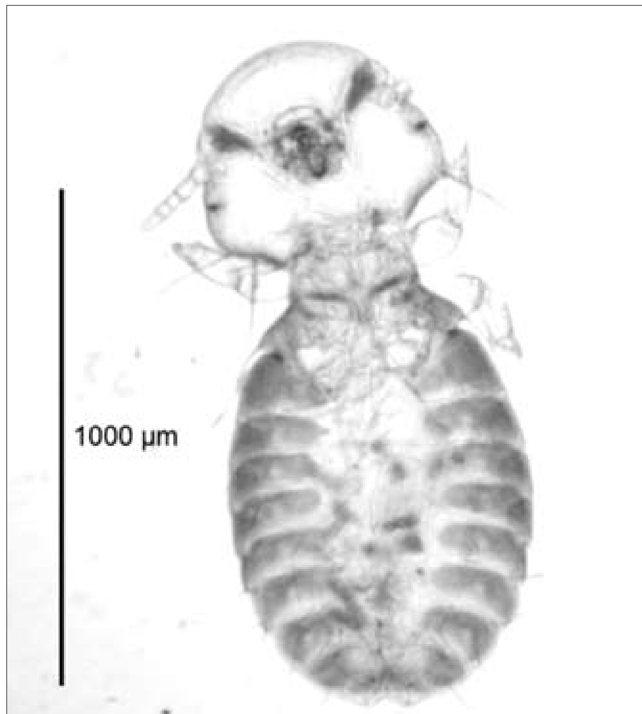

Fig 1. *Goniocotes megalocephalus,* famele, original
Şekil 1. *Goniocotes megalocephalus,* dişi, orijinal

Fig 2. *Actornitophilus piceus lari,* famele, original
Şekil 2. *Actornitophilus piceus lari,* dişi, orijinal

Laemobothrion tinnunculi (Linnaeus, 1758): Studied material: 1 ♀, July, 2014, Van. Host: Lesser Kestrel *(Falco naumanni)*.

Kurodaia fulvofasciata (Piaget, 1880): Studied material: 1 ♂, Van; 2 ♀, 9 ♂, 8 N, July, 2014, Van. Host: Long-legged Buzzard *(Buteo rufinus)*. It was recorded for the first time on Long-legged Buzzard *(Buteo rufinus)* in Turkey.

Meromenopon meropis (Clay & Meinertzhagen, 1941): Studied material: 2 N, September, 2014, Erciş,Van. Host: European Bee-eater *(Merops apiaster)*.

Trinoton anserinum (Fabricius [J.C.], 1805): Studied material: 2 ♀, December, 2013, Özalp, Van. Host: Greylag Goose *(Anser anser)*. It was recorded from Greylag Goose *(Anser anser)* for the first time in Turkey.

Trinoton querquedulae (Linnaeus, 1758): Studied material: 3 ♀, December, 2013, Özalp, Van. Host: Mallard *(Anas platyrhynchos)*.

DISCUSSION

Studies on the louse of birds and mammals in Turkey has not been completed. A total of 109 lice species belonging to 50 genera have recorded from animals and

humans in Turkey up to date [19]. Although approximately 500 bird specises were recorded from Turkey, all of the louse fauna infested these birds are still unknown. Up to this time, more than 100 chewing louse species have been reported from birds in Turkey [16].

This study is the first comprehensive study conducted on wild birds in Van region. In a previous study conducted on chewing lice on birds in Van, two species; *S. lari* and *Q. punctatus* had been reported from Yellow-legged Gull *(Larus michahellis)* [15]. In the present study, a total of 108 wild birds were examined for chewing lice fauna and 15 out of these (14.95%) were found to be infested with at least one chewing louse species and 19 lice species belonging to 15 genera were identified on the infested birds. Lice species detected in our study also have been reported from several hosts, previously, such that *Anaticola anseris* from Greylag Goose [12], *Anaticola crassicornis* from some duck species [9,22,31], *Chelopistes meleagridis* from Turkey [31], *Degeeriella fusca* from Marsh Harrier [13], *Degeeriella rufa* from Common Kestrel [16], *Meropoecus meropis* from European Bee-eater [10], *Meropsiella apiastri* from European Bee-eater [10], *Neophilopterus incompletus* from White Stork [10,16,17], *Quadraceps punctatus* from Yellow-legged Gull [15], *Saemundssonia lari* from Slenderbilled Gull [10] and Yellow-legged Gull [15], *Laemobothrion tinnunculi* from Kestrel [18], *Meromenopon meropis* from European Bee-eater [10], *Trinoton anserinum* from Wild Swans [21], *Trinoton querquedulae* from Ruddy Shelduck, Teal, Garganey, Northern Shoveller and Pintail [30].

In terms of host-parasite relationship, six new host records was reported in this study. One of the species found in this study is the new host record from Helmeted Guineafowl for both Turkey and the World. *G. megalocephalus* on Helmeted Guineafowl which is a new host record for the lice fauna of the World, is recorded for the first time in Turkey. The data in the previous study, reported only Hazelgrouse *(Tetrastes bonasia)* [32].

Other new host record was *A. piceus lari* which is one of the species identified in Armenian Gull. This new host record from Armenian Gull is recorded for the first time both in Turkey and in the World. This species has been previously reported from Yellow-legged Gull in Red Sea [33], Kelp gull (*Larus dominicus*) and Franklin's gull *(Larus pipixcan)* in Chile [34].

Kurodaia fulvofasciata is an amblyceran louse infesting some raptor species such as Common Buzzard, Little Banded Goshawk (Shikra), Short-toed Snake Eagle, Rough-legged Buzzard, Bald Eagle etc [7]. In addition, a study conducted in Israel reported that *Kurodaia fulvofasciata* was identified from Long-legged Buzzard *(Buteo rufinus)* in Israel [35]. This species has been reported from Common buzzard in Konya, Turkey, previously [20]. *K. fulvofasciata* recorded from Long-legged Buzzard, is recorded for the first time in Turkey as a new host. This species was also detected in Marsh Harrier *(Circus aeruginosus)* in this study.

There is no study on chewing louse species found on Golden Eagle in Turkey. For this reason, *Laemobothrion* sp.collected from Golden Eagle was one of the louse species detected for the first time in Turkey.

Trinoton anserinum was recorded from Greylag Goose *(Anser anser)* for the first time in this study, in Turkey. This species serve as intermediate host *Sarconema eurycerca* in Swans [2] and have been reported from Wild Swans in Samsun, Turkey [21].

Two *Holomenopon* specimens from Greyland Goose and one *Holomenopon* specimen from Mallard were collected in this study. It has been stated that *Holomenopon leucoxanthum* (Burmeister, 1838) was found on Greylag Goose, Mallard and some other ducks [7,36]. In addition, *Holomenopon maxbeirei* (Eichler, 1954) and *Holomenopon transvaalense* (Bedford, 1920) are found on Mallard [7]. Nevertheless, some morphological characters such as metasternal plate, shapes of the seta in the ventral anal fringe of *Holomenopon* specimens collected from Greylag Goose and Mallard in this study were different from those species. For these reasons, the specimens were not identified to species.

In conclusion, more information on chewing lice species infested wild bird will may be obtained with the extensive new studies that will be performed around the Van Lake basin that hosts approximately half of the Turkey's bird species.

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Research Article

Effects of Sperm from Different Bulls on Developmental Competence, Blastosist Quality and Cell Number of Bovine Embryos *In Vitro*

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Abstract

The aim of this study was to investigate the effects of sperm from different bulls on the developmental competence, blastosist quality and cell number of bovine embryos *in vitro*. *In vitro* matured bovine oocytes were fertilized with frozen-thawed sperm from five different bulls of Austrian Simmental Fleckvieh and then cultured in Synthetic Oviduct Fluid (SOF) medium at 38.5°C, 5% CO_2, 5% O_2 and 90% N_2 atmosphere with high humidity for 8 days. In the present study, there was no significant effect of bull variations on cleavage, morula, morula/cleavage, blastocyst and blastocyst/cleavage rates of embryos. Additionally, ICM cell numbers and ICM/total cells ratio of blastocysts obtained from *in vitro* fertilized oocytes with five bull's sperm were similar. However trophectoderm and total cell numbers of blastocysts obtained from *in vitro* fertilized oocytes with bull 5 sperm were lower than other bulls (P<0.05). In the present study, blastocysts quality was affected by bull variations and the percentage of excellent or good quality blastosist (Grade I) were lower (P<0.05) in embryos from fertilized oocytes with bull 5 sperm than those of other bulls, but the percentage of low quality blastosist (Grade III) were higher (P<0.05). There was a positive correlation between quality and total cell numbers of blastocysts in experimental groups. Result of present study showed that developmental competence of embryos *in vitro* were not affected by bulls variations, but may be influence blastocyst quality and cell numbers of blastocyst.

Keywords: *Bovine, Bull, in vitro fertilization, Embryo development, Blastocyst quality*

Farklı Boğa Spermalarının Sığır Embriyolarının *İn Vitro* Gelişim Yetkinliği, Blastosist Kalitesi ve Hücre Sayısı Üzerine Etkileri

Özet

Bu çalışma farklı boğa spermalarının sığır embriyolarının *in vitro* gelişim yetkinliği, blastosist kalitesi ve hücre sayısı üzerine etkilerini belirlemek amacıyla yapılmıştır. İn vitro olgunlaştırılan sığır oositleri, 5 farklı Avusturyan Simental Fleckvieh boğasına ait dondurulmuş-çözdürülmüş boğa sperması ile fertilize edilmiş ve sonra sentetik ovidukt sıvısı medyumu içerisinde yüksek oranda nemlendirilmiş 38,5°C, %5 CO_2, %5 O_2 ve %90 N_2 içeren atmosferde 8 gün boyunca kültüre alınmıştır. Sunulan çalışmada, embriyoların bölünme, morula, morula/bölünme, blastosist ve blastosist/bölünme oranları üzerine boğa çeşitliliğinin etkisi tespit edilmemiştir. Ayrıca beş farklı boğa sperması ile *in vitro* fertilize edilmiş oositlerden elde edilen blastosistlerin İHK hücre sayısı ve İHK/toplam hücre oranı benzer bulunmuştur. Ancak boğa 5 sperması ile *in vitro* fertilize edilmiş oositlerden elde edilen blastosistlerin trofektoderm ve toplam hücre sayısı diğer boğalardan daha düşük olduğu belirlenmiştir (P<0.05). Sunulan çalışmada blastosist kalitesi boğa çeşitliliği tarafından etkilenmiş olup mükemmel ve iyi kaliteli (Grade I) blastosistlerin yüzdesi boğa 5 sperması ile fertilize edilmiş oositlerden elde edilen embriyolarda diğer boğalarınkilerden daha düşük (P<0,05), fakat düşük kalitedeki (Grade III) blastosistlerin oranı ise daha yüksek olduğu belirlenmiştir (P<0.05). Bütün deneme gruplarında blastosist kalitesi ile toplam hücre sayısı arasında pozitif bir korelasyon saptanmıştır (P<0.05). Mevcut çalışmanın sonuçları boğa çeşitliliğinin *in vitro* embriyo gelişim yetkinliğini etkilemediği, ancak blastosist kalitesinin ve blastosistlerin hücre sayılarını etkileyebileceğini göstermiştir.

Anahtar sözcükler: *Sığır, Boğa, in vitro fertilizasyon, Embriyo gelişimi, Blastosist kalitesi*

INTRODUCTION

In vitro embryo production is one of reproductive biotechnology applications and increases the speed of genetic improvement in farm animals [1]. Moreover this reproductive biotechnology has enabled large amounts embryo production of superior breeds in various domestic animals and the production of large numbers of embryos for scientific research purposes from slaughtered and/or live animals [2,3]. Production of embryos *in vitro* also

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has potential for revolutionizing in cattle breeding and husbandry around the globe. Although several decades of research have gone into *in vitro* embryo production, the process involve *in vitro* embryo production is yet to be standardized and ratio of *in vitro* fertilized oocytes reaching the blastocyst stage is still low, approximately 30-40% [1]. Probably, the reason of this situation is *in vitro* embryo production practices cannot mimic *in vivo* conditions, resulting in alterations of development, morphology and gene expression in embryos [4].

Many factors can impact efficiency of *in vitro* embryo production [5,6] and one of these factors is use of sperm from different bulls [7]. Fertility ability of sperm is one of the main factors for success of *in vitro* fertilization, and previous studies reported that there are differences in *in vitro* embryo production rates among bulls [8-10]. When success rate of bulls in in-vitro fertilization is determined, *in vitro* embryo production may be standardized and decrease the differences in rate of embryos reach to blastocyst stage among bulls. Additionally genetic potential of sperm from different bull should be considered due to genetic information transmitted by sperm to the embryo. Moreover, embryo quality and success of *in vitro* embryo production applications can be influenced by genetic heritage of sperm [11]. The aim of the study was therefore to investigate the effects of sperm from different bulls on the developmental competence, blastosist quality and cell number of bovine embryos *in vitro*

MATERIAL and METHODS

All chemicals and media used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise indicated.

Collection and In Vitro Maturation of Oocytes

Cumulus-oocyte complexes (COCs) were obtained by aspirating antral follicles (2 to 5 mm in diameter) of bovine ovaries obtained from a local slaughterhouse. The COCs were collected in 3-4 ml Hepes-buffered Medium-199 containing Earle's salts and supplemented with 1% v/v antibiotic-antimycotic solution (10.000 IU penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml). COCs were assessed morphologically and only oocytes with compact, intact cumulus cells around and homogeneous cytoplasm were selected for maturation. COCs were washed three times in Hepes-buffered Medium 199, and then twice in maturation medium. Maturation medium were prepared as reported by Cevik et al.[12]. Maturation medium was sodium bicarbonate-buffered Medium-199 containing Earle's salts and L-glutamine supplemented with sodium pyruvate (5.5 µg/ml), antibiotic-antimycotic solution (1% v/v), heat-inactivated FCS (10% v/v), LH (5.0 µg/ml), FSH (0.5 µg/ml) and EGF (10 ng/ml). The COCs were placed in 500 µl of maturation medium (approximately 30-35 COCs per well) covered with 300 µl mineral oil in four-well dishes (Nunc, Roskilde, Denmark) and matured for 22 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

Sperm Preparation and In Vitro Fertilization

Cumulus expansion degree of COCs was assessed under a stereomicroscope at the end of the *in vitro* maturation period. COCs with full cumulus expansion considered as matured oocytes and immature COCs were discarded from experiment. The matured COCs were washed twice in Hepes-buffered Medium-199 and twice in fertilization medium. The fertilization medium was glucose-free modified TALP supplemented with 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/ml fatty acid-free BSA, 10 mg/ml heparin-sodium salt and 0.5 µl/ml antibiotic-antimycotic solution (pH 7.4 and 280-300 mOsm/kg). The matured COCs were then randomly separated and transferred into 45 µl fertilization drops (approximately 15 COCs per drop) covered with mineral oil. Frozen thawed semen from five different bulls (named; Bull 1, Bull 2, Bull 3, Bull 4 and Bull 5) of Austrian Simmental Fleckvieh was used for the *in vitro* fertilization in this study (Genovet L.T.D., S.T.I, Samsun, Turkey). Before *in vitro* fertilization frozen–thawed semen from five different bulls were separated by Percoll density gradient technique [13]. The percentage of sperm motility was visually evaluated using a phase-contrast microscope at a magnification of 400× (at least 80% progressively motile). The sperm concentration was determined by hemocytometer using a Thoma counting chamber. Sperm was then diluted to 2×10^6 sperm/ml with fertilization medium. COCs were fertilized randomly with 5 µl diluted sperm (approximately 13×10^3 sperm for per oocytes) from five bulls for 22 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

In Vitro Culture

After *in vitro* fertilization, the putative zygotes were washed three times in Hepes-buffered Medium 199 and vortexing for approximately 5 min, to remove the cumulus cells. The naked putative zygotes were then washed twice in Hepes-buffered Medium 199 and twice in synthetic oviduct fluid (SOF) embryo culture media. The SOF embryo culture media was supplemented with 20 µl/ml pyruvate, 8 mg/ml fatty acid-free BSA, 20 µl/ml MEM non-essential amino acids solution 100×, 10 µl/ml BME Amino Acids Solution 50× and 0.5 µl/ml antibiotic-antimycotic solution on the day of use. The naked putative zygotes were placed in 50 µl drops (approximately 15 zygotes per drop) of SOFaa media under mineral oil and cultured in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in air at 38.5°C. *In vitro* fertilization was considered as 0 day. On day 3 of development the proportion of zygotes cleaved was recorded. Morula and blastocyst development the proportion of zygotes were evaluated

on days 5 and 8, respectively. Experiments were repeated 5 times for each group.

Determination of Blastocyst Quality

The quality grading of the recovered blastocysts was done according to the morphological criteria of quality determined by International Embryo Transfer Society [14]. Blastocysts were classified as Grade I; excellent or good quality blastocysts with blastocoele filling the entire blastocyst, oval shaped and compact inner cell mass (ICM) and multicellular cohesive trophectoderm (TE), Grade II; moderate quality blastocysts with normal ICM, but non-optimal (fragmented or necrotic) TE, and Grade III poor quality blastocysts with very few cells or without ICM and with large vacuole instead of blastocoel.

Differential Staining of ICM and TE Cells

After determination of blastocyst quality, blastocysts were differential stained as described by Van Soom et al.[15] with some modifications. Briefly, the blastocysts were washed in PBS supplemented with 0.1 mg/ml poly-vinylalcohol and then incubated with picrylsulphonic acid diluted to with Ca^{2+}-free PBS (10 mM) for 5 min in the refrigerator (4°C). The blastocysts were then were washed in PBS supplemented with 0.1 mg/ml polyvinylalcohol and incubated for 30 min at 38.5°C in anti-dinitrophenyl antibody diluted to 30% (v/v) with Ca^{2+}-free PBS. The blastocysts were repeat washed in PBS supplemented with 0.1 mg/ml polyvinylalcohol and transferred into guinea pig complement (55852, ICN biochemicals, Irvine, CA, USA) diluted to 20% (v/v) with Ca^{2+}-free PBS supplemented with 50 µg/ml propidium iodide and 12.5 µg/ml bisbenzemide for 30 min at 38.5°C. Finally, blastocysts were fixed in 2% paraformaldehyde for 1-2 min at room temperature. Before mounting on slides with a 10 µl drop of 0.2 M 1.4 diazabicyclo-octanes in glycerol (50%, v/v) in Ca^{2+}-free PBS as an anti-fading solution. Blastocysts were examined under a fluorescence microscope (Nikon Invert Microscope Eclipse Ti-FL, 340-380 nm excitation and 430 nm suppression). ICM nuclei labelled with bisbenzimide appeared blue and TE nuclei labelled with both bisbenzimide and propidium iodide appeared pink to red *(Fig. 1)*. The numbers of ICM and TE nuclei were counted directly under the fluorescence microscope using a 345 nm ultraviolet light filter.

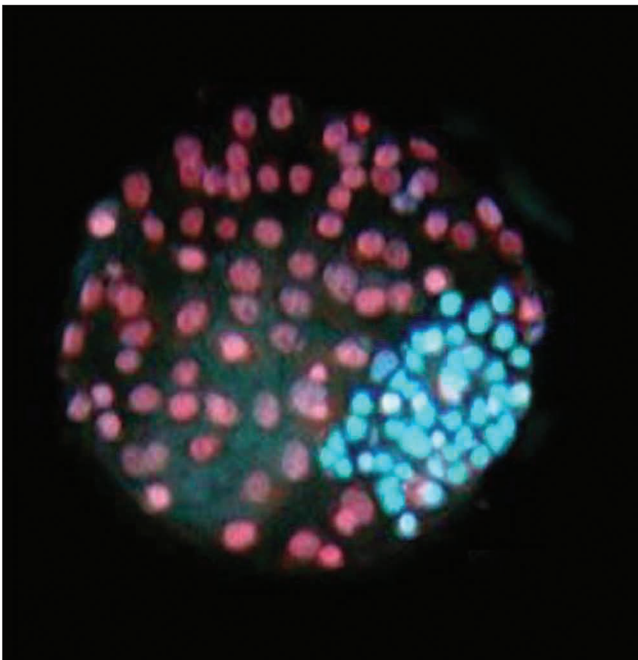

Fig 1. The picture of a blastocyst after differential staining (400× magnification)

Şekil 1. Diferansiyel boyama sonrası bir blastosist resmi (400× büyütme)

Statistical Analysis

Data were analyzed by one-way ANOVA after appropriate transformation where necessary (percentage of cleaved zygotes, morula and blastocyst yields, arcsine-transformation; cell numbers of blastocyst, log_{10} trans-formation). Data involved in blastocyst quality were analyzed by chi-square (χ^2) test. Relationships between quality and total cell numbers of blastocysts were determined with a Pearson correlation analysis at the 95% confidence interval. Significant differences between means were tested using Duncan's test with SPSS 20.0. The results are presented as untransformed mean ± SE values, and statistical significance was determined at the level of 0.05.

RESULTS

In the present study, total number of 2389 bovine cumulus-oocyte complexes (COCs) were used and maturated in standard maturation medium. Approximately 92% of COCs matured following *in vitro* maturation. *In vitro* matured oocytes were subjected to the *in vitro* fertilization procedure with sperm from various bulls and total numbers of 2198 bovine embryos were cultured in SOF media.

Developmental characteristics of *in vitro* produced bovine embryos obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 1*. There were no significant differences between bulls in terms of cleavage, morula and blastocyst formation rates. Similarly, there was no significant effect of bull variations on morula/cleavage and blastocyst/cleavage rates of embryos.

Inner cell mass, trophectoderm and total cell numbers of *in vitro* produced bovine blastocysts obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 2*. ICM cell numbers of blastocysts obtained from *in vitro* fertilized oocytes with five bull's sperm were similar, but trophectoderm and total cell

Table 1. *Developmental characteristics of in vitro produced bovine embryos obtained from in vitro fertilized oocytes with different bulls' sperm. Results are presented as untransformed mean values (± SEM) per groups for 5 replicates*

Tablo 1. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır embriyolarının gelişim özellikleri. Sonuçlar 5 tekrarlanmış grup başına dönüştürülmemiş ortalama değerler (± SEM) olarak sunulmuştur*

Bulls	Oocytes Fertilized (n)	Developmental Competence of Bovine Embryos (%)				
		Cleavage	Morula	Morula/Cleavage	Blastocyt	Blastocyst/Cleavage
1	420	73.8±4.03	34.2±4.23	46.3±4.03	25.37±3.79	34.02±4.71
2	498	69.5±2.08	44.6±2.84	64.7±5.54	29.23±1.73	42.34±3.03
3	476	73.9±1.69	39.3±4.18	52.5±5.23	29.26±4.63	39.48±6.02
4	424	74.8±4.11	34.1±3.47	45.4±3.17	26.76±3.95	34.93±3.86
5	380	73.1±2.86	37.4±1.63	51.8±3.87	25.38±0.91	34.92±1.62

Table 2. *Inner cell mass, trophectoderm and total cell numbers of in vitro produced bovine blastocysts obtained from in vitro fertilized oocytes with different bulls' sperm. Results are presented as mean values (± SEM) per groups for 5 replicates*

Tablo 2. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır blastosistlerinin iç hücre kitlesi, trofektoderm ve toplam hücre sayıları. Sonuçlar 5 tekrarlanmış grup başına ortalama değerler (± SEM) olarak sunulmuştur*

Bulls	Cell Numbers		
	Inner Cell Mass	Trophectoderm	Total
1	37.0±1.70	67.4±1.91^{a}	104.4±2.54^{a}
2	34.7±1.15	62.2±1.71^{a}	96.9±2.26^{a}
3	34.4±1.18	63.7±2.48^{a}	98.1±3.06^{a}
4	35.5±0.94	62.5±1.46^{a}	98.0±2.03^{a}
5	34.8±1.53	51.0±2.40^{b}	85.8±2.74^{b}

^{a,b} *Means in column with different superscripts are significantly different at P<0.05*

Table 3. *The percentages of blastocyst quality of in vitro produced bovine blastocyst obtained from in vitro fertilized oocytes with different bulls' sperm. Results are presented as mean values per (± SEM) groups for 5 replicates*

Tablo 3. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır blastosistlerinin blastosist kalite oranları. Sonuçlar 5 tekrarlanmış grup başına ortalama değerler (± SEM) olarak sunulmuştur*

Bulls	Blastocyst Quality		
	Grade I	Grade II	Grade III
1	36.5^{a}	60.4^{b}	3.1^{c}
2	24.4^{b}	73.5^{a}	2.1^{c}
3	21.7^{b}	72.4^{a}	6.9^{b}
4	26.9^{b}	71.4^{a}	1.7^{c}
5	12.5^{c}	78.3^{a}	9.2^{a}

^{a,b} *Means in column with different superscripts are significantly different at P<0.05*

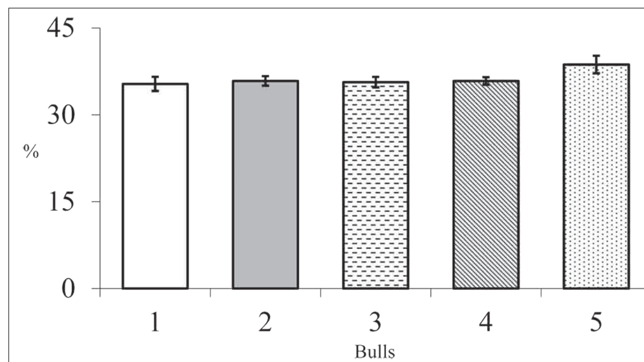

Fig 2. ICM/total cells number ratio of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm

Şekil 2. Farklı boğaların sperması ile *in vitro* fertilize edilen oositlerden elde edilen *in vitro* üretilmiş sığır blastosistlerinin İHK/toplam hücre sayısı oranlar

Table 4. *Pearson correlation coefficients (95% confidence intervals) between quality and total cell numbers of in vitro produced bovine blastocyst obtained from in vitro fertilized oocytes with different bulls' sperm*

Tablo 4. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır blastosistlerinin kalitesi ve toplam hücre sayısı arasındaki Pearson korelasyon katsayısı*

Bulls	Correlation Coefficients
1	.521*
2	.493*
3	.624*
4	.454*
5	.620*

** P<0.05*

numbers of blastocysts obtained from *in vitro* fertilized oocytes with bull 1 sperm were lower than those of other bulls (P<0.05). ICM/total cells number ratio of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Fig. 2*. There were no significant differences between bulls in terms of ICM/total cells ratio of blastocysts.

The percentages of blastocyst quality of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 3*. Blastocyst quality was dependent on bull variations. The percentage of excellent or good quality blastocyst (Grade I) were higher in embryos from fertilized oocytes with sperm of bull 1 than those of other bulls (χ^2 = 0.39; P<0.05), but moderate quality blastocyst

(Grade II) were lower in same bull than those of other bulls (χ^2 = 0.37; P<0.05). The percentage of poor quality blastocyst (Grade III) were higher in embryos from fertilized oocytes with sperm of bull 3 and 5 than those of bull 1, 2 and 4 (χ^2 = 0.32; P<0.05).

Pearson correlation coefficients (95% confidence intervals) between quality and total cell numbers of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 4*. There were positive correlations between quality and total cell numbers of blastocysts from *in vitro* produced bovine embryos obtained from *in vitro* fertilized oocytes with five bulls sperm (P<0.05).

DISCUSSION

The present study demonstrates that the use of semen from different bull for *in-vitro* fertilization did not affect developmental competence of embryos, but bull variations influenced trophectoderm and total cell numbers and quality of blastocyst derived *in vitro*. Additionally, excellent or good quality blastocysts had more total cell number than moderate or low quality blastocyst in the experimental groups.

The use of spermatozoa from different bulls during *in vitro* fertilization results in variable fertility rates [16]. Saeki et al.[17], Marquant-Le Guienne et al.[18] and Thara and Nair [19] showed that spermatozoa from different bulls have different rates of fertilization on *in vitro* matured bovine oocytes. Similarly, Totey et al.[20] and Jamil et al.[21] reported that fertilization rate was significantly different in buffalo oocytes inseminated with sperm from different buffalo bulls. Unfortunately, fertilization rates of *in vitro* fertilized oocytes with sperm from various bulls were not investigated in the present study, but Ciray et al.[22] indicated that early cleavage is a biological indicator of fertilization. Thus, it may be mentioned that the fertilization rates were not affected by sperm from various bulls in the present study.

Shamsuddin and Larsson [23], Zhang et al.[9] and Sudano et al.[16] reported that the use of sperm from various bulls or bulls with different fertility levels during *in vitro* fertilization leads to different cleavage rates of bovine embryos. Additionally, Al Naib et al.[10] found that *in vitro* fertilized oocytes with sperm from high fertility Holstein Friesian bulls had a higher rate of early cleavage compared with oocytes fertilized by sperm from low fertility Holstein Friesian bulls. Contrary to these studies, in the present study the cleavage rates were not affected by bull variations and average cleavage rate were 73% in the experimental groups. These finding is in agreement with the arguments of Schneider et al.[8] who reported that mean cleavage rates were similar in oocytes inseminated with semen from different bulls with different fertility levels. Also Galli and Lazzari [24] have compared cleavage rates of bulls and they have not seen a difference in the rates of cleavage between bulls. The differences in effects of bull variations on cleavage or fertilization rates with the above-mentioned studies may possibly be due to differences in age and breeds of bull, sufficient capabilities in *in-vitro* fertilization and metabolic characteristics of sperm cells.

Generally blastocyst yield is approximately 25-30% in *in-vitro* embryo production depending on embryo culture media and protocol [25]. In the present study, blastocyst development rates were approximately 27% in the experimental groups. Additionally developmental competence of embryos, obtained from *in vitro* fertilized oocytes with five bull's sperm, until the morula and blastocyst stage were similar in the present study. Moreover morula/cleavage and blastocyst/cleavage rates were similar among bulls. These results agree with the work of Shamsuddin and Larsson [23] who have demonstrated that the use of different bulls during *in vitro* fertilization have similar effect on embryo development until the morula and blastocyst stage. Similarly Sudano et al.[16] reported that although the cleavage rates observed between different bulls changed, differences were not observed among bulls in terms of blastocyst formation rates. On the contrary Akyol et al.[7] show that blastocyst development rate had a wide range among bulls. These differences may be explained with the variation at phenotypic traits and breed type of bulls.

Morphological observations are most widely used as indicator of blastocyst quality [26]. However the cell number of the blastocyst is a valid indicator of the viability of preimplantation embryos, while morphological criteria alone are poor indicators [27,28]. Additionally, Jiang et al.[29] reported that the cell number of *in vitro* derived blastocyst decreased with decreasing quality of the blastocyst. The analysis of distribution of ICM and trophectoderm cells by differential staining has been used as a technique to evaluate embryo quality in several species. Additionally ICM cells/total cell numbers ratio is an indicator of quality of blastocysts. In bovine embryos, poor quality is associated with blastocysts with low numbers of ICM or total cells [25,27,30]. In the present study blastocysts obtained from *in vitro* fertilized oocytes with five bulls's sperm had same cell numbers in ICM and the ICM/total cells ratios. However, trophectoderm and total cell numbers of blastocysts were different between bulls. Moreover quality of blastocysts obtained from *in vitro* fertilized oocytes with five bull's sperm quality of blastocysts varied between bulls. Generally, excellent or good quality blastocysts had more total cell number than moderate or low quality blastocyst in all bulls. Additionally the positive correlations observed between blastocysts quality and total cell number of blastocysts in all bulls. This result supports the view that total cell number influence blastocysts quality

and better quality blastocysts have more cell number than poor quality blastocysts.

The results of this study indicate that the use of sperm from various bulls in *in-vitro* fertilization does not have any effect on embryonic development. However, use of different bulls can influence the quality and cell numbers of the embryos reached the blastocyst stage in similar culture conditions. These results show that effects of bull have to be determined with preliminary studies in *in-vitro* researches and successful bulls should be selected for *in vitro* fertilization. Thus, the effects of bull on the success of *in vitro* embryo production may be eliminated. Moreover, determining the success rate after transfer of embryos obtained from *in vitro* fertilized oocytes with different bulls' sperm will help to identify the bull effect on *in vitro* embryo production more clearly.

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Research Article

Effects of Dietary Inactive Yeast and Live Yeast on Performance, Egg Quality Traits, Some Blood Parameters and Antibody Production to SRBC of Laying Hens

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Abstract

This study was carried out to determine the effects of dietary inactive yeast and live yeast on performance, egg quality traits, some blood parameters and antibody production to sheep red blood cell (SRBC) of laying hens during 16 weeks. A total of 96 Hyline Brown laying hens were allocated into one control group and three treatment groups each containing 24 hens. Each group had six replicate groups of 4 hens. A basal diet was supplemented with 1 g/kg inactive yeast (yeast autolysate, InteWall, *Saccharomyces cerevisiae*), 0.5 g/kg live yeast (InteSacc, *Saccharomyces cerevisiae*) and 1 g/kg inactive yeast + 0.5 g/kg live yeast in the diets of the first, second and third treatment groups, respectively. At the end of the study the results indicated that dietary treatments did not affect feed intake, interior and exterior egg quality characteristics. Dietary inactive yeast supplementation improved hen-day egg production (P=0.024) and feed conversion ratio (P=0.017) and decreased egg yolk cholesterol concentration (P=0.013). Antibody titers against SRBC and blood serum parameters were not affected by dietary treatments. The significant interaction was found in egg yolk cholesterol concentration (P=0.032) between inactive yeast and live yeast. As a result dietary inactive yeast at the level of 1 g/kg had beneficial effects in laying performance and in low cholesterol-egg production.

Keywords: *Blood characteristics, Egg quality, Inactive yeast, Live yeast, Performance*

Yumurta Tavuğu Karma Yemlerine İnaktif Maya ve Canlı Maya İlavesinin Performans, Yumurta Kalite Özellikleri, Bazı Kan Parametreleri ve SRBC'ye Karşı Antikor Üretimi Üzerine Etkileri

Özet

Bu araştırma yumurta tavuğu karma yemlerine inaktif maya ve canlı maya ilavesinin performans, yumurta kalite özellikleri, bazı kan parametreleri ve koyun eritrositine karşı (SRBC) antikor üretimi üzerine etkilerini 16 hafta süreyle incelemek amacıyla yapılmıştır. Toplam 96 adet Hyline kahverengi yumurta tavuğu her biri 24 adet içeren bir kontrol grubu ve üç deneme grubuna ayrılmıştır. Gruplar her birinde 4 tavuk bulunan altı tekerrür grubu kapsayacak şekilde düzenlenmiştir. Bazal karma yeme 1 g/kg inaktif maya (maya otolizatı, InteWall, *Saccharomyces cerevisiae*), 0.5 g/kg canlı maya (InteSacc, *Saccharomyces cerevisiae*) ve 1 g/kg inaktif maya+0.5 g/kg canlı maya ilave edilerek sırasıyla birinci, ikinci ve üçüncü deneme grupları karma yemleri oluşturulmuştur. Deneme sonucunda gruplar arasında yem tüketimi ile iç ve dış yumurta kalite özellikleri bakımından farklılık gözlenmemiştir. Karma yeme inaktif maya ilavesi yumurta verimini (P=0.024) ve yemden yararlanma oranını (P=0.017) olumlu yönde etkilemiş ve yumurta kolesterol konsantrasyonunu ise (P=0.013) azaltmıştır. Gruplar arasında SRBC'ye karşı antikor üretimi ve kan serum parametreleri bakımından farklılık gözlenmemiştir. İnaktif maya ve canlı maya arasında yumurta sarısı kolesterol konsantrasyonu bakımından önemli interaksiyon (P=0.032) bulunmuştur. Sonuç olarak inaktif mayanın 1 g/kg düzeyinde karma yeme ilave edilmesinin yumurta performansı ve düşük kolesterollü yumurta üretiminde yararlı olacağı kanısına varılmıştır.

Anahtar sözcükler: *Canlı maya, İnaktif maya, Kan parametreleri, Performans, Yumurta kalitesi*

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INTRODUCTION

Yeast products have been used increasingly in poultry diets as a feed additive after the ban on the use of antibiotic growth promoters in the EU. Inactive yeast as a prebiotics and live yeast as a probiotics are very important in growth promotion and disease resistance for poultry nutrition. Moreover, the effects of probiotics or prebiotics on the performance of livestock are contradictory with the improvements in some feeding trials [1].

There are some reports about the usage of various yeast and yeast products such as inactive dry yeast, yeast culture, yeast autolysate, yeast cell wall and live yeast in the diets of laying hens on performance [2-8]. Yalçın et al.[5] reported that yeast autolysate as inactive yeast at the levels of 2, 3 and 4 g/kg had beneficial effects on performance, egg cholesterol content and humoral immune response. However Sacakli et al.[3] concluded that inactivated brewer's yeast *(Saccharomyces cerevisiae)* had no beneficial effect on production parameters of hens fed with optimal diets and reared under proper management conditions. Hassanein and Soliman [9] concluded that dietary live yeast *(Saccharomyces cerevisiae)* supplementation at 4 and 8 g/kg can enhance the productive performance and nutrient utilization via the inhibitory effect of yeast against pathogenic bacteria. Chumpawadee et al.[10] observed that cassava yeast as probiotic source had positive effect on egg weight and egg shell thickness but had negative effect on egg production. Similarly, Dizaji and Pirmohammadi [11] reported that addition of yeast products to diets decreased egg production in laying hens. In the study of Ayasan et al.[12] dietary probiotic supplementation did not affect feed intake, feed conversion efficiency, egg weight, egg shell thickness and egg shape index but affected egg production and egg weight. However, as far as we know, there is no published report on the interaction of dietary inactive yeast and live yeast in laying hens. It was hypothesized that these two feed additives given in combination might enhance performance, egg traits and immune system.

Therefore the purpose of this study was to examine the effects of the dietary inactive yeast and live yeast on performance, egg quality traits, some blood characteristics and antibody titers to SRBC in laying hens.

MATERIAL and METHODS

Animals and Diets

A total of 96 Hyline Brown laying hens aged 54 wk were randomly assigned to one control group and three treatment groups each containing six replicate groups of 4 hens. They were housed in cages (30 cm x 44 cm x 44 cm) in a windowed poultry house with a 16/8 h light/dark regimen. Feed in mash form and water were provided *ad libitum* during the 16 wk experimental period. The diet was formulated to meet or exceed the nutrient requirements for Hyline Brown commercial layers [13]. The ingredients and chemical composition of the basal diet are shown in *Table 1*. The basal diet was supplemented with 1 g/kg inactive yeast, 0.5 g/kg live yeast and 1 g/kg inactive yeast + 0.5 g/kg live yeast in the diets of the first, second and third treatment groups, respectively. Inactive yeast (yeast autolysate, *Saccharomyces cerevisiae*, InteWall) and live yeast (InteSacc, *Saccharomyces cerevisiae*, $1x10^9$ cfu/g) derived from baker's yeast were obtained from Integro Food and Feed Manufacturing Company (İstanbul-Turkey). All animal use protocols were in accordance with the Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes [14]. This study was conducted by the researchers based on protocols by Ankara University Ethical Commission Report (No: 2008/18/72).

Measurements, Sample Collection and Laboratory Analysis

Nutrient composition of basal diet were determined according to the AOAC [15]. The samples were ashed in a muffle furnace prior to the analysis of calcium and total phosphorus [16,17]. Metabolizable energy levels of samples were estimated using the Carpenter and Clegg's equation [18].

Hens were observed daily for evaluating mortality during the experiment. Eggs were collected daily and egg production was expressed on a hen-day basis. All the eggs laid during the last two consecutive days of every week were collected and weighed individually to determine the egg weight. Feed intake was recorded biweekly and calculated as g per day per hen. The feed conversion ratio was calculated as g feed per g egg.

Table 1. *Ingredients and chemical composition of the basal diets*
Tablo 1. *Bazal karma yemlerin yapısı ve kimyasal bileşimi*

Ingredients (g/kg)		Chemical Composition (Analyzed)	
Corn	615.5	Metabolizable energy[b] (kcal/kg)	2750
Soybean meal, 44% CP	215.5	Crude protein (g/kg)	167.0
Full fat soya, 38% CP	50.0	Calcium (g/kg)	40.6
Limestone	95.0	Total phosphorus (g/kg)	6.2
Dicalcium phosphate	17.0		
Salt	2.5		
DL-Methionine	2.0		
Vitamin mineral premix[a]	2.5		

[a] Supplied the following per kilogram of diet: 12.000 IU vitamin A, 2.400 IU vitamin D_3, 30 mg vitamin E, 2.5 mg vitamin K_3, 2.5 mg vitamin B_1, 6 mg vitamin B_2, 4 mg vitamin B_6, 20 mg vitamin B_{12}, 25 mg niacin, 8 mg calcium-D-panthotenate, 1 mg folic acid, 50 mg vitamin C, 50 mg D-biotin, 150 mg choline chloride, 1.5 mg canthaxanthin, 0.5 mg apo carotenoic acid esther, 80 mg Mn, 60 mg Zn, 60 mg Fe, 5 mg Cu, 1 mg I, 0.5 mg Co, 0.15 mg Se; [b]Metabolizable energy content of diets was estimated according to the equation of Carpenter and Clegg [18]

To determine the egg internal and shell quality characteristics, 120 eggs laid at 09:00 to 12:00 h were collected randomly from each group (20 eggs from each replicate in total) during four consecutive days of last two weeks. Each egg was weighed and their shape index, shell breaking strength and shell thickness were measured. Then yolk height, albumen height, yolk width, albumen width and albumen length were determined. By using these values, yolk index, albumen index and Haugh units were calculated as shown with Yalçın et al.[6]. Egg internal and external quality analysis were completed within 24 h of the eggs being collected [6]. Egg quality evaluation was performed for individual eggs, as it was done in relation to egg weight.

At the end of the experiment, 18 eggs per each group (3 eggs from each replicate) were randomly chosen to determine yolk cholesterol. Eggs were boiled for 5 min. Egg yolk was blended with isopropyl alcohol with a volume of 10 ml per g of yolk [19]. Cholesterol content of this extract was determined according to the enzymatic method of TECO [20]. Yolk cholesterol was calculated and expressed as mg per g yolk.

At the 13th wk of the experiment, all hens were injected with 0.1 ml of 0.25% suspension of sheep erythrocytes (SRBC) in phosphate buffer saline. Circulating anti-SRBC antibody titers were determined by the microhem-agglutination technique from samples taken at 5 days after the immunization. All titers were expressed as the log_2 of the reciprocal of the the serum dilution [21].

Blood samples were collected from vena brachialis under the wing from all fed hens at the end of the experiment and centrifuged at 3.000 x *g* for 10 min. Serum was collected and stored at -20°C for determination of total protein, albumin, uric acid, triglyceride, cholesterol and levels of aspartate amino transferase (AST), alkaline phosphatase (ALP) and alanine amino transferase (ALT) by Vitros 350 autoanalyzer (New York, USA; Product code 680-2153) using their accompanying commercial kits [22].

Statistical Analysis

Statistical analysis were done using SPSS program (SPSS Inc., Chicago, IL, USA). The experimental unit was the cage (n=6). The normality of data distribution was checked using the Kolmogorov-Smirnov test. The effects of inactive yeast and live yeast were examined by two-way ANOVA. Values were reported as means ± SEM. When interaction between inactive yeast and live yeast was detected, one-way ANOVA with Duncan test was used to determine the differences among the groups [23]. Level of significance was taken as P<0.05.

RESULTS

The effects of dietary inactive yeast and live yeast on laying performance are shown in *Table 2*. Dietary treatments did not significantly affect feed intake and egg weight. However hen-day egg production (P=0.024) and feed conversion ratio (P=0.017) was improved by inactive yeast supplementation. No interactions were seen between inactive yeast and live yeast in feed intake, egg production and feed conversion ratio. No mortality was seen during the 16 wk experimental period.

The inclusion of inactive yeast or live yeast in the diet of laying hens had no significant effect (P>0.05) on the values of internal and external egg quality characteristics *(Table 3)*.

Table 2. *The effects of dietary supplementation of inactive yeast and live yeast on performance characteristics in laying hens*
Tablo 2. *Karma yemlere inaktif maya ve canlı maya ilavesinin yumurtacı tavuklarda performans ölçütleri üzerine etkileri*

Inactive Yeast (g/kg)	Live Yeast (g/kg)	Feed Intake (g/day per hen)	Hen-day Egg Production (%)	Egg Weight (g)	Feed Conversion Ratio (g feed/g egg)
0		112.7	88.20^b	66.12	1.94^a
1		113.4	91.39^a	67.03	1.85^b
	0	113.2	88.71	66.80	1.91
	0.5	112.9	90.88	66.40	1.88
0	**0**	112.8	86.39	66.04	1.98
1	**0**	113.5	91.03	67.47	1.85
0	**0.5**	112.5	90.01	66.20	1.89
1	**0.5**	113.3	91.74	66.58	1.86
SEM		0.9	1.31	0.53	0.03
Two way ANOVA (P values)					
Inactive yeast		0.413	0.024	0.102	0.017
Live yeast		0.778	0.113	0.495	0.225
Inactive yeast X Live yeast		0.936	0.279	0.334	0.158

$^{a-b}$*Means results within columns with different letters are significantly different (P<0.05); n = 6*

There were no interactions in these egg characteristics between inactive yeast and live yeast. Dietary inactive yeast supplementation decreased egg yolk cholesterol concentration (P=0.013). The interaction in egg yolk cholesterol content (P=0.032) between inactive yeast and live yeast was also observed.

Dietary supplementation of inactive yeast and live yeast did not affect blood serum parameters and antibody titers against SRBC *(Table 4)*. No interactions were also seen in these values.

DISCUSSION

Dietary inactive yeast supplementation improved hen-day egg production (P=0.024) and feed conversion ratio (P=0.017) and did not significantly affect feed intake and egg weight. However feed intake, hen-day egg production, egg weight and feed conversion ratio were not affected by live yeast inclusion. Some researchers also reported that inactive yeast [3,5,6,24,25] and live yeast [8,10,25,26] had no effect on feed intake of hens. Similarly, some researchers observed

Table 3. *The effects of dietary supplementation of inactive yeast and live yeast on egg quality characteristics and egg yolk cholesterol concentrations in laying hens*

Tablo 3. *Karma yemlere inaktif maya ve canlı maya ilavesinin yumurtacı tavuklarda yumurta kalite özellikleri ve yumurta sarısı kolesterol konsantrasyonu üzerine etkileri*

Inactive Yeast (g/kg)	Live Yeast (g/kg)	Shape Index (%)	Breaking Strength (kg/cm²)	Shell Thickness (µm)	Albumen Height (mm)	Albumen Index (%)	Yolk Index (%)	Haugh Unit (%)	Yolk Cholesterol (mg/g yolk)
0		76.60	2.61	385.5	7.31	9.18	42.08	83.90	16.45[a]
1		77.33	2.66	388.2	7.33	9.34	41.66	83.86	15.02[b]
	0	77.20	2.64	387.6	7.39	9.37	41.58	84.08	15.40
	0.5	76.73	2.64	386.1	7.25	9.15	42.16	83.68	16.10
0	0	76.85	2.60	385.1	7.37	9.25	41.89	83.90	16.69x
1	0	77.55	2.68	390.1	7.40	9.48	41.27	84.26	14.05y
0	0.5	76.34	2.62	386.0	7.24	9.11	42.27	83.89	16.22x
1	0.5	77.12	2.65	386.3	7.26	9.20	42.05	83.46	16.00x
SEM		0.38	0.07	3.90	0.08	0.14	0.29	0.48	0.52
Two way ANOVA (P-values)									
Inactive yeast		0.064	0.439	0.502	0.802	0.252	0.164	0.948	0.013
Live yeast		0.224	0.949	0.712	0.106	0.143	0.058	0.405	0.173
Inactive yeast X Live yeast		0.922	0.650	0.561	0.967	0.614	0.498	0.417	0.032

[a-b; x-y] Means results within columns with different letters are significantly different (P<0.05); n = 6

Table 4. *The effects of dietary supplementation of inactive yeast and live yeast on anti-SRBC titers and blood serum parameters in laying hens*

Tablo 4. *Karma yemlere inaktif maya ve canlı maya ilavesinin yumurtacı tavuklarda SRBC'ye karşı antikor düzeyi ve kan serum parametreleri üzerine etkileri*

Inactive Yeast (g/kg)	Live Yeast (g/kg)	Anti SRBC Titer (log_2)	Total Protein (g/l)	Albumin (g/l)	Uric Acid (mg/l)	Cholesterol (g/l)	Triglyceride (g/l)	ALT (U/l)	AST (U/l)	ALP (U/l)
0		5.85	58.4	26.0	49.9	1.59	16.02	16.04	160.0	140.3
1		6.42	58.9	25.0	48.3	1.48	14.98	16.26	165.0	143.4
	0	6.38	59.5	25.5	50.0	1.51	15.15	16.22	168.0	134.2
	0.5	5.89	57.8	25.4	48.1	1.56	15.86	16.08	157.0	149.5
0	0	5.83	58.9	25.8	50.0	1.58	16.02	16.08	159.6	138.1
1	0	6.93	60.1	25.2	50.1	1.45	14.27	16.36	176.4	130.2
0	0.5	5.86	57.9	26.1	49.8	1.60	16.02	16.00	160.5	142.6
1	0.5	5.92	57.7	24.8	46.4	1.52	15.69	16.17	153.6	156.5
SEM		0.30	1.70	1.00	2.90	0.05	0.68	0.65	7.20	7.00
Two way ANOVA (P-values)										
Inactive yeast		0.065	0.770	0.338	0.580	0.051	0.142	0.737	0.502	0.674
Live yeast		0.110	0.330	0.969	0.512	0.383	0.309	0.833	0.144	0.041
Inactive yeast X Live yeast		0.093	0.672	0.748	0.558	0.636	0.306	0.933	0.115	0.135

that egg weight [9,27] and feed conversion ratio [8,10] were not affected by live yeast supplementation. However Hassanein and Soliman [9] reported that feed conversion ratio was better when live yeast was added at 4 and 8 g/kg and concluded that adding live yeast *Saccharomyces cerevisiae* can enhance the productive performance of laying hens and nutrient utilization via the inhibitory effect of yeast against pathogenic bacteria which may cause mild enteritis and malabsorption of nutrients. However in this study the level of 0.5 g/kg live yeast may be too low to show these beneficial effects.

