Ankara Üniversitesi Veteriner Fakültesi Dergisi

Veterinary Journal of Ankara Univesity

E-ISSN 1308-2817 Cilt/Volume 69 ● Sayı/Number 3 ● 2022

Ankara Univ Vet Fak Derg - vetjournal.ankara.edu.tr - Open Acsess

E-ISSN 1308-2817

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Veterinary Journal of Ankara University

Cilt/Volume **69** • **Sayı**/Number **3** • **2022**

Ankara Üniversitesi Veteriner Fakültesi Dergisi
Cilt / *Volume*: 69 • Sayı / *Number*: 3 • 2022
Veterinary Journal of Ankara University
Üç ayda bir yayımlanır / *Published three monthly*
E-ISSN 1308-2817

Yönetim Yeri
Adres / *Address*

Ankara Üniversitesi Veteriner Fakültesi Dekanlığı
Yayın Alt Komitesi
06 110 Ankara, Türkiye
Tel: 90 312 317 03 15, **Fax:** 90 312 316 44 72
Sürdürüm ve iletişim: vfdergi@veterinary.ankara.edu.tr
URL: http://vetjournal.ankara.edu.tr
Yayın Türü: Yaygın süreli ve hakemli

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İnternet Adresi / *Web Address*
http://vetjournal.ankara.edu.tr
Yayım Tarihi: 30.06.2022

Ankara Üniversitesi Veteriner Fakültesi Dergisi
Cilt / *Volume*: 69 • Sayı / *Number*: 3 • 2022
Veterinary Journal of Ankara University

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Ankara Univ Vet Fak Derg, **69**, 241-249, 2022
DOI: 10.33988/auvfd.795175

Effects of pomegranate (*Punica granatum L.*) juice as a short-term water supplement during the peak production cycle in laying hens

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Received date: 20.10.2020 - Accepted date: 16.06.2021

Abstract: The objective of the study was to explore the effects of pomegranate juice (PJ) as a water supplement on performance, egg quality, and blood parameters in laying hens. For this purpose, a total of 72 Babcock laying hens, were divided into three groups, one control and two experiments (n = 24). Each main group consists of 4 subgroups, and each subgroup consists of 6 chickens. PJ was administered in drinking water at 0%, 5%, and 10% to the experimental groups for 4 weeks. Feed was offered to all groups *ad libitum.* The addition of PJ had no significant effect on performance parameters, Haugh unit, and egg breaking strength. The yolk color of the 5% PJ group was found to be lighter than the control and other experimental groups (P<0.05). The PJ did not affect serum glucose levels, serum lipid profile, liver enzyme levels, serum total protein levels, most of the hematological parameters, and serum Ca:P ratio in the present study, whereas there were linear and quadratic dose responses in AST, ALT, and HDL. Serum total Ca and P levels of 5% PJ supplemented birds were lower than 10% supplemented PJ birds, whereas it was similar to the control for both (P<0.05). Serum IgG levels were lower in both treatment groups than the control group (P<0.05). In conclusion, using 5% of PJ as a short-term water supplement have some specific effects on the cholesterol metabolism of laying hens rather than performance and immunity.
Keywords: Cholesterol, phenolic content, water additive, yolk color.

Kısa dönemli içme suyu katkısı olarak kullanılan nar (*Punica granatum L.*) suyunun pik verim dönemindeki yumurtacı tavuklara etkileri

Özet: Çalışmanın amacı, bir içme suyu katkısı olarak nar suyunun (NS) yumurtacı tavuklarda performans, yumurta kalitesi ve kan parametreleri üzerine etkilerini incelemekti. Bu amaçla, toplamda 72 Babcock yumurtacı tavuk; biri kontrol, ikisi deneme olmak üzere 3 gruba ayrıldı (n = 24). Her ana grup her biri 6 tavuk içeren 4 tekerrürden oluştu. Dört hafta boyunca deneme gruplarının içme sularına %0, %5 ve %10 NS katıldı. Yem ad libitum sağlandı. Nar suyu ilavesinin performans parametreleri, Haugh birimi ve yumurta kabuk kırılım mukavemeti üzerine önemli bir etkisi olmadı. %5 NS grubunda yumurta sarı rengi kontrol ve diğer deneme grubundan daha açık bulundu (P<0,05). Çalışmada NS, serum glikoz düzeylerini, serum lipit profilini, karaciğer enzim düzeylerini, serum total protein düzeylerini, hematolojik parametrelerin çoğunu ve serum Ca:P oranını etkilemezken; AST, ALT ve HDL'de lineer ve kuadratik doz yanıtları oluştu. %5 NS katkılı tavuklarda serum toplam Ca ve P düzeyleri %10 NS katkılı tavuklardan daha düşük olmuşken (P<0,05); kontrol her ikisine de benzer oldu. Serum IgG düzeyleri her iki uygulama grubunda da kontrol grubuna göre düşüktü (P<0;05). Sonuç olarak; kısa dönem içme suyu katkısı olarak %5 NS, yumurtacı tavuklarda performans ve bağışıklıktan daha ziyade kolesterol metabolizması üzerine bazı spesifik etkilere sahiptir.
Anahtar sözcükler: Fenolik içerik, kolesterol, su katkısı, yumurta sarı rengi.

Introduction

The poultry sector plays an important role in every country's economy and poultry is also one of the most economical and easily accessible protein sources. Many experiments are currently underway to make poultry products better for human health, while humans of all ages consume poultry products such as eggs and meat. In the past, many synthetic antibiotics have been used to protect the health and development of poultry. Currently, herbal extracts are also being explored for their potential as a substitute for synthetic antibiotics without affecting the profitability of poultry businesses in last two decades (5).

Pomegranate (*Punica granatum L.*) is one of the ancient fruit and belongs to the *Punicacea* family. It is mostly cultivated in subtropical regions. Turkey is known as one of the motherland of pomegranate and is among the top four in world production (22). Aril is the edible part of pomegranate and it consists of 52% (w/w) of the total fruit and comprises 78% juice and 22% seeds. The seed of pomegranate is rich in polyunsaturated fatty acids (PUFA), polyphenols, minerals, sugars, vitamins, and polysaccharides (19). Therefore, fruit and byproducts (pulp, juice, oil, and extract) of pomegranate are investigated as a phytochemical source in poultry nutrition (3) as well as human nutrition (30). Similarly, pomegranate juice (PJ) has some anti-inflammatory, antioxidant, anti-atherogenic, and antimicrobial effects due to abundant presence of anthocyanin, ellagic acid derivatives, and hydrolyzable tannins (9). Recently, the molecular effects of pomegranate on lipid metabolism have been reviewed in detail by Hou et al. (14). The authors stated that the active ingredients of pomegranate have widespread effects on a lot of key proteins and genes in the lipid metabolism. Although much research has been done on dietary pomegranate supplementation and its components, there are limited data available on the effects of the PJ supplement in laying hens.

Previous evidence suggests that the supplementation route of additives may lead to a different response in the poultry. Noy and Sklan (21) concluded that in the early stages of the life of the chicks, carbohydrates supplied with drinking water were more effective than dietary carbohydrates. Similarly, Ritzi et al. (23) noted that probiotics as a water supplement had a better impact on broiler feed efficiency compared with the same dietary probiotics. Recently, we observed some different effects of lemon juice, plant extracts, and pomegranate molasses as water additives for laying hens at different production cycles in our research lab, as compared to dietary counterparts of mentioned supplements (11, 12, 15).

Keeping in view the above points, the present study was conducted to investigate the effects of different levels of pomegranate juice on performance, egg quality trait, and blood parameters in laying hens. It was hypothesized that the PJ could sustain and improve the peak production phase of laying hens due to its high antioxidant potential. Also, it was hypothesized that water supplementation route could lead to an improvement on performance parameters on a short-term basis rather than extracts or byproducts in a long-term basis.

Materials and Methods

The current study was conducted at Afyon Kocatepe University Animal Research Center, Afyonkarahisar, following approval by the Local Ethics Committee on Animal Ethical Use under approval No:49533702/119, dated: 07/09/2016.

Birds, experimental design and management: A total of 72 white laying hens (Babcock) aged 30 weeks were divided into three groups with one control group and two treatment groups (n=24) and divided into four subgroups of 6 hens each. The conditions in cage were similar to the previous experiment of our research group (12). In summary, birds were housed in battery cages (width = 48 cm × depth = 45 cm × height = 45 cm) (2 birds/cage, 1080 cm/hen density). Throughout the experiment, the room temperature was recorded for 24 hours a day. The laying hens were provided with sixteen hours of light and eight hours of dark, along with ad libitum feed and free access water. Pomegranate juice was added to all treatment groups with different concentrations i.e. 0%, 5%, and 10% respectively for 4 weeks. In this study, a basal diet was fed to all treatment groups, which was formulated according to the bird's requirement (Table 1) (20).

Table 1. Composition of the basal diet.

Ingredients	%
Corn	54.90
Vegetable oil	0.34
Sunflower meal (%32 CP)	16.93
Full fat soya	10.00
Soyabean meal (%44 CP)	7.39
Limestone	7.87
Dicalcium phosphate	1.73
Salt	0.40
Vitamin-mineral mixture*	0.25
L-lysine HCl	0.10
DL-methionine	0.10
Calculated analyses (%)	
DM.%	87.5
CP.%	16.8
Ether ext.%	4.1
CF.%	5.6
Ash %	11.5
ME.kcal/kg**	2750
Ca.%**	3.71
Av.P.%**	0.38
Na.%**	0.20
Met+Cys%**	0.71
Lysine.%**	0.83
Threonine (%)**	0.61
Tryptophan (%)**	0.20
Linoleic acid (%)**	2.36

*Provided per kg of diet: Vitamin A: 12.000.000 IU, Vitamin D3: 3.000.000IU, Vitamin E: 35.000, Vitamin K3: 3.500, Vitamin B1: 2.750IU, Vitamin B2: 5.500IU, Nicotinamid: 30.000IU, Ca-D-Panthotenate: 10.000IU, Vitamin B6: 4.000IU, Vitamin B12-15IU, Folic acid: 1.000IU, D-Biotin: 50IU,Cholin clorid: 150.000IU, Manganese: 80.000mg, Iron: 60.000 mg, Zinc: 60.000 mg, Copper: 5.000 mg, Iodine: 2.000 mg, Cobalt: 500 mg, Selenium: 150 mg, Antioxidant: 15.000 mg
**Calculated

The experimental diet was prepared, mixed, and packaged at the Animal Research Center. The diet was offered as a mash form. Pomegranate juice (PJ) was prepared and poured in fresh drinking water regularly. The pomegranate fruit was purchased from a local fruit trader weekly and was stored at room temperature until obtaining the juice at the Animal Research Center. A manual hand press was used to obtain the PJ from arils, seeds, and pulp membranes together after removing the outer peels with a knife. After pressing, a metal strainer was used to remove any potential particle from rind, peel, seed or pulp of the fruit. Automatic nipple drinking system was used and each group has a separate water tank. Graduated cylinder glass was used for the scaling of the PJ. Then, PJ was mixed with tap water in a 20-liter water box between 1 p.m. to 2 p.m. every day. The product was easily soluble in the water and homogeneity was confirmed visually. During the study, the water in each tank (control 0%, 5%, and 10%) was refreshed after every 24 hours.

Data collection, calculations, and analyses: Hens were weighted at the beginning and at the end of the study to determine their body weights. No mortality was recorded during the study. Hen-day egg production (HDEP) was recorded daily and feed intake and egg weight of hens were recorded weekly. Egg mass (EM) was calculated as follows:

EM (g) = HDEP (%) × Egg weight (g).

Feed conversion ratio (FCR) values were calculated as follows:

FCR = feed intake (g) / egg mass (g).

At the end of the 4th week, three eggs from each subgroup were collected to determine the parameters of the egg quality. The eggs were kept for 24 hours at room temperature before the egg trait analyses. In these eggs, the eggshell breaking strengths were measured with a semi-automated egg force reader (Model EF 0468-2011, ORKA Food Technology Ltd., Israel) after weighting with a digital scale (two decimal place). The eggs were then poured individually onto a flat glass plate. The heights of the albumen were measured using a digital micrometer. Haugh Unit has been estimated according to: Haugh Unit = $100 \times \log (h - 1.7w^{0.37} + 7.6)$; where h is albumen height (mm) and w is egg weight (g). Three independent laboratory inspectors measured egg yolk color at the same time using Roch Improved Yolk Color Fan (15 bands) on the flat glass under daylight. At the end of the trial, 3 hens from each replicate were randomly selected, and blood was drawn from the heart into two separate vacutainers with and without the heparin as an anticoagulant. Blood samples arrived in the laboratory within 2 hours under a cold chain. Total blood analyser (BC 2800 Vet, Mindray Medical International Ltd., Shenzhen, China) analyzed the samples in heparinized tubes for hematological parameters (Total leukocyte count, WBC; Heterophil count, HC; Neutrophil count, NC; Monocyte count, MC; Red blood cell count, RBC; Hemoglobin, He; Mean corpuscular volume, MCV; Mean corpuscular hemoglobin, MCH; Mean corpuscular hemoglobin concentration, MCHC; Platelet, PLT; Mean platelet volume, MPV). The analyser determined hematocrit levels as follows: Ht = (MCV × RBC) × 0.1. For serum biochemical analyses, the samples in vacutainer tubes were centrifuged at 5 000 rpm for 10 minutes. Supernatants were transferred to Eppendorf tubes and stored at -20 °C till biochemical analyses. Serum glucose, total cholesterol (CHO), high density lipoprotein (HDL), low density lipoprotein (LDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), Gamma-Glutamyl Transpeptidase (GGT), total protein (TPRO), phosphorus, calcium and Immunoglobulin G (IgG) concentrations were determined by automated ELISA analyser (Elisys Uno, Human mbH, Wiesbaden, Germany) in a commercial diagnostic laboratory.

Phenolic content of the samples was determined using the Folin-Ciocalteu method (13) with slight modifications. Absorbance was read at 765 nm. The results were expressed as gallic acid equivalents (GAE) in mg GAE/L ± standard deviation. The data were obtained from the average of three determinations. Also, the effect of PJ on pH levels of drinking water was simulated *in vitro* with a portable digital pH meter at 0 h and 23rd h.

Statistical analysis: The data were analyzed in a randomized block design with cage as the experimental unit. The data was evaluated using the MIXED procedure of SAS (version 9.4, SAS Institute, Carry, NC) with the fixed effects of treatment. Cage and replicate (subgroup) were included as random effects. Degrees of freedom were calculated by using Kenward-Roger approach in the MODEL statement of SAS. The PDIFF statement was used for multiple comparisons as a post-hoc test. Outliers were deleted if studentized residuals > 4 or < -4 before the analysis. Then, normality tests (Shapiro-Wilk test, Kolmogorov-Smirnov test, Anderson-Darling test, and Cramer-von Mises test) were performed with UNIVARIATE statement of SAS and all data had a normal distribution. The polynomial contrasts (control vs. treatment diets, linear and quadratic trends) were used to evaluate responses to the PJ supplementation with different doses. Coefficients of contrasts were validated with IML procedure of SAS (17). All data are reported as LSMEANS with SEM in tables. The significance level was set at $P \leq 0.05$ for main effect and control *vs*. treatment contrast and it was assumed at $0.05 < P \leq 0.15$ for tendency (linear and quadratic).

Results

The total phenolic content of the PJ was 3661.9 ± 209.9 mg gallic acid equivalent (GAE)/L. Also, the average pH of tap water with 0%, 5%, 10% supplemental PJ were 6.92, 4.53, and 4.01, respectively.

Initial body weight (BW) was similar among the group before the study (P=0.64). Similarly, PJ supplementation to drinking water did not affect the final BW of the birds after 4 weeks. Also, feed intake, egg production, FCR, egg mass, and egg weights were not affected by PJ supplementation during the study. There was no difference in control *vs* treatments (5% and 10% supplemented PJ) contrasts or trend analysis for increased PJ levels in the drinking water (Table 2).

The PJ supplementation did not affect the breaking strength and Haugh Unit of the eggs (P=0.60 and P=0.42, respectively). Also, there was no linear or quadratic trend of the aforementioned parameters as a response to increased PJ drinking water supplementation. However, yolk colors of eggs were significantly lighter in 5% supplemented PJ group than the eggs from other groups (P=0.047). Although there was no significant difference for control *vs* treatments (5% and 10% supplemented PJ), there was a quadratic trend to respond to increased PJ supplementation (P=0.08) in yolk color (Table 3).

Supplementation of PJ to drinking water did not affect serum glucose levels, serum lipid profile, liver enzyme levels, and serum total protein levels in the present study. Also, there were no control *vs* treatments differences (5% and 10% supplemented PJ) (P>0.15) for the mentioned parameters. However, there was a linear trend for increased levels of PJ supplementation for both serum levels of AST and ALT (P=0.12 and P=0.10, respectively). Also, serum HDL levels had a quadratic trend for increased levels of PJ supplementation in the study (P=0.07). Serum total phosphorus and serum total calcium levels of 5% PJ supplemented laying hens were significantly lower than the 10% PJ supplemented birds (P=0.049 and P=0.04, respectively), while the control birds had no significant difference compared with both treatment groups for mentioned parameters. Also, both parameters had a quadratic response to increased PJ supplementation (P=0.06 for phosphorus and P=0.08 for calcium). Although PJ drinking water supplementation had no main effect on serum Ca:P (P=0.35), the ratio had a quadratic response to increased PJ supplementation (P=0.15). Serum IgG levels of both 5% PJ supplemented and 10% PJ supplemented birds were significantly lower than the control birds (P=0.03). Also, there was a significant difference for the control vs treatments (5% and 10% PJ supplementation) contrast. (P=0.02). Both a linear and a quadratic response were observed on serum IgG levels of birds during PJ supplementation to drinking water from 0% to 10% (P=0.06 and P=0.15, respectively) (Table 4).

Table 2. Effects of short-term water supplement of pomegranate juice on performance of laying hens during peak period[1].

	Treatments				P-values			
					Treatment	Contrast[6]		
Item	0%	5%	10%	SEM[5]		C *vs* T	Linear	Quadratic
Feed intake, g/day	104.38	98.00	103.13	5.31	0.68	0.57	0.87	0.40
Egg weight, g	59.68	59.88	59.39	0.78	0.91	0.96	0.80	0.73
HDEP, %[2]	80.66	77.88	78.89	4.00	0.89	0.65	0.76	0.71
Egg mass, g	48.10	46.56	46.83	2.17	0.87	0.61	0.69	0.74
FCR[3]	2.18	2.11	2.23	0.14	0.86	0.97	0.81	0.63
Initial BW[4], g	1591.43	1625.82	1630.28	31.72	0.64	0.35	0.39	0.70
Final BW[4], g	1480.38	1542.30	1536.24	37.62	0.44	0.21	0.30	0.46

[1]Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n=24).
[2]Hen day egg production.
[3]Feed conversion ration
[4]Body weight
[5]Standard error of the mean
[6]Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS
a,b,c Values with different superscripts in the same column are significantly different (P≤0.05) and for tendency declared at 0.05<P<0.15.

Table 3. Effects of short-term water supplement of pomegranate juice on egg traits of laying hens during peak period[1].

	Treatments				P-values			
					Treatment	Contrast[3]		
Item	0%	5%	10%	SEM[2]		C *vs* T	Linear	Quadratic
Breaking Strenght, kg/cm[2]	40.63	36.79	37.67	2.78	0.60	0.33	0.46	0.49
Haugh Unit	71.70	66.50	65.12	3.64	0.42	0.20	0.21	0.67
Yolk Color	6.41[a]	5.29[b]	5.88[ab]	0.39	0.05	0.09	0.34	0.08

[1]Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n=24).
[2]Standard error of the mean
[3]Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS
[a,b,c]Values with different superscripts in the same column are significantly different (P≤0.05) and for tendency declared at 0.05<P<0.15.

Table 4. Effects of short-term water supplement of pomegranate juice on serum biochemical parameters of laying hens during peak period[1].

	Treatments				P-values			
					Treatment	Contrast[9]		
Item	0%	5%	10%	SEM[8]		C *vs* T	Linear	Quadratic
Glucose, mg/dL	220.10	224.60	206.60	10.13	0.44	0.72	0.35	0.37
Cholesterol, mg/dL	84.50	82.60	93.00	12.23	0.82	0.83	0.63	0.68
HDL[2], mg/dL	21.10	27.70	21.90	2.70	0.19	0.27	0.84	0.07
LDL[3], mg/dL	35.70	29.90	36.50	7.55	0.80	0.79	0.94	0.51
AST[4], U/L	188.20	227.56	226.90	17.46	0.20	0.08	0.12	0.37
ALT[5], U/L	2.10	2.50	3.60	0.62	0.23	0.22	0.10	0.65
GGT[6], U/L	28.10	29.10	30.00	1.86	0.77	0.53	0.48	0.98
Total Protein, g/dL	4.81	4.99	4.92	0.32	0.92	0.72	0.81	0.75
Phosphorus, mg/dL	4.78^{ab}	4.01^{b}	5.18^{a}	0.40	0.05	0.71	0.49	0.06
Calcium, mg/dL	18.69^{ab}	17.18^{b}	20.64^{a}	1.11	0.04	0.87	0.22	0.08
Ca/P	4.03	4.66	4.07	0.33	0.35	0.42	0.93	0.15
IgG[7], mg/dL	48.26^{a}	17.19^{b}	21.42^{b}	9.64	0.03	0.02	0.06	0.15

[1]Data are represented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n=24).
[2]High density lipoprotein
[3]Low density lipoprotein
[4]Aspartate transaminase
[5]Alanine transaminase
[6]Gamma-glutamyl transferase
[7]Immunoglobulin G
[8]Standard error of the mean
[9]Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS
[a,b,c] Values with different superscripts in the same column are significantly different (P≤0.05) and for tendency declared at 0.05<P<0.15.

Table 5. Effects of short-term water supplement of pomegranate juice on hematology parameters of laying hens during peak period[1].

	Treatments					P-values			
					Reference	Treatment	Contrast[15]		
Item	0%	5%	10%	SEM[14]			C *vs* T	Linear	Quadratic
WBC[2], x10^{10}/L	220.10	224.60	206.60	10.13	1.2-3.0	0.44	0.72	0.35	0.37
HC[3], x10^{10}/L	84.50	82.60	93.00	12.23	0.7-1.76	0.82	0.83	0.63	0.68
NC[4], x10^{10}/L	21.10	27.70	21.90	2.70	0.3-0.6	0.19	0.27	0.84	0.07
MC[5], x10^{10}/L	35.70	29.90	36.50	7.55	0.01-0.20	0.80	0.79	0.94	0.51
RBC[6], x10^{12}/L	188.20	227.56	226.90	17.46	2.5-3.5	0.20	0.08	0.12	0.37
He[7], g/dL	2.10	2.50	3.60	0.62	7-13	0.23	0.22	0.10	0.65
Ht[8], %	28.10	29.10	30.00	1.86	32-36	0.77	0.53	0.48	0.98
MCV[9], fL	4.81	4.99	4.92	0.32	90-140	0.92	0.72	0.81	0.75
MCH[10], pg	4.78^{ab}	4.01^{b}	5.18^{a}	0.40	33-47	0.05	0.71	0.49	0.06
MCHC[11], %	18.69^{ab}	17.18^{b}	20.64^{a}	1.11	26-35	0.04	0.87	0.22	0.08
PLT[12], x10^{10}/L	4.03	4.66	4.07	0.33	n.a.	0.35	0.42	0.93	0.15
MPV[13], fL	48.26^{a}	17.19^{b}	21.42^{b}	9.64	n.a.	0.03	0.02	0.06	0.15

[1]Data are presented as least square means. The values are means of 4 replicate cages per diet with 6 hens (n=24).
[2]White blood cell, total leukocyte count
[3]Heterophil count
[4]Neutrophil count
[5]Monocyte count
[6]Red blood count
[7]Hemoglobin
[8]Hematocrit
[9]Mean corpuscular volume
[10]Mean corpuscular hemoglobin
[11]Mean corpuscular hemoglobin concentration
[12]Platelet
[13]Mean platelet volume
[14]Standard error of the mean
[15]Coefficients of contrast for unequally spaced particle size levels were calculated using PROC IML of SAS
[a,b,c] Values with different superscripts in the same column are significantly different (P≤0.05) and for tendency declared at 0.05<P<0.15.

Drinking water supplementation of PJ did not affect hematological parameters in the study, except mean corpuscular hemoglobin concentration (MCH) which was significantly higher in 5% PJ supplemented groups than the other groups (P=0.02). Also, there was a control vs treatments contrast in MHC levels (P=0.05). Quadratic trends for white blood cell count (WBC), MC, hemoglobin level, and MPV were observed as a response to increased PJ supplementation from 0% to 10% (P=0.11, P=0.06, P=0.15, and P=0.08, respectively) (Table 5).

Discussion and Conclusion

Researchers have been interested in pomegranate fruit for many years because of the abundance of polyphenolic flavonoid content and a broad range of health effects (30). Gozlekci et al (10) reported that the total phenolic content in PJ was lower than the PJ used in the current study (1218.2 *vs* 3661.9 mg GAE/L). The same researchers observed that pomegranate peels had the highest total phenolic content among the other parts of the fruit (10). In this study, pulp membranes were not removed during the hand pressing, although outer membranes (peels) were removed. The pulp membranes may have increase the total phenolic content of PJ used in the present study.

While much research has been done on the supplementation of pomegranate and its components, there is limited data on the effects of the PJ supplementation on laying hens. In the present study, the PJ supplementation did not affect feed intake, egg production, FCR, egg mass, and egg weight. The effects of dietary pomegranate by-products on the performance of laying hens were variable in previous reports. Kostogrys et al. (16) reported increased feed intake, egg production, and egg mass as a dose-dependent form of supplementation of pomegranate seed oil to laying hens' diet. Similarly, Abbas et al. (2) observed an improvement in egg production and feed intake of Japanese quails supplemented with pomegranate peel powder. However, Ghahtan et al. (8) recorded no effect of dietary supplementation of 1% pomegranate peel powder on egg production in laying quails until 10 weeks of the study, whereas 2% supplemented birds were lower in egg production than the control birds in the same research. Sharma et al. (27) also determined the effects of a commercial blend which includes active ingredients from thyme and garlic as well as pomegranate as a water supplement similar to the present study in laying hens. The authors stated that water supplementation of the blend included ellagic acid from pomegranate affected egg production and feed intake for two weeks, whereas there was no effect of the blend for other time points between 65th-73rd weeks of age. While Saki et al. (24) observed significantly higher egg production in laying hens supplemented with 5% dietary pomegranate seed pulp compared to control birds, the authors noted no difference between the groups supplemented with 10% and 15% dietary pomegranate seed pulp and the control birds. Similar to our findings, the authors stated that dietary pomegranate seed pulp had no effect on feed intake, egg weight, egg mass, and FCR. In an earlier study of our research lab, we observed that both 1% and 2.5% of drinking water supplementations of lemon juice were increased egg production of aged laying hens, whereas it did not affect the lowest or highest doses of supplementation (0.5% or 5%) inconsistent with our findings (11). However, water supplementation of lemon juice did not affect feed intake, egg weight, and FCR in the aforementioned study similar to our findings. Gil et al. (9) reported that pomegranate products' antioxidant capacity and active ingredients depend not only on the type of by-product (e.g. seed pulp, extract, juice) but also on the processing of the same type of products. The evidence suggests that variation of the effects of pomegranate by products in previous and current research on laying hens models may be explained in the aforementioned studies by different sources of pomegranate by products. Moreover, antioxidant activities of the different pomegranate by-products are variable because of different amounts of total phenolics which acts as hydrogen donors and reducing agents (10). However, the evidence from previous studies indicated that dietary supplementation of pomegranate by-products, similar to our findings, did not affect the body weight of laying hens or quails (2, 11, 24). Although there were no significant differences between the groups related to performance parameters, it is expected that BW should be from 1651 to 1683 grams at 30-34 weeks of age according to Production Guideline (4). However, the initial weights of our experimental birds were lower than expected weights in all groups. Also, the product guide is expected 60.5-61.7 grams egg weight at the same period. It is also higher than our experiment results. It may be explained by the effect of different lines/breeders. Although there was no water intake data in the current study as a weak point, we assumed that the PJ supplementation had no negative effect on the acceptability of the drinking water. Because the strong correlation between feed and water intake is a well-known phenomena.

In the present study, the addition of PJ drinking water did not affect the breaking strength of the eggshell. In agreement with our findings, Saki et al. (24) reported no effect of dietary pomegranate seed pulp on eggshell breaking strength from laying hens. Similarly, Sharma et al. (27) did not observe any effect of a commercial blend which includes active ingredients from thyme and garlic

as well as pomegranate as a water supplement on the eggshell breaking strength of supplemented laying hens. In an earlier study of our research lab, we stated that the addition of myrtle plant extract to drinking water did not affect the eggshell breaking strength of supplemented laying hens (12).

It is well known that eggshell thickness and specific gravity are important markers for eggshell breaking strength during the storage and processing of table eggs (29). The numerous evidence suggests that dietary supplementation of pomegranate byproducts does not affect specific gravity and eggshell thickness as an indirect marker of eggshell breaking strength in laying hens and quails (8, 27). Also, Saki et al. (25) reported that dietary supplementation of pomegranate by products did not affect the specific gravity of eggs in the study. Although the authors observed an increase for eggshell thickness in high dose groups (8% and 12% supplementation), there was no observed effect in the lower dose group (4% supplementation) in the aforementioned study. Furthermore, the previous evidence stated that water supplementation of lemon juice (11) and myrtle plant extract (12) did not affect eggshell thickness of the supplemented laying hens. In the present study, Haugh Unit was shown no improvement by supplementation of PJ in the drinking water of laying hens in agreement with the previous reports about dietary supplementation of pomegranate by products in laying hens and quails (8, 16, 24, 25). However, Sharma et al. (27) reported that a commercial blend as a water supplement containing the active ingredients of thyme, garlic, and pomegranate resulted in Haugh Unit improvement in laying hens. In an earlier study of our research lab, we observed that drinking water supplementation of myrtle plant extract did not affect Haugh Units of eggs (12). However, in a consecutive study, we observed that drinking water supplementation of lemon juice led to an improvement in Haugh Unit for 1% lemon juice, whereas there was no improvement for 0.5%, 2.5%, and 5% lemon juice (11). The evidence suggests that supplementation route (dietary or water supplement) of the same nutrients or additives may lead to a different response in poultry (21, 23, 28). For Haugh Unit, the conflicting results of pomegranate water supplements which were reported by Sharma et al. (27) with the others may be explained by having a different supplementation route.

In the present study, 5% of PJ water supplement resulted in lighter egg yolk. But the higher dose of PJ (10%) did not change the egg yolk color in the study. The effects of dietary pomegranate by products have been variable. Kostogrys et al. (16) found that dietary pomegranate seed oil improved yolk color scores at the study's highest dose (1.5%), while dietary supplementation did not alter color scores at lower doses (0.5% and 1%). Sharma et al. (27) also confirmed that a commercial dietary supplement that is a mixture of active ingredients of thyme, garlic, and pomegranate peel has resulted in darker yolk color in treatment groups than the control birds. Different routes of supplementations (dietary supplement vs. water supplement) may explain the conflicting results from the reported studies and our findings. In an earlier study of our research lab, we stated that the myrtle plant extract that is a weak acid/neutral characteristic (pH: 5-7) (26) did not change the laying hens' yolk color (12). In agreement with our findings in birds supplemented by 5 percent PJ, we also observed that supplementation of 5 percent lemon juice which has a strong acidic characteristic led to lighter yolk color in laying hens (11). Although PJ itself has a strongly acidic (pH: 2.9-3.10) property (7), PJ-supplemented drinking water samples did not show a strong acidic characteristic compared to plain water in the present study. The lighter egg yolk color scores of 5 percent PJ supplemented birds can be explained by the high phenolic content of PJ resulting in high antioxidant activity rather than the acidic characteristic of PJ. Furthermore, we observed that water supplementation of pomegranate molasses affected yolk color and yolk index (15).

The pomegranate has beneficial effects on lipid metabolism (14). Lv et al. (18) stated that pomegranate peel polyphenols, punicalagin, and pomegranate ellagic improved cholesterol catabolism via increasing total bile acid production in the liver cells. In the present study, we observed linear response of serum ALT and serum AST levels to PJ supplementation which may be explained as an indirect result of promoting total bile acid production in the liver. Liver X receptors and target gene of the mentioned receptors (ATP-binding cassette transporter A1 - ABCA1) have a key role in cholesterol metabolism of the body. Liver X receptors stimulate HDL production and the HDL contributes to cholesterol balancing by transporting peripheral tissue cholesterol to liver (14). Zhao et al. (31) observed that pomegranate polyphenol significantly increased mRNA expression of the Liver X receptor and ABCA1 in the hepatic cells. In our study, the PJ had higher total phenolic content than the previous reports (10). It may explain the observed quadratic response of HDL to PJ supplementation in the present study. A-Gonzalez et al. (1) stated that loss of the function of the Liver X receptors led to an increasing in IgG producing cells in the spleen. So, potentially enhanced expression of Liver X receptors with supplementation of PJ can explain lower serum IgG levels of both treatment groups than the control birds in the study. Furthermore, Caldas et al. (6) reported that enhanced Liver X receptors significantly decreased the relative abundance of intestinal

and renal sodium/phosphate (NaPi) cotransporters. Lacking the NaPi cotransporters causes hypophosphatemia and hypercalciuria (1). It may explain both lower serum phosphorus and serum calcium levels in PJ supplemented birds than the control birds. Also, the constant Ca:P ratio despite the decrease in serum Ca and serum P levels may explain why it had no negative effect on egg production and quality.

As a conclusion, using 5% of PJ as a short-term water supplement have some dose-dependent specific effects on the cholesterol metabolism of laying hens rather than performance and immunity of the birds due to the potential effects of abundant phenolic content on reverse cholesterol transport systems in the liver.

Acknowledgements

The authors thank the staff of Afyon Kocatepe University, Animal Teaching & Research Center (Afyonkarahisar, Turkey) for their assistance with this study.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

The current study was conducted at Afyon Kocatepe University Animal Research Center, Afyonkarahisar, following approval by the Local Ethics Committee on Animal Ethical Use under approval No:49533702/119, dated: 07/09/2016.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **A-Gonzalez N, Bensinger SJ, Hong C, et al** (2009): *Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR.* Immunity, **31**, 1276-1281.
2. **Abbas RJ, Al-Salhie KCK, Al-Hummod SKM** (2017): *The effect of using different levels of pomegranate (Punica granatum) peel powder on productive and physiological performance of japanese quail (Coturnix coturnix japonica)*. Livest Res Rural Dev, **29,** 1-7.
3. **Ahmed ST, Islam MM, Bostami ABMR, et al** (2015): *Meat composition, fatty acid profile and oxidative stability of meat from broilers supplemented with pomegranate (Punica granatum L.) by-products*. Food Chem, **188,** 481-488.
4. **Anonymus** (2020): Babcock White product guideline. Available at https://www.babcock-poultry.com/documents/562/Babcock_White_CS_cage_ English_guide.pdf (Accessed December 18, 2020).
5. **Bozkurt M, Hippenstiel F, Abdel-Wareth A, et al** (2014): *Effects of selected herbs and essential oils on performance, egg quality and some metabolic activities in laying hens – a review.* Eur Poult Sci, **78,** 1-15.
6. **Caldas YA, Giral H, Cortázar MA, et al** (2011): *Liver X receptor-activating ligands modulate renal and intestinal sodium–phosphate transporters.* Kidney Int, **80,** 535-544.
7. **Darvishi H, Khostaghaza MH, Najafi G** (2013): *Ohmic heating of pomegranate juice: Electrical conductivity and pH change.* J Saudi Soc Agric Sci, **12,** 101-108.
8. **Ghahtan N, Kohanmoo MA, Habibi H** (2019): *Evaluation of dietary medicinal plants and algae in laying Japanese quails.* J World's Poult Res, **9,** 82-88.
9. **Gil MI, Tomás-Barberán FA, Hess-Pierce B, et al** (2000): *Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing.* J Agric Food Chem, **48,** 4581-4599.
10. **Gozlekci S, Saracoglu O, Onursal E, et al** (2011): *Total phenolic distribution of juice, peel, and seed extracts of four pomegranate cultivars.* Pharmacogn Mag, **7**, 161-164.
11. **Gultepe EE, Iqbal A, Cetingul IS, et al** (2019): *Effects of lemon juice on performance, egg quality trait, and some blood parameters of laying hens in the late phase of production.* Acta Vet Eurasia, **45,** 56-62.
12. **Gultepe EE, Iqbal A, Cetingul S, et al** (2019): *Effect of Myrtus communis L. plant extract as a drinking water supplement on performance, some blood parameters, egg quality and immune response of older laying hens.* Kafkas Univ Vet Fak Derg, **25,** 1-8.
13. **Guvenc A, Akkol EK, Hurkul MM, et al** (2012): *Wound healing and anti-inflammatory activities of the Michauxia L'Hérit (Campanulaceae) species native to Turkey.* Journal of Ethnopharmacology, **139**, 401-408.
14. **Hou C, Zhang W, Li J, et al** (2019): *Beneficial effects of pomegranate on lipid metabolism in metabolic disorders.* Mol Nutr Food Res, **63,** 1800773.
15. **Iqbal A, Bayram I, Gültepe EE, et al** (2020): *Effect of different level of pomegranate molasses on performance , egg quality trait, serological and hematological parameters in older laying hens*. Prog Nutr, **22,** e2020052.
16. **Kostogrys RB, Filipiak-Florkiewicz A, Dereń K, et al** (2017): *Effect of dietary pomegranate seed oil on laying hen performance and physicochemical properties of eggs.* Food Chem, **221,** 1096-1103.
17. **Littell RC, Stroup WW, Freund RJ** (2002): SAS for Linear Models. 4th edition. SAS Institute Inc, Cary, NC, USA.
18. **Lv O, Wang L, Li J, et al** (2016): *Effects of pomegranate peel polyphenols on lipid accumulation and cholesterol metabolic transformation in L-02 human hepatic cells via the PPARγ-ABCA1/CYP7A1 pathway.* Food Funct, **7,** 4976-4983.
19. **Miguel G, Fontes C, Antunes D, et al** (2004): *Anthocyanin concentration of "Assaria" pomegranate fruits during different cold storage conditions.* J Biomed Biotechnol, **2004,** 338-342.
20. **National Research Council** (1994): Nutrient Requirements of Poultry. 9th edition. National Academy Press, Washington, D.C., USA.

21. **Noy Y, Sklan D** (1999): *Different types of early feeding and performance in chicks and poults.* J Appl Poult Res, **8,** 16-24.
22. **Ozkal N, Dinc S** (1993): *Valuation of the pomegranate (Punica granatum L.) peels from the standpoint of pharmacy.* Ankara Univ Eczac Fak Derg, **22,** 1-2.
23. **Ritzi MM, Abdelrahman W, Mohnl M, et al** (2014): *Effects of probiotics and application methods on performance and response of broiler chickens to an Eimeria challenge.* Poult Sci, **93,** 2772-2778.
24. **Saki AA, Rabet M, Zamani P, et al** (2014): *The effects of different levels of pomegranate seed pulp with multi-enzyme on performance, egg quality and serum antioxidant in laying hens.* Iran J Appl Anim Sci, **4,** 803-808.
25. **Saki AA, Shamsollah T, Ashoori A** (2019): *Egg iron enrichment in response to various levels of pomegranate by-product in laying hen diet.* Iran J Appl Anim Sci, **9,** 747-754.
26. **Şan B, Yildirim AN, Polat M, et al** (2015): *Chemical compositions of myrtle (Myrtus communis L.) genotypes having bluish-black and yellowish-white fruits.* Erwerbs-Obstbau, **57,** 203-210.
27. **Sharma MK, Dinh T, Adhikari PA** (2020): *Production performance, egg quality, and small intestine histomorphology of the laying hens supplemented with phytogenic feed additive.* J Appl Poult Res, **29,** 362-371.
28. **Torshizi MAK, Moghaddam AR, Rahimi S, et al** (2010): *Assessing the effect of administering probiotics in water or as a feed supplement on broiler performance and immune response.* Br Poult Sci, **51,** 178-184.
29. **Wells RG** (1967): *Egg shell strength.* Br Poult Sci, **8,** 193-199.
30. **Yilmaz B, Usta C** (2010). *Therapeutic use of pomegranate (Punica granatum).* Turkish J Fam Pract, **14,** 146-153.
31. **Zhao W, Li J, He X, et al** (2014): *In vitro steatosis hepatic cell model to compare the lipid-lowering effects of pomegranate peel polyphenols with several other plant polyphenols as well as its related cholesterol efflux mechanisms.* Toxicol Reports, **1,** 945-954.

Ankara Univ Vet Fak Derg, **69**, 251-257, 2022
DOI: 10.33988/auvfd.869204

Effects of medetomidine/ketamine and xylazine/ketamine anesthesia and their reversal by atipamezole on ocular parameters and monitored anesthesia care in cats

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Received date: 28.01.2021 - Accepted date: 08.07.2021

Abstract: The aim of this study was to investigate the impact of the general anesthetic drug ketamine and premedication agents medetomidine and xylazine, and their reversal by atipamezole, on monitored anesthesia care values and ocular parameters such as intraocular pressure, horizontal pupillar diameter, and Schirmer tear test in cats. A randomized, single-blinded study was conducted. Twenty intact female cats (weiging between 2.2 and 3.6 kg, and 0.5 to 5.5 years of age) referred for ovariohysterectomy (OHE) procedure by the owners at regular intervals over 4 months were included in the study. The cats were randomly divided into two groups containing 10 animals in each group. The cats were premedicated with medetomidine 80 µg/kg intramuscular in group 1 while the cats in the 2nd group were premedicated with xylazine hydrochloride 2 mg/kg intramuscular. After the OHE procedure was ended, anesthesia regimes were reversed by using atipamezole 200 µg/kg intramuscularly. Monitoring of respiration rate, heart rate, mean arterial pressure, peripheral arterial oxygen saturation, and body temperature were conducted using a patient monitor at T0, T1, T2, T3, and T4 time points. Both groups showed declines in intraocular pressure and increases in horizontal pupil diameter after anesthesia induction (T0 vs. T1, all, $P<0.05$); however, the chancing and recovery pattern of intraocular pressure and horizontal pupil diameter showed intergroup difference. In conclusion, xylazine/ketamine is more effective than medetomidine/ketamine in attenuating the intraocular pressure, increasing the horizontal pupil diameter, and alteration the monitored anesthesia care response in the general anesthesia.
Keywords: Anesthesia, atipamezole, cat, ocular.

Kedilerde medetomidin/ketamin ve ksilazin/ketamin anestezisinin ve bunların atipamezol ile etkilerinin ters çevrilmesinin oküler ve anestezi izlem parametreleri üzerindeki etkileri

Özet: Bu çalışmanın amacı, genel anestezik ilaç ketamin ve premedikasyon ilaçları medetomidin ve ksilazinin; ve bunların etkilerinin atipamezol ile tersine çevrilmesinin göz içi basıncı, yatay pupil çapı ve Schirmer gözyaşı testi gibi kedi oküler ve anestezi izlem parametreleri üzerindeki etkilerini araştırmaktı. Rastlantısal, tek tarflı kör bir çalışma yürütüldü. Sahipleri tarafından 4 aylık süre içerisinde ovaryohisterektomi operasyonu için başvurulan 20 erişkin dişi kedi (2,2 ila 3,6 kg ve 0,5 ila 5,5 yaş arasında) çalışmaya dahil edildi. Kediler, her grupta on kedinin olduğu iki gruptan birine rastlantısal olarak dahil edildi. Grup 1'de kediler kas içi 80 µg/kg medetomidin ile premedike edilirken grup 2 deki kediler kas içi ksilazin hidroklorür 2 mg/kg ile premedike edildi. Ovariohisterektomi işlemi bittikten sonra anestezik etki kasiçi atipamezol 200 µg/kg kullanılarak ters çevrildi. Solunum sayısı, kalp atım hızı, ortalama arter kan basıncı, periferik arteriyel oksijen düzeyi ve vücut ısının izlenmesi T0, T1, T2, T3 ve T4 zaman noktalarında hastabaşı monitör kullanılarak gerçekleştirildi. Her iki grup da anestezi indüksiyonundan sonra istatistiki olarak önemli olan göz içi basıncında düşüş ve yatay pupil çapında artış gösterdi (T0'a karşı T1, tümü, $P<0,05$); bununla birlikte, göz içi basıncı ve yatay pupil çapının anestezideki değişim ve başlangıç değerine dönüşler gruplar arası farklıydı. Sonuç olarak ksilazin/ketamin kombinasyonu, genel anestezide göz içi basıncını azaltmada, yatay pupil çapını artırmada ve izlenen anestezi bakım parametrelerini değiştirmede medetomidin/ketamin kombinasyonundan daha etkilidir.
Anahtar sözcükler: Anestezi, atipamezol, kedi, oküler.

Introduction

Anesthesia drugs and endotracheal intubation have vitally significant effects on the cardiovascular and pulmonary systems. At the same time, they cause sudden increases in intraocular pressure. This leads to severe complications occurring in surgical interventions performed on patients, in particular, those with ocular trauma or glaucoma (4, 18, 19, 23, 24, 37, 45).

Increasing intraocular pressure (IOP) in ophthalmic surgery has always been problematic for the surgeon and it is necessary to prevent the elevation of IOP (2, 32). Anesthesia for a patient with a penetrating eye injury and a full stomach is a challenge to the anesthesiologist. In these cases the aim of anesthesia is rapid sequence induction without increasing IOP. The anesthesiologist must plan carrefully the risk of aspiration against the risk of blindness in the injured eye that could result from elevated IOP and extrusion of ocular contents (2).

Monitored anesthesia care (MACr) has been utilized to ensure relief from anxiety, sedation, minimal memory loss, and comfort throughout diagnostic or therapeutic applications with analgesia and sedation (15, 26). Respiratory decrease is associated with the most significant case injuries in MACr (16, 26). The goal of MACr is the management of anesthesia and maintenance of optimal cardiac functions without intense respiratory down and airway obstruction. The ability to quickly modulate the depth of anesthesia when needed is also a significant aspect of MACr. Several narcotics, analgesics and sedatives are utilized to obtain these aims while minimizing side effects (26).

Anesthesia with the combination of medetomidine/ketamine (MED-KET) and xylazine/ketamine (XYZ-KET), each of which can be antagonized by atipamezole, has been described as a useful anesthetic technique for animals (1, 6, 31). Obvious benefits of these drug combinations are related to the competitive reversibility by atipamezole for all components, which leads to improved control of anesthetic depth, a shorter recovery phase and lower occurance of hypothermia (1, 23, 31).

While there has been extensive research on some general anesthesia and premedication agent pharmacodynamics and pharmacokinetics, information on their impact on MACr and ocular parameters, such as IOP, horizontal pupil diameter (HPD), and Schirmer's tear test (STT) in animals and particularly cats, are few, deficient and rather contentious (6, 26). To our knowledge, no articles have focused on the impact of medetomidine/ketamine and xylazine/ketamine anesthesia on ocular parameters and MACr values in cats. The aim of this study was to investigate the impact of the general anesthetic drug ketamine and premedication agents medetomidine and xylazine, and their reversal by atipamezole, on monitored anesthesia care values and ocular parameters such as intraocular pressure, horizontal pupillar diameter, and Schirmer tear test in cats.

Materials and Methods

The experimental procedure was authorized by the Local Ethics Committee of Kyrgyz Turkish Manas University (Approval Number: 2016-03/2). A randomized, single-blinded study was conducted.

Sampling: Twenty intact female cats (weighing between 2.2 and 3.6 kg, and 0.5 to 5.5 years of age) referred for ovariohysterectomy (OVH) procedure by owner at regular intervals over 4 months were included in the study.

Experimental procedure: The cats were randomly divided into two groups containing 10 animals in each group (T0, baseline). Carprofen (Rimadyl®, Zoetis, USA) 2 mg/kg IV was applied to all cats for analgesia. The cats were anesthesied using a combine with medetomidine (Tomidine®, Provet, Turkey) 80 µg/kg IM and ketamine hydrochloride (Alfamin®, Egevet, Turkey) 10 mg/kg IM in group 1 (T1: after 10 min combined anesthesia). Similarly, cats were anesthesied using a combine with xylazine hydrochloride (Alfazin®, Egevet, Turkey) 2 mg/kg IM and ketamine hydrochloride (Alfamin®, Egevet, Turkey) 10 mg/kg IM group 2. Electrocardiogram, oscillometric mean arterial blood pressure (MAP), respiratory rate (RR), heart rate (HR), hemoglobin oxygen saturation (SpO_2), and rectal body temperature (BT) were monitored by multiparameter monitor (G9000, Guoteng, China) with 5 min intervals throughout the anesthesia (T2: after 15 min starting operation; T3: after last suture). After the OHE procedure was ended, anesthesia regimes were reversed by using atipamezole (Reversal®, Provet, Turkey) 200 µg/kg (T4: after 10 min reversed) IM.

Animals breathed spontaneously during anesthesia and were not intubated in order to avoide a heightened effect on the IOP. Monitoring of heart rate, RR, MAP, SpO_2, and BT was conducted using a patient monitor at T0, T1, T2, T3, and T4 time points. Clinical data, including RR, HR, MAP, SpO_2, and BT were recorded concurrently.

Ocular exams: Ocular parameters were measured at T0, T1, T2, T3, and T4 time points. All ocular measurements were performed with the cat with the care taken not to occlude the jugular veins or place pressure on the globe while retracting the eyelids. The cats were in dorsal recumbency in T3 time point, and in lateral recumbency at other time points. One researcher who was blind to the anesthetic drug, performed all IOP controls using a rebound tonometry (Tonovet, Tiolat, Finland). Intraocular pressure was evaluated on the center of the cornea in accordance with the instruction manual of each tonometer. The tonometer was factory-calibrated before

the study and calibrated each day before data collection. Three readings were obtained in the right eye at each measurement time. The mean of the three readings was recorded. No local anesthetic drop was used before IOP measurement. PS was measured with a caliper horizontally at the same time points. Schirmer tear test I was performed using commercial Schirmer strips placed in the lower fornix for 1 min.

Ovariohysterectomy was carried out in cats after T1 time point. All surgeries were performed by the same gynecolog with assisstance from veterinary students. All cats were discharged 24 hours after the operation.

Statistical analysis: Mean±SE values were used to estimate the values of all data. Statistical analysis was performed using nonparametric tests since the sample size was small and the data were not normally distributed. In order to test whether the effects of the drug differed from the median values, the Friedman test was used to analyze the variables between the two groups. Comparisons between the groups were made employing the Mann-Whitney U test, and differences between measurements were taken over time using the Wilcoxon test. Statistical significance was set at a probability value of $P<0.05$ with a two-sided confidence interval and assumption of equal variance. The statistical analyses were run using SPSS statistical programme (22.0, IBM Company, USA).

Results

No statistically significant differences were observed between the treatment groups with regard to body weight (BW), body condition score (BCS), age and baseline measurements of HR, RR, SpO_2, and BT. All of baseline monitoring values were within the expected reference ranges for anesthetized cats (Table 1).

Table 1. Animal data and baseline (T0) physiological variables for both groups, Mean (±SE).

Patient variable	MED-KET	XYZ-KET	P
BW (kg)	2.66±0.13	2.80±0.99	0.223
BCS	2.78±0.15	3.00±0.0	0.072
Age (years)	1.43±0.20	2.10±0.53	0.067
Baseline HR (beats/min)	173.82±13.40	177.00±13.99	0.677
Baseline RR (breaths/min)	11.56±0.54	12.00±0.56	0.939
Baseline SPO_2 (%)	91.20±0.75	91.10±0.35	0.726
Baseline BT (C)	38.77±0.14	38.59±0.23	0.663

BW: body weight, BCS: body condition score, HR: heart rate, RR: respiratory rate, SpO_2: hemoglobin oxygen saturation, and BT: rectal body temperature, NS ($P>0.05$).

The serial monitorized data changes in MED-KET and XYZ-KET group are compared in Table 2. Within the MED-KET group, the HR decreased significantly after medetomidine and ketamine injection. There is a statistically significant difference between times ($P=0.005$). There was a statistically significant difference at T1, T2 and T3 versus at T0 (T1, T2, T3 vs. T0, $P=0.005$, $P=0.014$ and $P=0.005$, respectively); though values were not significanly different from baseline by T4 (T0 vs. T4, $P=0.508$). Even though the HR reduced temporarily, the quartile range was within normal values [T1:103.80 (60.00-148.00), T2: 124.41 (67.00-200.00), T3: 113.50 (68.00-175.00)].

Table 2. The serial monitorized data changes in MED-KET and XYZ-KET group.

	Group	T0	T1	T2	T3	T4	P-within group
HR (beats/min)	MED-KET	173.82±13.40	103.80±9.91*	124.41±10.72*	113.50±9.07*	158.80±21.25	0.940
	XYZ-KET	177.00±13.99	117.00±11.05*,‡	98.34±11.98*,‡	99.90±6.05*,‡	158.25±14.00	
P-within group by time		0.677	0.473	0.211	0.344	0.821	
SPO_2 (%)	MED-KET	91.20±0.75	87.80±2.24	89.07±2.15	88.60±2.63	87.40±2.14	0.140
	XYZ-KET	91.10±0.35	89.70±1.16	90.30±2.07	91.10±1.83	91.80±0.66	
P- within group by time		0.726	0.761	0.495	0.545	0.129	
RR (breaths/min)	MED-KET	11.56±0.54	12.70±0.83	12.04±0.81	12.20±0.85	12.60±0.90	0.120
	XYZ-KET	12.00±0.56	11.20±0.77	10.84±0.47	12.70±1.13	11.08±0.64	
P- within group by time		0.939	0.237	0.236	0.818	0.293	
MAP (mmHg)	MED-KET	108.50±4.96	104.00±3.33	113.82±4.98	117.90±3.84	110.72±7.38	**0.031**
	XYZ-KET	101.50±5.11	94.00±7.76	99.65±10.97	108.70±6.02	102.48±8.03	
P- within group by time		**0.040**	0.344	0.402	0.289	0.253	
BT (^{0}C)	MED-KET	38.77±0.14	38.23±0.33	36.62±0.36*,†,‡	34.83±0.37*,†	34.60±0.47*,†	0.364
	XYZ-KET	38.59±0.23	38.06±0.39	35.74±0.30*,†,‡	34.40±0.51*,†	34.73±0.39*,†	
P- within group by time		0.663	0.850	**0.043**	0.529	0.570	

* $P<0.05$: vs. T0 within the group, † $P<0.05$: vs. T1 within the group, ‡ $P<0.05$: vs. T4 within the group, HR: heart rate, SpO_2: hemoglobin oxygen saturation, RR: respiratory rate, MAP: mean arterial blood pressure, and BT: rectal body temperature.

Within the XYZ-KET group, the HR decreased significantly after xylazine and ketamine injection. There is a statistically significant difference between times (P=0.005). There was a statistically significant difference at T1, T2 and T3 versus at T0 (T1, T2, T3 vs. T0, P=0.005, P=0.014 and P=0.005, respectively); though values were not significanly different from baseline by T4 (T0 vs. T4, P=0.508). Even though the HR reduced temporarily, the quartile range was within normal values [T1: 117.00 (53-157), T2: 98.34 (32-160), T3: 99.90 (73-130)].

Comparison of the impact on monitorized changes data between MED-KET and XYZ-KET group is presented in Table 2. The HR values comparison between groups is illustrated in Figure 1. It is seen from the p values in the Table 2 that the monitorizing change data between the MED-KET and XYZ-KET groups were not statistically significant different over time.

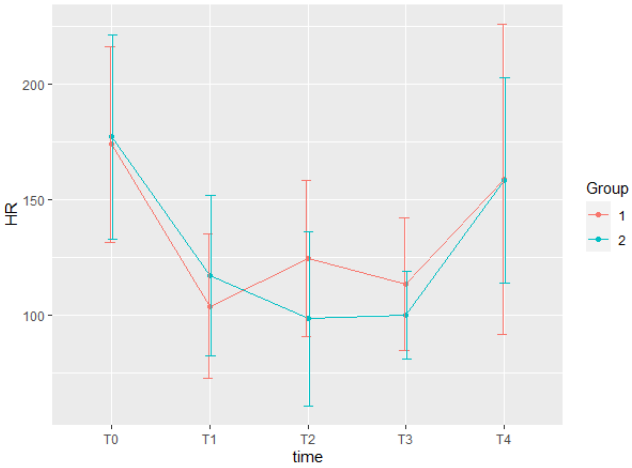

Figure 1. Comparison of HR values between MED-KET and XYZ-KET group.

The serial intraocular parameter changes in MED-KET and XYZ-KET group are compared in Table 3. Within the MED-KET group, the STT reduced significantly after medetomidine and ketamine injection. There is a statistically significant difference between times (P<0.001). There was a statistically significant difference at T1, T2 and T3 versus at T0 (T1, T2, T3 vs. T0, P=0.005, P=0.005 and P=0.007, respectively); though values were not significanly different from baseline by T4 (T0 vs. T4, P=0.233). Even though the STT decreased temporarily, the quartile range was within normal values [T1: 5.50 (2.00-15.00), T2:4.20 (3.00-13.00), T3:4.40 (2.00-8.00)].

Within the XYZ-KET group, the STT reduced significantly after xylazine and ketamine injection. There is a statistically significant difference between times (P=0.005). There was a statistically significant difference at T2 and T3 versus at T0 (T2, T3 vs. T0, P=0.035 and P=0.050, respectively); though values were not significanly different from baseline by T4 (T0 vs. T4, P=0.779). Even though the STT decreased temporarily, the quartile range was within normal values [T2: 6.54 (3-13), T3: 6.50 (2-13)].

Comparison of the impact on intraocular paramaters between MED-KET and XYZ-KET group is presented in Table 3. Both groups showed declines in IOP and increases in HPD after anesthesia induction (T0 vs. T1, all, P<0.05); however, the changing and recovery pattern of IOP and HPD showed intergroup difference. In IOP, the baseline value was similar between the two (P=0.939), with the first drop at T1 and T2 (P=0.790, P=0.705, respectively), however, after that point the MED-KET group showed more recovery and a higer level of IOP compared with those in the XYZ-KET group [T3: 23.60 (19.00-37.00) vs. 18.30 (9.00-33.00), P=0.019] (Table 3). In HPD, the baseline value was similar between the two (P=0.234). After that point the XYZ-KET group showed more recovery and a higher level of IOP compared with those in the MED-KET group [T1: 9.10 (7.00-11.00) vs. 7.10 (5.00-10.00), P=0.022; T2: 8.27 (5.00-10.00) vs. 7.10 (5.00-9.00), P=0.037; T3: 8.50 (7.00-10.00) vs. 6.90 (5.00-9.00), P=0.013 (Table 3).

Table 3. The serial intraocular parameter changes in MED-KET and XYZ-KET.

	Group	T0	T1	T2	T3	T4	P-within group
IOP	MED-KET	24.52±2.85	22.60±2.88	20.90±2.16	23.60±1.79	23.20±2.45	0.762
	XYZ-KET	22.70±1.30	21.90±1.23	19.70±1.31	18.30±2.06	24.69±2.79	
P- within group by time		0,939	0.790	0.705	**0.019**	0.910	
PS	MED-KET	6.60±0.40	7.10± 0.57	7.10±0.38	6.90±0.43	7.40±0.40	0.034
	XYZ-KET	6.40±0.40	9.10±0.46	8.27±0.43	8.50±0.34	8.39±0.43	
P- within group by time		0.234	**0.022**	**0.037**	**0.013**	0.113	
STT	MED-KET	12.84±1.59	5.50±1.23*,‡	4.20±0.59*,‡	4.40±0.56*,‡	10.00±1.67	0.325
	XYZ-KET	9.94±1.02	8.10±1.35	6.54±1.05*,‡	6.50±1.18*,†,‡	10.74±0.79	
P- within group by time		0.270	0.085	0.064	0.171	0.379	

* P<0.05: vs. T0 within the group, † P<0.05: vs. T1 within the group, ‡ P<0.05: vs. T4 within the group, IOP: Intraocular pressure, PS: pupil size, STT: Schirmer tear test.

Discussion and Conclusion

For ophthalmic surgery, to improve technique in general anesthesia, fixed cardiac parameters and intraocular pressure should be significant considerations (7, 10, 29). Some premedication agents and general anesthetics reduce IOP and decrease the HPD due to miosis. Ketamine, however, raises the IOP and causes enlargement of the pupilla in human subjects (12, 20, 29, 42).

Intraocular ophthalmic surgery in veterinary medicine has seen significant development over the last decades (13, 14, 28). Insufficient management of anesthesia can cause poor vision when the eyelids are unlocked throughout operation. The coaction of ophthalmic agents with premedicant agents must be considered prior to administration of anesthesia. Furthermore, as premedicants may have specific impacts on the physiology of the eye, this must be kept in mind for researchers overseeing anesthesia for ocular surgery. This is of extreme importance if requirements, such as immobility, decreased oculocardiac reflex, fixed eye, decreased intraocular pressure, minimal bleeding, smooth emergence without retching, nausea, or vomiting and postoperative analgesia are to be met for reliable ocular surgery (28, 35). Moreover, decrease in IOP and stabil bulbus oculi are preliminary conditions for smooth surgery and intraocular operation procedures (28, 35). The management of IOP is frequently of preliminary importance in intraocular operations. Premedicants that affect IOP have a determinative role in ensuring smooth surgery (5, 28, 38).

One of the problematic intraocular surgery operations for the surgeon is increasing IOP, so elevation of IOP and maintaining it within normal range is necessary (17, 33). IOP control before, during, and after the surgery is required for anesthesia management in ophthalmic surgery and one important aim in anesthetic management during ocular surgery is to provide adequate control of IOP (17, 43).

Strategies for induction of anesthesia are known to affect IOP in people (1, 23, 31). Abrupt increases in IOP in patients with near-perforating corneal trauma or glaucoma can cause dramatic effects. Prolapse of ocular contents complicates the surgical procedure and worsens the prognosis for recovery (9, 23). In animals with glaucoma, even minimal increases in IOP can lower axoplasmic flow within the optic nerve, predisposing to further injury (23, 44). Hence, any method that prevents elevation in IOP during induction of anesthesia may be of benefit in patients with ocular trauma or glaucoma and in those undergoing anesthesia for intraocular surgery.

One aim of managing of anesthesia throughout intraocular operation is to ensure a normotensive level of IOP. A rise in IOP may be disastrous in cases with glaucoma or a penetrating injury of the bulbus oculi. The IOP is determined by the incidence of vitreous capacity and manufacture of aqueous humour, external pressure, orbicularis oculi muscle tension and scleral rigidity (11, 27, 40). The ordinary IOP is around 15 mm Hg, with an accaptable value of 10±20 mm Hg. Former investigations have evaluated the impacts of premedicants and anesthetics, and related agents on IOP, though underlying factors impacting IOP are unclear.

Ocular and visual function may be impaired postoperatively if there are large variations in IOP in open-system ocular surgery. Mechanical and pharmacological stress during surgery must be avoided because these procedures can contribute to an increase in IOP (3, 30, 33). In general anesthesia in ocular surgery, short-acting anesthetic agents are commonly used. The central depressive effect on the diencephalic control of IOP, relaxing extraocular muscle tone and improving the aqueous humour outflow causes a reduction in IOP with the use of most anesthetics (21, 33, 34). It is shown that some agents, such as propofol, thiopental, halothane, isoflurane and desflurane, fentanyl, alfentanil and remifentanil decrease IOP (8, 18, 33, 34).

