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Research Article

Evaluation of the Effect of Different Doses of Butaphosphan and Cyanocobalamin Combination in Dairy Cattle with Subclinical Ketosis [1]

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Abstract

This study was conducted to assess the effects of different doses of butaphosphan-cyanocobalamin combination onbody condition score (BCS), beta-hydroxybutyrate (BHBA) and reproductive parameters in cows with subclinical ketosis (SCK). Holstein-Friesen cows (n=544) were checked for BHBA concentration. Cows with SCK (n=53, BHBA ranging from 1.00 to 3.00 mmol/L) were assigned randomly to receive saline (C0, n=18), 5 mL/100 kg BW (C5, n=18) or 10 mL/100 kg BW (C10, n=17) butaphosphan-cyanocobalamin combination. BHBA concentration was measured on d 0, 10, and 18 relative to treatment application. BCS was evaluated weekly until d 60 postpartum. For reproductive parameters cows were monitored until d 150 postpartum. The median reduction in blood BHBA concentrations was 28, 57, and 75% for C0, C5, and C10, respectively. NEFA and total bilirubin concentrations were significantly decreased in C10 group. The relative median change in BCS as compared to baseline was 17, 12, and 6% for C0, C5, and C10, respectively. Between d 15 and 25 postpartum uterine involution was completed in 44, 83, and 88% of cows in groups C0, C5, and C10, respectively. Interval from calving to first insemination in group C10 was shorter than control group. Overall pregnancy rate was not different among groups. In conclusion butaphosphan-cyanocobalamin combination decreased severity of hyperketonemia, stimulated uterine involution, shortened time to first insemination and increased pregnancy rate.

Keywords: *Beta hydroxybutyrate, Butaphosphan, Cyanocobalamin, Subclinical ketosis, Reproductive performance*

Subklinik Ketosisli Süt İneklerinde Farklı Dozlarda Butafosfan ve Siyanokobalamin Kombinasyonunun Etkisinin Değerlendirilmesi

Özet

Bu çalışma, subklinik ketozisli (SCK) süt ineklerinde farklı dozlarda butafosfan-siyanokobalamin kombinasyonunun vücut kondisyon skoru (VKS), beta-hidroksibütirat (BHBA) ve reprodüktif parametreler üzerine etkilerini değerlendirmek amacıyla gerçekleştirildi. Holştayn-Frizyan inekler (n=544) BHBA yönünden kontrol edildi. SCK'li inekler (n=53, 1.00-3.00 mmol/L BHBA); 4 gün süresince günlük salin uygulanan (C0, n=18) ve canlı ağırlığa 5 mL/100 kg dozunda (C5, n=18) ya da canlı ağırlığa 10 mL/100 kg dozunda (C10, n=17) butafosfan-siyanokobalamin kombinasyonunun uygulandığı gruplara rastgele olarak ayrıldı. BHBA düzeyleri; 0, 10 ve 18. günlerde ölçüldü. VKS postpartum 60. güne kadar haftalık değerlendirildi. Reprodüktif parametreler için inekler doğum sonrası 150. güne kadar takip edildi. Kan BHBA düzeyinde medyan azalma C0, C5 ve C10 grubunda sırasıyla %28, %57 ve %75 olarak gerçekleşti. NEFA ve total bilirübin düzeyleri C10 grubunda anlamlı şekilde azaldı. VKS'un relatif medyan değişimi baseline ile karşılaştırıldığında C0, C5 ve C10 grubunda sırasıyla %17, %12 ve %6 olarak belirlendi. Postpartum 15 ve 25. günler arasında uterus involüsyonu C0, C5 ve C10 gruplarında sırasıyla %44, %83 ve %88 tamamlandı. C10 grubunda buzağılama ilk tohumlama aralığı kontrol grubundan daha kısaydı. Ortalama gebelik oranı gruplar arasında farklılık göstermedi. Sonuç olarak butafosfan-siyanokobalamin kombinasyonu; hiperketonomi şiddetini azalttı, uterus involüsyonunu uyardı, ilk tohumlama zamanını kısalttı ve gebelik oranını arttırdı.

Anahtar sözcükler: *Beta hidroksibütirat, Butafosfan, Siyanokobalamin, Subklinik ketozis, Reprodüktif performans*

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INTRODUCTION

Subclinical ketosis is a consequence of transition period linked to negative energy balance (NEB), which leads lipolysis of body fat reserves and hyperketonemia [1]. Plasma BHBA greater than 1.2 mmol/L is considered ketosis [2]. However, definition of SCK is not consistent. In a study, SCK was considered whenblood concentration of BHBA higher than 1.00 mmol/L [3,4], while the other one indicated the SCK in the concentrations of BHBA above 1.40 mmol/L [5,6]. Negative energy balance is compensated by increased lipomobilization, which results in formation of BHBA produced by incomplete lipid oxidation in the liver immune system is adversely affected via hepatopathies-associated with ketotic metabolic status [7,8]. BCS loss resulting from fat mobilization is typical in ketotic cows [9]. This is often significant in cows with BCS greater than 3.25 during prepartum. These cows lose >0.75 BCS within 2 month after parturition [10,11]. Butaphosphan-cyanocobalamin combinationcontains both vitamin B12 and butaphosphan (alpha amino phosphonic acid). Researchers indicated that butaphosphan-cyanocobalamin combination could be supportive in correcting the metabolic status of high-producing cows [12,13] and controlling of SCK [14]. BCS loss was minimal in cows administered with 2 ml butaphosphan-cyanocobalaminon d 3 postpartum [12]. In other studies, injection of vitamin B12 [15] alone or in combination with butaphosphan [12] improved lactation yield. Cows with the high blood BHBA concentration between 2 and 15 day in milk (DIM) are more likely to reduce the first service conception rate and to yield less milk in the first 30 DIM [16] and to increase the odds of metritis, clinical ketosis, lameness and displaced abomasum [17]. Several reports indicate that this product had more likely positive effect to reduce blood BHBA concentration and to improve milk yield and general health status in animals suffering from SCK [18-20]. However, the short and long term impact of combined cyanocobalamin and butaphosphan treatment on reproductive measures parameters, such as uterine involution, ovarian function, days open and pregnancy rate has not been shown. Therefore, the objective of this study was to evaluate the effects of different dosages of combined cyanocobalamin and butaphosphan on BCS, blood BHBA concentration and reproductive parameters.

MATERIAL and METHODS

Ethical Approval

The ethics committee of the Ankara University (report no: 2009-45-214) approved the protocol used in this study.

Animals

This study was performed on 13 dairy farms in Turkey. All Holstein-Friesen cows were kept in loose housing with slatted floors and were milked twice daily. The milk yield was recorded after each milking. The feeding routine and composition in most part of the farms were generally standardized and calculated according to milk yield. The ingredients of ration for cows in lactation (kg/day/cow) were consisted of corn silage alfalfa hay, grass hay, straw, compound feed, barley grain, corn grain, calcium carbonate, salt, dicalcium phosphate, feed grade urea, and vitamin-mineral premix. The mean ME value of rations was 2600 kcal/kg DM. Water was provided *ad libitum*. The mean age in groups of C0, C5 and C10 were 4.83±2.13, 4.71±1.13, 4.82±1.24 respectively. The mean lactation number in groups of C0, C5 and C10 were also 2.5, 2.3 and 2.5, respectively.

Experimental Design

Five hundred and forty cows were screened with a hand-held meter (Precision Xceed, Abbott Diabetes Care®, Abingdon, UK) in whole blood from the coccygeal vein for BHBA concentration within 7-15 d postpartum *(Fig. 1)*. Of these, 53 cows with a blood BHBA concentration between 1.00-3.00 mmol/L were considered to have SCK at any point from d 7 to 15 following calving. They were randomly allocated into one of 3 study groups: group of cows administered intramuscularly with saline (10 mL/100 kg BW; C0, n=18) and with low (5 mL/100 kg/BW; C5, n=18) or high (10 mL/100 kg/BW; C10, n=17) dose of 10% butaphosphan and 0.005% cyanocobalamin combination (Catosal®, Bayer Animal Health, Leverkusen, Germany), every day for 4 days, starting from on d 11.4±2.79, 12.9±2.09 and 12.6±2.30 postpartum (pp) respectively, and it was not different among groups *(Fig. 1)*.

In addition, the concentrations of creatine kinase (CK), glutamate dehydrogenase (GLDH), cholesterol (CHO), aspartate amino transferase (AST) total bilirubin, indirect bilirubin (BID) (ERBA® Mannheim, Germany) and Non-esterified Fatty Acid (WAKO® Diagnostics NEFA-HR2) were measured spechtrophotometrically (ERBA® XL 600). The intra-assay and interassay CV for all the parameters were ≤3.4% and ≤7.8%, respectively. Blood samples were collected just before the first administration of butaphosphan and cyanocobalamin combinationand 10 and 18 d after the last administration. Animals diagnosed with clinical ketosis (BHBA >3 mmol/L) and secondary disease were not included to the study. Milk yield was recorded daily for 30 days. Body condition was evaluated by three person on d0, 10, 18, 30, 45 and 60.

The intervals from calving to first ovulation and from calving to morphologic uterine involution were categorized as d 15-25, 26-30, 31-35, 36-40, and ≥41 days due to weekly examination. The morphologic uterine involution was evaluated by rectal palpation if the uterus was returned to lie within the pelvic cavity [21]. Pregnancy diagnosis was performed twice by ultrasonographic examination 30 and 60 d after insemination. Cows that returned to estrus before the pregnancy check were re-inseminated.

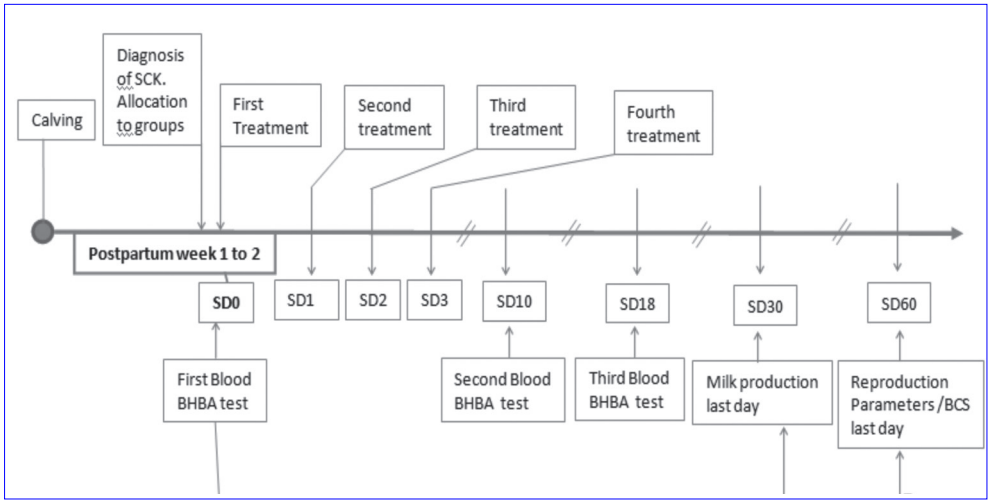

Fig 1. The experimental plan. Conception rate was observed up to 150 d postpartum. **SD:** Study day

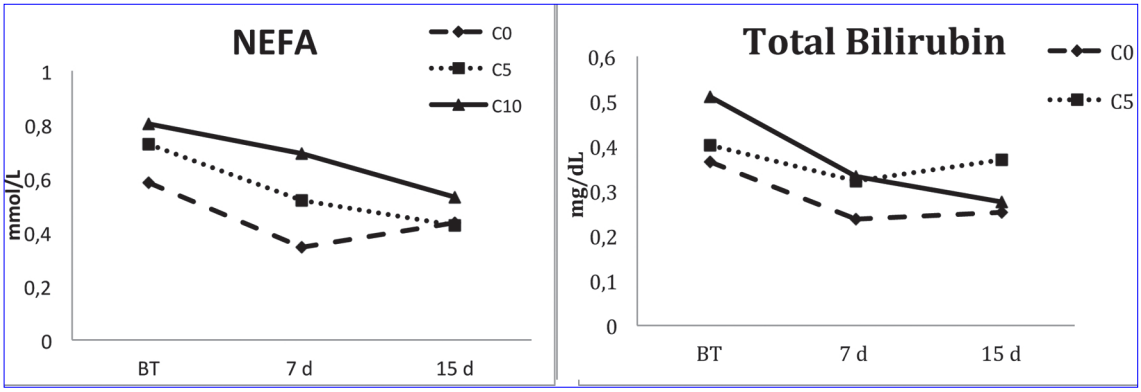

Fig 2. Effect of multiple injections of cyanocobalamin and butaphosphan administered on day 7 and 15 postpartum on the serum NEFA and Total Bilirubin concentrations of dairy cows. C0 = 10 mL/100 kg BW saline; C5 = 5 mL/100 kg/BW butaphosphan-cyanocobalamin combination; C10 = 10 mL/100 kg/BW butaphosphan-cyanocobalamin combination. **BT:** Before treatment, **7d:** Seven days after treatment, **15d:** Fifteen days after treatment *P=0.002, ** P<0.05

The reproductive parameter information was obtained until d 150, which covered intervals from calving to first ovulation, morphologic uterine involution, interval from calving to first insemination, the number of insemination, days open, and pregnancy rate (%, number of animals pregnant/number of inseminated animals).

Statistical Analysis

All data were subjected to the Mean Procedures for descriptive statistics. The non-parametric Wilcoxon-Mann-Whitney-U-Test was used for group comparisons. All tests were performed two-sided with an α value of 0.05. The medical relevance of the differences between groups was quantified using the Mann-Whitney superiority measure (MW). The MW-measure (0.0 to 1.0) reflects the probability that a randomly selected animal of the test group is better off than a randomly selected animal of the control group, while 0.5 denoting equality. If the hole confidence interval(CI) is above the equality line in the graphic, it is considered that superiority is proven (P<α). Wilcoxon-Mann-Whitney-U-Test also was applied for BHBA and BCS percent change from baseline without any LVCF-option.All statistical analysis were performed using the validated statistic program TESTIMATE Version 6.5 from IDV Datenanalyse und Versuchsplanung (Germany).

RESULTS

A total of 544 cows were screened for subclinical ketosis between d 7 and 15 postpartum. The SCK prevalence was 9.74% at the BHBA cut-off concentration of 1.0 mmol/L. The median and mean values of age, BW, and lactation numbers for cows with SCK were homogenous across the groups. The blood BHBA concentrations on the study days revealed insignificant alterations, except for decrease in BHBA for cows in C10 as compared to C0 on d 18 *(Table 1)*. The tendency to decrease in BHBA was highest in group C10, but was not different from that in C5 blood BHBA concentration for C0 did not change during the experiment. The percent change in blood BHBA concentration from baseline during sampling on d 10 and 18 for C5 (P=0.04 and P=0.005) and C10 (P=0.006 and P=0.00001) was significant. The serum NEFA and total bilirubin *(Fig. 2)* concentrations were lower in C10 group after treatment than C0 group

Table 1. *The median and mean blood BHBA levels (mmol/L) during experiment*

Groups *	d 0 Mean±SD (Min/Max) Median	d 10 Mean±SD (Min/Max) Median	d 18 Mean±SD (Min/Max) Median
C0 (n=18)	1.25±0.40 (1.0/2.6) 1.05	1.11±0.75 (0.2/3.0) 0.95	1.18±0.88 (0.4/3.0) 0.90
C5 (n=18)	1.76 ±0.74 (1.0/3.0) 1.40	1.07±0.85 (0.1/2.9) 0.75	0.85±0.78 (0.2/2.7) 0.60
C10 (n=17)	2.15±0.62 (1.2/3.0) 2.10	1.01±0.48 (0.2/1.8) 1.00	0.64±0.51 (0.2/2.4) 0.50**

**C0 = 10 mL/100 kg BW saline; C5 = 5 mL/100 kg/BW butaphosphan-cyanocobalamin combination; C10 = 10 mL/100 kg/BW butaphosphan-cyanocobalamin combination, ** P<0.05*

Table 2. *The median and mean blood parameters of liver function during experiment*

Groups *		GLDH (IU/L)			CHO (mg/dl)			AST (IU/L)			BID (mg/dl)		
		d0	d10	d18	d0	d10	d18	d0	d10	d18	d0	d10	d18
C0 (n=18)	Mean±SD (Min/Max) Median	24.19±15.38 (7.46/55.07) 18.60	32.21±19.65 (6.78/74.59) 21.07	22.54±17.86 (6.89/72.87) 18.37	128.53±42.29 (76/237) 131	150.06±39.86 (82/234) 150	166.33±43.38 (92/230) 166	99.81±44.98 (56.9/249.8) 88.5	133.61±185.41 (52.5/847.5) 93.1	85.35±23.66 (57.9/153) 82.7	0.15±0.09 (0.02/0.31) 0.19	0.12±0.06 (0.01/0.26) 0.11	0.14±0.08 (0.03/0.28) 0.14
C5 (n=18)	Mean±SD (Min/Max) Median	35.12±29.53 (5.74/89.20) 16.94	38.68±25.22 (4.71/75.50) 35.43	32.86±27.67 (7.41/89.92) 17.49	137.87±59.94 (63/301) 138	158.81±75.76 (77/385) 137	192.06±70.68 (71/289) 165.50	96.61±35.63 (58.30/198.20) 92.5	99.8±29.94 (55/171.60) 94.8	85.33±22.34 (50.5/132.8) 93.7	0.21±0.15 (0.06/0.47) 0.22	0.18±0.1 (0.02/0.32) 0.21	0.16±0.11 (0.01/0.37) 0.13
C10 (n=17)	Mean±SD (Min/Max) Median	37.95±27.49 (5.57/82.50) 27.39	43.77±28.41 (3.79/83.66) 42.44	49.37±28.75 (5.80/89.40) 52.71	119.36±33.61 (60/175) 117	154.07±65.64 (32/276) 157.50	193.21±64.76 (65/287) 198	102.65±39 (68.7/195.2) 85.5	96.17±33.02 (36.8/156) 101.15	88.1±20.72 (44.8/128.8) 85.55	0.22±0.12 (0.06/0.40) 0.17	0.19±0.09 (0.02/0.32) 0.21	0.18±0.11 (0.06/0.36) 0.17

** C0 = 10 mL/100 kg BW saline; C5 = 5 mL/100 kg/BW butaphosphan-cyanocobalamin combination; C10 = 10 mL/100 kg/BW butaphosphan-cyanocobalamin combination*

Table 3. *The median and mean values of milk production (first 30 d, kg)*

Groups*	Statistics**	Milk Production (kg)
C0 (n=18)	Mean±SD (Min/Max) Median (LQ/UQ)	640.8±201.2 (366/1182) 602.8 (532/720)
C5 (n=18)	Mean±SD (Min/Max) Median (LQ/UQ) W-Mann-Whit-U test (LB/UB)*** *P*<0.0096	863.2±201.2 (284/1313) 845.3 (714/1059.5) 0.75 (0.56/0.93)
C10 (n=17)	Mean±SD (Min /Max) Median (LQ/UQ) W-Mann-Whit-Utest (LB/UB)*** *P*<0.05	779.9±235.0 (302/1228) 811 (635.60/1002.50) 0.69 (0.50/0.87)

** **C0** = 10 mL/100 kg BW saline; **C5** = 5 mL/100 kg/BW butaphosphan-cyanocobalamin combination; **C10** = 10 mL/100 kg/BW butaphosphan-cyanocobalamin combination; ** **LQ** = Low Quarter; **UQ** = Upper Quarter; **LB** = Low Bar; **UB** = Upper Bar; *** **0.29/0.71** = large difference; **036/0.64** = medium sized difference; **0.44/0.56** = small difference; **0.50** = equality*

10 (*P*=0.002), 18 (*P*=0.02), 30 (*P*=0.02), 45 (*P*=0.0004), and 60 (*P*=0.0007) and in C5 on d 45 (*P*=0.01) and 60 (*P*=0.02). Percent change in BCS between C5 and C10 was different only on d 10 (*P*=0.05). The reproductive measures are summarized in *Table 5*. The percentage of uterine location into aperture pelvis at the interval 15-25 days pp was 83% and 88% in group C5 and C10, respectively, as compared to the control group. Morphologic uterine involution was notable at the pointed interval days pp (*P*=0.04 for C5 and *P*=0.007 for C10) in comparison with the control group.

The mean interval from calving to first insemination was lower by 15 days in group C10 than in group C0 (*P*=0.02) and by 7 days than in group C5 (*P*=0.19). There was no difference in the interval from calving to first ovulation among the groups. The mean days open for groups C5

Table 4. *The percent change in BCS relative to the baseline value during experiment*

Groups	SD-0	SD-10	SD-18	SD-30	SD-45	SD-60
C0 (n=18)						
Mean±SD (Min/Max)	3.08±0.31 (2.70/3.50)	-9.65±6.23 (-18.18/0)	-12.07±5.16 (-22.86/-3.57)	-13.83±7.83 (-28.57/0)	-19.12±10.54 (-42.86/-3.57)	-17.54±8.75 (-33.33/-3.57)
Median (LQ-UQ)	3.00 (2.80/3.50)	-10.00 (-14.29/-3.57)	-12.50 (-15.63/-7.41)	-14.29 (-18.18/-10.00)	-19.27 (-25.93/-10.00)	-16.67 (-24.24/-10.00)
C5 (n=18)						
Mean±SD (Min/Max)	3.05±0.52 (2.00/4.00)	-7.73±6.4 (-20/0)	-9.29±11.95 (-28.57/25)	-8.60±11.90 (-28.57/25)	-8.60±11.90 (-28.57/25)	-8.04±13.67 (-28.57/35)
Median (LQ/UQ)	3.00 (2.70/3.50)	-10.00 (-12.50/0)	-12.50 (-16.67 /0)	-12.50 (-16.67 / 0.00)	-12.50 (-16.67/0)	-12.50 (-16.67/0)
Man-Whit-U** (LB/UB)		0.40 (0.22/0.58)	0.45 (0.27/0.63)	0.37 (0.19/0.56)	0.26 (0.08/0.45)	0.28 (0.10/0.47)
P-Values*		0.3288	0.6542	0.2141	0.0155	0.0288
C10 (n=17)						
Mean±SD (Min/Max)	2.96±0.25 (2.50/3.50)	-2.02±6.97 (-16.67/11.11)	-4.20±10.95 (-16.67/20.00)	-4.94±12.59 (-28.57/20.00)	-4.39±12.30 (-28.57/20.00)	-4.39±12.30 (-28.57/20.00)
Median (LQ/UQ)	3.00 (2.80/3.00)	0.00 (-6.25/0)	-7.41 (-10.71/0)	-6.25 (-14.29/0)	-6.25 (-10.00/0)	-6.25 (-10.00/0)
Man-Whit-U** (LB/UB)		0.20 (0.01/0.38)	0.27 (0.08/0.45)	0.27 (0.08/0.46)	0.16 (0.00/0.35)	0.17 (0.00/0.37)
P-Values*		0.0015	0.0212	0.0223	0.0004	0.0007

** P value indicated the comparision with control group; ** Man-Whit-U = Wilcoxon-Mann-Whitney-U Test-Two -sided, **0.29/0.71** = largedifference; **036/0.64**= medium sized difference; **0.44/0.56** = small difference; **0.50** = equality. **LQ** = Low Quarter; **UQ** = Upper Quarter; **Min** = Minimum; **Max** = Maximum; **LB** = Low Bar; **UB** = Upper Bar; **SD** = StudyDay*

(P<0.05). In addition, the serum creatine kinase activity was significantly decreased in C10 group (P=0.047) with no differences between groups. There was no difference in the serum cholesterol, GLDH, AST and BID concentrations between groups *(Table 2)*. Treatment groups produced more milk than control group (*P*=0.01 for C5 and *P*=0.05 for C10; *Table 3*, but milk yield for C5 and C10 was not different (*P*=0.33). *Table 4* summarizes BCS during experiment and changes in BCS as compared to baseline. The cows in group C0 lost more BCS than those in group C10 on d and C10 were about 20 days shorter than that for C0 (*P*=0.07 for C5-C0; *P*=0.11 for C10-C0; and *P*=0.88 for C5-C10). The mean number of insemination and the overall pregnancy rate through 150 days DIM were similar across the groups.

DISCUSSION

The hyperketonemiain early lactation is a potential risk factor for general health status and the resumption of

Table 5. *Reproductive parameters in response to butaphosphan-cyanocobalamin combination administration*

Reproductive Measures	C0 (n=18)	C5 (n=18)	C10 (n=17)*	*P-value***
Interval from calving to first ovulation 15 to 25 days pp 26 to 30 days pp 31 to 35 days pp 36 to 40 days pp >41 dayspp	 3/18 (16.67%) 8/18 (44.44%) 2/18 (11.21%) 1/18 (5.56%) 4/18 (22.22%)	 8/18 (44.44%) 4/18 (22.22%) 4/18 (22.22%) 2/18 (11.11%) -	 3/16 (18.75%) 10/16 (62.50%) 2/16 (12.50%) 1/16 (6.25%) -	
Morphologic uterine involution 15 to 25 days pp 26 to 30 days pp 31 to 35 days pp 36 to 40 days pp	 8/18 (44.44%)^{c} 6/18 (33.33%) 4/18 (22.22%) -	 15/18 (83.33%)^{a} 1/18 (5.56%) 1/18 (5.56%) 1/18 (5.56%)	 15/17 (88.24%)^{b} 2/17 (11.76%) - -	^{a,c}0.04/^{b,c}0.007/1^{a,b}0.632
Interval from calving to first insemination (days)	74.9±20.59^{c}	66.66 ±26.70^{a}	59.75±23.14^{b}	^{a,c}0.075/^{b,c}0.017/^{a,b}0.188
Daysopen (days, mean±SD)	95.00±32.61^{c}	74.16±35.76^{a}	75.17±34.33^{b}	^{a,c}0.066/^{b,c}0.106/^{a,b}0.876
No of insemination (mean±SD)	2.0±1.19^{c}	1.8±1.20^{a}	1.6±0.80^{b}	^{a,c}0.614/^{b,c}0.335/^{a,b}0,955
Overall pregnancy rate, %	64.71^{a}	66.67^{a}	70.59^{a}	^{a}1.000

** One cow did not ovulate; ** Wilcoxon-Mann Whitney-U Test, 2-sided was used; $^{a,b,c:}$ The differences between groups in the same line with different letters is significant (P<0.05)*

reproductive function. The increased NEFA in prepartum, advanced parity, birth of a male calf, calving difficulty, precalving high BCS are important predictors of having hyperketonemia at any time from 3 to 16 DIM [22]. The prevalence of SCK within 10 European countries using a threshold ≥1.2 mmol/L of blood BHBA was 21.8%, ranging from 11.2 to 36.6% between 2 and 15 d in milk in Turkey and Italy and ranged from 8.9% to 34% during postpartum 2 month [23,24]. Significant decrease of blood BHBA in the treatment groups entire study days after drug applications compared to the control group confirmed the efficacy of the treatment on the blood BHBA in ketotic dairy cows. Our findings are consistent with the previous studies focused on the use of combined butaphosphan and cyanocobalamin in hyperketonemic animals at early postpartum period [14,25]. The results of a recent mode of action study [26] revealed that the same drug combination at a dose of 10 mL/100 kg applied IV in nonketotic dairy cows for 3 consecutive days reduced significantly in the liver the mRNA abundance of acyl coenzyme A synthetase long-chain family member 1, involved in fatty acid oxidation and biosynthesis. This can explain that lower fatty acid synthesis in the liver can result in less circulation of BHBA in the blood. However, this may not explain why the milk production in the treatment groups C5 and C10 was higher than in the control group. It can only be speculated that animals received treatments and consequently normalized blood BHBA had better metabolic balance and general health status. Interestingly, why lower dosage of the combination tended to produce more milk in group C5 is unknown. Increased milk production was also reported after butaphosphan and cyanocobalamine application in dairy cows [12,18,26]. The parallel fall of serum concentration of NEFA and total bilirubin in C10 group was showed the evidence of the treatment efficiency. It was expected results because; NEFA and bilirubin are utilizing the common hepatic pathways [9]. Ketones have a glucose-sparing effect to compensate milk production until reaching severe hypoglycemia [27]. There was a negative relationship between elevated BHBA concentrations and decreased milk production during wk 1-2 postpartum and that the loss of milk production at first week was 1.8 kg/day when blood concentration of BHBA was 1.4 mmol/L [6]. When blood concentration of BHBA increased to 2.0 mmol/L, loss of milk production at wk 2 increased to 3.3 kg/day due to decreased DMI. Body condition reflects energy balance in dairy cows [28]. In the present study, groups C10 had lowest BCS lost. The obese cows have sluggish appetite and are predisposed to SCK [29]. The mean duration of uterus involution is reported to 23.7-30.0 d [30,31]. In the present study, morphologic involution of uterus was completed by 83.3 and 88.2% in groups C5 and C10, respectively, by d 15-25 postpartum. These high involution successes in treatment groups could be related to improvement in the smooth muscle function in the uterus, due to supported the calcium-phosphor homeostasis or by the avoidance of hypotonia in response to butaphosphan administration. Hypotonic effect of SCK on uterine muscle could be a risk factor for the endometritis and delayed endometrium restoration resulting from immune suppression. The resumption of ovarian function in postpartum dairy cows depends on the reproductive and metabolic hormones levels as well as metabolite concentration. The failure of ovarian function is the most common pathology associated with the increased blood NEFA and BHBA in postpartum dairy cows [32]. In the present study, time from calving to first ovulation was similar across the groups, but time from calving to first insemination was shorter for 15 d in treated groups than nontreated groups. This could be related to activation of the up regulation of ovarian follicular competition and hypothalamic feedback systems. The earlier resumption of estrus cyclicity in treatment groups could also be

linked to the increased systemic IGF-1 concentration and enhanced hepatic gluconeogenesis in liver [32]. These could positively affect to maintain BCS and reduce blood BHBA concentration. Althougha study [19] explained the possible action of butaphosphan on the metabolism of dairy cows, the direct effect of butaphosphan on phosphorus and calcium homeostasis in dairy cows is still unknown. But, it has been shown that serum, liver, or kidney levels of IGF-1 are not increased during phosphate depletion in normal rats and the reduction in auto phosphorylation reaction of IGF-1 receptor blocks theaction of IGF-1 at the level of cytoplasmic and nuclear in target cell. Therefore, the cross interaction of IGF-1 and phosphate could positively affect to support the calcium homeostasis, improve the uterine muscle function and resume the ovarian function. Unfortunately, we had no data to support this suggestion in dairy cows and the future experimentations are necessary to clarify a possible the interrelationship between IGF-1 and phosphate on phosphorus and calcium homeostasis and on energy metabolism. Although the decreased BCS after calving in dairy cows did not affect the number of insemination, it delayed intervals from calving to first insemination [1,33] and of calving to pregnancy [34] as well as increased risk for uterine disease [35]. The decreased BCS during first 5 wk was associated with prolonged intervals from calving to first insemination and days open through effecting the frequency of LH pulsation and intrafollicular metabolism [36,37]. Improved reproductive parameters in treated groups, thus, could be related to less BCS as compared to control group. Cows with SCK have poor reproductive performance [38-41]. In cows administered with butaphosphan-cyanocobalamin, days open was 20 d shorter than cows administered with saline. The overall pregnancy rate at 150 DIM was about 7% higher in treatment groups than control group. The faster uterine involution could reduce veterinary cost and increase health status through decreasing time to first insemination and pregnancy rate.

In conclusion, injection (IM) of 10% butaphosphan and 0.005% cyanocobalamin combination at the level of 10 mL/100 kg BW everyday for 4 days starting from DIM 7-15 decreased severity of hyperketonemia, which were accompanied by less BCS lost and more milk production. This administration also stimulated uterine involution and shortened time to first insemination/days open as well as increased pregnancy rate. Further studies coping with more metabolic parameters would help explain the action mode of butaphosphan-cyanocobalamin combination.

REFERENCES

1. Rukkwamsuk T, Kruip TA, Wensing T: Relationship between overfeeding and over conditioning in the dry period and the problems of high producing dairy cows during the postparturient period. *Vet Q,* 21, 71-77, 1999. DOI: 10.1080/01652176.1999.9694997

2. Enjalbert F, Nicot MC, Bayourthe C, Moncoulon R: Ketone bodies in milk and blood of dairy cows: Relationship between concentrations and utilization for detection of SCK. *J Dairy Sci,* 84, 583-589, 2001. DOI: 10.3168/jds.S0022-0302(01)74511-0

3. Rehage J, Mertens M, Stockhofe-Zurwieden N, Kaske M, Scholz H: Post surgical convalescence of dairy cows with left abomasal displacement in relation to fatty liver. *Schweiz Arch Tierheilk,* 138, 361-368, 1996.

4. Staufenbiel R, Arndt G, Schröder U, Gelfert C: Körperkondition und Stoffwechselstabilität als Grundlage für eine hohe Milchleistung bei ungestörter Fruchtbarkeit von Milchkühen-ein beitrag zur ableitung von Referenzwerten. *Dtsch tierärztl Wochenschr*, 111, 173-228, 2004.

5. Gasteiner J: Ketose, die bedeutendste Stoffwechselerkrankung der Milchkuh. **In,** Gasteiner J (Ed): Viehwirtschaftliche Fachtagung, Gumpenstein, Bundesanstalt für alpenländische Landwirtschaft Gumpenstein. 27th edn., 11-18, Herausgeber, Irdning-Donnersbachtal, 2000.

6. Duffield TF, Lissemore KD, Mcbride BW, Leslie KE: Impact of hyperketonemia in early lactation dairy cows on health and production. *J Dairy Sci,* 92, 571-580, 2009. DOI: 10.3168/jds.2008-1507

7. Mehrzad J, Duchateau L, Burvenich C: Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation. *Vet Microbiol,* 134, 106-112, 2009. DOI: 10.1016/j.vetmic.2008.09.001

8. Van Kampen CU, Mallard BA: Effects of peripartum stress and health on circulating bovine lymphocyte subsets. *Vet Immunol Immunopathol*, 59, 79-91, 1997. DOI: 10.1016/S0165-2427(97)00069-X

9. Busato A, Faissler D, Küpfer U, Blum JW: Body condition scores in dairy cows: Associations with metabolic and endocrine changes in healthy cows. *J Vet Med A,* 49, 455-460, 2002. DOI: 10.1046/j.1439-0442.2002.00476.x

10. Sahal M, Vural R, Küplülü S, Polat IM, Özenç E: Metabolic diseases during postpartum period and their relationship with production diseases in dairy cows. *Türkiye Klinikleri J Vet Sci,* 2, 36-48, 2011.

11. Stengärde L, Traven M, Emanuelson U, Holteius K, Hultgren J, Niskanen R: Metabolic profiles around calving in five high-producing swedish dairy herds with a history of abomasal displacement and ketosis. *Acta Vet Scand,* 50, 31, 2008. DOI: 10.1186/1751-0147-50-31

12. Gengenbach S: Wirkungen von Catosal auf den Allgemeinzustand und den Stoffwechsel bei Kühen nach linksseitiger Dislocatioabomasi sowie bei Kühen im peripartalen Zeitraum, *MSc Thesis,* Vet Med Diss Uni, Leipzig, 2009.

13. Sahal M, Çolakoğlu EÇ, Alihosseini H: Ketosis and fatty liver syndrome: Current insights and causes of failure in treatment. *Türkiye Klinikleri J Vet Sci,* 2, 140-150, 2011.

14. Rollin E, Berghaus RD, Rapnicki P, Godden SM, Overton MW: The effect of injectable butaphosphan and cyanocobalamin on postpartum serum β-hydroxybutyrate, calcium, and phosphorus concentrations in dairy cattle. *J Dairy Sci,* 93, 978-987, 2010. DOI: 10.3168/jds.2009-2508

15. Girard C, Matte JJ: Effects of intramuscular injections of vitamin b 12 on lactation performance of dairy cows fed dietary supplements of folic acid and rumen protected methionine. *J Dairy Sci,* 88, 671-676, 2005. DOI: 10.3168/jds.S0022-0302(05)72731-4

16. Mcart JAA, Nydam DV, Oetzel GR: Epidemiology of subclinical ketosis in early lactation dairy cattle. *J Dairy Sci*, 88, 159-170, 2012. DOI: 10.3168/jds.2012-5443

17. Suthar VS, Canela-Raposo J, Deniz A, Heuwieser W: Prevalence of subclinical ketosis and relationships with postpartum diseases in European dairy cows. *J Dairy Sci,* 96, 2925-2938, 2013. DOI: 10.3168/jds.2012-6035

18. Cuteri V, Nisoli L, Attili AR, TejedaRA, Preziuso S, Fruganti A: Clinical field evaluation of a butafosfan + vitamin B12 compound (Phosphorum B12/Catosal) in the treatment of SCK in dairy cows. *Hungarian Vet J,* 130, 16-17, 2008.

19. Fürll M, Deniz A, Westphal B, Illing C, Constable PD: Effect of multiple intravenous injections of butaphosphan and cyanocobalamin on the metabolism of periparturient dairy cows. *J Dairy Sci,* 93, 4155-4164, 2010. DOI: 10.3168/jds.2009-2914

20. Sarasola P, Aramend U, Spiecker-Hauser U, Schmidt B: Preliminary results from an ongoing field study on the effect of catosal in the

treatment of sck in cows. *Hung Vet J*, 130, 26-27, 2008.

21. Sheldon IM, Barrett DC, Boyd H: The postpartum period. **In,** Andrews AH, Blowey RW, Boyd H, Eddy RG (Eds): Bovine Medicine Diseases and Husbandry of Cattle. 508-529, Blackwell Science, Oxford, UK, 2004.

22. Mcart JAA., Nydam DV, Oetzel GR: Dry period and parturient predictors of early lactation hyperketonemia in dairy cattle. *J Dairy Sci,* 96, 198-209, 2013. DOI: 10.3168/jds.2012-5681

23. Dohoo IR, Martin USW: Subclinical ketosis: Prevalence and association with production and disease. *Can J Comp Med*, 48, 1-5, 1984.

24. Kauppinen K: Correlation of whole blood concentrations of acetoacetate, ß-hydroxybutyrate, glucose and milk yield. *Acta Vet Scand*, 24, 337-348, 1983.

25. Deniz A, Westphal B, Illing C: Effects of prepartummetaphylactic treatment with Catosal on postpartum metabolic functions in cows. **In,** *Proc. 25th World Buiatrics Congr.* July, 6-11 2008, Budapest, Hungary, 26-31.

26. Kreipe L, Deniz A, Bruckmaier RM, Van Dorland HA: First report about the mode of action of combined butafosfan and cyanocobalamin on hepatic metabolism in nonketotic early lactating cows. *J Dairy Sci,* 94, 4904-4914, 2011. DOI: 10.3168/jds.2010-4080

27. Zarrin M, De Matteis L, Vernay MC, Wellnitz O, Van Dorland HA, Bruckmaier RM: Long-term elevation of β-hydroxybutyrate in dairy cows through infusion: Effects on feed intake, milk production, and metabolism. *J Dairy Sci,* 96, 2960-2972, 2013. DOI: 10.3168/jds.2012-6224

28. Heuwieser W, Bergmann J: Body conditionscoring. Kühe nach noten Füttern. *Top Agrar*, 3, 8-11, 1996.

29. Hayirli A, Grummer RR, Nordheim EV, Crump PM: Animal and dietary factors affecting intake during the prefresh transition period in Holsteins. *J Dairy Sci,* 85, 3430-3443, 2002. DOI: 10.3168/jds.S0022-0302(02)74431-7

30. Fonseca FA, Britt JH, Mcdaniel BT, Wilk JC, Rakes AH: Reproductive traits of holsteins and jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. *J Dairy Sci,* 66, 1128-1147, 1983. DOI: 10.3168/jds.S0022-0302(83)81910-9

31. Leslie KE: The events of normal and abnormal postpartum reproductive endocrinology and uterine involution in dairy cows: A review. *Can Vet J*, 24, 67-71, 1983.

32. Lucy MC: Growth hormone regulation of follicular growth. *Reprod Fert Develop,* 24, 19-28, 2012. DOI: 10.1071/RD11903

33. Pedron O, Cheli F, Senatore E, Baroli D, Rizzi UR: Effect of body condition score at calving on performance, some blood parameters and milk fatty acid composition in dairy cows. *J Dairy Sci,* 76, 2528-2535, 1993. DOI: 10.3168/jds.S0022-0302(93)77588-8

34. Markusfeld O, Galon N, Ezra E: Body condition score, health, yield and fertility in dairy cows. *Vet Res,* 141, 67-72,1997. DOI: 10.1136/vr.141.3.67

35. Schmausser M: Beziehungen zwischen verschiedenen Parametern des Energiestoffwechsels und Störungen der Fertilität beim Milchrind unter Berücksichtigung der individuellen Futteraufnahme. *MSc Thesis*, Vet Med Diss Univ, München, 2006.

36. Hayirli A, Çolak A: Managerial and nutritional strategies during the dry and transition periods in dairy cattle: Effects on postpartum metabolic profile, health and fertility. *Turkiye Klinikleri J Vet Sci,* 2, 1-35, 2011.

37. Roch JR, Friggens NC, Kay JK, Fisher M, Stafford KJ, Berry DP: Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. *J Dairy Sci,* 92, 5769-5801, 2009. DOI: 10.3168/jds.2009-2431

38. Bobe G, Ametaj BN, Nafikov RA, Beitz DC, Young JW: Relationships between mild fatty liver and health and reproductive performance in holstein cows. **In,** *Proc. 12th International Conference on Production Diseases in Farm Animals,* 2004, East Lansing, USA, 2004.

39. Reist M, Koller A, Busato U, Küpfer J, Blum W: First ovulation and ketone body status in the early postpartum period of dairy cows. *Theriogenology,* 54, 685-701, 2000. DOI: 10.1016/S0093-691X(00)00383-6

40. Ruegg PL, Milton URL: Body condition scores of holstein cows on prince edward island, canada: Relationships with yield, reproductive performances and disease. *J Dairy Sci,* 78, 553-564,1995. DOI: 10.3168/jds.S0022-0302(95)76666-8

41. Walsh RB, Kelton DF, Duffield TF, Leslie KE, Walton JS, Leblanc SJ: Prevalence and risk factors for postpartum anovulatory condition in dairy cows. *J Dairy Sci*, 90, 315-24, 2007. DOI: 10.3168/jds.S0022-0302(07)72632-2

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Research Article

Biomechanical Parameters of Asian Elephant *(Elephas maximus)* Walking Gait

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Abstract

Quadruped animals have a unique mechanism of movement that minimizes energy use and allows muscles to work effectively. Elephants are the biggest quadruped animals on earth and how they stabilize their body and use energy are of interest. This study aimed to analyze the characteristics of kinematic gait in Asian elephants trained to work with a mahout for tourism activities in Thailand. Twenty-one healthy adult Asian elephants were recorded by 2 digital cameras while walking at normal speed (average 1.1 m s-1.) along a 15-meter, solid-soil path. The temporospatial parameters evaluated for each limb consisted of stride length (cm), stride time (sec), swing time (sec), stance time (sec) and stance time percentage, using 2D motion analysis software. The result revealed that the average stride length was varied between 192-199 cm with no significant difference between fore and hindlimbs on either side but the stride length on the right side was significantly longer than that on the left in both forelimbs (right 197.5 cm; left 192.6 cm, $P<0.05$) and hindlimbs (right 198.9 cm; left 193.2 cm, $P<0.01$). The mean gait cycle time (stride time) was varied between 2.26 and 2.34 seconds for each limb and mean stance time was varied between 1.67-1.80 seconds, with both parameters were longer on the forelimbs than hindlimbs significantly ($P<0.01$). Hence, swing time for the forelimb was shorter than that for the hindlimb ($P<0.001$). The calculated stance time percentage for each limb was 72.64-76.09%. Data from this study confirmed that elephants walk with a lateral sequence and footfall pattern, and distribute the center of mass proportionally between all four limbs. Gait analysis is a valuable tool for identifying and understanding the pathogenesis of gait abnormality.

Keywords: *Elephant, Gait cycle, Stride length, Stance time, Swing time*

Asya Filinin *(Elephas maximus)* Yürüme Biyomekanik Parametreleri

Özet

Dört ayaklı hayvanlar hareket ederken enerji kullanımını kısıtlayan ve kasların etkili bir şekilde çalışmasını sağlayan özgün bir mekanizmaya sahiptir. Filler dünyadaki en büyük dört ayaklı hayvanlar olup, vücutlarını nasıl stabil tuttukları ve enerji kullanımları hususu ilgi konusudur. Bu çalışma Tayland'da turist aktiviteleri amacıyla bir fil seyisi ile eğitilmiş olan Asya fillerinde yürüme kinematiği özelliklerini analiz etmeyi amaçlamaktadır. Yirmi bir sağlıklı ergin Asya fili sert toprak zemin üzerinde 15 metre boyunca normal hızda (ortalama 1.1 m s-1) yürürken 2 dijital kamera ile kayıt edildi. 2 boyutlu hareket analiz yazılımı kullanılarak her bir ayak için değerlendirilen temporospasyal parametreler; adım uzunluğunu (cm), adım süresini (dak), salınım süresini (dak), duraklama süresini (dak) ve duraklama süresi yüzdesini içermektedir. Ortalama adım uzunluğu her iki tarafta da ön ve arka ayaklar için anlamlı bir fark olmaksızın 192 ile 199 cm arasında kaydedildi. Ancak hem ön (sağ 197.5 cm; sol 192.6 cm, $P<0.05$) hem de arka ayaklar (sağ 198.9 cm; sol 193.2 cm, $P<0.01$) için sağ taraftaki adım uzunluğu anlamlı derecede sol taraftakinden daha uzundu. Ortalama yürüme siklus süresi (adım süresi) her bir ayak için 2.26 ile 2.34 saniye arasında değişirken ortalama duraklama süresi 1.67 ile 1.80 saniye arasında değişim gösterdi ve her iki parametre için de değerler ön ayaklar için arka ayaklardan anlamlı oranda daha uzun olarak tespit edildi ($P<0.01$). Ön ayaklar için salınım zamanı arka ayaklar için olandan daha kısa idi ($P<0.001$). Her ayak için hesaplanan duraklama süre yüzdesi %72.64-76.09 olarak belirlendi. Bu çalışmadan elde sonuçlar göstermiştir ki filler lateral sekans ve ayak basım şekli ve vücut ağırlık merkezini orantısal olarak dört ayağa yayarlar. Yürüme analizi yürüyüş bozukluklarının patogenezini tespit etme ve anlamada değerli bir yöntemdir.

Anahtar sözcükler: *Fil, Yürüme siklusu, Adım uzunluğu, Duraklama süresi, Salınım süresi*

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INTRODUCTION

The biomechanics of locomotion identifies the kinetic and kinematic mechanisms of gait, and was first introduced in veterinary practice in the late 19th century [1]. Kinetics is the study of cause of motion, which is concerned with forces applied to the body, acceleration, energy and work, whereas kinematics is the study of changes in the position of body segments in space during a specified time. Both kinetic (i.e., potential and kinetic energy) and kinematic variables (i.e., displacement of center of mass, linear and angular variables, velocity) underlie mechanisms that minimize muscular work and the metabolic cost of locomotion, which also involves neural control strategies [1]. Motion analysis has been used widely to measure normal and pathological gait. Measuring kinematic parameters can help to identify any pathologic conditions that could affect the characteristics of gait, i.e., orthopedic or neurological conditions, and the degree of gait asymmetry were found to relate to the degree of lameness [1,2].

Gait has been defined as a complex and coordinated rhythmic and automatic movement of the limbs and entire body of an animal, which results in the production of progressive movements [1,3]. The footfall pattern of a quadruped gait can be categorized into two general types; symmetrical (i.e., walk and trot) and asymmetrical (i.e., canter and gallop). The symmetrical gait pattern is found usually at slow to moderate speeds, changing to an asymmetrical pattern from moderate to high speeds, in which a suspension phase can be found in the trot, pace, canter, and gallop of horses [1]. Whereas, dogs and cheetahs have a different footfall sequence, known as the rotary gallop [4].

As elephants are the biggest quadruped animals [5], with adults weighing over 2.5 tons and being 3 meters high, their walking mechanism is of interest, particularly in terms of how they stabilize their huge body and utilize energy. Previous studies have reported the footfall pattern of elephants as a lateral sequence, when a hindlimb on one side makes contact with the ground, followed in the pendulum mechanism by the forelimb on the same side [5-7]. Unlike other quadruped animals, elephants maintain this symmetrical pattern even at faster speeds, which are increased by increasing stride frequency rather than stride length, and so they do not trot or pace [5,8]. Elephants maintain stability by using the pendulum mechanism, despite their massive bodies, and they conserve energy with effective muscular work [9,10]. They maintain movements of mass per unit distance with only one-third of the average mass-specific mechanical work of other animals [5]. To date, biomechanical studies of elephant locomotion are still limited, due to complex methodology, and costly laboratory equipment and program analysis. Hence, this study aimed to focus on the temporospatial parameter of Asian riding elephants that were trained to work with a mahout for tourism activities in Thailand. Even formerly it had been done by Hutchinson et al.[6], this study used simple methodology, did not need high technology in a laboratory setting, therefore clinicians can use this technique further for field study and the subjects in this study were riding elephants for tourism, unlike those in a zoo or the natural environment. The research knowledge and database gained from this study will add information that can be applied to monitoring lifelong elephant health management.

MATERIAL and METHODS

Ethical Approval

This study was approved by The Animal Care and Use Committee of the Faculty of Veterinary Medicine (FVM-ACUC), Chiang Mai University, Research ID 9/ 2013.

Animals

One male and 20 female adult Asian elephants (*Elephas maximus*) from the Thai Elephant Conservation Center, National Elephant Institute, Forest Industry Organization, Lampang, Thailand, were evaluated by experienced veterinarians from the Center's elephant hospital and approved as being clinically healthy, i.e., appropriate body composition score, no neurological or musculoskeletal problems, prior injuries that affected movement, and no aggressive behavior. The elephants used in this study worked as riding elephants with a harness on their back, and they were guided by their own mahout. Practice trials were conducted to ensure that the elephant and mahout were familiarized with the experimental setting and able to walk at a comfortable and normal speed.

Video Recording and Temporospatial Analysis

Markers with reflexive tape were applied to the elephant at the joint landmarks of each limb by the same veterinarian, according to procedures described by Wijesooriya [7]. The elephant subjects walked in a straight line at a normal, comfortable speed for 15 meters up a walkway before turning back. Two digital cameras (Nikon: D3300, frame size 1280×720 pixels, frame rate of 30p) were set one on each side of the walkway and 10 meters away from it to record the movement of the elephant. Temporospatial parameters that evaluated each limb consisted of stride length (cm), stride time (sec), swing time (sec), and stance time (sec). The length of stride or gait cycle corresponded to the distance between two consecutive ground contacts by the same limb. Each cycle of limb movement included the stance, the phase during which a limb made contact with the ground and the swing when the limb is not contact with the ground [1]. Stance time percentage was calculated as (stance time/gait cycle time) x 100. Velocity or speed was calculated from the average of distance divided by

duration of the same limb. These parameters were digitized and calculated for three consecutive gait cycles using two-dimensional (2D) motion analysis Kinovea® software [11].

Statistical Analysis

Data were recorded as mean and standard deviation. Paired sample t-tests were used to analyze the differences of each parameter between forelimbs and hinlimbs of each side, with significant difference set at P<0.05.

RESULTS

Twenty-one elephants were enrolled into this study. Their average age was 32.7±10.2 years and weight 3.059.62±555.19 kg, and 20 of the 21 subjects were female. The demographic data are shown in *Table 1*.

The average stride length of each limb varied between 192-199 cm *(Table 2)*, with no significant difference between the fore and hindlimb on each side, but surprisingly, the average stride length on the right side was significantly longer than that on the left (right forelimb 197.45±29.06 cm vs left forelimb 192.64±28.29 cm; P<0.05; right hindlimb 198.94±29.97 cm vs left hindlimb 193.20±27.62 cm; P<0.01). Stride time or gait cycle time of both forelimbs was significantly longer than that of the hind limbs (left forelimb = 2.31±0.65 sec vs hindlimb = 2.26±0.63 sec; P<0.01 and right forelimb = 2.34±0.64 sec. vs hindlimb 2.26±0.59 sec, P<0.01). The stance time of both forelimbs was also significantly longer than that of the hindlimb (left forelimb = 1.76±0.62 sec. vs hindlimb = 1.67±0.58 sec; P<0.001 and right forelimb = 1.80±0.60 sec vs hindlimb 1.68±0.56 sec, P<0.001), which corresponded to the swing time of the forelimb being slightly shorter than that of the hindlimb, with significance on both the left and right side (P<0.001) *(Table 3)*. The stance time percentage for each limb was about 75%, with the right forelimb = 76.09%, left forelimb = 75.07%, right hindlimb = 73.28% and left hindlimb = 72.64%.

DISCUSSION

Locomotion in all animals takes place in order to transport the body, but in order to maintain such movement in various situations they need unique mechanisms with effective energy usage. Biomechanics studies of gait revealed that humans and animals use an "inverted pendulum" mechanism during walking

Table 1. *Demographic data of the Asian elephants (n =21)*

ID	Name	Age (year)	Sex	Weight (kg)	Shoulder height (cm)	Hip height (cm)	Body length (cm)	BCS
1	Jojo	25	M	4.020	267	272	420	4
2	Nue-oun	12	F	2.515	225	235	321	4
3	Prajuab	32	F	3.880	237	243	393	5
4	Kod	33	F	3.640	240	250	395	4.5
5	Pumpuang	40	F	3.970	253	258	381	5
6	Wanalee	20	F	3.090	205	207	346	4.5
7	Areena	12	F	2.280	242	238	325	3
8	Warunee	30	F	3.025	232	228	372	3
9	Taddao	35	F	3.130	312	234	340	4
10	Suwanan	33	F	3.060	257	241	356	4
11	Sankham	41	F	2.565	217	218	310	3
12	Pooky	41	F	2.930	230	227	330	4.5
13	Payom	30	F	2.590	278	273	365	3
14	Manao	24	F	2.395	332	234	340	3.5
15	Mali	41	F	2.890	253	257	344	4.5
16	Linda	32	F	2790	336	238	370	3
17	Kam-nguen	49	F	3.090	242	248	341	3
18	Kammoon	47	F	4.060	258	267	385	4.5
19	Kanjana	30	F	2.660	227	230	330	3
20	Boyo	45	F	3.155	240	240	350	3
21	Boonpeum	35	F	2.515	315	218	356	4.5
Mean		32.71		3.059.62	257.05	240.76	355.71	3.83
S.D.		10.17		**555.19**	**37.31**	**17.60**	**27.98**	**0.75**

BCS: *Body Condition Score*

Table 2. *Average stride length of the Asian elephants (n=21)*

Parameter		Forelimb (Mean ± S.D.)	Hindlimb (Mean ± S.D.)	P-value
Stride length (cm)	Left	192.64±28.29	193.20±27.62	0.64
	Right	197.45±29.06	198.94±29.97	0.20
P-value		0.03	0.005	

Paired samples t-test; significance level at P-value <0.05

Table 3. *Stride, swing, and stance time of the Asian elephants (n=21)*

Parameter		Forelimb (Mean ± S.D.)	Hindlimb (Mean ± S.D.)	P-value
Stride time (sec.)	Left	2.31±0.65	2.26±0.63	0.002
	Right	2.34±0.64	2.26±0.59	0.003
Swing time (sec.)	Left	0.55±0.06	0.59±0.06	<0.001
	Right	0.54±0.06	0.58±0.06	<0.001
Stance time (sec.)	Left	1.76±0.62	1.67±0.58	<0.001
	Right	1.80±0.60	1.68±0.56	<0.001
Stance time percentage (%)	Left	75.07	72.64	
	Right	76.09	73.28	

Paired samples t-test; significance level at P-value <0.05

gait [3,12-15]. Each limb generates ground force patterns that cause the fore and hind quarters to vault over their respective stance limbs, like inverted pendulums. This mechanism is a fundamental system that bipedal, quadrupedal, and even hoppers, like kangaroos, use to minimize muscular work and the metabolic cost of locomotion, with an effective exchange between potential gravitational and kinetic energy during the gait cycle. It is a cyclic exchange between gravitational potential energy and kinetic energy within each stride, aimed to maintain movements of the center of mass per unit distance [3-5,13,14]. At the start of a step, the center of mass becomes high; kinetic energy is converted into potential gravitational energy, and then moves forward and downward during the second half of a step, when potential gravitational energy is converted back into kinetic energy. Alternate transference of these two forms of energy results in some energy loss, but in the stance phase, leg performs like a pendulum that the hip moves along an arc and no moment acts, so with the knee kept rigid, muscles do not work. This inverted pendulum contributes to effective energy exchange, in which up to 70% of energy can be recovered [3,12,15].

In terms of the kinematic mechanism of an elephant, walking and running differ in the mechanics of center of mass motion [13]; walking and running types of gait are identified by several factors: duty factors (fraction of the stride duration in which each foot remains on the ground), Froude number (dimensionless speed parameter), phase relationship between kinetic and potential energies, and the slope of the vertical ground reaction force [5,13]. During running gaits, such as trotting, hopping and galloping, potential energy and kinetic energy are converted into elastic strain energy of a bouncing mechanism, like a mass-spring system [3,12-14]. A duty factor above 0.5 has been used to indicate walking, with feet on ground for more than half of each stride cycle, and a duty factor of below 0.5 indicates running [5].

Analysis of elephant locomotion is limited, however, the studies of Hutchinson et al.[6], Ren et al.[16], and Genin et al.[5] clarified the locomotor kinetic and kinematic characteristics of Asian elephants. They revealed that elephants, as with other quadrupeds, use a lateral sequence footfall pattern during walking to achieve the objectives of effective energy expenditure and minimal muscular work [5,6,16]. The temporospatial parameters reported in this study were consistent with those found in previous literature, even though all of the subjects were riding elephants guided by their own mahout and trained to work in tourism activities. The stride length of each limb was approximately 200 cm, ranging from 192.6 to 198.9 cm, with no significant difference between the fore and hindlimb on each side at a comfortable walking speed of average 1.1 ms^{-1}. This may confirm that elephants walk with a lateral sequence footfall pattern, and distribute their center of mass proportionally across all four limbs, as reported in previous studies [5-7,16].

It was somewhat surprising that in this study the average stride length on the right side was significantly longer than that on the left (P<0.05 for both fore limbs and hind limbs). Most of the subjects were adult elephants ridden by mahouts; and as domesticated Asian elephants, unlike zoo-captive elephants, their gait characteristics may have been influenced from a young age by training or developing lateralization behavior for a preferred side, as indicated by Haakonsson and Semple [17], who stated that left-side trunk movement bias was associated with feeding, swinging and self-touching. Thus, stride length in this study might reflect the lateral preference of Asian elephants.

With temporal parameters, the stride or gait cycle time of the forelimb was significantly longer than that of the hindlimb on both sides (P<0.01). Average stance time of all four limbs was between 1.67 and 1.80 sec., with the stance time of the forelimb significantly longer than that of the hind limb (P<0.001), as reflected in a shorter swing time of both fore limbs when compared to the hind limbs (P<0.001). The stance time percentage for each limb was about 75% (ranging from 72.64 to 76.09%). Temporal data from this study were consistent with those in the studies of Genin et al.[5] and Wijasooriya et al.[7] in that elephants may bear more weight on their forelimbs in order to support their massive body weight. This study also confirmed that Asian elephants spend a single limb swing phase of only approximately 25%, and distribute their body weight to the other three legs in order to maintain stability while accelerating the body forward. Therefore, even with their huge bodies, elephants are able to consume energy, reduce muscular work, and maintain their stability effectively.

The temporospatial parameters from this study confirmed those from earlier data, in that the biomechanics of the Asian elephant's low-speed walking is similar to that in other quadruped mammals. This study used simple technology and equipment, including two digital cameras, which veterinarians or researchers apply to field study. Understanding the biomechanics of elephant locomotion also can be used as objective measurement in clinical care and research. However, the normal kinematic parameter should be continued to study and compare walking in lifelong daily work, since it might provide other health parameters for elephant welfare and management.

Conflict of Interest

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

Authors' Contribution

Kongsawasdi S. was a major contributor, who designed, conducted, collected, and statistical analyzed the data in this study. Mahasawangkul S. and Boonprasert K. arranged in the process of evaluation of eligible subjects and data collection. Pongsopawijit P., Chuatrakoon B., Thonglorm N., and Kanta-in R. assisted in data collection. Tajarernmuang T. assisted in data analysis using Kinovea® software for motion analysis. Pongsopawijit P. and Nganvongpanit K. provided advice and support of information for discussion. Kongsawasdi S. wrote the manuscript and Pongsopawijit P. assisted in the discussions and writing of the manuscript.

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REFERENCES

1. Barrey E: Methods, applications and limitations of gait analysis in horses. *Vet J*, 157, 7-22, 1999. DOI: 10.1053/tvjl.1998.0297

2. Malikides N, McGowan T, Pead M: Equine and canine lameness. **In,** Catherine McGowan C, Goff L, Stubbs N (Eds): Animal Physiotherapy: Assessment, Treatment and Rehabilitation of Animals. 73-101, Wiley (E-Book), 2007.

3. Griffin TM, Main RP, Farley CT: Biomechanics of quadrupedal walking: How do four-legged animals achieve inverted pendulum-like movements? *J Exp Biol,* 207, 3545-3558, 2004. DOI: 10.1242/jeb.01177

4. Biancardi CM, Minetti AE: Biomechanical determinants of transverse and rotary gallop in cursorial mammals. *J Exp Biol*, 215, 4144-4156, 2012. DOI: 10.1242/jeb.073031

5. Genin JJ, Willems PA, Cavagna GA, Lair R, Heglund NC: Biomechanics of locomotion in Asian elephants. *J Exp Biol*, 213, 694-706, 2010. DOI: 10.1242/jeb.035436

6. Hutchinson JR, Schwerda D, Famini DJ, Dale RHI, Fischer MS, Kram R: The locomotor kinematics of Asian and African elephants: Changes with speed and size. *J Exp Biol*, 209, 3812-3827, 2006. DOI: 10.1242/jeb.02443

7. Wijesooriya PN, Abeykoon AHS, Udawatta L, Punchihewa A, Nanayakkara T: Gait pattern analysis of an Asian elephant. *IEEE 6th International Conference on Information and Automation for Sustainability (ICIAfS)*, 27-29 Sep., Beijing, China, 221-226, 2012.

8. Hutchinson JR, Famini D, Lair R, Kram R: Biomechanics: Are fast moving elephants really running? *Nature*, 422, 493-494, 2003. DOI: 10.1038/422493a

9. Heglund NC, Taylor CR: Speed, stride frequency and energy cost per stride: How do they change with body size and gait? *J Exp Biol*, 138, 301-318, 1988.

10. Hildebrand M: Rotations of the leg segments of three fast running cursors and an elephant. *J Mammal*, 65, 718-720, 1984. DOI: 10.2307/1380866

11. Klinhom S, Chaichit T, Nganvongpanit K: A comparative study of range of motion of forelimb and hindlimb in walk pattern and trot pattern of Chihuahua dogs affected and non-affected with patellar luxation. *Asian J Anim Vet Adv*, 2015. DOI: 10.3923/ajava.2015.247.259

12. Alexander RM: Energy-saving mechanisms in walking and running. *J Exp Biol*, 160, 55-69, 1991.

13. Biewener AA: Patterns of mechanical energy change in tetrapod gait: Pendula, springs and work. *J Exp Zool*, 305A, 899-911, 2006. DOI: 10.1002/jez.a.334

14. Cavagna GA, Heglund NC, Taylor CR: Mechanical work in terrestrial locomotion: Two basic mechanisms for minimizing energy expenditure. *Am J Physiol*, 233, R243-R261, 1977.

15. Kuo AD: The six determinants of gait and the inverted pendulum analogy: A dynamic walking perspective. *Hum Movement Sci*, 26, 617-656, 2007. DOI: 10.1016/j.humov.2007.04.003

16. Ren L, Butler M, Miller C, Paxton H, Schwerda D, Fischer MS: The movements of limb segments and joints during locomotion in African and Asian elephants. *J Exp Biol*, 211, 2735-2751, 2008. DOI: 10.1242/jeb.018820

17. Haakonsson JE and Semple S: Lateralisation of trunk movements in captive Asian elephants *(Elephas maximus). Laterality*, 14, 413-422, 2009. DOI: 10.1080/13576500802572442

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Research Article

The Effects of Intrathecal Administration of Bupivacaine or Ropivacaine Following Administration of Propofol in Dogs Undergoing Ovariohysterectomy [1]

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Abstract

The aim of this study was to compare intrathecal (IT) administration of bupivacaine and ropivacaine after propofol was given to dogs undergoing ovariohysterectomy operation. After propofol was administered to 16 dogs, IT anesthesia was performed with either 20 mg of bupivacaine (bupivacaine group, BG, n=8) or 30 mg ropivacaine (ropivacaine group, RG, n=8) in the lumbosacral space in this randomized, prospective, clinical trial. Noninvasively systolic, diastolic and mean arterial blood pressure, heart rate, respiratory rate and rectal temperatures were recorded after propofol and up to 150 min after IT injections. Onset, duration and the extent of sensory block were determined by using the needle prick during the ovariohysterectomy operation. The duration of IT anesthesia (BG: 89.5±7.6 min; RG: 64.75±6.8 min) was significantly different between the groups (P<0.05). The values in each group were within the reference range although there were differences in terms of arterial blood pressure (systolic, diastolic and mean), heart rate, respiratory rate and rectal temperatures. In conclusion, this study showed that IT bupivacaine and ropivacaine following administration of propofol can provide safe and effective anesthesia in dogs undergoing ovariohysterectomy operations.

Keywords: *Propofol, Intrathecal anesthesia, Bupivacaine, Ropivacaine, Dog*

Ovariohisterektomi Yapılan Köpeklerde Propofol Uygulamasını Takiben Bupivacaine ya da Ropivacainin İntratekal Uygulamasının Etkileri

Özet

Bu çalışmanın amacı ovariohisterektomi operasyonu yapılan köpeklere propofol verildikten sonra bupivacaine ve ropivacaine'in intratekal (IT) uygulamasının karşılaştırılması idi. Randomize ve prospektif olan bu klinik araştırmada 16 köpeğe propofol verildikten sonra IT anestezi lumbosakral boşluğa verilen ya 20 mg bupivacaine (Bupivacaine Grubu, BG, n=8) ya da 30 mg ropivacaine (Ropivacaine grubu, RG, n=8) ile sağlandı. Propofol uygulamasından sonra ve IT enjeksiyondan sonraki 150. dakikaya kadar noninvaziv olarak sistolik, diastolik ve ortalama arteriyel kan basınçları ile nabız, solunum sayısı ve rektal ısı kayıt edildi. Ovariohisterektomi operasyonu sırasında sensorik blokajın başlangıcı, süresi ve derinliği iğne pikürü ile yeterli derinlikte anestezi olup olmadığı değerlendirildi. Anestezi süresi (BG: 89.5±7.6 dk; RG: 64.75±6.8 dk) bakımından gruplar arasında istatistiki olarak önemli derecede bir farklı bulundu (P<0.05). Arteriyel kan basınçları (sistolik, diastolik ve ortalama) ile nabız, solunum sayısı ve rektal ısı açısından bazı farklılıklar olmasına rağmen her bir gruptaki değerler referans aralıkta idi. Sonuç olarak, bu çalışma ovariohisterektomi operasyonu yapılan köpeklerde propofol uygulamasını takiben IT bupivacaine ve ropivacainin güvenli ve etkili bir anestezi sağlayabildiğini gösterdi.

Anahtar sözcükler: *Propofol, İntratekal anestezi, Bupivacain, Ropivacain, Köpek*

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INTRODUCTION

Intrathecal (IT) or subarachnoid anesthesia providing sympathetic, sensory and motor nerve blockage with injection of local anesthetics into the subarachnoid space has become quite popular in recent years [1-3]. IT anesthesia is a simple and more economic approach to anesthesia as it can be administered without the equipment and devices required for inhalation anesthesia, but the experience during administration can be viewed as a disadvantage [1,3]. IT anesthesia is preferred to general anesthesia for hind limb surgery, flank surgery, ovariohysterectomy, cesarean section and tail surgery in many animal species [1-5].

In dogs, subarachnoid puncture at the lumbosacral junction is possible and intrathecal or spinal injections may be made with the patient lying in either sternal or lateral recumbency [6,7]. After the spinal needle enters into lumbosacral space by 90°, it should be moved towards the cranial by 45°. In this approach, cerebrospinal fluid (CSF) flow may not always be possible due to the cauda equina in dogs. If it cannot be sure that it has been entered, it should be abandoned. In this case, alternatively it can be entered in the L6-L7 range [8]. To perform the spinal puncture, the two tuberosities of the iliac bone and of the spinal process of the last lumbar vertebra are identified. The lumbosacral space is located immediately below. By sliding the finger in the cephalic direction, the next inter-vertebra space is L6-L7; this is the site where the puncture is performed using a disposable 20-25 G spinal needle which is inserted through median access at approximately 45°. After crossing the arachnoids membrane, the needle mandrel is removed to obtain free CSF leakage- proof of correct needle placement [1,3,6-9].

Propofol, a weak analgesic, has been preferred before anesthesia to ensure quick recovery and minimal side effects, patient comfort, safety and immobility. Although it may cause dose-dependent respiratory depression, advantages are the fact that it is an antiemetic, anti-convulsant and amnestic agent [10-15].

Bupivacaine and ropivacaine are both amide-type local anesthetics and effective, long-lasting, local anesthetics that are quite frequently used in IT anesthesia. Bupivacaine provides advantages over ropivacaine in terms of depth of anesthesia and duration of anesthesia, but it can cause ventricular arrhythmia and cardiotoxicity [2,3,16-18].

Animals are usually sedated before intrathecal anesthesia. However, during the spinal puncture, reactions that cause disgust may be seen. In addition, these reactions negatively affect the quality of anesthesia and it can even result in unwanted accidents. So, propofol may be an alternative to avoid these. The purpose of this study was to compare of the effects of intrathecal administration of bupivacaine and ropivacaine following propofol administration in dogs undergoing ovariohysterectomy.

MATERIAL and METHODS

The study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK: 2015/052). Consent was also obtained from the animal owners, who were informed about the study.

This study was conducted on 16 dogs brought to the Kafkas University, School of Veterinary Medicine, Department of Obstetrics and Gynecology for a routine ovariohysterectomy operation.

The dogs made general health examination were divided into two groups with one receiving bupivacaine (BG, n=8) and the other ropivacaine (RG, n=8). Before IT anesthesia, 6 mg/kg propofol (Propofol® 1%, 20 mL enj., Fresenious Kabi-Germany) was administered intravenously as a single dose to each dog. Five minutes later, dogs were placed in the prone position. IT anesthesia was performed under aseptic conditions with either 20 mg [1] (4 mL as total dose) bupivacaine (Marcaine®, 5 mg/mL Astra Zenaca) or 30 mg [1] (4 mL as total dose) ropivacaine (Naropin® 7.5 mg/mL, Astra Zenaca) injected into the L7-S1 or L6-L7 as previously described [1,3,5,7,8]. If the spinal puncture was suspicious or unsuccessful, it was not insisted and abandoned.

All dogs were laid in the supine position on the operating table with a slope of approximately 30° to prevent cranial progress of the local anesthetic. An ovariohysterectomy was routinely performed by entering the laparatomy incision, which began approximately 1 cm caudal to the belly button and extended for 5 cm to the posterior along the center line. During the ovariohysterectomy, regular checks were performed to determine whether or not the ovaries, uterus, and *ligamentum latum uteri* were sensitive to touch or dissection.

Each animal was monitored (Veterinary Monitor® MMED6000DP S6-V, Germany) by recording heart rate (HR), noninvasively systolic arterial blood pressure (SBP), diastolic arterial blood pressure (DBP), mean arterial blood pressure (MBP), electrocardiogram (ECG), respiratory rate (RR), and rectal temperature (RT) initially, at propofol administration, and during anesthesia at 5, 15, 30, 60, 90, 120, 150 min. Also, biochemical measurements were performed on venous blood at 0, 15, 30, 60, and 120 min with a colorimetric assay to determine levels of serum glucose, aspartate aminotransferase (AST), alanine amino-transferase (ALT), blood urea nitrogen (BUN), and creatine.

For ECG measurements, extremity leads were used. QT measurements were corrected for the heartbeat to calculate corrected QT (QTc) intervals. The formula $QTcF = QT/(RR)^{1/3}$ developed by Fridericia [19] was used for evaluation of the measurements.

Painful stimuli were created using a 23-gauge needle to assess superficial and deep pain ventral to the caudal abdomen, tail, perineum, and hind limbs. The needle prick

was evaluated on a scale of 0 to 3 as reported by some study [1,3,5]: 0 = no analgesia and normal strong reaction to stimulus, 1 = mild analgesia and depressed reaction to stimulus, 2 = moderate analgesia and no response to superficial needle-prick stimulation of the skin, and 3 = complete analgesia and no response to insertion of the needle deep into muscle tissue. The needle prick procedure was continued until the end of the anesthesia even if the operation ended early. In addition, it was checked for anesthesia by compressing the paw and tail end with a forceps during the anesthesia.

Before the ovariohysterectomy operation, a 22-G polyurethane catheter was aseptically placed into the ramus dorsalis of vena saphena and an electrolyte solution (saline 0.9 per cent) was administered intravenously at 10 ml/kg/hour for the duration of IT anesthesia.

Routine daily nursing procedures such as postoperative antibiotics (20 mg/kg, iv cefazolin sodium, Sefazol®, Mustafa Nevzat, for five days postoperatively) and analgesia (Carprofen, 4 mg/kg, im, Rimadyl, Pfizer, Turkey, for three days postoperatively) were provided for the dogs undergoing ovariohysterectomy.

Statistical evaluation of the data was performed with a normality test (Anderson-Darling) using the Minitab-16 packet program, followed by the Kruskal-Wallis test for non-parametric data and the ANOVA method (One-way Analysis of Variance-Tukey's pairwise comparisons), with $P<0.05$ accepted as significant.

RESULTS

All study findings were categorized as clinical, hemodynamic *(Table 1)* and biochemical *(Table 2)*.

There was no statistically significant difference between the groups in terms of body weight (BG: 21.5±6.9 kg RG: 25.13±5.0 kg), age (BG: 2.5±1.4 years, RG: 3.2±2.3 years) and ovariohysterectomy operation time (BG: 42.38±5.0 min, RG: 42.50±3.9 min). But, duration of IT anesthesia (BG: 89.5±7.6 min; RG: 64.75±6.8 min) was significantly different between the groups ($P<0.05$). Before recovery from IT anesthesia in all dogs, there was signs of waking up such as head and eye movements because it was more short-term effects of propofol. However, regardless of these symptoms, the duration of IT anesthesia was measured. Also, the effort for reach to the sternal position was not observed until the end of the IT anesthesia.

In both groups, the duration of IT anesthesia was sufficient for the ovariohysterectomy operation and adequate analgesia and muscle relaxation for this operation was achieved. Also, there was no need for additional local anesthetic doses in both groups.

No negative situations were encountered in the intraoperative period; in particular there was no response to any touch and pull of the *ligamentum latum uteri*.

All values for both groups were within the reference range despite some differences in hemodynamic values *(Table 1)*. After the administration of propofol, SBP, DBP, MBP and HR decreased, but this decrease was within the normal range. However, this decrease continued with IT anesthesia, but the decline was less in RG. Following propofol administration, apnea also occurred in all dogs and RR returned to baseline values within a short time. RT was similar in both groups, exhibiting a mild decline. No statistically significant differences were found between groups in ECG measurements.

Table 1. *Mean±sd for heart rate (HR), systolic arterial blood pressure (SBP), diastolic arterial blood pressure (DBP), mean arterial blood pressure (MBP), respiratory rate (RR), and rectal temperature (RT) with IT bupivacaine (BG) or ropivacaine (RG) following propofol administration in dogs*

Variables	Groups	Time (min)								
		0 (initially)	Propofol+1	IT +5	15	30	60	90	120	150
SBP (mm Hg)	BG	132.5±11.3^{a}	124.5±8.4^{ab}	118.1±8^{b}	115.2±8.6^{b}	123.8±6^{ab}	125±8.8^{ab}	126.5±9.3^{ab}	124±9.2^{ab}	130±9.2^{ab}
	RG	133.3±6^{a}	123.1±6.7^{b}	113.1±6.9^{c}	109.7±7.9^{c}	116.5±6.2^{bc}	126.5±3.3^{ab}	130.2±3.8^{ab}	132.5±5.1^{ab}	132.5±6.2^{ab}
DBP (mm Hg)	BG	96.5±6.8^{a}	92.2±6.3^{ab}	87.2±8.7^{b}	86.5±4.8^{b}	88.7±4.8^{ab}	92.2±4.33^{ab}	94±5.8^{ab}	95.1±6.6^{ab}	95.8±6.7^{a}
	RG	95.2±5.4^{a}	91±5.3^{ab}	86.2±3.9^{bc}	84.5±5.5^{bc}	83.2±3.2^{c}	87.1±3.5^{bc}	89.5±5.2^{abc}	94±5.9^{ab}	95.2±4.5^{ab}
MBP (mm Hg)	BG	113±3.8^{a}	107.8±3.4^{ac}	102±3.8^{b}	101.3±3.8^{b}	104.3±4.9^{bc}	108.5±5.4^{ac}	111.2±5.2^{ac}	111.7±3.9^{ac}	113.2±3.5^{ac}
	RG	111.5±2.9^{a}	106.3±3.8^{a}	99±3.5^{b}	94.6±2.9^{b}	94±3.5^{b}	100.7±5.5^{b}	108.5±5.9^{a}	110.7±3.5^{a}	111.7±3.2^{a}
HR (bpm)	BG	85.3±2^{a}	81.5±1.4^{b}	78.5±1.4^{c}	78±1.5^{c}	76.7±2.6^{c}	78.1±2.2^{c}	82±1.8^{b}	83.5±2.7^{ab}	85±2.6^{a}
	RG	85.6±2.5^{a}	81.2±2.1^{b}	78±1.8^{c}	77.8±1.1^{c}	78.8±2.2^{c}	81.3±1.4^{bc}	83.2±1.4^{ab}	84.2±1.9^{ab}	85.1±1.5^{a}
RR (bpm)	BG	26.5±3.3^{a}	17.8±1.8^{b}	16.8±2.2^{b}	16.6±1.9^{b}	18.6±1.9^{b}	21.2±1.9^{bc}	24.1±2.9^{ac}	25.2±3.1^{a}	26.7±3.9^{a}
	RG	27.7±3.2^{a}	16.8±3.9^{b}	17.7±2.8^{b}	17.2±3^{b}	19.6±2.3^{bc}	22.5±2^{c}	25.3±2.9^{abc}	26.5±3^{a}	27.2±3.9^{a}
RT (°C)	BG	38.5±0.1^{a}	38.4±0.1^{ab}	38±0.1^{b}	37.9±0.1^{b}	37.9±0^{b}	38.2±0.5^{b}	38.2±0.5^{ab}	38.2±0.1^{ab}	38.4±0.2^{ab}
	RG	38.6±0.1^{a}	38.2±0.2^{b}	38±0.1^{b}	37.9±0.1^{b}	37.9±0^{b}	38.2±0.1^{b}	38.4±0^{a}	38.5±0^{a}	38.5±0.1^{a}

a-c: *Differences between average values are shown by different letters on the same line ($P<0.05$)*

Table 2. *Mean ± sd for biochemical parameters according to anesthesia intervals*

Variables	Groups	Time (min)				
		0 (Initially)	15	30	60	120
Glucose (mg/dL)	BG	71.2±5.6 [a]	76.3±5.2 [a]	77.6±3.8 [ac]	79.8±4.8 [bc]	84.8±4.0 [c]
	RG	68.6±3.6 [a]	69.9±8.7 [a]	64.1±77.6 [a]	77.6±11.5 [a]	79.3±14.2 [a]
AST (IU)	BG	49.0±4.3 [a]	42.1±2.5 [b]	40.0±2.7 [b]	34.8±1.5 [c]	35.1±1.5 [c]
	RG	49.3±2.6 [a]	43.4±2.4 [b]	37.0±2.6 [c]	33.8±2.6 [c]	37.3±4.4 [c]
ALT (IU)	BG	64.7±9.6 [a]	44.6±4.8 [b]	38.8±6.9 [b]	28.8±3.3 [c]	25.6±2.8 [c]
	RG	58.6±9.8 [a]	43.2±4.7 [b]	36.1±3.3 [b]	28.0±3.0 [c]	34.6±5.8 [bc]
BUN (mg/dL)	BG	6.9±2.0 [a]	9.8±3.7 [a]	9.8±3.7 [a]	17.18±4.5 [b]	20.0±5.0 [b]
	RG	7.1±2.5 [a]	8.7±1.7 [a]	11.8±1.7 [a]	17.2±4.7 [b]	19.6±4.1 [b]
Creatine (mg/dL)	BG	0.64±0.1 [a]	0.76±0.1 [ab]	0.85±0.1 [b]	0.91±0.1 [bc]	1.07±0.1 [c]
	RG	0.71±0.1 [a]	0.71±0.0 [a]	0.78±0.0 [ab]	0.85±0.0 [b]	0.88±0.1 [b]

a-c: *Differences between average values are shown by different letters on the same line (P<0.05)*

In addition, recovery from depressed hemodynamic parameters lasted longer in BG but was more short-lived in RG.

All values were within the range of reference values in the groups *(Table 2)* although there were some statistically differences in terms of biochemical values.

DISCUSSION

Propofol is used with many different combinations to immobilize the patient, and maintain safe comfortable anesthesia and easy recovery from anesthesia with minimal side effects by administering before anesthesia despite its weak analgesic effect [10-15]. In our study, the patient was prepared for anesthesia by using propofol before IT anesthesia in ovariohysterectomy operations. An induction that is effective in a short time was achieved in all cases in this study. Therefore, difficulties were not encountered in the transition of the *ligamentum flavum* during subarachnoid or IT punctures. In addition, desensitization at this location does not require local anesthesia. Total immobility was provided for all of the dogs, and this situation was the positive contribution for IT anesthesia during surgery.

A dose-dependent depressive respiratory effect is expected with propofol administration [10-13]. There was apne in our study, but respiration rate after recovering apnea was within the range of normal physiological reference values [20]. Therefore, the lowest possible dose is recommended, and the intravenous injection should not be performed too rapidly.

It has been reported that neurological complications after subarachnoid or IT anesthesia can be caused by local anesthetic toxicity and that this complication varies depending on pH, oil solubility and the protein binding power of the local anesthetic [1,2]. The effective duration of a local anesthetic is determined by its protein binding power. Both bupivacaine and ropivacaine are members of the long-acting, amide class of local anesthetics [1,2,16,18]. It has been reported that the effect of bupivacaine lasts longer than ropivacaine, but bupivacaine may have cardio-pulmonary side effects [1,2,16]. A statistical difference was found between BG and RG with regard to the duration of IT anesthesia (BG: 89.5±7.6 min; RG: 64.75±6.8 min) (P<0.05). Other studies [1,5] have reported similar findings. In addition, recovery from depressed hemodynamic para-meters lasted longer in BG but it was more short-lived in RG. In our study, no complications of IT anesthesia that induced clinical findings or behavioral changees were observed in the dogs. Furthermore, abnormal symptoms in the dogs' general condition, such as inactivity, loss of appetite, moaning after the anesthesia or in the post-operative period were not observed. Moreover, the animal owners did not report any subsequent negative feedback.

Bupivacaine and ropivacaine are metabolized in liver and in kidney (up to 10%) within 24 h of administration [20]. Also, propofol is metabolized in the liver [11]. Even though there was a statistically significant difference (P<0.05) between the initial values and the values observed during anesthesia in biochemical parameters. However, the obtained values were in the range with the reference values [20]. Hence, it can be said that the side effects of drugs used in spinal anesthesia on the liver or kidney are less because they joined slowly in a long time to systemic circulation.

The cranial progression of local anesthetics in cerebro-spinal fluid throughout medullar canal is not a desirable situation during subarachnoid, IT or spinal anesthesia. It can lead to the development of hypotension, migration of local anesthetic towards the cranial area, and respiratory depression due to blockage of the diaphragm and inter-costal muscles, possibly resulting in death. Therefore, the choice of local anesthesia for spinal or IT anesthesia is critical [1-3,6-9].

There are many studies where the advantages of bupivacaine and ropivacaine are demonstrated [1,2,16,17]. However, these local anesthetics may extend to the cranial area. To prevent this from occurring, either the solution should be in hyperbaric concentrations, or the patient may be inclined on the operating table to take advantage of gravity [1-3,5]. In our study, the spread of local anesthetic agents to the cranial area was limited by tilting the operating table to elevate the animal's head. Additionally, hemodynamic values in both groups were within the physiological reference range despite a statistically significant difference compared to baseline values, and no life-threatening changes were observed. Therefore, we believe that tilting the operating table during spinal anesthesia should not be ignored.

Some authors have reported that an increase in heart rate, respiration rate and systolic blood pressure values of 20% or more during the operation are thought to be an indication of intraoperative pain [3,5,15,21]. In our study HR, RR and SBP were almost the same as baseline values. Hence, there was no evidence of intraoperative pain in this sense or any other indication during the operation.

The results of this study comparing IT bupivacaine and ropivacaine after propofol administration for ovario-hysterectomy operations in dogs demonstrate that safe and effective anesthesia can be achieved using IT ropivacaine and bupivacaine, and that it can be a practical and functional option after propofol administration.

Conflict of Interest

None of the authors of this paper have any financial or personal relationship with other individuals or organizations.

REFERENCES

1. Yayla S, Kilic E: The comparison of clinical, histopathological and some hemodynamic effects of spinal anesthesia applied in dogs through bupivacaine HCl and ropivacaine HCl in two different concentrations. *Kafkas Univ Vet Fak Derg*, 16, 835-840, 2010. DOI: 10.9775/kvfd.2010.1774

2. Yayla S, Kilic E, Aksoy O, Ozaydin I, Ogun M, Steagall PVM: The effects of subarachnoid administration of hyperbaric solutions of bupivacaine or ropivacaine in xylazine-sedated calves undergoing surgery. *Vet Rec*, 173, 580+, 2013. DOI: 10.1136/vr.101630

3. Yayla S, Kacar C, Kaya D, Merhan O, Aksoy O, Kilic E, Kaya S: Clinical, biochemical and haemodynamic effects of the intrathecal ketamine for ovariohysterectomy in bitches. *Bull Vet Ins Pulawy,* 56, 299-303, 2012. DOI: 10.2478/v10213-012-0054-9

4. Kilic E, Yayla S, Kamiloglu A, Baran V, Ogun M: Effects of intrathecal administration of ketamine HCl in young calves: A clinical trial. *Bull Vet Inst Pulawy*, 59, 155-159, 2015. DOI: 10.1515/bvip-2015-0023

5. Kacar C, Ozaydin I, Oral H, Kilic E, Gurbulak K, Aksoy O: Intrathecal slow infusion of isobaric bupivacaine HCl in low-dose for ovariohysterectomy in dogs. *Bull Vet Inst Pulawy*, 51, 89-92, 2007.

6. Skarda R: Local and regional anesthetic and analgesic techniques in dogs. **In,** Thurman JC, Tranquilli WJ, Benson GJ (Eds): Lumb and Jones' Veterinary Anesthesia, 3rd edn., 434-447. Baltimore: Williams and Wilkins, 1996.

7. Torske KE, Dyson DH: Epidural analgesia and anesthesia. *Vet Clin North Am: Small Anim Pract*, 30 (4): 859-874, 2000.

8. De Barros GAM, Marques MEA, Ganem EM: The effects of intrathecal administration of betamethasone over the dogs' spinal cord and meninges. *Acta Cir Bras*, 22 (5): 361-365, 2007. DOI: 10.1590/S0102-86502007000500007

9. Jones RS: Epidural analgesia in the dog and cat. *Vet J,* 161, 123-131, 2001. DOI: 10.1053/tvjl.2000.0528

10. Ozaydın I, Atalan G, Uzun M, Kilic E, Cenesiz M: Köpeklerde medetomidin, propofol ve ketamin kombinasyonunun anestezik özellikleri ile klinik, kardiyovasküler ve respiratorik etkilerinin değerlendirilmesi. *Kafkas Univ Vet Fak Derg*, 7 (1): 71-76, 2006.

11. Topal A: Enjektable anestezikler. **In,** Veteriner Anestezi. 125-126, Nobel&Güneş, İstanbul, 2005.

12. Mendes GM, Selmi AL: Use of combination of propofol and fentanyl, alfentanil, or sufentanil for total intravenous anesthesia in cats. *JAVMA*, 223, 1608-1613, 2003. DOI: 10.2460/javma.2003.223.1608

13. Morse Z, Sano K, Kanri T: Effect of a propofol-ketamine admixture in humans volunteers. *Pac Health Dialog*, 10, 51-54, 2003.

14. Pekcan Z: How can better anaesthetic combinations be performed? A review of current knowledge. *Kafkas Univ Vet Fak Derg,* 22, 631-638, 2016. DOI: 10.9775/kvfd.2016.15111

15. Kürüm B, Pekcan Z, Kalender H, Kumandaş A, Can-Mutan O, Elma E: Comparison of propofol-remifentanil and propofol-fentanyl anesthesia during ovariohysterectomy in dogs. *Kafkas Univ Vet Fak Derg,* 19 (Suppl-A): A33-A40, 2013. DOI: 10.9775/kvfd.2012.7548

16. Malinovsky JM, Charles F, Kick O, Lepage JY, Malinge M, Cozian A, Bouchot O, Pinaud M: Intrathecal anaesthesia: Ropivacaine versus bupivacaine. *Anesth Analg*, 91, 1457-1460, 2000. DOI: 10.1097/00000539-200012000-00030

17. Luck JF, Fettes PDW, Wildsmith AW: Spinal anaesthsia for elective surgery: A comparison of hyperbaric solutions of racemic bupivacaine, levobupivacaine, and ropivacaine. *Br J Anaesth,* 101, 705-710, 2008. DOI: 10.1093/bja/aen250

18. Liu SS, Lin Y: Local anesthetics. **In,** Barash PG, Cullen BF, Stoelting RK, Calahan MK (Eds): Clinical Anesthesia. 531-548, Lippincott Williams & Wilkins. 2009.

19. Fridericia LS: Die Systolendauer im Electrocardiogramm bei normalen Menchen und bei Herzkranken. *J Int Med,* 54, 17-50, 1921. DOI: 10.1111/j.0954-6820.1921.tb15167.x

20. Blue JT, Short CE: Preanesthetic evaluation and clinical pathology. **In,** Shot CE (Ed): Principles and Practice of Veterinary Anesthesia. 3-7, Williams & Wilkins, Baltimore, 1987.

21. Schuszler L, Igna C, Sala A, Brudiu I, Sabau M, Dascalu R: Intra-operative analgesic effect of butarphanol for ovariohysterectomy in bitches: A dose titration study. *Lucrari Ştiintifice Med Vet*, 2, 160-163, 2009.

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Research Article

Sequencing and Phylogenetic Analysis Reveal the Prevalence of Duck Hepatitis A Virus Genotype-3 in Vietnam [1]

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Abstract

Duck hepatitis A virus (DHAV) causes an acute and highly contagious disease in young ducklings worldwide. Despite the widespread use of the DHAV vaccine, many outbreaks still occur in Vietnam. In this study, we determined the full-length genome sequence of two DHAV isolates (NC and NT) obtained from infected ducks in 2009 and 2013, and compared them with the attenuated DHAV-1 vaccine strain (namely, VXXT) currently used in Vietnam. The NC and NT strains belong to the virulent DHAV-3 genotype, and their genomes consist of 7791 and 7790 nucleotides (nt), respectively. Both genomes contain one large open reading frame (ORF) of 6756 nt, encoding a polyprotein of 2251 amino acids and possess a typical picornavirus genome organization. The majority of predicted C-terminal cleavage sites in the polyprotein were Q/S or Q/G. Phylogenetic analysis of the nucleotide sequence of the full ORF revealed that all virulent Vietnamese strains are closely related to Chinese DHAV-3 strains. Noticeably, the virulent Vietnamese DHAV-3 strains had relatively low nucleotide identity with the DHAV-1 vaccine strain (73.5%). The study showed that an antigenicity-matched DHAV-3 vaccine is urgently required for use in Vietnam.

Keywords: *Duck hepatitis A virus, Genotype, Genome, Phylogenetic*

Sekans ve Filogenetik Analiz Vietnamda Ördek Hepatitis A Virüs Genotip-3'ün Prevalansını Ortaya Koymaktadır

Özet

Ördek Hepatitis A virüsü (DHAV) dünya çapında ördek palazlarında akut ve oldukça bulaşıcı bir hastalığa neden olmaktadır. DHAV aşısının yaygın kullanımına rağmen Vietnam'da hala birçok salgınlar oluşmaktadır. Bu çalışmada, 2009 ve 2013 yıllarında enfekte ördeklerden elde edilen iki adet DAHV izolatının (NC ve NT) tüm genom sekansı belirlenmiştir ve Vietnam'da mevcut kullanılan atenüe DHAV-1 aşı suşu (VXXT) ile karşılaştırılmıştır. NC ve NT suşları virulent DHAV-3 genotipine ait olup genomları sırasıyla 7791 ve 7790 nükleotid (nt)'ten oluşmaktadır. Her iki genom 6756 nt boyutunda bir büyük açık okuma çerçevesine (open reading frame ORF), sahip olup 2251 amino asit uzunluğunda bir poliprotein kodlar ve a tipik olarak picornavirus genom organizasyona sahiptir. Poliproteinde tahmini C-terminal ayrılma bölgelerinin çoğunluğu Q/S veya Q/G olarak belirlendi. Bütün ORF'nin nükleotid sekansının filogenetik analazi tüm virülent Vietnam suşlarının Çin DHAV-3 suşu ile yakından akraba olduğunu gösterdi. Virülent Vietnam DHAV-3 suşları göreceli olarak DHAV-1 aşı suşu ile düşük nükleotid benzerliğine (%73.5) sahipti. Bu çalışma antijen uyumlu bir DHAV-3 aşısının Vietnam'da acilen kullanılması gerekliliğini göstermektedir.

Anahtar sözcükler: *Ördek hepatitis A virüsü, Genotip, Genom, Filogenetik*

INTRODUCTION

Duck hepatitis virus (DHV), first described on Long Island, New York in 1949 [1], causes a rapidly spreading and often fatal disease in young ducklings [2]. Historically, three distinct serotypes of DHV (DHV-1, -2, and -3) have been described, among which there are no antigenic relationships [2-4]. Recently, according to the Ninth Report of the International Committee on Taxonomy of Viruses (ICTV), DHV-2 and DHV-3 were classified as duck astrovirus

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type 1 (DAstV-1) and duck astrovirus type 2 (DAstV-2), respectively, whereas DHV-1 was renamed as duck hepatitis A virus (DHAV) and classified as a member of the newly defined genus *Avihepatovirus* in family Picornaviridae [5]. DHAV has three genotypes, i.e., DHAV-1, DHAV-2, and DHAV-3 [6-9], are of the most virulent variants [2,10]. Currently, DHAV-1 distributes globally, whereas DHAV-3 has been reported only in China and Korea [6,7,11,12] and DHAV-2 is limited to Taiwan [8]. Recently, the *in vivo* distribution of both DHAV-1 and DHAV-3 viruses in clinically infected ducklings was reported in Chinese flocks [13,14].

To date, nearly 50 complete genome sequences of DHAVs have been published [7,12,15]. DHAVs have a positive single-stranded RNA genome with a single large open reading frame (ORF) of 6750 nucleotides, encoding a polyprotein of 2249 amino acids in DHAV-1 and DHAV-2 or 2251 amino acids in DHAV-3 (due to an additional 6 bp in VP1, encoding two extra amino acids).

