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RESEARCH ARTICLE

Omega-3 Fatty Acids Enriched Flaxseed Oil Effects on Meat Quality and Fatty Acid Profile of Broiler Chicks

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ABSTRACT

The study was planned to evaluate effect of adding flaxseed oil on meat quality, sensory attributes and fatty acids profile of broiler chicken meat. Further, optimization of supplementalflaxseed oil duration and level was also tested. A n=300 day-old broiler chicken (Ross-308) were procured from the hatchery and randomly divided into 5 treatments, T0 (control) T1 and T2 (3% and 4% flaxseed oil, 14-35 day), while T3 and T4 having (3% and 4% flaxseed oil, 21-35 day) groups respectively. Each treatment contained 6 replicates with 10 chicks per replicate. The T3 tested diet linearly raised n-3 (PUFA) contents. The n-6/n-3 ratio was also significantly lowered from 7.72 to 1.12%. Regardless of dietary flaxseed oil level, LC-PUFA contents were higher in the T2 group. Meat quality traits were not statistically affected while T1 exhibited a significantly increased meat tenderness value, but interestingly, all other sensory attributes remained statistically unaffected. An equivalent amount of n-3 LC-PUFA may be found in 3% dietary flaxseed oil for the last 21 days of rearing with optimized meat quality and sensory attributes. Healthy broiler meat enriched with n-3 fatty acids may be produced by dietary manipulation.

Keywords: Flaxseed oil, n-3 LC-PUFA, Meat quality, Sensory attributes, Broiler meat

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INTRODUCTION

The health benefits of dietary polyunsaturated fatty acids (PUFA) are ubiquitously recognized globally as they play a significant role in the development and maintenance of the human body. The empirical scientific studies indicate that proper intake of Omega-3 and Omega-6 PUFA or with a proper ratio of (n-6/n-3 PUFA) may generally inhibit the progression of various diseases but particularly control diabetes, coronary heart disease, rheumatoid arthritis and nervous system problems [1]. The World Health Organization (WHO) has also observed that Omega-3 long-chain PUFA may reduce cardiovascular diseases [2]. Omega-3 PUFA consumption also reduces plasma triglycerides, blood pressure, and resting heart rate and might also improve myocardial efficiency, lower inflammation, and improve vascular function. The content of Omega-3 PUFA has a significantly positive impact on public health nutrition, but unfortunately, their intake is still scarce for many reasons.

Marine products are the best source of Omega-3 fatty acids and are consumed predominantly in a few areas of the world [3]. However, their dietary inclusion is also rare due to their seasonal availability, consumer preferences and affordability worldwide. This scenario has compelled the scientific community to search for alternative sources for Omega-3 fatty acids, which may successfully serve the purpose. Chicken meat is the most consumed protein source in the world, and efforts are being made to produce broiler chicken meat containing high levels of omega-3 LC PUFA, particularly (C20:5 n-3, EPA) eicosapentaenoic and (C22:6 n-3, DHA) docosahexaenoic acid by the inclusion of natural flaxseed oil [4]. Although fish oil is the primary source of these fatty acids, its utilization is restricted in the broiler diet due to meat's high cost and poor sensory attributes. Furthermore, it was reported that dietary fish oil has some adverse effects on the growth performance of chicken and drip loss% of meat, making it less acceptable for processors and consumers [5].

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Flaxseed oil is the most valuable common plant-based feed ingredient to enrich n-3 fatty acid poultry products. The flaxseed contains an ample quantity of alpha linolenic acid (ALA) (>50%) and fat (~40-42%) contents along with other nutritional properties (high protein and metabolisable energy content) [6]. However, its high inclusion is also inhibited in poultry feed due to the high cost and presence of anti-nutritional factors such as mucilage, linatinedi-peptide, cyanogenic glycosides, phytic acid and trypsin inhibitor [7]. So, the rational utilization of flaxseed may show promising results on bird growth and may economize bird feed [3,8].

Therefore, the present study was planned firstly to evaluate the two levels of flaxseed oil for different feeding durations as a way to improve the functional value of chicken meat on meat quality, coloring index and fatty acid profile, especially the n-3 LC-PUFA contents and sensory analysis of broiler chicken meat: and Secondly to verify that how long dietary manipulation is suitable to achieve high quality nutritionally enriched chicken meat for human consumption.

Material and Methods

Ethical Approval

All the experimental procedures followed the Animal Care and Use Committee (dr/1376: 06-05-2019) Department of Poultry Production, University of Veterinary and Animal Sciences, Lahore, Pakistan.

Experimental Plan, Management and Slaughtering

A total of 300-day-old broiler chickens unsexed (Ross-308) were randomly distributed into 5 experimental groups according to a completely randomized design (each group contained 6 replicates and 10 broiler chickens per group) for 35 days. Five *isonitrogenous* and *isocaloric* diets were prepared in mash form *(Table 1)*. The experimental room was properly fumigated and disinfected before the arrival of broiler chickens. All these were raised under standard management conditions. A photoperiod of 23 h of light and 1 h of darkness was provided. Further, a temperature of 35-32°C was provided during the first week of rearing, which was gradually reduced to 5°C until 21°C. According to the schedule, birds were vaccined for Newcastle disease, infectious bursal disease and infectious bronchitis.

The diets were enriched with the two flaxseed oil levels and fed in two different durations to evaluate effective level of flaxseed. The T_0 was the control diet, but T_1 and T_2 basal diets contained 3% and 4% flaxseed oil provided from 14-35 days of production, while T_3 and T_4 were enriched with previously mentioned concentrations but fed from 21-35 days of production. Further, recipes and chemical composition of all the experimental diets have been shown in *Table 1* and formulated based on the recommendation [9].