Research of normotensive dogs and rabbits has noted that medetomidine reduces the IOP following its local administration to the bulbus oculi (25, 36, 42). Peripheral α-2 receptors in the bulbus oculi, both prejunctionally on bulbar sympathetic nerves and postjunctionally, are likely related in the physiological arrangement of IOP. This arrangement is obtained by lowing the alerted manufacture of cyclic AMP in the ciliary process (25, 36, 42).

Regarding the impacts of ketamine on intraocular pressure, a signifcant rise in IOP has been achieved in dogs. In a similar study with dogs, a dose of 10 mg/kg of ketamine in combination with xylazine or acepromazine was applied, but increase in IOP was not achieved (29). The effect of intravenous ketamine administration alone on the IOP in dogs was studied and a significant increase after 5 and 10 minutes at 5 mg/kg dose was determined (22, 25, 39, 41).

In conclusion, XYZ-KET is more effective than MED-KET in attenuating the IOP, increasing the HPD, and alteration of the MACr response in general anesthesia. This temporary reduction in IOP could likely be helpful in eye operations especially in animals with high IOP values. Additionally, dilated HPD could possibly aid in the avoidance of anretior sinechia during corneal surgery. Future investigations may focus on the impact of other premedicant or induction agents on the IOP, HPD, and MACr to confirm our results for medetomidine and xylazine, and on evaluating the effects on the IOP, HPD, and MACr of different doses of medetomidine or xylazine.

Acknowledgements

Some part of this article was summarized from the first author's master thesis.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study was approved by the Local Ethics Committee of Turkish Manas University (Approval Number: 2016-03/2).

Conflict of Interest

The authors are declared that there is no conflict of interest.

References

1. **Badrinath SK, Vazeery A, McCarthy RJ, et al** (1986): *The effect of different methods of inducing anesthesia on intraocular pressure.* Anesthesiol, **65**, 431–435.
2. **Banga PK, Singh DK, Dadu S, et al** (2015): *A comparative evaluation of the effect of intravenous dexmedetomidine and clonidine on intraocular pressure after suxamethonium and intubation.* Saudi J Anesth, **9**, 179-183.
3. **Bathija R, Gupta N, Zangwill L, et al** (1998): *Changing definition of glaucoma.* J Glaucoma, **7**, 165-169.
4. **Batista CM, Laus JL, Nunes N, et al** (2000): *Evaluation of intraocular and partial CO_2 pressure in dogs anesthetized with propofol.* Vet Ophthal, **3**, 17–19.
5. **Benson GJ, Manning JP, Hartsfield SM, et al** (1998): *Intraocular Tension of the Horse. Effects of Succinylcholine and Halothane Anesthesia.* Am J Vet Res, **42**, 1831-1832.
6. **Bruniges N, Taylor PM, Yates D** (2016): *Injectable anaesthesia for adult cat and kitten castration: effects of medetomidine, dexmedetomidine and atipamezole on recovery.* J Fel Med Surg, **18**, 860-867.
7. **Brunson DB** (1980): *Anesthesia in ophtalmic surgery.* Vet Clin North Am Small Anim Pract, **10**, 481-495.
8. **Butterworth JF, Mackey DC, Wasnick JD** (2013): Anesthesia for Ophthalmic Surgery. 759-771. In: JF Butterworth, DC Mackey, JD Wasnick (Eds), Morgan and Mikhail's Clinical Anesthesiology. McGraw-Hill Professional, New York.
9. **Chmielewski NT, Brooks DE, Smith PJ, et al** (1997): *Visual outcome and oular survival after iris prolapse in the horse: a review of 32 cases.* Equine Vet J, **29**, 31–39.
10. **Collins BK, Gress ME, Moore CP, et al** (1997): *Physiologic, pharmacologic, and practical considerations for anesthesia of domestic animals with eye disease.* J Am Vet Med Assoc, **207**, 220-230.
11. **Cunningham AJ, Barry P** (1986): *Intraocular pressure physiology and implications for anaesthetic management.* Can Anaesth Soc J, **33**, 195-208.
12. **Frischmeyer KJ, Miller PE, Bellay Y, et al** (1993): *Parenthal anticholinergics in dogs with normal and elevated intraocular pressure.* Vet Surg, **23**, 230-234.
13. **Gelat KN** (1991): Veterinary Ophthalmology. Lea & Fibiger, Philadelphia.
14. **Gelat KN** (2000): Essentials of Veterinary Ophthalmology. Lippincott, Philadelphia.
15. **Gelatt KN, Brooks DE, Kallberg ME** (2007): The Canine Glaucomas. 762-780. In: KN Gellatt (Ed), Veterinary Ophthalmology. Blackwell Publishing, Florida. 762.
16. **Gellatt KN, Gwin R, Peiffer RL, et al** (1977): *Tonography in normal and glaucomatous beagle*. Am J Vet Res, **38**, 515-520.
17. **Goepfert CE, Ifune C, Tempelhoff R** (2010): *Ischemic optic neuropathy: are we any further?* Curr Opin Anaesth, **23**, 582-587.
18. **Grosso A, Scozzari G, Bert F, et al** (2013): *Intraocular pressure variation during colorectal laparoscopic surgery: Standard pneumoperitoneum leads to reversible elevation in intraocular pressure.* Surg Endosc, **27**, 3370-3376.
19. **Güzel Ö, Erdikmen DO, Yıldar E, et al** (2013): *The effects of propofol and a diazepam/alfentanil combination in dogs aged 10 years and above on heart rate, respiratory rate, pulse oximetry data, intraocular pressure, and body temperature*. Turk J Vet Anim Sci, **37**, 170-176.
20. **Hahnenberger RW** (1976): *Influence of various anesthetic drugs on intraocular pressure in cats.* Alb. Won Graefes Arc Klin Exp Ophtalmol, **199**, 179-186.
21. **He Z, Vingrys AJ, Armitage JA, et al** (2011): *The role of blood pressure in glaucoma.* Clin Exp Optom, **94**, 133-149.
22. **Hofmeister EH, Mosunic CB, Torres BT, et al** (2002): *Effects of ketamine, diazepam and their combination of intraocular pressures in clinically normal dogs.* Am J Vet Res, **67**, 1136-1139.
23. **Hofmeister EH, Williams CO, Braun C, et al** (2006): *Influence of lidocaine and diazepam on peri-induction intraocular pressures in dogs anesthetized with propofolatracurium*. Can J Vet Res, **26**, 51–56.
24. **Hofmeister H, Weinstein WL, Burger D, et al** (2009): *Effects of graded doses of propofol for anesthesia induction on cardiovascular parameters and intraocular pressures in normal dogs.* Vet Anaesth Analg, **36**, 442–448.
25. **Jin Y, Wilson S, Elko EE, et al** (1991): *Ocular hypotensive effects of medetomidine and its analogs.* J Ocul Pharmacol, **7**, 285-296.
26. **Karabağlı M, Özer K, Şahin I** (2014): *The effects of xylazine-ketamine anesthesia on intraocular pressure in dogs*. J Fac Vet Med Istanbul Univ, **40**, 260-263.
27. **Katzenschlager SS, Deusch E, Dolezal S, et al** (2002): *Sevoflurane and propofol decrease intraocular pressure equally during non-ophthalmic surgery and recovery.* British J Anaesth, **89**, 764-766.
28. **Kılıç S, Ünsaldı S** (2009): *Effects of isoflurane and snflurane on ocular parameters in dogs.* YYU J Fac Vet Med, **20**, 1-3.
29. **Kovalcuka L, Birgele E** (2011): *The effects of some premedication and general anesthesia drugs on intraocular pressure and pupil diameter in Dog's eyes.* LLU Raksti, **26**, 77-83.
30. **Kymes SM, Kass MA, Anderson DR, et al** (2006): *Ocular Hypertension Treatment Study Group (OHTS). Management of ocular hypertension: a cost-effectiveness approach from the ocular hypertension treatment study.* Am J Ophthalmol, **141**, 997-1008.

31. **Lev R, Rosen P** (1994): *Prophylactic lidocaine use preintubation: a review*. J Emerg Med, **12**, 499–506.
32. **Miller RD** (2005): Miller's Anesthesia. Churchill Livingstone, USA.
33. **Montazeri K, Dehghan A, Akbari S** (2015): *Increase in intraocular pressure is less with propofol and remifentanil than isoflurane with remifentanil during cataract surgery: A randomized controlled trial.* Adv Biomed Res, **4**, 55.
34. **Moore D, Harris A, Wudunn D, et al** (2008): *Dysfunctional regulation of ocular blood flow: a risk factor for glaucoma?* Clin Ophthalmol, **2**, 849-861.
35. **Orkin FK, Cooperman LH** (1983): Complications in Anesthesiology. Lippincott, Philadelphia.
36. **Ogidigben MJ, Potter DE (1993):** *Comparative effects of alpha-2 and DA-2 agonists on intraocular pressure in pigmented and nonpigmented rabbits*. J Ocul Pharmacol, **9**, 187-199.
37. **Perk EC, Güzel Ö, Gülanber EG** (2002): *Etomidate/alfentanil anaesthesia in dogs and its effects on pulse oxymeter, electrocardiography and haematological parameters.* Turk J Vet Anim Sci, **26**, 1021–1024.
38. **Presbitero JV, Ruiz RS, Rigor BM, et al** (1980): *Intraocular pressure during enflorane and neurolept anesthesia in adult patients undergoing ophthalmic surgery.* Anesth Analg, **59**, 50-54.
39. **Pierce-Tomlin T, Shaughnessy MR, Hofmeister EH** (2020): *A systematic review of the effects of injectable sedative and anesthetic drugs and inhalant anesthetics on intraocular pressure in the dog.* Vet Anaesth Analg, **47**, 145-151.
40. **Sator S, Wildling E, Schabernig C, et al** (1998): *Desflurane maintains intraocular pressure at an equivalent level to isoflurane and propofol during unstressed nonophtalmic surgery.* British J Anaesth, **80**, 243-244.
41. **Smith MD, Barletta M, Diehl KA, et al** (2019): *Effect of propofol and ketamine-diazepam on intraocular pressure in healthy premedicated dogs.* Vet Anaesth Analg, **46**, 36-42.
42. **Verbruggen AMJ, Akkerdaas LC, Hellebrekers LJ** (2000): *Effects of intravenous medetomidine on pupil size and intraocular pressure in normotensive dog.* Vet Quarter, **22**, 179-180.
43. **Weber ED, Colyer MH, Lesser RL, et al** (2007): *Posterior ischemic optic neuropathy after minimally invasive prostatectomy.* J Neuroophthalmol, **27**, 285-287.
44. **Williams LW, Gelatt KN, Gum GG, et al** (1983): *Orthograde rapid axoplasmic transport and ultrastructural changes of the optic nerve. Part 1. Normotensive and acute ocular hypertensive beagles.* Glaucoma, **5**, 117–128.
45. **Zimmerman AA, Funk KJ, Tidwell JL** (1996): *Propofol and alfentanil prevent the increase in intraocular pressure caused by succinylcholine and endotracheal intubation during a rapid sequence induction of anesthesia.* Vet Anaesth Analg, **83**, 814–817.

Ankara Univ Vet Fak Derg, **69**, 259-264, 2022
DOI: 10.33988/auvfd.895959

Evaluation of acute partial unilateral ureteral obstruction based on the renal venous impedance index in Rabbit: An experimental study

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Received date: 12.03.2021 - Accepted date: 05.07.2021

Abstract: To investigate the feasibility of the venous impedance index (VII) in the diagnosis of acute partial unilateral ureteral obstruction (APUUO), in this study we aimed to measure and compare changes in intrarenal venous flow and arterial flow. In New Zealand rabbits (n=10) the left ureter was narrowed by ligation. Doppler ultrasonographic (US) examination of the interlobar arteries and veins in both kidneys was performed preoperatively and 3, 6, 12, 24, and 48 h after ureteral ligation. Resistive index (RI), RI difference (RIΔ) and the RI ratio (RIr), and the VII difference (VIIΔ) were calculated from RI and VII obtained from the obstructive and nonobstructive kidneys. The RI and VII were postoperatively compared between the left and right kidneys. Although the mean RI for obstructive kidneys was higher than those for nonobstructive kidneys, the difference was only statistically significant at 3 and 24 h (P=0.007, and P=0.049, respectively). In all postoperative measurement times, RIΔ was ≥0.08 and RIr was ≥1.169, and the mean VII values were significantly lower in the obstructive kidneys than the nonobstructive kidneys (P≤0.002), and the VIIΔ was equal to or greather than 0.159. The venous flow was more affected than arterial flow in the obstructive kidney. The renal RI alone was insufficient as a diagnostic criterion. The renal VIIΔ ≥0.16 can be enhanced the diagnostic accuracy of in APUUO if used as a combination of the RIΔ≥0.08 and RIr≥1.1.
Keywords: Kidney, partial unilateral ureteral obstruction, rabbit, resistive index, venous impedance index.

Tavşanlarda akut parsiyel unilateral üreteral obstruksiyonun renal venöz impedans indeks ile değerlendirilmesi: Deneysel çalışma

Özet: Akut parsiyel unilateral üreteral obstruksiyonda (APUUO)'nun tanısında venöz impedans indeksin (VII) uygulanabilirliğini araştırmak için bu çalışmada intrarenal venöz akım ve arteriyel akım arasındaki değişiklikleri ölçmeyi ve karşılaştırmayı amaçladık. Yeni Zelanda tavşanlarında (n=10) sol üreter ligasyonla daraltıldı. Her iki böbrekte interlobar arterlerin ve venlerin Doppler ultrasonografik (US) incelemesi preoperatif dönemde ve üreter ligasyonundan sonraki 3., 6., 12., 24. ve 48. saatlerde yapıldı. Resistif indeks (RI) farkı (RIΔ), RI oranı (RIr) ve VII farkı (VIIΔ) obstrüktif ve nonobstrüktif böbreklerden elde edilen RI ve VII'den hesaplandı. RI ve VII, bütün postoperatif ölçüm zamanlarında sol ve sağ böbrekler arasında karşılaştırıldı. Obstrüktif böbrek için ortalama RI, nonobstrüktif böbrekten daha yüksek olmasına rağmen, istatistiki fark sadece 3. ve 24. saatlerde önemliydi (sırasıyla P=0,007 ve P=0,049). Tüm postoperatif ölçüm zamanlarında RIΔ≥0,08 ve RIr≥1,169, ve ortalama VII değeri obstrüktif böbrekte nonobstrüktif böbrekten daha düşüktü (P≤0,002) ve VIIΔ değeri 0,159'a eşit veya daha yüksekti. Obstruktif böbrekte venöz akım, arteriyel akımdan daha fazla etkilendi. Tanısal bir kriter olarak renal RI'nin tek başına yetersiz olduğu belirlendi. Renal VIIΔ≥0,16 değeri, RIΔ≥0,08 ve RIr≥1,1 değerleri ile birlikte kullanılırsa, APUUO'nun tanısal doğruluğu arttırılabilir.
Anahtar sözcükler: Böbrek, parsiyel unilateral üreteral obstrüksiyon, resistif indeks, tavşan, venöz impedans indeks.

Introduction

Ureteral obstruction (UO) is one of the main pathologies of the urinary tract. The condition may be partial or complete, as well as unilateral or bilateral. If untreated it leads to reversible and irreversible injury to the kidneys and ureters. Etiological factors implicated in renal obstructive parenchymal injury include compromised blood flow, elevated intrapelvic pressure, and increased vasoactive and inflammatory mediators (30). Early diagnosis and treatment are essential if an irreversible injury to the obstructed kidney is to be averted (2).

UO can be diagnosed by several imaging techniques. Radiography and grayscale ultrasonography (US) are

usually employed in veterinary medicine. The grayscale US can reliably detect collector system dilatation in the kidneys, but it cannot by itself differentiate obstructive from nonobstructive dilatation (20). Changes in perfusions associated with dilatation of the collector system can be assessed using color, power, and duplex Doppler US. Duplex Doppler US examination of a renal arterial tree involves the resistive index (RI) (19, 27). The RI basically indicates impedance, defined as downstream as resistance and vessel compliance (7, 16). Renal RI is the most widely employed Doppler US index in the evaluation of renal (12, 26) and renovascular (6) diseases, including obstructive uropathy (4), and transplant kidney (23). Platt et al. (20) described renal RI of 0.70 as a good discriminatory value for obstructive uropathy, with an accuracy of 90%. Doppler US has been described as an accurate tool for distinguishing between obstructive and nonobstructive dilatation (20). However, the specificity and sensitivity of renal RI in showing a concurrent increase in resistance and decrease in compliance are poor. Low downstream resistance is usually observed on the venous side of the arterial tree because the veins operate as capacitance vessels. Venous impedance index (VII) is a term applied to the venous side of the vascular tree. Interstitial pressure takes place in acute, complete UO has been linked to a decrease in VII (5).

Although both experimental and clinical studies have investigated the diagnostic value of the RI in UO, there is little focus on the VII value (5, 18, 29). Only one experimental study has employed the VII value to assess complete UO (24).

The aims of this study were (i) to measure changes in intrarenal venous flow compared to arterial flow in experimentally induced-acute partial unilateral ureteral obstruction (APUUO) and (ii) to investigate the practicability of renal VII value.

Materials and Methods

The Animal Care Ethics Committee of Akdeniz University, Turkey, approved the study (No: B.30.2.AKD.0.05.07.00). Ten mature male New Zealand rabbits weighing 2.8-3.9 kg were housed at 25°C in a 12-h dark/light cycle. Standard rat chow and free access to water were provided.

Surgical procedure: All animals underwent laparotomy under general anesthesia. The protocol consisted of intramuscular administration of xylazine HCl (5 mg/kg, Rompun®, Bayer, Turkey) and ketamine (35 mg/kg, Ketalar®, Pfizer, Turkey) for pre-anesthesia, followed by induction and maintenance of general anesthesia using inhaled isoflurane (1.5 %, Forane®, Abbvie, Turkey) and 100% oxygen in an open system. Once the left ureter had been exposed and isolated, partial obstruction was induced through the insertion of a 24-gauge intravenous catheter (0.7 mm diameter) from the external aspect to the interior of the lumen of the left proximal ureter. The ureter was then ligated from the outside the lumen with 3-0 silk suture. The silicon part of the catheter remained outside the lumen (3). All animals received subcutaneous penicillin-G and subcutaneous Butorphanol (0.3mg/kg, Butomidor®, Richter Pharma AG. Austria).

Doppler US procedure: Rabbits were placed in the lateral position so that the kidney to be examined. First, the left kidney and then the right kidney were evaluated by means of Doppler US (MINDRAY, DC-6Vet Model, Shenzhen Mindray Bio-medical Electronics Co., LTD, China). Ultrasonographic images were taken using a microconvex, multifrequency probe (6.5-8.5 MHz) in longitudinal and dorsal planes. Color Doppler mode was used to identify intrarenal vessels. Sample volume was placed on the interlobar vascular structure where the best signal was obtained. Afterward, an arterial and venous distinction was made from spectral samples obtained by switching to Duplex Doppler mode. Both the wall filter and sample volume were maintained at minimum levels during the Doppler US examination. Spectral samples were obtained at an angle less than 60°.

Doppler US parameters: The renal RI and VII values were obtained preoperatively and postoperatively at 3, 6, 12, 24 and 48 h after ureteral ligation from both kidneys. The renal RI [*(peak systolic velocity-end diastolic velocity)/peak systolic velocity*] was estimated by means of peak systolic velocity and end diastolic velocity following compilation of similar wave forms (n=3-5) from interlobar arteries. Similar to the renal RI, the renal VII [*(peak flow signal-least flow signal)/peak flow signal*] was calculated from the spectrum obtained from interlobar veins (Fig 1). The mean renal RI and VII values, obtained from three different points for both kidneys for all measurement times, were calculated and statistically analyzed.

The RI difference (RIΔ) and RI ratio (RIr) from the RI values of obstructive and nonobstructive kidneys for all measurement times were calculated: *RIΔ = RI of the obstructive kidney-RI of the nonobstructive kidney* and *RIr = RI of the obstructive kidney/RI of the nonobstructive kidney.* The VII difference (VIIΔ) from the VII values of obstructive and nonobstructive kidneys for all measurement times were calculated: *VIIΔ = VII of the obstructive kidney-VII of the nonobstructive kidney*.

The renal RI> 0.70 (3), RIΔ ≥0.08 (10), and RIr ≥1.10 (15) were considered as the threshold values for APUUO's Doppler US diagnosis.

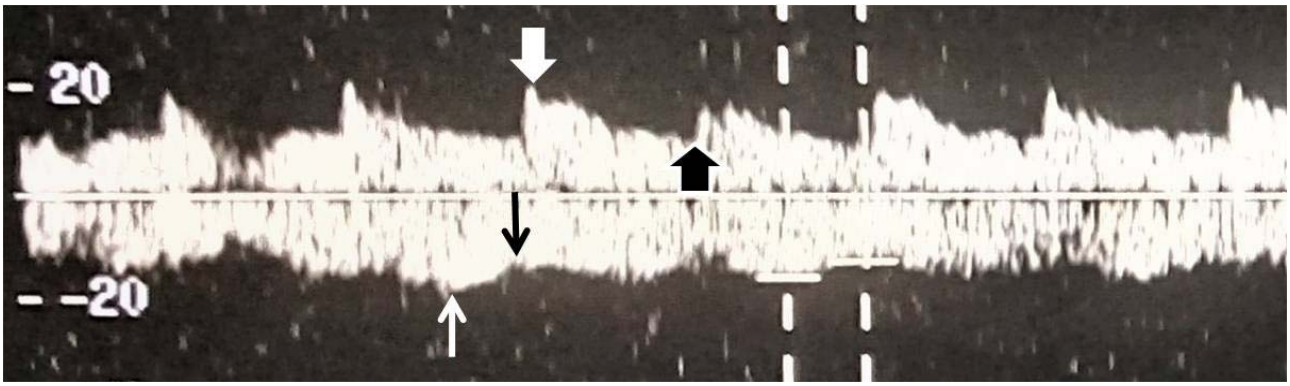

Figure 1. Since the interlobar artery and vein run side by side, arterial and venous Doppler spectra can be obtained simultaneously in the rabbit. The arterial spectrum is viewed as a monophasic wave above the baseline. The wide white arrow points to the peak systolic velocity and the wide black arrow indicates the end-diastolic velocity. In the venous spectrum that appears below the baseline with its wavy flow form, the white arrow indicates the peak flow signal and the black arrow indicates the latest flow signal.

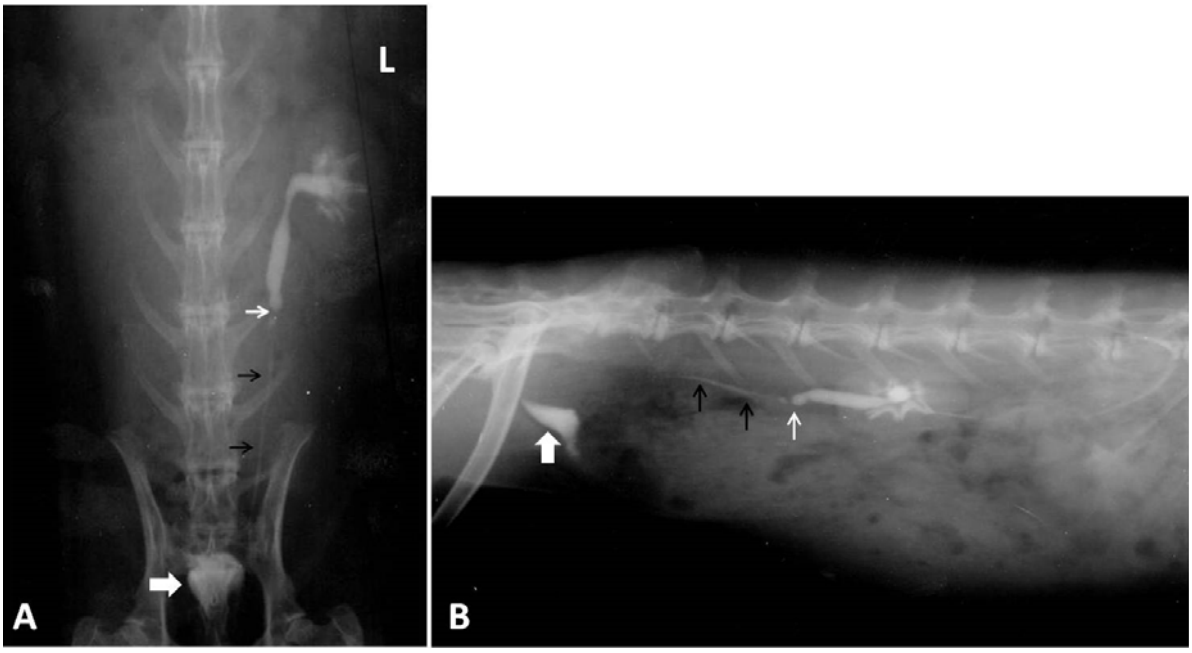

Figure 2. Percutaneous antegrade pyelography was performed to confirm partial unilateral ureteral obstruction showing the left dilated proximal ureter, the partial obstruction side (white arrow), the left distal ureter (black arrows), and bladder (wide arrow) in ventrodorsal (A) and left lateral (B) radiograms.

Confirmation of APUUO: Determination of the dilated renal pelvis and/or proximal ureter, obstruction site, distal ureter, and/or bladder were accepted as confirmation criteria of APUUO for US-guided percutaneous antegrade pyelography (UGPAP). Induction of APUUO was confirmed by UGPAP (13) at 72 h on all subjects following immobilization with propofol (10 mg/kg, IV, Pofol®, Sandoz, Turkey) (Fig. 2). UGPAP was only applied to the obstructive (left) kidney. A 25-gauge, 2.5-inch spinal needle was introduced into the renal cortex, perpendicular to the capsule, and advanced into the renal pelvis during ultrasonographic guidance after the kidney was visualized on the dorsal plane via US. The stylet of the spinal needle was removed and some urine was aspirated from pelvis renalis. Non-ionic contrast agent (ultravist 370, Schering, German) was injected into pelvis renalis as much as the amount of aspirated urine, and then the needle was removed. Left lateral and ventrodorsal abdominal X-rays were taken.

Statistical Analysis: Power and sample size analysis calculation (PS version 3.1.2., Vanderbilt University, USA) revealed that replication should be 11 when the mean difference in the renal VII of 0.25 between obstructed and non-obstructed groups to be significant, in a given standard deviation of 0.20 at the alpha error (Type I) of 0.05 and power (beta error or Type 2 error) of 0.95. Data were subjected to 2-way ANOVA with the repeated measures option was employed to compare the mean RI and mean VII difference between left and right kidneys as well as among time points (SPSS version 21.0, SPSS Inc., Chicago, IL). Results were expressed as mean ± standard deviation. Statistical significance was set at $P<0.05$.

Results

Renal RI, RIΔ, and RIr: The mean preoperative RI values were 0.533±0.09 and 0.565±0.12 for the left and right kidneys (P=0.565) (Table 1). The postoperative mean RI values decreased in the nonobstructive (right) kidney and increased in the obstructive (left) kidney (Fig. 3) compared to their mean preoperative values. However, this change in bilateral postoperative mean RI values was insignificant compared to the mean preoperative RI values. The mean RI in the obstructive kidney did not exceed 0.70 at any postoperative time point. The mean RI values in the obstructive kidneys were higher than those in the nonobstructive kidneys during all postoperative measurements, but the difference between nonobstructive and obstructive kidneys was only statistically significant at 3 and 24 h postoperation (P=0.007 and P=0.049, respectively).

The RIΔ was equal to or greater than 0.08, and the RIr was equal to or greater than 1.169 at all postoperative measurement times.

Renal VII and VIIΔ: The difference between mean preoperative VII values in the left (0.596±0.15) and right kidneys (0.644±0.12) was similar (P=0.392) (Table 1). The postoperative mean VII values in the obstructive kidney (Fig. 3) were lower than the mean preoperative VII values. This change in the obstructive kidney was significant at other postoperative time points, except for the 3rd and 24th hours (P≤0.015). The difference between preoperative and postoperative VII values of nonobstructive kidneys was statistically insignificant. The mean VII values at all postoperative time points were lower in the obstructive kidney than in the nonobstructive kidney (P≤0.002). The VIIΔ was equal to or greater than 0.159 at all postoperative measurement times.

Table 1. Renal Doppler indices before and after partial unilateral ureteral obstruction.

Doppler Indices	Kidney	Measurement Times					
		Preop	Postop (h)				
			3	6	12	24	48
RI	L	0.533±0.09	0.637±0.07	0.594±0.07	0.554±0.08	0.57±0.07	0.607±0.06
	R	0.565±0.12	0.502±0.07	0.498±0.09	0.474±0.08	0.467±0.1	0.508±0.07
P value		0.567	0.007	0.062	0.179	0.049	0.054
RIΔ		-	0.135±0.08	0.096±0.03	0.08±0.07	0.103±0.04	0.099±0.05
RIr		-	1.268±0.1	1.193±0.07	1.169±0.11	1.221±0.14	1.195±0.09
VII	L	0.596±0.15	0.466±0.13	0.387±0.12*	0.353±0.14*	0.472±0.13	0.413±0.13*
	R	0.644±0.12	0.625±0.13	0.699±0.15	0.601±0.19	0.722±0.14	0.672±0.15
P value		0.392	0.002	0.000	0.000	0.000	0.000
VIIΔ		-	0.159±0.1	0.313±0.13	0.248±0.15	0.25±0.13	0.259±0.14

Data present as mean±standard deviation. h: Hour; L: Left; R: Right; RI: Resistive index; RIΔ: Resistive index different between obstructive and nonobstructive kidneys. RIr: Resistive index ratio between obstructive and nonobstructive kidneys. VII: Venous impedance index. *Statistical difference between pre and postoperative values, P<0.05.

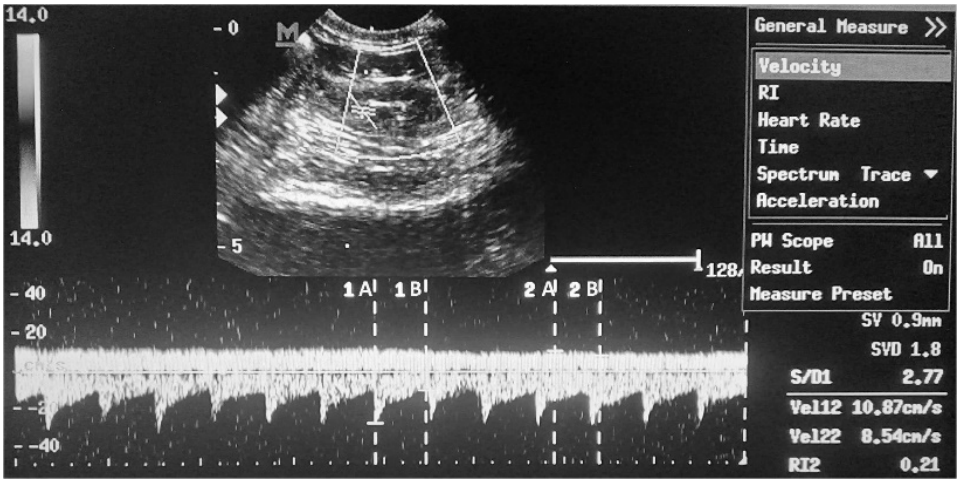

Figure 3. Arterial and venous flow patterns in obstructive (left) kidney at post-operative 6 hours. In obstructive kidney resistive index is 0.58 [peak systolic velocity (1A): 25.52 cm/s, end diastolic velocity (1B): 10.87 cm/s] and venous impedance index is 0.21 [peak flow signal(2A): 10.87 cm/s, least flow signal (2B): 8.54 cm/s]. The venous spectrum turned into a predominantly linear flow form.

Discussion and Conclusion

UO leads to renal parenchymal injury and even to permanent renal dysfunction unless being treated. Early diagnosis and release of the obstruction are essential if renal functions are to be preserved. Restoration of renal function is inversely correlated to the duration and degree of the obstruction (2).

The renal US is routinely employed for collecting system evaluation (31). The grayscale US is very sensitive in the detection of dilatation of the collecting system. Despite the high sensitivity of grayscale, it is insufficient in the diagnosis of acute UO due to the subsequent occurrence of pyelocaliectasis under conditions of UO (non-dilated obstruction). Additionally, in hydronephrosis and hydroureter formation associated with other nonobstructive conditions such as pyelonephritis, grayscale may lead to a false diagnosis of obstructive uropathy (11, 21). Specificity of 33% has been reported for the grayscale US in feline UO under similar conditions (1).

Grayscale US permits accurate visualization of the renal morphology, while Doppler US allows strategically important qualitative and quantitative data. One of these quantitative data is the renal RI value (11, 14). In addition to hemodynamic parameters, the renal RI also exhibits a strong correlation with histopathological parameters such as glomerular sclerosis, arteriolosclerosis, interstitial fibrosis/tubular atrophy, and interstitial infiltration (25). This index is only a specific marker of kidney damage and is not an indicator of renal function prognosis. However, it is significantly associated with systemic circulation (8). Although the renal RI is dependent on both renal vascular compliance and resistance, the dependence on resistance is reduced as compliance decreases (7). Experimental and clinical findings have also demonstrated that renal vascular resistance has little effect on the renal RI, but that renal (renal interstitial and venous pressure) and systemic (aortic stiffness and pulse pressure) determinants have a significant effect (28).

Platt et al. (20) first proposed the use of the RI in diagnosing acute urinary obstruction in 1989. Obstructive uropathy can be diagnosed by means of an acute unilateral increase in the renal RI, associated with hydronephrosis, observed 6-48 h from the onset of symptoms (11). In the present study, although postoperative renal RI values obtained from the obstructive (left) kidney were higher than its preoperative values but insignificant at all times. The mean RI values at postoperative measurement intervals were higher in the obstructive kidney than in the nonobstructive kidney, although only the differences at 3 h and 24 h were statistically significant (P=0.007, and P=0.049, respectively).

Our review of the literature revealed no clinical studies assessing UO in cats and dogs using Doppler US and revealing a threshold value for the renal RI. The mean RI values in the obstructive kidney have not exceeded the 0.70 thresholds in studies of experimentally induced partial UO (13, 31). The mean RI values in the obstructive kidney did not exceed 0.657 at any postoperative measurement interval in these studies. When a threshold limit for UO of the RIΔ>0.10 (20) was adopted, this was sufficient for diagnosis of APUUO obstruction at only two postoperative time points, while the RIΔ >0.06-0.08 (10) was significant at all measurement intervals (Table 1). Lim et al. (15) evaluated the benefit of the RIr in distinguishing between pediatric obstructive and nonobstructive upper urinary tract dilation. That study described a RIr cut-off value ≥1.10 as a useful parameter for the assessment and follow-up of pediatric unilateral obstructive hydronephrosis. Riahinezhad et al. (22) reported that the RIr ≤1.075 was 82.8% specific in differentiating nonobstructive hydronephrosis. The RIr ≥1.169 at all postoperative time points in that study.

Low sensitivity and specificity have been reported for the renal RI measurements for the concomitant increase in resistance and decrease in compliance (17). Since the renal RI was problematic in determining acute UO, Bateman and Cuganesan (5) focused on the venous aspect of the renal vascular tree in the diagnosis of acute obstruction. Decreased compliance observed in acute renal obstruction results in weakening of these renal venous signals, the impact on intraparenchymal venous flow being greater than that on arterial flow (5, 9). Bateman and Cuganesan (5) reported that, in contrast to the renal RI, the renal VII value is a more sensitive parameter reflecting physiological changes in simultaneously increased resistance and decreased compliance. However, this hypothesis has also been described as not yet proven (9). Similar to the present study, the VII value in the obstructive kidney being lower than in the nonobstructive kidney was also important in previous clinical (5, 18, 29) and rabbit experimental (24) studies. Combined evaluation of the renal RI and VII values in obstructive uropathy has been reported to improve the diagnostic accuracy of Doppler US (18, 29). In the present experimental study, we observed that a decreased VII value in the obstructive kidney compared to the nonobstructive kidney, especially if the VIIΔ ≥0.16, and the RIΔ ≥0.08, and the RIr ≥1.10 were sufficient for Doppler US diagnosis of APUUO. However, further clinical studies demonstrating a threshold limit, as with the renal RI value, are now needed for the renal VII value to be capable of use as a diagnostic criterion in obstructive uropathy.

In conclusion, the venous flow was more affected than arterial flow in the obstructive kidney, suggesting that the use of RI ≥0.70 alone as a diagnostic criterion for APUUO was insufficient. A decrease in the VII value in a single kidney may indicate an obstructive kidney. When the VIIΔ value is equal to or greater than 0.16, taking into account the RIΔ ≥0.08 and RIr ≥1.1 can increase the diagnostic accuracy of APUUO.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study was approved by the Animal Care Ethics Committee of Akdeniz University (No: B.30.2.AKD.0.05.07.00).

Conflict of Interest

We declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References

1. **Adin CA, Herrgesell EJ, Nyland TG, et al** (2003): *Antegrade pyelography for suspected ureteral obstruction in cats: 11 cases (1995–2001).* J Am Vet Med Assoc, **222,** 1576-1581.
2. **Apoku IN, Ayoola OO, Salako AA, et al** (2015): *Ultrasound evaluation of obstructive uropathy and its hemodynamic responses in southwest Nigeria.* Int Braz J Urol, **41**, 556-561.
3. **Ayyildiz A, Kaya M, Karaguzel E, et al** (2009): *Effect of tadalafil on renal resistivity and pulsatility index in partial ureteral obstruction.* Urol Int, **83**, 75-79.
4. **Azam A, Haq A, Beg MB** (2013): *Role of Renal Arterial Resistive Index (RI) in obstructive uropathy.* J Pak Med Assoc, **63**, 1511-1515.
5. **Bateman GA, Cuganesan R** (2002): *Renal vein Doppler sonography of obstructive uropathy.* Am J Roentgenol, **178**, 921-925.
6. **Boddi M** (2017): *Renal ultrasound (and Doppler sonography) in hypertension: An update.* Adv Exp Med Biol, **956**, 191-208.
7. **Bude RO, Rubin JM** (1999): *Relationship between the resistive index and vascular compliance and resistance.* Radiol, **211**, 411-417.
8. **Di Nicolo P, Granata A** (2017): *Renal Resistive index: not only kidney.* Clin Exp Nephrol, **21**, 359-366.
9. **Di Nicolo P, Granata A** (2018): *Renal intraparenchymal resistive index: the ultrasonographic answer to many clinical questions*. J Nephrol, **32**, 527-538.
10. **Granata A, Andrulli S, Bigi MQ, et al** (2009): *Predictive role of duplex Doppler ultrasonography in the diagnosis of acute renal obstruction in patients with unilateral renal colic.* Clin Nephrol, **71**, 680-686.
11. **Granata A, Zanoli L, Clementi S, et al** (2014): *Resistive intrarenal index: myth or reality?* Br J Radiol, **87**, 20140004.
12. **Grande D, Terlizzese P, Iacoviello M** (2017): *Role of imaging in the evaluation of renal dysfunction in heart failure patients.* World J Nephrol, **6**, 123-131.
13. **Kaya M, Bumin A, Sen Y, et al** (2010): *Comparison of Excretory Urography, Ultrasonography-Guided Percutaneous Antegrade Pyelography, and Renal Doppler Ultrasonography in Rabbits with Unilateral Partial Ureteral Obstruction: An Experimental Study.* Kafkas Üni Vet Fak Derg,**16**, 735-741.
14. **Kaya M** (2012): The evaluation of renal hemodynamics with Doppler ultrasonography. 1-30. In: Artis, AS (Ed), Hemodynamics - new diagnostic and therapeutic approaches. 1st ed. InTech, Rijeka, Croatia.
15. **Lim GY, Jang HS, Lee EJ, et al** (1999): *Utility of the resistance index ratio in differentiating obstructive from nonobstructive hydronephrosis in children.* J Clin Ultrasound, **27**, 187-193.
16. **Matos I, Azevedo P, Carreira M** (2018): *Pilot study to evaluate the potential use of the renal resistive index as a preliminary diagnostic tool for chronic kidney disease in cats.* J Feline Med Surg, **20**, 940-947.
17. **Mostbeck GH, Zontsich T, Turetschek K** (2001): *Ultrasound of the kidney: obstruction and medical diseases.* Eur Radiol, **11**, 1878-1889.
18. **Oktar SH, Cem Y, Ozdemir H, et al** (2004): *Doppler sonography of renal obstruction: value of venous impedance index measurements.* J Ultrasound Med, **23**, 929-936.
19. **Ostrowska J, Kielbowicz Z, Zaleska-Dorobisz U, et al** (2016): *Resistive index (RI) obtained in renal interlobar arteries of normal dogs and cats by means of Doppler ultrasonography.* Pak Vet J, **36**, 45-48.
20. **Platt JF, Rubin JM, Ellis JH** (1989): *Distinction between obstructive and nonobstructive pyelocaliectasis with duplex Doppler sonognaphy*. Am J Roentgenol, **153**, 997-1000.
21. **Platt JF, Rubin JM, Ellis JH** (1993): *Acute renal obstruction: Evaluation with intrarenal duplex Doppler and conventional US.* Radiology, **186**, 685-688.
22. **Riahinezhad M, Sarrami AH, Gheisari A, et al** (2018): *How may Doppler indices help in the differentiation of obstructive from nonobstructive hydronephrosis?* J Res Med Sci, **23**, 76.
23. **Schwarz C, Mühlbacher J, Böhming GA, et al** (2017): *Impact of ultrasound examination shortly after kidney transplantation*. Eur Surg, **49**, 140-144.
24. **Sohn KM, Chung SK, Lee SY** (2004): *Renal vein Doppler sonography in rabbits with acute ureteral obstruction: usefulness of impedance index of renal vein.* J Korean Radiol Soc, **50**, 369-374.
25. **Spatola L, Andrulli S** (2016): *Doppler ultrasound in kidney diseases: a key parameter in clinical long-term follow-up*. J Ultrasound, **19**, 243-250.
26. **Tipisca V, Murino C, Cortese L, et al** (2016): *Resistive index for kidney evaluation in normal and diseased cats.* J Feline Med Surg, **18**, 471-475.
27. **Tublin ME, Dodd G, Verdile V** (1994): *Acute renal colic: Diagnosis with duplex Doppler US.* Radiol, **193**, 697-701.
28. **Tublin ME, Bude RO, Platt JF** (2003): *The resistive index in renal Doppler sonography: where do we stand?* Am J Roentgenol, **180**, 885-892.
29. **Vadana BMK, Pasumarthy A, Penumalli N, et al** (2015): *Renal venous Doppler study in obstructive uropathy*. J Clin Diagn Res, **9**, TC13-15.
30. **Yasar A, Erdemir F, Parlaktas BS, et al** (2013): *The effect of carvedilol on serum and tissue oxidative stress parameters in partial ureteral obstruction induced rat model.* Kaohsiung J Med Sci, **29**, 19-25.
31. **Yokoyama H, Tsuji Y** (2002): *Diuretic Doppler ultrasonography in chronic unilateral partial ureteric obstruction in dog.* BJU Int, **90**, 100-104.

Ankara Univ Vet Fak Derg, **69**, 265-272, 2022
DOI: 10.33988/auvfd.871118

Proliferative and apoptotic evaluations of renal preventive effects of coenzyme Q10 in radioiodine-131 induced renal damage

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Received date: 30.01.2021 - Accepted date: 11.07.2021

Abstract: The aim of this study was to investigated anti-proliferative and anti-apoptotic effects of coenzyme Q10 (CoQ10) in the prevention of radioiodine-131 (RAI) (I^{131}) induced kidney damage. A total of 24 Wistar albino rats were separated into equal three groups (n = 8/group): Group 1 (control): untreated group; Group 2 (RAI): 3 mCi/kg RAI oral route; Group 3 (RAI+CoQ10): 3 mCi/kg RAI oral route and intraperitoneally 30 mg/kg/day CoQ10. CoQ10 treatment was started two days before RAI administration and was continued five days once daily after RAI. Pathomorphological parameters of kidneys were measured using hematoxylin–eosin and Masson's trichrome staining. Immunohistochemically; proliferating cell nuclear antigen (PCNA), caspase 8, caspase 9 and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) were used to determine proliferation and apoptosis. With the exception of the control group, varying degrees of inflammation, degeneration, necrosis, and interstitial/perivascular fibrosis were detected in the kidneys of all rats. This histopathological damage was found to be significantly less in CoQ10 group versus RAI group ($P<0.05$). The all immunohistochemical examinations demonstrated that administration of CoQ10 had reduced proliferation and apoptosis ($P<0.05$). The results of kidney histopathology and immunohistochemistry demonstrated that administration of CoQ10 had reduced inflammation, proliferation, and apoptosis. These findings show CoQ10 can play an important role in the radioprotection of kidneys against RAI-induced damage.

Keywords: Antiapoptotic, antiproliferative, coenzyme Q10, kidney, radioiodine-131.

Radioiodine-131 ile uyarılmış böbrek hasarının önlenmesinde koenzim Q10'un proliferatif ve apoptotik etkilerin değerlendirilmesi

Özet: Bu çalışmanın amacı radioioidine-131 (RAI) (I^{131}) tedavisine bağlı oluşan böbrek hasarının önlenmesinde koenzim Q10 (CoQ10)'nun anti-apoptotik ve anti-proliferatif etkisinin araştırılmasıdır. Yirmi dört Wistar albino sıçan rastgele olarak üç gruba ayrıldı (n=8/grup): Grup 1 (kontrol): tedavi uygulanmayan grup; Grup 2 (RAI): orogastrik yolla tek doz 3 mCi/kg RAI uygulanan grup; Grup 3 (RAI+CoQ10); tek doz 3 mCi/kg RAI ve intraperitonel olarak 30 mg/kg/gün CoQ10 uygulanan grup. CoQ10 tedavisi RAI uygulamasından 2 gün önce başladı ve RAI sonrası günde bir kez beş gün süreyle uygulandı. Son CoQ10 uygulamasından yirmi dört saat sonra sıçanların böbrekleri alınarak patomorfolojik incelemeler amacıyla hematoksilen-eosin ve Masson'un Trikrom metoduna göre boyandı. İmmunohistokimyasal olarak: proliferasyonun belirlenmesi amacıyla prolifere hücre nükleer antijen (PCNA); apoptozisin belirlenmesi amacıyla Caspase 8 ve Caspase 9; DNA hasarının belirlenmesi amacıyla TUNEL boyaması yapıldı. Çalışma sonucunda; kontrol grubu dışındaki sıçanların böbreklerinde değişen şiddette inflamasyon, ödem, dejenerasyon, nekroz ve fibrozis görülürken bu bulguların CoQ10 ile tedavi edilen grupta istatistiksel olarak belirgin düzeyde daha az olduğu dikkati çekti ($P<0,05$). İmmunohistokimyasal olarak profilerasyonun, apoptozisin ve DNA hasarının CoQ10 grubunda daha hafif olduğu istatistiksel olarak tespit edildi ($P<0,05$). Bu çalışma sonuçlarıyla radyoiyodin'in böbrekler üzerinde yaptığı hasarın önlenmesinde CoQ10'nun başlıca antienflamatuar, antiproliferatif ve antiapoptotik etkiyle radioprotektif olduğu belirlenmiştir.

Anahtar sözcükler: Antiapoptozis, antiproliferasyon, böbrek, koenzim Q10, radyoiyodin-131.

Introduction

Radioiodine–131 (RAI) (I^{131}) has been an important component in the treatment of thyroid dysfunction, hyperthyroidism, Graves' disease and differentiated thyroid cancer since the 1940s. It is applied as the continuation of treatment for the prevention of potential recurrences and undetected metastases following total thyroidectomy, particularly in patients with thyroid cancer (10). RAI, which is a radio–isotope, damages tumor cells by irradiating high energy beta (β) (0.61 MeV) and gamma (γ) (0.36 MeV) radiation in these cells and thereby stops tumor development (19).

RAI administered per oral as a part of the treatment (10). It is absorbed by gastrointestinal system cells and then it reaches to target cells through circulation and enters thyroid cells with active transport via Na+/I–Symporter (NIS), which is an integral plasma membrane glycoprotein found in the basolateral membrane of the cells (24). RAI is excreted from the salivary glands, lacrimal gland, mammary gland, and kidneys. Approximately 90% of the RAI administered for treatment is excreted from the kidneys in the first 48 hours (7). This excretion has been seen to continue until the 10^{th} week following the administration (30). During the excretion of RAI continue the uptake via NIS at these extrathyroidal tissues. RAI continue to emit ionizing radiation and leads to activation of chemical mediators of inflammation (Interleukin (IL), Tumor Necrosis Factor alpha (TNF-α), etc.) with the increase of reactive oxygen species (ROS) (superoxide, lipid peroxidase and hydrogen peroxide, etc.) in extra–thyroidal and extra–tumoral tissues of the treated patients (2). The ROS in the tissues causes impairment of the control mechanisms of the cell organelles, membrane, DNA and mitochondria and result in genetic destruction, proliferation, apoptosis and inflammation in the kidneys (3, 22). Therefore, there is a need for safe and effective protective agents to protect kidney during I^{131} therapy and prevent secondary renal complications. Recent studies have reported positive results with antioxidant supplements for the prevention of RAI–induced extrathyroidal tissue damage (4, 5, 13, 29).

CoQ10 is a lipid–soluble, nontoxic, vitamin like substance found in many natural foods. CoQ10, which is absorbed by chylomicrons from the gastrointestinal system. Then it goes to tissues which have high metabolic activity, such as the heart, liver, muscle and kidneys through lymphatics and blood. Most of it (40–50%) accumulates in the mitochondria of cells in these tissues, and the remainder accumulates in lysosome, Golgi apparatus, microsome and plasma membrane (11). It primarily acts as an electron transporter of mitochondrial respiratory chain and provides intracellular ion balance. It prevents oxidation and lipid peroxidation in the cell membrane and thereby plays a role in the control of apoptosis. In addition, it shows an anti–inflammatory effect by reducing inflammatory mediators like IL, cytokine and TNF-α (15, 23, 28). CoQ10 has been widely used for the prevention and treatment of various diseases, such as renal (18), cardiovascular (20) and neural diseases (16).

The aim of this study was to investigate the radioprotective effect of CoQ10, an easily available, nontoxic and inexpensive substance, in the prevention of RAI–induced kidney damage.

Materials and Methods

Animals: All stages of the study were conducted in the Animal Unit Laboratory after national and Local Ethics Committee approval had been obtained for the breeding and use of laboratory animals and accordance with the principles of the experimental ethical principles and animal protection laws according to the rules of EU Directive 2010/63/EU for animal experiments and in compliance with the ARRIVE guidelines and the AVMA euthanasia guidelines 2013 (Approval No: 2020-0059).

The study included a total of 24 male Wistar albino rats aged 4–5 months, each weighing 250–300 g. The sample size of the study was calculated with the G. Power software (ver. 3.1.9.7, Heinrich-Heine-Universität Düsseldorf, Germany) statistical analysis. The required total sample was calculated as 24 for 80% power, $\alpha = 0.05$ type I error, and f effect size= 0.80. The animals had a one-week adaptation period to the laboratory environment before the study. The rats were kept in polypropylene cages in room (65–70% humidity and of 21±2 °C temperature) 12–h light/dark cycle and were fed with ad libitum. A sterile pad sheath was used under an absorbable disposable cover to prevent radioactive spread.

Experimental design: The rats were randomly divided into equal three groups (n = 8/group). Group 1 (control) was given no treatment. For treatment-experiment groups (Group 2 and Group 3) were given standard therapy as according to previous similar studies (28). In Group 2 (RAI), radioiodine-131 (Mon–Iyot–131, Eczacıbaşı) was applied at 3 mCi/kg via orogastric route. Group 3 (RAI + CoQ10) was given 3 mCi/kg RAI and 30 mg/kg CoQ10 (Phytopharma, Turkey) via the intraperitoneal (ip) route. CoQ10 was started two days before the RAI, and was continued for five days once daily after RAI administration. At 24 hours after the last dosage of CoQ10, the animals were anesthetized with 50 mg/kg ip propofol and sacrificed. The bilateral kidneys were removed with standard methods for histomorphological and immunohistochemical examination.

Histopathological examination: Tissue samples were fixed in 10% formalin (pH 7.2-7.4) and taken for

routine pathology follow–up as described by Luna et al (17). The tissues, which were dehydrated in increasing degrees of alcohol in an automated tissue follow–up device, were applied with xylol to obtain transparency and embedded in paraffin blocks. Sections of 5–µm were taken with a Leica RM 2125 RT microtome, with the first three and every tenth sections taken onto lams. The preparations were passed through alcohol and xylol series and stained with Hematoxylin–Eosin (HE) and Masson's trichrome stains. All samples were examined under a light microscope (Olympus DP–73, Olympus BX53–DIC microscope; Tokyo, Japan).

All of the stained sections of kidneys were evaluated according to previously published grading system as 0 to 3 (score 0: none, score 1: mild, score 2: moderate and score 3: severe) by the presence and severity of the findings (30).

Immunohistochemical examination

Reagents: The reagents used in this study were: PCNA (PCNA15; Invitrogen, USA), Caspase 8 (ab4052; Abcam, USA), Caspase 9 (ab52298; Abcam, USA), and TUNEL assay (InSitu Cell DeathDetection Kit, POD; Roche, Germany); Proteinase K (Roche, Germany), for the chromogens; diaminobenzidine (DAB), 3–amino 9–ethylcarbasole (AEC), and Avidin Biotin Complex (ABC) Immunohistochemical compounds (Histostain Plus Kit; USA).

Staining: Standard Avidin Biotin Complex (ABC) protocol was followed to stain the tissue samples using the Histostain-Plus Kit. After routine deparaffinization and rehydration procedures, the Antigen retrieval was done in a microwave oven 700 W and 10 min with pH 6.0 citrate buffer solution. Endogenous peroxidase activation in the tissues was blocked for 15 min with 0.3% hydrogen peroxide (H_2O_2) in 0.01 mol/l Phosphate Buffered Saline (PBS) in methanol. After protein blocking with 5% normal goat serum for 20 min the sections were incubated with PCNA (1:100), caspase 8 (1:50), and caspase 9 (1:100) primer antibodies for one hour. Then, tissues were reacted with secondary antibody for 30 min and stained with DAB chromogen for 5 min. Finally, all of the sections were counterstained with hematoxylin. All steps were carried out at 37 °C and in a damp chamber. PBS was used as a washing solution during all the staining steps.

TUNEL assay procedures: TUNEL marking was performed in accordance with the instructions of the manufacturer. For this purpose, 5 µm sections were treated with Proteinase K (20 mg/ml) at room temperature and damp chamber for 20 min. The washed sections were kept in freshly prepared 50 µL of TUNEL reaction mixture (including TdT&dUTP) solution in the dark, at 37 °C for one hour. Afterwards, the sections were coated with 50 µL of antifluorescein antibody conjugated POD enzyme homogenate and kept at 37 °C for 30 min. Finally, the washed sections were stained with hematoxylin after treatment with AEC for 10 min.

Method of counting: The number of immunopositive cells in the sections were counted under the microscope magnified at 400X in 1 mm2, and ten fields were chosen randomly. The total number of cells in each field were counted. Staining indexes were calculated on the basis of the percentages of the stained nucleus for all markers. The staining intensity was scored from 0 to 3; the distribution of immunoreactive cells 0: for less than 10%, 1: for 10-50%, 2: for 50-80%, and 3: more than 80% of positive cells.

Statistical analysis: Data analysis was performed using Statistical Package for Social Sciences for Windows software (SPSS version 24.0, SPSS Inc. Chicago, IL). Descriptive statistics were used to determine the continuous variables and frequency distributions for categorical variables. The normality of distribution of continuous variables was tested by Shaphiro Wilk test. The groups were compared with nonparametric tests, as the data did not show a normal distribution. Statistical differences between the groups were assessed with the Kruskal Wallis tests. Tukey honestly significant difference or Dunn multiple comparison test was used to determine which group was different from the others. Results were given as Median [minimum–maximum] deviation. P value < 0.05 was accepted as statistically significant.

Results

Histopathological results: The histopathological results of the kidney tissues are summarized in the Table 1. The kidney tissues of the rats in control group were seen to be normal and no pathological changes were found. Whereas, the histopathological changes in tubules, glomeruli, interstitium and vessels were observed to be significantly milder in Group 3, where CoQ10 was applied, compared to Group 2 which was applied with RAI only (Fig. 1) ($P<0.05$). The severity of tubular hyperemia ($P=0.001$), inflammation ($P=0.002$), fibrosis ($P=0.001$), necrosis ($P=0.001$), degeneration ($P=0.002$), vacuolization ($P=0.002$), tubular dilation ($P=0.001$), and hyaline reabsorbs in tubular lumen ($P=0.001$) was found to be significantly lower in Group 3 compared to Group 2 ($P<0.05$). Of the pathological changes in glomerular structures, particularly periglomerular inflammation ($P=0.002$) and periglomerular fibrosis ($P=0.001$) were found to be milder in the group treated with CoQ10 compared to the group treated with RAI only ($P<0.05$). In addition, glomerular wall thickening ($P=0.001$) was found to be milder in Group 3 compared to Group 2 ($P<0.05$). Perivascular inflammation ($P=0.002$) was found to be statistically significantly milder in Group 3 compared to Group 2 ($P<0.05$).

Table 1. Distribution of histomorphological parameters in the groups and statistical significance levels.

Histopathological variables[a]	Group 1	Group 2	Group 3	P
Hyperemia	0[0–0]‡	3[2–3]	0[0–1]‡	0.001*
Inflammation	0[0–0]‡	2.5[2–3]	0.5[0–1]‡	0.002*
Fibrosis	0[0–0]‡	2[2–3]	0[0–1]‡	0.001*
Degeneration	0[0–1]‡	2.5[2–3]	0.5[0–1]‡	0.002*
Necrosis	0[0–0]‡	2[2–2]	0[0–1]‡	0.001*
Vacuolization	0[0–0]‡	2.5[2–3]	0.5[0–1]‡	0.002*
Tubular Dilatation	0[0–1]‡	2[2–3]	0[0–1]‡	0.001*
Reabsorb Drops in tubules	0[0–0]‡	2[2–3]	0[0–1]‡	0.001*
Periglomerular inflammation	0[0–0]‡	3[2–3]	0.5[0–1]‡	0.002*
Periglomerular fibrosis	0[0–0]‡	2[2–3]	0[0–1]‡	0.001*
Glomerular wall thickening	0[0–0]‡	2[2–3]	0[0–1]‡	0.001*
Perivascular inflammation	0[0–0]‡	2.5[2–3]	0.5[0–1]‡	0.002*

[a]Median [minimum–maximum]: 0 (none), 1 (mild), 2 (moderate), 3 (severe). *Significant at 0.05 level (Kruskal–Wallis test). ‡ Significant versus group 2. P shows the differences between all groups. All pairwise multiple comparison test.

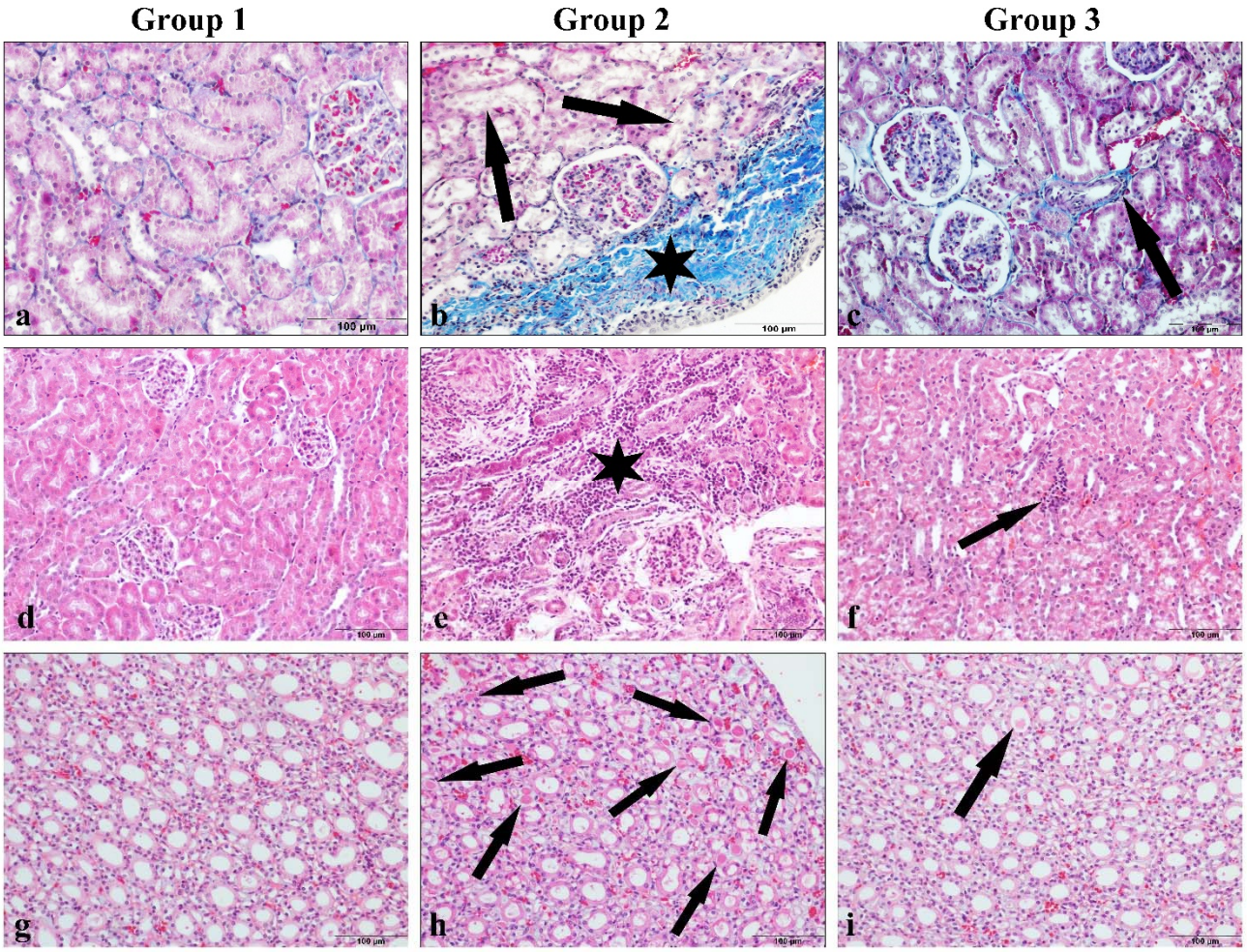

Figure 1. The histopathological appearances of the kidneys in different groups.
a., d., g. Normal kidney structures in control group. **b.** Diffuse fibrosis and severe inflammation (star), and tubular necrosis (arrows), trichrome. **c.** Mild interstitial fibrosis and inflammation (arrow), trichrome. **e.** Severe inflammation and fibrosis (star), HE. **f.** Few inflammatory cells in interstitium (arrow), HE. **h.** Numerous hyaline casts (arrows) in the collecting ducts, HE. **i.** Few hyaline casts in the ducts (arrow), HE.

Table 2. The statistically significant values of proliferation and apoptotic parameters of the kidneys of the groups.