The diversification of DHAV in recent years, and particularly the emergence of DHAV-3, may have been underestimated. It is possible that DHAV-3 exists in countries other than China and Korea; if so, the prevalence of this genotype in previously unreported locations remains unknown. Each year in Vietnam, 50 million ducks are raised in farms and in fields. The ducklings are all vaccinated with DHAV-1 vaccine(s), most commonly the VXXT vaccine strain produced by the Veterinary Vaccine Company of Vietnam (VETVACO, Hanoi, Vietnam). Nonetheless, severe annual outbreaks of DHAV still occur, suggesting an antigenic mismatch between the vaccine (DHAV-1 derivative) and the circulating virulent DHAV variants. The discovery of DHAV-3 in China and Korea [6,7] and DHAV-2 in Taiwan [8] raises questions about the existence of different genotypes of DHAVs in other countries. Therefore, in this study, we sequenced the complete genomes of two virulent DHAV strains and compared them with the vaccine strain currently used in Vietnam to determine whether DHAV-3 or DHAV-2 are occurring in this country. This is the first international report to analyze the full-length genome sequences of DHAVs in Vietnam.

MATERIAL and METHODS

Viruses Used in the Analyses

Totally 32 samples were collected including two DHAV-1 attenuated vaccine strains and 30 duckling liver clinical samples. Using primers for VP1 gene, 11 of them were identified as DHAV, and 10/11 were DHAV-3 [16]. Among them, four strains was chosen to sequence and analysis complete genome for this study, including one DHAV-1 vaccine strain (VXXT) and three field DHAV-3 strains (DN2, NC and NT) that isolated from outbreaks in Dong Nai (2009), Ho Chi Minh City (2009) and Ninh Thuan province (2013), respectively. The VXXT vaccine strain was produced by the VETVACO (Hanoi, Vietnam). The complete genome sequence of the DHAV-3 strain DN2 and the DHAV-1 vaccine strain VXXT were obtained and submitted in advance in GenBank (GenBank accession numbers JF914944 and JF914945). The complete genome sequence of the two remaining strains, DHAV-3 NC and NT, were obtained and analyzed in this study.

RNA Extraction and Reverse Transcription

Viral RNAs were extracted from samples using the RNeasy Mini Kit (Qiagen, Germany) and stored at -80°C until use. cDNA synthesis was conducted using the Maxima Reverse Transcriptase kit (Fermentas, EU) as follows: 4 µl of viral RNA (~100 ng of viral RNA), 1 µl of random hexamer primer (100 pmol/µl), 1 µl of dNTP mix (10 mM each), 5 µl of transcriptase buffer (5X), 0.5 µl of Ribolock™ RNase inhibitor (20 U), 1 µl of Maxima Reverse Transcriptase (20 U), and 8.5 µl of RNase-free water, in a final volume of 20 µl. The cDNA synthesis reaction mixture was incubated at 50°C for 60 min, and then for 5 min at 85°C to inactivate the enzyme. The cDNA samples were stored at -20°C for further use.

Primers and PCR Amplification

For preliminary detection of DHAV genotypes, three genotype-specific primer pairs were designed, based on the conserved sequences of each DHAV genotype, to specifically amplify the VP1 region *(Table 1)*. Next, five pairs of primers were designed to obtain full-length genome sequences of three DHAV-3 strains *(Table 2)*. The conditions used for amplification were as follows: initial denaturation at 94°C for 5 min; 35 cycles of 94°C/1 min, 55°C/1 min (annealing), and 72°C/2 min (extension); followed by 10 min/72°C (final extension). Negative controls (DEPC water) were included in every set of PCR. PCR products were visualized by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. After purification using the AccuPrep™ Gel Purification Kit (Bioneer Inc., South Korea), amplicons were sequenced by a commercial service (Macrogen, South Korea).

Nucleotide Sequencing and Computational Analysis

Nucleotide sequences were identified using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). Multiple nucleotide alignment of the Vietnamese and previously available DHAV sequences was performed using GENEDOC 2.7 (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). A phylogenetic tree was constructed using the MEGA 6.06 package, based on the neighbor-joining method with 1000 bootstrap replicas [17]. DNA contigs were assembled to generate the complete genome sequence in MacVector 8.2, and predicted polyprotein cleavage sites were determined using GENEDOC 2.7 with reference data from a previous analysis [7]. The DHAV sequences obtained in

this study were deposited in GenBank with the accession numbers KU860089 and KU860090. The GenBank accession numbers of the complete ORF sequences, sample codes, and geographical origins are shown in *Fig. 1*.

RESULTS

Sequence Analysis of the Whole Genomes of Four Vietnamese DHAV Strains

The VP1 region was chosen for preliminary species identification using three pairs of genotype-specific primers *(Table 1)*. All collected samples were subjected to PCR-coupled sequencing and BLAST-based genotype confirmation. The VP1 nucleotide sequence revealed that the field isolates (NC and NT strains) were of genotype 3 (DHAV-3). The cDNAs of these two strains were converted and used as templates for coupled primer-walking PCR and sequencing to obtain the complete genome nucleotide sequence. Primers are listed in *Table 2*.

The sizes of the complete genomes of the NC and NT strains (preliminarily classified as DHAV-3 by nucleotide analysis of VP1) were 7791 and 7790 nucleotides, respectively, including the 18-nucleotide poly-adenine (polyA) tail at the 3′ end *(Table 3)*. Each genome consisted of one large ORF flanked by 5′ and 3′ untranslated regions (UTRs). The complete ORF sequences were used in a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST), providing further confirmation that the NC and NT virulent strains were of the DHAV-3 genotype. Comparative sequence analyses revealed that both strains had typical picornavirus genome organization: 5′UTR–L–VP0–VP3–VP1–2A1–2A2–2B–2C–3A–3B–3C–3D–3′UTR, with a 3′ tail of 18 adenine nucleotides (polyA). Details of genomic features for each strain, including sequential gene arrangement, gene clusters, and characteristics of individual genes, are provided in *Table 3*.

All three Vietnamese DHAV-3 strains, including NC, NT, and DN2 (GenBank accession number JF914944), had an ORF of 6756 nt, a 5′UTR of 652 nt, and a 3′UTR of 366-369 nt. The DHAV-1 VXXT strain (GenBank accession number JF914945) had an ORF of 6750 nt, with a VP1 gene that was 6 nt shorter than in DHAV-3 strains (714 versus 720 nt in DHAV-3 viruses); a 5′UTR of 626 nt (26 nt shorter); and a 3′UTR of 315 nt (51 nt shorter) *(Table 3)*.

The three Vietnamese DHAV-3 strains had similar base compositions: adenine, 27.49-27.68%; uracil, 29.37-29.45%; guanine, 22.13-22.47%; and cytosine, 20.35-20.76%. The base composition of the DHAV-1 VXXT strain was as follows: adenine, 29.1%; uracil, 28%; guanine, 22.7%; and cytosine, 20.2%.

Analysis of the polyprotein sequence revealed that the sequential protein order was the same as in all picornavirus

Table 1. *Genotype-specific primers used for amplication of VP1 region for preliminary discrimination of DHAV-1, DHAV-2 and DHAV-3*

Primer Name	Sequence (5′-3′)	Amplicon Size (kb)	Identification of Genotype*
DHAV1F	GCCCCACTCTATGGAAATTTG	~ 0.8	DHAV-1
DHAV1R	ATTTGGTCAGATTCAATTTCC		
DHAV2F	CACCACTTGGAGGAAATCAACAG	~ 0.8	DHAV-2
DHAV2R	CCACCTGATGTTTTGTTGTGAGAG		
DHAV3F	ATGCGAGTTGGTAAGGATTTTCAG	~ 0.8	DHAV-3
DHAV3R	GATCCTGATTTACCAACAACCAT		

** **DHAV:** Duck hepatitis A virus*

Table 2. *List of primer pairs designed to obtain complete nucleotide sequence of the Vietnamese DHAV-3 viruses*

Primer Name	Sequence (5′-3′)	Nucleotide Positions	Amplicon Size (kb)
DK1F	TTTGAAAGCGGCTGTGGTGTAG	1-21	~ 1.7
DK1R	GAGAGCAAAAGTTGGCATTCG	1745-1725	
DK2F	GTGGGTGATTTTCAGTGGGC	1653-1672	~ 1.7
DK2R	AACCAACCTCGGTAAGTGAGCACG	3413-3390	
DK3F	TGGAATCACTTGTTCGTGCC	3286-3305	~ 1.7
DK3R	AGACTGCCATCCCTCATTGC	4987-4986	
DK4F	GCGTAGGTTTCCAATCCGAC	4892-4911	~ 1.8
DK4R	GCTAACAGATTGTCCCACTCAGC	6691-6669	
DK5F	TTTGACTACACAGTTGCGTTCC	6563-6585	~ 1.2
DK5R	AGGGTGGGGAGGAATAGTAAAG	7781-7760	

Table 3. *Sequential gene arrangement in clusters, comparison of the genomic features and amino acid residues at predicted C-terminal cleavage sites for NC, NT, DN2 (DHAV-3) and VXXT (DHAV-1) strains*

Gene/Region	Size (Nucleotide)[a]				Predicted C-terminal Cleavage Sites (Amino Acid)			
	DHAV3		DHAV1		DHAV3		DHAV1	
	NC	NT	DN2	VXXT	NC	NT	DN2	VXXT
5'UTR	652	652	652	626	-	-	-	-
ORF	6756	6756	6756	6750				
3'UTR	366	366	369	315				
PolyA	18	18	18	18				
Complete size	7791	7790	7789	7703				
Polyprotein	2251	2251	2251	2249	-	-	-	-
L	**30**	**30**	**30**	**30**	**L/G** (30–31)	**L/G**	**L/G**	**L/G** (30–31)
VP0	226	226	226	226	Q/G (256–257)	Q/G	Q/G	Q/G (256–257)
VP3	237	237	237	237	Q/G (493–494)	Q/G	Q/G	Q/G (493–494)
VP1	240	240	240	238	E/S (733–734)	E/L	E/L	E/S (733–734)
P1	**703**	**703**	**703**	**701**	**E/S**	**E/L**	**E/L**	**E/S**
2A1	20	20	20	20	NPG/P (753–754)	NPG/P	NPG/P	NPG/P (749–752)
2A2	285	285	285	285	Q/S (1038–1039)	Q/S	Q/S	Q/S (1036–1037)
2B	119	119	119	119	Q/S (1157–1158)	Q/S	Q/S	Q/S (1155–1156)
2C	333	333	333	333	Q/G (1490–1491)	Q/G	Q/G	Q/S (1488–1489)
P2	**757**	**757**	**757**	**757**	**Q/G**	**Q/G**	**Q/G**	**Q/S**
3A	93	93	93	93	Q/S (1583–1584)	Q/S	Q/S	Q/S (1581–1582)
3B	34	34	34	28	Q/G (1617–1618)	Q/G	Q/S	Q/S (1615–1616)
3C	181	181	181	187	Q/G (1798–1799)	Q/G	Q/G	Q/G (1796–1797)
3D	453	453	453	453	-	-	-	-
P3	**761**	**761**	**761**	**761**	-	-	-	-

[a] ***Size:*** *Number of nucleotides (genome) or amino acids (proteins); numbers in parentheses indicate position of amino acid residues at the possible predicted C-terminal cleavage sites. Only positions of sites in the NC (DHAV-3) and VXXT (DHAV-1) strains are indicated; these sites are the same in other DHAV-3 strains (NT and DN2)*

genomes, with four major clusters: L, P1 (VP0–VP3–VP1), P2 (2A1–2A2–2B–2C), and P3 (3A–3B–3C–3D). Except for VP1 in the DHAV-1 VXXT strain, which was two amino acids shorter, the other clusters (L, non-structural P2, and P3) and individual genes were the same sizes in the Vietnamese DHAV-1 and DHAV-3 strains examined in this study *(Table 3)*. Eleven protease-cleavage sites were identified in the polyprotein in all four Vietnamese strains and two reference strains isolated in China, resulting in generation of 12 independent proteins (i.e., L, VP0, VP3, VP1, 2A1, 2A2, 2B, 2C, 3A, 3B, 3C, and 3D). The majority of predicted C-terminal cleavage sites in the polyprotein were Q/S or Q/G, with the exception of L/G between L and VP0; E/L or E/S between VP1 and A1; and NPG/P between 2A1/2A2 *(Table 3)*.

Sequence Comparison

Table 4 shows a comparison between the nucleotide and amino acid sequences of the Vietnamese NC strain and those of other Vietnamese strains (NT, DN2, VXXT) and the genotype-reference GD and C80 strains from China. The NC Vietnamese genome had nucleotide identity of 93.8-99.0% (ORF) or 94.0-98.8% (complete genome) with other DHAV-3 strains (i.e., NT, DN2, and reference GD), but much lower identity with the DHAV-1 VXXT vaccine (73.5%, ORF) and C80 (73.6%, ORF) reference strains. The amino acid similarity was higher: 98.6-99.4% within DHAV-3 strains and 83.2-83.6% between DHAV-3 and DHAV-1 strains *(Table 4)*.

Next, we compared the complete ORF sequences of the three Vietnamese DHAV-3 isolates (NC, NT, and DN2; 6756 nt) and that of the DHAV-1 vaccine strain VXXT (6750 nt), as well as their deduced amino acid sequences, with 44 other DHAV sequences available in GenBank (16 DHAV-1, 2 DHAV-2, and 26 DHAV-3). The GenBank accession numbers, codes of samples, and geographical origin used in this study are shown in *Fig. 1*.

Pairwise comparison of the ORF nucleotide sequences of the 48 strains revealed 91-99% identity between the 16 DHAV-1 strains, 92-100% identity between the 26 DHAV-3 strains, and 100% identity between the two DHAV-2

Table 4. *Comparison of the nucleotide and amino acid sequences of the Vietnamese NC strain to those of other Vietnamese strains (NT, DN2, VXXT) and the genotype-reference Chinese GD (DHAV-3) and C80 (DHAV-1) strains*

Genes/Regions	Length (DN2 Strain)		Nucleotide Identity (%)					Amino Acid Homology (%)				
	nt	aa	DHAV-3			DHAV-1		DHAV-3			DHAV-1	
			NT	DN2	GD	VXXT	C80	NT	DN2	GD	VXXT	C80
5'UTR	652	-	97.1	94.9	98.3	70.7	68.9	-	-	-	-	-
L	90	30	96.7	97.8	97.8	80	77.8	100	100	96.7	86.7	83.3
VP0	678	226	97.1	93.2	97.9	70.8	71.8	99.1	98.2	99.1	78.8	79.2
VP3	711	237	97.5	93.4	99.4	70.5	70.0	99.6	96.6	99.6	79.7	79.7
VP1	720	240	97.8	92.6	99.0	71.2	71.5	97.5	93.3	99.2	76.2	76.7
2A	915	305	97.3	93.4	99.0	69.3	69.3	97.4	96.1	99.3	74.4	75.4
2B	357	119	96.9	95.5	98.9	80.4	80.1	98.3	97.5	99.1	91.6	92.4
2C	999	333	98.2	93.7	99.1	76.7	76.3	99.4	98.2	99.4	91.9	91.9
3A	279	93	98.2	93.5	99.3	67.0	68.1	97.8	93.5	98.9	72.0	74.2
3B	102	34	99.0	95.1	100	70.6	71.6	100	100	100	79.4	79.4
3C	543	181	96.9	93.5	99.1	75.9	77.1	98.9	98.3	100	89.5	89.5
3D	1362	454	97.3	94.9	99.0	75.6	75.5	98.7	99.1	99.8	88.1	88.3
3'UTR	366	-	97.5	96.1	97.9	76.2	75.9	-	-	-	-	-
Structural P1	2109	703	97.4	93.1	98.8	71.3	71.5	98.7	96.0	99.3	78.2	78.5
Non-structural P2	2271	757	97.6	93.9	99.0	73.9	74.1	98.4	97.2	99.3	84.8	85.3
Non-structural P3	2286	762	97.4	94.4	99.1	74.6	75.1	98.6	98.3	99.7	86.1	86.5
ORF	6756	2252	97.5	93.8	99.0	73.5	73.6	98.6	97.2	99.4	83.2	83.6
Complete genome	7791	2252	97.2	94.0	98.8	72.8	72.8	-	-	-	-	-

nt: *nucleoties; aa: amino acid, Reference strain GD: accession numbers GQ122332; strain C80: accession numbers DQ864514*

strains (data not shown). Among the four Vietnamese strains (NC, NT, DN2 of DHAV-3; VXXT of DHAV-1), the highest nucleotide identity (98.4-98.6%) was observed between the Vietnamese NT and Chinese 12-01 (GenBank: KC893553) and B-N (JX235698) strains, and between the Vietnamese NC and Chinese DHAV-3 SD1201 strains (KC993890). The Vietnamese vaccine strain VXXT had 99% identity with the Korean DHAV-1 DRL62 (DQ219396) strain.

Phylogenetic Analysis of DHAV Genotypes

Based on the full ORF nucleotide sequences, a phylogenetic tree was constructed to examine the relationships between four Vietnamese and 44 other DHAV strains of all three genotypes *(Fig. 1)*. The tree revealed three clear genetic groups representing the DHAV-1, DHAV-2, and DHAV-3 genotypes. The DHAV-3 genotype was represented by 21 isolates from China and Korea and three isolates from Vietnam. These 24 DHAV-3 isolates could be divided into three clades (Clades 1, 2, and 3). Almost all Chinese isolates (15/16) grouped into Clade 1, along with two Vietnamese isolates (NC and NT). Of those, the NC isolate was most closely related to two Chinese strains, SD1201 and G, and the NT isolate was most closely related to the B-N and 1201 strains. All five Korean DHAV-3 strains were closely related within Clade 2. Clade 3 contained the Chinese B-63 and Vietnamese DN2 strains *(Fig. 1)*. The average genetic similarity within DHAV-3 Clades 1, 2, and 3 was 94%, 93%, and 93.5%, respectively.

The DHAV-1 genotype contained 19 different strains, divided into four main subgroups (Clades 1–4). The Vietnamese vaccine strain VXXT was clustered in Clade 3 with two Korean strains, the DRL62 and HS isolates. Clades 1, 2, and 4 comprised 19 Chinese DHAV-1 isolates *(Fig. 1)*; Clade 2 consisted of the Taiwanese strain O3D (GenBank number DQ249299) and four Chinese strains (DQ864514, DQ886445, HQ232302, and GQ130377) *(Fig. 1)*. The average nucleotide sequence similarity within clades 1, 2, 3, and 4 was 96-99%.

The DHAV-2 genotype was represented by only two strains from Taiwan (04G and 90D), which were 100% similar *(Fig. 1)*.

DISCUSSION

To date, several DHAV sequences from Korea, China, and Taiwan have been published [7,11,12,18,19]. In this study, we reported for the first time in an international journal the complete genome sequences and analysis of three DHAV-3 strains isolated from outbreaks in Vietnam, including DN2 (isolated in Dong Nai, 2009), NC (isolated in Ho Chi Minh

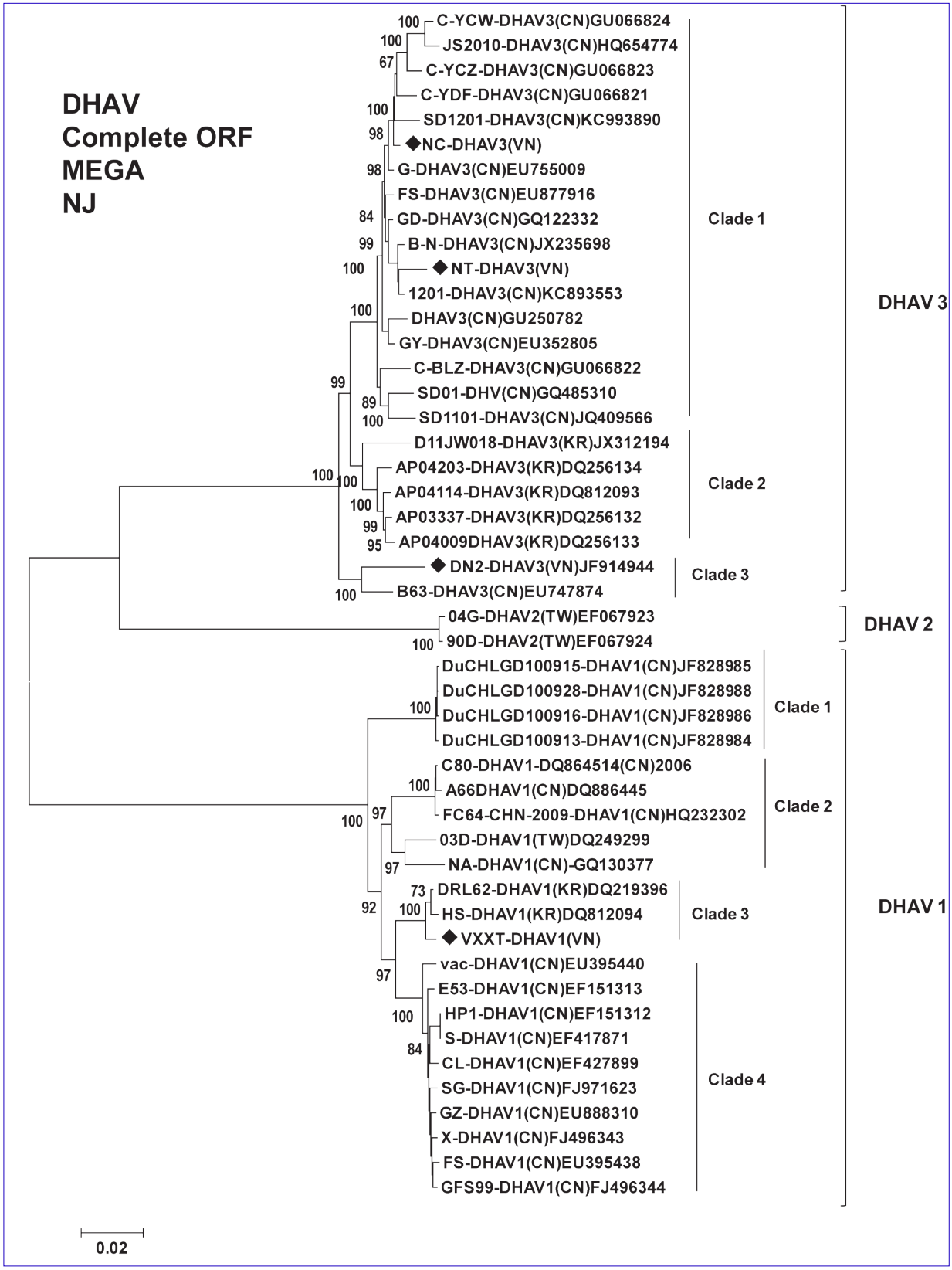

Fig 1. Phylogenetic tree showing the relationships between 4 Vietnamese and 44 DHAV strains of all three genotypes based on analysis of the full Open Reading Frame (ORF) nucleotide sequences. The phylogenetic tree was constructed with MEGA6.06 package using the neighbor-joining method [17] with bootstrap values of 1000 replicas (shown at each branch). Scale bar at bottom indicates the number of nucleotide substitutions per site. The isolates in this study are marked with bold letters and are indicated by a diamond symbol (♦). The accession numbers are given at the end of each sequence, where applicable. International country codes (https://countrycode.org/) in brackets; eg. VN, Vietnam; CN, China; TW: Taiwan; KR, South Korea.

City, 2009), and NT (isolated in Ninh Thuan province, 2013), as well as the DHAV-1 attenuated vaccine strain (VXXT) currently used for vaccination in Vietnam.

DHAVs were first detected in Vietnam in the 1980s. Some local reports based on epidemiological studies assumed that all DHVs in Vietnam were of the DHAV-1 genotype. However, none of the collected DHVs has been molecularly genotyped. In addition, despite efforts to understand the causes of this disease, no previous study has genetically characterized DHAV in Vietnam. Although a vaccine is used nationwide, severe outbreaks still occur in many areas throughout the country. In this study, we showed that the VXXT vaccine strain currently used in Vietnam is of the DHAV-1 genotype, whereas all three clinical samples collected from different provinces were of the DHAV-3 genotype. Our study of the complete genomes also revealed relatively low identity at the nucleotide (73%) and amino acid (83.1-83.6%) levels between strains of these two genotypes. This may explain the limited protection conferred by DHAV-1 vaccines against the circulating virulent DHAV-3 strains. Previous immunity studies in Taiwan and South Korea indicated that no cross-neutralization occurs in hosts infected with viruses of different genotypes [8,18].

Phylogenetic analysis of the complete ORFs confirmed that DHAVs in Vietnam were of two genotypes, DHAV-1 and DHAV-3. Noticeably, the Vietnamese vaccine strain (VXXT) was very closely related to two Korean strains (DRL62 and HS), forming a separate subgroup (Clade 3). This observation suggests that the VXXT strain may have originated in Korea. Our results also confirmed previous studies showing that the Chinese DHAV-1 strains, along with the Vietnamese strains used in this study, are highly diverse, forming three subgroups (Clades 1, 2, and 4) with nodal support (100%) in the phylogenetic tree [11].

Within the DHAV-3 genotype, all five Korean strains formed one subgroup (Clade 2). Clade 1 included most strains from China and two strains from Vietnam (NC and NT). The Vietnamese DN2 strain was closely related to the Chinese B-63 strain and formed a completely new subgroup, Clade 3. A previous study [15] reported a gene rearrangement between DHAV-1 and DHAV-3 in the Chinese DHAV-3 B-N strain, in which the 100 nt before the initiator codon has high identity with the corresponding sequence of DHAV-1. In this study, we demonstrated that the B-N strain was most closely related to another Chinese strain, 1201, and the Vietnamese strain NT. Moreover, we also paid special attention to the 5'UTR regions. Although the sequences of this region exhibit low similarity (66-72%) between genotypes 1, 2, and 3, all of them have some conserved regions, especially at 64 nt at the C-internal of the regions. In addition, the 34 amino acids at the N-terminus and the 24 amino acids from positions 65 to 88 of VP1 are extremely conserved among all genotypes. This information provides insight into the molecular features of DHAVs and hints at a transitional state between DHAV-1 and DHAV-3.

Our molecular investigation suggests that DHAV-3 viruses are predominantly spread in Vietnam. This is consistent with a previous study in China showing that DHAV-3 is more widespread than DHAV-1 [13], although other publications argued that DHAV-1 remains the most prevalent genotype in China [11,20]. Additionally, mixed infection with DHAV-1 and DHAV-3 in Chinese flocks has been in identified at various rates: 57.7% in one study [13] and 12% in another [14]. To date, however, we have not observed any mixed infections with these two genotypes in Vietnam. To clarify this issue further, more samples will be needed.

In summary, we report here the complete genome sequences of four DHAV samples: one attenuated vaccine of DHAV-1 genotype and three virulent strains of the DHAV-3 genotype currently circulating in Vietnam. These results will improve our understanding of the epidemiology and evolution of DHAVs in Vietnam. In addition to confirming the existence of DHAV-3 reported in previous studies, our observations emphasize the distribution of DHAV-3 viruses outside China and Korea and the significance of exploring DHAV-3 viruses in other countries where duck hepatitis is endemic and prevalent in ducklings. In light of these findings, an antigenicity-matched DHAV-3 vaccine is urgently required for use in Vietnam.

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REFERENCES

1. Levine P, Fabricant J: A hitherto-undescribed virus disease of ducks in North America. *Cornell Vet,* 40, 71-86, 1950.

2. Woolcock PR: Duck hepatitis. **In,** Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (Eds): Diseases of Poultry. 11th edn., 343-354, Iowa State University Press, Ames, 2003.

3. Haider SA, Calnek BW: *In vitro* isolation, propagation, and characterization of duck hepatitis virus typee III. *Avian Dis,* 23, 715-729, 1979. DOI: 10.2307/1589748

4. Toth TE: Studies of an agent causing mortality among ducklings immune to duck virus hepatitis. *Avian Dis,* 13, 834-846, 1969. DOI: 10.2307/1588590

5. Knowles NJ, Hovi T, Hyypia T, King AMQ, Lindberg AM, Pallansch MA, Palmenberg AC, Simmonds P, Skern T, Stanway G, Yamashita T, Zell R: Picornaviridae. **In,** King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (Eds): Virus taxonomy: Classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. 855-880, Elsevier, San Diego, California, 2012.

6. Kim MC, Kwon YK, Joh SJ, Lindberg AM, Kwon JH, Kim JH: Molecular analysis of duck hepatitis virus type 1 reveals a novel lineage close to the genus Parechovirus in the family Picornaviridae. *J Gen Virol,* 87, 3307-3316, 2006. DOI: 10.1099/vir.0.81804-0

7. Ding C, Zhang D: Molecular analysis of duck hepatitis virus type 1. *Virology*, 361, 9-17, 2007. DOI: 10.1016/j.virol.2007.01.007

8. Tseng CH, Tsai HJ: Molecular characterization of a new serotype of duck hepatitis virus. *Virus Res,* 126, 19-31, 2007. DOI: 10.1016/j.virusres.2007.01.012

9. Fu Y, Pan M, Wang X, Xu Y, Yang H, Zhang D: Molecular detection and typing of duck hepatitis A virus directly from clinical specimens. *Vet Microbiol*, 131, 247-257, 2008. DOI: 10.1016/j.vetmic.2008.03.011

10. Jin X, Zhang W, Zhang W, Gu C, Cheng G, Hu X: Identification and molecular analysis of the highly pathogenic duck hepatitis virus type 1 in Hubei province of China. *Res Vet Sci*, 85, 595-598, 2008. DOI: 10.1016/j.rvsc.2008.01.001

11. Gao J, Chen J, Si X, Xie Z, Zhu Y, Zhang X, Wang SJ, Jiang S: Genetic variation of the VP1 gene of the virulent duck hepatitis A virus type 1 (DHAV-1) isolates in Shandong province of China. *Virol Sin,* 27, 248-253, 2012. DOI: 10.1007/s12250-012-3255-8

12. Xu Q, Zhang RH, Chen LL, Yang L, Li JX, Dou PF, Wang H, Xie ZH, Wang Y, Jiang SJ: Complete genome sequence of a duck hepatitis A virus type 3 identified in Eastern China. *J Virol*, 86, 13848, 2012. DOI: 10.1128/JVI.02651-12

13. Chen LL, Xu Q, Zhang RH, Yang L, Li JX, Xie ZJ, Zhu YL, Jiang SJ, Si XK: Improved duplex RT-PCR assay for differential diagnosis of mixed infection of duck hepatitis A virus type 1 and type 3 in ducklings. *J Virol Methods,* 192, 12-17, 2013. DOI: 10.1016/j.jviromet.2013.04.012

14. Lin SL, Cong RC, Zhang RH, Chen JH, Xia LL, Xie ZJ, Wang Y, Zhu YL, Jiang SJ: Circulation and *in vivo* distribution of duck hepatitis A virus types 1 and 3 in infected ducklings. *Arch Virol,* 161, 405-416, 2016. DOI: 10.1007/s00705-015-2648-z

15. Wei CY, Su S, Huang Z, Zhu WJ, Chen JD, Zhao FR, Wang YJ, Xie JX, Wang H, Zhang G: Complete genome sequence of a novel duck hepatitis A virus discovered in Southern China. *J Virol*, 86, 10247, 2012. DOI: 10.1128/JVI.01643-12

16. Doan HTT, Le XTK, Do RT, Hoang CTM, Nguyen KT, Le TH: Molecular genotyping of duck hepatitis A viruses (DHAV) in Vietnam. *J Infect Dev Ctries*, 10, 988-995, 2016. DOI: 10.3855/jidc.7239

17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: MEGA 6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol,* 30, 2725-2729, 2013. DOI: 10.1093/molbev/mst197

18. Kim MC, Kwon YK, Joh SJ, Kim SJ, Tolf C, Kim JH: Recent Korean isolates of duckhepatitis virus reveal the presence of a new geno- and serotype when compared to duck hepatitis virus type 1 type strains. *Arch Virol,* 152, 2059-2072, 2007. DOI: 10.1007/s00705-007-1023-0

19. Wang L, Pan M, Fu Y, Zhang D: Classification of duck hepatitis virus into three genotypes based on molecular evolutionary analysis. *Virus Genes,* 37, 52-59, 2008. DOI: 10.1007/s11262-008-0233-1

20. Li J, Bi Y, Chen C, Yang L, Ding C, Liu W: Genetic characterization of duck hepatitis A viruses isolated in China. *Virus Res,* 178, 211-216, 2013. DOI: 10.1016/j.virusres.2013.10.007

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Research Article

Molecular Typing and Drug Resistance Analysis of *Salmonella spp.* Isolated from Pig Slaughterhouse in Shandong Province, China [1]

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Abstract

An epidemiological investigation of *Salmonella* enteritidis in pig and pork samples from eight slaughterhouses in Shandong Province, China, was conducted from December 2014 to October 2015. A total of 22.2% (142/640), of the slaughterhouse samples were recovered positive for *Salmonella spp.*. All Salmonella-positive were characterized using serotyping, antimicrobial resistance testing, pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Ten serotypes were shared by all isolates, with the most common serotypes being *Salmonella* Derby, *Salmonella* Typhimurium, and *Salmonella* Thompson. Antimicrobial sensitivity testing revealed that the highest antimicrobial resistance rate was against sulfisoxazole (91.55%) with many multidrug-resistant (MDR) isolates. MLST analysis showed that nine sequence type (ST) patterns were shared, ST40 was the most common (79 isolates) followed by ST19 (26 isolates) and ST26 (24 isolates). PFGE permitted the resolution of *XbaI* macrorestriction fragments of all the isolates, displaying the high similarity. Three clusters and 31 PFGE patterns were generated by PFGE analysis. Our results indicated that Salmonella spp. isolates from eight slaughterhouses were phenotypically and genetically homologous. These data could be used for further evolutionary analyses.

Keywords: *Salmonella spp., antimicrobial resistance, pulsed-field gel electrophoresis, multi-locus sequence analysis*

Çin'in Shandong Eyaletinde Domuz Mezbahasından İzole Edilen *Salmonella* spp.'nin Moleküler Tiplendirilmesi ve İlaç Dirençliliği Analizi

Özet

Çin'in Shandong Eyaletindeki sekiz kesimhaneden Aralık 2014 ile Ekim 2015 tarihleri arasında alınan domuz örneklerinde *Salmonella* enteritisi için epidemiyolojik bir çalışma yürütüldü. Kesimhane örneklerinin %22.2'sinden (142/640) *Salmonella* spp. tespit edildi. Tüm Salmonella pozitif örnekler serotiplendirme, antimikrobiyal dirençlilik testi, değişken alanlı jel elektroforez (PFGE) ve multilokus sekans tiplendirmesi (MLST) ile karakterize edildi. En yaygını Salmonella Derby, *Salmonella* Typhimurium ve Salmonella Thompson olmak üzere toplam on serotip tüm izolatlarda gözlemlendi. Antimikrobiyal hassaslık testi birçok çoklu ilaç direnciyle (MDR) birlikte en yüksek antimikrobiyal direncin sulfisoxazole (%91.55) karşı olduğunu gösterdi. MLST analizi dokuz sekans tipi (ST) şeklinin olduğunu gösterdi. Bu sekans tiplerinden en yaygını ST40 (79 izolat) daha az olarak ise ST19 (26 izolat) ve ST26 (24 izolat) olarak belirlendi. PFGE yüksek benzerlik göstererek tüm izolatların XbaI makrorestriksiyon parçalarının rezolüsyonunu sağladı. Üç küme ve 31 PFGE şekli PFGE analizi ile üretildi. Elde edilen sonuçlar, sekiz kesimhaneden izole edilen Salmonella spp.'nin fenotipik ve genotipik olarak homolog olduğunu gösterdi. Bu bulgular ileriki analizler için kullanılabilir.

Anahtar sözcükler: *Salmonella spp., Antimikrobiyal direnç, Değişken alanlı jel elektroforezi, Multilokus sekans analizi*

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INTRODUCTION

Salmonella enteritidis is an important group of bacterial pathogens which can cause severe foodborne disease in humans and animals, impacting health and productivity in worldwide [1]. In China, an estimated 70% to 80% of foodbome bacterial outbreaks were caused by *Salmonella spp.*[2]. Pigs have been recognized as one important reservoir for *Salmonella spp.*[3]. It also can be transferred to humans via pork along the food chain [4]. As one of the largest pork producersand consumption countries in the world, much more attention to the prevalence of *Salmonella spp.* in pork should be paid present in China.

Serotype and bacterial identification are important parts of outbreak investigation and epidemiological surveillance of *Salmonella spp.*[5]. Many DNA genotyping methods can be used to discriminate *Salmonella spp.* isolates beyond subspecies level and species, due to their high discriminative powers [6]. Pulse field-gel electrophoresis (PFGE) has been considered the "gold standard" for subtyping of all major foodbome pathogens because it is highly discriminatory [7]. However, this method can be difficult to compare across various laboratories for the same analysis or even among various runs within the same laboratory. To overcome these problems, multi-locus sequence typing (MLST) was developed as an alternative method for the analysis of bacterial populations. MLST results are easier to interpret and compare among laboratories and provide the best inferences of phylogenetic relationships [8]. Recent research indicated that MLST results generally prediction results of serotyping and that genotyping and serotyping can be complementary and also provide mutual authentication for Salmonella identification [9].

In recent years, the prevalence and characterization of *Salmonella spp.* along pork production chain were reported in many countries which the contamination rate of *Salmonella spp.* was 10% to 40% in pig slaughterhouse, the major serotypes were *Salmonella* Typhimurium and *Salmonella* Derby, and the serovars were diverse [10-13].

Though much attention has been focused on *Salmonella spp.* in pig slaughterhouse, intensive and simultaneous research regarding the prevalence, serotypes, antimicrobial resistance, pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) profiles of *Salmonella spp.* was limited, especially in China. Therefore, the objective of this study was to analyze the distribution, antimicrobial susceptibility profiles, and molecular characteristics of *Salmonella spp.* collected from eight pig slaughterhouse in Shandong Province, China, to determine the clonal relationships between isolates and provide data that can be used for further evolutionary analyses.

MATERIALS and METHODS

Salmonella Strains

The 640 fresh samples including five different production stages (n = 640), anal swab (n = 80), shower (n = 80), skinning or scalding (n = 120), removing the intestines (n = 80), cutting (n = 200) and product (n = 80) were collected from eight pig slaughterhouses (A~H) at different areas in Shandong Province, China. Pig slaughterhouse included five critical steps, such as shower, skinning or scalding, removing the intestines, cutting and product. Within a production cycle one compartment on five critical steps was included in the sampling process which was based upon ten time points for collecting *Salmonella spp.* contamination samples as presented *(Fig. 1)*.

For swab samples from slaughterhouse, the pre-enrichment step was performed by suspending each sample in 50 mL BPW, and incubating samples at 37°C for 16 h to 18 h. Then, 0.1 mL of the BPW suspensions was subcultured in 10 mL subpackaged Rappaport-

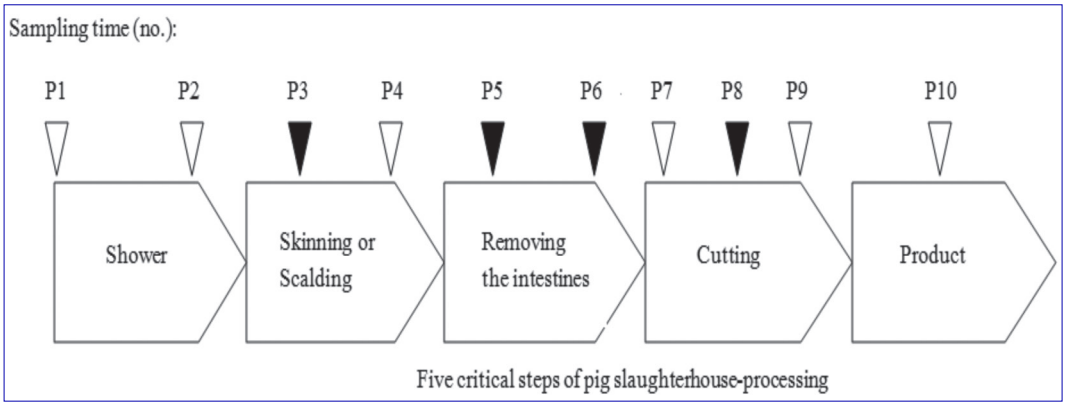


Fig 1. Graphic presentation of the sampling protocol throughout an pig slaughtering production line including one compartment at each production stage of f shower, skinning or scalding, removing the intestines, cutting and product in five China pig slaughtering production line. The numbers (no.) fromP1 to P10 above the white and black arrows are indicating the ten time points for sampling. white arrows: slaughtered each link of the samples (nos.: P1, P2, P4, P7, P9 and P10); black arrows: slaughtering production line used knives (nos.: P3, P5, P6 and P8))

Vassiliadis (RV) enrichment broth (Difco, USA) at 42°C for 24 h. One loopful of each RV broth culture was then streaked onto xylose lysine tergitol 4 (Difco, USA) agar plates, which were incubated at 37°C for 24 h to 48 h. One presumptive *Salmonella spp.* colony per plate was picked and biochemically confirmed using an API-20E test kit (bioMérieux, France). *Salmonella spp.* positive isolates were serotyped according to the White-Kauffmann-Le Minor scheme [14].

Antimicrobial Resistance

Susceptibility of all *Salmonella spp.* isolates to antimicrobial agents was evaluated according to Clinical Laboratory Standards Institute guidelines using the disc diffusion method on Mueller-Hinton agar [15]. Following 13 antimicrobial agents were tested, including Sulfisoxazole (SF, 30 μg), Doxycycline (DOX, 30 μg), Tetracycline (TE, 30 μg), Florfenicol (FFC, 5 μg), Ampicillin (AM, 10 μg), Gentamicin (GM, 10 μg), Spectinomycin (SPT, 10 μg), Sulfamethoxazole-trimethoprim (SXT, 1.25 and 23.75 μg), Enrofloxacin (ENR, 5 μg), Norfloxacin (NOR, 5 μg), Polymyxin (PME, 30 μg), Cefotaxime (EFT, 30 μg) and Amoxicillinpotassium clavulanate (AC, 30 μg). Results were interpreted using the Clinical and Laboratory Standards Institute (CLSI, 2013) breakpoints when available *E. coli* ATCC 25922 was used as quality control [16]. Descriptive statistical analysis of the results of these tests was accomplished using Epi Info 7.

Pulsed field-gel electrophoresis

PFGE was performed for *Salmonella* enterica using the Pulse Net protocol procedures described previously [17]. In brief, the chromosomal DNA of *Salmonella spp.* was digested with 50 U of *Xba* I (Takara, China) in 37°C water bath for 3 h. With a *Salmonella* serotype Braenderup strain (H9812) digested with *Xba* I (Takara, China) as the molecular weight standard, the DNA fragments were separated on a 0.8% agarose gel in 0.5 × TBE using CHEF-Mapper (Bio-Rad, USA). The experimental conditions were set up as follows: the initial and final switch time of 2.16 s and 63.8 s, respectively, an included angle of 120° and a gradient of 6 V/cm for 19 h at 14°C. The PFGE images were handled using the Gel Doc software (Bio-Rad, USA) according to the operation manual. PFGE was repeated twice to determine reproducibility. For untypable isolates, 50 μM thiourea (Sigma, USA) was added to the 0. 5 X TBE buffer prior to PFGE run as described by Römling and Tümmler [18]. The PFGE results of 142 isolates were disposed using the BioNumerics version 5.10 with the uniform marker normalization to record the strip position. A threshold of 85% homology was set to define clonal clustering of PFGE types.

Multi-locus Sequence Typing

Multi-locus sequence typing (MLST) method was performed according to the recommendations of the *Salmonella* enterica MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). An internal portion of seven housekeeping genes was amplified by PCR, including aspartokinase+homoserine dehydrogenase *(thrA)*, phosphoribosylaminoimidazole carboxylas *(purE)*, alpha ketoglutarate dehydrogenase *(sucA),* histidinol dehydrogenase *(hisD),* chorismate synthase *(aroC)*, uroporphyrinogen III cosynthase *(hemD*), and DNA polymerase III beta subunit (*dnaN*). DNA Taq premix (Promega, USA) was used with the amplification procedure: 94°C 5 min; followed by 30 cycles: 94°C 1 min, 55°C 30 s, 72°C 1min; and a final extension at 72°C 10 min. The amplification products were sent for bidirectional sequencing to Takara, China; and the sequences were analyzed using DNAStar Lasergene Version 7.1. We submitted the sequences to the UCC database for their allele and ST assignments.

RESULTS

The 142 out of 640 *Salmonella spp.* colonies were confirmed as *Salmonella*-positive samples. There into, 17 (21.3%) were recovered from anal swab, 13 (16.3%) from shower, 27 (22.5%) from skinning or scalding, 25 (31.3%) from removing the intestines, 47 (23.5%) from cutting and 13 (16.3%) from product *(Table 1)*. Ten majority serotypes were identified in all *Salmonella spp.* strains. *S. Derby* was

Table 1. *Prevalence data of Salmonella-positive samples by sampling in eight pig slaughterhouses line*

Sample from Production Line	No. of Positive Samples (Prevalence in %)								
	Pig Slaughterhouses								
	A n (%)	B n (%)	C n (%)	D n (%)	E n (%)	F n (%)	G n (%)	H n (%)	Weighted Mean n (%)
Anal swab	2 (20.0)	2 (20.0)	3 (30.0)	1 (10.0)	1 (10.0)	2 (20.0)	3 (30.0)	3 (30.0)	17 (21.3)
Shower	1 (10.0)	2 (20.0)	2 (20.0)	1 (10.0)	1 (10.0)	2 (20.0)	2 (20.0)	2 (20.0)	13 (16.3)
Skinning or scalding	4 (26.7)	5 (33.3)	2 (13.3)	3 (20.0)	2 (13.3)	3 (20.0)	4 (26.7)	4 (26.7)	27 (22.5)
Removing the intestines	3 (30.0)	2 (20.0)	4 (40.0)	4 (40.0)	3 (40.0)	2 (20.0)	4 (40.0)	3 (30.0)	25 (31.3)
Cutting	4 (16.0)	6 (24.0)	7 (28.0)	4 (16.0)	4 (16.0)	5 (20.0)	9 (36.0)	8 (32.0)	47 (23.5)
Product	2 (20.0)	2 (20.0)	1 (10.0)	1 (10.0)	2 (20.0)	1 (10.0)	2 (20.0)	2 (20.0)	13 (16.3)
Total	16 (20.0)	19 (23.8)	19 (23.8)	14 (17.5)	13 (16.3)	15 (18.6)	24 (30.0)	22 (27.5)	142 (22.2)

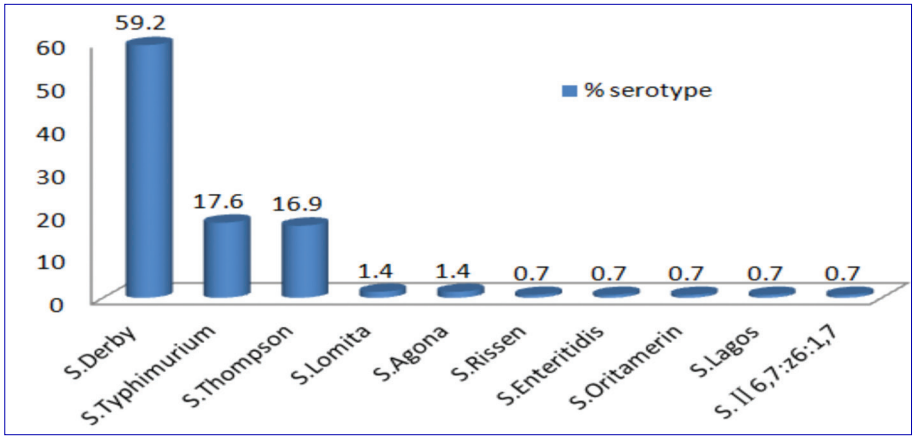
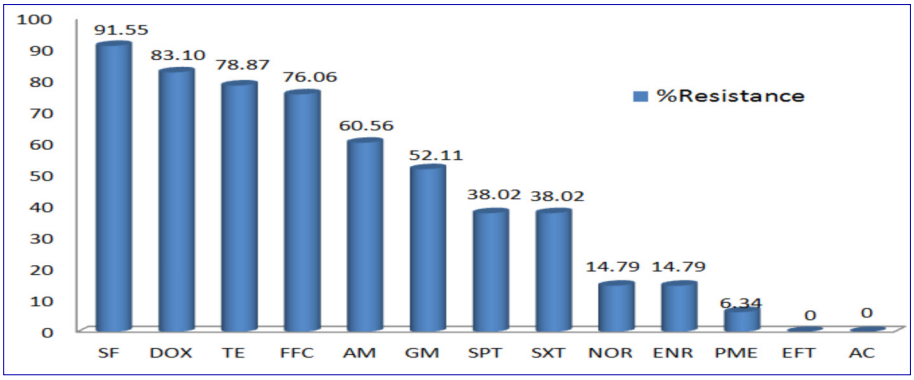
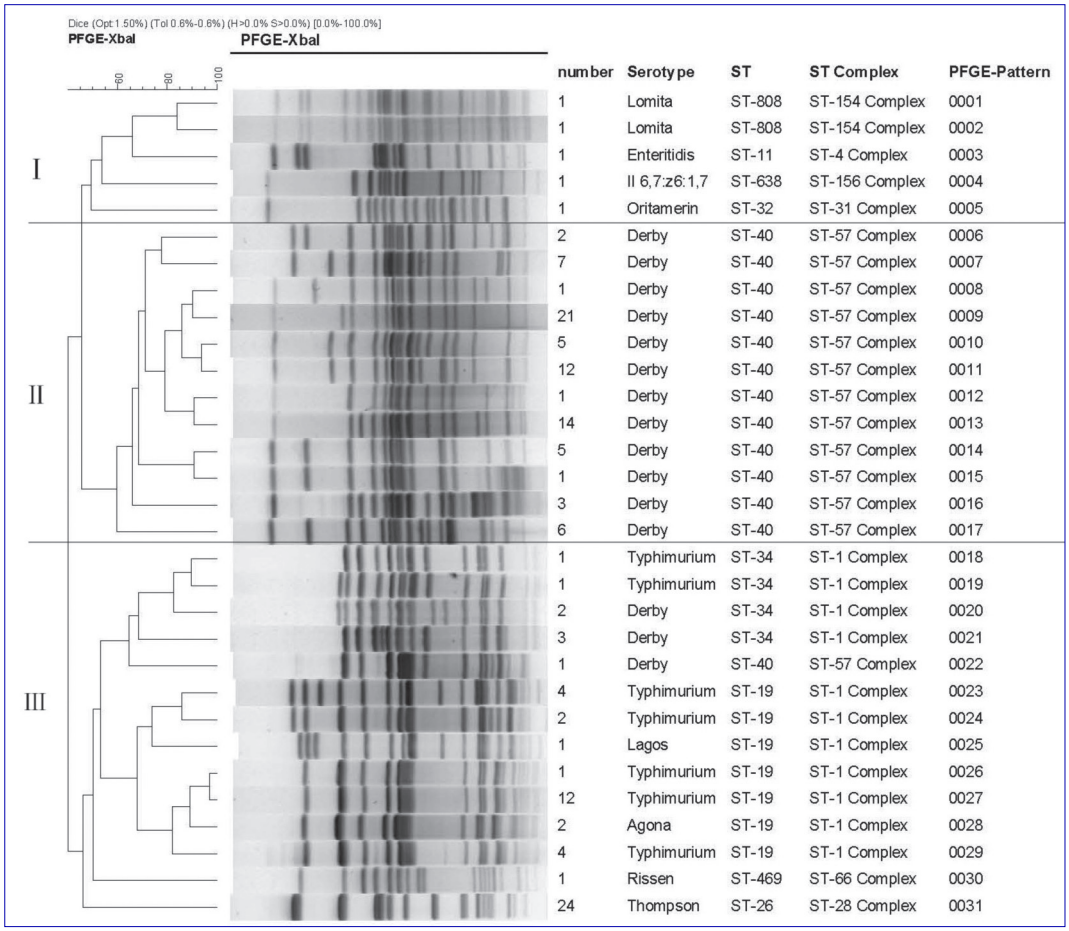


Fig 2. Serotype distribution of 142 *Salmonella* spp. isolate

Fig 3. Overview of antibiotic resistance ability of individual Salmonella strains

Antibiotic abbreviation: Sulfisoxazole (SF); Doxycycline (DOX); Tetracycline (TE); Florfenicol (FFC); Ampicillin (AM); Gentamicin (GM); Spectinomycin (SPT); Sulfamethoxazole-trimethoprim (SXT); Enrofloxacin (ENR); Norfloxacin (NOR); Polymyxin (PME); Cefotaxime (EFT); Amoxicillin potassium clavulanate (AC)





number	Serotype	ST	ST Complex	PFGE-Pattern
1	Lomita	ST-808	ST-154 Complex	0001
1	Lomita	ST-808	ST-154 Complex	0002
1	Enteritidis	ST-11	ST-4 Complex	0003
1	II 6,7:z6:1,7	ST-638	ST-156 Complex	0004
1	Oritamerin	ST-32	ST-31 Complex	0005
2	Derby	ST-40	ST-57 Complex	0006
7	Derby	ST-40	ST-57 Complex	0007
1	Derby	ST-40	ST-57 Complex	0008
21	Derby	ST-40	ST-57 Complex	0009
5	Derby	ST-40	ST-57 Complex	0010
12	Derby	ST-40	ST-57 Complex	0011
1	Derby	ST-40	ST-57 Complex	0012
14	Derby	ST-40	ST-57 Complex	0013
5	Derby	ST-40	ST-57 Complex	0014
1	Derby	ST-40	ST-57 Complex	0015
3	Derby	ST-40	ST-57 Complex	0016
6	Derby	ST-40	ST-57 Complex	0017
1	Typhimurium	ST-34	ST-1 Complex	0018
1	Typhimurium	ST-34	ST-1 Complex	0019
2	Derby	ST-34	ST-1 Complex	0020
3	Derby	ST-34	ST-1 Complex	0021
1	Derby	ST-40	ST-57 Complex	0022
4	Typhimurium	ST-19	ST-1 Complex	0023
2	Typhimurium	ST-19	ST-1 Complex	0024
1	Lagos	ST-19	ST-1 Complex	0025
1	Typhimurium	ST-19	ST-1 Complex	0026
12	Typhimurium	ST-19	ST-1 Complex	0027
2	Agona	ST-19	ST-1 Complex	0028
4	Typhimurium	ST-19	ST-1 Complex	0029
1	Rissen	ST-469	ST-66 Complex	0030
24	Thompson	ST-26	ST-28 Complex	0031

Fig 4. Dendrogram of the 31 patterns PFGE-XbaI identified with the frequency of each pattern from ten Salmonella serotypes isolated

Table 2. *STs and allele profile of isolates*

STs	acroC	dnaN	hemD	hisD	purE	SucA	thrE	No. of Isolates
11	5	2	3	7	6	6	11	1
19	10	7	12	9	5	9	2	26
26	14	13	18	12	5	18	1	24
32	17	18	22	17	5	21	19	1
34	10	19	12	9	5	9	2	7
40	19	20	3	20	5	22	22	79
469	92	107	79	156	64	51	87	1
638	47	98	36	152	174	7	171	1
808	10	71	43	12	190	20	18	2

the most serotype (59.2%, 84 of 142) strains. Salmonella group *Salmonella* Derby (n = 84), *Salmonella* Typhimurium (n = 25), *Salmonella* Thompson (n = 24), *Salmonella* Lomita (n = 2), *Salmonella* Agona (n = 2), *Salmonella* Rissen (n = 1), *Salmonella* Enteritidis (n = 1), *Salmonella* Oritamerin (n = 1), *Salmonella* Lagos (n = 1) and *Salmonella* II6, 7:z6:1,7 (n = 1), were identified in this study *(Fig. 2)*.

The individual antibiotic resistance profile of each of the 142 *Salmonella spp.* isolates was measured. Resistance to sulfisoxazole was the most prevalent among the *Salmonella* strains (130 strains, 91.55%) followed by doxycycline (118 strains, 83.10%), tetracycline (112 strains, 78.87%), florfenicol (108 strains, 76.06%), ampicillin (86 strains, 60.56%) and gentamicin (74 strains, 52.11%) *(Fig. 3)*. The isolates were not resistant to cefotaxime and amoxicillinpotassium clavulanate, respectively.

Pulse field-gel electrophoresis (PFGE) generated profiles of three major genotypic clusters(I- III) and 31 fingerprint-patterns with an 80% dice coefficient index cut-off of 12~20 DNA fragment bands *(Fig. 4)*. In this study, the 0031 PFGE pattern was the most common pattern, which included 24 strains of *Salmonella* Thompson, followed was 0009 PFGE pattern, which was composed of twenty-one *Salmonella* Derby isolates, and by 0013 PFGE patterns which was composed of fourteen *Salmonella* Derby isolates. In this study, the prevalence and characteristics of *Salmonella spp.* isolates are outlined in *Table 3*. Most patterns were within a single serotype and a single source, except 0014, 0017 and 0027 PFGE pattern. The 0014 PFGE pattern was

Table 3. *Origin and characterization of Salmonella spp. isolated from pig slaughter process*

No. of Isolates (n)	Serotype	Location	MLST Pattern	PFGE Pattern	Prevalence in %
A (16)	Derby (1) Derby (14) Rissen (1)	A A A	ST-40 ST-40 ST-469	0012 0013 0030	20.0
B (19)	II6,7:z6:1,7 (1) Derby (3) Typhimurium (1) Typhimurium (1) Typhimurium (9) Typhimurium (4)	B B B B B B	ST-638 ST-40 ST-34 ST-19 ST-19 ST-19	0004 0017 0019 0026 0027 0029	23.8
C (19)	Lomita (1) Lomita (1) Derby (1) Derby (12) Derby (1) Derby (1) Typhimurium (2)	C C C C C C C	ST-808 ST-808 ST-40 ST-40 ST-40 ST-40 ST-19	0001 0002 0008 0011 0014 0022 0024	23.8
D (14)	Enteritidis (1) Derby (3) Derby (1) Derby (3) Typhimurium (1) Typhimurium (3) Agona (2)	D D D D D D D	ST-11 ST-40 ST-40 ST-40 ST-34 ST-19 ST-19	0003 0014 0015 0017 0018 0027 0028	17.5
E (13)	Oritamerin (1) Derby (2) Derby (2) Derby (3) Typhimurium (4) Lagos (1)	E E E E E E	ST-32 ST-40 ST-34 ST-34 ST-19 ST-19	0005 0006 0020 0021 0023 0025	16.3
F(15)	Derby (7) Derby (5) Derby (3)	F F F	ST-40 ST-40 ST-40	0007 0010 0016	18.6
G(24)	Thompson (24)	G	ST-26	0031	30.0
H(22)	Derby (21) Derby (1)	H H	ST-40 ST-40	0009 0014	27.5

composed of five *Salmonella* Derby isolates. All recovered from various processing steps at three slaughterhouses, which were C (1), D (3) and H (1); The 0017 PFGE pattern was found to be composed of six *Salmonella* Derby, which were from B (3) and D (3) slaughterhouse, Similarly the 0027 PFGE pattern was composed of twelve *Salmonella* Derby, which were from two slaughterhouses of B (9) and D (3).

An interlinked dataset with partial sequencing of seven housekeeping genes at 399 bp to 501 bp revealed that 9 STs among the 142 isolates were found. 4 STs were represented a single isolates, and the others were represented more than one isolates (n=2 to 79). The predominant STs was ST40, ST19 and ST26, which contained 79 strains (55.6%), 26 strains (18.3%) and 24 (16.9%) strains isolates *(Table 3)*. Most of Salmonella-positive isolates were assigned to ST40 in the dedicated database (http://mlst.ucc.ie/mlst/dbs/Senterica). The isolates possessed identical alleles at all seven loci; *aroC* allele type19, *dnaN* allele type20, *hemD* allele type 3, *hisD* allele type 20, *purE* allele type 5, *sucA* allele type 22, and *thrE* allele type 22 *(Table 3)*. The reproducibility of PFGE and MLST showed that all isolates were consistent with before testing.

Salmonella spp. strains characterized as the same STs did not necessarily have the same PFGE pattern. 79 strains characterized as ST40 had 13 different PFGE patterns, the 26 strains characterized as ST19 had 7 different PFGE patterns *(Fig. 4)*. All strains which shared a PFGE pattern had the same ST. *Salmonella spp.* strains which shared a PFGE fingerprint-pattern had the same ST *(Fig. 4)*. Within clonal lineage 79 all *Salmonella* Derby isolates were of an identical MLST type (ST40) and revealed closely related PFGE patterns. The results showed that the character of *Salmonella spp.* strains in more detail, most STs were within a single serotype *(Table 2)*. ST40 distributed in seven slaughterhouses, except G slaughterhouse.

DISCUSSION

In the study, the overall prevalence of *Salmonella spp.* in pig slaughterhouse in Shandong Province was approximately 22.2%. For the slaughterhouse, the prevalence was significantly higher than that reported in Jiangsu Province, China (14.1%) [12], in Sichuan Province, China (10.7%) [3], and in Thailand (7.22%) [19], but it was lower than that reported in Jiangsu Province, China (71.8%) [13].

Although different methods of sampling, isolation, and identification could affect the overall results, the prevalence of *Salmonella spp.* observed in this study suggested that pig slaughterhouse examined exercised poor hygiene management. Meanwhile, the critical five steps of pig slaughterhouse, cutting is of the highest infection rates. We can focus on purification the step of cutting in pig slaughterhouse, to prevent the spread of salmonella food chain downstream.

We analyzed 142 *Salmonella spp.* samples to determine diversity within a strain *(Fig. 4)*. Two samples contained isolates with identical properties, suggesting they were the same strain, while the majority of the samples contained isolates belonging to the same sequence type but differing by one or more of the phenotypic or genetic properties tested, indicating that they were variants of the same clone. Most common variations were non-expression of the H antigen, variation in PFGE patterns. Thirty-one unique PFGE patterns were generated. All of the patterns were correlated with one serotype. The reason could also explain the arrangement position of PFGE and the genetically similar profiles [18]. Others have previously demonstrated PFGE pattern diversity within a serotype [20-22], but such diversity is a critical observation that is often overlooked. The diversity of PFGE pattern may explain why morbidity and mortality vary within a serotype and could be useful in assessing the effectiveness of control measures.

78 *Salmonella spp.* strains from seven slaughterhouses (A, B, C, D, E, F and H) in Shandong Province and two strains from Shanghai in group III displayed the same PFGE patterns and the same STs, which suggested they have a close genetic relationship. In view of the law, we should be paid to avoid further dissemination of *Salmonella* Derby, which has appeared in some areas [23].

In this study, the most tested isolates of *Salmonella spp.* isolates were assigned to MLST 40, according to the MLST database analysis. In previous studies using the same database [5,24-26], MLST was highly correlated to Salmonella serotype. The study targeting seven different housekeeping and virulence genes found that MLST was not able to discriminate clinically relevant serotypes of Salmonella [25]. The limited discriminatory ability of STs may be resulted from the moderate to slow rate of mutation accumulation within seven targeted housekeeping genes [26]. Therefore, the discriminatory performance of MLST needs to be increased if more variable gene targets are examined. Meanwhile, the reproducibility of the PFGE and MLST showed that all isolates were consistent with before testing. This showed that PFGE and MLST were the best method of DNA genotyping methods.

Antimicrobial resistance in *Salmonella spp.* has become a significant public health concern. The presence of antimicrobial-resistant pathogens in food and food products could enable the bacteria to spread via the food chain to humans, causing infections [27]. Our results indicated that all the *Salmonella spp.* isolates were resistant to at least one antimicrobial agent, with high levels of drug resistance in all eight pig slaughter process examined. This was somewhat expected because of its wide use in animal feed and was consistent with reports from slaughterhouses in China, Italy, Mexico, Vietnam, and the United States [12,13,28-31]. In the study, the highest rates of antimicrobial resistance were against sulfisoxazole (91.55%), which is one of the

most widely used antimicrobials in feed additives in livestock farming in China and other countries. Thus, this result was somewhat expected and agreed with previous reports from China. MDR Salmonella isolates were frequently observed among the slaughterhouse and retail market isolates in this study. This was posing great risk to public health if these MDR strains were transferred to humans via pork or pork-derived products [3].

In addition, *Salmonella* Derby was the primary serotype in H and F slaughterhouse, and in G slaughterhouse the main serotype is *Salmonella* Thompson. The strains from the slaughterhouse and tools had the different PFGE patterns as product isolates, except G slaughterhouse *(Table 2)*. Meanwhile, other slaughterhouse isolates have several serotype and PFGE pattern. The diversity of these strains showed that cross contamination among the pig slaughterhouse. This indicated a 'pig-slaughtered each link-product' transmission circulation for *Salmonella spp.* in local area. Previous research has shown that Salmonellosis outbreak is one of multiple Salmonella serotypes outbreaks caused by contaminated food [32,33]. Other study also indicated that *Salmonella spp.* had the 'patient-environment-food' transmission circulation [34]. So we should purify salmonella contamination in pork products, from the source control the spread of Salmonella, put an end to the spread of salmonella in the crowd. Study results suggested that Salmonella control programs should reduce *Salmonella spp.* loading on carcasses included good practices.

In conclusion, the study attempted to analyze the various molecular classification of Salmonella contamination, such as the serotype, PFGE pattern, ST pattern and drug resistance analysis, to minimize the *Salmonella spp.* contamination in pig slaughterhouse. The results of this study provide PFGE fingerprints of *Salmonella spp.* strains in Shandong Province, China and establish a good foundation for the realization of data sharing, which will help to realize active surveillance of Salmonellosis and tracing the source of infection in China.

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REFERENCES

1. Doorduyn Y, Van Den Brandhof WE, Van Duynhoven YT, Wannet WJ, Van Pelt W: Risk factors for *Salmonella* Enteritidis and Typhimurium (DT104 and non-DT104) infections in the Netherlands: Predominant roles for raw eggs in Enteritidis and sandboxes in Typhimurium infections. *Epidemiol Infect*, 134, 617-626, 2006. DOI: 10.1017/S0950268805005406

2. Wang J, Zheng R, Wang J: Risk assessment of Salmonella in animal derived food. *Chin J Anim Q*, 24: 23-25, 2007.

3. Li R, Lai J, Wang Y, Liu S, Li Y, Liu K, Shen J, Wu C: Prevalence and characterization of *Salmonella* species isolated from pigs, ducks and chickens in Sichuan Province, China. *Int J Food Microbiol*, 163, 14-18, 2013. DOI: 10.1016/j.ijfoodmicro.2013.01.020

4. Hauser E, Hebner F, Tietze E, Helmuth R, Junker E, Prager R, Schroeter A, Rabsch W, Fruth A, Malorny B: Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany. *Int J Food Microbiol*, 151, 141-149, 2011. DOI: 10.1016/j.ijfoodmicro.2011.08.020

5. Noda T, Murakami K, Asai T, Etoh Y, Ishihara T, Kuroki T, Horikawa K, Fujimoto S: Multi-locus sequence typing of *Salmonella enterica* subsp. *enterica* serovar Enteritidis strains in Japan between 1973 and 2004. *Acta Vet Scand*, 53, 38, 2011. DOI: 10.1186/1751-0147-53-38

6. Mürmann L, dos Santos MC, Cardoso M: Prevalence, genetic characterization and antimicrobial resistance of *Salmonella* isolated from fresh pork sausages in Porto Alegre, Brazil. *Food Control,* 20, 191-195, 2009. DOI: 10.1016/j.foodcont.2008.04.007

7. Galanis E, Lo Fo Wong DM, Patrick ME, Binsztein N, Cieslik A, Chalermchikit T, Aidara-Kane A, Ellis A, Angulo FJ, Wegener HC, World Health Organization Global Salm-Surv: Web-based surveillance and global *Salmonella* distribution, 2000-2002. *Emerg Infect Dis*, 12, 381-338, 2006. DOI: 10.3201/eid1205.050854

8. Urwin R, Maiden MC: Multi-locus sequence typing: A tool for global epidemiology. *Trends Microbiol*, 11, 479-487, 2003. DOI: 10.1016/j.tim.2003.08.006

9. Sukhnanand S, Alcaine S, Warnick LD, Su WL, Hof J, Craver MP, McDonough P, Boor KJ, Wiedmann M: DNA sequence-based subtyping and evolutionary analysis of selected *Salmonella enterica* serotypes. *J Clin Microbiol*, 43, 3688-3698, 2005. DOI: 10.1128/JCM.43.8.3688-3698.2005

10. Arguello H, Carvajal A, Collazos JA, Garcia-Feliz C, Rubio P: Prevalence and serovars of *Salmonella enterica* on pig carcasses, slaughtered pigs and the environment of four Spanish slaughterhouses. *Food Res Int*, 45, 905-912, 2012. DOI: 10.1016/j.foodres.2011.04.017

11. Bonardi S, Bassi L, Brindani F, D'Incau M, Barco L, Carra E, Pongolini S: Prevalence, characterization and antimicrobial susceptibility of *Salmonella enterica* and Yersinia enterocolitica in pigs at slaughter in Italy. *Int J Food Microbiol*, 163, 248-257, 2013. DOI: 10.1016/j.ijfoodmicro.2013.02.012

12. Li YC, Pan ZM, Kang XL, Geng SZ, Liu ZY, Cai YQ, Jiao XA: Prevalence, characteristics, and antimicrobial resistance patterns of *Salmonella* in retail pork in Jiangsu province, eastern China. *J Food Prot*, 77, 236-45, 2014. DOI: 10.4315/0362-028X.JFP-13-269

13. Cai Y, Tao J, Jiao Y, Fei X, Zhou L, Wang Y, Zheng H, Pan Z, Jiao X: Phenotypic characteristics and genotypic correlation between *Salmonella* isolates from a slaughterhouse and retail markets in Yangzhou, China. *Int J Food Microbiol*, 222, 56-64, 2016. DOI: 10.1016/j.ijfoodmicro.2016.01.020

14. Li W, Raoult D, Fournier PE: Bacterial strain typing in the genomic era. *FEMS Microbiol Rev*, 33, 892-916, 2009. DOI: 10.1111/j.1574-6976.2009.00182.x

15. Clinical and Laboratory Standards Institute: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 8th ed, Document M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA, 2009.

16. Clinical Laboratory Standards Institute (CLSI): Performance standards for antimicrobial susceptibility testing. Twenty-Third Informational Supplement, M100-S23, CLSI, Wayne, PA, 2013.

17. Harbottle H, White DG, McDermott PF, Walker RD, Zhao S: Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, and antimicrobial susceptibility typing for characterization of Salmonella enterica serotype Newport isolates. *J Clin Microbiol*, 44, 2449e57, 2006. DOI: 10.1128/JCM.00019-06

18. Adaska JM, Silva AJ, Berge ACB, Sischo WM: Genetic and phenotypic variability among *Salmonella enterica* serovar Typhimurium isolates from California dairy cattle and humans. *Appl Environ Microbiol, 72*, 6632-6637, 2006. DOI: 10.1128/AEM.01038-06

19. Tadee P, Boonkhot P, Patchanee P: Quantification of contamination levels and particular risk of *Salmonella* spp. in pigs in slaughterhouses in Chiang Mai and Lamphun provinces, Thailand. *Jpn J Vet Res*, 62, 171-199, 2014.

20. Gai W, Wang J, Wang J, Cui Z, Qu Z, Cui J, Du X, Huang X, Zhao J:

Molecular classification and drug resistance analysis of *Escherichia coli* isolated from poultry in China. *Int J Clin Exp Med*, 8, 836-844, 2015.

21. Sukhnanand S, Alcaine S, Warnick LD, Su WL, Hof J, Craver MP, McDonough P, Boor KJ, Wiedmann M: DNA sequence based subtyping and evolutionary analysis of selected *Salmonella enterica* serotypes. *J Clin Microbiol*, 43, 3688e98, 2005. DOI: 10.1128/JCM.43.8.3688-3698.2005

22. Liu F, Barrangou R, Gerner-Smidt P, Ribot EM, Knabel SJ, Dudley EG: Novel virulence gene and clustered regularly interspaced short palindromic repeat (CRISPR)multilocus sequence typing scheme for subtyping of the major serovars of *Salmonella enterica* subsp. *enterica*. *Appl Environ Microbiol*, 77, 1946-1956, 2011. DOI: 10.1128/AEM.02625-10

23. Torpdahl M, Skov MN, Sandvang D, Baggesen DL: Genotypic characterization of *Salmonella* by multilocus sequence typing, pulsed-field gel electrophoresis and amplified fragment length polymorphism. *J Microbiol Methods*, 63, 173-184, 2005. DOI: 10.1016/j.mimet.2005.03.006

24. Campioni F, Moratto Bergamini AM, Falcão JP: Genetic diversity, virulence genes and antimicrobial resistance of *Salmonella* Enteritidis isolated from food and humans over a 24-year period in Brazil. *Food Microbiol*, 32, 254-264, 2012. DOI: 10.1016/j.fm.2012.06.008

25. Ben-Darif E, De Pinna E, Threlfall EJ, Bolton FJ, Upton M, Fox AJ: Comparison of a semi-automated rep-PCR system and multilocus sequence typing for differentiation of *Salmonella enterica* isolates. *J Microbiol*, 81, 11e6, 2010. DOI: 10.1016/j.mimet.2010.01.013

26. Van Hoek AHAM, de Jonge R, van Overbeek WM, Bouw E, Pielaat A, Smid JH, Malorny B, Junker E, Löfström C, Pedersen K, Aarts HJ, Heres L: A quantitative approach towards a better understanding of the dynamics of *Salmonella spp.* in a pork slaughter-line. *In J Food Microbiol*, 153, 45-52, 2012. DOI: 10.1016/j.ijfoodmicro.2011.10.013

27. Witte W: Medical consequences of antimicrobial use in agriculture. *Science*, 279, 996-997, 1998. DOI: 10.1126/science.279.5353.996

28. Piras F, Brown DJ, Meloni D, Mureddu A, Mazzette R: Investigation of *Salmonella enterica* in Sardinian slaughter pigs: Prevalence, serotype and genotype characterization. *Int J Food Microbiol*, 151, 201-209, 2011. DOI: 10.1016/j.ijfoodmicro.2011.08.025

29. Miranda JM, Mondragón AC, Martinez B, Guarddon M, Rodriguez JA: Prevalence and antimicrobial resistance patterns of *Salmonella* from different raw foods in Mexico. *J Food Prot*, 72, 966-971, 2009. DOI: 10.4315/0362-028X-72.5.966

30. Thai TH, Hirai T, Lan NT, Yamaguchi R: Antimicrobial resistance profiles of *Salmonella* serovars isolated from retail pork and chicken meat in North Vietnam. *Int J Food Microbiol*, 156, 147-151, 2012. DOI: 10.1016/j.ijfoodmicro.2012.03.016

31. White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, McDermott PF, McDermott S, Wagner DD, Meng J: The isolation of antimicrobial-resistant *Salmonella* from retail ground meats. *N Engl J Med*, 345, 1147-1154, 2001. DOI: 10.1056/NEJMoa010315

32. Tadee P, Boonkhot P, Pornruangwong S, Patchanee P: Comparative phenotypic and genotypic characterization of *Salmonella* spp. in pig farms and slaughterhouses in two provinces in Northern Thailand. *PLo One,* 10, e0116581, 2015. DOI: 10.1371/journal.pone.0116581

33. Zou M, Keelara S, Thakur S: Molecular characterization of *Salmonella enterica* serotype Enteritidis isolates from humans by antimicrobial resistance, virulence genes, and pulsed-field gel electrophoresis. *Foodborne Pathog Dis*, 9, 232-238, 2012. DOI: 10.1089/fpd.2011.1012

34. Römling U, Tümmler B: Achieving 100% typeability of Pseudomonas aeruginosa by pulsed-field gelelectrophoresis. *J Clin Microbiol,* 38, 464-455, 2000.

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23 (3): 385-389, 2017
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Research Article

Genetic Characterization and Evolutionary Analysis of Emerging Newcastle Disease Virus Isolated from Tibetan Chickens

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Abstract

Newcastle disease (ND) is one of the highly contagious disease responsible for devastating outbreaks and considerable economic losses to poultry industry in China. However, no information is available about NDV in Tibet region; the aim of this study was to determine the genetic characterization and evolutionary analysis of NDV in Tibetan chickens. Four NDVs were isolated from an outbreak in Tibetan chickens. The pathogenicity of the isolates was determined by mean death time (MDT), intracerebral pathogenicity index (ICPI) and amino acid sequencing (112 to 117) of F protein. While the genetic characterization of F gene was determined by sequencing the isolated NDVs and phylogenetic relationship was established with the help of reference strains. Pathogenicity experiments revealed that XZ-F10 and XZ-F20 strains were lentogenic pathotype, while the remaining XZ-F2-1 and XZ-F4-6 strains were velogenic pathotype. The deduced amino acid sequences of the cleavage site of the F protein confirmed our results. Phylogenic analysis of these strains indicated that both XZ-F2-1 and XZ-F4-6 belong to genotype VII. However, XZ-F10 and XZ-F20 strains were assigned to genotypes II. The present study highlights the need for continuous surveillance of NDV in Tibetan chickens; moreover, this study will provide a reference for the local government to make the strategies and control emerging NDV in Tibet.

Keywords: *Newcastle disease viruses (NDVs), Fusion protein (F) gene, Molecular characterization, Pathogenicity, Tibetan chickens*

Tibet Tavuklarından İzole Edilen Newcastle Hastalığı Virüsünün Genetik Karakterizasyonu ve Kalıtsal Analizi

Özet

Newcatle hastalığı (ND) Çin'de önemli salgınlara ve ekonomik kayıplara neden olan oldukça bulaşıcı bir hastalıktır. Ancak Tibet bölgesinde NDV'ye ait bir bulgu bulunmamaktadır. Bu çalışmanın amacı; Tibet tavuklarında NDV'nin genetik karakterizasyonunu ve kalıtımsal analizi yapmaktır. Meydana gelen bir salgında Tibet tavuklarından dört NDV izole edildi. İzolatların patojenitesi ortalama ölüm zamanı (MDT), intraserebral patojenite endeksi (ICPI) ve F proteininin amino asit sekansı (112'den 117'e kadar) yöntemleriyle belirlendi. F geninin genetik karakterizasyonu izole edilen NDV'lerin sekansı ile belirlendi ve filogenetik akrabalık referans suşun yardımıyla ortaya konuldu. Patojenite deneyleri ile XZ-F10 ve XZ-F20 suşlarının lentojenik patotip, geri kalan XZ-F2-1 ve XZ-F4-6 suşlarının ise velojenik patotip oldukları belirlendi. F proteininin ayrılma bölgesinin amino asit sekansı sonuçlarımızı doğruladı. Bu suşların filogenetik analizi hem XZ-F2-1 hem de XZ-F4-6 suşlarının genotip VII'ye ait olduğunu gösterdi. XZ-F10 ve XZ-F20 suşları genotip II'ye aitti. Bu çalışma; Tibet tavuklarında NDV için sürekli takibin gerekli olduğunu ve lokal sorumlular için kontrol stratejileri geliştirmeleri gerekliliğini göstermiştir.

Anahtar sözcükler: *Newcastle hastalığı virüsü (NDV), Füzyon protein (F) geni, Moleküler karakterizasyon, Patojenite, Tibet tavuğu*

INTRODUCTION

Newcastle disease (ND) is one of the most contagious and devastating disease of poultry throughout the world [1]. NDV is the member of genus Avulavirus, family Paramyxoviridae, with six transcriptional proteins including fusion protein, phosphor protein, nucleocapsid protein, haemagglutinin-neuraminidase protein, matrix protein

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and polymerase protein [2,3]. It can cause severe economic losses to poultry industry, particularly chickens, and affects a range of other domestic species, including duck, wild bird, waterfowl and pigeon [4,5]. The main clinical signs are diarrhea, expiratory dyspnea, neurological symptoms, cloaca hemorrhage, focal glandular gastric bleeding or ulcers, intestinal mucosal bleeding and necrosis of the pancreas or spleen. The pathotype of NDV isolates are divided into three groups (lentogenic, mesogenic and velogenic) based on the intracerebral pathogenicity index (ICPI) and mean death time (MDT) [4]. Currently, basic amino acid sequence (from 112 to 117) of the fusion protein is used for classifying the virulence of NDV strains.

The Tibetan chickens have a very wide distribution at an altitude of 2.200 to 4.100 m on the Qinghai-Tibet Plateau, with a history of domestication of more than 1.000 year at high altitude [6,7]. Under special breeding conditions, this specie has been famous for disease resistance, easy breeding and increasingly vigorous market demand with high-quality meat production. This breed is of great economical importance to local herdsman, as the meat of Tibetan chickens is rich in protein, with a tender texture, and high content of amino acids [8]. In recent years, NDV has been reported with large-scale outbreaks and considerable economical losses to poultry industry in many provinces of China [9-11]. Until now, no information is available about the genetic characterization of NDV in Tibetan chickens. The present study reports the characterization of NDV isolates from the Tibetan chickens in Tibet.

MATERIAL and METHODS

Ethical Approval

All the experiments were approved by Animal Welfare and Research, Ethics Committee of Huazhong agricultural university and performed in accordance with the international guidelines for animal welfare.

The Study Site

The present study was carried out in Nyingchi Prefecture on Qinghai-Tibetan Plateau, China. This area is geographically isolated from Tibet and Sichuan Provinces by Himalayas, and shares border with Nepal in the south-west, and India and Myanmar in the south. The average elevation of the surveyed area is more than 3100 m above sea level, the largest continuous high elevation ecosystem *(Fig. 1)*.

Virus Isolation and Pathogenicity Test

Four NDVs were isolated from a flock of Tibetan chickens during an outbreak in Tibet, China and virus separation was carried out according to the guidelines provided by the World Organization for Animal Health [4]. Briefly, homogenized and clarified organ suspensions were made with streptomycin/penicillin in viral transportation media (VTM) overnight at 4°C and 0.2 mL suspensions were inoculated into the allantoic cavity of 9 to 11 day old specific pathogen free (SPF) chicken embryos, and incubated at 37°C for 72 h [12]. Allantoic fluid was harvested and the NDV was tested using haemagglutination inhibition (HI) and haemagglutination (HA) tests. The strains were designated names as XZ-F10, XZ-F20, XZ-F2-1 and XZ-F4-6. LaSota strain was used as a positive control in this study. The positive allantoic fluids were stored at -80°C until subsequent use and further analysis. The pathogenic potential for the isolates were evaluated by mean death time (MDT) using 10-days-old SPF embryonated chicken eggs and intracerebral pathogenicity index (ICPI) test using 1-day-old SPF chicks using standard procedures [13].

Viral RNA Purification, RT-PCR, and Sequencing of the F Gene

Viral RNA of the four isolated strains was extracted using TIANamp Virus DNA/RNA Kit (TianGen, China). Reverse transcription PCR amplification approach was performed using Quant One Step RT-PCR Kit (Tian Gen, China) with primers for the F gene (~1662bp). Based on the published F gene sequences in the GenBank database, we designed a pair of primers with the help of Primer Premier Software (version 5.0) and synthesized by Wuhan Qingke biotechnology CO., LTD (Wuhan, China). The primer forward: ATGGGCTCCAGAC CTTCTACCA and reverse CATTTTTGT AGT GGCTCTCATCTGAT was used and commercial RT-PCR Kit (TianGen, China) was used for reverse transcription. The RT-PCR mixture contained 14.2 µL RNase-

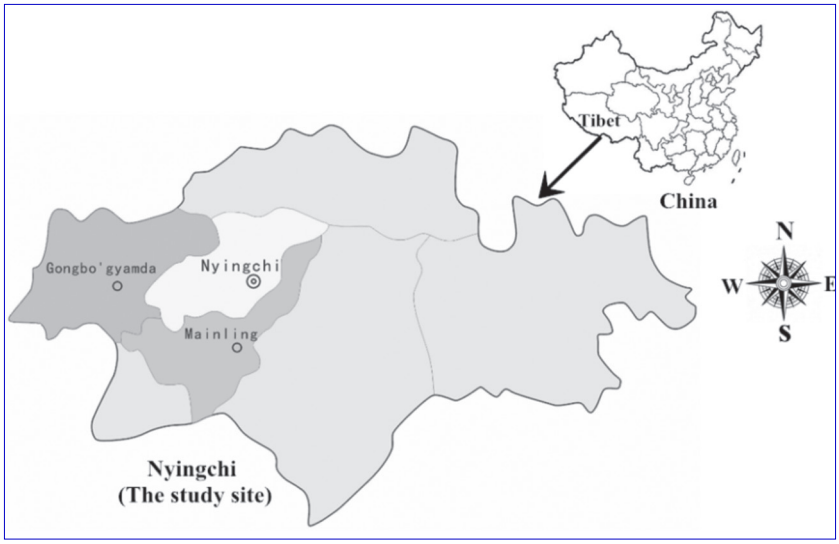

Fig 1. Collection sites of samples in Nyingchi, Tibet China

free water, 5 µL AMV Buffer, 0.5 µL dNTP, 0.8 µL $MgSO_4$, 0.5 µL Tfl DNA Polymerase, 2 µL RNA, 1 µL of each forward and reverse primer (working concentration: 10 µL mol/L) in a 25 µL reaction. For F gene: denaturation at 95°C for 40 s, and 72°C for 1.5 min of 35 cycles and annealing at 55°C for 50 s; and final extension at 72°C for 10 min. PCR products were separated on agarose gel (1%). The products were purified using a TaKaRa DNA Extraction Kit Ver.4.0 (Takara Biotechnology CO., LTD, Dalian, China) according to manufacturer's instructions, and then sequenced by a commercial company (Qingke Biosciences, Wuhan, China).

Sequence Analysis of F Protein Cleavage Site

Phylogenetic analysis, amino acid sequence prediction and nucleotide sequence similarity was performed by the Clustal W multiple alignment method (MegAlign program of the DNASTAR software, version 3.3.8) for the F gene (~1662bp). In addition to the 4 strains collected in this study, 15 previously reported F gene sequences representing different genotypes were obtained from GenBank database including vaccine strains, typically existing in China. The accession numbers of each of these NDVs are shown in the phylogenetic tree.

RESULTS

In present study, four NDVs were isolated from Tibetan chickens on SPF embryonated chicken eggs during different outbreaks at chicken's farm from 2012 to 2016. The details of the NDV isolates are shown in *Table 1*. As per classification, the virus of XZ-F10 and XZ-F20 strains were assigned to lentogenic pathotype, while XZ-F2-1 and XZ-F4-6 strains were classified as velogenic pathotype. As it is shown in *Table 2*, the F protein cleavage site motif sequence was 112G-R-Q-G-R-L117 in strain XZ-F10 and XZ-F20, which is the major determinant of lentogenic for NDV strains and 112R-R-Q-K-R-F117 in strain XZ-F2-1 and XZ-F4-6, that is considered as the velogenic.

Based on the complete F gene sequences derived through different years in Tibet, the XZ-F2-1 and XZ-F4-6 strains isolated in 2012, and XZ-F10 and XZ-F20 strains isolated in 2015 and 2016 were assigned to genotypes VII and II, respectively *(Fig. 2)*.

DISCUSSION

Newcastle disease can lead to high mortality and morbidity rates, and creates a big potential threat to the poultry industry in terms of serious economic losses in China [10]. In recent years, although many dynamic measures have been adopted to prevent and control this disease, however, it is still the major infectious disease, and as a big potential threat to the chicken industry in China. As a very wide distribution at altitudes of 2.200 to 4.100 m on the Qinghai-Tibet Plateau [6,7], no information is available on the pathogenicity and genetic characterization of NDV in Tibetan chickens. Fusion (F) protein is the key protein that measures the NDV pathogenicity and therefore, F gene was analyzed by different laboratories in recent decades [14,15]. The virulent strength of NDV can be classified through basic amino acid sequences from 112 to 117 of F protein [16]. As it is shown in table 2, the F protein cleavage site motif sequence was 112G-R-Q-G-R-L117 in strain XZ-F10 and XZ-F20, which is the major determinant of lentogenic for NDV strains [16], and 112R-R-Q-K-R-F117 in strains XZ-F2-1 and XZ-F4-6, that is considered as the velogenic [17,18]. This confirms that not only MDT and ICPI can be used to validate the pathogenicity, but as previous reports, the cleavage site motifs is also reliable way to predict the pathogenicity and virulence of NDV strains [16].

Table 1. *The characteristics description of four NDVs isolated from Tibetan Chickens in this study*

NDV Isolates	Year of Isolation	Location	MDT [b]	ICPI [c]	Pathotype
XZ-F10	2015	Tibet	>120	0.2	Lentogenic
XZ-F20	2016	Tibet	>120	0.2	Lentogenic
XZ-F2-1	2012	Tibet	53	1.62	Velogenic
XZ-F4-6	2012	Tibet	49	1.56	Velogenic

[a] Amino acid sequence (112 to 117) of F protein; [b] Mean death time in 9-day-old SPF embryonated chicken eggs (hours) (velogenic, <60; mesogenic, 60-90; lentogenic, >90); [c] Intracerebral pathogenicity index in 1-day-old chickens (lentogenic, <0.7; mesogenic, 0.7–1.4; velogenic, 1.4-2.0)

Table 2. *Fusion protein cleavage site description of NDVs isolated from Tibetan chickens*

Fusion Protein Cleavage Site From 112 to 117												NDV Isolates
109	110	111	112	113	114	115	116	117	118	119	120	
S	G	G	G	**R**	**Q**	G	**R**	L	I	G	A	XZ-F10
S	G	G	G	**R**	**Q**	G	**R**	L	I	G	A	XZ-F20
S	G	G	**R**	**R**	**Q**	**K**	**R**	**F**	I	G	A	XZ-F2-1
S	G	G	**R**	**R**	**Q**	**K**	**R**	**F**	I	G	A	XZ-F4-6

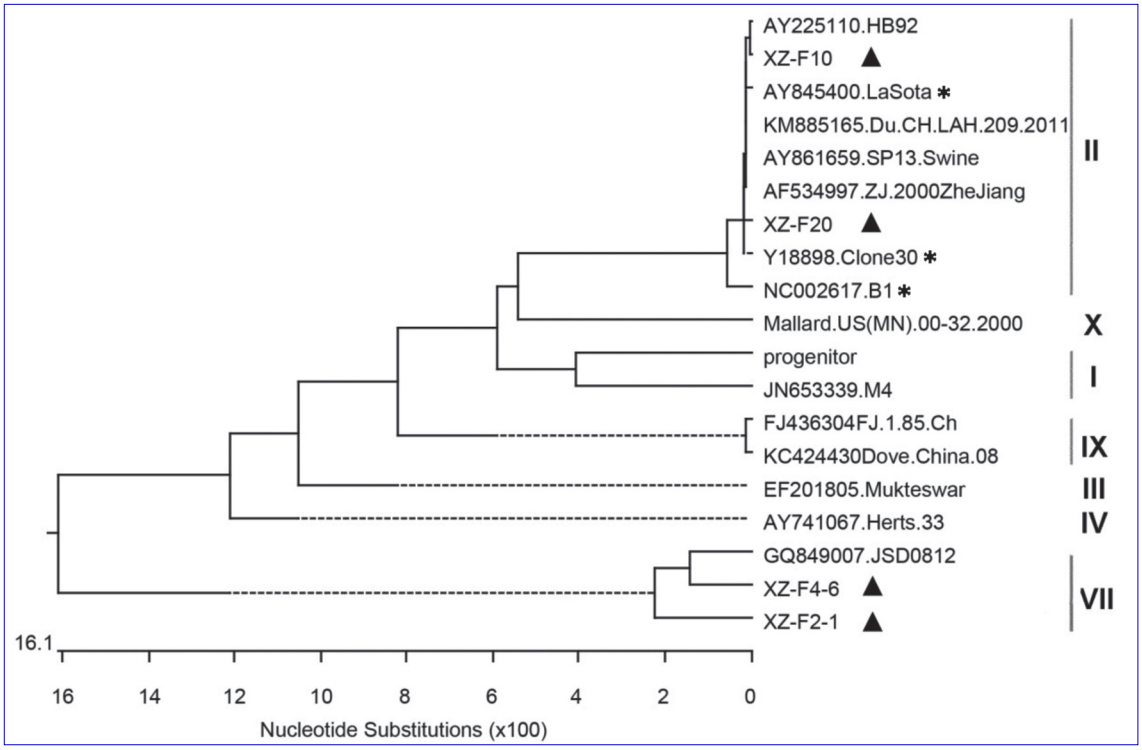

Fig 2. Phylogenetic tree constructed by the neighbor joining method using MegAlign software. * =Vaccine strains

The genotype VII NDVs have become the most prevalent strains since 1990s in most parts of China [19]. Because of the unique natural environment and high altitude in Tibet, NDV genotype and distribution is different from surrounding regions and countries. However, in recent years, with the continuous enhancement in tourism and transportation, more and more Tibetan chickens are exposing to the virus, this is an important reason that genotype VII was found in Tibetan chickens in 2012. In the past two years, genotypes II NDVs are reported from many countries including China, from numerous species [20]. We also found that XZ-F10 and XZ-F20 strains had a close genetic relationship with LaSota, HB92, SP13, CH.LAH209, ZJ.2000, Clone 30 and B1 (Accession number: AY845400, AY225110, AY861659, KM885165, AF534997, Y18898 and AF309418, respectively) strains that belongs to genotype II in terms of F gene analysis. The LaSota, Clone 30 and B1 were the attenuated strains, NDV attenuated strains (LaSota, Clone-30 and B1) are still used to make attenuated vaccine at a large scale in China [20-22]. Our results revealed that the NDVs isolated in Tibetan chickens showed a close phylogenetic relationship and evolutionary distance with strains of LaSota, Clone 30 and B1. There was significant similarity between the Tibetan chickens NDV strains and the current vaccine strains in their serology and genetics, which might be considered as the reasons for the ND outbreaks in Tibetan chickens.

In conclusion, we identified two genotypes (II and VII) in Tibetan chickens for the first time in our studied strains; moreover, results provided clear evidence that the non-standard use of vaccine may be the important reason that leads to epidemic outbreak of NDV in Tibetan chickens on Qinghai-Tibet Plateau, China. Furthermore, our study also highlights the need for continuous surveillance of NDV in Tibetan chickens, which will provide a reference for the local government to make the strategies for the control of emerging NDV in Tibet.

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Conflict of Interest

The authors declare that they have no competing interests.

REFERENCES

1. Aldous EW, Alexander DJ: Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathol*, 30, 117-28, 2001. DOI: 10.1080/03079450120044515

2. Mayo MA: A summary of taxonomic changes recently approved by ICTV. *Arch Virol,* 147, 1655-1663, 2002. DOI: 10.1007/s007050200039

3. Okwor E, Chukwudi E, Didacus C, Mary U: Newcastle disease virus shedding among healthy commercial chickens and its epidemiological importance. *Pak Vet J*, 8318, 2074-7764, 2011.

4. OIE: "Newcastle disease, chapter 2.3.14," in OIE Terrestrial Manual 2012:

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Paris: World Organisation for Animal Health), 576-589. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf; *Accessed:* 10.12.2016.

5. Sinkovics JG, Horvath JC: Newcastle disease virus (NDV): Brief history of its oncolytic strains. *J Clin Virol*, 16, 1-15, 2000. DOI: 10.1016/S1386-6532(99)00072-4

6. Zhang H, Wu C, Chamba Y, Ling Y, Luo Z: Adaptability to high altitude and NOS activity of lung in Tibetan chicken. *J China Agric Univ*, 11, 35-38, 2006.

7. Zhang H, Wu C, Chamba Y, Ma X, Tang X, Po B: Curve analysis of embryonic mortality in chickens incubated at high altitude. *J China Agric Univ*, 10, 109-114, 2005.