Similarly to this study observed with inactive yeast, some researchers observed considerable improvement in egg production [5,28,29] and feed conversion ratio [5,30,31] of hens fed yeast and yeast products. This improvement may partially be attributed to the improvement of the intestinal lumen health and nutrient absorption [4,32].

In agreement with this study, some researchers found that yeast and yeast products supplementation had no effect on egg weight in laying hens [3,8,24,25,33,34]. In contrast, others reported that egg weight was increased by dietary supplementation with yeast and yeast products [4,5,26]. It was also observed that there were no interactions in feed intake, egg production, egg weight and feed conversion ratio between inactive yeast and live yeast. The differences between the results of this study and previous studies may be the age of hens, dietary nutrient composition, type and level of yeast and yeast products.

Dietary inactive yeast or live yeast had no significant effect on the internal and external egg quality characteristics and no interactions were seen between inactive yeast and live yeast in these egg characteristics. In agreement with the present study some researchers [4,5,7] had not observed any effect on egg quality characteristics. However Chumpawadee et al.[10] reported that cassava yeast as probiotic source had positive effect on shell thickness of laying hens. Hassanein and Soliman [9] also observed that egg shell thickness was improved due to feeding various yeast levels and explained that this improvement may be attributed to the enhancement of calcium absorption and retention associated with adding yeast.

Egg yolk cholesterol concentration was decreased significantly with inactive yeast supplementation (P=0.013) but was not affected by live yeast supplementation in the present study. Inactive yeast supplementation in the absence of live yeast decreased egg yolk cholesterol concentration significantly compared to other groups. Some researchers also observed that egg yolk cholesterol was reduced by yeast probiotics [35,36] and yeast and yeast products [4-7]. The reduction in yolk cholesterol could be explained by the reduced absorption, synthesis or both of cholesterol in the gastrointestinal tract [36].

Antibody titers against SRBC were not affected by the supplementation of inactive yeast and live yeast and no interactions were seen in antibody titers. However inactive yeast supplementation tended to increase antibody titers against SRBC (P=0.065). Yalçın et al.[5] observed that greater antibody titer in laying hens fed diets containing 2, 3 or 4 g/kg yeast autolysate. Mohiti-Asli [25] reported that immune response of laying hens with multistrain probiotic and yeast supplementation was greater than the control group. Prebiotics would bind to macrophage reception sites by recognizing specific sugars found in glycoproteins of the epithelial surface, triggering a cascading reaction that would activate macrophages and release cytokines, thereby activating the acquired immune response and causing the higher antibody responses against antigens [37,38]. This may be an explanation for higher antibody titers in hens fed inactive yeast as prebiotics.

Dietary supplementation of inactive yeast and live yeast did not affect blood serum levels of total protein, albumin, uric acid, cholesterol, triglyceride, ALT, AST and ALP. In addition no interactions were seen in these blood serum parameters. However dietary inactive yeast tended to reduce serum yolk cholesterol (P=0.051). Yalçın et al.[5] observed that serum cholesterol and triglyceride was reduced with the addition of 2, 3 and 4 g/kg yeast autolysate. Krasowska et al.[39] reported that baker's yeast *Saccharomyces cerevisiae* can be the best organism for reducing cholesterol in the gastrointestinal system. Similar to the present study, Yalçın et al.[5] showed that yeast autolysate supplementation had no effect in the levels of serum total protein and AST. Saoud and Daghir [40] also reported that the level of serum uric acid was not affected with dietary single cell protein. In other study, Yalçın et al.[4] observed that serum levels of total protein, triglyceride, cholesterol, AST and ALP were not affected by yeast culture supplementation.

The differences between the results of the present study and those of previous studies may be due to the heterogeneity of the experimental protocol utilized: species and age of birds, dietary nutrient composition, type and dosage of yeasts in the diets, survivability of live microorganisms in probiotic yeasts and environmental conditions.

As a result dietary inactive yeast at 1 g/kg had beneficial effects in laying performance and in low cholesterol-egg production. No adverse effects were seen on other parameters. Further researches with high doses are required to see the effects of live yeast and to determine the mechanism of actions, evaluating inactive yeast and live yeast interaction.

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Research Article

Path Analysis for Body Measurements on Body Weight of Saanen Kids

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Abstract

The aim of this study was to examine the direct, indirect and total effects of some body measurements on body weight of Saanen kids by using path analysis. For this aim, relationship between body weight (BW) and four morpho-biometrical traits [chest girth (CG), height at withers (HW), height at sacrum (HS) and body length (BL)]were studied in 75 Saanen kids at one month of age using path analysis. However HW trait was excluded from analyze because of its multicollinearity with HS. The effects of CG, HS and BL on BW were found statistically significant. BL was found as variable with highest indirect effect (0.521) on BW. The direct effect of CG was higher than HS and BL. The total effect of BL was higher than those of HS and CG. These results showed that chest girth could be used to estimate the body weight of Saanen kids for practical purposes as well as for selection purposes.

Keywords: *Correlation, Path coefficient, Saanen kids, Body measurements*

Saanen Oğlaklarının Canlı Ağırlıkları Üzerine Vücut Ölçümleri İçin Path Analizi

Özet

Bu çalışmanın amacı Path analizi kullanılarak Saanen oğlaklarının canlı ağırlıkları üzerinde bazı vücut ölçümlerinin doğrudan, dolaylı ve toplam etkilerini incelemektir. Bu amaçla canlı ağırlık (BW) ve dört morfolojik-biyometrik özellikleri [Göğüs Çevresi (CG), Cidago Yüksekliği (HW), Sağrı Yüksekliği (HS) ve Vücut Uzunluğu (BL)] arasındaki ilişkiler Path analizi kullanılarak bir aylık yaştaki 75 adet Saanen oğlaklarında çalışıldı. Ancak HW özelliği HS özelliği ile çoklu bağlantıdan dolayı analizden çıkarılmıştır. BW üzerine CG, HS ve BL'nin etkileri istatistiksel olarak önemli bulunmuştur. BL, BW üzerine en yüksek dolaylı etkili (0.521) değişken olarak bulunmuştur. CG'in doğrudan etkisi, HS ve BL'den daha yüksektir. BL'nin toplam etkisi HS ve CG'ninkinden daha yüksektir. Bu sonuçlar seleksiyon seçimi yanında pratik seçim için Saanen oğlaklarının canlı ağırlığını tahmin için göğüs çevresinin kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Korelasyon, Path katsayısı, Saanen oğlakları, Vücut ölçüleri*

INTRODUCTION

In general, the aim of animal breeding is to genetically improve populations of livestock so that they produce more efficiently under the expected future production circumstances. Genetic improvement for economic traits is achieved by selecting the best individuals of the current generation and by using them as parents of the next generation [1]. In many cases, the animals with higher potential for body weight and body measurements are selected as breeding material or these criteriaare used to valorize the animals. To evaluate the data relational statistics such as regression and correlation are used. Generally, body weight is selected as response variable and body measurements are selected as explanatory variables. So, it is aimed to explain the response variable from explanatory variables. However, indirect effects of explanatory variables on response variable should be considered beside the direct effects [2]. Path analysis is used to describe the directed dependencies among a set of variables [3]. Therefore, application of path analysis in animal breeding practices began to increase [4-13]. Also, there are some studies which examined the direct and indirect effects of body measurements on body weight [14-17]. However there is not enough study for Saanen kids within this scope. In this study, direct, indirect and total effects of body measurements on body weight in Saanen kids were investigated.

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MATERIAL and METHODS

Material

This study was carried out at the private dairy goat farm in Bafra province of Samsun, Turkey (40°31'N, 36°53'E and 650 m above the sea level). Data was collected from 75 Saanen kids one month after birth and body weight (BW) was selected as response variable and body size parameters; body length (BL), height at sacrum (HS), height at withers (HW) and chest girth (CG) were selected as explanatory variables. While CG was measured with tape, other body size parameters were measured with stick (BL, HS and HW) and bascule (BW). SPSS [18] statistical software was used to analyze the data with the license of Ondokuz Mayıs University.

Method

Every linear model has a direct effect and amount of indirect effect which is number of explanatory variables minus one. The general expression of multiple regression model formed for the measurements (one response and p explanatory variables) is given in Eq. 1 [19].

$$y_k = \beta_0 x_{k_1}^{\beta_1} x_{k_2}^{\beta_2} x_{k_3}^{\beta_3} \ldots x_{k_p}^{\beta_p} e_i; \quad i = 1,2,\ldots,n \qquad (1)$$

The multiple linear regression model adopted was

$$\hat{y}_k = b_0 + b_1 x_{k1} + b_2 x_{k2} + b_3 x_{k3} \qquad (2)$$

where:

$\hat{y}_k$ = response variable (BW),

b_0 = intercept,

b_i = standardized regression coefficients,

x_{kp} = explanatory variables (CG, BL, HS)

A path coefficient (*P*) is a standardized regression coefficient (b) showing the direct effect of an independent variable on a dependent variable in the path model [20,21]. Path coefficient, which indicates the effect of one standard deviation change of any explanatory variable *X* versus on response variable *Y*, can be calculated as [22];

$$Pyx_k = b \frac{Sx_k}{S_y} \qquad (3)$$

Here; P_{yx} is the path coefficient which indicates the direct effect of *X* explanatory variable on response variable *Y*,S_x indicates the standard deviation of *X*, S_y indicates the standard deviation of *Y* and *b* indicates the partial regression coefficient.

Path coefficients can be shown with path diagrams. One way and two way arrows are used in path diagrams. One way arrows which named as direct effects are drawn from explanatory variable to response variable and two way arrows which showed correlations are drawn between explanatory variables [10]. Path diagram for this study was given in *Fig. 1*.

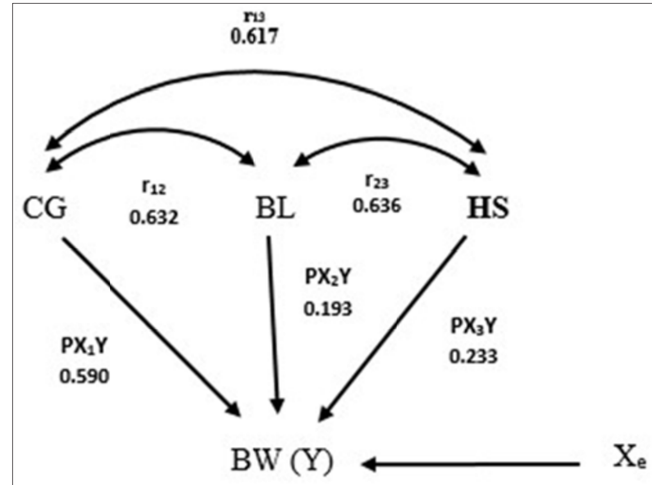

Fig 1. Path diagram
Şekil 1. Path diyagramı

To obtain the path coefficients should be replaced in linear equation system as given in equation 4.

$$\begin{bmatrix} P_{YX_1} \\ P_{YX_2} \\ P_{YX_3} \end{bmatrix} = \begin{bmatrix} 1 & r_{X_1X_2} & r_{X_1X_3} \\ r_{X_2X_1} & 1 & r_{X_2X_3} \\ r_{X_3X_1} & r_{X_3X_2} & 1 \end{bmatrix}^{-1} * \begin{bmatrix} r_{YX_1} \\ r_{YX_2} \\ r_{YX_3} \end{bmatrix} \qquad (4)$$

In the Eq. (4), coefficients given by P_{YX_i} were path coefficients (direct effects) between explanatory variable and response variable and $r_{x_ix_j}P_{YX_i}$ represented indirect effects of explanatory variable i^{th} on response variable via explanatory variable j^{th}, $r_{x_ix_j}$ represented pearson correlation coefficients between i^{th} an jth traits [23].

RESULTS

Descriptive statistics for body weight, chest girth, height at withers, height at sacrum and body length for Saanen kids were given in *Table 1*. Having normal distribution of obtained data was determined with Kolmogorov-Smirnov one sample test.

Pearson correlation coefficients between examined traits were given in *Table 2*. Results were not divided by sex factor because correlations among traits were similar in both sexes. All estimated correlation coefficients were positive and significantly (P<0.01) differ from zero.

Height at withers was removed from the analysis because multicollinearity was determined between HW and HS. HW was removed from the model because Type I error rate of HW (0.928) was higher than of HS (0.181). The highest and lowest relations on BW were observed with CG and BL, respectively. Standardized partial regression coefficients and significance levels were given in *Table 3*.

Standardized multiple regression equation was obtained, with 0.802 adjusted coefficient of determination, as follows;

$$BW = 0.590(CG) + 0.193(BL) + 0.233(HS)$$

In this equation constant was estimated as zero because of standardization. Partial regression coefficients of that equation stated direct effects of each explanatory variable on response variable. *Table 3* showed that all coefficients were statistically significant and there were no multicollinearity observed between variables, because VIF values were under the threshold of 10.

Path coefficients belong to direct and indirect effects of explanatory variables on body weight for Saanen kids were given in *Table 4*.

All direct effects were positive and CG had the highest direct effect on BW. Besides, the lowest indirect effect was observed between BL and CG. The lowest relation was determined between BW and BL which direct effect was 0.193. But, the highest total indirect effect on BW was obtained with BL. Also, direct effect of CG on BW was higher than total indirect effects of other explanatory variables.

Table 1. *Descriptive statistics of Saanen kids for examined traits*
Tablo 1. *İncelenen özellikler için Saanen oğlaklarının tanımlayıcı istatistikleri*

Traits	n	Mean	SD	Min	Max	*P**
BW	75	6.21	1.18	3.60	9.20	0.830
HW	75	42.85	3.19	36.00	49.00	0.334
HS	75	43.55	3.19	37.00	50.00	0.450
BL	75	38.47	3.06	31.50	43.00	0.249
CG	75	42.82	2.79	34.00	49.00	0.254

** Type I error rate for Kolmogorov-Smirnov one sample test*

Table 2. *Pearson correlation coefficients between traits*
Tablo 2. *Özellikler arasındaki Pearson korelasyon katsayıları*

Traits	BW	HW	HS	BL
HW	0.720**			
HS	0.720**	0.946**		
BL	0.714**	0.633**	0.636**	
CG	0.856**	0.638**	0.617**	0.632**

*** P<0.01*

Table 3. *Results of standardized regression analysis*
Tablo 3. *Standardize edilmiş regresyon analizi sonuçları*

Parameters	CG	BL	HS
Coefficients (b_i)	0.590	0.193	0.233
Significance (P)	<0.001	0.010	0.002
VIF value	1.914	1.991	1.930
Tolerance	0.523	0.502	0.518

DISCUSSION

Body weight is an important economic trait in the selection of animals [16]. So, some factors affecting body weight should be determined. Owing to this, the path analysis is very important for determining factors affecting body weight [17]. In this study, which aimed to investigate the direct, indirect and total effects of body measurements on body weight in Saanen kids, it was determined that there were positive relations between BW and CG, BL and HS. Although any study related to the effects of body measurements on body weight in Saanen kids, were not found, some results of this study (for example, CG had the

Table 4. *Direct and indirect effects of explanatory variables on body weight*
Tablo 4. *Canlı ağırlık üzerine açıklayıcı değişkenlerin doğrudan ve dolaylı etkileri*

Trait	Correlation Coefficient with BW	Direct Effect	Indirect Effect			
			HS	BL	CG	Total
HS	0.720**	0.233**	-	0.123	0.364	0.487
BL	0.714**	0.193**	0.148	-	0.373	0.521
CG	0.856**	0.590**	0.144	0.122	-	0.266

*** P<0.01*

direct and total effects on BW) were supported by some studies conducted with crossbreed kids of German Fawn X Turkish Hairy goats [17] and with Akkaraman lambs [24]. But, results of this study was not coherent with the results of Keskin et al.[25] who studied with male lambs of Anatolian merino. Similarly, BL was found to have the lowest direct and the highest total effect on body weight [6]. As a result, it was concluded that chest girth could be used for management decisions and as indirect selection criteria for selection on body weight due to CG had the highest direct and the lowest indirect effect on body weight.

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Research Article

Alt Solunum Sistemi Hastalığına Sahip Taylarda Bronkoalveolar Lavaj Sıvısının Bakteriolojik ve Sitolojik Değerlendirilmesi

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Özet

Taylarda alt solunum sistemi hastalıkları at yetiştiriciliğinde ekonomik kayıpların başında gelmektedir. Bu hastalıkta etkenin kesin tanısı için kullanılan birçok yöntem bulunmaktadır. Bronkoalveolar lavaj, hem bakteriyel hem de sitolojik muayenenin yapılabilmesi açısından en önemli tekniklerden biridir. Bu çalışmada, alt solunum sistemi hastalığı olan taylarda BAL örneklerinin sitolojik ve bakteriyolojik muayene bulgularının değerlendirilmesi amaçlandı. BAL örneklerinin sitolojik muayenelerine göre etken üreyen olgularda etken üremeyen olgulara göre nötrofil, makrofaj (P<0.001) ve lenfosit hücrelerinin (P<0.05) yüzde oranları arasında istatistiki olarak önem bulundu. Ayrıca alınan BAL sıvısından yapılan bakteriyolojik muayene sonucunda 20 tayda, Streptococcus equi subsp. zooepidemicus (10), Staphylococcus aereus (7), Escherichia coli (3), etkenleri çoğunlukta olmak üzere toplamda 25 adet aerobik/fakültatik gram (+) kok ve gram (-) basil grubunda bakteri üremesi tespit edildi. Sonuç olarak, alt solunum yolları hastalıklarında, özellikle endoskopi eşliğinde yapılan BAL tekniğinin gerek sitolojik değerlendirme, gerekse bakteriyolojik muayene amacıyla uygulanmasının tanı ve tedavi protokolünün oluşturulması açısından büyük önem taşıdığı sonucuna varıldı.

Anahtar sözcükler: *Alt solunum yolları hastalığı, Bakteriyolojik muayene, Sitolojik muayene, Bronkoalveolar lavaj tekniği, Tay*

Bacteriological and Cytological Findings of Bronchoalveolar Lavage Fluids in Foals with Lower Respiratory Tract Diseases

Abstract

Lower respiratory tract disease is one of the most common causes of economic loss in foals. There are several methods used for the diagnosis of this disease. Bronchoalveolar lavage is one of the most important technique for bacteriological and cytological examination. The aim of this study was to evaluate BAL samples' cytologic and bacteriologic examination of foals with lower respiratory tract disease. In the comparison of cytologic examination of BAL samples with bacterial agents and without bacterial agents statistical differences in the percentage of neutophils, macrophages (P<0.001) and lymphocytes (P<0.05) were defined. Also according to the bacteriologic examination results of the BAL samples in 20 foals following agents were mainly detected: *Streptococcus equi* subsp. *zooepidemicus* (10), *Staphylococcus aereus* (7), *Escherichia coli* (3). Totally 25 aerobic/facultative gram (+) and gram (-) bacils were isolated. In conclusion, the BAL technic is useful for cytological and bacteriological examination in horses with lower respiratory tract diseases.

Keywords: *Lower respiratory tract disease, Bacteriological examination, Cytological examination, Bronchoalveolar lavage, Foal*

GİRİŞ

Solunum yolları hastalığı yarış atlarında kas iskelet sisteminden sonra zayıf performansa sebep olan en çok karşılaşılan hastalıklar grubundan olduğu [1], özellikle taylarda önemli ekonomik kayıplara neden olduğu da ayrıca belirtilmektedir [2]. Yapılan çalışmalar alt solunum yolları hastalıklarına neden olan birçok dispozisyon faktörlerin olduğunu göstermektedir. Bunlar arasında viral, bakteriyel, paraziter etkenler gibi enfektif faktörlerin yanı sıra, mevsimsel faktörler, stres, ventilasyon, barınak koşulları gibi çevresel faktörler de yer almaktadır [3,4].

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Akciğerlerde, normal koşullarda klirens (mukosilier transport) mekanizması ile patojen bakteriler uzaklaştırılarak etkisiz hale getirilmektedirler fakat akciğer savunma mekanizmasında oluşan aksaklıklarla birlikte orofarenks bölgesinden aspire edilmiş olan bakteriler ilerleyerek çoğalırlar ve bunun sonucunda pnömoni oluşturdukları bilinmektedir [5]. Atlarda ve taylarda alt solunum yollarının hastalıklarında birçok etken izole edilmekle birlikte, bunların içerisinde bazı etkenler sıklıkla saptanmaktadır. Örneğin streptococcal pnömoniler ya da *Rhodococcus equi* enfeksiyonları tek başlarına çok ciddi enfeksiyonlar oluşturabilmektedir [6,7]. Atlarda alt solunum yolları hastalıklarında birçok etken izole edilmekle birlikte bunların içerisinde bazı etkenlere daha sık rastlanmaktadır. *Streptococcus equi* subsp. *zooepidemicus* (β-hemolitik), *Staphylococcus aureus* ve *Streptococcus pneumoniae* (α-hemolitik) en sık izole edilen gram (+) bakteri türleri iken, en sık izole edilen gram (-) bakteriler ise *Pasteurella, Actinobacillus* spp., *Escherichia coli, Klebsiella pneumonia, Bordetella bronchiseptica* ve *Enterobacter spp.*'dir [2,4,8-16]. Bu etkenler tek başlarına hastalık oluşturmakla birlikte miks enfeksiyonlara da neden olabilmektedir [11,17-20].

Hastaların anamnez bilgileri çok dikkatli alınmalı daha önce aynı şikayeti geçirip geçirmediği, tedavi uygulanıp uygulanmadığı, uygulandı ise kullanılan ilaçlar ve tedavi zamanı ile ilgili bilgiler alınmalıdır. Pnömoni şekillenen taylarda dikkatli bir şekilde klinik ve hematolojik değerlendirme yapılarak; özellikle, öksürük, burun akıntısı, vücut sıcaklığında artış, toraksın oskültasyon ve perküsyonunda anormal sesler değerlendirilmelidir. Solunum sistemi problemi olan atlarda hematolojik muayene enfektif problemlerle non-enfektif problemleri birbirinden ayırt etmede yardımcı olabilir. Bununla birlikte non-enfektif yangısal hastalıklarda ve stres durumlarında da hematolojik tabloda değişimler olabileceği unutulmamalıdır [21].

Atlarda alt solunum yolu hastalılarına tanısal yaklaşımda, torasik oskültasyon, endoskopik muayene, BAL ve toraks radyografisi kullanılan tekniklerdir [22]. Günümüzde, at hastanelerinde ve bazı kliniklerde atların alt solunum yolu hastalıklarında kullanılan tanı yöntemi endoskopik muayenedir. Uygulaması diğer metodlara göre ekonomik olup, saha şartlarında kullanımı da diğer tanı metodlarına göre daha pratiktir. Endoskopik muayene dışında yapılan BAL tekniği de hastalığın seyri ve tedavisi için sık kullanılan yöntemler arasındadır. Alınan örneklerin bakteriyolojik ve sitolojik değerlendirmesi [22] tedavi protokolü oluşturulmasına yardımcı olmaktadır. BAL yöntemi ile alınan örneklerin sitolojik muayenesinde, solunum yollarının farklı anatomik bölgelerine ait hücreler ile yangı hücreleri değerlendirilerek, yangının şiddeti daha net anlaşılmaktadır [22,23].

Bu çalışmada, alt solunum sistemi hastalığı olan taylarda BAL örneklerinin sitolojik ve bakteriyolojik muayene bulgularının değerlendirilmesi amaçlandı.

MATERYAL ve METOT

Çalışma Materyali

Çalışmanın materyali olarak, Mayıs-Ağustos 2013 ayları arasında hayvan hastanemize getirilen solunum sistemi sorunları olan, farklı yaş (2-6 ay) ve canlı ağırlıklardaki (50-100 kg) 20 adet İngiliz ırkı tayı kullanıldı. Taylar, düzenli olarak havalandırılan, birbiriyle bağlantısı olmayan ayrı padoklarda barındırılmakta, altlık olarak ince kıyılmış saman veya talaş kullanılmaktaydı. Hayvanlar serbest olarak su tüketimi imkânıyla birlikte ihtiyaçlarına göre özel olarak oluşturulan rasyonlarla beslenilmekteydi.

Endoskopik Muayene ve BAL Sıvı Örneklerinin Alınması

Endoskopi uygulamasının rahat yapılabilmesi için öncelikle taylar, üst dudak bölgelerine yavaşa uygulanarak zapt-ı rapta alındı. Endoskopik muayenede ilk olarak üst solunum yollarına (burun boşluğu, hava keseleri, farenks) ilişkin anatomik bölgeler muayene edilerek anormal bulgular not edildi. Üst solunum yollarının detaylı muayenesinden sonra alt solunum yollarına ilişkin muayeneler de yapılarak, elde edilen bulgular kayıt edildi. Sadece üst solunum yollarına ilişkin problemi olup alt solunum yollarına ilişkin problemi olmayan taylar belirlenerek çalışmaya dahil edilmedi. Endoskopi için 3 m uzunluğunda ve 9.9 mm çapında olan endoskop ile (vet-vu) burun boşluğundan ilerleyerek farenkse ve daha sonrada rima glottis'te ilerleterek trakeaya girildi. BAL steril katateri ile endoskopun çalışma kanalından 60 ml steril %0.9'luk izotonik NaCl solüsyonu verildi ve kısa bir süre sonra solüsyon steril enjektörlerle aspire edilerek bronchoalveolar lavaj uygulandı. En son olarak BAL sıvısı steril tüplere alındı. Alınan örnekler aynı gün analiz edilmek üzere 30 dak. içerisinde laboratuvara ulaştırıldı. Mikrobiyolojik analiz için birkaç gün bekletilmesi gereken örnekler ise -20ºC'de muhafaza edildi. Sitolojik muayene için ayrılan BAL sıvısından aynı gün içersinde froti hazırlandı.

Mikrobiyolojik Analiz

Soğuk zincirde laboratuvarımıza ulaştırılan BAL örneklerinin dikkatli bir şekilde genel, selektif ve diferensiyal besiyerlerine separe ekimleri yapıldı. Bakteriyel izolasyonlar için genel besiyeri olarak Kanlı Agar, Tryptic Soy Agar (T.S.A.), diferansiyel besi yeri olarak MacConkey Agar (M.C.) ve Eozin Metilen Blue Agar (E.M.B.) ve selektif besiyeri olarak Caz-N.B. Agar, Chocolate Agar, Edward's besiyerleri kullanıldı. Mantar izolasyonu için ise Saboraud Dekstroz Agar (S.D.A.)'a ekim yapıldı. Tüm ekimler optimal sürede ve 37°C'de etüvde bekletildi. Üreyen kolonilerden Gram boyama yapıldı. Saf kültürler hazırlanarak rutin biyokimyasal testler ile identifikasyon yapıldı.

Sitolojik Analiz

BAL örneklerinden froti hazırlamak amacıyla ilk olarak

dilue edilmemiş örnekler santrifüj edildi (Nüve HN075). Çekilen frotiler kurutulduktan sonra Wright-Giemsa boyası [GBL HEMADİFF (Wright's eosin methylene blue solution), MERCK (Giemsa's azur eosin methylene blue solution)] ile boyandı. Hazırlanan froti kurutulduktan sonra üzerine immersiyon yağı damlatılarak, mikroskobun (OLYMPOS cover-015) 100'lük objektifi ile incelendi. Yangı hücrelerini de içeren formül lökosit sayımında frotinin farklı bölgeleri incelenerek 100 adet hücre sayımı yapıldı. Daha sonra çıkan hücrelerin yüzde oranları belirlendi.

İstatistiksel Analiz

Varyansların homojenliği test edilerek, etken üreyen ve etken üremeyen grupların karşılaştırılmasında t-testi (independent samples t-test) uygulandı. Önemlilik düzeyi olarak P≤0.05 seçildi. İstatistik analizler S.P.S.S. 13 istatistik programından yararlanılarak yapıldı.

BULGULAR

Endoskopik Muayene Bulguları

Yapılan endoskopik muayenede bazı hayvanlarda farenks bölgesinde hiperemik ve ödematöz mukoza varlığı belirlendi. Özellikle öksüren hayvanlarda trakeal lümenin hiperemik olduğu saptandı. Çalışmayı oluşturan bütün olgularda; özellikle trakeanın karina bölgesinde mukoid ya da purulent karakterde akıntının mevcut olduğu belirlendi. Bu bulgular doğrultusunda: olguların 4'ünde (%20) üst solunum yollarına ilişkin bulgular, 13'ünde (%65) trakeada hiperemi bulgusu, 30'unda (%18) mukoid akıntı, 14'ünde (%70) purulent akıntı ve 2'sinde (%10) ödem tablosuna rastlanıldı.

Mikrobiyolojik Muayene Bulguları

Yapılan mikrobiyolojik muayenede, *Streptococcus equi* subsp. *Zooepidemicus* (10), *Staphylococcus aereus* (7), *Escherichia coli* (3), bakterileri izole edildi. Bunların dışında bazı olgularda *Aspergillus fumigatus, Candida spp.* etkenleri de saptandı. Yapılan analiz sonucunda 7 hayvanın BAL sıvısında üreme tespit edilmedi. Hastalardan 3 tanesinde 3, 5 tanesinde 2, 5 tanesinde 1. mikrobiyolojik analiz sonucunda üreyen etkenlerin izolasyon oranları *Tablo 1*'de belirtilmiştir.

Sitolojik Muayene Bulguları

Yapılan mikrobiyolojik muayene sonucunda bakteri saptanan örneklerde hücre morfolojileri genel anlamda irdelendiğinde makrofajların oldukça büyük olduğu ve vakuolizasyon şekillendiği, büyük çoğunlukla reaktif lenfositlerin ve nötrofillerin tabloya egemen olduğu belirlendi. Yine bu örneklerin bazılarında daha önceki bir kanamanın varlığını işaret eden hemosiderinlere ve bakteri izole edilen olgularda bakteri kümeleri saptandı.

Fungal etkenlerin saptandığı örneklerde ise genel olarak hücre morfolojilerinde herhangi bir anormallik saptanmadı.

Tablo 1. *Bakteriyolojik değerlendirmede üreme görülen 13 tayda izole edilen bakteri türleri*

Table 1. *Bacterial specments isolated from 13 culture positve foals in bacteriological evaluation*

Aerobik/FA Bakteri	Bakteri Sayısı (n)
Aerobik/FA Gram (+) Kok	
Streptococcus equi subsp. zooepidemicus	10
Staphylococcus aeurus	7
Streptococcus pyogenes	2
Rhodococcus equi	1
Aerobik/FA Gram (-) Basil	
Eschericia coli	3
Pseudomonas aeruginosa	2
Toplam izole edilen aerobik/FA bakteri sayısı	25

Tablo 2. *Etken üreyen ve etken üremeyen olgulardaki sitolojik örneklere ait lökositer bulguların % değerlerinin karşılaştırılması*

Table 2. *Comparison of bacterial isolated and non-bacterial isolated cases about the percentage of leukocytes in cytological samples*

Lökositler	Etken Üreyen (n=13) Ort. Değer± SE	Etken Üremeyen (n=7) Ort. Değer± SE
Nötrofil (%)	42.23±1.9*	13.29±0.83*
Lenfosit (%)	16.61±2.85**	24±2.72**
Eozinofil (%)	0.84±0.33	1.71±1.08
Makrofaj (%)	39.84±2.79*	60.14±2.97*
Mast (%)	0.46±0.46	0.85±0.7

** Etken üreyen ve etken üremeyen olgulardaki sitolojik örneklere ait lökositer bulguların % değerleri arasındaki P<0.05 ve ** P<0.001 düzeyindeki farkı ifade eder*

Froti muayenesinde hücre oranları hesaplandıktan sonra etken üreyen ile etken üremeyen örneklerin lökositer hücre yoğunlukları istatiksel yönden anlamlılığı *(Tablo 2)* karşılaştırıldı.

TARTIŞMA ve SONUÇ

At yetiştiriciliğinde, solunum sistemine ilişkin hastalıkların dünya genelinde en sık karşılaşılan hastalıklar arasında olduğu belirtilmektedir [24,25]. Solunum sistemi hastalıklarının oluşmasında barınak koşulları ve mevsim şartları önemli yere sahiptir. Kalabalık ortamda bulunan atların, bakteriyel ve viral solunum patojenlerine maruz kalma olasılığı daha yüksektir [21]. Solunum sistemi hastalıklarına predispozisyon hazırlayan önemli çevresel faktörler arasında saman üzerindeki mantar sporları, mikotoksinler, yataklık ve havadaki amonyak seviyesi, izolasyon, güneş ışığı ve ventilasyon sayılabilir [3,4]. Başarılı çevresel kontrol ve barınak koşullarının iyileştirilmesi solunum sistemi hastalıklarının önlenmesi açısından önemli rol oynamaktadır. Sunulan çalışmada taylar kendi bireysel padoklarında

barınmasına karşın altlık olarak ince kıyılmış saman veya talaş kullanılması, daha fazla tozlanmaya neden olarak solunum sistemi hastalıklarının oluşmasına predispoze bir faktör olarak rol oynadığı düşünülmektedir.

Atlarda ve taylarda alt solunum yolları hastalıklarında en sık başvurulan yöntemlerden biri de BAL tekniğidir. Özellikle kronik solunum sistemi hastalıklarında tercih edilse de gerek bakteriyolojik gerekse sitolojik muayene açısından değerlendirmede önemli bir rolü bulunmaktadır [15,26-33]. Diğer bir tanı yöntemi olan trakeal aspirasyon tekniği de alt solunum yolları hastalıklarında sıklıkla kullanılan yöntemlerdendir fakat BAL ile karşılaştırıldığında aralarında birçok farklılık bulunmaktadır [34,35]. Öncellikle trakeal aspirasyon yönteminde kullanılan %0.9 İzotonik NaCl solüsyonunun miktarı BAL yöntemine göre daha az miktardadır [35]. Yapılan çalışmalardaki iki tekniğin karşılaştırılmalı olarak yapılan sitolojik değerlendirmelerinde BAL tekniği ile alınan örneklerin anatomik konumunun değişik olmasından kaynaklanan farklılıklar bulunmaktadır [34,35]. Ayrıca trakeal aspirasyon yöntemi saha şartlarında daha pratik ve daha ekonomik bir yöntem olsa da tanısal açıdan BAL muayenesi sitolojik ve bakteriyolojik açıdan daha detaylı bilgi verdiği yapılan diğer çalışmalarda belirtilmiştir [36,37]. Yapılan bu çalışmada BAL tekniği ile alınan sıvının bakteriyolojik ve sitolojik değerlendirmesi hastalığın tedavi ve seyrini olumlu yönde etkilemiş ve yapılan diğer çalışmalar ile paralellik gösterdiği görülmüştür.

Atların ve tayların solunum sistemi hastalıkları kompleksi içerisinde yapılan çalışmalarda trakeabronşial aspirasyon (TBA) ve BAL teknikleri ile alınan örneklerde birçok etken identifiye edilmiştir [2,15,31,37-39]. *Streptococcus equi* subsp. *Zooepidemicus* (β- hemolitik), *Staphylococcus aureus* ve *Streptococcus pneumoniae* (α-hemolitik) en sık karşılaşılan gram pozitif bakteri türleri iken, en sık izole edilen gram negatif bakteri türleri ise *Pasteurella, Aktinobasillus* spp., *Escherichia coli, Klebsiella pneumonia, Bordetella bronchiseptica* ve *Enterobacter spp.*'dir [2,4,8-16,31]. *Pseudomonas* türleri ise atlarda karşılaşılan pnömonilerde etkili bir patojen değildir. Bu etken çoğunlukla trakeabronşial aspirasyon sırasında endoskop gibi ekipmanlar ile kontaminasyon sonucu izole edilir. Bu etkenler arasında *Str. zooepidemicus* en sık izole edilen bakteri olarak tanımlanmıştır [38-40]. Hoffman ve ark.'nın [31] taylarda yapmış oldukları bir çalışmada BAL tekniği kullanılarak alınan sıvı örneğinde *Streptococcus zooepidemicus* etkeninin en çok üreyen ikinci bakteri türü olduğu bulunmuştur. Bununla birlikte, Laus ve ark.[40] *Streptococcus zooepidemicus* ve *Streptococcus pneumoniae* etkeninlerinin neden olduğu yangısal solunum sistemi hastalıklarının yaşı ilerledikçe görülme oranının azaldığını belirtmektedirler. Bu çalışmada da *Streptococcus zooepidemicus* izole edilen atlarda yaş dağılımı incelendiğinde 10 atın da yaş aralığının 4-5 ay arasında olması bu veriler ile uygunluk göstermektedir.

Sağlıklı atların BAL sıvısı sitolojisinde nötrofil oranının %0-17 arasında olduğu bildirilmiştir [41]. Alt solunum yolu hastalıklarında BAL sıvısı sitolojisi ile histopatoloji bulguları arasında yüksek bir korelasyon olması nedeniyle transtracheal aspirasyon sitolojisinden daha fazla tercih edilmektedir [26]. Hastalık süresince BAL sıvısında nötrofil ve makrofaj oranlarında değişen derecelerde (%20-90 nötrofil) artış gözlenmektedir [26,41]. Hoffman ve ark.'nın [15] taylarda yapmış oldukları bir çalışmada nötrofil oranlarının bakteriyel üreme ile doğru orantılı olarak arttığı belirtilmiştir. Bu çalışmada bakteri izolasyonu yapılan olguların sitolojik örneklerinde bakteri izole edilmeyen olgulardaki sitolojik örneklere göre nötrofil yüzdesinin fazla olması bu görüşü desteklemektedir ($P<0.001$). Ayrıca, sitolojik değerlendirmede nötrofil oranının fazla olması, bakteriyel enfeksiyon varlığını da destekler niteliktedir. Bu çalışmada solunum sistemi şikayeti olan 20 olgunun17'sinde etken izolasyonu yapılarak olguların %85'inde neden ortaya konmuştur.

Sonuç olarak, öksürük ve burun akıntısı şikayeti ile gelen taylara uygulanan endoskopik muayenede alt solunum sistemine ait bulguları olan 20 adet tay çalışmaya alındı. Bu bulgular doğrultusunda: olguların 4'ünde (%20) üst solunum yollarına ilişkin bulgular, 13'ünde (%65) trakeada hiperemi bulgusu, 30'unda (%18) mukoid akıntı, 14'ünde (%70) purulent akıntı ve 2'sinde (%10) ödem tablosuna rastlanıldı. Taylara sırasıyla klinik ve BAL sıvısının sitolojik ve bakteriyolojik muayenesi yapıldı. Çalışmada, bakteri izole edilen BAL sıvısı örneklerin sitolojik değerlendirmelerinde, bakteri izole edilmeyenlere göre belirgin bir fark olması tanı aşamasında sitolojik değerlendirmenin önemini vurgulamaktadır. Çalışmanın sonunda endoskopi eşliğinde uygulanan BAL tekniğinin taylarda alt solunum sistemi hastalıklarının tanısal yaklaşımında gerek etken identifikasyonu açısından gerekse sitolojik değerlendirme açısından önemli ve faydalı olduğu saptanmıştır.

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Research Article

Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by Real-Time PCRs and *Mycoplasma gallisepticum*-antibody Detection by an ELISA in Chicken Breeder Flocks

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Abstract

This study aimed to determine the prevalence of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in breeder flocks showing respiratory symptoms. A total of 77 flocks (2153 tracheal swabs and blood samples) were sampled and all were tested by MG real time PCR (MG-rPCR) and MG-ELISA, and 32 flocks were tested by MS real time PCR (MS-rPCR). In the first part of this study covering 28 flocks, all samples from chickens with marked clinical symptoms and high MG-antibody levels gave negative results with MG-rPCR$_1$. Therefore, the MG-lipoprotein gene-specific primers (MG-rPCR$_1$) of this PCR were replaced with MG-16S rRNA primers (MG-rPCR$_2$), as were the MS-16S rRNA primers (MS-rPCR), thus the study was pursued accordingly. All of the first 28 flocks, which were 100% positive by MG-ELISA, were MG-rPCR$_1$ negative, whereas in the second part of the study, other 49 flocks, which were 87.8% MG seropositive, were found 42.9% positive by MG-rPCR$_2$. In addition, 5 selected flocks from the first 28 were negative, whereas 7.4% of the 27 selected flocks from the second 49 were positive by MS-rPCR. Overall, 81 out of 432 MG-rPCR$_{1-2}$ (18.7%) performed from 77 flocks, and 13 out of 187 MS-rPCRs (6.9%) in 32 flocks were determined as positive. ELISA results indicated that there could be significantly high false-positives in serological tests, thus results should not be relied upon one test system. Also, this study revealed that, for the confirmation of *Mycoplasma*-infected flocks in laboratories, rPCR is a reliable method as long as suitable primers are selected, and that MG and MS prevalence is considerably high in winter season.

Keywords: *Mycoplasma gallisepticum, Mycoplasma synoviae, Real time PCR, ELISA, Breeder chicken*

Damızlık Tavuk Kümeslerinde *Mycoplasma gallisepticum*ve *Mycoplasma synoviae*'nin Gerçek Zamanlı PCR'lar ile ve *Mycoplasma gallisepticum* Antikorlarının ELISA ile Tespiti

Özet

Bu çalışmada, *Mycoplasma gallisepticum* (MG) ve *Mycoplasma synoviae* (MS)'nın solunum sistemine ait semptomlara sahip damızlık tavuk kümeslerindeki yaygınlığını tespit etmek amaçlandı. Toplam 77 kümes (2153 trakeal svab ve kan örneği)'in toplanan tüm örnekleri MG gerçek zamanlı PCR (MG-rPCR) ve MG-ELISA ile, 32 kümes ise MS-rPCR ile test edildi. Çalışmanın birinci bölümünü kapsayan ilk 28 kümeste MG-antikorları yüksek bulundu ve belirgin klinik semptomlar gösteren bu tavukların örnekleri MG-rPCR$_1$ ile negatif sonuç verdi. Bu nedenle, bu PCR'da kullanılan MG-lipoprotein geni-spesifik primerler (MG-rPCR$_1$), MS tespitinde kullanılan MS-16S rRNA primerlerine (MS-rPCR) benzer olarak, MG-16S rRNA primerleri (MG-rPCR$_2$) ile değiştirildi ve çalışmaya bu primerler ile devam edildi. MG-ELISA ile %100 pozitif olan ilk 28 kümesin tümü MG-rPCR$_1$ ile negatif iken, çalışmanın 2. bölümünde %87.8 MG seropozitif olarak saptanan diğer 49 kümes MG-rPCR$_2$ ile %42.9 pozitif bulundu. Ayrıca, bu ilk 28 kümesten seçilen 5 adetinde MS-rPCR negatif iken, daha sonraki 49 kümesten seçilen 27'si MS-rPCR ile %7.4 pozitif olarak tespit edildi. Genel olarak, toplam 77 kümeste uygulanan 432 MG-rPCR$_{1-2}$'den 81'i (%18.7) ve bu kümeslerden 32 adetinde yapılan 187 MS-rPCR'ın 13'ü (%6.9) pozitif bulundu. ELISA sonuçları ise serolojik testlerden önemli oranda yanlış-pozitif sonuçlar alınabileceğini ve tek bir test sistemine güvenilmemesi gerektiğini işaret etti. Bu çalışma ile, aynı zamanda, *Mycoplasma*-enfekte kümeslerin laboratuvarlarda doğrulanabilmesi için doğru primerler seçildiğinde rPCR'ın güvenli bir metot olduğu, damızlık tavuk kümeslerinde MG ve MS prevalansının özellikle kış mevsiminde oldukça yüksek olduğu ortaya kondu.

Anahtar sözcükler: *Mycoplasma gallisepticum, Mycoplasma synoviae, Gerçek zamanlı PCR, ELISA, Damızlık tavuk*

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INTRODUCTION

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are infectious agents of chronic respiratory disease in chickens [1]. Multi-age commercial egg complexes are mostly positive for MG and MS and in some parts of the world, both infections are widespread in commercial chicken and turkey production [2]. MG and MS cause important primary and secondary bacterial poultry diseases [2,3].

Due to the persistent nature of the *Mycoplasma* infection and its vertical mode of transmission, monitoring and eradication is a preferable strategy for the achievement of a long-lasting *Mycoplasma*-free poultry stock [4]. Isolation and identification of the organism is the 'gold standard' for diagnosis of *Mycoplasma* infections. However, pathogenic avian mycoplasmas are slow growing (might require up to 3 weeks for detectable growth) and relatively fastidious organism, which are suppressed by use of antibiotic therapy, and, commonly overgrown by commensals such as *Mycoplasma gallinarum* and *Mycoplasma gallinaceum* [2].

Serology including ELISA is much faster than culturing, but nonspecific reactions and cross-reactions between bacterial species, misinterpretations due to recent vaccination, and high cost are all disadvantage of serology [1,5]. MG strains of low virulence typically produce a poor antibody response, and isolation from clinical specimens may be difficult [6]. Variability in strains and clinical responses were noted both for MG and MS [1,5], and, Feberwee et al.[1] indicated encountering flocks exhibiting low levels of serological response.

PCR is a rapid, sensitive and specific method, and is often used to complement culture to detect the presence of specific *Mycoplasma* DNA. There are different PCR procedures such as conventional and real time PCRs (rPCR) for MG or MS detection and their advantages and disadvantages have previously been discussed [1,4,5,7-10].

The objective of this study was rapid detection of MG and MS using rPCRs with three different primer pairs in the chicken breeder flocks, which were screened for antibody to MG by ELISA during the winter and summer seasons.

MATERIAL and METHODS

Standard MG and MS Strains

The MG S6 and the MS K1858 strains were kindly provided by *Mycoplasma* Laboratory, Pendik Veterinary Research Institute, Istanbul, Turkey, and were used as positive controls.