Immunohistochemical variables[a]	Group 1	Group 2	Group 3	P
PCNA	0[0–1]‡	2.5[2–3]	0.5[0–1]‡	0.001*
Caspase 8	0[0–0]‡	2[2–3]	0[0–1]‡	0.001*
Caspase 9	0[0–0]‡	2[2–3]	0[0–1]‡	0.001*
TUNEL	0[0–1]‡	2.5[2–3]	1[0–2]‡	0.004*

[a]Median [minimum–maximum]: 0 (<1% positive), 1 (1–25% positive), 2 (>25–75% positive), 3 (>75% positive) according to the percentage of positive staining cells. *Significant at 0.05 level (Kruskal–Wallis test). ‡ Significant versus group 2. P shows the differences between all groups. All pairwise multiple comparison test.

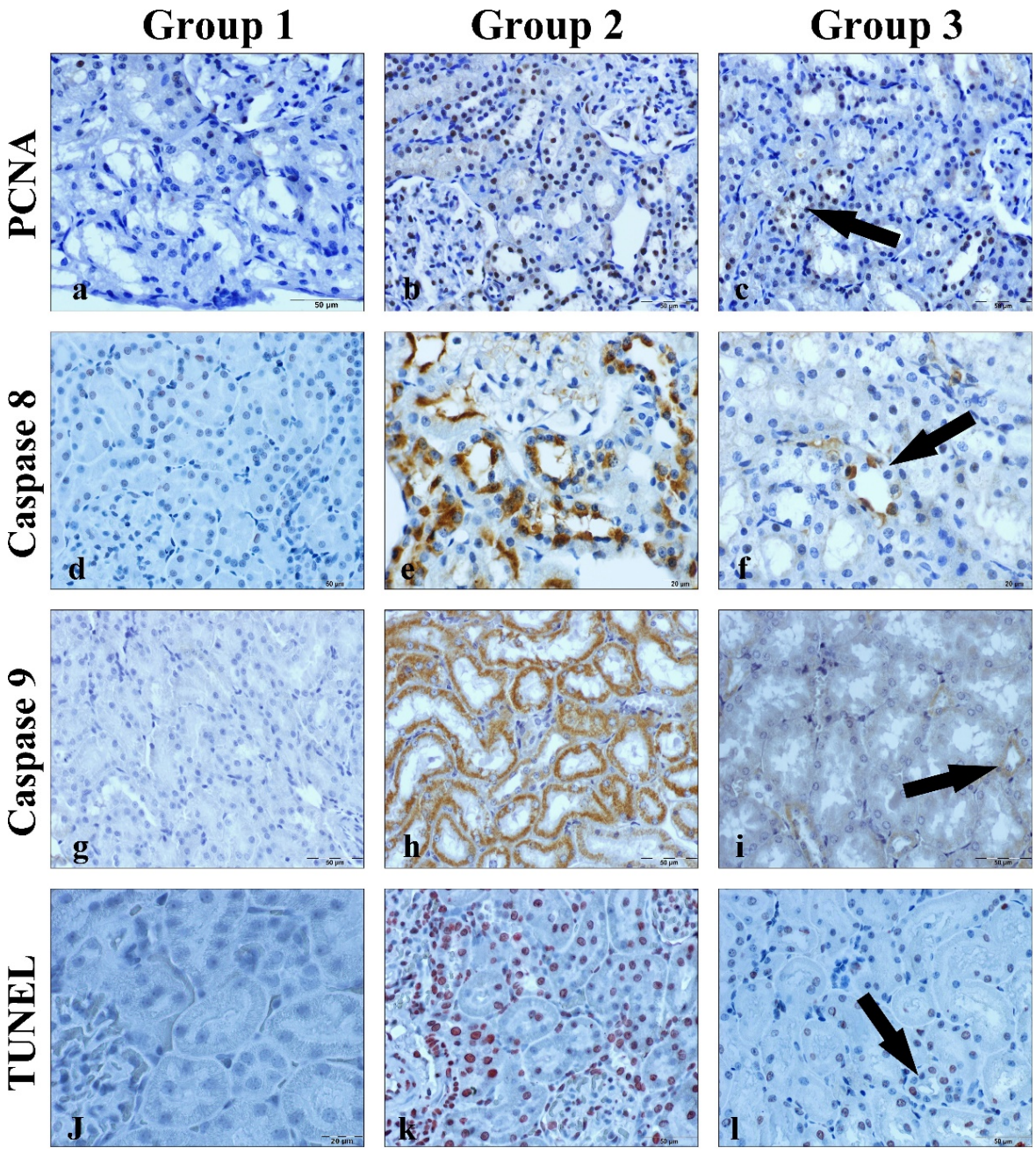

Figure 2. Evaluation of immunohistochemistry for cell proliferation and apoptosis.
a., d., g., j. All antibodies were immunonegative. Dark nuclear staining was considered to indicate positive immunoreactivity for PCNA; **b.** Severe nuclear immunopositive cells, **c.** Few cells with immunoreactivity, (DAB chromogen). In the caspases staining, yellow cells indicate positivity; **e.** Caspase 8, **h.** Caspase 9 diffuse cellular positivity, **f.** Caspase 8, **i.** Caspase 9 both antibodies were mildly positive, (DAB chromogen). In the TUNEL assay, the red nucleus was considered to indicate positivity; **k.** Severe red nuclear immunopositive cells, **l**. a few little positive immunoreactivity (AEC chromogen).

Immunohistochemical findings: The immunohistochemical findings are summarized in Table 2. The kidney tissues of Group 1 were found to be immune negative in respect of all three antibodies and TUNEL (Fig. 2a, d, g, j). In PCNA staining applied for the detection of tissue proliferation, the intense positive reactions in cell nuclei of the kidney tissues in the rats in Group 3, which were applied with RAI only, were found to be significantly lower compared to those in Group 2 (Fig. 2c) ($P<0.05$). PCNA was found to be more intensely positive in the collecting ducts compared to distal tubules in Group 2 (Fig. 2b). Positive cells in glomerulus were seen to be less intense in Group 3 compared to Group 2.

In the comparison of cytoplasmic marking intensities performed with caspase 8 ($P=0.001$) and caspase 9 ($P=0.001$) antibodies for the detection of apoptosis in the kidneys, a less intensive positive reaction was determined in Group 3 compared to Group 2 (Fig. 2e, h) ($P<0.05$). The staining severity in distal tubules in particular was detected to be more intensive than in proximal tubules in both groups. The staining in collecting ducts were seen to be more severe compared to that in distal and proximal tubules in Group 2 and Group 3. Immune positive cell density for caspase 8 and caspase 9 antibodies was found to be milder in Group 3 compared to Group 2 (Fig. 2f, i). While the glomerulus in Group 2 were detected to be mildly immune positive against both antibodies, mildly positive cells were not observed in the few cells in Group 3. Strong immune positivity was determined in interstitial cells in the kidneys in Group 2, and fewer and milder cytoplasmic positive cells were observed in the same regions in Group 3.

In TUNEL assay marking applied for the detection of DNA damage, positive cell intensity was found to be significantly lower in Group 3, which was applied CoQ10, compared to Group 2, which was applied with RAI only (Fig. 2k, l) ($P=0.004$). TUNEL staining was detected to be intensely positive in the nuclei of the collecting ducts of the kidneys in both groups. Distal tubules demonstrated a more severe reaction compared to proximal tubules. Positive reactions were observed to be less intensive in Group 3 compared to Group 2 ($P<0.05$).

Discussion and Conclusion

The RAI treatment is successfully used in the treatment of hyperthyroidism and thyroid cancer. Some side effects may occur despite the advantages of being easily applicable, inexpensive and not requiring monitoring after treatment. Particularly the clinical symptoms of nephropathy negatively affect the treatment process and the psychology of the patient (3, 7, 10). There is insufficient available data about RAI–induced nephropathy.

As mentioned earlier, several experimental studies have investigated the pathogenesis of this nephropathy. Yumuşak et al. (30) investigated the pathological findings in the kidneys of rats applied with RAI and detected that degeneration, necrosis, inflammation, proliferation and apoptosis. Kanter et al. (13) reported focal atrophy, cytoplasmic vacuolization and intercellular detachment in renal tubules. Caloğlu et al. (5) vascular congestion and dilation of Bowman's capsule were reported in the acute period. Sürücü et al. (25) reported endothelial destruction and perivascular fibrosis following RAI treatment. The present study also revealed similar histomorphological damage in the rats which were applied with RAI. Pathomorphological changes in tubular, glomerular, interstitial and vascular structures, and intensive hyaline drops in collecting duct lumen were particularly noticeable. Toxicity findings were seen to be more severe in the pelvic canal and medullary region.

The pathogenesis of RAI–induced kidney damage has not been completely explained to date. The general sense is that RAI increases the release of inflammatory mediators and many ROS by entering renal cells via NIS during its excretion (5, 13). The increased ROS leads to cellular proliferation by damaging organelles. Proliferation induces apoptosis by activating DNA breaks and caspase pathways. The caspase pathway mechanism mainly occurs in two ways as mitochondrial (intrinsic) and death receptor (extrinsic) cell death (22). The mitochondrial pathway, which is controlled by caspase 9, is initiated when activated by various forms of cellular stress such as hypoxia, ischemia, oxidative stress, anticancer drugs and DNA damage. The death receptor pathway, which is controlled by caspase 9, is activated by death ligands such as cytochrome and TNF-α (27).

In the present study, severe cell proliferation and DNA damage were seen to occur in the kidneys of the rats which were applied with RAI. It has been suggested that caspase 8 and caspase 9 positive intensity in these regions leads to significant damage in the mitochondria and is an indicator of activation of chemical inflammatory mediators in the tissue. These findings indicate that RAI leads to apoptosis both by mitochondrial and death receptor pathways. The more severe histomorphological and immunohistochemical damage in the pelvic canal and collecting ducts than in the medullary and cortical tubules are thought to develop in parallel with urinary excretion. It has been suggested that the pelvic canal and medulla are exposed to radiation for longer as RAI contaminated urine remains for a longer period in these structures. Tissue damage in these structures may lead to hyaline accumulation in tubule lumen due to impaired urinary excretion and urine accumulation in ducts.

Many animal models and human trials in recent years have indicated that CoQ10, a potent antioxidant,

suppressed inflammatory mediator release and prevented damage in the mitochondria and cell membrane (23). Therefore, it has begun to be commonly prescribed for the prevention and treatment of renal diseases. Zahed et al. (31) reported that CoQ10 administration in chronic kidney disease patients undergoing hemodialysis significantly reduced of inflammatory markers. Ishikawa et al. (12) detected that CoQ10 significantly reduced the urinary albumin level and superoxide anion in the prevention of salt–induced chronic kidney disease. Carrasco et al. (6) reported that CoQ10 regulated the serum albumin/creatinine ratio and beta 2 microglobuline levels against kidney damage and showed an anti–inflammatory effect by reducing mediators such as IL6 and cytokine in patients treated for urolithiasis. In a rat model experimental study by Fatima et al. (8), it was reported that CoQ10 reduced oxidant levels, cytokine levels such as TNF-α and IL6, and significantly reduced caspase activity immunohistochemically. In another study by Fatima et al. (9) CoQ10 was shown to be effective in the prevention of tubular necrosis, glomerular congestion and cast formation in diabetic nephropathy. Saiki et al. (21) conducted a study of PDSS2 deficient mice and reported that CoQ10 had protective effects against proteinuria, interstitial inflammation, tubular dilation and glomerular crescents. Abitoğlu et al. (1) also stated that CoQ10 had anticongestive and anti-necrotic effects in the prevention of septic damage in the kidneys of mice. Kırdağ et al. (14) determined that CoQ10 prevented oxidation, particularly interstitial inflammation, glomerular atrophy, degeneration and apoptosis in kidneys in an experimental ureteral obstruction study in rats. Üstüner et al. (26) reported significant success of CoQ10 in the prevention of tubular necrosis and hyaline accumulation and in the prevention of apoptosis detected with TUNEL in gentamicin–induced kidney damage. In the present study, histomorphological damage was seen to be significantly prevented by CoQ10. In particular, fibrosis and inflammation seen in perivascular and interstitial regions were significantly prevented by CoQ10. These results demonstrated that CoQ10 prevented inflammatory reactions and inhibited fibrosis formation. In addition, hyaline drops were seen to form less in tubule lumens. The most important findings of the study were that cell proliferation, DNA damage and apoptosis were suppressed with CoQ10 and CoQ10 showed an anti–apoptotic and anti–proliferative effect.

In conclusion, severe proliferative, apoptotic and histomorphological damage was seen to develop in the kidneys of rats applied with RAI. In the present study, an important antioxidant CoQ10, was determined to have a radioprotective effect through histomorphological and immunohistochemical examinations. These results demonstrated that especially anti–proliferative, anti–apototic, and anti–inflammatory activities are involved in the mechanism underlying the radioprotective effects of CoQ10.

Acknowledgements

Some part of this article was summarized from the first author's master thesis.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

All stages of the study were conducted in the Animal Unit Laboratory after national and Local Ethics Committee approval had been obtained for the breeding and use of laboratory animals and accordance with the principles of the experimental ethical principles and animal protection laws according to the rules of EU Directive 2010/63/EU for animal experiments and in compliance with the ARRIVE guidelines and the AVMA euthanasia guidelines 2013 (Approval No: 2020-0059).

Conflict of Interest

The authors are declared that there is no conflict of interest.

References

1. **Abitagaoglu S, Akinci SB, Saricaoglu F, et al** (2015): Effect of coenzyme Q10 on organ damage in sepsis. Bratisl Med J, **116**, 433–439.
2. **Almeida IV, Dusman E, Heck MC, et al** (2013): *Cytotoxic and mutagenic effects of iodine–131 and radioprotection of acerola (Malpighia glabra L.) and beta–carotene in vitro.* Genet Mol Res, **12**, 6402-6413.
3. **Baradaran–Ghahfarokhi M** (2012): *Radiation–induced kidney injury.* J Renal Inj Prev, **1**, 49-50.
4. **Barlas A, Bag Y, Pekcici M, et al** (2017): *Melatonin: a hepatoprotective agent against radioiodine toxicity in rats.* Bratisl Med J, **118**, 95-100.
5. **Caloglu M, Caloglu VY, Altun GD, et al** (2009): *Histopathological and scintigraphic comparisons of the protective effects of L–carnitine and amifostine against radiation–induced late renal toxicity in rats.* Clin Exp Pharmacol Physiol, **36**, 523-530.
6. **Carrasco J, Anglada FJ, Campos JP, et al** (2014): *The protective role of coenzyme Q 10 in renal injury associated with extracorporeal shockwave lithotripsy: a randomised, placebo-controlled clinical trial.* BJU Int, **113**, 942-950.
7. **Driver I, Packer S** (2001): *Radioactive waste discharge quantities for patients undergoing radioactive iodine therapy for thyroid carcinoma.* Nucl Med Commun, **22**, 1129-1132.
8. **Fatima S, Al–Mohaimeed N, Al–Shaikh Y, et al** (2016): *Combined treatment of epigallocatechin gallate and Coenzyme Q10 attenuates cisplatin–induced nephrotoxicity via suppression of oxidative/nitrosative stress,*

inflammation and cellular damage. Food Chem Toxicol, **94**, 213-220.

9. **Fatima S, Al-Mohaimeed N, Arjumand S, et al** (2015): *Effect of Pre-and Post–Combined Multidoses of Epigallocatechin Gallate and Coenzyme Q10 on Cisplatin-Induced Oxidative Stress in Rat Kidney.* J Biochem Mol Toxicol, **29**, 91-97.
10. **Haugen BR, Alexander EK, Bible KC, et al** (2016): *Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. The American thyroid association guidelines task force on thyroid nodules and differentiated thyroid cancer.* Thyroid, **26**, 1-133.
11. **Hemmi NB, Raj KC (2006):** *Coenzyme Q10: Absorption, tissue uptake, metabolism and pharmacokinetics.* Free Radic Res, **40**, 445-453.
12. **Ishikawa A, Kawarazaki H, Ando K, et al** (2011): *Renal preservation effect of ubiquinol, the reduced form of coenzyme Q10.* Clin Exp Nephrol, **15**, 30-33.
13. **Kanter M, Tarladacalisir YT, Uzal C** (2011): *Role of amifostine on acute and late radiation nephrotoxicity: A histopathological study.* In Vivo, **25**, 77-86.
14. **Kirdag MK, Tuglu D, Yuvanc E, et al (2019):** *The effect of coenzyme Q and selenium on kidney in rats with partial unilateral ureteral obstruction.* Turk J Urol, **45**, 70-77.
15. **Kwong LK, Kamzalov S, Rebrin I, et al** (2002): *Effects of coenzyme Q10 administration on its tissue concentrations, mitochondrial oxidant generation, and oxidative stress in the rat.* Free Radic Biol Med, **33**, 627-638.
16. **Li X, Zhan J, Hou Y, et al** (2019): *Coenzyme Q10 Regulation of Apoptosis and Oxidative Stress in H2O2 Induced BMSC Death by Modulating the Nrf–2/NQO–1 Signaling Pathway and Its Application in a Model of Spinal Cord Injury.* Oxid Med Cell Longev, **12**, 6493081.
17. **Luna LG** (1968): Manual of histologic staining methods of the armed forces. Institute of Pathology. New York: Blakiston, 1-46.
18. **Mahfoz AM** (2019): *Renal protective effects of coenzyme Q10 against chromate induced nephrotoxicity in rats.* J Applied Sci, **19**, 453-458.
19. **Marx K, Moka D, Schomacker K, et al** (2006): *Cell death induced by 131I in a differentiated thyroid carcinoma cell line in vitro: Necrosis or apoptosis?* Nucl Med Commun, **27**, 353-358.
20. **Okudan N, Revan S, Balci SS**, **et al** (2012): *Effects of CoQ10 supplementation and swimming training on exhaustive exercise–induced oxidative stress in rat heart.* Bratisl Med J, **113**, 393-399.
21. **Saiki R, Lunceford AL, Shi Y, et al** (2008): *Coenzyme Q10 supplementation rescues renal disease in Pdss2 kd/kd mice with mutations in prenyl diphosphate synthase subunit 2.* Am J Physiol Renal Physiol, **295**, 1535-1544.
22. **Savitskaya MA, Onishchenko GE** (2015): *Mechanisms of apoptosis.* Biochemistry, **80**, 1393-1405.
23. **Soltani R, Alikiaie B, Shafiee F, et al** (2020): *Coenzyme Q10 improves the survival and reduces inflammatory markers in septic patients*. Bratisl Med J, **121**, 154-158.
24. **Spitzweg C, Morris JC** (2002): *The sodium iodide symporter: its pathophysiological and therapeutic implications.* Clin Endocrinol (Oxf), **57**, 559-574.
25. **Surucu E, Bekis R, Sengoz T, et al** (2013): *The effect of radioiodine on the intima media thickness of the carotid artery.* Mol Imaging Radionucl Ther, **22**, 85-89.
26. **Ustuner MA, Kaman D, Colakoglu N** (2017): *Effects of benfotiamine and coenzyme Q10 on kidney damage induced gentamicin.* Tissue Cell, **49**, 691-696.
27. **Vakifahmetoglu–Norberg H, Ouchida AT, Norberg E** (2017): *The role of mitochondria in metabolism and cell death.* Biochem Biophys Res Commun, **482**, 426-431.
28. **Yakin M, Eksioglu U, Sadic M, et al** (2017): *Coenzyme Q10 for the protection of lacrimal gland against high–dose radioiodine therapy–associated oxidative damage: histopathologic and tissue cytokine level assessments in an animal model.* Curr Eye Res, **42**, 1590-1596.
29. **Yumusak N, Sadic M, Akbulut A**, **et al** (2019): *Beneficial effects of vitamin E on radioiodine induced gastrointestinal damage: an experimental and pathomorphological study.* Bratisl Med J, **120**, 263-269.
30. **Yumusak N, Sadic M, Yucel G**, **et al** (2018): *Apoptosis and cell proliferation in short–term and long–term effects of radioiodine–131 induced kidney damage: an experimental and immunohistochemical study.* Nucl Med Commun, **39**, 131-139.
31. **Zahed NS, Ghassami M, Nikbakht H** (2016): *Effects of coenzyme Q10 supplementation on C–reactive protein and homocysteine as the inflammatory markers in hemodialysis patients; a randomized clinical trial.* J Nephropathol, **5**, 38-43.

Ankara Univ Vet Fak Derg, **69**, 273-280, 2022
DOI: 10.33988/auvfd.866431

Effect of probiotic on mast cell density and expression of tryptase, chymase, and TNF-α in the urinary bladder of rats with high cholesterol

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Received date: 22.01.2021 - Accepted date: 07.07.2021

Abstract: In this study, it was aimed to immunohistochemically evaluate the possible effect of probiotic supplementation on rats fed a high cholesterol diet on mast cell heterogeneity and density in the urinary bladder and on the expression of chymase, tryptase and tumor necrosis factor-alpha (TNF-α) cytokine in the urinary bladder. Three groups were formed in the study. For 8 weeks, Group 1 (control group) was fed regular rat diet. Food for Group 2 (feeding +2% cholesterol) (high cholestrol group) was provided for 8 weeks. For the last four weeks, Group 3 (%2 cholesterol + *Lactobacillus acidophilus*) (probiotic group) was administered *L. acidophilus* probiotics. Levels of total cholesterol (TC) was measured by using a spectrophotometric autoanalyzer. Serum TC levels were remarkably increased in group 2 compared to the group 1 ($P<0.05$). TC decreased significantly ($P <0.05$) in group 3 compared to group 2. The number of mast cells in the control group and the probiotic group were close to each other. Only safranin O (SO) (+) mast cells was increased in the probiotic group. While there was no difference between control and high cholesterol groups, an increase in the number of tryptase mast cells was found in the probiotic group. The number of chymase mast cells declined in the probiotic group compared to control and high cholesterol groups. There was no significant difference in TNF-α immunoreactivity between the three groups. In conclusion, in this study, possible effects of cholesterol and probiotics on mast cell heterogeneity and expression of chymase, tryptase, TNF-α were revealed.

Keywords: Bladder, high cholesterol, mast cell, probiotic, TNF-α.

Probiyotiğin, yüksek kolesterolü olan sıçanların idrar kesesinde mast hücre yoğunluğu ve triptaz, kimaz ve TNF-α ekspresyonu üzerine etkisi

Özet: Bu çalışma da yüksek kolesterollü diyet ile beslenen ratlara probiyotik ilavesinin ratların idrar kesesindeki mast hücre heterojenitesi ve yoğunluğuna olası etkisi ile mesanede kimaz, triptaz ve tümör nekroz faktör-alfa (TNF-α) sitokinin ekspresyonuna etkisinin immunohistochemically değerlendirilmesi amaçladı. Grup 1 (kontrol grubu) 8 hafta boyunca standart fare yemi ile beslendi. Grup 2 (+%2 kolesterolle beslenen) (yüksek kolestrol grubu) 8 hafta boyunca verildi. Grup 3 (%2 kolesterol + *Lactobacillus acidophilus*) (probiyotik grubu): Son 4 hafta *L. acidophilus* probiyotiği verildi. Total kolesterol (TC) düzeyi spektrofotometrik yöntemle otoanalizör kullanılarak ölçüldü. Grup 2 serum TC düzeylerinin grup 1'e göre önemli ölçüde arttığı belirlendi ($P<0,05$). TC düzeyi Grup 3' te ise Grup 2'ye göre önemli düzeyde azaldığı belirlendi ($P <0,05$). Kontrol grubu ile probiyotik grubundaki mast hücre sayılarının birbirine yakın olduğu belirlendi. Gruplar arasında probiyotik verilen grupta sadece safranin O (SO) (+) mast hücrelerinin arttığı görüldü. Kontrol ve yüksek kolesterol grupları arasında sayısal bir fark olmamasına karşın, probiyotik verilen grupta triptaz mast hücresi sayısında bir artış bulundu. Probiyotik grubunda kimaz mast hücre sayısının kontrol ve yüksek kolesterol grubuna göre azaldığı görüldü. Üç grup arasında TNF-α immünoreaktivitesinde önemli bir fark yoktu. Sonuç olarak, bu çalışmada, kolesterol ve probiyotiklerin mast hücre heterojenliği ve kimaz, triptaz, TNF-α ekspresyonu üzerindeki olası etkileri gösterilmeye çalışılmıştır.

Anahtar sözcükler: İdrar kesesi, mast hücre, probiyotik, TNF- α, yüksek kolestrol.

Introduction

Cholesterol is an essential lipid for maintaining cellular homeostasis. Cell membranes, hormones, and vitamin D are created by your body using cholesterol. It is also enriched in lipid rafts and plays a key role in intracellular signal transduction (19). Dietary cholesterol is linked to an increased risk of lung, stomach, pancreatic, rectum, colon, kidney, and bladder cancers (16). Probiotics can be regarded as an alternative choice for the prevention and treatment of chronic infection (9). They can produce antimicrobial agents and also can keep the host away from infections by attaching to specific adhesion sites on the epithelial surface of the urinary tract (15). It has been reported that chronic hyperlipidemia leads to detrusor overactivity and detrusor inadequate activity, which cause developmental mechanisms of urinary bladder dysfunction (39).

Mast cells located in areas associated with the external environment detect potentially harmful substances (31). Mast cells are commonly found in systems such as the skin, respiratory and digestive systems. They're among the first cell groups in the defense mechanism against pathogens and foreign object entry because of this distribution. These cells can also be found in the connective tissue of the genital and urinary systems and around blood vessels and peripheral nerves (12). Mast cells can also function as antigen-presenting cells by processing bacteria and antigens. The most distinctive morphological feature of mast cells is secretory granules found in their cytoplasms such as biogenic amines, proteoglycans, and cytokines. When mast cells are stained with granule-specific dyes such as alcian blue and safranin O, they are divided into three subgroups according to their histochemical differences: alcian blue (AB) (+), safranin O (SO) (+), and AB/SO (+) (mixed) (13). Mast cells have a large number of co-stimulatory molecules. Among these mediators is the tumor necrosis factor-alpha (TNF-α), which allows them to interact with different cell populations (34).

Mast cells are immunohistochemically classified into two subgroups based on the distribution of neutral proteases, tryptase positive (MC_T) and chymase-positive mast cells (MC_{TC}). Tryptase, which is used as a marker for mast cell activation, is the most abundant secretory granule-derived serine proteinase contained in mast cells. Tryptase is known to participate in angiogenesis, the degradation of the fibrous and amorphous component of the extracellular matrix of connective tissue, and indirectly the release of growth factors, including matrix metalloproteinases (3). Chymases, which are neutral serine proteases, are significant components of mast cell granules. Chymase plays a role in remodeling the extracellular matrix of the connective tissue, inflammation, allergy, and angiogenesis mechanisms. Also, it inhibits the production of some pro-inflammatory cytokines while promoting neutrophil and eosinophil migration (32). TNF-α is an inflammatory cytokine responsible for a diverse range of signaling events within cells. TNF-α has various therapeutic functions in the body, including immunostimulation, infection resistance, tumor resistance, sleep regulation, and embryonic development. Also, TNF-α has the ability to cause necrotic or apoptotic cell death (18).

The purpose of this study was to investigate the possible effects of high cholesterol and probiotics on mast cell heterogeneity and density in the rat urinary bladder. Besides, chymase, tryptase neutral proteases and TNF-α cytokine expression in the urinary bladder were evaluated immunohistochemically.

Materials and Methods

The experimental protocol and all animal procedures were approved by the Experimental Ethics Committee. In the study, a power analysis was conducted by using the G*Power (3.1.9.3) software, and according to the result of the power analysis (95% power, α=0.05), 3 groups rats (n=7). In our study, we used a total of 21 Sprague Dawley adult male rats, 7 in each group. Rats were engaged under standard experimental laboratory conditions (temperature: 24°C; dark/light cycle: 12/12 hours; free arrival to food and water; relative humidity: 60%). Experimental animals were fed ad libitum throughout the study. The rats were randomly assigned to three groups.

In order to form high cholesterol, 2% cholesterol was added to standard rat feed and it was pelleted again after mixing homogeneously. The probiotic *Lactobacillus acidophilus* to be used for treatment in the study was purchased as lyophilized. After diluting the lyophilized bacteria with De Man, Rogosa ve Sharpe Agar (MRS Agar) broth, it was inoculated on 5% sheep blood agar for viability and purity control. It was added in an amount of 1 ml to the tube containing 9 ml MRS from the culture determined to be alive and pure and left to incubate at 37°C for 18 hours. At the end of the period, suspensions were prepared with physiological saline up to 10^9 from liquid culture. Three of these suspensions were seeded on blood agar and left incubation for 18 hours at 37°C. After the incubation, bacterial colonies grown on the plates were counted and the number of bacteria in the main culture was calculated. After the calculation, the suspension was made to contain 10^9 kob/ml bacteria in the main culture and *Lactobacillus acidophilus* probiotic suspension to be used for treatment was prepared.

Group 1 (control group): The male adult 7 rats were fed with standard pellet rat feed for 8 weeks. Group 2 (feeding +2% cholesterol) (high cholestrol group): The male adult 7 rats were fed with a ration of 2% cholesterol added to the standard feed for 8 weeks and high

cholesterol was formed (28). Group 3 (2% cholesterol + *Lactobacillus acidophilus*) (probiotic group): The male adult 7 rats were fed for 8 weeks with a ration containing 2% cholesterol in the standard feed, and in the last 4 weeks of the trial, *Lactobacillus acidophilus* probiotics containing $2x10^8$ kob/ml/day were administered via oral gavage (37).

At the end of the eight-week experimental period, the rats were fasted for 12 hours before weighed one by one, allowing only normal drinking water. Rats were anesthetized with 10% ketasol (0.8-1.3ml/kg) and 2% basilazine (2-5 mg/kg) via IP and blood samples were taken from the heart. Blood samples were leaved to stand for 20 minutes in the laboratory for allowed clotting, and then centrifuged for 10 min at 1550 xg. The sera were extracted and divided into aliquots. Sera were stored at -80 °C until used for the analyses.

The rats in all groups were sacrificed after 8 weeks later their urinary bladder tissues were taken. The urinary bladders of the rats were fixed in 10% formaldehyde solution (Sigma-Aldrich, S2BF1830V) for histological examination. Following this, they were blocked in paraffin after undergoing routine tissue processing procedures.

Serum total cholesterol: Total cholesterol level was measured by serum biochemistry autoanalysers (Autolab, AMS srl, Aotuanalyzer, Netherlands) using commercial autoanalyzer test kits (Audit Diagnostics, Ireland).

Mast cell histochemistry: In the urinary bladder blocks, 10 serial sections of 5µm thickness were taken at 30 µm intervals. Cross-sections were stained with toluidine blue (Sigma-Aldrich, 92-31-9) (0.5% and pH=0.5) for 10 minutes. In order to determine subtypes of mast cells, 5 µm thick sections with 30µm intervals were taken from each block and stained with alcian blue (Sigma-Aldrich, 22864-99-2)/safranin O (Sigma-Aldrich, 477-73-6) (AB/SO) combined staining method (11).

In the serial sections prepared to find out the numerical distribution of mast cells, cell counts were performed with 100 squares ocular micrometer. The mast cells at 100 square units of the ocular micrometer were counted with a magnification of X40. Cell count was performed at 10 randomly chosen different areas of the sections receipt from bladder and the arithmetic mean of the results was taken. All the data obtained by calculating the square of 100 square ocular micrometer for X40 objective magnification with the help of micrometric lame were turned into mast cell number within a unit area of 1 mm^2.

Immunohistochemistry: The urinary bladder sections 5 µm thick taken from paraffin blocks were stained immunohistochemically by using anti-rabbit polyclonal chymase (1/200 dilution, Biorbyt, orb11030), mouse monoclonal tryptase (1/200 dilution, Abcam, ab2378) and rabbit polyclonal TNF-α (1/200 dilution, Abcam, AB-9739) primary antibodies with Streptavidin biotin complex method (35). Histostain Plus (Zymed kit: 85-6743) kit was performed as secondary antibody. After deparaffinization, sections were heated in a microwave oven of 700 watts within citrate buffer (pH=6) solution for proteolysis. In order to block endogenous peroxidase activity, the tissues were incubated in 3% hydrogen peroxide solution. Following washing with phosphate buffer solution (PBS), serum in the kit was instilled to prevent nonspecific protein binding in sections. Primary antibody was applied on sections and they were stored at +4 ^{0}C for overnight. Only PBS solution was process on negative control group tissues. Following the washing procedure, biotinylated secondary antibody was instilled into sections and incubated at streptavidin-horseradish peroxidise complex after washing. As the last stage, 3, 3'-diaminobenzidine (DAB) (Abcam, ab64264) was used as chromogen and the preparations were covered with entellan by counterstaining was performed with hematoxylin.

Statistical analysis: The number of mast cell were analyzed with one-way ANOVA and determination of the significance of differences between the groups was done with Duncan's test. Differences among the groups $P<0.05$ was accepted to be significant. SPSS statistical software was used for analyses (IBM – Company, Armonk, NY-USA, version 21).

AB (+), SO (+), AB/SO (+) (mixed), MC_T and MC_{TC} distribution was evaluated semiquantitatively. In semiquantitative evaluation following criteria were used; no positive cell in the scanned area (-), 1-2 cells (±), 3-4 cells (+), and 5-6 cells (++). Quantitative evaluation was made according to the positive staining intensity. Staining intensity of TNF-α expression was scored as; 0, no immunoreactive detected; +/-, weakly positive staining present; +, moderate positive staining present; ++, strongly positive staining present.

Results

Serum total cholesterol levels: Total cholesterol (TC) level of Group 1, Group 2 and Group 3 were 51.14±1.56, 76.71±1.97, 62±1.3 (mg/dl), respectively. It was determined that TC level increased significantly in Group 2 ($P<0.05$) and begun to decrease in Group 3.

Histochemical findings

Toluidine blue staining: Mast cells showed metachromasia and their granules could not selected individually. They were in different sizes with round, oval or spindle shaped. Nuclei of the cells were covered by granules in most of the cells (Figure 1A). Mast cells observed in urothelium, submucosa, lamina propria (Figure 1B), and detrusor muscle layer. It was noteworthy that they were localized around capillaries in the lamina propria (Figure 1C), and close to detrusor smooth muscle

cells. When the groups were evaluated, the number of mast cells were lower in the control group. The number of mast cells in the control group and the probiotic group were close to each other. The change in the number of mast cells in the urinary bladder was not statistically significant (P> 0.05) (Table 1).

AB/SO combine staining: The application of the alcian blue/safranin O staining technique demonstrated that three types of cells, including in blue AB (+) cells, in pink-red SO (+) cells, and red-blue AB/SO (+) (mixed) cells, were present in the urinary bladder (Figures 2A and 2B). It was seen that mast cells stained SO (+) in the urinary bladder were numerically higher than AB (+) and AB/SO (+) stained mast cells. Among the groups, only the SO (+) mast cells increased in the probiotic given group (Figure 2C) (Table 2).

Table 1. Mast cell counts after staining with toluidine blue in three groups (P>0.05).

Groups	n	X ±Sx
Group 1	7	12.38±0.09
Group 2	7	14.03±0.18
Group 3	7	13.11±0.65

Table 2. Mast cell counts after staining with alcian blue/safranin O combined staining and tryptase and chymase positive cell reaction in bladder. No positive cell (-), 1-2 cells (±), 3-4 cells (+), and 5-6 cells (++).

Mast Cells	Control group	High cholesterol group	Probiotic group
AB (+)	±	±	±
SO (+)	+	+	++
AB/SO (+)	±	±	±
Tryptase-mast cell	+	+	++
Chymase-mast cell	++	++	+

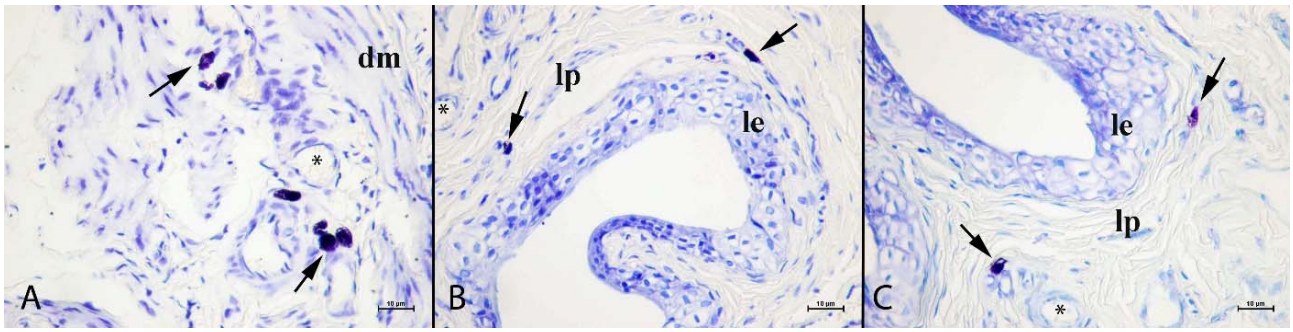

Figure 1. A Group 1, B group 2, C group 3, toluidine blue staining; le (lamina epithelialis), lp (lamina propria), dm (detrusor muscle), arrow: metachromatic mast cells, blood vessel (asterix), original magnification X40; range bar, 10 µm.

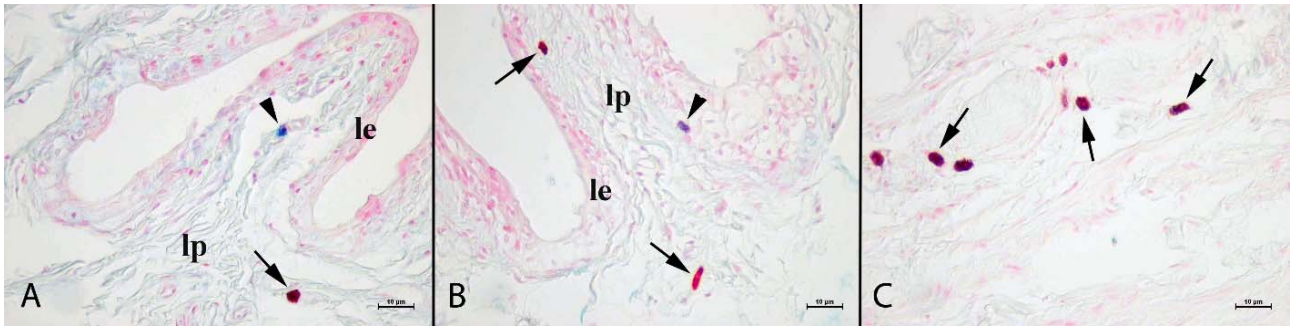

Figure 2. Alcian blue/safranin O combined staining method; A group 1, arrow: SO (+) mast cell, arrowhead: AB (+) mast cell, B group 2, arrow: SO (+) mast cell, arrowhead: mixed type mast cell, le (lamina epithelialis), lp (lamina propria), C group 3, arrow: SO (+) mast cell, original magnification X40; range bar, 10 µm.

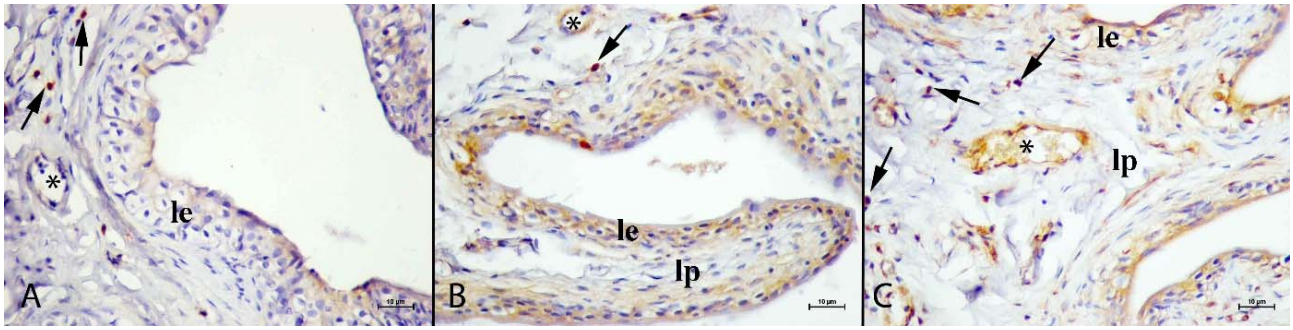

Figure 3. Tryptase immunostaining A group 1, B group 2, C group 3, le (lamina epithelialis), lp (lamina propria), dm (detrusor muscle), arrow: tryptase-positive mast cells, blood vessel (asterix), original magnification X40; range bar, 10 µm.

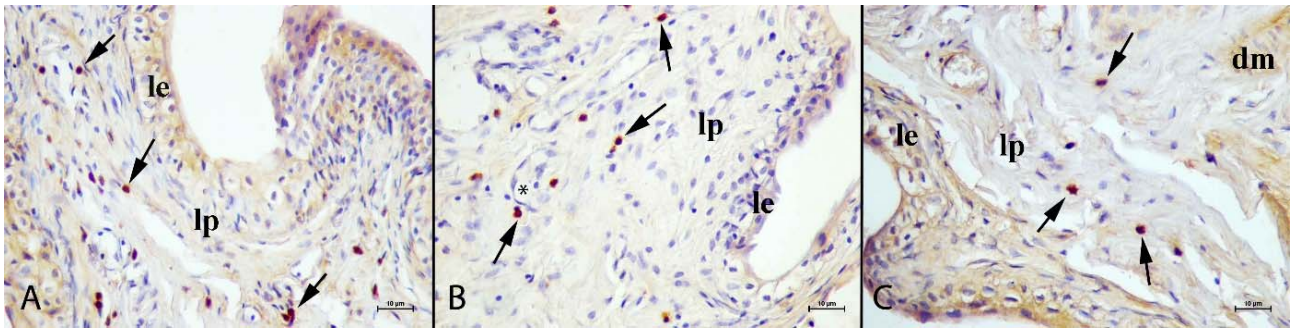

Figure 4. Chymase immunostaining A group 1, B group 2, C group 3, le (lamina epithelialis), lp (lamina propria), dm (detrusor muscle), arrow: chymase-positive mast cells, blood vessel (asterix), original magnification X40; range bar, 10 µm.

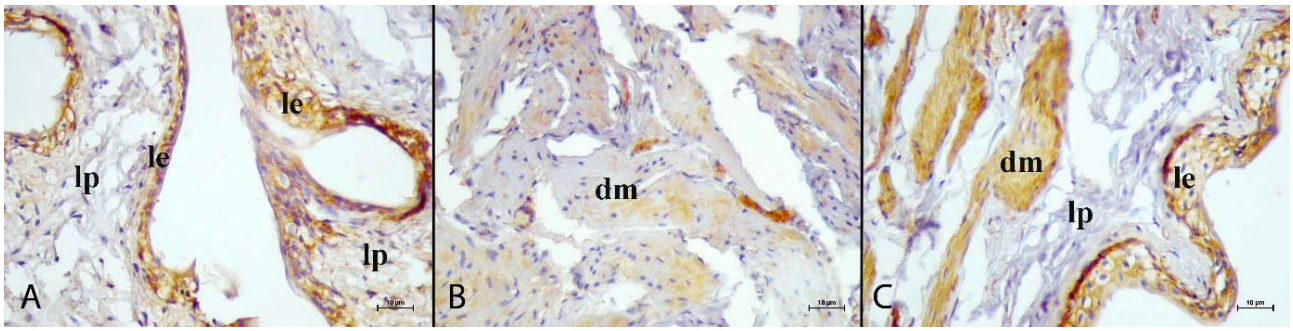

Figure 5. Tumor necrosis factor alpha immunostaining A group 1, B group 2, C group 3, TNF-α immunoreactivity, le (lamina epithelialis), lp (lamina propria), dm (detrusor muscle), original magnification X40; range bar, 10 µm.

Immunohistochemical findings

Tryptase-positive mast cell expression: In all groups, oval and round-shaped MC_TS were observed in the urinary bladder (Figure 3A). MC_TS were present in the urothelium and submucosa (Figure 3B), including the lamina propria, as well as for the detrusor smooth muscle layer. MC_TS were seen especially around the blood vessels in the submucosa (Figure 3C). While there was no numerical difference between the control and high cholesterol groups, an increase in the number of MC_TS was found in the probiotic given group (Table 2).

Chymase-positive mast cell expression: In the urinary bladder oval, round, or spindle-shaped $MC_{TC}S$ were detected in all layers, especially around the blood vessels (Figures 4A and 4B). $MC_{TC}S$ were localized in the mucosa especially in the lamina propria (Figure 4C). In the detrusor layer, they were predominantly detected close to smooth muscle cells (Figure 4). The number of $MC_{TC}S$ decreased in the probiotic group compared to the control and high cholesterol group (Table 2).

TNF-α expression: TNF-α immunoreactivity was observed throughout the transitional epithelium as a brown color in the urinary bladder. Membrane like and intracytoplasmically immunopositive staining were determined epithelial cells (Figures 5A and 5B). Analysis of the tissue preparations of each of the groups revealed the immunoreactivity of TNF-α antibodies in detrusor muscle cells intracytoplasmic staining (Figure 5C). There was no significant difference in TNF-α immunoreactivity between the three groups (Table 3).

Table 3. Immunoreactivity of TNF-α in bladder; +/-, weakly positive staining present; +, moderate positive staining present; ++, strongly positive staining present.

	Control group	High cholesterol group	Probiotic group
TNF-α	++	++	++

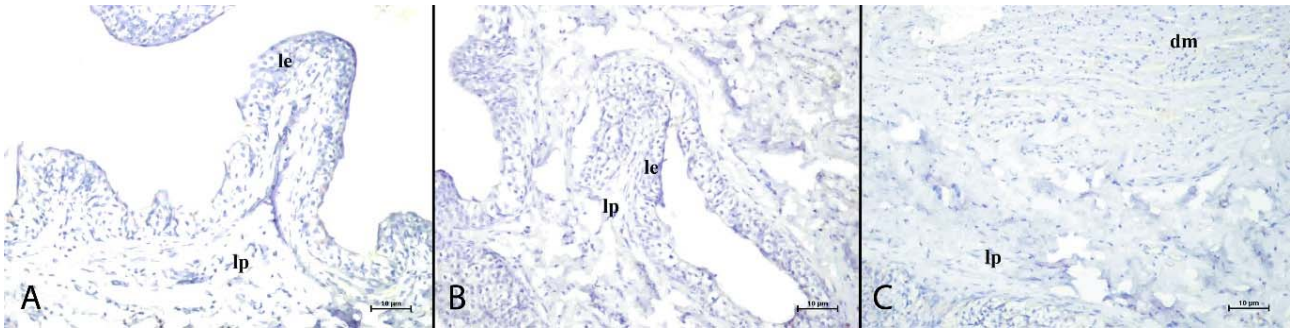

Figure 6. Immunohistochemistry staining negative control A tryptase immunostaining, B chymase immunostaining, C TNF-α immunostaining, le (lamina epithelialis), lp (lamina propria), dm (detrusor muscle), original magnification X20; range bar, 10 μm.

Also, tryptase, chymase, and TNF-α immunoreactions were not observed in the negative control groups when immunostaining was performed on urinary bladder tissue for control purposes (Figure 6).

Discussion and Conclusion

Studies have shown that certain probiotic strains can reduce inflammation and significantly impact immune cells and inflammation (34). Bacteria and mast cells are thought to interact with each other via useful stabilizing agents. The Histamine H1 receptor blocker diphenhydramine, for example, was shown in one study to inhibit the increase of cytokines from mast cells stimulated by bacteria (27). Probiotic VSL # 3 is known to have a reducing effect on mast cells that increase in visceral hypersensitivity (23). Cholesterol can cause very high levels of mast cell activation in mice fed a high cholesterol diet (40). Previous studies have shown that the probiotic bacteria *Bifidobacterium bifidum* BGN4 and *Lactobacillus casei* 911 reduce ovalbumin-induced mast cell degranulation in ear and tongue tissue samples of mice (21). Several studies in rodent models have shown that *L. rhamnosus* GG and some other probiotic strains have a decreasing effect on mast cell numbers (4). Furthermore, following oral treatment of *E. faecalis*, which reduced mast cell infiltration in a murine model, the role of commensal microorganisms in controlling mast cell activation was demonstrated (7). In addition, some broad-spectrum antibacterial agents are known to inhibit mast cell activation and degranulation. Also, it was demonstrated that different strains of Lactobacilli can suppress mast cell degranulation (34). In the study investigating the effect of probiotics on aspirin-induced gastric mucosal lesions, it was observed that the number of mast cells was not significantly different between the control and probiotic plus aspirin groups (33). In the light of the findings of our studies and previous studies, we suggest that probiotics have the ability to affect mast cell numbers as well as beneficial effects in the organism.

Two subtypes of mast cells were defined based on their physiological, staining characteristics, functional variety, and morphology. These cells are stained differently in terms of the proteoglycan type and protease content (11). In a study investigating the phenotypic heterogeneity of mast cells in rat kidney, three kinds of mast cells have been determined with AB/SO staining method. In studies examining mast cell subtypes in rat kidney (12) and rat small intestine (8), SO (+) mast cells were the most common subtype. It has been reported that more SO (+) mast cells were detected in the ovarian tissue of rats treated with experimental capsaicin (36). In our study, we found three subtypes of mast cells with SO (+), AB (+), and AB/SO (+) staining in all groups. We observed that feeding a high-cholesterol diet had no direct effect on mast cell heterogeneity in our research. On the other hand, the probiotic group had the highest number of SO (+) stained mast cells. Based on our findings, we hypothesized that probiotics may influence mast cell heterogeneity in the rat urinary bladder.

Previous work has reported that MC_TS were observed in urothelium and submucosa, including the lamina propria, as well as for the detrusor muscle layer in the bladder (29). It was found that MC_TS, which increased in urinary bladder damage caused by protamine sulfate, decreased when montelukast was given (6). Ketamine caused a significant increase in the numbers of MC_TS in the urinary bladder (22). In studies investigating the tryptase expression, it was found that in the bladder, montelukast against stress (10) and non-Hunner type interstitial cystitis (1) did not cause a significant increase

in the number of mast cells. We postulated that the high cholesterol diet did not directly affect the number of MC_Ts in the rat urinary bladder, whereas probiotics might have an effect on the number of MC_Ts.

In the study of hyperlipidemia on the heart tissue, the expression of chymase increased in the experimental group compared to the control group (5). Studies have shown that MC_{TC}s increase in the urinary bladder lamina propria (25), mucosa, and detrusor layer as a result of interstitial cystitis (38). Nedocromil treatment can reduce the number of chymase positive cells in diabetic mice (17). In previous studies, it was found that hormonal mechanism changes (14) and capsaicin administration (36) did not cause a change in MC_{TC} count. It was observed that the number of MC_{TC}s in the urinary bladder of rats fed with probiotics decreased. Furthermore, it was found that high cholesterol did not lead any change in chymase expression. Based on the findings we obtained in this study, we speculated that probiotics may play an important role in the attitude of MC_{TC}s in the rat urinary bladder.

In a study examining the immunomodulatory and anti-inflammatory capacities of probiotics, it has been reported that there was no difference in TNF-α densities between the control and experimental groups (26). It has also been shown that TNF-α expression was elevated in bladder inflammation (30). It has been observed that the expression intensity of TNF-α, one of the main active substances produced by mast cells, was significantly reduced in mast cell-deficient mice (24). The previous study reported an increase in mast cell count, strong mast cell tryptase, and TNF-α staining in allergic conjunctivitis (20). Besides, Pycnogenol has been reported to reduce both the number of mast cells and TNF-α staining (2). In our study, we could not find differences in TNF-α expression in the bladder among three groups. Although there was a difference in mast cell numbers between the groups in our study, it was statistically insignificant. Also, there was no difference between the control and high cholesterol groups in semi-quantitative evaluations of tryptase and chymase mast cells. The results obtained from this study show that the probiotic cannot directly affect the TNF-α expression in the urinary bladder.

In conclusion, to the best of our knowledge, this study is the first to show that cholesterol and probiotics may induce mast cell heterogeneity and chymase, tryptase, and TNF-α expression in the rat urinary bladder. The results of this study display that cholesterol and probiotic administration may cause changes, although not significantly, in mast cell heterogeneity and numbers in the urinary bladder. Other important findings of this study were that high cholesterol and probiotics may not affect TNF-α expression in the bladder. Taken together, these findings show how cytokines and mast cells respond to active substances in metabolism. However, this study is based on an animal model, and more clinical studies are needed to evaluate the effects of high cholesterol and probiotics on the urinary bladder.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

In the presented study, increasing samples of the project numbered PYO.VET.1904.16.019, which was approved by the Ondokuz Mayıs University Animal Experiments Local Ethics Committee (2016/27) and supported by the Scientific Research Projects of Ondokuz Mayıs University, were used.

Conflict of Interest

The authors declared that there are no conflicts of interest. The authors are responsible for the content and writing of the paper.

References

1. **Akiyama Y, Maeda D, Morikawa T, et al** (2018): *Digital quantitative analysis of mast cell infiltration in interstitial cystitis.* Neurourol Urodyn, **37**, 650-657.
2. **Akyuz AI, Kocaturk UT, Gunel C, et al** (2018): *Effect of Pycnogenol® on an experimental rat model of allergic conjunctivitis.* Graefes Arch Clin Exp Ophthalmol, **256**, 1299-1304.
3. **Atiakshin D, Buchwalow I, Samoilova V, et al** (2018): *Tryptase as a polyfunctional component of mast cells.* Histochem Cell Biol, **149**, 461-477.
4. **Cassard L, Lalanne AI, Garault P, et al** (2016): *Individual strains of Lactobacillus paracasei differentially inhibit human basophil and mouse mast cell activation.* Immun Inflam Dis, **4**, 289-299.
5. **Cheng Y, Zhu Y, Zhang J, et al** (2017): *Large accumulation of collagen and increased activation of mast cells in hearts of mice with hyperlipidemia.* Arq Bras Cardiol, **109**, 404-409.
6. **Çetinel Ş, Çanıllıoğlu YE, Çikler E, et al** (2011): *Leukotriene D4 receptor antagonist montelukast alleviates protamine sulphate-induced changes in rat urinary bladder.* BJU Int, **107**, 1320-1325.
7. **De Zuani M, Dal Secco C, Frossi B** (2018): *Mast cells at the crossroads of microbiota and IBD.* Eur J Immunol, **48**, 1929-1937.
8. **Demirbağ E, Çınar K, Kutlar MH, et al** (2012): *Distribution and heterogenity of mast cells in small intestine of rat (Rattus rattus).* SDÜ Fen Dergisi, **7**, 92-99.
9. **Doron S, Gorbach SL** (2006): *Probiotics: Their role in the treatment and prevention of disease.* Exp Rev Anti Infect Ther, **4**, 261-275.
10. **Dülger EÇ, Çanıllıoğlu YE, Çetinel Ş, et al** (2018): *Protective Effects of Montelukast Against Stress-Induced Degeneration of the Urinary Bladder.* Clin Exp Health Sci, **8**, 211-216.

11. **Enerback L** (1966): *Mast cells in rat gastrointestinal mucosa: 1. Effects of fixation.* Acta Pathol Microbiol Scand, **66**, 289-302.
12. **Ertuğrul T, Çiftci G, Tütüncü Ş** (2017): *Heterogeneity and distribution of mast cells in renal tissues of rats exposed to formaldehyde.* Vet Hekim Der Derg, **88**, 26-37.
13. **Ertugrul T, Tutuncu S, Kabak M, et al** (2018): *The distribution and heterogeneity of mast cells in tongue from five different avian species.* Anat Histol Embryol, **47**, 306-312.
14. **Hayıroğlu AE, Karaca T, Demirtaş S** (2016): *Streptozotocin-Induced diabetic that histochemical and immunohistochemical examination of mast cells distribution in ovary and uterus during different stages of estrous cycle in rats.* Kafkas J Med Sci, **6**, 29-37.
15. **Homayouni A, Bastani P, Ziyadi S, et al** (2014): *Effects of probiotics on the recurrence of bacterial vaginosis: a review.* J Low Genit Tract Dis, **18**, 79-86.
16. **Hu J, La Vecchia C, de Groh M, et al** (2012): *Dietary cholesterol intake and cancer.* Ann Oncol, **23**, 491-500.
17. **Huang ZG, Jin Q, Fan M, et al** (2013): *Myocardial remodeling in diabetic cardiomyopathy associated with cardiac mast cell activation.* PLoS One, **8**, e60827.
18. **Idriss HT, Naismith JH** (2000): *TNF and the TNF receptor superfamily: structure-function relationship(s).* Microsc Res Tech, **50**, 184-195.
19. **Ikonen E** (2008): *Cellular cholesterol trafficking and compartmentalization.* Nat Rev Mol Cell Biol, **9**, 125-38.
20. **Kocatürk T, Ünsal AİA, Günel C, et al** (2020): *The effect of thymoquinone on experimental model of allergic conjunctivis.* KOU Sag Bil Derg, **6,** 17-22.
21. **Kim H, Kwack K, Kim YD, et al** (2005)*: Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model.* FEMS Immunol Med Microbiol, **45**, 259-267.
22. **Li M, Yang K, Wang X, et al** (2015): *Mast cells infiltration and decreased E-cadherin expression in ketamine-induced cystitis.* Toxicol Rep, **2**, 205-209.
23. **Li YJ, Li J, Dai C** (2020): *The Role of Intestinal Microbiota and mast cell in a rat model of visceral hypersensitivity.* J Neurogastroenterol Motil, **6**, 529-538.
24. **Liu C, Ouyang W, Xia J, et al** (2018): *Tumor Necrosis Factor-α Is Required for Mast Cell-Mediated Host Immunity Against Cutaneous Staphylococcus aureus Infection.* J Infect Dis, **218**, 64-74.
25. **Malik ST, Birch BR, Voegeli D, et al** (2018). *Distribution of mast cell subtypes in interstitial cystitis: implications for novel diagnostic and therapeutic strategies?* J Clin Pathol, **71**, 840-844.
26. **Oksaharju A, Kooistra T, Kleemann R, et al** (2013): *Effects of probiotic Lactobacillus rhamnosus GG and Propionibacterium freudenreichii ssp. Shermanii JS supplementation on intestinal and systemic markers of inflammation in ApoE∗3Leiden mice consuming a high-fat diet.* Br J Nutr, **119**, 77-85.
27. **Oksaharju A, Kankainen M, Kekkonen, et al** (2011): *Probiotic Lactobacillus rhamnosus downregulates FCER1 and HRH4 expression in human mast cells.* World J Gastroenterol, **17**, 750-759.
28. **Onody A, Csonka C, Giricz Z, et al** (2003): *Hyperlipidemia Hinduced by a cholesterol-rich diet leads to enhanced peroxynitrite formation in rat hearts.* Cardiovasc Res, **58**, 663-670.
29. **Oottamasathien S, Jia W, Roundy LM, et al** (2013): *Physiological relevance of LL-37 induced bladder inflammation and mast cells.* J Urol, **190**, 1596-1602.
30. **Ozguven AA, Yılmaz O, Taneli F, et al** (2014): *Protective effect of ketamine against hemorrhagic cystitis in rats receiving ifosfamide.* Indian J Pharmacol, **46**, 147-151.
31. **Palm NW, Rosenstein RK, Medzhitov R** (2012): *Allergic host defences.* Nature, **484**, 465-472.
32. **Pejler G** (2020): *Novel ınsight into the in vivo function of mast cell chymase: lessons from knockouts and ınhibitors.* J Innate Immun, **12**, 357-372.
33. **Şenol A, Işler M, Karahan AG, et al** (2011): *Effect of probiotics on aspirin-induced gastric mucosal lesions.* Turk J Gastroenterol, **22**, 18-26.
34. **Traina G** (2019): *Mast Cells in Gut and Brain and Their Potential Role as an Emerging Therapeutic Target for Neural Diseases.* Front Cell Neurosci, **13**, 345.
35. **True LD** (1990): Principles of immunohistochemistry. Gower Medical Publishing, New York.
36. **Tutuncu Ş, Ertugrul T** (2019): *Immunohistochemical expression of tryptase- chymase and mast cell heterogeneity in capsaicin-treated rat ovaries.* IJVAR, **2**, 25-31.
37. **Wang L, Zhou B, Zhou X, et al** (2019): *Combined lowering effects of rosuvastatin and l. acidophilus on cholesterol levels in rat*. J Microbiol Biotechnol, **29**, 473-481.
38. **Yamada T, Murayama T, Mita H, et al** (2000): *Subtypes of bladder mast cells in interstitial cystitis.* Int J Urol, **7**, 292-297.
39. **Yoshida M, Kudoh J, Masunaga K, et al** (2010): *The effects of chronic hyperlipidemia on bladder function in myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits.* Neurourol Urodyn, **29**, 1350-1354.
40. **Zhanga X, Huang Q, Wang X, et al** (2019): *Dietary cholesterol is essential to mast cell activation and associated obesity and diabetes in mice.* Biochim Biophys Acta Mol Basis Dis, **1865**, 1690-1700.

Ankara Univ Vet Fak Derg, **69**, 281-288, 2022
DOI: 10.33988/auvfd.833094

Dietary supplementation of protexin and artichoke extract for modulating growth performance and oxidative stress in broilers

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Received date: 29.11.2020 - Accepted date: 05.07.2021

Abstract: In this study, the effects of Protexin and artichoke extract (AE) were evaluated on the performance and oxidative stress of chickens. Totally, 300 chicks were divided into 4 groups that were fed a basal diet, a diet containing Protexin, AE, and Protexin plus AE all over the growing period. The growth indices were measured weekly and analyzed at 21 and 42 days of age. At 42 days of age, blood samples were collected from all chickens. The concentrations of liver enzymes, lipid profiles, and antioxidant status were measured in blood samples. Results showed that the weight gain (WG) was significantly higher and the feed conversion ratio (FCR) significantly lower in chickens that received Protexin, or Protexin plus AE, in comparison with chickens that received AE and control chickens ($P<0.05$). Furthermore, the addition of AE plus Protexin can significantly increase the activity of blood Glutathione peroxidase (GPx) and total antioxidant status (TAS) with respect to chickens that were fed Protexin and AE alone. The triglyceride (TG), cholesterol (CHL), and low-density lipoprotein (LDL) was lower and high-density lipoprotein (HDL) were higher in chickens that received AE or Protexin plus AE, with a comparison of chickens fed Protexin and control chickens ($P<0.05$). The levels of aspartate transferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) show a significant decrease in chickens that get Protexin plus AE. In conclusion, continuous utilization of Protexin along with artichoke extract in broiler diets can promote growth performance and modulate oxidative stress in broilers.

Keywords: chicken, performance, artichoke extract, protexin.

Introduction

Formerly, antibiotics were commonly used in the poultry industry to prevent and treat infectious diseases. Some antibiotics promote growth and reduce mortality by decreasing gastrointestinal pathogens and ultimately declining the absorption of bacterial toxins, improving digestion, and absorption of nutrients (24). In addition, some of which also play a role in boosting the immune system and are thought to stimulate the immune response. Continued use of antibiotics in livestock production induces antibiotic resistance in microbial strains which the possibility of transferring via food to humans can increase (13). Furthermore, antibiotic residues in meat and by-products can be transferred to humans via the food chain, which can cause many side effects for humans. Antibiotics are linked to an increase in allergies, genomic mutations, and cancer (12). Humans have always thought of a suitable alternative to minimize the need for antibiotics, and today, those are less popular in livestock diets (25).