8. Zhang H, Wu CX, Yangzom C, Ling Y, Tang XH: Cardiac enzymes related to high-altitude hypoxic adaptation in tibetan chicken. *Chinese J Appl Physiol*, 24, 233-236, 2008.

9. Qin ZM, Tan LT, Xu HY, Ma BC, Wang YL: Pathotypical characterization and molecular epidemiology of Newcastle disease virus isolates from diffenrent hosts in China from 1996 to 2005. *J Clin Microbiol*, 46, 601-611, 2008. DOI: 10.1128/JCM.01356-07

10. Kang Y, Xiang B, Yuan R, Zhao X, Feng M: Phylogenetic and pathotypic characterization of Newcastle disease viruses circulating in South China and transmission in different birds. *Front Microbiol*, 7, 119, 2016. DOI: 10.3389/fmicb.2016.00119

11. Zhang R, Pu J, Su JL, Zhao JX, Wang XT, Zhang SP, Li XJ, Zhang GZ: Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001-2009. *Vet Microbiol*, 141, 246-257, 2010. DOI: 10.1016/j.vetmic.2009.09.020

12 Alexander DJ: Newcastle disease and other avian paramyxoviruses. *Rev Sci Tech*, 19, 443-462, 2010.

13. Kim SH, Xiao S, Shive H, Collins PL, Samal SK: Replication, neurotropism, and pathogenicity of avian paramyxovirus serotypes 1-9 in chickens and ducks. *PLoS One*, 7, e34927, 2012. DOI: 10.1371/journal.pone.0034927

14. Diel DG, Silva LHAD, Liu H, Wang Z, Miller PJ: Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infect Genet Evol*, 12, 1770-1779, 2012. DOI: 10.1016/j.meegid.2012.07.012

15. Samal S, Khattar SK, Kumar S, Collins PL, Samal SK: Coordinate deletion of N-glycans from the heptad repeats of the fusion F protein of Newcastle disease virus yields a hyperfusogenic virus with increased replication, virulence, and immunogenicity. *J Virol*, 86, 2501-2511, 2012. DOI: 10.1128/JVI.06380-11

16. Kim LM, King DJ, Guzman H, Tesh RB, Travassos DA: Biological and phylogenetic characterization of pigeon Paramyxovirus serotype 1 circulating in wild North American pigeons and doves. *J Clin Microbiol*, 46, 3303-3310, 2008. DOI: 10.1128/JCM.00644-08

17. Mehrabanpour MJ, Khoobyar S, Rahimian A, Nazari MB, Keshtkar MR: Phylogenetic characterization of the fusion genes of the Newcastle disease viruses isolated in Fars province poultry farms during 2009-2011. *Vet Res Forum*, 5, 187-191, 2014.

18. Sun C, Wen H, Chen Y, Chu F, Lin B, Ren G, Song Y, Wang Z: Roles of the highly conserved amino acids in the globular head and stalk region of the Newcastle disease virus HN protein in the membrane fusion process. *Biosci Trends*, 9, 56-64, 2015.

19. Wu S, Wang W, Yao C, Wang X, Hu S, Cao J, Wu Y, Liu W, Liu X: Genetic diversity of Newcastle disease viruses isolated from domestic poultry species in Eastern China during 2005-2008. *Arch Virol*, 156, 253-61, 2010. DOI: 10.1007/s00705-010-0851-5

20. Gaikwad S, Kim JY, Lee HJ, Jung SC, Choi KS: Genetic characterization and evolutionary analysis of Newcastle disease virus isolated from domestic duck in South Korea. *Gene*, 579, 34-40, 2015. DOI: 10.1016/j.gene.2015.12.040

21. Hu S, Ma H, Wu Y, Liu W, Wang X: A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine*, 27, 904-910, 2015. DOI: 10.1016/j.vaccine.2008.11.091

22. Kim SH, Xiao S, Collins PL, Samal SK: LaSota fusion (F) cleavage motif-mediated fusion activity is affected by other regions of the F protein from different genotype Newcastle disease virus in a chimerical virus: implication for virulence attenuation. *J Gen Virol*, 97, 1-8, 2016. DOI: 10.1099/jgv.0.000439

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Research Article

Gingivitisli Köpeklerde Bazı Sistemik Yangı Parametrelerinin Değerlendirilmesi [1]

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Özet

Bu çalışmada, CRP (C reaktif protein), albumin ve total protein değerleri ve CBC (tam kan sayımı) düzeyleri kullanılarak, hastanın sistemik inflamatuar durumunun değerlendirilmesi amaçlandı. Bu çalışmada, gingivitisli 10 ve sağlıklı 10 olmak üzere toplam 20 köpek kullanıldı. Gingivitis tanısı, klinik bulgulara (diş plakları, diş etinde kızarıklık ve ödem) dayalı olarak konuldu. Anamnezde, köpeklerde beslenme sonrası ağız bakımı (su içirme, diş fırçalama veya diş sağlığı gıdası veya kemirme malzemesi verme) yapılıp yapılmadığı ve hastaların ağızla ilgili şikayetlerinin bulunup bulunmadığı (çiğneme güçlüğü, aşırı salya akışı, ağız kokusu) soruldu. Fiziksel muayenede beden sıcaklığı, nabız ve solunum sayıları saptandı. Submandibular lenf yumrusu muayene edildi. Hasta ve sağlıklı her köpekten antikoagulansız tüplere 5'er mL kan ve EDTA'lı tüplere 2'şer mL kan alındı. Hastalardaki CRP, albumin, total protein değerleri ve CBC sağlıklı olgularla karşılaştırılarak değerlendirildi. Hastalarda klinik muayene bulgularına göre hafif-orta derecede gingivitis tanısı konuldu. Hastalarda her yemek sonrası ağız bakımı ile ilgili hiçbir özel uygulamanın yapılmadığı, hastalarda ağız kokusunun bulunduğu ve kuru gıdaları çiğnemede güçlük çektikleri öğrenildi. Yapılan fiziksel muayenede; hastaların tamamında diş plağı, diş eti kızarıklığı ve ödem ile ağız kokusu belirlendi. Beden sıcaklığı, solunum ve nabız sayıları normal sınırlardaydı. Submandibular lenf yumruları büyümemişti. Sağlıklı köpeklerde diş eti ile ilgili bir sorun bulunmamaktaydı. Hafif-orta derecede gingivitisli ve submandibular lenf yumrusu büyümemiş köpeklerde, CRP, albumin, total protein ve CBC de istatistiksel olarak önemli değişiklikler bulunmadı.

Anahtar sözcükler: *Albumin, CBC, CRP, Gingivitis, Köpek, Total protein*

Evaluation of Some Systemic Inflammatory Parameters in Dogs with Gingivitis

Abstract

In this study, it is aimed to evaluate the patient's systemic inflammatory condition, by estimating CRP, albumin, total protein values and CBC. These results were compared with inflammatory condition of the gums. In this study, a total of 20 dogs, including 10 dogs with gingivitis, and 10 healthy dogs, were used. Diagnosis was established based on the clinical findings (dental plaque, redness and edema in the gum). In history, the owners was asked if any oral care application was performed after each meal (drinking water, brushing dog's teeth, eating dental care food or giving chewing material), and if there is any mouth-related complaints (chewing difficulty, excessive saliva flow and halitosis). On physical examination; body temperature, pulse, and respiration rates were detected, submandibular lymph node was examined. From each patient and healthy dog, 5 mL blood to the tubes without coagulant, and 2 mL blood to the tubes with EDTA were taken. CRP, albumin and total protein values, and CBC were evaluated by comparing with those in healthy ones. Based on the clinical findings, mild-to-moderate gingivitis was diagnosed in patients. It was learned that patients have difficulty in chewing hard food, have halitosis and no special oral care application was done in patients after each meal, and have dental plaques. In physical examination, all of the patients have dental plaque, gingival redness and edema and halitosis. Body temperature, respiratory and pulse rates are within normal limits. There was no adenopathy in submandibular lymph node and no problems with the gums in healthy dogs. In the dogs with mild-to-moderate gingivitis and with no submandibular lymph node growth, no statistically significant changes were found in CRP, albumin, total protein values and the CBC.

Keywords: *Albumin, CBC, CRP, Dogs, Gingivitis, Total protein*

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GİRİŞ

Gingivitis, periodontitisin bir ön belirtisi olarak küçük-orta boyutlu köpeklerde orta yaşlardan itibaren görülmeye başlayan, yaşlılarda daha sık ortaya çıkan diş eti yangısıdır. Bozukluğun ortaya çıkışında beslenme tarzının önemi fazladır. Köpeklerde beslenme sonrası ağız bakımıyla ilgili bazı uygulamaların ihmal edilmesi hastalığın hazırlayıcı faktörlerindendir. Gingivitis diş plağı oluşumu ve bölgede bakteri üremesi ile başlar, periodontitise ve sistemik yangısal organ bozukluklarına yol açar [1-4]. Gingivitisli hastalarda ağzın fiziksel muayenesinde fark edilebilecek ilk bulgular diş plaklarının oluşumu ve hafif diş eti yangısına özgü belirtilerdir. Hastalığın başlangıcında beslenme esnasında bazen hafif bir güçlük fark edilir. Diş etinde hafif kızarıklık ve duyarlılık artışı ile arada bir fark edilen pis ağız kokusu ile bazen klinik olarak anlamlandırılması mümkün olmayan iştah azalması, durgunluk gibi sistemik bulgular ortaya çıkabilir. Gingivitisin şiddetlendiği hastalarda huzursuzluk, ağızda ağrı, aşırı salya akışı, salyada kan izi, ağızda pis koku, gıdayı çiğnemede güçlük, diş taşlarının oluşumu, diş etinde şiddetli yangısal belirtiler, diş eti kanaması ve kanda sistemik yangıyı yansıtan bazı laboratuar bulguları ortaya çıkar. Ancak bu dönemde bireysel immünolojideki farklılık ile sistemik yangıdan etkilenen organ çeşidi nedeniyle hastalar arasında klinik ve sistemik yangıya ilişkin laboratuar bulgularında farklılıklar ortaya çıkar [5,6].

Gingivitisli hastaların klinik tablosu 3 kategoride değerlendirilebilir. Bunlardan birincisi; sistemik yangısal parametrelerin sık ortaya çıkmadığı hafif-orta derecede gingivitis, ikincisi; sistemik yangısal kan parametrelerinin yükselmeye başladığı ve değişik derecede diş taşı oluşumuyla da karakterize şiddetli derecede gingivitis ve üçüncüsü; sistemik yangının laboratuvar bulgularının belirginleştiği ve bazen organ bozukluklarının da saptandığı periodontitis olarak isimlendirilen hastalığa ait ileri dönem klinik tablodur [5,6]. Gingivitisin başlangıcında hasta sahiplerinden; köpeklerin son zamanlarda sert ve kuru gıdaları dikkatli çiğnediği, ağzının bazen pis koktuğu ve/veya pis kokulu ve bazen içinde kan izi bulunan salya akıttıkları ve ara sıra kısmi iştah kaybı gösterdikleri anamnezi alınır. Fiziksel muayenede başlangıçta ağızda pis koku, salya birikmesi, diş etinde kızarıklık, ödem, duyarlılık artışı, ileri dönemde kanama ve diş eti cebinde gevşeme ve değişik derecede diş taşı oluşumu dikkati çeker. Diş taşı oluşumunun yoğunluğu ve şiddeti gingivitisin şiddeti ile doğru orantılıdır [5,6].

Gingivitisli hastalarda oluşan yangının şiddetine ve süresine ve vücudun yangıya karşı verdiği yanıta bağlı olarak öncelikle akut faz proteinlerinde olmak üzere albumin ve globulin düzeylerinde değişiklikler olur. Ayrıca lökositer kan hücrelerinde yangının şiddetiyle ilişkili olarak inflamatorik lökogram olarak isimlendirilen tablo ortaya çıkabilir [7-9]. Vücuttaki yangısal durumlarda kanda değişikliği fark edilecek ilk parametrenin serum (C-reaktif protein) CRP düzeyindeki artış olduğuna, CRP değişikliği ile toplam lökosit ve granülositler arasında doğru orantı olmadığına da işaret edilmektedir [10]. CRP enfeksiyon ve lokal doku yıkımlanmaları gibi çeşitli yangısal uyarımlarda ortaya çıkan ve esas olarak karaciğerde sentezlenen bir akut faz proteinidir. CRP ölçümünün esas amacı vücuttaki sistemik yangısal durumu belirlemek ve hastalığın seyrini yapılacak seri ölçümlerle izlemektir. LifeAssays® Canine CRP kiti ile yapılan ölçümlerde sağlıklı köpeklerdeki dCRP konsantrasyonu <35 mg/L'dir. Yangının nedenine ve şiddetine bağlı olarak CRP konsantrasyonu 600 mg/L'nin üzerine çıkabilir. Hafif derecede olan yangılarda ve lokal enfeksiyonlarda CRP konsantrasyonunda aşırı artış belirlenmeyebilir. Sistemik yangı albuminin katabolizasyonunu artırırken, karaciğerde albuminin sentezi azalır ve kan serumu albumin düzeyi düşer. Aksine yangısal globulinlerin üretimi arttığından kan serumu total globulin düzeyi yükselir. İnflamatorik lökogramda lokal yangının şiddetine, yaygınlığına, yangının süresine ve yangıya karşı vücudun verdiği selüler yanıta göre değişmek üzere nötrofil miktarında azalma veya artış ortaya çıkar. CBC tablosunda nötrofil miktarındaki artışa parelel olarak monosit miktarının da arttığı dikkati çeker. Yangının ortaya çıkış nedenine bağlı olarak lenfositlerde azalma veya artış ile eozinofillerde artış belirlenebilir. Hafif veya orta şiddetteki gingivitiste toplam lökosit sayısının ve granülositlerin normal hastalardaki aralığın üst sınırına doğru yükseldiği, gingivitisin şiddetlendiği periodontitisli hastalarda ise bu değerlerin hızla azalarak normal hücre aralığının altına düştüğü belirlenmiştir [7,9-11]. Bu çalışmada, gingivitisli köpeklerde CRP, albumin ve total protein miktarları ile CBC değerleri saptanarak hastanın sistemik enflamatuar durumunun incelenmesi ve sonuçların diş etinin enflamasyon durumu ile karşılaştırılması amaçlandı.

MATERYAL ve METOT

Bu çalışmada hayvan sahibinin izni ve Ankara Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nun karar numarası 2015-2-36 ile oluru alınarak gingivitisli 10 ve sağlıklı 10 olmak üzere toplam 20 köpek kullanıldı. Anamnez ve fiziksel muayene bulgularına bakılarak hastalarda gingivitis tanısı konuldu ve hastalığın şiddeti klinik bulgular dikkate alınarak kategorize edildi.

Anamnezde, köpeklerde beslenme sonrası ağız bakımı (su içirme, diş fırçalama veya diş sağlığı gıdası veya kemirme malzemesi verme) yapılıp yapılmadığı ve hastaların ağızla ilgili şikayetlerinin bulunup bulunmadığı (çiğneme güçlüğü, aşırı salya akışı, ağız kokusu, salyada kan) soruldu. Fiziksel muayenede beden sıcaklığı, nabız ve solunum sayıları saptandı. Submandibular lenf yumrusu muayene edildi. Hasta ve sağlıklı her köpekten antikoagulansız tüplere 5'er mL kan ve EDTA'lı tüplere 2'şer mL kan alındı. Hastalardaki CRP LifeAssays® Canine CRP kiti ile, otomatik cihazla, Random Access XL-600 klinik biyokimya otoanalizörü, Erba Türkiye kiti kullanılarak albumin, total protein değerleri ve Bouble Exigo Vet Hematology Analyse pr

1504215, s 52285 cihazı kullanılarak CBC otomatik kan sayımı cihazı kullanılarak belirlendi.

Önemlilik testlerine geçilmeden önce tüm veriler parametrik test varsayımlarından normallik yönünden Shapiro-Wilk, varyansların homojenliği yönünden Levene testi ile değerlendirildi. Sağlıklı ve hasta gruplarından elde edilen değişkenlerin karşılaştırılmasında student-t testi kullanıldı. Tüm istatistiksel hesaplamalar minimum %5 hata payı ile incelendi. SPSS 14.01 paket programından yararlanıldı.

BULGULAR

Bu çalışmada kullanılan 10 hasta ve 10 sağlıklı köpeğin küçük ve orta boy ırklardan, orta-ileri yaşta ve her iki cinsiyetten olduğu belirlendi. Anamnezde hasta köpeklerin tamamının hazır ticari köpek maması ile beslendikleri, köpeklere beslenme sonrası ve beslenme aralarında ağız bakımı için yararlı olabilecek bakım ve uygulamaların yapılmadığı bilgileri alındı. Köpeklerin sert gıda yerken zaman zaman durakladıkları, gıdayı çiğneyemedikleri, ağrı duyduklarına işaret olabilecek ses çıkardıkları ve ayaklarını ağzına sürdükleri, zaman zaman salya akıttıkları ve ağızlarından bazen kötü koku geldiği bilgisi alındı.

Klinik muayenede hastalarda iştah azalması ve durgunluğun seyrek ve anlık olarak ortaya çıkması, beslenme sırasındaki ağrı ve güç çiğneme şikayetlerinin her zaman görülmemesi ve aşırı şiddette olmaması, diş etinde hafif-orta şiddette kızarıklık, ödem ve hafif diş plaklarının varlığı ve ara sıra kötü ağız kokusu bulguları dikkate alınarak gingivitisli hastanın durumu; hafif-orta şiddette gingivitis olarak tanımlandı. Hastalarda ve sağlıklı kontrol köpeklerde beden sıcaklığı, solunum ve nabız sayıları normal sınırlardaydı. Submandibular lenf yumruları büyümemişti. Sağlıklı ve gingivitisli grupların CRP, Ig (Tp-Alb), WBC, lenfosit, monosit, nötrofil ve eozinofil değerleri *Tablo 1* ve *Tablo 2*'de sunuldu.

Tablo 1. *Sağlıklı ve gingivitisli grupların biyokimyasal parametrelerden CRP, Ig (Tp-Alb), değerleri*

Gruplar	Biyokimyasal Parametreler	
	CRP mg/L	Ig (Total protein-Albumin) (g/dL-g/dL)
Sağlıklı	24.90±9.29	2.57±0.30
Gingivitisli	20.50±8.49	2.70±0.37
p	0.283	0.377

Değerler ortalama±standart sapma olarak verilmiştir. Sağlıklı ve hasta gruplarından elde edilen CRP ve Ig (Tp-Alb), ölçümleri arasındaki fark istatistiksel olarak anlamlı değildir (P>0.05)

Hafif-orta derecede gingivitisli köpeklerde ve sağlıklı kontrol köpeklerde belirlenen değerler incelendiğinde; gingivitisli ve sağlıklı köpeklerdeki kan serumu CRP konsantrasyonları <35 mg/l bulundu *(Tablo 1)*. Gingivitisli köpeklerdeki kan serumu CRP konsantrasyonu istatistiksel olarak önemli olmasa da sağlıklı köpeklerdekinden daha yüksekti. Gingivitisli köpeklerdeki albumin, total protein ve toplam lökosit, nötrofil, monosit, lenfosit ve eozinofil değerlerinde sağlıklı köpeklerden elde edilen değerlerle karşılaştırıldığında istatistiksel olarak önemli değişiklikler ortaya çıkmadı.

TARTIŞMA ve SONUÇ

Gingivitise küçük-orta boyutlu ırklarda orta yaşlı-yaşlı hayvanlarda sık rastlanır. Beslenme alışkanlıkları periodontal hastalıkların oluşmasından sorumludur ve genel olarak yumuşak besinlerle beslenme hastalığın oluşma olasılığını artırır. Beslenme sonrası ağız bakımını sağlayacak uygulamaların yapılmaması periodontal bozukluk riskini artırır. Bazı bakteriler direk veya indirek olarak periodontal hastalıkların etiyolojisinde rol oynar [1,12,13]. Çalışmadaki hasta köpeklerin orta yaşlı ve yaşlı katagorisinden ve küçük-orta boyutlu ırktan olması, beslenmeler sonrası ağız bakımı uygulanmaması yukarıda belirtilen klasik literatür bulgularıyla uyumludur.

Ağız içindeki dokuların diyet ve salya ile temas süresi, dokulardaki kan dolaşımı ve dokuların immünolojik özellikleri ortam pH'ını ve mikroorganizma florasını etkiler ve dişte önce plak oluşur [2,14]. Başlangıçta yüzlek ve hafif derecede, sonra derin dokularda ve şiddetli derecede diş eti yangısı ortaya çıkar. Hastalarda bireye göre değişen sürelerde önce yakın dokularda sonra uzak organlarda akut veya kronik yangısal reaksiyonlar ve dejeneratif bozukluklar ortaya çıkar [15,16]. Bu araştırmadaki 10 köpekte diş plağı ve diş etinde hafif-orta şiddette yangı belirtileri bulguları yukarıdaki araştırmalarda belirtildiği üzere periodontitisin başlangıcında rastlanan lokal yangıya işaret bulgulardı. Hastalarda çiğneme güçlüğü, ağız kokusu, dişte

Tablo 2. *Sağlıklı ve gingivitisli grupların hematolojik bulgularından WBC (lökosit), lenfosit, monosit, nötrofil ve eozinofil değerleri*

Gruplar	Hematolojik Parametreler				
	WBC (Lökosit) 10^9/L	Lenfosit 10^9/L	Monosit 10^9/L	Nötrofil 10^9/L	Eozinofil 10^9/L
Sağlıklı	10.47±3.94	2.86±1.26	0.89±0.40	1.47±1.33	5.25±2.13
Gingivitisli	12.13±2.69	3.35±1.47	0.99±0.30	1.39±1.03	6.40±1.61
P	0.285	0.434	0.536	0.190	0.882

Değerler ortalama±standart sapma olarak verilmiştir. Sağlıklı ve hasta gruplarından elde edilen WBC, lenfosit, monosit, nötrofil, eozonofil ölçümleri arasındaki fark istatistiksel olarak anlamlı değildir (P>0.05)

plak oluşumu, yangının şiddetiyle doğru orantılı olarak diş etinde kızarıklık, şişlik ve duyarlılık artışı, ağızdan salya akması ve diş etinde kanamaya eğilim ve diş taşı oluşumu bulgularından [17,18] büyük kısmı çalışmadaki tüm hasta köpeklerde ortaya çıktı. Ağız içinde yangısal reaksiyonun ciddiyetinin göstergesi olan mandibular lenf yumrusu büyümesine [19] çalışmadaki olgularda rastlanmadı.

Gingivitisli hastalarda oluşan yangının şiddetine ve süresine ve vücudun yangıya karşı verdiği yanıta bağlı olarak CRP'de ortaya çıkan ve sistemik yangıya [8,20,21] işaret eden değişikliğin bu 10 köpekteki kan tablosunda istatistiksel olarak önemli çıkmaması yangının lokal oluşuna, yangısal reaksiyonun şiddetinin hafif olmasına ve metastazik yangısal durumların ortaya çıkmamasına bağlandı. CRP'nin gingivitisli köpeklerde kanın alındığı dönemde <35 mg/L olarak belirlenmesi hastalarda sistemik yangısal durumun gelişmediğini gösterdi.

Gingivitisli hastalarda sisteme yansımayan yangısal durumlarda albumin ve globulin düzeylerinin [8,9] normal sınırlarda bulunması bu araştırmadaki bulguyla uyumluydu. Aynı şekilde lokal yangının şiddetine, yaygınlığına, yangının süresine ve yangıya karşı vücudun verdiği selüler yanıta göre değişeceği belirtilen nötrofil, lenfosit, monosit, toplam lökosit ve eozinofil sayılarındaki değişikliklere [7,22] bu çalışmadaki hasta köpeklerde rastlanmaması lokal gingivitisin hafif-orta şiddette olmasına ve sistemik hücresel reaksiyona yol açamamasına bağlandı.

Hafif-orta şiddette gingivitisli ve submandibular lenf yumrusu büyümemiş köpeklerden elde edilen verilerin sağlıklı köpeklerden elde edilen verilerle karşılaştırmasında; CRP, albumin, toplam protein, toplam lökosit, nötrofil, monosit, lenfosit ve eozinofil değerlerinde istatistiksel olarak önemli değişiklikler ortaya çıkmadığı belirlenmiştir. Bu değerler temel alındığında, hafif-orta şiddette gingivitisi bulunan köpeklerde diş etinde bu şiddetteki bir yangının sistemik yangısal bir uyarıma neden olmadığı sonucuna varılmıştır.

KAYNAKLAR

1. Gorrel C: Periodontal disease and diet in domestic pets. *J Nutrition*, 128 (2): 2712-2714, 1998.

2. Harvey CE: Periodontal disease in dogs. Etiopathogenesis prevalence, and significance. *Vet Clin North Am: Small Anim Pract*, 28, 1111-1128, 1998. DOI: 10.1016/S0195-5616(98)50105-2

3. Marshall MD, Wallis CV, Milella L, Colyer A, Tweedie AD, Harris SA: Longitudinal assessment of periodontal disease in 52 Miniature Schnauzers. *Vet Res*, 10, 166, 2014. DOI: 10.1186/1746-6148-10-166

4. Watson AD: Diet and periodental disease in dogs and cats. *Aust Vet J*, 71, 313-318, 1994. DOI: 10.1111/j.1751-0813.1994.tb00905.x

5. Campos X, Manuel V, Izquierdo Cordova-Jimenez A, Alejandro C, Jimenez C, Silvia M, Salinas A, Saul K, Ueno KM, Suarez C: Periodontitis and characteristics associated dogs slaughtered in a canine control center and anti-rabies from Mexico City. *J Anim Vet Adv,* 8 (5): 977-979, 2009.

6. Kahn CM, Line S: Digestive System, Dentistry: Periodontal Diseases in Small Animals. **In**, Kahn CM, Line S (Eds): The Merck Veterinary Manual. 9th edn., 143-147, Merck and Co. Inc Merial LTD, USA, 2005.

7. Gaunt SD: Interpretation of the leukogram, **In**, Cowell RL (Ed): Veterinary Clinical Pathology Secrets. 38-44, Elsevier Mosby, Missouri, USA, 2004.

8. Kouki MI, Papadimitriou SA, Kazakos GM, Savas I, Bitchava D: Periodontal disease as a potential factor for systemic inflammatory response in the dog. *J Vet Dent,* 30 (1): 26-29, 2013. DOI: 10.1177/089875641303000103

9. Tyler RD, Cowell RL, Meinkoth JH: Disorders and laboratory evaluation. **In,** Cowell RL (Ed): Veterinary Clinical Pathology Secrets. 51-60, Elsevier Mosby, Missouri, USA. 2004.

10. Rai B, Kaur J, Kharb S, Jain R, Anand SC, Singh J: Peripheral blood and C-reactive protein levels (CRP) in chronic periodontitis. *Afr J Biochem Res,* 3 (4): 150-153, 2009.

11. Nakamura M, Takahashi M, Ohno K, Koshino A, Nakashima K, Setoguchi A, Fujino Y, Tsujimoto H: C-reactive protein concentration in dogs with various diseases. *J Vet Med Sci*, 2008 70 (2): 127-131, 2008.

12. Bradley WQ: Oral health benefits of a daily dental chew in dogs. *J Vet Dent,* 30 (2): 84-87, 2013. DOI: 10.1177/089875641303000203

13. Kyllar M, Witter K: Prevalence of dental disorders in pet dogs. *Vet Med Czech*, 50 (11): 496-505, 2005.

14. Gawor JP, Reiter AM, Jodkowska K, Kurski G, Wojtacki MP, Kurek A: Influence of diet on oral health in cats and dogs. *J Nutr*, 136, 2021-2023, 2006.

15. Logan EI, Finney O, Hefferren JJ: Effects of a dental food on plaque accumulation and gingival health in dogs. *J Vet Dent*, 19, 15-18, 2002. DOI: 10.1177/089875640201900102

16. Pavlica Z, Petelin M, Juntes P, Erzen D, Crossley DA, Skaleric U: Periodontal disease burden and pathological changes in organs of dogs. *J Vet Dent*, 25, 97-105, 2008. DOI: 10.1177/089875640802500210

17. Ingham KE, Gorrel C: Assessing oral health and hygiene in dogs. *Waltham Focus,* 9 (4): 32-33, 1999.

18. Logan EI, Boyce EN: Oral health assessment in dogs: Parameters and methods. *J Vet Dent*, 11 (2): 58-63,1994.

19. Anderson GB, Caffesse RG, Nasjleti CE, Smith BA: Correlation of periodontal probe penetration and degree of inflammation. *Am J Dent*, 4 (4): 177-183, 1991.

20. Rawlinson JE, Goldstein RE, Reiter AM, Attwater DZ, Harvey CE: Association of periodontal disease with systemic health indices in dogs and the systemic response to treatment of periodontal disease. *J Am Vet Med Assoc*, 238, 601-609, 2011. DOI: 10.2460/javma.238.5.601

21. Negro VB, Ciappesoni JL, Sorbello C, Rodríguez D, Juarez M, Mas J, Hernández SZ: Periodontal disease in dogs: Correlation between degree of disease, blood parameters, and systemic inflammatory response, and its response to treatment. *EVDS*, 14-21, 2015.

22. Kouki MI, Papadimitriou SA, Kazakos GM, Savas I, Bitchava D: Periodontal disease as a potential factor for systemic ınflammatory response in the dog. *J Vet Dent*, 30, 26-29, 2013. DOI: 10.1177/089875641303000103

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Research Article

The Effects of the Supplementation of Lamb Rations with Oregano Essential Oil on the Performance, Some Blood Parameters and Antioxidant Metabolism in Meat and Liver Tissues [1]

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Abstract

In this study, the effects of oregano essential oil, on performance, some blood parameters, the antioxidant metabolism such as lipid oxidation (LPO) and glutathione (GSH) levels with superoxide dismutase (SOD) and catalase (CAT) enzyme activities in the liver and the *Musculus longissimus dorsi* (meat) tissue were investigated. The study consisted of three groups; the control group fed a basal ration, the OEO1 group fed a basal ration added 200 mg/kg Orego-Stim and the OEO2 group fed a basal lamb ration added 400 mg/kg Orego-Stim. The groups did not differ for performance parameters. In groups control and OEO2, serum calcium and potassium levels were significantly higher than the group of OEO1 (P<0.05). However, the serum magnesium level were significantly higher in OEO1 group (P<0.05). While there are no meaningful changes on superoxide dismutase activity, LPO and GSH rates with CAT activity had significant changes in liver tissue (P<0.05). LPO and GSH levels with SOD and CAT enzyme activities were significantly affected in *M. longissimus dorsi* tissue (P<0.05). As a result, while oregano essential oil did not affect the performance parameters and lipid profile, it significantly affected the antioxidant metabolism in meat and liver tissues.

Keywords: *Oregano essential oil, Lamb, Performance, Antioxidant enzyme, Liver, Meat*

Kuzu Rasyonuna İlave Edilen Kekik Yağının Performans, Bazı Kan Parametreleri İle Et ve Karaciğer Dokularında Antioksidan Metabolizma Üzerine Etkisi

Özet

Bu çalışmada kekik yağının performans ve bazı kan parametreleri ile karaciğer ve *Musculus longissimus dorsi* dokularında lipid oksidasyon (LPO) ve glutatyon (GSH) oranları ile süperoksit dismutaz (SOD) ve katalaz (CAT) enzim aktivitelerinin antioksidan metabolizma üzerine etkileri araştırılmıştır. Çalışma üç grup halinde yürütülmüştür. Gruplandırma ise bazal rasyon verilen kontrol grubu, bazal rasyona ilave olarak 200 mg/kg Orego-Stim verilen OEO1 grubu ve bazal rasyona ilave olarak 400 mg/kg Orego-Stim verilen OEO2 grubu şeklinde yapılmıştır. Grupların performans parametreleri arasında fark bulunmamıştır. Kontrol ve OEO2 gruplarının serum kalsiyum ve potasyum seviyeleri OEO1 grubuna göre yüksek bulunmuştur (P<0.05). Ancak serum magnezyum seviyesi OEO1 grubunda daha yüksek olduğu tespit edilmiştir (P<0.05). Karaciğer dokusunda süperoksit dismutaz aktivitesine önemli bir etkisi olmazken, LPO ve GSH oranları ile CAT aktivitesini önemli olarak değiştirmiştir (P<0.05). *M. longissimus dorsi* dokusunda LPO ve GSH oranları ile SOD ve CAT enzim aktivitelerinin önemli oranda etkilendiği belirlenmiştir (P<0.05). Sonuç olarak kekik yağının performans parametreleri ve lipid profilini etkilemediği, karaciğer ve kas dokularında antioksidan metabolizma üzerine önemli etkilerinin olduğu tespit edilmiştir.

Anahtar sözcükler: *Kekik yağı, Kuzu, Performans, Antioksidan enzim, Karaciğer, Et*

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INTRODUCTION

Feed additives are used to ensure healthy growth and increase the yield and quality of animals. Therefore, for many years antibiotics have been used as feed additives. However, the use of these substances as feed additives bearing a risk to human health due to the development of resistance in microorganisms against antibiotics. That is why, the practice of using vitamins and plant extracts as feed additives has started to become commonplace [1-3]. Plant extracts are shown to stimulate the secretion of enzymes in the digestive system and regulate the microbial eco-system the effect on the performance of animals [4]. Already, plant extracts are used commonly in the pharmaceutical, cosmetics, perfumery, and food sectors, owing to their various biological effects. Both thyme itself and its extracts (thyme essential oil) contain substances that induce more than 60 effects, such as antiseptic, antioxidant, antimicrobial and aroma-regulating effects. It has been reported that thyme contains phenols, thymol (68.1%), carvacrol (3.5%), monoterpene hydrocarbons, p-cymene (11.2%) and γ-terpinene (4.8%) [5].

The free radicals occur as a result of metabolic activities and they generate oxidative stress and create damage to cells. Conversely, cells have several mechanisms that are capable of repairing harmful effects or are capable of preventing the effects harmful effects of reactive oxygen species (ROS). By the enzymes as primarily [SOD, CAT, myeloperoxidase (MPx), glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx)] and by secondary antioxidant vitamins, GSH, plant extracts and many macro and micro molecules have reduced the formed damage by free radicals and is kept low and the specific concentrations of free radicals in cells [6].

The antioxidant substances in the composition of oregano were determined in the laboratory analysis [7]. These substances explain the effect on the living organisms given to animals and full disclosure of the antioxidant metabolism in cells. Unal and Kocabagli [8] reported that, dietary supplementation of oregano to lambs' ration had no effects on performance parameters. Information related to oregano growth promoting effects when added to sheep diets is scarce [9].

In this study, the effect of Orego-Stim was determined on the performance and some blood parameters in the ruminants to the determined details of the effect on the antioxidant metabolism through LPO and GSH rates with SOD and CAT activities in the meat and liver tissue.

MATERIAL and METHODS

Animals, Experimental Design and Feed

The study was conducted at the Animal Husbandry Research and Application Unit of Atatürk University, Faculty of Veterinary Medicine. 24 male Akkaraman lambs (which were weaned when they were 3 months old on average) were divided to 3 groups as control and two treatment (OEO1 and OEO2) groups, each consisting of four replicates of eight lambs. The lambs two animals were allocated to each compartment measuring 280×200×120 cm. This study was approved by the ethic committee of Faculty of Veterinary Medicine in Ataturk University [Decision No: 2010/700 (Decision No: 2006/5g)].

In this study the control group was fed a basal lamb diet, the OEO1 group was fed a basal lamb diet + 200 mg/kg Orego-Stim and the OEO2 group was fed a basal lamb diet + 400 mg/kg Orego-Stim *(Table 1)*. The Orego-Stim was added in place of bran (Orego-Stim was obtained from Ecopharm Hellas S.A., Kilkis, Greece. Orego-Stim was contains 5% essential oil of *Origanum vulgare subsp. Hirtum* plants and 95% natural feed grade inert carrier). Concentrate feed was specially produced on a monthly basis in a factory, in order to prevent the spoiling of oregano essential oil while it was stored.

The animals were provided with pre-weighed feed, twice a day at 08:00 am and 05:00 pm. The study lasted for 70 days (14 days were used as adaptation followed by

Table 1. *Composition of lamb diets used in the study, %*

Ingredient	Groups		
	Control	OEO1	OEO2
Barley	30	30	30
Corn	20	20	20
Sunflower seed meal	13.33	13.33	13.33
Cotton seed meal	13.2	13.2	13.2
Bran	9.70	9.68	9.66
Corn gluten	5	5	5
DDGS[1]	5	5	5
Marble powder	2.05	2.05	2.05
Molasses	1.12	1.12	1.12
Salt	0.5	0.5	0.5
Vitamin mineral premix[2]	0.1	0.1	0.1
Orego-Stim[3]	-	0.02	0.04
Rates of nutrient, %			
Crude protein	18.53	18.52	18.51
Crude fibre	12.74	12.74	12.73
Crude ash	7.2	7.2	7.2
Acid detergent fibre	13.67	13.67	13.67
Neutral detergent fibre	27.3	27.3	27.3

[1] DDGS: Dried Distillers Grains with Solubles; [2] The vitamin & mineral premix provided the following (per kg): 4.000.000 IU vitamin A, 800.000 IU vitamin D_3, 5.000 IU vitamin E, 400 mg vitamin B_2, 2 mg vitamin B_{12}, 5.000 mg vitamin PP, 1.000 mg D-pantothenic acid, 20.000 mg choline, 50 mg Co, 5.400 mg Fe, 185 mg I, 6.900 mg Mn, 800 mg Cu, 6.400 mg Zn, 14 mg Se; [3] Orego-Stim was contains 5% essential oil of Origanum vulgare subsp. Hirtum plants and 95% natural feed grade inert carrier

56 days for data collection). The daily amount of roughage provided to the lambs was 125 g of wheat straw per animal (the chemical composition of wheat straw on the basis of dry matter content was as follows: crude protein: 3.1, crude ash: 6.69, Neutral Detergent Fiber (NDF): 77.45, Acid Detergent Fiber (ADF): 50.32) Concentrate feed and water was supplied *ad libitum* during the trial.

Feed Analysis

The rations fed to the animals were formulated in accordance with the recommendations of the NRC [10] *(Table 1)*. The raw feed materials used in the study were crude ash, crude protein, crude oil analysis according to the Weende Analysis System of AOAC [11] and if the crude cellulose according to Crampton and Maynard [12]. NDF and ADF analysis were according to Soest and Robertson [13] with Goering and Van Soest [14].

Determination of Performance Parameters

The body weight of the animals was measured at the beginning of the trial, and on days 14, 28, 42 and 56 (final) in the morning, before they were given feed. Daily feed intake was determined by weighing the concentrate remaining in the feeders prior to morning feeding. As the subgroups included two animals, individual daily feed intake was calculated by dividing the daily feed intake values by two. Feed efficiency was calculated as the proportion of daily feed intake to daily weight gain (kg/kg).

Collection of Blood, Liver and Muscle Tissues Samples

Blood samples were collected from jugular vein of the twenty-four animals on the last day of the trial, prior to morning feeding, for use in biochemical analyses. The blood samples, collected in volumes of 5 cc into dry tubes (Becton Dickinson Co. USA),were centrifuged at +4°C and 4.000 rpm for 10 min in a cooled centrifuge (Hettich 38R, Hettich Zentrifugen, Tuttlingen, Germany). The harvested serum samples were stored at -80°C until use.

At the end of the study 6 animals were slaughtered from each group. Longissimus muscle and liver tissues were homogenised using liquid nitrogen and then stored at -80°C until the biochemical investigations.

Biochemical Analyses

- Determination of Serum Biochemical Parameters

Serum concentrations of glucose, urea, uric acid, triglyceride, calcium, phosphorus, magnesium, sodium, potassium and chlorine and were measured with an automatic analyzer using commercial test kits (Cobas 8000 Analyzer, Roche).

- Determination of the Lipid Profile (Thin Layer Chromatography)

Thin layer chromatography was performed using a 20 x 10 cm Silica Gel 60 F254 High Performance Thin Layer Chromatography (HPTLC) Plate. 1 ml of serum homogenate or serum was added 1 ml of n-hexane/iso-propanol (2:1 (v/v)) mixture in a tube. After being mixed thoroughly, the tube content was maintained for 10 min and mixed once again. This procedure was repeated for a further two times [15]. Subsequently, the tubes were centrifuged at 8.000 rpm for 10 min and the upper phases were loaded onto the HPTLC plate. The plates were developed in a hexane: diethyl ether: formic acid (80:20:2 (v/v/v) mixture for 15 cm and then dried. The spots on the dry plates were made visible by means of treatment with 3% $CuSO_4$ in 8% phosphoric acid followed by burning on hot plates [16]. The hydrocarbon, triacylglycerol, steroid and polar lipid parameters were measured for lipid profile.

- Antioxidant Enzymes

Superoxide dismutase and CAT enzyme activities and the amounts of GSH and LPO in the tissues were determined. To prepare the tissue homogenates, the muscle tissues were ground with liquid nitrogen in a mortar. 0.5 g of tissue was then treated with 4.5 ml of appropriate buffer (SOD: pH 7.4/0.2mMTris-HCl buffer, CAT: pH 7/50 mM phosphate buffer, GSH: pH 7.4/50mMTris-HCl buffer, LPO: 10% KCl solution). The mixtures were homogenised on ice using an ultra-turrax homogeniser for 15 min. Homogenates were filtered and centrifuged at 4°C. These supernatants were then used for biochemical measurements. All biochemical measurements were carried out using a UV-Vis spectrophotometer.

- SOD Activity

SOD activity was measured according to Sun et al.[17]. SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which reacts with Nitro Blue Tetrazolium (NBT) to form formazan. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction, and was expressed as mmol/min/mg of tissue.

- CAT Activity

Decomposition of H_2O_2 in the presence of CAT was followed at 240 nm [18]. The CAT activity was defined as the amount of enzyme required to decompose 1mmol of H_2O_2 per minute at 25°C at pH 7.8. Results were expressed as mmol/min/mg of tissue.

- Total GSH

The amount of GSH in the tissues was measured according to the method described by Sedlak and Lindsay [19]. The muscles tissues were homogenised in 2 ml of 50mMTris-HCl buffer containing 20mM EDTA, at pH 7.5. After adding 2 ml ethanol (to precipitate the proteins), the homogenate was centrifuged at 3.000 g for 40 min at 4°C. The supernatant was used to determine GSH level using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB). The absorbance was measured at 412 nm. Following the GSH level of the muscles was expressed as nmol/g tissue.

- Determination of LPO

The level of LPO in the tissues was determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test [20]. The muscles were scraped, weighed and homogenised in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added with a solution containing 0.2 mL of 80 g/L sodium lauryl sulphate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate and 0.3 mL of distilled water. The mixture was incubated at 98°C for 1 h Upon cooling, 5 mL of n-butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4.000 rpm. The absorbance of supernatant was measured at 532 nm. The standard curve was obtained using 1,1,3,3-tetramethoxypropane. The recovery rate was over 99%. The results were expressed as nmol MDA/g tissue.

Statistical Analysis

Data were analysed using SPSS version 20.00 package software [21]. All values measured were tested with One-way ANOVA (total). Differences between the groups were determined by the Duncan Multiple Comparison Test with the P<0.05 value for significance.

RESULTS

Oregano essential oil had no significant effect on the performance parameters of the lambs *(Table 2)*. In *Table 3*, the serum levels of glucose, urea, uric acid and triglyceride were similar in all three groups. Furthermore, while serum phosphorus, sodium and chlorine levels were found to be similar in all three groups, in group OEO1 serum calcium and potassium levels were determined to have significantly decreased, when compared to with groups control and OEO2 (P<0.05) *(Table 3)*. The serum magnesium levels were ascertained to have significantly increased in group OEO1 (P<0.05) and to have slightly increased in group OEO2. The investigation of the lipid profile that, the hydrocarbon, triacylglycerol, steroid and polar lipid levels of all three groups were found to be similar *(Table 3)*.

In *Table 4*, oregano essential oil has a noticeable effect on the LPO rate in liver and meat tissues. While the LPO rate of the OEO1 group was significantly lower than the control and the OEO2 groups (P<0.01), LPO rates were similar in the control and OEO2 groups in the liver. LPO rates of the OEO1 and OEO2 groups were significantly lower than the control group in the *Musculus longissimus dorsi* (P<0.01).

The effects of oregano essential oil has on the SOD activity in liver and meat tissues are presented *(Table 4)*. Oregano essential oil has no determined significant effect on SOD activity in the liver. However, SOD activity of the OEO1 group was significantly lower than the control and OEO2 groups in meat tissue (P<0.01).

In *Table 4*, the oregano essential oil has given effects on the CAT activity in the liver and longissimus muscle tissue. Oregano essential oil has a determined significantly increase the CAT activity in the liver and the meat tissues (P<0.01).

Oregano essential oil has a determined significant effect on the GSH rates in the liver and the meat tissues *(Table 4)* (P<0.01).

DISCUSSION

Oregano essential oil has positive effects on performance via the assessment of food in the digestive system. Approximately 15% of the digested nutrients, converted to methane and CO_2 gas in particularly at the end of microbial digestion in the rumen, can not be used by the animals. The antimicrobial properties of the oregano oils modify the rumen metabolism by regulating/developing microbial activity in the rumen [22,23]. Oregano leaves [9] or extracts [24] supplementation of lamb ration has no known effects on the performance of animals. It is also reported that dietary supplementation of oregano oil to lambs'

Table 2. *The effects of the oregano essential oil on body weight, feed intake and feed efficiency of the lambs*

Variable Parameters	Groups			*P*-value
	Control	OEO1	OEO2	
Initial body weight, kg	34.73±4.45	34.11±3.51	34.58±3.98	0.953
14th day body weight, kg	37.10±5.16	37.91±4.20	38.46±4.82	0.858
28th day body weight, kg	40.38±5.12	40.08±3.48	39.57±7.34	0.960
42nd day body weight, kg	43.20±5.00	43.34±4.30	44.94±4.30	0.728
Final body weight, kg	46.06±4.47	46.75±4.74	48.61±3.86	0.526
Body weight gain, g/day	203±46.83	226±66.08	250±38.75	0.226
Feed intake, g/day	1813	1773	1702	-
Feed efficiency, kg/kg	8.93	7.85	6.81	-

All values are given as mean ± standard error of mean (SEM), (n=8); ***Control:*** *basal ration alone, OEO1: basal ration+200 mg/kg of Orego-Stim, OEO2: basal ration+400 mg/kg of Orego-Stim*

Table 3. *The effects of the oregano essential oil on some serum biochemical parameters the lambs*

Parameters	Groups			*P*-value
	Control	OEO1	OEO2	
Glucose, mg/dL	90.00±7.91	81.63±9.27	84.88±10.60	0.213
Urea, mg/dL	42.00±5.95	44.38±5.21	40.75±4.10	0.376
Uric acid, mg/dL	0.15±0.05	0.16±0.09	0.13±0.12	0.707
Triglyceride, mg/dL	23.50±7.50	22.38±6.70	34.50±16.25	0.074
Calcium, mg/dL	11.27±0.35^{a}	10.40±0.98^{b}	11.02±0.50^{a}	0.043
Phosphorus, mg/dL	8.52±0.72	8.49±1.68	8.39±1.11	0.977
Magnesium, mg/dL	2.43±0.22^{b}	2.97±0.54^{a}	2.56±0.30^{b}	0.026
Sodium, mmol/L	147.88±2.03	147.88±1.36	148.13±1.25	0.936
Potassium, mmol/L	4.82±0.22^{a}	4.56±0.14^{b}	4.89±0.25^{a}	0.010
Chlorine, mmol/L	107.88±1.81	108.63±2.33	108.38±1.92	0.756
Lipid profile, %				
Hydrocarbon	66.06±2.65	66.15±1.50	65.03±3.15	0.611
Triacylglycerol	3.45±0.52	4.09±1.15	4.44±0.55	0.062
Steroid	14.10±1.16	14.49±2.05	13.59±1.62	0.707
Polar lipid	16.81±2.22	15.61±2.01	17.43±1.01	0.376

^{a,b} Different letters in the same column represent a statistical significance between the groups; All values are given as mean ± standard error of mean (SEM), (n=8)

Table 4. *The effects of the oregano essential oil on LPO and GSH rates with SOD and CAT activities in the liver and M. longissimus dorsi muscle tissues*

Parameters	Groups			*P*-value
	Control	OEO1	OEO2	
Liver				
LPO, nmol/g tissue	2.669±0.112^{a}	2.089±0.09^{b}	2.646±0.113^{a}	0.003
SOD, mmol/min/mg tissue	0.223±0.005	0.218±0.003	0.222±0.004	0.095
CAT, mmol/min/mg tissue	0.721±0.045^{c}	1.337±0.056^{a}	1.083±0.068^{b}	0.005
GSH, nmol/g tissue	0.163±0.002^{b}	0.180±0.004^{a}	0.180±0.004^{a}	0.002
M. longissumus dorsi				
LPO, nmol/g tissue	0.263±0.018^{a}	0.197±0.025^{b}	0.220±0.025^{b}	0.004
SOD, mmol/min/mg tissue	0.366±0.018^{a}	0.312±0.025^{b}	0.348±0.025^{a}	0.003
CAT, mmol/min/mg tissue	1.006±0.207^{b}	1.571±0.050^{a}	1.466±0.050^{a}	0.005
GSH, nmol/g tissue	0.077±0.002^{b}	0.094±0.002^{a}	0.084±0.004^{a}	0.001

All values are given as mean ± SEM, (n=6); $^{a,b,c:}$ Different letters in the same column represent a statistical significance between the groups

ration did not affect the performance parameters [8,25]. In this study, no statistically significant effect was seen, although a certain improvement revealed daily live weight gain, feed consumption and feed efficiency *(Table 2)*. Our findings are in agreement with the previous literatures [9,24].

Unal and Kocabagli [8] has also reported that, thyme essential oil added to lamb rations did not affect the cholesterol, triglyceride, HDL and LDL ratios of the blood serum among the groups but changed within the groups depending on the time. Effects of oregano essential oil on blood metabolites in lambs have not been investigated widely. Vakili et al.[26] established that thyme essential oil in the diets of feedlot calves (5 g/day/calf) resulted in no changes in values of plasma triglyceride. Whereas in another study it has been reported that, concentrations of trigycerides can be influenced by oregano essential oil supplementation via changing of feed intake [27]. In the present study, while serum glucose, urea, uric acid and triglyceride levels were found to be similar in all three groups, in group OEO1 serum Mg levels were determined to have significantly increased, when compared to with groups control and OEO2. Also, in group OEO1 serum calcium and potassium levels were determined to have significantly decreased.

Today LPO is accepted as an important indicator of oxidative stress as well as tissue damages [28]. Many studies have determined the reduction at a significant level the lipid oxidation in the tissues, the antioxidants found in the structure of thyme essential oil [29-31]. In this study, either dose of oregano essential oil support by information the literature decrease the LPO rate in meat tissue. However, while it is similar with the literature information the 200 mg Orego-Stim is significant level reduced the rate of LPO in liver tissue, it is interesting that there was no effect on the rate of LPO of the group that were given 400 mg Orego-Stim. These results show different the effects antioxidative of oregano essential oil the rate of LPO in the liver and meat tissues. The study showed a high rate of LPO in the liver tissue of group OEO2 that probably can to come forward from fatty acids in the structure of the oregano essential oil. Also, more detailed studies are suggested to obtain more detailed information.

Almost all of the studies made to determine the antioxidant properties of oregano essential oil have been related to the LPO ratio [32,33]. In order to fully explain the mechanism the antioxidant effect of oregano essential oil must determine the rates of LPO and GSH with the SOD and CAT activities.

SOD enzyme, the first step of antioxidant defense, plays crucial role on the elimination of superoxide radical. The enzyme SOD catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Catalase enzyme constitutes the second step of the defense mechanism which prevents the accumulation of hydrogen peroxides and catalyzes the conversion of hydrogen peroxide to water and molecular oxygen. An increased activity of any enzyme could be linked with an enhanced substrate production during the metabolic processes. Therefore, both SOD and CAT activities inform us both about how the defense system works and the situation of oxidative stress [28].

In addition, it has been reported that the SOD activities are different according to the tissues [34]. But stress usually increases the SOD activity [28]. In the present study the SOD activity of group OEO1 is lower and it has been shown that the rate of superoxide radical is less than other groups and the most effective dose was 200 ppm of oregano oil in the *M. longissimus dorsi*. If it is not significant among the groups of activity SOD in liver tissue is shows different levels of superoxide radical formation in tissues and activity of SOD in the liver and *M. longissimus dorsi*. The free radicals rate has been known to cause an increase in the rate of free radicals in favour of the antioxidant balance between free radicals with antioxidants in the tissues. The rate of the SOD activity in diabetic rats was reported to be lower than in healthy animals [35].

Antioxidant strategies designed to either inhibit free radical formation or to scavenge free radicals may provide protection. An increased activity of any enzyme could be linked to enhanced substrate production during the metabolic processes. Indeed, the increased CAT activity would also suggest that the accumulation of H_2O_2 might be responsible for an increased LPO following heat stress. One may consider that the heat stress application increased the H_2O_2 formation; therefore, increased CAT activity could discharge these radicals from the medium [28]. Orageno essential oil increased significantly the CAT activity in the liver and meat tissues. These results of the oregano essential oil have been shown to increase in CAT activity for removed occurs intensive of hydrogen peroxide radicals in cells.

GSH forms an important part of the antioxidant defence system and plays an important role for the prevention of damage of many harmful molecules and compounds. Glutathione is the substrate of GSH-dependent enzymes such as GSH-peroxidase and GSH S-transferase. Organisms use GSH to eliminate hydrogen peroxide and other peroxides. These enzymes will not show any activity in the absence of GSH. Conversely, these enzymes use GSH against ROS, which leads to a decrease in the amounts of GSH. Briefly, GSH is a marker used to explain how the defence system acts directly, and the ROS amount indirectly. When GSH levels are insufficient, peroxides may accumulate, therein causing damage. Oregano essential oil has antioxidant components higher than GSH levels groups of OEO1 and OEO2 than the control group remove free radicals in liver and meat tissue. Glutathione (GSH) content was higher depending on the amount of essential oils obtained from plants [33].

As a result, Orego-Stim added as a feed additive in ration provides a significant effect on the rates of LPO and GSH with activities of CAT and SOD in *M. longissimus dorsi*, while it increased the activity of CAT with the rate of GSH in liver tissue. Also, the results show the different effect of different doses on SOD activities in the *M. longissimus dorsi* and on the rate of LPO in the liver. On the other hand, Orego-Stim was determined to have no significant effects on performance parameters. This study, after determining in the effect of antioxidants on the metabolism, will find more efficient and correct use areas, particularly in the food and pharmaceutical industry. There is a need for studies on different doses in order to obtain detailed information about oregano essential oil or derivatives added as a feed additive in ration.

REFERENCES

1. Imik H, Ozlu H, Gumus R, Atasever MA, Urcar S, Atasever M: Effects of ascorbic acid and α-lipoic acid on performance and meat quality of broilers subjected to heat stress. *Br Poult Sci,* 53, 800-808, 2012. DOI: 10.1080/00071668.2012.740615

2. Gumus R, Imik H: The effect of *Yucca schidigera* powder added to lamb feed on fattening performance, some blood parameters, the immune system, and the antioxidative metabolism of the hepatic tissue. *Turk J Vet Anim Sci,* 40, 263-270, 2016. DOI: 10.3906/vet-1504-92

3. Karadağoğlu Ö, Önk K, Şahin K, Bingöl SA, Aksu Elmalı D, Durna Ö:

Effects of different levels of essential oil mixed (peppermint-thyme-anise oil) supplementation in the drinking water on the growth performance, carcass traits and histologic structure of terminal ileum in quails. *Kafkas Univ Vet Fak Derg,* 22, 253-260, 2016. DOI: 10.9775/kvfd.2015.14390

4. Khaksar V, Krimpen MV, Hashemipour H, Pilevar M: Effects of thyme essential oil on performance, some blood parameters and ileal microflora of japanese quail. *J Poultry Sci,* 49, 106-110, 2012. DOI: 10.2141/jpsa.011089

5. Grosso C, Figueiredo AC, Burillo J, Mainar AM, Urieta JS, Barroso JG, Coelho JA, Palavra AMF: Composition and antioxidant activity of *Thymus vulgaris* volatiles: Comparison between supercritical fluid extraction and hydrodistillation. *J Sep Sci,* 33, 2211-2218, 2010. DOI: 10.1002/jssc.201000192

6. Sies H: Oxidative stress: oxidants and antioxidants. *Exp Physiol,* 82, 291-295, 1997. DOI: 10.1113/expphysiol.1997.sp004024

7. Alma MH, Mavi A, Yildirim A, Digrak M, Hirata T: Screening chemical composition and *in vitro* antioxidant and antimicrobial activities of the essential oils from *Origanum syriacum* L. growing in Turkey. *Biol Pharm Bull,* 26, 1725-1729, 2003.

8. Ünal A, Kocabağli N: Effect of different dosages of oregano oil on performance and some blood parameters in lambs. *Ankara Üniv Vet Fak Derg,* 61, 199-204, 2014. DOI: 10.1501/Vetfak_0000002629

9. Bampidis VA, Christodoulou V, Florou-Paneri P, Christaki E, Spais AB, Chatzopoulou PS: Effect of dietary dried oregano leaves supplementation on performance and carcass characteristics of growing lambs. *Anim Feed Sci Tech,* 121, 285-295, 2005. DOI: 10.1016/j.anifeedsci.2005.02.002

10. NRC: Nutrient Requirements of Small Ruminants. The National Academy Press, Washington D.C., USA, 2006.

11. AOAC: Official Methods of Analysis of AOAC International. 18th edn., Gaithersburg, USA, 2005.

12. Crampton E, Maynard L: The relation of cellulose and lignin content to the nutritive value of animal feeds. *J Nutr,* 15, 383-395, 1938.

13. Soest PJV, Robertson JB: Analysis of Forages and Fibrous Foods: A Laboratory Manual for Animal Science. Ithaca: Cornell University, 613, 202, 1985.

14. Goering H, Van Soest PJ: Forage fiber analyses (apparatus, reagents, procedures, and some applications), US Agricultural Research Service, Washington, USA, 1970.

15. Hara A, Radin NS: Lipid extraction of tissues with a low toxicity solvent. *Anal Biochem,* 90, 420-426, 1978. DOI: 10.1016/0003-2697(78)90046-5

16. Sherma J, Fried B: Thin layer chromatographic analysis of biological samples. A review. *J Liq Chrom Relat Tech,* 28, 2297-2314, 2005. DOI: 10.1080/10826070500187491

17. Sun Y, Oberley LW, Li Y: A simple method for clinical assay of superoxide dismutase. *Clin Chem,* 34, 497-500, 1988.

18. Aebi H: Catalase *in vitro.* **In,** Lester P (Ed): Methods in Enzymology. Academic Press, 121-126, 1984.

19. Sedlak J, Lindsay RH: Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem,* 25, 192-205, 1968. DOI: 10.1016/0003-2697(68)90092-4

20. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem,* 95, 351-358, 1979. DOI: 10.1016/0003-2697(79)90738-3

21. SPSS: Statistical Packages for the Social Sciences, 20th edn., IBM Inc., Chicago, USA, 2011.

22. Garcia V, Catala-Gregori R, Madrid J, Hernandez F, Megias MD, Andrade-Montemayor HM: Potential of carvacrol to modify *in vitro* rumen fermentation as compared with monensin. *Animal,* 1, 675-680, 2007. DOI: 10.1017/S1751731107730781

23. Canbolat O, Karaman S, Filya I: The Effect of different doses of oregano oil on corn silage digestive and rumen fermentation. *Kafkas Univ Vet Fak Derg,* 16, 933-939, 2010. DOI: 10.9775/kvfd.2010.1959

24. Simitzis PE, Deligeorgis SG, Bizelis JA, Dardamani A, Theodosiou I, Fegeros K: Effect of dietary oregano oil supplementation on lamb meat characteristics. *Meat Sci,* 79, 217-223, 2008. DOI: 10.1016/j.meatsci.2007.09.005

25. Unal A, Kocabagli N: Kekik uçucu yağının ruminant beslemede kullanımı. *İstanbul Univ Vet Fak Derg,* 40 (1): 121-130, 2014.

26. Vakili AR, Khorrami B, Danesh Mesgaran M, Parand E: The effects of thyme and cinnamon essential oil on performance, rumen fermentation and blood metabolites in Holstein calves consuming high concentrate diet. *Asian Australas J Anim Sci,* 26, 935-944, 2013. DOI: 10.5713/ajas.2012.12636

27. Yang WZ, Ametaj BN, He ML, Benchaar C, Beauchemin KA: Cinnamaldehyde in feedlot cattle diets: Intake, growth performance, carcass characteristics, and blood metabolites. *J Anim Sci,* 88, 1082-1092, 2010. DOI: 10.2527/jas.2008-1608

28. Halici M, Imik H, Koc M, Gumus R: Effects of α-lipoic acid, vitamins E and C upon the heat stress in Japanese quails. *J Anim Physiol Anim Nutr,* 96, 408-415, 2012. DOI: 10.1111/j.1439-0396.2011.01156.x

29. Mastromatteo M, Danza A, Conte A, Muratore G, Del Nobile MA: Shelf life of ready to use peeled shrimps as affected by thymol essential oil and modified atmosphere packaging. *Int J Food Microbiol,* 144, 250-256, 2010. DOI: 10.1016/j.ijfoodmicro.2010.10.002

30. Karabagias I, Badeka A, Kontominas MG: Shelf life extension of lamb meat using thyme or oregano essential oils and modified atmosphere packaging. *Meat Sci,* 88, 109-116, 2011. DOI: 10.1016/j.meatsci.2010.12.010

31. Nieto G, Bañón S, Garrido MD: Effect of supplementing ewes' diet with thyme *(Thymus zygis* ssp. *gracilis)* leaves on the lipid oxidation of cooked lamb meat. *Food Chem,* 125, 1147-1152, 2011. DOI: 10.1016/j.foodchem.2010.09.090

32. Giannenas IA, Florou-Paneri P, Botsoglou NA, Christaki E, Spais AB: Effect of supplementing feed with oregano and/or a-tocopheryl acetate on growth of broiler chickens and oxidative stability of meat. *J Anim Feed Sci,* 14, 521-535, 2005.

33. Mohamed AA, El-Emary GA, Ali HF: Influence of some citrus essential oils on cell viability, glutathione-S-transferase and lipid peroxidation in Ehrlich ascites carcinoma cells. *J Am Sci,* 6, 820-826, 2010. DOI: 10.7537/marsjas061010.96

34. Dincer Y, Telci A, Kayali R, Yilmaz IA, Cakatay U, Akcay T: Effect of α-lipoic acid on lipid peroxidation and anti-oxidant enzyme activities in diabetic rats. *Clin Exp Pharmacol Physiol,* 29, 281-284, 2002. DOI: 10.1046/j.1440-1681.2002.03642.x

35. Obrosova IG, Fathallah L, Greene DA: Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: Effect of dl-α-lipoic acid. *Eur J Pharmacol,* 398, 139-146, 2000. DOI: 10.1016/S0014-2999(00)00286-7

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Research Article

An Novel Strategy for Brucella Differential Vaccine Combined with Colloidal Gold Immunochromatographic Strips Based on Mutants of *Brucella melitensis* M5-90

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Abstract

There is a requirement to develop an novel vaccine to distinguish between vaccinated and infected animals after vaccination. Two mutants of *Brucella melitensis* M5-90Δ*bp*261 and M5-90Δ*bp*262, knockout of the fragments amino acids 55-152 and 22-185 respectively, were primarily generated. Then the two mutants were inoculated 7 weeks old BALB/c mice with 3.0×10^6 CFU/0.2 mL parent or mutant strains, Serum samples were evaluated by Rose Bengal plate tests, serum agglutination tests, colloidal gold immunochromatographic strips (ICS) coated with VirB5 or BP26 and indirect enzyme-linked immunosorbent assays. Spleen tissue samples were used to determine *B. melitensis* abundance at 10, 20, 30, and 40 days post-immunization (dpi). The serological results showed that the parent and mutant strains elicited a weak immunological reaction at 10–30 dpi. Fewer colony forming units (CFUs) were recovered from spleens in mutant strains group than the parent strain group during 20-40 post-immunization (dpi). The ICS were able to distinguish between the sera from mice immunized with either a parent or mutant strain of *B. melitensis*. These results indicate that the two mutants strain of *B. melitensis* have potentially perspectives in distinguishing infected animals from vaccination combined with ICS in this study.

Keywords: *Brucella melitensis, Distinguish, Vaccine, Colony forming units, ICS*

Brucella melitensis M5-90 Mutantları Temelli Kolloidal Altın İmmunokromatografik Şerit İle Kombine Brucella Ayırıcı Aşı İçin Yeni Bir Strateji

Özet

Aşı sonrası aşılı ve enfekte hayvanları ayırt edebilecek yeni bir aşının geliştirilmesine ihtiyaç vardır. Öncelikli olarak *Brucella melitensis*'in iki mutantı, sırasıyla 55 ile 152 arası ve 22 ile 185 arası amino asitleri çıkarılmış olan M5-90Δbp261 ve M5-90Δbp262, üretildi. Sonrasında, her iki mutant da 7 haftalık BALB/c farelere 3.0×10^6 CFU/0.2 mL miktarında inokule edildi. Serum örnekleri Rose Bengal lam testi, serum aglutünasyon testi, VirB5 veya BP26 ile kaplı kolloidal altın immunokromatografik şerit (ICS) ve indirek enzim bağlı immunsorbent assay ile değerlendirildi. Dalak doku örnekleri immunizasyon sonrası 10, 20, 30 ve 40. günlerde *B. melitensis* varlığını belirlemek amacıyla kullanıldı. Serolojik sonuçlar doğal ve mutant suşların inokulasyon sonrası 10-30. günler arasında zayıf immünolojik reaksiyon oluşturduğunu gösterdi. İnokulasyon sonrası 20-40 günler arasında dalaktan mutant suşların gruplarından doğal suş grubuna oranla daha az koloni oluşturan birim (CFU) belirlendi. ICS *B. melitensis*'in doğal ve mutant suçlar ile immunize edilmiş farelerin serumlarını ayırt edebildi. Elde edilen sonuçlar, her iki *B. melitensis* mutant suşun enfekte hayvanlar ile aşılanmış hayvanların ayırt edilmesinde ICS ile kullanıldığında başarılı olabileceğini göstermiştir.

Anahtar sözcükler: *Brucella melitensis, Ayırt etme, Aşı, Koloni oluşturan birim, ICS*

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INTRODUCTION

Brucellosis is a major bacterial zoonosis caused by members of the *Brucella* genus [1]. This infectious disease occurs worldwide, has resulted in significant economic losses, and also is a public health concern to humans, particularly in developing countries [2]. In 2012, the Chinese Center for Disease Control and Prevention reported 39,515 new cases of human brucellosis, with this number increasing by 10% each year. Approximately 85% of cases were caused by *Brucella melitensis via* direct contact with infected sheep or goats, or their products [3]. Vaccination is considered the most effective strategy for preventing brucellosis in animals. In China, the *B. melitensis* M5-90 strain has been widely used as a live attenuated vaccine for sheep and goats.

Application of the *B. melitensis* M5-90 vaccine strain is considered to be an important factor that led to a rapid decline in the incidence of brucellosis in animals and humans in China from the 1970s to the 1990s [4]. The vaccine M5-90 strain has been shown to confer protective effects against brucellosis [5]. However, it is difficult to distinguish between vaccinated and infected animals.

Clinical tests have revealed that the whole *Brucella* antigen is associated with false-positive results, due to serological cross-reactivity with other Gram-negative bacteria [6]. Various *Brucella*-dominant antigens have been used to increase the specificity of brucellosis diagnoses. As an example, the BP26 protein of *Brucella* species is a periplasmic protein that is highly immunogenic [7]. Seco-Mediavilla and colleagues characterized the antigenicity of BP26 using a panel of 18 BP26-specific monoclonal antibodies and recombinant fragments of the protein itself [8]. They found that amino acids 55-152 and 1-191 of BP26 were the immunodominant epitopes of this protein.

Previously, we generated *B. melitensis* strain M5-90r*bp26*, in which BP26 was deleted. This *B. melitensis* mutant elicited a weak serological reaction in mice and sheep [9]. To develop better candidate vaccines, and to differentiate infected and vaccinated animals, here we generated mutants of *B. melitensis* M5-90 based on BP26 partial fragments, the amino acids 55-152 and 22-185 respectively, and evaluated the immune responses of the BP261 and BP262 mutants in mice. Additionally, we sought to develop immunochromatographic strips (ICS) to discriminate between vaccinated and infected animals.

MATERIAL and METHODS

Bacterial Strains and Culture Conditions

We used *B. melitensis* strain M5-90 (Xinjiang Tiankang Animal Biotechnology Co. Ltd., China) as the parental vaccine strain, while *Bacillus subtilis* strain BAA12545 was used for *sacB* engineering and to screen for mutant strains by counter-selection. Bacteria were grown on *Brucella* agar or *Brucella* broth (BD Co., Sparks, MD, USA) at 37°C. To generate the *B. melitensis* M5-90 mutants, the medium was supplemented with ampicillin (100 mg/mL). Sucrose medium was used for *sacB* counter-selection as previously described [10]. *Escherichia coli* was used in the generation of M5-90Δ*bp261* and M5-90Δ*bp262* mutants, with cultures grown on Luria-Bertani (Difco/Becton Dickinson) plates or broth overnight at 37°C, with or without ampicillin (100 mg/L). *Brucella* cultures were harvested from plates using phosphate-buffered saline (PBS; pH 7.2) after 3 days. Based on optical density (OD) readings from a Klett meter and a standard curve, bacteria were centrifuged (8000 rpm) and resuspended to a final concentration (OD_{260nm}=0.18). The viability of bacteria was confirmed by serial dilution, plating, and enumeration.

Generation of BP26 Truncation Mutants

Genomic DNA from *B. melitensis* M5-90 was used as a template for the polymerase chain reaction (PCR) amplification of *bp26* fragments. The specific oligonucleotide primers which we used are summarized in *Table 1*. Flanking sequences were amplified separately, and overlap extension PCR [11] was used to combine sequences and generate fragments *bp261* (2445 bp including the DNA fragment encoding amino acids 55-152 of BP26) and *bp262* (2250 bp including the DNA fragment encoding amino acids 22-185 of BP26). Each fragment was cloned into the pMD18-T vector (TIAGEN, Beijing, China). Recombinant plasmids were digested with *Sph*I and *Sac*I, and the resulting fragments were introduced into pGEM-7zf (+) (Promega, Madison, USA). The *sacB* gene of *B. subtilis* was amplified by PCR and then inserted into pGEM-7zf (+) containing either fragment to yield pGB261 and pGB262.

The pGB261 and pGB262 vectors were introduced separately into *B. melitensis* M5-90 by electroporation as previously described, with ampicillin-resistant and sucrose-sensitive integrants selected for [12]. Two identified mutants were designated M5-90Δ*bp261* and M5-90Δ*bp262*, subcultured for 15 generations, and then subjected to western blotting analysis and various microbiological tests [12].

Bacteriological and Typing Methods

Conventional bacteriological and typing methods were carried out as described previously by Alton et al.[13]. Briefly these includes lysis by Tb, Wb, Iz, and R/C phages; urease test; CO_2 requirement; H_2S production; growth on dyes (thionin, basic fuchsin, safranin O) and oxidative metabolism tests. Then the characteristics of the two mutants strain were assessed by above methods.

Expression and Purification of BP261 and BP262

The nucleotide sequences encoding the BP261 and BP262 proteins were cloned into the pET-32a expression vector to generate pET32a-BP261 and pET32a-BP262,

Table 1. *PCR primers used for the construction and identification of the M5-90Δbp261 and M5-90Δbp262 mutants*

Primer	Primer Sequences (5'-3')
bp261-left flanking-forward	*GCATGC*TTTCTAAGCGCAGACCTTCGGG (*Sph* I)
bp261-left flanking-reverse	GTTCAAATCACCGCCCTGATTCATATCGGGCGAGGCCGTCAT
bp261-right flanking- forward	AATCAGGGCGGTGATTTGAACCT
bp261-left flanking-reverse	*GAGCTC*TACTGGTGGCATCCCCTTGTTTC (*Sac* I)
bp262-left flanking-forward	*GCATGC*TTTCTAAGCGCAGACCTTCGGG (*Sph* I)
bp262-left flanking-reverse	AGCGTCGGCAAGCGTCTTAGCGCCGACGAGCATGATT
bp262- left flanking-forward	AAGACGCTTGCCGACGCT
bp262-left flanking-reverse	*GAGCTC*TACTGGTGGCATCCCCTTGTTTC (*Sac* I)
sacB-forward	*GAGCTC*GGGCTGGAAGAAGCAGACCGCTA (*Sac* I)
sacB-reverse	*GAGCTC*GCTTATTGTTAACTGTTAATTGTCC (*Sac* I)
detecting rbp261/rbp262 -forward	TCCACAATCATGCTCGTCG
detecting rbp261/rbp262 -reverse	GCGTTTTGTATCAGGTGGC
expressing BP261-forword	*GAATTC*GCCATTCTCAATCTCTCGGTGC (*EcoR* I)
expressing BP261-reverse	*GTCGAC*AACACCGAGCGTGACGGATTC (*Sal* I)
expressing BP262-forword	*GAATTC*TTCAGCCTGCCCGCTTTCG (*EcoR* I)
expressing BP262-reverse	*GTCGAC*CGCTTGGCAATGGCATTG (*Sal* I)

respectively. Plasmids were introduced into *E. coli* strain BL21, and expression induced with isopropyl β-D-1-thiogalactopyranoside. BP261 and BP262 contained a 6×His tag, therefore Ni-NTA column chromatography was used to purify these proteins from their lysates.

Vaccination of Mice

Female BALB/c mice (7 weeks old) were obtained from the Xinjiang Center for Disease Prevention and Control and allowed to acclimate for 3 days prior to vaccination. Three groups of mice (n = 20 per group) were inoculated intraperitoneally with 3.0×10^6 CFU/0.2 mL of either *B. melitensis* M5-90, M5-90Δ*bp261*, or M5-90Δ*bp262* in 200 µL of PBS. The fourth group was injected with 200 µL of PBS as negative control.

Detection of Immunoglobin G (IgG)

Peripheral blood samples were collected by tail incision from five mice which selected randomly in each group at 0, 10, 20, 30, and 40 days post-inoculation (dpi). Serum samples were tested for *Brucella* spp. antibodies by Rose Bengal plate test (RBPT; Qingdao Yebio Bioengineering Co., Ltd, China) as described previously [14], and using a serum agglutination test, which has also been described previously [15]. Levels of serum IgG titers with specificity to *Brucella melitensis* M5-90, M5-90Δ*bp261* and M5-90Δ*bp262* were determined by indirect enzyme-linked immunosorbent assay (iELISA) according to manufacturer's instructions (RD, Minneapolis, Minnesota, USA). Subsequently the absorbance (450 nm) was recorded using a microplate reader (Molecular devices, Sunnyvale,CA). Then the measurement value was obtained through standard curve which is used to determine the amount in an unknown sample and the standard curve is generated by plotting the average OD (450 nm) obtained for each of the 6 standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis. The actual value of the sample is 5-fold to measurement value. The sensitivity by this assay is 1.0 µg/mL. The cutoff value for the assay was calculated as the mean optical density at a 1:5 dilution. All of these data was analyzed using SPSS version 17.0 software and the concentration of IgG titers in serum samples were determined. Meanwhile the serum samples were tested in duplicates.

Assessment of Brucella Abundance

Five mice from each group in randomly were euthanized using diethyl ether at 10, 20, 30, and 40 dpi. Spleens were removed aseptically. Then suspended in 1 mL of sterile PBS and were homogenized using tissue grinder [16]. Ten-fold serial dilutions of the tissue homogenates were prepared in saline, and 100 µL of each dilution was plated on *Brucella* agar medium. After 3-5 days of incubation at 37°C, the *Brucella* CFUs were counted and the bacterial burden per spleen was calculated.

Preparation and Evaluation of Colloidal Gold ICS

Colloidal gold ICS were coated with either VirB5 or BP26, as outlined in our patent [No: 201410405061.6]. For each test, the ICS was immersed in diluted serum samples obtained from *B. melitensis*-infected sheep, and from mice that immunized with *B. melitensis* M5-90, M5-90Δ*bp261*, or M5-90Δ*bp262* respectively. In addition, all serum samples were evaluated by RBPT and serum agglutination tests.

Ethics

All animals used in our experiment were treated humanely and in accordance with institutional animal care guidelines. Our study was approved by the Animal Care and Use Committee of Shihezi University.

RESULTS

Generation of B. melitensis M5-90Δbp261 and M5-90Δbp262

The *B. melitensis* mutants which we generated *(Table 1)* were identified using PCR and the relevant inserted nucleotide sequences were confirmed by sequencing analysis (data not shown). The serum samples collected from mice which immunized with *B. melitensis* M5-90 were analyzed by Western blotting. We found that they reacted strongly with BP261 and BP262, while serum from mice immunized with either M5-90Δ*bp261* or M5-90Δ*bp262* failed to react with BP261 or BP262 *(Fig.1)*.

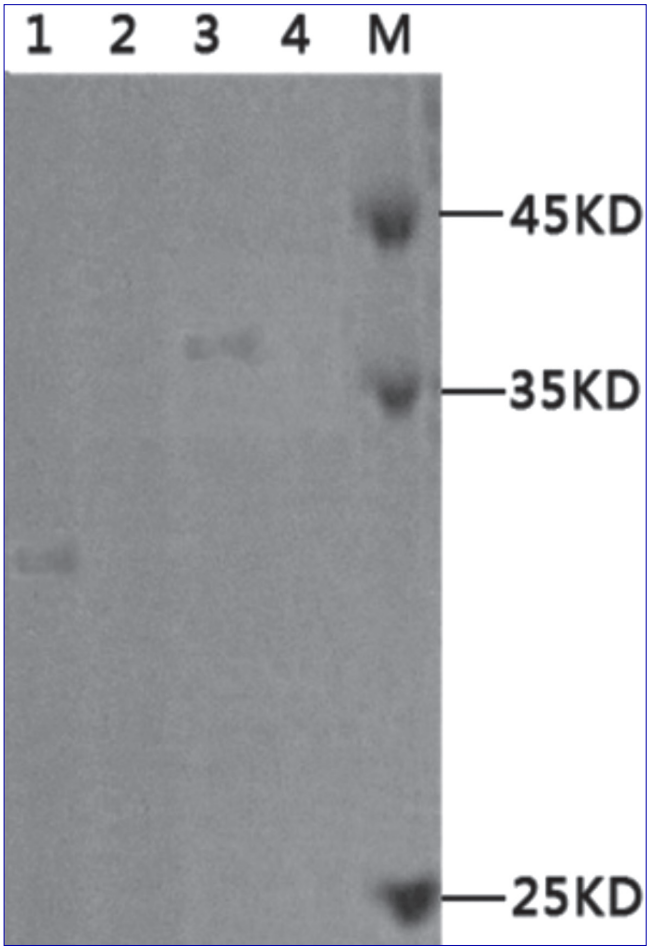

Fig 1. The result of Western blot of sera from animals which were infected with the parent or mutant strains that reacted with BP261 and BP262 (Lane 1 and Lane 3: The results from sera induced by the *M5-90* vaccine strain that reacted with BP261 and BP262 proteins; Lane 2 and Lane 4: The results from sera induced by the *Brucella* M5-90Δ*bp261* and M5-90Δ*bp262* mutants that reacted with BP261 and BP262 proteins. Lane M: Protein Molecular Weight Marker)

Phenotypic Characterization of Mutants

Microbiological testing indicated that mutants M5-90Δ*bp261* and M5-90Δ*bp262* were *B. melitensis* biotype 1. Growth rates and staining profiles for the mutants were similar to those of the parental strain.

Serum IgG Profiles

According to our iELISA results, the level of serum specific IgG titers in immunized mice ranged from 11.49 to 27.32 μg/mL. However, mice immunized with M5-90Δ*bp261* or M5-90Δ*bp262*, IgG titers were not significantly lower than those in mice immunized with M5-90 during 10 - 40 dpi [17] *(Fig. 2)*.

Residual Virulence of M5-90Δbp261 and M5-90Δbp262 Mutants

We compared the levels of viable bacteria recovered from the spleens of mice immunized with the parent or mutant strains of *B. melitensis*. Levels of bacteria in spleen tissues were slightly lower in mice immunized with the mutant strains of *B. melitensis* (M5-90Δ*bp261* and M5-90Δ*bp262*) than mice immunized with the parent strain (M5-90) *(Fig. 3)*. Bacteria recovered from spleen tissues proliferated at low levels at 0-10 dpi, peaking at 10-20 dpi for all three strains we investigated. Levels of bacteria decreased rapidly between 20 and 40 dpi, approaching 3.93-5.66 log units *(Fig. 3)*.

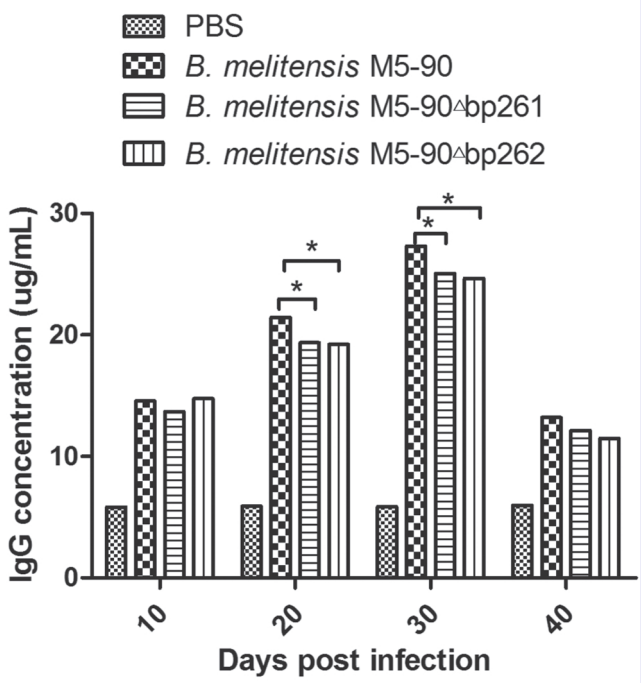

Fig 2. IgG antibody serum profiles at different time spots after immunization of BALB/c mice with *B. melitensis* M5-90 mutants

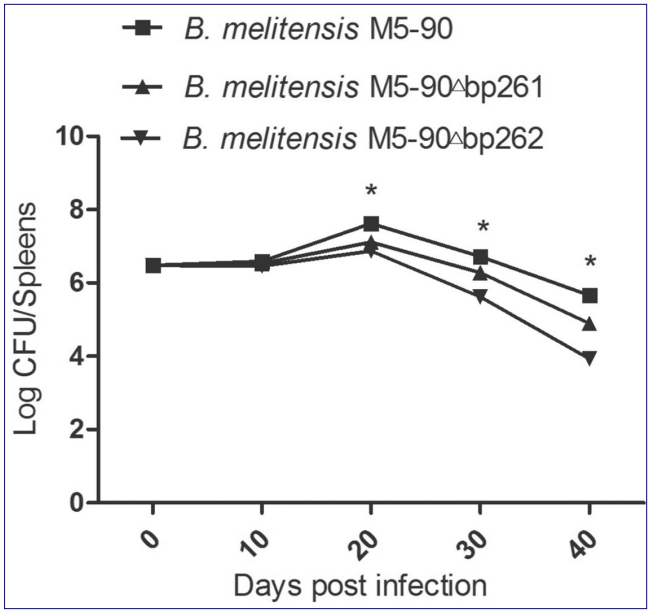

Fig 3. Replication profiles of *B. melitensis* M5-90 mutants in mice spleens

Screening Serum Samples with ICS

The colloidal gold ICS coated with VirB5 reacted with the sera from mice immunized with *B. melitensis* M5-90, M5-90Δ*bp261*, and M5-90Δ*bp262* at 10 and 30 dpi. However, ICS coated with BP26 failed to react with the sera from mice immunized with either the parent or mutant strains *(Fig. 4)*.

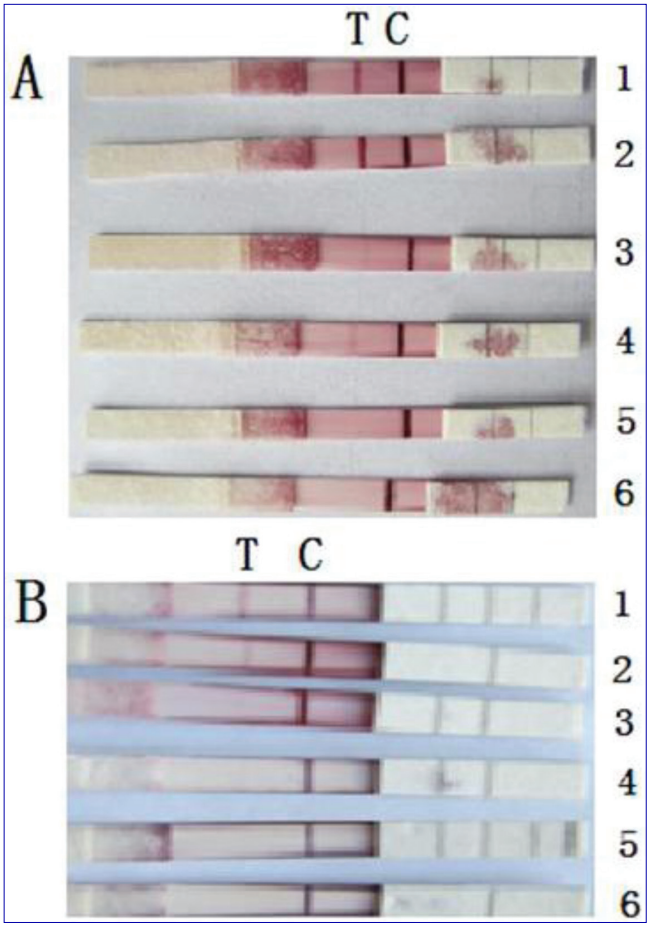

Fig 4. The result of using immunochromatographic test strips coated with VirB5 (A) or BP26 (B) protein. A. Lane 1: positive control (serum from sheep infected with *B. melitensis*). Lane 2,3,4: positive results (serum from mice immunized with *B. melitensis* M5-90, *B. melitensis* M5-90Δ*bp261* and *B. melitensis* M5-90Δ*bp262* respectively pi. 30d). Lane 5,6: negative results (serum from mice injected with PBS and brucellosis-free sheep). B. Lane 1: positive control (serum from sheep infected with *B. melitensis*). Lane 2: positive result (serum from mice immunized with *B. melitensis* M5-90 pi. 30d). Lane 3,4,5,6: negative results (serum from mice immunized with *B. melitensis* M5-90Δ*bp261*, *B. melitensis* M5-90Δ*bp262*, PBS and brucellosis-free sheep respectively pi. 30d). T = test zone, C = control zone

DISCUSSION

In previous studies, researchers have used attenuation or differential diagnostic markers to interrupt or delete genes associated with virulence or antigenicity to investigate novel vaccine strains [18,19]. The *B. abortus* vaccine strain S19, where *bp26* and *bmp18* are deleted, is attenuated and it is possible to differentiate between vaccinated and naturally infected animals [12]. In a separate study, *bp26* and/or *omp31* were found to be deleted in *B. melitensis* vaccine strain *Rev.1*. These genes encode the BP26 and OMP31 antigens and allow for serological differentiation between infected and vaccinated animals [20]. However, due to a lack of appropriate differential diagnostic methods and intellectual property issues, a candidate vaccine for brucellosis utilizing a mutant strain has not been commercially produced and marketed.

The M5-90 vaccine strain of *B. melitensis* was derived by a process that was significantly different from that used to generate the *S19* or *Rev.1* vaccine strains. The M5-90 strain was attenuated through the use of acriflavine and by consecutive subculturing in chickens and chicken embryo fibroblasts. *B. melitensis* M5-90 has been commonly used to vaccinate sheep and goats in China for at least two decades. Previously, a series of *B. melitensis* M5-90 mutants were developed where *virB2*, *WboA*, and/or *pgm* were deleted [21, 22]. To identify suitable methods for differential diagnosis, VirB2, VirB5, BP26, and OMP31 proteins were expressed, purified and coated onto ICS. Clinical tests revealed that ICS coated with VirB5, BP26, or OMP31 were specific and sensitive [23]. Evaluation of the humoral immune response in sheep and mice infected with *B. melitensis* M5-90 mutants where entire genes had been deleted revealed that only weak *Brucella*-specific antibodies were identified. In the current study, we generated truncated forms of BP26, with two fragments corresponding to epitopes omitted from the expressed proteins. However, similar results identified with the parent M5-90 strain, a weak humoral immune response was elicited in BALB/c mice immunized with M5-90Δ*bp261* or M5-90Δ*bp262*.

In a previous study, VirB5 was reported to be a

conserved protein and a serological marker suitable for brucellosis diagnosis [24]. The use of colloidal gold ICS to diagnose brucellosis is rapid and convenient [25]. The convective mass transfer of the immunoreactant to the binding partner allows the assay to be performed with no reagent handling [26]. An ICS coated with VirB5 and/or BP26 can used to test serum samples, and allowed us to differentiate between naturally infected mice and those immunized with M5-90Δ*bp261* and M5-90Δ*bp262*. We found that the sensitivity of ICS was around two-to-four fold higher than that for RBPT. Our developed methodology requires further confirmatory studies in humans and other animals, but could potentially be used in resource-poor rural communities.

Acknowledgments

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REFERENCES

1. Bercovich Z: The use of skin delayed-type hypersensitivity as an adjunct test to diagnose brucellosis in cattle: A review. *Vet Quart*, 22, 123-130, 2000. DOI: 10.1080/01652176.2000.9695040

2. Corbel MJ: Brucellosis: An overview. *Emerg Infect Dis*, 1997, 3, 213-221, 1997. DOI: 10.3201/eid0302.970219

3. Hai J, Fan M, Chen J: MLVA genotyping of Chinese human *Brucella melitensis* biovar 1, 2 and 3 isolates. *BMC Microbiol*, 2011, 11, 17043-17048, 2011. DOI: 10.1186/1471-2180-11-256

4. Shang D, Xiao D, Yin J: Epidemiology and control of brucellosis in China. *Vet Microbiol*, 2002, 90, 165-182, 2002. DOI: 10.1016/S0378-1135(02)00252-3

5. Research Group: Study on the *Brucella melitensis* strain M5-90 vaccine. *Chin J Cont End Dis*, 1991.

6. Schoerner C, Wartenberg K, Röllinghoff M: Differentiation of serological responses to *Yersinia enterocolitica* serotype O9 and *Brucella* species by immunoblot or enzyme-linked immunosorbent assay using whole bacteria and *Yersinia* outer membrane proteins. *J Clin Microbiol*, 28 (7): 1570-1574, 1990.

7. Debbarh SA, Zygmunt MS, Dubray G: Competitive enzyme-linked immunosorbent assay using monoclonal antibodies to the *Brucella melitensis,* BP26 protein to evaluate antibody responses in infected and *B. melitensis,* Rev.1 vaccinated sheep. *Vet Microbiol*, 53, 325-337, 1997. DOI: 10.1016/S0378-1135(96)01265-5

8. Secomediavilla P, Verger JM, Grayon M: Epitope mapping of the *Brucella melitensis* BP26 immunogenic protein: Usefulness for diagnosis of sheep brucellosis. *Clin Diagn Lab Immunol*, 10, 647-651, 2003. DOI: 10.1128/CDLI.10.4.647-651.2003

9. Cheng TT, Ren XL, Chen CF: Construction and studies on the effect upon liver of Brucella epitope-deleted strain M5-90 bp26. *Biotechnol*, 23 (6): 59-63, 2013.

10. Pelicic V, Reyrat JM, Gicquel B: Generation of unmarked directed mutations in mycobacteria, using sucrose counter-selectable suicide vectors. *Mol Microbiol*, 20, 919-925, 1996. DOI: 10.1111/j.1365-2958.1996.tb02533.x

11. Higuchi R, Krummel B, Saiki R: A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res*, 16, 7351-7367, 1988. DOI: 10.1093/nar/16.15.7351

12. Campos E, Cravero SL, Delgui L: *Brucella abortus* INTA2, a novel strain 19 *Δbp26::lucΔbmp18* double mutant lacking drug resistance markers. *Vet Microbiol*, 87, 1-13, 2002. DOI: 10.1016/S0378-1135(02)00016-0

13. Alton GG, Jones LM, Angus RD: Techniques for the brucellosis laboratory. *Vet Res Commun,* 13, 420, 1988. DOI: 10.1007/BF00402562

14. Morgan WJ: The serological diagnosis of bovine brucellosis. *Vet Rec*, 80, 612-620, 1967. DOI: 10.1136/vr.80.21.612

15. Ariza J, Pellicer T, Pallarés R: Specific antibody profile in human brucellosis. *Clin Infect Dis*, 14, 131-140, 1992. DOI: 10.1093/clinids/14.1.131

16. Dabral N, Martha-Moreno-Lafont, Sriranganathan N: Oral immunization of mice with gamma-irradiated *Brucella neotomae* induces protection against intraperitoneal and intranasal challenge with virulent *B. abortus* 2308. *Plos One*, 9 (9): e107180-e107180, 2014. DOI: 10.1371/journal.pone.0107180

17. Pakzad I, Rezaee A, Rasaee MJ: Immunogencity of HSA-L7/L12 *(Brucella abortus* ribosomal protein) in an animal model. *Iran J Immunol*, 6, 12-21, 2009.

18. Cloeckaert A, Jacques I, Grilló MJ: Development and evaluation as vaccines in mice of *Brucella melitensis* Rev.1 single and double deletion mutants of the *bp26* and *omp31* genes coding for antigens of diagnostic significance in ovine brucellosis. *Vaccine*, 22, 2827-2835, 2004. DOI: 10.1016/j.vaccine.2004.01.001

19. Grilló MJ, Blasco JM, Gorvel JP: What have we learned from brucellosis in the mouse model? *Vet Res*, 43, 1487-1494, 2012. DOI: 10.1186/1297-9716-43-29

20. Grilló MJ, Marín CM, Barberán M: Efficacy of *bp26* and *bp26/omp31 B. melitensis*, Rev.1 deletion mutants against *Brucella ovis* in rams. *Vaccine*, 27, 187-191, 2008. DOI: 10.1016/j.vaccine.2008.10.065

21. Li TS, Zhang H, Zhang Y: Construction and immune evaluation on mutation of phosphoglucomutase gene *(pgm)* attenuates of *Brucella melitensis* vaccine M5-90. *J Agric Biotechnol*, 21, 448-455, 2013.

22. Zhen LI, Chen C, Wang Y: Change of antibody and effect of related cytokines for mice vaccined by the Brucella *M5-90ΔvirB2* vaccine strain. *J Shihezi Univ*, 2011.

23. Cheng TT, Shi F, Chen CF: Establishment of a gold immunochromatographic assay for rapid detection of Brucella antibodies. *J Pathogen Biol*, 9 (2): 122-121, 2014.