Table 1. *Ingredients and chemical composition of the diets*

Ingredients%	Control[1]	Flaxseed Oil[2]	
		3%	4%
Corn grain	58.49	59.50	56.00
Wheat bran	3.00	2.30	4.70
Canola meal	4.77	4.76	4.86
Corn gluten 60%	1.00	1.00	1.00
Soybean meal	24.0	24.0	24.0
Soybean oil	3.30	00	0
Flaxseed oil	00	3.00	4.00
$CaCO_3$	1.10	1.10	1.10
Dicalcium phosphate	1.80	1.80	1.80
Lysine-SO_4	0.40	0.40	0.40
DL-methionine	0.16	0.16	0.16
Threonine	0.98	0.98	0.98
Ajinomot	0.10	0.10	0.10
NaCl	0.30	0.30	0.30
$NaHCO_3$	0.10	0.10	0.10
Vitamin premix	0.30	0.30	0.30
Minerals premix	0.20	0.20	0.20
Total	100	100	100
Calculated Composition, %			
Protein	19.3	19.3	19.3
Metabolizeable energy (MJ/Kg)	2988	2979	2985
Ether extract	6.03	5.70	6.70
Ash	6.10	6.08	6.10
Crude fiber	3.90	3.90	4.10
Calcium	0.91	0.91	0.91
Available-phosphorus	0.42	0.42	0.42

[1] *Diet: T_0: Control group*
[2] *Diets containing 3 and 4% flaxseed oil fed either during the last 21 days of production (T_1 and T_2, respectively) or during the last 14 days of production (T_3 and T_4, respectively)*

At the end of the trial, birds were slaughtered according to the local standard for Halal slaughtering (PS 3733:2016) in compliance with the Ethical Review Committee's institutional guidelines, UVAS (dr/1380: 13/06/ 2019). The birds were slaughtered on 35 days of production at the Meat Science and Technology department's meat processing plant, UVAS. The carcasses were subjected to an ice-water bath for 30 min, packed in a polystyrene tray, wrapped with cling film, and finally placed in a chiller at 0-4°C for 4 h before deboning.

pH

After 24 h of slaughtering, the pH was measured from breast muscle *(Pectoralis major)* for all the treatments of each group using a calibrated portable pH meter (WTW, pH 3210 SET 2, Germany) with a meat penetrating probe (WTW, SenTix® Sp, pH electrode, Germany) as described by [10]. The pH meter was calibrated on 4.0, 7.0 and 10.0 buffer solutions at the start of eachtrial.

Color

The color values were measured from breast fillets *(Pectoralis major)* with the help of a chroma meter (Konica Minolta® CR-410, Japan) 50 mm port size and D65 illuminant [10]. The chroma meter was calibrated using a standard white tile (L* 94.93, a* 0.13, b* 2.55) and color scale [11]. The color parameters were comprised of lightness (L*), redness (b*), yellowness (a*), chrome (c) and hue angle (h). After deboning, breast fillets were placed in food-grade polystyrene trays, wrapped with a commercially available 250 mm thick cling film and stored in a chiller (Model: S80100VVC, Tecnodom S.P.A., Vigodarzere, PD, Italy) at 0-4°C. Color values were taken from the center of the breast muscle, avoiding the visible fascia.

Cooking Loss (%)

The samples from each treatment were weighed with the help of an electronic weighing balance (SF-400, capacity 7000±1 g, China) and vacuum-packed in plastic bags using a C300 twin vacuum packer (Multivac®, Ltd., Serial no. 219528, Germany) in plastic bags (SR 150×200, PA/PE 90). The samples were cooked in a water bath (Memmert, WNB45, Germany), operating at 80°C to determine the cooking loss. The samples were cooked until the internal core temperature reached 72°C. The cooked meat temperature was recorded with the help of a thermometer (TP101, CixiSinco, China, -50°C to 300°C) [12]. The samples were cooled down to room temperature and again weighed to calculate the cooking loss by the following formula:

% Cooking Loss = $(W_1\text{-}W_2/W_1) \times 100$

W_1 = weight of meat before cooking

W_2 = weight of meat after cooking

Drip Loss (%)

After the deboning, breast fillets were randomly selected from each treatment to calculate the drip loss percentage. The drip loss percentage was calculated using the suspension method as described [13]. A digital compact weighing balance (SF-400, 7000±1 g) was used to weigh the samples. The drip loss percentage was calculated by the formula given below

% Drip Loss = $(W_1 - W_2/W_1) \times 100$

W_1 = weight of meat before the suspension

W_2 = weight of meat after suspension (24 h)

Warner-Bratzler Shear Force Value

The texture analysis was performed with the help of a texture meter (TA.XT plus®, Stable Micro System, Ltd., Surrey, UK, Serial no. 41851) fitted and calibrated with a 5 kg load cell. From the cooked breast filet samples, stripes of the meat were obtained and the fillets were cut down in a direction parallel to the muscle fiber orientation (2×1×1 cm^3) with scalpel blades [14]. The breast fillets were placed in polystyrene trays and cooled at 0-4°C in a display chiller (ALVO, Model MD-12, Technosight, 72″ × 42″ × 48). A minimum of 3 values for the shear force were recorded from each sample and the force required to cut the muscle fiber was N/cm^2.

Fatty Acid Analysis

The breast muscle *(Pectoralis major)* samples were randomly selected from each treatment group to extract and measure the lipid contents to determine fatty acids. Total lipid contents were extracted according to the method [15]. A finely minced meat sample of 50 g was added to 400 mL of organic solvent chloroform/methanol (v/v 2:1). The solvent and the minced meat sample were homogenized (6000 rpm, 2 times, 30 sec each time) using a homogenizer (DAIHAN Scientific, HG-15D-Set-A, South Korea). The homogenate was filtered and shaken by adding a 0.2 mL volume of 0.9% NaCl solution. The mixture was centrifuged (Eppendorf, 5810, Germany) at a low speed (3000 rpm, 15 min) to separate the two phases. A rotatory evaporator separated the lower organic phase from the upper layer (Daihan Scientific, WEV-1001L, 25W, South Korea).