Probiotics have long been used as a live beneficial microbe in livestock and poultry diets that improve the microbial balance of the gastrointestinal tract and have positive effects (47). According to the definition made by FAO/WHO, probiotics are living microorganisms that can positively affect the host (21). Probiotics may contain one or more strains of bacteria or yeast. The types of bacteria used as probiotics include *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, and yeasts, mainly *Saccharomyces cerevisiae* and *Saccharomyces* species (42). Probiotics alter the gastrointestinal tract by reducing the dominance of pathogenic bacteria and microbial populations, and stimulate the immune response, lower lipids, and increase vital organ health (47).

Another alternative to antibiotics can be found in herbs and their derivatives. The use of medicinal plants in poultry production provides beneficial effects for poultry due to the presence of valuable compounds in plants (25). Phytobiotics include a wide range of plant-derived products, including herbs, essential oils, spices, and extracts, which are added to livestock and poultry diets to enhance performance and increase product quality (52). The active substances and chemical composition of phytobiotics depend on the part of the plant used (flowers, leaves, seeds, etc.), geographical origin, and harvest season. The active ingredients in various plants belonging to distinct families are also different, and therefore their effects are expected to be different (11). However, these phytobiotics can have immunostimulatory, antimicrobial, anti-inflammatory, and antioxidant properties (31).

Due to the antimicrobial properties of medicinal plants, herbal medicine has recently become popular for poultry production without antibiotics.The use of medicinal plants in poultry production eliminates the antibiotic residual concerns in the meat and eggs. Phytobiotics can even increase the total antioxidant capacity of meat and have positive effects on consumer health (25). Antioxidant compounds in plants help the antioxidant system to remove free radicals, preventing the oxidants from damaging the vital organs and improving the performance of poultry (11).

Based on literature, phytobiotics can increase growth by improving taste and enhancing the secretion of digestive enzymes (17). In addition, there is evidence of the effect of phytobiotics on the modulation of pathogenic bacteria and inhibition of their attachment to the intestinal wall. Also, phytobiotics can increase secretion of the digestive tract and excrete the pathogenic bacteria and their toxic metabolites. It seems that all the mechanisms are involved in improving performance following phytobiotic supplementation in the poultry diet (25). Therefore, in this recent study, the performance of a commercial standard probiotic (Protexin) with a phytobiotic (artichoke extract) was compared to evaluate the effectiveness of each product in increasing performance and health indices in broiler chickens.

Materials and Methods

Study design: In this study, 300 broiler chicks (Ross 308) were randomly divided into 4 groups with 5 replicates each, so that 15 chickens were allocated to each replicate until 42 days of age. All chickens were fed and watered freely (ad libitum) and raised under the same growing conditions, which included a continuous lighting program, mechanical ventilation, at least 50% air humidity, and a comfortable temperature. All chickens were immunized with the Newcastle disease (ND) vaccine at 7, 18, and 35 days of age. The basal diet of all groups was balanced according to the Ross 308 production manual (Table 1) (44). Chickens in the first group received a commercial probiotic (Protexin, Probiotics International Ltd., UK) according to the manufacturer's recommendation; one gram per liter of drinking water in the first week, 150 grams per ton in the starter, 100 grams per ton in the growing, and 50 grams per ton in the final diet. The chickens in the second group were fed phytobiotic (artichoke extract, Tichoke, Goldaru, Iran), 100 mg/L. Chickens in the third group were given probiotic and phytobiotic supplements (Protexin and Tichoke), while chickens in the fourth group were given no supplements at all. The weight gain (WG), feed intake (FI), and feed conversion ratio (FCR) were measured weekly and calculated in two steps (at 21 and 42 days of age).

Table 1. The diet ingredients and nutrients value.

Ingredients	Starter diet (1-3 weeks)	Finisher diet (4-6 weeks)
Corn	57.00	57.65
Soybean meal	36.80	34.00
Vegetable oil	2.70	4.10
Salt (Sodium chloride)	0.30	0.30
Dicalcium Phosphate	1.40	1.80
Shell	1.20	1.60
Methionine	0.10	0.05
Commercial Premix*	0.50	0.50
Total	100	100
Calculated values	**Starter diet (1-3 weeks)**	**Finisher diet (4-6 weeks)**
Metabolic Energy (Kcal/Kg)	2970	3050
Protein (%)	21.30	20.00
Calcium	1.00	1.10
Available phosphate	0.45	0.55
Methionine+Cysteine	0.80	0.70
Lysine	1.20	1.10

At 42 days of age, all chickens were weighed and non-heparinized and heparinized blood samples were taken from the wing vein. The non-heparinized ones were used for the preparation of serum samples. The serum samples were utilized for the measuring of humoral antibodies against ND vaccine according to Allan and Gough (3) based on 4 haemagglutinin units. The blood samples were divided into two identical parts to measure the biochemical parameters in whole blood and plasma. For separation of plasma, the heparinized blood samples were centrifuged at 3,000 × g for 15 min at 4°C. All samples were stored at –80°C until analysis was carried out.

The concentration of plasma total protein (TP), triglyceride (TG), cholesterol (CHL), high-density lipoprotein (HDL), low-density lipoprotein (LDL),

aspartate transferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels were determined spectrophotometrically, using commercial kits (Technicon RA1000, H83014 model, Technicon Industrial Systems, Tarrytown, NY), according to the instructions of manufacture (Pars-Azmoon Co., Tehran, Iran). Furthermore, plasma total antioxidant status (TAS) was assayed using the Randox total antioxidant capacity test kit (Randox Laboratories Ltd., Crumlin, UK) as described by Miller et al. (35). Blood superoxide dismutase (SOD) activity was measured by the Ransod spectrophotometric kit (Ransod, Randox Laboratories Ltd., Crumlin, UK), according to the Woolliams et al. (53) method. Blood Glutathione peroxidase (GPx) activity was assessed by the Ransel spectrophotometric kit (Ransel, Randox Laboratories Ltd., Crumlin, UK) as described by Paglia and Valentine (41).

Protexin®: This commercial probiotic is presented at a concentration of 2 × 10^9 CFU/g and contains *Streptococcus feacium*, *Streptococcus termophilus*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium bifidum, Aspergillus ourozai,* and *Candida pentolopsy*. This probiotic is a product of Probiotics International Ltd., UK.

Tichoke ®: Tichoke is a commercial dried artichoke extract (AE) prepared from *Cynara scolymus* that contains 5% caffeoylquinic acid as chlorogenic acid. Based on the DPPH method (43), the radial scavenging activity (RSA) in the samples was on average 40%. This phytobiotic is a product of Goldaru, IRAN.

Statistical analysis: All data were analyzed with the One-way ANOVA method, using SPSS (version 22) statistical package (SPSS Inc., Chicago, IL, US). Significant differences among the treatments were recognized at P<0.05, using the Tukey test.

Results

Growth performance: The growth index at 21 and 42 days of age indicates that there were no statistical differences in the FI of chickens in different groups at all over the growing period, while the WG and FCR were influenced by adding probiotic, phytobiotic, and probiotic plus phytobiotic to the diet.

At 21 days of age, the WG was significantly higher in chickens fed probiotics, or probiotic plus phytobiotic in comparison with chickens receiving phytobiotic or control diets (P<0.05).

The comparison of WG in all treatment groups at 42 days of age showed the highest WG was obtained in chickens receiving probiotic plus phytobiotic, while it did not show significant differences with chickens fed probiotic.

The FCR data at 21 and 42 days of age represent same pattern and the lowest FCR was illustrated in chickens get probiotic plus phytobiotic that possess no significant difference with chickens fed probiotic (Table 2).

Biochemical parameters: The TP was statistically higher than the control group in treated chickens, except for the group that received phytobiotics. In chickens that received probiotic plus phytobiotic, the TG, CHL, and LDL were lower, but HDL was higher than in chickens fed probiotic or control chickens, significantly (P<0.05) (Table 3).

The comparison of ALT and AST in different treatment groups reveals that chickens that received diets supplemented with phytobiotics or probiotics did not have any significant differences with control chickens or chickens that received probiotic plus phytobiotic. The comparison of ALP in different treatment groups shows that chickens that were fed a diet supplemented with phytobiotics or probiotics did not have any significant differences with control chickens, but there was a significant difference when chickens received probiotic plus phytobiotic. In terms of ALP parameters, the value obtained in the probiotic plus phytobiotic group is lower than in all other groups. The chickens that received probiotic plus phytobiotic had significantly lower ALT, AST, and ALP levels than the control group (P<0.05) (Table 3).

Table 2. The growth parameters in broiler chickens fed probiotic and phytobiotic at 21 and 42 days of age.

Index/ Groups	Feed intake (g)		Weight gain (g)		FCR	
	21 days of age	42 days of age	21 days of age	42 days of age	21 days of age	42 days of age
Protexin	862±22^a	3610±163^a	731±11^a	2230±80^a	1.17±0.03^b	1.61±0.02^b
Artichoke extract	860±32^a	3500±190^a	700±27^b	2000±81^b	1.23±0.02^a	1.75±0.02^a
Protexin plus Artichoke extract	870±35^a	3600±181^a	740±24^a	2280±92^a	1.17±0.03^b	1.57±0.02^b
Control	880±28^a	3580±200^a	703±20^b	2050±123^b	1.25±0.02^a	1.74±0.02^a
P value	0.75	0.78	0.12	0.02	0.01	0.01

- The different superscript in each column represents significant differences between treatment group (P<0.05).

Table 3. The biochemical parameters in broiler chickens fed probiotic, phytobiotic and probiotic plus phytobiotic at 42 days of age.

Index/Group	Protexin	Artichoke extract	Protexin plus Artichoke extract	Control	P value
TP (g/dl)	4.24±0.50^{a}	3.52±0.42^{ab}	4.00±0.45^{a}	3.05±0.22^{b}	0.022
TG (mg/dl)	96±11^{b}	70±15^{c}	56±19^{c}	128±14^{a}	0.0014
CHL (mg/dl)	150±26^{a}	129±20^{b}	130±22^{b}	167±41^{a}	0.034
HDL (mg/dl)	68±19^{b}	73±21^{ab}	80±12^{a}	63±20^{b}	0.025
LDL (mg/dl)	62±18^{a}	42±12^{b}	40±11^{b}	77±19^{a}	0.012
ALT (U/L)	4.62±0.8^{ab}	4.22±0.7^{ab}	3.95±0.6^{b}	5.04±0.7^{a}	0.046
AST (U/L)	150±27^{ab}	139±40^{ab}	135±48^{b}	160±36^{a}	0.032
ALP (U/L)	2.98±0.26^{a}	2.70±0.39^{a}	2.50±0.20^{b}	2.85±0.22^{a}	0.038
SOD (U/mg Hb)	1135±99^{a}	1178±133^{a}	1190±107^{a}	1096±95^{a}	0.671
GPx (U/mg Hb)	181±16^{b}	189±17^{b}	220±19^{a}	100±21^{c}	<0.001
TAS (mmol/L)	0.64±0.05^{b}	0.67±0.07^{b}	0.79±0.09^{a}	0.45±0.07^{c}	0.003
HI titer (NDV)	4.55±1.01^{a}	4.2±1.22^{a}	4.8±1.35^{a}	2.77±1.30^{b}	0.046

- The different superscript in each line represents significant differences between treatment group (P<0.05).
- TP: Total Protein, TG: Triglyceride, CHL: Cholesterol, HDL: high-density lipoprotein, LDL: low-density lipoprotein, AST: Aspartate transferase, ALT: Alanine aminotransferase, ALP: alkaline phosphatase, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, TAS: Total antioxidant Staus, HI: Haemaglutination inhibition.

The concentration of SOD in all treatment groups did not have any significant difference. The blood GPx and TAS concentrations were significantly higher in chickens fed probiotic, phytobiotic, and probiotic plus phytobiotic, while the addition of phytobiotic plus probiotic can significantly increase the activity of blood GPx and TAS with respect to chickens that received probiotic or phytobiotic alone (P<0.05) (Table 3).

The IgG antibody titer against ND vaccine in the control group was significantly lower than in other groups (P<0.05). There is no significant difference in ND titer in chickens that received probiotic, phytobiotic, or probiotic plus phytobiotic (Table 3).

Discussion and Conclusion

The results of the study have shown that continuous utilization of probiotics (protexin) or probiotics plus phytobiotics (protexin plus Tichoke) in poultry diets increases growth by improving FCR and WG. Consumption of Tichoke as a phytobiotic has no effect on growth indices, alone. Therefore, it seems that the phytobiotic of Tichoke has no effect on growth indices in broiler chickens. A review of previous studies on the effect of probiotics on growth indices showed that the results of using probiotics in the diet are very diverse and the range of results varies from not affected on growth indices to improvement in all growth indices (27, 30, 48). Gunal et al. (27) and Shargh et al. (48) reported that the use of probiotics has no effect on growth indices, while Khosravi et al. (30) showed that it has no effect on FI and final weight but increases food efficiency. In another report, Murry et al. (38) stated that *lactobacillus*-based probiotics increase food efficiency but reduce FI. Also, Awad et al. (8) showed improvement in all growth indices following dietary supplementation with a *lactobacillus*-based probiotic in chickens. Variation in the results of studies on probiotics seems to be affected by growing conditions, diet ingredients, type of probiotic, gastrointestinal pH, stress, dose, and period of probiotic administration (51).

Various studies have been performed on the effects of the artichoke plant on poultry. Previously, the role of this plant in liver protection of Japanese quails (29, 40), improving the performance index of laying hens (39, 55), and lowering cholesterol (1, 20) has been studied. There are many studies that evaluated the effects of artichoke in the form of dried leaf powder or extract on FI, WG, and FCR in broilers, although the results on the effect of artichoke on growth indices are very diverse. These results varied from no effect on growth indices (36, 50) to decreased FCR (45, 49), increased WG (1, 15, 32, 49), and elevation of FI (15, 49). However, the results of the present study are consistent with some studies that indicated the administration of artichoke had no effect on growth indices (36, 50). Certainly, the geographical area of cultivation, plant chemical composition, harvest season, dose, duration of administration, and phytobiotic type can influence the results.

Various studies have been performed on the effects of probiotics on the antioxidant system. Amaretti et al. (5) showed that the use of probiotics increases antioxidant capacity and decreases oxygen radicals. In addition, several studies have shown that the use of probiotics reduces oxidative stress. In fact, probiotics produce butyric acid and hydrogen, which may play a stimulating

role in the production of antioxidants and free radical scavenging (56). In the recent study, the use of probiotics had no effect on the SOD but increased the GPx and TAS significantly. Cross et al. (16), and Erdogan et al. (19) observed that probiotics had no effect on GPx levels. In addition, Aluwong et al. (4) demonstrated that the use of yeast probiotics significantly increased GPx activity without affecting SOD in broilers. In another study, a *Bacillus subtilis* based probiotic increased antioxidants in broiler pectoral muscle. This is associated with increased mRNA expression of antioxidant genes and decreased oxidative damage in the pectoral muscle (9). Also, in another study, Bai et al. (10) showed that the higher expression of SOD and GPx genes in the mitochondria of the liver was related to feeding with probiotics in chickens. It seems that the strains of probiotics can be effective in achievingantioxidant results.

In the recent study, the use of artichoke extract could increase GPx and total antioxidant capacity, and it even seems that the combined use of probiotics and phytobiotics of artichoke extract has synergistic activity in increasing GPx and TAS. There is a significant difference in GPx and TAS values in chickens fed probiotic or phytobiotic compared with chickens receiving symbiotic. There is limited clinical research on the antioxidant properties of artichoke in poultry, and most studies have been in laboratory animals. Previously, Jimene-Escrig et al. (28) studied the antioxidant properties of artichoke and showed that this plant has acceptable antioxidant properties in vitro. Also, feeding of this plant for 3 weeks at 14% of feed in normal rats increased blood GPx levels and had no effect on catalase and SOD, which is consistent with the findings of the present study. In broilers, Mirderikvandi et al. (36) stated that adding 500 mg/L of artichoke extract in drinking water for 2 weeks did not influence malondialdehyde and reduced GPx. However, the increase in antioxidant activity in the present study after the use of AE is related to the level of scavenging activity of AE. The RSA of about 40% and phenolic content of about 5% (in the form of chlorogenic acid) are mainly contributed to by the effect of AE on antioxidant capacity. The role of chlorogenic acid as a potent antioxidant has been previously demonstrated in vivo and in vitro (46).

Until now, the various properties of probiotics in chickens have been studied. Positive effects on growth indices, increasing the level of specific and non-specific immunity, gastrointestinal health (33), increasing performance in layers and bone strength (54) are some of the cases that have been reported in various studies, but there is little information about the effect of probiotics in improving metabolic function, especially liver health in chickens. The results of the recent study show that continuous consumption of a commercial probiotic (Protexin) can improve liver enzymatic activity, reduce total cholesterol, and increase total protein. There are several studies on the effect of probiotics on serum lipids in poultry. Ashayerizadeh et al. (7) demonstrated that adding probiotics to the broiler diet decreased cholesterol levels when compared with control chickens or chickens fed prebiotics or antibiotics. Also, dietary supplementation with probiotics containing *Saccharomyces cerevisiae* reduced cholesterol in egg yolk (2) and serum in chickens (37). In a study performed by Amer and Khan (6), it was illustrated that the supplementation with probiotics containing *Lactobacillus acidophilus*, *Bacillus subtilis*, *Saccharomyces cerevisiae,* and *Aspergillus oryzae* significantly decreased cholesterol in serum after 6 weeks. Previously, it was explained that *L. acidophilus* absorbs cholesterol and decreases the cholesterol level in medium (26). Apart from their ability to eliminate lipids, probiotic microorganisms can also adsorb and detoxify microbial toxin in the gastrointestinal tract (GIT) and prevent its intestinal adsorption. Detoxification of poisons in the GIT inhibits the effect of toxins on hepatocytes (34). The increase of TP in the present study may be related to the influence of probiotics on secretory function of the GIT that leads to increased digestion and absorption and, subsequently, can elevate total protein in plasma. It seems that the positive effects of probiotics on physiological function lead to an increase in growth indices in chickens.

There are few studies on the effect of probiotics on liver enzymes. Bityutsky et al. (14) showed that the use of probiotics in quail could reduce the levels of liver enzymes of ALT and AST. Damage to the liver cell membrane causes these enzymes to be released into the bloodstream (23). Therefore, no changes in liver enzymes indicates no liver damage following probiotic supplementation.

In this study, AE was able to affect to serum lipid profile, reduce CHL, TG, and LDL, and increase HDL. Regarding the effect of artichoke on lipid metabolism, various studies have been performed in humans, animals, and poultry, and in most of these studies, there is an agreement that artichoke can affect lipid metabolism. Rouzmehr et al. (45) reported that although the addition of 200 g per ton of dried artichokes to the diet can increase WG and FI, it reduces abdominal fat and blood CHL. Also, Abdo et al. (1) stated that consumption of 6% dried artichoke leaves in the diet causes weight loss, and this finding is consistent with reducing the amount of abdominal fat. However, AE seems to reduce plasma cholesterol levels by increasing bile secretion and decreasing cholesterol biosynthesis (18). In addition, there is evidence that the active ingredients in artichokes have the ability to inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (22). Decreased hepatic enzymes including ALT, AST, and ALP and

decreased plasma lipid profiles may support this hypothesis because if lipids accumulated in hepatocytes, hepatic complications would manifest as elevated hepatic enzymes in plasma. Due to the fact that in liver problems, liver enzymes usually leak out of liver cells and increase in the blood (23), a decrease in liver enzymes following the use of phytobiotics or the simultaneous use of probiotics and phytobiotics can be a marker of liver health. Certainly, this increase in the level of liver health is in line with the increase in antioxidant capacity and can be due to the increased ability of antioxidants to protect liver cells against toxins and oxidants.

In conclusion, continuous use of probiotic (Protexin) along with phytobiotic (Artichoke extract) in broiler diets can improve growth indices, increase antioxidant capacity, reduce serum lipids and improve liver function in broiler chickens.

Acknowledgements

The authors are grateful to the staff of IAU, Shahrekord Branch Research Farm for their assistance during the research work.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study was confirmed and approved by Ethics Committee of Sharekord Branch, Islamic Azad University for care and use of animal for research (IR. Shk. 99. 128).

Conflict of Interest

The authors declare no conflict of interest.

References

1. **Abdo MA, Radwan NL, Selim NA** (2007): *The effect of artichoke leaves meal on the utilization of dietary energy for broiler chickens*. Int J Poult Sci, **12**, 973-982.
2. **Abdulrahim SM, Haddad MSY, Hashlamoun EAR, et al** (1996): *The influence of Lactobacillus acidophilus and Bacitracin on layer performance of chickens and cholesterol content of plasma and egg yolk.* Br J Poult Sci, **37**, 341-346.
3. **Allan WH, Gough RE** (1976): *A comparison between the haemagglutination inhibition and complement fixation tests for Newcastle disease*. Res Vet Sci, **20**, 101-103.
4. **Aluwong T, Kawu M, Raji M, et al** (2013): *Effect of Yeast Probiotic on Growth, Antioxidant Enzyme Activities and Malondialdehyde Concentration of Broiler Chickens.* Antioxidants, **2**, 326-339.
5. **Amaretti A, Di Nunzio M, Pompei A, et al** (2013*): Antioxidant properties of potentially probiotic bacteria, in vitro and in vivo activities.* Appl Microbiol Biotechnol, **97**, 809-817.
6. **Amer MY, Khan SH** (2012): *A comparison between the effects of a probiotic and an antibiotic on the performance of Desi chickens.* Vet World, **5**, 160.
7. **Ashayerizadeh A, Dabiri N, Mirzadeh KH, et al** (2011): *Effect of dietary supplementation of probiotic and prebiotic on growth indices and serum biochemical parameters of broiler chickens*. J Cell Anim Biol, **5**, 152-156.
8. **Awad WA, Ghareeb K, Abdel-Raheem SM, et al** (2009): *Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens.* Poult Sci, **88**, 49-56.
9. **Bai WK, Zhang FJ, He TJ, et al** (2016): *Dietary Probiotic Bacillus subtilis Strain Increases Antioxidant Capacity and Oxidative Stability of Chicken Breast Meat during Storage.* PLoS ONE, **11**, e0167339.
10. **Bai K, Huang Q, Zhang J, et al** (2017): *Supplemental effects of probiotic Bacillus subtilis on growth performance, antioxidant capacity, and meat quality of broiler chickens.* Poult Sci, **96**, 74–82.
11. **Bakkali F, Averbeck S, Averbeck D, et al** (2008): *Biological effects of essential oils-a review*. Food Chem Toxicol, **46**, 446–475.
12. **Barton MD, Hart WS** (2001): *Public health risks: antibiotic resistance-review.* Asian-Australs J Anim Sci, **14**, 414-422.
13. **Ben Y, Fu C, Hu M, et al** (2019): *Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: a review*. Environ Res, **169**, 483-493.
14. **Bityutskyy V, Tsekhmistrenko S, Tsekhmistrenko O, et al** (2019): Effects of Different Dietary Selenium Sources Including Probiotics Mixture on Growth Performance, Feed Utilization and Serum Biochemical Profile of Quails. 623-632. In: V Nadykto (Ed), Modern Development Paths of Agricultural Production. Springer, Cham.
15. **Boroumandnia Z, Mohammadzadeh S, Azizi M, et al** (2014): *The effect of adding artichoke extract to drinking water on performance, carcass characteristics and blood lipoprotein concentration in broiler chickens*. Iranian J Appl Anim Sci, **3**, 71–79.
16. **Cross ML** (2002): *Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens.* FEMS Immunol Med Microbiol, **34**, 245-253.
17. **Czech A, Kowalczuk E, Grela ER** (2009): *The effect of an herbal extract used in pig fattening on the animal performance and blood components*. Ann Univ Mariae Curie Sklodowska, **27**, 25–33.
18. **Edwards SE, da Costa Rocha I, Williamson EM, et al** (2015): Phytopharmacy: An evidence-based guide to herbal medicinal products. John Wiley and Sons, London.
19. **Erdoğan Z, Erdoğan S, Aslantaş Ö, et al** (2010): *Effects of dietary supplementation of synbiotics and phytobiotics on performance, caecal coliform population and some oxidant/antioxidant parameters of broilers*. J Anim Physiol Anim Nut, **94**, e40-e48.
20. **Fallah R, Kiani A, Azarfar A** (2013): *Effect of artichoke leaves meal and mentha extract (Mentha piperita) on immune cells and blood biochemical parameters of broilers*. Global Vet, **10**, 99–102.

21. **FAO/WHO** (2001): Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. FAO/WHO Publishing, Cordoba.
22. **Gebhardt R** (1998): *Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (Cynara scolymus L.) extracts*. J Pharmacol Exp Ther, **286**, 1122–1128.
23. **Gholami-Ahangaran M, Rangsaz N, Azizi S** (2016): *Evaluation of turmeric (Curcuma longa) effect on biochemical and pathological parameters of liver and kidney in chicken aflatoxicosis.* Pharm Biol, **5**, 780-787.
24. **Gholami-Ahangaran M, Peimani N, Dastgerdi AA** (2019)*: The effect of thyme (Thymus daenensis) supplement on growth and hygienic parameters of broilers meat*. Iraqi J Vet Sci, **33**, 87-92.
25. **Gholami-Ahangaran M, Ahmadi-Dastgerdi A, Karimi-Dehkordi M** (2020): *Thymol and carvacrol, as antibiotic alternative in green healthy poultry production.* Plant Biotechnol Persa, **2**, 22-25.
26. **Gilliland SE, Nelson CR, Maxwell C** (1985): *Assimilation of cholesterol by Lactobacillus acidophilus.* Appl Environ Microbiol, **49**, 377-381.
27. **Gunal M, Yayli G, Kaya O, et al** (2006): *The effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers*. Int J Poult Sci, **5**, 149-155.
28. **Jimene-Escrig A, Dragsted LO, Daneshvar B, et al** (2003): *In vitro antioxidant activities of edible artichoke (Cynara scolymus L.) and effect on biomarkers of antioxidant in rats.* J Agric Food Chem, **51**, 5540–5545.
29. **Khoramshahi M, Samadi F, Ganji F, et al** (2015): *The effect of artichoke leaf powder on performance, internal organs weight and some blood parameters of Japanese quail poisoned with carbon tetrachloride.* Anim Sci Res, **25**, 57–67.
30. **Khosravi A, Boldaji F, Dastar B, et al** (2010): *Immune response and performance of broiler chicks fed protexin and propionic acid.* Int J Poult Sci, **9**, 188-191.
31. **Kumar M, Kumar V, Roy D, et al** (2014): *Application of herbal feed additives in animal nutrition-a review*. Int J Lives Res, **4**, 1-8.
32. **Lertpatarakomo R, Jaipeng P, Sirimongkolworaku S, et al** (2015): Effect of artichoke (Cynara scolymus L.) extract supplementation in drinking water on growth performance of broiler chickens. 220-222. In: Proceeding of 20th European Symposium on Poultry Nutrition. Berlin, Germany.
33. **Makled MN, Abouelezz KFM, Gad-Elkareem AEG, et al** (2019): *Comparative influence of dietary probiotic, yoghurt, and sodium butyrate on growth performance, intestinal microbiota, blood hematology, and immune response of meat-type chickens.* Trop Anim Health Prod, **51**, 2333-2342.
34. **Markowiak P, Śliżewska K, Nowak A, et al** (2019): *Probiotic microorganisms detoxify ochratoxin A in both a chicken liver cell line and chickens.* J Sci Food Agricult, **99**, 4309-4318.
35. **Miller JK, Brzezinska-Slebodzinska E, Madsen FC** (1993): *Oxidative stress, antioxidants and animal function.* J Dairy Sci, **76**, 2812–2823.
36. **Mirderikvandi M, Kiani A, Khaldari M, et al** (2016): *Effects of artichoke (Cynara scolymus L.) extract on antioxidant status in chicken thigh meat*. Iranian J Vet Med, **10**, 73-81.
37. **Mohan B, Kadrivel R, Natarajan A, et al** (1996): *Effect of probiotic supplementation on growth, itrogen utilization and serum cholesterol in broilers*. Br Poult Sci, **37**, 395-401.
38. **Murry AC, Hinton Jr A, Buhr RJ** (2006): *Effect of botanical probiotic containing lactobacilli on growth performance and populations of bacteria in the ceca, cloaca, and carcass rinse of broiler chickens*. Int J Poult Sci, **5**, 344-350.
39. **Nadia LR, Zeinab M, Abdo A, et al** (2007): *Effect of feeding artichoke leaves meal on productive and reproductive performance of Mandarah hens*. Int J Poult Sci, **6**, 826–834.
40. **Nateghi R, Samadi F, Ganji F, et al** (2013): *Hepatoprotective effects of Cynara scolymus L. extract on CCl4 induced liver injury in broiler chickens*. Int J Agricult Sci Res, **3**, 678–688.
41. **Paglia DE, Valentine WN** (1967): *Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase.* J Lab Clin Med, **70**, 158-169.
42. **Pandey KR, Naik SR, Vakil BV** (2015): *Probiotics, prebiotics and synbiotics-a review*. J Food Sci Technol, **52**, 7577-7587.
43. **Rai S, Wahile A, Mukherjee K, et al** (2006): *Antioxidant activity of Nelumbo nucifera (sacred lotus) seeds*. J Ethnopharm, **104**, 322-327.
44. **Ross** (2019): Nutrition Specification. Aviagan, US.
45. **Rouzmehr F, Mohit A, KoshSekeh M, et al** (2014): *The effect of the additive containing artichoke extract (APC) on growth, blood cholesterol level, carcass characteristics and immune system of broiler chickens*. Iranian J Vet Clin Pathol, **8**, 357–366.
46. **Sato Y, Itagaki S, Kurokawa T, et al** (2011): *In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid*. Int J Pharm, **403**, 136-138.
47. **Sekhon BS, Jairath S** (2010): *Prebiotics probiotics and synbiotics: an overview*. J Pharm Edu Res, **1**, 13.
48. **Shargh MS, Dastar B, Zerehdaran B, et al** *(*2012*): Effects of using plant extracts and a probiotic on performance, intestinal morphology, and microflora population in broilers*. J Appl Poult Res, **21**, 201-208.
49. **Shokri A, Akbari Gharaei M, Varmaghani S, et al** (2018): *Effect of Cynara scolymus powder on performance and ascites syndrome in broiler chickens.* J Anim Prod, **20**, 55–67.
50. **Tajodini M, Samadi F, Hasani S, et al** (2015): *Influence of Artichoke (Cynara scolymus) Leaf Powder on Growth Performance; Carcass Traits and Blood Parameters in Broiler Chickens.* Iranian J Appl Anim Sci, **5**, 141-146.
51. **Wang Y, Wu Y, Wang Y, et al** (2017): *Antioxidant properties of probiotic bacteria.* Nutrients, **9**, 521.
52. **Windisch W, Schedle K, Plitzner C, et al** (2008): *Use of phytogenic products as feed additives for swine and poultry.* J Anim Sci, **86**, 140–148.
53. **Woolliams JA, Wiener G, Anderson PH, et al** (1983): *Variation in the activities of glutathioneperoxidase and superoxide-dismutase and in the concentration of copper in the blood in various breed crosses of sheep.* Resp J Vet Sci, **34**, 253-256.

54. Yan FF, Murugesan GR, Cheng HW (2019): *Effects of probiotic supplementation on performance traits, bone mineralization, cecal microbial composition, cytokines and corticosterone in laying hens.* Animal; an Int J Anim Biosci, **13**, 33.

55. Yildiz G, Sacakli P, Gungor T (2006): *The effect of dietary Jerusalem artichoke (Helianthus tuberosus L.) on performance, egg quality characteristics and egg cholesterol content in laying hens*. Czech J Anim Sci, **51**, 349–354.

56. Zheng W, Dawei Z, Wenjun Y, et al (2019): *Probiotic Cocktails Alleviate Heat-induced Intestinal Barrier Dysfunction and Oxidative Stress in Broiler Chickens*. Anim Husb Feed Sci, **11**, 107-114.

Ankara Univ Vet Fak Derg, **69**, 289-295, 2022
DOI: 10.33988/auvfd.842816

Comparison of different mathematical functions for fitting growth curves of ascitic and healthy broiler chickens

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Received date: 18.12.2020 - Accepted date: 11.08.2021

Abstract: Ascites syndrome (AS) causes major economic losses in commercial meat-type chickens. The objectives of the current study were to select the best non-linear growth curve functions (GCFs) of the ascitic and healthy chickens, and to investigate the association of ascites incidence with the growth pattern. A total of 5584 body weight (BW) records belonging to 823 chickens (381 male and 442 female) from a paternal pure Arian broiler line were used. The birds were categorized into; healthy male and female, ascitic male and female. Five GCFs including Logistic, Gompertz, Lopez, Richards, and Von baretanalffy were fitted to the BW records of all groups, separately. After the estimation of growth curve parameters for all the chicks individually, the effect of sex and health status on the growth curve parameters were assessed. The results revealed that the Richards function is the best for all the groups. Comparison of the growth curves showed that the ascitic chickens reach the inflection point of the curve earlier than their healthy counterparts ($P<0.05$). The average growth rate of the healthy birds in the rearing period was significantly higher than that of the ascitic birds ($P<0.05$), thereby suggesting that there is no direct relationship between the rapid growth rate and the incidence of ascites. Therefore, genetic improvement of the used population for both rapid growth rate and reduced ascites incidence may be possible and the utilization of growth curve parameters in the selection index might be beneficial.

Keywords: Ascites, broiler, growth pattern, non-linear functions.

Introduction

Ascites syndrome (AS) (also called pulmonary hypertension syndrome) is a metabolic disorder in modern meat-type chickens that are associated with pathological accumulation of excessive fluid in the abdominal cavity. AS is affected by both environmental and genetic factors (4, 9). The incidence of AS in commercial meat-type chickens is more prevalent and the range of mortality can rate from 0 to 30 %, therefore, AS could result in massive economic losses to poultry producers (15, 16). There is a direct association between the local elevation of broiler houses (sea level above 3500 m) and the prevalence of AS, so that it has historically been called high altitude disease (5, 10). However, it is now widely seen even at low altitude areas, as well (6). In such a condition, the incidence of AS is attributed to the rapid growth rate, especially in male chickens (18). The growth rate of broiler chicken is higher at early ages and the slope of the growth curve is steeper as compared to later ages. The growth rate subsequently decreases progressively to finally reach zero at maturity (8). The growth rate of ascitic birds is much slower than that of healthy ones and their final weight is lighter at the end of the rearing period (4). Nowadays, the growth curve has various applications and could be used in the optimization of management practices, evaluation of nutritional requirements, and improvement of breeding programs (1). Growth curves functions (GCFs) are nonlinear regression equations that predict body weight (BW) at different stages of animal life (12, 14). Understanding the biological meanings of the growth curve parameters and their relationship with other important economic traits may pave the way for experts to use this information in breeding plans (23). To this end, the study of the growth curves of the birds is necessary for the breeder to genetically evaluate the birds (2). Besides, the growth curves can be used as an appropriate tool by breeders to make important decisions about the nutritional and management strategies of farms (1, 2, 12, 26). The GCFs that are used more commonly are Gompertz, Richards, Von Bertalanffy, Brody, Logistic, Negative

Exponential, Morgan - Mercer - Flodin, and recently the Hyperbolastic models (12, 14, 25). These GCFs have been developed to model both the unprecedented and intraspecific population dynamics and more general biological growth. Most predictive models are based on variations of Verhulst's classic logistic growth equation (25). The Gompertz function, which was first presented for the prediction of animal growth, is one of the best models to describe the growth patterns of birds (3, 13, 21). The parameters of the Gompertz function are flexible, however, the Richards model has variable flexibility (19). The intense genetic selection of modern meat-type broilers for fast growth rate, high marketing weight, maximum white meat yield, and heavy final BW has augmented the incidence of AS (5, 15, 16). It seems that internal organs of modern broiler chickens such as heart, lungs, and liver, especially those with AS characteristics, are not well developed as compared with those of healthy birds or layer hens (6, 8, 18). Because of the inherent differences between the growth patterns of the healthy and ascitic birds (8), we aimed to select the best non-linear GCFs including Gompertz, Logistic, Lopez, Richards, and Von Bertalanffy in the fitting of the growth curves of four groups including healthy male, healthy female, ascitic male, and ascitic female and to investigate the association of ascites incidence with the growth pattern.

Materials and Methods

In this research, we examined different GCFs and the incidence of ascites under standard rearing conditions using 5584 body weight records of 823 chicks belonging to one paternal commercial broiler line of Arian. All birds were wing-banded at the hatchery for pedigree registration. Then, Chicks were immediately transformed to the Poultry Research Unit, located in Khalatposhan Agricultural Research Station of University of Tabriz, Tabriz, Iran and were raised on the deep floor according to Arian management guidelines to 45 d of age. The lighting schedule was near-continuous lighting (23L: 1D) throughout the experimental periods. The broiler chickens were housed in pens in a litter floor and reared under similar managerial and hygienic conditions. The temperature of rearing room was controlled at 32˚C for first 3 d, then decreased gradually to 20˚C until 21 d of age and maintained as such until 42 d. The water and feed were supplied *ad libitum* throughout the experimental period. The water was supplied by a bell-shaped drinker, and feed was provided to each pen by a plate feeder to 10 days of age and then by an 8 kg grower feeder from 10 to 45 days of age. The metabolizable energy (kcal/kg) and crude protein (%) contents of the starter (1 to 14d), grower (15 to 28d), and finisher (29 to 45d) diets were 2900 kcal/kg and 20.5 %, 3075 kcal/kg and 20 %, and 3120 kcal/kg and 18 %, respectively. The ingredients and chemical composition of the basal diets were listed in Table 1. The

Table 1. The ingredients and nutrient composition of the basal diet (%, as-fed bases).

Ingredients (%)	Starter, d 0-10	Grower, d 11-24	Finisher, d 25- 42
Yellow corn	62.10	69.00	71.73
Soybean meal (440 g/kg CP)	34.10	28.30	25.70
Wheat bran	0.98	-	-
Dicalcium phosphate	0.20	0.20	0.19
$CaCO_3$	1.10	1.00	0.95
DL-Methionine	0.33	0.29	0.26
L- Lysine	0.22	0.20	0.17
l- Threonine	0.08	0.07	0.07
NaCl	0.22	0.24	0.23
$NaCO_3$	0.17	0.15	0.15
Vitamin premix [1]	0.25	0.25	0.25
Mineral premix [2]	0.25	0.25	0.25
Coccidiostat	-	0.05	0.05
Total	100	100	100
Analyzed nutrient levels			
Metabolizable energy, kcal/kg	2900	3075	3120
Crude protein (CP), %	20.50	20.00	18.00
Ether exact, %	3.37	3.35	3.29
Calcium, %	1.00	0.95	0.95
Available phosphorus, g/k	0.45	0.43	0.43
Lysine, g/kg	1.17	1.10	1.08
Methionine, g/kg	0.55	0.51	0.45

[1] Provided per kilogram of diet: vitamin A, 8000 IU; vitamin D3, 1600 IU; vitamin E, 10 IU; vitamin B1, 0.8 mg; vitamin B2, 2.5 mg; vitamin B6, 0.1 mg; vitamin B12, 0.009 mg; D-pantothenic acid, 2.2 mg, folic acid 0.25 mg; nicotinic acid 20 mg; biotin 0.1 mg.
[2] Provided per kilogram of diet: 6 mg (MnO_2); 80 mg ($ZnSO_4 \cdot 7H_2O$); 8 mg ($CuSO_4 \cdot 5H_2O$); 60 mg ($FeSO_4 \cdot 7H_2O$); 0.35 mg (KI) and 0.3 mg ($Na_2SeO_3 \cdot 5H_2O$).

body weights of the chicks were individually taken with a precision digital scale sensitive to ±0.01 g by weekly and the dead birds were necropsied for determination of the cause of death. All birds with yellow fluid at the peritoneal cavity or in pericardium were considered as ascitic, while the remaining birds were considered as healthy. After the end of the rearing period, the birds were divided into four groups including healthy male (n=317), ascitic male (n=64), healthy female (n=424), and ascitic female (n=18). The sex determination was done by gender appearance differences. In this method, male broilers have a larger body size, comb, and wattle in comparison with female broiler chicks.

The effects of sex and health status on BW at different ages were studied using the GLMSELECT procedure of SAS software version 9.2 (22) and found to be significant (P<0.05) on weekly recorded BW (results not shown). For this reason, the fitting of the different GCFs was done separately for ascitic males, ascitic females, healthy males, and healthy females, using the nonlinear (NLIN) procedure of SAS software (22). Consequently, in the first step five nonlinear models including Gompertz (G), Logistic (L), Lopez (Lo), Richards (R), and Von Bertalanffy (VB) were employed to fit the average weekly weighed BW records. The mathematical equations of the functions are shown in Table 2.

Table 2. Growth curve functions used to fit bodyweight records.

Model	Function*
Gompertz	$Wt=W0\times\exp((1-\exp(-b\times t))\times(\log(Wf/W0)))$
Logistic	$Wt = (W0\times Wf) / (W0+(Wf - W0)\times\exp(-b\times t))$
Lopez	$Wt = ((W0 \times k^{b}) + (Wf \times t^{b})) / (k^{b} + t^{b})$
Richard	$Wt = (W_0\times W_f) / ((W_0^n)+(W_f^n - W_0^n)\times\exp(-b\times t))^{(1/n)}$
Von Bertalanffy	$W_t = a\times(1-b\times\exp(-k\times t))^3$

* Wt is the body weight (g) at age t (day), W0 is the initial weight, Wf is body weight at maturity, b the maturity index, k and n are the parameters of the functions.

The adjusted coefficient of determination (Adj-R^2), Akaike information criterion (AIC_c), and Durbin-Watson (DW) criteria were used to assess the goodness of fits of the functions. The calculation formula of these criteria were as follow:

1- Adj-R^2 statistic:

$$\text{Adj} - R^2 = 1 - \frac{(n-1)}{(n-p)} \times (1 - R^2)$$

In which, R^2 is the explanation factor, n is the number of observations, and p is the number of parameters of the model.

2- AICc statistic:

$$AICc = n\log(RSS/n) + 2p(n/(n-p-1))$$

In which, SSR is the sum of squares corresponds to the models, and n and p are the number of observations and the number of parameters of the model, respectively.

3- Durbin-Watson (DW) statistic:

$$DW = \frac{\Sigma_{t=2}^{n}(e_t - e_{t-1})^2}{\Sigma_{t=1}^{n} e_t^2}$$

In which, e_t and e_{t-1} are the residual values at days t and t-1.

Comparison of the different goodness of fit criteria proved that the Richards function as the best for all of the groups and, therefore, in the second step this model was employed to fit the individual BW records of the birds.

After estimating the parameters of the Richards function for individual birds, the parameters were used to calculate the time (T_{IP}) and weight (W_{IP}), at the inflection point of the growth curve (as T_{IP} and W_{IP}, respectively) as below:

$$T_{IP} = \frac{1}{b}\ln[\frac{W_f - W_0}{nW_f}]$$

$$W_{IP} = \frac{W_f}{(n+1)^{1/n}}$$

Then, the GLM procedure of SAS software was used to assess the effect of sex and health status (healthy or ascites) on the growth curve parameters, using the following model:

$$y_{ijk} = \mu + S_i + HS_j + e_{ijk}$$

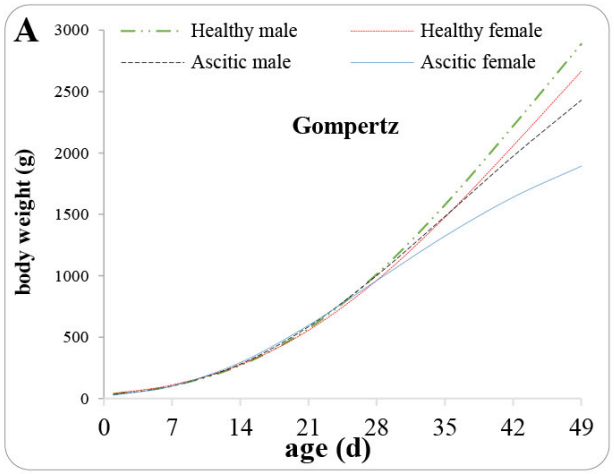
In which, y_{ijk} is any parameters of Richards function, μ is overall mean, S_i and HS_j are the fixed effects of sex and health status (healthy or ascitic), respectively, and e_{ijk} is the random residual.

At last, the correlation coefficients between the growth curve parameters were estimated using the CORR procedure of SAS software (22).

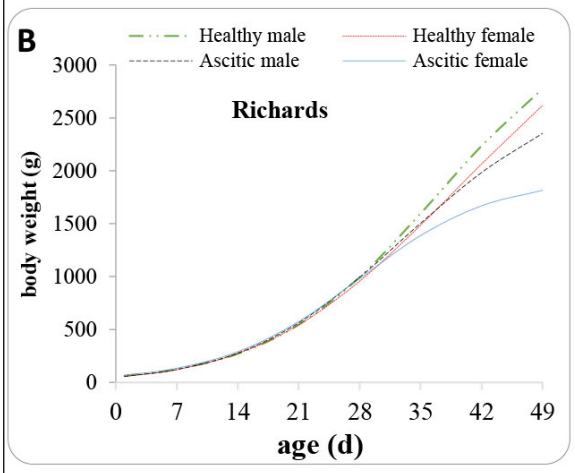
Results

Descriptive statistics and selection of the best growth model: Table 3 describes the descriptive statistics of the used data. The growth curves of the four groups were fitted using all of the studied functions and the resulted curves are shown in Figure 1 (labelled as A, B, C, D and E). As can be seen in Figure 1, before the age of 28 days, all groups had almost similar growth patterns, with the ascitic birds being slightly heavier than their healthy counterparts. After the age of 28 days, the growth rate of all groups diminished gradually. But, the reduction of growth rate was more considerable for the ascitic male and ascitic female groups than their corresponding healthy groups.

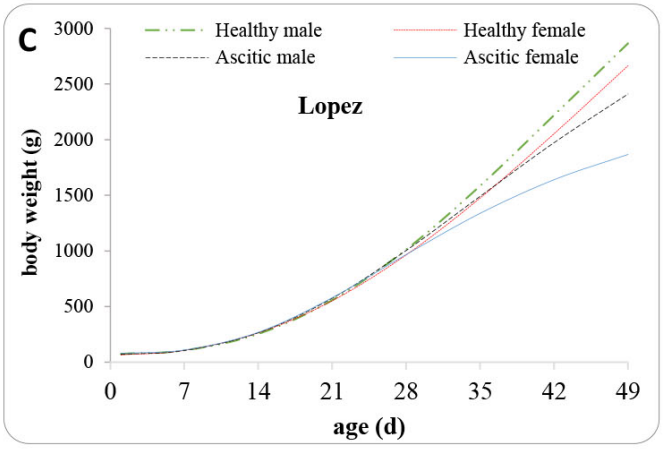
The goodness of fit of the GCFs was assessed using Adj-R^2, AIC_C, DW statistics, and the results are shown in Table 4. Function with the highest Adj-R^2, the lowest AIC_C, and DW around 2 was considered as the most suitable.



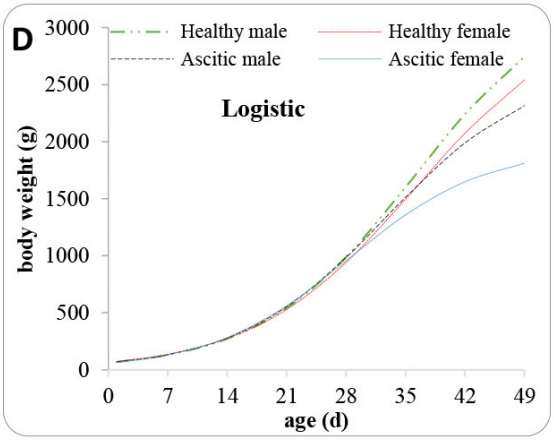
A: Fitting growth curve of the Gompertz model for healthy and ascitic male and female broiler chickens.



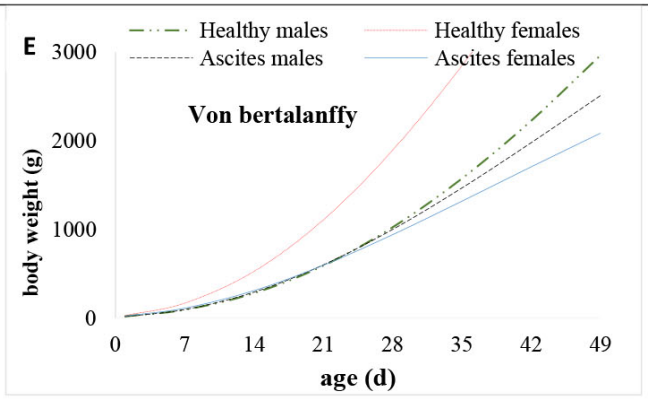
B: Fitting growth curve of the Richards model for healthy and ascetic male and female broiler chickens.



C: Fitting growth curve of the Lopez model for healthy and ascetic male and female broiler chickens.



D: Fitting growth curve of the Logistic model for healthy and ascetic male and female broiler chickens.



E: Fitting growth curve of the Von Bertalanffy model for healthy and ascetic male and female broiler chickens.

Figure 1. Fitting five different growth curves for healthy and ascitic male and female broiler chickens.

Table 3. Descriptive statistics of used BW records in different ages.

Age (Day)	Mean (g)	SD[1] (g)	CV[2] (%)	Minimum (g)	Maximum (g)
1	45	3.9	8.8	33	60
7	127	75.1	14.6	68	184
14	274	53.4	19.4	104	443
21	574	98.7	17.1	230	885
28	972	164.1	16.8	420	1485
35	1519	242.8	15.9	470	2200
42	2133	301.7	14.1	1070	3025
45	2363	338.4	14.3	1205	4425

[1]SD: standard deviation, [2] CV: coefficient of variance.

Table 4. The goodness of fit criteria of Richards, Lopez, Logistic, Gompertz and Von Bertalanffy functions for fitting the growth curves of healthy and ascitic male and female birds.

Criteria	R^2_{adj} [1]					AIC$_C$ [2]					DW [3]				
Function* / Group	G	L	Lo	R	VB	G	L	Lo	R	VB	G	L	Lo	R	VB
Healthy males	0.998	0.975	0.998	0.999	0.998	78.2	68.62	78.9	67.8	83.94	2.04	2.07	2.15	2.51	1.69
Healthy females	0.997	0.995	0.994	1	0.999	65.5	70.42	70.6	54.2	76.14	1.82	1.22	2.12	2.38	1.46
Ascites males	0.998	0.995	0.997	0.999	0.998	76.6	69.57	76.4	66.2	83.28	1.91	2.37	2.28	3.07	1.58
Ascites females	0.995	0.997	0.995	0.999	0.995	85.4	78.34	84.3	78.2	89.10	1.97	2.83	2.30	2.85	1.72

*G: Gompertz, L: Logistic, Lo: Lopez, R: Richards, and VB: Von Bertalanffy.
[1] adjusted coefficient of determination, [2] Akaike information criterion, [3] Durbin-Watson criteria.

Table 5. Least square means (LSM) comparison of growth curve parameters among different groups of gender and health status.

	Healthy male	Ascitic male	Healthy female	Ascitic female
W_0	55.7 [a]	56.5 [a]	47.9 [b]	63.9 [a]
W_f	3967 [b]	2877 [c]	5001 [a]	2112 [c]
b	0.219 [b]	0.666 [a]	0.118 [b]	0.414 [ab]
n	2.34 [b]	7.01 [a]	1.06 [b]	4.08 [ab]
W_{IP}	1862 [b]	1457 [c]	2043 [a]	1158 [c]
T_{IP}	37.3 [b]	33.4 [c]	40.6 [a]	29.7 [c]

W_0 is the hatching weight, W_f is the weight at maturity, b the maturity index, n is the parameters of the model, W_{IP} and T_{IP} are weight- and age at the inflection point of the growth curve, respectively.
LSM within the same row with different superscripts are significantly different ($P<0.05$).

Table 6. Correlation between the growth curve parameters in healthy (above diagonal) and ascitic chickens (below diagonal).

Parameters*	W_0	W_f	b	n	W_{IP}	T_{IP}
W_0	-	-0.35	0.35	0.37	-0.18	-0.25
W_f	-0.37[a]	-	-0.20	-0.19	0.96	0.90
b	0.60	-0.19	-	0.99	-0.10	-0.07
n	0.61	-0.19	0.99	-	-0.09	-0.06
W_{IP}	-0.01	0.90	0.08	-0.08	-	0.92
T_{IP}	-0.25	0.89	-0.10	-0.09	0.85	-

* W_0 is the initial weight, W_f is the maturity, b the maturity index, n is the parameters of the model, W_{IP} and T_{IP} are weight- and age at the inflection point of the growth curve, respectively.
[a] All correlations are statistically significant ($P<0.05$).

Effect of sex and health status on growth curve parameters: Generally based on the mentioned criteria in table 4, Richards's function was revealed to be the best for all of the four groups which followed by, Logistic, Gompertz, Lopez and VB (based on AIC criterion). Therefore, the Richards function was employed to be fitted to the BW records of all birds, individually. The growth curves of 115 chickens did not reach the convergence criterion or possessed inappropriate shapes and were excluded from further analyses. Therefore, the number of fitted curves reduced to 708 (330 male and 378 female). The growth curve parameters, as well as time and weight at an inflection point of the curve, were estimated for these birds.

In Table 5, the comparison of least-square means of Richards' growth curve parameters among the four groups are shown. There were significant differences between the estimated hatch weight (W_0) of the four groups ($P<0.05$). The mature weights (W_f) of the healthy males and healthy females were significantly higher than those of the ascitic males and ascitic females, respectively ($P<0.05$). There were significant differences between the b and n parameters of the four groups ($P<0.05$). The healthy chickens reached the inflection point of the growth curve later and at a heavier weight than the ascitic chickens ($P<0.05$). This indicates that the growth pattern of the ascitic birds is not as similar to that of the healthy birds.

Correlation between the growth curve parameters within the ascitic and healthy chickens: Table 6 shows the correlation coefficients between the growth curve parameters of healthy and ascitic chickens. The correlation coefficients are almost similar in the healthy and ascitic chickens with the highest correlation between W_f and W_{IP} parameters and the lowest correlation between T_{IP} and n parameters.

Discussion and Conclusion

AS, a serious metabolic disorder in commercial modern broilers, is one of the most important non-infectious causes of losses in the broiler production industry and the incidence of it has increased worldwide over the past several years (7). Meat-type chickens are sensitive to AS due to their genetic improvement history of intense genetic selection for production traits such as rapid growth rate and efficient feed conversion. Furthermore, non-genetic factors are also blamed for predisposing the broilers to AS. These factors are for example high ambient temperature, continuous lighting, improper ventilation, high altitudes, and pellet form of high-energy diets (4, 7, 15). Many studies have suggested that mortality due to AS could be attributed to metabolic burdening. These conditions have raised great challenges in meat-type chickens during rapid growth for fulfilling tissue demands for oxygen, resulting in relative hypoxemia, with a decrease in arterial oxygen saturation and high hematocrit values with increased red blood cell production (erythropoiesis). Although the pathological progression of AS has been known for years, the underlying molecular mechanism during AS development is still not fully understood (18).

As shown in figure 1, the growth rate of ascitic birds was slightly higher than that of healthy ones before the age of 28 days. Thus, we investigated the possible relationship of early rapid growth rate with the incidence of ascites. We observed no visible, statistical differences between the BW records of healthy and ascites birds before age 28 (results not shown). Therefore, it seems that the slightly faster growth rate of the ascitic birds may not be the causative factor predisposing them to ascites. As such, we should look for other reasons to explain their susceptibility to ascites. The results concluded by Julian (10), was consistent with our results that report the rapid growth of broilers were no the pathogenic mechanism of cardiovascular or musculoskeletal defects. At the end of the rearing period, the BW of the ascitic chickens was much lower than that of healthy birds which was the case for both males and females. This is inconsistent with the previous findings that considered a positive relationship between the rapid growth rate and the incidence of ascites (6, 9, 20).

The high correlation of both WIP and TIP with Wf indicates that birds with later inflection point have heavier weight at an inflection point as well as at maturity (Table 6). As mentioned above, healthy chicks have growth curves with later TIP and heavier WIP. Therefore, genetic selection for later TIP can assist in indirectly select for reduced ascites incidence (13, 17, 23, 24, 26). Indeed, poultry farmers frequently plan to slower the early growth rate of broiler chickens to postpone the inflection point of the growth curve and to help the chicken to cope with the high metabolic pressure which they experience during the early ages (11). Furthermore, more recently there have been some attempts to develop broiler strains that grow slowly during early ages but grow more rapidly during later ages, something that is so-called compensatory growth (9, 25, 27).

The birds that suffered from AS showed different and inappropriate growth patterns as compared to the healthy birds. Despite the previous findings, in the studied population, the ascitic birds grew as similar to healthy birds in early ages but at a slower rate at later ages. The inflection point of the growth curve of the ascites birds happened earlier than that of healthy birds indicating that the hindrance of inflection point, via genetically or management strategies, might cause the incidence of ascites to be reduced.

Acknowledgement

We cordially appreciated the Arian Farm staff for their technical assists and for providing the pedigreed chickens for the current study.

Ethical Statement

This study does not present any ethical concerns.

Financial Support

This research was financially supported by the University of Tabriz grants (project no. 312, Master of Science thesis at University of Tabriz).

Conflict of Interest

The authors declare no conflicts of interest.

References

1. **Aggrey S** (2002): *Comparison of three nonlinear and spline regression models for describing chicken growth curves*. Poult Sci, **81**, 1782-1788.
2. **Aggrey S** (2009): *Logistic nonlinear mixed effects model for estimating growth parameters*. Poult Sci, **88**, 276-280.
3. **Arango J, Van Vleck LD** (2002): Size of beef cows: early ideas, new developments. Faculty Papers and Publications in Animal Science, 237.
4. **Baghbanzadeh A, Decuypere E** (2008): *Ascites syndrome in broilers: physiological and nutritional perspectives*. Avian Pathol, **37**, 117-126.
5. **Balog J, Anthony N, Cooper M, et al** (2000): *Ascites syndrome and related pathologies in feed restricted broilers raised in a hypobaric chamber*. Poult Sci, **79**, 318-323.
6. **Currie RJ** (1999): *Ascites in poultry: recent investigations.* Avian Pathol, **28**, 313-326.
7. **Gupta A** (2011): *Ascites syndrome in poultry: a review*. Worlds Poult Sci J, **67**, 457-468.
8. **Hasanpur K, Nassiry M, Salekdeh GH, et al** (2015): *Influence of ascites syndrome on growth pattern of chickens reared at normal or cold ambient temperature*. Ann Anim Sci, **15**, 373-385.
9. **Hassanzadeh M, Buyse J, Toloei T, et al** (2013): *Ascites syndrome in broiler chickens: A review on the aspect of endogenous and exogenous factors interactions*. J Poult Sci, **51**, 229-241.
10. **Julian RJ** (1993): *Ascites in poultry*. Avian Pathol, **22**, 419-454.
11. **Kaplan S, Gürcan EK** (2018): *Comparison of growth curves using non-linear regression function in Japanese quail*. J Appl Anim Res, **46**, 112-117.
12. **Kaufmann KW** (1981): *Fitting and using growth curves*. Oecologia, **49**, 293-299.
13. **Kuhi HD, Kebreab E, Lopez S, et al** (2003): *An evaluation of different growth functions for describing the profile of live weight with time (age) in meat and egg strains of chicken*. Poult Sci, **82**, 1536-1543.
14. **Lee L, Atkinson D, Hirst AG, et al** (2020): *A new framework for growth curve fitting based on the von Bertalanffy Growth Function*. Scientific Reports, **10**, 1-12.
15. **Nurmeiliasari N** (2010): *Ascites Incidence in Broilers*. J Sain Peternak Indones, **5**, 59-64.
16. **Pavlidis H, Balog J, Stamps L, et al** (2007): *Divergent selection for ascites incidence in chickens*. Poult Sci, **86**, 2517-2529.
17. **Porter T, Kebreab E, Kuhi HD, et al** (2010): *Flexible alternatives to the Gompertz equation for describing growth with age in turkey hens*. Poult Sci, **89**, 371-378.
18. **Rabinovitch M** (2008): *Molecular pathogenesis of pulmonary arterial hypertension*. J Clin Invest, **118**, 2372-2379.
19. **Richards F** (1959): *A flexible growth function for empirical use*. J Exp Bot, **10**, 290-301.
20. **Riddell C, Springer R** (1985): Cardiomyopathy and ascites in broiler chickens. Proceedings of the 34th Western Poultry Disease Conference, Austria.
21. **Sakomura N, Longo F, Oviedo-Rondon E, et al** (2005): *Modeling energy utilization and growth parameter description for broiler chickens*. Poult Sci, **84**, 1363-1369.
22. **SAS S** (2009). STAT user's guide, version 9.2. Cary, NC, USA: SAS Inst, Inc.
23. **Segura-Correa J, Santos-Ricalde R, Palma-Avila I** (2017): *Non-Linear Model to Describe Growth Curves of Commercial Turkey in the Tropics of Mexico*. Braz J Poult Sci, **19**, 27-32.
24. **Şengül T, Kiraz S** (2005): *Non-linear models for growth curves in Large White turkeys*. Turk J Vet Anim Sci, **29**, 331-337.
25. **Winkel BJ** (2012): *Sourcing for parameter estimation and study of logistic differential equation*. Int J Math Educ Sci Technol, **43**, 67-83.
26. **Yang Y, Mekki D, Lv S, et al** (2006): *Analysis of fitting growth models in Jinghai mixed-sex yellow chicken*. Int J Poult Sci, **5**, 517-521.
27. **Zubair A, Leeson S** (1996): *Compensatory growth in the broiler chicken: a review*. Worlds Poult Sci J, **52**, 189-201.

Ankara Univ Vet Fak Derg, **69**, 297-302, 2022
DOI: 10.33988/auvfd.796785

Conventional and molecular identification of *Brucella* isolates from livestock in Turkey

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Received date: 28.09.2020 - Accepted date: 03.08.2021

Abstract: The main object of the study is to demonstrate that Bruce-ladder multiplex PCR, which is capable of identify all *Brucella* species including vaccinal strains, is a safe and practical method that is alternative to bacteriological culture methods. Brucellosis is a zoonosis of great socio-economic importance which is endemic in Turkey. In three-year period from 2009 to 2011, a total of 281 *Brucella* spp. isolates from cattle, sheep and goats were identified and characterized by both conventional biotyping procedures and the PCR based method called Bruce-ladder. Species identification from both phenotypic testing and Bruce-ladder was concordant for all isolates tested. The results showed preferred host pattern in *B.abortus* isolates. In terms of species identity, it was found that 94% of the isolations derived from cattle were *B.abortus* and were exclusively biotype 3. *B.melitensis* biotype 3 and biotype 1 were the prevelant biotypes in sheep and goats. Two isolates from sheep were identified as *B.melitensis* Rev.1 vaccine strain. Surprisingly, one of the goat isolates was identified as *B.abortus* S19 vaccine strain. Other interesting observations were the isolation of penicilin-sensitive non vaccine strains of *B.abortus* and *B.melitensis* as well as an isolation of an atypical *B.abortus* biotype 3 strain on the basis of growth inhibition in the presence of basic fuchsin dye. At the end of this study, it was concluded that monitoring *Brucella* isolates and identify them are indispensable epidemiological tool for brucellosis control
Keywords: Atypical *Brucellae,* bruce-ladder PCR, *Brucell*a typing, Rev.1.