24. Tan W, Wang XR, Nie Y: Recombinant VirB5 protein as a potential serological marker for the diagnosis of bovine brucellosis. *Mol Cell Probe*, 26, 127-131, 2012. DOI: 10.1016/j.mcp.2012.02.003

25. Paek SH, Lee SH, Cho JH: Development of Rapid one-step immunochromatographic assay. *Methods*, 22, 53-60, 2000. DOI: 10.1006/meth.2000.1036

26. Zhang GP, Guo JQ, Wang XN: Development and evaluation of an immunochromatographic strip for trichinellosis detection. *Vet Parasitol*, 137, 286-293, 2006. DOI: 10.1016/j.vetpar.2006.01.026

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Research Article

Effects of Thymoquinone Supplementation on Somatostatin Secretion in Pancreas Tissue of Rats [1] [2]

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Abstract

In this study, it was aimed to investigate the effects of thymoquinone (TQ), which is the bioactive phytochemical constituent of the seeds oil of *Nigella sativa*, on somatostatin secretion in the rat pancreatic tissue by immunohistochemical method. Animals (n=30) were divided into 3 groups as follows: control, sham and thymoquinone. While Thymoquinone group received 8 mg/kg of TQ which injected for 14 days, only isotonic saline was injected to the sham group for same time. The control group received nothing. Crossman's triple staining was applied to tissue sections to examine histology. Streptavidin-Biotin-Peroxidase Complex method was used to investigate somatostatin immunoreactivity in the pancreatic tissue. Specific somatostatin immunoreactivities were observed in endocrine cells (islets of Langerhans) in all groups. It was determined that somatostatin secretion increased in the thymoquinone group compared to control and sham. In conclusion, thymoquinone administration was concluded to increase the secretion of somatostatin that is known to regulate certain hormones such as growth hormone, insulin, glucagon, secretin and gastrin.

Keywords: *Imunohistochemistry, Nigella sativa, Pancreas, Thymoquinone*

Timokinon Uygulamasının Ratların Pankreas Dokusunda Somatostatin Salgısı Üzerine Etkileri

Özet

Bu çalışmada, *Nigella sativa* (Çörek otu) çekirdeğinin biyoaktif fitokimyasal bileşeni olan timokinonun (TQ), rat pankreas dokusundaki somatostatin salgısı üzerine etkisinin, immünohistokimyasal yöntem kullanarak incelenmesi amaçlandı. Hayvanlar (n=30) kontrol, sham ve timokinon olmak üzere üç gruba ayrıldı. TQ grubuna, 8 mg/kg TQ 14 gün boyunca enjekte edilirken sham grubuna aynı süre sadece serum fizyolojik enjekte edildi. Kontrol grubuna ise herhangi bir uygulama yapılmadı. Histolojik incelemeler için doku kesitlerine Crosman'ın üçlü boyama yöntemi uygulandı. Pankreas dokusunda somatostatin immunoreaktivitesini incelemek için Streptavidin-Biotin-Peroxidase Complex metodu kullanıldı. Spesifik somatostatin immünoreaktivitesi tüm grupların endokrin hücrelerinde (langerhans adacıkları) tespit edildi. Somatostatin sekresyonunun kontrol ve sham grupları ile karşılaştırıldığında thymoquinone grubunda arttığı tespit edildi. Sonuç olarak, TQ uygulamasının, büyüme hormonu, insulin, glukagon, sekretin, ve gastrin gibi hormonların salınımını düzenlediği bilinen somatostatinin salgısını arttırdığı sonucuna varıldı.

Anahtar sözcükler: *İmmünohistokimya, Nigella sativa, Pankreas, Timokinon*

INTRODUCTION

The use of medicinal plants as therapeutics drugs is as old as mankind itself [1]. *Nigella sativa* (black seed) is an annual flowering plant in the family Ranunculaceae and is among the most promising medicinal plants. It is used as a natural remedy in the Southern Europe, Northern Africa, Middle East, Saudi Arabia, and Southern and Southwestern

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Asia [2]. Black seeds contain carbohydrates, fixed oils, vitamins, minerals, proteins [3], calcium, iron and potassium [4]. The bioactive constituents of the volatile oil of black seeds are thymoquinone (TQ), dithymquinone (DTQ), thymohydroquinone (THQ) and thymol (THY) [3].

TQ has the most important bioactive components and antioxidant [5], antihistaminic [6], antiinflammatory [7], immunomodulatory [8], anti-microbial [9], anti viral [10], antihelminthic [11], anti-bacterial [12], hepatoprotective [13] and antitumoral [2] effects. Furthermore, several studies have examined the effects of TQ in diabetes, and found that it decreased levels of insulin and restored glucose homeostasis [14,15].

Somatostatin has two biologically active forms that are referred to as somatostatin-14 and somatostatin-28, and it was first isolated from ovine hypothalamus [16]. It can act as a neurotransmitter [17]. It has suppressive effects on growth hormone in the pituitary [18], glucagon, insulin and pancreatic polypeptide in the pancreas [15,19], and secretin and gastrin in the gastrointestinal tract [20].

In this study, it was aimed to examine the effect of TQ on the somatostatin positive cells in the pancreatic tissue of thymoquinone-treated rats.

MATERIAL and METHODS

Animals and Care Condition

Ethics approval was obtained from Kafkas University Local Ethics Committee for Animal Experiments (KAU-HADYEK/2015-38).

A total of 30 rats of the same species *(Sprague Dawley)*, which were 40 days old, weighted approximately 250-300 g. The animals were not used in any previous studies and did not mate before. The rats were housed in standard cages at ambient temperature of 22±2°C and were maintained on a 12-h light/dark cycle with free access to water and pellet food. The amount of TQ used in our study was based on the study conducted by Hawsawi et al.[21].

Experimental Design

The rats in the control group (n=10) were fed on standard *ad labitum* and normal drinking water. One mL of isotonic saline was administered intraperitoneally to sham group (n=10). Eight mg/kg of TQ (274666, ALDRICH), which was dissolved in 1 mL of isotonic saline, was intraperitoneally injected daily for 14 days to thymoquinone group (n=10). The control group received nothing.

End of the experiment, pancreatic tissue samples were collected under euthanasia after deep ether anesthesia.

Histological Procedures

Pancreatic tissue samples were fixed in 10% formalin solution for 48 h, dehydrated through graded alcohols and cleared in xylene. Tissues were infiltrated and embedded in paraffin. Sections (5 µm) from the paraffin blocks were stained with Crossman's triple staining.

Immunohistochemical Procedures and Statistical Analysis

Streptavidin-Biotin-Peroxidase Complex method was used to investigate somatostatin immunoreactivity in the pancreatic tissue. Following deparaffinization and re-hydration, sections were rinsed with Phosphate Buffer Solution (PBS) and incubated in 3% H_20_2 (prepared in 0.1 M PBS) for 15 min. After rinsing with PBS, sections were processed in citrate buffer solution in microwave oven (800 watt) for 10 min in order to expose the antigenic sites. After rinsing with PBS again, sections were incubated in primary somatostatin antibodies (ab183855) (1:1000 dilution ratio) in humid environment at room temperature for 1 h. After rinsing with PBS, sections were kept at room temperature for 15 min with added streptavidin-horse radish peroxidase (HRP) (Invitrogen Histostain plus Broad Spectrum Ref. 85.9943). Sections were rinsed again with PBS and 3,3'-Diaminobenzidine tetrahydrochloride (Dako Corp.) was used for chromogen application. Finally, the sections were counterstained with Mayer's hematoxylin. Negative control sections were incubated only in PBS.

Somatostatin immunoreactivities in tissues were graded with 40X lens zoom from 0 to +3 (0: no reaction; 1: minimal reaction, 2: moderate reaction; 3: strong reaction). Cells were evaluated by two different observers. Intensity of staining of somatostatin positive cells were determined in six sections chosen randomly from six islets of Langerhans of each animals.

Somatostatin positive cells were counted by 100 square ocular micrometer (eye piece graticule) at 100X magnification under Olympus microscope (CX22-type I). All the obtained data was converted to number of somatostatin positive cells per 1 mm^2 unit area [22,23]. Numerical distribution of somatostatin positive cells were observed in six sections chosen from six unit area of Langerhans of each animals.

Statistical Package for the Social Sciences 16.0 (SPSS) software was used. Possible differences were determined by using One-Way ANOVA and Duncan's multiple range test and means were considered significantly different at $P<0.001$.

RESULTS

Histological Results

The histological examination of pancreatic tissues in all groups exhibited normal findings. The obtained pancreatic tissues contained endocrine (islets of Langerhans) and exocrine cells *(Fig. 1)*.

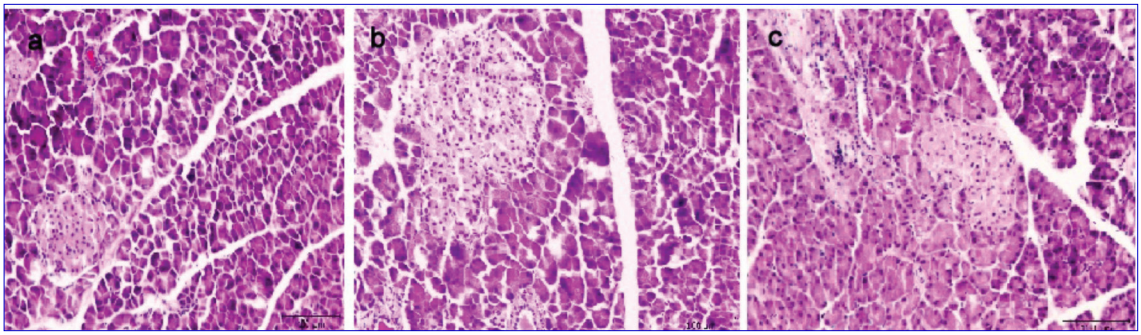

Fig 1. Rat pancreas. (a) Control group, (b) Sham group, (c) Thymoquinone group. Triple staining. Bar = 100 µm

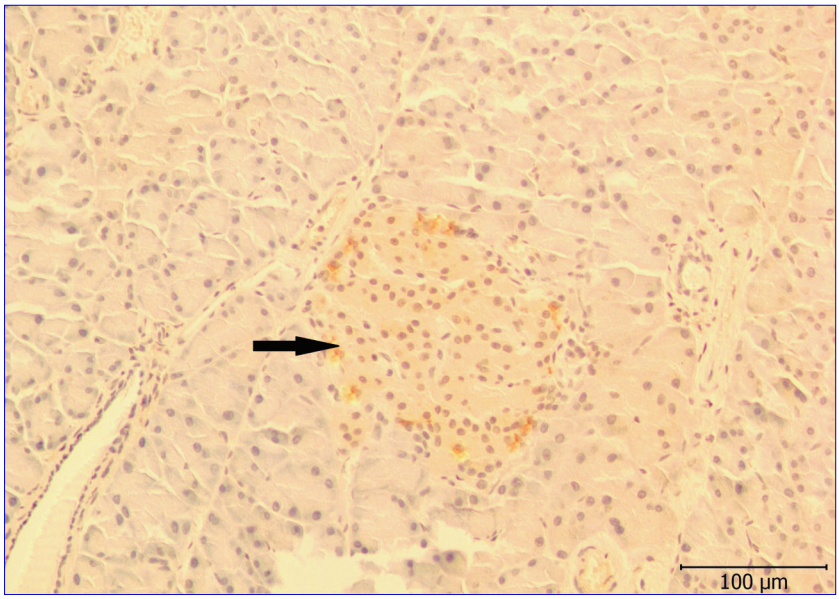

Fig 2. Somatostatin immunoreactivity in rat pancreas in control group. Somatostatin containing cell *(arrow)*. Streptavidin-Biotin-Peroxidase Complex method. Bar = 100 µm

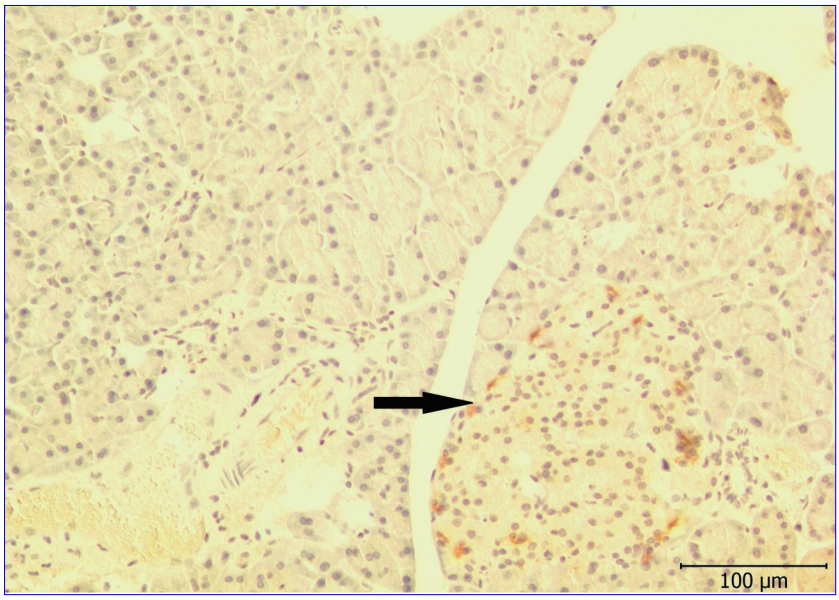

Fig 3. Somatostatin immunoreactivity in rat pancreas in sham group. Somatostatin containing cell *(arrow)*. Streptavidin-Biotin-Peroxidase Complex method. Bar = 100 µm

Immunohistochemical Results

Somatostatin expression was observed only in the endocrine cells (islets of Langerhans) of all groups. This cells was abundant especially in the peripheral areas of islets of Langerhans *(Fig. 2, 3, 4)*.

When all groups compared with each others regarding intensity of staining of somatostatin expression in islets of Langerhans, values of somatostatin expression in thymoquinone group was significantly higher ($P<0.001$) than control and sham groups. Intensity of immunohistochemical staining were summarized in *Table 1*.

When all the groups were compared with each others in terms of count of somatostatin positive cells. Thymoquinone group were observed more than both control and sham groups. And numerical distribution of this cells were determined statistically significant ($P<0.001$). Count of somatostatin positive cells in islets of Langerhans among groups were summarized in *Table 2*.

DISCUSSION

Pharmacological plants are used in the preparation of herbal medicines, which are known safer than modern medicines. *N. sativa,* which has been used for many diseases, is one of the famous pharmacological plants [24].

Somatostatin is known to regulate the endocrine system, cell proliferation and neurotransmission [17]. Somatostatin-immunoreactive cells among endocrine cells (islets of Langerhans) in the pancreas were determined by immunohistochemical

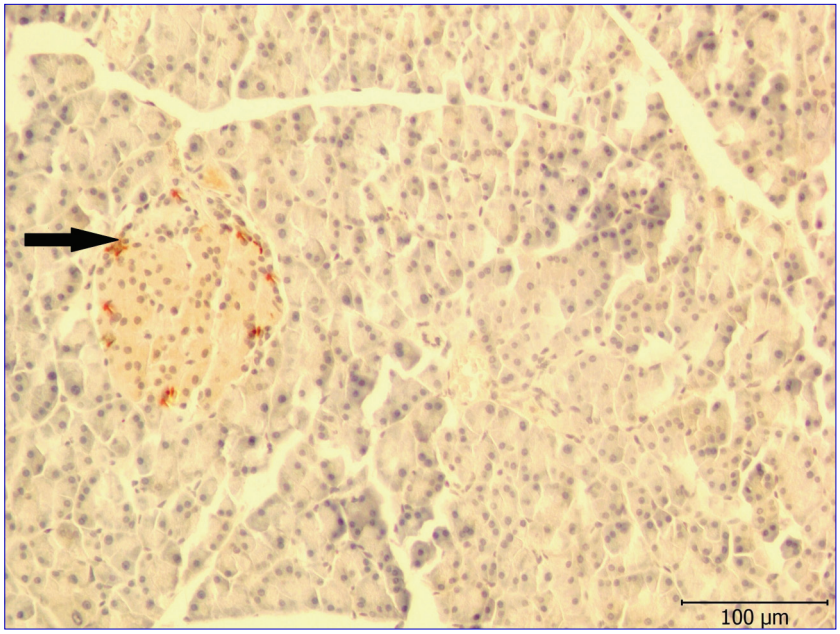

Fig 4. Somatostatin immunoreactivity in rat pancreas in thymoquinone group. Somatostatin containing cell *(arrow)*. Streptavidin-Biotin-Peroxidase Complex method. Bar = 100 µm

Table 1. *Comparison of intensity of immunohistochemical staining in islets of Langerhans among groups*

Groups	Number (islets of Langerhans)	M+SD	P Value
Control	360	1.62±0.48[a]	N.S
Sham	360	1.66±0.47[a]	N.S
Thymoquinone	360	2.79±0.40[b]	< 0.001

M: *mean;* ***SD:*** *standard deviation;* [a,b] *Different superscripts in the same column indicate significant differences between groups (P<0.001);* ***N.S:*** *Not significant*

Table 2. *Comparison of count of somatostatin positive cells in islets of Langerhans among groups*

Groups	Number (unit area)	M+SD	P Value
Control	360	11.13±3.2[a]	N.S
Sham	360	11.27±4.1[a]	N.S
Thymoquinone	360	12.24±3.6[b]	< 0.001

M: *mean;* ***SD:*** *standard deviation;* [a,b] *Different superscripts in the same column indicate significant differences between groups (P<0.001);* ***N.S:*** *Not significant*

and morphometric studies. It was claimed that somatostatin-immunoreactive cells were rare among endocrine cells (islets of Langerhans) in the pancreas and that these cells were localized especially in the peripheral regions of islets of Langerhans [25,26]. Our study showed that somatostatin-immunoreactive cells were localized in the peripheral region of the endocrine parts and TQ administration increased somatostatin release in the endocrine cells.

The black seed and TQ were determined to have anti-tumoral and anti-diabetic effects on the pancreatic tissue [2,14]. It was reported that TQ have cytotoxic effects on several tumoral cells, including pancreatic adenocarcinoma [27]. It was demonstrated that TQ triggers apoptosis by Bcl-2 protein [28].

Several studies have been focused on *N. sativa* and TQ administration in diabetes [2,14,29]. A study showed that TQ administration in diabetic rats led to significant decrease in blood glucose levels [29]. It was argued that TQ managed to reduce insulin resistance and increase β-cell function [14]. Due to its anti-tumoral and anti-diabetic effects, somatostatin acts as a regulator in some diseases. It also suppresses hormone secretion, growth and proliferation, and triggers apoptosis [30].

Somatostatin is a regulatory hormone for insulin and glucagon, and it inhibits insulin and glucagon release from pancreatic islets. An immunohistochemical study showed an increase in the number of somatostatin-secreting cells in patients with diabetes [31]. TQ administration also increased both intensity of staining and count of somatostatin positive cells in our study. Diabetes is known as a metabolic disorder and involves an imbalance between stimulatory (insulin) and inhibitory (glucagon, somatostatin) pancreatic islet hormones [32]. Based on our findings, it is possible to consider that TQ administration may be effective in the treatment of some conditions such as diabetes and tumors by increasing somatostatin expression in the pancreatic tissue.

In conclusion, somatostatin expression was present in the pancreatic islets in all groups, somatostatin expression was observed only in the peripheral region and it was determined TQ administration increased both intensity of immunohistochemical staining and numerical distribution of somatostatin positive cells in this study. Our findings may be useful for other studies on somatostatin .

REFERENCES

1. Rahmani AH, Aly SM: *Nigella Sativa* and its active constituents thymoquinone shows pivotal role in the diseases prevention and treatment. *Asian J Pharm Clin Res,* 8, 48-53, 2015.

2. Worthen DR, Ghosheh OA, Crooks PA: The *in vitro* anti-tumor activity of some crude and purified components of blackseed, *Nigella sativa* L. *Anticancer Res,* 18, 1527-1532, 1998.

3. Omar A, Ghosheh S, Abdulghani A, Houdi A, Crookscor PA: High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed *(Nigella sativa* L). *J Pharm Biomed Anal*, 19, 757-762, 1999. DOI: 10.1016/S0731-7085(98)00300-8

4. Al-Gaby AM: Amino acid composition and biological effects of supplementing broad bean and corn proteins with *Nigella sativa* (black cumin) cake protein. *Nahrung,* 42, 290-294, 1998. DOI: 10.1002/(SICI)1521-

3803(199810)42:05<290::AID-FOOD290>3.0.CO;2-Y

5. Erkan N, Ayranci G, Ayranci E: Antioxidant activities of rosemary *(Rosmarinus officinalis* L.) extract, black seed *(Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chem*, 110, 76 -82, 2008. DOI: 10.1016/j.foodchem.2008.01.058

6. Kalus U, Pruss A, Bystron J, Jurecka M, Smekalova A, Lichius JJ, Kiesewtter H: Effect of *Nigella sativa* (black seed) on subjective feeling in patients with allergic diseases. *Phytother Res,* 17, 1209 -1214, 2003. DOI: 10.1002/ptr.1356

7. Houghton PJ, Zarka R, de la Heras B, Hoult JR: Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. *Planta Med,* 61 (1): 33-36, 1995.

8. Swamy SM, Tan BK: Cytotoxic and immunopotentiating effects of ethanolic extract of *Nigella sativa* L. seeds. *J Ethnopharmacol*, 70, 1-7, 2000. DOI: 10.1016/S0378-8741(98)00241-4

9. Hanafy MS, Hatem ME: Studies on the antimicrobial activity of *Nigella sativa* seed (black cumin). *J Ethnopharmacol*, 34, 275-278, 1991. DOI: 10.1016/0378-8741(91)90047-H

10. Akhter MS, Riffat S: Field trail of *Saussurea lappa* roots againt nematodes and *Nigella sativa* seed againt cestodes in children. *J Pak Med Assoc*, 4, 185-187, 1991.

11. Khan MA, Ashfaq MK, Zuberi HS, Mahmood MS, Gilani AH: The *in vivo* antifungal activity of the aqueous extract from *Nigella sativa* seeds. *Phytother Res,* 17, 183-186, 2003. DOI: 10.1002/ptr.1146

12. El-Fatatry HM: Isolation and structure assignment of an antimicrobial principle from the volatile oil of *Nigella sativa* L. seeds. *Pharmazie,* 30, 109-111, 1975.

13. Gani MS, John SA: Evaluation of hepatoprotective effect of *Nigella Sativa* L. *Int J Pharm Pharm Sci*, 5, 428-430, 2013.

14. Kanter M: Protective effects of thymoquinone on β-cell damagein streptozotocin induced diabetic rats. *Tıp Araştırmaları Derg*, 7, 64-70, 2009.

15. Bamosa AO, Kaatabi H, Lebdaa FM, Elq AM, Al-Sultanb A: Effect of *Nigella sativa* seeds on the glycemic control of patients with type 2 diabetes mellitus. *Indian J Physiol Pharmacol*, 54, 344-354, 2010.

16. Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, Guillemin R: Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science,* 179, 77-79, 1973. DOI: 10.1126/science. 179.4068.77

17. Patel YC: Somatostatin and its receptor family. *Front Neuroendocrinol,* 20, 157-198, 1999. DOI: 10.1006/frne.1999.0183

18. Koerker D, Ruch W, Chideckel E, Palmer J, Goodner C, Ensinck J, Gale C: Somatostatin: Hypothalamic inhibitor of the endocrine pancreas. *Science,* 184, 482-484, 1974. DOI: 10.1126/science.184.4135.482

19. Alberti KG, Christensen NJ, Christensen SE, Hansen AP, Iversen J, Lundbaek K, Seyer-Hansen K, Orskov H: Inhibition of insulin secretion by somatostatin. *Lancet,* 2, 1299-1301, 1973. DOI: 10.1016/S0140-6736 (73)92873-0

20. Polak JM, Pearse A, Grimelius L, Bloom SR, Arimura A: Growth-hormone release inhibiting hormone in gastrointestinal and pancreatic D-cells. *Lancet,* 1, 1200-1222, 1975. DOI: 10.1016/S0140-6736(75)92198-4

21. Hawsawi ZA, Basil AA, Bamosa AO: Effect of *Nigella sativa* (Black Seed) and thymoquinone on blood glucose in albino rats. *Annals Saudi Med*, 21, 3-4, 2001.

22. Böck P: Romeis Microskopische Technik. 17th edn., 561, Urban and Schwarzenhferg Verlag, München, 1989.

23. Elis Yildiz S, Yigit F, Duman Aydin B, Karadag Sari E, Deprem T, Koral Taşçi S: Effects of kefir, koumiss, milk and yoghurt administration on distribution of plasma cells and mast cells in mice spleen. *Kafkas Univ Vet Fak Derg,* 21, 195-201, 2015. DOI: 10.9775/kvfd.2014.12015

24. Ali BH, Blunden G: Pharmacological and toxicological properties of *Nigella sativa. Phytother Res*, 17, 299-305, 2003. DOI: 10.1002/ptr.1309

25. Gulmez N, Kocamis H, Aslan S, Nazli M: Immunohistochemical distribution of cells containing insulin, glucagon and somatostatin in the goose *(Anser anser)* pancreas. *Turk J Vet Anim Sci*, 28, 403-407, 2004.

26. Kocamis H, Karadag Sari E, Nazli M, Gulmez N, Aslan S, Deprem T: Immunohistochemical distribution of ınsulin, glucagon, and somatostatin containing cells in the pancreas of the rat *(Wistar albino). Kafkas Univ Vet Fak Derg,* 15, 611-614, 2009. DOI: 10.9775/kvfd.2009.086-A

27. Islam SN, Begum P, Ahsan T, Huque S, Ahsan M: Immunosuppressive and cytotoxic properties of *Nigella sativa. Phytother Res*, 18, 395-398, 2004. DOI: 10.1002/ptr.1449

28. Gali-Muhtasib H, Diab-Assaf M, Boltze C, Al-Hmaira J, Hartig R, Roessner A, Schneider-Stock R: Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. *Int J Oncol*, 25, 857-866, 2004.

29. Al-Logmani A, Zari T: Long-term effects of *Nigella sativa* L. oil on some physiological parameters in normal and streptozotocin-induced diabetic rats. *J Diabetes Mellitus,* 1,46-53, 2011. DOI: 10.4236/jdm.2011.13007

30. De Herder WW, Hofland LJ, Van Der Lely AJ, Lamberts SW: Somatostatin receptors in gastroentero-pancreatic neuroendocrine tumours. *Endoc Relat Cancer*, 10, 451-458, 2003.

31. Orci L, Baetens D, Rufener C, Amherdt M, Ravazzola M, Studer P: Hypertrophy and hyperplasia of somatostatin-containing D-cells in diabetes. *Proc Natl Acad Sci USA,* 73, 1338-1342, 1976.

32. Keller J, Aghdassi AA, Lerch MM, Mayerle JV, Layer P: Tests of pancreatic exocrine function-clinical significance in pancreatic and non-pancreatic disorders. *Best Pract Res Clin Gastroenterol,* 23, 425-439, 2009. DOI: 10.1016/j.bpg.2009.02.013

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Research Article

Effect of Energy Sources and Levels on Caecal Microbial Population, Jejunal Morphology, Gene Expression of Jejunal Transporters (SGLT1, FABP) and Performance of Broilers Under Heat Stress

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Abstract

The present study was conducted to evaluate the effects of energy sources and levels on microbial population, jejunal morphology, gene expression of glucose transporter (SGLT1), fatty acid binding protein (FABP) and performance in broilers under heat stress. In a completely randomized design, 600 one-day-old Cobb broiler chickens were assigned to five dietary treatments and four replicates. Chicks were fed diet based on corn as main energy source and energy level based on Cobb standard considered as control (C), corn based diet with 3% lesser energy than control (T1), corn based diet with 6% lesser energy than control (T2), corn and soybean oil based diet according to Cobb standard (T3), corn and soybean oil based diet with 3% upper energy than control (T4). Temperature was increased to 34°C for 8 hours daily from day 21 to 41 to induce heat stress. Chickens in T3 and T4 had higher *Lactobacillus* population and lower Escherichia coli population than C group (P<0.05). Chickens in T1 and T2 had shorter jejunal villi, deeper crypts, and lower villus height: crypt depth than those fed C, T3 and T4 diets (P<0.05). There were no significant differences among treatments for gene expression of both nutrient transporters (P>0.05). Chickens in T3 and T4 had higher weight gain compared to C, T1 and T2 (P<0.05). Feed intake in T3 was lower than C, consequently, feed conversion ratio of chicks fed T3 was better than C group (P<0.05). In conclusion, replacement a part of dietary energy source with soybean oil might improve intestinal parameters and performance of broilers under heat stress.

Keywords: *Energy, Lipid, Intestinal morphology, Transporter, Broiler*

Isı Stresi Altında Enerji Kaynakları ve Seviyelerinin Sekum Mikrobiyal Popülasyonuna, Jejunum Morfolojisine, Jejunal Transporterlerin (SGLT1, FABP) Gen Ekspresyonuna ve Broiler Performansına Etkileri

Özet

Bu çalışma; enerji kaynakları ve seviyelerinin ısı stresi altında mikrobiyal popülasyona, jejunum morfolojisine, glukoz transporteri (SGLT1) ve yağ asitleri bağlayıcı protein (FABP) gen ekspresyonlarına ve broiler performansına etkilerini araştırmak amacıyla yürütülmüştür. Rastgele örneklemeyle, 600 adet 1 günlük Cobb broiler civciv, 4 tekrar olmak üzere 5 farklı beslenme uygulanmasına alındı. Civcivler ana enerji kaynağı olarak mısıra dayalı diyetle beslendi. Kontrol grubuna (C) bazal seviyede Cobb standardına göre yem verilirken, T1 grubuna kontrole göre %3 daha düşük enerjili yem, T2 grubuna kontrole göre %6 daha düşük enerjili yem, T3 grubuna Cobb standardına göre mısır ve soya fasulyesi yağı tabanlı diyet ve T4 grubuna kontrole göre %3 daha fazla enerjili mısır ve soya fasulyesi yağı tabanlı diyet uygulandı. Isı stresi oluşturmak amacıyla 21. günden 41. güne kadar günde 8 saat boyunca sıcaklık 34°C'ye çıkarıldı. T3 ve T4 grubundaki civcivlerde kontrol grubuna oranla *Lactobacillus* popülasyonu daha yüksek iken Escherichia coli popülasyonu daha düşüktü (P<0.05). T1 ve T2 grubundaki civcivlerde C, T3 ve T4 grubundakilere kıyasla daha kısa jejunal villi, daha derin kriptler ve daha düşük villus yüksekliği:derinliği belirlendi (P<0.05). Her iki besin transporteri için de gen ekspresyonlarında farklı uygulamalar için herhangi bir fark gözlemlenmedi (P>0.05). T3 ve T4 grubundaki civcivlerde C, T1 ve T2 gruplarına kıyasla daha yüksek kilo kazanımı belirlendi (P<0.05). Kontrol grubuna oranla T3 grubundaki civcivlerin yem tüketimi daha düşük ve dolayısıyla yem konversiyon oranı daha iyiydi (P<0.05). Sonuç olarak, diyetteki enerji kaynağının bir bölümünün soya fasulyesi yağı ile değiştirilmesinin bağırsak parametrelerini ve ısı stresi altındaki broilerlerin performansını iyileştirebileceği belirlendi.

Anahtar sözcükler: *Enerji, Yağ, Bağırsak morfolojisi, Transporter, Broiler*

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INTRODUCTION

Heat stress is considered as a major problem in poultry production on subtropical and tropical regions, where major broiler farms exists [1]. Heat stress occurs when a negative balance between the amount of heat energy produced by the animal and the amount of energy flowing from the broiler body to environment exists. This condition affects negatively the health and performance of broilers [2], In terms of changes in metabolic pathways [3], microbial population in the small intestine [4], hormonal levels [5], low feed consumption, body weight gain, high feed conversion ratio (FCR) [6] and damage to small intestine structure [7,8]. These changes not only could affect nutrient absorption, but also the merit of substrates for metabolism [9,10].

Intestinal bacterial flora are considered important for priming and maintaining an active immune system [11]. The microbiota composition can be affected by environmental factors, genetics and substrate availability within the gut [12]. Development of the intestinal villi in the early chicken`s life could increase efficiency of nutrient utilization and enhance the growth performance. Furthermore, an increase in villi height may increase the intestinal surface area and nutrient absorption [13]. Mitchell and Carlisle [14] reported that chronic heat stress decreased small intestinal villus height and wet and dried small intestinal weight of birds.

The process of absorption of carbohydrates into the enterocytes of the small intestine is mediated by sugar transporters, such as sodium-glucose transporter 1 (SGLT-1) [15], which diffuses monosaccharides into the extra-cellular fluid and then into the blood [16]. Triglycerides (TGs) are broken into glycerol and free fatty acids and then absorbed in the intestine and transported across the apical membrane of the enterocytes. Fatty-acid-binding proteins (FABPs) are intracellular lipid chaperones that transport lipids to a specific component in the cell [17]. Nutrient transporters in the small intestine are responsible for dietary nutrient assimilation; therefore, heat stress-related changes in the expression of these transporters affect the availability of nutrients and energy to the animal for growth and development. However, the effects of heat stress on the expression of nutrient transporters in the small intestine of broiler chickens are unclear. Some studies showed that starvation stress caused the increased expression of sodium glucose co-transporter 1 (SGLT1) mRNA in the small intestine of chickens and rats [18,19].

Wang et al.[20] showed that high apparent metabolizable energy in the diets fed to broilers improved their feed conversion ratio, emphasizing the potential role of nutrient density as an important factor that may affect animal intestine development. Chickens fed a higher nutrient density diet grow faster throughout all growing phases [21]. There are evidences that a higher dietary fat content contributes to improved heat tolerance in broiler chickens [22]. Zulkifli et al.[23] reported that providing diets containing high levels of palm oil enhanced growth performance and survivability of heat-stressed broiler chickens.

To our knowledge, there was no report concerning the effect of energy sources and levels on intestinal transporters gene expression, morphology and microbiology, especially in the context of heat stress condition. It was hypothesized that in the heat stress condition, lipid addition with higher energy level to diet could improves the small intestine morphology, gene expression of transporter and beneficiary bacteria population compared to diet containing main energy source from carbohydrate. Therefore, the main objective was to evaluate the effects of energy sources and levels on microbial population, small intestine morphology, gene expression of glucose transporter (SGLT1), fatty acid binding protein (FABP) and performance in broiler chickens under heat stress conditions.

MATERIAL And METHODS

Chickens Management

All animal procedures were approved by the Animal Care Committee of the Animal Sciences Research Institute of Iran. The use of broilers in this study was approved by the Animal Care Committee (Protocol 17-16-5-10938; 90-11-15).

A total of 600 one-day-old Cobb 500 male broiler chicks with an average weight of 39±0.50g was obtained from a local hatchery and randomly allocated to 20 floor pens (200 cm × 180 cm) covered with pine shaving. Chicks were randomly assigned to five dietary treatments with four replicates and 30 chicks per each. Chicks were raised under environmentally controlled conditions and lighting program based on Cobb 500 broiler guides (Cobb Broiler Management Guide, 2010), except temperature. Feed intake and live body weight were recorded in the beginning and at the end of experiment, and the feed conversion ratio was then calculated. Dead chicks were collected daily and weighed at the time of carcass removal; carcass weights were included in the feed conversion ratio calculations.

Experimental Design

Dietary treatments were: control group (C) which broilers fed diet with main energy from corn and energy level; T1: broilers fed diet with main energy from corn and 3% lesser energy; T2: broilers fed diet with main energy from corn and 6% lesser energy; T3: broilers fed diet with main energy from corn and soy oil and energy level T4: broilers fed diet with main energy from corn and soy oil and 3% upper energy. The experimental diets were formulated based on Cobb standard (Cobb instruction manual, 2012) *(Table 1)*. Washed sand as filler was used to balance for dietary metabolizable energy levels. Chicks were fed a starter diet from day 1 to 10, grower diet from day 11 to 28, followed by a finisher diet from day 29 to 42 of age. Feed

Table 1. *Composition (measured in %) of the experimental diets for broiler chickens*

Ingredients	Starter (0-10 days old)					Grower (11-28 days old)					Finisher (29-42 days old)				
	C	T1	T2	T3	T4	C	T1	T2	T3	T4	C	T1	T2	T3	T4
Corn	63.35	63.33	62.13	58.24	54.26	69.22	68.8	67.7	65.31	59.31	70.18	71.47	71.5	65.07	62.4
Soybean meal	22.57	28.86	31.48	31.90	32.21	18	23.43	26.2	24	28.45	19.3	20	24.2	25.8	25
Soybean oil	--------	-------	------	2.5	4.14	------	------	------	2	5	------	----	-----	3.5	5
Corn gluten meal	9.17	3	-----	2.7	4.7	8.2	3.24	------	4.2	2.9	6.23	4.2	-----	1.5	3.5
Di-calcium phosphate	2.07	2.06	2.06	2.05	2.05	1.9	1.9	1.9	1.9	1.9	1.7	1.7	1.7	1.7	1.7
Calcium carbonate[1]	1.06	1.03	1.01	1.01	1.01	1.05	1.05	1.05	1.05	1.05	0.92	0.92	0.92	0.90	0.90
NaCl	0.38	0.38	0.38	0.38	0.38	0.37	0.37	0.37	0.37	0.37	0.32	0.32	0.32	0.32	0.32
DL - Methionine	0.27	0.33	0.38	0.33	0.30	0.22	0.27	0.30	0.25	0.25	0.18	0.22	0.27	0.22	0.20
L – Lys HCl	0.53	0.40	0.35	0.31	0.36	0.44	0.34	0.28	0.32	0.22	0.36	0.36	0.27	0.21	0.22
L - Threonine	0.10	0.11	0.13	0.08	0.09	0.10	0.10	0.12	0.10	0.05	0.09	0.09	0.10	0.06	0.04
Vitamin & Mineral Permix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride	-----	------	-----	------	------	-----	-----	-----	-----	-----	0.22	0.22	0.22	0.22	0.22
Filler[3]	-----	-----	1.58	-----	-----	-----	-----	1.58	----	----	----	----	----	----	---
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Analyzed Nutrient content															
ME (kcal/kg)	3035	2934	2853	3035	3120	3108	3014	2921	3108	3201	3185	3085	2990	3185	3275
Digestible Methionine%	0.59	0.59	0.59	0.59	0.59	0.53	0.53	0.53	0.53	0.53	0.48	0.48	0.48	0.48	0.48
Digestible Lysine%	1.18	1.18	1.18	1.18	1.18	1.05	1.05	1.05	1.05	1.05	0.95	0.95	0.95	0.95	0.95
Digestible Threonine%	0.77	0.77	0.77	0.77	0.77	0.69	0.69	0.69	0.69	0.69	0.65	0.65	0.65	0.65	0.65
Calcium%	0.90	0.90	0.90	0.90	0.90	0.84	0.84	0.84	0.84	0.84	0.76	0.76	0.76	0.76	0.76
Available phosphorus%	0.45	0.45	0.45	0.45	0.45	0.42	0.42	0.42	0.42	0.42	0.38	0.38	0.38	0.38	0.38
Na%	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.16	0.16	0.16	0.16	0.16

[1] per kg contains:Ca , 23% and P, 18.5%; [2] Supplied by Razak Co., Tehran, Iran, and provided per kilogram of diet: vitamin A, 360.000 IU; vitamin D3, 800.000 IU; vitamin E, 7.200 IU; vitamin K3, 800 mg; vitamin B1, 720 mg; vitamin B9, 400 mg; vitamin H2, 40 mg; vitamin B2, 2.640 mg, vitamin B3, 4.000 mg; vitamin B5, 12.000 mg; vitamin B6, 1.200 mg; vitamin B12, 6 mg; Choline chloride, 200.000 mg, Manganese, 40.000 mg, Iron, 20000 mg; Zinc, 40.000 mg, copper, 4000mg; Iodine, 400 mg; [3] Inert filler used to complete diet formulations to 100%

Table 2. *Primers sequences used in RT-PCR*

Primers	Sequence
SGLT1 F	5-GATGTGCGGATACCTGAAGC-3
SGLT1 R	5-AGGGATGCCAACATGACTGA-3
FABP F	5-AGAAAGTTAGGAGGAGCCCACG-3
FABP R	5-TCGGTCACGGATTTCAGC-3
β-actin F	5-CCACCGCAAATGCTTCTAAAC-3
β-actinR	5-AAGACTGCTGCTGACACCTTC-3

and water were provided for *ad libitum* intake. From day 21 to 41, all the chickens were exposed to 34±1°C and 60-70% relative humidity for 8 hours per day from 08:00 to 16:00, and then raised at 24±1°C. Feed and water were provided throughout the heat challenge period.

Sample Collection

On day 28 of age, five chicks from each pen were weighed and euthanized by cervical dislocation. After excising jejunum, as described by Uni et al.[24] segments were washed with cold phosphate buffer saline, sectioned and immediately frozen in liquid nitrogen, and stored at -80°C. 2 cm sections were selected for histo-morphology examination. The sections were flushed with phosphate buffer solution and then fixed in buffered formalin solution (10%). The entry of ceca of five chicks from each pen was sealed, removed and placed in ice and the contents used immediately for microbial assays.

Total RNA Extraction and Reverse Transcription

The frozen jejunum was crushed in a sterile mortar, and the powder was applied for total RNA extraction using a suitable kit (Bioneer Co., Seoul, South Korea). The integrity of the RNA was verified by optical density (OD) absorption ratio 1.97>OD260 nm/OD280 nm>1.9 Ribonucleic acid integrity was determined by gel electrophoresis on a 1% agarose gel. Extracted RNA was stored at -80°C. Then, cDNA for each transporter gene was synthesized based on reverse transcription technique using kit (Bioneer Co., Seoul, South Korea) and stored at -20°C.

Quantitative Real-Time PCR

The relative abundance of SGLT1 and FABP mRNA was determined by quantitative real-time PCR. Quantitative real-time PCR was conducted using a Real-time PCR systems (Applied Biosystems). Each reaction contained the followings: 2 µL of cDNA, 10 µL of 2X SYBR Green Master Mix (Applied Biosystems), 0.4 µL each of the forward primer (4 µM) and reverse primer (4 µM), and 7.2 µL of nuclease-free water. Primers were designed by using Primer software (primer-BLAST) at website (www.ncbi.nlm.nih.gov). PCR reactions were performed with primers designed and synthesized for both transporters and the house-keeping gene β-actin *(Table 2)*. Amplification for transporter in the jejunum (SGLT1 and FABP) was performed for 45 cycles, which consisted of an initial activations step (95°C, 5 min), denaturation cycle (95°C, 30s) and annealing at 54°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were electrophoresed on a 1% agarose gel. Average gene expression relative to the endogenous control for each sample was calculated using the $2^{-\Delta\Delta CT}$ method [25].

Intestinal Microbial Populations

Immediately, samples of ileum and ceca were collected into glass containers under CO_2 sealed and put on ice until enumeration of microbial populations. Ten grams of mixed contents were blended under CO_2 in 90 mL of anaerobic dilution solution. Further serial dilutions were made in anaerobic dilution solution for anaerobic bacterial enumeration. The initial dilution in anaerobic dilution solution was also used as a source for serial dilutions in phosphate buffered saline for enumeration of aerobic bacterial populations. Triplicate plates were then inoculated with 0.1mL samples and incubated at 37°C aerobically or anaerobically as appropriate. Three dilutions were plated for each medium. Bacteria were enumerated on MRS agar (Merck, Germany) for *Lactobacillus* and MacConkey's (Merck, Germany) for *Escherichia coli*. Colony forming units (cfu) were defined as being distinct colonies measuring at least 1 mm in diameter [26].

Intestinal Morphological Analysis

Formalin fixed tissue sections were processed by dehydration through a series of graded alcohol solutions (50, 70, 80, 90, 95 and 100%), cleared with xylene, and embedded in paraffin. For each segment, a 5-µm cross-section was sectioned using a microtome and placed on a glass slide. The slides were stained using routine procedures for Mayer´s hematoxylin and eosin. Villus height, villus length and crypt depth were measured using a light microscope (Olympus attached camera) using the method presented by Wang et al.[27]. Double-stained of samples with Periodic Acid-Schiff and hematoxylin was done to determine the goblet cell count according to the method of Wang et al.[27]. The goblet cells were counted in the scale of 300 µm of epithelium length.

Statistical Analysis

Statistical analyses were carried out using ANOVA of SAS for Windows version 9.1 (SAS Institute Inc., Cary, NC) [28]. The Kolmogorov-Smirnov test was used to test the normality of the data before ANOVA was performed. Tukey test was used to compare the means. Statistical differences were declared at $P<0.05$.

RESULTS

Effect on Performance

The effect of energy sources and levels on daily body weight gain (WG), daily feed intake (FI) and feed conversion ratio (FCR) is presented in *Table 3*. Chickens fed T1 and T2 diets as compared to C group had lower WG, higher FI and higher FCR ($P<0.05$). Chickens in T3 and T4 that received soybean oil and energy level equal to or greater than guideline of Cobb had higher WG compare to those received equal to or lower energy with main energy source of corn grain ($P<0.05$). Chicks in C and T3 group received the same amount of energy with different sources, had difference in FI and FCR, but had no significant differences in weight gain ($P>0.05$). Feed intake in T3 was lower than C, consequently, FCR of chicks fed T3 was better than C group. Although chicks in T3 and T4 groups received diets containing soybean oil, chicks in T4 received 3% higher energy than T3. Chicks in T3 also had lower FI than, same FCR and weight gain as group T4. The highest BW was for T4 and the lowest for T2 group. No difference on BW was observed between C and T3 groups consuming the same energy level with different energy source ($P>0.05$).

Effect on Small Intestine Morphology

Dietary treatments significantly influenced jejunal villus height, crypt depth and villus height: crypt depth ratio *(Table 4)*, but had no effect ($P>0.05$) on villus width and goblet cell count. The smallest villus height, deeper crypt and lowest their ratio was related to chicks in T2 group, and in contrast respectively the largest, shallowest and highest of these parameters was related to T4 group. Chickens received diets with lower energy level had poorer jejunal morphological parameters than those fed with diet in sufficient or over energy level. There were no differences for villus height and crypt depth between T3 and T4, but their ratio was higher ($P<0.05$) in T4 than T3. Chicks in C and T3 group received the same amount of energy with different sources, had difference in villus height and villus height: crypt depth ratio, but difference for crypt depth was not significant ($P>0.05$).

Effect on Microbial Population

Effect of energy sources and levels on microbial population of broiler chicks is shown in *Table 5*. There

were no significant differences among C, T1 and T2 for *Lactobacillus* population (P>0.05), whereas addition of soybean oil to diet in T3 and T4 groups increased (P<0.05) *Lactobacillus* population. The highest population of *Escherichia coli was* seen in C group and the lowest in chickens received soybean oil. Chickens in T2 group that received the lowest energy level had less *Escherichia coli* population than T1 and C group (P<0.05). Chicks in T3 group that received soybean oil in comparison with C group that received the same amount of energy main from corn grain had higher (P<0.05) *Lactobacillus* population and lower *Escherichia coli* population.

Effect on SGLT1 and I-FABP Gene Expression

Effect of dietary treatment on jejunal gene expression of SGLT1 and FABP in broiler chickens is shown in *Table 6*. There were no significant differences (P>0.05) among treatments for gene expression of both nutrient transporters.

Table 3. *Effect of energy sources and levels on performance parameters of broiler chickens at total period* [1]

Treatments	Daily Weight Gain (g/bird)	Daily Feed Intake (g/bird)	Feed Conversion Ratio	BW (g)
C	51.42^{b}	93.07^{c}	1.81^{c}	2160^{b}
T1	49.83^{bc}	96.67^{b}	1.94^{b}	2093^{c}
T2	48.33^{c}	99.55^{a}	2.06^{a}	2030^{d}
T3	52.09^{ab}	89.59^{d}	1.72^{d}	2188^{b}
T4	53.80^{a}	93.61^{c}	1.74^{d}	2260^{a}
SEM	0.490	0.579	0.029	18.690
P-value	0.002	0.004	0.001	0.001

^{a,b,c} Means within a column with different superscripts are significantly different (P<0.05); [1] Data are means of 20 pens of 30 broilers each

Table 4. *Effect of energy sources and levels on jejunal morphological parameters of broiler chickens at d 28 of age* [1]

Treatments	Villus Height[2] (mm)	Villus Width[2] (mm)	Crypt Depth[2] (mm)	Height:Crypt Depth[1]	Goblet Cell (count/mm)[2]
C	1.01^{b}	0.74	0.40^{ab}	2.52^{c}	140
T1	0.90^{bc}	0.75	0.43^{ab}	2.09^{d}	138
T2	0.70^{c}	0.75	0.50^{a}	1.4^{e}	137
T3	1.13^{a}	0.74	0.38^{b}	2.97^{b}	147
T4	1.19^{a}	0.72	0.36^{b}	3.30^{a}	146
SEM	0.23	0.651	0.09	0.08	0.606
P-value	0.001	0.07	0.001	0.04	0.08

^{a,b,c} Means within a column with different superscripts are significantly different (P<0.05); [1] Data are means of 100 birds (5 birds from each pen); [2] Data were obtained from transmission electron microscopy

Table 5. *Effect of energy sources and levels on viable counts of Lactobacilli and Escherichia coli (log_{10}CFU/g of digesta) in cecal digesta of broiler chickens at d 28 of age* [1]

Microflora	Experimental Treatments					SEM	P-value
	C	T1	T2	T3	T4		
Lactobacillus	3.68^{c}	3.72^{c}	3.50^{c}	4.67^{b}	5.83^{a}	0.539	0.008
Escherichia coli	5.00^{a}	4.82^{a}	2.20^{b}	1.30^{c}	1.10^{c}	0.500	0.008

^{a,b,c} Means within a row with different superscripts are significantly different (P<0.05); [1] Data are means of 100 birds (5 birds from each pen)

Table 6. *Effects of energy sources and levels on SGLT1 and FABP gene expression in jejunal of broiler chickens at 28 d of age*

Relative Gene Expression[1]	Experimental Treatments					SEM	P-value
	C	T1	T2	T3	T4		
SGLT1	1.000	1.074	1.264	1.255	1.187	0.22	0.06
FABP	1.000	1.112	1.127	1.181	1.413	0.33	0.06

Means within a row without superscripts are not different (P>0.05); [1] Relative gene expression ($2^{-\Delta\Delta C_T}$) ±SEM was calculated using the $\Delta\Delta^{CT}$ method

DISCUSSION

Chicken health and performance are greatly influenced by diet quality and nutrient availability [29]. These traits are more dependent on the intestine health to assimilate nutrients efficiently as the bird shifts from the lipid-rich yolk as the main source of energy to a carbohydrate-based diet [30]. To improve the health and performance, addition of lipids to diet in the early life of chicks and later was recommended by many researchers [31-33]. In stress condition, plasma corticosterone level tends to increase and this hormone has negative effect on intestinal morphology and consequently performance [34]. A recent study revealed that birds exposed to chronic heat stress had poor growth performance and increased corticosterone concentrations [6]. Deteriorated performance resulted from heat stress in broilers can be attributed to a greater expenditure of energy for physiological adaptation to the stressful situation instead of growth enhancement [35]. Alternatively, it is believed that less weight gain in the heat stress groups is due to a smaller appetite and lower feed intake, as it maybe seemingly a defense mechanism to help reduce heat production.

The main objective of this study was to evaluate the effect of energy sources and levels on microbial population, SGLT1 and FABP gene expression and small intestinal parameters in broiler chickens under heat stress. In the literature, study about this subject under heat stress was not found; hence comparison of our findings with others was done on non-stressed condition.

The results of this study indicate that the source and level of metabolizable energy in broilers under heat stress had a statistically significant influence on intestinal morphology and microbiology and consequently performance ($P<0.05$), but it had no effect on gene expression of nutrient transporters of glucose and fatty acid binding protein. Maintenance of normal microarchitecture of the small intestine is very important for proper growth and development. Quite a few studies have reported that stress hampered the development of intestinal morphology and function [14]. Sohail et al.[6] observed that heat stress decreased villus height and width, crypt depth, and villus surface area. Stressors such as fasting and nutrient deficiency or corticosterone injections have noxious effects on the intestinal microarchitecture, resulting in reduction in the absorptive surface area [36,37]. Feeding ration with lower energy level to chickens resulted in short villus height and poor performance. The villi play a crucial role in the digestion and absorption processes of the small intestine, as is the first to make contact with nutrients in the lumen [38]. A shortening of the villus height may lead to poor nutrient absorption along with lower performance [26].

In contrast, substitution of a part of corn energy with soybean oil in the present study improved the intestinal parameters. The positive effect of lipids on intestinal health status and growth performance of broilers is well documented [39]. The extra caloric effect of added soybean oil resulted in an improved body weight gain [40]. Inclusion of soybean oil and delivery of higher energy than Cobb standard (T4) resulted in the highest villus height, the shallowest crypt depth, lowest *E. coli* and highest lactic acid bacteria population and finally higher body weight and the highest feed efficiency. It was reported that dietary soybean oil supplementation significantly increased villus height of the jejunum [41].

In this experiment, addition of soybean oil in diet T3 and T4 enhanced the growth of *Lactobacillus*, but inhibited that of *E. coli* in the small intestine. Freitas et al.[42] reported that addition of soybean oil could maximize the growth of the bacterial population. Intestinal microflora plays an important role in digestive health. Microbial population is dependent on food rations as the ultimate source of the organic substrate metabolism [43]. It is known that dietary fats cause changes in the intestinal microflora composition with a direct effect on digestion and absorption of nutrients by the birds. Innis et al.[44] reported that canola oil supplementation increased lactic acid bacteria population in the gut. The effect of fat on the microbial flora of the gastrointestinal tract may be due to its effect on digestion, viscosity, pH level and the transport of nutrients in the gastrointestinal tract [45].

No differences were found among treatments for gene expression of SGLT1 and FABP in this study. It was demonstrated that regulation of nutrient transporters by dietary substrate appears to occur by increasing mRNA stability or by increasing gene transcription rate [46]. It seems that metabolizable substrates are not necessary for its regulation. Sun et al.[47] reported that the expression levels of FABP were significantly decreased by heat exposure, but heat stress had no significant effect on gene expression of SGLT1. In Shepherd et al.[48] mentioned study has suggested that environmental stress decreases GLUT-2 expression at the brush border membrane level but does not alter SGLT-1 expression. Moreover, corticosterone-induced stress does not alter the expression of SGLT-1 in the jejunum of broiler chickens [49].

Moreover, an increased body temperature during heat stress conditions can lead to increased maintenance energy requirements [50] to keep body temperature around a normal level. Different levels of energy significantly affected body weight, weight gain, feed intake and feed conversion ratio [51]. The final live weight was significantly highest in broiler chickens fed dietary treatment with normal energy and was lowest in those fed dietary treatment with low energy [52]. Increased live weight was mostly due to higher metabolizable energy consumption in the same unit of diets by chickens, similarly supplementation of oil caused a positive trend in cumulative live weight gain (g/bird) of broilers at different ages [53]. Fats and vegetable oils

has been frequently included in broiler diets to increase the energy density of the diet, to improve efficiency and to increase nutrient digestibility in broilers [54]. Lipid supplementation increases the energetic efficiency in two ways. It increases density of energy and it has a lower heat increment or greater net energy. Increased energy and nutrient density of the diet and replacing carbohydrate calories reduced feed intake almost proportionally but increased live weight gains in hot weather. Nitsan et al.[40] stated that feed conversion ratio were significantly improved with addition of 3% soybean oil in the diet.

The present study has demonstrated that the levels and sources of dietary energy have significant effects on intestinal populations of *Lactobacillus* and *E. coli* in broiler chickens. It can be concluded that providing high levels of dietary energy with oil in broiler nutrition, increases beneficial intestinal flora and reduction of noxious microbes and simultaneously cause an increase intestinal villus height and decrease crypt depth and intestinal absorption that finally can ensure the intestinal health of broilers. The results showed that the higher energy level than nutritional needs based on Cobb broiler chickens requirements as specified in the manual was affective on microbial population and morphological parameters that can cause increasing weight gain and final body weight under heat stress.

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REFERENCES

1. Windhorst HW: Changes in poultry production and trade worldwide. *World's Poult Sci J*, 62, 585-602, 2006. DOI: 10.1079/WPS2006114

2. Lin H, Jaio HC, Buyse J, Decuypere E: Strategies for preventing heat stress in poultry. *World's Poult Sci J*, 62, 71-85, 2006. DOI: 10.1079/WPS200585

3. Schlesinger MJ, Ryan C, Chi MM, Carter JG, Pusateri ME, Lowry OH: Metabolite changes associated with heat shocked avian fibroblast mitochondria. *Cell Stress Chaperones*, 2, 25-30, 1997.

4. Lan PT, Sakamoto M, Benno Y: Effects of two probiotic *Lactobacillus* strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by molecular analysis of 16S rRNA genes. *Microbiol Immunol*, 48, 917-929, 2004. DOI: 10.1111/j.1348-0421.2004.tb03620.x

5. Tao X, Zhang ZY, Dong H, Zhang H, Xin H: Responses of thyroid hormones of market-size broilers to thermoneutral constant and warm cyclic temperatures. *Poult Sci*, 85, 1520-1528, 2006. DOI: 10.1093/ps/85.9.1520

6. Sohail MU, Hume ME, Byrd JA, Nisbet DJ, Ijaz A, Sohail A, Shabbir MZ, Rehman H: Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poult Sci*, 91, 2235-2240, 2012. DOI: 10.3382/ps.2012-02182

7. Yu J, Yin P, Liu F, Cheng G, Guo K, Lu A, Zhu X, Luan W, Xu J: Effect of heat stress on the porcine small intestine: a morphological and gene expression study. *Comp Biochem Physiol*, 156, 119-28, 2010. DOI: 10.1016/j.cbpa.2010.01.008

8. Quinteiro-Filho WM, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Sakai M, Sa LRM, Ferreira AJP, Palermo-Neto J: Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poult Sci*, 89, 1905-1914, 2010. DOI: 10.3382/ps.2010-00812

9. Menten JFM, Barbosa Filho JAD, Silva MAN, Silva IJO, Racanicci AMC, Coelho AAD, Savino VJM: Physiological responses of broiler chickens to pre-slaughter heat stress. *World´s Poult Sci J*, 62, 254-257, 2006.

10. Aksit M, Yalcin S, Ozkan S, Metin K, Ozdemir D: Effects of temperature during rearing and crating on stress parameters and meat quality of broilers. *Poult Sci*, 85, 1867-1874, 2006. DOI: 10.1093/ps/85.11.1867

11. Kohl KD: Diversity and function of the avian gut microbiota. *J Comp Physiol B,* 182,591-602, 2012. DOI: 10.1007/s00360-012-0645-z

12. Apajalahti JHA, Kettunen A, Bedford MR, Holben WE: Percent G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. *Appl Environ Microbiol*, 67, 5656-5667, 2001.DOI: 10.1128/AEM.67.12.5656-5667.2001

13. Soltan M: Influence of dietary glutamine supplementation on growth performance, small intestinal morphology, immune response and some blood parameters of broiler chickens. *Int J Poult Sci*, 8, 60-68, 2009. DOI: 10.3923/ijps.2009.60.68

14. Mitchell MA, Carlisle AJ: The effect of chronic exposure to elevated environmental temperature on intestinal morphology and nutrient absorption in the domestic fowl *(Gallus domesticus)*. *Comp Biochem Physiol*, 101, 137-142, 1992. DOI: 10.1016/0300-9629(92)90641-3

15. Thorens B: Glucose transporters in the regulation of intestinal, renal and liver glucose fluxes. *Am J Physiol*, 270, 541-553, 1996.

16. Wright EM, Turk E: The sodium/glucose co-transporter family SLC5. *Eur J Physiol*, 447, 510-518, 2004. DOI: 10.1007/s00424-003-1063-6

17. Furuhashi M, Hotamisligil GS: Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov*, 7, 489-503, 2008. DOI: 10.1038/nrd2589

18. Gal-Garber OS, Mabjeesh J, Sklan D, Uni Z: Partial sequence and expression of the gene for and activity of the sodium glucose transporter in the small intestine of fed, starved and refed chickens. *J Nutr*, 130, 2174-2179, 2000.

19. Naruhashi K, Sai Y, Tamai I, Suzuki N, Tsuji A: PepT1 mRNA expression is induced by starvation and its level correlates with absorptive transport of cefadroxial longitudinally in the rat intestine. *Pharm Res,* 19, 1417-1423, 2002. DOI: 10.1023/A:1020436028194

20. Wang X, Peebles ED, Zhai W: Effects of protein source and nutrient density in the diets of male broilers from 8 to 21 days of age on their subsequent growth, blood constituents, and carcass compositions. *Poult Sci*, 93, 1463-1474,2014. DOI: 10.3382/ps.2013-03838

21. Zai W, Peebles ED, Zumwalt CD, Mejia L, Corozo A: Effects of dietary amino acid density regimens on growth performance and meat yield of Cobb×Cobb 700 broilers. *J Appl Poult Res,* 22, 447-460, 2013. DOI: 10.3382/japr.2012-00658

22. Daghir NJ: Broiler feeding and management in hot climates. **In**, Daghir NJ (Ed): Poultry Production in Hot Climate. 185-218, CAB Intl, Wallingford, UK, 1995.

23. Zulkifli I, Liew PK, Israf DA, Omar AR, Hair-Bejo M: Effects of early age feed restriction and thermal conditioning on heterophil/lymphocyte ratio, heat shock protein 70 and body temperature of male broiler chickens subjected to acute heat stress. *J Thermal Biol*, 28, 217-222, 2003. DOI: 10.1016/s0306-4565(02)00058-x

24. Uni Z, Noy Y, Sklan D: Post-hatch development of smallintestinal function in the poultry. *Poult Sci*, 78, 215-222, 1999. DOI: 10.1093/ps/78.2.215

25. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. *Methods,* 25, 402-408, 2001. DOI: 10.1006/meth.2001.1262

26. Xu ZR, Hu CH, Xia MS, Xia X, Zhan A, Wang MQ: Effects of dietary fructo-oligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poult Sci*, 82, 1030-1036, 2003. DOI: 10.1093/ps/82.6.1030

27. Wang X, Peebles ED, Morgan TW, Harkess RL, Zhai W: Protein source and nutrient density in the diets of male broilers from 8 to 21 d of age: Effects on small intestine morphology. *Poult Sci*, 94, 61-67, 2015. DOI: 10.3382/ps/peu019

28. SAS Institute Inc: SAS/STAT 9.13 User's Guide. *SAS Inst Inc*, Cary, NC, 2004.

29. Geyra A, Uni Z, Sklan D: Enterocyte dynamics and mucosal development in the post-hatch chick. *Poult Sci*, 80, 776-782, 2001. DOI: 10.1093/ps/80.6.776

30. Sklan D, Geyra A, Tako E, Gal-Gerbert O, Uni Z: Ontogeny of brush border carbohydrate digestion and uptake in the chick. *Br J Nutr*, 89, 747-753, 2003. DOI: 10.1079/BJN2003853

31. Yaqoob P: Fatty acids and the immune system: from basic science to clinical applications. *Proceed Nutr Soc*, 63, 89-104, 2004. DOI: 10.1079/PNS2003328

32. Sadeghi A, Mirmohseni A, Shawrang P, Aminafshar M: The effect of soy oil addition to the diet of broiler chicks on the immune response. *Turk J Vet Anim Sci*, 37, 264-270, 2013. DOI: 10.3906/vet-1109-24

33. Cherian G: Nutrition and metabolism in poultry: Role of lipids in early diet. *J Anim Sci Biotechnol*, 6, 28-35, 2015. DOI: 10.1186/s40104-015-0029-9

34. Geraert PA, Padilha JCF, Guillaumin S: Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: Biological and endocrinologicalvariables. *Br J Nutr*,75, 205-216, 1996. DOI: 10.1017/BJN19960125

35. Lei KY, Slinger SJ: Energy utilization in the chick in relation to certain environmental stresses. *Can J Anim Sci*, 50, 285-292, 1970. DOI: 10.4141/cjas70-043

36. Yamauchi K, Yamamoto K, Ishiki Y: Morphological alterations of the intestinal villi and absorptive epithelial cells in each intestinal part in fasted chickens. *Jpn J Poult Sci*, 32, 241-251, 1995. DOI: 10.2141/jpsa.32.241

37. Hu XF, Guo YM: Corticosterone administration alters small intestinal morphology and function of broiler chickens. *Asian-Australas J Anim Sci*, 21, 1773-1778, 2008. DOI: 10.5713/ajas.2008.80167

38. Gartner LP, Hiatt JL: Color Textbook of Histology. 2nd edn., WB Saunders, Baltimore, MD, USA, 2001.

39. Griffiths L, Leeson S, Summers JD: Influence of energy system and level of various fat sources on performance and carcass composition of broiler. *Poult Sci*, 56, 1018-1026, 1977. DOI: 10.3382/ps.0561018

40. Nitsan Z, Dvorin A, Zoref Z, Mokady S: Effect of added soybean oil and dietary energy on metabolisable and net energy of broiler diets. *Br Poult Sci*, 38, 101-106,1997. DOI: 10.1080/00071669708417948

41. Markovic R, Sefer D, Krstic M, Petrujkic B: Effect of different growth promoters on broiler performance and gut morphology. *Archiv Med Vet,* 60 (2): 442-448, 2009.

42. Freitas KC, Gabriel JE, Leite LC, de Armas RD, Lanna DPD Madeira HMF: Molecular characterization of ruminal bacterial diversity. *Acta Scientific Anim Sci*, 30, 187-192, 2008. DOI: 10.4025/actascianimsci.v30i2.4699

43. Choct M, Hughes RJ, Wang J, Bedford M, Morgan AJ, Annison G: Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. *Br Poult Sci*, 37, 609-621, 1996. DOI: 10.1080/00071669608417891

44. Innis SM, Pinsk V, Jacobson K: Dietary lipids and intestinal inflammatory diseases. *J Peds*, 58, 89-95, 2006. DOI: 10.1016/j.jpeds.2006.06.058

45. Laflamme DR, Xu H, Long GM: Effect of diets differing in fat content on chronic diarrhea in cats. *J Vet Intern Med*, 25, 230-235, 2011. DOI: 10.1111/j.1939-1676.2010.0665.x

46. Adibi S: Regulation of expression of the intestinal oligopeptide transporter (PepT-1) in health and disease. *Am J Physiol-Gastrointestinal Liver Physiol*, 285, 779-788, 2003. DOI: 10.1152/ajpgi.00056.2003

47. Sun X, Zhang H, Sheikhahmadi A, Wang Y, Jiao H, Lin H, Song Z: Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens *(Gallus gallus domesticus)*. *Intl J Biometeorol*, 59, 127-135, 2015. DOI: 10.1007/S00484-014-0829-1

48. Shepherd EJ, Helliwell PA, Mace OJ, Morgan EL, Patel N, Kellett GL: Stress and glucocorticoid inhibit apical Glut2-trafficking and intestinal glucose absorption in rat small intestine. *J Physiol*, 560, 281-290, 2004. DOI: 10.1113/jphysiol.2004.072447

49. Hu XF, Guo YM, Huang BY, Bun S, Zhang LB, Li JH, Liu D, Long FY, Yang X, Jiao P: The effect of glucagon-like peptide 2 injection on performance, small intestinal morphology, and nutrient transporter expression of stressed broiler chickens. *Poult Sci*, 89, 1967-1974, 2010. DOI: 10.3382/ps.2009-00547

50. Hurwitz S, Weiselberg M, Eisner M, Bartov I, Riesenfeld G, Sharvit M, Niv A, Bornstein S: The energy requirements and performance of growing chickens and turkeys as affected by environmental temperature. *Poult Sci*, 59, 2209-2299, 1980. DOI: 10.3382/ps.0592290

51. Min YN, Shi JS, Wei FX, Wang HY, Hou XF, Niu ZY, Liu FZ: Effects of dietary energy and protein on growth performance and carcass quality of broilers during finishing phase *J Anim Vet Advances*, 11, 3652-3657, 2012. DOI: 10.3923/javaa.2012.3652.3657

52. Dairo F, Adesehinwa ASAOK, Oluwasola TA, Oluyemi JA: High and low dietary energy and protein levels for broiler chickens. *Afr J Agri Res*, 5 (15): 2030-2038, 2010.

53. Das GB, Hossain ME, Akbar MA: Effects of different oils on productive performance of broiler. *Iranian J Appl Anim Sci*, 4 (1): 111-116, 2014.

54. Monfaredi A, Rezaei M, Sayyahzadeh H: Effect of supplemental fat in low energy diets on some blood parameters and carcass characteristics of broiler chicks. *S Afr J Anim Sci*, 41, 24-32, 2011.

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Research Article

Antimicrobial Peptides in Housefly Larvae *(Musca domestica)* Affect Intestinal *Lactobacillus acidophilus* and Mucosal Epithelial Cells in *Salmonella pullorum*-infected Chickens

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Abstract

Pullorum disease, which is one the most serious intestinal diseases in poultry production, is generally treated by adding antibiotics to the feed of infected chickens. Although antibiotics are generally quite effective against the disease, they can harm small intestinal flora and mucosa. The objective of this experiment was to determine if antimicrobial peptides (AMPs) from housefly (*Musca domestica*) larvae can be used to treat pullorum disease. The study included AMPs extracted from *Salmonella enteric* serovar Pullorum-infected larvae as well as non-infected ones (referred to as induced-AMPs and non-induced AMPs, respectively). Tests were then conducted to determine (i) the activity of these AMPs against *S. pullorum* and (ii) the effects of the AMPs on intestinal *Lactobacillus acidophilus* and mucosa epithelial cells in *S.* pullorum-infected chicks. The results showed that *S.* pullorum-induced AMPs and non-induced AMPs both exhibited antimicrobial activity against *S. pullorum*. Small intestinal *L. acidophilus* populations in convalescent chicks that had been treated with induced AMPs showed similar patterns to those in healthy chicks. Induced AMPs also had relatively little effect on the number of mast cells, lymphocyte cells, and goblet cells in the small intestine of convalescent chicks compared with healthy chicks. In contrast, treatment with antibiotics generally reduced the number of all three cell types, especially in the duodenum. In conclusion, AMPs from housefly larvae offer potential for effective treatment of *S.* pullorum-infected chickens without the harmful side effects of antibiotics.

Keywords: *Larvae, Antimicrobial peptide, Salmonella Pullorum, Intestinal, Epithelial cells*

Karasinekteki *(Musca domestica)* Antimikrobiyal Peptidler *Salmonella pullorum* ile Enfekte Tavuklarda Bağırsak *Lactobacillus acidophilus* ve Mukozal Epitel Hücrelerini Etkiler

Özet

Kanatlı üretiminde en ciddi bağırsak hastalıklarından birine neden olan Pullorum hastalığı genellikle enfekte tavukların yemlerine antibiyotik ilavesi ile tedavi edilir. Antibiyotikler genellikle hastalığa karşı oldukça etkili olmakla birlikte ince bağırsak florasına ve mukozaya zarar vermektedir. Bu çalışmanın amacı; karasinek *(Musca domestica)* larvasındaki antimikrobiyal peptidlerin (AMP) pullorum hastalığının tedavisinde kullanılıp kullanılmayacağının belirlenmesidir. Çalışmada *Salmonella enteric* serovar Pullorum-enfekte (indüklenmiş AMP) ve enfekte olmayan (indüklenmemiş AMP) larvalardan ekstrakte edilen AMP kullanıldı. Çalışmada; (i) *S. pullorum*'a karşı AMP aktivitesi ve (ii) *S. pullorum*-enfekte civcivlerde bağırsak Lactobacillus acidophilus ve mukoza epitel hücrelerinde AMP etkileri araştırıldı. Elde edilen sonuçlar *S. pullorum* indüklenmiş AMP ve indüklenmemiş AMP'in her ikisinin de *S. pullorum*'a karşı antimikrobiyal aktivite gösterdiğini ortaya koymuştur. İndüklenmiş AMP uygulanarak tedavi edilen civcivlerin ince bağırsak *L. acidophilus* popülasyonu sağlıklı civcivlerinki ile benzerlik göstermekteydi. İndüklenmiş AMP; tedavi edilen civcivlerin ince bağırsak mast hücre, lenfosit ve goblet hücre sayılarında sağlıklı civcivler ile karşılaştırıldığında göreceli olarak az miktarda etkiye neden oldu. Aksine antibiyotik uygulaması özellikle duodenumda olmak üzere her üç hücre tipi sayısında genellikle düşmeye neden oldu. Sonuç olarak, karasinekten elde edilen AMP *S. pullorum* ile enfekte tavukların tedavisinde zararlı yan etkileri olmaksızın kullanılabilecek potansiyele sahiptir.

Anahtar sözcükler: *Larva, Antimikrobiyal peptid, Salmonella Pullorum, Bağırsak, Epitel hücresi*

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INTRODUCTION

Salmonella infection is caused by a variety of *Salmonella* species [1]. More than 2.550 *Salmonella* serotypes have been reported, mostly belonging to *S. enterica* [2]. Pullorum disease caused by *S. enterica* serovar. Pullorum is one of the most serious poultry diseases in the world. *S.* Pullorum first infects the intestinal tract of chickens. The main clinical symptoms of pullorum disease in chickens are listlessness and white diarrhea [3]. Chickens can be infected at any age. Two to three weeks old chicks have the highest morbidity and mortality. Pullorum disease tends to be either chronic in adult chickens or latent without obvious symptoms [4-6]. The disease is extremely difficult to cure because the bacteria can be carried for long periods, resulting in persistent infection [7].

Intestinal floraisvital to chicken health [8]. *S.* Pullorum infection can cause lesions and damage villi in the small intestine. Antibiotics are the main means of controlling pullorum disease [9]. However, bacterial resistance to antibiotics has increased due to long-term use and overuse [10]. More than 2000 antibiotic resistant strains of *S.* Pullorum were identified worldwide between 1962 and 2007 [11].

The substitution of antimicrobial peptides (AMPs) for antibiotics is one way to prevent the development of resistant microbial strains [12-15]. However, several studies have shown that AMPs have no real advantage compared with traditional antibiotics [16-18]. In recent years, AMPs have been used as a feed additive to prevent *S.* Pullorum infection.

AMPs are polypeptides produced by an organism to protect it from infection by pathogenic microorganisms [19]. AMPs have broad spectrum antimicrobial activity and, furthermore, resistance to AMPs is not easily developed [12,20-22]. AMPs have been isolated from a variety of organisms including insects, plants, and vertebrates. Insects, which have the greatest number of species in the animal world, can secrete many kinds of AMPs [23]. The housefly *(Musca domestica)* is surrounded from the larval to adult stages by many different pathogens [24]. Some researchers have attributed the unique pathogen resistance of houseflies to AMPs which they secrete [25,26].

The purpose of this study was to determine the efficacy of AMPs from housefly larvae for treatment of chicks with pullorum disease. The specific objectives were (i) to determine the bacteriostatic activity and minimum inhibitory concentration (MIC) of the AMPs toward *S. pullorum* and (ii) to confirm that the AMPs have therapeutic effect by comparing intestinal *L. acidophilus* populations and mucosal epithelial cell numbers in healthy chicks with those in *S. pullorum*-infected chicks treated with AMPs. The effects of the AMPs were also compared with those of gentamycin sulfate, an antibiotic that is commonly used to treat pullorum disease. The results of this experiment should provide information about the potential use of AMPs from housefly larvae as a feed additive.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University. All chickens were housed and euthanized in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Bacteria

S. Pullorum (CVCC578) was purchased from the China Institute of Veterinary Drug Control. The standard strains were cultured in Luria broth (LB) at 37ºC until the logarithmic growth phase was reached. The bacterial cells were collected by centrifugation (8.000 g, 5 min) and the cell concentration was adjusted to 1×10^7 CFU/mL.

Crude Extractions of Housefly AMPs

The housefly larvae used in this study were obtained from the Insect Laboratory, College Agronomy, Shihezi University. To induce the production of *S.* Pullorum-specific AMPs, the larvae were pricked with a needle that had been dipped into the suspension of *S.* Pullorum cells described above. The AMPs from this group will be referred to as *S.* Pullorum-induced AMPs [27]. A second group of larvae were pricked with a needle that had been dipped into distilled water. The AMPs from this group will be referred to as non-induced AMPs. The larvae were then put into an incubator for 24 h at 25ºC and 60% relative humidity.

The AMP was crudely extracted from the larvae using a modification of the method described by Gang et al.[28]. Briefly, the larvae were surface sterilized in 75% ethanol, washed with sterile water, and then dried. The larvae were homogenized in a mixture of 0.05 mol/L of ammonium acetate buffer (pH 5.0), 0.35 µg/mL PMSF, 0.2 mg/L EDTA, and 2% β-mercaptoethanol at a ratio of 1 mg larvae to 3 mL solution. The homogenate was centrifuged twice at 12000g for 30 min at 4ºC. The supernatant was decanted and then heated in a boiling water bath for 10 min. After rapid cooling, the samples were centrifuged at 12.000 g for 30 min at 4ºC in ultrafiltration tubes (molecular weight cut-off of 3 kDa). The supernatant was then stored at -80ºC.

Antibacterial Activity Assays

Antimicrobial activity was determined by the standard agar plate method [29]. Paper disks were soaked for 30 min in solutions containing either (i) *S.* Pullorum-induced AMPs, (ii) non-induced AMPs, or (iii) gentamycin sulfate antibiotic. *S.* Pullorum cells were spread evenly onto the surface of solid LB nutrient medium with a sterile glass-spreading

rod. The paper disks were placed on the surface of the medium after they were completely dry. The inhibition zones were measured after 24 h culture at 37°C. The areas of the inhibition zones were calculated to quantify the relative activity of each treatment against *S.* Pullorum. The interpretive criteria were as follows: low susceptible, inhibition zone diameter ≤10 mm; intermediate, 10 to 14 mm; susceptible, 14 to 19 mm; and highly susceptible ≥19 mm [28].

The minimum inhibitory concentration (MIC) of the *S.* Pullorum-induced AMPs was determined using the broth-double dilution method [30]. Normal saline solution (2.5 mL of 0.9% NaCl), and LB medium (2.5 mL) were added to sterile tubes containing 10^4 colony forming units (CFU) of *S.* Pullorum. *S.* Pullorum-induced AMPs were added to the tubes in 0.5 mg/L increments from 0 to 5.0 mg/L. In the control group, gentamycin sulfate was substituted for the AMPs. The tubes were incubated at 37°C for 48 h on a rotary shaker. The MIC was defined as the lowest peptide concentration causing the complete inhibition of *S.* Pullorum growth.

Artificial Infection Experiment

Specific pathogen-free (SPF) male chicks were purchased from a local hatchery. The chicks were raised in cages with *ad libitum* access to food and water. When they were 14-days-old, the chicks were randomly divided into five treatment groups of 50 chicks each. The chicks in four groups were injected with 2 mL of *S.* Pullorum suspension (1×10^7 CFU/mL) into the chest cavity [31]. All of the chicks presented symptoms of pullorum disease (i.e., diarrhea) 24 h after injection. The fifth group (referred to as the healthy group) was not injected with *S.* Pullorum, and *S.* Pullorum was replaced by injected the normal saline with the same dosage.

The *S.* Pullorum-infected chicks were treated in four different ways. One group was treated with *S.* Pullorum-induced AMP. Another group was treated with non-induced AMP. The chicks in these two groups were given 3 mL of the crude AMP extract (1 mg AMP/mL) daily. A third group was fed live housefly larvae. The AMP content of the housefly larvae was 0.5 μg AMP/g fresh weight. The AMP dosage was adjusted so that it was the same as that in the two AMP extract treatments. The fourth group was treated with 100 mg/L gentamycin sulfate antibiotic in the drinking water according to the manufacturer's instruction. These four treatments continued for 3-5 d until the disease symptoms disappeared. The healthy chicks (i.e., the fifth group) received normal food and water. The chicks were slaughtered 3, 5, and 7 d after the above treatments were started and their intestinal tracts were examined as described below.

Sample Collection

- *Isolation of Lactobacillus acidophilus: L. acidophilus* was isolated by washing the contents from the small intestine of each chick with normal saline solution under aseptic conditions. The samples were serially diluted 7 to 9 fold with saline solution and then plated onto De Man, Rogosave Sharpemrs(MRS) culture medium. The cultures were incubated for 24 h at 37°C.

- *Small Intestine Tissue Sections:* Four cm long sections of the duodenum, jejunum, and ileum were excised and then immediately put into 4% formalin and fixed for 72 h. The samples were then paraffin-embedded according to methods described by Watters et al.[32] and Alketa et al.[33]. Briefly, the tissue specimens on the surface of the formaldehyde were washed with tap water, dehydrated with graded alcohol, washed twice within xylene, and then embedded in paraffin. Xylene was used to remove wax and then the samples were rehydrated with graded alcohol. Tissue samples were cut into 5 μm thick sections using a histotome.

Five sections from each sample were dyed. The tissue sections were floated on distilled water, collected onto clean glass slides, dried in an oven, and then stained with hematoxylin and eosin (HE) and toluidine bluestain (0.8% toluidine blue, 0.6% potassium permanganate, dissolved in boil distilled water). The samples were decolorized and then sealed with neutral gum. The morphology of the small intestine sections was observed under an optical microscope. Five visual fields (1392 nm ×1040 nm) were randomly selected. The average positive cell number was regarded as the total cell number.

Statistical Analysis

Statistical analyses were performed using SPSS software version 17.0 (IBM, Armonk, NY). Independent *t*-tests and one-way ANOVA were used to analyze changes in the inhibition zone diameter, intestinal *L. acidophilus* populations, and the numbers of intestinal mucosal epithelial cells. Differences were considered to be significantly different when $P<0.05$.

RESULTS

Antibacterial Activity of AMP

The antibacterial activity of AMP against *S.* Pullorum was confirmed using the disc diffusion method. The inhibition zone diameters decreased significantly in the order antibiotic >*S.* Pullorum-induced AMPs>non-induced AMPs *(Table 1)*. The inhibition zone diameter of gentamycin sulfate was 11.76% greater than that of *S.* pullorum-induced AMP group ($P<0.05$) and 11.84% greater than that of non-induced AMP ($P<0.01$).

The MIC of AMPs against *S.* Pullorum was determined using liquid LB agar containing different concentrations of *S.* Pullorum-induced AMP and gentamycin sulfate *(Table 2)*. The MIC of induced AMP was 3.0 mg/L whereas that of gentamycin sulfate was 2.0 mg/L.

Changes in Intestinal L. acidophilus Populations

The populations of *L. acidophilus* in different parts of the small intestine are shown in *Fig. 1*. *L. acidophilus* numbers in the duodenum increased in the healthy, induced-AMP, and non-induced AMP groups between d 3 and 7. There was no significant difference between the healthy and non-induced groups on d 3. Moreover, the temporal changes in *L. acidophilus* numbers were similar in the induced AMP group and the healthy group. In contrast to the AMP groups, *L. acidophilus* numbers in the larvae-fed and antibiotic groups decreased with time. This meant that antibiotic reduced *L. acidophilus* numbers in the duodenum of convalescent chicks compared with healthy chicks, whereas AMP had little effect.

L. acidophilus numbers in the jejunum of the induced AMP group were similar to those in healthy chicks. *L. acidophilus* numbers remained steady or decreased with time in the other three treatments groups. In the ileum, *L. acidophilus* numbers increased with time in the induced-AMP, larvae, and healthy groups. The pattern of change in the induced-AMP was similar to that in healthy chicks. In conclusion, *S.* Pullorum induced AMP had relatively little effect on intestinal *L. acidophilus* in convalescent chicks ($P>0.05$).

Table 1. *Inhibition of S. Pullorum by antimicrobial peptides (AMPs) and gentamycin sulfate antibiotic*

Group	Diameters of Inhibition Zone
Non-induced AMPs (control)	22.8 ± 1.47^{Aa}
S. Pullorum-induced AMPs	25.5 ± 0.87^{b}
Gentamycin sulfate	28.5 ± 0.96^{Ba}

$^{A-B}$ *Values with different superscripts are significantly different at P<0.05*

Table 2. *The colony forming ability of S. Pullorum as affected by antimicrobial peptides (AMPs) and gentamycin sulfate antibiotic*

Concentration (mg/L)	*S.* Pullorum
S. Pullorum-induced AMPs	
5.0	-
4.5	-
4.0	-
3.5	+
3.0	+ +
2.5	+ +
2.0	+ +
1.5	+ +
1.0	+ +
0.5	+ +
0	+ +
Gentamycin sulfate	
5.0	-
4.5	-
4.0	-
3.5	-
3.0	-
2.5	+
2.0	+ +
1.5	+ +
1.0	+ +
0.5	+ +
0	+ +

Note:"-":no colony; "+":microcolony; "+ +":normal colony

Intestinal Morphology

Mast Cells: The number of mucosal mast cells increased from the duodenum to the jejunum to the ileum on all sample dates *(Fig. 2)*. In the duodenum, the healthy group had the most mucosal mast cells in the duodenum among all treatments. The induced-AMP group had the second most mast cells on d 3 and 5 (10.14%-11.59% less than the healthy chicks). The larvae group had the fewest mast cells on d 3 and 5. There was no significant difference in mast cell number among the four groups of *S.* Pullorum-infected chicks (i.e, gentamycin sulfate, larvae, non-induced AMPs, and induced-AMPs) on d 7. In the jejunum, there was no significant difference in mast cell number between the induced-AMP group and the healthy chicks on any date. The induced-AMP group had significantly more mast cells than (i) the non-induced AMP group on d 3 (5.15% more) and (ii) the larvae group on d 3 and 5 (13.97%-18.38% more). In the ileum, the gentamycin sulfate group and the induced-AMP group had as many or significantly more mast cells than the healthy group. There was no significant difference in mast cell number between the non-induced AMP group and the healthy chicks on d 5 and 7.

Lymphocyte Cells: The number of intestinal lymphocyte cells increased from the duodenum to the jejunum to the ileum on all sample dates *(Fig. 3)*. There was no significant difference in lymphocyte cell numbers between the induced-AMP group and the healthy chicks in any section of the small intestine on any sampling date. Lymphocyte cell numbers in the non-induced AMP group were as great as or greater than those in the induced-AMP and healthy groups. The exception was in the duodenum on d 3. Lymphocyte cell numbers in the duodenum and the jejunum were lowest in the larvae group on all sampling dates. Lymphocyte cell numbers in the gentamycin sulfate group were intermediate between the AMP groups and the larvae groups in the duodenum and the jejunum.

Goblet Cells: The number of goblet cells increased from the duodenum to the jejunum to the ileum on all sample dates *(Fig. 4)*. In the duodenum, goblet cell numbers in the induced-AMP group were significantly less (1.10%-15.38% less) than those in the healthy group on all sampling dates.

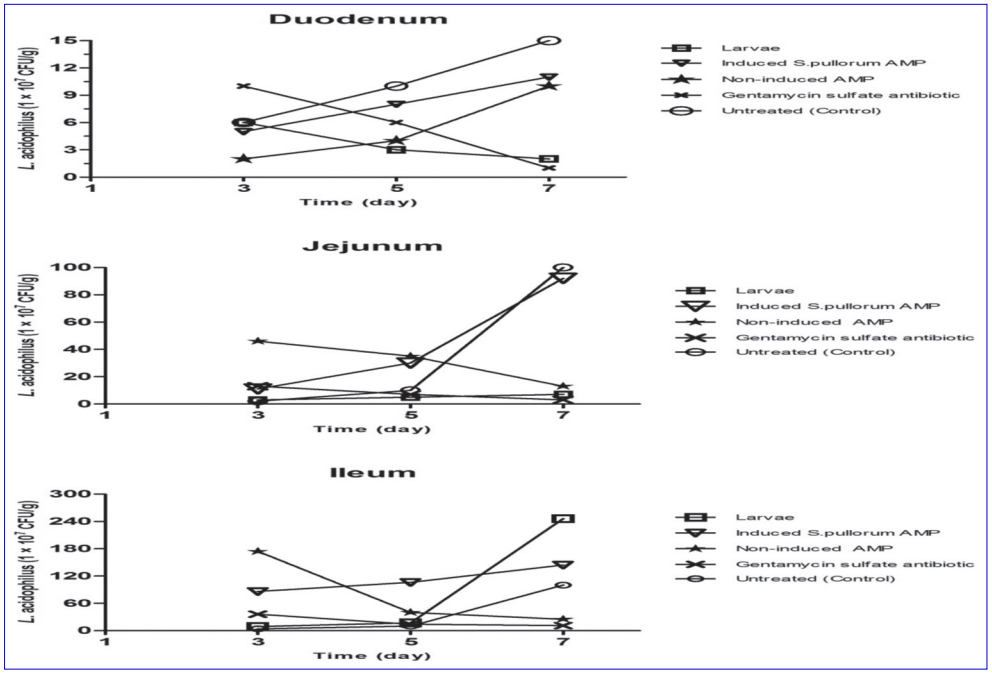

Fig 1. Intestinal *L. acidophilus* among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

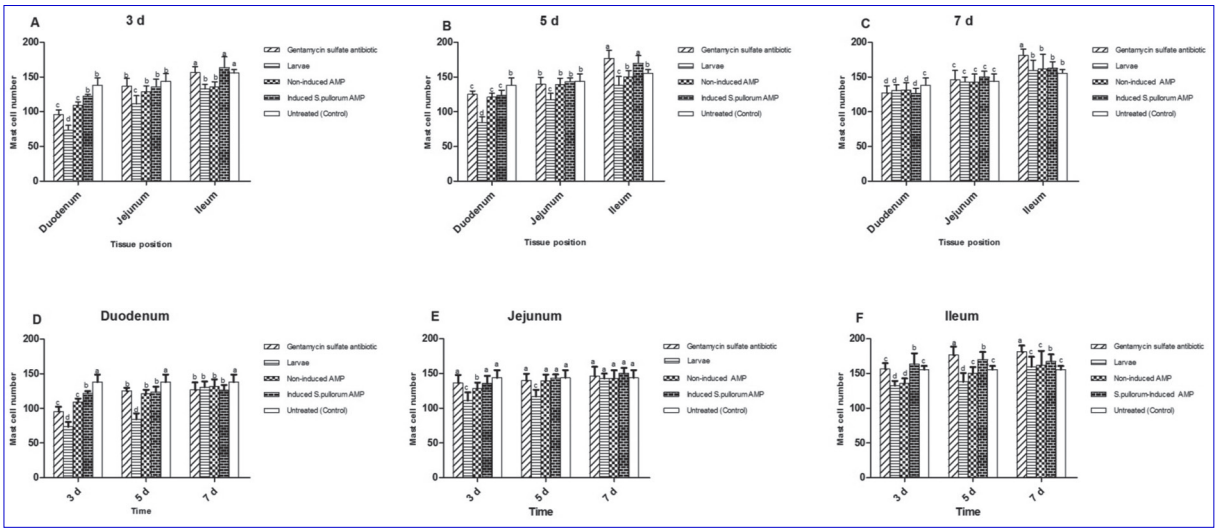

Fig 2. Number of intestinal mucosal mast cells among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

The non-induced AMP group had 3.33% fewer goblet cells than the induced-AMP group on d 3; however there was no significant difference between the two groups on d 5 and 7. The larvae group had the fewest goblet cells in the duodenum on d 3 and 5. There was no consistent pattern to the differences among the treatments in the jejunum and the ileum.

DISCUSSION

AMPs are small, biologically active molecular polypeptides produced by biological organisms after induction by pathogenic microorganisms [12]. AMPs have broad-spectrum antimicrobial activity against a vast variety of foreign pathogens including bacteria, fungi, and viruses. These pathogens are not inclined to develop resistance to AMPs [34]. In contrast, widespread antibiotic use in recent years has led to the development of antibiotic-resistant bacteria. Because of their potential for preventing and treating infections by drug-resistant bacteria, AMPs have received a great deal of research interest in recent years [35].

The activity of housefly AMPs against *S.* Pullorum was tested using the agar plate method *(Table 1)*. *S.* Pullorum-induced and non-induced AMPs both inhibited *S.* Pullorum. The inhibition zone diameter of *S.* Pullorum-induced AMPs was greater than that of non-induced AMPs and

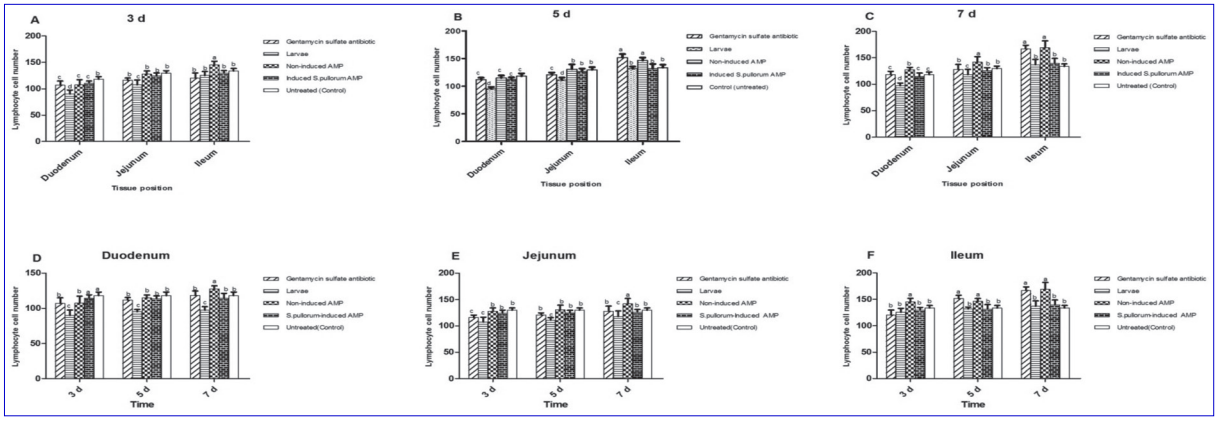

Fig 3. Number of intestinal lymphocyte cells among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

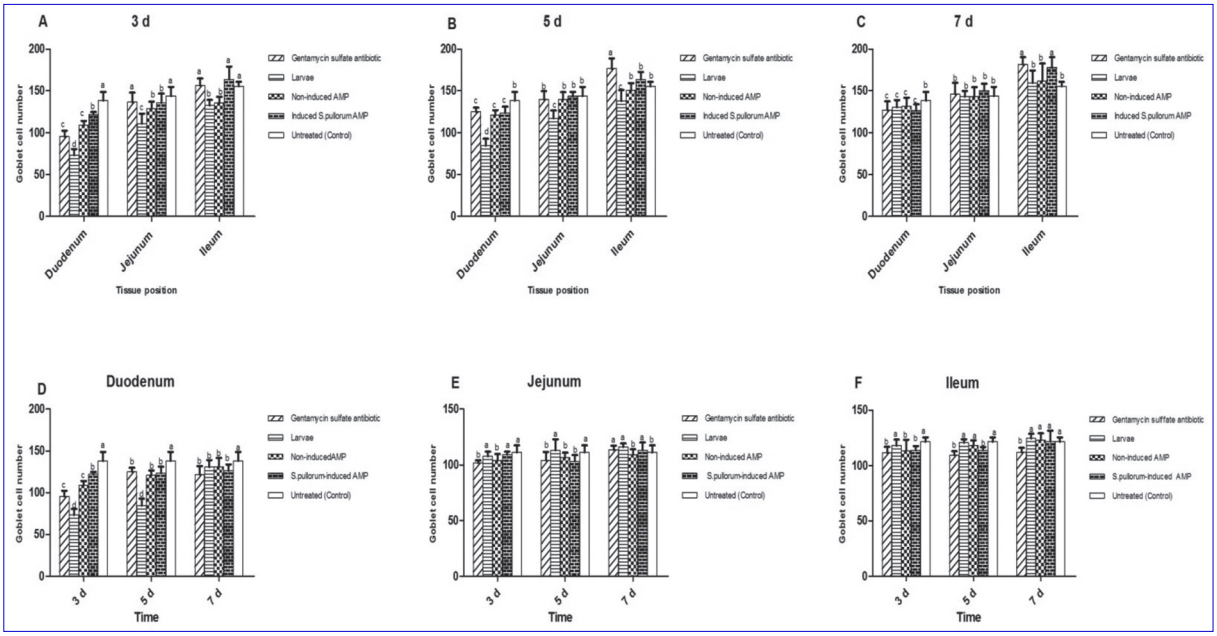

Fig 4. Number of intestinal goblet cells among groups given antibiotic and AMPs to treat *S.* Pullorum infection. The untreated group consisted of healthy (i.e, non-infected) chicks. Error bars represent standard deviation. Different letters indicate significant differences at P<0.05

close to that of gentamycin antibiotic. This indicated that *S.* Pullorum infection induces housefly larvae to produce AMPs with increased bioactivity against *S.* Pullorum. This agrees with a previous report that bacterial infection and injury induced AMP secretion in *Calliphora vicina* larvae [36].