Tracheal Swap and Blood Samples

During 16 months trial period (including one winter season) a total of 432 live chicken tracheal swab samples, comprised of 2153 individual samples, each pooled into 4-5, from 77 breeder flocks, with no antibiotic treatment, belonging to 13 companies, for MG were sampled as described [11], and transferred to the laboratory for rPCR test. Thirty-two of these 77 breeder flocks' same tracheal swab samples, belonging to 5 of these 13 companies, pooled into 127 tracheal samples, comprised of 935 individual tracheal swab samples, also used for MS-rPCR. That is, 77 breeder flocks were sampled for MG-rPCR and the same templates from 32 of these 77 breeder flocks' samples were also tested for MS-rPCR. All flocks had respiratory symptoms and tracheal swab samples were taken from chickens with marked respiratory symptoms. Concurrently, 2153 blood samples from same chickens were collected and tested by MG specific ELISA.

MG-ELISA

ELISA was performed using *Mycoplasma gallisepticum* antibody test kit (Synbiotics, catalog no: AUCMG900, Zoetis) following the instructions described by the manufacturer.

DNA Extraction

DNA was extracted from pooled tracheal swab samples, suspended and vortexed in 1 ml of sterile PBS in 1.5 µl tubes. The suspension was centrifuged for 30 min at 14.000 x g at 4ºC, and the swabs were discarded. The supernatant was carefully removed and the pellet was resuspended in 25 µl sterile deionized water. Then, this mix was boiled at 95-100ºC for 30 min, and kept on ice for 10 min before centrifugation at 14.000 x g for 5 min. The supernatant was used as template in rPCRs.

Primers

Primers used in this study for detection of MG and MS genes, references, amplified product size, name's of the PCR, number of the PCR worked with these primers, company's cod worked with these primers were shown in *Table 1*.

MG and MSrPCRs

rPCR reactions (MG-rPCR$_1$, MG-rPCR$_2$, MS-rPCR) were performed with the same conditions and cycling parameters by a LightCycler™ 2.0 system (Roche Diagnostics, Mannheim, Germany) using FastStart DNA Master SYBR Green I (SGI) PCR mix and reagents (Roche, catalog no: 03 003 230 001). Each reaction was performed in 20 µl volumes, including 18 µl of reaction mixture containing 2 µl 1X LC FastStart DNA SGI Master Mix, 4 mM $MgCl_2$, 0.5 mM of each primer and 2 µl of template DNA. Cycling parameters were as follows: initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 10 s, annealing at 50ºC for 10 s and extension at 72ºC for 20 s.

Table 1. *Primers used in this study for detection of MG and MS genes, references, amplified product size, name's of the PCR, number of the PCR worked with these primers, company's cod worked with these primers*

Tablo 1. *MG ve MS genlerinin tespiti için çalışmada kullanılan primerler, alındıkları literatürler, primerlerin amplifikasyon büyüklüğü, PCR'ın adı, primerlerle yapılan PCR sayısı, primerlerle çalışılan şirketlerin kodu*

Gene	Sequence (5'-3')	Reference	Amplified Product Size (bp)	Name's of the PCR	Number of the PCR, Worked These Primers	Company's Cod, Worked with These Primers
MG- lipoprotein gene	GATTTCGAAGAATCAACTGT AAGGGATTAATATTCCCAAC	[8]	400	MG-rPCR$_1$	159	1, 2, 3, 4, 5
MG-16S rRNA gene	GAGCTAATCTGTAAAGTTGGTC GCTTCCTTGCGGTTAGCAAC	[12]	185	MG-rPCR$_2$	273	6, 7, 8, 9, 10, 11, 12, 13
MS-16S rRNA gene	GAGAAGCAAAATAGTGATATC TCGTCTCCGAAGTTAACAA	[10]	207	MS-rPCR	187	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13

RESULTS

MG and MS rPCRs

No MG was detected by MG-rPCR$_1$ and MS-rPCR from the 28 and 5 of the flocks, respectively. Number of pooled samples for MG and MS detection and seasonal evaluation were demonstrated in *Table 2*. Flocks were found 42.9% (21/49) and 7.4% (2/27) positive by both MGr-PCR$_2$ and MS-rPCR, respectively. Among pooled samples, 12.8% (35/273) and 10.2% (13/127) were positive by MG-rPCR$_2$ and MS-rPCR, respectively. There was no MGr-PCR$_2$ positivity in summer, whereas this rate increased to 44% positivity in winter. Similarly, MS-rPCR detection rate was 0% in summer opposed to 10.8% positivity in winter *(Table 3)*. Overall, 81 out of 432 (18.7%) samples were positive by MG-rPCR$_{1-2}$ in 77 flocks, and 13 out of 187 (6.9%) samples were positive by MS-rPCR in 32 flocks. In flock-based evaluation, rPCR positive flock rate was 23.4% (18/77) and 8.1% (2/32) for MG and MS, respectively. Overall, MG-rPCRs were 33% positive during winter and 0% in summer season. MS detection rate was 8.1% and 0% in winter and summer, respectively *(Table 4)*.

MG-ELISA

Seropositive flock rate was 68.8% (53/77) by MG-ELISA. Individually, 453 of the 2153 (21.0%) samples were positive by MG-ELISA. Seroprevalence of MG infection was higher during winter season (27.6%) than in summer season (12.7%) *(Table 4)*.

DISCUSSION

Initial MG-rPCR$_1$ yielding consecutively negative results in contrast to 100% seropositivity in flocks by MG-ELISA from 28 flocks *(Table 2)* was resolved after replacement of MG-lipoprotein primers with MG-16S rRNA primers in the study. Results from this new rPCR, which was designated as MG-rPCR$_2$ were consistent with MG-ELISA results: flocks were found 42.9% (21/49) positive by MG-rPCR$_2$ and 87.8% (43/49) positive by MG-ELISA *(Table 3)*. There are earlier reports on PCR assays targeting the 16S rRNA gene regions [1,9,10], whereas more recent assays attempted to target more species-specific gene regions [4,5,8,13]. rRNA genes are present in all prokaryotes and include regions that are highly conserved among bacteria. Raviv and Kleven have reported [4] that PCR assays target the 16S rRNA gene might cross-react with other mollicutes and prokaryotes. In our study, when we replaced the MG-lipoprotein-based primers with 16S rRNA-based primers, our results started to match up with our serological data, which is in contrast to the findings of Raviv and Kleven [4]. Garcia et al.[13] compared the 16S rRNA, *mgc*2, lipoprotein and *gap*A surface protein genes for MG detection and found that *mgc*2 and the 16S rRNA methods had similar and the best detection limits. Hess et al.[14] reported that MG 16S rRNA gene-based PCR, which was developed by Lauermann [12], had higher analytical sensitivity than other PCR methods tested. It has been reported that the 16S rRNA-based PCR can amplify DNA from *Mycoplasma imitans* (MI), a phylogenetically related avian *Mycoplasma* with very similar

Table 2. *Results of tracheal swab and sera samples of 5 companies (company cod: 1-5) by MG-rPCR$_1$ (with primers of lipoprotein gene), MS-rPCR and MG-ELISA*

Tablo 2. *Beş şirkete ait (şirket kodu: 1-5) trakeal svab ve serum örneklerinin MG-rPCR$_1$ (lipoprotein geni primerleri ile), MS-rPCR ve MG-ELISA sonuçları*

Company	Total Flock/ Positive by MG-rPCR$_1$	Number of Total Tracheal Swap and Sera	Number of Pooled Samples/ MG Positive Samples by MG-rPCR$_1$ - (%)	Total MG-rPCR$_1$/ Positive %		Number of MS-rPCR/ Positive Samples - (%)	Total MS-rPCR/ Positive %		Number of MG-ELISA/ Positive Samples - (%)	Total MG-ELISA Positive Samples %		Season
1	5	80	16/0 - (0)	0 (Summer)	0 (Winter)	-	0 (Summer)	0 (Winter)	80/9 - (11.25)	18.3 (Summer)	16.1 (Winter)	Summer
2	8	200	40/0 - (0)			-			200/17 - (8.5)			Summer
3	6	128	27/0 - (0)			-			128/45 - (35.1)			Summer
4	5	300	60/0 - (0)			60/0 - (0)			300/78 - (26)			Winter
5	4	80	16/0 - (0)			-			80/5 - (6.2)			Winter
Total	28	788	159/0 - (0)			60/0 - (0)			788/154 - (19.5)			

Table 3. *Results of tracheal swab and sera samples of 8 companies (company cod: 6-13) by MG-rPCR$_2$ (with primers of 16S rRNA gene), MS-rPCR and MG-ELISA*
Tablo 3. *Sekiz şirkete ait (şirket kodu: 6-13) trakeal svab ve serum örneklerinin MG-rPCR$_2$ (16S rRNA geni primerleri ile), MS-rPCR ve MG-ELISA sonuçları*

Company	Total Flock/ Positive Flockby MG-rPCR$_2$	Number of Total Tracheal Swap and Sera	Number of Pooled Samples/ MG Positive Samples by rPCR$_2$ - (%)	Total MG-rPCR$_2$/ Positive %		Number of MS-rPCR/ Positive Samples - (%)	Total MS-rPCR/ Positive %		Number of MG-ELISA/ Positive Samples - (%)	Total MG-ELISA Positive Samples %		Season
6	5/5	100	20/16 - (80)			20/0 - (0)			100/62 - (62)			Winter
7	5/5	100	20/20 - (100)			-			100/42 - (42)			Winter
8	3/2	350	70/10 - (14.3)			-			350/35 - (10)			Winter
9	10/7	200	40/35 - (70)			40/0 - (0)			200/103 - (51.5)			Winter
10	6/2[MS+*]	200	40/0 - (0)	0 (Summer)	44 (Winter)	40/13 - (32.5)	0 (Summer)	10.8 (Winter)	200/35 - (17.5)	4,2 (Summer)	31.5 (Winter)	Winter
11	6	135	27/0 - (0)			27/0 - (0)			135/0 - (0)			Summer
12	10	200	40/0 - (0)			-			200/17 - (8.5)			Summer
13	4	80	16/0 - (0)			-			80/5 - (6.25)			Winter
Total	49/21	1365	273/35 - (12.8)			127/13 - (10.2)			1365/299 - (21.9)			

** 6 flocks of 10. company had no positivity for MG-rPCR$_2$, but 2 flocks had positivity with MS-rPCR*

Table 4. *Total evaluation of worked two MG-PCRs, MS-rPCR and MG-ELISA results*
Tablo 4. *Çalışılan 2 MG-PCRs, MS-rPCR ve MG-ELISA sonuçlarının birlikte değerlendirilmesi*

Total Flock/ Positive by MG-rPCR$_{1-2}$	Total Flock/ Positive by MS-rPCR	Number of Tracheal Swap for MG/MS	Number of Pooled Samples for MG-rPCR$_{1-2}$/ Positive	Total MG-rPCR$_{1-2}$ Positive Samples According to Season %		Number of Pooled Samples for MS-rPCR/ Positive	Total MS-rPCR/ Positive Samples According to Season %		Number of MG-ELISA/ Positive	Total MG-ELISA Positive Samples According to Season %	
77/18	32/2	2153/935	432/81	0 (Summer)	33 (Winter)	187/13	0 (Summer)	8.1 (Winter)	2153/453	12.7 (Summer)	27.6 (Winter)
28%	8.1%		18.7%			6.9%			21.0%		

16S rRNA genes to MG [15]. However, since MI has been only isolated from ducks, geese and partridges, it is considered of limited significance in diagnosis in samples from chickens [16].

We found that the overall rPCR positive flock rate was 23.38% (18/77) and 8.12% (2/32) for MG and MS, respectively *(Table 3)*. In a study from Turkey [5], the MG prevalence in MG positive flocks was found 16.1% (5/31) and 29% (9/31), by MG culture and PCR, respectively. However in other studies in Turkey, the positive results are higher than our results. Cengiz et al.[17] have reported that 14 of 26 (53.8%) investigated flocks were MG-PCR positive. Dakman et al.[18] have found 87.5% MS-PCR positive from 1.200 tracheal swabs and serum samples. Tuzcu et al.[19] have determined 80% MG-PCR positive results from 3 different broiler breeder farms. Aras and Sayın [20] have found that 5 out of 20 (25%) layer flocks were MS positive by PCR. Similarly, the incidence of MS infection detected by PCR in commercial flocks in Brazil and Netherlands has been found as 72.7% and 73%, respectively [7,21]. Also, MG-PCR positive results have been reported as 73.64% and 58.1% in Brazil and Vietnam, respectively [7,22].

In this study, seropositive flock rate was found 68.8% (53/77) by MG-ELISA. Four hundred fifty three individual tracheal samples out of 2153 (21.04%) were positive by MG-ELISA *(Table 4)*. In a previous study [5] MG-seropositive flock rate was reported as 48.4% (15/31) and 32.3% (10/31) by Rapid Plate Agglutination (RPA) and Hem-agglutination Inhibition (HI) tests, respectively. In our study, flocks belonging to companies 1, 2, 3, 4, and 5 had high MG-antibody (6-35%) levels; despite their MG-rPCR$_1$ negative results *(Table 2)*. This could be related either to the possibly lower specificity of the PCR primers used in MG-rPCR$_1$ or to the lower sensitivity of the MG-ELISA. Additionally, we found a high Infectious Bronchitis reverse transcriptase PCR-positive rate [23] from Company 12's same tracheal swab samples (data not shown), which were negative for MG and MS, but MG-antibody rate was 8.5% by MG-ELISA. Feberwee et al.[1] have found that the number of cross-reactions (false positives) in serological tests was lower in infection with the ATCC strains than in infections with the MG and MS field strains. In their study, they compared different commercial ELISA tests for serological identification of MG, and reported that a certain level of false positive results could be expected in any serological test. They also implied that the level of false positive results varied between several serological tests, and concluded that it was not advisable to rely completely on one test (system) only. Likewise, Aras and Sayın [20] indicated that genetic similarity between field MS strains from Turkey could vary 53% to 100% by RAPD analyses. This fact could also have an important impact on the sensitivity and specificity of a particular serological test. In our study, flocks of Companies 6, 7, and 9 had low MG-antibody rate (42-62%) in contrast to their high MG-rPCR$_2$ positivity rates (70-100%) *(Table 3)*. This instance could be explained by the possibility of a recently starting MG infection, which could not yet gave rise to detectable MG-antibody levels. Consequently, we should note here

that serological tests as ELISA are widely used screening methods with known shortcomings as cross reactivity, high sensitivity and low specificity. Therefore, ELISA can only be used in rapid primary screening of the flocks, and should be complemented by culture and PCR for confirmative and definitive diagnosis.

When our results are evaluated on seasonal basis, we found that MG and MS infections were more prevalent especially at winter season than summer season by both PCRs and ELISA *(Table 4)* similar to previous reports [24-26].

In this study, we used two rPCRs with the same cycling parameters and SGI without using specific probes for MG and MS detection primarily to reduce the cost of detection. Secondly, SGI rPCR has considerably higher detection capability than probe-based PCRs, since probes can detect only when all bases match up with the target DNA. Therefore, if there are (point) mutations, as widely seen in field *Mycoplasma* strains, probes may not match up, leading to false negative results. Therefore, SGI rPCR has no match up problem with mutated DNA yielding slightly shifted melting temperatures, which can be determined by melting curve analysis of the PCR product after rPCR.

Previously, the 16S rRNA gene-specific primers used in this study were well studied and evaluated by Lauerman et al.[10] for MS detection, where they found 100% correlation between their MS-PCR on both specificity and sensitivity. When evaluated MG and MS together, two primers sets utilized in this work were previously published with a known sensitivity of 70-100 colony-forming units (CFU) ml^{-1} [10]. Feberwee et al.[1] also reported that culture and PCR tests had comparable sensitivity in detecting both MG and MS with the same primers. Also, Jarquin et al.[9] used these primers in both conventional PCR and SYBR Green I (SGI) rPCR and found that there was no difference between two PCRs. Also, they determined that the SGIrPCR assay developed in their study was more rapid than all three methods tested and more sensitive and specific than culturing or serology. They have found that 13 cases were found positive by PCR and in only 9 cases culture was positive.

Infection of breeder poultry flocks with MG and MS can be economically devastating to producers especially at cold and wet winter seasons. The accurate and the timely diagnosis of MG and MS infections are essential to control these infections in poultry. The rPCR surveillance tool used in this study has the potential to save producers from these large losses on the basis of a reduced detection time, allowing producers to act quickly and prevent spread of disease. Our results showed that suitable primers selected for correct PCR assays with serological method (ELISA) for primary screening of the flocks is a considerably economical approach in diagnosis of MG and MS infections in breeder chicken flocks which are important for providing MG/MS-free progeny.

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Research Article

The Protective Effects of Peganum harmala Extract on Lung and Kidney in Sepsis Induced by Cecal Ligation and Perforation in Rats

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Abstract

Sepsis is characterized by multiple organ dysfunction, tissue damage and hyper-inflammation. Peganum harmala (PH) is a plant considered for its antibacterial, antioxidant, anticarcinogen and antiinflammatory properties. This study was aimed to evaluate the protective effects of PH extract on tissues and cytokines in sepsis induced by cecal ligation and perforation (CLP) in rats. Forty rats were divided into five groups. Groups were sham-operated (control), CLP, 90 mg/kg PH-treated CLP, 180 mg/kg PH-treated CLP and 180 mg/kg PH-treated control healthy. Animals were sacrificed at the 16th h of the study. Biochemical and histopathological analyses were performed in lung, kidney and blood samples. Both 90 mg/kg and 180 mg/kg doses of PH decreased the level of interleukin-1 beta (IL-1β) and interleukin-10 (IL-10), and high dose of PH reduced the tumor necrosis factor-alpha (TNF-α) in the serum compared to CLP group. The PH also increased the activity of superoxide dismutase (SOD) and the total levels of glutathione (GSH) in the lung and kidney tissues of septic rats. The level of malondialdehyde (MDA) in the lung and kidney tissues was reduced in both PH treated CLP groups. The histopathological results were in accordance with the biochemical results. The CLP + 180 mg/kg PH group had the lowest inflammation score in the lung. In conclusion, the administration of PH has prevented the oxidative stress, the cytokine response and the inflammation in CLP-induced septic rats.

Keywords: *Inflammation, polymicrobial sepsis, oxidative stress, cytokine, tissue damage*

Peganum harmala Ekstraktının Ratlarda Çekal Bağlama ve Delme ile İndüklenen Sepsiste Akciğer ve Böbrek Üzerine Koruyucu Etkileri

Özet

Sepsis organlarda fonksiyon bozukluğu, doku hasarı ve hiper-inflamasyon ile karakterizedir. Peganum harmala (PH) antibakteriyel, antioksidant, antikarsinojenik ve antiinflamatuar özellikleri olduğu kabul edilen bir bitkidir. Bu çalışma çekal bağlama ve delme (CLP) ile sepsis oluşturulan ratlarda PH ekstraktının dokular ve sitokinler üzerine koruyucu etkilerinin değerlendirilmesi amacıyla yapılmıştır. Kırk rat 5 gruba ayrıldı. Gruplar sham-operasyon (kontrol) CLP, 90 mg/kg PH-uygulanan CLP, 180 mg/kg PH-uygulanan CLP ve 180 mg/kg PH-uygulanan sağlıklı kontroldür. Hayvanlar çalışmanın 16. saatinde sakrifiye edildi. Akciğer, böbrek ve kan örneklerinde biyokimyasal ve histopatolojik analizler yapıldı. CLP grubuna kıyasla PH'nın 90 mg/kg ve 180 mg/kg her iki dozu da serum interlökin-1 beta (IL1β) ve interlökin-10 (IL-10) düzeylerini azalttı ve yüksek doz PH tümör nekrozis faktör-alfa (TNF-α) düzeyini düşürdü. PH uygulaması septik ratların akciğer ve böbrek dokularındaki süperoksit dismutaz (SOD) aktivitesini ve total glutatyon (GSH) düzeyini de arttırdı. Akciğer ve böbrek dokularındaki malondialdehit (MDA) düzeyi PH uygulanan CLP gruplarında azaldı. Histopatolojik bulgular biyokimyasal bulgular ile uyumluydu. CLP grupları içerisinde, CLP + 180 mg/kg PH grubu akciğerde en düşük inflamasyon skoruna sahipti. Sonuç olarak, PH uygulaması ratlarda CLP ile indüklenen sepsiste oksidatif stres, sitokin yanıtı ve inflamasyonu önledi.

Anahtar sözcükler: *Yangı, polimikrobiyal sepsis, oksidatif stres, sitokin, doku hasarı*

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INTRODUCTION

Sepsis, a complex syndrome, is a systemic response of an organism against microorganisms and/or their toxins in the bloodstream [1]. It is one of the major causes of mortality in worldwide [2]. Sepsis leads to multiple organ dysfunction, coagulopathy, hypoglycemia, systemic inflammation, metabolic acidosis, hypotension, and oxidative damage [3-6]. During sepsis, hyper-inflammation and oxidative damage contribute to the immune response with mainly effects on tissues of lungs, liver, kidney, and intestines [7-9]. Sepsis is currently treated with specific antibiotics and other pharmacological agents that antioxidants and anti-inflammatory reagents [10-12].

Peganum harmala (PH) is a traditional plant that has a long history of use as a folk medicine in Turkey, Iran and China to treat diseases. The PH contents chemical ingredients such as alkaloids, steroids, flavonoids, anthraquinones, amino acids, and polysaccharides from its seeds, leaves, flowers, stems and roots. Among these compounds, the alkaloids, mostly β-carbolines such as harmine, harmaline, harmalol, harmol and harman [13,14]. These alkaloids were found to be the main substances responsible for the analgesic, antiinflammatory, anti-bacterial, anti-parasitic, antioxidant, insecticidal, anti-tumoral and vaso-relaxant activities of PH [15-25].

However, to the best of our knowledge, the effects of PH on pro-inflammatory mediators and oxidative response have not been documented in rat experimental sepsis model, until now. Due to high alkaloids content and anti-oxidative and anti-inflammatory effects of PH, we hypothesized that PH may protect the organism against sepsis mortality. Thus, the purpose of this study was to evaluate biochemically and histopathologically the protective effects (antioxidative/anticytokine properties) of PH extract on lung and kidney tissues and blood samples in sepsis induced by cecal ligation and perforation (CLP) in rats.

MATERIAL and METHODS

Animals

In the present study, a total of 40 male Wistar rats were used for the experiments. The rats were housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22±1°C under lighting controls (14 h light/10 h dark cycle). Standard rat ration and tap water were given ad libitum. Each rat weighed 230-250 g, and all were obtained from Experimental Animal Laboratory of Medicinal and Experimental Application and Research Center (ATADEM). Animal experiments and procedures were performed in accordance with the national guidelines for the use and care of laboratory animals and they were approved by the university's local animal care committee (Decision No: 75/2014).

Chemicals

All chemicals were purchased from Sigma Chemical Co (Germany). ELISA kits were supplied from Invitrogen. IL-1β, IL-6 and TNF-α from each sample were measured with highly sensitive ELISA kits; Invitrogen-KRC0011, Invitrogen -KRC0101 and Invitrogen-KRC3011 (Grand Island, USA), respectively.

Preparations of PH Extract

The plant material was collected in July 2012 from Kayseri, a city in Middle Anatolia region of Turkey. The PH seeds were dried and grounded. Then, it was extracted with methanol at 40ºC for 4 h. The mixture was filtered and allowed for phase separation. The resultant supernatant was concentrated by using a rotary evaporator.

Experimental Design

The rats were allocated into five groups, each composed of 8 individual rats as shown below.

Group I (Sham): sham operated control group,

Group II (CLP): CLP group,

Group III (CLP+PH1): CLP + 90 mg/kg PH (oral administration),

Group IV (CLP+PH2): CLP + 180 mg/kg PH (oral administration),

Group V (Sham+PH2): 180 mg/kg PH (oral administration).

Sepsis Model

A CLP polymicrobial sepsis model was applied to the rats. Polymicrobial sepsis was induced through cecal ligation and two-hole puncture [7]. Briefly, rats were not fasted prior to the procedure and anesthesia was induced through intraperitoneal administration of thiopental sodium 25 mg/kg. After the abdomen was shaved, the peritoneum was opened. Once the diaphragm exposed the abdominal organs, the cecum was isolated and ligated with a 3/0 silk ligature just distal to the ileo-cecal valve. Two punctures were made with a 16-gauge needle through the cecum distal to the point of ligation, and the cecum was placed to the peritoneal cavity. The muscle and skin of abdominal incision was then closed with a 4/0 sterile synthetic, absorbable suture. The wound was bathed in 1% lidocaine solution to ensure analgesia.

The sham-operated groups received laparotomies, and the cecum were manipulated, but not ligated or perforated. All of the animals were given 2 ml/100 g body weight of normal saline subcutaneously at the time of surgery and 6 h after the operations, for fluid resuscitation. Immediately after the surgical procedure, the rats in the PH2-sham and the PH-treated CLP groups received 90 and 180 mg/kg doses of PH extract, which were administered with an oral gavage. An equal volume of saline was administered to the sham group and the CLP group. The rats were deprived

of food postoperatively but had free access to water for the next 16 h until they were sacrificed.

All groups were sacrificed 16 h later with an overdose of a general anesthetic (thiopental sodium, 50 mg/kg), and whole blood samples were withdrawn via the intracardiac method. The serum was immediately separated by centrifugation at 2.500 g for 10 min at +4°C, and stored at –80°C. The lungs and kidney were then quickly removed from all of the rats and washed in ice-cold saline. Half of the tissue was transferred to biochemistry laboratory and kept at –80°C for biochemical analyses, while the other half were fixed in a 10% formalin solution for histopathological analyses.

Biochemical Analyses of Cytokine Levels in the Serum

Cardiac blood samples were collected immediately and transferred to the laboratory to facilitate the estimation of the inflammatory cytokines, IL-1β, IL-10 and TNF-α levels in the serum. Sera from the five rat groups were separated and stored at –80°C until they were thawed for the assay. IL-1β, IL-10 and TNF-α from each sample were measured with highly sensitive ELISA kits; rat IL-1β immunoassay Kit (Invitrogen, Cat. No: KRC0011), IL-10 Elisa Kit (Invitrogen, Cat. No: KRC0101), TNF-α Elisa Kit (Invitrogen, Cat. No: KRC3011), respectively. Kits were specific for rat cytokines, and all measurements were performed according to the manufacturer's instructions by using Bio-Tek µQuant (USA) multi plate spectrophotometer. Cytokine assays for each animal and its correlated control were run in the same lot.

Biochemical Analyses of Lung and Kidney Tissues

After macroscopic analysis, the lung and kidney tissues of the rats were kept at –80°C. First, 100 mg of tissue from each rat was perfused with phosphate buffered saline (PBS)/heparin (137 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 + heparin (1.000 units/L), pH 7.2). After grinding in liquid nitrogen, tissues were homogenized in buffers specific for each parameter on ice bath by a tissue homogenizer.

Malondialdehyde (MDA): MDA level was determined according to the methods of Ohkawa et al.[26]. Tissue samples were homogenized in 2.5 ml 10% KCl over 25 mg sample using an ultraturrax homogenizer (IKA-Germany). Then, homogenates were centrifuged at 4000g and 4°C for 30 min. The supernatant was used to determine the MDA level. Within the capped tubes, 250µl homogenate, 100 µl 8% sodium lauril sulphate, 750 µl 20% acetic acid, 750 µl 0.08% thiobarbituric acid and 150µl distilled water were vortexed for 1 min. This mixture was incubated in 100°C for 60 min, centrifuged at 4.000 rpm for 10 min after adding 2.5ml n-butanol/pyridine and 200 µl supernatant was pipetted into microplates. The occurrence of red color was measured at 532nm by an ELISA reader (µQuant, BioTek). Standard curve was generated using 1, 1, 3, 3–tetramedhoxypropane. All samples were measured in triplicate. The results were expressed as nanomol MDA per milligram tissue (nmol/mg tissue).

Total Gluthathione (GSH): GSH analysis was measured according to methods described by Sedlak and Lindsay [27]. Twenty five mg tissue sample was homogenized by the ultraturrax homogenizer with 2.5 ml buffer (50 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 3.000g and 4°C for 30 min to obtain supernatant for determination of GSH level. In capped tubes, 125µl supernatant, 375 µl buffer (200 mM Tris-HCl including 0.2 mM EDTA, pH 8.2) + 25 µl DTNB + 1975µl methanol mixture was incubated at 37°C for 30 min. The resultant yellow color was measured at 412nm by the ELISA reader. The results were expressed as GSH nmol/mg tissue.

Superoxide Dismutase (SOD): SOD activity was analyzed according to the method described by Sun et al.[28]. Twenty five mg tissue sample was homogenized by the ultraturrax homogenizer with 2.5 ml buffer (0.2 mM Tris-HCl, pH 7.4). The samples were centrifuged at 5.000 g and 4°C for 60 min to obtain the supernatant. 200 µl supernatant, 980 µl measurement mixture (0.3 mM xanthine, 0.6 mM EDTA, 150 µM NBT, 0.4M Na_2CO_3, 1g/L BSA) and 20 µl xanthine oxidase incubated at 25°C for 20 min. The reaction was inhibited by $CuCl_2$ and measured at 560 nm. The results were expressed as SOD mmol/min/mg tissue.

Histopathology Process

For histopathology, tissue samples from lung and kidney were obtained and fixed in 10% buffered formalin solution. After the routine histopathology process, paraffin sections in 5 µ stained with hematoxylin and eosin (HE). All slides were examined under the light microscopy (Olympus BX52 with DP72 camera system).

Inflammation Scoring in Tissues

For inflammation scoring, histopathological changes in lung and kidney tissues were semi quantitatively assessed. Ten different areas were examined under 40X magnification. Histopathological changes in lung (hyperemia, vasculitis, alveolar or bronchiolar exudate, desquamation of bronchiolar epithelium) and kidney (glomerulitis, degeneration of tubulary epithelium, interstitial cellular infiltration and hyaline casts within the tubulary lumina) were graded as follows: none: -, mild: +, moderate: ++, and severe: +++.

Statistical Analyses

The IBM SPSS Statistics 20 computer program package was used for statistical calculations. Data for the serum cytokine levels measured by the ELISA and the oxidant and antioxidant enzymes were subjected to one-way ANOVA followed by Tukey's post hoc test and were considered significant at $P<0.05$. All data were expressed as mean ± standard deviation (SD) in each group.

RESULTS

The Effects of PH on Cytokines in Serum

The levels of IL-1β, IL-10, and TNF-α of serum were shown in *Table 1*. The cytokine levels significantly increased in the CLP groups (IL-1β: 84.40 ± 10.40 pg/ml, IL-10: 494.33±79.28 pg/ml, and TNF-α: 18.46±5.33 pg/ml) 16 h after sepsis, when compared to the sham operated rats (control group) that the IL-1β, IL-10, and TNF-α were 34.53±3.06 pg/ml, 18.70±4.36 pg/ml, and 2.95±0.62 pg/ml, respectively ($P<0.05$). On the contrary, the serum levels of IL-1β, IL-10, and TNF-α decreased as a result of the administration of both PH1 and PH2 in CLP induced rats ($P<0.05$). The administrations of PH1 and PH2 decreased the serum levels of the IL-1β to 52.60±4.24 pg/ml and 49.84±17.95 pg/ml, respectively. The administrations of PH1 and PH2 decreased the serum levels of the IL-10 to 236.20±182.15 pg/ml and 141.70±70.18 pg/ml, respectively. The administration of PH1 and PH2 decreased the serum levels of the TNF-α to 24.96±1.88 pg/ml and 12.16±6.00 pg/ml, respectively. As shown in *Table 1* the administration of PH2 in the sham control rats did not affect the serum levels of cytokines (IL-1β; 40.79±2.15 pg/ml, IL-10: 20.22± 3.73 pg/ml, and TNF-α: 3.96±0.24pg/ml), when compared to the control group.

The Effects of PH on Oxidants and Antioxidants in Tissues

The levels of MDA, SOD, and GSH in lung tissues were shown in *Table 2*.

The MDA level significantly increased in the CLP groups (17.00±0.58 nmol/mg), when compared to the sham operated rats that the MDA level was 12.93±1.19 nmol/mg. The administrations of PH1 and PH2 decreased the MDA levels in the lung tissue to 15.85±0.75 nmol/mg and 13.98±0.69 nmol/mg respectively. On the contrary, the SOD and GSH levels in lung tissue decreased in the CLP group (110.05±7.56 U/mg protein and 2.30±0.37 nmol/mg protein, respectively), when compared to the sham operated rats that the SOD and GSH levels were 129.79±5.77 U/mg protein and 4.29±0.41 nmol/mg protein, respectively. However, the levels of SOD and GSH were significantly increased in lung tissues of PH1+CLP and PH2+CLP group, respectively (SOD: 150.79±5.58 U/mg protein, GSH: 4.52±0.56 nmol/mg protein and SOD: 166.14±5.50 U/mg protein, GSH: 4.79±0.53 nmol/mg protein). The administration of PH2 in the sham control rats did not significant affect the lung levels of oxidant and antioxidants, when compared to the control group.

The levels of MDA, SOD, and GSH in kidney tissues were shown in *Table 3*.

The MDA level significantly increased in the CLP groups (25.26±2.06 nmol/mg), when compared to the sham operated rats (control group) that the MDA level was 18.81±0.71 nmol/mg. The administrations of PH1 and PH2 decreased in the kidney tissue levels of the MDA to 20.33±1.84 nmol/mg and 17.91±1.13 nmol/mg,

***Table 1.** Effects of Peganum harmala extract treatments on changes in serum levels of interleukin-1β (IL-1β) , interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) in sera of rats*

***Tablo 1.** Peganum harmala ekstraktı uygulamalarının rat serumlarındaki interlökin-1β (IL-1β), interlökin-10 (IL-10) ve tümör nekrosis faktör-α (TNF-α) üzerine etkileri*

Groups	IL-1β (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
Sham	34.53±3.06	18.70±4.36	2.95±0.62
CLP	84.40±10.40*	494.33±79.28*	18.46±5.33*
CLP + PH1	52.60±4.24#	236.20±182.15	24.96±1.88*
CLP + PH2	49.84±17.95#	141.70±70.18#	12.16±6.00*#
Sham + PH2	40.79±2.15	20.22±3.73	3.96±0.24

** Significantly different from Sham rat group (P<0.05), # Significantly different from CLP rat group (P<0.05)*

***Table 2.** Effects of Peganum harmala extracts treatments on changes in levels of malondialdehyde (MDA), activities of superoxide dismutase (SOD) and total glutathione (GSH) in lung tissues of rats*

***Tablo 2.** Peganum harmala ekstraktı uygulamalarının rat akciğer dokularında malondialdehit (MDA), süperoksit dismutaz (SOD) aktivitesi ve total glutatyon (GSH) düzeyleri üzerine etkileri*

Groups	MDA (nmol/mg)	SOD (nmol/mg)	GSH (nmol/mg)
Sham	12.93±1.19	129.79±5.77	4.29±0.41
CLP	17.00±0.58*	110.05±7.56*	2.30±0.37*
CLP + PH1	15.85±0.79*	150.79±5.58*#	4.52±0.56#
CLP + PH2	13.98±0.69#	166.14±5.50*#	4.79±0.53#
Sham + PH2	13.16±0.81	130.73±7.68	3.89±0.27

** Significantly different from Sham rat group (P<0.05), # Significantly different from CLP rat group (P<0.05)*

respectively. On the contrary, the SOD and GSH levels in kidney tissue decreased in the CLP group (SOD:103.14±8.64 U/mg protein and GSH: 2.44±0.22 nmol/mg protein), when compared to the sham operated rats that the SOD and GSH levels were 126.35±10.13 U/mg protein and 3.37±0.19 nmol/mg protein, respectively. However, the levels of SOD and GSH were significantly increased in kidney tissues of PH1+CLP and PH2+CLP group, respectively (SOD: 118.39±8.31 U/mg protein, GSH: 2.95±0.25 nmol/mg protein and SOD: 173.39±7.64 U/mg protein and GSH: 4.08±0.48 nmol/mg protein).

Histopathologic Findings

No histopathologic changes were observed in sham group *(Fig. 1)*. There were hyperemia, vasculitis, severe alveolar and bronchiolar exudation *(Fig. 2a)*, glomerulonephritis, tubular degeneration and severe hyaline cast formation *(Fig. 2b)* in the septic group. Similar histopathologic changes were observed in smaller areas in PH treated groups. There were no exudation within the alveoli and bronchiolar lumina in these groups *(Fig. 3a, Fig. 4a)* but vasculitis in some lung sections was observed. Nephrosis with hyaline casts was observed in many areas in group III *(Fig. 3b)*. However, hyaline formation in limited areas was observed in CLP + 180 mg/kg PH *(Fig. 4b)*. Inflammation scoring was demonstrated in Table 4.

DISCUSSION

Sepsis is an important health problem with high

Table 3. *Effects of Peganum harmala extracts treatments on changes in levels of malondialdehyde (MDA), activities of superoxide dismutase (SOD) and total glutathione (GSH) in kidney tissues of rats*

Tablo 3. *Peganum harmala ekstraktı uygulamalarının rat böbrek dokularında malondialdehit (MDA), süperoksit dismutaz (SOD) aktivitesi ve total glutatyon (GSH) düzeyleri üzerine etkileri*

Groups	MDA (nmol/mg)	SOD (nmol/mg)	GSH (nmol/mg)
Sham	18.81±0.71	126.35±10.13	3.37±0.19
CLP	25.26±2.06*	103.14±8.64*	2.44±0.22*
CLP + PH1	20.33±1.84#	118.39±8.31#	2.95±0.25#
CLP + PH2	17.91±1.13#	173.37±7.64*#	4.08±0.48*#
Sham + PH2	19.75±1.33	131.33±9.38	3.76±0.16

** Significantly different from Sham rat group (P<0.05), # Significantly different from CLP rat group (P<0.05)*

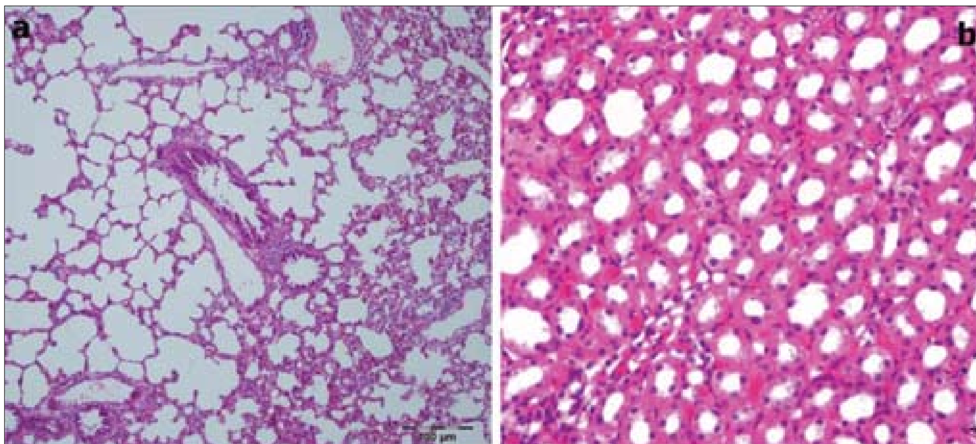

Fig 1. Group I (Sham). Normal histology of lung and kidney tissues in control group. There was no alveolar or bronchiolar exudate accumulation in lung (a) and no hyaline casts within the tubuli (b); HE

Şekil 1. Grup I (Sham). Kontrol grubunda akciğer ve böbrek dokularının normal histolojisi. Alveol ve bronşiol lümenlerinde eksudat yok (a) ve tubülüs lümenlerinde hiyalin kastları yok (b); HE

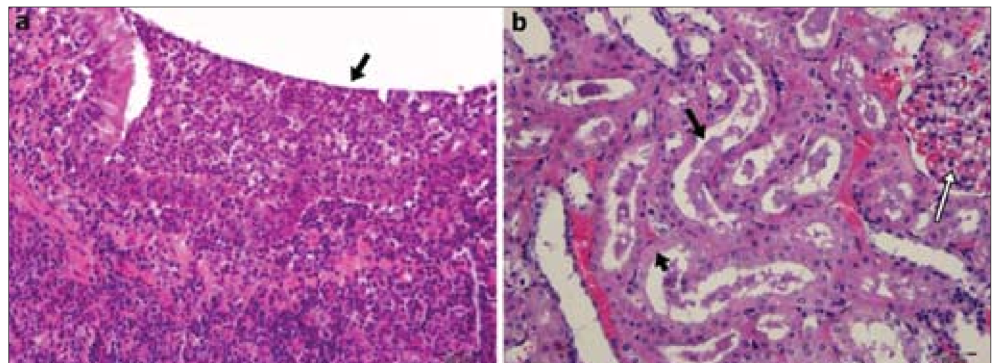

Fig 2. Group II (CLP). Severe bronchopneumonia and alveolar and bronchiolar exudate accumulation *(arrow in a)*, Glomerulitis *(white arrow in b)*, Hyaline casts *(long black arrow in b)* and tubulary degeneration *(short arrow in b)*; HE

Şekil 2. Grup II (CLP). Şiddetli bronkopnömoni, alveolar ve bronşiolar eksudat *(ok, a)*, Glomerulitis *(beyaz ok, b)*, Hyalin kastları *(uzun siyah ok, b)* ve tubüler dejenerasyon *(kısa ok, b)*; HE

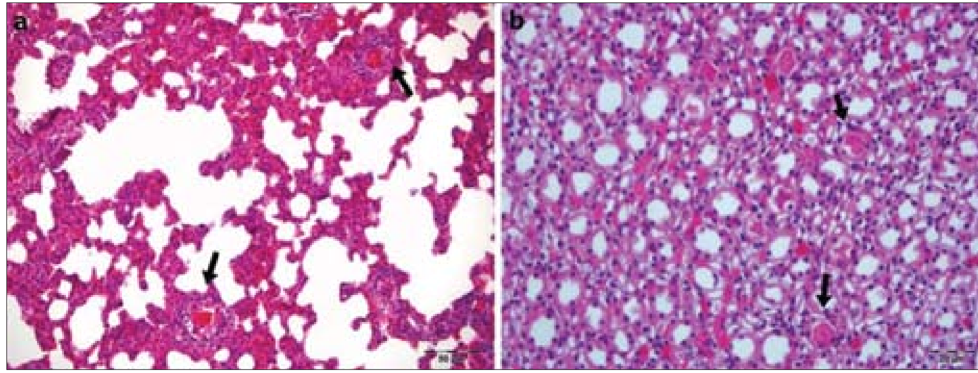

Fig 3. Group III (CLP + 90 mg/kg PH). Prominent decreasing of alveolar and bronchiolar exudate accumulation with vasculitis *(arrow in a)*, Hyaline casts *(arrows in b);* HE

Şekil 3. Grup III (CLP + 90 mg/kg PH). Alveolar ve bronşiolar eksudat birikiminde belirgin azalma görülürken vaskülitis devam etmekte *(ok, a)*, Hiyalin kastları *(oklar, b)*; HE

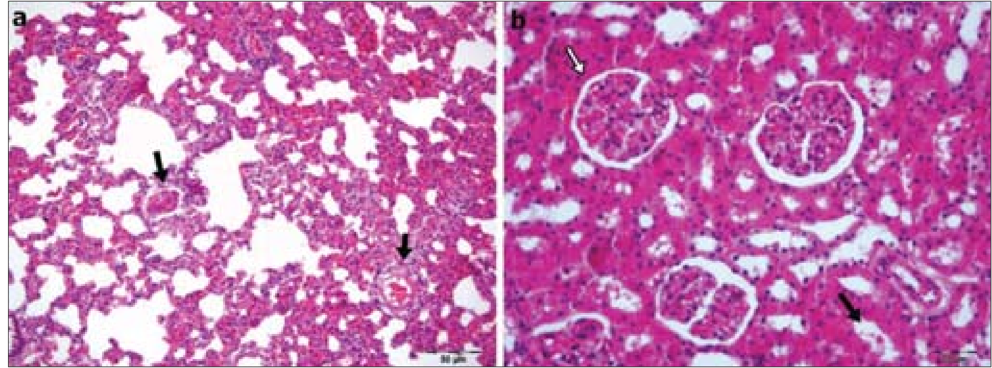

Fig 4. Group IV (CLP +180 mg/kg PH). Prominent decreasing of alveolar and bronchiolar exudate accumulation with vasculitis *(arrows in a)*, Glomerulitis *(white arrow in b)* and decreasing in hyaline cast formation *(black arrow in b);* HE

Şekil 4. Grup IV (CLP + 180 mg/kg PH). Alveolar ve bronşiolar eksudat birikiminde belirgin azalma ile birlikte devam eden vaskülitis *(oklar, a),* Glomerulitis *(beyaz ok, b)* ve hiyalin kast oluşumu *(siyah ok, b);* HE

Table 4. *Inflammation scoring in lung and kidney tissues*
Tablo 4. *Akciğer ve böbrek dokularında inflamasyon skorları*

Tissue Lesions	Group I (Sham)	Group II (CLP)	Group III (CLP +90 mg/kg PH)	Group IV (CLP +180 mg/kg PH)	Group V (Sham+180 mg/kg PH)
Lung					
Hyperemia	-	++	++	++	-
Alveolar exudation	-	+++	+	+	-
Bronchiolar exudation	-	+++	+	+	-
Desquamation of bronchiolar epithelium	-	++	++	++	-
Vasculitis	-	++	++	++	-
Kidney					
Hyperemia	-	+++	++	++	-
Glomerulitis	-	+++	++	++	-
Degeneration of tubulary epithelium	-	++	++	+	-
Hyaline casts	-	+++	+++	+	-
Interstitial cellulary infiltration	-	+	+	+	-

mortality and morbidity. Therefore, most of studies have been focused on prevention and treatment of sepsis by researchers. In the present study, the effects of PH extract were investigated on tissues (lung and kidney) and cytokines in sepsis induced by CLP in rats. Two different doses of PH were evaluated.

In light of literatures, the CLP is commonly used as a model in animals. Sepsis resulting from it in animals is described the clinical situation of bowel perforation and bacterial infection during both early and late phases. Wang et al.[29] reported that the period of 16-20 h is the late phase of sepsis induced by CLP in intra- abdominal sepsis model. In the current study, the late phase of sepsis was used (after 16 h) for experimental model.

As is known, microorganisms and their toxins cause the activation of inflammatory systems and the release of cytokines in sepsis syndrome [30]. Cytokines are small cell-signaling protein molecules that play major roles

in immune system response to inflammation and multi organ deficiencies. These cytokines are predominantly pro-inflammatory (TNF-α and IL-1ß) and the releasing of these pro-inflammatory mediators are characterized as the initial phase of sepsis. However, other cytokines are called anti-inflammatory (IL-10) and they leads to the compensatory antagonistic mechanism and the development of a balanced state of immunity [11,31-34]. TNF-α is produced primarily by activated macrophages, although it can be produced by many other cell types such as lymphoid, mast, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons [35,36]. It has an important role in coordinating of the inflammatory response and the releasing of other cytokines [37]. In previous studies, it was reported that the TNF-α infusion caused pulmonary hypertension, hypoxemia, decreased lung compliance, and increases in pulmonary micro-vascular permeability [38,39]. IL-1β is also produced by activated macrophages as a pro-protein, which is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [40]. Endo et al.[41] reported an increase in TNF-α and IL-1β levels of plasma in septic shock. In the present study, IL-1ß, IL-10 and TNF-α levels significantly increased in the serum of the CLP group when compared to the sham group. These results are in accordance with previous studies [7,42,43]. However, PH1 and PH2 treated CLP groups decreased the cytokine (IL-1ß, IL-10 and TNF-α) levels when compared to the CLP group. The current data suggests that the PH has an ability to produce less inflammatory cytokines in response the CLP-induced sepsis in rats and in part, it can prevent the cytokine-related organ injury. Previous studies have shown that PH has anti-inflammatory and antioxidant effects [19,23].