Türkiyede çiftlik hayvanlarından izole edilen *Brusella* izolatlarının konvansiyonel ve moleküler identifikasyonu

Özet: Çalışmanın başlıca amacı, izole edilen *Brusella* suşlarının identifikasyonunda, aşı suşları dahil tüm türleri saptayan Bruce-ladder multipleks PCR'nin bakteriyolojik kültür yöntemlerine güvenli ve kolay uygulanabilir bir alternatif sunduğunu göstermektir. Bruselloz, Türkiye'de yaygın olarak görülen ve büyük sosyo-ekonomik etkiye sahip bir zoonozdur. 2009 yılından 2011'e, 3 yıllık bir dönemde, sığır, koyun ve keçilerden izole edilen toplam 281 *Brucella* spp. izolatı, konvansiyonel biyotiplendirme ve PCR temelli Bruce-Ladder metodları birlikte kullanılarak tanımlandı ve karakterize edildi. Fenotipik test ve Bruce-ladder metodlarıyla tür identifikasyonu sonuçları, tüm izolatlar için birbirleriyle uyumluydu. Sonuçlar *B.abortus* izolatları için, tercih edilen konakları belirledi. Tür düzeyinde identifikasyona göre sığırlardan elde edilen izolatların %94'ü *B.abortus* ve çoğunlukla biyotip 3'tü. *B.melitensis* biyotip 3 ve biyotip 1, koyun ve keçilerdeki yaygın biyotiplerdi. Koyunlardan iki izolat, *B.melitensis* Rev.1 aşı suşu olarak identifiye edildi. Keçi izolatlarından biri, beklenmedik şekilde *B.abortus* S19 aşı suşu olarak tanımlandı. Diğer ilginç gözlemler, penisiline duyarlı olan ancak aşı suşu olmayan *B.abortus* ve *B.melitensis* suşları ile 3 adet Bazik fuksin boyasına duyarlı *B.abortus* biyotip 3 suşunun izolasyonlarıydı. Çalışmanın sonunda, *Brucella* izolatlarını izlemek ve onları tanımlamanın brusellozun kontrolünde vazgeçilmez bir epidemiyolojik araç olduğu kanısına varıldı.
Anahtar sözcükler: Atipik *Brusellalar*, bruce-ladder PCR, *Brusell*a tiplendirme, Rev.1.

Introduction

Brucellosis is a zoonosis of global importance that causes reproductive problems in ruminants and serious economic losses in the livestock industry (10). The genus *Brucella* currently consists of 11 species based on mainly host specificity, of which six (*B.abortus* (cattle),

B.melitensis (sheep and goats), *B.suis* (Swine but also hares, reindeer, rodents), *B.ovis* (sheep), *B.canis* (dogs), *B.neotomae* (wood rats)) are classical species. Newly described species include *B.ceti* (dolphins, porpoises), *B.pinnipedialis* (seals), *B.microti* (voles), *B.inopinata* (human breast implants) and *B.papionis* (baboons). *B.abortus, B. melitensis* and *B.suis* can be further subdivided into biotypes based on cultural, biochemical, and serological differences (10, 14, 19, 23).

From the previous studies centered on *Brucella* spp. characterizations in Turkey, it was shown that whilst brucellosis in sheep and goats was attributable to infection by either *B.melitensis* biotype 3 or biotype 1 (6, 11, 12, 16), brucellosis in cattle could be attributable to exclusively *B.abortus* biotype 3 (12, 18, 26).

In the case of control measures for brucellosis in endemic regions, it is advisable to vaccinate livestock to control the disease. *B.abortus* S19 and *B.melitensis* Rev.1 are the vaccine strains used to vaccinate cattle and sheep, and goats, respectively, in Turkey. Although vaccination offers protection from brucellosis in animals, sometimes these vaccine strains can cause abortions if especially administered to pregnant livestock (19).

Accurate identification of the *Brucella* species and biotypes is critical for the success of the control and eradication programs since it clearly demonstrates the prevailing *Brucella* species and biotypes in any given region or country as well as monitors of vaccine strains where vaccination is undertaken. Such information can also be used to trace and control newly introduced strains and it is indispensable for obtaining an accurate evaluation of the epidemiological status of herds and countries (7, 10).

Conventional biotyping procedures rely upon phenotypic differences seen in the culture of isolated bacteria and they include CO_2 requirement, H_2S and urease production, degrees of growth inhibition in the presence of certain dyes, agglutination with mono-specific antisera specific for A and M *Brucella* surface antigens, susceptibility to antibiotics and lysis by *Brucella* phages (2, 8). More recently, with advances in molecular techniques and a better understanding of genomes from a variety of *Brucella* species, several PCR-based assays have been published that can identify *Brucella* isolations to strain level as well as differentiate field strains from vaccine strains (5, 15, 20). *B.abortus* RB51 can be distinguished from field strains by serological means through its rough phenotype, but the smooth strain vaccines, *B.abortus* S19 and *B.melitensis* Rev.1 cannot be distinguished by this methodology. However, phenotypic characteristics and, more recently, molecular tests like Bruce-ladder can provide the means to identify these smooth vaccines from field strains. Although the use of these tests has been limited to identifying bacterial isolations, there is a potential use of these techniques directly on DNA extractions from clinical materials, thus circumventing the need for bacterial isolation for characterization of the causative agent (22). Further, as molecular assays do not require viable organisms and can work directly from heat-inactivated material, they are safer than conventional tests and do not require the expert who works at handling facilities required to work with live *Brucella* cultures.

The present study aimed to identify 281 *Brucella* isolates by conventional biotyping and molecular methods, verify the capability and usefulness of Bruce-ladder multiplex PCR for characterizing these isolates, and compare both typing methods. The study's second aim was to monitor prevalent biotypes, atypical *Brucella* strains, and vaccine strains in the field; therefore, it was expected to generate valuable epidemiological data, especially for the brucellosis control programs

Materials and Methods

***Reference and test strains*:** Reference *Brucella* strains (*B.abortus* 544, *B.melitensis* 16 M, *B.suis* 1330, *B.canis* RM6/66, *B.ovis* 63/290, *B.neotomae* K33, *B.abortus* S19, *B.melitensis* Rev.1, *B.abortus* RB51) were from the culture collection kept at Pendik Veterinary Control Institute, Istanbul. *B.pinnipedialis*, *B.ceti*, *B.microti* and *B.inopinata* were kindly obtained by Animal Health and Veterinary Laboratory Agency (AHVLA), Weybridge, UK. A total of 281 test strains were the isolates submitted to National Brucella Reference Laboratory for species and biotype determination in three years period from 2009 to 2011.

***Classical biotyping procedures*:** *Brucella* cultures were examined by classical biotyping procedures for the identification at species and biotype level (2, 19). Tryptic soy agar (L007516, BD) supplemented with heat-inactivated bovine serum (Biochrom, S0115, Germany) (5%, v/v) (TSA) was employed as the basal medium for all culture work. Inoculated plates were incubated at 37ºC in normal atmospheric conditions and with the addition of 10% CO_2 for 4-5 days. Submitted cultures were identified in a 3-stage procedure.

Stage 1. Cultures were examined for purity and colonial morphology. For this, cultures should be in a smooth phase for typing (2, 19). For differentiation smooth and rough isolates, they were checked for colonial morphology by stereomicroscope (Olympus, VZM 294769) and for agglutination with neutral acriflavin (0.1%, w/v) (Sigma A8126). Any possible agglutination rendered any given strain untypeable.

Stage 2. For species determination, the following tests were performed on all isolates; serum requirement for growth, oxidase and urease production and lysis with Tbilisi phage at routine test dilution (RTD) and 10^4 x RTD and R/C phage at RTD.

Stage 3. For biotyping, production of H_2S, CO_2 requirement for growth, growth in the medium containing thionine (T3387, Sigma) (20 mg/ml), basic fuchsin (115937, Merck) (20 mg/ml), and safranin O (S2255, Sigma) (100 mg/ml) dyes, agglutination with A and M monospecific antisera and R antiserum were investigated. To distinguish between field strains and vaccine strains, growth on medium containing penicillin (13750, Fluka) (5IU/ml), streptomycin (S6501, Sigma) (2.5 mg/ml), thionine blue (R310360, Sigma) (2 mg/ml) and erythritol (E7500 Sigma) (1 mg/ml) were tested.

Molecular typing of Brucella species by multiplex PCR (Bruce-ladder): This assay was undertaken using a published protocol (20). For extracting genomic DNA, a loopful of bacterial culture was taken from the petri plate and resuspended in 200 µl sterile distilled water. This was mixed and incubated at 99ºC for 10 minutes and centrifuged at 12 000 g for 20 seconds. The supernatant was used as DNA template for Bruce-ladder. The assay was carried out in a 25µl reaction mixture containing 2× Qiagen Multiplex Master Mix (Qiagen, Germany), 2µM of each primer in cocktail of nine primer sets and 1µl template DNA. Amplifications were initiated by denaturing the sample for 15 min at 95°C was followed by template denaturation at 94°C 30 s, primer annealing at 58°C for 90 s and primer extension at 72°C for 180 s for a total of 25 cycles. After the last cycle, samples were incubated for an additional 10 min at 72°C (Palm Cycler, C61-96). Amplification products were separated by electrophoresis on 1.5% agarose gels.

Results

Results of conventional biotyping: Table 1 and Table 2 show the distribution of *Brucella* biotypes based on the animal species from which they were isolated. A total of 281 *Brucella* field isolates were processed. Of the 125 isolates from sheep and goats, 98 isolates were found to be *B.melitensis* biotype 3, 20 were identified as *B.melitensis* biotype 1 and only one isolate confirmed as *B.melitensis* biotype 2. In addition to these, 3 isolations in sheep and goats were found to be *B.abortus* biotype 3. Apart from field strains, 3 vaccine strains (2 isolations of *B.melitensis* Rev.1 in sheep and a single isolation of *B.abortus* S19 in a goat) were also identified by conventional biotyping.

Regarding to cattle isolates, 147 out of 156 isolates were found to be *B.abortus biotype 3*, whilst the remainder (9/156) were identified as *B.melitensis* strains. No vaccine strains was identified in any of the cattle isolations using conventional biotyping. Deviations from classical biotype profiles in the characterized isolates included sensitivity to penicillin and it was determined 10 *B.abortus* and 6 *B.melitensis* field isolates. Single isolation of *B.abortus biotype 3* showed basic fuchsin sensitivity.

Table 1. Distribution of *Brucella* species and biotypes isolated from sheep and goats.

Biotype	Number of isolates	Percentage of biotype
B.melitensis biotype 3	98	78.4%
B.melitensis biotype 1	20	16%
B.melitensis biotype 2	1	0.8%
B.melitensis Rev.1	2	1.6%
B.abortus biotype 3	3	2.4%
B.abortus S19	1	0.8%
Total	125	

Table 2. Distribution of *Brucella* species and biotypes isolated from cattle.

Biotype	Number of isolates	Percentage of biotype
B.abortus biotype 3	147	94.2%
B.melitensis biotype 1	2	1.3%
B.melitensis biotype 3	7	4.5%
Total	156	100%

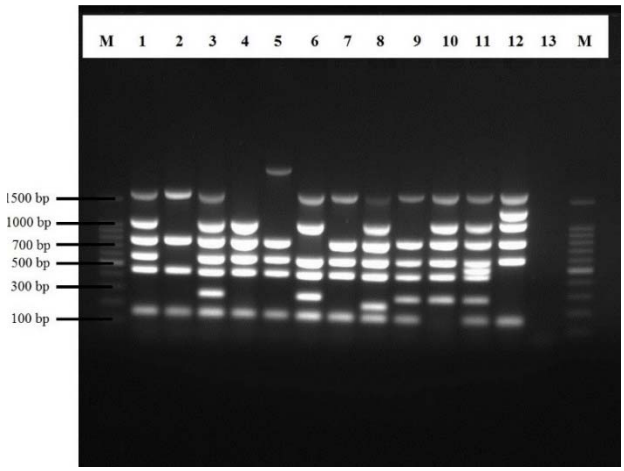

Figure 1. Bruce ladder PCR for reference *Brucella* strains. Lane M: 1500 bp ladder, Lane 1: *B.melitensis*, Lane 2: *B.abortus* S19, Lane 3: *B.suis*, Lane 4: *B.ovis*, Lane 5: *B.abortus* RB51, Lane 6: *B.canis*, Lane 7: *B.abortus*, Lane 8: *B.melitensis* Rev.1, Lane 9: *B.inopinata*, Lane 10: *B.neotomae*, Lane 11: *B.microti*, Lane 12: *B.ceti*, Lane 13: Negative control.

Results of Bruce-ladder multiplex PCR: Using the Bruce ladder protocol described by Mayer Scholl et al. (16), reference strains of all *Brucella* species that are currently known and three vaccine strains, namely *B.melitensis* Rev.1, *B.abortus* RB51 and *B.abortus* S19 displayed correct band profiles described for this test (Figure 1) Regarding to test strains, it was found that there was some degree of "host specificity" with 95% of isolates (119/125) from sheep and goats being *B.melitensis* field strains whilst 94% of isolates (147/156) from cattle were

found to be *B.abortus* field strains. However, there was a very small degree of "spill over" from "host species" with 2% (3/125) of sheep and goat isolates was identified as *B.abortus* field strains and 6% (9/156) of cattle isolates was identified as *B.melitensis*. To this end, whilst no isolations from cattle were found to be of vaccine origin, 2 isolates from sheep and goats were found to be *B. melitensis* Rev.1 and single isolation from a goat was found to be *B.abortus* S19 (Figure 2).

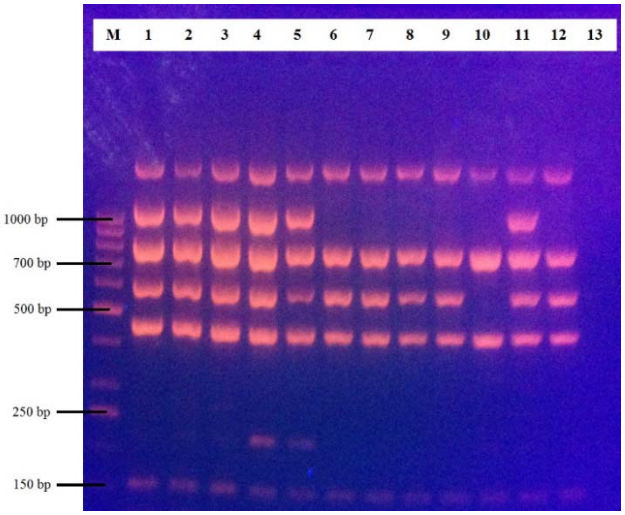

Figure 2. Bruce ladder PCR for field *Brucella* strains. Lane M: 1000 bp ladder, Lane 1-3: *B.melitensis* field isolates, Lane 4 and 5: *B.melitensis* Rev.1, Lane 6-9: *B.abortus* field isolates, Lane 10: *B.abortus* S19, Lane 11: Reference *B.melitensis*, Lane 12: Reference *B.abortus*, Lane 13: Negative control.

Discussion and Conclusion

A total of 281 *Brucella* field isolates were identified using conventional and molecular typing methods. *B.melitensis* biotype 3 is the dominant causative agent for brucellosis in sheep and goats, although other biotypes exist. However, in the case of bovine brucellosis caused by *B.abortus* biotype 3 overwhelmingly predominates in cattle. These observations are in keeping with the data from previous studies in Turkey (6, 11, 12, 16, 18, 26).

Of 125 *Brucella* isolates from sheep, 2 were identified as *B.melitensis* Rev.1 vaccine strain. This strain is known to often cause abortion and is excreted in milk when animals are vaccinated during pregnancy (4). The isolation of vaccine strains further emphasises the point that adult animals be vaccinated when they are not pregnant or during the lambing season (4, 19).

Surprisingly, one isolate from goats was identified as *B.abortus* S19 vaccine strain. This was very unusual when considering the host and the strain in question. We were informed that this goat had been reared on a goat farm where there were no other animals nearby. So, it was assumed that this animal might have been vaccinated with a full dose of *B.abortus* S19 accidentally when she was pregnant.

The most field isolations tested exhibited typical phenotypic characteristics in keeping with conventional biotyping. Nevertheless, in the course of this study, some atypical variants were identified. All biotypes of *B.melitensis* except vaccine strains are resistant to penicilin (2, 8). However, of the 128 *B.melitensis* field isolations from sheep and goats, 6 penicillin-sensitive non-vaccine strains were identified. In the case of *B.abortus* isolations tested from all livestock, 10 were found to be penicillin sensitive non vaccine strains. In addition, one isolate of *B.abortus* biotype 3 showed sensitivity to basic fuchsin dye. Several authors have reported atypical *Brucella* isolates that do not fit into the conventional *Brucella* biotyping scheme (3, 9, 13, 25). Occasional isolates exhibit atypical characteristics that can be useful in epidemiological studies. It was thought that penicillin susceptibility might show the source of infection and could help trace back the infection (25).

In this study, nine isolates from cattle were identified as *B.melitensis*, a species more commonly found in sheep and goats. Although, there have been several reports of isolation of *B.melitensis* from cattle from different parts of the world (1, 17). It is quite reasonable to think that wherever brucellosis is enzootic in sheep and goats, the cattle living in the neighborhood have likely to acquire the infection from these species. Three of the sheep isolates were shown to be *B.abortus* biotype 3 (2.4%). Although infection with *B.abortus* is rare in sheep, there have been several reports of sheep abortion caused by *B.abortus* in various countries (21, 24). It is probable that the sheep and goats acquired *B.abortus* from the cattle on the farm, possibly from using a field that had been infected by the cattle. It has often been assumed that each *Brucella* species is known to have a definite host preference. However, inappropriate management conditions like different species of animals being kept together may allow the disease to be transferred out with traditionally accepted host species. Because of interaction between agent infectivity and host immunity and also structure of animal populations, *B.melitensis* infection in cattle may occur more frequently than *B.abortus* infection in sheep and goats. These issues should be taken into consideration when planning effective disease control programs. Animal movements also should be controlled by appropriate regulations and legislation.

Molecular typing of all the reference strains showed the correct amplicon sizes described for Bruce-ladder multiplex PCR (Figure 1). There was concordance in the results generated by both classical biotyping and Bruce-ladder in regards to species and vaccine identities. Conventional biotyping is time-consuming, requires a

high level of bacteriological expertise, safe laboratory infrastructure for both workers and environment and also hazardous to personnel who work with these agents. In addition, whilst there are selective media, such as Farrell's that limit the growth of contaminating agents (19), it can be challenging to isolate pure *Brucella* cultures from field material. These issues can be overcome by using PCR-based molecular typing tools (5, 15, 16, 20). Molecular testing does not require viable material for testing which allows for samples to be inactivated prior to DNA extraction. Furthermore, molecular tests such as Bruce-ladder have been shown to specifically work on *Brucella* spp. DNA and so would not be adversely affected by non-target DNA in contaminating agents. Further, smooth *Brucella* strains that show transition to a rough state cannot be typed conventionally because of rough lipopolysaccharides on its cell wall that render them incapable of to be agglutinated by A and M mono-specific sera and being lysed by smooth *Brucella* phages (2, 8).

Although conventional biotyping has these drawbacks, there is currently no molecular typing test capable of differentiating biotypes of *B.abortus, B.melitensis*, and *B.suis* (19), which is essential to understanding strain diversity within a population. In addition, it is crucial to monitor atypical strains based on phenotypical characteristics (3, 9).

In spite of the diagnostic sensitivity and specificity decreased in molecular tests when used directly from tissues to diagnose brucellosis (22), the diagnostic success of molecular tests from direct bacterial cultures is quite impressive (20, 22). Our findings offer that molecular typing is a practical test that detects all currently known *Brucella* species and can be used conveniently in diagnostic laboratories, especially where a quick diagnosis is required. Conventional and molecular typing tests have their own advantages, so it would be efficient to use both together, especially in reference laboratories and centers. Present study results explicitly demonstrated that monitoring *Brucella* isolates at regular intervals serves as a powerful epidemiological tool allowing us to know which *Brucella* species and biotypes are prevalent and the presence of any atypical *Brucella* and vaccine strains in the field. Such studies are critical in the control and accurate diagnosis of brucellosis.

Acknowledgements

Part of this study has been presented in Brucellosis 2011 International Research Conference, Including 64th Research Conference, 21-23 September, Buenos Aires, Argentina.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

Ethical approval was not required in this study.

Conflict of interest

The authors declared that there is no conflict of interest.

References

1. **Aldomy FMM, Jahans KL, Altarzi YH** (1992): *Isolation of Brucella melitensis from aborting ruminants in Jordan*. J Comp Path, **107**, 239-242.
2. **Alton GG, Jones LM, Angus RD, et al** (1988): Techniques for the Brucellosis Laboratory. INRA, Paris, France.
3. **Banai M, Mayer I, Cohen A** (1990): *Isolation, identification, and characterization in Israel of Brucella melitensis biovar 1 atypical strains susceptible to dyes and penicillin, indicating the evolution of a new variant*. J Clin Microbiol, **28**, 1057-1059.
4. **Blasco JM** (1997): *A review of the use of B.melitensis Rev 1 vaccine in adult sheep and goats*. Prev Vet Med, **31**, 275-283.
5. **Bricker BJ, Halling SM** (1994): *Differentiation of Brucella abortus bv. 1,2, and 4, Brucella melitensis, Brucella ovis, and Brucella suis bv. 1 by PCR*. J Clin Microbiol, **32**, 2660-2666.
6. **Büyükçangaz E, Şen A, Kahya S** (2009): *Isolation and biotyping of Brucella melitensis from aborted sheep and goat fetuses*. Turk J Vet Anim Sci, **33**, 311-316.
7. **Corbel MJ** (1989): Brucellosis: epidemiology and prevalence worldwide. 25-40. In: Young EJ, Corbel MJ (Ed), Brucellosis: Clinical and Laboratory Aspects. CRC Press 1 st ed., Boca Raton, FL, USA.
8. **Corbel MJ** (1989): Microbiology of the genus Brucella. 54-67. In: Young EJ, Corbel MJ (Ed), Brucellosis: Clinical and Laboratory Aspects. CRC Press 1st ed., Boca Raton, FL, USA.
9. **Corbel MJ** (1991): *Identification of dye-sensitive strains of Brucella melitensis.* J Clin Microbiol, **29**, 1066-1068.
10. **Corbel MJ** (1997): *Brucellosis:an overwiev*. Emerg Infect Dis, **3**, 213-221.
11. **Erdenlig S, Şen A** (2000): *Koyun atıklarından izole edilen Brucella cinsi mikroorganizmaların izolasyonu ve biyotiplendirilmesi*. Pendik Vet Mikrobiol Derg, **31**, 31-42.
12. **Erdenlig S, Iyisan AS, Baklan EA, et al** (2007): Biovar distrubution of Brucella isolates from livestock in Turkey, 1999 to 2006. 27-28. In: Proceedings of the 15th International Congress of Mediterrenean Federation for Health and Production of Ruminants. Kuşadası, Turkey.
13. **Ewalt DR, Forbes LB** (1987): *Atypical isolates of Brucella abortus from Canada and the United States characterized as dye sensitive with M antigen dominant*. J Clin Microbiol, **25**, 698-701.
14. **Foster G, Osterman BS, Godfroid J, et al** (2007): *Brucella ceti sp. nov. Brucella pinnipedialis sp. nov. for Brucella strains with cetaceans and seals as their preferred hosts*. Int J Syst Evol Microbiol, **57**, 2688–2693.
15. **Garcia-Yoldi D, Marin CM, De Miguel MJ, et al** (2006): *Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis Rev1*. Clin Chem, **52**, 779–781.

16. **Güler L, Gündüz K, Ok U** (2003): *Comparison of polymerase chain reaction and bacteriological culture for the diagnosis of sheep brucellosis using aborted fetus samples*. Vet Microbiol, **93**, 53-61.
17. **Hemashettar BM, Patil CS, Jayakumar K, et al** (1987): *Isolation of Brucella melitensis biotype 1 from a cow and two of its attenders*. Indian Vet J, **64**, 822-825.
18. **Ica T, Aydın F, Erdenliğ S, et al** (2008): *Characterisation of Brucella abortus biovar 3 isolates from Turkey as biovar 3b*. The Vet Rec, **29**, 660-662.
19. **Manual of Diagnostic Tests and Vaccines for Terrestrial Animals** (2019): Chapter 3.1.4. Brucellosis (Brucella abortus, B. melitensis and B. suis) (infection with B. abortus, B. melitensis and B. suis) (NB: Version adopted in May2016), Available at https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf; [Accessed August 19, 2020].
20. **Mayer-Scholl A, Draeger A, Göllner C, et al** (2010): *Advancement of a multiplex PCR for the differentiation of all currently described Brucella species*. J Microbiol Method, **80**, 112-114.
21. **Ocholi RA, Kwaga JKP, Ajogi I, et al** (2005): *Abortion due to Brucella abortus in sheep in Nigeria*. Rev Sci Tech Int Epiz, **24**, 973-979.
22. **Saytekin AM, Ak S** (2018): *Direct diagnosis of Brucella species through multiplex PCR formed by a new method*. J Microbiol Method, **154**, 86-94.
23. **Scholz HC, Hubalek Z, Sedlacek I, et al** (2008): *Brucella microti sp. nov., isolated from the commonvole Microtus arvalis*. Int J Syst Evol Microbiol, **58**, 375–382.
24. **Shaw WB** (1976): *Brucella abortus infection in sheep. I. Field case*. Br Vet J, **132**, 18-26.
25. **Stinebring WR, Kunkel JR** (1982): *In vitro susceptibility of selected isolates of Brucella abortus to penicilin*. Am J Vet Res, **43**, 545-547.
26. **Şahin M, Genç O, Ünver A, et al** (2008): *Investigation of bovine brucellosis in the Northeastern Turkey*. Trop Anim Health Product, **40**, 281-286.

Ankara Univ Vet Fak Derg, **69**, 303-311, 2022
DOI: 10.33988/auvfd.824882

Investigation of deformed wing virus, black queen cell virus, and acute bee paralysis virus infections in honey bees using reverse transcriptase-polymerase chain reaction (RT-PCR) method

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Received date: 16.11.2020 - Accepted date: 02.07.2021

Abstract: Viruses are one of the most potential risk factors that negatively affect the different life stages of honey bees. This study was conducted to determine the presence of infections caused by the deformed wing virus (DWV), black queen cell virus (BQCV), and acute bee paralysis virus (ABPV) in honey bees in the beekeeping regions of Burdur, along with obtaining information about their prevalence in this particular region. In our study, the adult bees were taken from 31 different beehives and comb samples that had different honey bee breeds and were sampled randomly from 15 beekeeping areas within the region. The collected samples were analyzed using the reverse transcriptase-polymerase chain reaction (RT-PCR), and the prevalence of DWV, BQCV, and ABPV infections were determined to be 74.19% (23/31), 25.81%, (8/31) and 74.19% (23/31), respectively. In this study, the distribution of positive samples and the rates of multiple infections were determined in the colonies. Of the positive honey bee samples, 12 (%38.71) were detected only for one virus, 9 (%29.03) were positive for two viruses (DWV-ABPV) and 8 (%25.81) were positive for all three viruses. In the present study, the presence of the three bee viruses that caused significant damage to the colonies by multiple infections in the Burdur region was determined with RT-PCR. To our knowledge, this is the first report of three mentioned bee infections in honey bees in the Burdur region. The revealed epidemiological conditions lead to the conclusion that serious measures are needed to control these infections in this region.
Keywords: Acute bee paralysis virus, black queen cell virus, deformed wing virus, honey bee, RT-PCR.

Bal arılarında deforme kanat virus, siyah kraliçe hücre virus ve akut arı felci virus enfeksiyonlarının reverz transkriptaz-polymerase chain reaction (RT-PCR) metodu kullanılarak araştırılması

Özet: Viruslar bal arılarının farklı yaşam evrelerini olumsuz yönde etkileyen potansiyel risk faktörlerinden birisidir. Bu çalışma, Burdur yöresinde arıcılık işletmelerinde bulunan bal arılarında deforme kanat virus (DKV), siyah kraliçe hücre virus (BKHV) ve akut arı felci virus (AAFV) enfeksiyonlarının varlığının virolojik olarak saptanması ve bu yöredeki yaygınlıkları hakkında bilgi edinilmesi amacıyla yapılmıştır. Araştırmada söz konusu yörede bulunan 15 arıcılık işletmesinden tesadüfi örnekleme ile farklı arı ırklarından 31 farklı kovandan ergin arı ve yavrulu petek örneği usulüne uygun olarak alındı**.** Toplanan numuneler reverse transcriptase-polymerase chain reaction (RT-PCR) metodu ile analiz edildi ve DKV, BKHV, AAFV enfeksiyonlarının prevalansı sırasıyla %74,19 (23/31), %25,81 (8/31), %74,19 (23/31) oranlarında belirlendi. Çalışmanın yürütüldüğü kolonilerde tespit edilen pozitifliğin ırklara göre dağılımı ve çoklu enfeksiyon oranları tespit edildi. Pozitif örneklerin 12'si (%38,71) sadece bir virusa, 9'u (%29,03) iki virusa (DKV-AAFV) ve 8'ide (%25,81) üç virusa karşı pozitif bulundu. Bu çalışmada Burdur yöresinde çoklu enfeksiyonlarla kolonilercde önemli hasara neden olan üç arı virusunun varlığı RT-PCR ile belirlendi. Bu çalışma, Burdur bölgesindeki bal arılarında adı geçen üç virusun varlığına ilişkin bilinen ilk raporu sunmaktadır. Tespit edilen epidemiyolojik sonuçlara göre yörede bu enfeksiyonların kontrolü amacıyla ciddi önlemlerin alınması gerektiği kanaatine varıldı.
Anahtar sözcükler: Akut arı felci virus, bal arısı, deforme kanat virus, siyah kraliçe hücre virus, RT-PCR.

Introduction

Viruses are one of the most potential risk factors that negatively affect the different life stages of honey bees. About 26 honey bee viruses have been reported so far in the literature (16, 27, 40). Many studies conducted in beekeeping have shown that the deformed wing virus (DWV), black queen cell virus (BQCV), and acute bee paralysis virus (ABPV) are the main viruses causing

heavy losses in bees (41, 43, 44). The viral nucleic acid of DWV is a single-stranded positive polarity RNA (25, 28), and the structure is approximately 10.1 kb long. The DWV virus belongs to the *Iflavirus* genus of the *Iflaviridae* family. Iflaviruses differ from the dicistroviruses through a single ORF region present in their genome (45). The deformed wing virus of honeybees is closely associated with characteristic wing deformities, paralysis, abdominal bloating, and rapid mortality of emerging adult bees (25).

The *Varroa destructor* is a major vector for DWV. This ectoparasitic mite receives the virus through the hemolymph of the infected bee and transmits the virus while feeding on another bee. DWV can be transmitted across bee colonies through both vertical and horizontal transmission. Vertical transmission takes place through drone sperm and queen bee eggs while horizontal transmission takes place through larval foods (9, 33, 48, 49). DWV causes colony effects such as bee deformity, malformed appendages (crumpled/vestigial wings), shortened abdomen, weight loss (11, 38), a probable decline in lifespan (23), and consequently irregular and decreased bees (40).

BQCV is the most common (approximately 80%) honey bee virus, followed by the deformed wing and sacbrood viruses (43). It is an RNA virus that belongs to the *Triatovirus* genus of the *Dicistroviridae* family (42). This virus has a non-enveloped structure with cubic symmetry and carries a single-stranded positive polarity nucleic acid (ssRNA) (26). Being an etiological agent of a fatal disease in honeybee queen larvae and pupae, the BQCV infection is observed on the sealed cell wall of the queen's pupa along with the presence of dead pupae (5). The *Nosema apis* and *Nosema ceranae* microorganisms act as a vector in the epidemiology of the BQCV infection (1, 4, 7, 44). Also, the transmission may occur through feeding or contaminated foods (2). BQCV is one of the most common but least known honey bee pathogens, with the most common clinical symptoms consisting of blackened cells and the death of queen larvae or pupae (42). It is observed that the dead pupae in the cell take up a dark color and appear brownish to black. Simultaneously, the blackening of the cell wall occurs on the larvae and pupae as well. This virus causes diarrhea in adult bees (28), where the infected queen bee becomes weak and contaminated (14).

Acute bee paralysis is frequently seen in honey bees and is one of the causes of collapsing colonies. Acute bee paralysis virus (ABPV) is an RNA virus that belongs to the *Aparavirus* genus of the *Dicistroviridae* family (12). It has a cubic symmetry and non-enveloped structure that carries single-stranded viral nucleic acid (ssRNA) (46).

The disease caused by ABPV can be seen across all the biological phases of honey bees. In its natural condition, the virus spreads through the oral secretions (royal jelly) of infected adult bees, which are transmitted to young pupal bees during the feeding process. Bees display the symptoms of the viral disease through their feces. The parasitic mite *V. destructor* is the vector of this virus (30). Depending on the ABPV infection, acute or subclinical diseases are formed in bees. Symptoms and deaths connected to acute bee paralysis occur mostly in colonies infested with *Varroa* mites. Adult bees show symptoms of paralysis as trembling after 5–6 days of incubation. Progressively, some of the bees may appear dark and hairless, while flightless honey bees die within 1–2 days (28). The incidence of the ABPV infection varies in different countries based on their colony capacities and *Varroa* control programs.

Although there is a considerable amount of honey bee population in our country and the world, the data on bee viruses is a limited reference. This study aimed to determine the presence, prevalence, and distribution rates of the bee colonies infected with the DWV, BQCV, and ABPV, causing significant colony losses in the Burdur region using the RT-PCR method.

Materials and Methods

Samples and isolation procedure: In this study, sampling was carried out between June and September 2019 within the Burdur region. The sample size was calculated at a 90-95% confidence interval to determine the number of samples in this research (13). Thirty-one (from 15 apiaries) adult bees and pupa samples were taken from beehives and brought to the laboratory in the cold chain. The collected honey bee samples consisted of different races, including ten Anatolian races (five Muğla ecotypes and five Ege ecotypes), three Syrian, two Belfast, seven Italian, two Carpathian, and seven Carniolan races**.**

The presence of a clinically deformed wing in some honey bees was observed during our field studies (Figure 5). Also, all honey bees were checked during the laboratory stage of the research for *Varroa* mites under a stereomicroscope.

Twenty adult bees and honeycomb samples were grouped in each sample. The samples were homogenized in a sterile mortar with 4-5 mL of phosphate buffer saline (PBS) along with 1000 µg of streptomycin and 1000 IU of penicillin per mL. Following this process, the samples were transferred to 15 mL sterile tubes and centrifuged for 30 min at 4000 rpm. Supernatants were stored at –80 °C until further extraction of RNA.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR): Total RNA extraction was performed by placing the supernatants obtained from homogenate samples into the automatic extraction appliance (Roche, Magna Pure, Germany). The

obtained 31 supernatants were further used for the experiment, out of which 200 µL was used for each bee sample. RNA samples, thus, obtained were stored at –80 °C until required for reuse. As per the procedure, a separate primary concentrate was prepared for all three viruses using a high pure viral nucleic acid kit elution buffer (no: 11858874001). Further, OneStep RT-PCR Kit (Grisp, Xpert OneStep RT-PCR Kit GK64.0100 Portugal) was used for reverse transcription, where the RT-PCR mix for each sample was prepared in a sterile Eppendorf tube as follows: 12.5 µL of Fast PCR Master mix, 1 µL each of forward GSP (10 p/mol) and reverse GSP (10 p/mol), 5 µL of extracted RNA sample, 1.25 µL of RTase Mix, and 4.25 µL of RNase-free water.

RT-PCR was performed using specific primers for the VP1-VP2 gene of DWV, the VP3 gene of BQCV, and the VP2 gene for ABPV. The specific primers are shown in Table 1. The thermal cycling conditions were as follows: one cycle for reverse transcription at 45 °C for 10-15 min followed by initial denaturation at 95 °C for 3 min. Then, 35 cycles of denaturation step at 95 °C for 10 sec, annealing at 56 °C for 10 sec, and finally an extension step at 72 °C for 15 sec with a final extension of one cycle at 72 °C for 1 min. The annealing temperature used to amplify all the viruses was the same. Viral RNA was amplified using the Techne TC-412 device.

We used the positive RNA controls from the Izmir Bornova Veterinary Control Institute to achieve optimization in PCR. The products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and then visualized under a UV transilluminator.

***Varroa destructor* determination:** The honey bees brought to the laboratory were analyzed for the presence of *Varroa* mite. The average size of the mature *Varroa* mites is 1.2 mm while the width is 1.6 mm, and the male mite's size is smaller than the size of the female mite. Mature female *Varroa* mites are reddish brown, but male mites are tan in color (21, 30). The sedimentation method was used to separate the *Varroa* from the wastes of the hive wood floor. One part of waste was mixed with 10 parts of oil. *Varroa* mites that accumulated on the surface of the oil were collected after the waste had precipitated. The bees were placed in jars of warm water at 8-12°C to gain a clear result and one drop of detergent was dropped on them. The honey bees were then examined for *Varroa* mite approximately 5-10 min. after being removed from the jars (21).

Results

The prevalence of all three viruses among the 31 samples collected from 15 apiaries is shown in Table 2. Based on the results of the viral-specific PCRs, out of 31 samples, twenty-three (74.19%) were found to be coinfected with DWV and ABPV, while eight (25.81%) were found to be positive for BCQV nucleic acid. As predicted, the DWV primers amplified a 269 bp fragment from the VP1-VP2 gene of DWV, while a 460 bp amplicon was amplified from the VP2 gene of ABPV and 536 bp amplicon was obtained from the VP3 gene of BQCV. The gel images obtained from the RT-PCR results are presented in Figures 1, 2, and 3.

Table 1. Primers used for all three viruses and the size of their amplicons.

Primer	Nucleotide sequences	Target gene	Product length (bp)	Reference
DWV-F	TGGTCAATTACAAGCTACTTGG	VP1-VP2	269 bp	39
DWV-R	TAGTTGGACCAGTAGCACTCAT			
BQCV-F	CTTTATCGAGGAGGAGTTCGAGT	VP3	536 bp	39
BQCV-R	GCAATAGATAAAGTGAGCCCTCC			
AIV-F	GGTGCCCTATTTAGGGTGAGGA	VP2	460 bp	39
ABPV-R	ACTACAGAAGGCAATGTCCAAGA			

Table 2. Prevalence distribution of all three viruses based on the bee races.

Bee race	Number of samples	DWV (+)/%	BQCV (+)/%	ABPV (+)/%
Muğla	5	5 / 100	3 / 60	5 / 100
Syria	3	3 / 100	-	1 / 33.33
Belfast	2	2 / 100	-	1 / 50
Italian	7	5 / 71.43	3 / 42.86	7 / 100
Carpathian	2	2 / 100	-	2 / 100
Ege	5	4 / 80	2 / 40	2 / 40
Carniole	7	2 / 28.57	-	5 / 71.43
Total	**31 Samples**	**23 / 74.19**	**8 / 25.81**	**23 / 74.19**

Figure 1. DWV RT-PCR gel image [M: Marker, 1–31: Samples, NK: Negative Control, PK: Positive Control, PK1: Positive control] (269 bp).

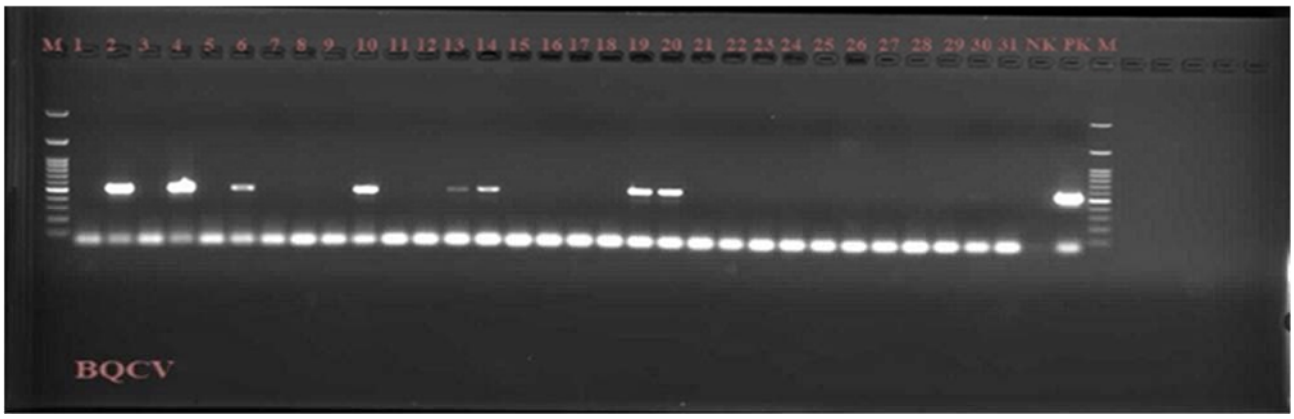

Figure 2. BQCV RT-PCR gel image [M: Marker, 1–31: Samples, NK: Negative Control, PK: Positive Control] (536 bp).

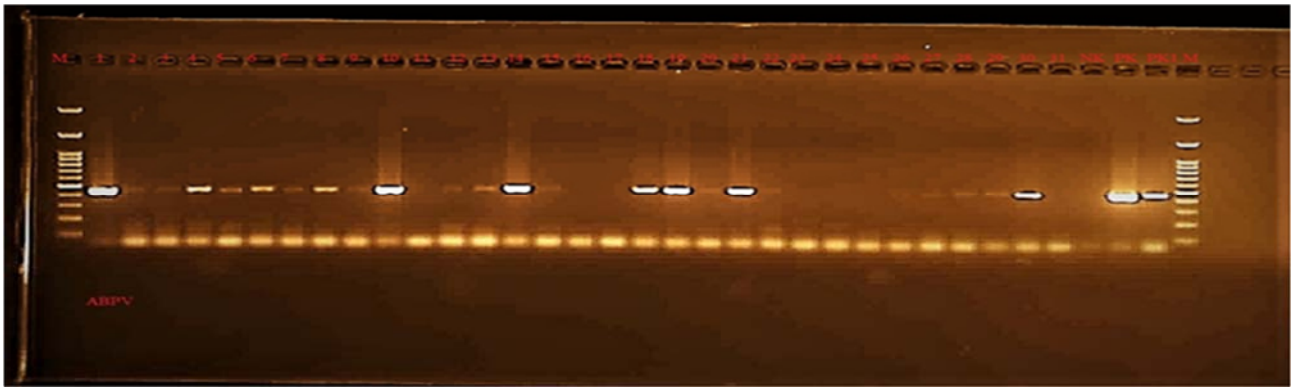

Figure 3. ABPV RT-PCR gel image [M: Marker, 1–31: Samples, NK: Negative Control, PK: Positive Control, PK1 Positive control] (460 bp).

The distribution of the resultant data was evaluated according to the races and is as follows: twenty-three DWV positive samples were observed in the Anatolian race of Muğla ecotype, four in the Anatolian race of Ege ecotype, three in Syria, two in Belfast, five in Italian, two in Carpathian and two in Carniole bee races, respectively. Out of the eight BQCV positive samples, three were determined to be of the Anatolian race of Muğla ecotype, two were from the Anatolian race of Ege ecotype, and three were from the Italian bee race. Out of twenty-three ABPV positive samples, five were found to be of the Anatolian race of Muğla ecotype, two were of the Anatolian race of Ege ecotypes, one of Syrian and Belfast races, seven of the Italian race, five of Carniole, and two were of the Carpathian bee race (Table 2).

Positive results were evaluated in the colonies in terms of DWV, BQCV, and ABPV, with multiple infection rates also being determined. When the distribution of study data by race is evaluated; 23 DWV positive samples; have been observed in Muğla, 4 in Ege

ecotype, 3 in Syria, 2 in Belfast, 5 in Italian, 2 in Carpathian and 2 in Carniole, respectively. 8 BQCV positive for example; 3 of them were determined in Muğla, 2 of them in Ege ecotype and 3 of them in Italian bee race. 23 ABPV positive samples were found to be 5 in Muğla, 2 in Ege ecotypes, 1 in Syria and Belfast, 7 in Italian, 5 in Carniole and 2 in the Carpathian bee race (Table 2).

In addition, positive results in terms of DWV, BQCV and ABPV were evaluated and multiple infection rates in colonies were determined. When these results were evaluated, 2 (6.45%) honey bee samples were found negative for all the viruses controlled. Of the positive honey bee samples, 12 (38.71%) were detected only for one virus, 9 (29.03%) were positive for two viruses (DWV-ABPV) and 8 (25.81%) were positive for all three viruses (Figure 6).

The obtained samples were identified as *Varroa destructor* by morphological analysis (Figure 4).

Figure 4. *Varroa* spp.-infested bee samples encountered during the research are marked with red signs (Original).

Figure 5. Images with deformed wings detected in bees at some sampled apiaries (Original).

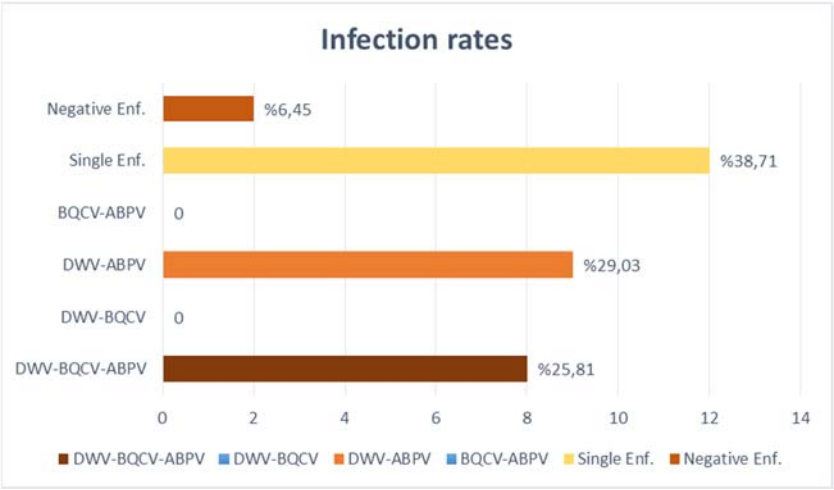

Figure 6. Multiple infections were observed in the controlled colonies.

Discussion and Conclusion

Honey bee products such as royal jelly, honey, and pollen are very valuable in terms of nutrition and health and also in the industries. Additionally, bees are critical for the continuity of the ecosystem. Therefore, it is very important to protect the health of honey bees and protect them against diseases. Especially, it is essential to prevent the unexplained colony losses in beehives or minimize the losses for the future and continuation of our world (3, 7, 18, 29).

The existence of healthy bees and bee colonies in our country and the world is very important in terms of both natural life and human health. One of the most important criteria is to raise the bees and their colonies free from the viral agents. Hence, it is necessary to conduct accurate and rapid diagnoses along with combating these diseases by proper protection measures in place. In this way, we can protect the health of honey bees and contribute to future generations and the ecosystem (17, 20, 31, 36, 44). Difficulties are encountered while assessing the epidemiological status of countries because it is not compulsory to report the DWV, ABPV, and BQCV infections in disease reporting systems such as Animal Disease Notification System (ADNS) and World Organization for Animal Health (OIE). However, when the literature related to these viruses was examined, the diseases were observed to be spread worldwide. Depending on the honey bee colony capacities of various countries and the *Varroa* struggle programs, the incidence of these infections varies between 2% and 91% (3, 30, 34, 44, 47).

DWV causes unusually high winter deaths and disease symptoms and is detected at high prevalence in adults and pupae in many countries using molecular diagnostic techniques such as RT-PCR methods (3, 6, 15, 24, 31). The prevalence of DWV in the world is as described below: the most common honey bee virus is found in Thailand (37) and is closely related to the infestation of *Varroa* (8) and *Tropilaelaps* mites (22). A study conducted in Germany showed that all German bees infested by the endemic *Varroa destructor* parasite species were compared with the Swedish bees brought from Sweden. However, this *Varroa* mite has not been reported to date. The German bees were 100% positive for the DWV nucleic acid, while only 40% of Swedish bees were positive for DWV (50). A study was carried out using the RT-PCR method in the Aclun region of Jordan, where there were losses observed in the bee colonies, and it was determined that there was a high rate of DWV in these colonies (19). Shumkova et al. (41) reported the prevalence of DWV in the bee colonies of Bulgaria at a rate lower than that found in other countries. In the study conducted on honey bees located in different climatic regions of Argentina, 35% of the colonies were found to be DWV positive. However, in the same study, approximately 25% of the bees showed binary and triplet viral infections (30).

Many studies have been conducted in our country related to DWV. One study was conducted in the queen bee colonies, where approximately 50% of the hives were reported to be infected with this virus (17). Karapınar et al. (21) detected a high rate of DWV disease, i.e., 69.23% in the province of Van, which was also similar to the prevalence of *Varroa* parasite in these colonies. A large-scale study was conducted in apiaries in the Aegean region which used the multiplex RT-PCR method and determined the DWV rate to be 25.2%. Especially in some apiaries, where there were DWV disease symptoms or high colony loss, the prevalence of viral agents was reported to be higher in the bee population (10). Rüstemoğlu also reported the prevalence of DWV at a rate of 23.3% in a total of 90 apiaries collected from Hakkari province in 2015, which was conducted on the bee samples using the RT-PCR method (36). Kalaycı et al. reported that DWV was the most prevalent virus in the Turkish apiaries with a rate of 44.7% (20).

In this study, DWV was detected in twenty-three (74.19%) of 31 samples collected from 15 apiaries. Our results were parallel with those obtained by Karapınar et al. (21). But, our percentage was found to be higher than the percentages found by Çağırgan (10), Kalaycı et al. (20), and Rüstemoğlu (36). The reason for high DWV prevalence may be attributed to this province being a transit/accommodation area for migratory beekeeping. Considering that the transmission of the virus happens through the *Varroa* mite, the evaluation that the *Varroa destructor* is seen on the bees in many sampled apiaries makes the fight against it not completely realized.

BQCV is one of the most common but least known honey bee pathogens, which causes a disease that leads to blackness and death of the queen, larvae, and pupae in high titers (42). The prevalence of BQCV in Uruguay was found to be 91% using the RT-PCR method in the bee colonies, while the research was being reported as the first case of BQCV in South America (3). Tentcheva et al. (44) screened 36 adult bees and pupa samples collected from apparently healthy colonies during the spring, summer, and autumn seasons using the RT-PCR method and found 86% positive BQCV colonies. A study conducted in Denmark (31) revealed the presence of BQCV in the colony where winter deaths were unusually high. In Australia, 65% of the bee colonies were reported to be BQCV positive (33). Also, the BQCV infection was found at a high prevalence (81%) in Chile province in South America (35). However, another research was conducted by the same researchers in Chile in the following years,

which showed the prevalence of infection to be decreased by 10% (34). However, in the studies conducted in England, Croatia, and Syria, the prevalence of BQCV was reported to be of a lower rate (6, 15, 24).

In terms of the studies related to BQCV infection in our country, Gümüşova et al. observed higher BQCV positive rates in adult bees (53%) compared to the larvae (33%) using the RT-PCR method in 2010 (18). Oğuz et al. reported the presence of BQCV nucleic acid in the bees of the Van province at a rate of 88.5% using the RT-PCR method, which was sampled during April and May 2017 (32). In another study conducted in the same province, BQCV was found to be positive in 88.46% (23/26) of the sampled hives. In a study conducted using the multiplex RT-PCR method in apiaries in the Aegean region, the BQCV infection rate was determined to be 25.2% in the bees (10). Rüstemoğlu determined the rate of positive BQCV as 32.2% in the bee samples collected from Hakkari province in 2015 (36).

In our study, the presence of BQCV nucleic acid was detected in eight (25.81%) samples out of 31 bee samples collected from the Burdur region. This rate is lower than the positive rates found in the black sea and eastern provinces of our country (18, 32, 36). However, the BQCV positive rates in our results were corresponding exactly with the rate determined in the Aegean region (10), which is also closer to our area of study. The reason for the lower rate of BQCV-positive bee colonies in our study compared to the DWV and ABPV infections was attributed to the low rate of *Nosema* infection in bee colonies in our area.

Although ABPV presence was not detected in eastern bee colonies of China's Yunnan province, its presence was reported as 2% in Chile and 9% in Uruguay (3, 34, 47). Also, the studies conducted in Europe determined ABPV positive rates to be higher (6, 15, 31, 44) and were accepted as one of the important causes of bee losses.

The presence of ABPV infections in Turkey was reported at different rates since the studies were conducted in different provinces. For the first time in our country, the presence of ABPV (2.2%) was demonstrated using the RT-PCR method in bee samples collected from Hakkari (36). In the following years, Çağırgan (10) reported the prevalence of ABPV as 3.6% in the samples collected from apiaries in the Aegean region while Karapınar et al. (21) did not observe any ABPV infection in their screening of bee colonies in Van province.

In this study, we determined the prevalence of ABPV in the Burdur region to be at the rate of 74.19%. Compared to the other studies conducted in Turkey, our work in the beehive indicated the reason for a high rate of ABPV infection in the bee colonies, commonly consisting of *V. destructor* infestations. An increase in the spread of infectious agents was attributed to the fact that the Burdur region is an accommodation/transit area for migratory beekeeping. Additionally, in our study, varroasis was observed in all of the sampled bees. This is an important finding in terms of the epidemiology of viral pathogens transmitted by the *Varroa* vector.

Honeybee colonies are commonly infected by many viruses simultaneously, often without exhibiting overt signs (15, 28). Mixed viral infections were detected in honeybee samples, which were shown in Figure 6. These results were consistent with those obtained by Kalaycı et al. (20) and Çağırgan (10). Our mixed viral infection ratios were 25.81% and 29.03%, which might indicate the presence of colony losses in this study. But in all cases, multiple viral infections were observed simultaneously along *Varroa*. Also, multiple factors may have led to colony collapses depending on the quality of nutrition and pathogens/pathogen titers.

In conclusion, the data of this research revealed a very high prevalence of pathogenic viruses in the bee population of the Burdur province, which was thought to be the probable reason for the loss of colonies in the hives of Burdur. It is required to detect and use bee breeds to protect the health of the bees that are resistant to viral disease. Also, feeding/hosting conditions, compliance with sanitation, and hygienic measures should not be ignored. In addition to these, it may be beneficial to carry out screening/struggle programs for viral and parasitic factors to protect bees' health. We conclude that conducting regional and national major studies along with serious measures to protect the bees against viral diseases is very important for animal health and our country's economy and ecosystem.

Acknowledgments

We thank Dr. A. Anıl Çağırgan and İzmir/Bornova Veterinary Control Institute for their contributions. This study represents part of the thesis submitted by A. Usta to the Virology Department of Veterinary Medicine Faculty of Burdur Mehmet Akif University, Burdur, Turkey, to fulfill the requirements for a master's degree in veterinary medicine.

Financial Support

This research was supported by Burdur Mehmet Akif Ersoy University Scientific Research Projects Unit with project number 0586-YL-19.

Ethical Statement

This study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (2019-61-493).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Allen MF, Ball BV** (1996): *The incidence and World distribution of honey bee viruses.* Bee World, **77**, 141–162.
2. **Anderson D** (2005): Triggering virus replication in honey bees. Bee Research And Virus in Europe. 24-26. In: Proceedings of the meeting in Sophia-Antipolis, France**.**
3. **Antúnez K, D'Alessandro B, Corbella E, et al** (2006): *Honey bee viruses in Uruguay*. J Invertebrat Pathol, **93**, 67-70.
4. **Bailey L, Ball BV, Perry JN** (1983): *Association of viruses with two protozoal pathogens of the honey bee*. Ann of Appl Biol, **103**, 13-20.
5. **Bailey L, Woods RD** (1977): *Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses.* J Gen Virol, **37**, 175-182.
6. **Baker AC, Schroeder DC** (2008): *Occurrence and genetic analysis of picorna-like viruses infecting worker bees of Apis mellifera L. populations in Devon, South West England.* J Invertebr Pathol, **98**, 239-242.
7. **Berényi O, Bakonyi T, Derakhshifar I, et al** (2006): *Occurrence of six honey bee viruses in diseased Austrian apiaries*. Appl Environ Microbiol**, 72,** 2414-2420.
8. **Chantawannakul P, Ward L, Boonham N, et al** (2006): *A scientific note on the detection of honey bee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honey bee (Apis mellifera) apiary.* J Invertebr Pathol, **91,** 69-73.
9. **Chen YP, Evans J, Feldlaufer M** (2006): *Horizontal and vertical transmission of viruses in the honey bee (Apis mellifera).* J Invertebr Pathol, **92,**152-159.
10. **Çağırgan AA** (2018): Ege Bölgesinde Virus Nedenli Arı Hastalıklarının Multipleks Polimeraz Zincir Reaksiyonu İle Araştırılması. Doktora Tezi. Ondokuz Mayıs Üniversitesi Sağlık Bilimleri Enstitüsü, Samsun.
11. **De Jong D, De Jong PH, Gonçalves LS** (1982): *Weight loss and other damage to developing worker honey bees from infestation with Varroa jacobsoni.* J Apic Res, **21**, 165-167.
12. **De Miranda JR, Cordoni G, Budge G** (2010): *The Acute bee paralysis virus–Kashmir bee virus–Israeli acute paralysis virus complex.* J Invertebr Pathol, **103**, 30–47.
13. **Erganiş O** (1993): Veteriner Epidemiyoloji (Temel Bilgiler). 74-79. Mimoza Yayınları:16, Sağlık Bilimleri Dizisi: 2, Konya.
14. **Fievet J, Tentcheva D, Gauthier G, et al** (2006): *Localization of deformed wing virus infection in queen and drone Apis mellifera L.* Virol J, **3,** 16.
15. **Gajger I, Kolodziejek J, Bakonyi T, et al** (2014): *Prevalence and distribution patterns of seven different honey bee viruses in diseased colonies: a case study from Croatia.* Apidologie, **45**, 701-706.
16. **Garigliany M, Agrebi NE, Franssen M, et al** (2018): *Moku virus detection in honey bees Belgium, 2018*. Wiley Transbound Emerg Dis, **66**, 43-46.
17. **Gülmez Y, Bursalı A, Tekin Ş** (2009): *First molecular detection and characterization of Deforme dwing virus (DWV) in honey bee (Apis melifera) in Turkey*. African J Biotechnol, **8**, 3698-3702.
18. **Gümüşova SO, Albayrak H, Kurt M, et al** (2010): *Prevalence of three honey bee viruses in Turkey.* Veterinarski Arhiv, **80**, 779-785.
19. **Haddad N, Brake M, Migdadi H, et al** (2008): *First detection of honey bee viruses in jordan by Rt-Pcr*. Jordan J Agricul Sci, **4**, 3.
20. **Kalaycı G, Cagırgan AA, Kaplan M, et al** (2020): *The Role of Viral and Parasitic Pathogens Affected By Colony Losses in Turkish Apiaries.* Kafkas Univ Vet Fak Derg, **26,** 671-677.
21. **Karapınar Z, Oğuz B, Dinçer E, et al** (2018): *Phylogenetic analysis of black queen cell virus and deformed wing virus in honey bee colonies infected by mites in Van, Eastern Turkey.* Med Weter, **74**, 460-465.
22. **Khongphinitbunjong K, De Guzman LI, Tarver MR, et al** (2015): *Interactions of Tropilaelaps mercedesae, honey bee viruses and immune response in Apis mellifera.* J Apic Res, **54**, 40-47.
23. **Kovac H, Crailsheim K** (1988): *Life span of Apis mellifera Carnica Pollm. infested by Varroa jacobsoni in relation to season and extent of infestation*. J Apic Res, **27**, 230-238.
24. **Kubaa RA, Molınatto G, Khaled BS, et al** (2018): *First detection of black queen cell virus, Varroa destructor macula-like virus, Apis mellifera filamentous virus and Nosema ceranae in Syrian honey bees Apis mellifera syriaca.* Bull Insectology, **71**, 217-224.
25. **Lanzi G, De Miranda JR, Boniotti MB, et al** (2006): *Molecular and biological characterization of deformed wing virus of honey bees (Apis mellifera L).* J Virology, **80**, 4998-5009.
26. **Leat N, Ball B, Govan V, et al** (2000): *Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees.* Journal of General Virology, **81**, 2111-2119.
27. **Levin S, Sela N, Erez T, et al** (2019): *New Viruses from the Ectoparasite Mite Varroa destructor Infesting Apis mellifera and Apis cerana.* Viruses, **11,** 94.
28. **Maramorosch K, Shatkin A** (2007): Honey bee viruses. Advances in Virus Research, Academic Press, 33-80.
29. **Molineri AI, Pacini A, Giacobino A, et al** (2017): *Prevalence of honey bee (Apis mellifera) viruses in temperate and subtropical regions from Argentina*. Rev Argent Microbiol, **49**, 166-173.
30. **Moore PA, Wilson ME, Skinner JA** (2015): *Honey bee viruses, the deadly Varroa Mite Associates*. Bee Health, **19**, 2015**.**
31. **Nielsen SL, Nicolaisen M, Kryger P** (2008): *Incidence of Acute bee paralysis virus, Black queen cell virus, Chronic bee paralysis virus, Deformed wing virus, Kashmir bee virus and Sacbrood virus in honey bees (Apis mellifera) in Denmark.* Apidologie, **39**, 310-314.
32. **Oğuz B, Karapınar Z, Dinçer E, et al** (2017): *Molecular detection of Nosema spp. and black queen-cell virus in honey bees in Van Province, Turkey.* Turk J Vet Anim Sci, **41**, 221-227.
33. **Roberts JMK, Anderson DL, Durr PA** (2017): *Absence of deformed wing virus and Varroa destructor in Australia provides unique perspectives on honey bee viral landscapes and colony losses.* Sci Rep, **7**, 6925**.**

34. **Rodríguez M, Vargas M, Antúnez K, et al** (2014): *Prevalence and phylogenetic analysis of honey bee viruses in the Biobío Region of Chile and their association with other honey bee pathogens*. Chilean J Agricu, **74**, 170-177.
35. **Rodriguez M, Vargas M, Gerding M, et al** (2012): *Viral infeciton and Nosema ceranae in honey bees in Chile*. J Apicul Res, **51**, 285-287**.**
36. **Rüstemoğlu M** (2015): Hakkari İli Bal Arılarında (Apis Mellifera L.) Görülen Önemli Arı Viruslarının RT-PCR Yöntemi İle Araştırılması Ve Moleküler Karakterizasyonlarının Yapılması. Doktora Tezi. Van Yüzüncü Yıl Üniversitesi Fen Bilimleri Enstitüsü, Van.
37. **Sanpa S, Chantawannakul P** (2009): *Survey of six bee viruses using RT-PCR in Northern Thailand.* J Invertebr Pathol, **100**, 116-119.
38. **Schatton-Gadelmayer K, Engels W** (1988): *Hamolymphproteine und Korpergewicht frischgeschlupfter Bienen-Arbeiterinnen nach unterschiedlich starker Parasitierung durch Brutmilben*. Entomol Gen, **14**, 93-101.
39. **Sguazza GH, Reynaldi FJ, Galosi CM, et al** (2013): *Simultaneous detection of bee viruses by multiplex PCR.* J Virol Methods, **194**, 102-106.
40. **Shimanuki H, Calderone NW, Knox DA** (1994): *Parasitic mite syndrome: The symptoms.* Am Bee J, **134**, 827-828.
41. **Shumkova R, Neov B, Sirakova D, et al** (2018): *Molecular detection and phylogenetic assessment of six honey bee viruses in Apis mellifera L. colonies in Bulgaria*. Peer J, **20**, 6:e5077.
42. **Spurny R, Pridal A, Pálková L, et al** (2017): *Virion structure of black queen cell virus, a common honey bee pathogen*. J Virol, **91**, e02100-16.
43. **Tapaszti Z, Forgach P, Kovágó C, et al** (2009): *Genetic analysis and phylogenetic comparison of Black queen cell virus genotypes.* Vet Microbial, **139**, 227-234.
44. **Tentcheva D, Gauthier L, Zappulla N, et al** (2004): *Prevalence and Seasonal Variations of Six Bee Viruses in Apis mellifera L. and Varroa destructor Mite Populations in France*. Appl Environ Microbiol, 7185-7191**.**
45. **Valles SM, Chen YP, Firth AE, et al** (2017): *ICTV virus taxonomy profile: iflaviridae.* J Gen Virolo, **98**, 527–528.
46. **Valles SM, Chen YP, Firth AE, et al** (2017): *ICTV virus taxonomy profile: dicistroviridae.* J Gen Virolo, **98**, 355–356.
47. **Wang M, Bi J, Wang L, et al** (2016): *Prevalence of Four Common Bee RNA Viruses in Eastern Bee Populations in Yunnan Province, China.* J Veterinar Sci Technol, **7**, 1.
48. **Weinberg KP, Madel G** (1985): *The inxuence of the mite Varroa jacobsoni Oud. on the protein concentration and hemolymph volume of the blood of the worker bees and drones of the honey bee, Apis mellifera.* Apidologie, **16**, 421-436.
49. **Yang X, Cox-Foster DL** (2005)*: Impact of an ectoparasite on the immuni and pathology of an invertebrate: evidence for host immunosuppression and viral amplification.* Proc Natl Acad Sci, **102**, 7470-7475.
50. **Yue C, Genersch E** (2005): *RT-PCR analysis of Deformed wing virus (DWV) in honey bees (Apis mellifera) and mites (Varroa destructor).* J Gen Virol, **86**, 3419-3424.