L. acidophilus is an important intestinal bacteriumin healthy chickens [37]. This probiotic bacterium improves and adjusts the balance among intestinal microflora, thereby enhancing immunity, preventing infection, and preventing inflammation in small intestinal mucosa [38]. The present study showed that feeding chicks with *S.* Pullorum-induced AMP not only cured *S.* Pullorum-infected chickens but also had no significant effect on intestinal *L. acidophilus* populations. In contrast, the antibiotic, larvae, and non-induced AMP treatments reduced *L.acidophilus* in the small intestine, perhaps by damaging small intestinal mucosa. Additional study needs to be done to confirm this hypothesis.

The intestinal mucosal barrier includes both a mechanical barrier and an immunological barrier. Mucosal immune cells include mast cells, lymphocytes, and goblet cells. Mast cells originate from hematopoietic stem cells in bone marrow [39]. Mast cells can modulate the host's innate immune response for phagocytesis of Gram-negative bacteria. Mast cells may alter intestinal homeostasis and enhance intestinal permeability during parasite infections of the gastrointestinal tract [40]. In this test, AMPs, antibiotics, and larvae were administered orally to *S.* Pullorum-infected chickens and then changes in mast cell numbers were

observed across time. The results showed no significant difference in mast cell numbers between convalescent chicks after treatment with AMPs and healthy chicks ($P>0.05$). In contrast, the antibiotic and larvae treatments significantly reduced mast cells numbers in convalescent chicks ($P<0.01$). Overall, the results indicate that the AMPs had no significant effect on intestinal mucosal mast cells after treatment.

Lymphocyte cells protect intestinal mucosal immunity. Many autoimmune diseases as well as intestinal diseases are related to either declines in the number of lymphocyte cells or to their dysfunction [41-43]. In our study, the number of lymphocyte cells increased from the duodenum to ileum in all treatments on all sampling dates. Furthermore, the number of lymphocyte cells in the convalescent chicks increased from d 3 to 7. This indicated that *S.* Pullorum infection increased lymphocyte cell numbers in the small intestine, enhancing the immunity of the chicks. There was no significant difference in the number of lymphocyte cells between the induced-AMP group and the healthy group on d 7.

Goblet cells are glands which secrete glycoprotein. Goblet cells protect the intestinal epithelium and play an important role in the gut immunity of neonatal animals before passive immunization [44]. Goblet cells are sentinel cells which help to expel bacteria by stimulating mucus secretion from adjacent crypt cells [45]. In this study, the number of goblet cells increased from the duodenum to the ileum in all treatments. Furthermore, the number of goblet cells in each section of the small intestine increased slightly between d 3 and 7 in convalescent chicks. Goblet cell numbers were much less in convalescent chicks in the antibiotic and larvae groups than in healthy chicks. In contrast, goblet cell numbers in the AMP groups were similar to those in healthy chicks.

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REFERENCES

1. LutfulKabir SM: Avian colibacillosis and salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int J Environ Res Public Health*, 7, 89-114, 2010. DOI: 10.3390/ijerph7010089

2. Calenge F, Kaiser P, Vignal A, Beaumont C: Genetic control of resistance to salmonellosis and to salmonella carrier-state in fowl: A review. *Genet Sel Evol*, 42, 1-11, 2010. DOI: 10.1186/1297-9686-42-11

3. Volf J, Stepanova H, Matiasovic J: *Salmonella enterica* serovar typhimurium and enteritidis infection of pigs and cytokine signalling in palatinetonsils. *Vet Microbiol*, 156, 127-135, 2012. DOI: 10.1016/j.vetmic.2011.10.004

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

5. Shivaprasad HL: Fowl typhoid and pullorum disease. *Rev Sci Tech*, 19 (2): 405-424, 2000.

6. Gong JS, Xu M, Zhu CH: Antimicrobial resistance, presence of integrons and biofilm formation of *Salmonella pullorum* isolates from eastern China (1962-2010). *Avian Pathol*, 42, 290-294, 2013. DOI: 10.1080/03079457.2013.788129

7. Pan ZM, Geng SZ, Zhou YQ: Prevalence and antimicrobial resistance of *salmonella* sp. isolated from domestic animals in eastern China. *J Anim Vet Adv*, 9, 2290-2294, 2010. DOI: 10.3923/javaa.2010.2290.2294

8. Chambers JR, Gong J: The intestinal microbiota and its modulation for *Salmonella* control in chickens. *Food Res Int*, 44, 3149-3159, 2011. DOI: 10.1016/j.foodres.2011.08.017

9. Barrow PA, FreitisNeto OC: Pullorum disease and fowl typhoid-new thoughts on old diseases: A review. *Avian Pathol,* 40, 1-13, 2011. DOI: 10.1080/03079457.2010.542575

10. Sarmah, AK, Meyer MT, Boxall, AB: A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics in the environment. *Chemosphere*, 65, 725-759, 2006. DOI: 10.1016/j.chemosphere.2006.03.026

11. Pan ZM, Wang XQ, Zhang XM, Liu XF: Changes in antimicrobial resistance among *Salmonella enteric* subspecies *enteric* serovar Pullorum isolates in China from 1962 to 2007. *Vet Microbiol*, 136, 387-392, 2009. DOI: 10.1016/j.vetmic.2008.11.015

12. Zasloff M: Antimicrobial peptides of multicellular organisms. *Nature*, 415, 389-395, 2002. DOI: 10.1038/415389a

13. Hancock RE, Sahl HG: Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol*, 24, 1551-1557, 2006. DOI: 10.1038/nbt1267

14. Hull R, Katete R, Ntwasa M: Therapeutic potential of antimicrobial peptides from insects. *Biotechnol Mol Biol Rev*, 7, 31-47, 2012.

15. Joerger RD: Alternatives to antibiotics: Bacteriocins, antimicrobial peptides and bacteriophages. *Poult Sci*, 82, 640-647, 2003. DOI: 10.1093/ps/82.4.640

16. Peschel A, Sahl HG: The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol*, 4, 529-536, 2006. DOI: 10.1038/nrmicro1441

17. Nizet V: Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr Issues Mol Biol*, 8, 11-26, 2006.

18. Chernysh S, Gordya N, Suborova T: Insect antimicrobial peptide prevent resistance development in bacteria. *PlosOne*, 10, e0130788, 2015. DOI: 10.1371/journal.pone.0130788

19. Seo MD, Won HS, Kim JH, Lee BJ: Antimicrobial peptides for therapeutic applications: A review. *Molecules*, 17, 12276-12286, 2012. DOI: 10.3390/molecules171012276

20. Marr AK, Gooderham WJ, Hancock RE: Antibacterial peptides for therapeutic use: Obstacles and realistic outlook. *Curr Opin Pharmacol,* 6, 468-472, 2006. DOI: 10.1016/j.coph.2006.04.006

21. Mygind PH, Fischer RL, Schnorr KM: Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature*, 437, 975-980, 2005. DOI: 10.1038/nature04051

22. Hof W, Veerman EC, Helmerhorst EJ, Amerongen AV: Antimicrobial peptides: Properties and applicability. *Biol Chem*, 382, 597-619, 2001. DOI: 10.1515/BC.2001.072

23. Yi HY, Huang YD, Yu XQ: Insect antimicrobial peptides and their applications. *Appl Microbiol Biotechnol*, 98, 5807-5822, 2014. DOI: 10.1007/s00253-014-5792-6

24. Pei ZH, Sun XN, Ma HX: Cloning, expression, and purification of a new antimicrobial peptide gene from *Musca domestica* larva. *Gene*, 549, 41-45, 2014. DOI: 10.1016/j.gene.2014.07.028

25. Ai H, Wang FR, Xia YQ, CL Lei: Antioxidant, antifungal and antiviral activities of chitosan from the larvae of housefly, *Musca domestica*. *Food Chem*, 132, 493-498, 2012. DOI: 10.1016/j.foodchem.2011.11.033

26. Karen M, Elke C, Liliane S: Identification of 1-lysophosphatidylethanol-

amine (C (16:1)) as an antimicrobial compound in the housefly, *Musca domestica*. *Insect Biochem Mol Biol*, 34, 43-49, 2004. DOI: 10.1016/j.ibmb.2003.09.001

27. Anne-Kathrin P, Heiko V, Jochen W, Andreas V: Antimicrobial peptides expressed in medicinal maggots of the blow fly *Lucilia sericata* show combinatorial activity against bacteria. *Antimicrob Agents Chemother*, 59, 2508-2514, 2015. DOI: 10.1128/AAC.05180-14

28. Zhou G, Wang JG, Shen H: Induction of maggot antimicrobial peptides and treatment effect in *Salmonella pullorum*-infected chickens. *J Appl Poultry Res*, 23, 376-383, 2014. DOI: 10.3382/japr.2013-00804

29. Afonin S, Glaser RW, Berditchevskaia M: 4-fluorophenylglycine as a label for 19FNMR structure analysis of membrane-associated peptides. *Chembiochem*, 4, 1151-1163, 2003. DOI: 10.1002/cbic.200300568

30. Guo BL, Han P, Guo L: The antibacterial activity of ta-doped ZnO nanoparticles. *Nanoscale Res Lett*, 10, 1047, 2015. DOI: 10.1186/s11671-015-1047-4

31. Guo ZH, Ma X, Zhang GZ: Therapeutic effect of Baitouweng San on chicken artificially infected with pullorosis. *J Tradit Chin Vet Med,* 3, 29-31, 2013. DOI: 10.13823/j.cnki.jtcvm.2013.03.009

32. Watters AD, Bartlett MS: Fluorescence in situ hybridization in paraffin tissue sections. *Mol Biotechnol*, 21, 217-220, 2002. DOI: 10.1385/MB:21:3:217

33. Alketa Q, Letizia P, Luljeta D: Spontaneous skin canine tumors: Toluidine blue stain detection of mast cells in tissue section. *Albanian J Agric Sci*, 13, 391-394, 2014.

34. Pal L, Brahmkhatri VP, Bera S: Enhanced stability and activity of an antimicrobial peptide in conjugation with silver nanoparticle. *J Colloid Interf Sci*, 483, 385-393, 2016. DOI: 10.1016/j.jcis.2016.08.043

35. Silva JP, Appelberg R, Gama FM: Antimicrobial peptides as novel anti-tuberculosis therapeutics. *Biotechnol Adv*, 34, 924-940, 2016. DOI: 10.1016/j.biotechadv.2016.05.007

36. Sergey C, Natalia G, Tatyana S: *Salmonella* pullorum infection can induce the AMP production. *Plos One*, 10, 1-15, 2015. DOI: 10.1371/journal.pone.0130788

37. Jahromi MF, Altaher YW, Shokryazdan P, Ebrahimi R: Dietary supplementation of a mixture of *Lactobacillus* strains enhances performance of broiler chickens raised under heat stress conditions. *Int J Biometeorol*, 60, 1099-1110, 2016. DOI: 10.1007/s00484-015-1103-x

38. Dongarra ML, Rizzello V, Muccio L, Fries W, Cascio A: Mucosal immunology and probiotics. *Curr Allergy Asthma Rep*, 13, 19-26, 2013. DOI: 10.1007/s11882-012-0313-0

39. Murakami S, Yoshino H, Ishikawa J, Yamaguchi M: Effects of ionizing radiation on differentiation of murine bone marrow cells into mast cells. *J Radiat Res,* 56, 865-871, 2015. DOI: 10.1093/jrr/rrv061

40. Rashaun AP, Caitlin MT, Shirley L: Mast cells and histamine alter intestinal permeability during malaria parasite infection. *Immunobiol*, 221, 468-474, 2016. DOI: 10.1016/j.imbio.2015.11.003

41. Luo X, Zheng YY, Wen RY, Liao HF: Effects of ceftriaxone induced intestinal dysbacteriosis on lymphocytes in different tissues in mice. *Immunobiol*, 221, 994-1000, 2016. DOI: 10.1016/j.imbio.2016.04.003

42. Maglio M, Florian F, Vecchiet M: Majority of children with type 1 diabetes produce and deposit anti-tissue transglutaminase antibodies in the small intestine. *Diabetes*, 58, 1578-1584, 2009. DOI: 10.2337/db08-0962

43. Fuchs A, Vermi W, Lee JS: Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12 and IL-15 responsive IFN-γ-producing cells. *Immun*, 38, 769-781, 2013. DOI: 10.1016/j.immuni.2013.02.010

44. Knoop KA, McDonald KG, Newberry RD: Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol*, 8, 198-210, 2015. DOI: 10.1038/mi.2014.58

45. Charlotte R: Sentinel goblet cells flush out bacteria from crypts. *Nat Rev Gastroenterol Hepatol*, 13, 438, 2016. DOI: 10.1038/nrgastro.2016.117

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Research Article

Evaluation of Virulence Factors and Phylogrouping of *Escherichia coli* Strains Isolated from Acute Bovine Mastitis in Turkey

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Abstract

The objective of this study was to determine the phylogenetic distribution of commonly studied virulence factors of 155 E. coli isolated from acute bovine mastitis. In the study, A, B1, B2, C, D, E, F phylogroups, and Clade I were defined. B1 and C were found to be the most prevalent phylogroups covering commensal strains with a ratio of 45.2% and 37.4%, respectively. The other phylogroups determined in the study were F, D, A, and E with a range of 0.6%-8.9%. Commonly known virulence genes; *clpg, F17, afa, iucC, iucD, cnf1, cnf2, kps,* and *traT* were selected to determine the virulence factors. The only virulence factor gene existing within the strains was *traT* (66.4%). However, any relation between phylogroups and virulence factors was not determined. Phylogroups C, E and F were found to be new phylogroups for the first time in acute bovine mastitis cases.

Keywords: *Escherichia coli, Bovine mastitis, Phylogroup, Virulence factor*

Akut Sığır Mastitislerinden İzole Edilen *Escherichia coli* Suslarının Filogruplandırılması ve Virulens Faktör Değerlendirmesi

Özet

Çalışmanın amacı akut sığır mastitinden izole edilen 155 E. coli izolatında, yaygın olarak çalışılan virulens faktörlerinin filogenetik dağılımını belirlemekti. İzolatların filogruplandırması yapıldığında, A, B1, B2, C, D, E, F grupları ve Sınıf I tespit edildi. Kommensal suşları oluşturan, B1 ve C sırasıyla, %45.2 ve %37.4 oranla en yaygın filogruplar olarak bulundu. Bu çalışmada, diğer filogruplar, %0.6-%8.9 aralığındaki oranda F, D, A, and E olarak tespit edildi. Virulens faktörlerini belirlemek için en çok bilinen virulens genleri; *clpg, F17, afa, iucC, iucD, cnf1, cnf2, kps* ve *traT* seçildi. *traT*, suşların büyük çoğunluğunda (%66.4) tespit edilen virulens genidir. Filogruplar ve virulens faktörleri arasında herhangi bir ilişki belirlenmedi. Akut sığır mastitis olgularında, C, E. F filogrupları ilk kez tespit edildi.

Anahtar sözcükler: *Escherichia coli, Sığır mastitis, Filogrup, Virulens faktörü*

INTRODUCTION

Escherichia coli is the most common pathogen of acute bovine mastitis worldwide [1-3]. Clinical signs of *E. coli* mastitis differ from severe or even fatal forms to mild mastitis. Association between the virulence factors of the isolate and the clinical severity of mastitis has not been clarified, yet [3].

Escherichia coli have a clonal genetic structure with a low level of recombination which leads *E. coli* strains to be grouped. These groups also have the same phenotypic and genotypic characteristics, ecological niche and ability to cause disease which helps researchers to understand the epidemiology of *E. coli*. According to rapidly expanding multi locus sequence data for *E. coli* isolated from different hosts and habitats, at first mainly four phylogenetic groups A, B1, B2 and D were discovered [4,5]. Most of the commensal and diarrheagenic strains belong to group A and B1 whereas extra-intestinal *E. coli* strains belong to group B2 and D [6,7]. Bovine *E. coli* mastitis isolates had been shown to belong to phylogroup A, mostly constituted commensal (non-pathogenic) strains [8-10]. For determining the new phylogroups in 2013, Clermont *et al.*[11] improved

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their former method [4] and developed a new quadruplex-PCR by using additional *arp*A gene target to *chu*A, *yja*A, TspE4.C2 target genes, and also *trp*A target genes, and enabled to differentiate A, B1, B2, C, D, E, F groups, and Clade I.

Several virulence factors such as adhesion, invasion, capsule production, ability to resist serum complement, siderophores were determined from pathogenic *E. coli* isolated in case of diarrhea, septicemia and meningitis, infection of urinary tract, and mastitis. Virulence factors of *E. coli* have different functions which formed against to host defense system to colonize, multiply and survive in the udder [12]. Several adhesins were detected in *E. coli* isolated from bovine mastitis. The family of F17 fimbriae and *afa* adhesin facilitate the adherence of pathogenic *E. coli* to various host tissues and mammary gland as well. The family of F17 (F17A) fimbriae comprising F17a-A, F17b-A, F17c-A and F17d-A also fimbrial adhesin *afa* facilitate the adherence of *E. coli* to various host tissues were frequently detected in *E. coli* from bovine mastitis [13-15]. CS13A adhesin encoding *clp*G gene has been detected in *E. coli* isolates from bovine mastitis and bovine with septicemia or diarrhea [16]. Siderophores are the important components for the virulence of *E. coli* strains. The presence of virulence genes (*iuc*ABCD) encoding aerobactin iron uptake system was reported significantly higher in *E. coli* isolates from bovine mastitis than fecal isolates [17]. The *tra*T gene encoding an outer membrane protein plays an important role in serum resistance was reported in mastitic milk in high proportions [18]. Capsular polysaccharide gene reported in coliform mastitis is known to be related to the resistance to serum bactericidal activity and opsonization [19]. Cytotoxic necrotizing factors (CNF) are putative virulence factors of pathogenic *E. coli* strains. CNF1 and CNF2 producing isolates termed as necrotoxigenic *E. coli* (NTEC) are associated with enteritis and intestinal infections, mastitis, pneumonia and metritis [20,21].

The purpose of this study was to determine the phylogenetic groups and the most common virulence genes (*tra*T, *F17*, *afa*, *iuc*C, *iuc*D, *cnf*1, *cnf*2, *clpg*, *kps*) of *E. coli* strains isolated from acute bovine mastitis.

MATERIAL and METHODS

Escherichia coli Strains

A total of 155 *E. coli* strains was isolated from acute bovine mastitis and identified according to conventional biochemical tests [22]. *Escherichia coli* positive control strains for phylogrouping were obtained from culture collection of Ankara University Faculty of Veterinary Medicine Department of Microbiology. *Salmonella* Typhimurium 2696 for *tra*T, *E. coli* AVMC95-03 for *F17*; *E. coli* AVMC95-07 for *cnf*1; *E. coli* S5 for cnf2; *E. coli* 31A for *clpg*; *E. coli* AVMC95-05 for *afa*; *E. coli* U9-41 for *kps*; *E. coli* AVMC95-18 for *iuc*D and *iuc*C were used as positive control strains for PCR.

Determination of Phylogroups and Virulence Genes

DNA extraction from *E. coli* strains was performed by DNA isolation kit (QIAamp DNA Mini Kit; Qiagen, Cat no: 51104) according to the manufacturer's instructions. Isolated DNAs were served as template for determining phylogroups and virulence genes of *E. coli*. PCR for determining the phylogroups of *E. coli* was performed as previously described by Clermont *et al*.[11].

The genes of *iuc*D, and *cnf*1 were examined by a multiplex PCR as described by Yamamoto *et al*.[23]. The *iuc*C gene was analyzed by PCR as described by Bingen *et al*.[24]. The presence of *F17* and *clp*G genes were analyzed by PCR as stated by Bertin *et al*.[14] and Bertin *et al*.[16], respectively. C*nf*2 gene was determined by the procedure described by Kaipainen *et al*.[12]. The PCR procedures described by Yamamoto *et al*.[23] and Johnson *et al*.[25], previously were used to determine *afa* and kps, respectively. The PCR products were analyzed in 1.5% agarose gel containing ethidium bromide. The oligonucleotide primers used for amplification of the genes and expected size of products were presented in *Table 1*.

RESULTS

Of 155 *E. coli* isolates examined by Quadruplex-PCR [11] from bovine mastitis, 71 (45.2%) were found to belong to group B and 58 (37.4%) were found to belong to group C. Nine (5.7%) *E. coli* isolates were determined in group A, 2 (1.2%), 14 (8.9%), and 1 (0.6%) *E. coli* isolates were found to belong to groups D, E, and F, respectively *(Table 2)*.

Among the isolates, 148 out of 155 possess either one or more virulence factors examined in the study. The virulence genes associated with adhesion *(clpg, F17, afa)*,

Table 1. *Oligonucleotide primers used to determine the virulence genes*

Gene	Primer Sequence	PCR Product Size (bp)	References
clpG	GGGCGCTCTCTCCTTCAAC CGCCCTAATTGCTGGCGAC	402	Bertin *et al*.[18]
cnf1	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	Yamamoto *et* al.[23]
cnf2	ACTGAAGAAGAAGCTGGGAATA ATAAGTTGAGCCGAGCGAGG	654	Kaipainen *et al*.[12]
F17	GCAGAAAATTCAATTTATCCTTGG CTGATAAGCGATGGTGTAATTAAC	537	Bertin *et al*.[14]
*iuc*D	TACCGGATTGTCATATGCAGACCG AATATCTTCCTCCAGTCCGGAGAAG	602	Yamamoto *et al*.[23]
*iuc*C	AAACCTGGCTTACGCAACTGT ACCCGTCTGCAAATCATGGAT	269	Bingen *et al*.[24]
*tra*T	GATGGCTGAACCGTGGTTATG CACACGGGTCTGGTATTTATGC	307	Kaipainen *et al*.[12]
kps	GCGCATTTGCTGATACTGTTG CATCAGACGATAAGCATGAGCA	272	Johnson *et al*.[25]
afa	GCTGGGCAGCAAACTATAACTCTC CATCAAGCTGTTTGTTCGTCGCCG	750	Yamamoto *et al*.[23]

Table 2. *Phylogroup distribution of E. coli strains (number 'N' and percentage '%')*

Parameter	Phylogroups					
	A	B1	C	D	E	F
N of isolates and (%)	9 (5.73)	71 (45.2)	58 (37.4)	2 (1.27)	14 (8.91)	1 (0.63)

Table 3. *Frequency of virulence genes among each phylogroups of E. coli*

Gene	Number Positive Isolates (n=155) and Percentage (%)	Number of Virulence Genes Among Each Phylogroups					
		A	B1	C	D	E	F
traT	103 (66.4)	4	52	36	1	9	1
cnf2	4 (2.5)	0	3	0	0	1	0
afa	15 (9.6)	1	8	4	0	1	1
iucD	9 (5.8)	0	4	5	0	0	0
iucC	9 (5.8)	1	3	5	0	0	0
clpg	1 (0.6)	0	0	0	0	0	1
kps	2 (1.2)	0	0	0	1	1	0
F17	5 (3.2)	0	2	2	0	1	0
cnf1	0 (0)	0	0	0	0	0	0

Table 4. *Patterns of virulence factors within each phylogroups*

Group	Number of Isolates	Patterns of Virulence Genes
B1	1	*traT*, *iucC*, *iucD*, *afa*,
B1	1	*traT*, *iucC*, *afa*
B1	1	*traT*, *iucC*, *iucD*
B1	5	*traT*, *afa*
B1	2	*traT*, *F17*
B1	2	*traT*, *cnf2*
C	2	*traT*, *iucC*, *iucD*, F17
C	3	*traT*, *iucC*, *iucD*
C	4	*traT*, *afa*
C	1	*traT*, *iucD*
E	1	*traT*, cnf2, *afa*
E	1	*traT*, kps
E	1	*traT*, F17
A	1	*traT*, *afa*, *iucC*
F	1	*traT*, *afa*, *clpg*

aerobactin production *(iucC, iucD)*, cytotoxic necrotizing factors *(cnf1, cnf2)*, capsule production *(kps)*, and serum resistance *(traT)* were found to be with a range of 0.6%-66.4%. None of the isolates were had *cnf*1 gene. The most prevalent virulence gene from acute bovine mastitis was *traT*, coding serum resistance with a percentage of 66.4. The genes associated with adhesion were ranging from 0.6% to 9.6%. Each aerobactine production genes were determined to be 5.8%. The remaining virulence genes, *cnf2* and *kps* were determined as 2.5% and 1.2%, respectively. The distribution of virulence genes among the phylogroups were summarized in *Table 3* in detail. The combination of virulence factors with phylogroups determined in the study was shown in *Table 4*.

DISCUSSION

The most prevalent phylogroups determined in this study were B1 and C according to Clermont *et al*.[11]'s phylogrouping. Following dominant phylogroups from acute bovine mastitis in this study were determined as E and A phylogroups. The least two phylogroups determined from acute bovine mastitis were found to be D and F. Most of the commensal and intestinal strains have been reported as group A and B1, extra-intestinal *E. coli* strains belonged to group B2 and D in other studies [6,7]. In the previous studies, bovine mastitis isolates have been generally belonged to A and B1 groups [8,9,26-28]. However, phylogroup C was defined to be closely related to, but distinct from B1 [29,30]. Phylogroup E was designated as comprising the unassigned strains of O157:H7 which is enterohemorrhagic classified under intestinal pathogenic *E. coli* [11]. According to the results of this study, most of *E. coli* isolates from bovine acute mastitis were determined as commensal and intestinal origin in compatible with the previous studies [17,26,30]. Newly designated phylogroup F has been termed as a sister group to phylogroup B2 [30]. Hence, two isolates in pylogroup D and 1 isolate in phylogroup F were found to be extra-intesinal *E. coli* in this study. Due to being the first time for reporting phylogroups C, E, F from acute bovine mastitis, we could not compare the newly designated phylogroups of mastitic bovine *E. coli*.

Several panels of virulence factors have been studied in the previous studies [3,12,18,28,31]. The most common studied virulence genes representing pathogenic potential were

choosen in the study. Among the virulence genes examined, except *tra*T, the remaining virulence factor genes; *cnf*2, *cnf*1, *iuc*D, *iuc*C, *afa*, F17, clpg, *kps*, were found to be uncommon among the *E. coli* isolates.

Serum resistance is the most studied and commonly reported virulence factor related to bovine mastitis with a range of 16.7% and 99.5% [12,18,28,31-33]. In our study, 66.4% of *E. coli* isolates were determined to have *tra*T gene which causes serum resistance. There are two controversial views about *tra*T gene. One of them assumes that the presence *tra*T gene in a high proportion of mastitis isolates may indicate a role of *tra*T in the pathogenesis of mastitis caused by *E. coli* and other mastitis pathogen species as well [34]. The second one assumes that serum resistance has not been attributed only to mastitis, also environmental strains may carry *tra*T gene [11]. In this respect, the first assumption is more logical that *tra*T is necessary for virulence in the mammary gland.

The prevalence of *cnf*2 found in *E. coli* strains isolated from bovine mastitis was with a range of 3%-17% [3,12,27,35]. In this study, *cnf*1 gene was not detected in *E. coli* isolates from bovine mastitis whereas 2.5% of the isolates were found to be *cnf*2 positive. Similar result was previously described by Kaipainen *et al*.[12] that *cnf*1 was far less prevalent than *cnf*2 in bovine *E. coli* strains. Although *cnf*2 and *cnf*1 genes were investigated in mastitic bovine *E. coli* isolates, the role of *cnf* toxins in the pathogenesis of bovine mastitis is not clarified, but supposed be associated with urinary tract infections and meningitis.

Siderophores are one of the most commonly studied virulence factors [12,36]. Each *iuc*D and *iuc*C genes, responsible for aerobactin production were found to be 5.80% positive. In previous studies, ratio of the presence of aerobactin genes were determined to be approximately 20% [12,17,27,36]. Taking into account of iron acquisition in the pathogenesis of systemic infections, siderophores are well-defined virulence factors in Gram(-) bacteria acquiring iron and enhances the pathogenicity of the microorganism [37]. Aerobactins enables *E. coli* to acquire iron from lactoferrin and iron binding protein in milk, subsequently multiply and cause mastitis. In this study, aerobactin rate was expected to be high within the *E. coli* isolates due to importance acquisition of iron from lactoferrin. However, our findings overlapping a previous study performed by Linggood *et al*.[38] showed that aerobactin is probably not involved in the virulence of *E. coli* isolated from bovine mastitis. We also examined whether the genes *(F17*, *clpg*, and *afa)* encoding adhesion proteins were necessary for the pathogenesis of *E. coli* isolated from acute bovine mastitis or not. *F17* and *clpg* genes were reported to be relevant in *E. coli* isolates from bovine mastitis [14,16,39]. The prevalence of *F17* related gene in other countries from bovine mastitis were reported to be 1% [12] to 20.4% [27]. The prevalence of *clpg* gene from bovine mastitis was determined between 0.78%-29.62% in the previous studies [3,27,28]. In the study, virulence genes, *afa*, *clpg, F17* encoding adhesions were found with a range of 0.6%-9.6%. The low prevalence of these genes encoding adhesion proteins from acute bovine mastitis was interpreted as attachment of bacteria to mammary epithelium may have a less importance in the pathogenesis of acute bovine mastitis [40]. Unless adherence to epithelial tissue of mammary gland, *E. coli* can adjust metabolically to mammary secretion. The last virulence gene, *kps*, examined in the study was determined in two *E. coli* isolates. In compatible with our result, Fernandes *et al*.[28] reported only one isolate possessing *kps* gene.

The agent features of *E. coli* comprising ability to utilize lactose as an energy source and survive in the mammary gland though the oxygen tension is very low, makes itself possible to cause mastitis. Peracute infections due to *E. coli* will not be explained with the role of expected virulence factors such as adhesion proteins, siderophores, cytotoxic necrozan factors, in the pathogenesis of infection. The possible candidate virulence factor for the pathogenesis of bovine mastitis is endotoxin which does not directly effect the secretory cell but distrups the blood flow [41] and cause decreased milk production by systemic and local effects of itself [42].

As a conclusion, the results obtained in this study indicate that neither a specific set of virulence factors nor any phylogroup-virulence factor association was determined in compatible with the former studies [3,12,13,27]. Phylogroups C, E, F were found to be as new phylogroups for the first time in acute bovine mastitis cases.

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REFERENCES

1. Blum S, Heller ED, Krifucks O, Sela S, Hammer-Muntz O, Leitner G: Identification of a bovine mastitis *Escherichia coli* subset. *Vet Microbiol*, 132, 135-148, 2008. DOI: 10.1016/j.vetmic.2008.05.012

2. Correa MGP, Marin JM: O-serogroups, *eae* gene and *EAF* plasmid in *Escherichia coli* isolates from cases of bovine mastitis in Brazil. *Vet Microbiol*, 85, 125-132, 2002.

3. Wenz JR, Barrington GM, Garry FB, Ellis RP, Magnuson RJ: *Escherichia coli* isolates serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. *J Dairy Sci*, 89, 3408-3412, 2006. DOI: 10.3168/jds.S0022-0302(06)72377-3

4. Clermont O, Bonacorsi S, Bingen E: Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*, 66, 4555-4558, 2000. DOI: 10.1128/AEM.66.10.4555-4558.2000

5. Escobar-Paramo P, Le Menac'h A, Le Gall T, Amorin C, Gouriou S, Picard B, Skurnik D, Denamur E: Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environ Microbiol*, 8, 1975-1984, 2006. DOI: 10.1111/j.1462-2920.2006.01077.x

6. Escobar-Paramo P, Clermont O, Blanc-Potard AB, Bui H, Le Bouguenec C, Denamur E: A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. *Mol Biol Evol*, 21, 1085-1094, 2004. DOI: 10.1093/molbev/msh118

7. Girardeau JP, Dalmasso A, Bertin Y, Ducrot C, Bord S, Livrelli V, Vernozy- Rozand C, Martin C: Association of virulence genotype with phylogenetic background in comparison to different seropathotypes of Shiga toxin producing *Escherichia coli* isolates. *J Clin Microbiol*, 43, 6098-6107, 2005. DOI: 10.1128/JCM.43.12.6098-6107.2005

8. Dogan B, Klaessiga S, Rishniw M, Almeida RA, Oliver SP, Simpson K, Schukken YH: Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. *Vet Microbiol*, 116, 270-282, 2006. DOI: 10.1016/j.vetmic.2006.04.023

9. Dosogne H, Meyer E, Sturk A, van Loon J, Massart-Leën AM, Burvenich C: Effect of enrofloxacin treatment on plasma endotoxin during bovine *Escherichia coli* mastitis. *Inflamm Res*, 51, 201-205, 2002. DOI: 10.1007/PL00000293

10. Picard B, Garcia JS, Gouriou S, Duriez P, Brahimi N, Bingen E, Elion J, Denamur E: The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect Immun*, 67, 546-553, 1999.

11. Clermont O, Christenson JK, Denamur E, Gordon DM: The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep*, 51, 58-65, 2010. DOI: 10.1111/1758-2229.12019

12. Kaipainen T, Pohjanvirta T, Shpigel NY, Shwimmer A, Pyörälä S, Pelkonen S: Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. *Vet Microbiol*, 85, 37-46, 2002. DOI: 10.1016/S0378-1135(01)00483-7

13. Lehtolainen T, Pohjanvirta T, Pyörälä S, Pelkonen S: Association between virulence factors and clinical course of *Escherichia coli* mastitis. *Acta Vet Scand*, 44, 203-205, 2003. DOI: 10.1186/1751-0147-44-203

14. Bertin Y, Martin C, Oswald E, Girardeau JP: Rapid and specific detection of F17-related pilin and adhesin genes in diarrheic and septicemic *Escherichia coli* strains by multiplex PCR. *J Clin Microbiol*, 34, 2921-2928, 1996.

15. Girardeau JP, Lalioui L, Said AM, De Champs C, Le Bouguenec C: Extended virulence genotype of pathogenic *Escherichia coli* isolates carrying the *afa-8* operon: Evidence of similarities between isolates from humans and animals with extraintestinal infections. *J Clin Microbiol*, 41, 218-226, 2003.

16. Bertin Y, Martin C, Girardeau JP, Pohl P, Contrepois M: Association of genes encoding P fimbriae, CS31A antigen and EAST 1 toxin among CNF1-producing *Escherichia coli* strains from cattle with septicemia and diarrhea. *FEMS Microbiol Lett*, 162, 235-239, 1998. DOI: 10.1111/j.1574-6968.1998.tb13004.x

17. Nemeth J, Muckle C, Gyles C: *In vitro* comparison of bovine mastitis and fecal *Escherichia coli* isolates. *Vet Microbiol*, 40, 231-238, 1994. DOI: 10.1016/0378-1135(94)90112-0

18. Nemeth J, Muckle CA, Lo RY: Serum resistance and the traT gene in bovine mastitis-causing *Escherichia coli*. *Vet Microbiol*, 28, 343-351, 1991. DOI: 10.1016/0378-1135(91)90069-R

19. Kremer WDJ, Noordhuizen-Stassen EN, Lohuis JACM: Host defence mechanisms and characteristics of coliform bacteria in coliform mastitis in bovine: A review. *Vet Q*, 12 (2): 103-113, 1990. DOI: 10.1080/01652176.1990.9694252

20. De Rycke J, Milon A, Oswald E: Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. *Vet Res*, 30(2-3): 221-233, 1999.

21. Van Bost S, Bâbe MH, Jacquemin E, Mainil J: Characteristics of necrotoxigenic *Escherichia coli* isolated from septicemic and diarrheic calves between 1958 and 1970. *Vet Microbiol*, 82 (4): 311-320, 2001.

22. Quinn PJ, Carter ME, Markey BK, Carter GR: Mastitis. **In**, Quinn PJ, Carter ME, Markey BK, Carter GR (Eds): Clinical Veterinary Microbiology. 327-344, Wolfe Publishing, London, 1994.

23. Yamamoto S, Terai A, Yuri K, Kurazono H, Takeda Y, Yoshida O: Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Immunol Med Microbiol*, 12, 85-90, 1995. DOI: 10.1016/0928-8244(95)00053-A

24. Bingen E, Bonacorsi S, Brahimi N, Denamur E, Elion J: Virulence patterns of *Escherichia coli* K1 strains associated with neonatal meningitis. *J Clin Microbiol*, 35, 2981-2982, 1997.

25. Johnson JR, Adam LS: Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J Infect Dis*, 181, 261-272, 2000. DOI: 10.1086/315217

26. Blum SE, Leitner G: Genotyping and virulence factors assessment of bovine mastitis *Escherichia coli*. *Vet Microbiol*, 163, 305-312, 2013. DOI: 10.1016/j.vetmic.2012.12.037

27. Ghanbarpour R, Oswald E: Phlogenetic distribution of virulence genes in *Escherichia coli* isolated from bovine mastitis in Iran. *Res Vet Sci*, 88, 6-10, 2010. DOI: 10.1016/j.rvsc.2009.06.003

28. Fernandes JBC, Zanardo LG, Galvão NN, Carvalho IA, Nero LA, Moreira MAS: *Escherichia coli* from clinical mastitis: serotypes and virulence factors. *J Vet Diagn Invest*, 23, 1146-1152, 2011. DOI: 10.1177/1040638711425581

29. Moissenet D, Salauze B, Clermont O, Bingen E, Arlet G, Denamur E, Merens A, Mitanchez D, Vu-Thien H: Meningitis caused by *Escherichia coli* producing TEM-52 extended-spectrum beta-lactamase within an extensive outbreak in a neonatal ward: epidemiological investigation and characterization ofthe strain. *J Clin Microbiol*, 48, 2459-2463, 2010. DOI: 10.1128/JCM.00529-10

30. Clermont O, Olier M, Hoede C, Diancourt L, Brisse S, Keroudean M, Glodt J, Picard B, Oswald E, Denamur E: Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infect Genet Evol*, 11, 654-662, 2011. DOI: 10.1016/j.meegid.2011.02.005

31. Suojala L, Pohjanvirta T, Simojoki H, Myllyniemi AL, Pitkälä A, Pelkonen S, Pyörälä S: Phylogeny, virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated in clinical bovine mastitis. *Vet Microbiol*, 147, 383-388, 2011. DOI: 10.1016/j.vetmic.2010.07.011

32. Carrol EJ, Jasper DE: Bactericidal activity of standard bovine serum against coliform bacteria isolated from udders and the environment of dairy cows. *Am J Vet Res*, 38, 2019-2022, 1977.

33. Fang W, Pyörälä S: Mastitis-causing *Escherichia coli*: Serum sensitivity and susceptibility to selected antibacterials in milk. *J Dairy Sci*, 79, 76-82, 1996. DOI: 10.3168/jds.S0022-0302(96)76336-1

34. Rainard P: The complement in milk and defense of the bovine mammary gland against infections. *Vet Res*, 34, 647-670, 2003. DOI: 10.1051/vetres:2003025

35. Acik MN, Yurdakul NE, Cakici L, Onat N, Dogan O, Cetinkaya B: traT and cnf2 genes of *Escherichia coli* isolated from milk of healthy cows and sheep. *Res Vet Sci*, 77, 17-21, 2004. DOI: 10.1016/j.rvsc.2004.01.003

36. Lin J, Hogan JS, Smith KL: Inhibition of *in vitro* growth of coliform bacteria by a monoclonal antibody directed against ferric enterobactin receptor FepA. *J Dairy Sci*, 81, 1267-1274, 1998. DOI: 10.3168/jds.S0022-0302(98)75688-7

37. Brock JH, Pickering MGG, Mcdowall MC, Deacon AG: Role of antibody and enterobactin in controlling growth of *Escherichia coli* in human milk and acquisition of lactoferrin- and transferrin-bound iron by *Escherichia coli*. *Infect Immun*, 40 (2): 453-459, 1983.

38. Linggood MA, Roberts M, Ford S, Parry SH, Williams PH: Incidence of the aerobactin iron uptake system among *Escherichia coli* isolates from infections of farm animals. *J Gen Microbiol*, 133, 835-842, 1987. DOI: 10.1099/00221287-133-4-835

39. Lipman LJA, de Nijs A, Gaastra W: Isolation and identification of fimbriae and toxin production by *Escherichia coli* strains from cows with clinical mastitis. *Vet Microbiol*, 47, 1-7, 1995. DOI: 10.1016/0378-1135(95)00108-M

40. Opdebeeck JP, Frost AJ, O'Boyle D: Adhesion of *Staphylococcus aureus* and *Escherichia coli* to bovine udder epithelial cells. *Vet Microbiol*, 16, 77-86, 1988. DOI: 10.1016/0378-1135(88)90128-9

41. Shuster DE, Harmon RJ, Jackson JA, Hemken RE: Suppression of milk production during endotoxin-induced mastitis. *J Dairy Sci*, 74, 3746-3774, 1991. DOI: 10.3168/jds.S0022-0302(91)78568-8

42. Bouchard L, Blais S, Desrosiers C, Zhoa X, Lacasse P: Nitric oxide production during endotoxin-induced mastitis in the cow. *J Dairy Sci*, 82, 2574-2581, 1999. DOI: 10.3168/jds.S0022-0302(99)75512-8

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Research Article

An Investigation on Protective Effect of *Viburnum opulus* L. Fruit Extract Against Ischemia/Reperfusion-Induced Oxidative Stress After Lung Transplantation in Rats [1] [2]

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Abstract

The aim of this study was to investigate the protective effect of *Viburnum opulus* L. fruit extract against ischemia/reperfusion (I/R)-induced oxidative stress during the lung transplantation. For this purpose, 30 female rats were firstly randomized to form of donor and recipients. After then, the rats were divided into three groups named as control, I/R, and *V. opulus* + I/R. Experimental rats were subjected to lung transplantation with ischemia followed by 2 h of reperfusion. Transplantation-related lung injury was evaluated by multiple parameters. A significant decrease was observed in the enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and the levels of glutathione and total antioxidant status (TAS), whereas the levels of malondialdehyde (MDA), total oxidant status (TOS), and protein carbonyl were significantly increased in lung tissue samples of I/R group in comparison to the control group. However, treatment with *V. opulus* fruit extract resulted in significant reduction of MDA and protein carbonyl levels and increment of the antioxidant system. In conclusion, *V. opulus* fruit extract showed protective effects against I/R-induced oxidative stress during lung transplantation probably by the radical scavenging and antioxidant activity. Therefore, this fruit extract can be efficient in the prevention of I/R-related lung toxicity.

Keywords: *Oxidative Stress, Transplantation, Ischemia/Reperfusion, Antioxidant*

Ratlarda Akciğer Transplantasyonda Iskemi/Reperfüzyonun İndüklediği Oksidatif Strese Karşı *Viburnum opulus* L. Meyve Ekstresinin Koruyucu Etkisinin Araştırılması

Özet

Bu çalışma, akciğer transplantasyonu sırasında iskemi/reperfüzyonun indüklediği oksidatif hasara karşı *Viburnum opulus* L. meyve ekstresinin koruyucu etkisini araştırmak amacıyla yapılmıştır. Bu amaçla, ilk olarak donör ve alıcı oluşturmak için 30 dişi rat rastgele seçildi. Daha sonra, ratlar kontrol, I/R ve *V. opulus* + I/R olarak adlandırılan üç gruba ayrıldı. Deneysel ratlara iskeminin ardından 2 saat reperfüzyon ile akciğer transplantasyonu yapıldı. Transplantasyon ile ilişkili akciğer hasarı, farklı parametrelerle incelendi. Kontrol grubu ile karşılaştırıldığında I/R grubunun akciğer doku örneklerinde süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx) ve katalaz (CAT) enzim aktiviteleri, glutatyon ve total antioksidan durum (TAS) düzeylerinde önemli bir azalış, malondialdehit (MDA), total oksidan durum (TOS) ve protein karbonil düzeylerinde anlamlı artış belirlendi. Bununla birlikte, *V. opulus* meyve ekstresi ile uygulama sonrası MDA ve protein karbonil düzeylerinde önemli azalma ve antioksidan sistemde artma saptanmıştır. Sonuç olarak, akciğer transplantasyonu sırasında I/R'nin indüklediği oksidatif strese karşı *V. opulus* meyve ekstresinin muhtemel radikal temizleme ve antioksidan aktivitesi ile koruyucu etkilerinin olduğunu göstermiştir. Bu nedenle, bu meyve ekstresi I/R ilişkili akciğer hasarının önlenmesinde etkili olabilir.

Anahtar sözcükler: *Oksidatif Stres, Transplantasyon, İskemi/Reperfüzyon, Antioksidan*

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INTRODUCTION

Lung transplantation has become an effective therapeutic option in the treatment of patients with various end-stage pulmonary diseases [1] such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis, bronchiectasis, and primary pulmonary hypertension [2]. Lung injury induced by ischemia-reperfusion (I/R) can lead to severe primary graft failure with about 60% mortality after lung transplantation [3-5]. Although, there are significant improvements in surgical techniques, lung preservation, donor management and immunosuppressive strategies [6,7], I/R injury occurs in up to 22% of patients and is still the main cause of death within the first month of surgery [8]. I/R injury causes oxidative stress characterized by production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and hypochlorous acid [9,10], which potentially can be more detrimental than its local effects [11].

In recent times, there is an attention for the significance of antioxidant substances such as phenolic compounds present in the plants [12], which provide protection against toxic free radicals due to their radical scavenger activities and have therapeutically beneficial effects on human health [13].

Viburnum opulus L. is known as "Gilaburu" in Turkey and "Guilder rose" in Europe [14]. *V. opulus* growing in Turkey have been so far reported to possess several biological activities, including antioxidant, hepatoprotective, hypoglycemic, antinociceptive, and antiinflammatory effects [15]. It has been traditionally used to prevent the formation of kidney stone [16]. Phenolic acids such as hydroxybenzoic acids, tannins, coumarins, cathechols, iridoid glycosides, antocyanins, ascorbic acid, chlorogenic acid, salicin, (+)-catechin, (-)-epicatechin, cyanidin-3 glucoside, cyanidin-3-rutinoside, and quercetin [15-17] were determined in the composition of *V. opulus* fruit extract.

V. opulus fruit is known to have an important antioxidant effect, including strong radical scavenging activity [12,18]. Therefore, the objective of this study was to investigate the possible protective effects of *V. opulus* fruit extract against I/R-induced oxidative stress during the lung transplantation. Lung injuries were assessed by multiple parameters, including measurements of oxidative stress markers [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), total glutathione, malondialdehyde (MDA), protein carbonyl, total antioxidant status (TAS), total oxidant status (TOS)], oxygenation index (arterial oxygen tension/inspired oxygen concentration ratio-PaO_2/FiO_2), wet/dry weight ratio of transplanted lungs, and histologic examination.

MATERIAL and METHODS

Chemicals

All reagents used in this study were analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany). TOS and TAS kits were purchased from Rel Assay Diagnostic (Turkey). Total glutathione and protein carbonyl kits were purchased from Cayman (Ann Arbor, MI, USA).

Plant Material and Preparation of Extract

V. opulus fruits were collected after full maturation in September 2014 from city of Kayseri in Turkey. Air-dried and powdered fruits (100 g) were macerated in methanol for 3 days. Sample was then filtered through Whatman No 1 papers in a Buchner funnel. The macerates were evaporated in-vacuo until dryness and lyophilized.

Estimation of Total Phenolic Content and Identification with LC-MS/MS

Total phenolic content was estimated quantitatively using the method described by Jindal and Singh [19]. A standard curve was prepared by using different concentrations (0.1-100 mg/mL) of gallic acid and used for the determination of total phenolic compounds content (mg gallic acid equivalent-GAE/g extract).

In order to identify the active phenolic compounds present in *V. opulus* aqueous methanol (70%) extract, UPLC-MS/MS with an electrospray ionisation (ESI) was utilized. UPLC-ESI-MS/MS analyses were performed on an UPLC instrument with Q-trap mass spectrometer (Shimadzu, Japan). The system was operated using LabSolutions software. The flow rate was 0.2 mL/min and mobile phase was mixture of A (1% acetic acid in water) and B (1% acetic acid in methanol). Ultrahigh pure helium (He) and high purity nitrogen (N_2) were used as collision and nebulising gases, respectively. The mass spectrometer was operated in negative ionisation mode, and mass spectra were recorded between 100 and 1.000 amu.

Animals and Experimental Groups

All procedures performed on animals were in accordance with the European Union Directive 2010/63/EU for care and use of laboratory animals. The experiment protocol was approved by the Ethical Committee for Animal Research at Erciyes University (Approval date: 13.03.2013; no: 13/52). Thirty female Wistar albino rats (weighing 250-300 g) were used in our study. They were housed in individual cages under standard laboratory conditions (12 h/12 h light/dark cycle, 22-24°C temperature and 55-60% relative humidity). A commercial pellet diet (2.600 kcal/kg metabolic energy, 7% crude fiber and 23% crude protein) and fresh drinking water were given *ad libitum*.

At the beginning, 30 female Wistar albino rats were randomized to form of donor (n=12) and recipients (n=18). After then, the rats were divided into groups as follows; control group (n=6), I/R (n=6), and *V. opulus*-treated I/R (VO+I/R) (n=6). In the control group, we obtained samples from the left lung lower lobe without any additional procedure for biochemical and histopathological evaluation.

Surgical Procedures

Lung transplantation was performed as reported previously by Yucel et al.[20]. In the donor group, left upper lobectomy was performed and the left lower lobe was removed as an allograft with the following procedures: The rats were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 10 mg/kg xylazin combination. The rats were shaved after induction and fixed in a supine position. Each rat was heparinized intravenously (15 U). Following midline incision, tracheostomy was performed. Ventilation was maintained with tidal volume of 1.5 mL room air at 60 breaths/min and the positive end expiratory pressure (PEEP) of 2 cm-H_2O. Median sternotomy incision was used for opening the thoracic cavity. Left pulmonary artery, vein and bronchus were exposed by dissecting left hilar region. The upper lobe branches of pulmonary artery and vein were tied with 5/0 free silk suture and cut. The left upper lobe bronchus was tied with 4/0 free silk suture and cut. So, left upper lobectomy was completed. Following left upper lobectomy, pulmonary artery and vein of left lower lobe were catheterized in the peripheral direction. Placed catheters were fixed with 4/0 free silk suture and then left lower lobe artery, vein, and bronchus were tied and cut. After completion of the removal of allograft lung tissue, a catheter was placed in the left lower lobe bronchus to provide a three-way flow. The first line was fixed in the left lower lobe bronchus. The second line is attached to the intrabronchial pressure gauge. The last line was fixed to ventilation balloon. The washing process of the left lower lobe was performed by giving Euro-Collins solution at pressure of 1.5 cm H_2O to artery catheter of left lung lower lobe. During the washing process, it was manually reventilated with not exceeding 0.2 mL of room air, 60 breathes/min, and PEEP of 1.5 cm H_2O from the left lower lobe bronchus. The allograft lung tissue was immersed in the +4°C Euro-Collins solution for 24 h in a half-inflated manner. Donor group were sacrificed by infusing a lethal dose of anesthetics afterwards. Six of the donor lungs were reperfused and reventilated in I/R group and the other six were reperfused and reventilated in VO + I/R group.

I/R Group: Left lower lobectomy was performed in I/R group, after left upper lobectomy. Thus, left pneumonectomy was completed. Unlike the subject of the donor group, pulmonary artery and vein were catheterized in the proximal direction. Placed catheters were fixed with 4/0 free silk suture and then left lower lobe of artery, vein, and bronchus were tied and cut. Thus, the catheterization was completed. After catheterization, the allograft left lower lobe was reventilated and reperfused for two hours. Catheter in the pulmonary artery of lung obtained from donor was combined with the placed one in the pulmonary artery of left lung lower lobe. Again, catheter in the pulmonary vein of left lower lobe obtained from donor was combined with the placed one in the pulmonary vein of left lung lower lobe. After the anastomosis process, reperfusion was allowed. Simultaneously, the animals were manually reventilated not to exceed 1.2 mL of room air, 60 breaths/min, and PEEP pressure of 1.5 cm H_2O with the help of ventilation device. Also, allograft lung tissue was manually reventilated not to exceed 0.2 mL of room air, 60 breathes/min, and PEEP pressure of 1.5 cm H_2O with the help of ventilation device. The reperfusion and ventilation was continued for 2 h. After 2 h, the allograft lungs were sampled for histopathological and biochemical tests.

VO+I/R Group: After the left upper lobectomy as in I/R group, the subjects' left lower lobe pulmonary artery and vein were catheterized. Unlike subjects in I/R group, *V. opulus* fruit extract (200 mg/kg) was administered intraperitoneally to both this group (the recipient) and six subjects of the donors group 3 h before anesthesia [3]. Immediately after the lung transplantation, recipients was given again the same dose of the fruit extract intraperitoneally. The rats were reperfused and reventilated as in the I/R group and tissue sampling was performed for pathologic and biochemical examinations.

Tissue and Blood Sample Collection and Preparation

After 2 h reperfusion, blood was sampled from the right carotid artery after 10 min of ventilation, followed by immediate blood gas analysis. Blood samples were centrifuged at 1.000 g for 10 min to separate plasma. The plasma samples were stored at -80°C until analysis of TAS, TOS, and protein carbonyl using ELISA reader (Biotek Synergy HT, Vermont, USA).

Lung samples were homogenized in ten volumes of ice-cold 140 mM KCl and Tris-HCl buffer (50 mM, pH 7.6) using a homogenizer (IKA Ultra-Turrax T10 basic model, Germany) for 2 min at 13.000 rpm. The total glutathione and MDA levels were determined in the tissue homogenates by using spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan). The homogenates were then centrifuged at 5.000 g for 60 min to remove debris. Clear supernatant was used for SOD, GPx, and CAT enzyme activity assays. Protein content in tissue homogenate and supernatant was measured according to the method of Lowry et al.[21].

Analyses of Oxidative Stress Parameters

SOD activity was measured as described by Fitzgerald et al.[22]. Briefly, the tissue samples were diluted with 10 mM phosphate buffer, pH 7.0. 25 µL aliquots were mixed with 850 µL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L

2-(4-iodophenyl)-3-(4-nitrophenol) 5-phenyltetrazolium chloride (INT) in a buffer solution containing 50 mmol/L CAPS (3-(cyclohexylaminol)-1-propanesulfonic acid) and 0.094 mmol/L EDTA (pH 10.2). To the mixture, 125 µL xanthine oxidase (80 U/L) was added and then the increase of absorbance was recorded by spectrophotometer at 505 nm for 3 min. SOD activity was expressed in U/mg protein.

GPx activity was measured as previously described by Pleban et al.[23]. Briefly, a reaction mixture containing 1 mmol/L Na_2EDTA, 2 mmol/L reduced glutathione, 0.2 mmol/L NADPH, 4 mmol/L sodium azide and 1.000 U glutathione reductase in 50 mmol/L Tris buffer (pH 7.6) was prepared. 20 µL of tissue samples and 980 µL of the reaction mixture were mixed and incubated for 5 min at 37°C. The reaction was initiated by adding 8.8 mmol/L hydrogen peroxide (H_2O_2) and the decrease of absorbance recorded at 340 nm was monitored by spectrophotometer for 3 min. GPx activity was expressed in U/mg protein.

CAT activity was measured in tissue samples at 25°C by the method of Aebi [24]. The decomposition rate of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm for 30 sec. The activity was expressed as U/mg protein.

Lipid peroxidation was estimated by measurement of MDA in tissue samples by the method described by Ohkawa et al.[25]. After reaction of MDA with thiobarbituric acid reactive substances (TBARS), the reaction product was followed spectrophotometrically at 532 nm, using tetramethoxypropane as a standard. The results were expressed as nmol/mg protein.

For the estimation of glutathione (GSH) levels in tissue samples, a commercial kit based on the enzymatic recycling method was utilized. The assay was based on the reaction of sulfhydryl group of glutathione with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent) resulting in the production of 5-thio-2-nitrobenzoic acid (TNB). The disulfide formed between GSH and TNB was then reduced by glutathione reductase to recycle the GSH and produce TNB. Since the rate of TNB production is directly proportional to concentration of GSH in the sample, the absorbance of TNB was measured at 412 nm to evaluate total glutathione. Tissue homogenates were deproteinated using 10% metaphosphoric acid and pH was adjusted with 4M triethanolamine before assay. The results were expressed as µmol/g tissue.

Protein carbonyl levels were analyzed in the plasma samples based on the reaction between 2,4-dinitrophenylhydrazine and protein carbonyls using a commercially available kit. The absorbance of resulting protein-hydrazone was measured at 370 nm as described by the manufacturer. Following standardization to the related protein concentration for each sample, the carbonyl content was expressed as nmol/mg protein.

TAS and TOS assays developed by Erel [26] were carried out by commercially available kits in plasma samples. Principle of the assay for TAS levels is as follows; anti-oxidants in the samples reduce dark blue-green colored 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related with total antioxidant level of the sample. The results were expressed as µmol Trolox equivalent (eq)/mg protein. TOS measurement is based on the oxidation of the ferrous ion-o-dianisidine complex to ferric ion by the oxidants present in the sample. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity, measured spectro-photometrically, is related to the total amount of oxidant molecules present in the sample. The results were expressed µmol H_2O_2 eq/mg protein. The percent ratio of TOS to TAS was used as OSI, an indicator of the degree of oxidative stress. The OSI value was calculated as follows: OSI = [(TOS, µmol H_2O_2 eq/mg protein)/(TAS, µmol Trolox eq/mg protein) x 100].

Oxygenation Index (PaO_2/FiO_2)

Oxygenation index was measured by blood gas analysis of blood samples from either the right carotid artery or the left pulmonary vein.

Wet/Dry Weight Ratio of Transplanted Lungs

Transplanted left lungs removed at 2 h after the reperfusion were sliced into 3 parts. After the upper one third was weighed immediately, it was dried at 80°C for 48 h to calculate the wet/dry weight ratio.

Histologic Examination

A tissue sample from the middle of the transplanted lung was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. The severity of lung injury was scored based on alveolar edema and congestion, interstitial edema and congestion, neutrophil infiltration. Histopathological changes were graded as follows: (0) absent, (1) mild, (2) moderate, (3) severe injury, respectively. For each lung tissue sample, the final value was the mean of the scores for the 3 separate slides [27].

Statistical Analysis

Analysis of the data was performed by using Statistical Package for the Social Sciences (SPSS version 18.0 for Windows, Chicago, IL, USA). The comparison of the results among different groups was carried out by one-way ANOVA and followed by Tukey multiple comparisons test. The results were expressed as aritmetic mean±standard deviation (SD). The values were considered statistically significant if the P value was less than 0.05.

RESULTS

Total Phenolic Content and Identified Compounds of V. opulus Fruit Extract

In fruit extract, total phenolic amount was found as 67.73 mg GAE/g extract.

Compound 1-8 were identified based on the MS fragmentation data in negative ionisation mode compared with literature [28,29] as shown in *Table 1*.

Oxidative Stress Parameters

As presented in *Fig. 1A*, the SOD activity in group of I/R (1.65±0.33 U/mg protein) was significantly lower than that in groups of control (3.6±0.37 U/mg protein) and VO+I/R (3.31±0.43 U/mg protein) (P<0.05). However, treatment with *V. opulus* markedly improved this effect as compared with I/R group (P<0.05). The GPx activity in I/R group (1.42±0.29 U/mg protein) was markedly lower than that of the control (3.18±0.25 U/mg protein) group and VO+I/R (2.84±0.28 U/mg protein) group (P<0.05). However, this effect was significantly ameliorated by *V. opulus*-treatment as compared to I/R group (P<0.05) as presented in *Fig. 1B*. There was a significant depletion in the activity of CAT in group of I/R (17.97±1.62 U/mg protein) compared to control (27.02±1.41 U/mg protein) and VO+I/R (24.5±1.72 U/mg protein) groups (P<0.05) as demonstrated in *Fig. 1C*. I/R resulted in a significant decrease of glutathione levels (1.32±0.43 µmol/g tissue) compared with control group

Table 1. *Identified compounds of V. opulus fruit extract by LC-MS/MS*

No	Molecular Mass	[M-H]⁻ m/z	Compound	Amount (%)*
1	133	115, 71	Malic acid	24.18
2	179	89, 68	Caffeic acid	3.31
3	191	111, 85	Quinic acid	5.31
4	295	179, 133, 89	Caffeic acid derivative	27.09
5	337	277, 174	Coumaroyl-quinic acid	1.64
6	353	191	Chlorogenic acid	5.36
7	451	341, 133	Not identified	1.91
8	613	295, 133, 89	Not identified	2.01

** Relative amounts from total ion chromatogram*

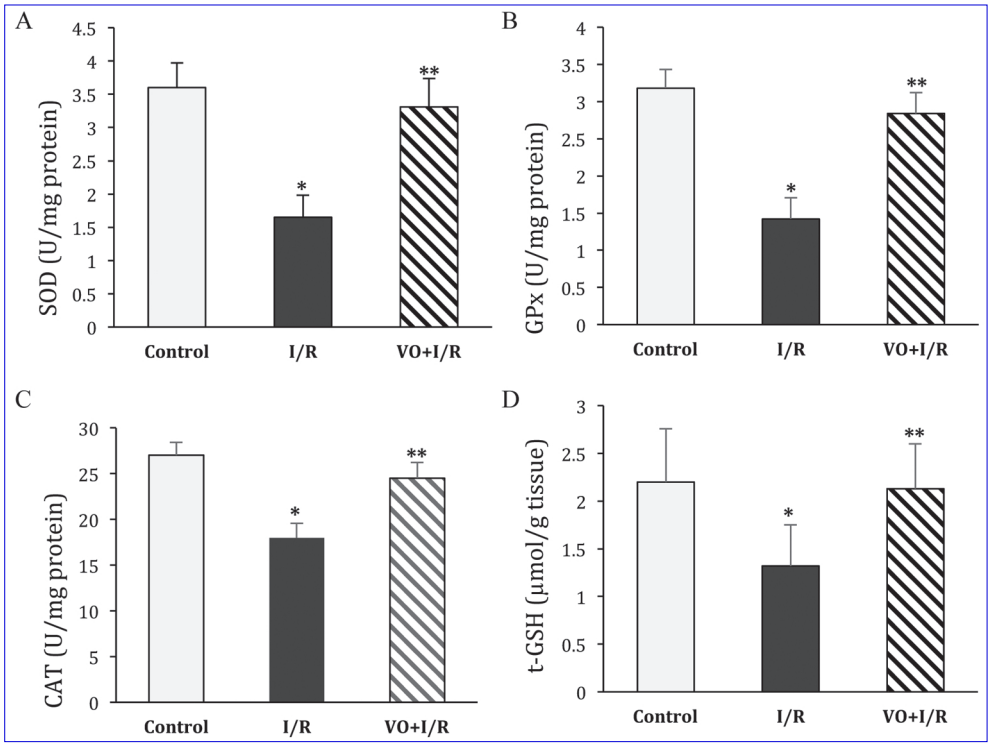

Fig 1. Effects of *V. opulus* fruit extract treatment on (A) SOD, (B) GPx, (C) CAT enzyme activities, and (D) t-GSH. Values are indicated by mean±SD. * P<0.05 vs. control group; ** P<0.05 vs. I/R group

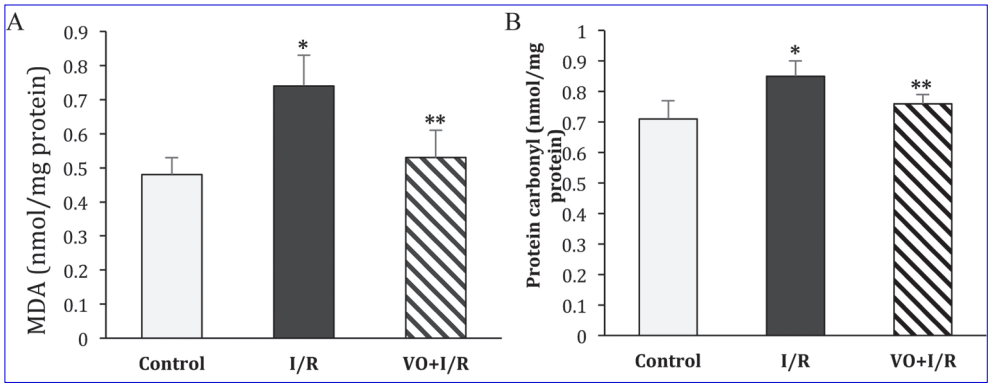

Fig 2. Effects of *V. opulus* fruit extract treatment on (A) MDA and (B) protein carbonyl levels. Values are indicated by mean±SD. * P<0.05 vs. control group; ** P<0.05 vs. I/R group

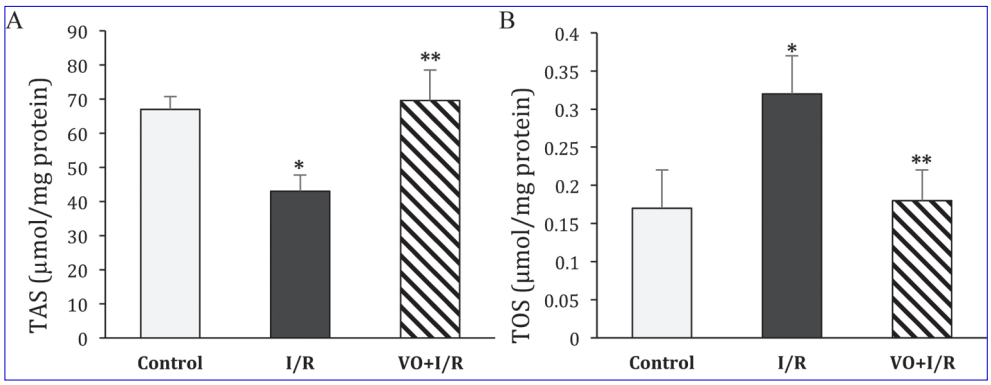

Fig 3. Effects of *V. opulus* fruit extract treatment on (A) TAS and (B) TOS levels. Values are indicated by mean±SD. * P<0.05 vs. control group; ** P<0.05 vs. I/R group

Table 2. *The comparison of the parameters among the groups*

Parameters	Control (mean±SD)	I/R (mean±SD)	VO+I/R (mean±SD)
PaO_2/FiO_2	454±18.64	354±32.54*	386±12.53*
Wet/dry weight ratio	4.98±0.23	5.66±0.91	5.39±0.69
Histological score	0.83±0.98	2.16±0.40**	0.16±0.40

** P<0.05 vs. control group; ** P<0.05 vs. VO+I/R*

(2.20±0.56 µmol/g tissue), while this effect was efficiently compensated by *V. opulus* treatment (2.13±0.47 µmol/g tissue) (P<0.05) as shown in *Fig. 1D*.

Fig. 2A shows that the lung tissue levels of MDA in I/R group (0.74±0.09 nmol/mg protein) were significantly higher than that in the control group (0.48±0.05 nmol/mg protein) and VO+I/R group (0.53±0.08 nmol/mg protein). However, *V. opulus* fruit extract treatment resulted in a significant reduction in MDA levels as compared to I/R group (P<0.05). As expected, the plasma protein carbonyl level was significantly elevated in I/R group (0.85±0.05 nmol/mg protein) as compared with control (0.71±0.06 nmol/mg protein), and we observed that *V. opulus* fruit extract-treatment counteracted this effect (0.76±0.03 nmol/mg protein) *(Fig. 2B*, P<0.05).

Our results indicated that plasma TAS levels were significantly lower (P<0.05) in the group of I/R (43.02±4.75 µmol Trolox eq/mg protein) as compared to groups of control (66.98±3.8 µmol Trolox eq/mg protein) and VO+I/R (69.59±8.9 µmol Trolox eq/mg protein) as presented in *Fig. 3A*. We observed that plasma TOS levels were significantly higher (P<0.05) in I/R group (0.32±0.05 µmol H_2O_2 eq/mg protein) as compared with groups of control (0.17±0.05 µmol H_2O_2 eq/mg protein) and VO+I/R (0.18±0.04 µmol H_2O_2 eq/mg protein), while administration of *V. opulus* fruit extract significantly decreased the TOS levels in VO+I/R group as shown in *Fig. 3B*. The comparison of control and I/R groups in terms of OSI% did reveal a statistically significant difference between these groups (P<0.05). The similar results were also observed between I/R and VO+I/R groups.

Oxygenation Index (PaO_2/FiO_2)

As shown in *Table 2*, compared with the control group, the PaO_2/FiO_2 level was significantly decreased by 22% at 2 h after reperfusion in I/R group (P<0.05). However, when compared with I/R group, administration of *V. opulus* fruit extract was able to elevate the PaO_2/FiO_2 level by 9% (P<0.05).

Wet/Dry Weight Ratio of Transplanted Lungs

As an indicator of pulmonary edema, wet/dry weight ratios were calculated. As demonstrated in *Table 2*, it was seen a slight increase by 14% in wet/dry weight ratio in group of I/R compared with the control group, but a statistical association was not detected. On the other hand, there was a slight decrease level by 5% in wet/dry weight ratio in group of VO+I/R compared with I/R group.

Histopathological Findings

Histological examination of the lung tissue subjected to I/R process showed the distinctive pattern of ischemic injury, which included interstitial distinct congestion and edema, widespread edema and congestion in the alveoli. In the control group, lung tissue sections had a normal morphology. As indicated in *Table 2*, treatment with *V. opulus* fruit extract significantly decreased the histological injury score in VO+I/R group compared with I/R group ($P<0.05$).

DISCUSSION

In our study, I/R injury resulted in decrease of SOD, GPx and CAT enzyme activities as well as glutathione levels as compared with control group, while this effect was compensated by *V. opulus* treatment. In line with our findings, a report of an experimental model has shown the beneficial effect of *V. opulus* treatment on the levels of total thiols and glutathione [30]. MDA elevation shows increased lipid peroxidation due to the interaction between lipid components of cellular membranes and ROS [31]. In the present study, we indicated that MDA level was increased in I/R group. Administration of *V. opulus* fruit extract resulted in significant reduction of tissue MDA levels in VO+I/R group compared with I/R group. In this study, we carried out total oxidant and antioxidant status at the same time to more accurately evaluate oxidative stress. We observed that plasma TOS levels were significantly higher and TAS levels were significantly lower in I/R group as compared to groups of control and VO+I/R. The comparison of control and I/R groups in terms of OSI did reveal a statistically significant difference between these groups. The similar results were also observed between I/R and VO+I/R groups.

Similar to other biomolecules, proteins are prone to attacks during oxidative stress conditions. In this context, protein carbonyls represent an irreversible form of protein modification and are relatively stable; therefore determination of protein carbonyl levels serves as an overall marker of protein oxidation [32]. Our study has revealed the beneficial effect of *V. opulus* fruit extract-treatment in I/R-induced oxidative stress as evidenced by the compensation of the increase in plasma protein carbonyl level.

Transplantation-related lung injury was showed by decreased levels of PaO_2/FiO_2. The level of PaO_2/FiO_2 was significantly decreased at 2 h after reperfusion in I/R group compared with the control group. However, administration of *V. opulus* extract prevented the decrease in PaO_2/FiO_2 level. Sun et al.[3] found similar result with curcumin at the early stage of post-transplantation.

In our study results were in agreement with the previous ones. Sun et al.[3] suggested that curcumin, which is an active component of *Curcuma longa*, can be an alternative therapy for protecting lung transplantation-related I/R injury. They found that there was a significant increase in MDA level and a significant decrease in TAS levels in I/R group compared with the control group. Pretreatment with curcumin significantly prevented the increase in MDA level and the decreased TAS level. In our previous study [1], we demonstrated that donor treatment with taurine protected the lungs of rats against post-transplantation I/R injury in respect to histopathological and biochemical findings. Following treatment with taurine, we observed increased activities of SOD and CAT, and decreased MDA levels in I/R group compared with taurine-treatment group.

It was found that *V. opulus* fruit extract has strong radical scavenging activity and antioxidant effects due to its phenolic substance contents [12,16,18]. We also determined the active phenolic compounds such as caffeic acid derivative and malic acid in our *V. opulus* fruit sample. Our results indicated that *V. opulus* fruit extract administration to animals before/during lung transplantation significantly reduced I/R-related lung injury by preventing oxidative damage. These results may explain that the active compounds determined with LC-MS/MS in *V. opulus* fruit extract provided the preventive effect on oxidative stress. Some herbal drugs have been used to prevent certain diseases due to their capacity of radical scavenging activity [12]. Liu et al.[33] showed that *Ginkgo biloba* extract had a protective effect on lung injury induced by I/R, which may be related to its antioxidant property. *Ginkgo biloba* extract markedly increased SOD activity, reduced MDA levels. He et al.[34] suggested the protective effects of triptolide (Tripterygium extracts of the Chinese herb) on I/R-induced injury of transplanted rabbit lungs. Zayachkivska et al.[35] also identified that pretreatment with *V. opulus* proanthocyanidins exhibited a potent gastroduodenoprotective effect by the suppression of lipid peroxidation and an enhancement of SOD, CAT activities in rats.

In conclusion, our findings showed that *V. opulus* xtract indicated protective effects against I/R-induced oxidative stress during lung transplantation probably by the radical scavenging and antioxidant activities. Therefore, *V. opulus* fruit extract can be alternative therapeutic strategy for the prevention of I/R-related lung toxicity.

REFERENCES

1. Guler L, Tavlasoglu M, Yucel O, Guler A, Sahin MA, Kurkluoglu M, Sirin Y, Eken A, Gamsızkan M, Dakak M, Gurkok S, Genc O: Taurine attenuates lung ischemia-reperfusion injury after lung transplantation in rats. *J Anesth*, 28, 347-353, 2014. DOI: 10.1007/s00540-013-1741-2

2. Abreu MM, Pazetti R, Almeida FM, Correia AT, Parra ER, Silva LP, Vieira RP, Pego-Fernandes PM, Jatene FB: Methylene blue attenuates ischemia-reperfusion injury in lung transplantation. *J Surg Res,* 192, 635-641, 2014. DOI: 10.1016/j.jss.2014.07.043

3. Sun J, Guo W, Ben Y, Jiang J, Tan C, Xu Z, Wang X, Bai C: Preventive effects of curcumin and dexamethasone on lung transplantation-associated lung injury in rats. *Crit Care Med,* 36, 1205-1213, 2008. DOI: 10.1097/CCM.0b013e31816a06fc

4. Hengst WA, Gielis JF, Lin JY, Van Schil PE, De Windt LJ: Lung ischemia-reperfusion injury: a molecular and clinical view on a complex pathophysiological process. *Am J Phys Heart Circ Physiol*, 299, H1283-H1299, 2010. DOI: 10.1152/ajpheart.00251.2010

5. Sugimato R, Okamoto T, Nakao A, Zhan J, Wang Y, Kohmoto J, Tokita D, Farver CF, Tarpey MM, Billiar TR, Gladwin MT, McCurry KR: Nitrite reduces acute lung injury and improves survival in a rat lung transplantation model. *Am J Transplant*, 12, 2938-2948, 2012. DOI: 10.1111/j.1600-6143.2012.04169.x

6. Okazaki M, Kreisel F, Richardson SB, Kreisel D, Krupnick AS, Patterson GA, Gelman AE: Sphingosine 1-phosphate inhibits ischemia reperfusion injury following experimental lung transplantation. *Am J Transpl*, 7, 751-758, 2007. DOI: 10.1111/j.1600-6143.2006.01710.x

7. Guo W, Ge D, Wang Q, Xu S, Xue L, Lu C, Tan L: Diazoxide decreases ischemia-reperfusion injury in a rat model of lung transplantation. *Transplant Proc*, 43, 2510-2516, 2011. DOI: 10.1016/j.transproceed.2011.04.015

8. Ito K, Shimada J, Kato D, Toda S, Takagi T, Naito Y, Yoshikawa T, Kitamura N: Protective effects of preischemic treatment with pioglitazone, a peroxisome proliferator-activated receptor-ɣ ligand, on lung ischemia-reperfusion injury in rats. *Eur J Cardiothorac Surg*, 25, 530-536, 2004. DOI: 10.1016/j.ejcts.2003.12.017

9. Ovechkin AV, Lominadze D, Sedoris KC, Robinson TW, Tyagi SC, Roberts AM: Lung ischemia-reperfusion injury: Implications of oxidative stress and platelet-arteriolar wall interactions. *Arch Physiol Biochem*, 113, 1-12, 2007. DOI: 10.1080/13813450601118976

10. Xu JZ, Shen BZ, Li Y, Zhang T, Xu WH, Liu XW, Lu HG: Edaravone attenuates ischemia-reperfusion injury by inhibiting oxidative stress in a canine lung transplantation model. *Chin Med J*, 121, 1583-1587, 2008.

11. Esme H, Fidan H, Koken T, Solak O: Effect of lung ischemia-reperfusion on oxidative stress parameters of remote tissues. *Eur J Cardiothorac Surg*, 29, 294-298, 2006. DOI: 10.1016/j.ejcts.2005.12.008

12. Altun ML, Çitoğlu Saltan G, Sever Yılmaz B, Çoban T: Antioxidant properties of *Viburnum opulus* and *Viburnum lantana* growing in Turkey. *Int J Food Sci Nutr*, 59, 175-180, 2008. DOI: 10.1080/09637480701381648

13. Yaylacı-Karahalil F, Şahin **H:** Phenolic composition and antioxidant capacity of cherry laurel *(Laurocerasus officinalis* Roem.) sampled from Trabzon region, Turkey. *Afr J Biotechnol*, 10, 16293-16299, 2011.

14. Cam M, Hisil Y, Kuscu A: Organic acid, phenolic content, and antioxidant capacity of fruit flesh and seed of *Viburnum opulus*. *Chem Nat Comp*, 43, 460-461, 2007. DOI: 10.1007/s10600-007-0161-7

15. Erdogan-Orhan I, Altun ML, Sever-Yılmaz B, Saltan G: Anti-acetylcolinesterase and antioxidant assets of the major components (salicin, amentoflavone, and chlorogenic acid) and the extracts of *Viburnum opulus* and *Viburnum lantana* and their total phenol and flavonoid contents. *J Med Food*, 14, 434-440, 2011. DOI: 10.1089/jmf.2010.0053

16. Soylak M, Elçi L, Saraçoğlu S, Divrikli U: Chemical analysis of fruit of European cranberrybush *(Viburnum opulus)* from Kayseri-Turkey. *Asian J Chem*, 14, 135-138, 2002. DOI: 10.1111/1750-3841.12836

17. Velioglu YS, Lutfiye E, Poyrazoglu ES: Phenolic composition of European cranberrybush *(Viburnum opulus* L.) berries and astringency removal of its commercial juice. *Int J Food Sci Techn*, 41, 1011-1015, 2006. DOI: 10.1111/j.1365-2621.2006.01142.x

18. Sagdıc O, Aksoy A, Ozkan G: Evaluation of the antibacterial and antioxidant potentials of cranberry (Gilaburu, *Viburnum opulus* L.) fruit extract. *Acta Alimentaria*, 35, 487-492, 2006. DOI: 10.1556/AAlim.35.2006.4.12

19. Jindal KK, Singh RN: Phenolic content in male and female *Carica papaya:* A possible physiological marker for sex identification of vegetable seedlings. *Physiol Plantarum*, 33, 104-107, 1975. DOI: 10.1111/j.1399-3054.1975.tb03774.x

20. Yücel O, Güler A, Şirin YS, Gamsızkan M, Şahin MA, Eken A, Arslan S, Çubuk S, Genç O: Akciğer transplantasyonunda kolay uygulanabilir iskemi reperfüzyon modeli. *J Clin Anal Med*, 1, 13-20, 2010.

21. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem*, 193, 265-275, 1951.

22. Fitzgerald SP, Campbell JJ, Lamont JV: The establishment of reference ranges for selenium. The selenoenzyme glutathione peroxidase and the metalloenzyme superoxide dismutase in blood fractions. *The Fifth International Symposium on Selenium Biology and Medicine.* July 20-23, Tennessee, USA, 1992.

23. Pleban PA, Munyani A, Beachum J: Determination of selenium concentration and glutathione peroxidase activity in plasma and erythrocytes. *Clin Chem*, 28, 311-316, 1982.

24. Aebi H: Catalase *in vitro. Methods Enzymol*, 105, 121-126, 1984. DOI: 10.1016/S0076-6879(84)05016-3

25. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animals and tissues by thiobarbituric acid reaction. *Anal Biochem*, 95, 351-358, 1979. DOI: 10.1016/0003-2697(79)90738-3

26. Erel O: A novel automated direct measurement method for total anti-oxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem*, 37, 277-285, 2004. DOI: 10.1016/j.clinbiochem.2003.11.015

27. Bancroft GD, Stevens A, Turner DR: Theory and practice of technique. 4th ed., New York: Churchill Livingston, 1996.

28. Karaçelik AA, Küçük M, İskefiyeli Z, Aydemir S, De Smet S, Miserez B, Sandra P: Antioxidant components of *Viburnum opulus* L. determined by on-line HPLC-UV-ABTS radical scavenging and LC-UV-MS methods. *Food Chem*, 175, 106-114, 2015. DOI: 10.1016/j.foodchem.2014.11.085

29. Kraujalyte V, Venskutonis PR, Pukalskas A, Cesoniene L, Daubaras R: Antioxidant properties and polyphenolic compositions of fruits from different European cranberrybush *(Viburnum opulus* L.) genotypes. *Food Chem*, 141, 3695-3702, 2013. DOI: 10.1016/j.foodchem.2013. 06.054

30. İlhan M, Ergene B, Süntar I, Ozbilgin S, Saltan Çitoğlu G, Demirel MA, Keleş H, Altun L, Küpeli Akkol E: Preclinical evaluation of antiurolithiatic activity of *Viburnum opulus* L. on sodium oxalate-induced urolithiasis rat model. *Evid Based Compl Alternat Med*, 2014, 1-10, 2014. DOI: 10.1155/2014/578103

31. Eren S, Esme H, Balci AE, Cakir O, Buyukbayram H, Eren MN, Erdinc L, Satici O: The effect of aprotinin on ischemia-reperfusion injury in an in situ normothermic ischemic lung model. *Eur J Cardiothorac Surg*, 23, 60-65, 2003. DOI: 10.1016/S1010-7940(02)00623-1

32. Weber D, Davies MJ, Grune T: Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions. *Redox Biol*, 5, 367-380, 2015. DOI: 10.1016/j.redox.2015.06.005

33. Liu K-X, Wu W-K, He W, Liu C-L: Ginkgo biloba extract (EGb 761) attenuates lung injury induced by intestinal ischemia/reperfusion in rats: Roles of oxidative stress and nitric oxide. *World J Gastroenterol*, 13, 299-305, 2007.

34. He JK, Yu SD, Zhu HJ, Wu JC, Qin ZH: Triptolide inhibits NF-$_{\kappa}$B activation and reduces injury of donor lung induced by ischemia/reperfusion. *Acta Pharmacol Sin*, 28, 1919-1923, 2007. DOI: 10.1111/j.1745-7254.2007.00701.x

35. Zayachkivska OS, Gzhegotsky MR, terletska OI, Lutsyk DA, Yaschenko AM, Dzhura OR: Influence of *Viburnum opulus* proanthocyanidins on stress-induced gastrointestinal mucosal damage. *J Physiol Pharmacol*, 57 (Suppl. 5): 155-167, 2006.

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Research Article

Protective Effects of Rutin on Acute Lung Injury Induced by Oleic Acid in Rats [1]

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Abstract

The purpose of this study was to explore the protective effects of different doses of rutin with the antioxidant and anti-inflammatory properties on acute lung injury (ALI) induced by oleic acid (OA) in rats. Thirty-five Sprague-Dawley male rats were randomly separated into five groups comprising control, rutin 150 mg, OA, rutin 75 mg + OA and rutin 150 mg + OA. In the rutin 75 mg + OA and rutin 150 mg + OA groups, the lung malondialdehyde level (MDA) was significantly lower than that of the OA group. In the rutin 75 mg + OA and and rutin 150 mg + OA groups, the lung GPx (glutathione peroxidase), CAT (catalase) and SOD (superoxide dismutase) activities and GSH (glutathione) levels were significantly higher than those of the OA group, and significantly lower than those of the control group. iNOS expressions in the interstitial parts of the lungs were significantly lower than those of the OA group. The iNOS expression was lower in the 150 mg + OA group compared to the rutin 75 mg + OA group. It was concluded that on the ALI induced by OA, rutin had protective effects through the antioxidant and anti-inflammatory properties and that the treatment of rutin as a supportive treatment in ALI was found to be practically useful.

Keywords: *Acute lung injury, Oleic acid, Oxidative stress, Rutin*

Ratlarda Oleik Asit Kaynaklı Akut Akciğer Hasarı Üzerine Rutinin Koruyucu Etkileri

Özet

Bu çalışmanın amacı ratlarda rutinin farklı dozlarının antioksidan ve anti-inflamatuar özellikleri yoluyla oleik asit (OA) ile oluşturulan akut akciğer hasarı (ALI) üzerine koruyucu etkilerini araştırmaktı. 35 adet Sprague-Dawley erkek rat kontrol, rutin 150 mg, OA, rutin 75 mg + OA ve rutin 150 mg + OA olmak üzere rasgele 5 gruba ayrıldı. Rutin 75 mg + OA ve rutin 150 mg + OA gruplarında akciğer malondialdehit (MDA) düzeyi OA grubununkine göre önemli düzeyde düşüktü. Rutin 75 mg + OA ve rutin 150 mg + OA gruplarında akciğer GPx (glutasyon peroksidaz), CAT (katalaz), SOD (süperoksit dismutaz) aktiviteleri ile GSH (glutatyon) düzeyleri OA grubununkilere göre önemli düzeyde yüksekti ve kontrol grubununkilere göre önemli düzeyde düşüktü. Rutin 75 mg + OA ve rutin 150 mg + OA gruplarında akciğerlerin interstisyel kısımlarında iNOS ekspresyonları OA grubunkilere göre önemli düzeyde düşüktü. OA ile oluşturulan ALI'de rutinin antioksidan ve antiinflamatuvar özellikler aracılığıyla koruyucu etkilere sahip olduğu, ALI'de destekleyici tedavi amacıyla rutin uygulamasının pratik olarak yararlı olduğu tespit edildi.

Anahtar sözcükler: *Akut akciğer hasarı, Oleik asit, Oksidatif stres, Rutin*

INTRODUCTION

Acute lung injury (ALI) is a disease characterized by edema due to intra or extra pulmonary risk factors, hypoxemia resistant to oxygen treatment, alveolar hemorrhage, development of hyaline membrane, increase in the alveolar wall thickness and histopathologic changes containing pulmonary inflammation [1], and clinically characterized by pulmonary edema and respiratory distress with an acute onset [2]. Sepsis, pneumonia, shock, aspiration, pancreatitis, blood transfusion, severe trauma and the inhalation of toxic gases are all factors creating

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predisposition against ALI [1,3]. The mortality rate of this disease changes between 34-68% [1], and still has no effective pharmacological treatment [4]. For this reason, it is indicated that there is a necessity for the development of new and different treatment alternatives [5].

In ALI, an increase in the alveolar capillary permeability, inflammatory reactions such as polymorphic nuclear neutrophil infiltration and proinflammatory cytokine release [1] and the release of reactive oxygen species (ROS) such as superoxide and hydroxyl radicals occur. Even though there are little amounts ROS formed in the physiological period, the formation of ROS in excess amounts may result in the peroxidation of the membrane lipids and the dysfunctioning of the biologic membranes [6]. It is indicated that ROS and hence oxidative stress have a very important role in the development of endothelial damage in ALI [7-9]. The lung tissue is protected against oxidative stress through antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and hemeoxygenase (HO)-1 [10].

Rutin (quercetin-3-rhamnosylglucoside) is a natural polyphenolic flavonoid found in buckwheat germ, citrus fruits, vegetables and herbal drinks such as wine and tea [11]. It has a wide range of biologic and pharmacologic activities such as antiinflammatory, antihypertensive, anti-carcinogenic, vasoprotective, antioxidant, antidiabetic and cardioprotective [11,12]. These beneficial effects of rutin are because of its high radical scavenging activity and antioxidant capacity [13]. Various methods in order to create experimental ALI types have been developed on laboratory animals. The primary ones of these methods are lipopolysaccharide (LPS) [4], acid aspiration [14], surfactant consumption with saline lavage [15], pulmonary ischemia/reperfusion [16], cecal ligation and puncturing [5] and application of oleic acid (OA) [17]. OA applied intravascularly cause damage in pulmonary vascular endothelial cells and inflammation in the lungs [6].

The purpose of this study is to explore the protective effects of different doses of rutin with the antioxidant and anti-inflammatory properties on ALI induced by OA in rats.

MATERIAL and METHODS

Animal Material

In the presented study, 35 mature male Sprague-Dawley rats between the weights of 200-220 g were used as animal material. The rats that were subject to normal living standards (temperature: 24±1°C, humidity: 45±5% and 12 h light/dark cycle, feeding with standard laboratory food and ad libitum water) were provided by Ataturk University Medical Experimental Application and Research Center. This study was approved by Ataturk University Animal Experiments Local Ethical Committee (Desicion Number: 2016-1/15). Furthermore, the experimental procedure was carried out in accordance with the International Guidelines for the Care and Use of Laboratory Animals.

Experimental Procedure

The rats were randomly divided into five groups and each group consisted of seven rats. The rats in Group I were given intravenous (i.v.) sterile saline once (control group). The rats in Group II were given rutin (rutin hydrate, Sigma Chemical Company, USA) orally in the doses of 150 mg/kg/day for 7 days. The rats in Group III were given i.v. 50 µl OA (Cis-9-octadecenoic acid, Sigma Aldrich, Germany) dissolved in 250 µl 1% BSA (Bovine Serum Albumin, Sigma Aldrich, Germany) through their tail veins once. The rats in Group IV were given rutin orally in the doses of 75 mg/kg/day for 7 days and on the 7th day the rats were given 50 µl OA i.v. through the tail veins once. The rats in Group V were given rutin orally in the doses of 150 mg/kg/day for 7 days and on the 7th day the rats were given 50 µl OA i.v. through the tail veins once. The rats in all of the groups were sacrificed with decapitation under sevoflurane anesthesia (Sevorane liquid 100%, Abbott Laboratory, Istanbul, Turkey) 24 h after the last application.

Analysis of Oxidants and Antioxidants

The homogenization of lung tissues was performed in a Teflon-glass homogenizer with the use of a buffer of 1.15% KCl in order to obtain a 1:10 (w/v) homogenate. The malondialdehyde (MDA) levels in the lung homogenate were measured by the thiobarbituric acid reaction according to the method of Placer et al.[18]. The lung CAT activity was measured by the decomposition of hydrogen peroxide at 240 nm according to the method of Aebi [19]. The measurement of protein concentration in the supernatant was also performed according to the method of Lowry et al.[20]. The measurement of lung SOD activity was performed by superoxide radical production via xanthine and xanthine oxidase, following the reaction of nitro blue tetrazolium and the formation of formazan dye [21]. The measurement of GSH content according to the method of Sedlak and Lindsay [22]. The GPx activity was measured according to the method of Matkovics et al.[23] and is expressed as U/g of protein in the lung tissue.

Histopathological Examination

Rats were killed by decapitation. The lungs were immediately removed, fixed in 10% neutral formalin solution for 24-48 h, then processed to obtain paraffin blocks. Paraffin-embedded blocks were routinely processed. 5-µm thick sections were stained with hematoxylin-eosin, and examined under a microscope under 20X magnification. Slides in the sections were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe).

Immunohistochemical Examination

After deparaffinization, the slides were immersed

in antigen retrieval solution (pH 6.0) and heated in a microwave for 15 minutes to expose antigens. The sections were then dipped in a 3% H_2O_2 for 10 minutes to block endogenous peroxides. Afterwards, they were incubated at room temperature with polyclonal rabbit inducible nitric oxide synthase (iNOS) antibody (cat. no. Ab48394, dilution 1/400; Abcam, UK) for inflammation. Mouse and rabbit specific HRP/DAB detection IHC kit was used as follows: sections were incubated with goat anti-mouse antibody, then with streptavidin peroxides, and finally with 3,3′ diaminobenzidine + chromogen. Slides were counterstained with hematoxylin. Immunoreactivity in the sections were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe).

Statistical Analysis

The biochemical, histopathologic and immunohistochemical parameters were analyzed with one-way ANOVA using SPSS package program (version 20.0; SPSS, Chicago, IL). The Duncan test was used in the comparison of the groups. All data were presented in mean (±) standard error of means (SEM). Differences in histopathologic and immunohistochemical measured parameters among the five groups were analyzed with a nonparametric test (Kruskal-Wallis). Dual comparisons between the groups exhibiting significant values were evaluated with a Mann–Whitney U-test ($P<0.05$).

RESULTS

The lung tissue MDA levels were significantly higher in the OA group compared to the control and rutin 150 mg groups. The lung tissue MDA levels significantly decreased in the rutin 75 mg + OA and rutin 150 mg + OA groups compared to the OA group. But there were no significant changes in the lung tissue MDA levels between the rutin 150 mg + OA group and the control group. The lung tissue MDA levels were significantly higher in the rutin 75 mg + OA group compared to the rutin 150 mg + OA group *(Table 1)*.

The lung tissue GPx activity significantly decreased in the OA group compared to the control and rutin 150 mg groups. The lung tissue GPx activity significantly increased in the rutin 150 mg + OA group compared to the OA group. But there were no significant changes in the lung tissue GPx activities between the rutin 150 mg + OA group and the control group. The lung tissue GPx activities were significantly higher in the rutin 150 mg + OA group compared to the rutin 75 mg + OA group *(Table 1)*.

The lung tissue GSH level significantly decreased in the OA group compared to the control and rutin 150 mg groups. The lung tissue GSH levels significantly increased in the rutin 150 mg + OA group compared to the OA group, but significantly decreased compared to the control group. There were no significant changes in the lung GSH levels between the rutin 75 mg + OA group and the rutin 150 mg + OA group *(Table 1)*.

The lung tissue CAT activity significantly decreased in the OA group when compared to the control and rutin 150 mg groups. The lung tissue CAT activity significantly increased in the rutin 150 mg + OA group compared to the OA group. There were no significant changes in the lung tissue CAT activity between the rutin 150 mg + OA group and the control group. Furthermore, there were no differences in the lung tissue CAT activity between the rutin 150 mg + OA group and the rutin 75 mg + OA group *(Table 1)*.

The lung tissue SOD activity significantly decreased in the OA group compared to the control and rutin 150 mg groups. The lung tissue SOD activity significantly increased in the rutin 150 mg + OA group compared to the OA group. There were no significant changes in the lung tissue SOD activity between the rutin 150 mg + OA group and the control group. There was a significant increase in the lung SOD activities in the rutin 150 mg + OA group compared to the rutin 75 mg + OA group *(Table 1)*.