The extracted lipid contents were methylated as fatty acids methyl esters (FAME) by gas chromatography (Agilent Technologies, GC System 7890B, USA) to determine the fatty acid quantity. Each fatty acid was identified as a methyl ester by comparing the retention time with a standard.

Sensory Analysis

The Sensory analysis of poultry breast fillets was performed at the sensory analysis lab, Central Laboratory Complex (CLC), UVAS, by a trained panel of 20 judges [16]. Before starting the sensory trial, all the panelists were aware of the experiment. After opening the tray, samples from each treatment were cooked without salt and spices on a hot plate until they attained the core temperature of 72°C. The core temperature was recorded using a digital food-grade thermometer (TP101, CixiSinco, China, -50°C to 300°C). Each specimen was sub divided into uniform parts to

serve panelists in warm conditions. All the samples were coded and served to the panel and in between subsequent samples, the panelists had a facility to rinse their mouths to remove any carry-over effect. The panelists evaluated the samples for odor, tenderness, juiciness, oiliness, flavor, overall acceptability and shallowness on a 9-point hedonic scale.

Statistical Analysis

The collected datawere subjected to the One-way analysis of variance (ANOVA) technique. The means were compared using Duncan's multiple range test with the help of SAS 9.1.The means were considered significantly different at P≤0.05 [17].

Results

Meat Quality Parameters

Table 2 shows the results of meat quality parameters, including colors (redness, yellowness, lightness, chroma and hue). The pH, cooking loss, tenderness and drip loss have been shown in *Table 3*. The diets enriched with flaxseed oil exhibited non-significant change (P>0.05) in meat pH values among the treatments during both dietary durations. The meat quality parameters, including color, cooking and drip loss percentage, and shear force, were closely linked to the pH of meat. Consequently, no potential statistically significant difference was observed among them. Moreover, flaxseed oil's supplemental level and duration exhibited no negative impact on broiler meat quality parameters.

Results of Fatty Acids Analysis

The fatty acid composition of broiler breast meat is shown in *Table 4* and *Table 5*. The crude fat (CF) percentage among all the dietary treatments was statistically non-significant. The supplementation of flaxseed oil showed

Table 2. *Effect of varying dietary flax seed oil levels on the color values of breast fillets*

Treatment[1]	a* (Redness)	b* (Yellowness)	L* (Lightness)	C (Chroma)	h (Hue)
T_0	14.6	19.5	56.4	24.4	53.0
T_1	16.3	19.7	54.9	25.7	51.8
T_2	13.9	19.5	55.7	25.3	50.8
T_3	15.1	19.9	55.4	25.2	52.4
T_4	14.9	18.5	56.7	23.9	50.9
SEM	0.553	0.211	0.337	0.229	0.500
P-Value	0.63	0.62	0.70	0.34	0.76

[1] *T_0 = Control group, T_1 and T_2 = 3 and 4% flaxseed oil, respectively, fed during the last 21 days of production, while T_3, and T_4 contained 3 and 4% flaxseed oil, respectively, fed during the last 14 days of production*
Different alphabets on means showing significant differences (P≤0.05) among treatments

Table 3. *Effect of varying dietary flax seed oil levels on the meat quality*

Treatment[1]	pH	Drip loss %	Cooking loss %	Tenderness
T_0	5.93	4.85	11.9	19.4
T_1	6.04	4.87	12.1	19.4
T_2	5.99	5.15	11.1	19.7
T_3	6.01	4.55	11.4	19.6
T_4	5.94	5.11	11.6	20.0
SEM	0.013	0.205	0.142	0.218
P-Value	0.15	0.88	0.46	0.97

[1] *T_0 = Control group, T_1 and T_2 = 3 and 4% flaxseed oil, respectively, fed during the last 21 days of production, while T_3, and T_4 contained 3 and 4% flaxseed oil, respectively, fed during the last 14 days of production*
Different alphabets on means showing significant differences (P≤0.05) among treatments

Table 4. *Effect of varying dietary flax seed oil levels on fatty acid[1] profiles of breast fillets*

Treatment[2]	Crude fat	SFA	MUFA	PUFA	n-6 PUFA	n-3 PUFA	n-6/n-3
T_0	1.77	35.4^a	42.9^a	21.8^e	19.3^a	2.50^e	7.72^a
T_1	1.83	32.8^b	39.8^d	27.4^b	15.4^c	11.9^b	1.29^b
T_2	1.83	31.9^c	39.5^d	28.7^b	15.2^d	13.5^a	1.12^b
T_3	1.80	32.7^b	41.6^b	25.7^a	15.8^b	9.87^d	1.60^b
T_4	1.87	32.8^b	40.6^c	26.6^d	15.3^d	11.3^c	1.36^b
SEM	0.016	0.047	0.076	0.045^c	0.024	0.045	0.092
P-Value	0.48	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] *SFA Saturated fatty acids; MUFA Monounsaturated fatty acids; PUFA Polyunsaturated fatty acids; n-6 PUFA polyunsaturated fatty acid; n-3 PUFA n-3 polyunsaturated fatty acid; n-6/n-3 Ratio of polyunsaturated fatty acid*
[2] *T_0 = Control group, T_1 and T_2 = 3 and 4% flaxseed oil, respectively, fed during the last 21 days of production, while T_3, and T_4 contained 3 and 4% flaxseed oil, respectively, fed during the last 14 days of production*
Different alphabets on means showing significant differences (P≤0.05) among treatments

Table 5. *Effect of varying dietary flax seed oil levels on fatty acid profiles of breast fillets*