Ankara Univ Vet Fak Derg, **69**, 313-318, 2022
DOI: 10.33988/auvfd.876578

Use of essential oil mixture to improve antioxidant capacity and concentrations of cecum short-chain fatty acids in Turkish domestic geese (*Anser anser*)

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Received date: 08.02.2021 - Accepted date: 20.05.2021

Abstract: The aim of this study is to investigate the effect of essential oil mixture supplemented in drinking water on antioxidant capacity and intestinal health in geese. One hundred eight chicks (which were 3 days old) were randomly allocated to 3 groups and each group was allocated to 6 subgroups. Research groups have been as follows: C (Control; without supplementation); E1 (0.4 ml/L essential oil mixture supplementation) and E2 (0.8 ml/L essential oil mixture supplementation). The duration of the experiment was 13 weeks. In the first 4 weeks of the trial, the animals were fed for the chick period. In the last 9 weeks of the trial, geese were fed in the pasture under the conditions of Kars province. In the 4th week and at the end of the experiment, GSH exhibited a linear response (P=0.008 and P=0.004, respectively). However, MDA, GSH, SOD, GPx, CAT, nitric oxide, ceruloplasmin, albumin, total protein and globulin were not affected. At the end of the experiment, acetic acid, butyric acid, isocaproic acid and total short-chain fatty acid concentrations were linearly affected with the gradued level of essential oil mixture. There were no significant differences in propionic acid, isobutyric acid, valeric acid, isovaleric acid, caproic acid and BCFA concentrations. In conclusion, water containing essential oil mixture in geese can be used to improve antioxidant capacity and intestinal health.

Keywords: Antioxidant capacity, cecum short-chain fatty acid concentrations, essential oil mixture, Turkish domestic goose (*Anser Anser*).

Türk yerli kazlarında (*Anser anser*) uçucu yağ karışımının antioksidan kapasitesi ve sekum kısa zincirli yağ asidi konsantrasyonlarının iyileştirilmesi için kullanımı

Özet: Bu çalışmanın amacı, içme suyuna eklenen uçucu yağ karışımının kazlarda antioksidan kapasite ve bağırsak sağlığı üzerindeki etkisini araştırmaktır. Yüz sekiz civciv (3 günlük) rastgele 3 gruba ve her bir grup 6 alt gruba ayrılmıştır. Araştırma grupları şu şekildedir: C (Kontrol; Katkı takviyesiz); E1 (0,4 ml / L uçucu yağ karışımı takviyeli) ve E2 (0,8 ml / L uçucu yağ karışımı takviyeli). Deneme süresi 13 haftadır. Denemenin ilk 4 haftasında hayvanlar civciv dönemi besin madde ihtiyaçlarına gore beslenmiştir. Denemenin son 9 haftasında Kars ili şartlarında merada beslenmiştir. Denemenin 4. haftası ve bitiminde, GSH linear bir artış göstermiştir (sırasıyla P=0,008 ve P=0,004). Ancak, MDA, GSH, SOD, GPx, CAT, nitrik oksit, seruloplazmin, albümin, toplam protein ve globulin konsantrasyonları muameleleren önemli düzeyde etkilenmemiştir. Deneme sonunda asetik asit, butirik asit, izokaproik asit ve toplam kısa zincirli yağ asidi konsantrasyonları dereceli uçucu yağ karışımı ile doğrusal olarak etkilenmiştir. Propiyonik asit, izobütirik asit, valerik asit, izovalerik asit, kaproik asit ve BCFA konsantrasyonları açısından önemli bir farklılık bulunmamıştır. Sonuç olarak, kazlarda içme sularında bulunan uçucu yağ karışımı, antioksidan kapasite ve bağırsak sağlığını iyileştirmede kullanılabilir.

Anahtar sözcükler: Antioksidan kapasite, sekum kısa zincirli yağ asidi konsantrasyonları, uçucu yağ karışımı, Türk yerli kazı (*Anser Anser*).

Introduction

The prolonged use of antibiotics as growth factor has prevented the growth of beneficial microorganisms in digestive tract of animals as well as pathogenic microorganisms (20). Use of antibiotics in feeds has promoted development of resistance against bacteria. In addition, residues in animal products have become a risk to human health (1). For this negative reasons, antibiotics have been prohibited as growth factor in animal feed. After this ban probiotics, prebiotics, enzymes, organic acids and some product, such as essential oils, started to be used as an alternative feed additive to antibiotics (7, 15, 41).

Rosemary is in the *Lamiaceae* family (5, 34). Its leaves contain strong antioxidants such as *carnosol, rosmarinic acid* and *carnosic acid*. Mint is in the *Labiatae* family. Its active compounds are flavones, r*osmaniric acid, chlorogenic acid* and triterpenic substances (30). Juniper is in the *Cupressaceae* family (16). In juniper; there are inositis, flavonoids, glycosides, resin, invert sugar, katesin, organic acids, volatile oil, terpenic acids, lacoanthocyanidine substances (22). Oregano is in the *Lamiaceae* family (containing carvacrol and phenolic monoterpenoids) (9, 29). These plant have appetite-enhancing, digestive- stimulanting, anticoccidial, antihelmintic, antiviral, antimicrobial and antioxidant effect (19).

Recent studies have focused on the use of essential oil blends and aromatic herbs in animal nutrition. In this study, it was aimed to investigate the effect of essential oil mixture on blood antioxidant capacity and intestinal health in geese.

Materials and Methods

Animals, experimental design and feed: Ethical approval for this study was obtained from the Kafkas University Animal Experiments Local Ethics Committee (Decision No: KAU-HAYDEK /2018-054/2019-001). One hundred eight chicks (which were 3 days old) were randomly allocated to 3 groups and these groups were allocated to 6 replicate pens (100x100 cm) (6 chicks in each subgroup). The animals were fed with a basal diet based on corn and soybean meal (Table 1). All diets were determined according to NRC standards (28). Nutrient analyses of the feed were performed according to AOAC (3). The duration of the experiment was 13 weeks. In the first 4 weeks of the trial, the animals were fed for the chick period. Each subgroup was equipped with manual feeders and automatic nipple drinkers. Water and feed were given *ad libitum*. In the last 9 weeks of the trial, geese were fed in the pasture under the conditions of Kars province. Goose breeding widely takes place in Kars province because of the suitable climatic and geographic conditions (10). The geese were exposed to natural daylight and kept with a room temperature of 24±3°C. The animals were taken to the pasture between 8-12 am and 13-18 pm. Research groups have been designed as follows: C (Control; without supplementation); E1 (0.4 ml/L essential oil mixture supplementation in drinking water) and E2 (0.8 ml/L essential oil mixture supplementation in drinking water). The essential oil mixture (Mintofarm®) used in the research was obtained from a private company (FARMAVET A.Ş.). Composition of Mintofarm used in the study is shown in Table 2.

Table 1. Composition of basal diets used in experiment (%)[1].

Feed materials	**%**
Corn	56.35
Soyben meal (CP, 46%)	36.10
Corn gluten (CP, 60%)	4.35
Limestone	1.45
Dicalciumphosphate	1.00
DL- Methionine	0.08
L-Lysine Hydrochloride	0.07
Vitamin- mineral premix	0.40
Salt	0.20
Total	100.00
The calculated value	
Crude protein, %	23.00
ME (kcal/kg)	2909.33
Ca, %	0.90
Total P, %	0.59
Analysis Values	
ME (kcal/kg)	2915.25
Crude protein, %	23.11
Ca, %	1.01
Total P, %	0.49

[1] As-fed basis
[2] Vitamin-mineral premix provided per kg diet: Vit. A 8000 IU, Vit. D3 1000 IU, Vit. E 20 IU, Vit. K 0.5 mg, Vit. B1 3 mg, Vit. B2 9 mg, Vit. B6 7 mg, Vit. B12 0.03 mg, niacin 35 mg, D-pantothenic acid 10 mg, folic acid 0.55 mg, biotin 0.18 mg, Fe 100 mg, Cu 8 mg, Zn 100 mg, Mn 120 mg, I 0.7 mg, and Se 0.3 mg.

Table 2. Chemical composition essential oil mixture used in experiment (%).

Product Composition*	**%**
Mint oil	2
Juniper Oil	2
Rosemary Oil	2
Oregano Vulgare oil	2
Surfactants and Stabilizers	15
Water (transporter)	77

*Mintofarm.

Blood antioxidant capacity: Blood samples were taken from the wing veins of the animals into anticoagulant (EDTA) tubes at the 4th week and at the end of the experiment. After a sufficient amount of blood sample was separated as whole blood, plasma of the remaining blood was obtained. The samples taken were centrifuged at 3000 rpm for 15 minutes and stored at -20 ºC until analysis. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) antioxidant enzyme activities in plasma were determined by ELISA device (Epoch, Biotek, USA) using commercial kits (Cayman Chemical Company, USA). Whole blood reduced glutathione (GSH) analysis was determined colorimetrically (Epoch, Biotek, USA) according to the method of Beutler et al. (4). The malondialdehyde (MDA) in plasma was determined by the method of Yoshoiko et al.(42), ceruloplasmin by the method of Colombo and Ricterich and albumin and total protein levels by a commercial test kit (Biolabo, Maizy, France) (8). Globulin value was determined by subtracting albumin from total protein (11). Nitric oxide levels in serum Miranda et al. (26) was determined according to the method they reported.

Cecal short-chain fatty acid concentrations: The cecal digesta that was obtained after sacrificing the animals, was used for the determination of acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic and isocaproic acid with a gas chromatography (Shimadzu GC, Shimadzu Co., Kyoto, Japan), a flame ionization detector **(**FID) and colons (Teknokroma; TR-151035, TRB-FFAP 30m×0.53 mm×0.50 µm). At the end of the study, the cecum content were stored at -18ºC and then were dissolved at + 4ºC before analysis. The contents were centrifuged at 4000 rpm for 15 min at +4°C for homogenization. The supernatant was taken into an Eppendorf tube and mixed with 0.2 mL ice-cold 25% metaphosphoric acid solution. After that, the tubes were kept in ice for 30 min to ensure the collapse of proteins. Subsequently, tubes were centrifuged for 10 min at 11000 rpm at + 4°C. Supernatants were analyzed using GC. The analysis was performed according to Zhang et al. (43). Helium was used for the carrier gas and the colon temperature was programmed so that it was increased stepwise from 110°C to 180°C. Also, the FID and injector block temperature was set to 250°C.

Statistical Analysis: The one-way analysis of varience (ANOVA) method was used for the statistical calculations of the groups and polynomial contrast test was used to determine the dose effect of the essential oil mixture used at different levels in the groups. Statistical differences and tendence analysis were considered significant at P≤0.05. The statistical analysis was done with the SPSS software package (35).

Results

Blood antioxidant capacity: In the 4th week and at the end of the experiment, the increase by essential oil mixture, GSH exhibited a linear response (P=0.008 and P=0.004, respectively). However, MDA, GSH, SOD, GPx, CAT, nitric oxide, ceruloplasmin, albumin, total protein and globulin were not affected by essential oil mixture added. The blood antioxidant capacity parameters of the study at the 4th week and at the end of the experiment are shown in Table 3 and Table 4.

Table 3. Influence of essential oil mixture on antioxidant capacity in the 4th week of the experiment.

	Groups					
Blood Parameters	**C**	**E1**	**E2**	**SEM**	**Significance**	
	x̄	**x̄**	**x̄**		**L**	**Q**
MDA (µmol/L)	7.11	7.27	7.34	0.22	0.699	0.936
Nitric oxide (µmol/L)	31.72	33.58	35.15	1.82	0.475	0.973
GSH (mg/dL)	18.73	24.11	26.51	1.25	0.008	0.513
SOD (U/mL)	47.27	52.60	53.33	8.27	0.226	0.588
CAT (nmol/min/mL)	1.19	1.20	1.23	0.02	0.529	0.875
GPx (nmol/min/mL)	261.48	286.73	288.86	6.22	0.073	0.362
Ceruloplasmin (mg/dL)	19.27	19.31	19.28	0.58	0.991	0.980
Albumin (g/dL)	2.76	2.72	2.71	0.08	0.838	0.957
Total protein (g/dL)	5.91	5.90	5.87	0.10	0.907	0.969
Globulin (g/dL)	3.15	3.17	3.16	0.11	0.970	0.942

[1] Data represent mean values of 6 replicates per treatment,
[2] Groups; C: Control without supplementation drinking water, E1: 0.4 mL essential oil mixture supplementation in drinking water and E2: 0.8 mL essential oil mixture supplementation in drinking water,
[3] Polynomial contrasts: L=linear and Q=quadratic effect of supplemental essential oil mixture.

Table 4. Influence of essential oil mixture on antioxidant capacity at the end of the experiment.

	Groups					
Blood Parameters	**C**	**E1**	**E2**	**SEM**	**Significance**	
	x̄	**x̄**	**x̄**		**L**	**Q**
MDA (µmol/L)	7.19	7.30	7.37	0.18	0.717	0.951
Nitric oxide (µmol/L)	32.41	34.17	34.93	1.19	0.418	0.852
GSH (mg/dL)	20.04	24.58	27.36	1.09	0.004	0.640
SOD (U/mL)	49.14	51.65	54.72	2.09	0.308	0.953
CAT (nmol/min/mL)	1.20	1.21	1.22	0.02	0.691	0.914
GPx (nmol/min/mL)	267.41	289.03	291.59	9.42	0.322	0.648
Ceruloplasmin (mg/dL)	19.51	19.53	19.48	0.54	0.985	0.977
Albumin (g/dL)	2.74	2.72	2.71	0.03	0.721	0.990
Total protein (g/dL)	5.89	5.89	5.87	0.09	0.949	0.971
Globulin (g/dL)	3.14	3.16	3.16	0.10	0.967	0.976

[1] Data represent mean values of 6 replicates per treatment,
[2] Groups; C: Control without supplementation drinking water, E1: 0.4 mL essential oil mixture supplementation in drinking water and E2: 0.8 mL essential oil mixture supplementation in drinking water,
[3] Polynomial contrasts: L=linear and Q=quadratic effect of supplemental essential oil mixture.

Table 5. Effect of essential oil mixture on cecal short-chain fatty acid concentrations at the end of the experiment (µmol/g).

	Groups					
SCFA Parameters	**C**	**E1**	**E2**	**SEM**	**Signifiance**	
	x̄	**x̄**	**x̄**		**L**	**Q**
Acetic acid	50.09	58.68	72.06	3.70	0.012	0.732
Propionic acid	21.88	20.62	26.30	1.32	0.167	0.219
Isobutyric acid	0.86	1.01	1.07	0.07	0.258	0.774
Butyric acid	9.46	15.97	17.30	1.35	0.013	0.317
Isovaleric acid	1.07	1.17	1.27	0.08	0.355	0.996
Valeric acid	1.47	1.49	1.74	0.09	0.270	0.602
Isocaproic acid	0.04	0.10	0.09	0.00	0.011	0.042
Caproic acid	0.08	0.08	0.08	0.00	0.317	0.946
BCFA	3.41	3.68	4.09	0.23	0.265	0.898
Total SCFA	84.86	98.96	119.76	5.66	0.008	0.750

[1] Data represent mean values of 6 replicates per treatment,
[2] Groups; C: Control without supplementation drinking water, E1: 0.4mL essential oil mixture supplementation in drinking water and E2: 0.8 mL essential oil mixture supplementation in drinking water,
[3] Polynomial contrasts: L=linear and Q=quadratic effect of supplemental essential oil mixture.
[4] BCFA (Branched Chain Fatty Acids): isobutyric acid+isovaleric acid+valeric acid.
[5] Total SCFA (Short Chain Fatty Acids): acetic acid+propionic acid+isobutyric acid + butyric acid+isovaleric acid+valeric acid.

Cecal short-chain fatty acid concentrations: Cecal short-chain fatty acid concentrations (µmol/g) measured at the end of the experiment are given in Table 5. Acetic acid, butyric acid, isocaproic acid and SCFA were linearly affected by the gradued level of essential oil mixture (P=0.012, P=0.013, P=0.011 and P=0.008, respectively). There were no significant differences in propionic acid, isobutyric acid, valeric acid, isovaleric acid, caproic acid and BCFA concentrations.

Discussion and Conclusion

The sources of natural antioxidant compounds are plants. Plants show antioxidant properties with the phenolic compounds they contain (25). Free oxygen radicals damage the organism and this situation is kept under control by antioxidant systems. In pathological conditions, the oxidant and antioxidant balance changes. Major phenolic antioxidants prevent cell death under oxidative stress (31). Phenolic compounds in the plant exhibit antioxidant effects, especially due to their redox properties. Therefore, redox agents act as hydrogen donors, oxygen inhibitors, and metal chelators (36). Oxidative stress and balance between antioxidant capacity organ or organ determines the susceptibility of their systems to oxidative stress. GSH, antioxidant vitamins, antioxidant enzymes cells against oxidative damage plays an important role in protection (33). GSH is used for therapeutic purposes in preserving antioxidant capacity (2). In our study, GSH exhibited a linear response with the increase of the levels of essential oil mixture. Due to the insufficient number of studies using aromatic plants and extracts in goose, in the discussion has also been benefited

from studies in other different breeds and species. Chen et al. (6) showed that flaxseed improved the antioxidant status in the 1-day-old gosling (Huoyan Geese). The use of aromatic plant oil mixture in quail breeders' drinking water has been protective against oxidative stress (12). In a study, the use of thyme oil in Tuj lamb improved blood oxidant-antioxidant balance (13). In another study, Habibi et al. (17) reported that serum total antioxidant capacity was affected the addition of ginger essential oil in broiler. In a study using phytogenic feed additives in broilers, antioxidant capacities increased in liver and jejunum and decreased liver lipid peroxidase level (27). Overall, these results show that essential oil mixture could be considered as strong natural antioxidants in poultry diets.

Short chain fatty acids are formed by bacterial fermentation. Short-chain fatty acids stimulate cell growth and differentiation in the gut, thereby improving intestinal integrity, as well as preventing the growth of pathogenic microorganisms by lowering the digestive system pH (21). Researchers reported that there is a close relationship between the composition of cecum microflora and SCFA concentration (24). Increased SCFA concentrations have been shown to have beneficial effects on energy, metabolism, microflora and immune responses (37). The contribution of the use of barley in goose rations to the metabolizable energy of SCFA formation in secum is 2.7 kJ g^{-1} (18). Weng et al. (40) reported that the value of isocaproic acid decreases in the investigation of the relationship of metabolic and microbiota variables with diet in the case of intestinal inflammation. The decrease in isocaproic acid value shows that pH acidity in the intestine is active and pathogenic microorganisms are active in inflammation. In the light of these studies, the increase in secum short chain fatty acids can be interpreted as having a positive effect on intestinal health. Among SCFAs, butyric acid is a primary energy source for enterocytes. In cellular differentiation and takes part in proliferation in the intestinal mucosa (32). In our study, acetic acid, butyric acid, isocaproic acid and SCFA were linearly affected with the gradued level of by essential oil mixture. There were no significant differences in propionic acid, isobutyric acid, valeric acid, isovaleric acid, caproic acid and BCFA concentrations. The number of studies investigating cecum short-chain fatty acids in intestinal health in poultry is very limited. Moreover, in the literature search, no articles investigating geese or SCFAs were found. In one study, the addition of bilberry to rat diets enriched cecum SCFAs (23). The presence of green tea extract and black tea extract in rat rations stimulates cecum SCFA production (38). In a different study, limonene caused significant changes in short-chain fatty acids in mice (39). On the other hand, use of thyme and black cumin oil in broiler rations did not affect cecum short chain fatty acids (14). The differences in the results of these studies can be explained by the type and dosage of the plant extracts added, the ratio of volatile fatty acids and active ingredients, house conditions and the influence of environmental factors.

In conlusion, the addition of essential oil mixture to drinking water has been found to be effective in protecting geese against oxidative stress and improving gut health according to the blood antioxidant capacity and cecum SCFA results. Further studies are needed to illuminate the investigated parameters.

Acknowledgements

Some part of this article was summarized from the first author's master thesis.

Financial Support

This study was funded by the Kafkas University Coordination of Scientific Research Projects with 2019-TS-12 project number.

Ethical Statement

Ethical approval for this study was obtained from the Kafkas University Animal Experiments Local Ethics Committee (Decision No: KAU-HAYDEK /2018-054/2019-001).

Conflict of interest

The authors declared that there is no conflict of interest.

References

1. **Aarestrup FM, Kruse H, Tast E, et al** (2000): *Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among Enterococcus faecium from broilers and pigs in Denmark, Finland, and Norway.* Microb Drug Resist, **6**, 63-70.
2. **Aksoy Y** (2002): *Antioksidan mekanizmada glutatyonun rolü*. T Klin Tıp Bilimleri, **22**, 442-448.
3. **AOAC** (2000): Official methods of analysis. 1-41. In: 17th Ed. Association of Official Analytical Chemists. AOAC International, Maryland.
4. **Beutler E, Duron O, Kelly BM** (1963): *Improved method for the determination of blood glutathione*. J Lab Clin Med, **61**, 882-888.
5. **Carvalho RN, Moura LS, Rosa PTV, et al** (2005): *Supercritical fluid extraction from rosemary (Rosmarinus officinalis): Kinetic data, extract's global yield, composition and antioxidant activity.* J Supercrit Fluids, **35**, 197-204.
6. **Chen W, Jiang YY, Wang JP, et al** (2015): *Effect of flaxseed on the fatty acid profile of egg yolk and antioxidant status of their neonatal offspring in Huoyan Geese*. Animal, **9**, 1749-1755.
7. **Cimrin T, Tunca RI, Avsaroglu MD, et al** (2020): *Effects of an antibiotic and two phytogenic substances (cinnamaldehyde and 1,8-cineole) on yolk fatty acid profile and storage period-associated egg lipid peroxidation level*. R Bras Zootec, **49**, e20190270.
8. **Colombo JP, Richterich R** (1964): *Zur bestimmung des caeruloplasmin im plasma [on the determination of*

ceruloplasmin in plasma]. Schweiz Med Wochenschr, **23**, 715-720.

9. **D'antuono LF, Gallet, GC, Bocchini P** (2000): *Variability of essential oil content and composition of origanum vulgare I. populations from a north mediterranean area (Liguria Region, Northern Italy)*. Annals of Botany, **86**, 471– 478.
10. **Demir P, Aksu Elmalı D** (2012): *Economical analysis of goose breeding commercially by small family farms*. World's Poultry Science Journal, **68**, 5-10.
11. **Doumas BT, Watson WA, Biggs HG** (1971): *Albumin standards and the measurement serum albumin with bromcresol green*. Clinica Chimica Acta, **31**, 87-96.
12. **Durna Aydın O** (2020): *Effect of drinking water supplementation of different aromatic plant essential oils on performance and some blood parameters in quail breeders (Coturnix coturnix japonica).* Eurasian J Vet Sci, **36**, 16-21.
13. **Durna Aydın O, Merhan O, Yıldız G, et al** (2020): *The effect of oregano oil (Origanum vulgare) on the fattening performance and blood oxidantantioxidant balance in post-weaned Tuj lambs*. Kafkas Univ Vet Fak Derg, **26**, 59-64.
14. **Durna Aydın O, Yıldız G** (2020): *The effects of thyme oil and black cumin oil in broiler feeding on growth performance, intestinal histomorphology, and cecal volatile fatty acids*. Turk J Vet Anim Sci, **44**, 17-25.
15. **Durna Aydin O, Yıldız G, Merhan O** (2021): *Effects of probiotic (Lactobacillus farciminis) supplementation in quail (Coturnix coturnix japonica) rations on growth performance, blood antioxidant capacity and cecal some short-chain fatty acid concentrations.* Kafkas Univ Vet Fak Derg, **27**, 15-20.
16. **Gauquelin T, Bertaudiere-Montes A, Badri W, et al** (2002): *Sex ratio and sexual dimorphism in mountain dioecious thuriferous juniper (Juniperus thurifera L., Cupressaceae*). Bot J Linn Soc, **138**, 237-244.
17. **Habibi R, Sadeghi GH, Karimi A** (2014): *Effect of different concentrations of ginger root powder and its essential oil on growth performance, serum metabolites andantioxidant status in broiler chicks under heat stress*. Br Poult Sci, **55**, 228-237.
18. **Jamroz D, Jakobsen K, Knudsen KEB, et al** (2002): *Digestibility and energy value of non-starch polysaccharides in young chickens, ducks and geese, fed diets containing high amounts of barley*. Comp Biochem Phys, **131**, 657-668.
19. **Jamroz D, Kamel C** (2002): *Plant extacts enhance broiler performance. In non ruminant nutrition: Antimicrobial agents and plant extracts on immunity, health and performance*. J Anim Sci, **80**, 41.
20. **Kahraman R, Alp M, Kocabağlı N, et al** (1996): *The effects of fastrack and sodium bicarbonate on performance of broilers.* Turk J Vet Anim Sci, **20**, 383 - 386.
21. **Knudsen KEB, Hedemann MS, Lærke HN** (2012): *The role of carbohydrates in intestinal health of pigs*. Anim Feed Sci Technol, **173**, 41–53.
22. **Koç H** (2002): Lokman Hekimden Günümüze Bitkilerle Sağlıklı Yaşama. T.C. Kültür Bakanlığı Yayınları, Ankara.
23. **Liu HY, Walden TB, Cai D, et al** (2019): *Dietary fiber in bilberry ameliorates pre-obesity events in rats by regulating lipid depot, cecal short-chain fatty acid formation and microbiota composition*. Nutrients, **11**, 1350.
24. **Meimandipour A, Shuhaim M, Soleiman AF, et al** (2010): *Selected microbial roups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of Lactobacillus*. Poult Sci, **89**, 470-476.
25. **Merken HM, Merken CD, Beecher G** (2001): *Kinetics method for the quantitation of anthocyanins, flavonal and flavones in food*. J Agric Food Chem. **49**, 2727-2732.
26. **Miranda KM, Espey MG, Wink DA** (2001): *A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite*. Nitric Oxide, **5,** 62-71.
27. **Muller K, Blum NM, Kluge H, et al** (2012): *Influence of broccoli extract and various essential oils on performance and expression of xenobiotic- and antioxidant enzymes in broiler chickens*. Brit J Nutr, **108**, 588–602.
28. **NRC** (1994): Nutrient Requirements of Poultry. 34 – 45. In: 9th rev. ed. National Academy Press, Washington DC.
29. **Olivier GW** (1976): The world market of oregano. 142–146. In: Padulosi S, (ed.) Oregano, 14. Proceedings of the IPGRI International Workshop. Rome, Italy.
30. **Öztürk B, Konyalıoğlu S, Baykan LS** (2002): Türkiye'de doğal yayılış gösteren bazı Thymus L. taksonlarının uçucu yağlarının karşılaştırmalı antioksidan etkileri. 387-388. 14. Bitkisel İlaç Hammaddeleri Toplantısı, Eskişehir.
31. **Parihar MS, Hemnani T** (2003): *Phenolic antioxidants attenuate hippocampal neuronal cell damage against kainic acid ınduced excitotoxicity*. J Biosci, **28**, 121-128.
32. **Rinttilla T, Apajalahti J** (2013): *Intestinal microbiota and metabolites Implications for broiler chicken health and performance*. J Appl Poult Res, **22**, 647–658.
33. **Saracila M, Panaite T, Tabuc C, et al** (2020): *Dietary ascorbic acid and chromium supplementation for broilers reared under thermoneutral conditions vs. high heat stress*. Scientific Papers-Animal Science Series: Lucrări Ştiinţifice - Seria Zootehnie, **73**, 41-47.
34. **Sevim B, Gümüş E, Harman H, et al** (2020): *Effects of Dietary Rosemary Essential Oil on Growth Performance, Carcass Traits and Some Hematological Values of Chukar Partridge*. TURJAF, **8**, 430-435.
35. **SPSS** (2011): Statistical Packages for the Social Sciences, 20th ed. IBM Inc, Chicago, USA.
36. **Summanen J, Vuorela P, Rauha JP, et al** (2001): *Effect of simple aromatic compounds and flavonoids on Ca+2 fluxes in rat pituitary GH4C1 Cells*. Eur J Pharmacol, **414**, 125-133.
37. **Tan L, Yuan D, Chen YB** (2012): *Differ Bacillus probiotics affect growth perform ammon emission excreta broilers.* Chinnese J Anim Nutr, **24**, 877-885.
38. **Unno T, Osakabe N** (2018): *Green tea extract and black tea extract differentially influence cecal levels of short chain fatty acids in rats*. Food Sci Nutr, **6**, 728–735.
39. **Wang L, Zhang Y, Fan G, et al** (2019): *Effects of orange essential oil on ıntestinal microflora in mice*. J Sci Food Agric, **99**, 4019-4028.
40. **Weng YJ, Gan HY, Li X, et al** (2019): *Correlation of diet, mic-robiota and metabolite networks in ınflammatory bowel disease*. J Dig Dis, **20**, 447–459.
41. **Wiseman M** (2012): Evaluation of Tasco® as a candidate prebiotic in broiler chickens. Master of Science Thesis. 39-43. Dalhousie University, Nova Scotia.
42. **Yoshioka T, Kawada K, Shimada T, et al** (1979): *Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood*. Am J Obstet Gynecol, **135**, 372-376.
43. **Zhang WF, Li DF, Lu WQ, et al** (2003): *Effects of isomalto-oligosaccharides on broiler performance and intestinal microflora*. Poult Sci, **82**, 657-663.

Ankara Univ Vet Fak Derg, **69**, 319-328, 2022
DOI: 10.33988/auvfd.851447

Investigation of the effects of Pine and Chestnut Honey on wound healing

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Received date: 04.01.2021 - Accepted date: 12.07.2021

Abstract: This study aimed to investigate the effect of creams containing pine and chestnut honey on wound healing in rats. The animals were divided into the following four groups: a control group (A), a group treated with only basic cream ingredients (B), a group treated with pine honey cream (C), and a group treated with chestnut honey cream (D). Full-thickness skin wounds were created on the back of each rat (14 per group) with a 10-mm diameter punch instrument. In each group, skin samples were taken from seven rats on day 7 and from the remaining seven rats on day 14. The effects of the creams on wound healing were assessed by histopathological examination and hydroxyproline assays. The histopathological examination showed that chronic inflammation significantly decreased in group D compared to the control group ($P<0.05$). Furthermore, the levels of granulation tissue and granulation tissue/fibroblast maturation in skin samples taken on day 14 were also significantly lower in group D compared to the control group ($P<0.05$). It was observed that the neovascularization values of skin samples taken on day 7 were lower in group D than in the control group ($P<0.05$). Also, the difference in hydroxyproline levels between groups B and D was statistically significant ($P<0.05$). The results showed that the cream containing chestnut honey had a positive effect on wound healing.
Keywords: Apitherapy, chestnut honey, pine honey, rat, wound healing.

Çam ve Kestane Balı'nın yara iyileşmesi üzerine etkilerinin araştırılması

Özet: Bu çalışmada, ülkemize özgü çam balı ve kestane balı içeren krem formülasyonlarının yaralar üzerine iyileştirici etkilerinin inclenmesi amaçlanmıştır. Çalışmada, herhangi bir uygulama yapılmayan kontrol grubu (A), sadece krem taşıt maddesi uygulanan grup (B), Çam Balı kremi uygulanan grup (C) ve Kestane balı kremi uygulanan grup (D) olmak üzere 4 farklı denek grubu oluşturuldu. Her grupta (n=14) yer alan sıçanların sırt kısmına 10 mm çapında punch aparatı ile tam kat yara oluşturuldu. Farklı gruplarda yer alan sıçanların yarısının deri örnekleri yara oluşumunu takip eden ilk 7. günde alınırken geri kalanların deri örnekleri 14. günde alındı. Alınan bu deri örneklerine histopatoloji incelemesi ve hidroksiprolin analizi yapıldı. Histopatolojik değerlendirme sonucuna göre, kronik yangının, kestane balı kremi uygulanan grupta kontrol grubuna göre önemli derecede azaldığı tespit edildi ($P<0,05$). Buna ek olarak, granülasyon doku ve granülasyon dokusu/fibroblast olgunlaşmasının da kestane balı kremi uygulanan ve 14. gün alınan deri örneklerinde, kontrol grubuna göre önemli derecede azaldığı belirlendi ($P<0,05$). Kestane balı kremi uygulanan grubun 7. günde alınan deri örnekleri neovaskülarizasyon değerlerinin, kontrol grubu neovaskülarizasyon değerlerine göre daha düşük değerlerde olduğu gözlendi ($P<0,05$). Ayrıca, hidroksiprolin düzeyleri çalışma grupları yönüyle değerlendirildiğinde B ve D grupları arasındaki farklılık istatistiksel açıdan önemli bulundu ($P<0,05$). Sonuçlar bütün halinde ele alındığında, kestane balı içeren kremin yara iyileşmesi üzerine katkısının olumlu yönde olduğu belirlendi.
Anahtar sözcükler: Apiterapi, çam balı, kestane balı, sıçan, yara iyileşmesi.

Introduction

Honey contains sugar, enzymes, flavonoids, minerals, and other nutrients that have exert antioxidant, antibacterial, and anti-inflammatory effects and has been used as a wound dressing to support quick and improved recovery (2, 43). Honey effectively treats foot and mouth wounds in cattle and horses and chemical eye injuries in rabbits (47). In addition to the epithelium, myofibroblasts, collagen, and angiogenesis play important roles in wound healing (1, 12). Fibroblasts play a crucial role in wound healing; the division and migration of epidermal cells into the surrounding wound area is of secondary importance (22). Collagen is the main structural component in the extracellular matrix and is vital in ensuring all tissues' integrity and wound healing (9, 12). Angiogenesis refers to forming new blood vessels from existing blood vessels

and is a necessary component of the healing process due to the increased nutrient requirement (10). Activated platelets, neutrophils, and macrophages play a significant role in wound healing (16). Wound care gels, creams, and dressing materials containing honey that are approved by the U.S. Food and Drug Administration (FDA) are being used in wound healing. Products containing Manuka honey are especially recommended to treat minor wounds, cuts, burns, diabetic foot ulcers, leg ulcers, pressure ulcers (bed sores), partial- and full-thickness wounds, first- and second-degree partial burns, and traumatic and surgical wounds (37).

Honey is important for health since it possesses antimicrobial properties and contains antioxidants varyingly depending on its botanical source. Its antimicrobial effect results from its high osmotic pressure and low pH, as well as the presence of compounds, such as hydrogen peroxide, flavonoids, and phenolic compounds (caffeic and ferulic acids) (23, 25, 46). The antimicrobial properties of honey are determined by the diversity and concentrations of these components that depends on various factors, such as its floral origin and nectar combination (flower type, single/many), source (flower/honeydew), climatic characteristics (dry/humid), and color (light/dark) (3, 24, 44). Besides its antibacterial activity, honey has antifungal and antiviral effects (11, 25, 31). Chestnut honey (CH), important flower honey, is obtained from trees belonging to the Castanea genus of the Fagaceae family. CH is distinguished from the other types of honey by its taste, aroma, and color (21). A study in Spain established that the taste and aroma of CH collected from different regions differed (7). Another study found that the number of essential elements in dark-colored honey, such as CH, was higher than in light-colored honey (6). The pine scale *Marchalina hellenica*, which plays an important role in the production of pine honey (PH), is found in Turkey on the following pine species: *Pinus brutia* (Turkish red pine), *P. halepensis* (Aleppo pine), *P. silvestris* (Scotch Pine), and *P. pinea* (Stone pine) (48).

Studies on treating wound bacteria with specific regional honey indicate that the antimicrobial activity of honey depends on the dose (11, 25). Another scientifically promising finding is that honey inhibits the growth of antibiotic-resistant bacteria and also prevents the development of resistance to antibiotics (25). The antibacterial and healing effects of honey can lead to less painful, quicker, natural, and cost-effective treatment of wounds. This study examines the effects of creams containing honey from Düzce and Muğla provinces on wound healing in rats.

Materials and Methods

***Animal materials*:** Fifty-six healthy male Wistar albino rats (250–350 g) aged 6–8 weeks were used in the study. Ethical consent was obtained from the Animal Experiments Local Ethics Committee of Kırıkkale University on 31/03/2016 (Meeting Number: 16/03, Meeting Decision: 16/44). Food (standard rat feed) and water were provided ad libitum for the duration of the experiment. The animals were kept in rooms with a 12-hour light/dark cycle at a suitable humidity and temperature.

***Obtaining and analyzing honey samples*:** The CH and the PH were collected from the Beekeepers Association in Düzce and Muğla Province for this study.

The properties and antibiotic residue (sulfacetamide, sulfadiazine, sulfamethoxazole, sulfamerazine, sulfisoxazole, sulfamethizole, sulfabenzamide, sulfamethazine, sulfachloropyridazine, sulfadimethoxine, sulfathiazole, sulfameter, sulfamethoxypyridazine, sulfadoxine, methacycline, epitetracycline, doxycycline, tetracycline, oxytetracycline, epioxytetracycline, chlortetracycline, and chloramphenicol) analysis were performed at the Muğla Sıtkı Koçman University Food Analysis Application and Research Center.

DIN 10760 was used in pollen analysis, while the in-house method was used to measure the moisture, conductivity, pH, free acidity, (hydroxymethiylphurfural (HMF), sugar profile, concentrations of proline, diastase, and naphthalene, and antibiotic residues; the TS 13262 method was used in protein and raw honey analysis.

***Sterilization of honey*:** The honey samples were sterilized using Cobalt-60 (^{60}Co) gamma radiation at a dose of 5 kGy.

Preparation of cream formulation: The process of the sterilized honey into a pharmaceutical product in cream form was carried out by Nehar Chemicals, Istanbul, Turkey. Glycerin, methylparaben, sodium benzoate, liquid paraffin, and cetearyl alcohol were used as auxiliary substances in the preparation of the cream formulations; honey was added to 25%.

***Formation of experimental groups and the experimental protocol*:** The model defined by Park et al. (34) was used as an experimental wound model. After the rats were anesthetized with diethyl ether, the prospective wound area was shaved and cleaned with povidone-iodine (betadine). Using the punch biopsy instrument, two 10-mm diameter full-thickness excision wounds, each approximately 1 cm away from the midline, were created on the dorsal thoracic region in all rats. At the same time, the animals used in the study were divided into four groups, with 14 in each group. Group A (control) did not receive any treatment to the wound area. Plain (common) cream (group B) was treated with a cream containing basic ingredients. Group C was treated with a cream containing PH. Group D was treated with a cream containing CH. The animals were kept in individual cages throughout the test period, and the wounds were fully covered with the appropriate creams. Two subgroups of seven animals were

formed in each group. At the end of the first seven days, the animals in one subgroup from each group were sacrificed, and skin samples were taken for histopathological evaluation. The animals in the second subgroup of each group continued to be treated with the creams for a total of 14 days. All animals were sacrificed with an overdose of general anesthetics at the end of day 14, and skin samples were collected from the wound area. Depending on the nature of the tests, the samples were fixed in buffered formalin or kept at -80ºC until analyzed.

***Morphometric parameters*:** The wounds on the rats were checked daily from the beginning of the study until the practical applications were completed. The skin samples collected from the animals sacrificed on days 7th and 14th of the study were placed in cryotubes and stored in freezers with dry ice. All clinical findings in rats were also noted during the experimental studies. The Greenhalgh method was modified for histopathological evaluation (35).

***Calculation of the wound area*:** To determine the healing process, the rats were anesthetized, and the size of the wounds was drawn on acetate paper with a permanent marker with a diameter tip of 0.3 mm; this application was repeated at intervals of two days. The drawings were transferred to a computer and evaluated using the SketchAndCalc program. The image was scaled and measured using the ruler feature, and the size was recorded in mm/cm.

***Hydroxyproline analysis*:** A Hydroxyproline Assay Kit (MAK008) from Sigma was used to measure hydroxyproline levels and assess the collagen level in tissue samples taken from the wound areas.

***Statistical analysis*:** All variables were examined using the Shapiro–Wilk test of normality, followed by the Levene test for the homogeneity of the variances, before performing significance tests. Descriptive statistics were calculated and shown as "Arithmetic mean ± Standard error of mean (SEM)" or "Median (Minimum-Maximum)" where necessary. The effects of the group (A, B, C, D), time (Day 7 and Day 14), and their interactions on wound measurement area were analyzed using the MIXED procedure. The animals in the groups were included in the model as random effects, and the group, time, and their interactions were included as fixed effects. The post-hoc Bonferroni test was used for multiple comparisons. Histopathological score changes between 7-day and 14-day were analyzed using the Wilcoxon signed-rank test. The Kruskal–Wallis test was performed to determine the differences in scores between the groups on each day. Dunn-Bonferroni test was used as a post-hoc test for parameters that were found to be significant. In the hydroxyproline analysis, statistical control of the differences between variables was measured using the analysis of variance (one-way ANOVA). The Duncan test was used as a post-hoc test for variables that showed significant differences between groups. $P<0.05$ was the criterion used for all statistical analyses. All analyses were performed using the SPSS (V22.0) software package.

Results

***Honey analysis*:** The PH and CH analyses did not find antibiotic and naphthalene residues. The content analysis results were confirmed to be within the limits specified in the Communiqué on Honey; the origin of the honey was also confirmed. Pollen analysis results for CH and PH are presented in Table 1. The CH and PH contents are presented in Table 2.

Table 1. Pollen analysis results of chestnut and pine honey.

Analysis performed	Results		Measurement Limit (LOQ)	Analysis Method
	Chestnut	**Pine**		
Isolated Dominant Pollen (>15%)	*Castanea sativa* Miller (Fagaceae)	*Pinus brutia* (Pinaceae)	50%	DIN 10760
	-	Astragalus subsp. (Fabaceae)	18%	DIN 10760
Isolated Significant Pollen (>1%)	*Helianthus annuus* L. (Asteraceae)	-	10%	DIN 10760
	-	*Erica manipuliflora* Salisb. Ericaceae)	12%	DIN 10760
	Astragalus odoratus L. (Asteraceae)	Asteraceae	5%	DIN 10760
Isolated Pollen (<1%)	Apiaceae	Apiaceae	1%	DIN 10760
	-	Asteraceae	1%	DIN 10760
	Poaceae	Poaceae	1%	DIN 10760

Table 2. Content analysis results of chestnut and pine honey.

Analysis performed	Result		Measurement Unit	Analysis Method
	Chestnut	Pine		
Moisture	20.28	17.96	%	IHC
Conductivity	1.72	1.24	mS/cm	IHC
Proline	855.08	801.9	mg/kg	IHC
pH	4.78	4.61	pH	IHC
Free Acidity	19.82	21.85	mmol/kg	IHC
Diastase	9.80	19.97		IHC
HMF	24.05	1.09	mg/kg	IHC
Fructose+Glucose	59.87	62.44	g/100g	IHC
Fructose/Glucose	1.62	1.19		IHC
Saccharose	N.D.	N.D.	g/100g	IHC
Naphthalene	N.D	N.D	mg/kg	In-House Method
Protein (Delta 13C)	-26.40	-26.34		TS 13262
Raw Honey (Delta 13C)	-26.56	-27.19		TS 13262
Difference in protein and raw honey delta C13 values in honey	0.17	0.85		TS 13262
C4 Sugar Ratio calculated from Delta C13 Value	N.D	N.D	%	TS 13262

N.D. None detected.

***Histopathological examinations*:** On day zero, 14 tissue samples taken from groups A, B, C, and D were examined. It was observed that the epidermis was intact, and the blood vessels were hyperemic in all tissue samples. The histopathological examinations of all groups on days 7 and 14 are presented in Figures 1, 2, and 3, and the evaluation scoring is presented in Table 3. The change in acute inflammation over time between days 7 and 14 was statistically significant ($P<0.05$), although the differences in acute inflammation between the groups on days 7 and 14 were not statistically significant ($P>0.05$). Similarly, the change in chronic inflammation over time between days 7 and 14 was statistically significant ($P<0.05$), although the differences in chronic inflammation between the groups on day 7 were not statistically significant ($P>0.05$); however, the differences between the groups on day 14 were statistically significant ($P<0.05$). Group D was similar to groups B ($P>0.05$) and C ($P>0.05$), but it was statistically different from group A ($P<0.05$). Chronic inflammation decreased significantly in the group treated with CH-containing cream (group D) compared to the control group ($P<0.05$).

The change in the amount of granulation tissue over time between days 7 and 14 was statistically significant ($P<0.05$). Although the differences in the amount of granulation tissue between the groups on day 7 were not statistically significant ($P>0.05$), the differences between the groups on day 14 were found to be statistically significant ($P<0.05$). Group D was similar to groups B ($P>0.05$) and C ($P>0.05$), but it was statistically different from the control group ($P<0.05$). The change in granulation tissue/fibroblast maturation over time between days 7 and 14 was statistically significant ($P<0.05$). Although the differences in granulation tissue/fibroblast maturation between the groups on day 7 were not statistically significant ($P>0.05$), the differences between the groups on day 14 were found to be statistically significant ($P<0.05$). Group D was similar to groups B ($P>0.05$) and C ($P>0.05$), but it was statistically different from the control group ($P<0.05$).

The change in the amount of collagen over time between days 7 and 14 was statistically significant ($P<0.05$), although the differences in the amount of collagen between the groups on days 7 and 14 were not statistically significant ($P>0.05$). The change in epithelialization over time between days 7 and 14 was statistically significant ($P<0.05$), although the differences in epithelialization between the groups on days 7 and 14 were not ($P>0.05$). The change in neovascularization over time between days 7 and 14 was statistically significant ($P<0.05$). Although the differences in neovascularization between the groups on day 14 were not statistically significant ($P>0.05$), the differences between the groups on day seven were found to be statistically significant ($P<0.05$). Group D was similar to groups B ($P>0.05$) and C ($P>0.05$), but it was statistically different from the control group ($P<0.05$).

Table 3. Histopathological examination in all groups.

Groups		A		B		C		D		P
Variable	**Time**	**Mean ± SEM**	**Median (Min - Max)**	**Mean ± SEM**	**Median (Min - Max)**	**Mean ± SEM**	**Median (Min - Max)**	**Mean ± SEM**	**Median (Min - Max)**	
Acute Inflammation	Day 7	0.57 ± 0.2	1 (0 - 1)	0.29 ± 0.18	0 (0 - 1)	0.43 ± 0.2	0 (0 - 1)	0.29 ± 0.18	0 (0 - 1)	0.662
	Day 14	0 ± 0	0 (0 - 0)	0 ± 0	0 (0 - 0)	0 ± 0	0 (0 - 0)	0 ± 0	0 (0 - 0)	1
	P	0.046		0.157		0.083		0.157		
Chronic Inflammation	Day 7	2.57 ± 0.2	3 (2 - 3)	2 ± 0	2 (2 - 2)	2.14 ± 0.26	2 (1 - 3)	2 ± 0.22	2 (1 - 3)	0.144
	Day 14	1.14 ± 0.26	1 (0 - 2)^{a}	0.29 ± 0.18	0 (0 - 1)^{ab}	1.14 ± 0.51	1 (0 - 3)^{ab}	0 ± 0	0 (0 - 0)^{b}	0.012
	P	0.023		0.014		0.038		0.014		
Granulation Tissue	Day 7	2.57 ± 0.3	3 (1 - 3)	2.57 ± 0.2	3 (2 - 3)	2.86 ± 0.14	3 (2 - 3)	2.43 ± 0.3	3 (1 - 3)	0.636
	Day 14	1.29 ± 0.36	1 (0 - 3)^{a}	0.71 ± 0.47	0 (0 - 3)^{ab}	0.71 ± 0.42	0 (0 - 3)^{ab}	0 ± 0	0 (0 - 0)^{b}	0.032
	P	0.024		0.026		0.024		0.016		
Granulation Tissue/ Fibroblast Maturation	Day 7	2 ± 0.31	2 (1 - 3)	2.14 ± 0.14	2 (2 - 3)	2.29 ± 0.29	2 (1 - 3)	2.29 ± 0.29	2 (1 - 3)	0.824
	Day 14	1.43 ± 0.37	1 (0 - 3)^{a}	0.71 ± 0.47	0 (0 - 3)^{ab}	0.57 ± 0.43	0 (0 - 3)^{ab}	0 ± 0	0 (0 - 0)^{b}	0.024
	P	0.157		0.04		0.04		0.016		
Collagen	Day 7	0.86 ± 0.34	1 (0 - 2)	1.29 ± 0.18	1 (1 - 2)	1 ± 0.31	1 (0 - 2)	1.29 ± 0.42	1 (0 - 3)	0.744
	Day 14	2.71 ± 0.29	3 (1 - 3)	2.57 ± 0.3	3 (1 - 3)	2.43 ± 0.43	3 (0 - 3)	3 ± 0	3 (3 - 3)	0.476
	P	0.026		0.034		0.047		0.026		
Reepithelization	Day 7	1 ± 0	1 (1 - 1)	1.71 ± 0.36	1 (1 - 3)	1.86 ± 0.4	1 (1 - 3)	2 ± 0.38	2 (1 - 3)	0.158
	Day 14	2.71 ± 0.29	3 (1 - 3)	3 ± 0	3 (3 - 3)	3 ± 0	3 (3 - 3)	3 ± 0	3 (3 - 3)	0.392
	P	0.014		0.034		0.046		0.059		
Neovascularization	Day 7	3 ± 0	3 (3 - 3)^{a}	2.86 ± 0.14	3 (2 - 3)^{a}	2 ± 0.31	2 (1 - 3)^{b}	1.86 ± 0.34	2 (1 - 3)^{b}	0.007
	Day 14	0.71 ± 0.29	1 (0 - 2)	0.43 ± 0.3	0 (0 - 2)	0.71 ± 0.47	0 (0 - 3)	0 ± 0	0 (0 - 0)	0.208
		0.016		0.014		0.071		0.017		

a,b indicates the differences between groups in the same row at $P<0.05$.

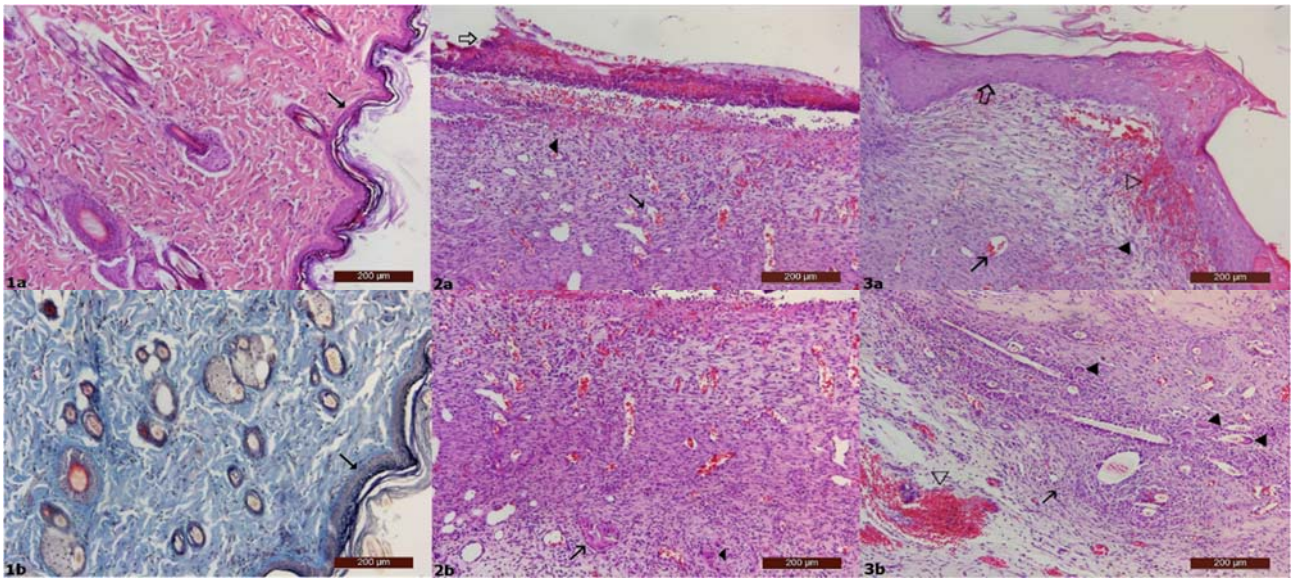

Figure 1. 1a,b: Histopathological examination of rat tissues on day zero. Intact epidermis (arrows) and dermis tissue. a: HxE b: Masson Trichrome (day 0). **2a,b:** Group A Histopathological examination on day 7. Ulcer in the epidermis (White arrow), mononuclear inflammatory cells (black arrowhead) and newly formed blood vessels, neovascularization (Black arrow). b: Foreign body giant cells (Black arrowhead) and amorphous substance with grayish material in the middle (Black arrow), HxE. **3a,b:** Group B Histopathological examination of the wound area on day 7. a: Reepithelialization in the epidermis (White arrow), mononuclear inflammatory cells (black arrowhead), bleeding site (White arrowhead) and newly formed blood vessels, neovascularization (Black arrow). b: Foreign body giant cells in free form and around the hair follicles (Black arrowheads), bleeding site (White arrowhead) and mononuclear inflammatory cells (Black arrow), HxE.

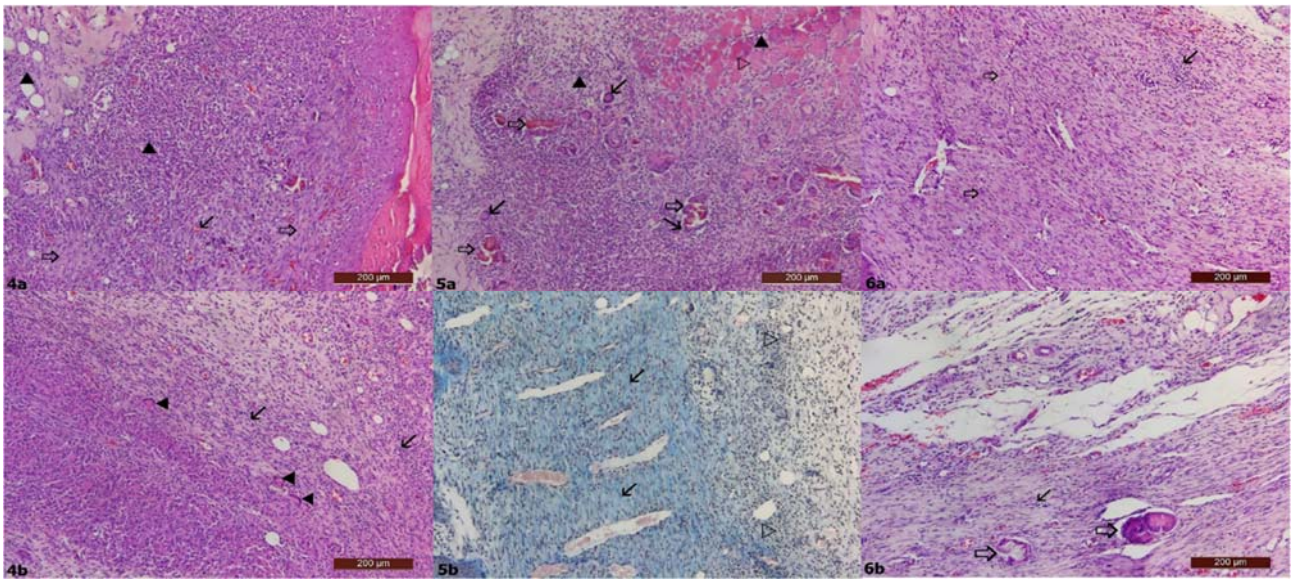

Figure 2. 4a,b: Group C Histopathological examination of the wound area on day 7. a: Mononuclear inflammatory cells infiltrated up to the muscles in the dermis (black arrowheads), granulation areas (White arrows) and newly formed blood vessels, neovascularization (Black arrow). b: Free foreign body giant cells (Black arrowheads) and diffuse mononuclear inflammatory cells (Black arrow), HxE. **5a,b:** Group D Histopathological examination of the wound areas on day 7. a: Mononuclear inflammatory cells infiltrated up to the muscles in the dermis (black arrowheads), foreign body giant cells in free form or around a pinkish amorphous structure (White arrows) (Black arrows), HxE. b: Increase in granulation tissue and collagen amount (Black arrows) and diffuse mononuclear inflammatory cells (White arrowheads), Masson Trichrome (x200). **6a,b:** Group A Histopathological examination of the wound area on day 14. a: Ongoing mononuclear inflammatory cells (black arrows) in the dermis and the increase in the amount of granulation tissue and fibroblasts (White arrows). b: pinkish/grayish amorphous structures (White arrows) and diffuse mononuclear inflammatory cells (black arrows), HxE.

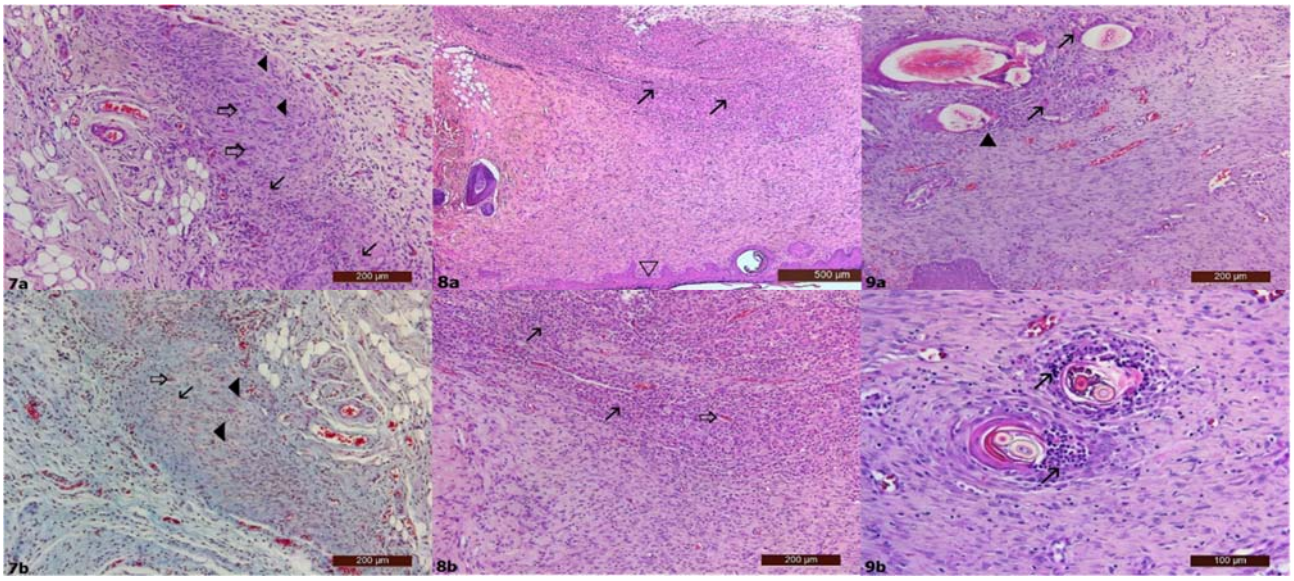

Figure 3. 7a,b: Group B Histopathological examination of the wound area on day 14. a-b: Initiation of reparation in the muscles and the placement of connective tissue cells between the muscles (black arrowheads), multinuclear cell formations (regenerative muscle cells) (White arrows) and the separation of muscle bundles and loss of myofibril lines (Black arrows), a: HxE. b: Masson Trichrome. **8a,b:** Group C Histopathological examination of the wound area on day 14. a: Reepithelialization shaped in the epidermis (white arrowhead) and mononuclear inflammatory cells infiltrated up to the muscles in the dermis (black arrows). b: Newly formed blood vessels, neovascularization (White arrows) and diffuse mononuclear inflammatory cells (Black arrows), HxE. **9a,b:** Group D Histopathological examination of the wound area on day 14. a: Mononuclear inflammatory cells (black arrowhead) and foreign body giant cells (black arrows) formed around the hair follicles in the dermis. b: Mononuclear inflammatory cells formed around the hair follicles in the dermis (Black arrows), HxE.

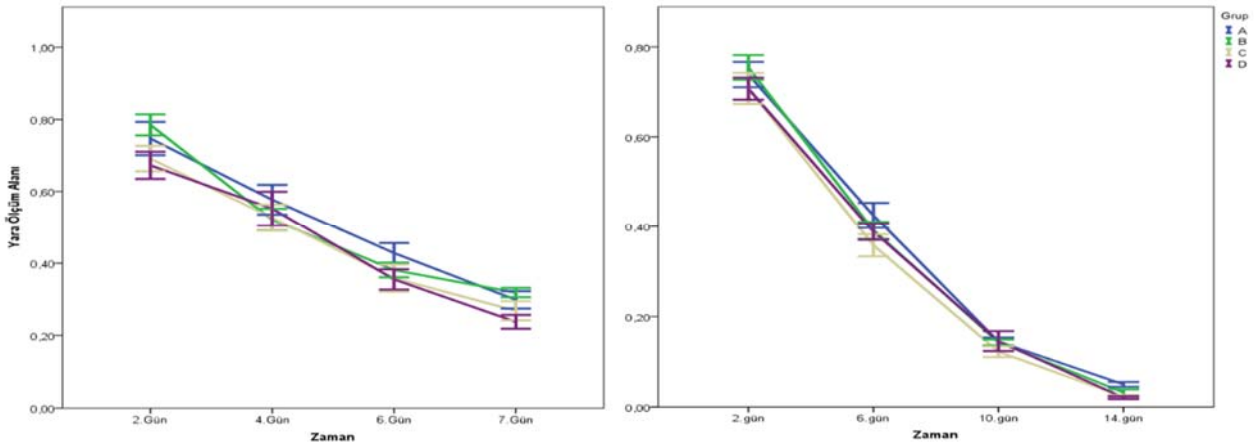

Figure 4. Display of the first 7-day and 14-day wound measurement areas of the experimental groups.