Histopathologic Evaluation

It was observed that the lung structures in the control and rutin 150 mg groups were normal *(Fig. 1A-B)*. Severe inflammatory cell infiltrations and hemorrhagia were observed in the interstitial areas of the OA group *(Fig.*

Table 1. *The MDA, GSH levels and GPx, CAT, SOD activities in the lung tissue of the rats in the groups*

Parameter	Groups				
	Group I (Control)	Group II (Rutin 150 mg)	Group III (OA)	Group IV (75 mg rutin + OA)	Group V (150 mg rutin + OA)
MDA (nmol/g tissue)	51.26 ± 1.02^{c}	55.95 ± 1.41^{c}	146.22 ± 3.05^{a}	63.34 ± 1.97^{b}	53.08 ± 0.87^{c}
GPx (U/g protein)	5.90 ± 0.22^{a}	5.67 ± 0.15^{a}	3.08 ± 0.17^{c}	4.68 ± 0.19^{b}	5.41 ± 0.13^{a}
GSH (nmol/g tissue)	34.08 ± 1.02^{a}	35.04 ± 0.84^{a}	20.73 ± 0.58^{c}	24.96 ± 0.75^{b}	27.45 ± 1.39^{b}
CAT (katal/g protein)	45.74 ± 1.40^{b}	49.16 ± 0.89^{a}	30.81 ± 0.62^{d}	41.08 ± 0.72^{c}	41.99 ± 1.26^{c}
SOD (U/g protein)	26.93 ± 0.33^{b}	30.33 ± 0.47^{a}	17.36 ± 0.46^{d}	22.16 ± 1.51^{c}	26.07 ± 0.70^{b}

MDA: *malondialdehyde,* ***SOD:*** *superoxide dismutase,* ***CAT:*** *catalase,* ***GPx:*** *glutathione peroxidase,* ***GSH:*** *glutathione.* ^{a,b,c,d} *Means in rows with different superscripts differ significantly at $P<0.01$. All the values are expressed as the mean±SEM of seven rats in each group*

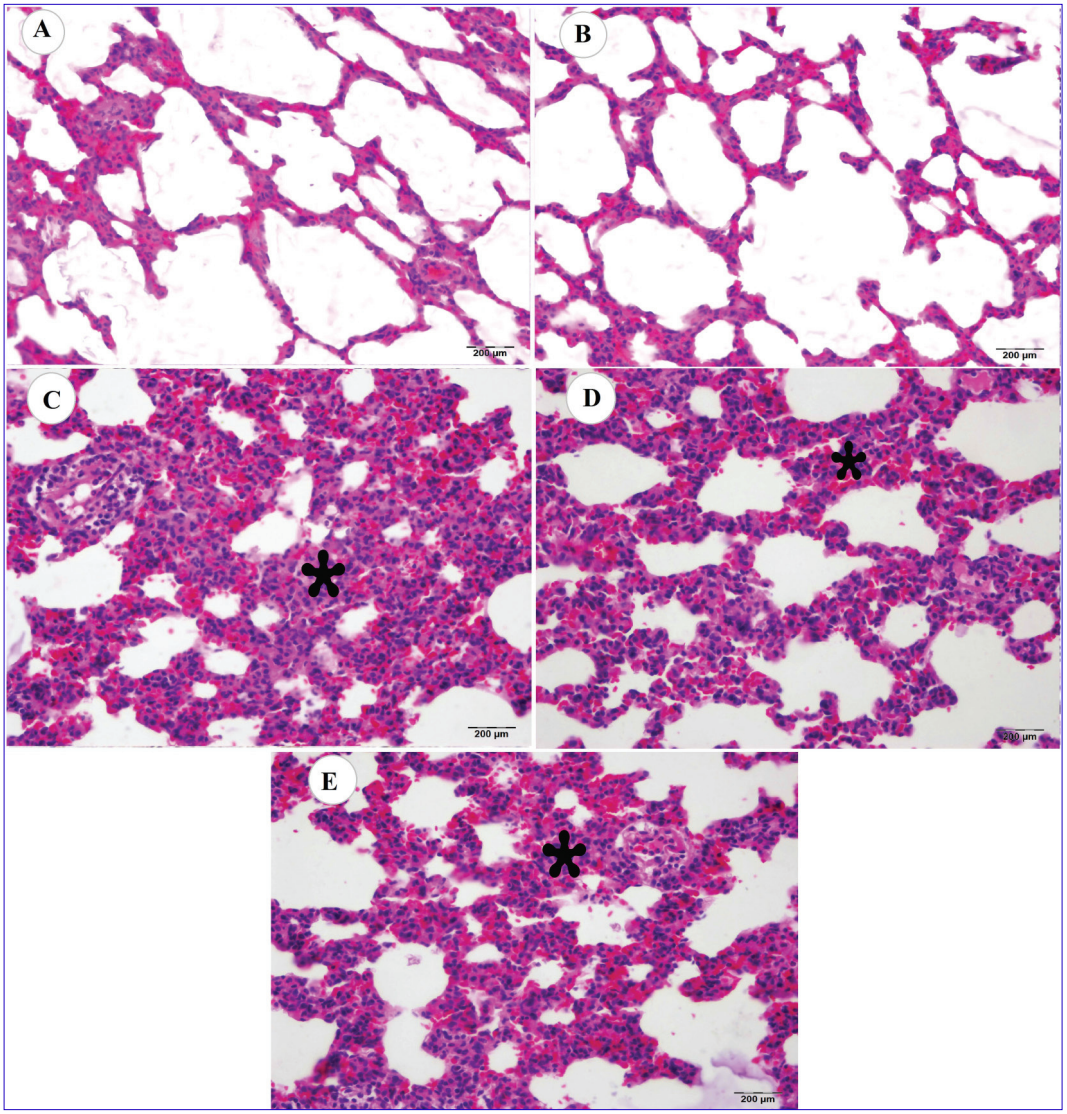

Fig 1. Normal histologic appearance in the control group (Group I) (A). Normal histologic appearance in the rutin 150 mg group (Group II) (B), Severe inflammatory cell infiltrations in the interstitial areas (*) of groups that have been treated with oleic acid (Group III) (C), Moderate inflammatory cell infiltrations in the interstitial areas (*) of the rutin 75 mg + OA group (Group IV) (D), Moderate inflammatory cell infiltrations in the interstitial areas (*) of the rutin 150 mg + OA group (Group V) (E), (H-E)

Table 2. *Evaluation of the inflammatory cell infiltrations and hemorrhage in the lung tissue samples of the groups under a light microscope with x20 magnification: 0 (none), 1 (light), 2 (moderate), 3 (severe)*

Histopathologic Findings	Groups				
	Group I (Control)	Group II (Rutin 150 mg)	Group III (OA)	Group IV (75 mg rutin + OA)	Group V (150 mg rutin + OA)
Inflammatory cell infiltrations and hemorrhage	0.14±0.14[a]	0.14±0.14[a]	2.85±0.14[b]	2.14±0.14[c]	2.28±0.18[c]

All data were presented in mean (±) standard error of means (SEM). [a,b,c] *P<0.05 versus other groups*

1C). The severity of the inflammatory cell infiltrations and hemorrhage in ALI induced by OA were significantly decreased in the rutin 75 mg + OA and rutin 150 mg + OA groups *(Fig. 1D-E, Table 2)*.

Immunohistochemical Evaluation

The lung iNOS, which is used as an inflammatory marker, were expressed in low levels in the lungs of the rats in the control and rutin 150 mg groups *(Fig. 2A-B, Table 3)*. The lung iNOS was severely expressed in the interstitial areas in the OA group, and the lung iNOS expression in the interstitial areas was significantly decreased in the rutin 75 mg + OA and rutin 150 mg + OA groups compared to the OA group. The lung iNOS expression was lower in the rutin

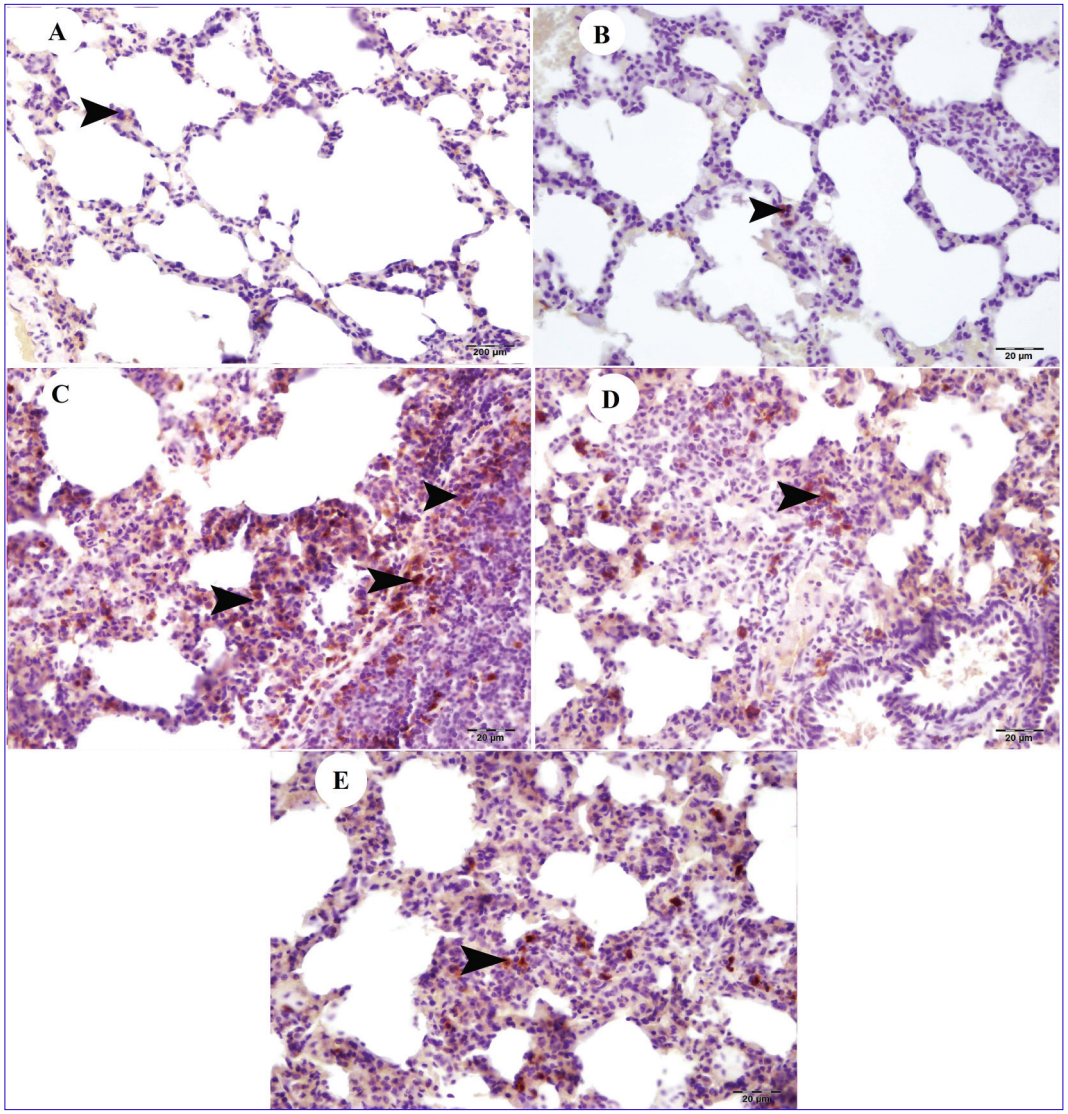

Fig 2. iNOS expressions. Low in the control group (Group I)(A), low in the rutin 150 mg group (Group II) (B), Severe in OA group (Group III) (C), Moderate in the rutin 75 mg + OA group (Group IV) (D), Low in the rutin 150 mg + OA group (Group V) (E) (arrowheads) (IHC)

Table 3. *Immunopositivity of iNOS in the lung tissues of rats*

Immunostaining	Groups				
	Group I (Control)	Group II (Rutin 150 mg)	Group III (OA)	Group IV (75 mg rutin + OA)	Group V (150 mg rutin + OA)
iNOS	1.00±0.21[a]	1.14±0.14[a]	2.85±0.14[b]	2.14±0.14[c]	1.42±0.20[d]

All data were presented in mean (±) standard error of means (SEM) [a, b, c, d] *P<0.05 versus other groups*

150 mg + OA group compared to the rutin 75 mg + OA *(Fig. 2C-E, Table 3)*.

DISCUSSION

Because the morphologic, cellular and functional changes caused by the intravenous treatment of OA are similar to those of ALI, OA treatment in experimental studies in order to research the therapeutic effects of different agents on ALI is quite a common method [24]. There are many studies where OA is used to form ALI in many species such as rat [25], mouse [17], dog [26] and rabbit [27]. Nonenzymatic lipid peroxidation is an important point in the oxidative stress related cellular damage caused by free radicals. MDA, which is the last product of lipid peroxidation, is a good indicator of cellular damage caused by free radicals and oxidative stress [28]. Koksel et al.[25] have indicated the increased MDA levels and the development

of oxidative stress in rats with ALI induced by OA. The same results have been determined in various other studies [6,29]. In the present study in conformity with the results of Koksel et al.[25] it was determined that the MDA level in the OA group was significantly higher than that of the control group. The MDA increase in the rat brains and kidneys caused by ischemia-reperfusion has been reported to be lessened by rutin [13,30]. Furthermore, it was determined that the increase in MDA level caused by β-amyloid 42 in BV-2 cells reduced by rutin [31]. It was found in this study that although the lung MDA level of the rutin 75 mg + OA and rutin 150 mg + OA groups significantly decreased compared to that of the OA group, it still was very high compared to that of the control group. Similarly, Yeh et al.[32] have determined that rutin has an inhibiting effect on the lipid peroxidation of rats with ALI induced by LPS.

Under normal physiological circumstances, cellular defense against oxidative damage is provided through various mechanisms and antioxidant molecules such as SOD, CAT, GSH, and GPx [33]. It is indicated that the antioxidant enzymes are consumed during ALI [34]. This result was supported in the presented study where the GSH level and the GPx, CAT and SOD activities of the lung tissue in the OA group were significantly lower compared to those in the control and rutin 150 mg groups. It is indicated that rutin is an antilipoperoxidant agent [35] and a strong free radical scavenging [36]. Rutin has been indicated to increase the levels of SOD and CAT in rats with cerebral ischemia-reperfusion [37]. Furthermore, rutin treatment has been indicated to increase the SOD, CAT and GPx activities in neurotoxicity [38], liver damage [39], renal damage [40] and testicular damage [41]. Similarly, Yeh et al.[32] have determined that rutin increases the SOD, CAT and GPx activities in rats with ALI induced by LPS. Also, Martinez et al.[42] have revealed that quercetin has anti-inflammatory effect via antioxidant activity on lung injury induced by bleomycin in hamster. Inducible nitric oxide synthase (iNOS), which is used as an inflammatory indicator, is a basic enzyme responsible for the production of high concentration nitric oxide in immune and inflammatory responses [43]. In accord with the reported studies, in the presented study there was a slight increase of iNOS expression in the lung tissue, especially in the interstitial areas, of the rats in the control and rutin 150 mg groups and there was a severe iNOS expression in the interstitial areas in the OA group treated with OA alone. In accord with the results in the presented study, Yi-Chun et al.[44] have found that rutin shows the protective effect by inhibiting neutrophil infiltration and the expression of iNOS and vascular cell adhesion molecule (VCAM)-1 in ALI model induced by LPS in mice. The studies have revealed that rutin has the protective effect via anti-inflammatory properties in ALI model [45,46]. Also, Kandemir et al.[40] have reported that iNOS is expressed in the glomeruli and mesangial cells in rats with renal damage induced by gentamicin, and that the treatment of rutin decreases the iNOS expression and thus decreases inflammation.

As a result, it was concluded that rutin has a protective effect through its antioxidant and anti-inflammatory properties in the ALI induced by OA and that this protective effect is higher in the 150 mg dosage than the 75 mg dosage and that it would be beneficial to use rutin in the supportive treatment of ALI patients.

REFERENCES

1. Ware LB, Matthay MA: The acute respiratory distress syndrome. *N Engl J Med*, 342, 1334-1349, 2000. DOI: 10.1056/NEJM200005043421806

2. Mouratis MA, Magkrioti C, Oikonomou N, Katsifa A, Prestwich GD, Kaffe E, Aidinis V: Autotaxin and endotoxin-induced acute lung injury. *Plos One*, 10 (7):10:e0133619, 2015. DOI: 10.1371/journal.pone.0133619

3. Brun-Buisson C, Fartoukh M, Lechapt E, Honore S, Zahar JR, Cerf C, Maitre B: Contribution of blinded, protected quantitative specimens to the diagnostic and therapeutic management of ventilator-associated pneumonia. *Chest,* 128, 533-544, 2005. DOI: 10.1378/chest.128.2.533

4. Zhang Y, Liang D, Dong L, Ge X, Xu F, Chen W, Dai Y, Li H, Zou P, Yang S, Liang G: Anti-inflammatory effects of novel curcumin analogs in experimental acute lung injury. *Respir Res,* 16, 43-55, 2015. DOI: 10.1186/s12931-015-0199-1

5. Wang SY, Li ZJ, Wang X, Li WF, Lin ZF: Effect of ulinastatin on HMGB1 expression in rats with acute lung injury induced by sepsis. *Genet Mol Res,* 14, 4344-4353, 2015. DOI: 10.4238/2015.April.30.7

6. Yang C, Moriuchi H, Takase J, Ishitsuka Y, Irikura M, Irie T: Oxidative stress in early stage of acute lung injury induced with oleic acid in guinea pigs. *Biol Pharm Bull*, 26, 424-428, 2003. DOI: 10.1248/bpb.26.424

7. Tampo Y, Tsukamoto M, Yonaha M: Superoxide production from paraquat evoked by exogenous NADPH in pulmonary endothelial cells. *Free Radic Biol Med,* 27, 588-595, 1999. DOI: 10.1016/S0891-5849(99)00110-0

8. Chow CW, Abreu MTH, Suzuki T, Downey GP: Oxidative stress and acute lung injury. *Am J Respir Cell Mol Biol,* 29, 427-431, 2003. DOI: 10.1165/rcmb.F278

9. Ward PA: Oxidative stress: Acute and progressive lung injury. *Ann N Y Acad Sci,* 1203, 53-59, 2010. DOI: 10.1111/j.1749-6632.2010.05552.x

10. Kuo MY, Liao MF, Chen FL, Li YC, Yang ML, Lin RH, Kuan YH: Luteolin attenuates the pulmonary inflammatory response involves abilities of anti- oxidation and inhibition of MAPK and NFκB pathways in mice with endotoxin-induced acute lung injury. *Food Chem Toxicol,* 49, 2660-2666, 2011. DOI: 10.1016/j.fct.2011.07.012

11. Guo R, Wei P: Studies on the antioxidant effect of rutin in the microenvironment of cationic micelles. *Microchim Acta*, 161, 233-239, 2008. DOI: 10.1007/s00604-007-0888-7

12. Sharma S, Asgar A, Javed A, Jasjeet KS, Sanjula B: Rutin: Therapeutic potential and recent advances in drug delivery. *Expert Opin Investig Drugs,* 22, 1063-1079, 2013. DOI: 10.1517/13543784.2013.805744

13. Korkmaz A, Kolankaya D: Protective effect of rutin on the ischemia/reperfusion induced damage in rat kidney. *J Surg Res,* 164, 309-315, 2010. DOI: 10.1016/j.jss.2009.03.022

14. Maniatis NA, Sfika A, Nikitopoulou I, Vassiliou AG, Magkou C, Armaganidis A, Raussos C, Kollias G, Orfanos S, Kotanidou A: Acid-induced acute lung injury in mice is associated with P44/42 and c-Jun N-terminal kinase activation and requires the function of tumor necrosis factor α receptor I. *Shock*, 38, 381-386, 2012. DOI: 10.1097/SHK.0b013e3182690ea2

15. Kamiyama J, Jesmin S, Sakuramoto H, Shimojo N, Islam M, Khatun T, Oki M, Kawano S, Mizutani T: Assessment of circulatory and pulmonary endothelin-1 levels in a lavage-induced surfactant-depleted lung injury rabbit model with repeated open endotracheal suctioning and hyperinflation. *Life Sci,* 118, 370-378, 2014. DOI: 10.1016/j.lfs.2014.04.001

16. Wu SY, Tang SE, Ko FC, Wu GC, Huang KL, Chu SJ: Valproic acid attenuates acute lung injury induced by ischemia-reperfusion in rats. *Anesthesiology,* 122, 1327-1337, 2015. DOI: 10.1097/ALN.0000000000000618

17. Dong HY, Xu M, Ji ZY, Wang YX, Dong MQ, Liu ML, Xu DQ, Zhao PT, Liu Y, Luo Y, Niu W, Zhang B, Ye J, Li ZC: Leptin attenuates lipopolysaccharide or oleic acid-induced acute lung injury in mice. *Am J Respir Cell Mol Biol,* 49, 1057-1063, 2013. DOI: 10.1165/rcmb.2012-0301OC

18. Placer ZA, Cushmanni LL, Johnson BC: Estimation of products of lipid peroxidation (as malondialdehyde) in biochemical systems. *Anal Biochem,* 16, 359-364, 1966. DOI: 10.1016/0003-2697(66)90167-9

19. Aebi H: Catalase *in vitro. Methods Enzymol,* 105, 121-126, 1984. DOI: 10.1016/S0076-6879(84)05016-3

20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem,* 193, 265-275, 1951.

21. Sun Y, Larry WO, Ying L: A simple method for clinical assay of superoxide dismutase. *Clin Chem,* 34 (3): 497-500, 1998.

22. Sedlak J, Lindsay RHC: Estimation of total protein bound and nonprotein sulfhydryl groups in tissue with Ellmann's reagent. *Anal Biochem*, 25, 192-205, 1968. DOI: 10.1016/0003-2697(68)90092-4

23. Matkovics B, Szabo I, Varga IS: Determination of enzyme activities in lipid peroxidation and glutathione pathways. *Lab Diagn*, 15, 248-249, 1988.

24. Golbidi S, Moriuchi H, Yang C, Irikura M, Irie T, Hamasaki N: Preventive effect of phosphoenolpyruvate on hypoxemia induced by oleic acid in guinea pigs. *Biol Pharm Bull,* 26, 336-340, 2003. DOI: 10.1248/bpb.26.336

25. Koksel O, Ozdulger A, Ercil M, Tamer L, Ercan B, Atik U, Cinel L, Cinel İ, Kanik A: Effects of N-acetylcysteine on oxidant antioxidant balance in oleic acid-induced lung injury. *Exp Lung Res,* 30, 431-446, 2004. DOI: 10.1080/01902140490476319

26. Gaio E, Silva CAM, Brito F, Firmino MAP, Storck R, Freitas E: Stability of the animal model of oleic acid-induced acute lung injury. *J Bras Pneumol,* 35, 759-766, 2009. DOI: 10.1590/S1806-37132009000800007

27. Vadasz I, Morty RE, Kohstall MG, Olschewski A, Grimminger F, Seeger W, Ghofrani HA: Oleic acid inhibits alveolar fluid reabsorption. A role in acute respiratory distress syndrome. *Am J Respir Crit Care Med,* 171, 469-479, 2005. DOI: 10.1164/rccm.200407-954OC

28. Ayala A, Munoz MF, Argüelles S: Lipid Peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxid Med Cell Longev,* 2014, 1-31, 2014. DOI: 10.1155/2014/360438

29. Chen HI, Hsieh NK, Kao SJ, Su CF: Protective effects of propofol on acute lung injury induced by oleic acid in conscious rats. *Crit Care Med,* 36, 1214-1221, 2008. DOI: 10.1097/CCM.0b013e31816a0607

30. Annapurna A, Ansari MA, Manjunath PM: Partial role of multiple pathways in infarct size limiting effect of quercetin and rutin against cerebral ischemia-reperfusion injury in rats. *Eur Rev Med Pharmacol Sci,* 17, 491-500, 2013.

31. Wang SW, Wang YJ, Su YJ, Zhou WW, Yang SG, Zhang R. Zhao M, Li Y, Zhang Z, Zhan D, Liu R: Rutin inhibits β-amyloid aggregation and cytotoxicity, attenuates oxidative stress, and decreases the production of nitric oxide and proinflammatory cytokines. *Neurotox,* 33, 482-490, 2012. DOI: 10.1016/j.neuro.2012.03.003

32. Yeh CH, Yang JJ, Yang ML, Li YC, Kuan YH: Rutin decreases lipopolysaccharide-induced acute lung injury via inhibition of oxidative stress and the MAPK-NF-κB pathway. *Free Rad Biol Med,* 69, 249-257, 2014. DOI: 10.1016/j.freeradbiomed.2014.01.028

33. Halliwell B: Antioxidant defense mechanism: From the beginning to the end (of the beginning). *Free Radic Res,* 31, 261-272, 1999. DOI: 10.1080/10715769900300841

34. Hosakote YM, Liu T, Castro SM, Garofalo RP, Casola A: Respiratory syncytial virüs induces oxidative stress by modulating antioxidant enzymes. *Am J Respir Cell Mol Biol,* 41, 348-357, 2009. DOI: 10.1165/rcmb.2008-0330OC

35. Negre-Slvayre A, Affany A, Hariton CR: Additional antilipoperoxidant activities of alpha-tocopherol and ascorbic acid on membrane- like systems are potentiated by rutin. *Pharmacol,* 42, 262-272, 1991. DOI: 10.1159/000138807

36. Oomah BD, Mazza G: Flavonoids and antioxidant activities in buckwheat. *J Agric Food Chem,* 44, 1746-1750, 1966. DOI: 10.1021/jf9508357

37. Victor VM, Rocha M, Esplugues JV, Dela Fuente M: Role of free radicals in sepsis: antioxidant therapy. *Curr Pharm Des,* 11, 3141-3158, 2005. DOI: 10.2174/1381612054864894

38. Magalingam KB, Radhakrishnan A, Haleagrahara N: Rutin, a bioflavonoid antioxidant protects rat pheochromocytoma (PC-12) cells against 6- hydroxydopamine (6-OHDA)-induced neurotoxicity. *Int J Mol Med,* 32, 235-240, 2013. DOI: 10.3892/ijmm.2013.1375

39. Lee CC, Shen SR, Lai YJ, Wu SC: Rutin and quercetin, bioactive compounds from tartary buckwheat, prevent liver inflammatory injury. *Food Funct,* 4, 794-802, 2013. DOI: 10.1039/C3FO30389F

40. Kandemir FM, Ozkaraca M, Yildirim BA, Hanedan B, Kirbas A, Kilic K, Aktaş E, Benzer F: Rutin attenuates gentamicin-induced renal damage by reducing oxidative stress, inflammation, apoptosis, and autophagy in rats. *Ren Fail,* 37, 518-525, 2015. DOI: 10.3109/0886022X.2015.1006100

41. Abarikwu SO, Iserhienrhien BO, Badejo TA: Rutin-and selenium-attenuated cadmium-induced testicular pathophysiology in rats. *Hum Exp Toxicol*, 32, 395-406, 2013. DOI: 10.1177/0960327112472995

42. Martinez JAB, Ramos SG, Meirelles MS, Verceze AV, Rodrigues de Arantes M, Vannucchi H: Effects of quercetin on bleomycin-induced lung injury: A preliminary study. *J Bras Pneumol,* 34, 445-452, 2008. DOI: 10.1590/S1806-37132008000700003

43. Anggard E: Nitric oxide: Mediator, murderer and medicine. *Lancet,* 343, 1199-1206, 1994. DOI: 10.1016/S0140-6736(94)92405-8

44. Yi-Chun H, Hong CT, Chen ST, Lee SS, Yang ML, Lee CY, Kuo WH, Yeh CH, Kuan YH: Rutin improves endotoxin-induced acute lung injury via inhibition of iNOS and VCAM-1 expression. *Environ Toxicol*, 31, 185-191, 2016. DOI: 10.1002/tox.22033

45. Feng L, Wang D, He J, Qi D: Protective effect of rutin against lipopolysaccharide-induced acute lung injury in mice. *Nan Fang Yi Ke Da Xue Xue Bao*, 34 (9): 1282-1285, 2014.

46. Chen WY, Huang YC, Yang ML, Lee CY, Chen CJ, Yeh CH, Pan PH, Horng CT, Kuo WH, Kuan YH: Protective effect of rutin on LPS-induced acute lung injury via down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation. *Int Immunopharmacol,* 22, 409-413, 2014. DOI: 10.1016/j.intimp.2014.07.026

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Research Article

Evaluation of Paraoxonase Activity, Total Sialic Acid and Oxidative Stress in Sheep with Ecthyma Contagiosa [1]

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Abstract

Zoonoses are defined by the world health organization as diseases and infections which are transmitted naturally between vertebrate animals and human. Undestanding the zoonotic risk posed by pox viruses in companion animals is important for both human and animal health. Contagious ecthyma is highly contagious, zoonotic, viral skin disease that affects sheep, goats and some other domesticated and wild animals. In this present study was detected and evaluated levels of plasma paraoxonase activity (PON1), high-density lipoprotein (HDL), total sialic acid (TSA), malondialdehyde (MDA), nitric oxide (NO) and total blood glutathione (GSH) concentrations in healthy sheep and natural infected sheeps with ecthyma. In healthy sheep, laboratory results were determined as PON1 218.54±17.93 U/L, TSA 59.89±5.59 mg/dL, HDL 48.4±4.88 mg/dL, MDA 8.58±0.80 µmol/L, NO 7.78±1.02 µmol/L and GSH 21.11±3.70 mg/dL. These values were found 174.92±18.68 U/L, 70.1±6.56 mg/dL, 37.9±6.47 mg/dL, 11.26±1.06 µmol/L, 12.44±1.90 µmol/L, 7.79±0.90 mg/dL respectively in sheeps wich are infected by ecthyma. As a result, it was concluded that there is oxidative stress due to imbalance between pro-oxidant and antioxidant molecules in sheep which are infected by ecthyma, and this imbalance is shaped by increasing oxidant levels.

Keywords: *Ecthyma, Paraoxonase activity, Total sialic acid, Oxidative stress, Sheep*

Ecthyma Contagiosa'lı Koyunlarda Paraoksonaz Aktivitesi, Total Sialik Asit ve Oksidatif Stresin Değerlendirilmesi

Özet

Zoonozlar, dünya sağlık organizasyonu tarafından omurgalı hayvanlar ve insanlar arasında doğal olarak iletilen hastalıklar ve enfeksiyonlar olarak tanımlanmaktadır. Pox virüslü hayvanların oluşturduğu zoonotik riskin saptaması ve bertaraf edilmesi hem insan hem de hayvan sağlığı için önemlidir. Bulaşıcı ektima koyun, keçi, diğer bazı evcil ve vahşi hayvanları etkileyen bulaşıcı, zoonotik, viral cilt hastalığıdır. Sunulan bu çalışmada sağlıklı ve ektima ile doğal enfekte koyunlarda plazma paraoksonaz aktivitesi (PON1), yüksek dansiteli lipoprotein (HDL), toplam sialik asit (TSA), malondialdehit (MDA), nitrik oksit (NO) ve toplam kan glutatyonu seviyeleri tespit edildi ve sonuçları değerlendirildi. Sağlıklı koyunlarda PON1 218.54±17.93 U/L, TSA 59.89±5.59 mg/dL, HDL 48.4±4.88 mg/dL, MDA 8.58±0.80 µmol/L, NO 7.78±1.02 µmol/L ve GSH 21.11±3.70 mg/dL olarak belirlendi. Ektima ile enfekte koyunlarda ise bu değerler sırasıyla 174.92±18.68 U/L, 70.1±6.56 mg/dL, 37.9±6.47 mg/dL, 11.26±1.06 µmol/L, 12.44±1.90 µmol/L, 7.79±0.90 mg/dL tespit edildi. Sonuç olarak, ektima ile enfekte koyunlarda, pro-oksidan ve antioksidan moleküller arasındaki dengesizliğe bağlı oksidatif stres oluştuğu, bu dengenin artan oksidan seviyelerine bağlı olduğu saptandı.

Anahtar sözcükler: *Ektima, Paraoksonaz aktivitesi, Total sialik asit, Oksidatif stres, Koyun*

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INTRODUCTION

Ecthyma contagiosum *(Contagious ecthyma, Orf)* mainly occures in sheep and goats. The disease is caused by an epitheliotropic virus *(Parapoxvirus -Orfvirus-* of the family Poxviridae), and is common disease in Türkiye, it can affects many small ruminant spicies between spring and autumn. Orf has been reported in people who handled infected animals or there tissues. Contagious ecthyma results from infection by the orf virus, a member of the genus parapox virus in the sub family chordopox viridae. Ecthyma contagiosum mainly occures in sheep and goats. Orf has been reported ın people who handled infected animals or there tissues. It is mandatory to report this zoonotic disease that causes significant economic loss in animal husbandry and can be transmitted to humans from animals [1-3]. Ecthyma contagiosum disease is known as orf, contagious ecthyma, contagious pustular stomatitis, infectious labial dermatitis, infectious pustular dermatitis, sore mouth, and scabby mouth [4].

Orf virus can be transmitted by direct contact with sick animals or indirectly from materials such as dried skin that fall on the grass during grazing. Infection can also be transmitted as a result of the administration of salt blocks or licking stones, which have contacted with infected wild goats. It has also known that the rate of spread of the disease and the size of the lesions are related to the labor exposure which causes injuries on the mouths of animals during grazing [3]. It has been reported by different researchers that the disease affects all age groups equally, without racial and gender discrimination between animals [5-7].

In ecthyma contagiosum, lesions are mainly seen around the lips and around. In addition, typical lesions can be seen in mouth, nose, oral mucosa, tongue, gingiva and palate. Similar lesions are observed in the lambs as small erythematous and ulcerated papules on the gums, tongue and palate [2,3,5,8,9]. Skin lesions of the disease are frequently seen in the head, face, ears, neck, chest, legs and inguinal region [10]. The lesions can usually be cauliflower-like papillomatosis, proliferative, granulomatous, dry crusted, embossed and erosive [5]. The average incubation period in ecthyma contagiosum disease is 2-3 weeks [11,12]. Diagnosis of the disease is easily possible with clinical findings, characteristic skin lesions and histopathological examination findings of these lesions as well as biopsy of the infected skin and visualization of the agent itself in electron microscopy or advanced laboratory techniques like PCR [11].

Reactive oxygen species or free radicals are released from dendritic cells, neutrophils and macrophages in response to an inflammatory agents. These materials are highly reactive because they contain unpaired electron or non-static bonds. Free radicals and antioxidant mechanisms are in balance in normal physiological condition. In order to limit an effective immune response and tissue damage with the living organism, it is vital that these substances are in balance [13,14]. The increase in the level of reactive oxygen species in cells leads to oxidative damage in protein, lipid and DNA. In this case, loss of enzyme activity, inhibition of protein synthesis and DNA damage occur, resulting in cell death [14].

The aim of this study is to determine the levels of plasma paraoxonase activity (PON1), total sialic acid (TSA) and some oxidative stress markers in Akkaraman sheep that are determined to be infected by ecthyma, a natural and important zoonotic viral disease, and to reveal possible changes of these parameters in ecthyma contagiosum disease.

MATERIAL and METHODS

Experimental Animals

Twenty Akkaraman sheep were used in the study at the age of 1 year in the flock which was examined due to the suspicion of ecthyma disease in the Erzurum (Türkiye) region. According to the clinical and laboratory studies animals divided into two groups: Those who were infected with the ecthyma virus (n=10) and those who were healthy (n=10). Blood samples were taken from the vena jugularis of the sheep for laboratory diagnosis of the disease and were centrifuged at 3.000 rpm for 10 min to separate the plasmas. The obtained plasmas were kept at -25°C until biochemical analyses.

Biochemical Assays

PON1 activity measurement was performed according to the methods of Eckerson [15] and Gülcü and Gürsu [16]. PON1 activity was determined by spectrophotometric measurement of the absorbance of the colored compound, 4-nitrophenol, from the enzyme hydrolysis product paraoxone (Sigma) at 25°C and 412 nm. For PON1 activity, enzyme in 1 mL of serum was identified as the enzyme activity unit that converts 1 nmol of paraoxon to 4-nitrophenone in 1 min and the results are given as U/L. TSA values were measured using a spectrophotometer (PowerWave XS, BioTek®, USA) according to the colorimetric method of Sydow [17] and the results were expressed in mg/dL. High-density lipoprotein (HDL) was studied in the autoanalyzer using the Biotrol trademark kit and the results were shown in mg/dL. Plasma nitric oxide (NO) levels were determined by the method reported by Miranda et al.[18]. In this method, nitrate is converted to nitrite with vanadium (III) chloride. Reaction of N- (1-Naphthyl) ethylenediaminedihydrochloride with nitrite sulfanil-amine in acidic medium gave the resultant complex diazonium compound. The resulting colored complex was measured at 540 nm. After nitrate and nitrite levels were determined separately, the sum of the two was determined as NO amount. The level of malondialdehyde (MDA) was determined according to the method reported by

Yoshioka et al.[19]. The MDA formed in this method forms a pink complex with thiobarbituric acid (TBA) and the absorbance of this solution is measured spectrophotometrically at 535 nm (PowerWawe XS, Biotek®, Instruments, USA) to determine the degree of lipid peroxidation. The MDA levels obtained were calculated as µmol/l. The level of glutathione (GSH) was determined from whole blood according to the method reported by Beutler [20].

DNA Extraction

Scabs were collected from the lip lesions on affected animals and were used for DNA extraction. DNA was extracted from scabs and by using QIAamp DNA Mini kit™ (Qiagen). Scabs were separately placed in a 2 mL screw capped tubes including glass particles with lysis buffer and proteinase K supplied with the kit and homogenised using Magna Lyser™ instrument (Roche). Further extraction protocol was applied according to the manufacturer's instructions, eluted with 100 µL elution buffer and stored at -20°C.

Polymerase Chain Reaction (PCR)

PCR reaction was done with 045-F (5'-CCT ACT TCT CGG AGT TCA GC-3') and 045-R (5'-GCA GCA CTT CTC CTC GTA G-3') primers encoding 392 bp of VLTF-1 gene as described before [19]. PCR was conducted in a 50 µL reaction mixture comprising 25 µL 2X PCR Master Mix (Promega) containing: 50 units/mL of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$; 20 pmol each forward and reverse primer, 2 µL template DNA and remained amount of PCR grade water. The reaction was carried out in conditions 40 cycles of denaturation at 95°C for 10 s, annealing at 47°C for 10 s, extension at 74°C for 10 s followed by final extension at 78°C for 10 min.

PCR products were electrophoresed on 2% agarose gel (Sigma®) in Tris-acetate EDTA (TAE) buffer and stained with ethidium bromide (1 µg/mL). Gel was analyzed with a gel documentation system (GeneLine, Spectronics Corp. NY, USA) [21].

Reference control of *Orfvirus* which was previously identified with PCR from field epizooties was kindly provided by Veterinarian Ömer Faruk KÜÇÜKKALEM (Virology Department, Veterinary Control and Research Institute, Erzurum, Turkey). Negative control samples were collected from slaughtered sheep in abattoir without any clinical history and Orf symptoms.

Statistics

The SPSS program was used to evaluate the data obtained from the study. First, the Kolmogorov-Smirnov test was performed and the normal distribution of the groups was assessed and the Student's t test was used to compare these groups with normal distribution. The results are expressed as "mean value (X)±standard deviation (SD)".

RESULTS

As a result of clinical examination of the animals evaluated in the diagnosis of this disease, which is mandatory to report, clinical symptoms were observed as fatigue and hypersalivation as well as typical lesions in the mouth, lips, gums, tongue and nose *(Fig. 1)*.

PON1 activity, TSA, HDL, MDA, NO and GSH levels were measured in the blood samples taken from the animals and PCR evaluation was performed to confirm the diagnosis. In biochemical evaluations; plasma PON1 activity, HDL, and GSH levels obtained from the animals in the contagiosum disease group were statistically significantly low (P<0.001)

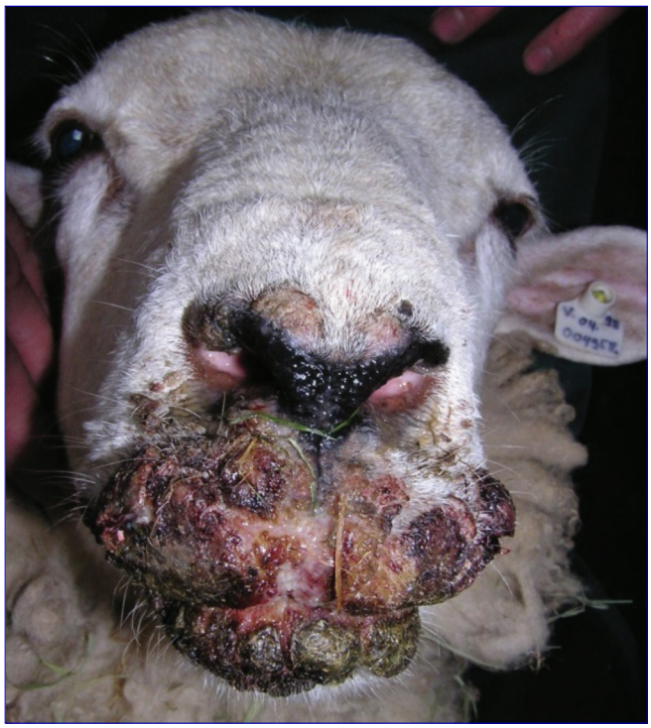

Fig 1. Clinically view of the ecthyma contagiosa in a Akkaraman sheep

Table 1. *Plasma PON1 activity, HDL, TSA, NO, MDA, and whole blood GSH levels in healthy and sheeps with ecthyma*

Parameters	Healty Group (n=10)	Ecthyma Contagiosum (n=10)
TSA (mg/dL)	59.89±5.59[a]	70.1±6.56[b]
PON 1 (U/L)	218.54±17.93[a]	174.92±18.68[b]
HDL (mg/dL)	48.4±4.88[a]	37.9±6.47[b]
MDA (µmol/L)	8.58±0.80[a]	11.26±1.06[b]
NO (µmol/L)	7.78±1.02[a]	12.44±1.90[b]
GSH (mg/dL)	21.11±3.70[a]	7.79±0.90[b]

[a,b] The difference between the averages with different letters in the same line is important (P<0.001)

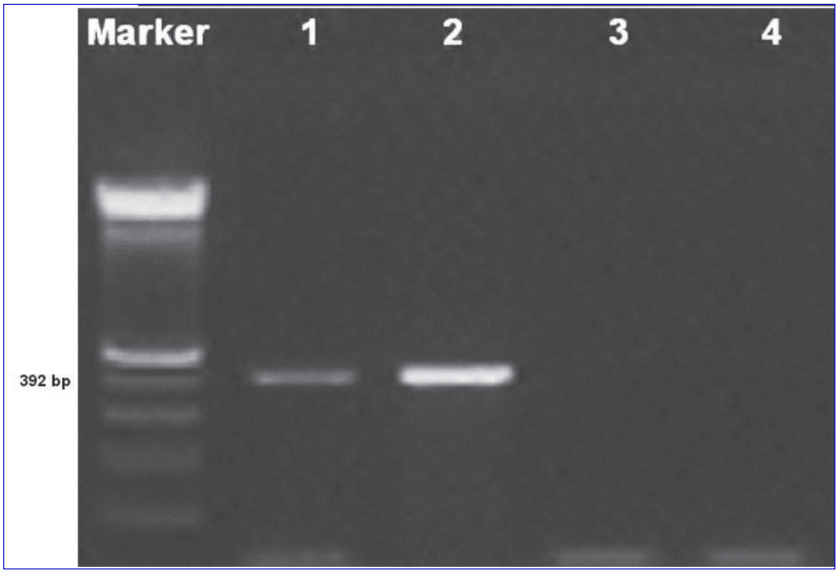

Fig 2. Agarose gel electrophoresis of PCR products: Marker 100 bp DNA ladder (Gene Ruler™, Fermentas), lane 1: Field sample collected in this study, lane 2: Positive control, lane 3: Negative control, lane 4: Water

compared to the healthy animal group. On the other hand, on the ecthyma contagiosum group TSA, MDA and NO levels were found to be statistically significantly ($P<0.001$) higher than the control group *(Table 1)*.

All of the clinical samples were detected positive with PCR *(Fig. 2)* in ecthyma group. Targeted 392 bp of PCR product was visualised from the DNA extracts of positive control sample and scab materials collected in this study. Targeted specific PCR amplicons were not detected from negative control samples and water.

DISCUSSION

It has been reported that in the case of ecthyma contagiosum disease, typical lesions such as papules, pustules, nodules have been observed following clinical manifestations of weakness, anorexia, hypersalivation, tongue and swelling and redness in the gums [2,11,12]. Typical skin lesions in the clinical examination of the animals used in our study were observed in the group of patient sheep, suggesting similar symptoms in addition to the usual symptoms.

NO is a biological mediator synthesized from L-arginine by nitric oxide synthase catalysis and cytotoxic to agents such as bacteria, fungi, protozoa and viruses [3,14,22]. Free radicals or reactive oxygen species are released from neutrophils and macrophages during inflammatory conditions [13]. These species are toxic to biomembranes. Unless removed by free radical cleansing enzymes such as glutathione peroxidase (GSH-px), they cause peroxidation of lipids. In such cases, antioxidants act to purify free radicals by converting them into less harmful molecules [23]. It has been reported that the paratoxyl-orf virus influences neutrophil, basophil, and mast cells from the inflammatory cells, in spite of the early response of multiplying neutrophils in the early period of viral replication and acts against disease, the number of cutaneous mast cells does not alter [24,25]. In our study, the level of NO obtained from sick animals was higher than that of control group, and the level of GSH obtained from whole blood was found to be low. This increase in NO levels in ecthyma infection is thought to be caused by release of too much free radicals due to the increase in neutrophils that became active because of the disease which are reported by researchers [3]. The decrease in the level of GSH that we have found is in line with the view that researchers previously reported that free radicals, which are found in inflammatory conditions, strongly consume antioxidants.

The increase in the level of reactive oxygen species in the cells results in oxidative damage to the structure of proteins, lipids and DNA [14]. MDA, the final product of lipid peroxidation and the most important indicator, is the most important molecule effective in cellular degeneration caused by free radicals [26]. In the present study, it was determined that the MDA concentration obtained from the group of sheeps with ecthyma was significantly higher than that of the other group and lower GSH and HDL levels. It has been determined that high MDA and low GSH and HDL levels from the group of sheeps with ecthyma were found to be an important indicator of lipid peroxidation in ruminants, and are particularly compatible with studies conducted by researchers in poxvirus, some other viral, bacterial and parasitic agents [23,26-30].

Sialic acid, an acetylated derivative of neuraminic acid, is an important cell surface component found in biological membranes of bacteria and animals. Many investigators have reported that TSA concentration increases in cases of severe inflammatory, cellular degeneration or proliferation situations [30-32]. In this study, it was determined that the TSA concentration obtained from parapox-orf virus-infected sheep was statistically significantly higher than healthy sheep. The high level of TSA in infected sheep is thought to be due to severe cellular degeneration and proliferation caused by inflammation in these animals.

PON1 is an antioxidant enzyme found in liver, kidney, intestine and HDL on serum and its activity can change as part of the inflammatory response [32,33]. In this presented study, a decrease in both HDL and PON1 activity was detected in the sheeps with ecthyma. The cause of this reduction may be the lipid peroxidation, which the severity is evidenced by other markers. This has been confirmed

in agreement with investigators who report that oxidized lipids inhibit PON1 activity in elevated lipid peroxidation [33-35].

In conclusion, low PON1 activity, HDL, GSH levels and high MDA and NO levels, which were obtained from the sheeps were infected by ecthyma. That indicate the disease caused significant oxidative stress in sheep. Along with that, it was concluded that the high TSA level determined in the group consisting of patient sheeps was an important indicator of oxidative stress-induced cell and tissue damage in the disease.

REFERENCES

1. Nadeem M, Curran P, Cooke R, Ryan CA, Connolly K: Orf: Contagious pustular dermatitis. *Ir Med J*, 103, 152-153, 2010.

2. Issi M, Gulacti I, Bulut H, Gul Y: Severe persistent contagious ecthyma cases in twin goats. *J Anim Vet Adv*, 9, 2477-2481, 2010. DOI: 10.3923/javaa.2010.2477.2481

3. Kandemir FM, Issi M, Benzer F, Gul Y, Basbug O, Ozdemir N: Plasma nitric oxide concentrations and erythrocyte arginase activities in lambs with contagious ecthyma. *Revue Med Vet,* 162 (6): 275-278. 2011.

4. de Wet C, Murie J: Lamb pays lip service: Two cases of ecthyma contagiosum *(orf)*. *Scott Med J*, 56, 59, 2011.

5. Kumar R, Trivedi RN, Bhatt P, Khan SH, Khurana SK, Tiwari R, Karthik K, Malik YS, Dhama K, Chandra R: Contagious pustular dermatitis *(orf disease)*-epidemiology, diagnosis, control and public health concerns. *Adv Anim Vet Sci*, 3, 649-676, 2015. DOI: 10.14737/journal.aavs/2015/3.12.649.676

6. Billinis C, Mavrogianni VS, Spyrou V, Fthenakis GC: Phylogenetic analysis of strains of *Orf virus* isolated from two outbreaks of the disease in sheep in Greece. *Virol J*, 9, 24, 2012. DOI: 10.1186/1743-422X-9-24

7. Bouznach A, Hahn S, Stram Y, Menasherov S, Edery N, Shicaht N, Kenigswald G, Perl S: Case report: Contagious ecthyma-deviations in the anatomical appearance of lesions in an outbreak in lambs in Israel. *Israel J Vet Med*, 68, 246-251, 2013.

8. Gökce Hİ, Genç O, Gökçe G: Sero-prevalence of contagious ecthyma in lambs and humans in Kars, Turkey. *Turk J Vet Anim Sci*, 29, 95-101, 2005.

9. McElroy MC, Bassett HF: The development of oral lesions in lambs naturally infected with Orf Virus. *Vet J*, 174, 663, 2007. DOI: 10.1016/j.tvjl.2006.10.024

10. Alian S, Ahangarkani F, Arabsheybani S: A case of orf disease complicated with erythema multiforme and bullous pemphigoid-like eruptions. *Case Rep Infect Dis*, Article ID: 105484, 2015. DOI: 10.1155/2015/105484

11. Coates JW, Hoff S: Contagious ecthyma: An unusual distribution of lesions in goats. *Canadian Vet J*, 31, 209-210, 1990.

12. Zamri-Saad M, Al-Ajeeli KSA, Ibrahim AL: A severe outbreak of Orf involving the buccal cavity of goats. *Trop Anim Health Prod,* 24, 177-178, 1992. DOI: 10.1007/BF02359612

13. Salman KA, Ashraf S: Reactive oxygen species: A link between chronic inflammation and cancer. *As Pac J Mol Biol Biotechnol*, 21, 42-49, 2013.

14. Özcan A, Öğün M: Biochemistry of reactive oxygen and nitrogen species. **In,** Gowder SJT (Ed): Basic Principles and Clinical Significance of Oxidative Stress. *InTech*, 2015. DOI: 10.5772/61193

15. Eckerson HW, Romson J, Wyte CM, La Du BN: The human serum paraoxonase polymorphism: Identification of phenotypes by their response to salts. *Am J Hum Genet*, 35, 214-227, 1983.

16. Gülcü F, Gürsu MF: The standardization of paraoxonase and arylesterase activity measurements. *Turk J Biochem*, 28, 45-49, 2003.

17. Sydow G: A simplified quick method for determination of sialic acid in serum. *Biomed Biochem Acta*, 44, 1721-1723, 1985.

18. Miranda KM, Espey MG, Wink DA: A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, 5, 62-71, 2001. DOI: 10.1006/niox.2000.0319

19. Yoshioka T, Kawada K, Shimada T, Mori M: Lipid peroxidation in maternal and cord blood and protective mechanism aganist actived oxygen toxicity in the blood. *Am J Obstet Gynecol*, 135, 372-376, 1979. DOI: 10.1016/0002-9378(79)90708-7

20. Beutler E, Duran O, Kelley BM: Improved method for determination of blood glutathione. *J Lab Clin Med*, 61, 882-888, 1963.

21. Kottaridi C, Nomikou K, Lelli R, Markoulatos P, Mangana O: Laboratory diagnosis of contagious ecthyma: Comparison of different PCR protocols with virus isolation in cell culture. *J Virol Meth,* 134, 119-124, 2006. DOI: 10.1016/j.jviromet.2005.12.005

22. Khazan M, Hdayati M: The role of nitric oxide in health and diseases. *Scimetr*, 3 (1): e20987, 2014. DOI: 10.5812/scimetr.20987

23. Issi M, Gul Y, Yilmaz S: Clinical, haematological and antioxidant status in naturally poxvirus infected sheep. *Rev Med Vet*, 159 (1): 54-58, 2008.

24. Jenkinson DM, McEwan PE, Onwuka SK, Moss VA, Elder HY, Hutchinson G, Reid HW: The polymorphonuclear and mast cell nephrosis in ovine skin infected with orf virus. *Vet Dermatol,* 1, 71-77, 1990. DOI: 10.1111/j.1365-3164.1990.tb00082.x

25. Jenkinson DM, McEwan PE, Onwuka SK, Moss VA, Elder HY, Hutchinson G, Reid HW: The pathological changes and polymorpho-nuclear and mast cell responses in skin of specific pathogen free lambs following primary and secondary challenge with orf virus. *Vet Dermatol*, 1, 139-150, 1990. DOI: 10.1111/j.1365-3164.1990.tb00092.x

26. Nisbet C, Çenesiz S, Açıcı M, Umur Ş: Determination of the serum malondialdehyde, ceruloplasmin, adenosine deaminase levels in cattle with cystic echinococcosis. *Erciyes Üniv Vet Fak Derg*, 5, 1-4, 2008.

27. Kırmızıgül AH, Öğün M, Özen H, Erkılıç EE, Gökçe E, Karaman M, Kükürt A: Oxidative stress and total sialic acid levels in sheep naturally infected with poxvirus. *Pak Vet J*, 36, 312-315, 2016.

28. Deveci HA, Güven A: Investigation of blood MDA and GSH levels in cow with mastitis. *Kafkas Univ Vet Fak Derg*, 14, 63-66, 2008. DOI: 10.9775/kvfd.2008.05-A

29. Bozukluhan K, Merhan O, Öğün M, Cihan M, Gökçe G: Determination of the levels of some oxidative stress parameters in calves with omphalitis. *FÜ Sağ Bil Vet Derg,* 30, 79-81, 2016.

30. Uzlu E, Karapehlivan M, Erdoğan HM, Kızıltepe Ş, Erkılıç EE, Deveci HA, Gökçe E, Kaya İ, Çitil M: Serum and saliva sialic acid and oxidative stress parameters changes in bulls with foot and mouth disease. *Kafkas Univ Vet Fak Derg*, 22, 321-325, 2016. DOI: 10.9775/kvfd.2015.13114

31. Pancarcı SM, Gürbülak K, Oral H, Karapehlivan M, Tunca R, Çolak A: Effect of immunomodulatory treatment with levamisole on uterine inflammation and involution, serum sialic acid levels and ovarian function in cows. *Kafkas Univ Vet Fak Derg*, 15, 25-33, 2009. DOI: 10.9775/kvfd.2008.47-A

32. Karapehlivan M, Atakişi E, Çitil M, Kankavi O, Atakişi O: Serum sialic acid levels in calves with pneumonia. *Vet Res Commun*, 31, 37-41, 2007. DOI: 10.1007/s11259-006-3312-6

33. Marsillach J, Camps J, Beltran-Debón R, Rull A, Aragones G, Maestre-Martínez C, Sabench F, Hernández M, Castillo DD, Joven J, Mackness M, Mackness B: Immunohistochemical analysis of paraoxonases-1 and 3 in human atheromatous plaques. *Eur J Clin Invest*, 41, 308-314. 2011. DOI: 10.1111/j.1365-2362.2010.02411.x

34. Deveci HA, Karapehlivan M, Kaya İ, Kükürt A, Alpay M: Akut klorprifos-etil zehirlenmesine karşı kafeik asit fenetil ester'in koruyucu etkisi. *Ankara Üniv Vet Fak Derg*, 62, 255-260, 2015. DOI: 10.1501/Vetfak_0000002689

35. Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, Newton RS, La Du B: Human serum paraoxonase (PON1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic Biol Med*, 26, 892-904, 1999. DOI: 10.1016/S0891-5849(98)00272-X

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Research Article

Changes in Lipid Peroxidation, Glutathione and Fertility in Tuj Sheep After Combined Administration of Vitamin A and E and Passive Immunization with Testosterone Antibodies [1]

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Abstract

This study investigated the effect of testosterone antibodies and a combination of vitamin A and E on reproductive performance and lipid peroxidation in Tuj sheep during the oestrus period. Two castrated Tuj rams were used to produce an ovine testosterone antibody. To perform the experiment, 30 clinically healthy adult Tuj sheep were divided into three groups, in each group had 10 sheep. The Control group were given a placebo, Group I was injected with the testosterone antibody alone and Group II was injected with testosterone antibody plus a combination of vitamins A and E in Freund's incomplete adjuvant. The testosterone antibody and vitamin combination were administered at synchronization and 1 week before synchronization. To synchronize the sheep, 2.5 ml GnRH was injected to sheep in Control, Group I and Group II. Control, Group I and II were subsequently given 600 IU PMSG with 2 ml $PGF_{2\alpha}$ at 5th day of synchronization. Progesterone levels were higher in the two treatment groups than in the control group as pregnancy progressed. Plasma malondialdehyde levels were higher during initial drug application and prior to mating but were lower in the experimental groups than in control during pregnancy and after parturition. Erythrocyte glutathione levels remained significantly higher in experimental groups than in Control during pregnancy. The number of offspring and the lambing rates in Group I and Group II was higher than the Control. There were no stillbirths in Group I. The number of non-pregnant sheep was lowest in Group II. In summary, injections of testosterone antibody and a combination of vitamins A and E led to an increased incidence of multiple pregnancies in sheep and a greater number of lambs were born. These data indicate that the immunoneutralization of testosterone combined with a reduction in free radicals via the antioxidant activities of vitamins led to increased rates of conception and twinning. Also, it is thought that to allow the growth of the herd in a shorter time, testosterone antibody and combination of vitamins A and E can be applied.

Keywords: *Testosterone antibody, Vitamin A, Vitamin E, Tuj sheep, MDA, GSH, Progesterone, Fertility*

Testosteron Antikoru ile Pasif İmmunizasyon ve A-E Vitamini Kombinasyonu Uygulanmış Tuj Koyunlarında Döl Verimi, Glutatyon ve Lipid Peroksidasyonda Meydana Gelen Değişikler

Özet

Bu çalışmada Tuj koyunlarına östrus döneminde testosteron antikoru ile yapılan pasif immunizasyonun ve A ve E vitamini kombinasyonu uygulamalarının üreme döneminde döl verimi ve oksidatif stres üzerine etkileri araştırıldı. Bu amaçla, 30 Tuj koyunu her grupta 10 koyun olmak üzere 3 gruba ayrıldı. İlk grup Kontrol grubu olarak değerlendirildi ve senkronizasyondan 7 gün önce plesebo uygulandı. Grup I'deki koyunlara testesteron antikoru (AnT), Grup II'deki koyunlara AnT ve Freund's adjuvant incomplate içinde A-E vitamini kombinasyonu uygulandı. Vitamin ve AnT uygulamaları senkronizasyon günü ve senkronizasyondan bir hafta önce yapıldı. Hayvanları senkronize etmek için, Kontrol, Grup I ve Grup II'deki koyunlara 2.5 ml GnRH enjekte edildi. Kontrol, Grup I ve Grup II'deki koyunlara senkronizasyonun 5. günü 600 IU PMSG ile 2 ml $PGF_{2\alpha}$ uygulandı. Deney gruplarının plazma progesteron düzeyleri gebelik süresince kontrol grubuna göre yüksek olarak belirlendi. Deney gruplarının Plazma malondialdehit düzeyleri ilk ilaç uygulamaları yapıldığında ve koç katımından önce yüksekken, gebelik süresince ve doğumdan sonra kontrol grubuna göre düşük olarak tespit edildi. Deney gruplarının, kontrol grubuna göre yüksek eritrosit glutatyon düzeylerini gebelik döneminde ve doğumdan sonra koruduğu gözlemlendi. I. ve II. Grupların bir batında doğan yavru sayılarının ve kuzulma oranının kontrol grubuna göre daha yüksek olduğu gözlendi. I. Grupta hiç ölü doğum olmazken, II. Grupta gebe kalmayan hayvan sayısı diğer gruplardan daha düşüktü. Sonuç olarak, testosteron antikorunun serbest testosteron düzeyini düşürmesi ve vitaminlerin antioksidan etkileri ile serbest radikal düzeylerinin azalmasının koyunlarda gebelik performansını ve bir batında doğan yavru sayısını arttırdığı tespit edilmiştir. Ayrıca, testosteron antikoru ve testosteron antikoru ile A ve E vitamin kombinasyonlarının büyük sürülerde uygulanması ile işletmelerde daha kısa zamanda sürülerin büyütülmesinin mümkün olabileceği düşünülmektedir.

Anahtar sözcükler: *Testosteron antikoru, Vitamin A, Vitamin E, Tuj koyunu, MDA, GSH, Progesteron, Fertilite*

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INTRODUCTION

The number of offspring per parturition is the most important factor in terms of managing the productivity of sheep. To manipulate the number of offspring, sheep are selectively crossed with others, using techniques such as flashing and a variety of different synchronisation protocols. Earlier studies have described the development of effective ways to improve fecundity in sheep as well as the number of offspring. These include both active [1,2] and passive [3] immunization against a variety of steroid hormones. Immunization against specific steroids represents a useful management option to increase reproductive performance in sheep via a relatively simple treatment. Increased levels of fertility were reported by many authors following passive immunization against testosterone [4-6]. Immunization against testosterone leads to changes in the concentration of biologically active progesterone as a result of cross-reacting with antibodies. Antibodies are known to bind efficiently with their appropriate endogenous circulating steroid [2,3]. The effect of immunization upon receptivity and fertility is via reductions in the amount of unbound and biologically inactive hormones [7]. In addition, sheep that were passively immunized against testosterone showed induced changes in the secretion of gonadotrophins [3,8]. Furthermore, this procedure leads to increased rates of ovulation and lambing [1,5].

Recent research [9-16] has aimed, first, to develop reproductive technologies to produce high-yielding lambs in large numbers and, second, to create supplementation strategies to protect sheep and embryos from oxidative damage by free radicals. If the antioxidant system is impaired, reactive oxygen species (ROS) can initiate lipid peroxidation and DNA damage, leading to cell death [9]. Therefore, excessive oxidative stress during the mating and gestation periods of sheep can be controlled by the administration of antioxidants [10]. Within the prepartum period, the administration of vitamin A and E, scavengers of free radicals, can protect oocytes and embryos from oxidative damage during gestation [11-14]. Furthermore, levels of antioxidant enzymes, such as glutathione, glutathione peroxidase, superoxide dismutase, can be elevated via the combined administration of vitamin A and E, leading to reduced levels of lipid peroxidation and ROS generation in oviductal and follicular fluid [13,15,16].

Vitamin A and E play important roles in a variety of biological processes, including fertility, the regulation of embryonic growth and cell differentiation. In addition, vitamin A and E play key roles in the patterns of cellular differentiation occurring during embryonic and foetal development and are responsible for proximodistal patterning, limb development and regeneration, neural differentiation and axon outgrowth [15-17]. Micronutrient deficiencies have also been associated with major reproductive risks, ranging from foetal structural defects to infertility. The periconceptional period consists of a number of critical stages, including preconception, conception, implantation, placentation and embryo organogenesis. These phases are critical in determining successful foetal development and health and can be influenced by maternal nutrition, particularly imbalances in micronutrients [13]. Embryonic and foetal development, implantation and placentation are particularly vulnerable to maternal micronutrient levels. Micronutrient supplementation may also play a role in altering development of the placenta, a structure that is critical for nourishing the foetus throughout pregnancy. In addition, evidence indicates a role for micronutrient supplementation in preventing some pregnancy disorders [5]. In fact, despite initial normal growth cycles, foetuses may develop impaired growth during the second part of gestation as a result of nutrient deprivation occurring early in gestation. Furthermore, sheep in poor body condition are typically less productive and the supplemental injections of vitamin A and E have been shown to increase viability of embryos and lambs [18].

The present study investigated the influence of supplementary injections of testosterone antibody and a combination of vitamin A and E, upon reproductive performance, lipid peroxidation, glutathione and progesterone levels in Tuj sheep.

MATERIAL and METHODS

Animal Treatment

Thirty clinically healthy, weighing average 55±5 kg, 3-5 years of age Tuj sheep were randomly divided into three groups. Applications were initially made during estrus period of ewes. The first group was used as the Control (n=10) and were given a placebo, Group I (n=10) was injected with testosterone antibody alone, whereas Group II (n=10) was injected with testosterone antibody and a combination of 100.000 IU of vitamin A [31.58 mg all-trans retinol (Sigma®, R2500, USA) dissolved in 0.5 ml Freund's adjuvant incomplete (Sigma®, F5506, USA)] and vitamin E [18.22 mg DL-α-Tocopherol acetate (Sigma®, T3376, USA)] dissolved in 0.5 ml Freund's adjuvant incomplete (Sigma®, F5506, USA)]. Study design in experimental groups are shown in *Fig 1*. Testosterone antibodies and the vitamin combination were administered 1 week before synchronization (-7th day) and at the point of synchronization (premating). To synchronize the sheep, Control, Group I and Group II were given an IM injection of 2.5 ml GnRH (0.004 mg Bucerelin acetate, Receptal®, MSD, Turkey). Control, Group I and II were subsequently administered with 600 IU PMSG (Chronogest/PMSG, 6000 IU, MSD, Turkey) with 2 ml PGF 2α (5 mg Dinoprost, Dinolytic®, Zoetis, Turkey) at 5th day of synchronization. Rams were then added to the sheep enclosures and oestrus monitored in the sheep for 6 days *(Fig. 1)*. The rams used in the study were examined andrologically and macroscopic

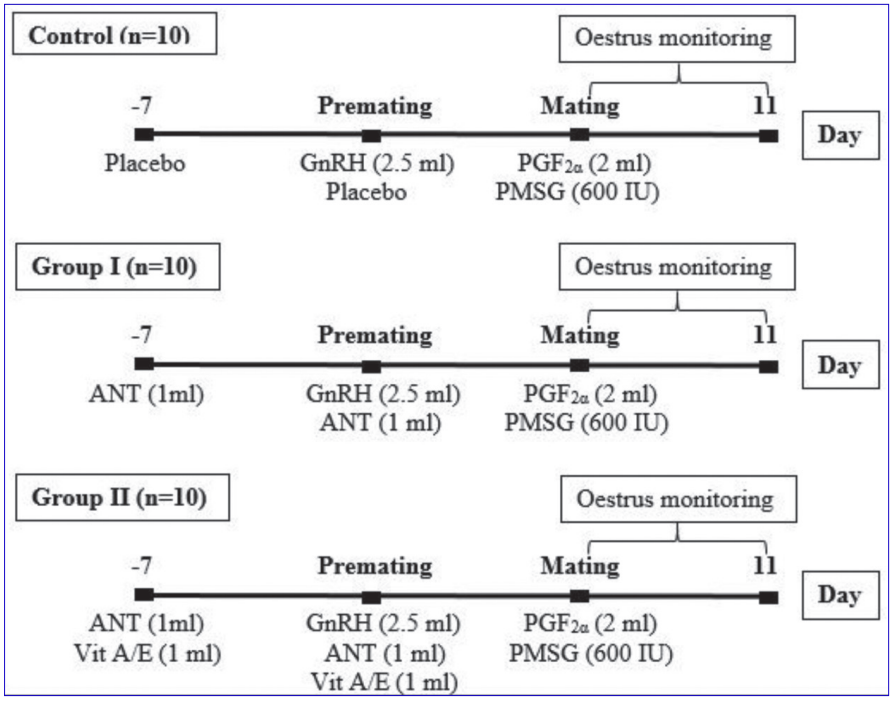


Fig 1. Study design in experimental groups

and microscopic sperm examinations were performed at the same time. Sheep were assessed for pregancy after 35 days by ultrasonography (Sonosite, Vet 180 Plus, USA).

Antibody Production

Two castrated healthy Tuj rams were used to produce ovine anti-testosterone antiserum. Rams were given five injections with an interval of 3 weeks between each injection. For the first injection, 5 mg of testosterone-3-carboxymethyloxime-bovine serum albumin (T-3-CMO-BSA, Sigma®, T3392, USA) conjugate in 2.5 ml of non-ulcerative complete Freund's Adjuvant (Sigma®, F5881, USA) was injected into different areas of dorsal skin in an intra-cutaneous manner. After 3 weeks, a booster dose of 3 mg of the batch of conjugate in incomplete Freund's adjuvant (Sigma®, F5506, USA) was injected via the same route and blood samples taken from the jugular vein 7 days later. Samples were taken from the rams every 2 weeks when antibody titres were appropriate. Testosterone antibody levels were determined by ELISA. Plasma was separated by centrifugation at 4°C and 3000 × *g* for 10 min and frozen at -20°C.

Sample Collection

To measure levels of progesterone and lipid peroxidation in the plasma, blood samples were obtained from the jugular vein either at synchronization or 1 week previously, before and after mating, and once a month during pregnancy and after giving birth. Blood was sampled using heparinized vacutainer tubes. Plasma was then separated by centrifugation (3000 × *g*, for 10 min at 4°C) and frozen (-20°C) to await further analysis.

Analytical Procedures

Lipid peroxidation contents were assessed by measuring thiobarbituric acid reacting substance (TBARS) in plasma according to the method of Placer et al.[19]. TBARS was determined in terms of malondialdehyde (MDA) content, which served as a standard of 1,1,3,3-tetraethoxy-propane (Sigma Chemical Company, T9889, USA). The values of MDA reactive material were expressed in terms of TBARS (nmol/ml plasma). Glutathione (GSH) levels of haemolysed red blood cells were measured spectrophotometrically using Ellman's reagent [20]. Haemoglobin concentration in lysed erythrocytes was also determined by the cyanmet haemoglobin method [21].

Progesterone Measurements

Progesterone levels in blood samples were determined by radioimmunoassay (RIA) using commercial kits (Immunotech®, France). Intra- and inter-assay coefficients for these kits were 6.5% and 7.2% respectively.

Statistical Analysis

Data were analysed by analysis of variance (ANOVA) using SPSS 16.0 software. Tukey's test was used to separate and compare mean data. Pregnancy and lambing rates were compared with the chi-square test. All results were expressed as the mean ± standard deviation (SD). P value <0.05 was considered to be statistically significant.

RESULTS

Plasma Progesterone Levels

Changes in plasma progesterone levels of the sheep are shown in *Fig. 2*. Progesterone levels began to increase after mating. Levels of progesterone were higher in treatment groups than the control group after this time (P<0.05) and increased as pregnancy progressed (P<0.001). In the first month of pregnancy, progesterone levels were highest in Group II (P<0.001), whereas those were higher in the Group I than in the other groups during the 3rd and 4th month of pregnancy (P<0.001).

Plasma MDA Levels

Changes in plasma MDA levels of the sheep are

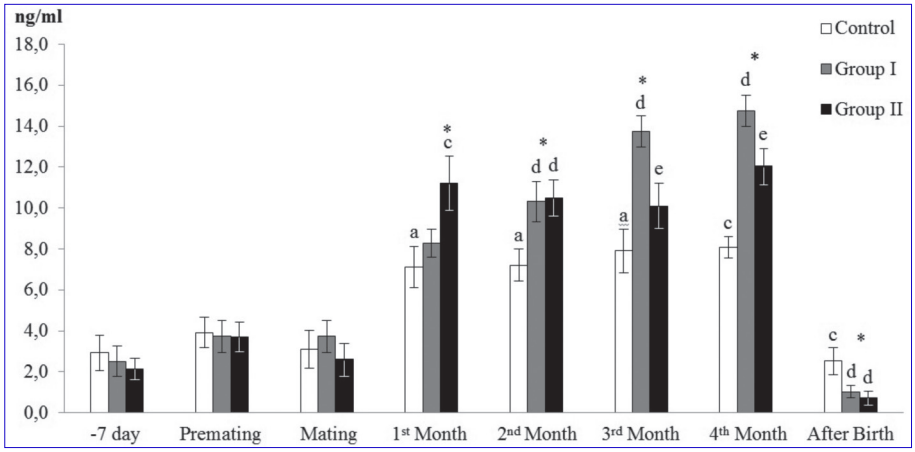
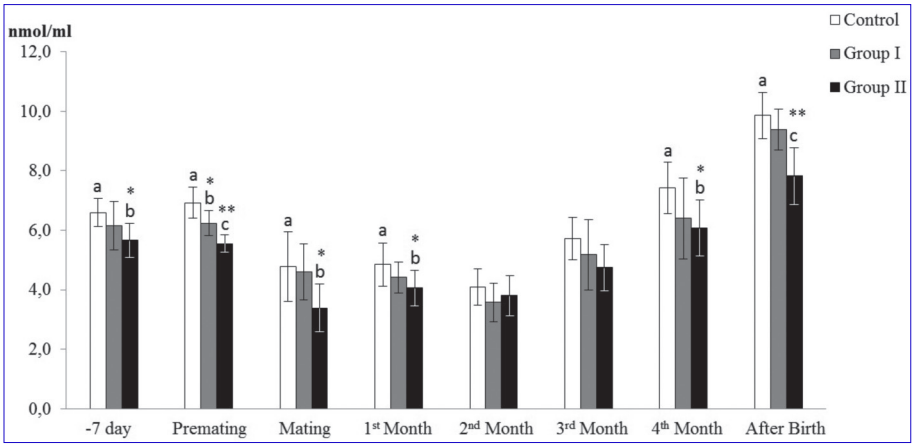


Fig 2. Levels of plasma progesterone (ng/ml) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A-E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.001

Fig 3. Levels of Plasma MDA (nmol/ml) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A-E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.05, ** P<0.01



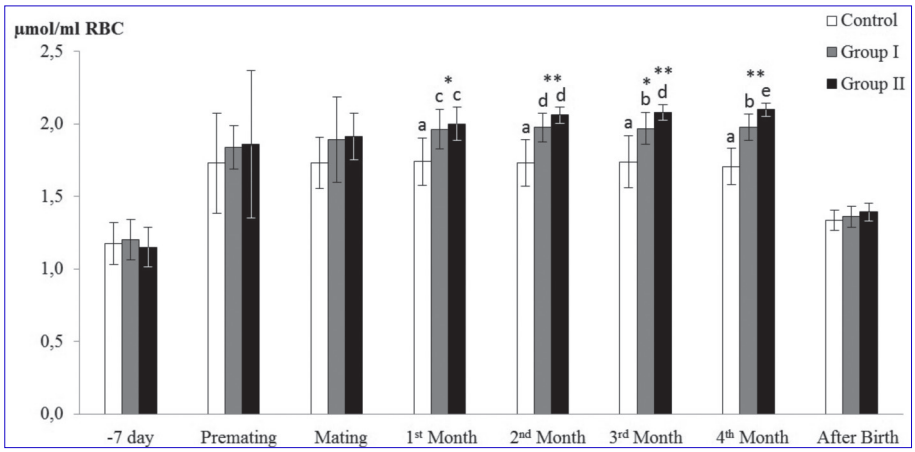


Fig 4. Levels of Plasma GSH (µmol/ml RBC) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A–E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.01, ** P<0.001

shown in *Fig. 3*. Plasma MDA levels were high during drug application and prior to mating (P<0.01) but were lower in the experimental groups than in the control group during the 1st and 4th month of pregnancy (P<0.05) and after parturition (P<0.001). Although the levels of MDA in the plasma were higher in the control group than in the experimental groups, no significance was observed during the 2nd and 3rd month of pregnancy.

GSH Levels in Erythrocytes

Changes in the GSH levels of erythrocytes isolated from blood samples are shown in *Fig. 4*. Levels of erythrocyte GSH levels did not change following injections in the experimental groups, although a significant increase was observed during pregnancy (P<0.01). Erythrocyte GSH levels remained significantly higher in experimental groups than in the control group during pregnancy (P<0.001) and levels increased as pregnancy progressed.

Reproductive Performance

The effect of a combination of vitamins A and E and AnT injections on the reproductive performance of sheep

Table 1. *Effect of vitamin A-E combination and AnT injections on the reproductive performance of sheep*

Determined Measurements	Control (n=10)	Group I (n=10)	Group II (n=10)	P-value
Rate of Lambing (%)	66	125	100	-
Number of singlelambs	4	7	7	-
Number of tweens	-	-	1	-
Number of triplet	-	1	-	-
Number of offsprings	4	10	9	-
Number of stillbirths	2	-	1	-
Number of non-pregnant sheep	4	2	1	-
Ram joining - start of estrus (h)	67.20	64.44	62.29	-
First estrus response (%)	70^{b} (7/10)	90^{a} (9/10)	90^{a} (9/10)	a:b: 0.001
Pregnancy rate (%)	60^{b} (6/10)	80^{ac} (8/10)	90^{a} (9/10)	ac:b: 0.003; a:b: 0.001
Fecundity rate	0.7 (7/10)	1.2 (12/10)	1.0 (10/10)	

Oestrus rate *= number of sheep showing estrus × 100/total number of sheep;* ***Pregnancy rate*** *= number of pregnant sheep × 100/total number of sheep;* ***Lambing rate*** *= number of foetuses × 100/number of pregnant sheep;* ***Fecundity rate*** *= number of foetuses/total sheep number*

and formula for determining key fertility indices are given in *Table 1*. The number of offspring in Group I and Group II were higher than that in the Control group. In addition, there were no stillbirths recorded for Group I. The number of non-pregnant sheep was lowest in Group II. As shown in *Table 1*, the number of lambs and the rate of lambing in Group I and Group II were 125% and 100% higher, respectively, than those in the Control group. In addition, twins and triplets were seen in Group I and Group II. The time when the ram joined the sheep at the start of estrus, first estrus response and pregnancy rate were higher in Group II than in the other groups. However, fecundity rate was higher in Group I than in other groups.

DISCUSSION

Several authors have shown that gonadal hormones can be manipulated by both active and passive immunization methods and thus increase fertility in sheep [3,6]. However, the responses of sheep to active immunization against a variety of steroids have tended to be variable and have resulted in reduced rates of conception. Other studies have shown that passive immunization against testosterone leads to excessive ovarian stimulation and increased secretion of steroids into the ovarian vein and the follicullar fluid of ewes [7,22]. It is possible that the removal of biologically active local androgens using a testosterone antibody may result in an increased ovulation rate and improved fecundity in both sheep and cattle [3,23]. In the present study, we used passive immunization to testosterone, along with a combination of vitamin A and E to improve reproductive traits in sheep. Lambing rate and the number of live lambs were higher in experimental groups than in control groups. We also achieved a high conception rate and increased the proportion of twins and triplets, indicating that the use of testosterone antibody, with a vitamin combination, can markedly improve reproductive capacity in sheep.

Pregnancy involves anabolic states that are directed via hormones to produce nutrients in the maternal tissues and their transfer to the developing foetus via the placenta. Nevertheless, reproductive loss during pregnancy is the most significant problem in sheep breeding and it is known that progesterone plays a key role in the establishment and maintenance of pregnancy. As the hormone of pregnancy, progesterone stimulates maintenance of the early uterine environment and also development of the placenta that takes over progesterone production after week 5-8 of gestation and causes the smooth muscle of the uterus to relax. Studies of the application of vitamins A and E [24,25] and AnT [4,26] have previously shown an effect upon plasma levels of progesterone, embryonic viability and twinning rate [3,22]. However, to date, there has not been any research focussed upon the precise effects of their use together, or actually direct comparison of their actions. The present results suggest that the combination of vitamin A and E along with AnT significantly increased plasma progesterone level after mating until the 1st month of gestation. After the 2nd month of pregnancy, the application of AnT increased and maintained high progesterone levels. This may be due to the synergistic effect of antioxidants with AnT.

In pregnancy, the synthesis of hormones and changes in the partial pressure of oxygen in the placenta, leads to the formation of ROS in both the placenta and foetus. Furthermore, the multiplication and proliferation of cells, and their high rates of metabolism, cause the formation of ROS after electrons escape from mitochondria within the embryo and foetus [15]. Oxidative stress via the release of ROS, and lipid peroxidation, would be highly detrimental to the viability of both the mother and foetus. Stores of

vitamins and minerals in gestating females protect the mother and foetus from ROS fluxes and lipid peroxidation, which is essential to create an imbalance between ROS production and scavenging activity [11,27]. A study indicated that higher levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT) activities or total antioxidant power (TAP) and lower levels of oxidative stress markers such as lipid peroxidation (LPO) in the endometrial secretions were associated with successful in vitro fertilization outcome [28]. During specific states of pregnancy, vitamin A and E deficiency can lead to a failure to express some genes and is also followed by increased release of MDA and of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (a marker of DNA peroxidation) and by a reduction in mitochondrial GSH content [29]. Also, Nawiota et al.[16] suggested that pregnancy constituted the most oxidative stress and lipid peroxidation facing the grazing and concentrated diet feed sheep and goats under arid and saline conditions. In this study, achieved increase in oxidative stress and decrease in GSH levels during mating and pregnancy reduced by application of the combination of vitamin A-E and ANT.

Vitamin A and vitamin E have a synergic effect upon ROS trapping and the placental transport of vitamins A and E between the mother and foetus is sufficient enough to protect them from the destructive affects of lipid peroxidation. Inhibition of lipid peroxidation and the trapping of ROS via the actions of vitamin A and E have been reported to protect the integrity of mitochondria in the placenta and thus prevent extensive oxidative degradation [30]. Reduction in nutrient intake and mineral and vitamin requirements, especially vitamin A and E, from 28 to 78 days of gestation are highly likely to reduce growth and development of the ovine foetus [9]. Reports have stated that it is therefore necessary to provide supplements during the mating period of sheep in Autumn months, when the quality of grass declines and the vitamin requirements of grazing sheep increases [24,25,31]. Thomas and Kott [31] also concluded that unsupplemented ewes on rangeland lost a significant amount of weight during early to mid-gestation and that even after supplementation during late gestation, the health of their lambs was compromised. In addition, a recent report indicate that daily supplementation of vitaminE during the last 6-7 weeks before lambing decreases the stillbirth rate of ewes [14]. Low levels of maternal vitamin A and E has been shown to be associated with intrauterine growth retardation of both the embryo and foetus [24,32]. In a similar fashion, Johansson et al.[33] showed that cows in organic dairy production can fulfill their requirements of vitamins A and E without any supplementation of synthetic vitamins, except at the time around calving, when the requirements are high. Collectively, these findings support the need for vitamin supplementation during mating and pregnancy to protect both the mother and offspring from the deleterious effects of lipid peroxidation and to maintain a healthy pregnancy. Moreover, researchers offer many programs for nutrition and reproduction support to enhance reproductive performance, on the basis that increased nutritional requirements can support foetal growth and development, as well as improve ROS trapping and antioxidant levels [3,11,22,30]. The present research investigated the application of vitamins and E alone, or in combination with AnT, upon lipid peroxidation and GSH activity, when the quality of the pasture deteriorated during times of short day length. Data showed reductions in both lipid peroxidation and MDA levels, along with increased activity of GSH, an enzyme associated with ROS trapping.

Consequently, our present data indicate that the application of AnT and a combination of vitamin A and E increased fertility by reducing stress generated by mating and pregnancy in sheep. The passive immunization procedure is already progressing to farm trials. The present study suggests that the combination of AnT and vitamins A and E may result in further improvements in reproductive performance. Further research should aim to establish optimized ways of applying such techniques under practical conditions.

REFERENCES

1. Cox RI, Wilson PA: Proceedings: Effects of immunizing sheep to steroid oestrogens and phyto-oestrogens. *J Reprod Fertil*, 46 (2): 524,1976.

2. Elsaesser F: Effects of active immunization against oestradiol-17β, testosterone or progesterone on receptivity in the female rabbit and evaluation of specificity. *J Reprod Fertil*, 58, 213-218, 1980. DOI: 10.1530/jrf.0.0580213

3. Land RB, Morris BA, Baxter G, Fordyce M, Forster J: Improvement of sheep fecundity by treatment with antisera to gonadal steroids. *J Reprod Fertil*, 66, 625-634, 1982. DOI: 10.1530/jrf.0.0660625

4. Alabart JL, Folch J, Cocero MJ, Cognie Y: Effect of immunization on reproductive performance, embryo quality and progesterone in rasa aragonesa ewes actively immunized against androstenedione or passively immunized against testosterone. *Theriogenology*, 35, 799-813**,** 1991. DOI: 10.1016/0093-691X(91)90421-9

5. Kaçar C, Kamiloğlu NN, Gürbulak K, Pancarcı ŞM, Güngör Ö, Güvenç K, Saban E: Üreme mevsimi dışındaki Tuj ırkı koyunlarda testosteron antikoru ile β-karoten ve E vitamini uygulamalarının çoğul gebelik ve MDA (Malondialdehit) üzerine etkisi. *Kafkas Univ Vet Fak Derg*, 14, 51-56, 2008. DOI: 10.9775/kvfd.2008.03-A

6. Lermite V, Delaby L, Thimonierx J, Dufour R, Terquil M: Effect of passive immunization against testosterone on reproductive hormone secretion and ovarian function in dairy cows and pubertal beef heifers. *Theriogenology*, 39, 507-526, 1993. DOI: 10.1016/0093-691X(93)90393-J

7. Scaramuzzi RJ, Baird DT, Martensz ND, Turnbull KE, Van PFA: Look ovarian function in the ewe after active immunization against testosterone. *J Reprod Fertil*, 61, 1-9, 1981. DOI: 10.1530/jrf.0.0610001

8. Pathiraja N, Carr WR, Fordyce M, Forster J, Land RB, Morris BA: Concentration of gonadotrophins in the plasma of sheep given gonadal steroid antisera to raise ovulation rate. *J Reprod Fertil*, 72, 93-100, 1984. DOI: 10.1530/jrf.0.0720093

9. Ashworth CJ, Antipatis C: Micronutrient programming of development throughout gestation. *Reproduction,* 122, 527- 535, 2001. DOI: 10.1530/rep.0.1220527

10. Kamiloğlu NN, Beytut E, Aksakal M: Alteration in antioxidant status and lipid peroxidation of sheep previously injected with vitamin A and

ß-Carotene during the periparturient period. *Bull Vet Inst Pulawy,* 50, 171-177, 2006.

11. Aurousseau B, Gruffat D, Durand D: Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant. *Reprod Nutr Dev*, 46, 601-620, 2006. DOI: 10.1051/rnd:2006045

12. Hirschi KK, Keen CL: Nutrition in embryonic and fetal development. *Nutrition*, 16, 495- 499, 2000. DOI: 10.1016/S0899-9007(00)00362-2

13. Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ, Rose BI: Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. *Fertil Steril*, 81, 973-976, 2004. DOI: 10.1016/j.fertnstert.2003.11.021

14. Dønnema I, Randbya ÅT, Hektoenb L, Avdemc F, Melingd S, Vågeb ÅØ, Ådnøya T, Steinheima G, Waage S: Effect of vitamin E supplementation to ewes in late pregnancy on the rate of stillborn lambs. *Small Ruminant Res,* 125, 154-162, 2015. DOI: 10.1016/j.smallrumres.2015.02.012

15. Agarwal A, Gupta S, Sharma RK: Role of oxidative stress in female reproduction. *Reprod Biol Endoc*, 3, 1-21, 2005. DOI: 10.1186/1477-7827-3-28

16. Nawito MF, Abd El Hameed AR, Sosa ASA, Mahmoud KGM: Impact of pregnancy and nutrition on oxidant⁄antioxidant balance in sheep and goats reared in South Sinai, Egypt. *Vet World*, 9, 801-805, 2016. DOI: 10.14202/vetworld.2016.801-805

17. Maden M: Retinoic acid in the development, regeneration and maintenance of the nervous system. *Neuroscience*, 8, 755-765, 2007. DOI: 10.1038/nrn2212

18. Kamiloğlu NN, Beytut E, Güven A, Altınsaat Ç: Changes in the erythrocyte anti-oxidant system of offspring of dams treated with vitamin A and ß-carotene during gestation. *Small Ruminant Res,* 65, 142-148, 2006. DOI: 10.1016/j.smallrumres.2005.06.001

19. Placer ZA, Cushman LL, Johnson BC: Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems. *Anal Biochem*, 16, 359-364, 1966. DOI: 10.1016/0003-2697(66)90167-9

20. Sedlak J, Lindsay RH: Estimation of total protein-bound and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem*, 25, 192-205, 1968. DOI: 10.1016/0003-2697(68)90092-4

21. Drapkin DL: Spectrophotometric studies: XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. *J Biol Chem*, 164, 703-708,1946.

22. Cox RI, Wilson PA, Scaramuzzi RJ, Hoskinson RM, George JM, Bindon BM: The active immunization of sheep against oestrone, androstenedione or testosterone to increase twinning. *Anim Prod Aust*, 14, 511-514, 1982.

23. Clayton J, Rhind SM, Groves DJ, Morris BA: Pharmacokinetic studies of passively administered ovine anti-testosterone antibodies given to cattle by the subcutaneous and intravenous routes. *Immunology*, 69, 603-609, 1990.

24. Vonnahme KA, Hess BW, Hansen TR, McCormick RJ, Rule DC, Moss GE, Murdoch WJ, Nijland MJ, Skinner DC, Nathanielsz PW, Ford SP: Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. *Biol Reprod*, 69, 133-140, 2003. DOI: 10.1095/biolreprod.102.012120

25. Raasch GA, Morrical DG, Youngs CR: Effect of parenteral vitamin e and/or a on reproductive performance of ewes mated on pasture or in drylot. *Sheep Res Report*, 1997, 10, 1998.

26. Rangel PL, Rodriguez A, Gutierrez CG: Testosterone directly induces progesterone productionand interacts with physiological concentrations of LH to increase granulosa cell progesterone production inlaying hens *(Gallus domesticus). Anim Reprod Sci*, 102, 56-65, 2007. DOI: 10.1016/j.anireprosci.2006.09.029

27. Rani V, Deep G, Singh RK, Palle K, Yadav UCS: Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. *Life Sci*, 148, 183-193, 2016. DOI: 10.1016/j.lfs.2016.02.002

28. Rahiminejad ME, Moaddab A, Ganji M, Eskandari N, Yepez M, Rabiee S, Wise M, Ruano R, Ranjbar A: Oxidative stress biomarkers in endometrial secretions: A comparison between successful and unsuccessful in vitro fertilization cycles. *J Reprod Immunol*, 116, 70-75, 2016. DOI: 10.1016/j.jri.2016.05.003

29. Barber T, Borras E, Torres L, Garcia C, Cabezuelo F, Lloret A, Pallardo FV, Vina JR: Vitamin A deficiency causes oxidative damage to liver mitochondria in rats. *Free Radic Biol Med*, 29, 1-7, 2000. DOI: 10.1016/S0891-5849(00)00283-5

30. Tesoriere L, Bongiorno A, Pintaudi AM, D'Anna R, D'Arpa D, Livrea MA: Synergystic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes. *Arch Biochem Biophys*, 326, 57-63, 1996. DOI: 10.1006/abbi.1996.0046

31. Thomas VM, Kott RW: A review of Montana winter range ewe nutrition research. *Sheep Goat Res J*, 11, 17-24, 1995.

32. Picciano MF: Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements. *J Nutr*, 133, 1997-2002, 2003.

33. Johansson B, Waller KP, Jensen SK, Lindqvist H, Nadeau E: Status of vitamins E and A and β-carotene and health in organic dairy cows fed a diet without synthetic vitamins. *J Dairy Sci,* 97, 1682-1692, 2014. DOI: 10.3168/jds.2013-7388

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Research Article

Microsatellite Analysis for Parentage Verification and Genetic Characterization of the Turkmen Horse Population

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Abstract

Microsatellites are a class of genetic markers commonly used for parentage verification and population studies. This study determined the efficiency of microsatellite markers for identification and pedigree analysis in horses based on the example of Turkmen horse population. For this purpose, 748 Turkmen horse samples including 574 adults (92 stallions, 345 males and 137 mares) and 174 foals (98 colts and 76 fillies),were genotyped by using seventeen microsatellite markers recommended by ISAG. The number of allele per locus varied from 5 (HMS01 and HTG07) to 10 (HTG10) with an average value of 7.65. The observed heterozygosity and the expected heterozygosity ranged 0.365-0.953 (mean 0.703), from 0.617-0.884 (mean 0.792) respectively. PIC value ranged from 0.586 (HTG7) to 0.873 (HTG10) with average 0.767. The total exclusion probability of the 17 microsatellite loci was 0.9999. The pedigree study of the Turkmen horse using microsatellite markers was efficient in detecting mistakes during genealogical records. These results suggested that the DNA typing method had high potential for systematic control of the genealogical registrations and genetic resources to improve genetic aspects in Turkmen horses.

Keywords: *Parentage verification, Genetic characterization, Microsatellite markers, Turkmen horse*

Türkmen At Popülasyonunda Soy Tespiti Amacıyla Mikrosatellit Analiz ve Genetik Karakterizasyon

Özet

Mikrosatellitler yaygın olarak soy tespiti amacıyla ve popülasyon çalışmalarında kullanılan bir sınıf genetik belirteçlerdir. Bu çalışma, Türkmen at popülasyonunda identifikasyon ve soy analizinde mikrosatellit belirteçlerin kullanılabilirliğini tespit etmek amacıyla yapılmıştır. Bu amaçla, 574 ergin (92 aygır, 345 beygir ve 137 kısrak) ve 174 tay (98 erkek tay ve 76 dişi tay) içeren toplam 748 Türkmen ata ait örnekler ISAG tarafından önerilen 17 mikrosatellit belirteç kullanılarak genotiplendirildi. Her bir lokusta allel sayısı 5 (HMS01 ve HTG07) ile 10 (HTG10) arasında olmak üzere ortalama 7.65 olarak tespit edildi. Gözlemlenen heterozigotluk ve beklenen heterozigotluk sırasıyla 0.365-0.953 (ortalama 0.703) ve 0.617-0.884 (ortalama 0.792) olarak belirlendi. Polimorfizm bilgi içeriği ortalama 0.767 olmak üzere 0.586 (HTG7) ile 0.873 (HTG10) arasında değişim gösterdi. 17 mikrosatellit bölgenin total dışlama olasılığı 0.9999 idi. Mikrosatellit belirteçler kullanılarak yapılan Türkmen atlarındaki soy araştırması soy kayıtlarındaki hataları tespit etmede etkiliydi. DNA tiplendirme metodu soy kayıtlarının sistemik kontrolünde yüksek potansiyele sahip olup Türkmen atlarının genetik kaynağını artırmada kullanılabilir.

Anahtar sözcükler: *Soy tespiti, Genetik karakterizasyon, Mikrosatellit belirteçler, Türkmen atı*

INTRODUCTION

Horses are belonging to Equidae family; the horse's influence on human history and civilization make it one of the most important animals [1]. Iran has a long history of horse domestication and breeding [2]. Iranian horse breeds may be classified into 4 main groups according to their origins and habitats: North alluvial plains such as Caspian breed, northeast fields such as Turkmen breed, and west highlands such as Kurd breed and southwest and central plateau such as Persian-Arab breed [2]. Turkmen horse is one of the oldest breeds in the world and always achieves high ranks in courses and jumping competitions [3]. Studbook data includes some errors in the registration of the Turkmen studbook. Those data important to the conservation of the breed and correct lines of ancestral might be essential for breeding of Turkmen horse. This information might be verified by a molecular data. It

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is necessary, because the genetic characterization of a breed is the first step in the conservation of breeds, determination of future breeding strategies, and is important to protect breed integrity [4]. Identification of genetic variation among various horse breeds requires the development of genetic markers [5]. Microsatellite markers are useful to diversity studies in the animal. They are tandemly repeated sequences which can be genotyped by PCR techniques [6]. Even in the time of wide genome scanning with the use of SNP microarrays, microsatellites are still used in the construction of linkage maps, when narrowing down the regions of QTLs [7]. Microsatellite markers were first characterized in Swedish horse [8,9]. Commonly, in horses set of seventeen basic microsatellites loci are used (Equine Genetics and Parentage Analysis Workshop, 2012). This horse genotyping panel has been designed to provide high discrimination power (PD), with minimum time need to sample preparation and minimum use of reagents [10]. Those markers constitute a panel of loci recommended by International Society for Animal Genetics (ISAG) in horses parentage testing. In Iran, the polymorphism of these markers has been proved to be useful in Caspian horse [11]; Kurd [12] and Iranian-Arab horse [13]. Application of microsatellite markers in evaluation of the genetic structure in Turkmen horses has not been done yet and this is the first research for parentage verifications based on seventeen microsatellites loci recommended by ISAG's in this breed. The purpose of this study was to evaluate these microsatellite loci in Turkmen horse population and design a marker system for future low-cost genotyping, which will give high combined exclusion probabilities (EPs).