Oxidative stress is the imbalance between oxidants and antioxidants at the cell. This imbalance can cause oxidative damage [44]. Malondialdehyde (MDA) is resulted from lipid peroxidation of polyunsaturated fatty acids and used as a marker for oxidative stress. The degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues. Superoxide dismutase (SOD) and Glutathione (GSH) are important antioxidants in the intracellular protective mechanisms caused by reactive oxygen species such as free radicals and peroxides [45]. The SOD shows the antioxidant effect by converting toxic superoxide radicals into nontoxic hydroxyl peroxide and molecular oxygen [46]. Previous studies reported that, the level of GSH decreased in septic shock [47-49]. Ritter et al.[50] showed that MDA and SOD levels are markers of early mortality in septic rats. It has been reported that in CLP-induced sepsis, increasing oxidative stress in tissue in parallel with plasma are important mechanisms due to the output of free radicals [51]. In addition to, endotoxin administration caused to increase in cytokines along with lipid peroxide formation and membrane damage in animals [52,53]. Starkopf et al.[54] reported an increase in lipid peroxidation levels and a decrease in serum antioxidant capacity in sepsis. Our study showed increased tissue MDA level and decreased GSH and SOD levels after CLP, consistent with the literatures [7,43,55]. We observed a significant decrease in MDA and an increase in SOD and GSH in the PH-treated CLP rats compared to the sham groups. These results show the protective capacity of PH on lung and kidney tissues of septic rats.

In the present study, any histopathologic changes were not observed in sham group. However, we found significant histopathological changes in lung and kidney after the CLP-induced sepsis. There were dense inflammatory cell infiltrations with diffuse and nodular forms displayed remarkable findings at first glance. When the histopathological changes were evaluated in both PH1+CLP and PH2+CLP application groups, the inflammatory cell infiltrations decreased when compared to the CLP group. It was observed an inhibition of exudation in lung that in both PH1 and PH2 treated CLP groups, but the kidney had limited hyaline in only PH2 treated CLP group. According to our histopathological analysis, significant differences were determined in terms of inflammation scores between the sepsis group and the other groups. The CLP+PH1 and the CLP+PH2 groups had the same inflammation score in lung, finally, the effect of PH is not depend on dosage in lung, however, the high dose of PH had the lowest inflammation score in kidney.

As a result, the PH is a highly protective agent in preventing lung and kidney damage caused by CLP-induced sepsis via maintenance of alteration in the tissue levels of SOD, GSH, and MDA and alteration in serum levels of inflammatory cytokines such as TNF-α, IL-1β, and IL-10. Moreover, the administration of PH in CLP-induced septic rats has prevented the oxidative stress, cytokine response and the inflammation along with the protection of vital organ tissues.

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Research Article

The Effects of Pasture Characteristics and Seasonal Differences on Sheep Foot Diseases: A Field Study on the Kars and Iğdır Regions - Turkey [1] [2]

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Abstract

With this study, in which the incidences of diseases seen in sheep feet raised in the Kars and Iğdır regions were evaluated clinically and radiologically according to two different seasons, a total of 8.000 sheep were examined in the pasture (n=4230) and pen/stall (n=3770) seasons; problems were detected in 1.080 (25.51%) of them in the pasture season and 520 (13.76%) of them in the pen/stall season. It was determined that the herd in general suffered primarily from horn and hoof deformations in the pasture season with 17.70% and in the pen/stall season with 11.78%. Osteophyte formations and rotation were detected in the phalangeal bones of 20 of the sheep, and osteolysis was detected in the third phalanx of 8 of the sheep. In the aforementioned seasons, the rate of foot rot was 2.83% and 0.82% respectively. It was detected that according to the breed of sheep, 54.69% of the foot problems were seen in Akkaraman, 39.81% in Morkaraman, 1.43% in Tujin, and 4.07% in other sheep breeds (Merino, Kıvırcık). In addition, it was concluded that animal owners do not have sufficient knowledge concerning foot and hoof care, or do not care much about this issue. In conclusion, it was determined that the most important foot problem for sheep raised in the Kars and Iğdır regions is hoof deformation, and it was concluded that this could largely be avoided with simple precautions.

Keywords: *Sheep, Kars, Iğdır, Foot diseases, Hoof deformation*

Mera Özellikleri ve Dönemsel Farklılıkların Koyun Ayak Hastalıkları Üzerine Etkileri: Kars ve Iğdır Yöresine Ait Saha Çalışması

Özet

Kars ve Iğdır Yöresinde yetiştirilen koyunlarda görülen ayak hastalıklarının klinik, radyolojik olarak değerlendiği ve farklı iki döneme göre insidensinin belirlendiği bu çalışmada, mera (n=4230) ve ağıl/ahır döneminde (n=3770) toplam 8.000 koyun muayene edilmiş olup; bunlardan mera döneminde 1.080 (%25.51), ağıl/ahır döneminde ise 520 (%13.76)'sinde problem saptanmıştır.Sürü genelinde boynuz tırnak deformasyonlarının mera döneminde %17.70, ağıl/ahır döneminde ise %11.78 ile ilk sırayı aldığı tespit edildi. Koyunlardan 20'sinde phalangeal kemiklerde osteofitik üremeler ve rotasyon, 8'inde ise üçüncü phalanksta osteoliz saptandı. Aynı dönemler içinde piyeten oranı sırasıyla %2.83 ve %0.82 olarak saptandı. Koyun ırklarına göre ayak problemlerinin %54.69'unun Akkaraman; %39.81'inin Morkaraman; %1.43'ünün Tuj, %4.07'sinin diğer (Merinos, Kıvırcık) koyun ırklarında görüldüğü tespit edildi. Ayrıca hayvan sahiplerinin ayak ve tırnak bakımı konusunda yeterince bilgi sahibi olmadıkları ya da konuyu fazla önemsemedikleri kanaatine varıldı. Sonuç olarak, Kars ve Iğdır yöresinde yetiştirilen koyunlar için en önemli ayak probleminin tırnak deformasyonlarının olduğu saptanmış olup, bunların basit önlemlerle önemli ölçüde önlenebileceği sonucuna varılmıştır.

Anahtar sözcükler: *Koyun, Kars, Iğdır,Ayak hastalığı,Tırnak deformasyonu*

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INTRODUCTION

Sheep and goat farming play an important role in meeting the need for safe, affordable and easy to access [1] animal protein required for an adequate and balanced diet. The fact that most of the milk, wool/cashmere and skins needed by industries is obtained from these animals has made sheep and goat farming an important sector in our country, as well as in the world [1-5]. Despite certain negative factors, the fact that it is sustainable has allowed it to keep its place in the agricultural economy throughout history.

Sheep foot diseases, which directly impact profitability due to the negative impact on animal welfare, pose a significant threat [6-11]. Because sheep have such a high tolerance of pain, as long as they do not have extreme foot lesions, they will not limp [9,12,13]. Thus, the fact that there is no limping does not mean there are no foot/hoof problems in the herd [14-17].

The factors affecting sheep foot diseases and the incidence rate of the disease may differ according to region. The main reasons for this are the characteristics of climate [18-20], nutrition [21,22], caretaking and the pasture on which they are grazed, brought about by varying geographical conditions [18,23]. Varying results have been obtained from studies done on the role of regional differences in the occurrence of sheep foot diseases. In a study carried out on 10.327 sheep in the Afyon region [24], it was detected that the foot disease incidence rate was 2.62%, and 66.44% of the lesions were in the springtime and 33.94% were in autumn. In studies carried out by different researchers in the Burdur [25], Konya [26] and Elazığ [2] regions, foot diseases found in sheep were determined to be at the corresponding rates of 16.30%, 19.9% and 20.26%.

No widespread studies were found which focus on sheep foot diseases in the Kars and Iğdır regions, provinces where most of the country's pasture-fed animals are raised. The aim of this study was to determine the incidence rate of sheep foot diseases according to two different seasons, and classify the lesions in a comprehensive manner. In addition, an attempt has been made to determine the effects of climate and environmental conditions on the occurrence and variation of foot diseases.

MATERIAL and METHODS

The study was carried out on a total of 8.000 sheep of different breed, gender and age (ages 1-4 year), raised in the central district and surrounding villages of the Kars and Iğdır provinces/Turkey.

Four pre-planned trips were made to the regions between June 2012 and July 2013, during both the pasture season (the middle and end of the season) and the pen/stall season (the middle and end of the season). First, the number of animals in each herd was noted, as well as their age, breed, type of shelter and nutrition, and caretaking environments. In addition, a specific method of examination was developed, so that the sheep in the herd could be inspected. Animals which were limping or which had abnormalities in their foot regions were identified. The problems were determined by conducting systematic foot examinations on each of the animals in question. The information for each animal found to have a problem was noted on special forms, and the forms were collectively evaluated at the end of each visit. Also, all of the data that was collected was statistically evaluated using X^2 test. The foot regions of sheep with extreme hoof deformation and severe limping were X-rayed. A portable 50mA 100kV X-ray unit was used for this purpose.

At the end of each pasture season, soil samples were taken from each pasture area for pH analysis using a specific sampling method.

Using a pre-defined method [27], distilled water was added to the bottles in which there was 100 g of soil each, and the bottles were shaken using a horizontal shaking machine (180 rpm) for 2 h. The pH values of the solutions were measured at room temperature using a pH meter.

RESULTS

It was determined that of the 8.000 sheep examined in both seasons, 50% were of the Akkaraman breed, 37.50% were Morkaraman, 2.50% were Tuj and 10% were of other breeds such as Merino and Kıvırcık. Foot lesions were detected in 1.080 of the sheep examined in the pasture season and 520 of the sheep examined in the pen/stall season. It was understood that the existing problems were not a high priority for the owners and caretakers of the animals.

Statistical and acquired results according to the seasons are shown in *Table 1*. It is apparent from the table that different foot lesions were detected in a total of 1.600 (20%) of the 8.000 sheep. The *Paries ungulae* growing too long and curving over the top of the *Solae ungulae*, as well as the growth in which both the beak and corkscrew hoof disorders occur together were the most common horn and hoof deformations (74.70%). It was determined that the deformations took place more in the front feet/hooves as opposed to the hind *(Table 2)*. It was understood that the animal owners were not knowledgeable concerning foot care and hoof trimming, and that in isolated cases of limping, instead of consulting a veterinarian or trimming the hooves, the owners preferred to kill the animal. Of the 1.600 sheep spotted with foot lesions, it was determined that 54.69% were Akkaraman, 39.81% Morkaraman, 1.43% Tujin, and 4.07% were of other sheep breeds (Merino, Kıvırcık).

Table 1. *Distribution of foot diseases and hoof deformities according to the pasture and pen seasons*
Tablo 1. *Ayak hastalıkları ve tırnak deformitelerinin mera ve ağıl dönemine göre dağılımı*

Season	Foot Diseases						Hoof Deformities								Other		Total	
	Sinusitis Interdigitalis		Foot Rot		Foot Abscess		Beak/Sharp Hoof		Corkscrew Hoof		Scissor-Shaped Hoof		Fractured/Broken Hoof		Interdigital Dermatitis etc.			
	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics	n (%)	Statistics
Pasture season (April-December)	20 (0.47)	X^2: 1.70 P>0.05 OR: 1.78 %95 CI: 0.79-4.09	120 (2.83)	X^2: 42.60 P<0.001 OR: 3.50 %95 CI: 2.30-5.30	110 (2.60)	X^2: 52 P<0.001 OR: 5 %95 CI: 3.90-8.30	452 (10.68)	X^2: 24 P<0.001 OR: 1.40 %95 CI 1.30-1.70	91 (2.15)	X^2:15.50 P<0.001 OR: 2 %95 CI 1.40-3.0	20 (0.47)	X^2:001 P>0.05 OR:0.99 %95 CI 0.50-1.90	187 (4.42)	X^2:15 P<0.001 OR:1.60 %95 CI 1.20-2.10	80 (1.89)	X^2: 39 P<0.001 OR: 5 %95 CI 2.90-9.50	1080/4230 (25.51)	X^2:170 P<0001 OR: 2.10 %95 CI 1.90-2.40
Pen season (December-April)	10 (0.26)		31 (0.82)		20 (0.53)		283 (7.50)		39 (1.03)		18 (0.47)		105 (2.78)		14 (0.37)		520/3770 (13.76)	
Total	30		151		130		735		130		38		292		94		1600/8000	

Table 2. *Distribution of detected foot diseases according to front and hind feet*
Tablo 2. *Saptanan ayak hastalıklarının ön ve arka ayaklara göre dağılımı*

Season	Feet	Number of Animals Infected	
		n	%
Pasture Season (April - December)	Front	784	72.50
	Hind	296	27.40
Pen Season (December - April)	Front	326	62.60
	Hind	194	37.30

During the radiographic examinations of 20 sheep with extreme hoof deformations and severe limping, osteophyte formations and rotation were detected in the phalangeal bones, and in 8 cases, osteolysis was detected in the *Phalanx tertia*.

It was noticed that the floors of the pens and stalls were mostly either concrete or dirt, and that the canals for urine and excrement were inadequate or did not exist at all. It was learned that every two to three months the accumulated feces were made into dried dung cakes, which are used for fuel.

It was understood that some herds were made to walk long distances during the pasture season due to insufficiency of vegetation. It was found that the sheep were forced to migrate to the high regions of Kars due to the especially hot summers in Iğdır. It was observed that these herds had a higher number of fractured and broken hooves. It was found that beak, corkscrew and scissor-shaped hoof deformations were more common in sheep which were grazed on pastures rich in vegetation or on prairies. It was noted that meeting the water demand for the sheep was especially difficult during the pasture season, and that mostly man-made ponds were utilized.

It was determined that all the pH values of the dirt samples were slightly alkaline (7.2-8.0).

DISCUSSION

In Kars and Iğdır provinces, where sheep herding is common, the animals spend two thirds of the year grazing on pastures. Because the physical circumstances in the winter allow for it, the sheep were fed outdoors (on snow or in pens) during the day and brought into the stalls in the evenings. It was noted that animal husbandry is done as a family business, and in both provinces the sheep taken to the pasture were joined with the sheep of other families in herds numbering between 1.000 and 2.000. It was concluded from the visits that neither the animal owners nor the shepherds knew much or cared much about foot/hoof diseases other than foot rot. It is apparent from studies carried out in other regions of Turkey that herders are generally like-minded regarding this issue [2,3,25,28].

Turkey has an important place among the world's countries in terms of its sheep population [3,5,22]. Just as the profitability of meat, milk and fleece production drops due to foot diseases, serious economic losses are also experienced due to premature births and culling animals from the herds [11,29-32]. It was observed that the general health of the animals visited, especially in the pen/stall season, was weak, and that the wool had fallen out from many different regions of their bodies. It was found that most of the sheep that had experienced wool loss were those with feet problems.

In order for the animals' feeding and shelter conditions to be evaluated correctly, visits were conducted twice each, once mid-season and once at the end of the season. It was found that it was quite common for sheep grazed on meadows to have extremely long growths of scissor-shaped and corkscrew hooves, and for a deformed hoof structure to occur in which the *Paries ungulea* grows extremely long and curves over the top of the *Solea ungulea*. It was found that it was relatively more common for herds grazed on arid land to have cases of foot abscess and fractured or broken hooves. Though it has been noted in studies focusing on the effects of environmental factors, including pastoral characteristics, on foot formations and diversity, that similar results have been observed, the fact that it is wrong to use meadows for sheep herding has not been stressed. It is very important that sheep, just like cattle, be grazed on land which allows for their hooves to be regularly worn down in order to prevent hoof deformations, as well as foot diseases caused by them, from occurring. Animals grazed on meadows do not have to walk far to feed, nor are their hooves properly worn down on the rich vegetation and soft soil. This explains the formation of an extra layer due to the *Paries ungulea* growing on top of the *Solea ungulea* in sheep grazed in such places.

It is observed that when hoof deformities, caused either by extreme growth or due to other reasons, are compared according to season, the lesions detected in the pasture season (25.51%) are nearly double the rate of those detected in pen/stall season (13.76%). For the same two categories in the same order, İzci et al.[28] reported their results as 12.80%-25.30% and Yadav et al.[33] reported their results as 9.31%-29.40%. When these results are considered, it is easy to see there is a clear discrepancy between the results of this study and the data of previous literature. Many environmental factors such as the layout, hygiene and floor of the stalls, as well as the physical characteristics of the pasture on which the animals are grazed, play an important role in the occurrence of foot diseases. On visits conducted during the middle and end of the pen/stall season, it was observed that the animals were fed outdoors during the day and brought into the stalls in the evening. It was found that the stalls are aired out during the day while the animals are outdoors. In addition, considering the fact that sheep farming is done as a family business, it is easy to imagine that during the pasture season the number of animals in a herd is extremely high with only one shepherd looking after them, and that the whole family takes care of the sheep in the stall season. It was also determined that the sheep are roughly examined upon entering the stall season, and that the sheep detected to have serious problems are disposed of.

Sağlıyan [2], detected a rate of 31.16% front and 68.84% hind foot lesions, İzci et al.[28] detected a rate of 23.40% front and 76.60% hind foot lesions, and Mahin [34] detected

39.50% front and 60.50% hind foot lesions. In this study, the rate of detection for front foot lesions during the pasture season was determined to be 72.50%, while the rate for hind foot lesions was found to be 27.40%. During the pen/stall season a rate of 62.6% was detected in front foot lesions and 37.30% in hind foot lesions. These results also show a difference between our findings and those of the aforementioned authors. Though it is not possible to reach a definite conclusion on the reasons behind the differences, the sheep belonging to the region in question satisfy their need for water mostly from ponds or rivers. When drinking water, the front hooves of animals often come into contact with water or mud. It is thought that this dampness plays a role in the fact that the front hooves of the animals given access to water at least twice a day remain softer compared to the hind hooves.

It has been noted that foot rot is one of the most important foot diseases with a rate of 10-21% in habitats with mild and rainy climate conditions and other environmental factors, and that it is infectious [4,26,31,35]. In this study, foot rot was detected at a rate of 2.83% in the pasture season and 0.82% at the end of the pen/stall season ($P<0.001$). The results of studies done throughout various provinces of the Eastern Anatolia Region, including the provinces of Kars and Iğdır, were found to be quite high with rates of 8.30%-19.89%. In one of two studies [6] conducted in and around Konya, a rate of 28.12% was detected, and in the other, the average of both seasons was determined to be 0.39%. Avki et al.[25], on the other hand, in their study done on sheep in the Burdur region, noted that they found foot rot to have a rate of 2.55%. It is apparent that with our average rate of both seasons at 1.83%, our results are different from those of other studies done throughout the provinces of the Eastern Anatolia Region. It is obvious that more detailed studies are needed in order to clarify the reasons for this discrepancy.

It has been noted that foot and hoof lesions are seen more often in domestic sheep breeds [25]. İzci et al.[28] have determined in their study done on sheep in the Konya region that foot diseases are more common among the breeds of Merino and Ivesi. Our study found that lesions are found relatively more often in the breed types Akkaraman (54.69%) and Morkaraman (39.81%). The fact that lesions are found relatively more often in the Akkaraman and Morkaraman breeds has been attributed, in our study, to the fact that both breeds are the region's most well-fed breeds.

It has been reported that the fact that the pH value of the soil is acidic directly affects the hoof and causes structural deformations in the horn and hoof quality, and thus also paves the way for many problems related to the foot [15,19,26]. It was determined in this study that all the pH values of the soil samples were slightly alkaline (7.2-8.0). In this regard, it cannot be said definitively whether or not the soil of the region plays a role in the foot/hoof problems.

This study, in which the effects of caretaking, feeding and other environmental effects as well as the effects of seasonal difference on the occurrence and diversity of foot/hoof diseases were researched, has the distinction of being the first study done in our region. With this study it was concluded that the foot/hoof diseases of the sheep in the region were found to be at a higher rate in the pasture season, that the most widespread lesions were hoof deformations mostly in the front feet, that the rate of foot rot was very low compared to the average rates in both Turkey and throughout the world, that the animal owners were not knowledgeable in the areas of foot care and hoof trimming or that they did not care much about these issues [7,10,19].

In conclusion, though it does not seem possible to completely avoid foot diseases, which are the cause of important losses in sheep farming in many ways, it can be said that foot/hoof diseases can be significantly decreased by paying attention to practices such as herd management and the deliberate utilizing of pastures.

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Research Article

Levels of Cardiac Biomarkers and Coagulation Profiles in Dogs with Parvoviral Enteritis [1]

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Abstract

The first aim of this study is to determine the changes of cardiac biomarkers and coagulation profiles in parvoviral enteritis and present the importance of these parametres for prognosis of the disease. The second aim is to determine the presence of myocarditis in the enteritis form of the disease via cardiac biomarkers. Twenty seven dogs with parvoviral enteritis (experiment group) and 6 healthy dogs (control group) which were aged between 1.5 and 6 months, weighted between 5 - 15 kg were used as a material of this study. Anorexia, fever, depression, lethargy, vomitting and haemorrhagic diarrhea were determined in dogs with parvoviral enteritis. Parvovirus infection in dogs were verified via feces parvovirus antigen test. Blood samples were collected from all dogs and electrocardiographies (ECG's) were performed. Standard treatment is applied for dogs with parvoviral enteritis. Twenty three of these dogs were treated successfully; however four of them died. Mild to intermediate acute myocarditis were determined in the histopathological examination of the dead dogs. Plasma protrombin (PT), actived parsiel tromboplastin time (aPTT), antitrombin III (AT-III), fibrinogen and D-dimer concentrations and serum creatin kinase-MB (CK-MB), cardiac troponin I (cTnI) and brain natriuretic peptid (BNP) concentrations were measured. Increase in levels of plasma PT ($P<0.001$) and aPTT ($P<0.001$), fibrinogen ($P<0.001$) and D-dimer ($P<0.05$), and decrease in level of III AT-III ($P<0.05$) were detected in dogs with parvoviral enteritis. Increased level of serum CK-MB ($P<0.05$) and BNP ($P<0.001$) were also determined. No important change detected in serum cTnI levels. As results, in dogs with parvoviral enterit there were increase in PT, APTT, fibrinogen and D-dimer levels, on the other hand there was a decrease on AT III level resulting in DIC. In addition to this an increase was observed on plasma serum CK-MB and BNP levels. Considering the increase on CK-MB and BNP levels a long with results of histopathological dead dogs, it should be taken into account that acute miocarditis also occures simultaneous with hemoragic parvoviral enterit.

Keywords: *Dog, Cardiac biomarkers, Parvoviral Enteritis, Coagulation profile*

Parvoviral Enteritli Köpeklerde Pıhtılaşma Profilleri ve Kalp Biyomarkır Düzeyleri

Özet

Bu çalışmanın birinci amacı, köpeklerin parvoviral enteritinde kalp biyomarkırları ve pıhtılaşma profilindeki değişimleri belirlemek, hastalığın tanı ve prognozunda bu parametrelerin önemini ortaya koymaktır. İkinci amacı ise enterit formunda miyokart hasarı gelişip gelişmediğini kalp biyomarkırları ile belirlemektir. Bu çalışmanın materyalini yaşları 1.5 ile 6 ay, canlı ağırlıkları 5-15 kg arasında değişen 27 parvoviral enteritli (deney grubu) ve 6 sağlıklı köpek (kontrol grubu) oluşturdu. Parvoviral enteritli köpeklerde anoreksi, ateş, depresyon, latherji, kusma ve kanlı diyare belirlendi. Köpeklerin parvoviral enfeksiyonu dışkı parvovirus antijeni testi ile doğrulandı. Bütün köpeklerden kan örnekleri alındı ve kalp EKG traseleri çekildi. Parvoviral enteritli köpeklere standart tedavi uygulandı. Bu köpeklerin 23'ü iyileşti, 4'ü ise öldü. Ölen hayvanların histopatolojik muaynesinde hafif veya orta derecede akut miyokardit saptandı. Bütün köpeklerin plazma protrombin zamanı (PT), active edilmiş parsiyel tromboplastin zamanı (aPPT), antitrombin III (AT-III), fibrinojen ve D-dimer düzeyleri ve serum kretin kinaz-MB (CK-MB), beyin natriüretik peptit (BNP) ve kardiak troponin I (kTnI) düzeyleri ölçüldü. Parvoviral enteritli köpeklerde plazma PT ($P<0.001$) ve APTT ($P<0.001$) süreleri ile fibrinojen ($P<0.001$) ve D-dimer ($P<0.05$) düzeylerinde artış, AT-III ($P<0.05$) düzeyinde ise azalma belirlendi. Serum CK–MB ($P<0.05$) ve BNP ($P<0.001$) düzeylerinde artış, kTnI düzeyinde ise önemli bir farklılık saptanmadı. Sonuç olarak köpeklerin parvoviral enteritinde PT, APPT, fibrinojen ve D-dimer düzeylerinde artış, AT-III düzeyinde azalma ve buna ilişkin DİK meydana gelmektedir. Ayrıca parvoviral enteritli köpeklerde plazma CK-MB ve BNP düzeyindeki artış tespit edildi. Gerek CK-MB ve BNP düzeyindeki artış gerekse ölen hayvanların histopatolojik sonucuna göre köpeklerde hemorajik enterit formuyla birlikte akut miyokardit formununda gelişebileceği göz önünde bulundurulmalıdır.

Anahtar sözcükler: *Köpek, Kalp biomarkırları, Parvoviral enterit, Pıhtılaşma profili*

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INTRODUCTION

Canine parvovirus infection is an acute, high contagious and mortal viral disease of dogs. Although the disease may develop in dogs within all age ranges, it may cause severe and mortal infections in dogs that are younger than 12 months. Infection has two clinical forms as acute hemorrhagic enteritis and myocarditis. Acute enteritis form is mostly seen in puppies up to 12 months of age. Typical symptoms include depression, vomiting and small bowel hemorrhagic diarrhea. Myocarditis form can develop from infection in utero or in puppies less than 8 weeks old. In this form, the infected puppies may be found dead without any symptoms within 24 h [1-3]. Virus affinites predominantly cript epitels in the small intestine, precursor cells of bone marrow, lymphoid cells and cardiac muscle cells due to fast reproduction speed of the virus [4]. Disseminated intravascular coagulation is a severe problem that threats lives of both people and animals. In this syndrome, disseminated intravascular micro trombosises shape and cause the circulation disorders, thus multiple organ failure develops and leads to death [5,6]. Protrombin time, activated parsiyel tromboplastin time, D-dimer, fibrinogen levels, activity of Anti-trombin III and trombosit count should be considered regarding DIC [7]. It is pointed that, important changes can be seen in CPV infections due to gastrointestinal hemorhagia and bone marrow depression [2]. On the other hand it is argued that the reason of hemorrhagic enteritis which occures in CPV may be due to endotoxemia caused by coliform bacteries and increased level of cytokines [8].

Acute cardiomyopathies are rooted from bacterial, viral, parasitical, metabolic disorders, toxication and deficiency of vitamins and elements [9]. One of the important reason of the acute cardiomyopathies is parvoviral infections. It is noticed that cardiac enzymes and neurohormons are the most confidental methods for the diagnosis of myocardial damage [10-15]. Troponins (cTnI, cTnT), creatin kinase-MB (CK-MB) and brain natriuretic peptid (BNP) are the leading cardiac biomarkers which are used in the diagnosis of myocard damages [10-12]. Vartner et Ingwall [16] indicated that the first symptoms is the increase in the level of serum CK-MB when the cardiac muscle is damaged. Yılmaz et Şentürk [17] noticed that significiant increase in the level of serum CK-MB of dogs with parvoviral enteritis. On the other hand, Burgener et al.[18] determined that cTnI level increases significantly in the first 24 to 48 h in the case of acute miocardial damage in dogs. The cTnI level returns to it's normal level after 48 h. Another caridac biomarker used to diagnose ventriculer damage is B type natriuretic peptid [19-21]. It is noticed that especially BNP can be used to evaluate the response to therapy and prognosis of disease in patients with congestive heart failure [22]. Macdonald et al.[23] noticed that BNP level signicantly increase in dogs with cardiac failure, Donker et al.[24] also reports that BNP level increases within several days in dogs through the artifically generated atrio-ventricular block.

The first aim of this study is to determine the changes in some selected cardiac biomarkers such as BNP, CK-MB and cTnI and coagulation profiles in parvoviral enteritis and present the importance of these parametres for prognosis of the disease. The second aim is to determine the presence of myocarditis in the enteritis form of the disease via cardiac biomarkers.

MATERIAL and METHODS

Animals and Clinical Examination

Authorization to conduct this study has been taken from S.U. Faculty of Veterinary Medicine Animal Ethics Comittee (2011/10). The materials of the study consist of twenty seven dogs with parvoviral enteritis (experiment group) and 6 healthy dogs (control group) which were aged between 1.5 and 6 months, weighted between 5 - 15 kg, were brought into University of Selcuk, Faculty of Veterinary Medicine, Department of Internal Medicine. History of diseases of dogs were found to be 1 to 2 days. First, routin clinical examinations performed for all dogs. Feaces samples were collected from clinically parvovirus suspected dogs (anorexia, depression, lethargy, vomiting and hemorhhagic diarrhea) by using rectal swab. Feaces samples were evaluated by the quick parvovirus antigen detection test (CPV Ag test, rapidy test kits, Vettek Medical İstanbul/Türkiye). The dogs with positive results were incorporated into the study. Twenty two of these dogs had not been vaccinated and 5 others had been. Electrocardiogram examination is performed to all healthy and parvoviral enteritis infected dogs to determine any ritmic problems in the heart. Standard therapy is applied to dogs with parvoviral enteritis.

Colection of Blood Samples

Blood samples with and without anti-coagulant (Na-citrat) collected from dogs with parvoviral enteritis and healthy dogs; plasma and serums were separated. Samples, which would be used in the evaluation of coagulation profiles and cardiac biomarkers, were kept in -80°C deep freeze until the measurement is completed.

Measurement of Coagulation Profile

Protrombin time (Kat no: OUHP-49) and APPT (Kat no:OQGS-29), AT III activity (Kat no: OWWR-15), fibrinogen level (Kat no: OWZG-15) and D-dimer (Kat no: OPBP-03) levels were measured by coagulomethric method in Sysmex CA 1500 device (Siemens, A-7799, Germany).

Measurement of Cardiac Biomarkers

CK-MB (ADVIA Centaur for human, Kat no: RF420) and cTnI (ADVIA Centaur for human, Kat no: RF421C) levels were measured by ELISA method in Dimension Xpand

Plus device (Siemens, 2004080651, Germany), BNP (ADVIA Centaur for human, Kat no: 02816634) level was measured by ELISA method in Immunassay Systems device (Siemens, TRL 93450905, Germany) [25,26]. Measurable sensitivity and test interval of CK-MB enzym level is 0.18 ng/mL and 300 ng/mL, measurable sensitivity and test interval of cTnI enzym level is 0.006 pg/mL and 50 ng/mL, and for BNP, it is 1 pg/mL and 5.000 pg/ml.

Histopathological Examination

Necropsy performed for all dead dogs. Tissue samples collected from ilio-secal valvule, some areas of small intestines where bleeding detected, cardiac tissue and other organ tissues such liver and spleen to control if any other pathological findings appear.

Statistical Analysis

Unpaired student test was used to determine the differences between groups. SPSS 19.0 for Windows® was used to perform the test. The test procedure is designed according to Akgül [27].

RESULTS

Clinical Results

Anorexia (27 case), lethargy (27 case), depression (27 case), fever (10 case >39.5°C), vomiting (23 case) and hemorrhagic diarrhea (21 case) were seen in dogs which formed the experiment group of the study. Twenty three of these dogs responsed well to the therapy and four of the dogs did not. No anormal condition was seen in ECG examination of all healthy and 23 of the sick dogs. However, sinus tachicardia was diagnosed in 3 of 4 dead dogs.

Coagulation Profile Results

Statistically, significant increase in PT (P<0.001) and APTT (P<0.001) times, fibrinogen (P<0.001) and D-dimer (P<0.05) levels, and significant decrease in AT-III (P<0.05) activity were observed in dogs with parvoviral enteritis compared with healthy dogs. No significant change was seen in trombosit count *(Table 1)*.

Cardiac Biomarker Results

Statistically, significant increase in CK-MB (P<0.05) and BNP (P<0.001) levels were measured in dogs with parvoviral enteritis compared to healthy dogs. No significant change was detected in cardiac troponin I level *(Table 2)*.

Histopathological Findings

Villous atrophy in small bowels, mono nucleer cell infiltration in lamina propria and mild fibrosis, degeneration in intestinal cript epitels and mild or intermediate acute miocarditis were detected during the histopathological examination of the dead dogs.

Table 1. *Mean values and statistical importance of coagulation parametres in healthy and infected dogs*

Tablo 1. *Sağlıklı ve enfekte köpeklerde pıhtılaşma parametrelerinin ortalama değeri ve istatistiksel önemi*

Parameter	Control Group (n:6)	Experiment Group (n: 27)	P Value
PT (sn)	7.03±0.14	8.05±0.23	***
APTT (sn)	17.0±1.20	93.3±7.50	***
Fibrinojen (mg/dL)	122±11.0	257±18.0	***
AT III (%)	90.1±5.60	74.6±2.90	*
D-dimer (mg/dL)	0.69±0.15	1.31±0.20	*
Trombosit (x10³/µL)	214±28.2	270±21.8	

** P<0.05, ** P<0.01, *** P<0.001*

Table 2. *Mean values and statistical importance of cardiac biomarkers in healthy and infected dogs*

Tablo 2. *Sağlıklı ve enfekte köpeklerde kalp biyomarkırlarının ortalama değeri ve istatistiksel önemi*

Parameter	Control Group (n:6)	Experiment Group (n: 27)	P Value
CK–MB (ng/mL)	2.20±1.00	8.51±1.25	*
cTnI (ng/mL)	0.06±0.02	0.10±0.03	
BNP (pg/ mL)	1.40±0.05	30.1±2.68	***

** P<0.05, ** P<0.01, *** P<0.001)*

DISCUSSION

Anorexia, depression, lethargy, fever, tachicardia and tachipnea, vomiting, diarrhea (that can change to mucoid or hemorrhagic) are the common symptoms among dogs with parvoviral enteritis. Severe hypovolemia, due to fluid and electrolit loss caused by vomiting and diarrhea, leads to death if not treated [17,28-31]. Anorexia, depression, lethargy, fever, tachicardia and tachipnea, vomiting, diarrhea and hemorrhagic diarrhea were diagnosed in this study as well. The diagnosis was finalized after the parvovirus antigen test was resulted positive. Twenty three of these dogs responded well to the therapy and four of them could not survive. Villous atrophy in small bowels, mono nuclear cell infiltration in lamina propria and mild fibrosis, degeneration in intestinal cript epitels and mild or intermediate acute miocarditis were detected during the histopathological examination of the dead dogs.

Disseminated intravascular coagualtion may develop due to viremia, septisemi, parazitic infection, severe tissue damage, toxication, intravascular hemolizis, autoantibody, hepatitis, pancreatitis and neoplasma [7,32-34]. Protrombin time, activated parsiyel tromboplastin time, D-dimer, fibrinogen levels, activity of Anti-trombin III and trombosit count should be considered in DIC [7]. It is pointed that, important changes due to coagulopathie can be seen in CPV infections due to gastrointestinal hemorrhagia and bone marrow depression [2]. At the same time, it is argued that the reason of hemorrhagic enteritis which occures

in CPV may be due to endotoxemia caused by coliform bacteries and increased level of sitokins [8]. Otto et al.[35] noticed that DIC may occure in dogs with parvoviral enteritis. For an accurate diagnosis, prolonged PT and APTT, decrease in AT-III activity, trombosit count and fibrinogen level, increase in FDP or D-dimer level must be detected. At least three of these changes must be detected for a suspicious DIC case [36]. Feldman et al.[37] indicated that prolong in PT and APTT, increase in FDP level, decrease in AT-III activity and trombosit count, develop in dogs with DIC. In recent years D-dimer is used instead of FDP and it is proved that D-dimer is more sensitive and spesific incompared to FDP [7,38]. On the other hand, Carr et al.[39] noticed that increased level of D-dimer does not always indicate the presence of DIC. Laforcade et al.[40] noticed that they had found prolong in PT and APTT, increase in D-dimer, decrease in AT-III level and no changes in trombosit count. Esmon [41] and Wada [42] noticed that fibrinogen level may increase proportional to inflammation if there is no high consumption.

Prolonged PT and APTT, decreased AT-III and increase in D-dimer level may show the development of DIC in the dogs with parvoviral enteritis in this study. Although prolonged PT and APTT, increased D-dimer level and decreased AT-III activity were detected as other authors also noticed [7,36,43]; however, compared to other studies, in this study there were no decrease in fibrinogen and trombosit levels. On the other hand, Otto et al.[35] noticed that they found increase in fibrinogen level and no change in D-dimer level and trombosit count. In this study, detection of prolonged PT and APTT, decreased AT-III and increased D-dimer level shows the coagulation activation (PT↑, APTT↑, AT-III↓) which is the first step of DIC and fibrinolitic activation (D-dimer↑) which is the last step of DIC. To conclude the findings coincides with the findings of Otto et al.[35] and Laforcade et al.[40].

Some authors [35,41,42] report that fibrinogen level may increase in the first period of DIC due to inflamatory response; then, the level decreases due to fibrinolisis; some other authors [7,43] report significant decrease in fibrinogen level. In this study, increase in fibrinogen level was observed. Increase in fibrinogen level may be due to the increase of acute phase proteins in spite of DIC. Our findings on fibrinogen verifies the studies of most of the other mentioned authors [35,41,42].

The most important method to determine the myocardial damage is to measure cardiac biomarkers [10-15]. Troponins, CK-MB and BNP are the most common used biomarkers in the diagnosis of acute myocardial damage [10-12,15,44]. CK-MB is a first degree trusted and spesific enzyme in acute cardiac tissue damage [45]. Smithline et al.[46] indicate that serum CK-MB level starts to increase within 4 h following the myocardial damage, and reaches it's peak level in approximately within the 12th h then, decrease to normal level in 24 to 72 h. Troponin I is considered a reliable serum biomarker for myocardial ischemia and necrosis in human and animals. After acute myocardial injury, cTnI is released from the cytoplasmic pool, resulting in increased blood concentrations within 2 h, with a peak after 12-24 h. Persistently increased cTnI blood level suggest irreversible and active on going damage to cardiomyocytes [25,47]. On the other hand, Mair et al.[48] report that cTnI starts to increase in 4 to 6 hours and it may take 7 to 10 days to reach the basal level. Vartner et Ingwall [16] noticed that CK-MB is the first enzyme that can be detected in serum following to myocardial damage. Yılmaz and Şentürk [17] noticed the significant increase of CK-MB serum level in dogs with parvoviral enteritis. Burgener et al.[18] showed that cTnI level significantly increase in 24 to 48 h and then returns to basal level. Bastan et al.[49] found that increased serum cTn-I levels were consistent with short survival times in dogs with CPV-2. B type natriuretic peptid is also an important indicator of ventricular damages [19-21]. Especially, BNP level may inform the clinician about the prognosis and response to treatment of patient with congestive heart failure [22]. Macdonald et al.[23] report that BNP level significantly increase in dogs with congestive heart failure. Donker et al.[24] report that BNP level through artifical atrio ventricular block increase in several days. Haßdenteufel et al.[50] determined that NT-proBNP represents a useful additional diagnostic parameter in veterinary clinical cardiology to assess the severity of cardiac disease.

In this study, CK–MB ($P<0.05$) and BNP ($P<0.001$) levels statistically increased in dogs with parvoviral enteritis compared to the healthy dogs. Although not statistically No significant, cTnI concentration was numerically increased in dogs with parvoviral enteritis compared to the healthy dogs *(Table 2)*. Increase in CK-MB and BNP concentrations may show that mild to intermediate myocarditis might be ocurred in the enteritis form of CPV. On the other hand, possible reason of numerically increased in cTnI level may be that there is no severe damage in myocard. Detection of mild to intermediate myocarditis in histopathological examination of 4 dead dogs, increase in CK-MB (15-28 ng/ml) and BNP (44-58 pg/ml) levels, and numerically increase in cTnI (0.1 ng/ml) levels of these dogs may be confirm the result. It must be considered that CK-MB level may increase in blood serum rapidly, in mild to intermediate myocarditis thus it may be more diagnostic than cTnI. CK-MB, BNP and cTnI result of this study were appropriated with findings of some authors [12,17,18,22-25,44,46-49].

In conclusion, PT, APTT, fibrinogen and D-dimer levels increase, AT-III level decreases in parvoviral enteritis of dogs due to DIC. In addition, increase in serum CK-MB and BNP levels were detected. It is concluded that acute myocarditis form may develop in the enteritis form of disease according to histopathological results of dead animals and increase in CK-MB and BNP levels.

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Research Article

Detection of Multiple Anthelmintic Resistance of *Haemonchus contortus* and *Teladorsagia circumcincta* in Sheep and Goats of Northern Punjab, Pakistan

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Abstract

The present study was conducted to investigate the efficacy of albendazole, levamisole and ivermectin against gastrointestinal nematodes of sheep and goats. One hundred and sixty small ruminants comprised four breeds, sheep (Salt range and Pak Karakul) and goats (Jattal and Beetal) were selected for this experiment. These animals were artificially infected with nematodes (*Haemonchus contortus* and *Teladorsagia circumcincta*). FECs were done to confirm the presence of adult stages of *H. contortus* and *T. circumcincta*. The selected sheep and goat were divided into four groups. First group was treated with albendazole, second with levamisole, third with ivermectin and fourth kept as untreated to serve as control. Faecal samples were collected on day first prior to administration of treatment followed by day 14 post treatment. The efficacy of each anthelmintic was measured using the faecal egg count reduction test and egg hatch test. The percentage reduction in FECs (95% CI) for albendazole, levamisole and ivermectin for salt range sheep were 88%, 89% and 99%, for Pak karakul sheep, 91%, 90% and 97%, for Jattal goat 91%, 91% and 98%, and for Beetal goat 91%, 92% and 99%. All results showed the presence of multiple anthelmintic resistances in selected sheep and goats flocks. All flocks were found resistance against the albendazol and three were resistance against levamisole. The arithmetic faecal egg count reduction with albendazole, levamisole and ivermectin recorded for *H. contortus* and *T. circumcincta* indicated the resistance against albendazole and levamisole and susceptibility of these worms against ivermectin. The results revealed that a significant difference (P<0.05) of FECRT were found on pre-treatment and post-treatment with different anthelmintic drug as compared to control group in all the flocks. The results of egg hatch test indicated that LC_{50} were ranged from 0.138 µg/mL to 0.141 µg/mL for four breeds, which were more than 0.1ug/ml of albendazole. Result indicates that resistance was found in the eggs with albendazole. The egg hatch assay also confirmed the result of faecal egg count reduction test.

Keywords: *Sheep, Goat, Anthelmintic resistance, Albendazole, Levamisole, Ivermectin, Northern Punjab*

Pakistan'ın Kuzey Punjab Eyaletindeki Koyun ve Keçilerde *Haemonchus contortus* ve *Teladorsagia circumcincta*'nın Anthelmentiklere Karşı Direncinin Belirlenmesi

Özet

Bu çalışma albendazol, levamizol ve ivermektinin koyun ve keçilerdeki gastrointestinal nematodlara karşı etkinliğini araştırmak amacıyla yürütüldü. Salt range ve Pak Karakul cinsi koyunlar ile Jattal ve Beetal cinsi keçilerinden oluşan toplam 160 adet küçük ruminant bu çalışmada kullanıldı. Bu hayvanlar suni olarak *Haemonchus contortus* ve *Teladorsagia circumcincta* nematodları ile enfekte edildi. *H. contortus* ve *T. circumcincta*'nın olgun evrelerini teyit etmek amacıyla dışkı yumurta sayımı yapıldı. Seçilen koyun ve keçiler 4 gruba ayrıldı. Birinci gruba albendazol, ikinci gruba levamizol, üçüncü gruba ivermektin uygulanırken dördüncü grup kontrol olarak tutularak herhangi bir uygulama yapılmadı. Uygulamalardan önce ve uygulamanın 14. gününde dışkı örnekleri toplandı. Herbir anthelmentiğin etkinliği dışkı yumurta sayımı azalma testi ve yumurta açılımı testi ile belirlendi. Albendazol, levamizol ve ivermektin için dışkı yumurta sayımında % azalma Salt range koyununda sırasıyla %88, %89 ve %99, Pak karakul koyununda %91, %90 ve %97, Jattal keçisinde %91, %91 ve %98, ve Beetal keçisinde %91, %92 ve %99 olarak tespit edildi. Çalışmanın sonuçları seçilen koyun ve keçi sürülerinde anthelmentik direncin mevcut olduğunu göstermiştir. Tüm sürüler albendazola karşı dirençli bulunurken üçü levamizole karşı dirençliydi. *H. contortus* ve *T. circumcincta* için albendazol, levamizol ve ivermektin ile tespit edilen aritmetik dışkı yumurta sayımı azalma albendazol ve levamizole karşı direnci gösterirken ivermektine karşı duyarlılığı ortaya koymuştur. Tüm sürülerde kontrol grubu ile karşılaştırıldığında uygulama öncesi ve sonrası arasında dışkı yumurta sayımı azalma testinde anlamlı derecede fark (P<0.05) belirlendi. Yumurta açılımı testinin sonuçları 4 cins için LC_{50} değerinin 0.138 µg/mL ile 0.141 µg/mL arasında, albendazol için 0.1ug/ml'den daha fazla, değiştiğini gösterdi. Bu sonuçlar albendazol için yumurtada direncin varlığını göstermektedir. Yumurta çıkma testi dışkı yumurta sayımı azalma testinin sonuçlarını destekler yöndedir.

Anahtar sözcükler: *Koyun, Keçi, Anthelmintik direnç, Albendazol, Levamizol, İvermektin, Kuzey Punjab*

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INTRODUCTION

The widespread prevalence of gastrointestinal nematodes (GINs) infections in tropical and sub-tropical areas has worried the production potential of livestock development by causing countless deaths and indirect economic losses [1]. About seventy percent nematode species have been isolated from small ruminants, with over thirty percent nematode species being isolated from the digestive system worldwide [2]. The most common ovine nematode genera include *Haemonchus contortus (H. contortus), Teladorsagia circumcincta (T. circumcincta)* and *Trichostrongylus* species [3].

Parasitism leads to lowered productivity of livestock [4]. Developing countries are much more affected due to very favorable conditions for helminths [5,6]. As a result small ruminant production in the tropics and elsewhere is greatly reduced due to diseases caused by helminths [1], and helminth infection is shown in up to 95 percent of small ruminants [1]. In Pakistan, prevalence of anthelmintic ineffectiveness, in sheep and goats, has been reported by several authors [8-11]. Recently, in Pakistan, one of the important factors of high prevalence of GINs infections in small ruminants might be due to the loss of anthelmintic activity [8,9,12-14]. Resistance to the major classes of anthelmintics (albendazol, levamisol and ivermectin) in all of the economically important nematodes of sheep and goats, in particular *H. contortus*, *T. circumcincta* and *Trichostrongylus colubriformis* [14] has been recorded in Canada [15], North America [16], Latin America [17], Europe [18], Australia [19], Asia [20], Pakistan [21] and even throughout the world [22]. Development of multiple resistance has been reported due to frequent treatments with one family and thus a change to alternative families of anthelmintics [23]. Anthelmintic resistance is becoming a main constraint in small ruminant production throughout the world [24] and has serious implications due to non-availability of new drugs.

Methodologies for the examination and detection of anthelmintic resistance (AR) from the field and in the laboratory such as controlled efficacy tests (CET, drench and slaughter), faecal egg count reduction tests (FECRT), egg hatch test (EHT) and larval development test (LDT). Standardization of general parasitological methods used in the detection of AR has been reported for sheep and goats [25]. General parasitological based techniques such as CET, FECRT, EHT and LDT have been used in the initial reporting and subsequent surveying of benzimidazole and Ivermectin resistance throughout the world in ruminants.

Therefore the present study has been designed to evaluate the development of resistance against commonly used anthelmintics in sheep and goat from Punjab, Pakistan.