Table 4. Hydroxyproline levels in the experimental groups (µg/µl).

Number	Intact skin	Group A	Group B	Group C	Group D
Mean±SD	103.86±5.13^{d}	59.34±4.52^{c}	42.14±2.41^{b}	39.53±4.16^{b}	24.63±2.61^{a}

The values refer to mean ± standard deviation. (a,b,c,d) indicates the differences between groups in the same row, P<0.05

***Follow-up of wound areas in rats*:** After day 7, the incision area was covered with scabs in the control animals, and the epithelialization was insufficient. Skin repair was better in all rats treated with the creams, and it was difficult to distinguish the wound area from normal skin. Concerning the wound area, an observable difference was detected between the groups treated with creams for 14 days and the control group.

***Analysis of the wound areas in rats*:** The changes in the wound areas in rats on different days are presented in Figure 4. No significant differences were found between the groups regarding wound measurement areas

(P=0.424). The measured wound areas similarly decreased with time in all groups (P<0.001). There were differences between the groups for each time. Although the least mean wound measurement on day 7 was in group D, this difference was insignificant (P<0.005).

***Determination of hydroxyproline*:** Significant differences (P<0.005) were observed in hydroxyproline levels between the groups (Table 4). When hydroxyproline levels were evaluated on day 14, the highest level was found in intact skin, followed by group A (no cream), group B (treated with basic-ingredients cream), group C (treated with PH cream), and group D (treated with CH cream). The difference between groups B and D was found to be statistically significant (P<0.005).

Discussion and Conclusion

In the experimental burn model study conducted by Zohni et al. (26), the inflammatory response decreased significantly on day 7 in the group treated with hydrogel dressing preparation containing honey. In the current study, it was determined that inflammation decreased significantly in all groups on day 7. The results revealed that CH has an anti-inflammatory effect. The antibacterial activity of honey results from its high osmolarity, low pH (3.2-4.5), and the presence of hydrogen peroxide (5, 33, 42). Hydrogen peroxide creates free radicals that mediate the entry of leukocytes into areas of inflammation. It promotes the production of pro-inflammatory cytokines by leukocytes (17). It was reported that Manuka honey significantly increased the production of pro-inflammatory cytokines (45).

A previous study indicated that the epithelialization of wounds treated with Manuka honey and Indonesian honey (18) was higher than that of untreated wounds in control groups. Zohni et al. (26) reported that honey-based hydrogel dressing preparation accelerated epithelialization in the experimental burn model in rats. In the full-thickness wound healing study performed in rabbits, three types of honey (chestnut, flower, and rhododendron) were applied to the wound, and epithelialization increased significantly (P < 0.05) on day 7 in the groups treated with honey; the wounds of all trial groups were almost completely epithelialized on day 21 (32). In the current study, no differences were observed in epithelialization between the groups on days 7 and 14. Epithelialization was complete on day 14. Similar results have been reported by Nisbet et al. (32) and Haryanto et al. (18). Based on the day seven results, no differences were observed between the groups regarding epithelialization in the current study, unlike the results reported by Nisbet et al. (32) and Zohni et al. (26).

Several studies have reported that materials containing Acacia honey (19), Malaysian honey (26), Indonesian honey (18), Manuka honey (18, 30), and other types of honey (32, 38) enhance granulation tissue formation, capillary formation, and collagen synthesis in wound areas. In the current study, although the differences in the amounts of granulation tissue and granulation tissue/fibroblast maturation between the groups on day 7 were not statistically significant, the differences between the groups on day 14 were found to be statistically significant. The granulation tissue in the CH group decreased rapidly by day 14 day compared to the other groups. In this study, the differences in the amount of collagen between the groups on days 7 and 14 were not statistically significant. The collagen results of this study are similar to the results of Zohdi et al. (26), Nisbet et al. (32), Mukai et al. (30), and Iftikhar et al. (19). Sugar in honey can be used as an energy source for the synthesis of collagen, which can be demonstrated by fibroblast proliferation and collagen synthesis on day 8 (43). In the current study, the thickness of the wound tissue did not significantly increase; studies by Ghaderi and Afshar (14) and Haryanto et al. (18) also showed the effectiveness of Indonesian and Manuka honey on the formation of collagen fibers was almost the same. Honey is mildly acidic, with a pH between 3.2 and 4.5 (28). Topical acidification of wounds also supports healing (20). The hydrogen peroxide produced by honey stimulates tissue growth. It was demonstrated that hydrogen peroxide stimulates fibroblast growth in cell culture at micromolar and nanomolar concentrations (39). Furthermore, honey is a rich source of carbohydrates. It can provide the environment and energy required for fibroblastic proliferation and maturation and collagen formation, increasing wound shrinkage and consequently increasing the tensile strength of wounds (5, 11). Honey in low concentrations delivers hydrogen peroxide to the wound area in a slow-release manner, which promotes angiogenesis and the growth of fibroblasts (27, 28). The increase in collagen production and fibroblast maturation with the CH-containing cream may be due to the hydrogen peroxide produced by the honey and the energy it provides to the wound area. In this study, the differences between the groups on day seven were found to be statistically significant. Group D was similar to groups B and C but statistically different from group A. It is possible that the lower level of neovascularization in group D on day 7 compared to the other groups and the complete disappearance of vascularization on day 14 were due to the healing effects of CH. The increased healing rate can also be attributed to the lymph-draining osmotic effect of honey and may result from a deeper flow of nutrients from working capillaries (29).

Nisbet et al. (32) evaluated the effects of three different types of honey (chestnut, flower, and rhododendron) on the healing areas of full-thickness wounds in rabbits on days 7 and 14; they could not find significant differences in the wounds during the healing

process. The flower honey was found to be the least effective in this regard. In another study, the effects of Manuka honey and Indonesian honey on full-thickness wounds in rats were compared; according to the study, wounds did not differ significantly between the Indonesian and Manuka honey groups 11 days after wound creation. In the control group, the decrease in the wound area was very slow for the first five days; however, the reduction in the control group's wound areas was the same as that for the honey-treated groups on day 11 (18). In the current study, wound healing at day 14 was found to be similar in all groups, similar to the results reported by Nisbet et al. (32) and Haryanto et al. (18).

Increased hydroxyproline content in granulation tissue is indicative of an increased collagen cycle and shows the maturation and proliferation of collagen during wound healing (22). Although some studies have shown that applying honey to the wound area increases the production of hydroxyproline (19, 32, 36), one study has indicated that honey has no effect on hydroxyproline production (40). In the current study, the hydroxyproline levels of the groups treated with CH- and PH-containing creams were found to be significantly lower on day 14 compared to the control group. These results differ from the results of previous studies. Furthermore, the current study found that auxiliary substances in creams also decreased the hydroxyproline level in the wound area. The histopathological examination showed no significant differences in the amount of collagen between the groups on days 7 and 14.

More oxygen is released from oxyhemoglobin at an acidic pH (41). Therefore, the absence of necrosis of the wound edges in treated animals may be associated with improved tissue oxygenation due to the lower pH of the honey. The lack of necrosis provides increased wound contraction, lower inflammatory responses, better tissue organization, and improved mechanical properties of treated wound lesions. Thus, honey shortens the inflammatory phase of wound healing due to its antibacterial effect, while its acidic pH supports the delivery of O_2 to the healing tissue. The high concentration of carbohydrates in honey provides a rich source of nutrients and energy for the healing tissue, while amino acids play a significant role in collagen formation and maturation, improving contraction and epithelialization, helping in fibroblastic division and maturation, reshaping and organizing collagen fiber, and consequently increasing tensile strength. The organization of collagen fibers may be due to reduced edema and inflammatory exudates, which may result from the high osmolarity of this high-energy source (8, 33). Furthermore, honey can contain many medicinal compounds, including essential oils, flavonoids, terpenes, and polyphenols, depending on the plant from which the pollen is taken (4, 13, 15). These various components of honey also affect wound healing.

In conclusion, the results from this study show that creams containing CH were more effective in wound healing than those containing PH. Thus, the botanical origin of honey is significant in terms of efficiency. The application period also affects wound healing. Based on the results obtained from this study, new forthcoming studies with different types of honey, animal models, and dosages can be planned.

Acknowledgments

This manuscript is derived from the Ph.D. thesis of the first author.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study was approved by the Kırıkkale University Animal Experiments Local Ethics Committee (Meeting Number:16/03, Meeting Decision: 16/44).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Akershoek JJ, Brouwer KM, Vlig M, et al** (2017): *Differential effects of Losartan and Atorvastatin in partial and full thickness burn wounds.* PLoS One, **12**, e0179350.
2. **Al-Manary M, Al-Meeri A, Al-Habori M** (2002): *Antioxidant activities and total phenolics of different types of honey.* Nutrition Research, **22,** 1041-1047.
3. **Al-Waili NS** (2004): *Investigating the antimicrobial activity of natural honey and its effects on the pathogenic bacterial infections of surgical wounds and conjunctiva.* J Med Food, **7,** 210-222.
4. **Azeredo LC, Azeredo MAA, Souza SR, et al** (2003): *Protein contents and physiochemical properties in honey samples of Apis mellifera of different floral origins.* Food Chem, **80**, 249–254.
5. **Bergman A, Yanai J, Weiss J, et al** (1983): *Acceleration of wound healing by topical application of honey: An animal model.* Am J Surg, **145**, 374-376.
6. **Bogdanov S, Haldimann M, Luginbühl W, et al** (2007): *Minerals in honey: environmental, geographical and botanical aspects.* J Apicult Res, **46,** 269–275.
7. **Castro-Vázquez L, Díaz-Maroto MC, De Torres C, et al** (2010): *Effect of geographical origin on the chemical and sensory characteristics of chestnut honeys.* Food Res Int, **43**, 2335-2340.
8. **Cooper R, Molan P, Harding KG** (1999): *Antibacterial activity of honey against strains of Staphylococcus aureus from infected wounds*. J R Soc Med, **92**, 283–285.

9. **Czubryt MP** (2012): *Common threads in cardiac fibrosis, infarct scar formation, and wound healing.* Fibrogenesis & Tissue Repair, **5**, 1-11.
10. **Delavary BM, Veer WM, Egmond M, et al** (2011): *Macrophages in skin injury and repair.* Immunobiology, **216**, 753–762.
11. **Efem SEE, Iwara CI** (1992): *The antimicrobial spectrum of honey and its clinical significance*. Infection, **20**, 227-229.
12. **Enoch S, Leaper DJ** (2005): *Basic science of wound healing.* Surgery, **23**, 37–42.
13. **Ferreres F, Garcia-Viguera C, Tomas-Lorente F, et al** (1993): *Hesperetin: a marker of the floral origin of citrus honey.* J Sci Food Agric, **61**, 121–123.
14. **Ghaderi R, Afshar M** (2004): *Topical application of honey for treatment of skin wound in mice.* Int J Mol Sci, **29**, 185–188.
15. **Gheldof N, Engeseth NJ** (2002): *Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples.* J Agric Food Chem, **50**, 3050–3055.
16. **Ghosh PK, Gaba A** (2013): *Phyto-extracts in wound healing.* J Pharm Sci, **16**, 760-820.
17. **Grimble GF** (1994): *Nutritional antioxidants and the modulation of inflammation: theory and practice.* New Horiz, **2**, 175–185.
18. **Haryanto TU, Mukai K, Suriadi JS, et al** (2012): *Effectiveness of Indonesian honey on the acceleration of cutaneous wound healing: an experimental study in mice.* Wounds, **24,** 110-119.
19. **Iftikhar F, Arshad M, Rasheed F, et al** (2010): *Effects of acacia honey on wound healing in various rat models.* Phytother Res, **24**, 583-586.
20. **Kaufman T, Eichenlaub EH, Angel MF, et al** (1985): *Topical acidification promotes healing of experimental deep partial thickness skin burns: a randomised doubleblind preliminary study.* Burns, **12**, 84–90.
21. **Kolayli S, Aliyazıcioglu R, Ulusoy E, et al** (2008): *Antioxidant and Antimicrobial Activities of Selected Turkish Honeys.* Hacettepe J Biol & Chem, **36**, 163-172.
22. **Kumar R, Katoch SS, Sharma S** (2006): *β-Adrenoceptor agonist treatment reverses denervation atrophy with augmentation of collagen proliferation in denervated mice gastrocnemius muscle*. Indian J Exp Biol, **44**, 371-376.
23. **Kwakman PHS, Zaat SAJ** (2012): *Antibacterial components of honey*. IUBMB Life, **64**, 48-55.
24. **Mcloone P, Warnock M, Fyfe L** (2016): *Honey: A realistic antimicrobial for disorders of the skin.* J Microbiol Immunol Infect, **49,** 161-167.
25. **Mercan N, Guvensen A, Celik A, et al** (2007): *Antimicrobial activity and pollen composition of honey samples collected from different provinces in Turkey*. Nat Prod Res, **21**, 187-195.
26. **Mohd ZR, Abu Bakar Zakaria Z, Yusof N, et al** (2012): *Gelam (Melaleuca spp.) honey-based hydrogel as burn wound dressing.* J Evid Based Complementary Altern Med, 1-7.
27. **Molan PC** (1999): *The role of honey in the management of wounds.* J Wound Care, **8**, 415–418.
28. **Molan PC** (2001): *Potential of Honey in the Treatment of Wounds and Burns.* Am J Clin Dermatol, **2**, 13-19.
29. **Molan PC** (2002): *Re-introducing honey in the management of wounds and ulcers—theory and practice.* Ostomy Wound Manag, **48**, 28–40.
30. **Mukai K, Koike M, Nakamura S, et al** (2015): *Evaluation of the effects of a combination of japanese honey and hydrocolloid dressing on cutaneous wound healing in male mice.* J Evid Based Complementary Altern Med, **2**, 1-9.
31. **Mutlu C, Erbaş M, Tontul SA** (2017): *Bal ve diğer arı ürünlerinin bazı özellikleri ve insan sağlığı üzerine etkileri.* Akademik Gıda, **15**, 75-83.
32. **Nisbet HO, Nisbet C, Yarim M, et al** (2010): *Effects of Three Types of Honey on Cutaneous Wound Healing.* Wounds, **22**, 275-283.
33. **Oryan A, Zaker SR** (1998): *Effects of topical application of honey on cutaneous wound healing in rabbits.* J Vet Med Series A, **45**, 181-188.
34. **Park SI, Sunwoo YY, Jung YJ, et al** (2012): *Therapeutic effects of acupuncture through enhancement of functional angiogenesis and granulogenesis in rat wound healing*. Evid Based Complement Alternat Med, Volume 2012.
35. **Paydar S, Akrami M, Dehghanian A, et al** (2016): *A Comparison of the Effects of Alpha and Medical-Grade Honey Ointments on Cutaneous Wound Healing in Rats*. Int J Pharm, Volume 2016.
36. **Rozaini MZ, Zuki ABZ, Noordin M, et al** (2004): *The effects of different types of honey on tensile strength evaluation of burn wound tissue healing*. Int J Appl Res Vet Med, **2**, 290–296.
37. **Sami K. Saikaly SK, Khachemoune A** (2017): *Honey and Wound Healing: An Update.* Am J Clin Dermatol, **18**, 237-251.
38. **Sazegar G, Reza S, Hosseini A, et al (**2011): *The Effects of Supplemental Zinc and Honey on Wound Healing in Rats.* Iran J Basic Med Sci, **4**, 391-398.
39. **Schmidt RJ, Chung LY, Andrews AM** (1993): Hydrogen peroxide is a murine (L929) fibroblast cell proliferant at micro- to nanomolar concentrations. 20–23. In: Harding KG, Cherry G, Dealey C, Turner TD (eds). Proceedings of the Second European Conference on Advances in Wound Management; London, UK.
40. **Sell SA, Wolfe PS, Spence AJ, et al** (2012): *A preliminary study on the potential of manuka honey and platelet-rich plasma in wound healing.* Int J Biomater, 1-14.
41. **Silver A** (1982): *Basic physiology of wound healing in the horse.* Equine Vet J, **14**, 7-15.
42. **Subrahmanyam M** (1998): *A prospective randomized clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine.* Burns, **24**, 157–161.
43. **Sumitra M, Manikandam P, Gayathri VS, et al** (2009): *Influence of honey on energy metabolism during wound healing in rats*. Sch Res Exch, 1–6.
44. **Taormina PJ, Niemira BA, Beuchat LR** (2001): *Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power.* Int J Food Microbiol, **69,** 217-225.
45. **Tonks A, Cooper RA, Price AJ, et al** (2001): *Stimulation of TNF-alpha release in monocytes by honey.* Cytokine, **14**, 240–242.
46. **Tutun H, Kahraman HA, Aluc Y, et al** (2019): *Investigation of some metals in honey samples from West Mediterranean region of Turkey.* Vet Res Forum, **10**, 181.
47. **Ulusoy E** (2012): *Bal ve apiterapi.* U Bee J, **12,** 89-97.
48. **Yeşil A, Gürkan B, Saraçoğlu Ö, et al** (2005): *Effect of the pest Marchalina hellenica Gennadius (Homoptera, Margarodidae) on the growth parameters of Pinus brutia Ten. in Muğla region (Turkey).* Pol J Ecol, **53,** 451-458.

Ankara Univ Vet Fak Derg, **69**, 329-336, 2022
DOI: 10.33988/auvfd.865840

An evaluation of the efficiency of beekeeping enterprises in Turkey: The case of Mersin City

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Received date: 21.01.2021 - Accepted date: 01.07.2021

Abstract: Beekeeping contributes significantly to both beekeeping enterprises and the country's economy, as it provides jobs, income, and nutrition for the rural population of developing countries. Mersin City is an essential region for beekeeping, both in terms of honey production and migratory beekeeping in Turkey. In this study, the efficiency of beekeeping enterprises was revealed, and the factors causing inefficiency were examined. The efficiency measure of enterprises was determined using data envelopment analysis (DEA). According to the findings obtained, beekeeping enterprises' technical efficiency, allocation efficiency, and economic efficiency were calculated as 0.89, 0.84, and 0.81, respectively. The economic efficiency score showed that inefficient enterprises could effectively reduce their production costs by 19%. When the factors causing inefficiency are examined, it is revealed that income per hive, subsidy rate, and credit use have adverse effects on efficiency, education level, experience, number of honey frames used per hive, and migratory beekeeping effects. According to the results, it is thought that increasing education and extension activities, improving marketing opportunities, legal regulations in using agricultural credits, and extending consultancy services can also help to increase economic efficiency in the research field.
Keywords: Beekeeping, data envelopment analysis, efficiency, honey production, Tobit model.

Türkiye'deki arıcılık işletmelerinin etkinliğinin değerlendirilmesi: Mersin ili örneği

Özet: Arıcılık, gelişmekte olan ülkelerin kırsal nüfusu için iş, gelir ve beslenme olanağı sağlaması nedeniyle hem işletmelere hem de ülke ekonomisine önemli katkılar sağlamaktadır. Mersin, Türkiye'de hem bal üretimi hem de gezginci arıcılık açısından önemli bir il konumundadır. Bu çalışmada arıcılık işletmelerinin etkinliği ortaya konulmuş, ayrıca etkinsizliğe neden olan faktörler incelenmiştir. İşletmelerin etkinlik ölçümü veri zarflama analizi (VZA) kullanılarak belirlenmiştir. Elde edilen bulgulara göre, arıcılık işletmelerinin teknik etkinliği 0,89, tahsis etkinliği 0,84 ve ekonomik etkinliği ise 0,81 olarak hesaplanmıştır. Ekonomik etkinlik skoru dikkate alındığında, etkin olmayan işletmelerin üretim maliyetlerini %19 oranında azaltarak etkin hale gelebileceğini göstermektedir. Etkinsizliğe neden olan faktörler incelendiğinde ise kovan başına gelir, sübvansiyon oranı ve kredi kullanımının etkinlik üzerinde negatif, eğitim düzeyi, deneyim, kovan başına kullanılan bal çerçevesi sayısı ve gezginci arıcılığın ise pozitif etkileri olduğu ortaya çıkmaktadır. Elde edilen sonuçlara göre, eğitim ve yayım faaliyetlerinin artırılması, pazarlama olanaklarını geliştirilmesi tarımsal kredi kullanımında yasal düzenlemeler ve danışmanlık hizmetlerinin yaygınlaştırılması da araştırma alanında ekonomik etkinliğin artırılmasına yardımcı olabileceği düşünülmektedir.
Anahtar sözcükler: Arıcılık, bal üretimi, etkinlik, Tobit model, veri zarflama analizi.

Introduction

Beekeeping is a common agricultural activity with significant differences in its economic structure, contribution to rural development, and technical features. In recent years, migratory beekeeping activities have increased, and bee products have diversified. Therefore, productivity and quality features have come to the forefront in Turkey.

Turkey contains 20% of the world's bee races (5, 7). Turkey has 75% of the world's honeyed plant flora, and it has a tremendous opportunity for beekeeping in terms of rich flora, habitats, colony life, and genetic diversity in the bee population (16, 24, 25, 27). This potential can be activated by studies that will help beekeepers choose suitable bees for honey and other bee product production and suitable feeding places. Thus, it is possible to increase the revenue of beekeepers in Turkey (14, 20).

Mersin City is one of the most suitable regions, mainly for migratory beekeeping in Turkey (17), and ranks 7th with 2270 beekeeping enterprises in Turkey. The honey production accounts for 2.15% of the total production amount with 2352 tones and 1.69% of the production amount with 67 tones in wax production (28).

There are several benefits of beekeeping, i.e., extra profits over a shorter period, fulfilling the families' daily needs, pollination, and the productive use of family labor (18), and biodiversity conservation (4). Furthermore, honey production is unavoidable for producing healthy and intelligent future generations as well as developing rural development (3). However, the Turkish beekeeping sector has faced technical and economic challenges, such as low hive productivity, bee diseases, pests, failure to increase export potential, difficulties in marketing, an inadequate degree of industrial organization, and unexpected migratory beekeeping. In addition to the sector's challenges, competition in the globalizing environment has become a vital aspect that the Turkish apiculture sector needs to recognize (12). These results show that it is necessary to increase production and make the current production more efficient.

This paper's objective was to assess enterprise-level efficiency (technical, allocative, and economic efficiency) and the determinants of cost efficiency in beekeeping enterprises in Mersin City. There are two approaches to measuring efficiency: data envelopment analysis (DEA) and the stochastic frontier model. Data envelopment analysis (DEA) was preferred in this study since measurement errors were desired to be a minimum in efficiency studies. In the Tobit model, the number of dependent variables is limited compared to linear regression models. In various studies about efficiency analysis in beekeeping, it was seen that Tobit regression was used with analyzing parameters, which were considered to be influential on efficiency (5, 6, 8, 12, 22). In this study, the Tobit model was used as only the variables related to the production of beekeeping enterprises were reached.

Materials and Methods

Research data: The research materials were the survey data obtained from enterprises registered with the Mersin Beekeepers Union. Beekeeping enterprises with a total of 30 or more hives are affiliated with the Union. The stratified random sampling method was used in the formula below to improve the precision of the results obtained from enterprises and ensure sufficient population representation (30).

$$n = \frac{\Sigma (N_h S_h)^2}{N^2 D^2 + \Sigma N_h S_h^2}, \qquad D^2 = \frac{e^2}{t^2} \qquad (5)$$

The enterprises were split into three strata, including 30-100 hives classified as small, 101-180 hives classified as medium, and 181 hives and above classified as large enterprises. The total number of beekeeping enterprises was calculated as 81 in the study, with a 10% sampling error margin and 95% confidence level (t=1.645). The number of enterprises surveyed was 41, 19, and 21 in terms of scales, respectively.

Within the study's scope, all data, including the beekeeping enterprises' socio-economic characteristics (age of the beekeeper, size of the household, beekeeping experience, education level, variable and fixed costs in beekeeping activities), were obtained through questionnaires.

DEA and TOBIT models for beekeeping enterprises: The data envelopment analysis (DEA) method was used to analyze the study's beekeeping enterprises' efficiency analysis. There is no need to define a function type for the output component or the cost function using the DEA model. Also, there is no need to specify a distributional form for the inefficiency term (10).

It was suggested that the enterprise's efficiency could be made up of two components: technical efficiency that represents the enterprise's ability to achieve full performance from a defined collection of inputs, and allocative efficiency that reflects the enterprise's ability to use inputs in optimum proportions, provided their respective prices and production technology (13). Using the current technology, an input-oriented technical efficiency calculation can be determined as the linear programming (LP) solution for the ith enterprise:

$$\begin{aligned} &\text{Min}_{\theta,\lambda}\ \theta, \\ &\text{Subject to:}\ -y_i + Y\lambda \geq 0 \\ &\qquad\qquad \theta_{x_i} - X\lambda \geq 0 \qquad (1) \\ &\qquad\qquad \lambda \geq 0, \end{aligned}$$

where θ is the technical efficiency value between 0 and 1. The enterprise is on the frontier if the value equals 1. The vector λ is a N x 1 weight vector that determines the ith enterprise's linear combination. The input-based minimum cost for the ith enterprise can be achieved by solving the following LP problem:

$$\begin{aligned} &\text{Min}_{\lambda,xi^*}\ \ w_i^t x_i^*, \\ &\text{Subject to:}\ -y_i + Y\lambda \geq 0 \quad (2) \\ &\qquad\qquad \lambda \geq 0, \end{aligned}$$

where w is a vector of input prices for the ith enterprise; x_i^* is the input quantity cost-minimizing vector for the ith enterprise, determined by the LP, and λ is the N*1 constant vector. The BCC model will be a CCR-model (Eq. 2) representing a CRS condition without this convexity constraint.

Scale efficiency expresses whether a firm is operating at its optimal size. Based on the CCR and BCC-scores, scale efficiency is defined by (11):

$$SE = \frac{TE_{CCR}}{TE_{BCC}} \quad (3)$$

Efficiency measures under CRS and VRS were calculated by using the program DEAP 2.1 (9). The efficiency level was carried out according to Aydın et al. (5), where TE = 1 is fully efficient, 0.90 ≤ TE ≤ 0.949 is efficient, 0.90 ≤ TE ≤ 0.949 is less efficient, and TE ≤ 0.899 is inefficient.

The Tobit model is used to explain the relationship between the inefficiency scores and variables for demographic and socio-economic characteristics. It defines the Tobit model as follows: (15).

$$Y_{ij} = \beta_0 + \sum_{j=i}^{N} \beta_i X_i + u_i \quad if \quad u_i > -\beta_0 - \sum_{j=i}^{N} \beta_i X_i$$

$$Y_{ij} = 0 \quad if \quad u_i \leq -\beta_0 - \sum_{j=i}^{N} \beta_i X_i \quad (4)$$

Y_i is the measure of inefficiency for enterprise i; X_i is the explanatory variables that influence the enterprises' inefficiencies, N is the number of explanatory variables; and β, and u are the model random error term parameters, respectively (23). For the estimation of the Tobit model, the LIMDEP 7.0 statistical and data analysis program was used.

Results

In the research, one output (honey income), the number of hives, and the costs of feed, drug, fuel-transport, honeycomb, jar-tin, temporary labor, accommodation, and repair-maintenance inputs were used to estimate the efficiency of the beekeeping enterprises. Descriptive statistics of the inputs and output are given in Table 1. It was observed that there were high deviations in honey income and production costs according to enterprise scale. The reason for these deviations was to the input and output levels used within each enterprise scale.

The average number of hives was calculated as 77, 145, and 312 on the enterprise scale. In terms of honey income, it was determined as 5981.49, 11548.79, and 27971.06 US$, respectively. One of the most critical components of cost for bees to feed is sugar. According to the enterprise scale, it was determined that the sugar costs were 213.47, 521.97, and 1352.50 US$, drug costs were 75.21, 186.66, and 686.94 US$, fuel-transport costs were 302.67, 537.62, and 1186.21 US$, honeycomb costs were 241.11, 524.03, and 1066.77 US$, respectively. Beekeepers sell their honey in jar or tin. According to the enterprise scale, the jar-tin costs were determined at 158.13, 242.68, and 399.00 US$. However, there is often a need for temporary employment, and in terms of enterprise scales, temporary labor costs were determined at 53.76, 210.87, and 465.41 US$, respectively. The accommodation has essential effects on honey quality. In this context, accommodation costs were determined in terms of enterprise scale at 33.45, 60.65, and 123.94 US$, respectively. Besides, repair and maintenance costs were 47.73, 50.91, and 132.35 US$.

The study's findings will increase beekeepers' efficiency and contribute to policymakers, beekeepers' unions, and extension services. According to the input-oriented efficiency analysis results, pure technical efficiency was found at 0.96 for VRS. This value suggests that inefficient beekeeping enterprises could decrease inputs by 4% without output reduction to ensure efficiency. The pure technical efficiency values were 0.94, 0.97, and 0.97, respectively (Table 2). It can also be said that medium and large enterprises were practically more effective than small enterprises.

Table 1. Descriptive statistics of the output and inputs by enterprise scale.

	Small		Medium		Large	
Output and inputs	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**
Honey income (US$)	5891.49	2321.38	11548.79	2767.18	27971.06	11090.19
Number of hives (piece)	77.07	20.27	144.89	17.93	312.14	108.83
Feed (sugar) costs (US$)	213.47	88.48	521.97	116.92	1352.50	430.47
Drug costs (US$)	75.21	16.04	186.66	57.44	686.94	204.73
Fuel-transport costs (US$)	302.67	93.10	537.62	131.69	1186.21	452.75
Honeycomb costs (US$)	241.11	208.47	524.03	567.39	1066.77	498.44
Jar-tin costs (US$)	158.13	40.30	242.68	28.07	399.00	153.73
Temporary labor costs (US$)	53.76	51.92	210.87	119.87	465.41	294.90
Accommodation costs (US$)	33.45	20.46	60.65	24.41	123.94	78.85
Repair-maintenance costs (US$)	47.73	13.14	50.91	10.11	132.35	44.70

Table 2. Descriptive statistics of technical efficiency scores by enterprise scale.

	Small			Medium			Large			Average		
Technical efficiency scores	CRS	VRS	SE	CRS	VRS	SE	CRS	VRS	SE	CRS	VRS	SE
Average	0.88	0.94	0.94[a]	0.86	0.97	0.89[b]	0.92	0.97	0.96[a]	0.89	0.96	0.93
Standard deviation	0.12	0.09	0.09	0.15	0.05	0.13	0.10	0.06	0.08	0.13	0.08	0.10
Minimum	0.57	0.70	0.57	0.59	0.85	0.59	0.67	0.74	0.71	0.57	0.70	0.57
Maximum	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[a,b] shows the statistically significant differences between the enterprise scale at a level of 5%.
CRS: constant return to scale; VRS: variable return to scale; SE: Scale efficiency.

Table 3. Classification of technical efficiency scores by enterprise scale.

	Small		Medium		Large		Average	
Technical efficiency scores	**Number**	**%**	**Number**	**%**	**Number**	**%**	**Number**	**%**
Fully efficient (TE = 1)	26	63.41	12	63.16	13	61.90	51	62.96
Efficient (0.95 ≤ TE ≤ 1)	2	4.88	2	10.53	3	14.29	7	8.64
Less efficient (0.90 ≤ TE ≤ 0.949)	2	4.88	3	15.79	2	9.52	7	8.64
Inefficient (TE ≤ 0.899)	11	26.83	2	10.53	3	14.29	16	19.75
Total	41	100.00	19	100.00	21	100.00	81	100.00

P=0.599

Table 4. Returns to scale by enterprise scale.

	Small		Medium		Large		Total	
Return to scale	**Number**	**%**	**Number**	**%**	**Number**	**%**	**Number**	**%**
Increasing return to scale	25	60.98	11	57.9	10	47.62	46	56.79
Constant return to scale	14	34.15	7	36.84	10	47.62	31	38.27
Decreasing return to scale	2	4.88	1	5.26	1	4.76	4	4.94
Total	41	100	19	100	21	100	81	100

P=0.886

The average scale efficiency was estimated at 0.93. This score means that almost all the enterprises in the region were based on technical efficiency. Beekeeping enterprises have been categorized according to technical efficiency in Table 3. Enterprises were determined fully efficient on enterprise scales of 63.41%, 63.16%, and 61.90%, respectively (Table 3). The chi-square test results revealed that technical efficiency does not differ by enterprise scale, indicating that enterprises use their production resources at the same level (P=0.599).

On average, 56.79% of enterprises had an increasing return to scale, 38.27% had a constant return to scale, and 4.94 % of the beekeeping enterprises had a decreasing return to scale (Table 4). It was determined that the ratio of constant return to scale in large enterprises was higher than in small and medium enterprises. The chi-square test results showed that returns to the scale did not vary by the scale of the enterprise, and this showed that the relationship between the amount of input and output was similar across all enterprise scales (P=0.886).

Allocative efficiency values varied on a scale between 0.50 and 1.00 and were found to be 0.84 on average (Table 5). This value suggests that many enterprises produced an inaccurate input combination at the current technology level when the current input prices were considered. The enterprises' costs were 16% more than the input combination with the minimum costs. Allocative efficiency coefficients varied by enterprise scale (F=3.547, P=0.034).

Table 5. Descriptive statistics of allocative efficiency scores by enterprise scale.

	Average	**Std. dev.**	**Minimum**	**Maximum**
Small	0.82[a]	0.11	0.50	1.00
Medium	0.87[b]	0.09	0.69	1.00
Large	0.88[b]	0.07	0.76	1.00
Average	0.84	0.10	0.50	1.00

[a,b] Means within these comparisons with different letters are significantly different at P<0.05.

Based on the enterprises' average, allocative efficiency was determined to be 20.69% of the enterprises allocating the resources fully efficient, and that value was found to be 7.32%, 15.79%, and 14.29% in terms of scales, respectively (Table 6). The chi-square test findings showed that allocative efficiency varied in enterprise scale, and small enterprises used less technology level and input combination (P=0.053).

On average, economic efficiency was determined at 0.81. The value indicated that inefficient beekeeping enterprises had to minimize operating costs by 19% to become efficient enterprises. According to enterprise scale, economic efficiency values were calculated at 0.77, 0.84, and 0.85, respectively (Table 7). Due to the number of hives, it was observed that the large enterprises were more efficient than the small and medium enterprises. There were statistically significant differences in economic efficiency scores between the enterprise scales, and this revealed that small enterprises should provide the optimum combination of inputs by reducing their costs (F=4.3737, P=0.016).

Based on the beekeeping enterprises' average, economic efficiency was calculated to be 7.32% of small enterprises, 15.79% of medium enterprises, and 14.29%of full-efficient. This value was considered 11.11% on average (Table 8). The chi-square test findings revealed that economic efficiency varied with enterprise scale (P = 0.070), and the rate of efficient enterprises is high in medium-scale enterprises.

The Tobit model was used in the second stage to determine the effect of some socioeconomic factors on economic inefficiency, and the results are shown in Table 9. The Tobit analysis results showed that the gross honey income subsidy rate is negative and statistically significant at all enterprise scales.

Table 6. Classification of allocative efficiency scores by enterprise scale.

	Small		Medium		Large		Average	
Allocative efficiency scores	**Number**	**%**	**Number**	**%**	**Number**	**%**	**Number**	**%**
Fully efficient (TE = 1)	3	7.32	3	15.79	3	14.29	9	11.11
Efficient (0.95 ≤ TE ≤ 1)	1	2.44	3	15.79	0	0.00	4	4.94
Less efficient (0.90 ≤ TE ≤ 0.949)	4	9.76	1	5.26	6	28.57	11	13.58
Inefficient (TE ≤ 0.899)	33	80.49	12	63.16	12	57.14	57	70.37
Total	41	100.00	19	100.00	21	100.00	81	100.00

P=0.053

Table 7. Descriptive statistics of economic efficiency scores by enterprise scale.

	Average	**Std. dev.**	**Minimum**	**Maximum**
Small	0.77[a]	0.13	0.50	1.00
Medium	0.84[b]	0.12	0.66	1.00
Large	0.85[b]	0.10	0.59	1.00
Average	0.81	0.12	0.50	1.00

[a,b] Means within these comparisons with different letters are significantly different at P<0.05.

Table 8. Classification of economic efficiency scores by enterprise scale.

	Small		Medium		Large		Average	
Economic efficiency scores	**Number**	**%**	**Number**	**%**	**Number**	**%**	**Number**	**%**
Fully efficient (TE = 1)	3	7.32	3	15.79	3	14.29	9	11.11
Efficient (0.95 ≤ TE ≤ 1)	1	2.44	3	15.79	0	0.00	4	4.94
Less efficient (0.90 ≤ TE ≤ 0.949)	3	7.32	1	5.26	5	23.81	9	11.11
Inefficient (TE ≤ 0.899)	34	82.93	12	63.16	13	61.90	59	72.84
Total	41	100.00	19	100.00	21	100.00	81	100.00

P=0.053

Table 9. Tobit analysis results by enterprise scale.

	Small		Medium		Large	
Variables	**Coefficient**	**Standard error**	**Coefficient**	**Standard error**	**Coefficient**	**Standard error**
Constant (C)	0.523[c]	0.162	2.489[c]	0.837	1.973[c]	0.633
Beekeepers' age (year)	0.002[b]	0.001	-0.007[b]	0.003	-0.002	0.005
Education period (year)	-0.003	0.003	-0.007	0.015	-0.004	0.004
Family size (person)	0.004	0.006	-0.021	0.019	0.006	0.010
Beekeeping experience (year)	0.002[b]	0.001	0.005[a]	0.003	0.005	0.005
Land size (da)	0.001[b]	0.000	0.003	0.004	0.002	0.002
Income per hive (US$)	0.007[c]	0.001	0.011[c]	0.004	0.013[c]	0.004
Honey-frame per hive (number)	0.013[a]	0.007	-0.062	0.041	-0.012	0.015
Subsidy rate in gross honey income (%)	-0.020[b]	0.008	-0.182[a]	0.099	-0.219[b]	0.099
Information source (number)	0.028[b]	0.014	0.053[a]	0.030	0.021	0.028
Migratory beekeeping (if yes 1; others 0)	0.064[c]	0.021	-0.024	0.067	0.057	0.064
Off-farm income (if yes 1; others 0)	-0.044[a]	0.023	0.045	0.072	-0.137[b]	0.063
Credit usage (if yes 1; others 0)	-0.038	0.058	-0.178[b]	0.076	-0.197[b]	0.079

[a,b,c] Values indicate the significance at the 10%, 5% and 1% level, respectively.

Discussion and Conclusion

This study revealed that Data Envelopment Analysis (DEA) would be more appropriate to determine the efficiency level of beekeeping enterprises due to diseases and pests on production and changing input and honey prices over the years.

Technical, allocative, and economic efficiency means were estimated at 0.89, 0.84, and 0.81, respectively. Besides, total technical efficiency was found to be 0.89 and pure technical efficiency was 0.96 on average. In the study on beekeeping conducted in Turkey (8), the average allocative efficiency and economic efficiency of beekeeping in Turkey were found to be 0.75 and 0.62, respectively. In the study on beekeeping conducted in Turkey (12), the researcher identified the technical efficiency score as 0.84, and the economic efficiency score as 0.63. The efficiency scores obtained from the study have higher values than other studies because beekeeping in Mersin City is a more favorable and economic activity for reasons such as climate, flora, and low wintering losses.

The average efficiency scores in beekeeping enterprises were calculated as 0.89 in Tolon Kumbungu-Ghana (1), as 0.55 in Oyo State-Nigeria (2), as 0.89 in Çanakkale-Turkey (5), as 0.97 in Hatay-Turkey (19), as 0.57 in all of Greece (21) and as 0.85 in Adana-Turkey (22). Among the 81 enterprises in the region, 63% were technically full-efficient, 11% were allocatively full-efficient, and 11% were economically full-efficient.

Scale efficiency shows that many enterprises are at an adequate level in their production and that their size is optimal. A study in Çanakkale-Turkey determined technical, allocative, and economics scores of 52.87%, 20.69%, and 17.24% efficient, respectively (5). A study in Greece determined that technical, allocative, and economics scores were 3.14%, 8.36%, and 3.14% full-efficient, respectively (21).

The definition of pure technical efficiency demonstrates the optimal utilization of inputs on the basis of the return on the variable scale. 56.79% of beekeeping enterprises increase returns to scale, 38.27% maintain constant returns to scale, and 4.94% decrease returns to scale. This situation showed that the production unit had scale efficiency. Other study percentages determined were 77.01%, 18.39%, and 4.60%, respectively (5). Since honey production in beekeeping is affected by many factors, especially climatic conditions and input prices, it varies according to years among the enterprises' scale returns.

Experience is significant to maintaining beekeeping effectively. Because experience will enable beekeepers to make reliable and accurate production (29), extension activities should be carried out in a way that will strengthen the communication between beekeepers and publication sources, especially the diagnosis of bee diseases and pests (5).

Subsidies should be included in the factors that affect yield per hive (17), and beekeepers should be aimed at solving the problems in marketing. The study revealed that small-scale enterprises were influenced by social and economic factors, while large-scale enterprises were only economic. Especially for large-scale beekeeping enterprises, providing marketing support instead of subsidies per hive will solve marketing problems.

If appropriate credit opportunities cannot be provided to the beekeepers, they try to provide the funds they need by working in different jobs or sectors (18). It was noted that the rise in the use of credit harms economic efficiency. In other respects, the subsidy rate in gross honey income was also another variable that adversely impacts economic efficiency.

Agricultural credit is an essential factor in production. Credits for agricultural production need to be restructured for beekeepers who wish to use credit for more productive processing. However, the credit reduces financial restrictions on cash inputs, boosts technical efficiency, and increases resource allocation and profitability (26).

According to the results, subsidies were merely additional revenue, not intended to raise the production amount. For this purpose, new subsidy instruments should be implemented to make more efficient production, improve development technology for beekeepers, and increase rural welfare.

Acknowledgements

This study was carried out in cooperation with Mersin Beekeepers Union.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The author declared that there is no conflict of interest.

References

1. **Abdul-Malik A, Mohammed A** (2012): *Technical efficiency of beekeeping farmers in Tolon-Kumbungu district of Northern region of Ghana*. J Dev Agric Econ, **4**, 304-310.
2. **Aburime IL, Omotesho OA, Ibrahim HY** (2006): *An analysis of technical efficiency of beekeeping farms in Oyo state, Nigeria*. Eur J Soc Sci, **4**, 1-8.
3. **Aksoy A, Ertürk YE, Eyturan, E, et al** (2018): *Estimation of Honey Production in Beekeeping Enterprises from Eastern Part of Turkey Through Some Data Mining Algorithms*. Pakistan J of Zoo, **50**, 2200-2207.
4. **Al-Ghamdi AA, Adgaba N, Herab AH, et al** (2017): *Comparative analysis of profitability of honey production using traditional and box hives*. Saudi Journal of Biological Sciences, **24**, 1075–1080.
5. **Aydın B, Aktürk D, Arsoy D** (2020): *Economic and Efficiency Analysis of Beekeeping Activity in Turkey: Case of Çanakkale Province*. Veterinary Journal of Ankara University, **67**, 23-32.
6. **Barlovic N, Kezic J, Benedic ON, et al** (2009): *Economic efficiency of beekeeping in Croatia*. Agriculturae Conspectus Scientificus, **74**, 51-54.
7. **Borum AE** (2017): *A study of beekeeping survey in Southern Marmara region of Turkey*. U Bee J, **17**, 24-34.
8. **Ceyhan V, Canan S, Yıldırım Ç, et al** (2017): *Economic structure and services efficiency of Turkish Beekeepers' Association*. Eur J Sustain Dev, **6**, 53-64.
9. **Coelli TJ** (1996): A guide to DEAP Version 2.1: a data envelopment analysis (Computer) program, CEPA Working Paper, No. 8/96, ISBN 1 86389 4969, Department of Econometrics, University of New England, p. 49.
10. **Coelli TJ, Rao DSP, Battese GE** (1998): An Introduction to Efficiency and Productivity Analysis. Kluwer Academic Publishers, Boston.
11. **Cooper WW, Seiford LM, Tone K** (2006): Introduction to DEA and its uses with DEA-Solver software and references. New York: Springer.
12. **Emir M** (2015): Exploring the socio-economic structure of beekeepers and their production efficiency in Turkey. PhD Thesis. Ondokuz Mayis University Department of Agricultural Economics, Samsun.
13. **Farrell MJ** (1957): *The Measurement of Productive Efficiency*. Journal of the Royal Statistical Society, **120**, 253–281.
14. **Fıratlı Ç, Karacaoğlu M, Gençer H, et al** (2010): Structural analysis of beekeeping Turkey. In Proceedings of Turkey Agricultural Engineering VII. Technical Congress (pp. 707-717). Ankara, Turkey.
15. **Gujarati DN** (1999): Basic Econometrics. 2nd Edition, Mc Graw Hill. Literatür Publishing, İstanbul.
16. **Güler A, Demir M** (2005): *Beekeeping potential in Turkey*. Bee World, **86,** 114-118.
17. **Gürer B, Akyol E** (2018): *An empirical analysis of technical efficiency determinants in beekeeping farms: evidence and policy implications from Niğde Province, Turkey*. JAEID, **112**, 343-359.
18. **Karadas K, Kadirhanogullari IH** (2017): *Predicting honey production using data mining and artificial neural network algorithms in apiculture*. Pakistan J Zool, **49**, 1611-1619.
19. **Kaya U, Gürcan S** (2021): *An evaluation of the efficiency of beekeeping enterprises in Hatay province with data envelopment analysis*. Ankara Univ Vet Fak Derg, Available at http://vetjournal.ankara.edu.tr/tr/pub/issue/48904/754619 (Accessed January 14, 2021).
20. **Kosoğlu M, Yucel B, Ozsoy N, et al** (2017): *Effects of Queen Bee on Colony Development and Management Economy in Turkish Beekeeping*. Turkish Journal of Agricultural Economics, **23**, 55–60.
21. **Makri P, Papanagiotou P, Papanagiotou E** (2015): *Efficiency and economic analysis of Greek beekeeping farms*. Bulg J Agric Sci, **21**, 479-484.
22. **Ören N, Alemdar T, Parlakay O, et al** (2010): Economic Analysis of Beekeeping Activity in Adana Province. Agricultural Economics Research Institute Edition No: 78, Ankara.
23. **Ramanathan R** (1998): Introductory Econometrics with Applications. The Dryden Press, USA.

24. **Sancak K, Zan Sancak A, Aygören E** (2013): *Beekeeping in the World and Turkey*. Arıcılık Araştırma Dergisi, **5**, 7-13.
25. **Sıralı R** (2002): *General Beekeeping Structure of Turkey*. U Bee J, **2,** 30-39.
26. **Sial MH, Awan MS, Waqas M** (2011): *Role of Institutional Credit on Agricultural Production: A Time Series Analysis of Pakistan*. International Journal of Economics and Finance, **3**, 126-132.
27. **Tamali H, Özkırım A** (2019): *Beekeeping Activities in Turkey and Algeria*. Mellifera, **19**, 30-40.
28. **TURKSTAT** (2020): Turkey Statistical Institute, Statistical Indicators. Available at https://biruni.tuik.gov.tr/medas/?kn=92&locale=tr (Accessed December 22, 2020).
29. **Vanengelsdorp T, Tarpy DR, Baylis K, et al** (2012): *The bee informed partnership: using beekeeper's real-world experience to solve beekeepers' real-world problems*. American Entomologist, **58**, 116-118.
30. **Yamane T** (1967): Elementary sampling theory. Englewood Cliffs, N.J.: Prentice Hall, Inc.

Ankara Univ Vet Fak Derg, **69**, 337-340, 2022
DOI: 10.33988/auvfd.1059346

Short Communication / *Kısa Bilimsel Çalışma*

Rumen fermentation characteristics of rams fed supplemental boric acid and humic acid diets

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Received date: 21.01.2022 - Accepted date: 16.06.2022

Abstract: The aim of the study is to investigate the effects of humic acid and boric acid as feed additives on rumen fermentation parameters in rams. For this purpose, 3 yearling rams were used in this experiment. Rams in each treatment; a control with no supplements (C), 180 ppm boric acid (B) and 5 ml/kg humic acid (H) with 65:35 forage to concentrate ratio. Each experimental period lasted 14 days, with 12 first days of diet adaptation. Totally, the experimental period lasted 42 days. In both time periods (0 and 3h after feeding), there were no significant differences (P>0.05) on rumen pH, ammonia, protozoa count, estimated methane production and volatile fatty acid composition. However, butyric acid concentration tended to be higher in experimental groups (P=0.08) compared with control. As a result of this study, these feed additives did not modify the rumen milieu and showed no negative effect in rams.
Keywords: Boron, humate, methane, volatile fatty acids.

Borik asit ve humik asit ilave edilen yemlerle beslenen koçların rumen fermentasyon karakteristikleri

Özet: Bu çalışmanın amacı, yem katkı maddesi olarak kullanılan humik asit ve borik asidin koçlarda rumen fermantasyon parametreleri üzerine etkilerini araştırmaktır. Bu amaçla, söz konusu denemede 3 adet damızlık koç kullanılmıştır. Her bir deneme grubu için gruplar; herhangi bir katkı maddesi içermeyen kontrol (C), 180 ppm borik asit (B) ve 5 ml/kg humik asit (H) içeren deneme grupları şeklinde oluşturulmuştur ve 65:35 kaba yem/konsantre oranı ile besleme gerçekleştirilmiştir. Her bir deneme periyodu 12 günlük adaptasyon ile beraber 14 gün olmak üzere deneme toplamda 42 gün sürmüştür. Denemenin sonunda elde edilen verilere göre, her iki zaman periyodunda da (beslenmeden 0 ve 3 saat sonra), rumen pH'sı, amonyak konsantrasyonu, protozoa sayısı, tahmini metan üretimi ve uçucu yağ asidi bileşimi üzerinde önemli bir fark (P>0,05) gözlemlenmemiştir. Ancak bütirik asit konsantrasyonun kontrol grubuna göre deneme gruplarında daha yüksek olma eğiliminde olduğu belirtilmiştir (P=0,08). Bu çalışmanın sonucunda, söz konusu yem katkı maddelerinin koçlarda rumen ortamını değiştirmediği ve herhangi bir olumsuz etki göstermediği ortaya konmuştur.
Anahtar sözcükler: Bor, humat, metan, uçucu yağ asitleri.

Efficiency of rumen fermentation can be regulated by manipulating the rumen milieu with chemical agents, feed additives, which modulate selected pathways of metabolism such as volatile fatty acids (VFA) and methane production (CH_4), by regulating ruminal pH (5). Fermentation provides not only the ruminant with VFA but also methane production that is a potential greenhouse gas, resulting in environmental pollution, and also energy loss for ruminants (6, 19). Recently, the use of feed additives has gained importance, modified rumen fermentation, especially after the prohibition of the use of synthetic additives such as antibiotics (10, 16, 17). Humic acid and boric acid are some of such feed additives.

Humic acid arises from the organic materials such as plant and animal matters that are naturally decomposed and modulates the rumen fermentation by changing the rumen fluid ammonia concentration (1, 8) as well as boric acid (16). It has been demonstrated that dietary boron had an influence on rumen microbial fermentation in yearling rams (16).

Little information is present on the effects of boric acid and humic acid supplementation to ram diets on rumen fermentation and methane production. Therefore, the current experiment aimed to evaluate the effect of humic acid and boric acid on rumen fermentation, and estimated methane production of rams. We hypothesized that these feed additives would alter ruminal fermentation, reduce potent methane production of rams.

Three 11-12 months' age Merinos rams, which weigh approximately 60 kg and are kept in individual pens. Feed and water were provided separately for each animal in plastic buckets which are installed onto the pens. Rams were fed alfalfa pellets (500 g/day), barley straw (400 g/day) and concentrates (500 g/day) (% 14 CP and 2.6 Mcal/kg ME). Forage concentrate ratio was 65:35 for all treatment groups. The chemical composition of the diet used in this experiment is given in Table 1.

The daily diet of animals was fed in two meals (9.00 am-16.30 pm), and water was provided *ad libitum*. Animals in each treatment; a control with no supplements (C), 180 ppm boric acid (B) and 5 ml/kg humic acid (H) were fed according to Bialek et al. (3) and Varadyova et al. (20). Each 180 mg of boric acid represents approximately 10 mg boron. The experiment was designed 3x3 Latin square in order to eliminate environmental differences. Each period was lasted 14 days resulted in 42 days of the experimental period.

Samples of rumen fluid were taken for 2 days following the 12-day adaptation period, before feeding (0 h) and 3 hours after feeding by using rumen catheter. Ruminal pH was measured using pH meter and NH_3 levels were measured using gas-sensitive ammonia electrode (Orion^R). Protozoan counts were performed in fresh rumen fluid by using Fuchs Rosenthal Lam (depth 0.1 mm) and a microscope (15). The VFA were determined using Gas Chromatography (GC; Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan). To calculate the estimated CO_2 and CH_4 based on the stoichiometry, CO_2 = (Acetic acid/2) + (Propionic acid/4 + 1.5 × Butyric acid) and CH_4 = (Acetic acid + 2 × Butyric acid) – CO_2 formula was used (4).

The data were analyzed with SPSS 14.01 program (SPSS Inc. Chicago, IL, USA) using the GLM procedures. The analysis included 3x3 between subject's factorial design and the main factors were different time sampling and humic acid, and boric acid supplementation. The Duncan's Multiple Range Test was used for the comparison of means. Statements of significance were based on P-value of equal to or less than 0.05.

The effects of additional humic acid or boric acid did not affect the dry matter intake (DMI), because of no residual feed remained between meals in any group. Fermentation parameters of the experimental rams' rumen fluid, pH value, ammonia concentration and protozoan numbers at 0h and 3 h post feeding were determined and summarized in Table 2. There were no statistically significant differences between the experimental groups ($P>0.05$), interaction of group and the sampling time, while the time effect altered the fermentation parameter ($P\leq0.001$), as expected. After 3 h of feeding, ruminal microbial fermentation patterns increased except for pH, isobutyrate, isovalerate, and protozoa counts.

In recent years, the use of boric acid and humic acid, which are thought to contribute economically, that is sourced from the country, has increased. Studies on the use of these feed additives in ruminant nutrition are being carried out and especially their effect on rumen microbial fermentation is tried to be revealed (9, 16). This study investigated the effect of supplemented humic acid and boric acid on microbial fermentation in rams. In the light of our previous study (16), which examined the effect of boric acid on the rumen environment, what kind of results would emerge in comparison with humic acid has been discussed. In the current study, additional humic acid and boric acid did not alter the rumen milieu in terms of fermentation. However, time sampling did significantly affect the pH, ammonia concentration, VFA, methane production and protozoa counts, as expected. It's well known that the fermentation parameters, in the expense of VFA, increased after feeding (14). The VFA concentration was not affected by using dietary used feed additives, whilst butyrate concentration tended to be higher both before and 3 h after feeding, albeit for total VFA and individual concentrations with ammonia level were exhibited relatively numerical increasing at 3h after feeding. In ruminants, persistent increase of butyric acid concentration in the digestive system have positive effects on gut development, nutrient utilization efficiency and the animal production (11). Conversely, Sizmaz et al. (16) reported that the higher level of boric acid increased the butyric acid concentration in rams. Our best knowledge is there is no other reporting on the effects of boric acid on rumen fermentation patterns. Similarly, some studies showed no effect (8; 20) on VFA with the using of humic acid in ruminant diets. Source of the ruminal proteins recycling by reactions of amino acids such as isoleucine and valine (13). Theoretically, increasing in ammonia level, which is formed by the breakdown of proteins as well as amino acids, manage to higher concentrations of iso-VFA in the milieu of rumen (12). This outcome coincides with the observed higher ammonia concentration in the treatment groups, which is similar to the previous study (16).

Table 1. Analyzed chemical composition of forages and concentrates used in the experiment (2).

	Forages		Concentrate[1]		
Nutrients	Alfalfa	Straw	C	B	H
DM, %	92.80	89.00	90.80	92.70	92.80
OM, %	92.00	94.70	82.50	84.94	85.78
CP, %	9.51	2.70	15.00	15.50	15.30
CF, %	30.80	39.60	6.40	6.60	5.97
EE, %	1.10	2.18	4.20	3.75	3.75

[1] The concentrate contained 25% corn, 24% barley, 3% soybean meal, 12% sunflower meal, 25% rasmol, 2% full fat soy, 4% molasses, 3% $CaCO_3$, 1% salt and 1% mineral-vitamin premix for yearling rams (contained per kg: retinol 3000 mg, cholecalciferol 75000 mg, tocopherol 30000 mg, thiamin 980 mg, niacin 99500 mg, biotin 20 mg, manganese 50000 mg, zinc 50000 mg, iron 50000 mg, copper 10000 mg, iodine 800 mg, cobalt 200 mg, selenium 300 mg, magnesium 250 mg).

Table 2. Effects of using boric acid and humic acid on rumen pH, NH_3 (ppm), VFA concentration (nM), estimated CO_2 and CH_4 (mmol/l) and protozoan counts (x10^4 number/ml) (Mean ± SD).

		Groups				**P values**		
Items	Time, h[1]	**C**	**H**	**B**	**Main effect**	**G**	**T**	**GxT**
pH	0	6.90±0.25	6.83±0.14	6.78±0.28	6.61	0.316	≤0.001	0.996
	3	6.44±0.16	6.37±0.12	6.33±0.24				
Ammonia	0	168.75±48.01	164.29±56.89	168.29±56.50	197.20	0.615	0.003	0.643
	3	210.63±70.73	218.75±78.32	252.50±64.81				
Acetate	0	34.05±11.29	37.58±7.64	40.61±11.95	45.42	0.250	≤0.001	0.979
	3	50.89±10.49	53.05±8.43	56.33±8.53				
Propionate	0	7.55±2.44	8.46±2.70	9.25±3.38	10.69	0.175	≤0.001	0.962
	3	12.13±2.26	12.71±2.64	14.04±2.42				
Isobutyrate	0	0.88±0.15	0.80±0.10	0.85±0.08	0.78	0.973	≤0.001	0.166
	3	0.69±0.10	0.76±0.11	0.71±0.05				
Butyrate	0	9.15±3.08	11.24±2.02	10.96±3.31	12.32	0.082	≤0.001	0.994
	3	12.85±2.98	14.90±2.36	14.83±2.71				
Isovalerate	0	1.03±0.18	0.88±0.17	1.02±0.12	0.81	0.780	≤0.001	0.092
	3	0.61±0.11	0.70±0.19	0.64±0.11				
Valerate	0	0.58±0.11	0.62±0.15	0.64±0.16	0.87	0.338	≤0.001	0.878
	3	1.04±0.13	1.14±0.28	1.17±0.22				
Total VFA	0	53.47±16.13	59.83±11.52	63.57±18.65	71.13	0.196	≤0.001	0.993
	3	78.46±15.31	83.51±12.88	87.96±13.81				
CO_2	0	42.64±10.27	37.77±6.92	39.06±11.70	43.86	0.163	≤0.001	0.992
	3	47.76±9.72	52.05±8.11	53.92±8.84				
CH_4	0	19.72±6.35	22.30±4.08	23.48±6.80	26.20	0.221	≤0.001	0.986
	3	28.84±5.92	30.80±4.80	32.07±5.03				
Protozoa	0	42.13±16.54	49.00±21.19	43.93±18.07	36.95	0.812	≤0.001	0.463
	3	31.19±16.64	27.8111.49	27.8111.49				

[a,b] Values within a row with different superscripts differ significantly at P<0.05
[1]0; before feeding, 3; 3h after feeding
G; group, T; time, GxT; group and time interactions.

Conversely, there are some studies with different results of using of different derivatives of humic acid on rumen fermentation in goats (7), lactating dairy cows (10), beef heifers (18) in vivo and in vitro (9) and effect of boric acid in previous study on protozoa number (16). This might be originated from the different type of diets, form of the feed additives and ruminants, and conditions in terms of in vitro and in vivo.

In conclusion, the current trial demonstrated that addition of humic and boric acid did not significantly

affect the ruminal fermentation patterns before and 3 h after feeding. However, tended to increase the parameters compared with control groups. It should be noted that the feed additive supplementation level, in vitro or in vivo conditions and using in big or small ruminants are significant when feed additives are applied. It would be useful to further assess the effects of these additives on detailed and molecular analysis under both conditions.

Financial Support

This work was supported by the Boron Research Institute (BOREN) (Project No. 2011.Ç0302).

Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee of the Ankara University (Ethical Approve Number: 200515).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. **Achard FK** (1986): *Rich soils for cures*. Cereals Chemical Ann, **11**, 391-403.
2. **AOAC** (2000): Official Methods of Analysis. 18th edn. Association of Official Analytical Chemists, Virginia, USA.
3. **Białek M, Czauderna M, Krajewska KA, et al** (2019): *Selected physiological effects of boron compounds for animals and humans. A review*. J Anim Feed Sci, **28**, 307-320.
4. **Blümmel M, Aiple KP, Steingaβ H, et al** (1999): *A note on the stoichiometrical relationship of short chain fatty acid production and gas formation in vitro in feedstuffs of widely differing quality*. J Anim Physiol Anim Nutr (Berl), **81**, 157-167.
5. **Chalupa W** (1977): *Manipulating rumen fermentation*. J Anim Sci, **45**, 585-599.
6. **Eckard RJ, Grainger C, De Klein CAM** (2010): *Options for the abatement of methane and nitrous oxide from ruminant production: a review*. Livestock Sci, **130**, 47–56.
7. **El-Zaiat HM, Morsy AS, El-Wakeel EA, et al** (2018): *Impact of humic acid as an organic additive on ruminal fermentation constituents, blood parameters and milk production in goats and their kids growth rate*. J Anim Feed Sci, **27**, 105-113.
8. **Galip N, Polat U, Biricik H** (2010): *Effects of supplemental humic acid on ruminal fermentation and blood variables in rams*. Italian J Anim Sci, **9**, e74.
9. **Ikyume TT, Sowande OS, Yusuf AO, et al** (2020): *In vitro gas production, methane production and fermentation kinetics of concentrate diet containing incremental levels of sodium humate*. Agric Conspec Sci, **85**, 183-189.
10. **Kholif AE, Matloup OH, EL-Bltagy EA, et al** (2021): *Humic substances in the diet of lactating cows enhanced feed utilization, altered ruminal fermentation, and improved milk yield and fatty acid profile*. Livestock Sci, **253**, 104699.
11. **Li RW, Wu S, Baldwin RL, et al** (2012): *Perturbation dynamics of the rumen microbiota in response to exogenous butyrate*. PloS one, **7**, e29392.
12. **Mathieu F, Jouany JP, Sénaud J, et al** (1996): *The effect of Saccharomyces cerevisiae and Aspergillus oryzae on fermentations in the rumen of faunated and defaunated sheep; protozoal and probiotic interactions*. Reprod Nutr Dev, **6**, 271–287.
13. **Miltko R, Rozbicka-Wieczorek JA, Więsyk E, et al** (2016): *The influence of different chemical forms of selenium added to the diet including carnosic acid, fish oil and rapeseed oil on the formation of volatile fatty acids and methane in the rumen, and fatty acid profiles in the rumen content and muscles of lambs.* Acta Vet Beogr, **66**, 373–391.
14. **Mwangi FW, Suybeng B, Gardiner CP, et al** (2022): *Effect of incremental proportions of Desmanthus spp. in isonitrogenous forage diets on growth performance, rumen fermentation and plasma metabolites of pen-fed growing Brahman, Charbray and Droughtmaster crossbred beef steers*. Plos One, **17**, e0260918.
15. **Ogimoto K, Imai S** (1981): Atlas of rumen microbiology. Japan Scientific Societies Press, Japan.
16. **Sizmaz O**, **Koksal BH, Yildiz G** (2017): *Rumen microbial fermentation, protozoan abundance, and boron availability in yearling rams fed diets with different boron concentrations*. J Anim Feed Sci, **26,** 59-64.
17. **Sizmaz O**, **Calik A, Gumus H, et al** (2020): *Effects of probiotics on in vitro ruminal fermentation, abundance of cellulolytic bacteria and estimated methane production.* Ankara Univ Vet Fak Derg, **67,** 249-255.
18. **Terr, SA, Ribeiro GDO, Gruninger RJ, et al** (2018): *Effect of humic substances on rumen fermentation, nutrient digestibility, methane emissions, and rumen microbiota in beef heifers.* J Anim Sci, **96**, 3863-3877.
19. **Ungerfeld EM** (2020): *Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions.* Front Microbiol, **11**, 589.
20. **Váradyová Z, Kišidayová S, Jalč D** (2009): *Effect of humic acid on fermentation and ciliate protozoan population in rumen fluid of sheep in vitro*. J Sci Food and Agri, **89**, 1936-1941.