MATERIALS and METHODS

Animals and DNA Extraction

The animals were randomly chosen by their breeders who were able to document their pedigrees (parents, offspring). Blood samples were collected from 748 Turkmen horses, 574 adults (92 stallions, 345 males and 137 mares) and 174 foals (98 colts and 76 fillies). Genomic DNA was extracted from blood samples using the salting-out method [14].

Microsatellite Markers Genotyping

Seventeen microsatellites were selected for this study that had been reported by ISAG for individual identification and parentage verification of Turkmen horses. Microsatellite markers *(Table 1)* were combined in multiplex PCR reaction using fluorescently labeled primers and amplified in a total volume of 20 µl of the following mixture: 40 ng of genomic DNA, 2 mM $MgCl_2$ (Fermentas, Canada), 250 µM of each dNTP (Roche Applied Science, Germany), 0.03 µM of both primers (Metabion, Germany), 1X PCR buffer (Fermentas, Canada) and 0.5U Taq DNA polymerase (Fermentas, Canada). Amplifications were performed using the GeneAmp PCR 9700 (Applied Biosystems, USA). PCR amplification was as follows: the first step was performed by initial denaturation for 5 min at 95°C, followed by 35 cycles at 95°C for 30 sec, 58°C or 60°C for 30 sec, and 72°C for 1 min then extension step of 72°C. The set of proofreading activity and fluorescently labeled 17 primers specific for STRs was tested. PCR products were further sequenced using capillary electrophoresis system on the 3130×l Genetic Analyser (Applied Biosystems). The GeneScan-500 LIZ Size Standard was used in each sample run for an application of automated DNA fragments analysis with four fluorescent dyes. Analysis of DNA profiles for 17 STR loci was conducted in GeneMapper 4.0 software (Applied Biosystems).

Data Analysis

Number of alleles (Na), Allele's frequencies for each locus, observed heterozygosity (HO), expected heterozygosity (He), Polymorphic information content (PIC) and combined probability of exclusion (PE), were computed using CERVUS version 3 software [15]. Deviations from HWE and inbreeding coefficient (F_{is}) were estimated by GENEPOP version 4.4 program [16].

RESULTS

Microsatellite Polymorphism

A total of 130 alleles were observed among the 748 animals and demonstrated that they were highly polymorphic in Turkmen horse populations. A number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content and exclusion probability (PE) in the Turkmen horse were shown in *Table 2*. The number of allele per locus varied from 5 (HMS01 and HTG07) to 10 (HTG10) with an average value of 7.65 in the Turkmen horse. The observed heterozygosity and the expected heterozygosity ranged 0.365-0.953 (mean 0.703), from 0.617-0.884 (mean 0.792) respectively. Microsatellite markers showing PIC values higher than 0.5 are commonly considered as informative in horse population [17]. All marker loci engaged in this study were informative since the average PIC value calculated at 0.767. The lowest PIC value was for HTG7 (0.586), while the highest value was for HTG10 (0.873). The within population inbreeding estimate (F_{is}) ranged between -0.246 and 0.482 with an average of 0.081. Thus, on an average, deficiency (8.1%) of heterozygote existed in the Turkmen horse population *(Table 2)*. Statistically significant deviation from Hardy-Weinberg equilibrium ($P<0.05$) was found at total loci, except (AHT5, ASB17, HTG4, LEX33and UCDEQ425) loci *(Table 2)*. The obtained PE for each polymorphic locus was ranged from 0.3098 for HTG07 to 0.803 for HTG10 with a combined average probability of exclusion of 0.99999 *(Table 2)*. The parentage testing

Table 1. *Characteristics of 17 Horse microsatellites DNA loci*

Loci	Primer Sequences 5'-3'	Dye	Size Range (bp)
AHT04	F: AACCGCCTGAGCAAGGAAGT R: CCCAGAGAGTTTACCCT	6-Fam	166 - 140
AHT05	F: ACGGACACATCCCTGCCTGC R: GCAGGCTAAGGGGGCTCAGC	VIC®	147 - 126
ASB02	F: CCTTCCGTAGTTTAAGCTTCTG R:CACAACTGAGTTCTCTGATAGG	VIC®	268 - 237
ASB17	F: GAGGGCGGTACCTTTGTACC R: ACCAGTCAGGATCTCCACCG	PET®	116 - 104
ASB23	F: GAGGTTTGTAATTGGAATG R: GAGAAGTCATTTTTAACACCT	VIC®	212 - 176
HMS01	F: CATCACTCTTCATGTCTGCTTGG R:TTGACATAAATGCTTATCCTATGGC	PET®	178 - 166
HMS02	F: ACGGTGGCAACTGCCAAGGAAG R:CTTGCAGTCGAATGTGTATTAAATG	NED™	236 - 215
HMS03	F: CCAACTCTTTGTCACATAACAAGA R: CCATCCTCACTTTTTCACTTTGTT	NED™	170 - 146
HMS06	F: GAAGCTGCCAGTATTCAACCATTG R: CTCCATCTTGTGAAGTGTAACTCA	VIC®	170 - 154
HMS07	F: CAGGAAACTCATGTTGATACCATC R:TGTTGTTGAAACATACCTTGACTGT	6-FAM™	187 - 167
HTG04	F: CTATCTCAGTCTTCATTGCAGGAC R: CTCCCTCCCTCCCTCTGTTCTC	6-FAM™	137 - 116
HTG06	F: CCTGCTTGGAGGCTGTGATAAGAT R: GTTCACTGAATGTCAAATTCTGCT	VIC®	103 - 74
HTG07	F: CCTGAAGCAGAACATCCCTCCTTG R: ATAAAGTGTCTGGGCAGAGCTGCT	NED™	128 - 114
HTG10	F: CAATTCCCGCCCCACCCCCGGCA R: TTTTTATTCTGATCTGTCACATTT	NED™	110 - 83
LEX33	F: TTTAATCAAAGGATTCAGTTG R: TTTCTCTTCAGGTGTCCTC	PET®	217 - 203
UCDEQ425	F: AGCTGCCTCGTTAATTCA R: CTCATGTCCGCTTGTCTC	PET®	247 - 224
VHL20	F: CAAGTCCTCTTACTTGAAGACTAG R: AACTCAGGGAGAATCTTCCTCAG	6-FAM™	102 - 83

of the 174 foals was verified by the compatibility of 17 microsatellite markers according to Mendelian laws and using likelihood based method. However, 26 foals did not inherit alleles from the registered sire and 9 foals did not inherit alleles from the registered dam.

DISCUSSION

The use of microsatellite markers for individual identification and parentage verification of horses is a routine method in several countries [18]. The present study describes the utility of seventeen microsatellite markers in parentage verification in Turkmen horse breeds. For a clear genetic differentiation between breeds, it has recommended a minimum of four alleles per locus by FAO [19]. Consequently, All 17 microsatellite markers applied in this study showed reliable polymorphism for evaluating genetic variation within the Turkmen horse population. The allele numbers and heterozygosity levels observed this study, indicate a presence of a reasonably high level of genetic variability in Turkmen horse population. The genetic structure of the Turkmen horse population revealed an increased allelic diversity for 17 microsatellites in relation to other studies. With the same set of microsatellite markers, Georgescu et al.[20], investigated the structure of indigenous Romanian Hucul horse breed. The observed and expected heterozygosity per breed ranged from 0.662 and 0.676, respectively. Genetic variation among four Italian horse breeds was assessed using a set of 11 microsatellites [21]. In the breed level, it was showed a high level of gene diversity (He) ranging from 0.71 in Sicilian Oriental Purebred to 0.81 in Sicilian Indigenous [21]. The Polymorphism Information Content (PIC) similar to heterozygosity and is calculated from allele frequencies. A high PIC value is indicative of a locus with high informativeness. In this study average PIC value was 0.767 which is moderate polymorphic. For linkage mapping, Dierks et al.[7], selected microsatellite markers with PIC values >0.5 as markers with values below this level are insufficient for parentage verification. In their study, the

Table 2. *Number of alleles (Na), observed heterozygosity (H_O), expected heterozygosity (H_E), Polymorphic information content (PIC), inbreeding coefficient (F_{is}), exclusion probabilities (PE) and Hardy Weinberg Equilibrium (HWE) of 17 microsatellites loci for Turkmen horse*

Loci	Na	H_O	H_E	PIC	F_{is}	PE	HWE
AHT04	9	0.755	0.825	0.826	0.092	0.701	**
AHT05	8	0.521	0.725	0.799	0.281	0.619	NS
ASB02	9	0.625	0.734	0.825	0.148	0.633	**
ASB17	8	0.452	0.758	0.810	0.403	0.629	NS
ASB23	8	0.852	0.725	0.815	-0.170	0.476	**
HMS01	5	0.425	0.821	0.664	0.482	0.476	**
HMS02	7	0.732	0.789	0.783	0.072	0.605	**
HMS03	9	0.786	0.725	0.829	-0.084	0.655	**
HMS06	7	0.724	0.821	0.730	0.118	0.532	*
HMS07	9	0.701	0.822	0.794	0.166	0.627	*
HTG04	7	0.671	0.727	0.593	0.077	0.310	NS
HTG06	6	0.809	0.712	0.703	-0.136	0.491	**
HTG07	5	0.733	0.726	0.586	-0.009	0.309	**
HTG10	10	0.753	0.604	0.873	-0.246	0.803	**
LEX33	8	0.609	0.793	0.836	0.232	0.701	NS
UCDEQ425	7	0.758	0.723	0.725	-0.048	0.555	NS
VHL20	8	0.794	0.801	0.832	0.008	0.713	*
Mean **± Sd**	7.65	0.690±0.18	0.731±0.19	0.767±0.15	0.081±0.03	0.99999	-

average PIC value was 0.596 with a maximum of 0.866 [7]. The inbreeding index Fis indicates moderate level of inbreeding in Turkmen horse population, but Fis for locus HMS1 and ASB2 was high in this population. The inbreeding detected in Turkmen horse population may be as a result of depauperate population size, small breeding areas and/or with an insufficient number of breeding males in the breeding region. However, high levels of heterozygousity, PIC and moderate level of inbreeding in Turkmen horse population reflect high genetic variability that can be exploited by horse breeders for planning breeding strategies and prioritizing the breed for its conservation. The International Stud Book Committee (ISBC) has required that the combined exclusion probability (CPE) value for paternity testing and an individual identification in a horse be higher than 0.9995 [22]. In this study, the CPE using 17 microsatellite markers was greater than the value required by the ISBC. Other studies reported similar values of total exclusion probability (0.999) in Thoroughbred and Arabian horse [23-25]. Ellegren et al.[8], proposed at least ten microsatellite loci should be used to gain maximum exclusion in horses. Marklund et al.[9], analyzed eight microsatellite loci in parentage testing to gain a combined exclusion probability of 0.96 to 0.99 in different breeds. At least five microsatellite loci with PE more than 97% should be used to obtain a high degree of excluding probability [26]. Seyedabadi et al.[11], also reported a total PE of 0.973 for seven microsatellite loci used in Caspian horse parentage control. These various results comparison with our results, shows that our selected microsatellites have greater power of exclusion. The prosperity of paternity testing is not only depends on the number of loci but on the level of informativeness that these markers provide. The level of informativeness of a microsatellite marker is specified by its values of heterozygosity, PIC, PE and genetic diversity and these values are dependent on the number and frequency of alleles in the population [27]. These values obtained for microsatellite markers used in our study indicated the high level of informativeness of these markers in Turkmen horse population. So, these microsatellite markers (ISAG), showed to be adequate to parentage verification and for individual identification in Turkmen horse. Our data showed decrepitude in the individual identification system and confirmed interest in using genetic markers in this system. Identification and parentage verification of the Turkmen horse population using a panel of microsatellite markers would be of great importance for the conservation program being applied to this breed.

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REFERENCES

1. Mahrous KF, Hassanane M, Abdel Mordy M, Shafey HI, Hassan N: Genetic variations in horse using microsatellite markers. *J Genet Eng*

Biotech, 9, 103-9, 2011. DOI: 10.1016/j.jgeb.2011.11.001

2. Rafeie F, Amirinia C, Nejati Javaremi A, Mirhoseini SZ, Amirmozafari N: A study of patrilineal genetic diversity in Iranian indigenous horse breeds. *Afr J Biotechnol*, 10, 75, 17347-17352. 2011.

3. Golshan A: Introduction to the Turkmen horse in Iran. 37-71 Tehran, Pejvake Keyhan (in Persian), 2005.

4. Burocziova M, Riha J: Horse breed discrimination using machine learning methods. *J Appl Genet*, 50, 375-77. 2009. DOI: 10.1007/BF03195696

5. Rukavina D, Hasanba D, Pojski N, Rami A, Zahirovi A: Analysis of genetic diversity among certain horse breeds from Bosnia and Herzegovina. *Veterinaria*, 64, 25-28, 2015.

6. Chauhan M, Gupta AK, Dhillon S: Genetic characterization of Indian Spiti horses. *J Genet*, 83, 291-295, 2004.

7. Dierks C, Lohring K, Lampe V, Wittwer C, Drogemuller C, Distl O: Genome-wide searchfor markers associated with osteochondrosi in Hanoverian warm blood horses. *Mam Genome*, 18, 739-747, 2007. DOI: 10.1007/s00335-007-9058-9

8. Ellegren H, Johansson M, Sandberg K, Andersson L: Cloning of highly polymorphic microsatellites in the horse. *Anim Genet*, 23, 133-142, 1992. DOI: 10.1111/j.1365-2052.1992.tb00032.x

9. Marklund S, Ellegren, H, Eriksson S, Sandberg K, Andersson L: Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. *Anim Genet*, 25, 19-23, 1994. DOI: 10.1111/j.1365-2052.1994.tb00050.x

10. Dimsoski P: Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses. *Croat Med J*, 44, 332-335, 2003.

11. Seyedabadi HR, Amirinia C, Banabazi MH, Emrani H: Parentage verification of Iranian Caspian horse using microsatellites markers. *Iran J Biotechnol*, 4, 260-264, 2006.

12. Rafeie F: Genetic Relationships between Iranian Caspian Horses and Other Iranian Horse Breeds. *PhD Thesis,* SRBIAU, Tehran, Iran, 2011.

13. Moshkelani S: DNA fingerprinting of Iranian Arab horse using fourteen microsatellites marker. *Res J Biol Sci*, 6, 402-405, 2011.

14. Javanrouh A, Banabazi MH, Esmaeilkhanian S, Amirinia C, Seyedabadi HR, Emrani H: Optimization on salting out method for DNA extraction from animal and poultry blood cells. *The 57th Annual Meeting of the European Association for Animal Production.* 17 to 20 September, Antalya, Turkey, 2006.

15. Kalinowski ST, Taper ML, Marshall TC: Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol*, 16, 1099-1106, 2007. DOI: 10.1111/j.1365-294X.2007.03089.x

16. Rousset F: GENEPOP' 007: A complete re-implementation of the GENEPOP software for Windows and Linu. *Mol Ecol Resour*, 8, 103-106, 2008. DOI: 10.1111/j.1471-8286.2007.01931.x

17. Botstein D, White RL, Skolnick M, Davis RW: Construction of a genetic linkage map in man using restriction fragment length polymorphism. *Am J Hum Genet*, 32, 314-331, 1980.

18. Seo JH, Park KD, Lee HK, Kong HS: Genetic diversity of Halla horses using microsatellite markers**.** *J Anim Sci Technol*, 58, 1-5, 2016. DOI 10.1186/s40781-016-0120-6

19. Pandey AK, Sharma R, Singh Y: Evaluation of genetic variability in Kenkatha cattle by microsatellite markers. *J Anim Sci*, 12, 1685-1690, 2006. DOI: 10.5713/ajas.2006.1685

20. Georgescu SE, Manea MA, Costache M: The genetic structure of indigenous Romanian Hucul horse breed inferred from microsatellite data. *Roum Biotechnol Lett*, 13, 4030-4036, 2008.

21. Zuccaro A, Bordonaro S, Criscione A, Guastella AM, Perrotta G, Blasi M, D'Urso G, Marletta D: Genetic diversity and admixture analysis of Sanfratellano and three other Italian horse breeds assessed by microsatellite markers. *Animal*, 7, 991-998, 2008. DOI: 10.1017/S1751731108002255

22. Tozaki T, Kakoi H, Mashima S, Hirota KI, Hasegawa T, Ishida N, Miura N, Choi-Miura NH, Tomita M: Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci. *J Vet Med Sci*, 63, 1191-1197, 2001. DOI: 10.1292/jvms.63.1191

23. Lee SY, Cho GJ: Parentage testing of Thoroughbred horse in Korea using microsatellite DNA typing. *J Vet Sci*, 7, 63-67, 2006. DOI: 10.4142/jvs.2006.7.1.63

24. Khanshour AM, Conat EK, Juras R, Cothran EG: Microsatellite analysis for parentage testing of the Arabian horse breed from Syria. *Turk J Vet Anim Sci*, 37, 9-14, 2013. DOI: 10.3906/vet-1104-8

25. Cho GJ: Microsatellite DNA polymorphism of Thoroughbred horses in Korea. *Korean J Genet*, 24, 177-182, 2002. DOI: 10.4142/jvs.2006.7.1.63

26. Jakabova D, Trandzik J, Chrastina J: Effectiveness of six highly polymorphic microsatellite markers in resolving paternity cases in Thoroughbred horses in Slovakia. *Czech J Anim Sci*, 47, 497-501, 2002.

27. Curi RA, Lopes CR: Evaluation of nine microsatellite loci and misidentification paternity frequency in a population of Gyr breed bovines, Braz. *J Vet Res Anim Sci*, 39, 129-135, 2002. DOI: 10.1590/S1413-95962002000300004

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Research Article

Effects of Flavonoids from Mulberry Leaves and *Candida tropicalis* on Performance and Nutrient Digestibility in Calves

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Abstract

Flavonoids from mulberry leave (FML) are natural flavonoids, and *Candida tropicalis* (CT) is yeast like microbe. In this study, forty-eight male calves were selected with born weight (40.5±0.7 kg) and 20±2 days age, divided into 4 groups randomly. FML was supplemented in a dairy calf starter at 2 g/d per calf before weaning, or 4 g/d per calf after weaning (FML group), while CT was added in a dairy calf starter at 1 g/d per calf (CT group). Our results showed that FML could be used to enhance body weight (BW) of calves through enhancing apparent digestibility of ether extract (EE) of the diet, and increased the levels of serum growth hormone (GH) and insulin-like growth factors 1 (IGF-1) in calves after the age of 56 days. CT enhanced the BW of calves before weaning through increasing the apparent digestibility of neutral detergent fiber (NDF) of the diet, and elevated the apparent digestibility of EE of the diet of calves after weaning through increasing the level of serum IGF-1 in calves. Furthermore, the mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment digestibility. In conclusion, FML and CT could be used as additives to increase growth and nutrient digestibility in calves.

Keywords: *Calves, Flavonoids from mulberry leave, Candida tropicalis, Nutriment digestibility, Hormone level*

Dut Yaprağından Elde Edilen Flavonoidler İle *Candida tropicalis*'in Buzağılarda Performans ve Besin Sindirilebilirliği Üzerine Etkileri

Özet

Dut yaprağından elde edilen flavonoidler (FML) doğal flavonoidler olup *Candida tropicalis* (CT) de mantar benzeri mikroplardır. Bu çalışmada, doğumda 40.5±0.7 kg ağırlığa sahip 20±2 günlük kırk sekiz erkek buzağı kullanılarak rastgele 4 gruba ayrıldı. FML sütten kesme öncesi her bir buzağı için 2 g/d olarak buzağı başlangıç yemi içerisinde veya her bir buzağı için 4 g/d olarak sütten kesme sonrası (FML grup), CT ise her bir buzağı için 1 g/d olarak buzağı başlangıç yemi içerisinde (CT grup) verildi. Çalışma sonucunda FML'nin diyetin eter ekstraktının (EE) sindirilebilirliğini ve serum büyüme hormonu (GH) ve insülin benzeri büyüme faktörü 1 (IGF-1)'in seviyelerini belirgin bir şekilde arttırmak yoluyla 56 günlükten sonrasında buzağıların vücut ağırlığını (BW) geliştirmek amacıyla kullanılabileceği tespit edilmiştir. CT; buzağıların vücut ağırlığını sütten kesme öncesinde diyetteki nötr deterjan fiberin sindirilebilirliğini belirgin ölçüde geliştirdi ve serum IGF-1'in seviyesini yükseltmek suretiyle sütten kesme sonrasında diyetteki eter ekstraktının sindirilebilirliğini belirgin ölçüde artırdı. FML ve CT karışımı büyümeyi geliştirme, gıda tüketimini ve besin sindirilebilirliğini iyileştirmekte sinerjistik bir rol oynadı. Sonuç olarak, FML ve CT büyüme ve besin sindirilebilirliğini artırmak amacıyla buzağılarda bir katkı olarak kullanılabilir.

Anahtar sözcükler: *Buzağı, Dut Yaprağı Flavonoidleri, Candida tropicalis, Besin sindirilebilirliği, Hormon seviyesi*

INTRODUCTION

It is well documented that there are a number of potential risks for human health in using antibiotics in food-producing animals, including drug residues in meat products, increasing bacterial resistance and environmental contamination [1]. Flavonoids are found in berries, tea, cocoa, soybeans, grains, and plant leaves, are a class of organic polyphenolic compounds [2]. It is through various mechanisms including protection against oxidative stress, and preservation of epithelial barrier function and immuno-modulatory properties that flavonoids are used in acute or chronic intestinal inflammation [3]. Dietary flavonoids (quercetin and morin) have marked effects on the fatty

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acid composition of blood plasma, liver, or breast muscle lipids in vitamin E-deficient chicks [4]. Condensed tannins (a polyflavonoid) can mitigate methane emission by feeding *Leucaena leucocephala* in sheep [5]. It is effective in improving rumen fermentation and reducing the incidence of rumen acidosis through supplementation of natural flavonoids extract from bitter orange and grapefruit [6]. Sainfoin which contains rich condensed tannins can increase beneficial fatty acids and reduce skatole content in lamb meat [7]. Flavonoids from mulberry leaves (FML) have positive effects on the hypoglycaemic, antihypercholesterolemic and anti-oxidative potential in rats [8].

Probiotics are live microorganisms including bacteria and yeasts, which can improve intestinal health and immune response, prevent acute and antibiotic-associated diarrhea [9]. It is reported that GSY10 is the most promising oleaginous yeast for microbial lipid production from molasses, and it can be used as feed supplement for microbial lipid production in dairy cattle [10]. The yeast culture extract can activate natural killer (NK) cells and B lymphocytes *in vitro*, which plays a role in the anti-inflammatory effects [11]. Yeast-based immunogen (EpiCor) possesses conspicuous anti-inflammatory activity, and can directly induce activation of chemotactic awareness of lymphocyte subsets *in vitro* [12]. After experimental challenged with *Salmonella*, pre-weaned dairy cows fed with *Saccharomyces cerevisiae* fermentation products have fewer bouts of diarrhea and fever, more beneficial microbe in rumens and higher weight gain comparing with no-fed group [13]. *Lactic acid bacteria* or *Bacillus* species generally target the lower intestine to stabilize the gut microbiota, which decreases the risk of pathogen colonization in young ruminants [14]. As a fungal organism, *Candida tropicalis* (CT) can grow as yeast morphology [15]. It is known that the somatotropic axis primarily consists of growth hormone (GH), insulin-like growth factors (IGF), as well as their associated carrier proteins and receptors, which plays a key role in the control of the protein anabolism, fat deposition, and growth rate in animals [16]. It was hypothesized that supplementation of FML and CT in starter of calves might improve consumption of nutriment, accelerate animal growth, and feed intake. Thereby, the aim of current study was to determine the effects of supplementation FML and CT in the starter on growth, performance and the concentrations of GH and IGF-1 in plasma during the first 80 days of age in calves.

MATERIALS and METHODS

Materials

The extract of FML was purchased from Xi'an Feida Biotechnology Co. Ltd., and there were 50 mg of FML per g of extract, which was analyzed by the manufacturer. CT was from Beijing Vano Biological Engineering Co., Ltd., and the concentration of live bacteria was 5×10^9 CFU/g, which was provided by the manufacturer. The milk replacer is provided by Beijing Jingzhun Animal Nutrition Center, and the starter is from Beijing Sanyuan Luhe Feed Factory. The basal diet consists of milk replacer and starter with no antibiotics and microbial preparation. The ingredients and nutrient levels of basal diet are shown in *Table 1*.

Animals and Experimental Design

Holstein male calves were managed on the first farm of Western Suburbs, Beijing Sanyuan Luhe Cow Breeding Center. The experimental protocol was approved by the Chinese Academy of Agricultural Sciences Animal Ethical Committee, and humane animal care and handling procedures were followed throughout the experiment. Forty-eight male calves with 20±2 days old, and 40.5±0.7 kg birth weight were selected from cows with natural childbirth and between 3 and 5 years old. The calves were fed adequate colostrum during the first 3 days and then fed milk replacer until 80 days of age. Calves randomly were divided into 4 groups (n=12) based on parity and birth weight. The control group (Ctrl group) was fed with basal diet, while the other three groups were added with FML (FML group, 2 g/d per calf before weaning, or 4 g/d per calf after weaning), with CT (CT group, 1 g/d per calf) and with the above two additives (FML + CT group, FML

Table 1. *Nutrient composition and levels of basal diet (air-dry basis) %*

Items	Starter	Milk Replacer
Corn	20.0	-
Extrude corn	22.9	
Soybean meal	20.0	
Extruded soybean	18.0	
Whey powder	5.00	
Wheat bran	10.0	
$CaHPO_4$	0.800	
Limestone	1.80	
Salt	0.500	
Premix*	1.00	
Total	100	
Nutrient levels		
DM (Dry matter)	85.4	95.4
OM (Organic matter)	92.2	94.9
CP (Crude protein)	19.1	24.3
EE (Ether extract)	2.21	12.9
NDF (Neutral detergent fiber)	18.6	4.02
ADF (Acid detergent fiber)	10.7	2.11
Ca	1.09	1.07
P	0.473	0.482
GE (Gross energy) MJ/kg	15.5	19.9

** Premix supplemented with VA 15.000 IU, VD 5.000 IU, VE 50 mg, Fe 90 mg, Cu 12.5 mg, Mn 30 mg, Zn 90 mg, Se 0.3 mg, I 1.0 mg and Co 0.5 mg for per kg starter*

2 g/d + CT 1 g/d per calf before weaning, or FML 4 g/d + CT 1 g/d per calf after weaning). The experiment began at 21 days old and lasting 60 days. At the age of 55 days old, calves were weaned. The diet for calves contained milk replacer and starter. Milk replacer was offered daily at 10% of body weight (BW) (adjusted weekly) and starter was offered *ad libitum* throughout the 60 days trial period. Each calf was housed in an individual hutch during the whole experiment period except the metabolic study period during which each calf was raised in an individual metabolic cage. There are two metabolic trials in the whole experiment period. One trial began at the age of 43 days lasting 5 days (preweaning), the other began at the age of 60 days lasting 5 days too (postweaning). BW was measured at 21, 28, 42, 56 and 80 days old during the whole trial period, and starter intake was recorded daily.

Sample Collection and Analysis

Every three calves that were from the correspond group, and reached the average BW were selected for the metabolic trial. Urinary and fecal excretions of every calf (4 groups in all, one group having 3 calves) were entirely collected daily for analyzing the apparent digestibility of dry matter (DM), ether extract (EE), neutral detergent fiber (NDF) and acid detergent fiber (ADF). The mixed sample was from 10% of total amount of feces, and 10 g mixed fecal sample was treated with 10 mL of 10% dilute hydrochloric acid for nitrogen fixation. Then a 500-g feces sample was taken, dried at 103°C for 48 h, ground in a Cyclotec 1093 mill (Tecator, Sweden). The digestion rate was calculated as previously described [17]. The blood samples (jugular venipuncture) were collected before the morning feeding at 28, 42, 56 and 80 days old, respectively. Plasma samples were stored at -19°C after centrifugation (3000×g, for 15 min, at 25°C) for analysis of GH and IGF-1 by radioimmunoassay (RIA) as previously described [18]. IGF-1 antibody (sc-1422, Santa Cruz Biotech, CA) was used to analyze the IGF-1 concentration, and the GH concentration was determined using an antibody (sc-10365, Santa Cruz Biotech, CA).

Statistical Analysis

The experimental design was a randomized complete block design. Continuous variables were analyzed by ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, 2003). The model included fixed effect and the random effect. Treatments, days (as a repeated effect), and their interaction were as the fixed effects, and the calf was as the random effects. Restricted Maximum Likelihood was used to estimate least square mean values. Where treatment effects were significant the means were analyzed using Tukey's procedure for multiple comparisons. The initial BWs were modeled as a Covariate to further control the experimental error. Differences were considered statistically significant at the 95% confidence level (P<0.05).

RESULTS

The Effect of FML on Performance and Plasma Hormone Level

It was shown in *Fig. 2* that the BW of FML group was significantly higher (P<0.05) than that of Ctrl group after weaning, but there was no significant effect of FML on BW before weaning. It was observed in *Fig. 1* that there was no significant effect of FML on starter intake throughout the experiment in calves (P<0.05). Meanwhile significant changes (P<0.05) were observed between FML and Ctrl group in the levels of plasma GH and IGF-1 at the age of 80 days *(Table 3)*, and the digestibility of EE in FML group was significantly higher (P<0.05) than that in Ctrl group after weaning *(Table 2)*.

The Effect of CT on Performance and Plasma Hormone Level

The diet supplementation with CT did not affect starter intake (P>0.05) throughout the experiment in calves *(Fig. 1)*, but the BW *(Fig. 2)* of calves of CT group was significantly higher than that of Ctrl group only at the age of 56 days (P<0.05), while the *apparent digestibility* of NDF

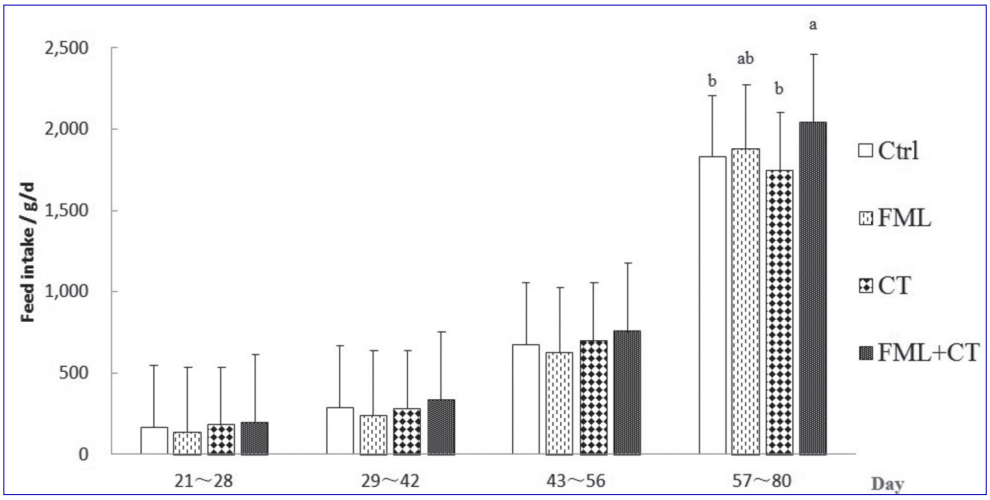

Fig 1. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on feed intake. Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences (P<0.05)

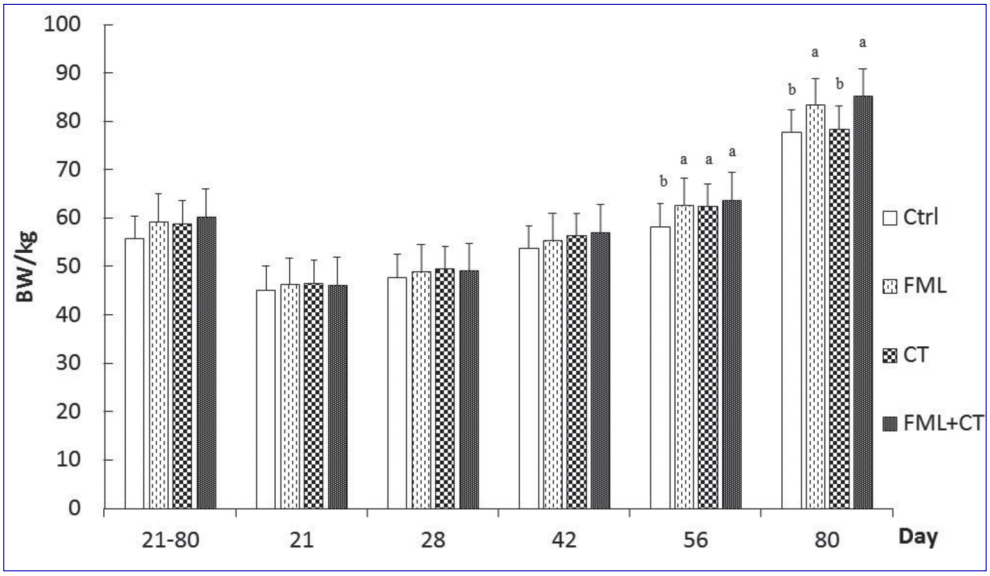

Fig 2. Effect of flavonoids from mulberry leaves and *Candida tropicalis* on body weight. Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, *Candida tropicalis* (1 g/d per calf); FML + CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + *Candida tropicalis* (1 g/d per calf). Within the same row with different superscripts indicated significant differences ($P<0.05$)

Table 2. *Effect of flavonoids from mulberry leaves and Candida tropicalis on nutrient digestibility*

Items	Treatment				SEM	*P*-value
	Ctrl	FML	CT	FML+CT		
Pre-weaning male calves						
Dry matter	79.3	79.2	79.0	80.1	1.09	0.976
Organic matter	81.3	80.5	80.6	81.5	1.07	0.883
Ether extract	79.3	75.8	79.1	69.5	2.55	0.905
Neutral detergent fiber	19.2^{b}	21.3^{b}	25.7^{a}	19.8^{b}	0.983	0.163
Acid detergent fiber	27.4	29.6	27.7	29. 4	1.62	0.0994
Post-weaning male calves						
Dry matter	79.3	84.7	81.6	84.9	1.52	0.342
Organic matter	81.3	86.4	82.2	86.9	1.34	0.328
Ether extract	33.7^{b}	53.5^{a}	46.3^{a}	62.7^{a}	3.16	0.0317
Neutral detergent fiber	49.3^{b}	57.5^{ab}	48.3^{b}	60.7^{a}	2.03	0.0287
Acid detergent fiber	63.7^{b}	66.3^{ab}	65.4^{b}	72.5^{a}	1.36	0.0322

Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, Candida tropicalis (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + Candida tropicalis (1 g/d per calf). Within the same row with different superscripts indicated significant differences (P<0.05)

of CT group was significantly higher than that of Ctrl group before weaning ($P<0.05$). The digestibility of EE in CT group was significantly higher ($P<0.05$) than that in Ctrl group after weaning *(Table 2)*, while the level of plasma IGF-1 in CT group was obviously higher ($P<0.05$) than that in Ctrl group post weaning *(Table 3)*.

The Effect of FML + CT on Performance and Plasma Hormone Level

The results of current study *(Fig. 1, Fig. 2)* indicated that BW and starter feed in FML + CT group were significantly higher ($P<0.05$) than that in Ctrl group at the age of 57-80 days. There was no significant change in BW between FML + CT and Ctrl group at the age of 21-42 days ($P>0.05$). The levels of plasma GH and IGF-1 in FML + CT group were significantly higher ($P<0.05$) than that in Ctrl group at the age of 80 days *(Table 3)*. The diet supplementation with FML+CT simultaneously enhanced digestibility of EE, NDF and *ADF* ($P<0.05$) significantly compared with that no supplementation after weaning in calves *(Table 2)*.

DISCUSSION

The use of antibiotics for growth promotion has been totally banned in many countries, owing to drug residues in meat products and increasing bacterial resistance by use and misuse of in-feed antibiotics in food-producing animals [19], so utilization of phytogenic compounds in feed for food animal production has good potential [20]. It is fed flavonoids extracted from propolis that calves have

Table 3. *Effect of flavonoids from mulberry leaves and Candida tropicalis on GH and IGF-1*

Items	Treatment				SEM	P-value		
	Ctrl	FML	CT	FML+CT		Treatment	age	Treatment × Age
GH (ng/mL)								
28-80	3.21	3.31	3.33	3.39	0.0526	0.613	0.00157	0.169
28	3.28	2.95	3.02	3.09	0.0618	0.645	-	-
42	3.23	3.18	3.25	3.16	0.0578	0.463		
56	3.25	3.43	3.67	3.71	0.293	0.0681		
80	3.23^{b}	3.72^{a}	3.31^{ab}	3.75^{a}	0.181	0.0357		
IGF-1 (ng/mL)								
28-80	162	225	211	221	9.35	0.0452	0.0132	0.246
28	173	171	206	189	13.2	0.383	-	-
42	167	195	194	158	13.5	0.336		
56	159	215	209	214	19. 5	0.0543		
80	177^{b}	295^{a}	277^{a}	294^{a}	20. 60	0.00219		

Ctrl, no additive; FML, FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning; CT, Candida tropicalis (1 g/d per calf); FML+CT, FML (FML 2 g/d per calf before weaning, or FML 4 g/d per calf after weaning) + Candida tropicalis (1 g/d per calf). Within the same row with different superscripts indicated significant differences (P<0.05)

higher BW than those fed no flavonoids until 120 days of age [21]. Results of our present study revealed that calves supplement with FML in the diet have higher BW than those fed no flavonoids after the age of 56 days, without significant difference in feed intake comparing to those fed no flavonoids, which is consistent with the previous reports. Flavonoids have beneficial effects on urinary tract infections, cognitive function and age-related cognitive decline, cancer and cardiovascular disease in human [22], and flavonoids and their metabolites modulated the expression and activity of several metabolic key enzymes, and are involved in regulation of lipid and carbohydrate metabolism [23]. Therefore, the higher BW in FML group may be due to its beneficial effects on several metabolic key enzymes.

Our results showed that the *apparent digestibility* of ADF and NDF was not affected by FML. It was also reported that feeding quebracho tannin extract, a diverse group of polymeric flavonoids, had no effect on ADF and NDF digestibility in Angus heifers [24], which was consistent with our results. However, there was significant effect on the EE of the diet of calves by supplementation with FML comparing Ctrl group after weaning in this study. It was reported that condensed tannins altered ruminal biohydrogenation process of unsaturated fatty acids [25], and the greater digestibility of EE in the FML group than that in Ctrl group may be due to the antioxidant capacities of FML after weaning.

Our results also indicated that there were higher levels of serum GH and IGF-1 in FML group comparing with that in Ctrl group at the age of 80 days. The binding of Genistein to estrogen receptors in the hypothalamus influences the production of GH and growth factors (GF), which lead to increasing the uterine weight, uterine wall thickness and ovarian weight in Sprague Dawley rats [26]. It is reported that flavanone 8-prenylnaringenin, as a phytoestrogen, increases serum GH, but decreases serum IGF-1 levels in rats [27], and IGF-1 has negative effect on GH gene expression in somatotroph cell line [28]. However, GH can strongly stimulate production of IGF-1 *in vivo*. Many tissues and cells can produce IGF-1, and IGF-1 is mainly secreted by the liver under the control of GH, meanwhile have effects on growth and development mediated partly by the effects of GH [29]. Our results may suggest that FML promotes the growth and development of calves by increasing the levels of serum GH and IGF-1. Therefore, FML enhanced apparent digestibility of EE of the diet, and increased the levels of serum GH and IGF-1 in calves, which led to the increased BW after the age of 56 days by supplemented with FML.

It is reported that yeast culture can enhance crude protein and cell wall digestibility, ruminal molar proportion of propionate and plasma glucose concentration in Baluchi lambs [30], and Jersey calves feed live yeast product have greater final BW at 63 days than calves fed none [31]. Our results indicated that there was significant effect on BW of calves supplemented with CT comparing with that no CT only at the age of 56 days, with no significantly difference in the feed intake during total experimental stage. Meanwhile the apparent digestibility of NDF of CT group was significantly higher than that of Ctrl group before weaning. Lesmeister *et al.*[32] reported that Holstein calves fed 2% yeast culture had greater BW at 42 days of age than calves with receiving no yeast. There are improvements in grain intake, BW gain, and blood parameters of calves when fed live yeast only during the pre-weaning period [33]. The calves fed *Saccharomyces cerevisiae* have greater BW during the

pre-weaning period, because yeast can improve growth and activity of fiber-degrading bacteria and fungi, stabilize rumen pH, prevent lactate accumulation, improve ruminal microbial colonization, and set up fermentative processes [34], which is consistent with our study. Our study suggested that CT enhanced the BW of calves before weaning through manipulating rumen fermentation and increasing the apparent digestibility of NDF of the diet.

Furthermore, the results of present study showed that the apparent digestibility of EE in CT group was significantly higher than that in Ctrl group after weaning, and CT have significant effect on the level of serum IGF-1 comparing with Ctrl group in post-weaning male calves. It is supplemented with *Lactobacillus plantarum* that serum IGF-1 can return to pre-challenge values by day 13 post-challenge orally with *Salmonella* in pigs [35]. It is treated with *Lactobacillus rhamnosus* that Zebrafish exhibits a high gene expression level for IGF-1 comparing to untreated group at 6 days post fertilization [36]. Therefore, CT enhanced the apparent digestibility of EE of the diet in calves after weaning through increasing the level of IGF-1 and manipulating rumen fermentation.

Results of the current experiment showed that mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment *digestibility*. It achieved the best effect among four treatments through simultaneously supplementation the two additives (FML + CT) to the starter of calves. BW, starter feed and the levels of plasma GH and IGF-1 were observed significantly higher ($P<0.05$) in FML + CT group compared with Ctrl group at the age of 80 days. Furthermore, the *digestibility of* EE, NDF and ADF in FML + CT group was significantly higher ($P<0.05$) than that in Ctrl group after weaning.

In conclusion, FML could be used to enhance BW of calves through enhancing apparent digestibility of EE of the diet, and increased the level of serum GH and IGF-1 in calves after the age of 56 days. CT enhanced the BW of calves before weaning through increasing the apparent digestibility of NDF of the diet, and elevated the apparent digestibility of EE of the diet of calves after weaning through increasing the level of serum IGF-1 in calves. Furthermore, the mixture of FML and CT plays a synergistic role in enhancing growth, improving feed intake and nutriment *digestibility*.

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REFERENCES

1. Redding LE, Cubas-Delgado F, Sammel MD, Smith G, Galligan DT, Levy MZ, Hennessy S: The use of antibiotics on small dairy farms in rural Peru. *Prev Vet Med,* 113, 88-95, 2014. DOI: 10.1016/j.prevetmed.2013.10.012

2. Bell L, Lamport DJ, Butler LT, Williams CM: A review of the cognitive effects observed in humans following acute supplementation with flavonoids, and their associated mechanisms of action. *Nutrients,* 7, 10290-10306, 2015. DOI: 10.3390/nu7125538

3. Vezza T, Rodríguez-Nogales A, Algieri F, Utrilla MP, Rodriguez-Cabezas ME, Galvez J: Flavonoids in inflammatory bowel disease: A review. *Nutrients,* 8, 211, 2016. DOI: 10.3390/nu8040211

4. Jenkins KJ, Atwal AS: Flavonoids increase tissue essential fatty acids in vitamin E-deficient chicks. *J Nutr Biochem,* 6, 97-103, 1995. DOI: 10.1016/0955-2863(94)00018-H

5. Soltan YA, Morsy AS, Sallam SM, Lucas RC, Louvandini H, Kreuzer M, Abdalla AL: Contribution of condensed tannins and mimosine to the methane mitigation caused by feeding *Leucaena leucocephala*. *Arch Anim Nutr,* 67, 169-184, 2013. *Arch Anim Nutr,* 2013. DOI: 10.1080/1745039X.2013.801139

6. Balcells J, Aris A, Serrano A, Seradj AR, Crespo J, Devant M: Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diets. *J Anim Sci,* 90, 4975-4984, 2012. DOI: 10.2527/jas.2011-4955

7. Girard M, Dohme-Meier F, Silacci P, Ampuero Kragten S, Kreuzer M, Bee G: Forage legumes rich in condensed tannins may increase n-3 fatty acid levels and sensory quality of lamb meat. *J Sci Food Agric,* 96,1923-1933. 2016. DOI: 10.1002/jsfa.7298

8. El-Sayyad HI, El-Sherbiny MA, Sobh MA, Abou-El-Naga AM, Ibrahim MA, Mousa SA: Protective effects of *Morus alba* leaves extract on ocular functions of pups from diabetic and hypercholesterolemic mother rats. *Int J Biol Sci,* 7, 715-728, 2011. DOI: 10.7150/ijbs.7.715

9. Wong A, Ngu DY, Dan LA, Ooi A, Lim RL: Detection of antibiotic resistance in probiotics of dietary supplements. *Nutr J,* 14, 95, 2015. DOI: 10.1186/s12937-015-0084-2

10. Paserakung A, Pattarajinda V, Vichitphan K, Froetschel MA: Selection and identification of oleaginous yeast isolated from soil, animal feed and ruminal fluid for use as feed supplement in dairy cattle. *Lett Appl Microbiol,* 61, 325-332, 2015. DOI: 10.1111/lam.12475

11. Jensen GS, Patterson KM, Yoon I. Yeast culture has anti-inflammatory effects and specifically activates NK cells. *Comp Immunol Microbiol Infect Dis,* 31, 487-500, 2008. DOI: 10.1016/j.cimid.2007.08.005

12. Jensen GS, Hart AN, Schauss AG: An antiinflammatory immunogen from yeast culture induces activation and alters chemokine receptor expression on human natural killer cells and B lymphocytes *in vitro*. *Nutr Res,* 27, 327-335, 2007. DOI: 10.1016/j.nutres.2007.04.008

13. Brewer MT, Anderson KL, Yoon I, Scott MF, Carlson SA: Amelioration of salmonellosis in pre-weaned dairy calves fed *Saccharomyces cerevisiae* fermentation products in feed and milk replacer. *Vet Microbiol,* 172, 248-255, 2014. DOI: 10.1016/j.vetmic.2014.05.026

14. Uyeno Y, Shigemori S, Shimosato T: Effect of probiotics/prebiotics on cattle health and productivity. *Microbes Environ,* 30, 126-132, 2015. DOI: 10.1264/jsme2.ME14176

15. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J: *Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev,* 36, 288-305, 2012. DOI: 10.1111/j.1574-6976.2011.00278.x

16. Renaville R, Hammadi M, Portetelle D: Role of the somatotropic axis in the mammalian metabolism. *Domest Anim Endocrinol,* 23, 351-360, 2002. DOI: 10.1016/S0739-7240(02)00170-4

17. Kammes KL, Allen MS: Nutrient demand interacts with grass particle length to affect digestion responses and chewing activity in dairy cows. *J Dairy Sci,* 95, 807-823, 2012. DOI: 10.3168/jds.2011-4588

18. Mejía M, Gonzalez-Iglesias A, Díaz-Torga GS, Villafañe P, Formía N, Libertun C, Becú-Villalobos D, Lacau-Mengido IM: Effects of continuous ivermectin treatment from birth to puberty on growth and reproduction in dairy heifers. *J Anim Sci,* 77, 1329-1334, 1999. DOI: 10.2527/1999.7761329x

19. Casewell M, Friis C, Marco E, McMullin P, Phillips I: The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J Antimicrob Chemother,* 52, 159-161, 2003. DOI: 10.1093/jac/dkg313

20. Yang C, Chowdhury MA, Huo Y, Gong J: Phytogenic compounds as alternatives to in-feed antibiotics: Potentials and challenges in application. *Pathogens* 4, 137-56, 2015. DOI: 10.3390/pathogens4010137

21. Yaghoubi SM, Ghorbani GR, Rahmani HR, Nikkhah A: Growth, weaning performance and blood indicators of humoral immunity in Holstein calves fed supplemental flavonoids. *J Anim Physiol Anim Nutr (Berl),* 92, 456-462, 2008. DOI: 10.1111/j.1439-0396.2007.00734.x

22. Rodriguez-Mateos A, Vauzour D, Krueger CG, Shanmuganayagam D, Reed J, Calani L, Mena P, Del Rio D, Crozier A: Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: An update. *Arch Toxicol,* 88, 1803-1853, 2014. DOI: 10.1007/s00204-014-1330-7

23. Kobayashi Y, Miyazawa M, Kamei A, Abe K, Kojima T: Ameliorative effects of mulberry *(Morus alba* L.) leaves on hyperlipidemia in rats fed a high-fat diet: Induction of fatty acid oxidation, inhibition of lipogenesis, and suppression of oxidative stress. *Biosci Biotechnol Biochem,* 74, 2385-2395, 2010. DOI: 10.1271/bbb.100392

24. Beauchemin KA, McGinn SM, Martinez TF, McAllister TA: Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *J Anim Sci,* 85, 1990-1996, 2007. DOI: 10.2527/jas.2006-686

25. Benchaar C, Chouinard PY: Short communication: Assessment of the potential of cinnamaldehyde, condensed tannins, and saponins to modify milk fatty acid composition of dairy cows. *J Dairy Sci,* 92, 3392-3396. 2009. DOI: 10.3168/jds.2009-2111

26. Zin SR, Omar SZ, Khan NL, Musameh NI, Das S, Kassim NM: Effects of the phytoestrogen genistein on the development of the reproductive system of Sprague Dawley rats. *Clinics (Sao Paulo),* 68, 253-262, 2013. DOI: 10.6061/clinics/2013(02)OA21

27. Böttner M, Christoffel J, Wuttke W: Effects of long-term treatment with 8-prenylnaringenin and oral estradiol on the GH-IGF-1 axis and lipid metabolism in rats. *J Endocrinol,* 198, 395-401, 2008. DOI: 10.1677/JOE08-0127

28. Romero CJ, Pine-Twaddell E, Sima DI, Miller RS, He L, Wondisford F, Radovick S: Insulin-like growth factor 1 mediates negative feedback to somatotroph GH expression via POU1F1/CREB binding protein interactions. *Mol Cell Biol,* 32, 4258-4269, 2012. DOI: 10.1128/MCB.00171-12

29. Zhou Y, Capuco AV, Jiang H: Involvement of connective tissue growth factor (CTGF) in insulin-like growth factor-I (IGF1) stimulation of proliferation of a bovine mammary epithelial cell line. *Domest Anim Endocrinol,* 35, 180-189, 2008. DOI: 10.1016/j.domaniend.2008.05.003

30. Malekkhahi M, Tahmasbi AM, Naserian AA, Danesh Mesgaran M, Kleen JL, Parand AA: Effects of essential oils, yeast culture and malate on rumen fermentation, blood metabolites, growth performance and nutrient digestibility of Baluchi lambs fed high-concentrate diets. *J Anim Physiol Anim Nutr (Berl),* 99, 221-229, 2015. DOI: 10.1111/jpn.12230

31. Hill SR, Hopkins BA, Davidson S, Bolt SM, Diaz DE, Brownie C, Brown T, Huntington GB, Whitlow LW: The addition of cottonseed hulls to the starter and supplementation of live yeast or mannanoligosaccharide in the milk for young calves. *J Dairy Sci,* 92, 790-798, 2009. DOI: 10.3168/jds.2008-1320

32. Lesmeister KE, Heinrichs AJ, Gabler MT: Effects of supplemental yeast *(Saccharomyces cerevisiae)* culture on rumen development, growth characteristics, and blood parameters in neonatal dairy calves. *J Dairy Sci,* 87, 1832-1839, 2004. DOI: 10.3168/jds.S0022-0302(04)73340-8

33. Galvão KN, Santos JE, Coscioni A, Villaseñor M, Sischo WM, Berge AC: Effect of feeding live yeast products to calves with failure of passive transfer on performance and patterns of antibiotic resistance in fecal *Escherichia coli. Reprod Nutr Dev,* 45, 427-440, 2005. DOI: 10.1051/rnd:2005040

34. Fonty G, Chaucheyras-Durand F: Effects and modes of action of live yeasts in the rumen. *Biologia,* 61, 741-750, 2006. DOI: 10.2478/s11756-006-0151-4

35. Gebru E, Lee JS, Son JC, Yang SY, Shin SA, Kim B, Kim MK, Park SC: Effect of probiotic-, bacteriophage-, or organic acid-supplemented feeds or fermented soybean meal on the growth performance, acute-phase response, and bacterial shedding of grower pigs challenged with *Salmonella enterica* serotype Typhimurium. *J Anim Sci,* 88, 3880-3886, 2010. DOI: 10.2527/jas.2010-2939

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, Carnevali O: *Lactobacillus rhamnosus* accelerates zebrafish backbone calcification and gonadal differentiation through effects on the GnRH and IGF systems. *PlosOne,* 7, e45572, 2012. DOI: 10.1371/journal.pone.0045572

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Research Article

Investigation of Acute Phase Reactants and Antioxidant Capacity in Calves Infected with *Cryptosporidium parvum* [1]

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Abstract

Cryptosporidiosis is a zoonotic infection contaminating via fecal-oral route. *Cryptosporidium parvum* has a wide host prevalance, but is more epidemic in calves. This disease courses with high morbidity and mortality resulting considerable economic losses. In this study, halofuginon (100 µg /kg/day for 7 days) was applied to calves infected with *C. parvum* and the effect of this treatment on acute phase proteins and antioxidant capacity were investigated. Study group was comprised of sera of 10 Holstein calves aged 1-3 weeks, infected with *C. parvum*. Blood samples were obtained from the animals before and after treatment of 7 days and serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), ceruloplasmin (CP), malondialdehyde (MDA) levels and superoxide dismutase (SOD) and adenosine deaminase (ADA) activities were measured in sera. Obtained data showed that there was no statistical difference between pre and post treatment SAA, CRP and MDA levels, but a decrease was determined in post treatment Hp ($P<0.001$) and CP ($P<0.05$) levels, with ADA ($P<0.05$) and SOD ($P<0.001$) activities. Eventually, it was determined that ADA and SOD activities and Hp and CP levels decreases by treatment in calves infected with *C. parvum*.

Keywords: *Acute phase reactants, Antioxidant capacity, Cryptosporidium parvum*

Cryptosporidium parvum ile Enfekte Buzağılarda Akut Faz Reaktanları ve Antioksidant Kapasitenin Araştırılması

Özet

Kriptosporidiozis, fekal-oral yolla bulaşan bir zoonoz enfeksiyondur. *Cryptosporidium parvum* yaygın prevalans göstermekle birlikte, buzağılarda daha epidemik olarak seyretmektedir. Hastalık yüksek morbidite ve mortalitesine bağlı olarak, ciddi ekonomik kayıplara neden olur. Bu çalışmada, *C. parvum* ile enfekte buzağılara halofuginon (100 µg /kg/gün-7 gün) tedavisi uygulanmış ve bu tedavinin akut faz proteinleri ile antioksidant kapasite üzerindeki etkileri araştırılmıştır. Çalışma grubu, *C. parvum* ile enfekte, 1-3 haftalık 10 Holstein buzağıdan oluşturulmuştur. Tedavi öncesi ve sonrası alınan kan numunelerinde, serum amiloid A (SAA), haptoglobulin (Hp), C-reaktif protein (CRP), seruloplazmin (CP), malondialdehid (MDA) seviyeleri ile süperoksid dismutaz (SOD) ve adenozin deaminaz (ADA) aktiviteleri tespit edilmiştir. Elde edilen veriler, tedavi öncesi ve sonrası SAA, CRP ve MDA seviyelerinde istatistiksel bir farklılık olmadığını, fakat tedavi sonrası Hp ($P<0.001$) ve CP ($P<0.05$) seviyeleri ile ADA ($P<0.05$) ve SOD ($P<0.001$) aktivitelerinde istatistiksel olarak anlamlı bir düşüş meydana geldiğini ortaya koymuştur. Sonuç olarak, *C. parvum* ile enfekte buzağılarda tedavi ile ADA ve SOD aktiviteleri ile Hp ve CP seviyelerinde düşüş sağlandığı tespit edilmiştir.

Anahtar sözcükler: *Akut faz reaktanları, Antioksidant kapasite, Cryptosporidium parvum*

INTRODUCTION

C. parvum is the most common enteral pathogen of neonatal calves and mortal cause of neonatal calf diarrhoea worldwide [1,2]. Nearby leading major economic losses in breeding, the agent is zoonotic and has a potential of public health concern. Humans gain the infection by direct contact with infected individuals or animals and ingestion

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of *Cryptosporidium* oocysts via contaminated food or water [3].

C*ryptosporidium* spp. directly affects the intestines by multiplying at the microvillus borders of the enteric epithelium, giving serious damage to the villi thereby reduction of the absorptive surface and maldigestion and malabsorption followed by diarrhoea [4]. Occasionally, the parasite may affect other tissues such as the respiratory and renal epithelia in all the species of its spectrum [5,6].

Majority of cattle infections are due to *C. parvum, C. bovis,* and *C. Andersoni*, but in pre-weaned calves *C. parvum* is the dominant species [2,7].

The sporulated oocysts spread by the faeces which has long survival capacity with high resistence to environmental conditions and this is the form frequently used in the diagnosis in practice [6]. Studies comparing the diagnostic methods for *Cryptosporidium parvum* in calf faeces revealed high sensitivity and specificity for the rapid tests [8].

There is no definite effective treatment assigned currently for the therapy and prevention in bovine cryptosporidiosis, but conventional therapy includes halofuginone lactate together with providing good husbandry. Studies revealed that halofuginone lactate is satisfactory both in the clinical and prophylactic aspects reducing the environmental contamination with Cryptosporidium oocysts [9].

Acute phase proteins are involved in the restoration of homeostasis and preventing bacterial growth before the acquired immunity development [10]. Haptoglobin, ceruloplasmin and α-1 acidglycoprotein are some of the acute phase proteins triggered by infection. It is concluded that, oxidative stress and reactive oxygen species (ROS) due to tissue damage has a crucial role in the enteric damage pathogenesis of farm animals [11]. Acute phase proteins are an alternative path for monitoring clinical course and may be useful for providing information in the severity and prognosis [12].

The aim of this study is to investigate the effects of conventional halofuginon (100 µg/kg/day for 7 days) treatment on acute phase proteins and antioxidant capacity by determining serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), ceruloplasmin (CP), malondi-aldehyde (MDA) levels and superoxide dismutase (SOD) and adenosine deaminase (ADA) activities in calves infected with *C. parvum*.

MATERIAL and METHODS

Animals

The study group was comprised of 10 Holstein calves aged 1-3 weeks, infected with *C. parvum*. The calves were housed in a 200 cow farm in which yellow, nasty smelling and watery diarrhoea was observed in the neonates. Stool specimen obtained directly from the rectum were analysed for cryptosporidium oocyts with rapid kit, BİO K 155 (1x10 strips- Bio X Diagnostics) according to the manufacturers instructions. All specimen were also analysed with carbol fuchsin method. Blood specimen were obtained from the calves before and after the treatment. Conventionel treatment included Halocur® (MSD), Baytril® (Bayer) nearby fluid therapy and supplemental vitamins.

Carbol Fuchsin Staining Method

Stool specimen obtained directly from rectum were transferred to the laboratory in sterile plastic containers in cold chain. All specimen were analysed with Heine's carbol fuchsin staining method. For this purpose, 50 µl of homogenised stool specimen were placed on slides cleaned with ether-alcohol mixture. Same amount of carbol fuchsin was added and a thin specimen smear was prepared. After drying, a drop of immersion oil was added and the slide cover was placed. Smears were examined at X40 aggrandizement at microscope for *Cryptosporidium* oocysts *(Fig. 1)*.

SAA, CRP, Hp Analyses

CRP, SAA and haptoglobuline values were analysed with ELISA, using phase bovine (Tridelta Development Limited, Ireland) kits. Tests were performed according to the standards and guidelines provided by the manufacturer. All samples were calculated with a spectrophotometer (Digital and Analogue Systems S.R.L.) at 450 nm.

Ceruloplasmin Analyses

Serum ceruloplasmin analyses were performed with spectrophotometrically revised modified Ravin method based on the oxidation of colorless phenilen diamine to a blue-purple color product [13].

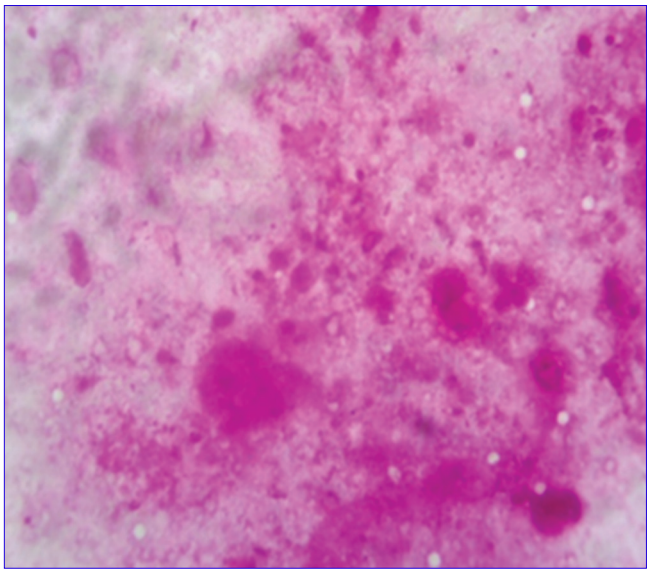

Fig 1. Cryptosporidium oocyts with carbol fuschin staining

MDA Analyses

MDA levels were determined according to the method of Yoshioko and Kawada [14], based on thiobarbuturic acid (TBA) reactivity. According to this method, a stabile red matter giving absorbans at 535 nm was formed by warming lipid content in low pH and thiobarbituric acid (TBA) containing medium resulting in unition of MDA and two TBA molecules was spectophotometrically determined. 1.1.3.3-tetraetoxypropan dissolved in 2.5-5-10 and 20 µmol/L concentration ethyl alcohol was used for calibration. MDA concentration was measured as an indirect marker of oxidative stress in terms of TBARS (thiobarbituric acid reactive substances), spectrophotometrically.

ADA Analyses

ADA in sera was determined at 37°C according to the method of Giusti and Galanti [15], based on the Bertholet reaction, formation of coloured indophenol complex from ammonia liberated from adenosine, and quantified colorimetrically with spectrophotometer (Thermo Scientific, Genesys 10S UV-Vis, USA). One unit of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia/min from adenosine at standard assay condition. Results were expressed as international unit of enzyme activity.

SOD Analyses

SOD analyses were performed according to the method of Podczasy and Wei [16]. The method is based on the principle of reduction of nitroblue tetrazolium (NBT) by xantine-xantinoxidase system which is a superoxide producer. Reducted NBT transforms to blue colored fomazon and measured spectrophotometrically at 560 nm (Thermo Scientific, Genesys 10S UV-Vis, USA). SOD activity was expressed as unit/g protein (U/g).

RESULTS

Obtained data revealed that there was no statistical difference between pre and post treatment SAA, CRP and MDA levels, but a decrease was determined in post treatment Hp ($P<0.001$) and CP ($P<0.05$) levels, with ADA ($P<0.05$) and SOD ($P<0.001$) activities *(Table. 1, Fig. 2)*. Eventually, it was determined that ADA and SOD activities and Hp and CP levels decreases by treatment in calves infected with *C. parvum*

Table 1. *Pre and posttreatment acute phase proteins and antioxidant capacity parameters*

Parameter	Pretreatment (n=10)	Posttreatment (n=10)
SOD	51.8±16.4	138.6±14.4
MDA	0.34±0.06	0.44±0.08
ADA	42.33±5.21	16.84±4.22
CP	20.65±1.25	18.41±0.68
SAA	426.3±52.7	339.4±26.6
HAPTO	1.26±0.13	0.54±0.07
CRP	0.29±0.03	0.22±0.04

DISCUSSION

Enteritis and diarrhoea are major causes of neonatal calf mortality and *C. parvum* is the most common enteral pathogen of neonatal calves [2] also having a zoonotic potential [17]. The disease is spread by the oocyts shed with stool and once ingested by the host, the endogenous phase starts with the invasion of target cells. Following biological steps are schizogony, gametogony, fertilization, and sporogony [6].

$CD4^+$ T cells has a crucial defense action in the immune response against *C. parvum* infection [18]. One other component of protective immunity is interferon (IFN)-γ action and Th2 cytokines [18]. Interleukin (IL)-12 is another part of defens against *C.parvum* [19]. The involvement of IL-12 and (IFN)-γ in host defense shows that a Th1 cell-mediated response is important [20]. IFN-c is also a significant element in the immune response against *C. Parvum* [21].

The acute phase response is a nonspecific reaction to tissue damage as the result of infection, inflammation, neoplasia and immunological disease [10]. It triggers the production of acute-phase proteins (such as a-1 acidglycoprotein, haptoglobin and ceruloplasmin) and involves local and systemic effects and acute phase proteins are also the components of innate immunity mediated by cytokines [10]. In the present study, elevation of Hp and ceruloplasmin in clinically ill calves infected with *C. parvum* and statistically significant decrease following recovery by the therapy is concordant with literature.

Oxidative stress is created as the result of insufficient antioxidant enzyme asset or overproduction of free radicals in the body. Free radicals and lipid peroxidation has detrimental effects to the cell [22]. MDA a product of lipid peroxidation is an important indicator of oxidative damage of cell membrane as it is the most abundant aldehyde formed [23]. Our findings was surprising in this aspect because MDA was the only parameter elevated following therapy. This may be attributed to the very abundant nature of the enzyme as the posttreatment specimen obtaining was just after the therapy and the animals were newly recovered from a devastating disease condition.

Superoxide dismutase (SOD) is a component of the compensatory reflex of the metabolism to oxidative damage targeting to neutralize the free radicals [24]. Our findings supported the literature as the high levels of SOD regressed by the treatment.

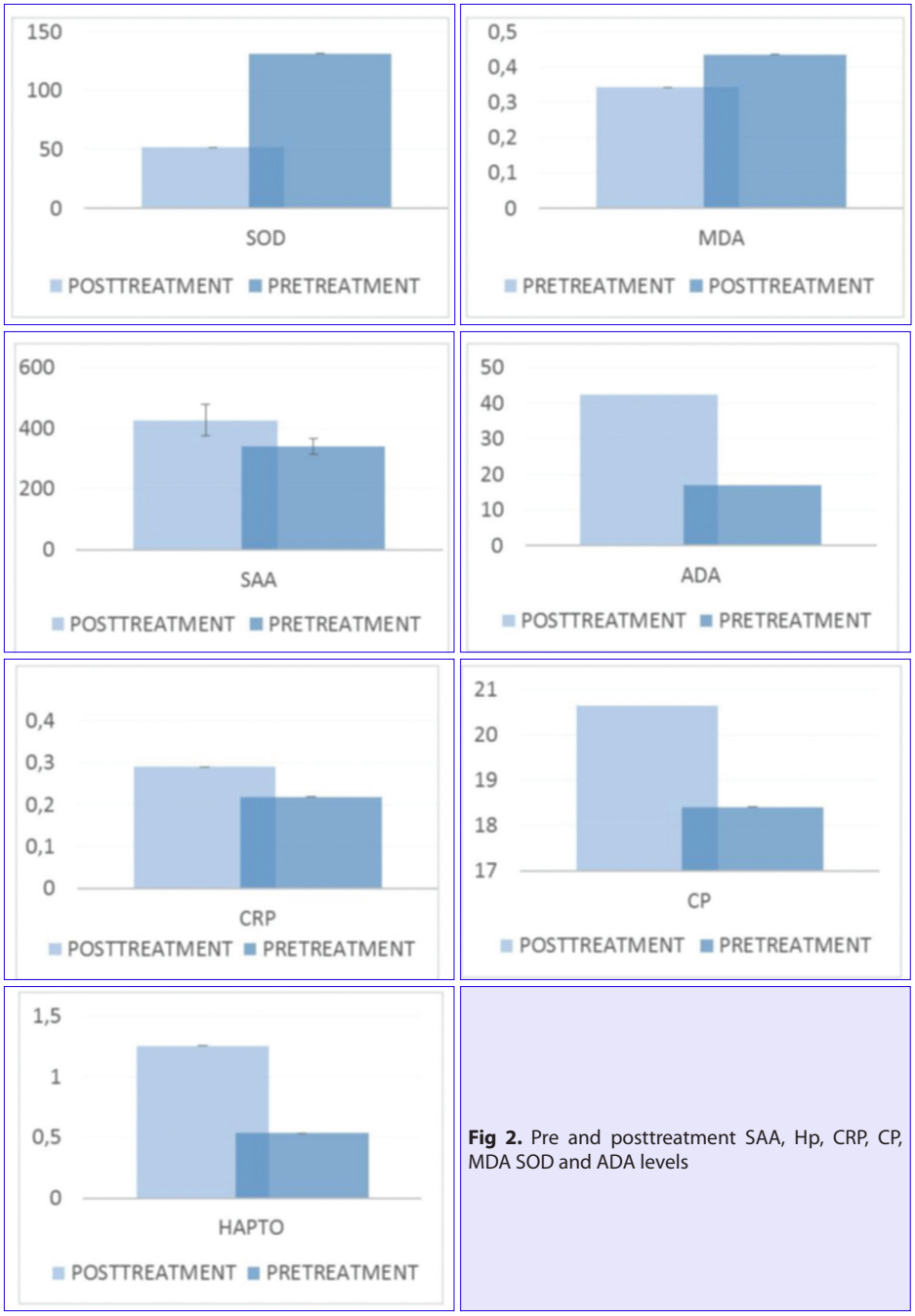

Fig 2. Pre and posttreatment SAA, Hp, CRP, CP, MDA SOD and ADA levels

ADA is important in cellular immunity and its major site of action is in the formation and differentiation of lymphocytes in lymphoid cells [25]. ADA binds cell surface receptors and promps T cells and ADA activity is directly related to the immune resonse [26], therefore the high ADA activity observed pretreatment in the present study reveals the potent immune response against *C. Parvum* infection and though statistically insignificant, a slight decrease in ADA level following therapy shows the regression of the infection.

The acute phase response SAA was characterised by a large individual variation [27], SAA is reported as one of the major acute phase proteins that increases significantly in diarrhoeic calves up to four weeks of age and could be used as a reliable indicator of clinical severity [28]. Concordantly, high SAA levels were determined in clinically infected neonatal calves in the present study, but decrease in SAA level after therapy was not statistically significant.

CRP increase during acute phase response like other acute phase proteins [29]. Our data showed high CRP levels in clinically ill calves whereas not a significant decrease was determined following therapy. This may be related to the regression period.

As the existence of oxidant activity is proven in *C. parvum* infection of calves, some authors reports that antioxidant

supplementation with standard treatment will promise a better therapeutic response [29,30], where on the other side some other researchers administration of antioxidants will exacerbate *C. parvum* infection [31,32]. Although a therapy attempt with antioxidants and evaluation of the effects was not aforementioned in the present study, obtained data on the acute phase response and antioxidant capacity in *C. parvum* infection of calves makes us think that the effects of antioxidant supplementation additional to the conventional therapy must be enlightened with the further studies.

In conclusion, obtained data of the present study showed that serum Hp and CP levels with ADA and SOD activities significantly decreased after treatment in calves clinically infected with *C. parvum* and screening for these values, though not sufficient for establishing a specific diagnosis, may be alternative indicators of the severity and the prognosis of the disease.

REFERENCES

1. Nydam DV, Susan EW, Stephanie LS, Hussni OM: Number of *Cryptosporidium parvum* oocysts or *Giardia* spp cysts shed by dairy calves after natural infection. *Am J Vet Res*, 62, 1612-1615, 2001. DOI: 10.2460/ajvr.2001.62.1612

2. Santin M, Trout JM, Fayer R: A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. *Vet Parasitol,* 155, 15-23, 2008. DOI: 10.1016/j.vetpar.2008.04.018

3. Okhuysen PC, Chappell CL: Cryptosporidium virulence determinants-Are we there yet? *Int J Parasitol*, 32 (5): 517-25, 2002. DOI: 10.1016/S0020-7519(01)00356-3

4. Gookin JL, Chiang S, Allen J, Armstrong MU, Stauffer SH, Finnegan C, Murtaugh MP: NF-kappaB-mediated expression of iNOS promotes epithelial defense against infection by *Cryptosporidium parvum* in neonatal piglets. *Am J Physiol Gastrointest Liver Physiol*, 290, G164-G173, 2006. DOI: 10.1152/ajpgi.00460.2004

5. Mercado R, Buck G, Manque P, Ozaki L: *Cryptosporidium hominis* infection of the human respiratory tract. *Emerg Infect Dis,* 1, 462-464, 2007. DOI: 10.3201/eid1303.060394

6. Fayer R: Taxonomy and species delimitation in *Cryptosporidium. Exp Parasitol,* 124, 90-97, 2010. DOI: 10.1016/j.exppara.2009.03.005

7. Feng Y, Ortega Y, He G, Das P, Xu M, Zhang X, Fayer R, Gatei W, Cama VLX: Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovines. *Vet Parasitol,* 144, 1-9, 2007. DOI: 10.1016/j.vetpar.2006.10.001

8. Klein D, Kern A, Lapan G, Benetka V, Möstl K, Hassl A, Baumgartner W: Evaluation of rapid assays for the detection of bovine coronavirus, rotavirus A and *Cryptosporidium parvum* in faecal samples of calves. *Vet J,* 182, 484-486, 2009. DOI: 10.1016/j.tvjl.2008.07.016

9. Waelea VD, Speybroeckc N, Berkvensc D, Mulcahyb G, Murphya TM: Control of cryptosporidiosis in neonatal calves: Use of halofuginone lactate in two different calf rearing systems. *Prevent Med,* 96, 143-151, 2010. DOI: 10.1016/j.prevetmed.2010.06.017

10. Petersen HH, Nielsen JP, Heegard PMH: Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res,* 35,163-187, 2004. DOI: 10.1051/vetres:2004002

11. Kim YJ, Kim EH, Hahm KB: Oxidative stress in inflammation-based gastrointestinal tract diseases: Challenges and opportunities. *J Gastroenterol Hepatol,* 27, 1004-1010, 2012. DOI: 10.1111/j.1440-1746.2012.07108.x

12. Gånheim C, Hultén C, Carlsson U, Kindahl H, Niskanen R, Waller KP: The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or Mannheimia haemolytica. *J Vet Med B Infect Dis Vet Public Health,* 50 (4): 183-190, 2003. DOI: 10.1046/j.1439-0450.2003.00658.x

13. Ravin HA: An improved colometric enzymatic ceruloplasmin assay. *J Lab Clin Med,* 85, 161-168, 1961.

14. Yoshioka T, Kawada K, Shimada T, Mori M: Lipid peroxidation in maternal and cord blood and protective mechanism aganist actived-oxygen toxicity in the blood. *Am J Obstet Gynecol*, 135, 372-376, 1979.

15. Giusti B, Galanti B: Adenosine Deaminase: Colorimetric Method. **In,** Bergmeyer HU (Ed): Methods of Enzymatic Analysis Weinheim: Verlac Chemie, 1984.

16. Özçelik S, Poyraz Ö, Kalkan K, Malatyalı E, Değerli S: The investigation of Cryptosporidium spp. prevalence in cattle and farmers by ELISA. *Kafkas Univ Vet Fak Derg*, 18 (Suppl-A): A61-A64, 2012. DOI: 10.9775/kvfd.2011.5981

17. Podczasy JJ, Wei R: Reduction of iodonitrotetrazolium violet by superoxide radicals. *Biochem Biophys Res Comm,* 150, 1294-1301,1988. DOI: 10.1016/0006-291X(88)90770-X

18. Schmidt W, Wahnschaffe U, Schafer M, Zippel T, Arvand M, Meyerhans A, Riecken EO, Ullrich R: Rapid increase of mucosal CD4 T cells followed by clearance of intestinal cryptosporidiosis in an AIDS patient receiving highly active antiretroviral therapy. *Gastroenterology*, 120, 984-997, 2001. DOI: 10.1053/gast.2001.22557

19. Hayward AR, Cosyns M, Jones M, Ponnuraj EM: Marrow-derived CD40-positive cells are required for mice to clear *Cryptosporidium parvum* infection. *Infect Immun,* 69, 1630-1634, 2001. DOI: doi: 10.1128/IAI.69.3.1630-1634.2001

20. McDonald SAC, Gardy JE, Elliott MB, Notley CA, Alexander J, Brombacher F, McDonald V: Protection against the early acute phase of *Cryptosporidium parvum* infection conferred by interleukin-4-induced expression of T helper 1 cytokines. *JID,* 190, 1019-1025, 2004. DOI: 10.1086/422761

21. Okazawa A, Kanai T, Nakamaru K, Sato T, Inoue N, Ogata H, Iwao Y, Ikeda M, Kawamura T, Makita S, Uraushihara K, Okamoto R, Yamazaki M, Kurimoto M, Ishii H, Watanabe M, Hibi T: Human intestinal epithelial cell derived interleukin (IL)-18, along with IL-2, IL-7 and IL-15, is a potent synergistic factor for the proliferation of intraepithelial lymphocytes. *Clin Exp Immunol,* 136, 269-276, 2004. DOI: 10.1111/j.1365-2249.2004.02431.x

22. Lobo V, Patil A, Phatak A and Chandra N: Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev,* 4, 118-126, 2010. DOI: 10.4103/0973-7847.70902

23. Petry F, Jakobi V, Tessema TS: Host immune response to *Cryptosporidium parvum* infection. *Exp Parasitol*, 126, 304-309, 2010. DOI: 10.1016/j.exppara.2010.05.022

24. Daudelin JF, Lessard M, Beaudoin F, Nadeau É, Bissonnette N, Boutin Y, Brousseau JP, Lauzon K, Fairbrother JM: Administration of probiotics influences F4 (K88)-positive enterotoxigenic *Escherichia coli* attachment and intestinal cytokine expression in weaned pigs. *Vet Res,* 42, 69, 2011. DOI: 10.1186/1297-9716-42-69

25. Olson ME, Handley RM, Ralston BJ, McAllister TA, Thompson RC: Update on *Cryptosporidium* and *Giardia* infections in cattle. *Trends Parasitol,* 20, 185-191, 2004. DOI: 10.1016/j.pt.2004.01.015

26. Zintl A, Mulcahy G, Waal T, Waele V, Byrne C, Clyne M, Holden N, Fanning S: An Irish perspective on *Cryptosporidium*. Part 2. *Irish Vet J,* 59, 495-500, 2006. DOI: 10.1186/2046-0481-59-9-495

27. Pourjafar M, Badiei K, Nazifi S, Naghib SM: Acute phase response in Holstein dairy calves affected with diarrhoea. *BJVM,* 14 (3): 142-149, 2011.

28. Murata H, Shimada N, Yoshioka M: Current research on acute phase proteins in veterinary diagnosis: An overview. *Vet J,* 168, 28-40, 2004. DOI: 10.1016/S1090-0233(03)00119-9

29. Nathan C, Shiloh MU: Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *PNAS,* 97, 8841-8848, 2000. DOI: 10.1073/pnas.97.16.8841

30. De UK, Mukherjee R, Nandi S, Bhushan B, Dimri U, Verma AK: Disturbance of oxidant/antioxidant balance, acute phase response and high mobility group box-1 protein in acute undifferentiated diarrhea in crossbred piglets. *Indian J Anim Sci,* 84 (8): 860-863, 2014.

31. Huang K, Yang S: Inhibitory effect of selenium on *Cryptosporidium parvum* infection *in vitro* and *in vivo. Biol Trace Elem Res*, 90, 261-272, 2002. DOI: 10.1385/BTER:90:1-3:261

32. Yassien NA, Khalifa EA, el-Nouby KA: Role of anti-oxidant preparation (oxi-guard) on *Cryptosporidium parvum* infection in albino mice. *J Egyp Soc Parasitol*, 31, 95-106. 2001.

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Short Communication

Comparison of Ultrasonographic Images Retrieved using Two Different Probes (Mechanical Sector and Linear Ones) and Macroscopic Features of Bovine Reproductive Organs: Biometric Studies [1]

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Abstract

Objective of the study was to assess whether there are some differences in biometric measurements of the reproductive organs using mechanical sector or linear array ultrasound probe in comparison to the macroscopic measurements. The results revealed no significant differences between ultrasonographic (USG) images in comparison to macroscopic features. High correlations between post - mortem biometric measurements of examined structures and monitored via USG in conscious animals using both probes were found (P<0.001). In conclusion, both USG systems can be effectively used as clinical and research tools in the field of examination of bovine reproductive tract status.

Keywords: *USG, Linear probe, Mechanical sector probe, Cow*

İki Farklı Prob (Mekanik Sektör ve Doğrusal Olanlar) Kullanarak Alınan Ultrasonografik Görüntülerin Karşılaştırılması ve Sığır Üreme Organlarının Makroskobik Özellikleri: Biyometrik Çalışmalar

Özet

Bu çalışmanın amacı, ineklerde üreme organlarının mekanik sektör veya linear ultrasonografi probu kullanılarak yapılan makroskopik biyometrik ölçümleri arasında bir farkın olup olmadığını karşılaştırmaktır. Çalışmadan elde edilen sonuçlar, makroskopik özellikler açısından ultrasonografik (USG) görüntüler arasında bir fark olmadığını göstermiştir. Post-mortem muayeneler ile canlı hayvanlarda yapılan muayeneler arasında, her iki USG probu ile incelenen yapılarda yüksek korelasyon bulundu (P<0.001). Sonuç olarak, ineklerde reprodüktif organların klinik muayenesinde ve bilimsel araştırmalarında her iki USG sistemi de etkin bir şekilde kullanılabilir.

Anahtar sözcükler: *USG, Lineer prob, Mekanik sektör prob, İnek*

INTRODUCTION

In veterinary practice, ultrasonography (USG) is the most profound technological advance to study changes in the ovarian morphology, including the characterization of bovine follicular waves and corpus luteum (CL) development during the estrous cycle and pregnancy [1]. The ultrasonographic examination is useful in the diagnosis of ovarian cysts and ovarian tumors in cattle [2]. Moreover, in the area of pregnancy diagnosis [3,4], fetal sex determination [4], characteristic of reproductive system disorders in cows (endometritis, pyometra), transvaginal oocyte retrieval (ovum pick up) [5], USG has proved to be particularly important technique [6,7]. Recent applications of USG in bovine reproduction includes color Doppler USG [8] and mammary gland USG [9]. Most ultrasound scanners routinely used in bovine reproduction are B-mode (brightness modality) real-time scanners, equipped with

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probes of varying frequencies. The commonly used frequencies in bovine reproduction are 3.5, 5.0 and 7.5 MHz, depending on the type of scanner [1]. The higher frequency probes create better USG images [6]. There are two types of probes used commonly in veterinary reproductive practice: linear (frequencies of 5-7.5 MHz) and the sector probe (frequencies of 3.5-7 MHz) [1]. The data comparing mechanical sector probe to the linear array probe are very limited [5,10].

In our study we assume that there are no differences between ultrasonographic features of bovine reproductive track structures and their macroscopic features. The purpose of present study was to compare biometric measurements of the reproductive organs using sector or linear probes against macroscopic measurements (post-mortem) in cows. The practical purpose of this study was to show the advantages or possible defects of both ultrasound scanners.

MATERIAL and METHODS

Animals

All animal procedures were approved by the Local Animal Care and Use Committee, University of Warmia and Mazury in Olsztyn, Poland (85/2012). This study was conducted in Pomerania, northern Poland during April 2014 to May 2015. Target population was consisted of Polish Holstein - Friesian cows (free of IBR/IPV, BVD/BM, EBL), which were under registration of the dairy herd improvement program, by Polish Federation of Cattle Breeders and Dairy Farmers. In the studied herd, the animals had non-seasonal reproductive programs and were bred routinely by artificial insemination. The farm had veterinary and nutrition consultants. Experimental cows (3 and more lactation) were culled from the farm because of the low milk production. The animals (n = 24) were housed in an intensive indoor barn system, milked twice a day and fed with a total mixed ration ad libitum to meet nutritional requirements of lactating cows (20-25 L per day), BCS (Body Condition Score) = 3.5.

Experimental Procedures

Comparison of ultrasonographic images retrieved using two different probes (mechanical sector and linear ones) and macroscopic features of bovine reproductive organs: biometric studies. For transrectal USG examinations two types of probes Draminski Animal Profi Scanner (Draminski Electronics in Agriculture, Poland) were used: (i) mechanical sector (3.5/5.0/7.0 MHz; 180°) and (ii) linear probe (7.5 MHz). All examined structures were imaged before animals slaughter in local abattoir (Zakłady Mięsne "Warmia" Biskupiec). Then not later than 1-h after ultrasonographic examination the genitalia were collected from slaughtered cows and transported to the laboratory within 40 min after collection. Ovaries were cut with the knife and observed in cross-section. Measurements of separated uteri were done in cross-section of the cranial tip of active uterine horn.

Statistical analysis

Data were analyzed using correlations analyses (GraphPad Prism, version 5.00; GraphPad Software). $P<0.05$ was considered significant.

RESULTS

In the experiment a representative USG images and macroscopic findings in a cross-section of the follicle, CL or uterine horn are present on *Fig. 1, Fig. 2* and *Fig. 3*. Corpora lutea (CL) were imaged in 16 ovaries, follicles (in various size) in 13 ovaries. Cysts were found in 6 ovaries, which were excluded for further correlation analyses. Uteri (n = 17) shown no pathological changes in their structures and were included for further correlation analyses. Additionally, we diagnosed endometritis (n = 5), pyometra (n = 1) and pregnancy (8-10 week; n = 1). These results were excluded for further correlation analyses. In respect to follicles *(Fig. 1),* high correlations between USG measurements of follicular diameter and assessed post-mortem were found (r = 0.88 and r = 0. 87, respectively; P<0.001). Similarly, we demonstrated correlations between USG and macroscopic measurements of the CL *(Fig. 2)* using sector or linear ultrasound probe compared to post-mortem findings (r = 0.94 and r = 0.91, respectively; P<0.001). Moreover, we found correlations between post-mortem biometric measurements and ultrasonographic images of uteri *(Fig. 3)* using sector or linear ultrasound probes (r = 0.96, r = 0.90; respectively; P<0.001).

DISSCUSION

Practical application of USG by veterinarians for reproductive organs examination is the most important method in livestock industry. Thus, the clarification of the ultrasonographic images is necessary to obtain a precision in the diagnosis of physiological and pathological ovarian structures and conditions of bovine uterus [11]. The USG examination of the ovaries and uterus in cows has been described in detail [11-13]. Moreover, previous studies have already compared the ultrasonographic features with macroscopic findings of examined structures [14]. However, in this study we compared ultrasonographic images obtained from both sector and linear probes. Therefore, practical purpose of our study was to show the advantages and similarity or possible defects of both probes.

In our experiment we found that the images of reproductive organs discernible by USG corresponded to their macroscopic features. Moreover, we determined the significant correlations between the size of examined structures measured by USG using both probes and related measurements assessed post-mortem. Similar results were

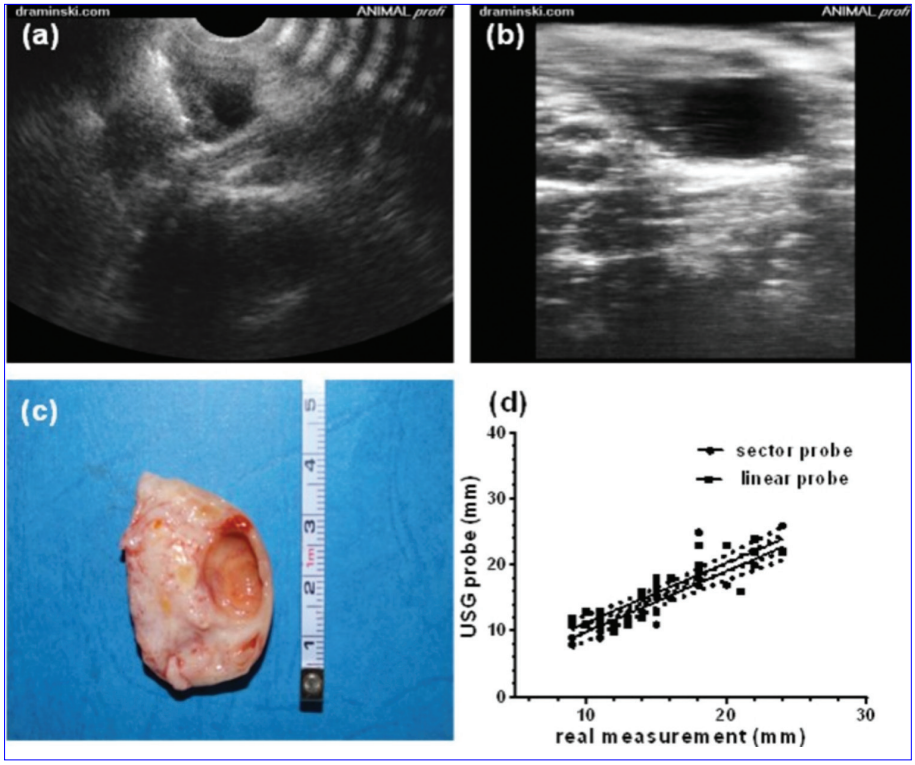

Fig 1. A representative ultrasonographic image and macroscopic finding in a cross-section of the follicle: **(a)** generated using a sector probe; **(b)** generated using a linear probe; **(c)** macroscopic finding of the follicle; **(d)** Correlation between diameter (mm) of the follicles evaluated using sector and linear probe in comparison to the real measurements (mm)

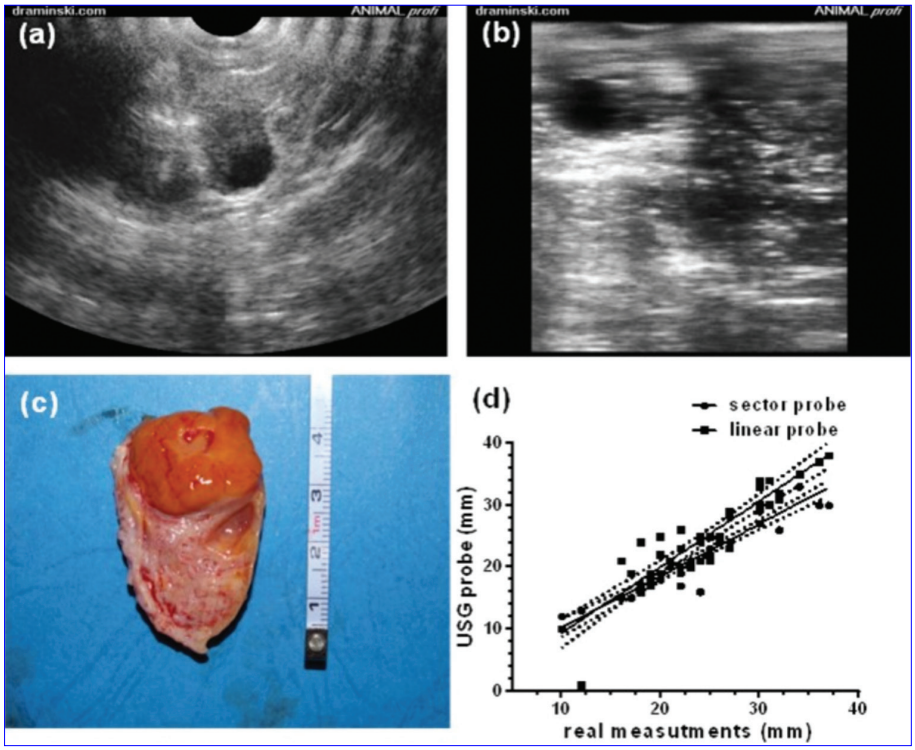

Fig 2. A representative ultrasonographic image and macroscopic finding in a cross-section of the CL: **(a)** generated using a sector probe; **(b)** generated using linear probe; **(c)** Macroscopic finding of the CL; **(d)** Correlation between diameter (mm) of the CL evaluated using sector and linear probe in comparison to real measurements (mm)

obtained by other authors who have found relationship between USG (using 5 MHz probe) and macroscopic measurements of follicles [13]. Moreover, Pierson and Ginther [13] have also reported high correlations between *in vivo* USG and post-mortem features of examined CL. In respect to the uteri similar findings were confirmed by Saito et al.[14].

In the practical purpose, both systems can be used for imagining of bovine reproductive organs. Our results showed that there were no differences between ultrasonographic features of reproductive organs achieved using both probes and their macroscopic features. Mechanical sector scanners offer multi-frequency capability, making them multifunctional and universal scanners. For early pregnancy diagnosis a 5 MHz or 7.5 MHz probe tends to provide more reliable results [3]. The linear probes using offer more detailed imaging of examined structures, which predisposes these probes for use in clinical trials (diagnosis of reproductive tract disorders). Moreover, Ribadu and Nakao [1] suggested that in routine bovine reproductive ultrasonography a 5 MHz linear rectal probe is the most effective.

In conclusion, the results of our experiment revealed no significant differences between ultrasonographic images retrieved by both probes in comparison to macroscopic features. Moreover, high correlations between post-mortem biometric measurements of examined structures and monitored via USG in conscious animals using both probes were found.

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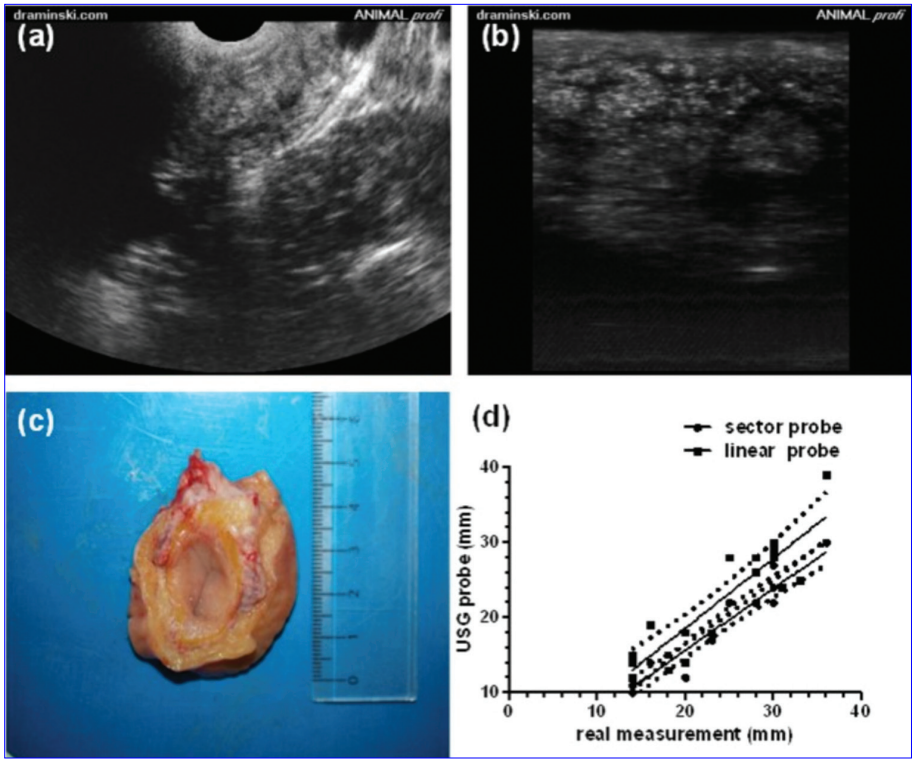

Fig 3. A representative ultrasonographic image and macroscopic finding in a cross - section of uterine horn: **(a)** generated using a sector probe; **(b)** generated using a linear probe; **(c)** Macroscopic finding of uterine horn; **(d)** Correlation between diameter (mm) of uterine horn generated using sector and linear probe in comparison to real measurements (mm)

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Conflicts of Interest

None of the authors have any conflicts of interest to declare.