Treatment[1]	ALA	n-3 LCPUFA	EPA	DPA	DHA
T_0	1.77^d	0.73^c	0.27^c	0.47^c	Trace
T_1	8.60^b	3.37^a	1.13^a	1.47^a	0.77^a
T_2	10.1^a	3.37^a	1.10^a	1.50^a	0.77^a
T_3	7.60^c	2.27^b	0.73^b	1.10^b	0.43^b
T_4	8.90^b	2.37^b	0.77^b	1.13^b	0.47^b
SEM	0.032	0.024	0.016	0.008	0.018
P-Value	<0.001	<0.001	<0.001	<0.001	<0.001

[1] *T_0 = Control group, T_1 and T_2 = 3 and 4% flaxseed oil, respectively, fed during the last 21 days of production, while T_3, and T_4 contained 3 and 4% flaxseed oil, respectively, fed during the last 14 days of production*
Different alphabets on means showing significant differences (P≤0.05) among treatments

Table 6. *Effect of varying dietary flax seed oil levels on sensory analysis of breast fillets*

Treatment[1]	Odor	Tenderness	Juiciness	Oiliness	Mouth Feel	Swallowness	Overall Acceptability
T_0	7.80	8.00^{ab}	7.50	7.10	7.60	8.10	7.80
T1	7.80	8.20^{a}	7.30	7.50	7.50	7.50	8.10
T_2	7.70	7.40^{ab}	7.80	7.10	7.20	7.30	7.20
T_3	7.70	7.70^{ab}	7.60	6.90	8.20	7.70	7.90
T_4	7.90	6.70^{b}	7.20	7.20	7.60	7.00	7.00
SEM	0.087	0.124	0.118	0.121	0.147	0.158	0.103
P-value	0.98	0.03	0.68	0.82	0.46	0.52	0.07

[1] *T_0 = Control group, T_1 and T_2 = 3 and 4% flaxseed oil, respectively, fed during the last 21 days of production, while T_3 and T_4 contained 3 and 4% flaxseed oil, respectively, fed during the last 14 days of production*
Different alphabets on means showing significant differences (P≤0.05) among treatments

no effect on the fat contents but only improved the fatty acids contents. The maximum level of saturated fatty acids (SFA) was observed in the control group. However, the SFA content was reduced with the increased dietary flaxseed oil. A linear relationship was found in the amount of PUFA contents in the chicken meat as the supplemental level and duration increased. It was found that adding flaxseed oil as a source of ALA linearly increased the Omega-3 PUFA contents and reduced Omega-6 PUFA contents, ultimately decreasing the Omega-6/3 PUFA ratios to 7.72 and 1.12 for the control and T_2 groups, respectively. The Omega-3 LC-PUFA contents were significantly different from each other.

The ALA contents in broiler breast meat were associated with the supplemental level of dietary flaxseed oil, as it deposits directly into the muscles. The highest level of Omega-3 LC-PUFA was observed in the T_1 and T_2 treatment groups as they were supplemented for 3 weeks before slaughtering *(Table 5)*.

Sensory Analysis Findings

The sensory interpretation showed a non-significant difference among all the treatments *(Table 6)*. However, tenderness was the only attribute having the least acceptance with a 4% dietary flaxseed oil treatment supplemented for 14 days. While observing odor, juiciness, oiliness, flavor, swallow and overall acceptability, the study showed no notable difference between the treatments.

Discussion

The current study highlights the effects of feeding varying flaxseed oil (Omega-3 PUFA) on, meat quality, coloring index, sensory attributes and fatty acids profile, especially the Omega-3 LC-PUFA contents of broiler meat. The broiler breast fillets' pH values showed a non-significant difference among all the treatments. Our results aligned with Lee et al.[18], who found statistically unchanged effect of feeding flaxseed oils on the egg albumen pH. Moreover, the quail's breast meat pH remained statistically unaffected when the birds were subjected to flaxseed oil containing diet and this finding correlateswith our results [19]. The findings related to the meat color, including lightness value (L*), correspond with the muscle biochemistry, as described by [11]. As all the dietary treatments pH values remained non-significant, the color values also showed non-significant results, including redness (a*) and yellowness (b*) values. The linear correlation in pH and redness (a*) values were present as a higher pH value shows higher redness (a*) value, as reported by El Rammouz [20]. Further, Qiao et al.[21] and Ribeiro et al.[22] described the relationship between the pH and yellowness (b*) values of meat. They stated that with the decline in the meat pH, the yellowness (b*) values increase, as observed in the current trial.

The non-significant results were recorded in cooking and drip loss percentage among all the treatments as these parameters are also associated with the pH. The effect of the pH value on the drip and cooking losses percentage was described by Ribeiro et al.[22], indicating that lower pH values may lead to more drip and cooking loss and vice versa.

Factually, meat tenderness is perceived as the most important quality factor determining the consumer's ultimate satisfaction. The data regarding the tenderness values illustrated a non-significant difference among various treatments and these findings justified the relationship between the tenderness and meat pH. Similar results were reported by Wang et al.[23] in which birds were supplemented with lipid and exhibited no adverse effect on the cooking loss % and meat tenderness. Moreover, our results correlate with the observation of Reddy et al.[24], who recorded significantly higher tenderness values

of all the treatments and remained non-significant with different dietary regimes.

Significant work has been accomplished to include dietary fish meals and oil to enrich broiler meat with omega-3 fatty acids. Though, the inclusion of these sources in the ration had adversely affected the sensory attributes of meat [25]. Sensory attributes of a product are vital as they directly affect the consumer's liking. Flax seed and flax seed oil has a high dietary ALA level and may be used as a replacement for similar marine products. In addition, replacing fish oil with vegetable sources rich in Omega-3 PUFA may significantly improve the meat's sensory attributes [3].

In this study, a high level of flaxseed oil rich in ALA did not affect broiler meat's overall sensory attributes. These results agree with Fletcher [26] also concluded the perpetual effects of dietary Omega-3 PUFA on broiler meat's sensory attributes. Similarly, López-Ferrer et al.[27] used 10 and 17% flaxseed as a dietary ALA source for 8 various durations in broilers. The results showed that high Omega-3 PUFA levels up to 20 days posed no unfavorable effect on chicken meat's sensory attributes, texture, flavor, after taste, liking and overall opinion. Further, meat tenderness was the only conflicting attribute affected by the increased long-duration supplementation of Omega-3 PUFA.