MATERIAL and METHODS

Experimental Design

Sheep (Salt range and Pak Karakul) and goats (Jattal and Beetal) were selected for the obtaining *H. contortus* and *T. circumcincta* eggs from northern Punjab, Pakistan. The infective stage (L_3) larvae of both *H. contortus* and *T. circumcincta* were cultured and recovered through Baermann procedure [26]. Two thousand infective larvae (L_3) of *H. contortus* and five thousand *T. circumcincta* were given to the above selected goats and sheep breed. Four weeks post infection FECs was done to confirm the presence of adult stages of *H. contortus* and *T. circumcincta*.

Grouping of Animals and Treatment

Forty goats and sheep which experimentally infected with *H. contortus* and *T. circumcincta* herd were selected separately, aged between 3 to 4 months, to conduct efficacy trial. Selected goats and sheep were divided into four groups with ten animals each group, on the basis of their equal egg per gram (EPG). Group- I were treated with albendazole (Valbazen®, Pfizer Animal Health, Exton, PA 19341, USA) by oral drench, Group- II were treated with levamisole (Levasole®, Schering-Plough, Animal Health Corp., Union, NJ 07083), Group- III were treated with Ivermectin (Ivomec®, Merial Limited Iselin, NJ, USA) by injecting subcutaneous and Group- IV were serve as control (distilled water), respectively.

Faecal Egg Count (FEC)

Faecal samples were taken from experimental animals 0 and 14 days directly from rectum with a two finger procedure in label plastic bags by using disposable gloves. All samples were screened individually for nematode eggs (FEC) at 0 and 14 days using the McMaster technique as described by MAFF [26].

Faecal Egg Count Reduction Test (FECRT)

The formulae recommended by the WAAVP guidelines for detecting anthelmintic resistant nematodes of ruminants were used to calculate mean faecal egg count, percentage reduction and 95% confidence interval. The difference between the mean faecal egg counts recorded at the first and second visit were used to calculate the percentage efficacy of each anthelmintic treatment according to the formula:

$$\text{Efficacy} = \frac{(\text{Pre-treatment mean} - \text{Post-treatment mean})}{\text{Pre-treatment mean}} \times 100$$

An efficacy of less than 90 percent and 95 percent upper confidence levels of less than 90 percent were taken as indicating the presence of anthelmintic resistant nematode.

Coproculture Analysis

Pooled faecal samples of all animals from each experimental group were used for coprocultures before (on day 0) and after treatment (on day 14). Baermann procedure was used to recover the larvae [26] and determine the relative composition of specific resistant nematode species. The identification of larvae (L_3) was carried out by following the keys and description given by MAFF [26].

Egg Hatch Assay (EHA)

The guidelines of WAAVP were used to carry out egg hatch assay for determining anthelmintic resistance [27].

Statistics

Data analysis by statistical package POST HOC TEST (Univariate analysis of variance) was performed. Fecal egg count and larval culture records were transformed before analysis [log10 (n+1)] to stabilize the variance. The data resulting from the study were analyzed for ANOVA. Data were also be analyzed by statistical package SPSS 16 were performed. Log probit analysis [28] was also done for EHA.

RESULTS

The present study results revealed post treatments mean FECs with different drug types in salt range sheep are 160±64.89, 140±51.53 and 15±7.63 for albendazol (ABZ), levamisol (LEV) and Ivermectin (IVM) respectively. Efficacy and confidence levels indicated presence of ABZ and LEV resistant nematodes in treated sheep. However, the nematodes were found susceptible to IVM *(Table 1)*. Coproculture revealed that *H. contortus* and *T. circumcincta* were resistant to ABZ and LEV. Nematode species were susceptible to IVM. The reduction in eggs per gram (EPG) of faeces (efficacy) was 87%, 87% and 99% respectively for *H. contortus* in salt range sheep treated with ABZ, LEV and IVM. The lower confidence interval was 69%, 72%, 95% and upper confidence interval was 95%, 94% and 100% respectively. The reduction in eggs per gram (EPG) of faeces (efficacy) was 88%, 92% and 99% respectively for *T. circumcincta* in salt range sheep treated with ABZ, LEV and IVM. The lower confidence interval was 73%, 81%, 93% and upper confidence interval was 95%, 96% and 100% respectively *(Table 2)*.

Post treatments mean FECs with different drug types in Pak karakul sheep are 120±40.27, 130±42.29, 40±12.47 for ABZ, LEV and IVM, respectively. Efficacy and confidence levels indicated presence of ABZ and LEV resistant nematodes in treated sheep. However, the nematodes were found susceptible to IVM *(Table 1)*. The EPG of faeces (efficacy) was 88%, 87% and 96% respectively for *H. contortus* in Pak karakul sheep treated with ABZ, LEV and IVM. The lower confidence interval was 78%, 82%, 93% and upper confidence interval was 94%, 90% and 98%

Table 1. *Post treatments Mean faecal egg counts of different species of nematodes in different breeds of sheep and goat before and after treatment with anthelmintics*

Tablo 1. *Değişik nematodlara karşı anthelmentikler ile uygulama öncesi ve uygulama sonrası ortalama dışkı yumurta sayımı*

Breeds	Drug Types	EPG (Mean±SE) n=10	Efficacy (%)	Lower Confidence Level	Upper Confidence Level	Remarks
Salt Range Sheep	ABZ	160±64.89	88	71	95	Resistance
	LEV	140±51.53	89	76	95	Resistance
	IVM	15±7.63	99	97	100	Susceptible
	Control	1570±100	---	---	---	---
Pak Karakul sheep	ABZ	120±40.27	91	82	95	Resistance
	LEV	130±42.29	90	80	95	Resistance
	IVM	40±12.47	97	94	98	Susceptible
	Control	1310±44.59	---	---	---	---
Jattal Goats	ABZ	115±30.77	91	84	95	Resistance
	LEV	115±23.62	91	86	94	Suspected
	IVM	25±8.33	98	96	99	Susceptible
	Control	1285±38.76	---	---	---	---
BeetalGujrati Goats	ABZ	110±41.36	91	81	96	Resistance
	LEV	105±24.09	92	87	95	Resistance
	IVM	15±7.63	99	96	100	Susceptible
	Control	1345±29.29	---	---	---	---

ABZ (Albendazole), LEV (Levamisole), IVM (Ivermectin)

Table 2. *Post treatments mean FECs of nematodes with different drugs in sheep and goats*
Tablo 2. *Farklı ilaç uygulanan koyun ve keçilerde uygulama sonrası nematodarın ortalama dışkı yumurta sayıları*

Animal Breed	Drug Type	Nematode Species	Pre Treatment Mean EPG n=10	Post Treatment Mean EPG n=10	Efficacy (%)	Lower Confidence Level	Upper Confidence Level	Remarks
Salt Range Sheep	ABZ	*H. contortus*	705	90	87	69	95	Resistance
		T. circumcincta	585	70	88	73	95	Resistance
	LEV	*H. contortus*	705	95	87	72	94	Resistance
		T. circumcincta	560	45	92	81	96	Resistance
	IVM	*H. contortus*	735	10	99	95	100	Susceptible
		T. circumcincta	530	5	99	93	100	Susceptible
Pak Karakul Sheep	ABZ	*H. contortus*	725	85	88	78	94	Resistance
		T. circumcincta	575	35	94	84	98	Resistance
	LEV	*H. contortus*	705	95	87	82	90	Resistance
		T. circumcincta	570	35	94	89	97	Resistance
	IVM	*H. contortus*	715	30	96	93	98	Susceptible
		T. circumcincta	615	10	98	94	100	Susceptible
Jattal Goats	ABZ	*H. contortus*	675	75	89	82	93	Resistance
		T. circumcincta	550	40	93	83	97	Resistance
	LEV	*H. contortus*	715	80	89	84	92	Resistance
		T. circumcincta	565	35	94	87	97	Resistance
	IVM	*H. contortus*	690	20	97	93	99	Susceptible
		T. circumcincta	580	5	99	93	100	Susceptible
Beetal Goats	ABZ	*H. contortus*	660	85	87	75	93	Resistance
		T. circumcincta	615	25	96	86	99	Resistance
	LEV	*H. contortus*	715	80	89	83	92	Resistance
		T. circumcincta	570	25	96	89	98	Suspected
	IVM	*H. contortus*	700	10	99	94	100	Susceptible
		T. circumcincta	585	5	99	93	100	Susceptible

ABZ (Albendazole), LEV (Levamisole), IVM (Ivermecti

respectively. The reduction in EPG of faeces (efficacy) was 94%, 94% and 98% respectively for *T. circumcincta* in Pak karakul sheep treated with ABZ, LEV and IVM, respectively. The lower confidence interval was 84%, 89%, 94% and upper confidence interval was 98%, 97% and 100% respectively *(Table 2)*.

Post treatments mean FECs with different drug types in Jattal Goats are 115±30.77, 115±23.62, 25±8.33 for ABZ, LEV and IVM, respectively. Efficacy and confidence levels indicated presence of ABZ resistant nematodes in treated goats. However, the nematodes were found susceptible to LEV and IVM *(Table 1)*. The reduction in EPG of faeces was 89%, 89% and 97% respectively for *H. contortus* in Jattal goats treated with ABZ, LEV and IVM. The lower confidence interval was 82%, 84%, 93% and upper confidence interval was 93%, 92% and 99% respectively. The reduction in EPG of faeces was 93%, 94% and 99% respectively for *T. circumcincta* in Jattal Goats treated with ABZ, LEV and IVM. The lower confidence interval was 83%, 87%, 93% and upper confidence interval was 97%, 97% and 100% respectively *(Table 2)*.

Post treatments mean FECs with different drug types in Beetal goats are 110±41.36, 105±24.09, 15±7.63 for ABZ, LEV and IVM, respectively. Efficacy and confidence levels indicated presence of ABZ and LEV resistant nematodes in treated goats. However, the nematodes were found susceptible to IVM *(Table 1)*. The reduction in EPG of faeces was 87%, 89% and 99% respectively for *H. contortus* in Beetal Goats treated with ABZ, LEV and IVM. The lower confidence interval was 75%, 83%, 94% and upper confidence interval was 93%, 92% and 100%, respectively. The reduction in EPG of faeces was 96%, 96% and 99% respectively for *T. circumcincta* in Beetal Goats treated with ABZ, LEV and IVM. The lower confidence

Table 3. *Percent eggs hatched at different concentrations of albendazol in sheep and goats*
Tablo 3. *Koyun ve keçilerde farklı albendazol konsantrasyonları için yumurtadan çıkma yüzdeleri*

Animal Breed	Conc. Albendazol µg/mL	Conc. Albendazol ng/mL	Log (Albendazol)	% age Hatch	Probit Hatching	Regression	LC_{50} µg/mL
Salt Range Sheep	0.001	1	0	97	6.88	7.1971	0.138
	0.05	50	1.699	82	5.915	5.336048	
	0.1	100	2	54	5.1	5.0063	
	0.3	300	2.4771	28	4.417	4.483661	
	1	1000	3	18	4.084	3.9109	
	2	2000	3.301	3	3.119	3.581152	
Pak Karakul Sheep	0.001	1	0	96.3333	6.774	7.1294	0.138
	0.05	50	1.699	81	5.877	5.253737	
	0.1	100	2	51	5.025	4.9214	
	0.3	300	2.4771	26.6667	4.375	4.394658	
	1	1000	3	16	4.005	3.8174	
	2	2000	3.301	2	2.946	3.485063	
Jattal Goats	0.001	1	0	96	6.75	7.1708	0.141
	0.05	50	1.699	84	5.994	5.242639	
	0.1	100	2	51.6667	5.041	4.901	
	0.3	300	2.4771	23	4.261	4.359515	
	1	1000	3	17	4.045	3.7661	
	2	2000	3.301	1.3333	2.773	3.424461	
Beetal Goats	0.001	1	0	95.3333	6.674	7.0455	0.141
	0.05	50	1.699	81	5.877	5.236777	
	0.1	100	2	51.6667	5.041	4.9163	
	0.3	300	2.4771	27.6667	4.405	4.408357	
	1	1000	3	15.6667	3.989	3.8517	
	2	2000	3.301	2.3333	3.004	3.531223	

interval was 86%, 89%, 93% and upper confidence interval was 99%, 98% and 100%, respectively *(Table 2)*.

Results of EHT revealed that all the four flocks found positive for resistance against ABZ had LC_{50} values more than 0.1 µg/mL. The LC_{50} value for salt range sheep was 0.138 µg/mL, Pak Karakul sheep 0.138 µg/mL, Jattal goats 0.141 µg/mL and Beetal goats 0.141 µg/mL which confirmed the results of FECRT *(Table 3)*.

DISCUSSION

Faecal egg count reduction (efficacy in percent) and confidence levels indicated that the presence of ABZ, LEV and IVM resistant nematodes in treated sheep and goats. Mostly, the nematodes were found susceptible to IVM. The results revealed that a significant difference (P<0.05) of FECRT were found on pre-treatment and post-treatment with different anthelmintic drug as compared to control group. These results are inconformity with that of Sheferaw *et al.*[29] suggested that it is safe to use these drugs because they all have safe efficacy. The efficacy values are for albendazole, tetramisole and ivermectin were 99.34%, 97.77% and 98.30%, respectively. Moreover, Gill [20] and Uppal *et al.*[30], verified similar findings in India but with low efficacy.

Copro-culture revealed that *H. contortus* and *T. circumcincta* were resistant to ABZ and LEV. Nematode species were susceptible to IVM. These results also similar with the other studies and are especially for the ABZ [23,31,32]. Explanation for the effectiveness of potent agent IVM among all sheep and goats might be introduction of susceptible parasite population which replaced the resistant parasites by presenting the susceptible status to the nematode parasite population within the host body. These results are inconformity with previous studies [23,31]. Moreover, similar findings were verified in India [30].

According to our coproculture analysis results, in sheep and goat which have shown *Haemonchus contortus* to have the greatest propensity for deworming resistance to ABZ. These findings are in accordance with Chandrawathani *et*

al.[33]. Moreover, sheep and goats are constantly at the high risk to acquire the nematodes infection, as a result farmers continue using drugs without the basic information of their dosage and administration hence resulting in a stern multiple anthelmintic resistances as indicated in our Salt range breed results. According to Coles and Roush [27], the optimal recommendation is to use anthelmintic different families one by one according to the demand of the host. Overall, the supporting fact regarding drug resistance against heterogeneous nematodes population within and outside the body of host in Northern region points towards existing poor farm management husbandry practices, illiteracy of farmer and lack of basic knowledge about drug, nematodes infection and administration to host. Similar trend for efficacy of ABZ and LEV in sheep and goat was also reported [22]. Basically emergence of AR is a result of many factors such as genetic, biological or operational.

Egg hatch test (EHT) was performed for confirmation of ABZ resistance detected by FECRT. Results of EHT revealed that all the four flocks found positive for resistance against ABZ had LC_{50} values more than 0.1 µg/mL. The LC_{50} values ranged from 0.138 µg/mL to 0.141 µg/mL, which confirmed the results of FECRT. The egg hatch assay was carried out using the WAAVP guidelines for determining anthelmintic resistance [34] with modifications that allowed the testing of these natural compounds. EHT was performed for confirmation of results of FECRT and found 100% correct. Logarithmic concentration (LC_{50}) value was calculated for the eggs by log probit analysis [28]. Eggs having LC_{50} value in excess of 0.1 µg anthelmintic per mL was indicative of anthelmintic resistance against ABZ [35].

The present study results revealed that, percent reduction in EPG of faeces was resistant with ABZ in all breeds of sheep and goat against *H. contortus* and *T. circumcincta* infection which are in agreements with Gill [20] who observed resistance to ABZ and LEV against sheep nematodes. These findings are against previous studies that reported no resistance against ABZ in sheep [36,37]. Sargison [38] reported multiple anthelmintic resistances in sheep and goat. Marian *et al.*[39] used egg hatch test for benzimidazole resistance and compared it with FECR test and declared resistance with two tests. The LD_{50} was higher than 0.1mg/ml thiabendazole indicating resistance, which in agreement to the present study. The economic significance of helminths throughout the World [40] necessitates immediate attention to launching an effective control program. Chemical control is the main part of all helminth control programs [22]. The other control programs includes an integrated approach based on the use of plants with modern anthelmintics, Mass education awareness program and most importantly pasture management (clean and alternated pasture).

The present study indicates the presence of anthelmintic resistance against nematode infections in native sheep and goats of Northern Punjab. Therefore it is recommended that the proper anthelmintic dose with 3-4 months interval and rotation of anthelmintics for minimized the nematode infection and enhance goat and sheep productivity.

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Research Article

Effect of *Maclura pomifera* Extract on Cisplatin-Induced Damages in Reproductive System of Male Rats [1]

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Abstract

In this study, we aimed to investigate the effect of *maclura pomifera* (MP) (known as cytotoxic, anti-tumoural, anti-inflammatoric, antinociceptive, and anti-oxidative) extract upon the spermatological parameters in Cisplatin (anti-tumoural chemotherapeutic with Reactive Oxygen Species (ROS)- generating side-effects)-treated rats. A total of seventeen male Sprague Dawley rats, aged eight weeks and weighing 250-300 g, were used. Three experimental groups were assigned, as follows; Group 1 (n=5) as control (C): the animals received intraperitoneal (IP) injection of physiological saline and further saline via oral gavage during 5 days before and after the injection, Group 2 (n=6): single IP injection of 7 mg/kg Cisplatin (CP) and saline via oral gavage as above, and Group 3 (n=6): referred as MP + CP, oral administration of 500 mg/kg/day MP extract during 5 days before and after cisplatin (CP) injection, as above. At the end of the experiment, rats were decapitated and their testes and cauda epididymis weights as well as epididymal sperm parameters were analysed. The CP administration caused a significant (P<0.05) decline in sperm motility and density, with a significant increase in dead spermatozoa. The MP + CP combination also led to a significant decline (P<0.05) in the weights of total cauda epididymis and of testes, but significantly improved (P<0.05) sperm survival as compared to the CP alone. In conclusion; the findings suggested that the MP plus CP combination had an adverse effect upon the total weights of testes and cauda epididymis, however the dead sperm rate was markedly lower than the CP alone.

Keywords: *Cisplatin, Maclura pomifera extract, Male rat, Sperm, Reproductive system*

Erkek Ratlarda *Maclura pomifera* Ekstraktının Sisplatin ile Oluşturulmuş Üreme Sistemi Hasarı Üzerine Etkisi

Özet

Bu çalışmanın amacı, *maclura pomifera* (MP) (sitotoksik, anti-tümöral, yangı önleyici, analjezik/ağrı giderici, ve antioksidan olarak bilinen) ekstraktının sisplatin (ROS (Reaktif oksijen türevleri) -oluşturucu yan etkili anti-tümöral ajan) uygulanan erkek ratların spermatolojik parametreleri üzerine etkisini incelemekti. Araştırmada 8 haftalık yaşta, 250-300 g canlı ağırlığa sahip 17 adet erkek Sprague Dawley ırkı rat kullanıldı. Üç deney grubu oluşturuldu; Grup 1 (n=5): kontrol grubu, hayvanlara intraperitonal (IP) serum fizyolojik enjeksiyonuyla birlikte, enjeksiyondan 5 gün öncesi ve sonrası boyunca oral gavaj yoluyla yine serum fizyolojik verildi. Grup 2 (n=6): Sisplatin (CP) grubu, hayvanlara bir kez IP 7 mg/kg CP enjeksiyonundan 5 gün öncesi ve sonrası boyunca oral yolla serum fizyolojik verildi. Grup 3 (n=6): MP + CP grubu, hayvanlara bir kez İP 7 mg/kg CP enjeksiyonuyla birlikte, enjeksiyondan 5 gün öncesi ve sonrası boyunca oral yolla günlük 500 mg/kg MP ekstraktı verildi. Deney sonunda ratlar dekapite edilerek testis ve kauda epididimis ağırlıkları ile epididimal sperm parametreleri incelendi. CP enjeksiyonu, sperm motilitesi ve yoğunluğunu önemli derecede (P<0.05) düşürdü ve ölü spermatozoa sayısını artırdı. MP + CP uygulaması, CP grubuna kıyasla toplam testis ağırlığı ve toplam cauda epididimis ağırlığını önemli derecede (P<0.05) azalttı, ancak canlı sperm sayısında önemli (P<0.05) bir artışa neden oldu. Sonuç olarak; MP + CP kombinasyonunun, CP grubuna kıyasla toplam testis ve cauda epididimis ağırlığı üzerine olumsuz bir etki göstermekle birlikte, ölü sperm sayısını önemli oranda azalttığı gözlendi.

Anahtar sözcükler: *Sisplatin, Maclura Pomifera ekstraktı, Erkek rat, Sperm, Üreme sistemi*

INTRODUCTION

Maclura pomifera (MP), a member of Moraceae family, is a dioecious tree that inhabits in the Southwestern United States. It is also a widely cultivated hardwood tree in Turkey. The plant is known as osage orange, horse apple, mock orange or hedge apple [1]. Numerous articles have indicated that the MP and its components have several

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biological activities including cytotoxic [2], anti-tumoural [3], anti-inflammatory and antinociceptive activities [1].

The MP contains lectins [2], xanthones [4] and flavone-type compounds [1]. *Maclura pomifera* has antioxidant capacity, attributable to flavonoid type components especially isoflavones, osajin and pomiferin [5]. The antioxidant activities showed marked difference between osajin and pomiferin. Vesela et al.[5] showed that the antioxidant profile of highly active pomiferin was comparable to the reference compounds used, while osajin showed only low activities. Pomiferin also has inhibitory effects for anti-cholinesterase [6] and histone deacetylase (HDAC) [7]. Chromatin is a dynamic macromolecular complex that consists of DNA, histones, and non-histone proteins. Histones are small basic proteins consisting of a globular domain and a more flexible and charged NH_2 -terminus protruding from the nucleosome [8].

The HDAC inhibitors act in dual way, as having inhibitory effect on the proliferation and inducing differentiation and/or apoptosis of tumour cells in culture and in animal models. Some HDAC inhibitors have been shown to have a potent anti-tumoural effect with a little toxicity *in vivo* in animal models [8]. The HDAC inhibitors exhibit an anti-proliferative activity on tumour cells [7]. Yang et al.[9] suggested that pomiferin has an anti-proliferative effect not only on transformed breast epithelial cells but also on the normal cells. Robert and Rasool [10] emphasised that the HDAC inhibitors are triggered in cancer and leukemia cells by widespread histone acetylation and actual increases in reactive oxygen species (ROS). Also, the HDAC inhibitors may increase DNA damage following treatment with DNA-damaging chemotherapies by inhibition of DNA repair mechanism.

Cisplatin (CP), known as one of the most effective anti-tumoural chemotherapeutic drugs, is widely used in the treatment of various cancer types [11-13]. Despite the fact that CP has a powerful effect on the destruction of neoplastic cells; it has also some dose-dependent side effects, including; cytotoxicity, nephrotoxicity [14,15], hepatotoxicity [16] and reproductive toxicity [17]. The CP exposure can break the redox balance of tissues, suggesting that biochemical and physiological disturbances may result from the oxidative stress [18]. The ROS, like singlet molecular oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide are normally produced in the subcellular compartments of testis, particularly within the mitochondria. They are then scavenged by the antioxidant defence system like the enzymes of the related subcellular compartments such as plasma membrane, cytosol, acrosome, nucleus, equatorial segment, midpiece and flagellum [19]. However, this balance can readily be broken by the chemicals like the CP, that disrupts the prooxidant-antioxidant balance, leading to cellular dysfunction [20]. Also, the CP can induce free radical toxic stress and spermatic DNA damage [21,22].

To protect the live cells within the body against the CP's deleterious effects, numerous studies have been conducted. Various studies suggested that some anti-oxidant substances like lycopene, ellagic acid [18], royal jelly [23], melatonin [24], roselle and ginger [25] and selenium [26] have all ameliorative effects against the CP-induced damages in the male reproductive system. However, in the literature, there appears no study available about the effect of maclura pomifera extract on the spermatological parameters in CP-exposed rats. Hence, the combined effects of *maclura pomifera* and CP administration upon the reproductive parameters of male rats were investigated in this study.

MATERIAL and METHODS

Chemicals

Cisplatin (50 mg/100 ml) was purchased from Ebewe Pharma® (Unterach-Austria). To obtain the MP extract, fruits of *maclura pomifera* were collected from Ankara province in September and then were washed and removed from the shell. After the slicing into 100 g portions, they were wrapped with a filter paper. The extraction was performed routinely in 250 ml distilled water on a magnetic mixer at room temperature during one day. By removing the water in the lyophilisator, the extract of raw fruit juice was obtained. This process was repeated twice with two-day interval and both extracts were pooled and used as *maclura pomifera* extract. The extract was kept at +4°C during the study.

Animals and Experimental Protocols

Seventeen male Sprague Dawley rats (Specific Pathogen Free) aged eight weeks old and weighing 250-300 g, were used in this study. The animals were obtained from Atatürk University Medical & Experimental Research & Application Centre (Erzurum, Turkey) and were housed therein under standard laboratory conditions (24±2°C, 40-60% humidity, a 12 h light: 12 h dark cycle). A commercial pellet chow (in the sack of 50 kg, Bayramoglu Food Co./Erzurum-TR) and fresh drinking water were available *ad libitum*. Rats were divided into 3 groups, as follows; In Group 1 (n=5), served as control (C), animals were exposed to intraperitoneal (IP) physiological saline injection along with further saline via oral gavage during 5 days before and after the injection. In Group 2 (n=6), referred to as Cisplatin (CP), rats were exposed to single dose (7 mg/kg) of IP injection of the CP along with saline via oral gavage for 5 days before and after the CP injection. In Group 3 (n=6), named as cisplatin + *maclura pomifera* (MP + CP), animals received single dose of IP injection of the CP (7 mg/kg) and oral administration of *MP* extract (500 mg/kg/day) during 5 days before and after the cisplatin injection.

Before the study started, the approval of Committee for Institutional Animal Care and Use was provided from

Atatürk University Local Board of Ethics (the approval number: 2013/141, dated on 22nd Oct., 2013).

Collection of Samples

Rats were sacrificed under ether anaesthesia. Both testes were then removed. Cauda epididymides were separated from both testicles and the connective tissues were cleansed by using anatomic scissors. Cauda epididymides and testicles both were then weighed. To obtain semen samples from both cauda epididymises, a modified method, previously described by Sonmez et al.[27] was used. Briefly, the epididymides were minced by anatomical scissors in 1 ml of physiological saline (0.9% w/v NaCl) in a petri dish. Afterwards, the pieces of epididymides were allowed to incubate at room temperature for 15 min to provide the migration of all spermatozoa from the epididymal tissue into the fluid. Epididymal tissues were removed from dishes by tweezers. The fluid obtained was used as semen sample.

Sperm Evaluation

To evaluate the percentage of sperm motility, light microscope equipped with heated stage was used. Briefly, a slide was placed on a light microscope with a heated stage warmed up to 37°C. A droplet of semen sample was dropped on the slide and the percentage of sperm motility was determined routinely by visual observation. The motility estimations were performed in three different fields for each sample. Then, the mean of the three consecutive estimations was used as the final motility score [27].

The epididymal sperm concentration was determined by using a slight modification of the method (using eppendorf tubes instead of routine haemocytometer), as described by Sonmez et al.[27]. The semen sample was drawn into the eppendorf tube at 10 µl volume with an automatic pipette and 990 µl eosin solution was added. The solution contained 5 g sodium bicarbonate, 1 mL formalin (35%, v/v) and 25 mg eosin per 100 mL distilled water. After vortexing the eppendorf tubes for 15 sec, approximately 10 µl of diluted sperm suspension was transferred into the counting chambers of Thoma chamber and allowed to settle down for 5 min. The sperm cells in both chambers were counted by using phase contrast microscope at the magnification of 400×.

To determine the percentage of morphologically abnormal spermatozoa, the methods described by Sonmez et al.[27] and Turk et al.[20] were used. Briefly, the slides were stained with eosin-nigrosin (1.67% eosin, 10% nigrosin and 0.1 M sodium citrate). The slides were then examined under a phase contrast microscope at 400x magnification. A total of 200 sperm cells were examined on each slide and the head, mid-piece, tail and total abnormality rates of spermatozoa were all expressed as percentage [20].

To determine the percentage of dead sperm, the stained slides prepared originally for determination of abnormal spermatozoa were used. The slides were examined under a phase contrast microscope at 400x magnification and 200 sperm cells were counted on each sample. According to the staining status (eosin uptake) of the head of sperm cells, they were classified as dead (stained head) or alive (unstained head).

Pathological Examinations

Tissue samples of testis were fixed in Bouine's solution. Tissues were exposed to alcohol-xylol series, embedded in paraffin blocks, sectioned 5 µm in thickness and stained with Hematoxylin-Eosin dye. The evaluation was performed according to Johnsen classification criteria with examination of 100 seminiferous tubules selected randomly in each sample under the light microscope [28].

Statistical Analyses

All values of sperm parameters and testicular traits studied were presented as mean ± standard error of means (S.E.M.). The differences were considered significant when P<0.05. Statistical analyses were performed using analysis of variance (One-way ANOVA) and post hoc Tukey test by using the SPSS/PC (Version 20) software programme. Also, data from spermatological parameters and testis traits were analysed further to find possible correlations exist between them with Pearson-correlation test.

RESULTS

Reproductive Organ Weights

Total testes weights (TTW) of rats in all groups were presented in *Table 1*. Although the TTW of MP + CP group was significantly (P<0.05) lower than in the control group, but there was no significant difference (P>0.05) either between the MP + CP and CP groups or between the control and CP groups.

Total cauda epididymis weights (TCEW) were presented in *Table 1*. The mean TCEW of MP + CP group was statistically (P<0.05) lower than those in the control group. But, no significant difference (P>0.05) was found either betweenthe MP + CP and CP groups or between the control and CP groups.

Table 1. *Total testes and cauda epididymis weights*
Tablo 1. *Toplam testis ve cauda epididymis ağırlıkları*

Groups	N	Total Testes Weights (g)	Total Cauda Epididymis Weights (g)
Control	5	2.660±0.082^{a}	0.414±0.0264^{a}
CP	6	2.605±0.038^{ab}	0.348±0.0187^{ab}
MP + CP	6	2.403±0.070^{b}	0.296±0.0165^{b}

^{a,b} *Different superscript letters show significant differences between groups (P<0.05)*

Epididymal Sperm Parameters

Sperm motility percentages were presented in *Table 2*. In terms of sperm motility rate, both the CP and MP + CP group had significantly (P<0.05) lower values than those in the control group. However, no significant difference (P>0.05) was found between the CP and MP + CP groups.

Sperm cell densities in all the groups studied were presented in *Table 2*. It was significantly (P<0.05) higher in the controls than both in the CP and in MP + CP groups. However, there were no differences (P>0.05) between the CP and MP + CP groups.

Epididymal sperm abnormalities were classified as head, mid-piece, tail and total sperm abnormality. There was no significant difference (P>0.05) among all the groups in terms of all abnormalities concerned. The values of all the sperm abnormalities of groups were presented in *Table 2*.

Dead sperm rates were presented in *Table 2*. The rate of CP group was significantly (P<0.05) higher than in the control and in the MP + CP groups. However, there was no difference (P>0.05) between the control and MP + CP groups.

Correlations between all the reproductive parameters studied were presented in *Table 3*.

Pathological Findings

The structures of seminiferous tubules were normal in the control group. In this group, the germ cells in the seminiferous tubules were arranged regularly and the normal course of spermatogenesis was evident *(Fig. 1)*. However, both in the CP and MP + CP groups although the borders of tubules appeared normal, the sequence of germ cells were disordered and there were significant (P<0.05) evidence of degeneration. Furthermore, oedema in the interstitial fields in these groups was evident *(Fig. 2* and *Fig. 3)*. Based on the Johnsen's Score, there was a significant (P<0.05) difference between the control and CP groups, between the control and MP + CP groups, but no differences (P>0.05) were found between treatment groups *(Table 4)*.

DISCUSSION

Despite the fact that the CP exhibited deleterious side effects, it is still one of the most effective chemotherapeutic agents that is widely used against various cancer types [11-13]. Cisplatin-based chemotherapy results in toxicological changes in other tissues such as liver, kidney and testes [17]. Chemotherapy-induced gonadal toxicity and the recovery of spermatogenesis are interrelated with the type of drugs, their doses and duration of therapy used [29]. Treatment with chemotherapeutic agents causes germinal

Table 2. *Semen and reproductive parameters of all the experimental groups*
Tablo 2. *Bütün grupların sperma ve reprodüktif parametreleri*

Groups	N	Motility (%)	Density (10^6/ml)	Head Abnormality (%)	Mid-piece Abnormality (%)	Tail Abnormality (%)	Total Abnormality (%)	Dead Sperm Rate (%)
Control	5	55.59±1.20^a	135.000±27.9^a	14.9±1.6	5±1.4	10.5±0.6	30.5±2	34.6±1.1^b
Cisplatin	6	42.12±1.29^b	55.338±6.26^b	11.9±1.3	4.2±1.9	12.7±0.7	28.9±1.2	42.3±1.2^a
MP + CP	6	44.04±0.96^b	52.343±7.59^b	13.2±1.1	3±1.4	9.3±1.7	25.4±2.6	38.3±0.8^b
Significance		P<0.05	P<0.05	NS	NS	NS	NS	P<0.05

^{a,b} *Different superscript letters show significant differences between groups (P<0.05);* ***NS:*** *Non-significant*

Table 3. *Correlations between all the reproductive parameters studied*
Tablo 3. *Çalışılan bütün reprodüktif parametrelerin arasındaki korelasyonlar*

Reproductive Parameters	Motility	Density	Dead sperm Rate	Head Abnormality	Mid-Piece Abnormality	Total Sperm Abnormality	Total Testis Weight	Total Cauda Epididymis Weight
Motility		(+)**	(-)**					(+)*
Density	(+)**		(-)*					(+)**
Dead sperm rate	(-)**	(-)*						
Head abnormality						(+)**		
Mid-piece abnormality						(+)*		
Total sperm abnormality				(+)**	(+)*			
Total testis weight								(+)**
Total cauda epidydimis weight	(+)*	(+)**					(+)**	

(+): *Positive correlation,* ***(-):*** *Negative correlation; ** Correlation is significant at 0.01 level; * Correlation is significant at 0.05 level*

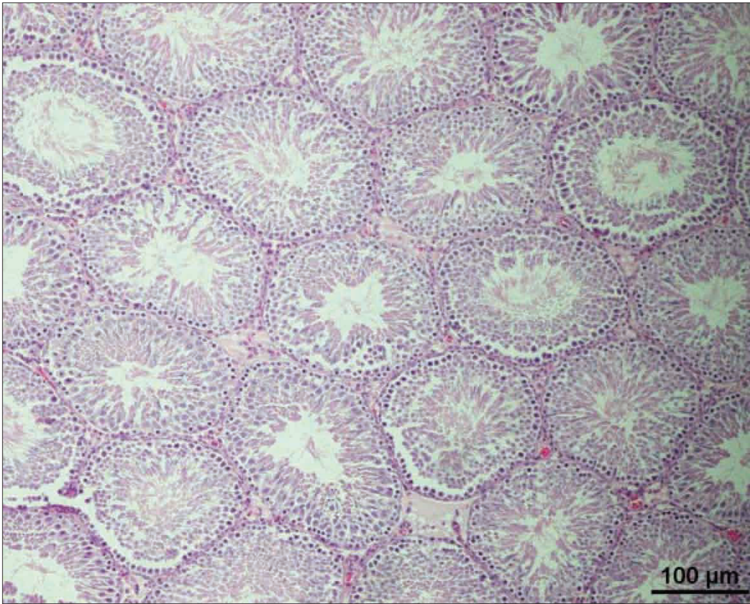
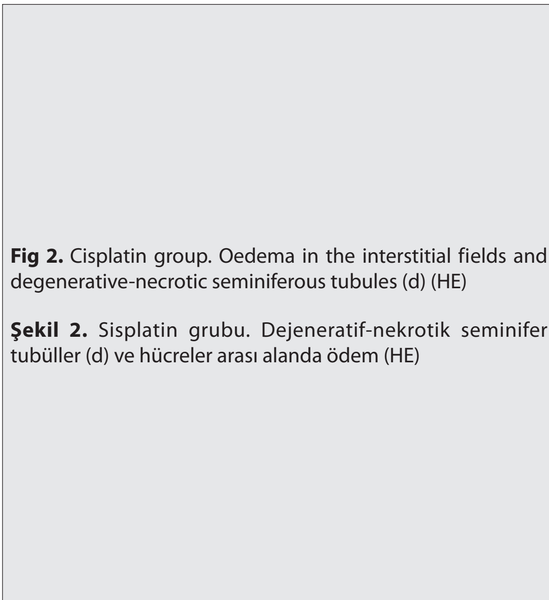


Fig 1. Control group. The structure appears normal (HE staining)

Şekil 1. Kontrol grubu. Yapı normal görünümde (HE boyama)



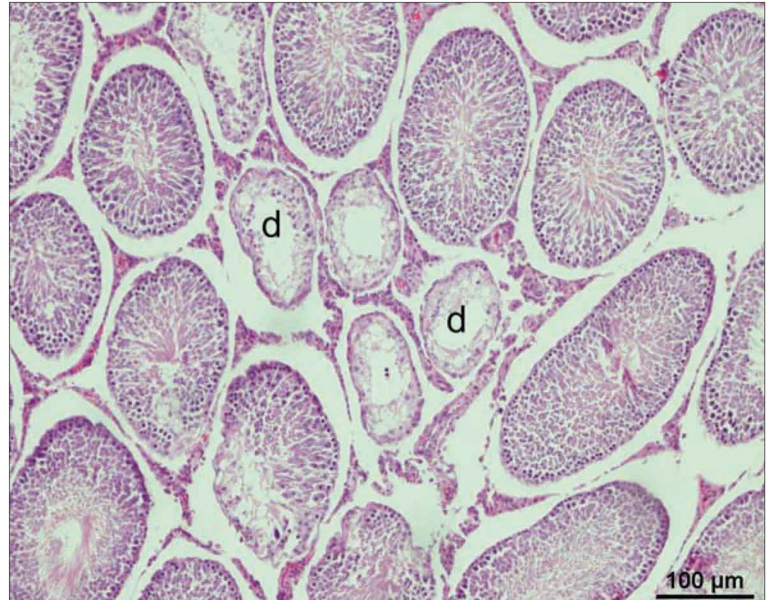


Fig 2. Cisplatin group. Oedema in the interstitial fields and degenerative-necrotic seminiferous tubules (d) (HE)

Şekil 2. Sisplatin grubu. Dejeneratif-nekrotik seminifer tubüller (d) ve hücreler arası alanda ödem (HE)

epithelial damage leading to oligo- or azoospermia [17]. Atessahin et al.[17] reported that the CP administration (7 mg/kg) resulted in a marked decrease in the concentration and motility together with an increase in the rates of all types of sperm abnormalities in rats. Similar findings with the epididymal sperm were reported by Salem et al.[26], using the 10 mg/kg dose. It was presumed that declines both in the motility and density of sperm cells observed herein might stem from lipid peroxidation induced by CP [17,18,26]. The latter workers also reported that the administration of CP further caused a marked decline in the TTW and TCEW. Present results showed that the administration of CP (7 mg/kg) caused a marked ($P<0.05$) decline in sperm motility and density, together with an increase in the number of dead sperm. These results were similar to other studies [17,26]. On the other hand, the CP treatment did not affect the TTW, TCEW and sperm cell abnormalities such as the head, mid piece, tail and total abnormalities. This effect might be dependent on the management, sensitivity or health conditions of rats.

Pomiferin, one of the components of *Maclura pomifera* used in this study has inhibitory effects for anti-cholinesterase [6] and histone deacetylase (HDAC) [7]. The HDAC inhibitors function as an anti-proliferatives on tumour cells [7]. Indeed, Yang et al.[9] suggested that pomiferin has anti-proliferative effect not only on transformed cells but also on the normal cells. Herein, the combination of *maclura pomifera* extract with the CP (MP + CP) caused a marked decline in the TTW and TCEW which might depend on the inhibitory effect of pomiferin. As known, the HDAC inhibitors act as anti-proliferative agent for tumour cells, but with a mild toxicity [8]. Normal body cells could also be affected by this undesirable effect [9]. Presumably, this

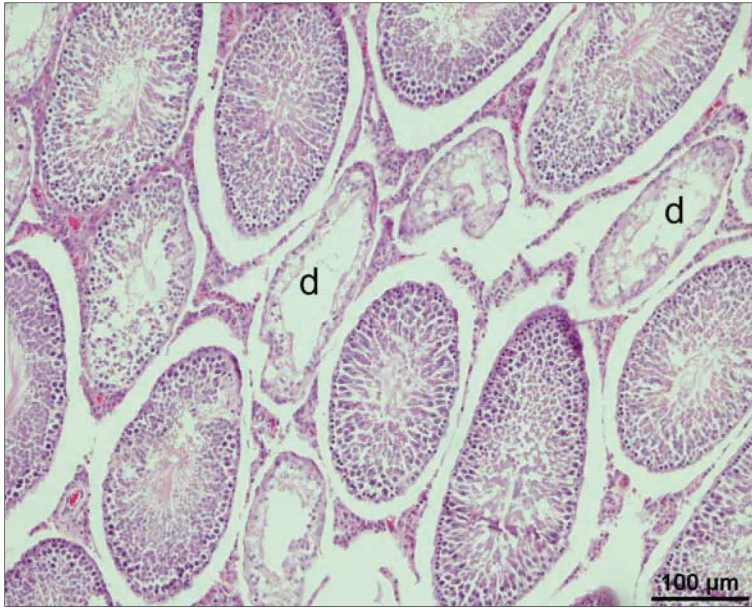


Fig 3. MP + Cisplatin group. Oedema in the interstitial fields and degenerative-necrotic seminiferous tubules (d) (HE)

Şekil 3. MP + Sisplatin grubu. Dejeneratif-nekrotik seminifer tubüller (d) ve hücreler arası alanda ödem (HE)

Table 4. *Jonhsen Score table of all the experimental groups*
Tablo 4. *Bütün deney gruplarının Johnsen Skor tablosu*

Johnsen Scores	Groups		
	Control	Cisplatin	MP + CP
Mean Score	9.6±0.24[a]	8.4±0.24[b]	8.6±0.24[b]

[a-b] Different superscript letters show significant differences between the groups (P<0.05)

phenomenon may be explained by a dual mechanism, as the inhibition of testicular tissue proliferation by the pomiferin's inhibitory effect for the HDAC and degenerative side effect of the CP administration simultaneously. While degenerative effects of chemotherapeutic agent might have been compensated for by the natural proliferation in the CP group, the anti-proliferative property of pomiferin might have blocked the cell proliferation in the MP + CP group. The positive correlation observed between the TTW and TCEW strongly indicates that the weights of these tissues are closely interrelated with each other.

Treatments of *Maclura pomifera* extract for 5 days pre- and post-treatment together with the CP had no effect on decreased sperm motility and density. These side effects could be the result of CP's side effects on the male reproduction system. Degeneration and oedema in testicular tissues both in the CP and in MP + CP could have led to the low sperm cell density and motility rate. The oedema in testicular tissue may have been caused an obstruction in the testicular tubules or epididymal lumens. In the one hand, a possible obstruction in this field could be the result of decreased sperm transport from the seminiferous tubules into the cauda epididymis. On the other hand, *maclura pomifera* extract might have had a protective effect on dead sperm rate in the MP + CP group. In this group, the rate of dead sperm was markedly lower than in the CP group but similar to that in the control group. This might have resulted from the protective effect of antioxidant component of *maclura pomifera* extract.

As expected, a positive correlation was observed between the abnormalities of head and mid-piece and the total sperm abnormality. Furthermore, a negative correlation was found between the sperm motility and dead sperm rate. Decreases in cauda epididymal sperm density had resulted neither from the atrophy in the seminiferous tubules nor from the oedema in the interstitial field. Instead, decreased production might have resulted from the acute necrosis in seminiferous tubules originating from the cisplatin administration. It could be speculated that, as this situation might decrease the sperm density, the TCEW decreased. Indeed, this hypothesis is supported by positive correlation observed between sperm density and the TCEW.

In conclusion, it appeared that pre- and post-treatment with *maclura pomifera* extract (500 mg/kg/day) for 5 days had a limited beneficial effect on the reproductive parameters in the CP-exposed male rats. According to our results, the usage of *maclura pomifera* extract together with the CP; i) had an unfavourable effect on the TTW and TCEW, ii) it was ineffective for protecting the motility rate and sperm density rates, while iii) being protective against the death of the sperm. However, for achieving more reliable results, it is recommended

that future studies should be planned using longer treatment days, larger numbers of rats in the different groups and different concentrations of *maclura pomifera* extract to be given.

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Research Article

Gebe Farelerde Desidua Bazalis Dokusundaki PAS-Pozitif Uterus Doğal Katil Hücrelerinin Dağılımı

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Özet

Bu çalışma, gebeliğin farklı dönemlerindeki fare desidua bazalis dokusunda periyodik-asit Schiff (PAS)-pozitif granüllere sahip uterus doğal katil (uNK) hücrelerinin dağılımının belirlenmesi amacıyla yapıldı. Bu amaçla, 12-14 haftalık fareler, gebe olmayan-kontrol grubu ile gebeliğin birinci, ikinci ve üçüncü haftalarının ortasına karşılık gelecek şekilde erken, orta ve geç gebelik dönemi (sırasıyla gebeliğin 3, 10. ve 17. günleri) olmak üzere 4 gruba ayrıldılar (n= 6). Plasentasyonun ilerlemesiyle birlikte metriyal bölge (MLAp; mesometrial lymphoid aggregate of pregnancy), desidua bazalis, bağlantı bölgesi ve labirint bölgesi olmak üzere dört farklı bölge ayırt edildi. Desidua bazalis ve bağlantı bölgesi arasında trofoblast dev hücreleri dikkati çekti. Endometriyum dokusunda kontrol grubunda 5 adet/10.000 μm^2 olarak tespit edilen PAS-pozitif uNK hücre sayısının, gebeliğin erken döneminde desidua bazalis bölgesinde arttığı dikkati çekti (26,5 adet/10.000 μm^2). En yüksek uNK hücre sayısı (56 adet/10.000 μm^2) gebeliğin ikinci haftasında tespit edildi. uNK hücrelerine maternal kan damarlarının içinde de rastlanırken, mesometriyal bölgedeki maternal kan damarlarının genişlediği ve tunika mediya katmanlarının belirgin bir biçimde inceldiği dikkati çekti. Gebeliğin PAS-pozitif uNK hücrelerinin sayı ve dağılımlarını etkilediği sonucuna varıldı.

Anahtar sözcükler: *Fare, Gebelik, PAS, uNK*

The Distribution of PAS-Positive Uterine Natural Killer (uNK) Cells in the Decidua Basalis of Pregnant Mice

Abstract

This study was carried out to determine the distribution of uterine natural killer (uNK) cells having periodic-acid Schiff (PAS) positive granules in the decidua basalis in pregnant mice. For this purpose, mice at 12-14 weeks of age were divided into four groups as non-pregnant control, and at the middle of the first, second, and the third week of the pregnancy, corresponding to early, middle, and late (3rd, 10th, and 17th days of pregnancy) gestational stages respectively (n= 6 for each group). After placentation, it was identified four different regions as metrial region (MLAp; mesometrial lymphoid aggregate of pregnancy), decidua basalis, junctional zone and labyrinth zone in mice. It was observed trophoblast giant cells between decidua basalis and junctional zone. The number of PAS-positive uNK cells was found as 5 in 10.000 μm^2 in endometrium of control animals while it was determined as 26.5 number/10.000 μm^2 in decidua basalis at early gestational period. The highest PAS-positive uNK cell numbers (56 number/10.000 μm^2) were detected at the mid-gestational period in decidua basalis. uNK cells were also observed in the lumen of the maternal blood vessels. In mesometrial region, the dilatation of maternal blood vessels and thinning of the media layer was distinct. It was concluded that the number and the distribution of the uNK cells having periodic-acid Schiff (PAS) positive granules was affected by pregnancy.