REFERENCES

1. Ribadu AY, Nakao T: Bovine reproductive ultrasonography: A review. *J Reprod Dev*, 45, 13-28, 1999. DOI: 10.1262/jrd.45.13

2. Farin PW, Youngquist RS, Parfet JR, Gaverick HA: Diagnosis of luteal and follicular ovarian cysts in dairy cows by sector scan ultrasonography. *Theriogenology,* 34, 633-642, 1990. DOI: 10.1016/0093-691X(90)90019-P

3. Rantanen NW: General considerations for ultrasound examinations. *Vet Clin North Am: Equine Pract,* 2, 29-32, 1986.

4. Kamimura S, Kondoh S, Enomoto S, Sameshima H, Goto K, Hamama K: Ultrasonic diagnosis of bovine fetal sex by the relative location of the genital tubercle in early pregnancy. *J Reprod Dev.* 40, 4, 1994. DOI: 10.1262/jrd.40.343

5. Bols PEJ, Leroy JLMR, Vanholder T, Van Soom A: A comparison of mechanical sector and a linear array transducer for ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. *Theriogenology,* 62, 906-914, 2004. DOI: 10.1016/j.theriogenology.2003.12.016

6. Reeves JJ, Rantanen NW, Hauser M: Transrectal real-time ultrasound scanning of the cow reproductive tract. *Theriogenology,* 21, 485-494, 1984. DOI: 10.1016/0093-691X(84)90410-2

7. Ginther OJ: How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. *Theriogenology,* 81, 112-125, 2014. DOI: 10.1016/j.theriogenology.2013.09.007

8. Lüttgenau J, Bollwein H: Evaluation of bovine luteal blood flow by using color doppler ultrasonography. *Reprod Biol,* 14, 103-109, 2014. DOI: 10.1016/j.repbio.2014.03.003

9. Franz S, Floek M, Hofmann-Parisot M: Ultrasonography of the bovine udder and teat. *Vet Clin North Am: Food Anim Pract,* 25, 669-685, 2009. DOI: 10.1016/j.cvfa.2009.07.007

10. Seneda MM, Esper CR, Garcia JM, Andrade ER, Binelli M, Oliveira JA, Nascimento AB: Efficacy of linear and convex transducers for ultrasound-guided transvaginal follicle aspiration. *Theriogenology,* 59, 1435-1440, 2003. DOI: 10.1016/S0093-691X(02)01188-3

11. Hanzen Ch, Pieterse M, Scenczi O, Drost M: Relative accuracy of the identification of ovarian structures in the cow by ultrasonography and palpation per rectum. *Vet J,* 159, 161-170, 2000. DOI: 10.1053/tvjl.1999.0398

12. Pierson RA, Ginther OJ: Reliability of diagnostic ultrasonography for identification and measurement of follicles and detection of the corpus luteum in heifers. *Theriogenology,* 28, 929-936, 1987. DOI: 10.1016/0093-691X(87)90043-4

13. Ribadu AY, Ward WR, Dobson H: Comparative evaluation of ovarian structures in cattle by palpation per rectum, ultrasonography and plasma progesterone concentration. *Vet Rec,* 135, 452-457, 1994. DOI: 10.1136/vr.135.19.452

14. Saito Y, Kamomae H, Tanaka T, Machida N, Kaneda Y: Correlations between ultrasonic imagines and macro-and microscopic features in the uterine horn of dairy cows. *J Reprod Dev,* 47, 1-17, 2001.

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Short Communication

Research on the Report of Professor Rostafinski as a Sample of Scientific Cooperation in Animal Breeding in the First Years of the Republic of Turkey [1]

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[1] *This study was presented as oral presentations at the 5th Congress of the Balkan Medical History and Ethics, 11-15 October 2011, Cerrahpasa Faculty of Medicine, İstanbul, Turkey*

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Abstract

Reconstructing the veterinary services was added to the agenda at the (National) Economy Congress in Izmir in 1923. Scientists from abroad were invited for their advice on animal diseases and animal breeding. Polish Professor Rostafinski was invited by Şükrü Kaya, the Minister of Agriculture during that period. Rostafinski came to Turkey in November 1924. After his researches on husbandry in Western Anatolia, he presented his opinions in a 31- page report to the Ministry of Agriculture. He advised that pedigree records of horses should be tracked, horse raising should be adopted as a government policy. For cattle, it is important to determine the needs of the peasants. This study aims to identify the husbandry conditions in that period concerning the report on the husbandry in Western Anatolia.

Keywords: *Rostafinski, Specialist report, Animal breeding, Husbandry in the Turkish Republic period, Veterinary history*

Türkiye Cumhuriyeti'nin İlk Yıllarında Hayvan Islahı Alanında Bilimsel İşbirliği Örneği Olarak Profesör Rostafinski'nin Raporu Üzerine Bir İnceleme

Özet

Cumhuriyetin ilânından sonra, İzmir'de 1923 yılında toplanan İktisat Kongresinde veteriner hekimlik hizmetlerinin de yeniden düzenlenmesi gündeme gelmiştir. Hayvan hastalıkları ve hayvan ıslahı konularına ilişkin görüşleri için yurtdışından bilim adamları davet edilmiştir. Dönemin Ziraat Vekili Şükrü Kaya tarafından davet edilen Polonyalı Profesör Rostafinski, 1924 yılı Kasım ayında Türkiye'ye gelerek Batı Anadolu'daki hayvancılık üzerinde incelemelerde bulunmuş ve görüşlerini Ziraat Vekâletine 31 sayfalık bir raporla sunmuştur. Raporda, atlar için pedigrilerinin tutulmasının, at yetiştirmenin devlet politikası olması, sığır içinse önce köylünün ihtiyacının belirlenmesi gerekliliğini gibi önerilerde bulunmuştur. Bu çalışma ile Batı Anadolu hayvancılığına ilişkin rapor temel alınarak dönemim hayvancılık alanındaki durumu belirlemek amaçlanmıştır.

Anahtar sözcükler: *Rostafinski, Mütehassıs raporu, Hayvan ıslahı, Türkiye Cumhuriyeti döneminde hayvancılık, Veteriner hekimliği tarihi*

INTRODUCTION

Between the late Ottoman and the early Turkish Republic period, husbandry, zootechnics and veterinary institutions activities became impossible under adverse circumstances because of the war [1,2]. Under these circumstances, in order to designate the economical restrictions and development procedures and principles of the newly established Turkish Republic, husbandry development and breeding subjects were evaluated at the 1st Economy Congress held in 1924. At the congress, it was emphasized on *'… takes pains over its animals as well as correcting their strains and augments*

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their quantity' under the act 8, National Economy Principles [3,4]. In addition, a five-year plan was formulated for the purpose of reconstructing the veterinary services, and it was put in practice in 1925 [1,5].

Animal breeding was detected in the curriculum of veterinary education during the Ottoman period [6,7]. During this period, it was seen that campaign activities on animal breeding and epidemic subjects were practiced. It is known that some studies were performed to build up a stud farm in Thessaloniki, a stallion warehouse in Monastery and in Kosovo in 1907, by Department of Veterinary Affairs and Animal Breeding (Islah-ı Hayvânat ve Umûru Baytâriye Şubesi), which was founded on 29th October, 1892. Along with the Proclamation of Constitutional Monarchy, animal breeding gained importance. The number of warehouses in Rumelia augmented to five, and 12 warehouses were established in Anatolia [5,8]. After the Proclamation of Republic, scientists from abroad were invited to study animal species in Turkey along with their breeding methods, and to advise on animal diseases. Within the scope of the advice of the scientists, laws and orders were imposed, whilst various studies were performed to develop animal husbandry in Turkey [5,9,10].

Prof. Jan Rostafinski (1882-1966) was one of the specialists who was invited to Turkey. He was a Polish scientist who studied animal husbandry and animal breeding and development. He was kept in captivity in Jewish prison camp [11].

In this study, it is aimed to present animal husbandry and veterinary conditions in Western Anatolia Dourineg that specific period related with the report of Professor Rostafinski.

MATERIAL and METHODS

Thirty-one pages of the report of Ministry of Agriculture Expertise Reports, Veterinary Part (Ziraat Vekâleti Mütehassıs Raporları, Baytar Kısmı) published, in 1927 forms the first chapter of the research material *(Fig. 1)*. The text was summarized whilst being translated from Ottoman Turkish to Modern Turkish, and it was evaluated through related surveyed documents and literature.

RESULTS

The report (31 pages) that Professor Rostafinski *(Fig. 2)* presented to the Ministry of Agriculture, which was written in Ottoman Turkish (240 pages) forms the first chapter of 'Ministry of Agriculture Expertise Reports, Veterinary Part'. The report begins with the clause 'A copy of the report presented by Professor Rostafinski' and states that upon the invitation received from Şükrü Kaya, term Minister of Agriculture, Rostafinski traveled to Western Anatolia for the purpose of studying and evaluating the animals in Western

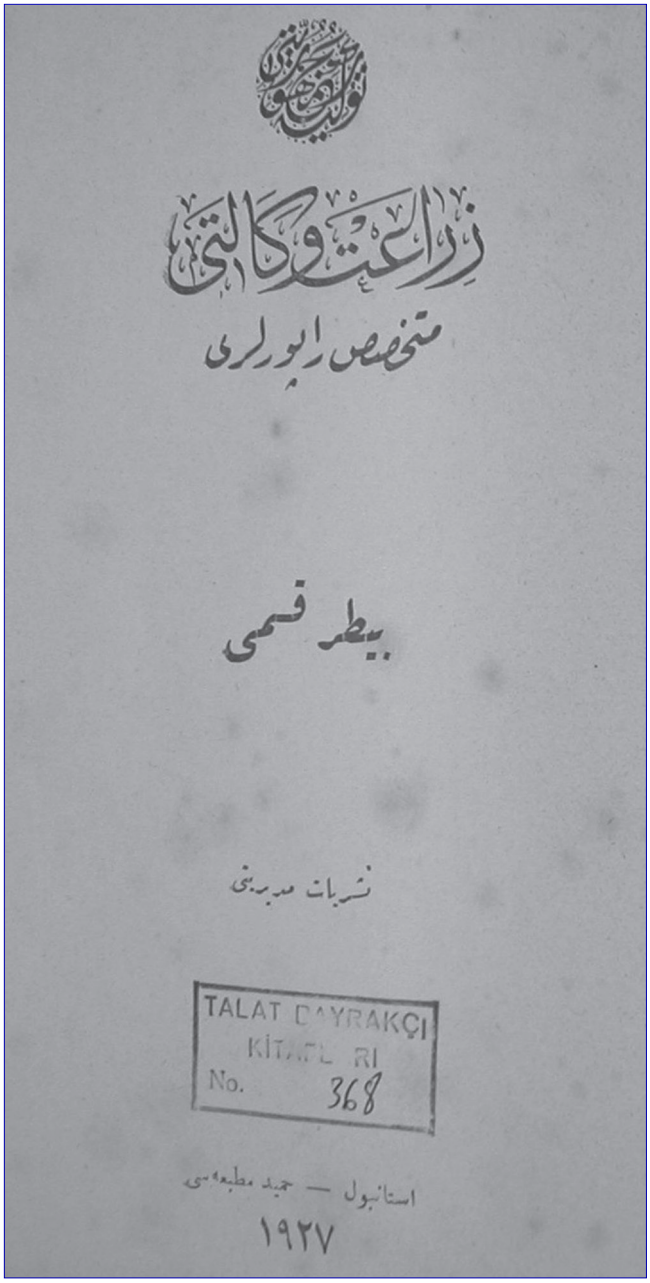

Fig 1. The first chapter of the research material

Anatolia with the help of Şefik (Kolayli), bacteriologist at Ministry of Agriculture and Mr. Yaşar, the Administrator of Agriculture in Izmir. Apart from Ankara, the journey includes stud horse warehouse in Eskisehir-Cifteler Farm, stud farm in Bursa-Karacabey, Buca-Izmir (draught animals, Izmir Agriculture School), Selcuk, Soke, Aydin, Omurlu, Denizli (stud horse warehouse), Dinar, Sandikli, Afyonkarahisar. Professor Rostafinski expressed his opinion in detail about the strains of horses, cattle and sheep. Even though he remarked that he added an animal breeding project to his report, that part does not appear in the book. In his report, Rostafinski gave wide publicity to Karacabey Farm, which was handed over to Ministry of Agriculture upon his arrival. The professor stated in his report that

Fig 2. Professor Rostafinski

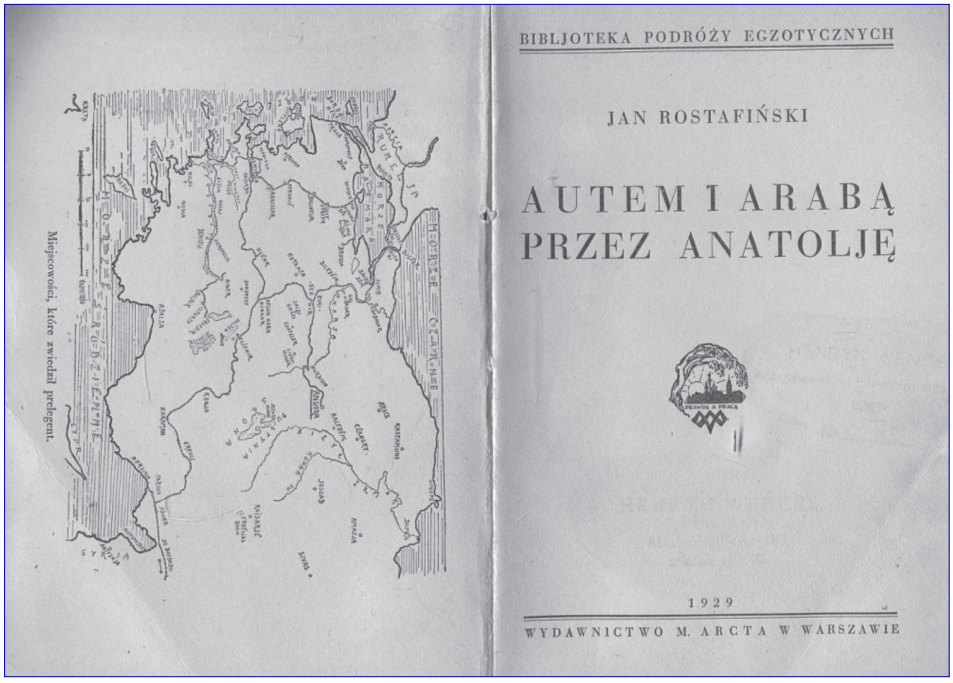

Fig 3. The book on Rostafinski's Anatolian observations

none of the animals in the Cifteler Stud Horse Warehouse had any pedigree and despite the misimplementation in the stud farm, it had a positive effect on the environment. Karacabey: Apart from the draught animals, it was stated that there were 12 studhorses, 28 mares in the warehouse, together with 7 mares, 23 studhorses and 70 foals, which were transferred from Cifteler warehouse. Since the most appropriate strain for Turkey is 'Karabag', he suggested that this strain should be bred and protected in Turkey unless it is hybridized with '*Nonius*'. He noted that the biggest problem of animal breeding was the reason that racing associations in Izmir, Istanbul and Karacabey were not subsidized. He specified that the field structure of Karacabey studfarm was suitable to be transform into a race track; and he suggested that the race horses should be bred in that place. He emphasized that if they work fast enough, Turkey could be a significant worldwide "east animal (probably Arabian horse) resource. He indicated that horse breeding should be adopted as a government policy and animal owners should be encouraged.

Suggestions of Professor for Western Anatolia region horse breeding follows as: *between 1.45 and 1.50 meters of pedigreed 'English-Arabian', 'Arabian-Karabag' or hybridized from amble stallions should be used in the coastal region. In the highlands without a pasture, Arabian or 'Arabian-Karabag' hybrids, mares or overweight stallions, with height of 1.48 should be used. Even if it is a 'Nonius' strain, it should not be imported from countries with severe climate conditions. Horse racing should be organized in the studfarms for Arabian, English and Karabag horse strains, even for short-mountain horses in Anatolia. Russian and Mongolian horses which were bred with tropical animals and have lost their pure race should not be used for breeding. Hybrids whose pedigree are not defined shall not be used for breeding. For an appropriate breeding programme, the pedigree must be determined and tracked decently.*' The qualities of broods in Çifteler Warehouse, Karacabey Studfarm and Denizli Warehouse were described one by one, with appendixes in the following part of the report about the horses. He suggested that the markers on animals should be changed so that the animals in Karacabey shall not be mixed with the animals in Çifteler farm.

He stated that the purpose was to distinguish the animals one from another. He said '*a horseshoe, with the endspointing down with a nail in the middle, which I present its drawing below can be considered as a marking. Acceptance of such marking method is important for state studhorses*'. He highlighted that the most important issue was that the stud farm had a budget and the amount determined directly from Ministry of Agriculture. He indicated that it was important that the stud farm should remain

interconnected to Ministry of Agriculture, which would help to gather the mandatory tools and materials quickly, this would prevent loss of money and time.

He argued that when it comes to cattle breeding, it is important to determine the needs of the peasants first. He stated that animal breeding in Turkey should be studied urgently on the animals at the coastal regions of Turkey first, and later on, it should be practiced on the grey and black cattle in the Central Anatolia.

In conclusion of this part of the report, he asserted that for cattle breeding and raising, Polona strain shall neither be raised nor used anymore, and this practice should stop immediately. In addition, in order to increase the population of red animals (cattle imported from Germany), it was advised to import 2-3 breeding cattle and that it is important to study and examine in Karacabey, which strain is viable for the coastal regions and Turkey's condition. He also mentioned that it is essential to study breeding the grey and black animals with 'Algav' and 'Svitch' hybrids. Moreover, it is essential to teach the peasants how to build and use a granary. He also added that the stud farms should be reformed.

He began his statement in sheep breeding chapter by expressing how sheep breeding is as important as horse breeding for Turkish economy to protect the borders. He asserted that the sheep stock in the country consists of curly-fleeced sheep, fat-tailed sheep and Karaman sheep which were imported from Rumelia. He indicated that all those strains are important for their milk, meat and fur. He remarked that there had been no considerable research for animal husbandry in Turkey; and animal husbandry studies should be carried on by the Ministry of Agriculture.

According to research, all his documents and photographs were destroyed during a bombardment in the war period [1]. However, one of his books about Turkey was published in Warsaw in 1929, which was named *'Autem i araba przez Anatolie' (Fig. 3)*.

DISCUSSION

In the evaluation section of the institution, previously called Çifteler Studhorse but later evacuated because of the incidence of Glanders and Dourine disease [12], and named Çifteler Stallion Ware House, he stated that despite the misimplementation of the stud management, the horses of villagers were in good condition. After 10 years, when invited for a related subject, the expert Professor Welleman emphasized in his report to the Ministry of Agriculture that Çifteler Studhorse made a positive impact on Turkish horse-breeding in 1934 [2,13].

In the assessment report of a 10-year study on animal husbandry in the Republican period [14], the other experts who were invited previously and Professor Rostafinksi were not mentioned; however, the subject about identification of races, which was also mentioned in his report, had been referred to. Professor made vital recommendations on how to improve presence of animals in future of Turkey. It can be said that in the experts' reports which were considered Dourineg the implementation of the Republic development policy [10], and in the above-mentioned report [13] conflicts with these recommendations.

His indication that the breeders should be encouraged through exhibitions and competitions in animal breeding, which highlights the impact of rewarding in good and quality animal husbandry by referring to the current situation can be accepted as an important guidance to be effective in animal breeding studies. In addition, considering an active participation of breeders on animal breeding, his suggestion on supporting the relevant non-governmental organizations - only if they are inspected - can be considered as an another dimension.

Professor Rostafinski offered to use a special marker for Karacabey Stud animals which was not used in European studs and stated that accepting such kind of marker is very important for a state stud. Professor indicated that he suggests a figure for this marker in his report However, it is not determined either in his report or in his studies.

In conclusion, before Professor Rostafinski's report, any scientific or actual study on the subject is not detected. It can be said that the report is important from the perspective of providing information about the origins and phenotypes of the animals in the related period. Also, when it is reviewed as the quality and quantity of Western Anatolia animals, it can be expressed as an important historical source.

[1] *E-mail correspondence with the grandson of Prof. Rostafinski in 2012*

REFERENCES

1. Dinçer F: Türk veteriner hekimliğinin 139. yılında Atatürk dönemi tarımsal politikası. *Ankara Üniv Vet Fak Derg (Atatürk'ün 100. Doğum yılı Özel Sayısı),* 28 (1-4): 307-310, 1981.

2. Yaşar A: Osmanlı Döneminde ve Cumhuriyet Tarihinde Haralar. *Doktora Tezi,* Ankara Üniv. Sağlık Bil. Enst., 1996.

3. İnan A: İzmir İktisat Kongresi. 17 Şubat-4 Mart 1923. Atatürk Kültür, Dil ve Tarih Yüksek Kurumu Türk Tarih Kurumu Yayınları XVI. Dizi, 2. Baskı, Türk Tarih Kurumu Basımevi, Ankara, 1989.

4. Ökçün G: Türkiye Kongresi 1923- İzmir Haberler- Belgeler- Yorumlar. A.Ü. Siyasal Bilgiler Fakültesi Yayın No: 262, 2. Baskı, Sevinç Matbaası, Ankara, 1971.

5. Ali Rıza: Beş Senelik Umur-u Baytariye Programı. Okut Matbaası, Ankara, 38 s. 1924.

6. Erk N, Dinçer F: Türkiye Veteriner Hekimlik Öğretimi ve Ankara Üniversitesi Veteriner Fakültesi Tarihi. Ankara Üniversitesi Veteriner Fakültesi Yayınları: 259, Ankara, 1970.

7. Godlewsky und Sommer: Türkiye'de Veteriner Hekimlik (19'uncu Yüzyıl Ortalarında). (Çev: N. Erk) Ankara Üniversitesi Veteriner Fakültesi Yayınları: 281, Ankara Üniversitesi Basımevi, Ankara, 1972.

8. Dinçer F: Türkiye'de Bilimsel veteriner hekimliğin başlaması, evreleri ve ilim tarihimizdeki yeri. *Ankara Üniv Elazığ Vet Fak Derg*, 2 (1): 81-91, 1975.

9. Bekman M: Veteriner Tarihi. Ankara Basım ve Cildevi, Ankara, 1940.

10. Osmanağaoğlu Ş, Melikoğlu B: Hayvancılık ve veteriner hekimlik alanlarında Türkiye ve Macaristan arasındaki ilişkilere bir bakış. *Türk Vet Hek Bir Derg*, 12, 3-4, 2012.

11. Rolniczy Magazyn Elektroniczny: Jan Rostafiński - profesorski ród. http://www.cbr.edu.pl/rme-archiwum/2006/rme15/rme.php?strona=48; *Accessed:* 05.04.2016.

12. Batu S: Türkiye'de Yetiştirme Çalışmaları ve Yetiştirme Kurumları. Ankara Üniversitesi Veteriner Fakültesi Yayınları, No: 74, Ankara, 1955.

13. Menteş Gürler A, Osmanağaoğlu Ş: Cumhuriyet'in İlk 10 yılının Veteriner Hekimlik Açısından Değerlendirilmesi: II. Kurumlar. *II. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyum Kitabı, 24-26 Nisan,* Konya, Türkiye, 2008.

14. T.C. Başbakanlık Cumhuriyet Arşivi: Cumhuriyet döneminde yapılan haralar, ahırlar ve hayvan hastalıkları ile mücadele merkezleri hakkında hazırlanan raporlar, grafikler ve fotoğraflar. Belge No: 030.1 0.187.285.1, 110 s. 1946.

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Case Report

A Case of *Trichophyton mentagrophytes* Infection in Rabbits Accompanied by Farm Staff Infection in China

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Abstract

Trichophyton (T.) mentagrophytes is common tineas infection with high incidence in rabbits and also a classical zoonotic infection in people exposed to infected animals. However, many cases never got a correct diagnosis, let alone considerable management and treatment, which could cause certain economic losses in rabbit farms. An outbreak of a highly contagious skin disease infecting rabbits and accompanying all farm staff on a large scale rabbit farm in China was investigated. The disease was diagnosed by routine physical examinations, and the etiology of the disease was identified as *T. mentagrophytes* by fungal culture, sequence of ribosomal internal transcribed spacer (ITS+) and chitin synthase-1 gene (pchs-1). To date, there is no report of Dermatomycoses infection in rabbits accompanied by farm staff infection in mainland China. In this case, the etiology is identified by using advanced molecular techniques and clinical, microbiological aspects and the histological features of the infection are described.

Keywords: *Dermatomycosis, Zoonosis, Trichophyton, Rabbits*

Çin'de Çiftlik Çalışanları ile Birlikte Tavşanlarda *Trichophyton mentagrophytes* Enfeksiyonu Olgusu

Özet

Trichophyton (T.) mentagrophytes tavşanlarda yüksek oranda gözlemlenen yaygın bir mantar enfeksiyonu olup enfekte hayvanlarla temas halindeki insanlarda da gözlemlenen klasik zoonotik bir enfeksiyondur. Tavşan çiftliklerinde çoğu vaka doğru teşhis edilemeyip bakım ve tedavi masrafları sebebiyle önemli ekonomik kayıpların oluşmasına neden olabilmektedir. Bu çalışmada Çin'de büyük ölçekli bir tavşan çiftliğindeki tavşanlarda ve çiftlik çalışanlarında gözlemlenen oldukça bulaşıcı bir deri hastalığı salgını incelendi. Hastalığın rutin fiziksel muayene ile tanısı konuldu ve hastalığın etiyolojisi fungal kültür, ribozomal internal transkript spacer (ITS+) ve kitin sentaz-1 gen sekansları ile *T. mentagrophytes* olarak belirlendi. Bugüne kadar Çin'de tavşanlarda ve çiftlik çalışanlarında aynı zamanlı olarak tespit edilen Dermatomycoses enfeksiyonuna dair bir rapor bulunmamaktadır. Bu vakada etiyoloji gelişmiş moleküler teknik, klinik, mikrobiyolojik ve hastalığın histolojik yapısı ile tanımlandı.

Anahtar sözcükler: *Dermatomikoz, Zoonoz, Trichophyton, Tavşan*

INTRODUCTION

Rabbit dermatophytoses are highly contagious zoonotic diseases. The organisms causing these dermatophytoses mainly include *Trichophyton, Microsporum* and *Epidermophyton genera* within the *Fungi Imperfecti*, most of which reproduce asexually by sprouting or budding [1]. Many types of *Trichophyton* can infect humans as well as animals. For instance, *Trichophyton rubrum, Trichophyton mentagrophytes (T. mentagrophytes), Trichophyton tonsurans, Trichophyton verrucosum* and *Trichophyton schoenleinii* are common in clinical infections. Among rabbits, *T. mentagrophytes* is one of the most important pathomycetes, which incubates and develops in keratin tissues and damages the skin, hair and nails of the animals. *T. mentagrophytes* infection is characterized by inflamed, itchy skin with irregular lumps, alopecia, and dermatitis [2]. Previous epidemiological studies have demonstrated a high incidence of *T. mentagrophytes* in rabbits and its transmission to humans [3-5], but there are no case reports in mainland China. In this study, we diagnosed a case of a highly contagious skin disease infecting rabbits and all farm staff on a large scale rabbit farm in this region as *T. mentagrophytes* by using advanced molecular techniques and clinical, microbiological aspects and the histological features of the infection are described.

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CASE HISTORY

During a technical visit to a large scale farm containing approximately 1000 rabbits in the Fujian Province of China, a case of highly contagious rabbit dermatophytoses was reported. Prior to our visit, over a 5 week period, approximately 70% of rabbits of all ages had developed or were developing hair loss, crusts and scales. Meanwhile, all farm staff, including 6 males and 2 females (20 to 30 years of age), who were exposed to the sick rabbits successively showed itching ringworm lesions. They did not realize they suffered from dermatophyte infection and treated the lesions themselves with topical applications of iodine tincture. The skin lesions of some of the staff had recovered while some were still suffering dermatoses. At the beginning, the veterinarian diagnosed this disease as bacterial infection, and antibiotics were administrated to sick rabbits. During the outbreak, environmental disinfection was conducted in the rabbit houses, although these measures had little effect. The disease spread to the entire farm, including accompanying staff and they asked us for assistance.

Dermatological examination revealed hair loss and scales on all body locations. A great deal of canary crusts was present on the top of the head of rabbits with alopecia *(Fig. 1A)*. Numerous canary scales were present around the eyes, the ears and the back. Hair loss appeared also on the abdomen and limbs. In infected staff, the lesion sites were mainly on the finger joints, palms, arms, necks and legs, with symptoms of itching, redness, peeling and blistering. An approximately 30 mm×30 mm circular lesion on the palm side of the left hand was sharply marginated erythema *(Fig. 1B)*.

Initially, direct microscopic examination of rabbit skin crusts and scales with 15% KOH showed septate, and branching hyphae by re-examination, confirming our hypothesis of dermatophyte infection. Rabbit skin samples of nine rabbits and cotton swab samples of three infected staff members were inoculated onto slants of Sabouraud glucose agar (Hangzhou Tianhe Microorganism Reagent Co, Ltd, China) with 10^6 U/L penicillin (Beyotime, China) and 10 mg/L of chloramphenicol (Shanhai Sangon Biotech, China). The cultures were incubated in an incubator at 28°C. After 7 d, white mycelium were seen at the beginning, turning gradually to irregularly shaped colonies with a powdery surface *(Fig. 2A, Fig. 2B)*. Under microscopic observation, elongated mycelium could be seen with septate and branching *(Fig. 2C)*. Two types of spores were observed. The large conidia were rod-like with 6-8 divisions and few in number *(Fig. 2D)*, while small conidia were round or pear-shaped with large numbers *(Fig. 2E)*. The morphology of colonies, the mycelium and spores from different samples looked very similar, suggesting the tiological agents of dermatophyte infection in the rabbits and farm staff were identical.

To molecularly identify the dermatophyte, a region (ITS+, 900-950 bp) spanning across the ITS-1, 5.8 S and ITS-2 of the nuclear ribosomal and part of chitin synthase 1 gene (CHS-1) gene (897 bp) were amplified separately using specific primers as the following: ITS+ (forward primer 5′-CCAGGGAGGTTGGAAACGACCG-3′; reverse primer 5′-CTA CAAATTACAACTCGGACCC-3′), CHS-1 (forward primer 5′-GACTGTCCCATTCCACCA-3′; reverse primer 5′-GTTCTTGTC ATTCTGTAGCG-3′). All ITS+ and CHS-1 amplicons produced from genomic DNA samples were purified using mini-columns (OMEGA bio-tek, USA) and automated nucleotide sequencing was performed using 3730XL DNA Analyzer with the BigDye terminator v3.1 by Shanghai SanGong Biological Engineering Technology Service Co., LTD, China. The sequences of CHS-1 from different DNA samples were identical, as were the ITS+ sequences. These results demonstrated that the rabbits must have been the source of the farm staff infection. Sequence data reported in this paper are available in GenBank under accession numbers KM355551 (ITS+) and KM355549 (CHS-1). The sequences were aligned with previously published sequences from GenBank by using the ClustalW method, identifying the dermatophyte infection as *T. mentagrophytes* and confirming that the strains isolated from nine rabbits and three staff are identical.

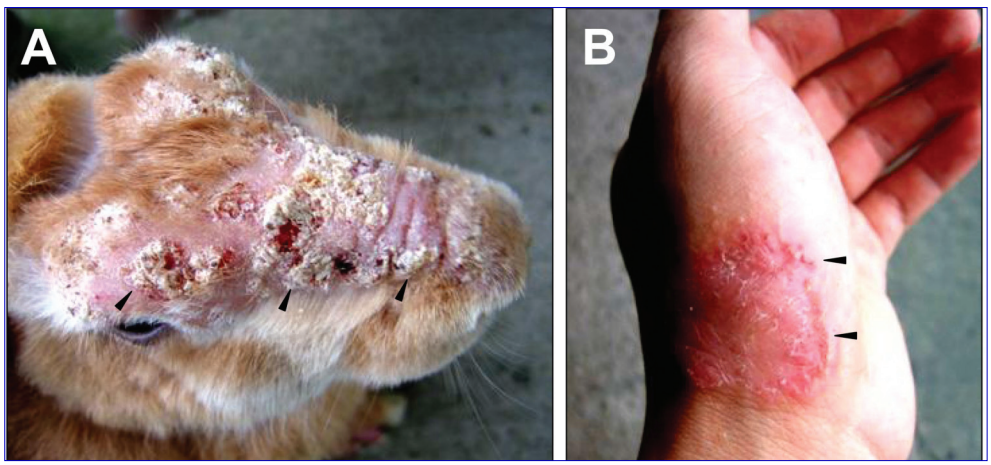


Fig 1. A- Numerous canary crusts and large scales were present on the head of rabbits with hair loss, **B-** An approximately 30 mm×30 mm circular lesion on the palm side of the left hand of an infected staff member was sharply emarginated erythema

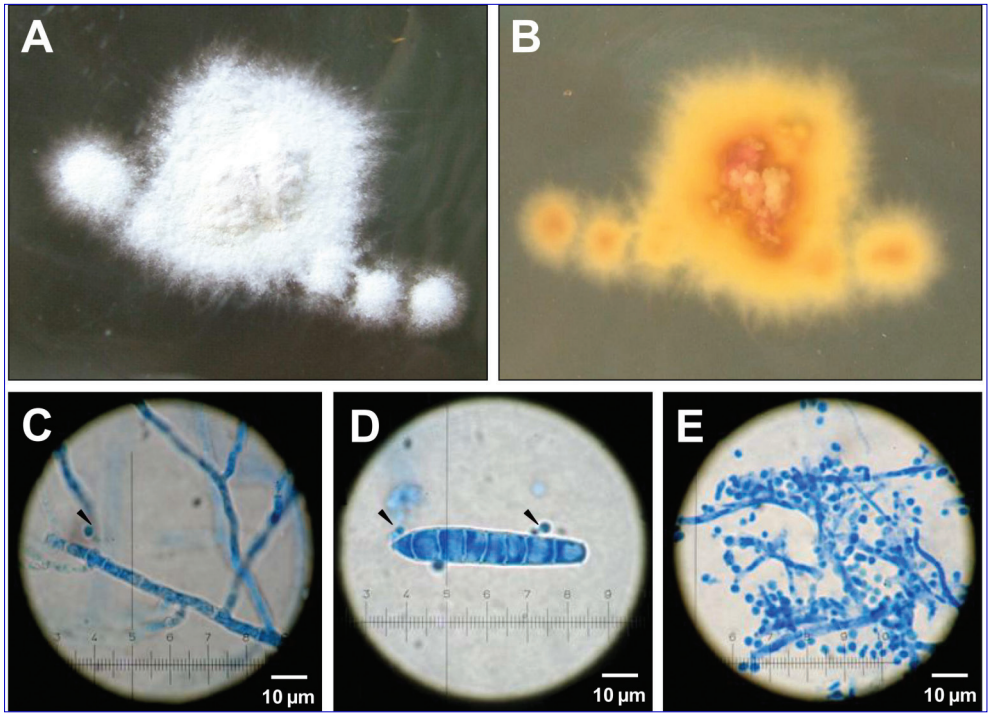
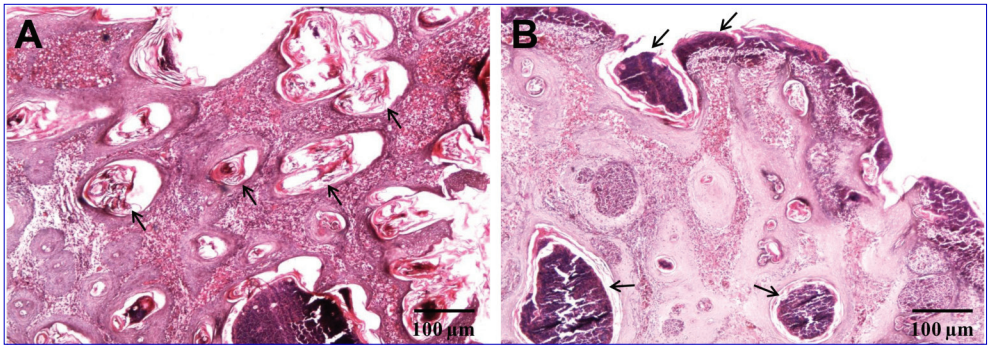


Fig 2. A- Cultured pure *T. mentagrophytes*: surface of colony show powder-like shape, white, loose irregular mycelium on the edge, **B-** Back side of Trichophyton mentagrophytes: pale yellow color, **C-** Mycelium: elongated, divisions and branches, **D-** Large conidia (size: 4-8×40-50 µm), E- Small conidia (size: 2-3×2-4 µm) and mycelium

Fig 3. A- Plentiful laminar orthokeratotic keratinization *(arrows)* covering the hair follicles, **B-** Compact hyperkeratosis *(arrows)* covering the stratum corneum and hair follicles (H&E, ×40)



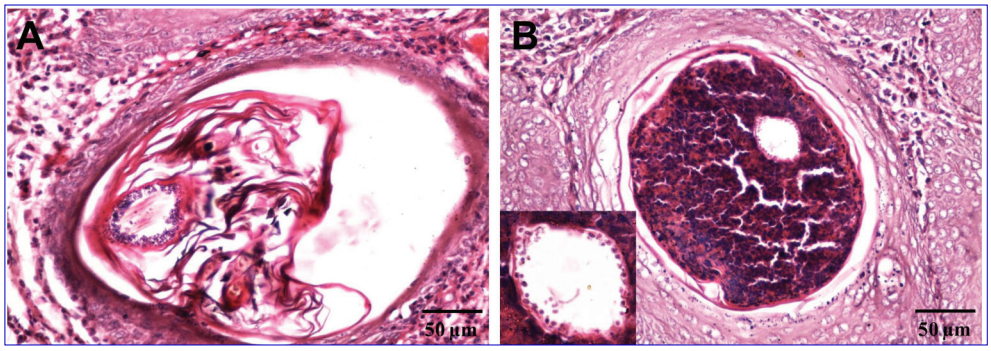


Fig 4. A- Numerous, round, fungal spores in laminar hyperkeratotic scale in the hair follicles, **B-** In compact hyperkeratotic scale in the hair follicles. *Arrows* show fungal spores. (H&E, ×200)

In order to understand histological features of *T. mentagrophytes* infection in rabbits, biopsy specimens from the back skin of a severe infected rabbit were obtained and submitted for histological examination. In the epidermis, the general changes were acanthosis, multifocal spongiosis, hyperplasia of epidermal cells, lymphocyte infiltration in the basal layer, and lamellar or compact hyperkeratosis present in the hair follicles and the stratum corneum *(Fig. 3A, Fig. 3B)*. By further examination, the typical characteristic of the fungal infection was revealed. In laminar hyperkeratotic scale *(Fig. 4A)* and compact hyperkeratotic scale *(Fig. 4B)* in the transverse section of hair follicles, at high power, numerous, round, fungal spores were observed. Furthermore, longitudinal section showed fungal spores with various shapes were widely distributed throughout the hair follicles. In addition, a large number of fungal spores were also observed in the laminar orthokeratotic keratinization in the stratum corneum

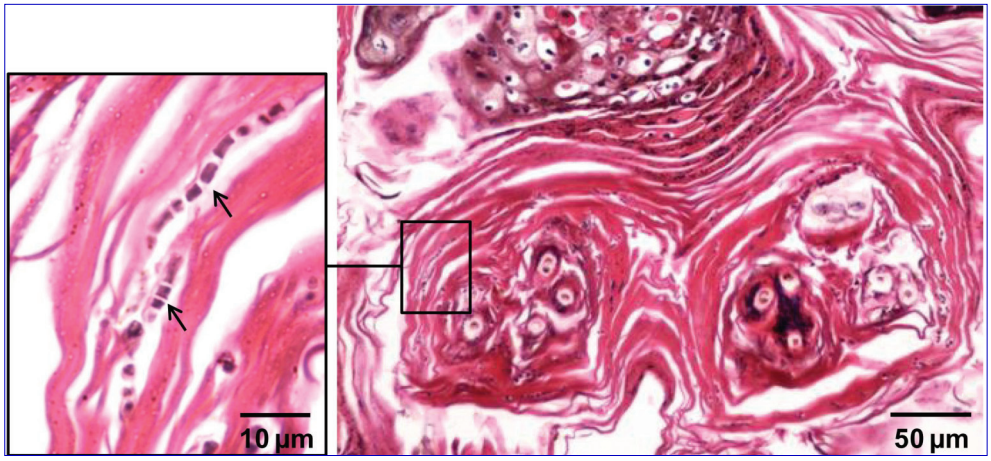


Fig 5. Stratum corneum on the back skin. Large number fungal spores and hyphae *(arrows)* were present in the laminar orthokeratotic keratinization (H&E, ×40) Inset, high-power photomicrograph shows elongated, septate mycelium *(arrows)*, ×1000)

(Fig. 5). However, fungal hyphae were mainly distrubuted in the laminar orthokeratotic keratinization in the stratum corneum, and inset showed mycelium were elongated and obviously septate.

After hyphae were observed by direct microscopic examination of rabbit skin scrapings, a miconazole nitrate cream (Trade name: Da Ke Ning, Xi'an Janssen Pharmaceutical Ltd, China) was applied on affected areas of the farm staff twice per day. The lesions disappeared gradually and the infection resolved completely after one week of treatment. In infected rabbits, the ten rabbits with the heaviest crusts were euthanized and disposed of appropriately. Those rabbits with heavier crusts were separated in a single room, given the oral antifungal drug griseofulvin (Beijing Zhong Xin Pharmaceutical Factory, China) for four weeks. Meanwhile, voriconazole was administered by intramuscular injection. Those rabbits with light visible skin lesions were given only griseofulvin. At the same time, the farm was disinfected and dehumidified with quicklime. The dermatophytoses were under control after one month of treatment and only sporadic cases were encountered thereafter.

DISCUSSION

This case describes the first case of rabbit dermatophytes accompanying farm staff infection in Fujian Province, China. The lack of epidemiological data on the rabbit tinea in this region hampers the understanding of its clinical significance. Before our technological visit, this dermatophyte was misdiagnosed and under reported. In this case, hyphae were observed by direct microscopic examination, confirming our hypothesis of fungal infection. Fungal culture is thought the most reliable technique for confirming dermatophytosis [6]. We performed the fungal culture and we found the morphology of colonies, the mycelium and spores from different samples looked very similar. Biomolecular tools have been used for the identification of rabbit dermatophytes by amplification of CHS-1 gene and ITS+ sequence [6]. By using molecular methods, we identified the dermatophytic infection as *T. mentagrophytes* and confirmed the strains isolated from nine rabbits and three staff are identical.

After the preliminary diagnosis of fungal infection, topical applications of miconazole nitrate cream was performed immediately and effectively treated human infections, but there were some problems with the control dermatophytic infections in rabbit. Griseofulvin was added into the fodder for oral administration and voriconazole was administered by intramuscular injection. After four weeks of continuous use, the epidemic situation was effectively controlled. However, the use of these drugs affected the growth and performance of the rabbits, especially females, whose reproductive performance decreased. Female rabbits developed symptoms like premature birth and stillbirth, and some of them did not come into estrus. After stopping medication, drug adverse reaction gradually disappeared.

Despite the epidemiological and molecular features of *T. mentagophytes* in rabbits having been documented [3-5,7], few studies have described histological features of rabbit infection. In this study, histological examination revealed a usual feature that lamellar or compact hyperkeratosis was widely distributed in the hair follicles and the stratum corneum. Most notably, we observed fungal hyphae and spores in large numbers in laminar hyperkeratotic scale in the stratum corneum and numerous spores were widely distributed throughout the hair follicles. Based on the above facts, it is reasonable to make predictions that hyperkeratosis in the hair follicles and the stratum corneum lead to the production of large numbers scales and hair loss for ensuring the transmission of the fungus, suggesting the main origination of transmission of this fungus in rabbits is the infected hairs and scales.

Similarly, *M. canis* infection in cats and dogs and *T. verrucosum* infection in calves show a usual feature of the production of abundant arthrospores [8,9]. By contrast, the histological feature of *T. mentagrophytes* infection in dogs shows hyphae and spores were usually sparse and often

difficult to observed [10]. This discrepancy of histological feature is presumably related to infection in adapted hosts or nonadapted hosts. Thus, rabbits are likely a reliable host for *T. mentagophytes* infection.

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Competing Interests

The authors declare that they have no competing interests.

REFERENCES

1. Weitzman I, Summerbell RC: The dermatophytes. *Clin Microbiol Rev*, 8, 240-259, 1995.

2. Degreef H: Clinical forms of dermatophytosis (ringworm infection). *Mycopathologia*, 166, 257-265, 2008. DOI: 10.1007/s11046-008-9101-8

3. Torres-Rodríguez JM, Dronda MA, Rossell J, Madrenys N: Incidence of dermatophytoses in rabbit farms in Catalonia, Spain, and its repercussion on human health. *Eur J Epidemiol*, 8, 326-329, 1992. DOI: 10.1007/BF00158563

4. Proverbio D, Perego R, Spada E, Bagnagatti de Giorgi G, Della Pepa A, Ferro E: Survey of dermatophytes in stray cats with and without skin lesions in Northern Italy. *Vet Med Int*, 2014. DOI: 10.1155/2014/565470

5. Cafarchia C, Camarda A, Coccioli C, Figueredo LA, Circella E, Danesi P, Capelli G, Otranto D: Epidemiology and risk factors for dermatophytoses in rabbit farms. *Med Mycol*, 48, 975-980, 2010. DOI: 10.3109/13693781003652620

6. Bond R: Superficial veterinary mycoses. *Clin Dermatol*, 28, 226-236, 2010. DOI: 10.1016/j.clindermatol.2009.12.012

7. Cafarchia C, Weigl S, Figueredo LA, Otranto D: Molecular identification and phylogenesis of dermatophytes isolated from rabbit farms and rabbit farm workers. *Vet Microbiol*, 154, 395-402, 2012. DOI: 10.1016/j.vetmic.2011.07.021

8. Gross TL, Walder EJ, Ihrke PJ: Feline and Canine Dermatophytosis. **In,** Veterinary Dermatopathology: A Macroscopic and Microscopic Evaluation of Canine and Feline Skin Disease. 243-245, Mosby, St Louis, 1992.

9. Yager JA, Wilcox BP: Dermatophytosis. **In,** Color Atlas and Text of Surgical Pathology of the Dog and Cat. 188-189, Mosby, St Louis, 1994.

10. Fairley RA: The histological lesions of *Trichophyton mentagrophytes* var erinacei infection in dogs. *Vet Dermatol*, 12, 119-122, 2001. DOI: 10.1046/j.1365-3164.2001.00235.x

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Case Report

Surgical Treatment of Dorsal Scapular Luxation in Cats: Six Cases (2010-2016) [1]

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Abstract

Dorsal luxation, avulsion or dislocation of the scapula is described as the dorsal displacement of the scapula, resulting from the rupture of the ventral serratus, rhomboideus and trapezius muscles. In this report, we aimed to suggest a different way for the surgical treatment of dorsal scapular luxation in cats. The study material comprised of 6 cats presented to our clinics. Intra-operatively, in all the patients it was determined that the ventral serratus and rhomboideus muscles were ruptured, however, the trapezius muscle was undamaged. In surgical intervention, cauterization of contact surfaces of two muscle to improve adhesion and suturing of serratus and rhombeideus muscles to infraspinatus and supraspinatus muscles was carried out respectively. In the clinical follow-up examination on post-operative day 15, dorsal luxation of the affected extremity during weight-bearing had completely disappeared in all of the patients. On the telephone consultation 2 months after operation, owners declared no complaints of lameness or recurrence of symptoms. In conclusion, the treatment model suggested in this study is quite simple and minimally invasive which could be applied in the treatment of the dorsal scapular luxation in cats.

Keywords: *Scapula, Dorsal luxation, Avulsion, Serratus ventralis, Rhombeideus, Cat*

Kedilerde Dorsal Skapular Lukzasyonun Cerrahi Sağaltımı: Altı Olgu (2010-2016)

Özet

Skapulanın dorsal luksasyonu, avulziyonu ya da dislokasyonu, m. serratus ventralis, m. rhombeideus ve m. trapezeus kaslarının rupturu sonucu skapulanın dorsale yer değiştirmesi olarak tanımlanır. Bu raporda, kedilerde dorsal skapular luksasyonun cerrahi sağaltımı için farklı bir metot önermek amaçlanmıştır. Çalışmanın materyalini kliniğimize getirilen 6 kedi oluşturdu. Operasyon esnasında tüm olgularda ventral serratus ve rhombeideus kaslarının yırtıldığı ancak trapezeus kasının hasar görmediği tespit edildi. Cerrahi müdehalede sırasıyla, temas yüzeyi bulunan iki kasın adezyonunu arttırmak için koterizasyonu ve serratus ve rhombeideus kaslarının, infraspinatus ve supraspinatus kaslarına dikilme işlemi gerçekleştirildi. Postoperatif 15. gün yapılan kontrolde hastaların tümünde basış esnasında ilgili ekstremitenin dorsale luksasyonunun tamamen ortadan kalktığı görüldü. Postoperatif 2. ay hasta sahipleriyle yapılan telefon görüşmelerinde hastaların hiçbirinde topallama şikayetinin ve nüks durumunun olmadığı öğrenildi. Sonuç olarak bu çalışmada, kedilerde skapulanın dorsal luksasyonu için önerilen sağaltım modelinin, daha kolay uygulanabilir, daha az invaziv ve tercih edilebilir bir metot olduğu sonucuna varıldı.

Anahtar sözcükler: *Skapula, Dorsal luksasyon, Avulziyon, Serratus ventralis, Rhombeideus, Kedi*

INTRODUCTION

The scapula is a flat bone located in the cranio-dorsal region of the lateral chest wall. This bone does not have a conventional joint with the torso, but is attached to the chest wall via a special arrangement of the muscles in the area (synsarcosis) [1]. These muscles that attach the scapula and front legs to the chest wall are called the shoulder girdle muscles. The girdle muscles are classified as superficial and deep. The trapezius muscle is superficial, while the ventral serratus and rhomboideus muscles are deep muscles [2]. The girdle muscles are among those commonly subjected

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to avulsion in cases of dorsal scapular luxation [3,4].

Dorsal scapular luxation, avulsion or dislocation is described as the dorsal displacement of the scapula as a result of the rupture of the ventral serratus, rhomboideus and trapezius muscles [1,4]. This rare condition is more commonly observed in cats than in dogs and no breed or age predilection has been determined [4]. Dorsal luxations of the scapula occur in relation to jumps, falls or bite wounds. Within a few days following the injury, a distinct soft tissue swelling is apparent in the region [3].

On clinical examination, dorsal displacement of the scapula is clearly visible when the affected extremity is weight-bearing. Also, adduction of the extremity causes the scapula to be displaced laterally. Comparing the position with the sound side simplifies the diagnosis [4]. As well as clinical examination, radiological examination can be used in diagnosing patients with this condition. Conditions resulting from thorax trauma such as rib fractures, pulmonary contusions and pneumothorax can be evaluated with radiological examination. The rate of concurrent scapula fracture is low [5].

In the surgical treatment of this condition, the techniques used are: where possible, repairing the ruptured muscles by suturing, or circling the 5th, 6th or 7th rib with cerclage wire and threading this through a hole drilled in the caudal end of the scapula and fixing it. In some acute cases in cats and small dogs, despite reports of recovery by placing the extremity in a Valpeau sling and cage rest, surgical intervention has been reported as necessary for a satisfactory functional and cosmetic end result [3,5].

In the present study, the authors performed the surgical treatment and post-operative follow-up of 6 cases presenting with dorsal scapular luxation. The compiled outcome was intended to suggest a treatment model to overcome the negative aspects that could arise from fixing the scapula to the ribs.

CASE HISTORY

Of the cats included in this study, 5 were female and 1 was male which were brought to our clinic between 2010-2016, with a complaint of lameness. Breed distribution was; 1 Turkish Angora cat and 5 mixed breed. In only 1 of the patients (Turkish Angora) the age and history regarding etiology could be obtained. This was a 1-year old cat and dorsal scapular luxation had occurred due to falling from a height. The remaining 5 cats had been found in the street and presented to the clinic for treatment by their new owners. Therefore, no definite information could be obtained regarding either etiology or the age of the cats. Prior to the operation, the cats' teeth were examined and all were determined to be adult cats. Scapular luxation had occurred unilaterally in all the cases. While the Turkish Angora cat had left-sided scapular luxation, 4 of the mixed breed cats had left-sided and 1 had right-sided scapular luxation.

On inspection, the scapula on the affected extremity was seen to displace dorsally during walking and weight-bearing *(Fig. 1a)*. Neither walking nor orthopaedic examination elicited any pain response in the patients. When the related extremity was held at the elbow area and rotated laterally, the medial surface of the scapula was seen to detach laterally from the chest wall with ease *(Fig. 1b)*.

Clinical examination did not reveal any swelling or ecchymotic areas on or around the scapula. None of the patients exhibited any respiratory distress or emphysema in the thorax region. In the radiological examination, while not encountering any scapular fractures, nor was there any evidence of atelectasis, emphysema or pneumothorax in the lungs. Pre-operative haemogram and serum biochemistry findings were normal in all the patients.

Patients were intubated following an induction with 4-6mg/kg IV propofol (Pofol®, Sandoz). Anaesthesia was maintained using 1.5-2.5% isoflurane (FORANE®, Abbott). Patients were placed in lateral recumbency with the affected extremity uppermost. The lateral thoracal wall was shaved up to the distal third of the humerus and the region was aseptically prepared.

A longitudinal skin incision was made along the caudal edge of the scapula *(Fig. 1c)*. Intra-operatively, in all the patients it was determined that the ventral serratus and rhomboideus muscles were ruptured, however, the trapezius muscle was undamaged. After that, latissimus dorsi and trapezius muscle was incised at the level of the caudal edge of the scapula, exposing the medial surface of the scapula and the thorax wall *(Fig. 1d)*. The proximal ends of the ventral serratus muscle were located. Since the patients had been presented in the chronic period, the proximal ends of this muscle were covered with granulation tissue. In the first stage of surgical treatment, in order to increase post-operative tissue adhesion, several different points were cauterized on the subscapular and ventral serratus muscles, which have a contact surface on a non-luxated scapula *(Fig. 1e)*. Following this, the ruptured section of the ventral serratus muscle was pulled across the lateral surface of the scapula and sutured to the supraspinatus and infraspinatus muscles using the horizontal mattress suture technique. Similarly, the rhomboideus muscle was pulled over the scapular cartilage and sutured to the infraspinatus muscle using the horizontal mattress suture technique *(Fig. 1f,g)*. The incised portion of the trapezius muscle, connective tissue and skin was closed using the simple interrupted suture technique. For all sutures, 2/0 monofilament absorbable suture material was used. The extremity was kept in a Valpeau sling for 1 week and the patient's movements were restricted for 15 days *(Fig. 1h)*.

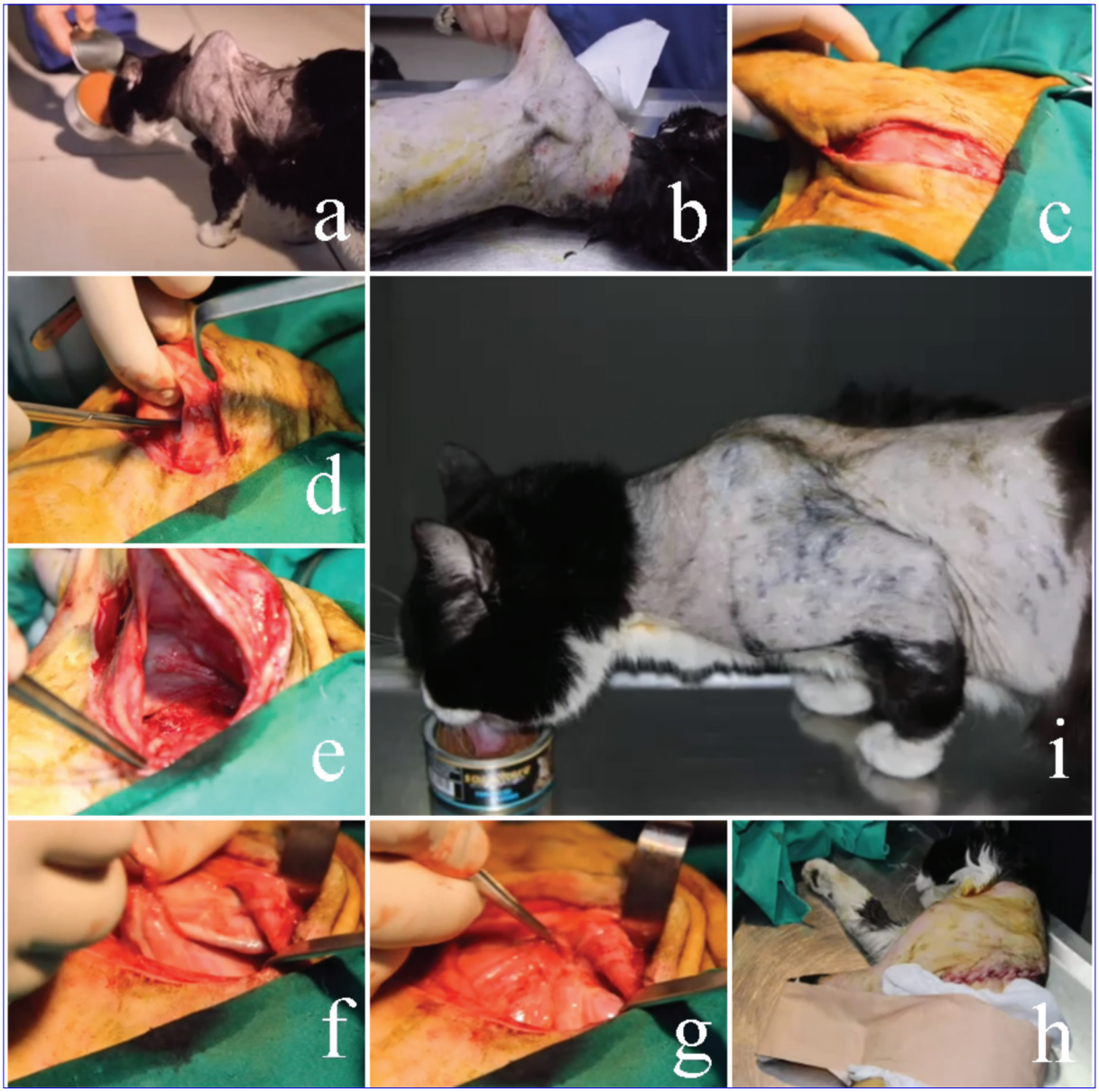

Fig 1. Preoperative-postoperative appearance of a patient during weight bearing and surgical treatment process. Preoperative appearance of dorsal scapular luxation, **a;** lateral rotation of effected limb and lateral displacement of scapula, **b;** longitudinal skin encision along the caudal border of scapula, **c;** incision of undamaged latissimus dorsi and trapezius muscles, **d;** post-cauterization apparence of the subscapularis and serratus ventralis muscles, **e;** pulling the ventral serratus muscle across the lateral surface of the scapula in order to suture to the supraspinatus and infraspinatus muscles using the horizontal mattress suture technique, **f-g;** postoperative Valpeu sling application, **h;** and postoperative 15th day appearance of the patient during weight bearing, **i**

The patients were re-examined on post-operative day 15 and discharged. Presence of dorsal scapular luxation was not determined by inspection *(Fig. 1i)* in all of the patients, while palpation was used to assess lateral movement of the scapula. Two months later, the patient owners were contacted by telephone and information regarding their latest condition was collected.

In the follow-up examination on post-operative day 15, dorsal luxation of the affected extremity was seen to have completely disappeared during weight-bearing in all the patients. On adduction of the extremity, it was observed that the scapula was not displaced laterally. There was no clinical lameness. On the telephone consultation 2 months later, patient owners reported that none of the patients had a complaint of lameness and there was no recurrence.

DISCUSSION

The ventral serratus muscle is a large muscle mass covering the caudal wall of the thorax and is attached to the proximal medial angle of the scapula. This muscle is the major muscle support of the front leg [5]. The dorsal part of the scapula has an unossified structure also known as the scapular cartilage. This situation increases the area

available for muscle attachment and with age, this area calcifies and becomes more rigid [2]. The ventral serratus and rhomboideus muscles attach to this cartilaginous area. The trapezius muscle, frequently mentioned in dorsal scapular luxations, originates from the spinal processes of cervical and thoracal vertebrae and attaches to the scapular spine. While all three of these muscles have been reported to be affected in cases of dorsal scapular luxation [3,4], rupture of the trapezius muscle was not encountered in any of the patients in this study. Nevertheless, all clinical examination findings required to make a diagnosis of dorsal scapular luxation were present.

Various methods are available for the treatment of dorsal scapular luxation. One technique is to fix the ruptured muscles and re-attach them to the scapula. However, this procedure has been reported to fail to achieve sufficient stabilization for the extremity to bear weight [4].

Another method is to restore the scapula to its normal anatomical position and attach it to the ribs. In this technique, firstly an inverted L-shaped incision is made along the dorsal and caudal edge of the scapula. If there are parts of the serratus, rhomboideus or trapezius muscles without avulsion, these are carefully separated from their attachment points enough to allow lateral retraction of the scapula and visualization of the caudal angle and caudal border of the scapula [4]. Stabilization is achieved with a 20-22G cerclage wire passed around the 5th, 6th or 7th rib threaded through holes drilled on the caudo-dorsal edge of the scapula, in the area of origin for the teres major muscle [5]. While the cerclage wire is tightened, the aim is to minimize dorsal movement of the scapula. In other words, dorsal movement is not completely restricted [4]. In addition, the ventral serratus muscle can be reconstructed using the holes drilled in the cranio-dorsal edge of the scapula [3,7]. Post-operatively, Valpeau slings, spica splint application [8,9] or carpal flexion bandage [3] is recommended for 2 weeks. During the 2 weeks after removal of the sling, splint or carpal flexion bandage, it is recommended that the duration of the patient using its leg is gradually increased [3]. However, once the patient starts using the affected leg, this technique has the possibility of causing intercostal muscle rupture, subcutaneous emphysema and breathing difficulties due to the cerclage wire passed around the rib. However, very long skin incision is another disadvantage. Therefore, recovery is thought to be achieved, by the Valpeau sling, splint or carpal flexion bandage applied post-operatively and kept in place for 2-3 weeks. Also use of cerclage wire for the procedure requires a second operation for wire removal following healing. The risks of non-removal of the cerclage wire include; pain and long period material reaction.

In this concept, relocation of the ventral serratus and rhomboideus muscles over the dorsal edge of the scapula and sutured to the infraspinatus and supraspinatus muscles, could be a more suitable option to prevent dorsal re-luxation of the scapula. Also, adhesion-increasing effect of point cauterization of subscapularis and serratus muscles and post-operative use of Valpeu slings together will decrease the operation time in this technique. In conclusion, the treatment model suggested in this study is quite simple and less invasive surgical intervention which could be applied in the treatment of the dorsal scapular luxation in cats.

REFERENCES

1. Perry KL, Rutherford L, Arthurs GI: A case of scapular avulsion with concomitant scapular fracture in a cat. *JFMS*, 14, 946-951, 2012. DOI: 10.1177/1098612X12459646

2. Dyce KM, Sack WO, Wensing CJG: The Locomotor Apparatus. **In,** Dyce KM, Sack WO, Wensing CJG (Eds): Textbook of Veterinary Anatomy. 4th edn., 32-100, Saunders Elsevier, Missouri, 2010.

3. DeCamp CE, Johnston SA, Dejardin LM, Schaefer SL: The shoulder joint. **In,** DeCamp CE, Johnston SA, Dejardin LM, Schaefer SL (Eds): Brinker, Piermattei, and Flo's Handbook of Small Animal Orthopedics and Fracture Repair. Fifth edn., 260-297, Elsevier, Missouri, 2016.

4. Johnson AL, Hulse DA: Diseases of joints. **In,** Fossum TW (Ed): Small Animal Surgery. 2nd edn., 1023-1150, Mosby, China, 2002.

5. Parker RB: Scapula. **In,** Slatter D (Ed): Textbook of small animal surgery. 3rd edn., 1891-1897, Saunders, Philadelphia, 2003.

6. Bozcek-Funcke A, Kuhtz-Buschbeck JP, Illert M: Kinematic analysis of the cat shoulder girdle during treadmill locomotion: An X-ray study. *EJN*, 8, 261-272, 1996. DOI: 10.1186/1746-6148-9-129

7. Piermattei DL, Flo GL, DeCamp CE: Fractures of scapula. **In,** Piermattei DL, Flo GL, DeCamp CE (Eds): Brinker, Piermattei, and Flo's handbook of small animal orthopedics and fracture repair. 4th edn., 255-261, Elsevier, St Louis, 2006.

8. Peck J: Scapula. **In,** Tobias KM, Johnston SA (Eds): Veterinary Surgery Small Animal, Vol. 1., 685-691, Elsevier Saunders, Canada, 2012.

9. Kano WT, Rahal SC, Mesquita LR, Agostinho FS, Faria LG: Gait analysis in a cat with scapular luxation and contralateral forelimb amputation. *Can Vet J*, 54, 990-991, 2013.

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Case Report

Corneal Impression Cytology for the Diagnosis of Limbal Stem Cell Deficiency in a Dog

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Abstract

A 14-year old, castrated, female terrier was referred to Near East Animal Hospital with a complaint of red eye. Ophthalmoscopic examination revealed superficial corneal vascularization originating from conjunctiva and encompassing proximal one-third of cornea in 10-02 o'clock position together with photophobia and vision loss. Schirmer tear test was normal and fluorescein staining was negative for the affected eye. Since combined treatment with topical corticosteroid, antibiotic and cycloplegic did not improve the vascular regression, rather exacerbated the symptoms, blood and serum samples were collected for hematologic, biochemical and serologic analyses in order to investigate possible etiologic factors in human LSCD and to define its association with prevalent local blood disorders. As hematologic, biochemical and serologic test results were within the normal ranges, corneal impression cytology was conducted. Upon the observation of goblet cells the gold standard for the diagnosis of LSCD and the indicative of conjunctivalization in cornea the diagnosed of idiopathic partial limbal stem cell deficiency was confirmed in the dog in which it was the first case with ocular surface defect in veterinary medicine.

Keywords: *Limbal stem cell deficiency (LSCD), Corneal impression cytology, Dog*

Bir Köpekte Limbal Kök Hücre Yetmezliğinin Tanısında Korneal İmpresyon Sitolojisi: Olgu Sunumu

Özet

Yakın Doğu Hayvan Hastanesine kırmızı göz şikayeti ile getirilen 14 yaşlı kısırlaştırılmış, dişi, Terrier ırkı bir köpekte oftalmoskopik muayene sonucu sağ gözde, saat 10-02 pozisyonunda korneanın proksimal 1/3'ünü kaplayan konjunktivadan köken alan korneal yüzeyel vaskülarizasyon, fotofobi ve görmede azalma saptandı. Schirmer göz yaşı testi normal ve florescein boyama negatif olan gözde, damarların regresyonu için uygulanan topical kortikosteroit, antibiotik ve sikloplejik kombinasyonundan olumlu bir sonuç alınamadı ve semptomların artması ve gerilememesi üzerine olgunun kan ve serum örnekleri, insanlardaki olası limbal kök hücre yetmezliği (LKHY) etiyololojik faktörlerini irdelemek ve bölgesel yaygın kan hastalıkları ile ilişkilendirmek amacı ile hematolojik, biyokimyasal ve serolojik analizler için toplandı. Test sonuçları normal sınırlarda olduğu gözlenen olguda korneal impresyon sitolojisi gerçekleştirildi. LKHY tanısında altın standart olan korneada konjunktivalizasyonun göstergesi goblet hücrelerinin gözlenmesi ile veteriner hekimlikte ilk kez oküler yüzey bozukluğu olan bir olguda idiopatik parsiyel LKHY tanısı kondu.

Anahtar sözcükler: *Limbal kök hücre yetmezliği, Korneal impresyon sitoloji, Köpek*

INTRODUCTION

Tunica fibrosa bulbi, outer layer of bulbus oculi consists of sclera and cornea which lacks pigment, vessels and cells. Cornea maintains its transparency with stem cells in limbus that have unlimited proliferation feature. In order to maintain its transparency and integrity, limbal stem cells (LSC) prevent conjunctival invasion of corneal surface. Limbus prevents the growth and migration of conjunctival epithelial cells towards corneal surface and it consists of

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Langerhans cells and melanocytes unlike cornea, and as well as it lacks goblet cell layer unlike conjunctiva [1,2]. Corneal epithelium do not regenerate in limbal stem cell deficiency (LSCD) [3-5] and conjunctivalisation (growth of conjunctival epithelia upon the cornea), vascularization, chronic inflammation, calcification, ulceration or persistent epithelial defects (PED) develop consequently [2,6,7]. In LSCD cases, the corneal surface is covered by conjunctival epithelium and goblet cells are observed in cornea [8]. In humans LSCD results in corneal opacity, vision loss, chronic pain, and photophobia and results in failure of topical medical treatment and/or keratoplasty operations.

Investigations in human LSCD patients yielded the identification of several factors in primary (congenital) etiology of the disease. Aniridia associated with *PAX6* mutations [9], ectrodactyl-ectodermal-dysplasia-clefting syndrome, keratitis-ichthyosis-deafness syndrome, xeroderma pigmentosum, dominant congenital keratitis and dyskeratosis congenita result in primary LSCD. On the other hand, inflammatory features of Steven-Johnsons Syndrome [10], ocular cicatricial pemphigoid [11], graft versus host disease, vernal keratoconjunctivitis [12] are implicated in the etiology of secondary LSCD. Several etiologic factors were defined for human LSCD patients day by day and beside the factors enumerated above, it was shown that neurotrophic keratopathy, bullous keratopathy, radiotherapy and systemic chemotherapy, topical chemotherapeutics, benzalkonium chloride toxicity during medical treatment of glaucoma [13], ocular surface tumors and pterygium [14] result in LSCD as well. Furthermore, chemical-thermal injuries, multiple surgical interventions including limbal region, chronic mechanical microtrauma induced by contact lens and inflammatory diseases of ocular surface are reported to cause LSCD [3,5,6,10,12]. Additionally, diabetes and vitamin A deficiency have a role in the development of LSCD [9]. LSCD develops in albino rats with induced type 2 diabetes mellitus as a result of diabetic keratopathy. Idiopathic LSCD was observed in humans and stem cell loss was shown to originate from direct cell and/or cellular microenvironment damages [2].

Clinical manifestations of LSCD in humans are epiphora, photophobia, vision loss, and erosion of palisades of Vogt observed under slit-lamp biomicroscopy, corneal neovascularization and conjunctivalization. Among the clinical symptoms of human LSCD, stippled late staining pattern with fluorescein stems from the loss of tight junctions between the cells and results in staining of basement membrane. Stippled staining may be observed as swirling around the center and fluorescein stain tends to pool on the conjunctivalised area due to relative thinness of epithelium [3,5,6]. While vision loss and photophobia develop in patients at this stage, precorneal tear film and corneal erosion at variable levels might also be observed. Disarrayed palisades of Vogt and perilimbal vascular archades are reported as early anatomical changes in mild LSCD, but the absence of pallisades of Vogt, which is observed only via slit lamp biomicroscope, may not be a sign of LSCD alone [15].

Observation of PED and superficial vascularization was reported in human cases having mild deficiency, and PEDs yield to scarring, ulceration, stromal neovascularization, corneal thinning and perforation. PEDs cause pain, photophobia and vision loss, and keratinization may develop if tear deficiency accompanies these symptoms.

Total LSCD is characterized by the total loss of LSC population together with conjunctivalization of the entire corneal surface. Neovascularization is also observed frequently but not in all LSCD cases. Similarly, conjunctiva is detected in many disorders such as chemical burns, Stevens-Johnson syndrome, mucous membrane pemphigoid, all of which result in LSCD. Chronic inflammation frequently yields to fibrosis and continues to damage LSCs. Sub-conjunctival fibrosis leads to symblepharon formation and consequently to goblet cell deficiency. Subsequent poor tear function further worsens the ocular surface in such patients [5]. Although clinical diagnosis of LSCD can be established with symptoms mentioned above, some of those may also be observed in other conditions without LSCD component. Especially, symptoms of partial deficiency may remain subtle and subclinical. It is known that impression cytology of the cornea is the gold standard in the diagnosis of LSCD in human medicine [5,10]. This method employs the removal of cells by impressing the cellulose acetate paper over the corneal surface and conduction of cytological analyses to observe goblet cells of which are the signs of the deficiency [16]. The removal of 1-3 cell layers by impressing nitrocellulose acetate filter paper over ocular surface makes only superficial cells available for the analyses. Beside cytology, immuno-histologic and molecular analyses can also be conducted on cells collected on the membrane. Epithelial morphology and goblet cells are evaluated [5,17]. Goblet cells are the sign of conjunctivalization which confirms limbal deficiency. However, these cells may be absent in severe burns and Stevens-Johnson syndrome. It should be kept in mind that absence of goblet cells may yield to false negative results in chemical and thermal damages since 36% of the goblet cells are also damaged in such cases [5,16].

This case report will explore the current challenges and future research directions that will be required to increase our understanding of corneal diseases in dogs and cats and consider the diagnosis of LSCD to veterinary ocular surface patients. Studies have been focused on the human LSCD with little attention being paid to animals. Developing a deep understanding of the limbus and corneal cell turnover in all species is of paramount importance. The successful treatment of LSCDs in humans and animals, and validation of comparative studies between species depends on this knowledge.

CASE HISTORY

A 14-years old, castrated, female, terrier was referred to Near East Animal Hospital with a complaint of red eye. In the ophthalmoscopic examination, corneal surface vascularization encompassing proximal one-third of cornea was observed in 10-02 o'clock position in the right eye together with photophobia, epiphora and vision loss *(Fig. 1)*. Schirmer tear test was normal and corneal fluorescein staining was negative. Dexamethasone (0.1% Onadron®), Ciprofloxacin (0.3% Siprogut®) and a pain killer, Cyclopentonate HCl (1% Sikloplejin®), were administered and the progress was monitored every other day. However, the recovery could not be achieved and symptoms were exacerbated.

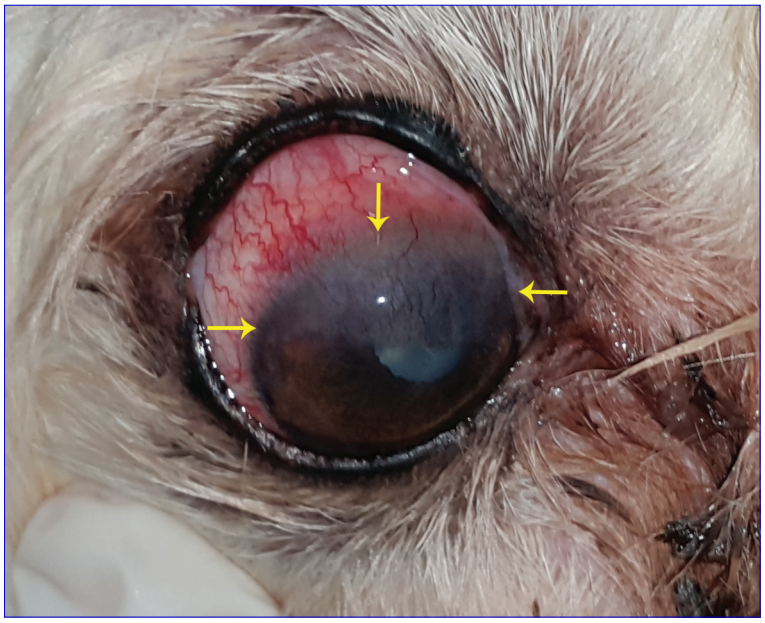

Fig 1. Conjunctivalization observed in 10-02 o'clock position of right eye of the dog and the region where corneal impression cytology was applied *(arrows)*

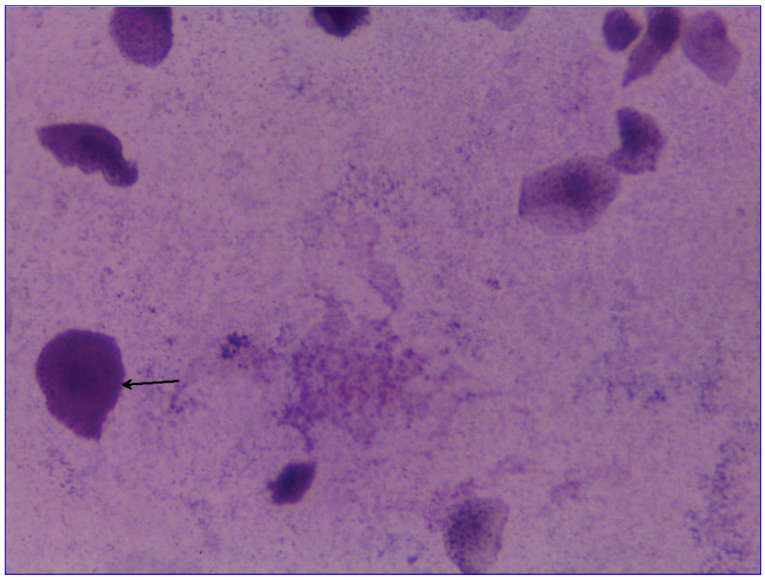

Fig 2. Observation of goblet cells in cornea as a result of corneal impression cytology *(arrow)*

The case was considered to be LSCD; therefore, blood and serum samples were collected in order to conduct hematologic, biochemical and serologic analyses for the evaluation of possible etiologic factors of human LSCD and local prevalent blood diseases (Leishmania, Ehrlichiosis). The haematological parameters were analyzed using an automatic analyzer (BC- 2800Vet, Mindray, Shenzen, China) and white blood cell (WBC) count, red blood cell (RBC) count, Hct, Hb, MCV, MCH, MCHC and RDW were recorded for this study. Serum biochemical analyses were measured by using commercial assay kits (Randox Laboratories Ltd., UK; Mindray Chemistry Reagents, Shenzen, China) and an automated blood chemistry analyzer (BS120, Mindray, Shenzen, China). The serum concentrations of albumin (bromocresol green method, in g/dL), creatinine (jaffe method, in mg/dL), total and direct bilirubin (vanadate oxidase method in mg/dL), phosphorus (P, phospho-molybdate method in mg/dL), glucose (glucose oxidase method in mg/dL), calcium (arsenozo III method in mg/dL), urea (urease method in mg/dL), total cholesterol (cholesterol oxidase-peroxidase method in mg/dL), magnesium (xylidyl method in mg/dL), total protein (biuret method in g/dL), triglycerides (glycerol kinase-peroxidase method in mg/dL), uric acid (uricase peroxidase method in mg/dL) and the enzyme activities of alanine amino-transferase (ALT, IFCC method in U/L), aspartate amino-transferase (AST, IFCC method in U/L), γ-glumatyltrans peptidase (γ-GT, IFCC method in U/L), alkaline phosphatase (ALP, IFCC method in U/L), creatine kinase (CK, IFCC method in U/L), lactate dehydrogenase (LDH, IFCC method in U/L), lipase (enzymatic colorimetric method in U/L) were noted for this study. Immunofluorescence assay (IFA) were performed for the detection of Leishmania infantum and Ehrlichia canis IgG antibodies. Standardized assay kits were used for this purpose which were supplied by MEGACOR Diagnostik GmbH, Austria. The results of hematological, biochemical and serological parameters were within normal limits.

Samples for corneal impression cytology were collected from right eye surface, suspected for partial LSCD, using cellulose acetate filter with 0.20 µm pore diameter under local ophthalmic anesthesia. Opaque surface of cellulose filter papers in 3x5 mm dimensions were placed on cornea using a forceps with a

smooth tip and removed after a soft impression for 5 sec. Collected samples were fixed with 95% ethanol. Flowingly, samples were stained with periodic acid Shiff (PAS) and Hemalun, and evaluated under light microscope. Goblet cells were observed as the indicator of conjunctivalization which is the gold standard of LSCD diagnosis *(Fig 2)*. Autologous limbal stem cell transplantation from healthy left eye to the damaged right eye was considered; however, pet owner did not approve the operation on the geriatric animal.

DISCUSSION

Unlike human medicine, limbal deficiency is defined under ocular surface defects but not further classified in veterinary medicine. However, eye diseases causing ocular surface defects in cats and dogs, such as Uberreiter's syndrome, keratoconjunctivitis sicca, feline corneal necrosis, and feline eosinophilic keratitis, disrupt the transparency of cornea and thus cells and pigments cover the surface of it. It is considered that these features show similarities with primary stem cell deficiency cases of humans. Similarly, corneal alkaline burns, frequently observed in humans, were shown to cause secondary stem cell deficiency in veterinary medicine [18].

In human and veterinary ophthalmology, topical medical treatment predominantly with corticosteroid and/or corneal transplantation, cornea-conjunctival transpositioning, conjunctival pedicle graft applications are routine procedures for the treatment of eyes with corneal transparency loss and without epithelial defect. It is known in human medicine that corneal epithelium cannot regenerate itself in LSCD condition, which consequently results in PEDs, corneal conjunctivalization, neovascularization, corneal scaring and chronic inflammation. This condition negatively influences both medical treatment and also corneal transplantation (keratoplasty) [7,12]. According to Sanchez and Daniels, comparatively to humans, what is known about the healthy limbus and corneal surface physiology of companion animals is still very little. Blinding corneal diseases in animals such as symblepharon in cats with Feline Herpes Virus-1 infections require a basic understanding of the functional companion animal limbus and corneal stem cells [19].

It is considered to evaluate the diseases causing corneal vascularization, conjunctivalization, pigmentation, keratinization, ulceration and erosion, and ultimately to loss of corneal transparency and vision in cats and dogs in terms of LSCD for veterinary medicine. It was reported for conditions in which the connection of corneal epithelial cells with basement membrane was weak, as in Boxer ulcus, events such as positive fluorescein staining, repeating epithelial erosions resulting in PEDs and consequently to corneal perforations may stem from LSCD. On the other hand, the development of fibrovascular pannus and predominant corneal scar formation were reported in cases with severe limbal deficiency [3,6]. Taking these facts into consideration, it was considered that in veterinary ophthalmology, definitive diagnosis of LSCD in cases irresponsive to traditional treatments was crucial and this study aimed to contribute to literature on this field. In human medicine, researches on LSCD contributed several factors of underlying etiology to literature. Similarly, further studies on the etiology of LSCD are highly recommended in veterinary medicine.

In present case, blood and serum samples were collected in order to conduct hematologic, biochemical and serologic analyses for the evaluation of possible etiologic factors of human LSCD and local prevalent blood diseases (Leishmania, Ehrlichiosis) and laboratory parameters were within the normal ranges. In this context, LSCD was diagnosed in a dog for the first time depending on the clinical signs, presence of goblet cells in corneal impression cytology and laboratory parameters.

Beside primary ocular surface disorders that are frequently observed in cats and dogs, that cause corneal vascularization, conjunctivalization, pigmentation, keratinization, ulceration and erosion, and ultimately to loss of corneal transparency and vision, association of ocular surface pathologies with LSCD should also be evaluated in cases of type-2 diabetes in which secondary corneal pathologies may also be observed, and in endemic systemic disorders such as Erchlisiosis and leishmaniosis. It should be kept in mind that the clinical signs of LSCD include epiphora, photophobia, vision loss, corneal vascularization and conjunctivalization, and corneal impression cytology should be performed in cases with ocular surface defect and irresponsive to medical treatment. Final results of cytology should be compared to laboratory analyses.

REFERENCES

1. Dua HS, Gomes JAP: Corneal epithelial wound healing. *Br J Ophthalmol*, 78, 401-408, 1994.

2. Li W, Hayashida Y, Chen YT, Tseng SC: Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res*, 17, 26-36, 2007. DOI: 10.1038/sj.cr.7310137

3. Dua HS, Azuara-Blanco A: Limbal stem cells of the corneal epithelium. *Surv Ophthalmol*, 44, 415-425, 2000. DOI: 10.1016/S0039-6257(00)00109-0

4. Osei-Bempong C, Figueiredo FC, Lako M: The limbal epithelium of the eye - A review of limbal stem cell biology, disease and treatment. *Bioessays*, 35, 211-219, 2013. DOI: 10.1002/bies.201200086

5. Sejpal K, Bakhtiari P, Deng SX: Presentation, diagnosis and management of limbal stem cell deficiency. *Middle East Afr J Ophthalmol*, 20, 5-10, 2013. DOI: 10.4103/0974-9233.106381

6. Dua HS, Azuara-Blanco A: Autologous limbal transplantation in patients with unilateral corneal stem cell deficiency. *Br J Ophthalmol*, 84, 273-278, 2000. DOI: 10.1136/bjo.84.3.273

7. Strungaru MH, Mah D, Chan CC: Focal limbal stem cell deficiency in Turner syndrome: Report of two patients and review of the literature. *Cornea*, 33, 207-209, 2014. DOI: 10.1097/ICO.0000000000000040

8. Dua HS: The conjunctiva in corneal epithelial wound healing. *Br J*

Ophthalmol, 82, 1407-1411, 1998. DOI: 10.1136/bjo.82.12.1407

9. He H, Yiu SC: Stem cell-based therapy for treating limbal stem cells deficiency: A review of different strategies. *Saudi J Ophthalmol*, 28, 188-194, 2014. DOI: 10.1016/j.sjopt.2014.06.003

10. Puangsricharern V, Tseng SC: Cytologic evidence of corneal diseases with limbal stem cell deficiency. *Ophthalmology,* 102, 1476-1485, 1995. DOI: 10.1016/S0161-6420(95)30842-1

11. Tsai RJ, Li LM, Chen JK: Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med*, 343, 86-93, 2000. DOI: 10.1056/NEJM200007133430202

12. Sangwan VS, Jain V, Vemuganti GK, Murthy SI: Vernal kerato-conjunctivitis with limbal stem cell deficiency. *Cornea,* 30, 491-496, 2011. DOI: 10.1097/ICO.0b013e3181cbf9d3

13. Kim BY, Riaz KM, Bakhtiari P, Chan CC, Welder JD, Holland EJ, Basti S, Djalilian AR: Medically reversible limbal stem cell disease: clinical features and management strategies. *Ophthalmology*, 121, 2053-2058, 2014. DOI: 10.1016/j.ophtha.2014.04.025

14. Tseng SC: Staging of conjunctival squamous metaplasia by impression cytology. *Ophthalmology*, 92, 728-733, 1985. DOI: 10.1016/S0161-6420(85)33967-2

15. Zarei-Ghanavati S, Ramirez-Miranda A, Deng SX: Limbal lacuna: A novel limbal structure detected by *in vivo* laser scanning confocal microscopy. *Ophthalmic Surg Lasers Imaging,* 42, e129-31, 2011. DOI: 10.3928/15428877-20111201-07

16. Liang L, Sheha H, Li J, Tseng SC: Limbal stem cell transplantation: new progresses and challenges. *Eye,* 23: 1946–1953, 2009. DOI: 10.1038/eye.2008.379

17. Fatima A, Iftekhar G, Sangwan VS, Vemuganti GK: Ocular surface changes in limbal stem cell deficiency caused by chemical injury: A histologic study of excised pannus from recipients of cultured corneal epithelium. *Eye*, 22, 1161-1167, 2008. DOI: 10.1038/SJ.EYE.6702895

18. Özgencil FE, Sancak İG, Alçığır İG, Ovali E: Comparison of amniotic membrane (AM) transplantation and mesenchymal stem cell (MSC) transplantation in corneal alkaline burn-induced stem cell deficiency in rabbits. *Ankara Üniv Vet Fak Derg*, 61, 99-106, 2014.

19. Sanchez RF, Daniels JT: Mini-review: Limbal stem cells deficiency in companion animals: Time to give something back? *Current Eye Res,* 41, 425-432, 2016. DOI: 10.3109/02713683.2015.1056801

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Letter to the Editor

Bladder Retroflexion in a Pointer Bitch During Pregnancy (Pointer Irkı Bir Köpekte Gebelik Sürecinde Karşılaşılan İdrar Kesesi Retrofleksiyonu)

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Dear Editor,

We would like to report a rare case of bladder retroflexion during pregnancy in a bitch.

A 4-year-old pregnant Pointer bitch was brought to Clinic of Obstetrics and Gynaecology Department, Faculty of Veterinary Medicine, Afyon Kocatepe University with the complaint of vaginal mass and dysuria. In patient's history, it was learnt that the bitch was mated 50 days ago. Owner also stated that the dog experienced a recent abdominal blunt trauma. On clinical examination, a protruded vaginal mass was observed *(Fig. 1a)*. In ultrasonography, a large amount of anechoic image was seen in the prolapsed tissue. The abdominal ultrasonography revealed that the dog was pregnant but two fetuses were not viable. In addition, urinary bladder was not in normal position. Then it was decided to carry out ovariohysterectomy and to reduce the retroflexed bladder into normal position concurrently. Within two days after surgical intervention, the urinary bladder was retroflexed once again *(Fig. 1b)*. A positive contrast cystourethrography was performed to reveal the bladder position *(Fig. 1c)*. In the second surgery, cystopexy of the retroflexed urinary bladder to the right abdominal wall was performed *(Fig. 1d)*. Ten days after the second surgery, the skin sutures were removed and no complications were observed *(Fig. 2)*. After ten months following second surgery recurrence of the retroflexion was not detected.

Retroflexion of the urinary bladder has been documented in the first stage of parturation in a bitch [1], postpartum three weeks in a cat [2], in the neutered bitch [3] and presence of follicular cyst in the ovary in a bitch [4]. Our literature search revealed that, there is one report of bladder retroflexion related to pregnancy and continuous barking [5]. However, our case differs from the study of Sontas et al.[5] since the retroflexion was associated with abdominal trauma leading to increased intra-abdominal pressure. One paper [6] describes urinary bladder retroflexion in two male dogs with a history of traffic accident. However, in our case the bladder retroflexion occured in the bitch by blunt trauma.

To our knowledge, this case was the first report in the literature describing an increased intra-abdominal pressure related to abdominal trauma in a pregnant bitch. As a result, urinary bladder retroflexion must be correctly evaluated in pregnant bitch and cystopexy can be considered as one of the choice to prevent recurrence.

REFERENCES

1. Canatan HE, Ergin I, Polat IM, Yazlık MO: Unusual cases of vaginal prolapse concurrent with cystocele in two dogs. *Revue Med Vet*, 166 (1-2): 43-46, 2015.

2. Risselada M, Kramer M, Van De Velde B, Polis I, Görtz K: Retroflexion of the urinary bladder associated with a perineal hernia in a female cat. *J Small Anim Pract,* 44, 508-510, 2003. DOI: 10.1111/j.1748-5827.2003.tb00112.x

3. Niles JD, Williams JM: Perineal hernia with bladder retroflexion in a female Cocker Spaniel. *J Small Anim Pract,* 40, 92-94, 1999. DOI: 10.1111/j.1748-5827.1999.tb03045.x

4. Ajadi TA, Ajayi OL, Adeniyi AA, Ajadi RA: Vaginal wall hyperplasia with fold prolapse in an eight-month-old Cane Corso dog. *Bulg J Vet Med,* 19 (3): 252-258, 2016. DOI: 10.15547/bjvm.917

5. Sontas BH, Apaydin SO, Toydemir TS, Kasikci G, Ekici H: Perineal

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hernia because of retroflexion of the urinary bladder in a rottweiler bitch during pregnancy. *J Small Anim Pract*, 49, 421-425, 2008. DOI: 10.1111/j.1748-5827.2008.00553.x

6. Lee H, Kang H, Kim M-S, Kim N, Lee K: Radiographic features of traumatically caused retroflexion of urinary bladder in two dogs. *J Vet Clin*, 25 (6): 553-556, 2008.

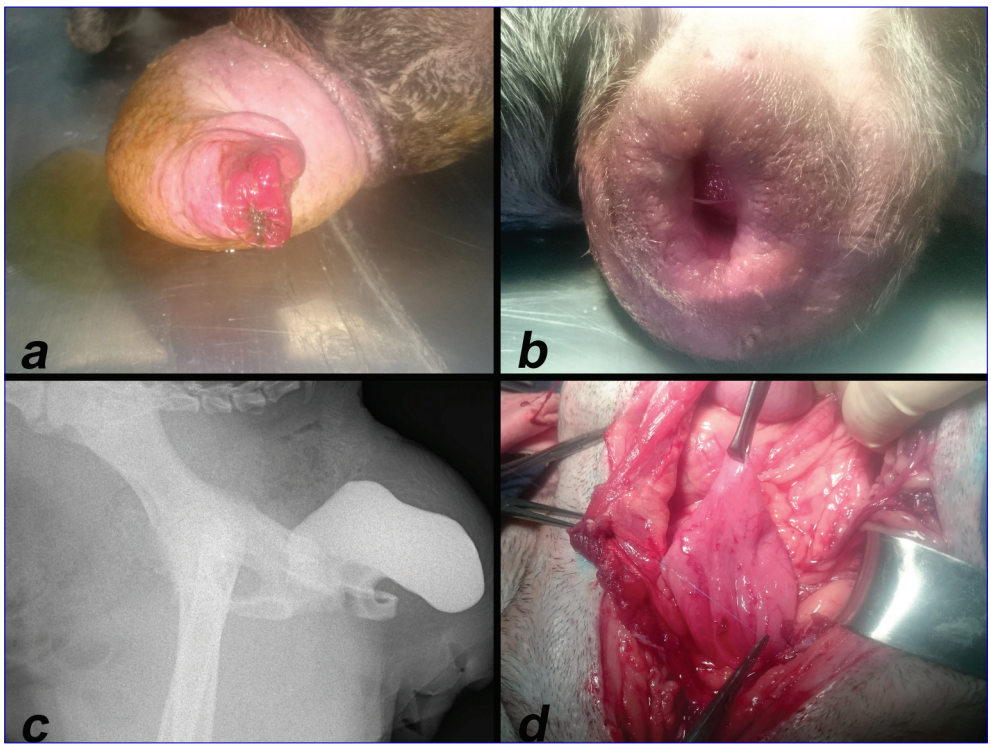

Fig 1. a- Prolapsed vagina in the pregnant Pointer bitch before the first surgery, **b**- Recurrence of the retroflexion before the second surgery, **c**- Positive contrast cystourethrogram (latero-lateral projection) **d**- Cystopexy

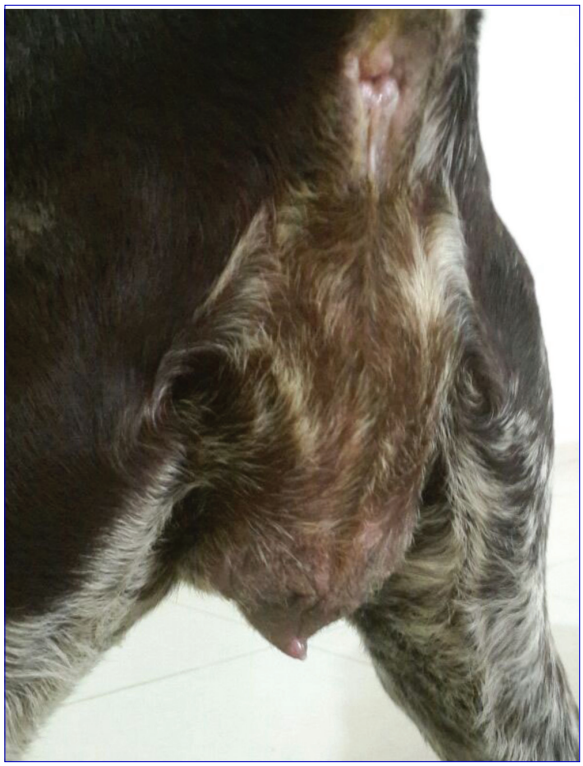

Fig 2. Ten days after the second surgery, full recovery was observed

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