Furthermore, Qiao et al.[21] and Zelenka et al.[28] evaluated the relationship between dietary Omega-3 PUFA and sensory attributes of broiler meat and found variable results. They observed that a higher level of n-3 PUFA did not affect the breast fillet's tenderness, texture and juiciness. However, the odor, taste, oily taste, and after-feel were the attributes that the higher dietary Omega-3 PUFA levels compromised. Moreover, linoleic acid (LA) should also be incorporated into the diet to produce finely fibrous, juicy and enhanced flavored meat. Gonzalez-Esquerraand Leeson [29] reported that the high dietary ALA compromised sensory attributes like aroma and after taste; the flavor was also influenced due to the production of volatile compounds that mediates the off-flavor production. Further, this might be associated with the level of Omega-3 PUFA contents, thermal degradation of lipids and the level of volatile compounds in the meat.

The crude fat (CF) percentage remained unchanged by the duration of the dietary addition of flaxseed oil which shows that the diet containing a higher level of ALA does not affect the fat contents of meat; nonetheless, it only modifies the fatty acids profile. Diets containing high ALA levels increased the n-3 PUFA contents of broiler breast meat and some of its fractions were also converted into biologically more active fatty acids, n-3 LC-PUFA [28].

The maximum level of Omega-3 LC-PUFA was determined in the birds fed with 3 and 4% dietary flaxseed oil for 21 days before slaughtering and processing; however, no difference was found in converting dietary ALA into Omega-3 LC-PUFA between T_1 and T_2 treatment. Duringthe last two weeks of dietary flaxseed oil supplementation, i.e, 3 and 4% T_1 and T_2, the level has shown a similar trend, as the conversion rate was found to be equal between T_3 and T_4. This indicates that supplementation duration is more important to convert the dietary ALA into biologically beneficial Omega n-3 LC-PUFA rather than the level of dietary flaxseed oil.

Arnesen et al.[3] reported that 4-week feeding of dietary flaxseed oil yielded 4.7%n-3 LC-PUFA in broilers. The author further added that the duration of supplementation of flaxseed oil for a 2 to 4 wk period effectively enriches broiler meat with Omega n-3 LC-PUFA compared to 6 wk duration. This might be because elongation enzymes like elongase and desaturase for the synthesis and incorporation of long-chain fatty acids into breast fillet's phospholipid fractions. The flaxseed oil supplementation of T_3 and T_4 (3 and 4%) during the last 2 wk before slaughtering was inadequate for the optimal conversion of ALA into n-3 LC-PUFA.

Broiler diets were enriched with 2.5 and 5% flaxseed oil during the starter and finisher stage of production and it was observed that 5% dietary flaxseed oil for 2.5 wk feeding optimized the Omega n-3 LCPUFA contents of broiler meat [30]. However, in the present study, the n-6 to n-3 PUFA ratio of broiler breast fillet was reduced with 2 wk supplementation, i.e. 1.60 and 7.72% of total fatty acids. Moreover, Fletcher [26] and Gonzalez-Esquerra and Leeson [29] also reported similar findings by concluding that 10% flaxseed oil may increase ALA and n-3 PUFA contents but seems unable to increase the Omega n-3 LC-PUFA content of broiler chicken meat.

Dietary manipulations showed that Omega3 PUFA/LC-PUFA enriched broiler meat might be produced. It is viable to effectively reduce the supplement level and duration of flaxseed oil in the broiler ration. Dietary flaxseed oil did not affect the sensory and quality parameters of chicken meat. Replacement of conventional fat sources with flaxseed oil may reduce the Omega-6 to Omega-3 PUFA ratio and increase the overall Omega-3 LC-PUFA contents in meat. Dietary flaxseed oil linearly increased Omega-3 PUFA content and significantly lowered the n-6/n-3 ratio in broiler meat. Furthermore, regardless of the dietary flaxseed oil level inclusion, Omega-3 LC-PUFA contents were observed to be higher in groups with a supplementation duration of 21 days before processing without significant difference in meat quality and sensory analysis parameters except for the tenderness value. The producer and consumer will accept the economical production of omega-3 enriched broiler meat with optimal sensory attributes and meat quality.

The study revealed that meat quality traits were not statistically affected while T_1 exhibited a significantly increased meat tenderness value. The sensory attributes remained statically unchanged. Similarly, amount of n-3 LC-PUFA may be found with 3% dietary flaxseed oil for the last 21 days of rearing with optimized meat quality and sensory attributes. Therefore, it is recommended that healthy broiler meat enriched with n-3 fatty acids may be produced by dietary manipulation.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author (S. Ali) at the University of Veterinary and Animal Sciences, Lahore, Pakistan.

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Competing Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication

Authors Contribution

S. A. and M. H. designed, planned and drafted the experiment and converted into the manuscript. N. A., conducted and collected the data. B. A., J. N. analysed the data. R. M. B., K. N. and I. B. performed interpretation of data, conception and reviewed the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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Research Article

The Efficacy of Thyme, Peppermint, Eucalyptus Essential Oils, and Nanoparticle Ozone on Nosemosis in Honey Bees

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ABSTRACT

Nosemosis is an intracellular fungus that seriously affects honeybees' health globally. This study aimed to evaluate and compare the efficacy of thyme, peppermint, eucalyptus essential oils, and nanoparticle ozone applied by spray on the frames against Nosemosis in adult honey bees under field conditions. Five treatment groups and two (negative and positive) control groups were assigned for this study. In treatment groups; the 3% solutions of thyme, peppermint, and eucalyptus, and the 1.000 and 2.000 ppm solutions of nanoparticle ozone, were applied four times each week, 125 mL per hive (500 mL solution in total). Average *Nosema* spore counts before and after the treatment were calculated on a Neubauer hemocytometer slide by the digestion method, and the percent reduction test determined the efficacy of the combinations. According to the results, the highest reduction in the number of *Nosema* spores was observed in the thyme essential oil group at 84%. Peppermint essential oil, eucalyptus essential oil, and 1.000 and 2.000 ppm nanoparticle ozone efficacies were found at 77.45%, 76.10%, 72.41%, and 71.21%, respectively. Findings from this study revealed that essential oils and nanoparticle ozone can reduce the *Nosema* spore load to a point under field conditions. Plant extracts would offer a non-antibiotic alternative for *Nosema* control and further studies of herbal extracts are required as potential *Nosema* control agents in honey bees.