Keywords: *Mice, PAS, Pregnancy, uNK*

GİRİŞ

İnsan ve fare gibi invaziv hemo-koryal ya da hemo-endotelyal plasentasyona sahip türlerde gebeliğin uterus dokusunda meydana getirdiği en önemli histolojik değişim desidualizasyondur. Bu süreçte endometriyumdaki fibroblast benzeri hücreler çoğalıp farklılaşarak desidual hücrelere dönüşürken; değişen endometriyum dokusu da desidua olarak adlandırılır [1]. Plasentanın maternal bölümü, desidualizasyondan sonra farklı dokular halinde kendini

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belli eder. Bunlardan birisi mezometrial üçgen tarafında uterus düz kas katmanları arasındaki geçici bir yapı olan "metrial gland" ya da "MLAp (Mesometrial Lymphoid Aggregate of pregnancy)" olarak bilinirken; bir diğeri yeni gelişmekte olan damarları içeren desidua bazalis dokusudur. Desidua bazalis bölgesine komşu olan bir diğer bölge ise spongiotrofoblastların çoğunluğu oluşturduğu bağlantı bölgesidir. Lümene komşu olan ve kan aralıklarında maternal ve fötal alyuvarların yakın ilişkisi ile dikkati çeken son bölge ise labirint bölge olarak adlandırılır [2]. Bu süreçte endometriyum dokusundaki pek çok hücre tipinin sayı ve dağılımında değişimler meydana gelir. Bununla birlikte asıl değişim uterus doğal katil (uterine natural killer-uNK) hücrelerinin sayısında karşımıza çıkmaktadır. uNK hücrelerinin 2 haftalık farelerde küçük ve granülsüz oldukları [3], fertilizasyon ve implantasyonu takiben gebeliğin yaklaşık 4. gününde, önce desidua bazalis, ardından komşu trofoblast tabakası ve daha sonra da metrial gland olarak adlandırılan bölgede toplandıkları bildirilmektedir [4]. Gebeliğin 12-14. günlerinde sayıları en yüksek seviyeye ulaşan uNK hücrelerinin, gebeliğin sonlarına doğru belirgin bir biçimde azaldıkları ifade edilmektedir [5].

Doğal katil (natural killer-NK) hücrelerinin, tümör hücreleri, virusla enfekte hücreler ve allojen antijenlere karşı organizmayı savundukları bilinmektedir. Bu hücrelerin, esas olarak kemik iliğinde pluripotent hemapoietik hücrelerin lenfoid öncül hücrelerine dönüşümü ile ortaya çıktıkları ve sekonder lenfoid organlarda pre-NK hücreler halini alarak farklı dokulara göç ettikleri bildirilmektedir. Uterus dokusuna gelen küçük ve granülsüz pre-NK hücreleri de uterusta olgunlaşarak büyük ve granüllü hücre formuna dönüşürler (Large Granulated Cells). Bu andan itibaren uNK hücreleri olarak adlandırılan söz konusu bu hücreler bazı araştırıcılar [6] tarafından "Granüllü Metriyal Bez Hücreleri" olarak da tanımlanır. Pre-NK hücrelerinin uterusa göçü ve uNK'lara dönüşümünün, IL-15 tarafından stimule edildiği ileri sürülmektedir [1].

Erken gebelik döneminde uterus dokusunda en bol bulunan hücre tipi olan uNK hücrelerinin, gerek fenotipik gerekse fonksiyonel açıdan sistemik dolaşımdaki NK'lardan farklı oldukları ve NK1.1, DX5 gibi belirteçleri taşımadıkları bildirilmektedir. Bu hücrelerin implantasyon ve plasentasyon sürecinde trofoblastların uterus arterlerine ulaşmalarını koordine ettiği düşünülmektedir. İnsanlarda olduğu gibi farelerde de uNK hücrelerinin periferal dolaşımdaki NK hücrelerinden farklı reseptörler taşımalarının, söz konusu kritik fonksiyonlarını yerine getirebilmede önemli rol oynadığı ileri sürülmektedir. İnsanlardaki KIR (Killer Inhibitory Receptor) gibi farelerde de Ly49 gen ailesinden gelen reseptörlerin, trofoblastlardaki MHC-I molekülüne bağlanarak bu hücreleri yönlendirdikleri düşünülmektedir [7]. Cerdeira ve ark.'nın [8] insanlarda yapmış oldukları bir çalışmada uNK hücrelerinin CD56$^+$ iken CD16$^-$ oldukları; bunun yanı sıra CD9 ve CD49a yüzey belirteçlerinin de yine uNK'lar için spesifik olduğu ve perifer kan NK (pNK) hücrelerinde bulunmadığı tespit edilmiştir. Araştırıcılar [8], uNK hücrelerinin pNK hücrelerinden fonksiyonel olarak farklı olduklarını ve uNK hücrelerinin sahip oldukları yüzey belirteçleri ile ilişkili olarak sitotoksik yönlerinin olmadığını, vasküler endotelyal büyüme faktörü (VEGF) gibi proangiojenik moleküller salgıladıklarını ve trofoblast invazyonunu düzenlediklerini ileri sürmüşlerdir.

Trofoblastların desidua dokusuna yayılmasını ve yerleşmesini kolaylaştıran uNK hücrelerinin özellikle spiral arterlerin yakınında ve hatta içlerinde yer almaları dikkat çekicidir. uNK hücrelerinin salgılamış olduğu gama interferonun (IFN-γ), uterus dokusundaki spiral arterlerdeki değişimden de sorumlu olduğu ileri sürülürken; mesometriyal bölgedeki spiral arterlerin duvarında, gelişmekte olan fötusun ihtiyaç duyduğu kanın sağlanmasına yönelik meydana gelen incelme ve dilatasyonların da, uNK hücresi kaynaklı IFN-γ etkisiyle oluştuğu bildirilmektedir [1].

Bu çalışma, gebeliğin farklı dönemlerindeki farelerde, desidua bazalis bölgesinde PAS-pozitif granülleri ile ayırt edilebilen uterus doğal katil (uNK) hücrelerinin dağılımında gebelikle birlikte meydana gelen değişimlerin belirlenmesi ve bundan sonra gerek gebelik immunolojisi ve gerekse gebelik patolojisi konularında yapılacak olan çalışmalara katkı sağlamak amacıyla yapıldı.

MATERYAL ve METOT

Hayvan Materyali

Çalışma, T.C. Selçuk Üniversitesi Meram Tıp Fakültesi Etik Kurulu'nun 31.08.2009 tarih ve 2009/50 sayılı kararı ile alınan Etik Kurul Onayı ile gerçekleştirildi. Çalışmanın materyalini erişkin (12-14 haftalık) dişi fareler oluşturdu. Dişi fareler ve erkek fareler, 1 adet erkek 4 adet dişi fare birlikte olacak şekilde gruplara ayrılarak aynı kafese alındılar. Bir gece boyunca çiftleşmeye bırakılan dişi fareler günlük olarak vajinal tıkaç oluşumu yönünden kontrol edildiler. Vaginal tıkaç oluşan dişi fareler gebeliklerinin 0. gününde kabul edilip ayrı kafeslere aktarılarak takibe alındılar [9]. Her grupta 6'şar adet olacak şekilde gebe olmayan-kontrol grubu ile gebeliğin birinci, ikinci ve üçüncü haftalarının ortalarına karşılık gelecek şekilde erken, orta ve geç gebelik dönemi (sırasıyla gebeliğin 3., 10. ve 17. günleri) olmak üzere 4 gruba ayrılan fareler, eter ile bayıltıldılar. Ardından servikal dislokasyonla sakrifiye edilen hayvanlardan uterus dokusu örnekleri alındı.

Histolojik İşlemler

Uterus dokusundan alınan parçalar %10'luk tamponlu formal-salin (pH: 7.4) solüsyonunda tespit edildi. Tespit edilen dokular rutin histolojik metotlarla takip edilerek parafinde bloklandı. Bloklardan 6 µm kalınlığında 36'şar µm aralıklarla seri kesitler alındı. Bu kesitlerden ilki Crossman'ın üçlü boyama yöntemi [10] ile boyanırken; diğer 3 kesite PAS-pozitif granülleri nedeniyle uNK olarak değerlendirilen

hücrelerin belirlenebilmesi için PAS reaksiyonu uygulandı [11].

Tüm preparatlar DFC-320 model kamera ataçmanı olan Leica DM-2500 model ışık mikroskobu ile incelendikten sonra gerekli bölgelerin dijital görüntüleri kaydedildi. Üçlü boyama yapılan preparatlarda normal uterus histolojisi değerlendirilirken; PAS reaksiyonunun gerçekleştirildiği kesitlerde ise uNK hücrelerinin sayı ve dağılımı belirlendi.

Hücre Sayımları

PAS-pozitif granül içeren uNK hücrelerinin sayımı amacıyla, aynı hücrenin iki kez sayımından kaçınmak için 36 µm aralıklarla alınan ve PAS reaksiyonu uygulanan 3 seri kesitin 10 farklı bölgesinden toplam 10.000 μm^2'lik alanda, uNK hücre sayımı gerçekleştirildi.

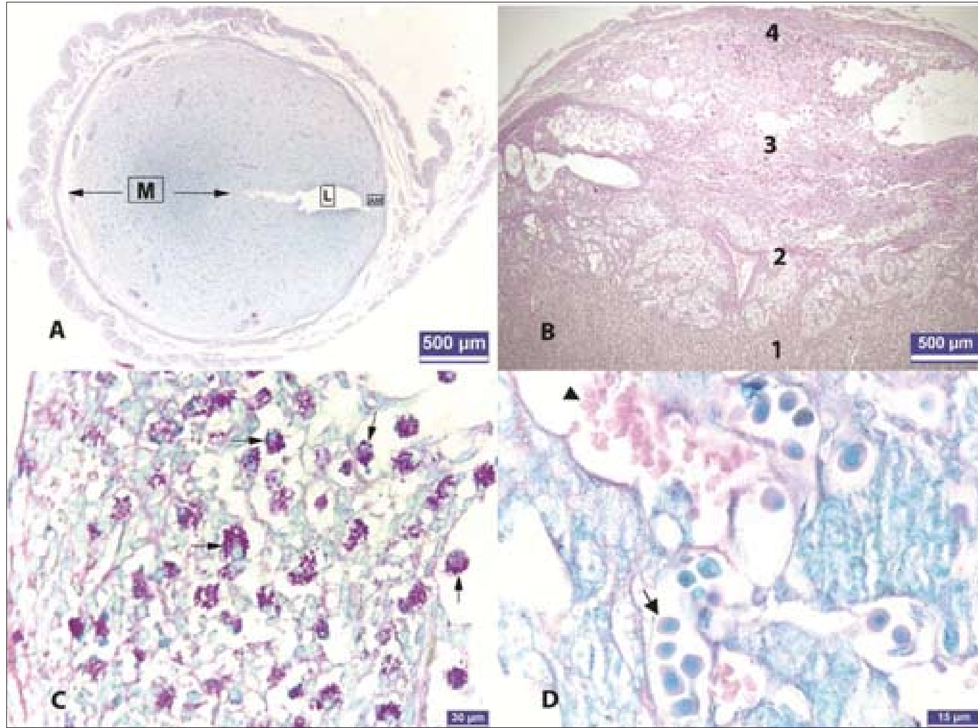
İstatistiksel Analizler

Elde edilen sayısal veriler SPSS 10 [12] programı yardımıyla One-WayAnova testi ve ardından çoklu karşılaştırma testi olan DUNCAN testiyle analiz edilerek grupların ortalama değerleri arasındaki farkların önem dereceleri belirlendi.

BULGULAR

Histolojik Bulgular

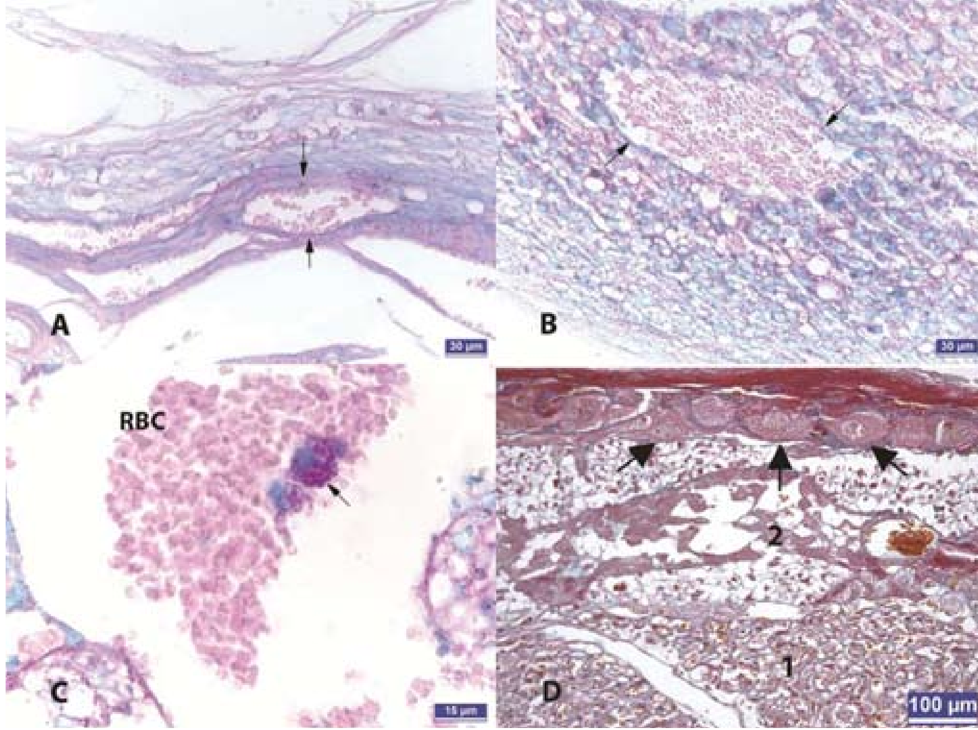
Üçlü boyama ve PAS reaksiyonun gerçekleştirildiği kesitler üzerinde yapılan incelemeler sonucunda, gebe olmayan farelerin uterus dokusunun normal histolojik yapı düzeninde olduğu görüldü. Gebeliğin 3. günündeki (erken dönem) farelerden elde edilen uterus dokusunda ise implantasyonun şekillendiği mesometriyal tarafta desidualizasyonun başladığı dikkati çekti *(Şekil 1. A)*. Gebeliğin ikinci ve üçüncü haftaları içinde desidualizasyonun ilerlemesi ve endometriyumda meydana gelen yapısal değişimlerle birlikte metriyal bölge, desidua bazalis, bağlantı bölgesi ve labirint bölgesi olmak üzere dört farklı bölge ayırt edildi *(Şekil 1. B)*. Özellikle metriyal bölge ve desidua bazalis dokusu içerisinde PAS pozitif granülleri ile kolaylıkla ayırt edilebilen uterus doğal katil (uNK) hücrelerinin sayıca arttığı dikkati çekti *(Şekil 1. C)*. Desidua bazalis bölgesindeki en yüksek uNK hücre sayısına gebeliğin orta dönemindeki farelerde rastlandı *(Tablo 1, Şekil 3)*. Gebeliğin geç döneminde labirint bölgedeki



Şekil 1. A- Gebeliğin erken dönemindeki bir fareye ait uterus dokusu kesiti. Üçlü boyama. M: Mesometriyal bölge, L: Uterus lümeni, AM: Anti-mesometriyal bölge, Büyütme çizgisi: 500 µm; **B-** Gebeliğinin orta dönemindeki bir fareye ait uterus dokusu kesiti. PAS reaksiyonu. 1: Labirint bölgesi, 2: Bağlantı bölgesi, 3: Desidua bazalis, 4: Metriyal bölge. Büyütme çizgisi: 500 µm; **C-** Gebeliğinin geç dönemindeki bir fareye ait uterus dokusu kesiti. PAS reaksiyonu. *Oklar:* PAS-pozitif granül içeren uNK hücrleri. Büyütme çizgisi: 30 µm; **D-** Gebeliğin geç dönemindeki bir farenin uterus dokusu kesiti. PAS reaksiyonu. *Ok:* Fötal alyuvarlar, *Ok başı:* Maternal alyuvarlar. Büyütme çizgisi: 15 µm

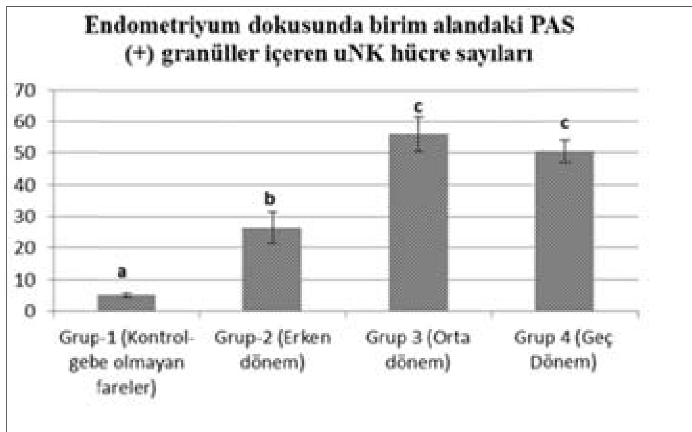
Fig 1. A- A section from uterus of animal at early gestational period. Triple stain. M: Mesometrial region, L: Lumen, AM: Anti-mesometrial region, Bar: 500 µm; **B-** A section from uterus of animal at midgestational period. PAS reaction. 1: Labyrinth zone, 2: Junctional zone, 3: Decidua basalis, 4: Metrial region. Bar: 500 µm; **C-** A section from uterus of animal at late gestational period. PAS reaction. *Arrows:* uNK cells having PAS positive granules, Bar: 30 µm; **D-** A section from uterus of animal at late gestational period. PAS reaction. *Arrow:* Fetal red blood cells, *Arrow head:* Maternal red blood cells. Bar: 15 µm

Tablo 1. *Gebeliğin farklı dönemlerinde endometriyum dokusundaki birim alanda PAS-pozitif granül içeren uNK hücre sayıları (adet/10.000 mm²)* ***Table 1.*** *The number of uNK cells having PAS-positive granules in endometrium of different gestational stages (number/10.000 mm²)*				
Gruplar (n=6)	**Kontrol (Gebe Olmayan Fareler)**	**Grup 2 (Erken Dönem)**	**Grup 3 (Orta Dönem)**	**Grup 4 (Geç Dönem)**
uNK ± SE	5.00±0.577[a]	26.50±5.155[b]	56.00±5.549[c]	50.66±3.602[c]
a-c: *Aynı satırda farklı harfler taşıyan gruplar arasındaki farklılıklar istatistiksel açıdan önemlidir, P<0.001*				



Şekil 2. A- Gebeliğinin orta dönemindeki bir fareye ait uterus dokusu kesiti. PAS reaksiyonu. *Oklar:* Tunika mediya katmanı, *Büyütme çizgisi:* 30 µm; **B-** Gebeliğinin orta dönemindeki bir fareye ait uterus dokusu kesiti. PAS reaksiyonu. *Oklar:* İncelmiş tunika mediya katmanı, *Büyütme çizgisi:* 30 µm; **C-** Gebeliğin geç dönemindeki bir fareye ait uterus dokusundaki kan damarı kesiti. PAS reaksiyonu. RBC: Maternal alyuvarlar, *Ok:* PAS-pozitif granül içeren uNK hücresi. *Büyütme çizgisi:* 15 µm; **D-** Gebeliğinin orta dönemindeki bir fareye ait uterus dokusu kesiti. Üçlü boyama. 1: Labirint bölgesi, 2: Bağlantı bölgesi, *Oklar:* trofoblast dev hücreleri. *Büyütme çizgisi:* 100 µm

Fig 2. A- A section from uterus of animal at midgestational period. PAS reaction. *Arrows:* Tunica media layer, *Bar:* 30 µm; **B-** A section from uterus of animal at midgestational period. PAS reaction. *Arrows:* Thinner tunica media layer, *Bar:* 30 µm; **C-** A section from blood vessel in uterus of animal at late gestational period. PAS reaction. RBC: Maternal red blood cells, *Arrow:* uNK cell having PAS positive granules, *Bar:* 15 µm; **D-** A section from uterus of animal at midgestational period. Triple stain. 1: Labyrinth zone, 2: Junctional zone, *Arrows:* Trophoblast giant cells. *Bar:* 100 µm



Şekil 3. Gebeliğin farklı dönemlerinde endometriyum dokusunda birim alanda PAS-pozitif granül içeren uNK hücre sayıları (adet/10.000 mm²)

Fig 3. The number of uNK cells having PAS-positive granules in endometrium of different gestational stages (number/10.000 mm²)

kan aralıklarında yer alan fötal ve maternal alyuvarların yakın ilişkisi dikkati çekerken *(Şekil 1. D)*; gebeliğin orta döneminden itibaren anti-mesometriyal bölgedeki kan damarlarına göre mesometriyal bölümde yer alan damarların genişlediği ve tunika mediya katmanlarının oldukça incelmiş olduğu tespit edildi *(Şekil 2. A,B)*. Desidua bazalis bölgesindeki maternal kan damarlarının içerisinde de uNK hücrelerinin varlığı göze çarptı *(Şekil 2. C)*. Desidua bazalis ve bağlantı bölgesi arasında yer alan trofoblast dev hücrelerinin gebeliğin orta döneminde arttığı dikkati çekerken *(Şekil 2. D)*; gebe olmayan farelerin endometriyum dokusunda ise son derece az sayıda uNK hücresine rastlandı.

TARTIŞMA ve SONUÇ

Uterus doğal katil hücreleri (uterine natural killer-uNK) desidualizasyon sürecinde endometriyum ve damarların yeniden yapılanmasında rol alan en belirgin hücre grubudur. Özellikle insanlardaki hemo-koryal ve kemiricilerdeki hemo-endotelyal plasenta gibi invaziv plasenta tiplerinde gebeliğin orta döneminde desidua bazalis bölgesinde sayılarının oldukça arttığı bildirilmektedir [2,9]. Perifer kan doğal katil hücrelerinden farklı reseptörler taşıyan ve implantasyonun başlangıcında sekonder lenfoid organlardan uterusa göç eden bu hücrelerin öncüllerinin, özellikle farelerde gebeliğin 6. gününde desidua bazalis bölgesinde spiral arterlerin çevresinde yerleşmeye başladıkları ileri sürülmektedir [13].

Sıçanlarda implantasyonun ilk üç gününde desidual alanda belirgin bir biçimde artan $CD_{56}^{+}CD_{16}^{-}$ uNK hücrelerinin aktivitesi tam olarak açıklığa kavuşturulamamış olsa da diğer NK'lar gibi tümöral hücrelere saldırdıkları, ancak tümör hücrelerini andırır biçimde yayılan trofoblastlara karşı daha toleranslı davrandıkları tespit edilmiştir. Zaman zaman artan IL–2 seviyesine paralel olarak aktivitelerini artıran bu hücrelerin, bir yandan trofoblastların uterus mukozası içerisinde ilerlemesini yönlendirirken, bir yandan da onların aşırı çoğalmalarını kontrol ettikleri düşünülmektedir [14].

uNK hücrelerinin implantasyon süreciyle birlikte farklılaşarak etkinleştikleri ileri sürülmektedir. Koç ve Kanter'in [15] gebe sıçanlarda yaptıkları bir çalışmada alfa naftil aseat esteraz (ANAE) pozitivitesi gösteren ve uterus doğal katil hücreleri (uNK) oldukları iddia edilen hücrelerin implantasyonun ikinci gününden itibaren arttığı ve 6. günde en yüksek seviyeye ulaştığı bildirilmektedir. Kokubu ve ark.'nın [16] yaptıkları bir başka çalışmada ise gebeliğin üçüncü gününde ovariyektomi yapıldıktan sonra düşük dozda progesteron hormonu verilerek implantasyonu geciktirilen farelerde PAS pozitif granüllere sahip uNK hücrelerinin kontrol grubu farelerdeki hücrelere kıyasla daha küçük oldukları ve farklılaşmalarının geciktiği tespit edilmiştir.

uNK hücrelerinin hiç çiftleştirilmemiş, gebe ve post-partum dönemlerde uterus endometriyumundaki sayılarında da farklılıklar söz konusudur. Delgado ve ark.'nın [17] farelerde yaptıkları bir çalışmada hiç çiftleştirilmemiş ve post-partum dönemdeki fare endometriyumlarında uNK hücrelerine rastlanmazken, gebe farelerde dönemsel farklılıklar olsa da desidua dokusunda PAS pozitif granüllere sahip çok sayıda uNK hücresinin varlığından söz edilmektedir. Araştırıcılar [17] özellikle post-partum dönemde uNK hücre sayılarındaki ani düşüşlerin nedeninin, gebeliğin sonlanması ile birlikte uNK hücrelerinde hızlanan apopitozis süreci ve plasental ayrılma olduğunu ileri sürmektedirler.

uNK hücrelerinin implantasyon ve plasentasyon sürecinde trofoblastların desidua dokusuna yayılmasını ve yerleşmesini kontrol ederken, uterus arterlerine ulaşmalarını da koordine ettiği bildirilmektedir. IFN-γ ve Vasküler Endotelyal Gelişme Faktörü (VEGF) gibi bir takım sitokinler salgılayan bu hücrelerin özellikle spiral arterlerin yakınında ve hatta içlerinde yer almaları oldukça dikkat çekicidir. Zira söz konusu sitokinlerin, gelişmekte olan yavrunun ihtiyaç duyduğu oksijen ve besin maddelerinin geçişini maksimum seviyede temin edecek olan damarsal değişimlere olanak sağladığı bildirilmektedir [1,18]. Yapılan deneysel çalışmalar, farelerde bu hücrelerin olmaması ya da yetersizliği durumunda uterus arteriollerinde arteriosklerozis, hipertansiyon ve bu patolojik değişimlere bağlı olarak fötal ölümlerin şekillendiğini ortaya koymuştur [19].

İnsanlarda ve rodentlerde uNK hücrelerinin gelişiminde IL-15'in rolünün önemli olduğu ileri sürülmektedir. Özellikle desidual spiral arterleri çevreleyen perivasküler hücreler ile gebeliğin erken dönemlerinde söz konusu arterlerin endotel hücrelerinde eksprese edilen IL-15 molekülünün implantasyon bölgesindeki yetersizliğinin uNK hücrelerinin bölgesel göçünde ve maturasyonunda aksamalara neden olduğu bildirilmektedir. Ashkar ve ark.'nın [18] farelerde yaptıkları bir çalışmada, IL-15 sentezi yapamayan $IL15^{-/-}$ farelerin implantasyon bölgesinde uNK hücrelerinin bulunmadığı ve buna bağlı olarak da bu farelerde spiral arter modifikasyonunda yetersizlik, desidua gelişiminde gerilik gibi gebeliğin devamını ve sağlıklı bir doğum sürecini olumsuz etkileyecek problemlerin gözlendiği bildirilmiştir.

Wang ve ark.'nın [5] farelerde PAS reaksiyonunun yanı sıra vasküler endotelyal büyüme faktörü (VEGF) ve vasküler hücre adezyon molekülünü (VCAM-1) immünohistokimyasal yöntemlerle gösterdikleri bir çalışmada, uNK hücrelerinin implantasyonun 12-14. günlerinde en yüksek sayıya ulaştıkları, total vasküler alanın da 13. günde en yüksek değerde olduğu bildirilmektedir. Araştırıcılar [5], içerdikleri PAS pozitif granüllerle kolayca ayırt edilebilen uNK hücrelerinin çoğunlukla damarların yakınında bulunduklarını ve söz konusu bu hücrelerin yüksek oranda VEGF eksprese ettiklerini de ileri sürmüşlerdir. Bu bulgularla uyumlu olarak gebe olmayan hayvanların endometriyal damarlarının endotel hücrelerinde tespit edilemeyen ve dolaşımdaki hücrelerin damar dışına çıkabilmeleri için

gerekli olan VCAM-1 immünoreaktivitesinin gebe hayvanlardaki pozitivitesi ise söz konusu çalışmanın [5] önemli bir diğer bulgusu olarak sunulmaktadır.

Başarılı bir gebelik için spiral arter modifikasyonunun son derece önemli olduğu ileri sürülmektedir. Spiral arterlerdeki söz konusu yeniden yapılanma sürecinde meydana gelebilecek aksaklıkların ise erken doğum, preeklampsi ve fötal gelişim geriliği gibi bir çok gebelik komplikasyonuna yol açabileceği tespit edilmiştir. Robson ve ark.'nın [20] insan plasentalarından elde ettikleri uNK hücreleri, damar düz kas hücreleri ve ekstra villöz trofoblast hücrelerini kültüre ederek yaptıkları çalışmada, salgıladıkları bir takım anjiyopoietik faktörler aracılığıyla uNK hücrelerinin özellikle gebeliğin erken dönemindeki spiral arter düz kas hücreleri üzerinde etkili oldukları ortaya konulmuştur. Araştırıcılar [20], bu modifikasyonun aksaması durumunda yukarıda bahsedilen gebelik komplikasyonlarının kaçınılmaz olduğunu ileri sürerek uNK hücrelerinin klinik önemine vurgu yapmışlardır. Wilkens ve ark.[21] da uNK hücrelerinin spiral arterler üzerindeki etkilerinin sadece gebelik sürecinde plasentasyonun şekillenmesi ve desidualizasyon aşamasında değil, aynı zamanda gebe olmayan siklik endometriyum dokusunda homeostasisin sağlanmasında da rol aldığını ortaya koymuşlardır. Araştırıcılar [21], gebe olmayan endometriyum dokusunda yaptıkları bir çalışmada, şiddetli endometriyal kanamaların kontrolünde kullanılan ve bir progesteron reseptör modülatörü olan asoprisnil'in, insan endometriyum dokusundaki stromal hücrelerde yer alan progesteron reseptör seviyesini azalttığını; buna bağlı olarak IL-15 seviyesinin ve dolayısıylada uNK hücre sayılarının azaldığını belirlemişlerdir. Çalışmanın sonunda, uNK hücrelerinin varlığında meydana gelen spiral arter duvarlarındaki incelmelerin asoprisnil kullanılan gruplarda şekillenmediğini tespit eden araştırıcılar [21], söz konusu ilacın etki mekanizmasını da progesteron/IL-15/uNK etkileşimi ile başlayan ve spiral arter modifikasyonu ile tamamlanan yolaktaki aksamalarla açıklamışlardır.

Sitotoksik özelliğe sahip perifer kan NK hücrelerinin çoğu CD16, immün sistemi düzenleyici sitokinler sentezleyen uNK hücrelerinin çoğu ise CD56 pozitiftir [8,13]. uNK hücrelerinin söz konusu moleküllerin immünohistokimyasal ya da flow-sitometrik yöntemlerle demonstrasyonları mümkün olmakla birlikte periyodik asit schiff (PAS) reaksiyonu da zaman zaman tercih edilen basit ve pratik bir metot olarak karşımıza çıkmaktadır [9,13,18,22]. Yadi ve ark.[2] da farelerde periferal NK hücrelerinde bulunmayan ancak uNK hücrelerinde bulunan N-asetil D-galaktozamin içeren glikokonjugatların gösterilmesinde kullanılan *Dolichus biflores* agglutinininin (DBA) demonstrasyonu ile PAS reaksiyonunu seri kesitlerde uygulamışlar ve PAS reaksiyonunun da uNK hücrelerinin tanınmasında alternatif bir yöntem olarak kullanılabileceğini ortaya koymuşlardır.

Bu çalışmada da uNK hücrelerinin 20-30 µm çapında hücreler oldukları ve PAS ile pozitif reaksiyon veren iri granülleri ile ışık mikroskobik düzeyde pratik olarak tanınabildikleri tespit edildi *(Şekil 1. C; Şekil 2. C)*. Gebe olmayan farelerin endometriyum dokusunda son derece az sayıda oldukları tespit edilen uNK hücrelerinin, gebelikle birlikte özellikle desidua bazalis bölgesinde sayılarının ileri derecede arttığı, gebeliğin orta döneminde ise en yüksek seviyelerine ulaştıkları gözlendi *(Tablo 1; Şekil 3)*. Gebeliğin son dönemlerinde labirint bölgedeki kan aralıklarında fötal ve maternal alyuvarların yakın ilişkisi dikkati çekerken yine bu dönemde sayıca azalan uNK hücrelerine desidua bazalis bölgesinde yer alan spiral arterlerin yakınlarında ve hatta lümenlerinde de rastlandı *(Şekil 2. C)*. Özellikle mesometriyal bölgede yer alan maternal kan damarlarının, anti-mesometriyal bölgedeki kan damarlarına nazaran genişlediği ve tunika mediya katmanlarının belirgin bir biçimde incelmiş olduğu gözlendi *(Şekil 2. A,B)*. Desidua bazalis ve bağlantı bölgesi arasında özellikle gebeliğin orta döneminde yan yana sıralanmış oldukça iri trofoblast dev hücreleri dikkati çekti *(Şekil 2. D)*. Bu bulgular, daha önce yapılan ve uNK hücrelerinin desidualizasyon sürecinde sayıları artan ve damarların yeniden yapılanmasında rol alan hücreler olduklarını ileri süren çalışmaları [1,23,24] destekler niteliktedir.

Sonuç olarak başarılı bir gebelik için gerekli olan uNK hücrelerinin, gerek etik gerekse ekonomik açıdan insanlardan ve diğer memeli hayvanlardan elde edilmesi son derece sınırlı olan sağlıklı gebe uterus dokusundaki yerleşimlerinin ve fonksiyonlarının anlaşılması, insanlarda ve hayvanlarda gebelik patolojisi ile birlikte diğer gebelik komplikasyonlarının etiyolojisinin ortaya konması ve tedavi prosedürlerinin geliştirilmesinde önemli yararlar sağlayacaktır. Ayrıca bu hücrelerin, uterusun lokal savunmasında görev alan diğer hücrelerle olan etkileşimlerinin de değerlendirildiği daha kapsamlı çalışmaların planlanmasının, sebebi bilinmeyen infertilite olgularının bir çoğunun sorumlusu olarak kabul edilen maternal tolerans yetmezliği ya da başka bir ifadeyle gebeliğin maternal kabulünde yaşanan problemlerin aydınlatılmasına da katkıda bulunabileceği düşünülmektedir.

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Short Communication

Effect of Orange Peel Essential Oil and Thermotolerance Acquisition on Oxidative Stress Parameters of Liver, Heart and Spleen in Heat Stressed Japanese Quails [1]

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Abstract

The aim of this study was to determine the effects of orange peel essential oil intake by feed and thermotolerance acquisition by early age treatments on antioxidant status of liver, heart and spleen in Japanese quails which subjected to acute heat stress. 168 seven-day-old quail chicks were randomly assigned to six groups as the control; thermal conditioning and fasting for 24 h fed with basal feed or 300 ppm orange peel essential oil added feed. Acute heat stress at marketing age was affected antioxidant systems of selected organs. Orange peel essential oil supplementation decreased Malondialdehyde (MDA) levels while no effect was observed on enzyme activities. Fasting and thermal conditioning at early age negatively affected the antioxidant status of selected organs. Orange peel essential oil may prevent tissues from lipid peroxidation and can be used as a natural antioxidant supplement in heat stress.

Keywords: *Antioxidant, Heat stress, Orange peel essential oil, Thermotolerance*

Portakal Kabuğu Esansiyel Yağı ve Termotolerans Kazandırmanın Sıcaklık Stresine Tabi Tutulan Japon Bıldırcınlarında Karaciğer, Kalp ve Dalakta Oksidatif Stres Parametrelerine Etkisi

Özet

Bu çalışmada, Japon bıldırcınlarında erken yaşta stres uygulamaları sonucu termotolerans kazandırılması ve portakal kabuğu esansiyel yağının yeme ilavesinin akut sıcaklık stresinde karaciğer, kalp ve dalak dokularının antioksidan durumlarına etkileri incelenmiştir. 168 adet 7 günlük yaşta Japon bıldırcınları, 24 saatlik açlık veya sıcaklık stresi uygulaması ve bunların yeme portakal kabuğu esansiyel yağı (300 ppm) katılan alt grupları olacak şekilde altı gruba ayrılmıştır. Kesim yaşına gelen bıldırcınlara uygulanan akut sıcaklık stresi organlarda antioksidan sistemleri etkilemiştir. Portakal kabuğu esansiyel yağı ilavesi Malondialdehit (MDA) düzeylerinde düşmeye neden olurken enzim aktiviteleri üzerine etki etmemiştir. Erken yaşta sıcaklık ve/veya açlık uygulamaları ile seçilen organlarda antioksidan sistemler olumsuz yönde etkilenmiştir. Portakal kabuğu esansiyel yağı dokuları lipid peroksidasyonundan koruyabilir ve sıcaklık stresinde doğal antioksidan yem katkısı olarak kullanılabileceği kanısına varılmıştır.

Anahtar sözcükler: *Antioksidan, Portakal kabuğu esansiyel yağı, Sıcak stresi, Termotolerans*

INTRODUCTION

Animals under heat stress simultaneously exhibit oxidative stress, which causes further cellular damage. Dietary modifications are among the most preferred and practical ways to alleviate the effect of high environmental temperature on poultry performance. Studies have shown that under stressful conditions, the requirements for antioxidants are thought to be increased to protect tissues from lipid peroxidation and antioxidant nutrient supplementation could be used to attenuate the negative effects of environmental stress [1]. Orange oil is an essential oil produced by cells within the rind of an orange fruit. Recently, the orange peel essential oil has been used as

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antioxidant, antimicrobial and growth promoter agent [2].

Thermal conditioning is one of the management tools that partially enable organisms to cope with extreme environmental conditions and has been experienced in many poultry species for economic losses due to heat stress by thermal conditioning or short-term fasting or feed restriction at an early age [3]. The objective of the present study was to test the effects of orange peel essential oil and acquisition of thermotolerance by early age thermal conditioning or fasting on the antioxidant function of liver, heart and spleen in quail chicks when they are subjected to a high ambient temperature in the finishing phase.

MATERIAL and METHODS

All procedures were approved by Firat University Institutional Animal Care and Use Committee (FUHADEK, verdict no: 04.04.2013/55). One hundred and sixty eight 7-d-old Japanese quail chicks were weighed individually and randomly assigned to six groups of 4 replicate pens (30×80 cm), containing 7 chicks using a completely randomized design (CRD). Then early age stress factors of fasting and thermal conditioning were applied to four of six groups for 24 h. The two groups of four were subjected to fasting and the other two groups of four were subjected to thermal conditioning at 36±1°C with 70-80% relative humidity for 24 h. Thermal conditioning was applied by electric heater with water vaporization to obtain heat stress. Then the experiment was continued as: 1) Unstressed and no added (Negative Control) Group (NC), 2) Fasted Group (F), 3) Thermal Conditioned Group (TC), 4) Unstressed but Orange Essential Oil added (Positive Control) Group (PC + OEO), 5) Fasted + Orange Essential Oil Group (F + OEO) and 6) Thermal Conditioned + Orange Essential Oil Group (TC + OEO).

The diet was formulated to meet the nutrient requirements of the Japanese quails according to the NRC [4] recommendations. The ingredients and compositions of the basal diet are presented in *Table 1*. Orange peel essential oil was mixed in a carrier (Zeolite), and added to the basal diet at a level of 10 kg/ton to obtain 300 ppm of essential oil concentration. The concentration of the volatile component of orange peel essential oil was shown at *Table 2*.

Chemical composition of feed ingredients (dry matter, crude protein, ash and ether extract) were analyzed according to the AOAC [5] procedures and crude fiber was determined by the methods of Crampton and Maynard [6].

At the end of the study, all groups were subjected to heat stress at 33±1°C for 6 h. Then 10 quails from each experimental group whose body weight near the group average were slaughtered by cutting the jugular vein. Then liver, heart and spleen weights were measured and kept at -20°C until laboratory analyses.

Table 1. *Ingredients and chemical composition of standard diet (g/kg)*
Tablo 1. *Bazal diyetin kompozisyonu ve bileşimi (g/kg)*

Feed Ingredients	Starting and Growing 0-42 Days	Calculated Analysis	
Maize	564.3	Crude protein	236.0
Soybean meal	315.0	ME, MJ/kg	12.74
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*	3.5		
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 groups (10 g zeolite/kg feed); 300 ppm orange peel essential oil added groups (3 g orange oil + 7 g zeolite/kg feed)*

Table 2. *The concentration of the volatile components in orange peel essential oil (%)*
Tablo 2. *Portakal kabuğu esansiyel yağındaki uçucu bileşenlerin konsantrasyonu (%)*

Analysis	Result*
Limonene	92.31
Beta Myrcene	3.25
Alpha Pinen	1.41
Linalool	0.89
Sabinen	0.61
Delta 3 Caren	0.22
Octanal	0.21
Undefined	1.10

** Obtained by GC-MS analysis*

Malondialdehyde (MDA) concentration of the tissue homogenates expressed as the thiobarbituric acid reactive substances (TBARS), were assayed spectrophotometrically according to the method of Placer *et al.*[7]. Catalase (CAT) activity was estimated by measuring the breakdown of H_2O_2 at 240 nm according to the method of Aebi [8]. Glutathione Peroxidase (GSH-Px) activity was determined using the method of Lawrance and Burk [9], which records at 340 nm, the disappearance of NADPH. Glutathione (GSH) concentrations of the tissue homogenates were measured by an assay using the dithionitrobenzoic acid recycling method described by Sedlak and Lindsay [10]. Superoxide

Dismutase (SOD) activity were evaluated in accordance with the method described by Sun *et al.*[11]. Tissue protein contents were determined by the method of Lowry *et al.*[12].

Data were subjected to two-way Anova by using GLM (General Linear Model) procedure. Significant differences were further subjected to Duncan's multiple range test [13].

RESULTS

Either early age treatments or essential oil supplementation were not influenced the liver, heart and spleen weights of quails *(Table 3)*.

The effect of orange peel essential oil supplementation and thermotolerance acquisition on antioxidant status of liver, heart and spleen are presented in *Table 4*. Essential oil supplementation was significantly decreased Malondialdehyde (MDA) levels of liver ($P<0.001$), heart ($P<0.05$) and spleen ($P<0.05$). It is strange that the unexpected values were occurred by thermotolerance acquisition. MDA ($P<0.001$), CAT ($P<0.01$), GSH ($P<0.01$) levels of liver and MDA ($P<0.05$), CAT ($P<0.001$), GSH-Px ($P<0.05$), GSH ($P<0.01$) levels of heart and MDA ($P<0.001$), CAT ($P<0.05$) levels of spleen were negatively affected by fasting and thermal conditioning at early age.

DISCUSSION

Organ weights were similar among groups in the current study. Similarly, Simsek *et al.*[14] and Ciftci *et al.*[15] have found no effect on relative weights of liver, heart

Table 3. *Effect of orange peel essential oil supplementation and thermotolerance acquisition on liver, heart and spleen weights,(g)*
Tablo 3. *Termotolerans kazandırma ve portakal kabuğu esansiyel yağı ilavesinin karaciğer, kalp ve dalak ağırlıklarına etkisi, (g)*

Organ	Basal Diet, No Added			Orange Peel Essential Oil, 300 ppm			P	
	NC	F	TC	PC	F	TC	O	T
Liver	4.88±0.68	4.01±0.44	3.99±0.35	4.34±0.33	4.05±0.28	4.32±0.51	NS	NS
Heart	1.72±0.12	1.62±0.08	1.67±0.06	1.76±0.08	1.63±0.06	1.79±0.07	NS	NS
Spleen	0.11±0.01	0.20±0.03	0.14±0.01	0.21±0.03	0.27±0.00	0.17±0.02	NS	NS

NS: *Not significant,* ***NC:*** *Negative control,* ***PC:*** *Positive Control,* ***F:*** *Fasted,* ***TC:*** *Thermal Conditioned,* ***O:*** *Oil,* ***T:*** *Treatment*

Table 4. *Effect of orange peel essential oil supplementation and thermotolerance acquisition on oxidative stress parameters of liver, heart and spleen*
Tablo 4. *Termotolerans kazandırma ve portakal kabuğu esansiyel yağı ilavesinin karaciğer, kalp ve dalak oksidatif stres parametrelerine etkisi*

Parameter	Basal Diet, No Added			Orange Peel Essential Oil, 300 ppm			P	
	NC	F	TC	PC	F	TC	O	T
Liver								
MDA	2.48±0.12^{c}	3.56±0.39^{b}	5.37±0.41^{a}	1.98±0.25^{B}	2.60±0.18^{AB}	3.14±0.20^{A}	***	***
CAT	1.52±0.16^{a}	0.75±0.15^{b}	0.71±0.12^{b}	1.51±0.09	1.15±0.06	0.98±0.21	NS	**
GSH-Px	0.05±0.00	0.08±0.04	0.03±0.00	0.05±0.00	0.04±0.00	0.03±0.00	NS	NS
GSH	0.07±0.01	0.06±0.00	0.04±0.00	0.10±0.01^{A}	0.07±0.00^{AB}	0.05±0.00^{B}	NS	**
SOD	33.30±3.63	30.03±2.05	30.85±5.65	28.70±1.72	29.87±0.83	29.52±2.29	NS	NS
Heart								
MDA	4.01±0.25^{b}	7.02±2.06^{a}	6.12±1.54^{ab}	2.26±0.85^{B}	6.67±0.86^{A}	5.09±0.82^{AB}	*	*
CAT	0.12±0.01^{a}	0.03±0.00^{b}	0.04±0.01^{b}	0.07±0.00^{A}	0.04±0.00^{B}	0.04±0.00^{B}	NS	***
GSH-Px	0.23±0.01^{a}	0.19±0.00^{b}	0.12±0.02^{b}	0.20±0.01	0.22±0.01	0.20±0.01	NS	*
GSH	0.20±0.01^{a}	0.19±0.01^{ab}	0.12±0.01^{b}	0.15±0.01^{B}	0.23±0.01^{A}	0.15±0.01^{B}	NS	**
SOD	111.39±9.22	95.54±4.14	73.70±22.21	104.56±6.73	100.03±10.31	102.60±8.06	NS	NS
Spleen								
MDA	14.33±1.00^{b}	18.10±1.02^{ab}	28.85±4.30^{a}	7.30±1.16^{B}	16.50±2.46^{A}	21.30±1.78^{A}	*	***
CAT	0.07±0.00^{a}	0.05±0.00^{ab}	0.03±0.00^{b}	0.05±0.00	0.04±0.00	0.04±0.00	NS	*
GSH-Px	0.05±0.00	0.05±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.06±0.00	NS	NS
GSH	0.05±0.00	0.04±0.01	0.04±0.00	0.06±0.00	0.04±0.00	0.06±0.01	NS	NS
SOD	32.42±8.04	33.15±2.06	21.54±5.38	21.96±3.96	20.51±2.59	33.88±2.51	NS	NS

NS: *Not significant, * P<0.05, ** P<0.01, *** P<0.001;* ***NC:*** *Negative control,* ***PC:*** *Positive Control,* ***F:*** *Fasted,* ***TC:*** *Thermal Conditioned,* ***O:*** *Oil,* ***T:*** *Treatment;* ***MDA:*** *Malondialdehyde, nmol/mg protein;* ***CAT:*** *Catalase, k/g protein;* ***GSH-Px:*** *Glutathione Peroxidase, U/g protein;* ***GSH:*** *Glutathione, nmol/mg protein;* ***SOD:*** *Superoxide Dismutase, U/g protein*

and spleen of broilers with dietary thyme and cinnamon essential oil supplementation, but it is reported that liver weights were increased by anise essential oil supplementation [16].

Increasing MDA levels of liver, heart and spleen in the current study might be due to the heat stress condition. Similarly, Yang *et al*.[17] mentioned about heat stress induced a significant production of reactive oxygen species (ROS), function of the mitochondrial respiratory chain, antioxidative enzymes such as SOD, CAT, GSH-Px activity and formation of MDA. Supplementation of orange peel essential oil to diet reduced MDA production in liver, heart and kidney, under heat stress condition. Likewise, Simsek *et al*.[18] reported that cinnamon oil supplementation decreased the MDA levels in liver, kidney and heart tissues of Japanese quails under heat stress condition. Faix *et al*.[19] observed that 0.1% level *C. zeylanicum* essential oil supplementation has significantly decreased the concentration of MDA in plasma and duodenal mucosa; however it had no significant effect on the concentration of MDA in the liver and kidney tissues.