Ankara Univ Vet Fak Derg, **69**, 341-345, 2022
DOI: 10.33988/auvfd.963458

Case Report / *Olgu Sunumu*

Pathologic findings of acantholytic squamous cell carcinoma coexisting with cutaneous cryptococcosis in a *Houbara bustard* (*Chlamydotis undulata*)

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Received date: 14.07.2021 - Accepted date: 16.06.2022

Abstract: A 2-year-old Asian *Houbara bustard* was presented with a solitary well-defined, firm cutaneous mass on the hock region. Grossly, the mass protruded from the surface was located on the hairless and unpigmented areas of the right hock joint with ulceration and dried hemorrhagic foci. On microscopic examination, ulceration, hemorrhage, as well as hyperkeratosis were observed. Large round, oval to polygonal neoplastic cells extended into the dermis were arranged to form cords, trabeculae, islands or glandular-like structures without keratin pearls. These pseudoglandular structures were composed of pseudolumina containing acantholytic and detached tumor cells. Necrosis of the neoplastic cells was accompanied by infiltration of inflammatory cells particularly heterophils. Unlike pleomorphic tumor cells, mitosis count was almost frequent. No evidence of other abnormalities and tumor metastasis was found. These gross and microscopic features appeared to be suggestive of a rare histologic variant of squamous cell carcinoma (SCC), acantholytic SCC.

Keywords: Acantholytic squamous cell carcinoma, *Chlamydotis undulata,* histopathology*, Houbara bustard.*

Squamous cell carcinoma (SCC) is one of the most common malignant skin tumors in all species of domestic animals. It occurs most commonly in dogs, cats, cattle, horses, less commonly in sheep, goats, and rarely in chickens (4, 7). Histopathologically, several subtypes of SCC are identified including well-differentiated, poorly differentiated, spindle cell, acantholytic and verrucous SCC (7, 15, 16). Acantholytic SCC, also known as adenoid or pseudoglandular SCC, is an uncommon intermediate- to high-risk subtype of SCC (15, 16). In addition to the skin, this uncommon subtype of SCC has been reported in non-dermal locations including the oral cavity, tonsil, maxilla, palate, esophagus, cecum, nasal cavity, lung, uterine cervix and vulva as well as breast. In the veterinary literature, there are only a few reported cases of acantholytic SCC in animals. It has not been previously reported in poultry unlike scattered reports of acantholytic SCC in dogs (4, 8, 21, 23) and cattle (12).

Cryptococcosis is a fungal infection mainly caused by *Cryptococcus neoformans*. *This* opportunistic pathogen affects immunocompromised hosts and invades the central nervous system and lungs (6, 9). Skin is the third most common tissue that may be infected. Cutaneous infection occurs third most common clinical manifestation of cryptococcosis. It is associated with the excreta of certain birds, including pigeons, canaries, and cockatoos (5).

The present study describes pathologic features of concurrent cryptococcosis and acantholytic SCC in a rare bird, Asian *Houbara bustard (Chlamydotis undulata*).

An approximately 2-year-old Asian *Houbara bustard* was referred to a veterinary hospital with a large solitary mass on the region of the hock joint. Grossly, the skin mass located on the right hock joint was sessile, firm, round to cauliflower-like, well circumscribed but non-encapsulated protruding from the surface (Figure 1). In addition, it measured three cm in diameter and the surface of the mass was slightly ulcerated with dried hemorrhagic

foci in some regions without secondary bacterial infections. In palpation, the mass had a firm consistency with a rough surface and gray to dark brown on cut section. The affected skin was unpigmented and hairless, and the mass did not invade the underlying bone and remained intact. No other abnormalities, tumor invasion and metastasis were found in necropsy. After removing the mass, taken samples were fixed in 10 % neutral buffered formalin (NBF) and then processed for paraffin embedding after fixation. The samples were sectioned at 5 µm and stained with hematoxylin-eosin (H&E). The number of mitotic figures was counted in 10 contiguous fields, with no overlapping, high-power field (HPF). Mitotic counts per field were calculated as:

$$\text{Mitotic count/field} = \frac{\text{total number of mitotic figures observed}}{\text{number of fields counted}}$$

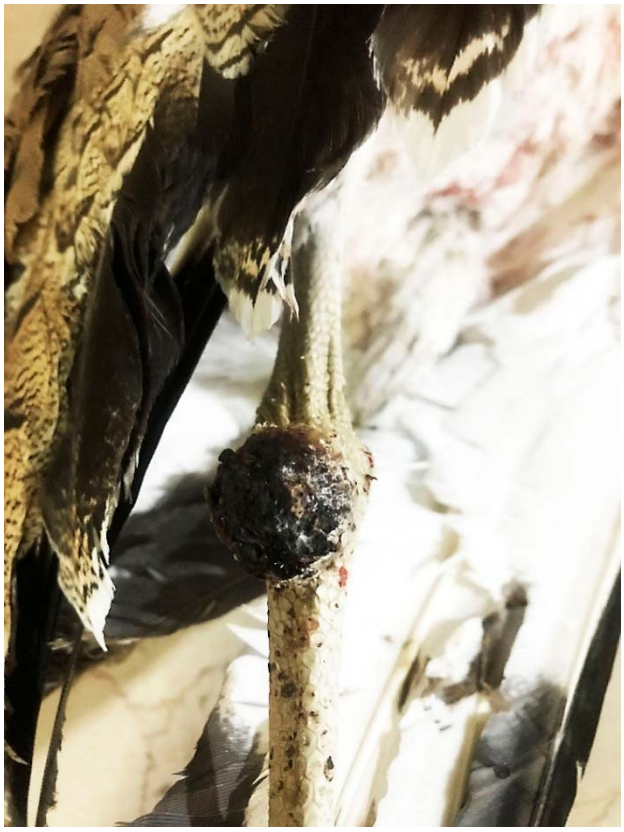

Figure 1. Right leg showing a large solitary skin mass attached to the hock joint in *Houbara bustard*. Hemorrhagic surface of firm, well-defined but non-capsulated mass with no secretion is evident.

Histologic examination revealed hyperkeratosis and parakeratosis over the epidermis surface of the mass. Moreover, the epidermal surface of the mass was ulcerated and hemorrhagic in some areas (Figure 2A). The tumor consisted of large round and oval to polygonal cells arranged in cords, trabeculae or islands extended into the underlying dermis. No evidence of intracytoplasmic eosinophilic keratin tonofilaments was observed and there were no keratin pearls characteristic of well-differentiated SCC. Interestingly, some neoplastic keratinocytes underwent dyskeratosis or filamentous degeneration. More importantly, there was marked dyshesion of the non-delimited neoplastic cells so that basal cells were the only cells attached to the basal lamina in some sections (Figure 2B, 2C). The gland-like or pseudoglandular structures were comprised of tumor islands with the cohesive outer layer and floating individual and/or clustered acantholytic or/and dyskeratotic keratinocytes in the center of false lumina (Figure 2C, 2D). In some areas of the tumor, numerous cells underwent extensive necrosis and the necrotic keratinocytes were infiltrated by inflammatory cells (Figure 3A). Inflammatory cells particularly heterophils were visible around the necrotic areas to phagocyte the dead cells and tissue remnants (Figure. 3B, 3C). The neoplastic cells had pale to brightly eosinophilic cytoplasm with a large round to ovoid vesicular nuclei containing loose chromatin. Some neoplastic cells had more than one prominent nucleoli (Figure 3C). Mitotic figures was frequent and different phases of the mitosis were observed (Figure 3D). Conversely, there was not considerable cellular and nuclear pleomorphism. In according to macroscopic and microscopic features, a diagnosis of acantholytic or pseudoglandular SCC was provided. PAS staining of tissue samples showed a lot of oval to spherical-shaped microorganisms with positive mucinous capsule in areas of proliferated squamous cells and also in the necrotic debris of pseudoglandular structures of the tumor (Figure 4). Based on morphology, *Cryptococcus neoformans* infection was diagnosed.

Acantholytic SCC is an uncommon histologic variant of SCC in which neoplastic cells form pseudoglandular structures with variably sized discohesive acantholytic cells (15). The histopathologic characteristics of the neoplasm in the present case are extremely consistent with those observed in the medical and veterinary literature (21, 23). The pathogenesis and precise mechanism responsible for the development of acantholytic SCC have not been entirely understood. However, it has been demonstrated that decreased expression or loss of at least one desmosomal protein such as the cadherin family and subsequent loss of cell-cell adhesions potentially contribute to 89 % of the pseudoglandular structures formed in acantholytic SCC. Likewise, it has been illustrated that two or more desmosomal proteins responsible for tissue cohesion have been lost in 65 % of the acantholytic SCC (10). On the other hand, although acantholytic SCC might arise at any site in different species, several factors can significantly influence the tumor development including overexposure to sunlight and ultraviolet light of hairless and lightly pigmented sites (15). As a result, receiving a large load of ultraviolet radiation can also be suggested as one of the probable etiologies for acantholytic SCC.

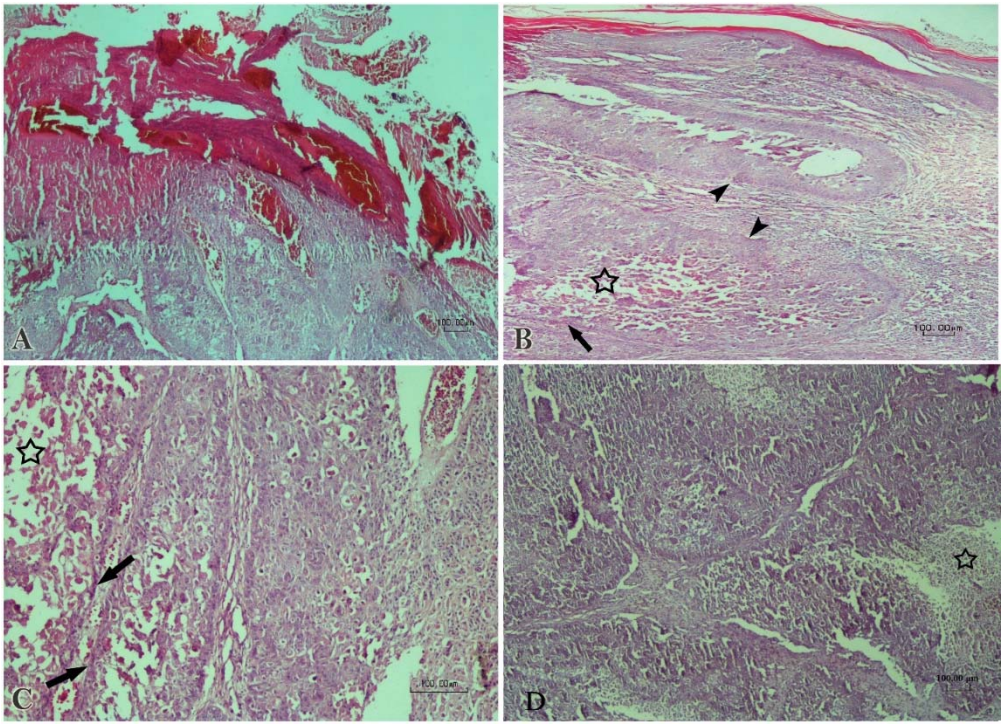

Figure 2. Acantholytic squamous cell carcinoma.
A. Occurrence of ulceration and hemorrhage on the epidermal surface of tumor. Islands of neoplastic squamous cells are typically extended into the dermis.
B. Mild hyperkeratosis with ulceration of the epidermis in the left side of the illustration. Pseudoglandular structures (arrowheads) with neoplastic keratinocytes (arrow) are obvious in the dermis. Some neoplastic cells are sloughed (empty star).
C and D. A higher magnification of glandular-like structures with clustered acantholytic keratinocytes floated in the lumen (empty star) so that the basal layer is the only remained layer (arrows). H&E staining. Bar = 100 µm.

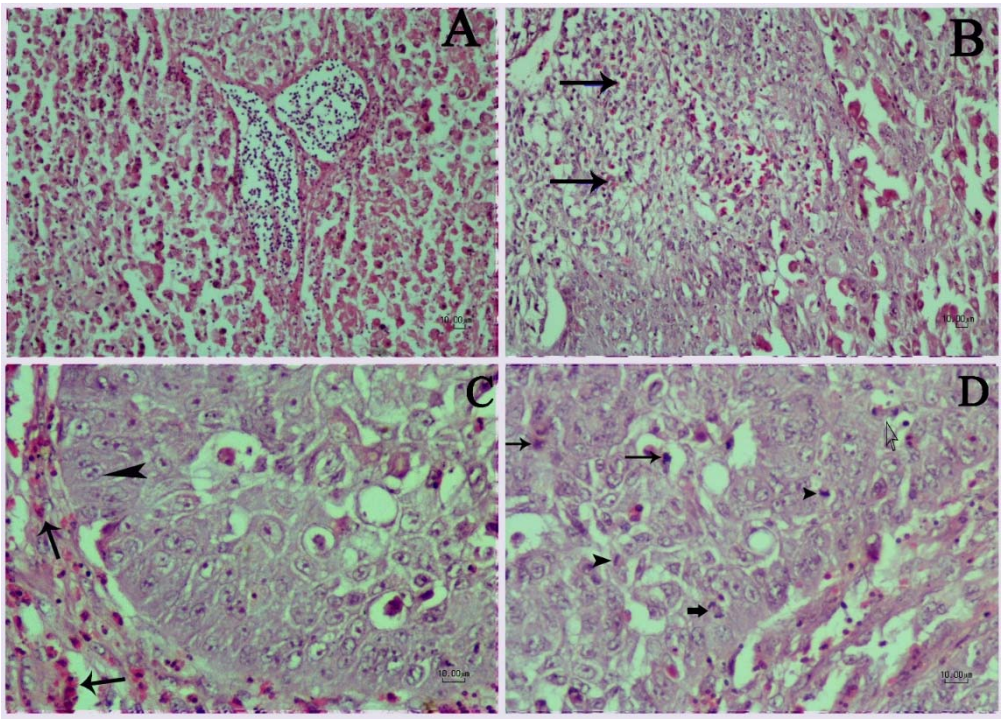

Figure 3. Acantholytic squamous cell carcinoma.
A. Extensive dermal degeneration and necrosis of the neoplastic cells.
B. Infiltration of heterophils in the stromal tumor (arrows).
C. Neoplastic cells with vesicular nuclei and prominent nucleoli (arrowhead) and also heterophils Infiltration in the stroma (arrows)
D. Different stages of mitotic figures including prophase (arrows), metaphase (arrowheads) and anaphase (empty arrow). H&E staining. Bar = 10 µm.

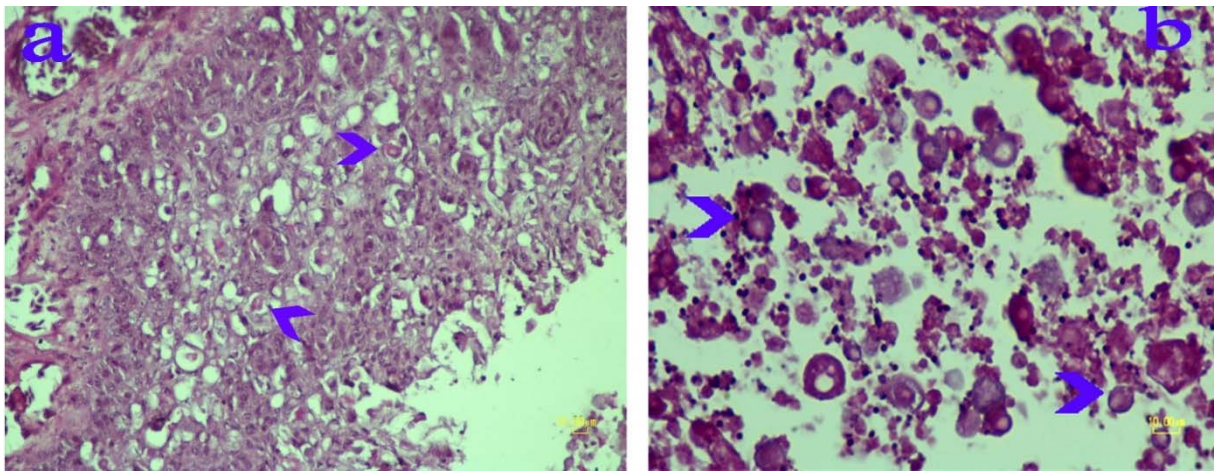

Figure 4. Periodic acid–Schiff (PAS) staining shows positive mucinous capsule of *Cryptococcus neoformans* (arrowheads) between proliferated squamous cells (a) and necrotic debris in the tumor (b).

Although the aggressiveness and malignancy of acantholytic SCC compared with conventional SCC are debated in the literature (8, 11, 23), the former has been proposed to display a more aggressive clinical behavior on the basis of the literature review conducted by Mohammad and Wilcox (16). Due to rarity of acantholytic SCC and low numbers of these cases, the biologic and aggressive behavior of the tumor has not been precisely determined in the literature. In accordance with the present research, three cases of acantholytic SCC reported in animals have not showed any evidence of metastasis (4, 8, 12). Nonetheless, metastasis to regional lymph nodes and manifestation of considerable clinical signs has been reported in 2 cases of the neoplasm (21, 23). Romanucci et al. (21) reported acantholytic SCC in the external ear canal of dogs with metastasis to the submandibular, parotid, retropharyngeal, cervical, prescapular lymph nodes and the lung. In another study, the tumor in the submandibular region had metastasis to the cervical and submandibular lymph nodes (11). Nevertheless, the prognosis of acantholytic SCC varies depending on the location size, and grade of the tumor as well as the host characteristics (10). The tumor location and its proximity to the regional lymph nodes are counted as critical factors influencing the tumor aggressive behavior. The tumor was well-demarcated from the surrounding tissues and the underlying joint and bones in the present research. Consequently, it did not show aggressive behavior and extension to the adjacent tissues. Nevertheless, mitotic figures were present (3-4 mitoses/400× field) and the frequency of mitotic figures might increase with the degree of malignancy (7).

As mentioned previously, there are few reports on the occurrence of acantholytic SCC in animals such as dogs and cattle to date, but here we reported this variant of SCC in a rare bird. The *Houbara bustard*, *Chlamydotis undulata* macqueenii, is a rare ground-dwelling steppe bird that inhabiting in desert and semi-desert areas (25). Thereby, the exposure to the strong and direct sunlight might be a possible risk factor for the neoplasm development in the hairless and unpigmented site of the hock region of the *Houbara* in our study. In the present case, concurrent cutaneous cryptococcosis (PCC) infection was observed. Cryptococcosis is an opportunistic fungi disease found throughout the world. PCC occurs as primary and secondary forms. Primary form mainly results from direct skin trauma and aberrations such as, papules, nodules, tumors, ulcerations, ecchymoses, pustules, abscesses and granulomas (3, 13, 17). Secondary PCC a more common type spreads from the brain, lung and other parts of the body. Immunodeficient patients such AIDS, malignant tumors, organ transplantation, anti-neoplastic and corticosteroid therapy and immunosuppressive drugs are the most susceptible cohort for cutaneous cryptococcosis (18).

Cutaneous cryptococcosis usually becomes visible as fluctuant or firm nodules that some of them may be ulcerated. Direct inoculation of organisms into the skin occasionally leads to the formation of solitary lesions (20).

Mallany et al. (2021) described laryngeal cryptococcus infections in an 83-year-old man with a history of chronic asthma and chronic obstructive pulmonary disease (COPD). Histopathologic results showed atypical squamous proliferation, acute inflammation, and scattered round-shaped microorganisms surrounded by clear halos. It was diagnosed as laryngeal squamous cell carcinoma (SCC), due to an overlying laryngeal squamous cell proliferation (14).

Squamous pseudoepitheliomatous hyperplasia (PEH) is a reactive epithelial proliferation that may occur secondary to different factors including infection, injury and inflammation. PEH in relation to fungal infection may

provide a potential mechanism for the overlying squamous cell carcinoma (SCC) mimicry (19).

Infections in birds are rare. The bird showed patchy feather loss, especially around the back and beak area. The feathers had a greasy appearance and disseminated a moldy odor (24). Bird faces particularly from pigeon is an environmental reservoir for *C. neoformans*. This yeast is present in decaying trees, wood, soil and waterways that contaminated with bird excrement (1, 2, 22).

In the present study, except for sunlight, cryptococcus infection can be a risk factor for acantholytic squamous cell carcinoma formation in this bird. Further investigation is needed until the pathogenesis of this tumor be illustrated in wild birds.

Acknowledgements

The authors are truly grateful to Mr. Saeed Hassanzadeh for his technical assistance.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

The current study is not an experimental part on living animals. Therefore, any approval from the ethic committee was not required.

Conflict of Interest

The authors declared no potential conflicts of interest.

References

1. **Abo El-Yazeed H, Ezz-Eldin N, Tawakkol W, et al** (2006): *Isolation and identification of Cryptococcus neoformans from bird droppings, with special reference to newly formulated differential media based on development of brown colonies*. J Egy Vet Med Assoc, **66**, 165-179.
2. **Abou-Elmagd Sh, Kotb H, Abdalla K, et al** (2011): *Prevalence of Candida albicans and Cryptococcus neoformans in Animals from Quena Governorate with Special Reference to RAPD-PCR Patterns.* J Am Sci, **7**, 20-31.
3. **Christianson JC, Engber W, Andes D** (2003): *Primary cutaneous cryptococcosis in immunocompetent and immunocompromised hosts*. Med Mycol, **41**, 177–188.
4. **Cushing T, Barnard S, Fleis R, et al** (2010): *Pseudoangiomatous squamous cell carcinoma in the oral cavity of a dog*. J Vet Diagn Invest, **22**, 299-302.
5. **Duperval R, Hermans PE, Brewer NS, et al** (1977): *Cryptococcosis, with emphasis on the significance of isolation of Cryptococcus neoformans from the respiratory tract*. Chest, **72**, 13-19.
6. **Gaylord EA, Choy HL, Doering TL** (2020): *Dangerous Liaisons: interactions of Cryptococcus neoformans with host phagocytes*. Pathogens, **9**, 891.
7. **Goldschmidt MH, Goldschmidt KH** (2017): Tumor of the skin and soft tissues. In: Meuten DJ, editor. Tumors in domestic animals. 5th edition, John Wiley and Sons, Inc, P. 88-141.
8. **Grandi F, Monteiro LN, Fernandes TR, et al** (2011): *What is your diagnosis? Cutaneous mass in the mammary region of a dog*. Vet Clin Pathol, **40**, 101-102.
9. **Gushiken AC, Saharia KK, Baddley JW** (2021): *Cryptococcosis*. Infect Dis Clin North Am, **35**, 493-514.
10. **Jurcic V, Kukovic J, Zidar N** (2015): *Expression of desmosomal proteins in acantholytic squamous cell carcinoma of the skin*. Histol Histopathol, **30**, 945-953.
11. **Kerawala CJ** (2009): *Acantholytic squamous cell carcinoma of the oral cavity: a more aggressive entity?* Br J Oral Maxillofac Surg, **47**, 123-125.
12. **Kiani K, Dezfoulian O, Sookhthezari A, et al** (2010): *Acantholytic squamous cell carcinoma in a cow*. Vet Clin Pathol, **19**, 397-400.
13. **Longhitano S, Peccerillo F, Odorici G, et al** (2019): *An unusual presentation of primary cutaneous cryptococcosis.* Dermatol Ther, **32**, e12942.
14. **Mallany HP, Curcin N, Borgia S, et al** (2021): *Squamous cell proliferation as a reactive mechanism to laryngeal cryptococcus infection: a case report*. Cureus, **13**, e12587.
15. **Mardi K, Singh N** (2014): *Acantholytic squamous cell carcinoma of the oral cavity: a rare entity*. J Oral Maxillofac Pathol, **18**, S128.
16. **Mohammad M, Wilcox R** (2018): *Morphologic mimicry: acantholytic squamous cell carcinoma in nondermal locations-a literature review*. Ann N Y Acad Sci, **1434**, 102-108.
17. **Neuville S, Dromer F, Morin O, et al** (2003): *Primary cutaneous cryptococcosis: a distinct clinical entity*. Clin Infect Dis, **36**, 337-347.
18. **Noguchi H, Matsumoto T, Kimura U, et al** (2019): *Cutaneous cryptococcosis*. Med Mycol J, **60**, 101-107.
19. **Pui J** (2006): *Distinguishing pseudoepitheliomatous hyperplasia from squamous cell carcinoma in mucosal biopsy specimens from the head and neck*. Arch Pathol Lab Med, **130**, 764.
20. **Refai MK, El-Hariri M, Alarousy R** (2017): *Cryptococcosis in animals and birds: A review*. Eur J Acad Essays, **4,** 202-223.
21. **Romanucci M, Malatesta D, Marinelli A, et al** (2011): *Aural carcinoma with chondroid metaplasia at metastatic sites in a dog*. Vet Dermatol, **22**, 373-377.
22. **Saleh H, Moawad AA, El- Hariri M, et al** (2011): *Prevalence of yeasts in human, animals and soil sample in el-fayoum governorate in Egypt*. Int J Microbiol, **2**, 233-239.
23. **Silva IC, Sarandy TB, Tamiasso NV, et al** (2018): *Metastatic submandibular acantholytic squamous cell carcinoma in a dog*. Braz J Vet Pathol, **11**, 19-23.
24. **Singh SD, Dash BB** (2008): *Spontaneous lesions of cryptococcosis in white Leghorn chicken*. Indian J Vet Pathol, **32**, 68-69.
25. **Tourenq C, Combreau O, Lawrence M, et al** (2005): *Alarming houbara bustard population trends in Asia*. Biol Conserv, **121**, 1-8.

Ankara Univ Vet Fak Derg, **69**, 347-354, 2022
DOI: 10.33988/auvfd.1019154

Review / *Derleme*

Aethina tumida (Small Hive Beetle; SHB) and *Tropilaelaps* spp. Mite; an emerging threat to Turkey Honey Bees

Levent AYDIN

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Received date: 04.11.2021 - Accepted date: 10.05.2022

Abstract: Turkey is the second largest honey producer in the world. However, the beekeeping sector, especially the export of honey and honey products, is not supported by income compatible with this asset. The main reasons for this discrepancy are the presence of honey bee diseases (predators) and their residues in honey. Due to the observation of *Aethina tumida* (small hive beetle; SHB) in Europe (Italy) and *Tropilealaps* spp. Iran's border regions close to Turkey, the Ministry of Agriculture and Forestry of the Republic of Turkey has raised its alert level for these two notifiable diseases. *Aethina tumida* is a fruit insect originally from South Africa and it has later adapted to bee colonies. In the early 2000s, the agent came to North America with imported fruits and caused serious bee losses. SHB, which has spread to 12 countries so far, caused serious economic losses in the countries it entered and could not be eliminated. *Tropilaelaps* spp. is an ectoparasite that cannot survive for long without honey bee brood it feeds on and needs to survive. *Tropilaelaps* mite even suppresses *Varroa* when found in a colony and extinguishes the colony in a short time. It is a fact that these two pests may spread rapidly in Turkey due to the practice of migratory beekeeping and they can cause serious damage to our beekeeping.
Keywords: *Aethina tumida*, honey bee, *Tropilaelaps* spp., Turkey.

Aethina tumida (Küçük Kovan Kurdu; KKK) ve *Tropilaelaps* spp. Akarı; Türkiye Bal Arıları için yakın bir tehdit

Özet: Türkiye, dünyanın en büyük ikinci bal üreticisidir; ancak arıcılık sektörü, özellikle bal ve bal ürünleri ihracatı bu varlıkla uyumlu gelirle desteklenmemektedir. Bunun başlıca nedeni bal arısı hastalık- zararlıları ve balda kalıntı bulunmasıdır. Son yıllarda, (Küçük kovan böceği; SHB) Avrupa'da (İtalya) ve *Tropilaelaps* spp. İran'ın Türkiye sınırında görülmesi nedeniyle bu iki bal arısı zararlısına karşı ilgi artmıştır. Türkiye Cumhuriyeti Tarım ve Orman Bakanlığı bu iki zararlıyı ihbarı zorunlu hastalıklar arasına almıştır. *Aethina tumida*, aslen Güney Afrika'dan gelen ve daha sonra arı kolonilerine adapte olmuş bir meyve böceğidir. 2000'li yılların başında ithal meyvelerle Kuzey Amerika'ya gelmiş ve ciddi arı kayıplarına neden olmuştur. Bugüne kadar 12 ülkeye yayılan SHB, girdiği ülkelerde ciddi ekonomik zararlara yol açmış ve eradike edilememiştir. *Tropilaelaps*, beslendiği ve yaşaması için gereken bal arısı kuluçkaları olmadan uzun süre yaşayamayan bir ektoparazittir. *Tropilaelaps* akarı, bir kolonide bulunduğunda *Varroa*'yı bile baskılar. Koloniyi kısa sürede söndürür. Ülkemizde gezici arıcılık uygulaması nedeniyle bu iki zararlının hızla yayılacağı ve arıcılığımıza ciddi zararlar vereceği bir gerçektir.
Anahtar sözcükler: *Aethina tumida*, bal arısı, *Tropilaelaps* spp., Türkiye.

Introduction

According to the statistics of the United Nations Food and Agriculture Organization (FAO) for 2019, there are approximately 90 million honey bee colonies all over the world. The highest numbers of colonies are found in Asia (48.4%), followed by Africa (19.3%), Europe (18.0%), America (12.9%) and Oceania (1.5%) (16). Considering TURKSTAT's 2020 data (17, 35), Turkey ranks third in the world in terms of the number of colonies (approximately eight million in total) and second in honey production (104,000 tons per year). Beekeeping activities are performed in 81 provinces of Turkey (36) by 82 662 registered professionals whose main source of income or side income is beekeeping, as well as families engaged in

hobby beekeeping. The most important risks of beekeeping in our country are breeding queen deficiency, bee diseases and pests, residue problems, and marketing (17, 36).

The occurrence of some diseases and predators in honey bees not only threatens the future of the colony and the apiary but also closely concerns human health (6, 16). As with all mammals, many factors threaten bees together with environmental conditions. Various arthropods such as *Varroa*, wax moth and *Tropilaelaps* play an important role in the transmission of various disease agents to colonies. Especially in recent years, global climate change and international trade pose an increasing threat to honey bee health. *Varroa* spp is the best example of many bee diseases, especially after the 1970s, the spread of other new bee pests all over the world (16).

In recent years, *Aethina tumida* (small hive beetle (SHB); South African origin) and *Tropilaelaps* spp. (Southeast Asian origin) spread rapidly around the world, SHB reaching Italy and *Tropilaelaps* western Iran. Both predators are known to be more pathogenic than *Varroa* spp. (4, 5, 7-9, 28, 29). One of the most important problems of beekeeping in our country is bee diseases and pests. Uncontrolled migratory beekeeping causes these diseases and pests to spread rapidly throughout the country in particular. Parasitic agents are at the forefront of these diseases and pests due to the damage they cause and the need for drug use-residue problems (7, 8, 10, 12, 13, 16).

Aethina tumida (Small Hive Bettle)

Small hive beetle, commonly known as *A. tumida*, is an insect located in the south of the Great Sahara in the African continent and is a close relative and/or one of the insects that attack plants in the form of strawberries, melons, and saplings and feed on debris. For this reason, it is estimated to be a fruit pest (7, 8, 12, 13).

Significance of the Disease: It was first described by Lundie in 1940 and detected in beehives in South Africa (24). It affects detrimentally honeycombs and weak colonies stored in the African continent, while strong colonies can deal with this disease. *A. tumida*, thought to have entered the United States through fruit and vegetable imports, was first identified in Florida in May 1998. Later, it was noticed in 20 regions within 2 years and spread to 7 states (18). Due to both the climate characteristics and the absence of natural enemies, it has become more harmful in America than the African continent and has reached a level that threatens beekeeping in this continent (26-28, 31-34). So far, the estimated economic loss has been around $200 million, and the insect has reportedly caused the extinction of about 20,000 colonies (27, 28). It should be kept in mind that epidemics in the United States may create major problems in the future, as these regions are similar to Turkey when the climatic characteristics of these regions are considered, and it should not be forgotten that it would be a very rational behavior to take precautions for this (31, 35).

Etiology:

Phylum: Artropoda
Subphylum: Antennata
Class: Insecta
Order: Coleoptera
Family: Nitidulidae
Genus: *Aethina*
Species: *Aethina tumida* (Murray) (30).

Adult *A. tumida* is 5-7 mm long, and has three pairs of legs, two pairs of wings, and a dark brown almost black color. The bees cannot sting these pests due to the strong chitin layer covering their body. The dorsal side of their body is covered with hair and spikes and they have strong wings (Figure 1A, B). They are able to fly up to 24 km, thus they can spread rapidly in the environment. Parasite eggs are fusiform and they are laid irregularly into the deep corners of the hives, which are hard to reach, and cells (Figure 1C). Its larvae are long, oval, and approximately 11 mm long, and have a whitish-light brown color (Figure 1D). Despite the larvae looking like a wax moth, they have three not well-developed legs and well-developed dorsal spikes, which distinguish them from the wax moth. The larvae feed on pollen and honey. They defecate in the cells (18, 20, 31).

Biology: *A. tumida* is brown and about the size of a mature insect. Matures emerging from the pupa attack the colonies again. Although both larvae and adults of *A. tumida* feed on honey and pollen, they also consume bee eggs from time to time. They do not only need beehives to stay alive and lay eggs; these insects also live in some fruits, especially melons, and they can lay their eggs on these fruits. Studies have shown that adults of *A. tumida* survive for up to two weeks without food and water. This development, which is in four periods, is completed in 38-82 days (Figure 2). Temperate climatic regions contribute to the formation of five generations per year. It can contribute to deflating of the hive as it can lay a large number of eggs (7, 8, 10, 18, 20, 31).

Distribution: Although *A. tumida* is known mainly as a fruit pest, it was first seen in beehives in South Africa in 1940 (18, 24). Seasonal conditions are crucial in the ecology of the parasite, and it is reported the existence of the pest in regions close to the tropical climate. Factors such as beekeeping, fruit growing and humidity in South Africa have greatly affected the development and breeding of *A. tumida* and increased its prevalence. The phenotypic characteristics of bees are important against this pest, and it poses a serious threat to bee colonies that are not weak and vicious (31).

Figure 1. *Aethina tumida* Adult A, B (dorsal and ventral), C Eggs and D Larva (Buss and Ratikan, University of Florida).

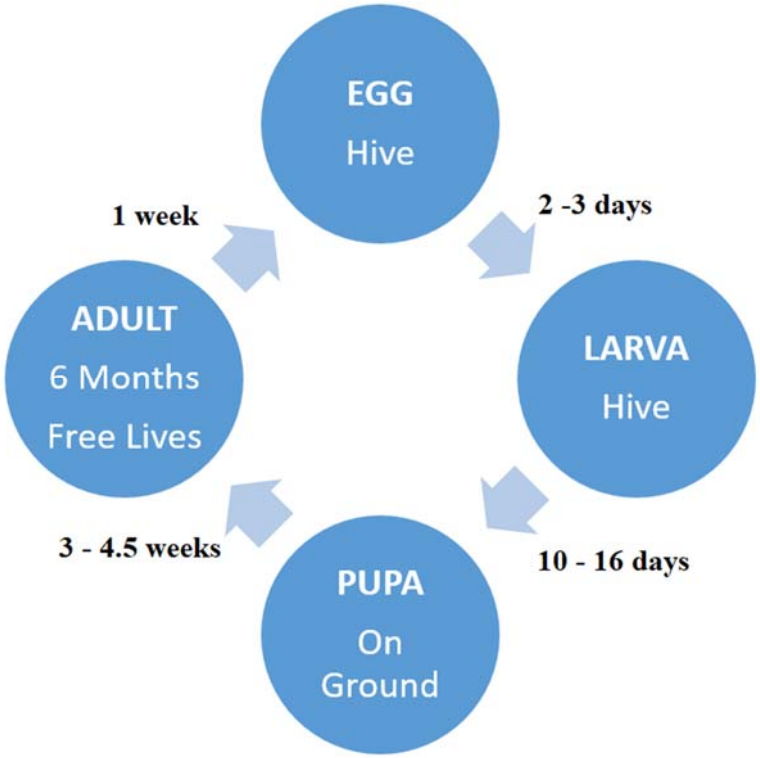

Figure 2. Biology in the ideal environment (Original).

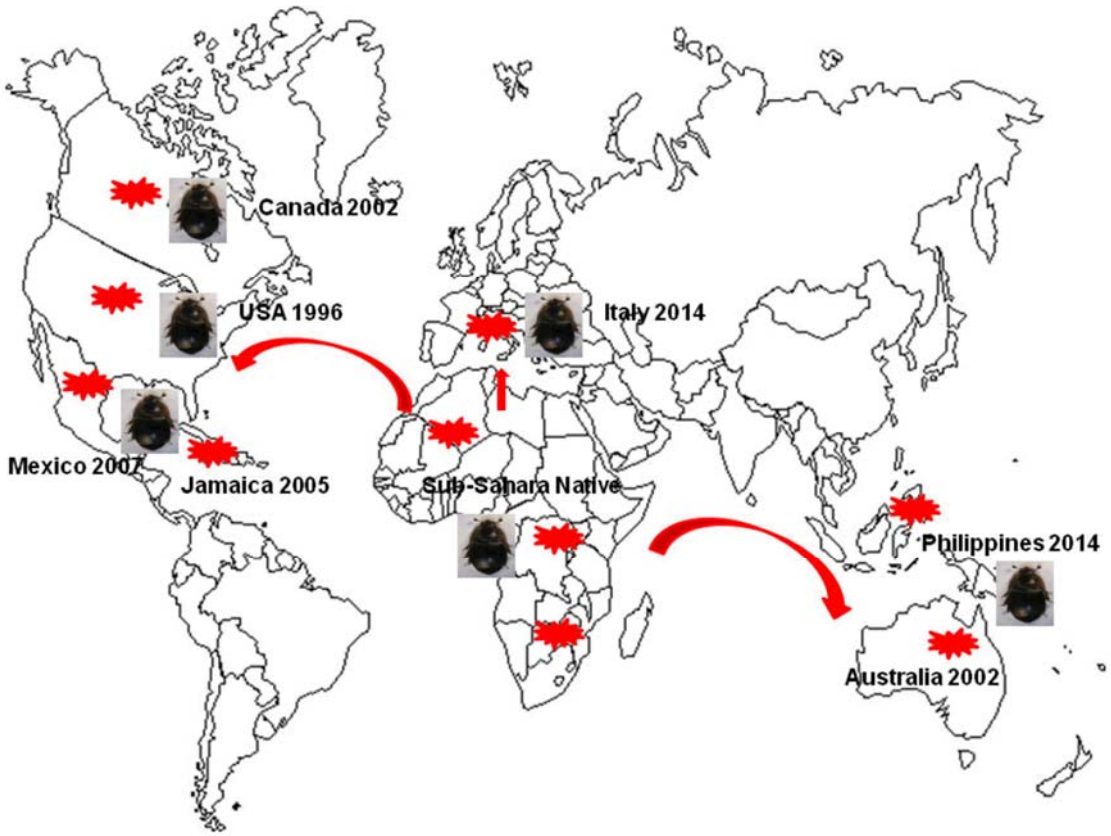

Figure 3. Prevalence of SHB (19).

According to the OIE, *A. tumida* has been recorded in North and Central America (USA, Canada, Mexico, Nicaragua, El Salvador), Cuba, Australia and southern Italy (5). There is no reliable information about its presence in Africa, but this pest is endemic in this region (7, 8, 10). Although this disease is not officially reported to the OIE, the OIE provides information on the presence of this pest in Egypt (2000) and Portugal (2004) in the Land Animal Health Code (5, 7). Finally, combined genetics data and beeswax trade data (FAO) for 12 confirmed small hive beetle (SHB) invasions (USA, Mexico, Jamaica, Cuba, Canada, Brazil, Costa Rica, USA [Hawaii], South Korea, Australia, Portugal and Italy) (Figure 3) (10). SHB has detected that the maximum entropy algorithm (Maxent Analysis) can damage the temperature factors in Southern Europe and North Africa in near future (21).

Clinical Symptoms: Due to their natural enemies and the hygienic behavior (aggressive) of African bees (*Apis mellifera scutellata*), great losses did not occur in the African continent and *A. tumida* population was kept under control. However, temperate countries (such as the USA) where it has been newly infected have become the primary pest (18, 20).

Pollution of honey and the use of pesticides in the control cause economic losses, and both the continuation of the hive and the vegetative production through pollination are endangered due to the weakening or extinction of the colony (37).

In the colonies of *A. tumida*, honeycombs are destroyed, bee larvae and eggs are consumed by the insect, a large number of larvae and eggs of *A. tumida* are found in the comb cells and a sour rotten orange (citrus) smell is felt as a result of fermentation due to the excrement they produce. Bees leave the honeycombs with brood and honey. Even when honey is left out of the hive, it is not consumed by other bees and insects (7, 10, 18, 31, 32).

Diagnosis: The diagnosis of infestation is made by identifying the larval and adult forms of the agent, as well as its eggs, which are much smaller (two-thirds) and few in number than bee eggs. Especially perception of the typical odor is suggestive of the disease and 15×15 cm honeycomb traps (which will remain for at least 3 days) can be used to catch the agent. If there are small hive beetles in a colony, they can be easily seen while the beekeeper is cleaning the hive. Insects are generally insusceptible to light conditions and will quickly seek shelter. Therefore, beekeepers can have an idea about the number of insects seen in the colony by checking the top of the hive. Once many insects are present at the top of the hive, brood chambers should be scrutinized to reveal the total insect population (7, 10, 20, 32, 34). To demonstrate

potential field use in recent years, the laboratory has tested sensitive loop-mediated isothermal amplification (LAMP) assay test program for rapid identification of *A. tumida* in honey bee hive ground debris and from crude extracts of samples or partial samples. In *A. tumida*, it is sensitive to an existing real-time polymerase chain reaction (PCR) and can be used worldwide as a useful biosafety tool for rapid detection of SHB (23, 32).

Control: Despite the use of many chemicals, complete success has not yet been achieved against *A. tumida*. However, strip applications of coumaphos have yielded relatively positive results. For protection, care should be taken to place the hives at least 20-30 cm above the ground with a forward inclination of 4°C. When combs with small hive beetles were kept at -12 °C for 24 hours or at -20 °C for 6-8 hours, it was observed that all developmental stages disappeared (28). The most assuring way to detect the presence of SHB is to use cardboard placed at the bottom of the hive. This method has been used successfully in the USA. This method is based on the SBH's tendency to search for dark habitats and holes and use them for hiding (10, 18, 20, 28).

Pesticide application containing 40% permethrin (40% EC) against larvae and pupae in the soil provides successful results. Spraying should be implemented late in the evening when the bees returned to the hive. The prepared medicine is applied to the area 45-60 cm wide in front of each hive. The application is repeated at 30-day intervals (18, 20, 31, 37).

Intra-hive drug control is one of the most dangerous and undesirable methods because of the risk of drug contamination to honey. Traps used to capture and destroy SHB in honey bee colonies are effective to reduce the contamination level (7, 10, 18, 22, 28). In addition, adult insects are susceptible to this fungus and three other general entomopathogenic fungi isolated (*M. anisopliae*, *Beauveria bassiana* and *Hirsutella illustris*). The results revealed that entomopathogenic fungi are an alternative way to control SHB (26, 28).

Traps; Beetle Eater Trap: It is the most effective plastic trap among the traps (Figure 4). It has a comb-shaped upper cover with 0.3 cm openings large enough for the SHB to pass through. SHB entering through this opening dies by suffocation (8, 28).

Beetle Barn Trap: This type of trap has a flat and rectangular structure. Made of black plastic, it is 9 cm long, 7.5 cm wide and 0.7 cm high. There are small openings of 1.3×0.3 cm on each side. SHB adults can pass through these openings, but not honey bees. A 2 cm^2 strip containing 10% coumaphos was placed in the middle of the trap. This trap can be put on the hive bottom board or the top of the frames (7, 8, 28).

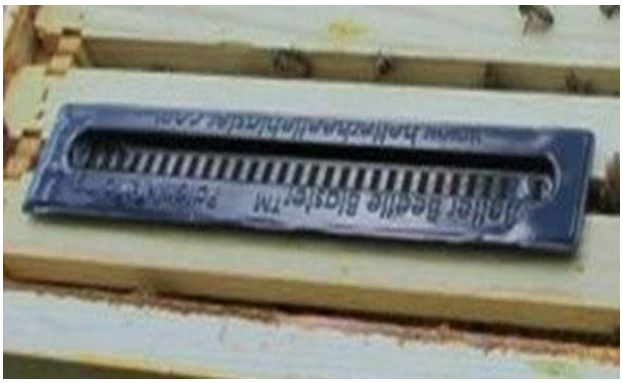

Figure 4. Beetle Eater Trap (Possum Catchers Pty Ltd.).

Figure 5. Beetle Barn Trap and Hood Trap (https://www.google.com.tr/Fbeetle-barn-reusable- beetle-hood trap).

Hood Trap: Oil or apple cider vinegar is placed in this trap made of transparent plastic, which is 15 cm long, 2.5 cm wide and 8 cm deep. The cover on it has a 12.8×0.3 cm opening so that the SHB adult can enter, but the adult bee cannot. This trap, which is attached to the bottom of an empty frame, is placed near the hatchery or on a hive floor as the first or tenth frame (8, 28).

Hive Bottom Board Trap, Full Pollen Traps: The SHB adult reaching the hive comes directly into the trap and falls into the tray with mineral oil at the bottom through the opening on the trap and disappears by suffocating (8).

What needs to be done to be protected our country?

- The purchase of all kinds of beekeeping materials and breeding stock from contaminated countries should be kept under control.
- Suspicious hives should be reported to the provincial and district directorates of agriculture, research institutions and universities.
- Manufacturers and organizations should be informed about this factor.
- Especially beekeepers' collection areas (Thrace, Muğla, etc.) should be kept under control during the season.
- The life cycle should be known in detail, and knowledge should be gained on how to recognize the larva and adult.

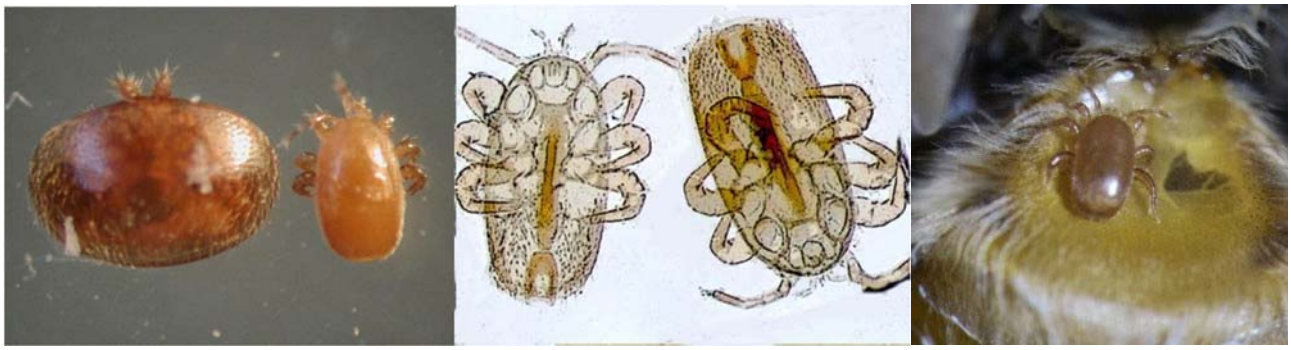

Figure 6. *Varroa* and *Tropilaelaps* spp. (ACBO), adult mite (Current in Insect Science) distributions and biology.

- Inspection of hives should be routinely performed to monitor the presence of SHB.

- In the importation of bumblebee colonies for pollination, control should be carried out in terms of SHB.

- Care should be taken in terms of the SHB factor when importing raw beeswax and honeycomb honey, as well as soil or compost planted/planted plants for commercial purposes.

- Honeycombs stored with honey or filtered should be checked regularly against the risk of SHB.

- A fluorescent light source placed on the ground at the honey extraction site will attract the larvae that seek soil to pupate. These larvae are then collected and destroyed.

- Since the SHB population develops easily in light sandy soils, apiary should not be established in these areas, it should be tried to be created on heavy clay soils (8, 10).

Tropilaelaps spp. mite

Significance of the Disease: *Tropilaelaps clareae* is a mesostigmatid parasite belonging to the Laelapidae family and is a parasite of both adult and juvenile honeybees. The agent, which is estimated to be transmitted from rat colonies, was first detected in *Apis dorsata* in the Philippines in 1961 (1-3, 9, 11, 13). After the European honeybee (*A. mellifera*) was brought to Asia, the mites infecting this species have become one of the most dangerous parasites of *A. mellifera* today. Today, although their spread is limited to the Asian continent and Kenya, they are a very serious threat to world beekeeping. Mites feeding on larvae and pupae of honeybees cause damage similar to *Varroa* infestations (15). After being observed in Iran, the mite was included in the list of notifiable diseases in our country in 2012 (9).

The place of the *Tropilaelaps* types in the system is as follows (9, 15, 29, 30);

Class: Arachnida
Underclass: Acari
Above knee: Parasitiformes Sequence: Mesostigmata
Family: Laelapidae
Genus: *Tropilaelaps*

Tropilaelaps clarae (*Apis mellifera, Apis dorsata*), *Tropilaelap mercedesae* (*Apis mellifera, Apis dorsata*), *Tropilaelaps koenigerum* (*Apis dorsata*), *Tropilaelaps thaii* (*Apis laboriosa*).

Adult mites are arthropods less than 1 mm long, dorsoventrally flattened, and longer than wide. Colors range from bright red to brown (Figure 6). It can be observed that they move quickly on honeycombs and on adult bees in the infected colonies. Their bodies are not able to divide into head, thorax, and abdomen and are covered with a hard layer of chitin (1, 9, 11, 15, 27, 30, 33).

Distributions and Biology: The natural geographic range of *Tropilaelaps* species is generally the tropical and subtropical regions of Asia where they cause very significant economic losses. The prevalence of the parasite has increased significantly in the last 50 years, although it does not have as much intercontinental spread as *Varroa destructor*. *Tropilaelaps* spp. was first seen in the Philippines in 1961, and has spread to honeybees in Vietnam, India and Afghanistan 7 years later (1, 15, 27, 29). Today, it has been detected in Afghanistan, Burma, China, Hong Kong, Java, Malaysia, Pakistan, Philippines, Taiwan, Thailand, Vietnam and Iran in Asia. It has been reported as a case report in Kenya. When *T. clareae* was above 46% when coexisting with *V. destructor* in *Apis mellifera* colonies, *V. destructor* remained below 5%. It was found more intensely in baby bee cells, showing that it reproduces faster than *Varroa* and colony destruction is more. Studies have shown that *T. clareae* infestations are faster, but they are affected more quickly by drug applications (3, 29, 33). No parasite has been found in our country in the studies carried out to date (6, 8, 36).

The development of *Tropilaelaps* spp. is very similar to the biology of *V. destructor*. As in *Varroa,* its life cycle has two periods: a) pre-reproductive (phoretic) period, b) reproductive (larval) period. In the development of the parasite, there are egg-larva-protonymph- deutonymph- and adult stages (6, 29, 30, 33).

Pathogenity and Clinical Symptoms: The pathogenicity of *Tropilaelaps* mites is similar to that of *Varroa*. As the mite larvae developing in the brood buds feed on the hemolymph of the honeybee fry (larva, pupa), they prevent them from getting the essential nutrients necessary for their development. As a result, damage and death occur in bee broods (6, 22, 29). Because the parasite is fully adapted to the young eyes, it is more dominant and disrupts the development environment of *Varroa* when co-existing with *Varroa destructor* in the same colony (6, 8, 22, 30).

- It causes rapid pup deaths and prepares the environment for secondary bacterial and viral factors. Although *Tropilaelaps* infestations are also seen in adult bees, they cannot be fed wellwith hemolymph because their mouth organelles are atrophic (6, 8).

- They exacerbate the pathogenicity by transmitting the deformed wing virus (DWV) to the honeybees (Figure 7). The most important signs are parasites in many different developmental stages when the brood eyes open, and bees without wings, deformed legs, or missing extremities in the hive exit hole. The abdomen is shortened (6, 8, 22).

- The number of hive colonies decreases rapidly. It is noteworthy that honey flow time yield islow. Especially in untreated colonies, the rapid decrease in the number of *Varroa* and as a result the extinction of the colony or the abandonment of the hive can be seen (6, 8, 30).

Diagnosis: It is done by detecting the cause of the disease. Morphologically, it should be differentiated from other ectoparasites, especially *Varroa*. The parasite moves longer and faster than *Varroa*. Approximately 100-200 adult bees are placed in powdered sugar, shaken linearly for 5 minutes, passed through 0.4 mm sieves, and the presence of the parasite can be revealed, as well as the presence of closed brood cells (6, 8). It is easier to reveal the presence of honey bees, especially in the pupal period. By placing a sticky light-colored paper on the bottom of the hive and giving smoke to the colony, parasites falling on the paper are seen (6, 8, 30).

Control: Successful results can be obtained with biological and chemical control methods.

- In the biological method, the queen bee is imprisoned in a small cell in the hive, preventing the formation of new brood cells and disrupting the breeding environment of the parasite. Removal of closed brood cells is a good option in colonies where the parasite is positive (8).

- In chemical control, fluvalinate vapor, flumethrin, coumaphos and amitraz treatment for *Varroa* treatment are used. In recent years, the use of thymol, acetic and formic acid has been tried. Positive results have been obtained from the use of formic acid for a total of 80 ml/4 weeks against larval infestation and acetic acid against adult beetles (6, 8, 27).

- The use of formic acid was found to be healthier. When the disease factor is detected, it should be reported, colonies should be strong and pollen-trapped, especially migratory beekeeping, which is an important fact of our country, should be controlled (8, 25).

- Beekeepers should be informed about this issue, as *T. clareae* is a mandatory factor. Breeding, and relocating the colony give successful results (8).

- Bee and bee products should not be bought from countries such as Iran, and colonies on the Iranian border should be pushed back 5-10 km from the border (6, 8, 33).

General Recommendations

- Establishing consulting and supporting institutions that could provide required information on standards, improved beekeeping, alternative products to honey, control of bee diseases and pests, availability of pastures, resources of nectar, etc.

- Enforcing practice of record-keeping on production and health at the apiary.

- Stimulating certified reproduction apiaries which will produce healthy reproduction material such as queen bees and colonies.

- Stimulating good bee husbandry, good hygiene practice, and apiary management.

- Further studies that will shed light on the above issues, and influence the future control programs of bee diseases have to be conducted.

- The eight basic IPM (Integrated pest control) beekeeper principles should be used when prevention is limited with protection measures against all bee diseases and pests:

a) Acceptable pest level
b) Preventive culture practices
c) Monitoring practices
d) Genetic control
e) Mechanical control
f) Physical control
g) Biologic control
h) Chemical control

- A control program starting with *Varroa* control will both be a measure to prevent other bee diseases and establish an early warning concept for diseases and pests which can be introduced later (*Tropilaelaps* spp., small hive beetle). Furthermore, the use of chemical medicines will be controlled (4, 5, 8, 17, 27, 33).

References

1. **Anderson DL, Morgan MJ** (2007): *Genetic and morphological variation of bee parasitic Tropilaelaps mites (Acari; Laelapidae) new and redefined species*. Exp Appl Acarol, **43**, 1- 24.
2. **Anderson DL, Roberts JMK** (2013): *Standard methods for Tropilaelaps mites research*. J Apic Res, **52**.

3. **Anonymous** (2008): Terrestrial Manual Chapter 2.2.6.- *Tropilaelaps* infestation of honey bees (*Tropilaelaps* spp.). OIE 419-423.
4. **Anonymous** (2013): Honeybee Disease in Europe Report to Syngenta Ltd, The Food and Environment Research Agency.
5. **Anonymous** (2013): Terrestrial Animal Health Code Offıcıal Health Control of Bee DiseasesArticle 4.14.1 Bee Diseases OIE.
6. **Aydın L** (2016): Bal arılarında *Tropilaelaps* spp. enfestasyonu. In: Veteriner Hekimliğinde Paraziter Hastalıkları. Türk Parasitol Dern. Yayın No: 24.
7. **Aydın L** (2016): Balarılarında Küçük kovan böceği *Aethina tumida* enfestasyonları. In: Veteriner Hekimliğinde Paraziter Hastalıkları. Türkiye Parazitoloji Derneği Yayın No: 24.
8. **Aydın L** (2016): Animal health Strategy. Analytical Study Honey bee diseases Final Report European and The Republic of Turkey, Technical assistance for preparation of the veterinary strategy document 60p.
9. **Aydın L, Doğanay A** (2021): Bal arılarında *Tropilealaps* spp. enfestasyonu. In: BALARISI Yetiştiriciliği Ürünleri Sağlığı. Ed: A. Doğanay, L. Aydın. Dora Yayınevi.
10. **Bakırcı S** (2021): *Aethina tumida* (Küçük kovan Böceği). In: BALARISI Yetiştiriciliği Ürünleri Sağlığı. Ed: A. Doğanay, L. Aydın. Dora Yayınevi.
11. **Buawangpong N, De Guzman LI, Khongphinitbunjong K, et al** (2015): *Prevalence and reproduction of Tropilaelaps mercedesae and Varroa destructor in concurrently infested Apis mellifera colonies*. Apidologie, **46**, 779-786.
12. **Chantawannaku LP, Guzman LI, Jilian LI, et al** (2015): *Parasites, pathogens, and pests of honeybees in Asia*. Apidologie, **47**, 301-304.
13. **Coffey MF** (2007): Parasites of honeybee. Ea-asc. Agriculture and food. UK-32-34.
14. **Dainat B, Ken T, Berthoud H, et al** (2009): *The ectoparasitic mite Tropilaelaps mercedesae [Acari, Laelapidae] as a vector of honey bee viruses*. Insectes Sociaux, **56**, 40-43.
15. **De Guzman LI, Williams GR, Khongphinitbunjong K, et al** (2017): *Ecology, life history and management of Tropilaelaps mites*. J Econ Entomol, **110**, 319-332.
16. **De Landa GF, Porrini MP, Revainera P, et al** (2021): *Pathogens Detection in the Small Hive Beetle (Aethina tumida Coleoptera: Nitidulidae)*. Neotropical Entomology, **50**, 312-316.
17. **Doğanay A, Aydın L** (2021): Dünya'da ve Türkiye'de Arıcılık. In: Balarısı Yetiştiriciliği Ürünleri Sağlığı. Ed: A. Doğanay, L. Aydın. Dora Yayınevi.
18. **Ellis JD** (2007): The ecology and small hive beetles. PhD Dissertation, Rhodes University. Grahamstown, South Africa.
19. **Giangaspero M, Turno P** (2015): *Aethina tumida*, *an exotic parasite of bees.* Clinical Microbiology: Open Access, **4**, e128.
20. **Hood MV** (2004): *The small hive beetle, Aethina tumida*. Bee World, **85**, 51-59.
21. **Jamal ZA, Abou-Sahara HF, Qamer S, et al** (2021) *Future expansion of small hive beetles, Aethina tumida, towards North Africa and South Europe based on temperature factors using maximum entropy algorithm.* J King Saud University-Science, **33**, 101242.
22. **Khongphinitbunjong K, Neumann P, Chantawannakul P, et al** (2016): *The ectoparasitic mite Tropilaelaps mercedesae reduces western honey bee, Apis mellifera, longevity and emergence weight, and promotes Deformed wing virus infections.* J Invertebr Pathol, **137**, 38-42.
23. **Li D, Waite DW, Fan QH, et al** (2018): *Molecular detection of small hive beetle Aethina tumida Murray (Coleoptera: Nitidulidae): DNA barcoding and development of a real-time PCR assay*. Sci Rep, **8**, 9623.
24. **Lundie, AE** (1940): The small hive bettle *Aethina tumida*, S.A. Depart Agric & Forestry Entomol, Series 3, Science Bulletin 220.
25. **Mahmood R, Wagohoure ES, Sarwar G, et al** (2011): *Effect of tymol and formic acid agains ectoparasitic brrod mite Tropilaelaps clareae in Apis mellifera colonies.* Pakistan J Zool, **43**, 91-95.
26. **Muerrle TM, Neumann P, Dames JF, et al** (2006): *Susceptibility of adult small hive beetle to entomopathogenic fungi*. J Economic Entomol, **99**, 1-6.
27. **Neumann P, Carreck N** (2010): *Honey bee colony losses*. J Apic Res, **49**, 1-6.
28. **Neumann P, Pettis JS, Schäfer MO** (2016): *Quo vadis Aethina tumida? Biology and control of small hive beetles*. Apidologie, **47**, 427-466.
29. **OIE** (2013): *Tropilaelaps* spp. Infestations.
30. **OIE** (2015): Morphological identification of *Tropilaelaps* spp. (adult form).
31. **Pettis JS, Shimanuki HA** (2000): *Observations on the small hive beetle, Aethina tumida, Murray in the United States*. Am Bee J, **140**, 152-155.
32. **Ponting S, Tomkies V, Stainton K** (2021): *Rapid identification of the invasive small hive beetle (Aethina tumida) using LAMP*. Pest Management Science, **77**, 1476-1481.
33. **Ritter W** (2014): Bee health and veterinarians. World Organization for Animals Health, 316 p.
34. **Spiewok S, Neumann P** (2006): *Cryptic low-level reproduction of small hive beetles in honeybee colonies*. J Apic Res, **45**, 47-48.
35. **Spiewok S, Pettis JS, Duncan M, et al** (2007): *Small hive beetle, Aethina tumida, populations I: Infestation levels of honeybee colonies, apiaries and regions*. Apidologie, **38**, 595-605.
36. **TURKSTAT** (2020): Tarım- Hayvancılık. Available at: http://www.tuik.gov.tr. (Accessed Nov 11, 2021).
37. **Valdovinos-Flores C, Gaspar-Ramírez O, Heras-Ramírez ME, et al** (2016): *Boron and coumaphos residues in hive materials following treatments for the control of Aethina tumida Murray*. PLoS One, e0153551.

Veterinary Journal of Ankara Univesity

E-ISSN 1308-2817 Cilt/Volume 69 ● Sayı/Number 3 ● 2022

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