Keywords: Honey bee, Essential oil, Eucalyptus, Peppermint, Thyme, Nanoparticle ozone, Nosemosis

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Introduction

Pollinator insects present essential contributions to agricultural production and the ecosystem. Among the insects, managed bees are known as the most important pollinators of crops worldwide. In addition, honey bees offer essential products such as honey, pollen, propolis, royal jelly, and bee venom [1-3]. In the USA, pollination provides an annual economic contribution of 16 billion dollars, and 12 billion dollars of this economy is obtained from honey bees [4]. Several factors cause the decline of honey bee colonies worldwide. Among these factors, parasitic mites, pathogens, poor nutrition, and pesticide exposure are in the foreground. [5,6]. Nosemosis in honey bees is caused by two species of microsporidian parasites, *Nosema apis* and *N. ceranae*. Although *N. ceranae* was originally discovered in the Asian honey bee *(Apis cerana),* it has outcompeted *N. apis* in the Western honey bee *(Apis mellifera)* in many regions [7]. Nosemosis is spread orally within the honey bee colony through contaminated food, pollen, and water. The disease is then spread by spores in the excrement of infected bees [8,9]. Nosemosis causes fatigue, a shorter lifespan, poor foraging, delayed immune response, and pheromone and hormone production problems. These findings lead to significant mortality, adult population loss, reduced honey production, and potential colony failure [10].

Different approaches are applied against Nosemosis infection [11]. Fumagillin, an antibiotic, has been used for over 50 years to treat Nosemosis [12]. Although this antibiotic is widely used in the USA, it is prohibited in Europe because the maximum residue limits have not been determined [13]. The use of antibiotics causes residue

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problems in honey bee products and has negative effects such as destroying intestinal bacteria that reduce immune function and increases vulnerability to *Nosema* infection [14]. Due to these problems arising from synthetic drugs, studies on natural origin treatment methods are carried out recently. Natural products have advantages such as regional availability, low resistance, and minimal toxicity. Microorganisms, phytotherapeutics, essential oils, and organic acids are the main natural products used to treat Nosemosis [15]. Among these natural products, essential oils are the most used organic compounds against Nosemosis. They are mixtures of fragrant and odorless substances extracted by steam distillation from raw plant materials [16]. Essential oils are mainly composed of terpenoids (isoprenoids), aromatic compounds (aldehydes, alcohols, phenols, methoxy derivatives, and others), and terpenes (monoterpenes and sesquiterpenes) [17]. Due to these components, essential oils have repellent and insecticidal properties [18]. Ozone (O_3), a highly reactive molecule, is one of the most potent oxidizers after fluorine and persulfate [19]. Ozone is an allotropic form of oxygen with many applications in medical and industrial fields. In addition, it has immunomodulatory, analgesic and anti-inflammatory properties [20], and is used as a supplement in treating various degenerative, infectious and autoimmune disorders. Ozone remains stable in oils for many years. Studies have shown that the anti-bacterial effects of ozonated oils with their liquefied formulas with nanotechnological methods remain the same for two years [21].

Ozone has been tested with the fumigant method on *Galleria mellonella*, *Ascosphaera apis*, and *Paenibacillus larvae*. Ozone killed *G. mellonella* adults within five h, and the egg form was destroyed by fumigation of 460-920 mg O_3/m^3 for 48 h. *A. apis* and *P. larvae* were removed with 3.200 O_3/m^3 and 8.650 O_3/m^3 ozone fumigation, respectively [22]. It was reported that applying ozone against *Nosema* spores on the honeycomb reduced the number of *Nosema* spores by 20.25% [23].

In the present study, alternative approaches to control *Nosema* disease were investigated by testing the anti-microsporidian activity of essential oils (thyme, peppermint, and eucalyptus), and nanoparticle ozone on *Nosema* spp. under field conditions.

Material and Methods

Ethical Statement

Insect studies do not require ethics committee approval.

Study Area

This study was carried out in Balıkesir province in August, September, and October 2022. There were 100 hives in the apiary where the study was carried out. Balıkesir is located in the southern part of the Marmara Region of Türkiye (39°40'N-26°28'E). August, September, and October 2022 weather information is shown in *Table 1*.

Table 1. *August, September, and October 2022 temperature, humidity, and rain condition*

Weather Parameters	August 2022	September 2022	October 2022
Minimum-maximum temperature values	19°C-31°C	15°C-29°C	21°C-14°C
Average humidity	50%	56%	67%
Sunny day	31 days	29 days	16 days
Rain condition	No rain	One day (heavy rain)	Four days (heavy rain)

Field Experiment

One-hundred hives were included in the study. Samples were collected individually from each hive from the outermost frames. The samples were placed into sterile containers, and the numbers of the hives and other necessary information were written on the containers. The honey bee samples were collected between 6:00 and 7:00 p.m.

Honey Bee Information

The Anatolian honey bee breed *(Apis mellifera anatoliaca)* was studied. The queen bees in all hives were one year old. During the period of the study, honey bee colonies average between 45.000 and 50.000 individuals. Before the study, the honey bees' owner confirmed that no chemical or plant extract was used to treat any pathogens, in that season.