In this study orange peel essential oil supplementation had no effect on antioxidant enzyme activities of liver, heart and spleen. But, these results are in contrast with the findings of Akbarian *et al*.[20] whom recorded that the dietary supplementation of the bird's diet with orange peel essential oil had significant differences in glutathione peroxidase and superoxide dismutase activity, when compared with the control group. It is possible that the antioxidant properties of essential oils are being utilized by the cells, thus sparing the intracellular antioxidant system. Barja de Quiroga *et al*.[21] suggested that cellular antioxidants are under homeostatic control and that dietary antioxidant supplementation depresses endogenous antioxidant synthesis so as to nullify the expected beneficial effect of the supplement.

In conclusion, orange peel essential oil supplementation has protective effects to tissues in heat stress, but early age thermal conditioning or fasting had no positive effects. However, future research experiments based on different doses of essential oils or in combination (to explore their possible synergic effects) and thermotolerance acquisition could be designed to elucidate more precisely their effects on the oxidative status of avian species.

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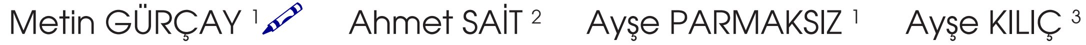
Short Communication

Türkiye'de Lumpy Skin Disease Virus Enfeksiyonunun Klinik Bulgular ve PCR Yöntemi İle Saptanması [1]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[1] *Bu çalışma XI. Veteriner Hekimleri Mikrobiyoloji Kongresi'nde (21-24 Ekim 2014, Antalya, Türkiye) sunulmuştur*

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Özet

Lumpy Skin Disease (LSD), sığır, bufalo ve bazı yabani türlerin derileri üzerinde nodüller ve ödem ile karakterize viral bir enfeksiyonudur. Bu çalışmada, LSD virus enfeksiyonunun, Türkiye'deki varlığının, enfekte sığırlarda gözlenen klinik bulgulara göre tanısı ve PCR yöntemi ile doğrulanması amaçlanmıştır. Bu amaçla, Eylül 2013-Haziran 2014 tarihleri arasında Türkiye'de yerleşik 8 sığır çiftliğinde klinik olarak LSD enfeksiyonu belirtileri gösteren 113 sığır incelendi. Daha sonra, bu sığırlardan 12 deri nodülü örneği alındı ve viroloji laboratuvarına getirildi. Bu örnekler OIE tarafından tanımlanan yönteme göre PCR ile test edildi. Enfeksiyonun klinik bulgularının PCR sonuçları ile uyumlu olduğu ortaya konuldu.

Anahtar sözcükler: *Lumpy Skin Disease, PCR, Epidemiyoloji*

The Detection of Lumpy Skin Disease Virus Infection by Clinical Findings and PCR Method in Turkey

Abstract

Lumpy Skin Disease (LSD) is a viral disease of cattle, buffalo and some wild species characterized by oedema and nodules on the skin. The aim of this study was to report the presence and the clinical signs of LSD in infected cattle in Turkey, and to confirm by PCR method. For this purpose, 113 cattle showing clinical signs of LSD were examined from 8 settled farms in Turkey between September 2013 to June 2014. Then, 12 skin nodule samples from these cattle were obtained, and brought to the virology laboratory. The samples were tested by PCR according to the method described by OIE. The clinical diagnosis was well correlated with PCR results.

Keywords: *Lumpy Skin Disease, PCR, Epidemiology*

GİRİŞ

Lumpy skin disease (LSD), *Poxviridae* ailesinin *Capripoxvirus* cinsinde bulunan lumpy skin disease virusunun sığırlarda neden olduğu bir hastalıktır [1-5]. Virusun taşınması özellikle artropod vektörler aracılığıyla gerçekleşir. Sığırdan sığıra bulaşma da olabilmektedir. Artropod vektörlerin varlığını ve çoğalmalarını etkileyen faktörlere (mevsim, iklim vb.) bağlı olarak önemli epizootik salgınlar ortaya çıkabilmektedir. Enfeksiyon, enzootik olmayan bölgelere artropodların vektörlüğünde taşınabilmektedir [2,6-8].

LSD, ilk defa 1929 yılında Afrika'da, 1989 yılında İsrail' de, daha sonraki yıllarda da Bahreyn, Kuveyt, Umman ve Yemen'de rapor edilmiştir [5]. Enfeksiyon, özellikle 2012 ve 2013 yıllarında Lübnan ve Ürdün'de bildirilmiştir [5]. LSD hastalığı, Türkiye'de ilk olarak 2013 yılında Kahramanmaraş'ın Göksun ilçesinde ortaya çıkmış, daha sonra Batman, Hakkari, Malatya, Hatay, Adıyaman, Osmaniye ve Adana gibi birçok ilde hastalığın gözlendiği ve kontrol/eradikasyon çalışmalarının yapıldığı bildirilmektedir [5,9].

Enfeksiyonun klinik belirtileri, subklinik enfeksiyondan ölüme kadar giden değişiklik gösterebilmektedir. Hastalığın şiddetinde, *Capripoxvirus* suşu ve konakçı sığır ırkının yanısıra vektör prevalansı, konakçının immunolojik yapısı ve virus izolatının virulensi gibi faktörler etkilidir [10]. İnkübasyon periyodu, doğal salgınlarda 1-4 hafta arasında değişmektedir. Çoğu vakalarda ilk klinik belirti gözyaşı akıntısı olmakla birlikte, ateş (40-41°C'ye kadar) ve pre-

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skapular lenf yumrularında büyüme de görülmektedir. Bazı vakalarda ateş görülmeyebilir. Ateş olan durumlarda, kısa bir süre sonra 1-5 cm çapında değişen sayılarda deri nodülleri ortaya çıkmaktadır. Ağır seyreden vakalarda, göz ve ağız-burun boşluğu mukozalarında ülseratif lezyonlar oluşmaktadır. Bu lezyonlara bağlı olarak burun boşluğunda kızarıklık, burun ve gözyaşı akıntısı görülür [4,10,11]. Bu sekresyonların tamamı virus içermektedir. Mermede ödematöz bir görüntü vardır ve hasta hayvanlar hareket etmek için isteksizdirler. Gebe hayvanlarda abort görülebilir. Genç boğalar geçici veya sürekli infertil kalabilirler. Virus semen ile uzun süre saçılabilir [8].

Bu çalışma, Lumpy skin disease (LSD) hastalığının, enfekte hayvanlarda gözlenen klinik bulgular yönünden değerlendirilmesi ve laboratuvar tanı bağlamında PCR ile teşhis edilmesi, ve ayrıca hasta hayvanların ırk ve fizyolojik durumlarına göre enfeksiyonun morbidite ve mortalite düzeyinin araştırılması amacıyla yapılmıştır.

MATERYAL ve METOT

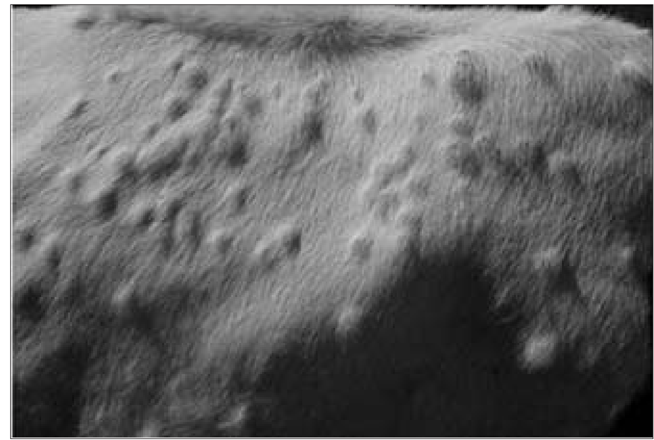
Çalışmada Kullanılan Sürüler ve Test Materyalleri

Çalışmada, Eylül 2013 - Haziran 2014 tarihleri arasında Hakkari Merkez, Batman Merkez, Şırnak Merkez, Malatya' nın Battalgazi, Akçadağ ve Darende ilçelerinde bulunan küçük aile işletmeleri ve Batman Gercüş' te bulunan bir entansif işletmeden sağlanan numuneler kullanıldı. Bu işletmelerde bulunan farklı ırk (Montafon, Simental, Holştayn ve melezleri), yaş ve fizyolojik durum özelliklerine sahip toplam 113 sığıra; klinik olarak iki fazlı ateş (40°C-41.5°C), depresyon, iştahsızlık, salivasyon, göz ve burunda değişiklikler, preskapular lenf yumrularında büyüme ve tüm vücudu kaplayan çoklu deri nodülleri *(Şekil 1)* bulgularına göre (n=113) LSD ön tanısı konuldu. Hastalığın laboratuvar teyidi için klinik belirti gözlenen 12 hayvandan deri nodülü biyopsi materyalleri aseptik şartlarda alındı *(Tablo 1)*.

Viral DNA ekstraksiyonu ve PCR

Test materyallerinden viral DNA izolasyonu ticari kit (QIAamp DNA Mini Kit, USA) kullanılarak ve üretici firmanın önerdiği prosedürüne göre yapıldı. Viral DNA izolasyonunda kullanılan primerlerin dizilimleri *(Tablo 2)* ve uygulanan prosedür OIE manuel esas alınarak uygulandı [8]. Test sonunda 192bp ürün büyüklüğünde bantlar elde edildi *(Şekil 2)*.

DNA amplifikasyonu 50 µl final hacimde olacak



Şekil 1. Lumpy skin disease virus ile enfekte bir sığırda karekteristik deri lezyonları

Fig 1. The characteristic skin lesions in a cattle infected with Lumpy skin disease virus

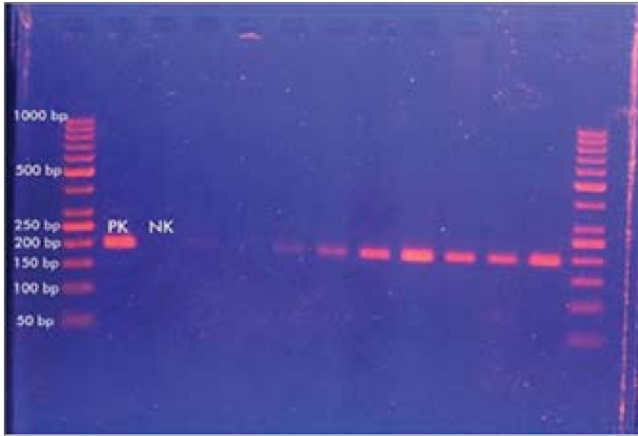
Tablo 1. *LSD belirtisi gösteren sığırların bulunduğu işletmeler, işletmelerin yeri, işletmedeki hayvan sayıları, hastalanan hayvan sayısı, ırk ve fizyolojik durumları, morbidite, mortalite, ve alınan deri biyopsi materyali sayıları*

Table 1. *The place where the enterprise of cattle showing signs of LSD, number of animals at the enterprise, number of diseased animals, race and physiological status, morbidity and mortality rate and the number of biopsies of skin taken*

İşletmenin Bulunduğu Yer	İşletmede Bulunan Hayvan Sayısı	Hastalanan Sığırın Irkı ve Fizyolojik Durumu	Hastalanan Hayvan Sayısı	Morbidite	Mortalite	Deri Nodülü Biyopsisi Materyali Sayısı
Hakkari Merkez	4	Montofon Dana	1	% 25	-	1
Malatya Battalgazi	2	Montofon Sağmal İnek	1	%50	-	1
Malatya Akçadağ	6	Simental Melezi Gebe İnek	2	%50	-	2
Batman Merkez	3	Holştain Sağmal İnek	1	%33	-	1
Batman Gercüş	203	Holştain Sağmal İnek	105	%51	28 (%26.66)	4
Şırnak Merkez	3	Holştain Gebe İnek	1	%33	-	1
Malatya Darende	26	Holştain Gebe İnek	1	%03.8	-	1
Malatya Darende	3	Holştain Melezi Sağmal İnek	1	%33	-	1

Tablo 2. PCR'da kullanılan primerlerin özellikleri
Table 2. The characteristics of the primers used in PCR

Primer (5'- 3')	Proteini Kodlayan Gen	Ürün Büyüklüğü	Referans
5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3' 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'	LSDV Viral bağlama proteinini kodlayan gen	192 (bp)	[8]



Şekil 2. LSDV'nin agaroz jel elektroforezisi, GeneRuler 50 bp DNA Ladder. 192 bp PCR amplifikasyon ürünü için pozitif, negatif kontrol ve numuneler

Fig 2. Agarose gel electrophoresis of LSDV. GeneRuler 50 bp DNA Ladder. 192 bp amplicon PCR product, positive and negative controls and specimens

şekilde Thermo-Scientific PCR Master mix (2X) kit (Cat No: KO171,USA) kullanılarak yapıldı. Kısaca, 25 µl PCR Master mix (2X), 1 µl (0.1 mM) forward primer, 1 µl (0.1 mM) reverse primer, 1 µl DNA template (~10 ng), 50 µl'ye nuclease free su ile tamamlanarak hazırlanan karışım kullanıldı. Karışım, başlangıçta 95°C'de 2 dak., daha sonra 95°C'de 45 sn, 50°C'de 50 sn ve 72°C'de 1 dak. olacak şekilde programlanarak thermal cycler cihazına konuldu ve 34 siklus olacak şekilde ayarlandı. Son siklus ise, 72°C'de 2 dak. olarak uygulandı. Elektroforez işlemi sonucunda LSD virus DNA'sının 192 bp moleküler uzunluğa sahip bantları UV transilluminatörde gözlemlendi [8]. Pozitif kontrol virusu olarak, Pendik Veteriner Kontrol Enstitüsü Çiçek Referans Laboratuvarı'nda izole edilen LSD saha suşu kullanıldı.

BULGULAR

LSD hastalığının varlığı Hakkari, Şırnak, Batman il merkezleri ve Batman/Gercüş, Malatya/Battalgazi, Akçadağ, Darende ilçesinde bulunan 8 sığır işletmesinde klinik bulgular ve laboratuvar teşhisi ile ortaya konuldu *(Şekil 1, Şekil 2)*. İşletmelerde bulunan hasta hayvanlardan alınan 12 adet deri nodülü biyopsi materyallerinin tamamında klinik teşhisi doğrulayacak şekilde 192 bp uzunluğunda pozitif bantlar elde edildi *(Şekil 2)*. LSD hastalıklı bir Montafon dana ve bir sağmal inek, iki Simental melezi gebe inek, bir Holştayn sağmal inek, iki Holştayn gebe inek ve bir Holştayn melezi gebe inek olmak üzere toplam 8 sığırın iyileştiği, ancak Holştayn sağmal ineklerin entansif yetiştiriciliğinin yapıldığı bir işletmede 105 hasta inekten 28 (%26)'inin öldüğü tespit edildi. Hastalığın işletmelerdeki morbidite oranı %3.8-51.0 ve mortalite oranı ise %0-26 olarak belirlendi *(Tablo 1)*.

TARTIŞMA ve SONUÇ

Bu çalışma ile LSD hastalığının varlığı Hakkari, Batman, Şırnak ve Malatya illerinde bulunan küçük aile işletmelerinde ve entansif yetiştiricilik yapılan Batman ili Gercüş ilçesindeki bir işletmede enfekte hayvanlarda gözlenen klinik belirtiler ve PCR yöntemi ile moleküler olarak ortaya konulmuştur.

Hastalığın çıktığı yerlerdeki endemilerde, sığırlarda orta ve şiddetli olmak üzere iki farklı formda klinik bulgular gözlenmiştir. Etçi ve sütçü özelliği olan Montofon ve Simental ırkları ile sütçü Holştayn melezleri orta şiddette klinik belirti gösterdiği halde, 203 Holştayn ırkı sütçü sağmal ineğin bulunduğu bir işletmede 105 ineğin şiddetli klinik belirtiler gösterdiği tespit edilmiştir. Bu klinik bulgular arasında özellikle deri lezyonları belirgin olarak görülmüş ve bir veya birden fazla ayakta ödematöz şişkinlik ve topallık saptanmıştır. Hastalık, hayvanlarda süt veriminde azalmayla birlikte, 28 (%26) ineğin de ölümüne yol açarak önemli ekonomik kayıplara neden olmuştur. Elde edilen verilere göre, hastalığın morbiditesi %3.8-51.0 arasında, bulunurken; sığırların ırkı ve fizyolojik duruma bağlı olarak mortalite oranı ise %0-26 olarak belirlenmiştir. Morbidite oranlarının düşük olması hastalığın yayılmasında sığırdan sığıra bulaşmanın çok etkili olmadığını, buna karşılık insekt vektörlerin bulaşmada daha fazla etkili olduğunu göstermektedir [7,8,10]. Hakkari, Malatya, Şırnak ve Batman illeri arasında en düşük rakıma sahip olanı Batman ilidir. Batman ili diğer illere göre daha sıcak ve nemli bir iklime sahiptir. Nemli ve sıcak iklim sinek populasyonunda ve dolayısı ile alınan virus miktarı üzerinde etkili olmaktadır [2]. Bu çalışmada mortalite oranlarının %0 ile %26 arasında değişken olmasını etkileyen nedenler arasında bölgenin coğrafyası, iklimi, işletmenin yönetim şartları, hayvanın ırkı ve bağışıklık durumu ile virus suşu sayılabilir. Hastalığın şiddetinin ise alınan virusun dozu, alınma yolu ve konakçının ırkına göre değiştiği bildirilmektedir [6,7]. Kuzey Omman'ın Al-Batinah bölgesinde Holştayn ırkı süt sığırcılığının yapıldığı bir işletmede hastalığın görülmesinden sonra, hayvanlarda süt veriminin %40-65 oranında düştüğü ve hastalığın %12 oranında mortaliteye neden olduğu bildirilmiştir [11].

Hastalığın enzootik olarak görüldüğü bölgelerde LSD

hastalığının teşhisi, çoğunlukla karakteristik klinik belirtilere göre yapılmaktadır. Ancak, etkili kontrol ve eradikasyon için geçici klinik teşhisin doğru ve hızlı laboratuvar teknikleri ile doğrulanması gerekmektedir. Laboratuvar teşhisinde, hücre kültüründen veya embriyolu tavuk yumurtasından izole edilen virusun elekton mikroskopi veya floresan antikor testi ile identifiye edilmesi gerekmektedir. Serolojik testlerle spesifik LSD antikorlarının ortaya konulması ile de teşhis yapılabilmektedir. Çabuk ve doğru laboratuvar teşhisi için, son zamanlarda çeşitli PCR metotları geliştirilmiştir [1,3,8,10,12]. Spesifik immunulojik testlerle karşılaştırıldığında, PCR'ın daha duyarlı olduğu belirtilmiştir [3]. Bu çalışmada, klinik olarak hastalık belirtisi gösteren hayvanlardan alınan, deri nodülü biyopsi materyallerine PCR uygulandı ve örneklerin tamamı pozitif olarak bulundu (%100). Çalışmamızda elde edilen PCR test sonuçları, sahada gözlenen klinik bulguları tamamen doğrulamıştır. El Kholy ve ark.[3], klinik belirti gösteren hayvanların deri biyopsi materyallerinden yaptıkları PCR testinde, klinik teşhisi doğrulayacak şekilde 80 numunenin tamamında pozitif sonuçlar elde etmişlerdir. Sharawi ve Abd [1], klinik belirti gösteren hayvanlardan alınan 10 adet deri biyopsi materyalinin tamamında PCR testi ile LSD teşhisi yapmışlardır. Bu çalışmamızda elde edilen sonuçlar ile El Kholy ve ark.[3] ve Sharawi ve Abd'ın [1] sonuçları arasında uyumluluk görülmüştür. LSD'nin laboratuvar teşhisinde materyal olarak deri nodülü biyopsi materyali, kan, semen ve süt kullanılmaktadır. Bunlardan deri biyopsi materyalinin, hastalığın başlangıcından hastalığın üçüncü ayına kadar olan devrede fazla miktarda virus içermesi nedeniyle PCR testi için en iyi teşhis materyali olduğu bildirilmektedir [1,8,11].

Endemik ülkelerde, LSD salgınlarının tek etkili kontrol yolu aşılamadır. Koyun çiçek, keçi çiçek ve LSD viruslarının antijenik homolojileri ve çapraz koruma oluşturma nedenlerinden dolayı bu virus suşlarından herhangi birisi, sığırları LSD'ye karşı korumak için kullanılabilmektedir [13,14].

Sonuç olarak, bu çalışma ile Doğu ve Güneydoğu Anadolu bölgelerinde yer alan Hakkari, Malatya, Şırnak ve Batman illerinde sığırlarda LSD hastalığının klinik teşhisi yapılmış ve uygulanan PCR yöntemi ile virus varlığının tespiti yapılarak klinik teşhis doğrulanmıştır. Ayrıca, bu çalışmada LSD hastalığının, hayvanların ırk, fizyolojik durum ve vücut direncine bağlı olarak Montofon dana ve inekler, Simental inekler ve Holştayn melezi gebe ineklerde ölüme neden olmadığı halde, sağmal Holştayn ırkı bir süt işletmesinde ölümlere neden olduğu gözlenmiştir. Hastalık, danaların besi performansında gerileme, sağmal ineklerin süt veriminde azalma ve ölümlere bağlı olarak önemli ekonomik kayıplara neden olmuştur.

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Short Communication

The Effect of Egg Shell Thickness on Some Hatching Traits of Broiler Breeders

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Abstract

This study examined the effect of egg shell thickness on hatching traits of broiler breeders. A total of 253 eggs from broiler breeders were classified into three groups according to shell thickness (thin, medium, thick). Eggs were weighed, and shell thicknesses were measured ultrasonically. Hatchability, chick weight and chick length were assessed at the end of the incubation period. The effect of egg shell thickness on hatchability was found to be insignificant for all groups. Moreover, shell thickness had no significant effect on chick weight or length.

Keywords: *Egg shell thickness, Hatchability, Incubation, Ultrasound*

Broiler Damızlıklarda Yumurta Kabuk Kalınlığının Bazı Kuluçka Özellikleri Üzerine Etkisi

Özet

Bu çalışma broiler damızlıklarda kabuk kalınlığının kuluçka sonuçları üzerine etkisini araştırmıştır. Broiler damızlık sürüden elde edilen toplam 253 yumurta kabuk kalınlıklarına göre gruplandırılmıştır (kalın, orta ve ince). Yumurtalar tartılmış ve kabuk kalınlıkları ultrasonik olarak ölçülmüştür. Çıkış gücü, civciv ağırlığı ve civciv uzunluğu kuluçka sonunda belirlenmiştir. Tüm gruplarda kabuk kalınlığının çıkış gücüne etkisi önemsiz bulunmuştur. Ayrıca, kabuk kalınlığının civciv ağırlığı ve civciv uzunluğu üzerine önemli bir etkisi de olmamıştır.

Anahtar sözcükler: *Yumurta kabuk kalınlığı, Çıkış gücü, Kuluçka, Ultrasonik*

INTRODUCTION

Incubation is one of the most important factors affecting profitability in poultry production. Egg physical characteristics play an important role in the processes of embryo development and successful hatching. Any abnormalities in these characteristics can lead to a collapse in embryo development [1]. While the shell must be thick enough to protect the embryo from external factors during incubation, it must also be thin and fragile enough not to act as a strong barrier to hatching [1]. Eggshell thickness is usually measured with or without membranes using a thickness measurer [2]. However, this method does not sufficiently reflect the effect of shell thickness on hatchability. Hence, some researchers have assessed egg shell thickness according to egg specific gravity [3], which is closely related to shell thickness [4]. Eggs with specific gravities of 1.080 or 1.075 have been classified as thin shelled, whereas those with specific gravities of 1.085 or higher have been classified as thick shelled [3]. Ar et al.[5] calculated eggshell thickness with a logarithm that used egg weight, and this logarithm was adopted by other researchers as well [6]. In yet another method, shell thickness determined after hatching [7]. However, all these methods assess egg shell thickness indirectly. Furthermore, whereas most studies investigate egg physical characteristics (egg weight, shape, length, etc.) in relation to chick measurements (chick weight and chick length), there is little information available about the relationship between egg shell thickness and hatchability. Therefore, this study evaluated the relationship between egg shell thickness and hatchability, chick weight and chick length.

MATERIAL and METHODS

This study was conducted at the Experimental Farm of the Ondokuz Mayis University Agricultural Faculty using a total of 253 eggs from two broiler breeder genotypes

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obtained from the farm's parent stock. Hens of the parent stocks were two-way crosses of ROSS x Rhode Island Red (RIR) and ROSS x Barred Plymouth Rock (BAR), and they were mated with ROSS males to obtain the eggs used in the study. Eggs were collected when the flock age was 43 wks, with 117 eggs collected from the ROSSx(ROSSxBAR) flock ('Genotype 1') and 136 from the ROSSx(ROSSxRIR) flock ('Genotype 2'). All eggs were collected on the same day. Eggs were numbered and weighed, and shell thicknesses were measured with an Egg Shell Thickness Gauge (ORKA Tech. Ltd., Israel) that uses precision ultrasound to gauge thickness without breaking the egg and is accurate to within 0.01 mm. The shell thicknesses were measured on blunted edge of eggs. Three measurements were performed for each egg and the mean of these measurements was assessed as egg shell thickness. The thinnest and thickest egg shell thickness values of the eggs were determined. The difference between thickest and thinnest eggs was divided to three (X_{max}-X_{min} /3). This value was added to mean egg shell thickness to determine the range of thick shell group; and deducted from mean egg shell thickness to determine the range of thin shell group. The eggs were classified to three egg shell thickness groups (thin, medium and thick) with this method *(Fig. 1)*.

Eggs were placed in a 2400-egg-capacity incubator (Cimuka Incubator Company, Turkey) and transferred to individual pedigree hatch baskets at 18 d of incubation to allow for chick measurement according to egg number. The eggs were randomly distributed to trays. But each genotype and shell thickness group was rationally represented on trays. Also, trays were replaced to each levels of incubation to eliminate the different conditions of incubation machine. Hatching was completed at 21.5 days. All chicks were weighed and chick length measured at hatch. Chick weight was assessed using a bascule with a sensitivity of up to 0.01 g. Length was determined by measuring each chick from the tip of the beak to the end of the middle toe, with the chick's dorsal surface extended over a ruler [8].

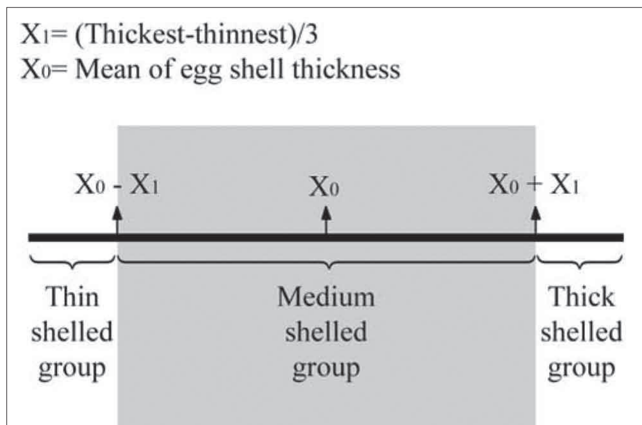

Fig 1. Classification method of egg shell thickness groups
Şekil 1. Yumurta kabuk kalınlığı gruplarını sınıflandırma metodu

Factorial analysis was conducted on a completely randomized design, with genotype and shell thickness as factors. Pearson correlation analysis was used to assess relationships between egg shell thicknesses and hatching traits, with differences in means evaluated for significance using Duncan's Multiple comparison test. R statistical software was used to analyze the data. A difference of $P<0.05$ was considered statistically significant.

RESULTS

Analysis results showed that Genotype x Egg Shell Thickness interaction was insignificant ($P>0.05$) on all traits, so only main effects were interpreted. Mean egg weight, shell thickness, chick weight and chick length at hatch are given in *Table 1*. Differences between all hatching traits were significant among genotypes ($P<0.05$). Chick weights at hatch for Genotypes 1 and 2 were 41.42 g and 43.18 g, respectively, and chick weight/egg weight ratios were 68.54 and 69.67, respectively.

Egg shell thicknesses ranged between 0.28-0.45 mm and included thin (≤0.34 mm), medium (0.35-0.38 mm) and thick (≥0.39) -shelled eggs, which were classified using the equation X_{max}-X_{min} /3. Infertile eggs (Genotype 1: 6/117; Genotype 2: 20/136) were not evaluated. Hatching rates, egg weights, chick weights and chick lengths of each genotype according to eggshell thickness classifications are given in *Table 2*.

Table 3 shows the relationships between shell thickness, egg weight, chick weight and chick length.

DISCUSSION

Mean egg weights for Genotype 1 and Genotype 2 were, respectively, 60.43 g and 61.98 g, which Abiola et al.[9] described as 'medium-sized' eggs. Their study found

Table 1. *Some hatching characteristics of genotypes (Mean ± SEM)*
Tablo 1. *Genotiplerin bazı kuluçka özellikleri (Ortalama ± Standart Hata)*

Genotype	Egg Weight (g)	Shell Thickness (mm)	Chick Weight (g)	Chick Length (cm)
1	60.4±0.44b	0.380±0.003b	41.4±0.4b	18.7±0.050a
2	62.0±0.48a	0.390±0.003a	43.2±0.41a	18.4±0.060b
Sig (P)	0.019	0.020	0.002	0.007

a, b: *Differences in superscript letters within columns represent significant differences between groups (P<0.05),* ***SEM:*** *Standard Error of Means*

Table 2. Hatching rates, egg weights, chick weights and chick lengths of genotypes 1 and 2, according to egg shell thickness classification (Mean ± SEM)

Tablo 2. Kabuk kalınlığı gruplandırmasına göre 1. ve 2. genotiplerin çıkış gücü, yumurta ağırlığı ve civciv ağırlığı ve civciv uzunlukları (Ortalama ± Standart Hata)

Genotype	Egg Shell Thickness Classification	Hatching Rate (%)	Egg Weight (g)	Chick Weight (g)	Chick Length (cm)
1	Thin	91.6	58.6±1.39	40.3±1.2	18.4±0.160
	Medium	84.6	60.5±0.630	41.8±0.51	18.7±0.090
	Thick	91.3	60.8±0.700	41.3±0.69	18.7±0.070
Sig. (P)		0.852	0.492	0.119	0.479
2	Thin	77.8	63.3±1.22	45.6±1.10	18.6±0.200
	Medium	95.0	61.5±0.840	42.4±0.640	18.5±0.100
	Thick	87.9	62.2±0.620	43.6±0.550	18.4±0.09
Sig. (P)		0.432	0.492	0.119	0.479

***SEM:** Standard Error of Means*

Table 3. Coefficient of correlations between egg shell thickness, chick weight and chick length

Tablo 3. Yumurta kabuk kalınlığı, civciv ağırlığı ve civciv uzunluğu korelasyon katsayıları

Egg and Chick Traits	Egg Shell Thickness	Chick Weight	Chick Length
Egg weight	0.108	0.862**	0.365**
Egg shell thickness		0.082	0.020
Chick weight			0.302**

*** P<0.01*

'medium-sized' eggs had the greatest hatchability. Shell thicknesses of Genotypes 1 and 2 were 0.38 and 0.39 mm, respectively. These figures are lower than those reported by Wolanski et al.[10], who reported shell thicknesses of eggs from 10 broiler breeder genotypes ranging from 0.325 to 0.370 mm. Chick weight/ egg weight ratios were similar to those obtained in previous studies [8,10]. Msoffe et al.[11] has shown that chick lengths at hatch are positively correlated with adult body weight (r = 0.96).

No significant differences were observed between groups for hatching rates, egg weights, chick weights and chick lengths; in other words, egg shell thickness had no effect on egg weight, chick weight, or chick length.

The highest correlation was found between egg weight and chick weight. The correlation between chick weight and chick length was also found to be significant. This result is similar to the result reported by Wolanski et al.[8], who found a correlation of 0.303 between chick weight and length. In line with the correlation found between chick weight and chick length, the correlation between egg weight and chick length was also found to be significant.

Bennet [3] found that eggs with thin shells had hatchability rates between 3%-9% lower than eggs with thick shells, whereas Tsarenko [12] reported a 30% difference in hatchability rates between thin- and thick-shelled eggs. A number of studies [13] examining hatchability in different poultry species including turkey and geese found hatchability of thick-shelled eggs to be 20%-40% higher than that of thin-shelled eggs, although a study by Andrews [14] found the hatchability of turkey eggs to be higher for eggs with thinner shells. Despite their differences in findings, all of these studies reported egg shell thickness to have an effect on egg hatchability. In contrast to this, however, our study found egg shell thickness had no effect on hatchability. Also, most of previous studies classified the eggs as thin or thick regardless measuring the average shell thickness of eggs. In our study, the mean shell thickness of the eggs was determined, and the differences between the thickest/thinnest shelled eggs and mean shell thickness were used to identify the limits of each thickness group. This method had more accurate classification than general classifying.

According to these results, it could be said that, after the embryo completed its development, it could crack the egg regardless to the thickness of shell. The insignificant correlation between egg shell thickness and chick weight supported this result.

In conclusion, after measuring shell thicknesses with an ultrasound gauge and classifying eggs into three groups accordingly, this study found no significant differences between egg weights, hatching rates, chick weights and chick lengths among egg shell groups.

It should be noted that although most studies determine egg shell thickness by using specific gravity, Sarica et al.[15] have shown that this parameter may not yield accurate results, as the correlation between specific gravity and egg shell thickness varies by age. Thus, future studies comparing measurement methods would be beneficial.

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Short Communication

Prevalence of Fungi in the Conjunctival Sac of Clinically Normal Sheep

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Abstract

Conjunctival swabs were obtained from both eyes of 50 healthy sheep to identify the fungal flora. Data were analyzed for effect of age and sex. Out of 100 samples cultured, 18 (18%) showed fungal growth with predominance of the genus *Cladosporium* (38.89%). Other isolated fungal genera were *Penicillium* (16.67%), *Rhodotorula* (16.67%), *Aspergillus* (16.67%) and *Curvularia* (11.10%). There was no significant effect of sex and age on frequency of fungal isolation. Results showed that fungi are not prevalent on the ocular surface of healthy sheep.

Keywords: *Fungal flora, Eye, Sheep, Cladosporium spp.*

Klinik Olarak Normal Koyunların Konjunktival Kesesinde Mantarların Yaygınlığı

Özet

Elli adet koyunda yapılan bu çalışmada hayvanların gözlerindeki konjunktival keselerden alınan sıvap örneklemesi ile normal fungal floranın belirlenmesi amaçlandı. Çalışmada yaş ve cinsiyetin etkisi analiz edildi. Kültürü yapılan 100 örneğin 18 (%18)'inde fungal üreme görüldü. *Cladosporium* cinsi (%38.89) en çok üreyen mantar idi. Diğer üreyen mantarlar; *Penicillium* (%16.67), *Rhodotorula* (%16.67), *Aspergillus* (%16.67) ve *Curvularia* (%11.10) idi. Cinsiyet ve yaşın fungal izolasyon üzerine etkisi saptanmadı. Sonuçlar sağlıklı koyunların göz yüzeyinde mantarların yaygın olarak bulunmadığını gösterdi.

Anahtar sözcükler: *Mantar florası, Göz, Koyun, Cladosporium spp.*

INTRODUCTION

In many animal species fungi are considered as a part of the normal ocular flora, but their existence can turn to pathogenic state when defense mechanisms of the outer eye are damaged. Knowledge on the fungal species which are most likely encountered in the conjunctival sac is important to select an antifungal drug as initial empirical treatment of corneal mycoses [1,2]. There is a paucity of information on the ocular fungal flora of ruminants [3-5], especially sheep [6]. Reportedly, *Cladosporium* spp, *Penicillium* spp and *Mucor* spp. are the predominant species. In order to increase knowledge of the ovine ocular fungal flora and to determine the effect of sex and age on the prevalence of isolates, we conducted present study on healthy sheep.

MATERIAL and METHODS

Fifty clinically healthy fat-tailed sheep presented at the Urmia abattoir (Iran) from May to July 2011 were swabbed. Animals were from both sexes (26 males and 24 females) and divided into two age groups based on dental formula; A: Under 2 years old (34.68%) and B: above 2 years old (16.32%).

Samples were taken from the lower conjunctival sac of both eyes (n=100) using dry sterile swabs per eye, avoiding the eyelid margins and eyelashes. Swabs were placed into sterile tubes containing 2 ml normal saline and immediately transferred to the laboratory in a chilled box. Samples were plated onto Sabouraud dextrose agar (Merck, Darmstadt, Germany) and malt extract agar (Quelab, Montreal, Canada) and incubated at 25°C for 3 weeks. Identification of isolates was achieved to the genus level on the basis of macroscopic and microscopic features [7].

The effect of age and sex on the frequency of fungal isolation was determined using Fischer's exact test. Significance was set at $P<0.05$.

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RESULTS

Five genera of fungi were cultured from a total of 13 sheep (26%) and 18 eyes. The fungi isolated and the isolation rates are listed in *Table 1*. *Cladosporium* spp (38.89%) was the most frequent isolate. Single fungi were isolated from each eye.

According to statistical analysis *(Table 2, Table 3)*, the prevalence of fungal isolates did not show significant differences between sexes and age groups (P>0.05).

Table 1. *Fungal genera, frequency of species isolated and number of positive culture eyes and sheep*

Tablo 1. *Mantar türleri, izole edilen türlerin bulunma sıklığı ile göz ve koyunlarda pozitif kültür sayıları*

Fungi	No. of Positive Sheep (%)	No. of Positive Eyes (%)	No. of Isolates (%)
Cladosporium spp.	6 (12)	7 (7)	7 (38.89)
Penicillium spp.	3 (6)	3 (3)	3 (16.67)
Rhodotorula spp.	3 (6)	3 (3)	3 (16.67)
Aspergillus spp.	3 (6)	3 (3)	3 (16.67)
Curvularia spp.	2 (4)	2 (2)	2 (11.10)

Table 2. *Frequency analysis of the conjunctival fungal isolates in relation to sex of sheep*

Tablo 2. *Koyunların cinsiyet ile ilişkili olarak konjuktuval mantar izolatlarının sıklık analizi*

Fungi	No. of Isolates (%)		P-value
	Males n=26	Females n=24	
Cladosporium spp.	2	5	0.239
Penicillium spp.	2	1	0.999
Rhodotorula spp.	1	2	0.602
Aspergillus spp.	1	2	0.602
Curvularia spp.	2	-	0.491
Total	8	10	0.557

Table 3. *Frequency analysis of the conjunctival fungal isolates in relation to age of sheep*

Tablo 3. *Koyunların yaş ile ilişkili olarak konjuktuval mantar izolatlarının sıklık analizi*

Fungi	No. of Isolates (%)		P-value
	<2 years old n=34	>2 years old n=16	
Cladosporium spp.	3	4	0.190
Penicillium spp.	2	1	0.999
Rhodotorula spp.	2	1	0.999
Aspergillus spp.	1	2	0.236
Curvularia spp.	1	1	0.542
Total	9	9	0.060

DISCUSSION

To our knowledge, this is the second published report of the ocular fungal flora in healthy sheep. In the current study, 26% of the sheep demonstrated positive cultures for fungi. This finding was consistent with those reported for goats (37.14%) [5], dogs (22%) and cats (40%) [3]. In the large animals this amount were reported to be 100% for cattle, 95% for horses [3], and 79.04% for donkeys [8]. It seems that results of the ocular fungal culture could be influenced by size of eye and its surface area exposed to the environmental fungi.

In the majority of studies on equine species *Aspergillus* spp is the most common isolate of the normal conjunctiva [2,8-10], but in domestic carnivores [3] and ruminants including cattle [3,4] and goats [5] generally *Cladosporium* spp and *Penicillium* spp were reported to be predominant. In our study, genus *Cladosporium* (38.89%) also reported as the most frequent isolates of conjunctiva. This finding was not similar to that reported for sheep in Italy [6], in which *Mucor* spp predominated (49%). It has been suggested that fungi are transitory inhabitants of the eye surface, and their prevalence can thus be influenced by geographic conditions [1,11].

In the present study there was no significant difference in isolation rates of fungi between sexes and age groups. In various studies have been showed that prevalence of the normal eye flora could be affected by these two host factors, as in male horses [1], male goats [5], female pigs [12] and younger horses [11] frequency of isolates were significantly higher.

Fungi are not prevalent on the ocular surface of healthy sheep. This could be considered as a reason for paucity of keratomycosis in this species.

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Short Communication

Treatment of Bath with Enrofloxacin in Red-Eared Sliders *(Trachemys scripta elegans)* Suffer from Conjunctivitis and Its Results [1]

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Abstract

Conjunctivitis is frequently seen in red-eared sliders *(Trachemys scripta elegans)*. If the diseases are untreated, it can cause starvation and death. This study was performed on eleven red-eared sliders with conjunctivitis. Clinical findings were closed eyes, chemosis and unilaterally or bilaterally tear secretion, loss of sight and anorexia. Conjuctival swap samples were taken for bacterial examination. *Aeromonas hydrophyla* and *Pseudomonas* spp. were detected in microbiological analysis. In treatment were performed bath with enrofloxacine solutions (5 mg/L), 3% boric acid solution and oral Vitamin A (5.000 IU/L). In all cases, full recovery was seen following the treatment. This is the first report about enrofloxacin bath for using conjuctivitis in red-eared sliders. As a result; bath with enrofloxacin solutions may be a suitable alternative treatment for red-eared sliders with conjunctivitis.

Keywords: *Conjunctivitis, Red-eared slider, Trachemys scripta elegans, Enrofloxacin, Bath treatment*

Kırmızı Yanaklı Su Kaplumbağaları *(Trachemys Scripta Elegans)* Konjunktivitis Olgularında Enrofloksasin ile Banyo Sağaltımı ve Sonuçları

Özet

Kırmızı yanaklı su kaplumbağalarında (*Trachemys scripta elegans*) sık görülen konjunktivitis vakaları tedavi edilmediği takdirde açlık ve ölümle sonuçlanabilir. Bu çalışma konjunktivitis teşhisi konan 11 kırmızı yanaklı su kaplumbağası üzerinde yapılmıştır. Klinik muayenede kaplumbağaların göz kapaklarında ödem, korneada bulanıklık, tek veya iki taraflı gözyaşı akıntısı ile görme kaybı ve iştahsızlık tespit edilmiştir. Göz konjunktivasından alınan sıvaptan yapılan mikrobiyolojik ekimlerde *Aeromonas hydrophyla* ve *Pseudomonas* türleri belirlenmiştir. Tedavide oral vitamin A (5.000 IU/L), %3'lük asit borik ve enrofloksasin (5 mg/L) solüsyonlarıyla banyo uygulandı. Tedavi sonrası tüm olgularda tam bir klinik iyileşme görüldü. Bu çalışmada banyo tarzında enrofloksasin uygulaması kırmızı yanaklı su kaplumbağalarının konjunktivitis tedavisinde ilk defa denenmiştir. Sonuç olarak; kırmızı yanaklı kaplumbağalar ve egzotik hayvanların göz hastalıklarında enrofloksasin solüsyonu ile banyo tedavisi alternatif bir tedavi metodu olabilir.

Anahtar sözcükler: *Konjunktivitis, Kırmızı yanaklı su kaplumbağası, Trachemys scripta elegans, Enrofloksasin, Banyo tedavisi*

INTRODUCTION

Conjunctivitis is a common disease in turtles and it can be the result of septicemia. For this reason it can be result in reptiles [1,2].

It was reported that the damage to soft tissue can cause infection from opportunistic microfloral bacteria in turtles [3]; vitamin A deficiency is seen as the most important predisposing factor [1]. Conjunctivitis treatment cover in turtles, antibiotic applications and vitamin A supplementation are recommended [1,4].

In this study, a total of eleven red-eared sliders with conjunctivitis, brought to our clinics by their owners, were cured with oral vitamin A and bath treatments with enrofloxacine, 3% boric acid.

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MATERIALS and METHODS

Animals and Management

A total of 11 red-eared sliders with conjunctivitis were evaluated at the Department of the Surgery Clinics, Faculty of Veterinary Medicine, University of Mustafa Kemal. All subjects were female. Out of four cases conjunctivitis was bilaterally affected *(Fig. 1)*. Ophthalmological examinations were performed via direct and indirect ophthalmoscopy, due to the subjects' eyes were closed and eye examination was very difficult *(Fig. 2)*. The ophthalmological examinations were repeated periodically over five days. Our patient is conjunctivitis, not seen keratitis. Another tissue of eye such as cornea is seen normally. When healing was observed, treatment was finished *(Fig. 3)*.

Bacterial Isolation

Samples were taken with non-invasive conjunctival swabs. For this purpose, the seared exudate layer on the eyelid was removed aseptically and conjunctival swab samples were collected. The conjunctival swabs were streaked across blood agar. For the each sample, two blood agar samples were used: one of which was incubated at 37°C, and the other was incubated at 25°C. Isolated strains were identified biochemically [5].

Fig 1. Apperance of bilaterally conjunctivitis in case 3
Şekil 1. Olgu 3'te bilateral konjunktivitisin görünümü

Therapy

A complete ophthalmological examination was made as part of the conjunctivitis therapy. Initially, a diet high in vitamin A, regular clean water and an appropriate

Fig 2. Apperance of eye examination in case 2
Şekil 2. Olgu 2'de göz muayenesinini Görünümü

Fig 3. Apperance post of the treatment in case 3
Şekil 3. Olgu 3'te tedavi sonrası görünüm

environment was provided. We are suggested use of water with distilled and ambient temperature. The treatment was planned in four stages. In the first stage, the turtles were kept in an empty box for five minutes in order to dry their skin. Second, the turtle's heads were flushed with a 3% boric acid solution. Third, the turtles were treated with baths of enrofloxacin solutions (5 mg/L, Baytril 2.5%, Bayer, Turkey) for five minutes. Fourth, the first applications were repeated, and the turtles were returned to their cages. Finally, the turtles were fed with oral Vitamin A (Deepfix reptivit 30 ml, Mar Kimya Ltd. Turkey). This treatment was applied twice daily.

RESULTS

Water pollution and infrequent water exchange were serious problem in our patient. We recommended of clean distilled and ambient temperature water and water changed daily. Excessive discharge, swollen and closed eyelids, anorexia and deadness were seen both unilaterally and bilaterally in patient. Our six patients is recovery in first five days, another patient is recovery second five days. Cornea is seen normally in repeated periodically over five days, there is not seen keratitis. In the result of the bacteriological examination, *Aeromonas hydrophyla* was isolated from all of the examples; at the same time, *Pseudomonas* spp. were isolated from three of the samples. Bath treatment with 3% boric acid and enrofloxacine were successfully. Eye problems in exotic animals can lead to serious health problems if left untreated. Treatment with enrofloxacin solutions may be suitable for wild and exotic animals, such as sea turtles, in this case red-eared sliders with conjunctivitis.

DISCUSSION

Eye problems are a common ailment among pet turtles. Conjunctivitis in reptiles may be due to foreign bodies, dirty water in its box, air currents, nutritional imbalances, trauma or genetic predisposition [6]. A total of eleven red-eared slider turtles with conjunctivitis were brought to our clinic. Clinical symptoms, bacterial isolations, water pollution and infrequent water exchange were same in our patient. Traumatic factors and air currents were caused unilaterally and another factors such as dirty water caused bilaterally conjunctivitis.

Conjunctivitis in turtles is not only an eye disease, but also a symptom of respiratory disease or septicemia. Intramuscular antibiotic implementations and vitamin A supplementation are an effective treatment [6-8]. In this study, turtles with conjunctivitis were treated by applying bath-style antibiotics and antiseptic. Furthermore, adding green vegetables to the diet as a source of vitamin A was recommended. We reached that the same conclusion that enrofloxacine use successfully exotic animals [2,5-8]. Bath treatment of antiseptic and antibiotic showed synergic effect. Our patients have been treated, and no recurrence of disease in the post-treatment examinations was observed.