Detection of Nosemosis

To detect Nosemosis, 50 honey bees were collected from the outer frame of 100 hives. To ensure the immobilization of the honey bees, the samples were kept in a deep freezer in the laboratory for one day. Then, 10 immobile honey bees per hive were examined for the existence of *Nosema* spp. spore (positive or negative) by the digestion method. *Nosema* spore forms are shown in *Fig. 1*. In this method, first, the abdomens of 10 honey bees were separated from their body with the help of a scalpel. The abdomens were put into a mortar, where they were crushed. One ml of distilled water was added per abdomen in the mortar. The honey bees' abdomens were crushed with the help of a baguette for about five min. The solution was homogenized via a Pasteur pipette, and one drop was examined at ×400 magnification under a light microscope (Nikon Eclipse E100, Japan) to detect *Nosema* spp. spores.

Counting of *Nosema* spp. Spores

Nosema spp. spores were counted in positive honey bee samples from 81 hives. Adult honey bees of each colony

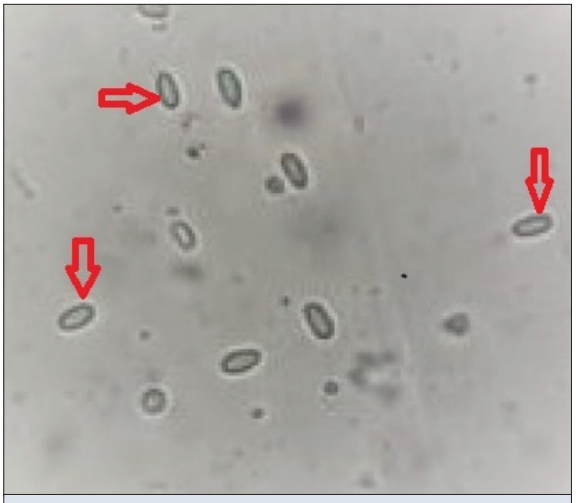

Fig 1. Nosemosis agents are shown red arrow

Fig 2. Spray application

were collected and counted at 0, 15, 30, 50, 65, and 90 days after initial treatment [24]. The digestion method evaluated the *Nosema* infection level from a pooled sample of 50 bees from each colony. A Neubauer hemocytometer slide was used for counting *Nosema* spp. spore loads. The results of these six days were assessed with the formula: $N=S\times4\times10^6/80$, where S is the spore count from all 80 cells of the hemocytometer (one cell represents $1/4\times10^6$ of the total volume) [25].

Treatment Groups

Before the field studies, no tests were made in vitro. This study was designed to determine the effectiveness of spray forms of compounds from thyme, peppermint, eucalyptus essential oils, and nanoparticle ozone against Nosemosis infection in live honey bees. Also, the spray application route is shown in *Fig. 2*.

Preliminary field studies were carried out with 1.000, 2.000, 3.000, 4.000, and 8.000 ppm nanoparticle ozone. A sharp odor formed in concentrations of the nanoparticle ozone of more than 2.000 ppm. Due to this sharp odor, the honey bees were irritated and failed to perform the associated self-licking cleaning process, which is in their normal biology, and the combinations were not fully consumed. Therefore, 1.000 and 2.000 ppm nanoparticle ozone solutions were included in the main field trials.

The information about the thyme essential oil; 100% purity, active ingredients: p-cymene (8.25%), γ-terpinene (31.35%) and thymol (48.50%), linear formula: $2\text{-}[(CH_3)2CH]C_6H_3\text{-}5\text{-}(CH_3)OH$, molecular weight: 150.20 g/mol, peppermint essential oil; 100% purity, active ingredients: menthol (32%), mentone (16.40%), menthofuran (10.6%), 1,8- cineole (6.5%), trans-ferulic acid (11.3 mg/g), hesperidin (8.2 mg/g), ellagic acid (7.5 mg/g), and sinapic acid (5.3 mg/g), linear formula: C10H20O|CID 1254, molecular weight: 960.5 g/mol, eucalyptus essential oil; 100% purity, active ingredients: eucalyptol (50.22%), α-pinene (24.78%), p-cymene (9%), and β-cymene (9.24%), linear formula: C10H18O| ID 2758, molecular weight: 153.20 g/mol.

Thyme, peppermint, and eucalyptus essential oils (Yeşilvadi Botanical Products, Türkiye) were homogenized with Poly Ethylene Glycol 400 (PEG-400: Alpha Lab®, United States) as an emulsifier in sugar syrup. A previous study has proven the fungicidal effect of ozone [23]. On this basis, whether it affects the Nosema spore development in spray form was investigated. Liquid nanoparticle ozone (Genoxyn Nanotech®, Farmoksi Drug, Türkiye) was used at 1.000 and 2000 ppm concentrations. Treatment groups are shown in *Table 2*. Thyme, peppermint, and eucalyptus essential oils were dissolved by mixing them with PEG-400 in a 1:1 ratio. The desired concentrations were obtained by adding sugar syrup to the essential oils dissolved in PEG-400. The preparation of essential oils is shown in *Table 3*. Eight hives containing seven or nine frames were used in

Table 2. *Treatment groups and the number of colonies included in the treatment*

Treatment Groups	Colony Number
125 mL-3% thyme (for each colony)	8
125 mL-3% peppermint (for each colony)	8
125 mL-3% eucalyptus (for each colony)	8
1.000 ppm-1.000 mL nanoparticle ozone (for each colony)	8
2.000 ppm-1.000 mL nanoparticle ozone (for each colony)	8

***Table 3**. Preparation of stock solutions of essential oils for treatment groups*

Percentage	Thyme	Peppermint	Eucalyptus
3%	30 mL thyme oil + 30 mL PEG-400 + 940 mL sugar syrup	30 mL peppermint oil + 30 mL PEG-400 + 940 mL sugar syrup	30 mL eucalyptus oil + 30 mL PEG-400 + 940 mL sugar syrup

PEG-400: Poli Etilen Glikol 400

each treatment combination. These combinations were applied at a ratio of 1000/8 mL (125 mL) to each hive on days 0, 7, 14, and 21.