Nowadays red-eared sliders *(Trachemys scripta elegans)* as a pet has been a significant increase in Turkey. For this reason we are noted this cases for the authors interest to the disease and turtles were noted present cases [9]. This study was first valuated turtle populations with conjunctivitis. In conclusion, the bath type of application was considered because, its ease of application, in addition to its reduces the effects of stress and it can eliminate dosing or overdosing risks. This study has concluded that for a turtle with conjunctivitis, simple and successful treatment may be performed with administrations of bath-style.

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Short Communication

Evaluation of Acrylic Pin External Fixation (APEF) System in Metacarpal Fractures of Newborn Calves: Cheap But Effective? [1]

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[1] *This article was presented at 14th Congress of Veterinary Surgery (23-26 October 2014, Antalya, Turkey)*
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Abstract

In this study, the effectiveness of acrylic pin external fixation (APEF) system was investigated in healing of newborn calves' metacarpal fractures. Six newborn calves with closed metacarpal fracture were used. Fracture healing was monitored by scoring "walking-weight bearing" "radiographic healing" and "pin tract infections" in postoperative days 10, 20, 40 and 80. Calves were able to walk without support at postoperative day 1 and able to walk normally at day 40. In all cases, radiological fracture healing was observed on day 40 and APEF system was removed. After day 10 controls, no pin track infection was observed in any case.

Keywords: *Calf, Metacarpal fracture, APEF, Fracture healing*

Neonatal Buzağıların Metakarpal Kırıklarında Akrilik Pin Eksternal Fikzasyon (APEF) Sisteminin Değerlendirilmesi: Ucuz Ama Etkili mi?

Özet

Bu çalışmada, akrilik pin eksternal fikzasyon (APEF) sisteminin neonatal buzağıların metakarpal kırıklarında etkinliği araştırıldı. Çalışmada kapalı metakarpus kırığı teşhis edilen 6 neonatal buzağı kullanıldı. Olguların kırık iyileşmesi, postoperatif 10, 20, 40 ve 80. günlerde "yürüme-ağırlık yüklenebilme", "radyografik iyileşme" ve "pin dibi enfeksiyonu" skorlamaları ile değerlendirildi. Postoperatif 1. günden itibaren desteksiz yürüyebilen olguların, 40. günde normal yürüyebildikleri dikkat çekti. Olguların tümünde 40. günde radyolojik olarak kırık iyileşmesi izlendi. Postoperatif 10. gün kontrollerinden itibaren hiçbir olguda pin dibi enfeksiyonu izlenmedi.

Anahtar sözcükler: *Buzağı, Metakarpal kırık, APEF, Kırık iyileşmesi*

INTRODUCTION

Metacarpal and metatarsal bone fractures are 50% of the bovine fractures [1]. Due to excessive fetal limb tractions during dystocia, this ratio is higher among newborn calves [2-5]. In treatment of newborn calf fractures, external or internal osteosynthesis techniques can also be utilized beside conservative methods [3-9]. For the choice of treatment technique, client's economic status is at least as decisive as the shape and location of the fracture [4,5]. Acrylic pin external fixation (APEF) system has significant advantages compared with conventional external skeletal fixators [6,10-12]: no restrictions of transcortical pin diameter, size, type and number; no need for a precise and complex planning for transcortical pin inserting points before operation; transcortical pins do not need to be placed in a specific vertical direction; no need for preassembly complex planning of fixator frames; minor risk of trans-cortical pin or connecting bar loosening; plastic tubes constructed for acrylic substance filling are flexible and lightweight; cheap and ease of use. In rural practices, APEF seems to be the cheapest external fixator system for the economic requirements of livestock production [6], and in this study, it was aimed to present the effectiveness of APEF in healing of newborn calves' metacarpal fractures.

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MATERIAL and METHODS

Six newborn calves brought to MAKU Surgery Clinic and had closed metacarpal fracture were used. Under general anesthesia (5% isoflurane-Aerrane Eczacıbaşı Baxter Istanbul and an O_2 flow of 5L min^{-1}), closed reduction was applied with transcortical pins (Ø2-4 mm, nonthreaded) placed by a low-speed drill in type-II (full-pin) configuration *(Fig. 1-A)*. Two flexible plastic tubes (Ø4 cm) were pushed over the lateral and medial pin ends in columnar form *(Fig. 1-B)*. As the plastic tubes were flexible, any adjustment power was provided to the exit direction of the pins. Before the tubes were filled with acrylic material, to ensure the stability between fracture fragments, fixation pins passing through the proximal and distal of the fracture line were tightened with plastic handcuffs *(Fig. 1-B)*. The lumen of the plastic tubes was filled with cold acryl (Pancryl, Rubydent, Istanbul) after each tip was closed and waited for hardening to take the form of a real column *(Fig. 1-B)*. While hardening is going on, a pair of glove filled with rendered ice was padded on the transcortical pins in both sides. After hardening, the pin ends protruding from plastic tubes and handcuffs between pins were cut. After surgery, animals were allowed for free movements without an extra bandage *(Fig. 1-C)*, and daily pin tract dressing (%10 polyvidone iode, Polyod, Drogsan Istanbul) and IM antibiotherapy (1 mg/kg IM ceftiofur HCL, Excenel, Pfizer, Istanbul) was applied for 10 days.

Fracture healing was monitored by scoring “walking-weight bearing”, “radiographic healing” and “pin tract infection” in postoperative days 10, 20, 40 and 80 *(Table 1)* [13-15].

RESULTS

The main “walking-weightbearing” scores of the cases recorded on postoperative days 10, 20, 40 and 80 were

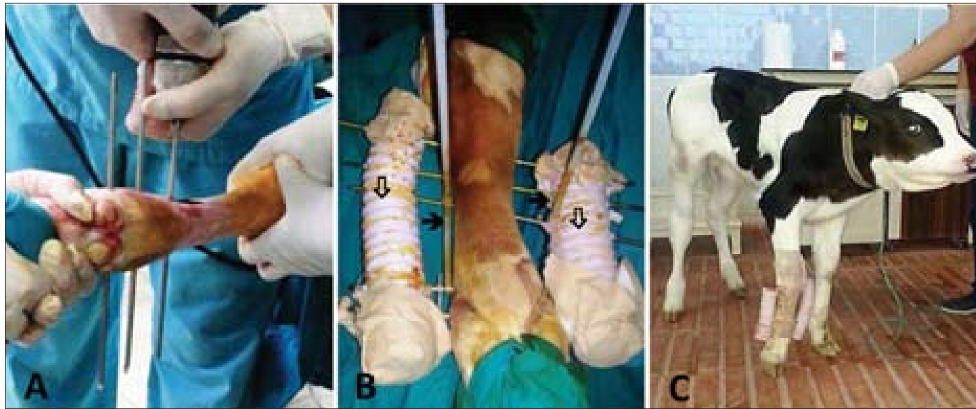

Fig 1. A- Placing nonthreaded transcortical pins by a low-speed drill in type-II configuration, **B-** Close-up view of a case waited for hardening of the acryl filled into the plastic tubes, ⇨ flexible plastic tubes passed into lateral and medial pin ends, ➔ plastic handcuffs applied between the pins passing through the proximal and distal of the fracture line, **C-** A case just after APEF application

Şekil 1. A- Vidasız transkortikal çivilerin, düşük devirli matkap yardımıyla II. tip konfigürasyonda yerleştirilmesi, **B-** Plastik tüplerin içine doldurulan akrilin sertleşmesi için beklenen bir olgunun yakından görünümü, ⇨ transkortikal pinlerin lateral ve medialdeki uçlarından geçirilmiş bükülebilir plastik tüpler, ➔ kırık hattının alt ve üstündeki pinler arasından geçirilerek sıkıştırılan plastik kelepçeler, **C-** Bir olgunun APEF uygulamasından hemen sonraki görünümü

Table 1. *Fracture healing monitoring parameters and scoring critters*
Tablo 1. *Kırık iyileşmesini değerlendirme parametreleri ve derecelendirme ölçütleri*

“Walking-Weightbearing” Scoring	“Radiographic Healing” Scoring	“Pin Tract Infection” Scoring
1: unable to stand and was brought on a stretcher	1: visible fracture line and no peri-/or endosteal callus	1: infection in all pin penetration points
2: can stand by support but keeps the fractured leg hanged	2: partially visible fracture line and a little peri-/or endosteal callus	2: infection in some pin penetration points
3: can stand by support and a little weightbear on the fractured leg	3: partially visible fracture line and peri-/or endosteal callus is present	3: no infection in any pin penetration points
4: can walk but keeps the fractured leg hanged	4: non visible fracture line and completed peri-/or endosteal callus	
5: can walk but weightbearing time of the fractured leg is shortened		
6: can walk normally and weightbear on the fractured leg		

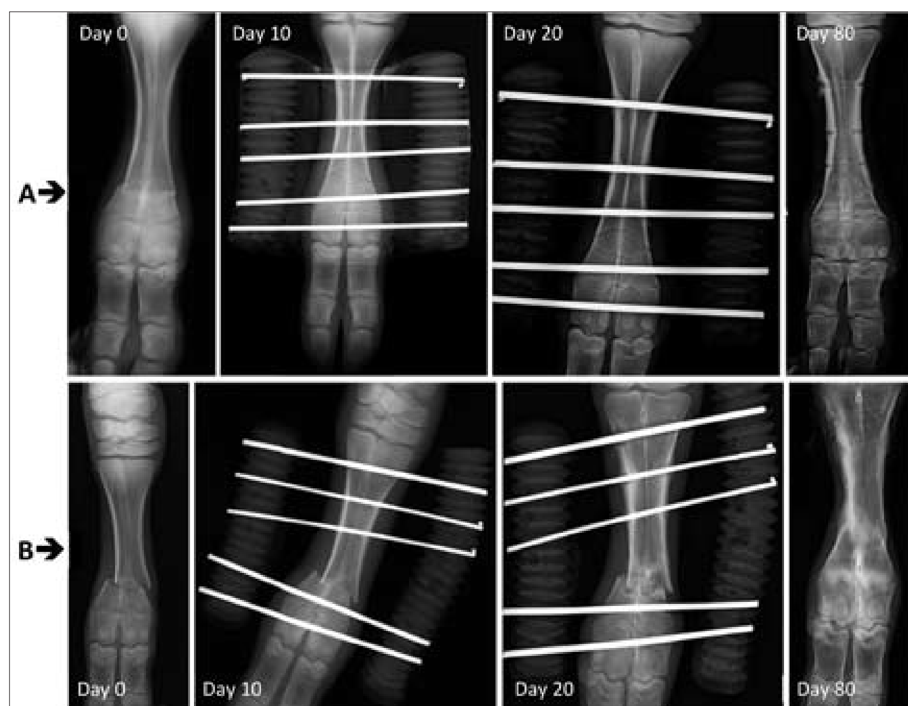

Fig 2. Radiographic appearances of fracture line of case 3 **(A)** and case 1 **(B)** on days 0 (preoperative), 10, 20 and 80. Note the fracture lines on day 80 in **(A)** mimics nearly primer fracture healing and in **(B)** shows secondary fracture healing

Şekil 2. Üç **(A)** ve 1 **(B)** nolu olguların 0 (preoperatif), 10, 20 ve 80. günlerdeki kırık hatlarının radyografik görünümleri. **(A)**'da kısmen primer kırık iyileşmesini andıran ve **(B)**'de sekunder kırık iyileşmesi şeklinde belirginleşen 80. gündeki kırık hatlarına dikkat ediniz

respectively 5, 5.25, 6 and 6. It was noted that calves can walk unassisted from day 1, and can walk normally and weightbear on the involved limb at day 40. The main "radiographic healing" scores for days 10, 20, 40 and 80 were respectively 2.25, 2.75, 3.5 and 4. Except for one case (case no 3), the fracture was healed with secondary bone healing *(Fig. 2-A,B)*. In case 3, the observed healing was near to primary bone healing *(Fig. 2-A)*. On radiographic controls, fracture healing was determined in all cases at day 40 and the APEF system was removed. From the day 10, pin tract infection was not observed in any case. The main pin tract infection scores for days 10, 20, 40 and 80 were respectively 2.75, 3, 3 and 3.

DISCUSSION

The fundamental reason of APEF system comes back on the agenda of bovine practitioners is higher cost of other internal and external fixation devices [6]. Treatment of a calf's metacarpal or metatarsal fracture may have excellent results with circular or linear external fixators but economic considerations of the farmers seem to be an obstacle for veterinary orthopedists. APEF system was really cheap than other external fixators, so the total cost of an APEF system per calf in this study was approximately 38 TL.

Premature pin loosening is an important problem for APEF as in other external fixators systems. One of the main causes for pin loosening in APEF system is heat necrosis of the bone. The source of heat is either polymerization of acryl or high speed drilling of the bone by transcortical pins [10-12]. In this study, transmission of the unavoidable heating during polymerization by transcortical pins into bone was prevented by padding pin-acryl column connections with gloves filled with rendered ice. On the other hand high speed drilling was avoided during placing the transcortical pins into metacarpus. The second cause for loosening of the transcortical pins in APEF system is pin tract infections [10-12]. Daily pin tract dressing with 10% polyvidone iode and IM antibiotherapy with ceftiofur HCL for 10 days seems to be effective results in our study for preventing these infections.

Fractures that occur shortly after or during birth in calves usually have a favorable prognosis unless the site is open and subsequently becomes infected. This favorable prognosis depends on the calves' great ability to produce periosteal new bone. Healthy and well vascularized bones heal to functional union as early as 3 weeks after treatment [1]. The 6 calves in this study had all closed metacarpal fractures and achieved functional unions recorded at day 40 controls. But more importantly, they could stand up, walk and suck without any support during the first 40 days of life. As a result, the APEF system in neonatal calves was thought to be an alternative technique in the treatment of closed metacarpal fractures.

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Case Report

Forensic Findings on Acute Mortality of Piglets after Ingestion of Aflatoxin

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Abstract

Aflatoxin (AF) is one of the most significant mycotoxins in pigs and results in a significant decrease of production performance. This paper describes the first case of acute effects of AF in suckling piglets in Serbia. Within 7 days, 420 suckling piglets aged 20±3 days died on a farm. Forensic findings included a medical history, clinical presentation with pathomorphological and histopathological confirmation of intoxication of the piglets with AF. Detection of AF in sows' milk (870 µg/kg), which was the primary nutriment for the piglets, confirmed the suspicion of AF intoxication. The piglets at that age also consumed solid feed, which was found to be contaminated with AF (960 µg/kg), and which accelerated the process of intoxication. After removal of the contaminated feed, the number of deaths began to reduce, as well as the clinical symptoms typical for AF.

Keywords: *Aflatoxin, Forensic finding, Intoxication, Piglet*

Aflatoksin Sindirilmesi Sonucu Akut Mortalite Şekillenen Domuz Yavrularında Adli Bulgular

Özet

Aflatoksin (AF) domuzlarda rastlanan en önemli mikotoksinlerden biri olup üretim performansında önemli azalmalara sebebiyet verir. Bu vaka takdimi süt emme dönemindeki domuz yavrularında AF'nin akut ektilerini gösteren Sırbistan'daki ilk çalışmadır. Bir çiftlikte 7 günlük bir süreçte yaşları 20±3 gün arasında değişen 420 adet domuz eniği ölü bulundu. Anamnez, klinik bulgular, patomorfolojik ve histopatolojik bulgular AF toksikasyonunu şüphelendirdi. Temel besin kaynağı olarak annelerin sütünde AF belirlenmesi (870 µg/kg) AF toksikasyonunu teşhisini doğruladı. Katı yem de tüketen yavruların bu yemlerinde de AF belirlenmesi (960 µg/kg) toksikasyonun hızlı seyrini açıklar nitelikteydi. Kontamine yemin uzaklaştırılmasından sonra ölümlerde ve AF'nin tipik klinik bulgularında azalma tespit edildi.

Anahtar sözcükler: *Aflatoksin, Adli bulgular, İntoksikasyon, Domuz eniği*

INTRODUCTION

Aflatoxins (AF) are toxic metabolites of fungi (*Aspergillus flavus*, *A. parasiticus* and *A. nomius*) commonly found on cereal grains, widespread contaminants of foods and feeds [1,2]. Ingestion of AF can harm the health of pigs. Depending on the ingested quantity and the age of the pig, changes can manifest themselves in acute, subacute or chronic forms. In the acute form, the liver is a major target-organ and widespread hemorrhage and jaundice and enlargement of gallbladder occur. Fatty buildup, centrilobular necrosis and hemorrhages can be observed microscopically on the liver. The immunotoxic potential of AF was detected in several animal species, including pigs [3], in which it decreases the blastogenesis response to mitogen, reduces the complement titers, decreases macrophage activation, and depresses delayed hypersensitivity [4]. The acute course of the disease occurs more frequently in younger age groups of pigs and the younger the pigs, the greater the risk of a lethal outcome.

CASE HISTORY

This paper describes forensic findings leading to confirmation of AF ingestion as the cause of death in

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piglets. On a commercial pig farm with the capacity to hold 2.000 sows, 420 suckling piglets aged 20±3 days died within seven days. The piglets were apathetic, depressed, moved reluctantly, cachectic and they were not febrile *(Fig. 1)*. Deaths occurred after a short period of inappetence, weakness and anorexia. The farm conducted regular immunoprophylaxis of sows and piglets (Aujeszky's disease (AD)), *E.coli* enterotoxaemia, necrotic enteritis in piglets (*Clostridium perfringens* (type C)), *Mycoplasma hyopneumoniae, Porcine circovirus* (PCV2), Classical swine fever (CSF) and porcine parvovirus (PPV)).

When the first symptoms of disease occurred in the piglets, they were treated with antibiotics, but treatment had no visible effect. Subsequently, bacterial, viral and serological testing of the sick piglets was conducted. None of the bacteria or viruses tested for were detected, while the presence of antibodies specific for AD, *E. coli* and *Clostridium*, CSF and PPV was established serologically. Considering the vaccination of sows, this finding suggests that stated specific antibodies in piglets of sows are the consequence of immunoprophylaxis sows.

Macroscopical examination revealed bright, yellowish color of the liver with subserous petechial bleedings and enlarged gallbladder *(Fig. 2)*. The tissues were fixed in 10% formalin and processed by routine paraffin technique. Microtome sections of 5 µm thickness, after deparaffinization, were stained using standard hematoxylin and eosin method. Vacuolization of hepatocytes, necrosis and fatty degeneration of the liver were determined histopathologically *(Fig. 3)*.

Fig 1. The clinical picture of piglets aged 20±3 days, after AF intoxication

Şekil 1. AF toksikasyonu sonrası 20±3 günlük domuz yavrularinin klinik tablosu

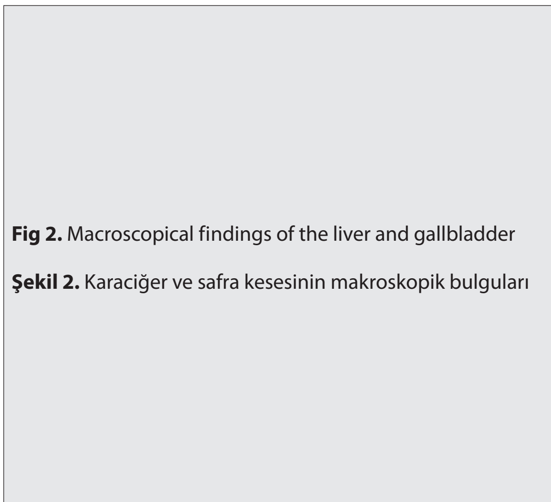
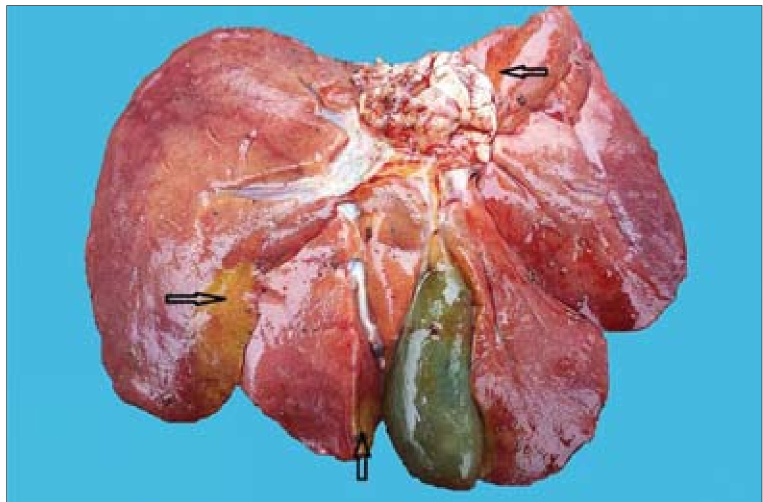


Fig 2. Macroscopical findings of the liver and gallbladder

Şekil 2. Karaciğer ve safra kesesinin makroskopik bulguları



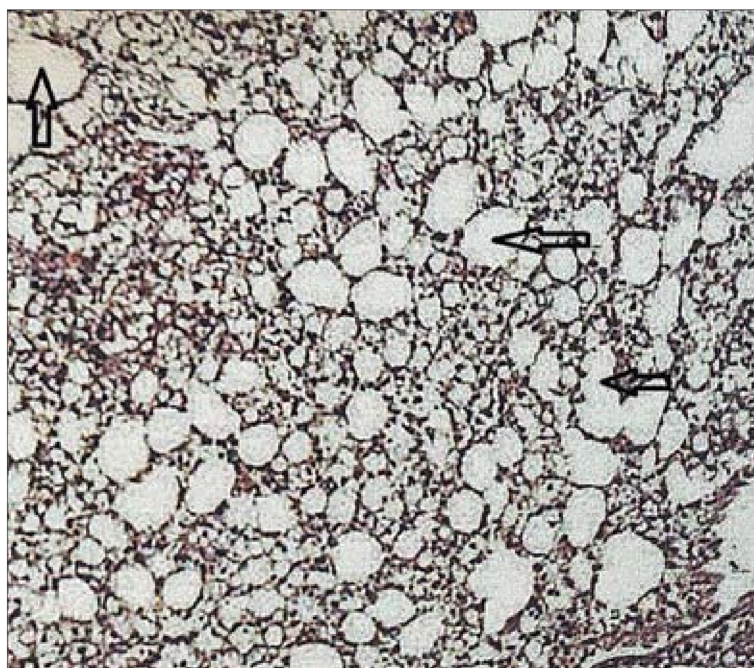


Fig 3. Microscopical findings of the liver

Şekil 3. Karaciğerde mikroskopik bulgular

Table 1. *Level of mycotoxins (µg/kg) in sows' milk and feeds on the farm*
Tablo 1. *Çiftlikteki domuz süt ve yemlerindeki mikotksin düzeyleri (µg/kg)*

Mycotoxin	Milk	Sows' Feed	Piglets' Feed
Aflatoxin	870	1710	960
Deoxynivalenol	52	201	74
Fumonisin	<50	<50	<50
Ochratoxin	<5	<5	<5
Zearalenone	<25	29	<25

The macroscopical examination of the stomachs of dead piglets revealed the presence of small amounts of milk and feed. After that, samples were taken from the sows' milk, sows' feed and piglets' feed for mycotoxin analysis (aflatoxin, deoxynivalenol, fumonisin, ochratoxin and zearalenone). Mycotoxins analysis were conducted using validated analytical method, enzyme-linked immunosorbent assay (ELISA) with good sensitivity and precision. Results are shown in *Table 1*.

After removal of the contaminated feed, the number of deaths began to reduce, as well as the clinical symptoms typical for AF.

DISCUSSION

Based on medical history, clinical, macroscopical and microscopical findings, the deaths of the suckling piglets were believed to likely be due to AF intoxication. Many infectious organisms (bacteria and viruses) can cause similar changes in piglets [5-7], but the forensic analysis excluded them. The final diagnosis was confirmed by the finding of AF in the sows' milk (870 µg/kg), which was a consequence of the presence of AF in the sows' feed. This is the first report of AF intoxication of piglets through the milk of sows, which is the primary nutriment of piglets of this age. Many other authors have reported the adverse effect of AF in pigs, and this effect is primarily reflected in the reduction of the production performance (average daily gain, feed conversion ratio and morbidity) and immunosuppressive effect depending on the ingested quantity of AF and the age of the individual piglets [8-11]. Their studies were conducted on older piglets exhibiting chronic effects [12,13], while in suckling piglets, much lower AF quantities can produce a lethal effect, as in our current report. Detection of 870 µg/kg of AF in sows' milk suggests that the level of AF in the feed was even higher than the determined level of 1710 µg/kg, because AF is catabolized in the body, and is excreted through the milk in lower dosage than that ingested. The piglets were aged 20±3 days and already consuming solid feed which was also found to be contaminated with AF (960 µg/kg); this only served to accelerate the process of intoxication.

After confirmation that the cause of death of piglets was intoxication with AF, the contaminated feed was withdrawn from use. Over the next few days the number of deaths among the piglets decreased, no new forms of clinical cases occurred and production performance began to increase.

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Case Report

An Athrax Outbreak in Wild Felidae Kept in a Local Zoo [1]

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[1] *Presented at VII. Veterinary Pathology Congress 08th-10th September 2014, Kars, Turkey*

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Abstract

This report presents cases of anthrax infection in the leopard *(Panthera pardus)*, tiger *(Panthera tigris)* and lion *(Panthera leo)*. Of wild Felidae kept in the same zoo, 7 animals (2 lions, 1 tiger, 2 leopards, 1 jaguar, and 1 puma) died within 2 days with clinical manifestations including swelling of the tongue and lips, bleeding from the mouth and nose, listlessness. Of the animals, 2 sick lions were examined cytologically from the haemorrhagic exudate in the mouth and nose. Macroscopic examination revealed the presence of anthrax carbuncles in the skin and lesion in the spleen of the dead leopards. The microscopic examination of the skin lesions demonstrated haemorrhage, oedema and the infiltration of neutrophils and mononuclear cells in the dermis and hypodermis. Microscopically, the splenic lesions consisted of widespread haemorrhagic areas, the atrophy of the white pulp and multifocal necrosis in the red pulp. *Bacillus anthracis* spores were observed in the cytological examination of the skin and spleen lesions and the haemorrhagic nasal exudate. *Bacillus anthracis* spores were observed in smears of the haemorrhagic mouth and nasal exudates, in histological sections of the skin and spleen. Based on clinical, macroscopical, microscopical and cytological examinations, infection of the animals was diagnosed as an anthrax infection.

Keywords: *Anthrax, Cytology, Histopathology, Wild felidae*

Yerel bir Hayvanat Bahçesindeki Yabani Kedigillerde Antraks Salgını

Özet

Bu raporda, Leopar *(Panther pardus)*, kaplan *(Panhera tigris)* ve aslanda *(Panthera leo)* antraks enfeksiyonu sunulmaktadır. Aynı hayvanat bahçesinde dilde ve dudaklarda şişkinlik, ağız ve burundan kan gelmesi, halsizlik ve 2 gün içerisinde ölüm şeklinde klinik bulgulara sahip 7 hayvan (2 aslan, 1 kaplan, 2 leopar, 1 jaguar ve 1 puma) ölmüştür. Hasta 2 aslanın ağız ve burundan gelen kanlı eksudat sitolojik yönden incelendi. Ölen leoparların makroskobik muayenesinde deride antraks karbunkellerine ve dalakta lezyona rastlandı. Deri lezyonunun mikroskobik muayenesinde dermis ve hipodermiste kanama, ödem ile nötrofil ve mononükleer hücre infiltrasyonları görüldü. Dalağın mikroskobik muayenesinde yaygın kanama alanları, beyaz pulpada atrofi ve kırmızı pulpada multifokal nekroz odakları gözlendi. Deri ve dalağın histolojik kesitlerinde, ağız ve burundaki kanlı eksudat frotilerinde *Bacillus anthracis* sporlarına rastlandı. Yapılan makroskobik, mikroskobik ve sitolojik muayeneler sonucunda leopar, kaplan ve aslanda antraks enfeksiyonu tanımlanmıştır.

Anahtar sözcükler: *Antraks, Sitoloji, Histopatoloji, Yabani Kedigil*

INTRODUCTION

Anthrax is a zoonotic disease caused by *B. anthracis* [1]. *Bacillus anthracis* can persist as a dormant spore in soil for many years, there is little evidence to suggest that it can multiply outside an infected animal. In natural environments it is thought that healty animals acguire the disease by grazing on the land contaminated with spores from infected animals [2]. Though the principal mode of transmission is ingestion of infective microorganisms, biting flies have been reported to transmit the disease from one animal to another [3]. In animals, the disease progresses rapidly, causes a high rate of mortality and generally courses with septicaemia. The principal lesions are those of oedema, haemorrhage and necrosis [4]. Illness is observed for one or two days, but it may last five days; symptoms are preceded by fever, with a period of excitement in which the animal may charge anyone nearby [5]. All herbivorous animals are particularly susceptible to this pathogen. Humans, suids and carnivores are considered incidental hosts [4]. Susceptibility and high fatality has been reported in cattle, sheep, goats, horses, donkeys, pigs, and many warm-blooded domestic animals. Although avian species are naturally immune to the disease owing to their body temperature, literature reports indicate that the ostrich, crow and duck can be experimentally infected by faecal contamination. Humans may become infected by handling contaminated hides or wool or by examining infected

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carcasses [5]. Wildlife with high rates of disease include antelope, bison, gazelles, impalas, elephants, hippopotami [6-8], red deer [9] black jackal [10]. Wild carnivores can also become infected through the consumption of dead animals infected with anthrax [7,8]. In this report, anthrax infection in the leopard *(Panthera pardus)*, tiger *(Panthera tigris)* an lion *(Panthera leo)* were described with clinic-patholojical findings.

CASE HISTORY

Two dead leopards, 1 dead tiger and 2 sick lions kept in the same zoo constituted the study material. Once all the necessary measures were taken to prevent the risk of contamination, smear samples were taken of all of the dead and live animals *(Fig. 1-A)*. For cytological examination were carried out from the skin and spleen lesions of the dead

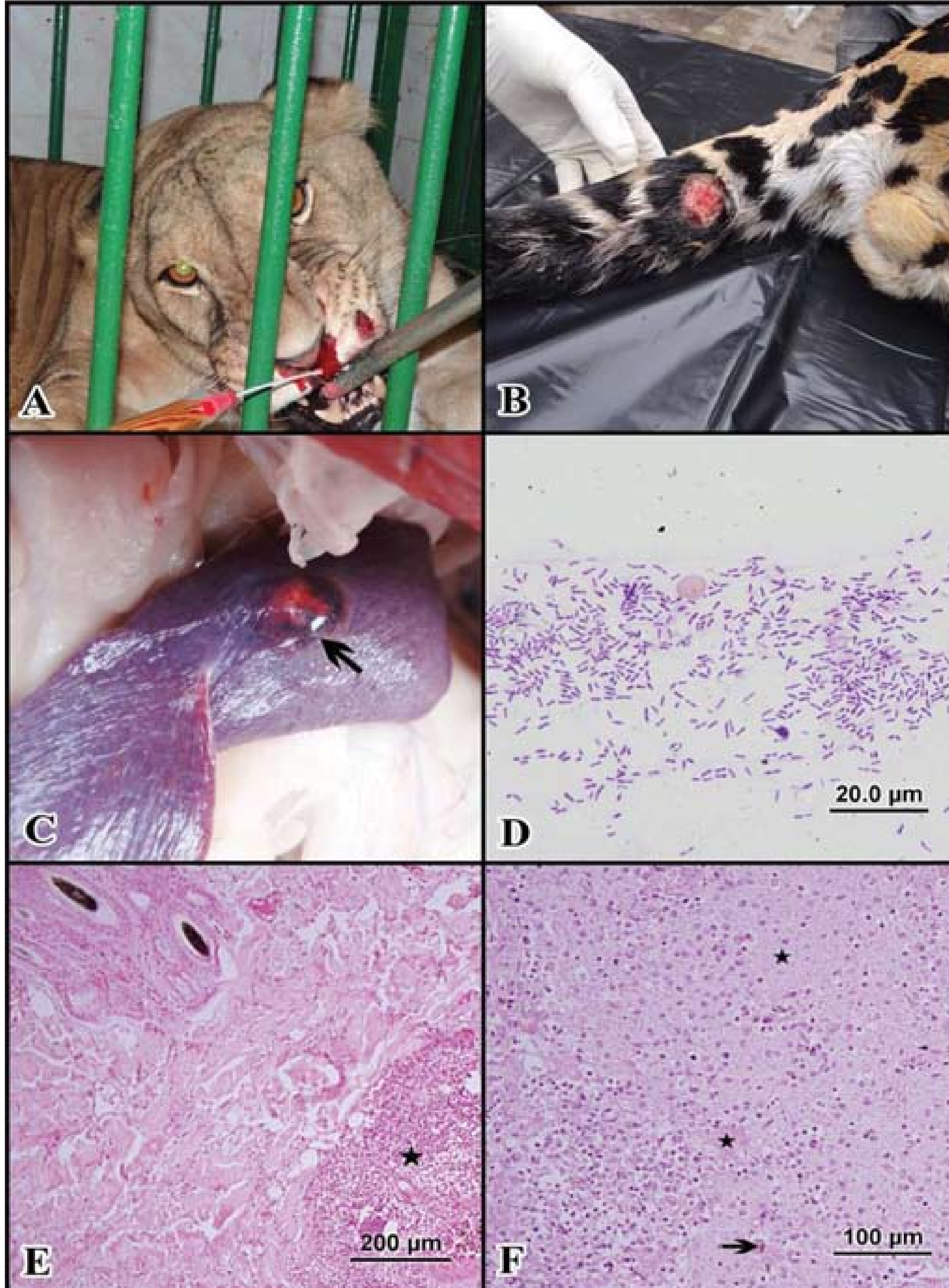

Fig 1. A- Bloody discharge the nose; **B**- Anthrax carbuncle on the tail; **C**- Lesion on the spleen; **D**- Spores of *Bacillus anthracis*, May Grünwald-Giemsa stain, x100; **E**- neutrophil infiltration in the dermis *(star)*, Haematoxylin&Eosin stain, x10, **F**- Spleen necrosis *(stars)* and siderosit *(arrow)*, Haematoxylin&Eosin stain, x20

Şekil 1. A- Burundaki kanlı eksudat; **B**- Kuyruktaki Antraks karbunkeli; **C**- Dalaktaki lezyon; **D**- *Bacillus anthracis* sporları, May Grünwald-Giemsa boyması, x100; **E**- Dermiste nötrofil infiltrasyonu *(yıldız)*, Hematoksilen&Eozin boyama, x10; **F**- Dalakta nekroz *(yıldızlar)* ve siderosit *(ok)*, Hematoksilen&Eozin boyama, x20

animals. The smears were stained with May Grünwald-Giemsa solution. Specimens taken from the ulcerous focus (2x3 cm) in the skin of the tail of one of the dead leopards *(Fig. 1-B)* and the 2 convex lesions, measuring 3x3 cm and of light cream colour in the centre and dark red colour in the periphery, in the dorsal and ventral surfaces of the spleen of the other dead leopard *(Fig. 1-C)*. The sections were fixed in neutral-buffered formalin and processed routinely. All sections were stained with Haematoxylin-Eosin (H&E). Because of there is no facility to bacteriological culture microbiologial evaluation could be added.

The microscopic examination of the smears revealed the presence of short chains of dark blue coloured rod-shaped bacteria, surrounded by a purplish-red capsule *(Fig. 1-D)*. The histopathological examination of the skin demonstrated widespread haemorrhage and oedema in the epidermis and dermis. The epidermis and dermis also presented with widespread and intense infiltration foci of normal, degenerated and necrotic neutrophils as well as with mononuclear cell infiltrations *(Fig. 1-E)*. The histopathological examination of the demonstrated wide-spread haemorrhagic areas beneath the splenic capsule. Light brown colored siderocytes containing the hemo-siderin pigment were dense in the haemorrhagic areas splenic tissue. The white pulp was observed to be atrophic. Disseminated multifocal necrosis was observed in the form of small foci in the red pulp *(Fig. 1-F)*.

DISCUSSION

All domestic and wild animal species susceptible to the disease are infected either by the consumption of soil, feed, milk and water contaminated with *B. anthracis* spores, the biting of blood sucking flies carrying the pathogen, or the direct contact of open wounds with the spores [3,8,11,12]. According to the information given by the staff of the zoo, the wild Felidae were fed mainly the meat of horses and donkeys obtained from the region where the zoo was located. It was declared that the horses and donkeys were isolated in a separate unit in the zoo for 2 weeks, observed for signs of disease, and slaughtered only in the absence of any such sign. Due to the entire horse and donkey meat stock having been consumed, it could not be confirmed whether the infection of the wild Felidae was caused by the consumption of contaminated meat. In order to prevent any further risk of anthrax infection, the remaining live horses and donkeys were killed under the necessary precautions, and were not slaughtered for being fed to the wild Felidae. Once having entered the body, *B. anthracis* spores are phagocyted by macrophages and transported to the local lymph nodes. Here the endospores germinate, outgrow into vegetative bacilli, and cause septicaemia after being released into the blood circulation. Inside the macrophage, the vegetative form of the causative agent produces 3 exotoxins, namely, the protective antigen, lethal factor and oedema factor. These toxins cause various clinical symptoms and lesions, including haemorrhage, oedema and necrosis [13,14]. Anthrax can be diagnosed on the basis of the identification of the causative agent by the examination of smears prepared from local lesions and blood, which are stained with the Gram, May-Grünwald Giemsa and methylene blue stains [15,16]. The direct or indirect contact of the pathogen with scratches and wounds in the skin, results in the formation of anthrax carbuncles. Skin lesions are observed as haemorrhagic pustules with a necrotic centre and surrounded by oedema, which may ulcerate with the course of the disease [1]. The internal organs affected by the disease vary with the transmission route of the pathogen [17]. As not performing a post-mortem examination of animals known or suspected to have died from anthrax prevents contamination [18], the animals included in the present study were not considered for necropsy. However, prior to the visit of the zoo for the on-the-spot examination of the animals, one of the dead leopards had been skinned and the abdominal cavity of the animal had been opened. The microscopic findings obtained for the spleen in the present study were in agreement with those reported in previous studies with in the experimentally infected [19]. In the present study, following an outbreak, anthrax infection was detected in wild Felidae kept in a zoo, on the basis of macroscopic, cytological and histopathological examinations. It was concluded that, food of animal origin provided to wild animals must be controlled for possible contamination with the anthrax pathogen in order to prevent the transmission of the infection.

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Letter to Editor

İneklerde Bir Biyosidal Dezenfektanın İntrauterin Kullanımı Üzerine Histopatolojik Bir Değerlendirme

(A Histopathological Assessment on the Use of an Intrauterine Biocidal Disinfectant in Cows)

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Sayın Editör,

İneklerde uterus enfeksiyonlarının tedavisinde; sistemik antibiyotik, intrauterin antibiyotik ve antiseptikler ile hormonlardan yararlanılmaktadır. Antiseptik olarak; lugol solüsyonu, povidon iyot, klorhekzidin, entozon, etakrinik asit (rivanol) yaygın uygulama alanı bulmuştur. Son yıllarda hidrojen peroksit de intrauterin kullanılan ajanlar arasında yer bulmuştur [1,2]. İrritan antibiyotik ve solüsyonların tekrarlı veya yüksek konsantrasyonda kullanımı uterusta fibrozise ve irreversibl etkilere yol açarak fertiliteyi olumsuz etkilemektedir [3,4].

Biz, T.C.Sağlık Bakanlığı'ndan biyosidal dezenfektan ruhsatı alınmış olan, Sodyum hipoklorit (%0.022) ve Hidrojen peroksit (%0.00005) aktif maddelerini içeren bir ürünün konsantre olarak intrauterin kullanımı sonucunda uterusta oluşabilecek histopatolojik değişikliklerin belirlenmesi düşüncesiyle bir ön deneme planladık ve Atatürk Üniversitesi Veteriner Fakültesi Araştırma ve Uygulama Çiftliğinde kesime sevkedilen 7 inekte bu preparatı intrauterin olarak kullandık. Bu amaçla, hayvanlardan 4'üne kesim tarihinden 15 gün, 3'üne ise 10 gün önce intauterin olarak 80 ml konsantre biyosidal dezenfektan uyguladık.

Kesim sonrası uteruslar alınarak histopatolojik muayene için Atatürk Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı Laboratuvarına iletildi. Laboratuvarda %10'luk nötral formalin solüsyonunda tespit edilen korpus ve kornu uteri örnekleri rutin işlemlerden geçirilerek parafin bloklara alındı. Bloklardan alınan 5 µ'lik kesitler rutin alkol, ksilol serilerinden geçirildi. Hematoksilen-Eozin ile boyanan kesitler, yangısal ve nekrotik-dejeneratif değişiklikler yönünden ışık mikroskobu altında incelendi.

Kesimden 15 gün önce biyosidal dezenfektan uygulanan 4 örneğin sadece 1'inde hafif düzeyde yangısal değişiklikler tespit edilirken, 3'ünde bu tablonun oldukça şiddetli düzeyde olduğu saptandı. Ayrıca bu 3 örnekte endometriyal bezlerde yoğun nekroz ve mukoza epitelinde deskuamasyon da mevcuttu *(Fig. 1, Fig. 2)*. Kesimden 10 gün önce biyosidal dezenfektan uygulanan 3 örnekte ise yangısal değişiklikler ile birlikte nekrotik ve dejeneratif değişiklerin daha hafif olduğu belirlendi *(Fig. 3)*.

Gerek histopatolojik sonuçlara ve gerekse bu biyosidal dezenfektan ile ilgili devam eden saha çalışmalarına göre; ürünün konsantre kullanılması durumunda irreversible sonuçlara yol açması nedeniyle, klinik endometritislerin tedavisinde konsantre değil, ½ oranında sulandırılarak kullanımı ile tatminkar sonuçlar alınabileceği sonucuna varıldı.

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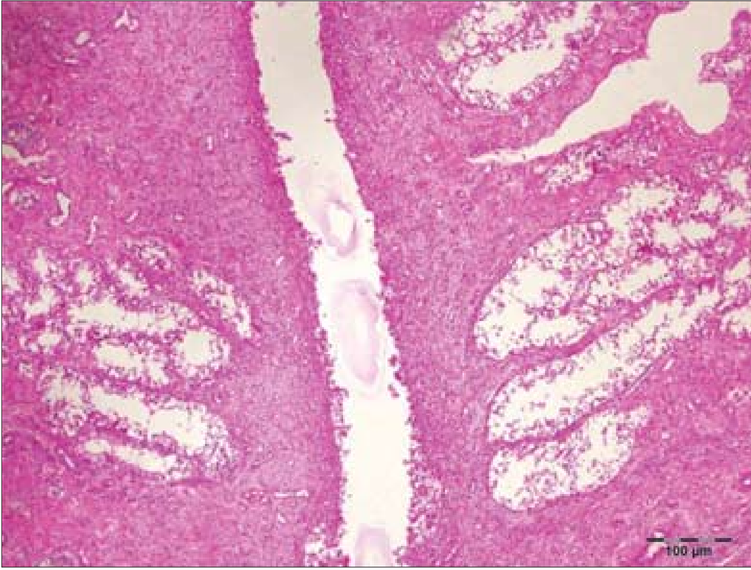
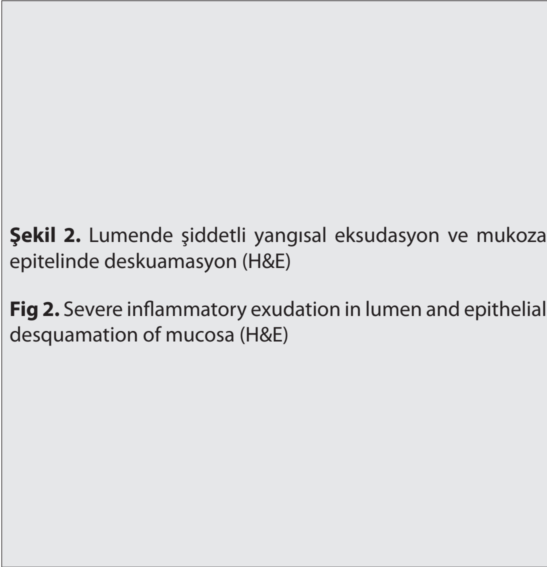


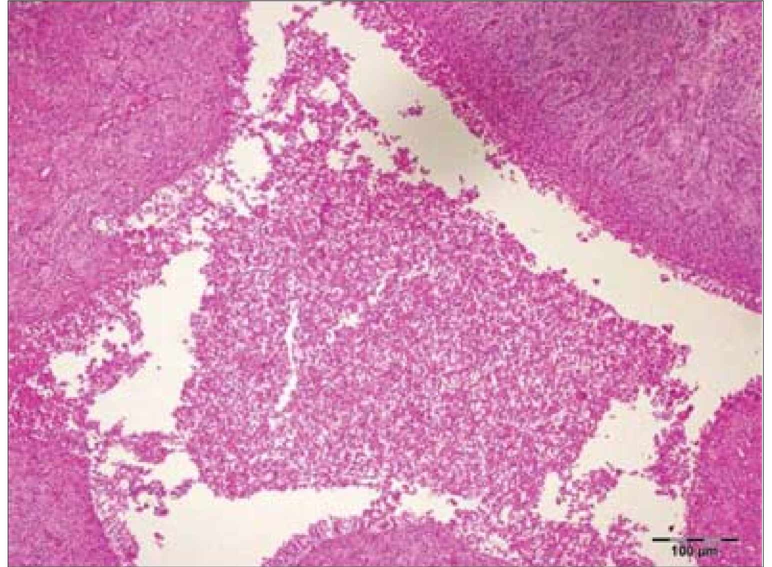
Şekil 1. Endometriyal bezlerde yoğun nekroz ve mukoza epitelinde deskuamasyon (H&E)

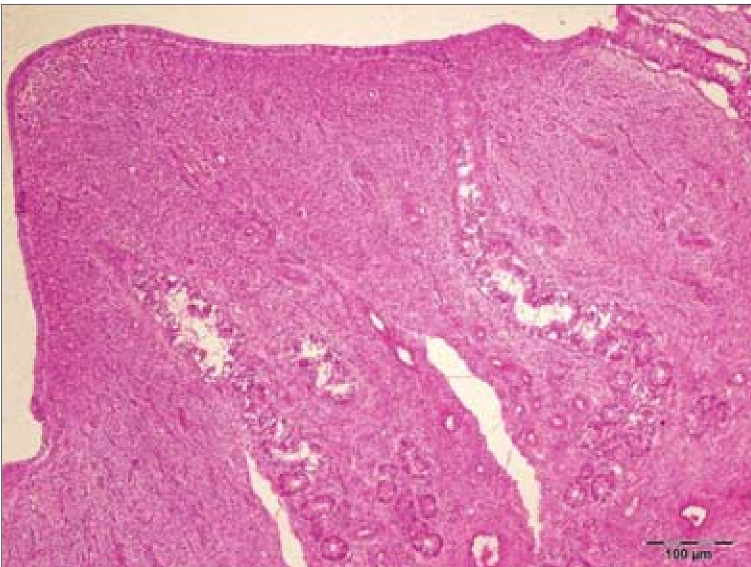
Fig 1. Intensive necrosis in endometrial glands and epithelial desquamation of mucosa (H&E)



Şekil 2. Lumende şiddetli yangısal eksudasyon ve mukoza epitelinde deskuamasyon (H&E)

Fig 2. Severe inflammatory exudation in lumen and epithelial desquamation of mucosa (H&E)





Şekil 3. Yangısal hücre infiltrasyonları ile endometriyal bez epitellerinde hafif düzeyde nekrotik ve dejeneratif değişimler (H&E)

Fig 3. Necrotic and degenerative changes in the endometrial glandular epithelium with inflammatory cell infiltration (H&E)

YAZIM KURALLARI

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3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.
4- ***Makale Türleri***
Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır. Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.
Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.
Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.
Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
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Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.
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Örnek: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.
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