Control Groups

The *Nosema*-positive and *Nosema*-negative control groups consisted of 8 hives, as in the treatment groups. Honey bee samples of positive and negative control groups were collected at 0, 15, 30, 50, 65, and 90 days as in the treatment groups and *Nosema* spore loads were determined.

Determination of Efficiency of Combinations

The efficiency of combinations was determined with the following formula: Percent reduction test = 100-($\frac{Final\ Number\ of\ Nosema\ spores}{Initial\ number\ of\ Nosema\ spores}$×100) [25]. Day 90 was accepted as the final count of the number of *Nosema* spores, while day 0 was accepted as the initial count (baseline) of *Nosema* spores.

Statistical Analysis

All data were evaluated by IBM®SPSS 20 software. The data were subjected to a Chi-square test of independence due to the number of *Nosema* spores in hives as observed counts. A 2x2 cross-tabulation chi-square test was applied to multiple comparisons for the agents. The days of the *Nosema* loads are compared with a paired-sample t-test. The data are presented as the number of *Nosema* counts in hives. Statistical significance level was considered when $P \leq 0.05$. [26]

Results

Eightyone of 100 hives were found to be positive, and 19 of them were negative. *Nosema* spp. spores were counted on days 0, 15, 30, 50, 65, and 90 after 5 different compounds had been applied to the treatment groups. The percent efficacy values of the treatment groups were calculated by applying the percent reduction test to the results of the counts on days 0 and 90. Treatment efficacies and the total *Nosema* spp. spore loads on the different days are given in *Table 4*. Eight negative hives at the first examination, separated from the positive hives, were found to be positive due to the microscopic examination fifteen days later. In addition, the *Nosema* spore load increased from day 0 to day 90 in the positive and negative control groups. Positive, negative, and treatment groups daily *Nosema* spp. spore loads, and SPSS results are given in *Table 5*. Statistical significance was determined by comparing the agents and *Nosema* loads ($P<0.001$). However, after 2 × 2 multiple comparisons, it was determined that the difference was sourced only days. All the agents effectively reduced the *Nosema* load in the hives up to day 50. The decrease was also found to be significant on days 65 and 90. The *Nosema* load on the 50th day was not statistically significant between the agents ($P>0.05$). According to the percent decrease test results, the highest decrease in *Nosema* spore load was observed in the thyme treatment group with 84%; however, this result was not statistically significant ($P>0.05$). The percentage increase and decrease in Nosemosis spore load daily are given in *Table 6*. The highest decrease in *Nosema* spore load was determined as 40.29% between days 65 and 90 in the thyme treatment group. The minimum decrease in *Nosema* spore load was determined as 8.10% between 15-30 days in the peppermint treatment group. In addition, an increase of 154.11% was observed in the Nosemosis spore load between days 30 and 50 in the peppermint treatment group.

The effectiveness of nanoparticle ozone applied to the frames by spray was investigated against Nosemosis in

***Table 4.** Positive, negative, and treatment groups Nosema spp. spore loads and treatments efficacies*

Treatment Groups	Day 0	Day 15	Day 30	Day 50	Day 65	Day 90	Efficiency (%)
Thyme	6.525×10^3	5.125×10^3	3.500×10^3	2.425×10^3	1.675×10^3	1.000×10^3	84
Peppermint	6.875×10^3	4.625×10^3	4.250×10^3	10.800×10^3	2.050×10^3	1.550×10^3	77.45
Eucalyptus	5.650×10^3	4.350×10^3	3.500×10^3	2.750×10^3	1.950×10^3	1.350×10^3	76.10
1.000 ppm nanoparticle ozone	7.250×10^3	5.200×10^3	3.600×10^3	3.800×10^3	2.850×10^3	2.000×10^3	72.41
2.000 ppm nanoparticle ozone	6.600×10^3	5.100×10^3	4.000×10^3	3.575×10^3	2.700×10^3	1.900×10^3	71.21
Positive	4.350×10^3	5.550×10^3	6.600×10^3	7.600×10^3	8.900×10^3	9.800×10^3	
Negative	0	3.200×10^3	3.750×10^3	4.600×10^3	5.850×10^3	7.000×10^3	

***Table 5**. Positive, negative, and treatment groups daily Nosema spp. spore loads, and SPSS results*

Treatment Groups	Day 0[a]	Day 15[b]	Day 30[c]	Day 50[abcdef]	Day 65[e]	Day 90[f]	df	X^2	P Value
Thyme	6.525.000	5.125.000	3.500.000	2.425.000	1.675.000	1.000.000	20	7481.854	<0.001
Peppermint	6.875.000	4.625.000	4.250.000	10.800.000	2.050.000	1.550.000			
Eucalyptus	5.650.000	4.350.000	3.500.000	2.750.000	1.950.000	1.350.000			
1.000 ppm nanoparticle ozone	7.250.000	5.200.000	3.600.000	3.800.000	2.850.000	2.000.000			
2.000 ppm nanoparticle ozone	6.600.000	5.100.000	4.000.000	3.575.000	2.700.000	1.900.000			
Positive	4.350.000	5.550.000	6.600.000	7.600.000	8.900.000	9.800.000			
Negative	0	3.200.000	3.750.000	4.600.000	5.850.000	7.000.000			

The data presented is the number of Nosema counts in hives. df: Degree of freedom; X^2: Table chi-square value. Superscripts of the columns indicate differences between the days

***Table 6.** Percent increase and decrease of Nosemosis spore load daily*

Day	Thyme	Peppermint	Eucalyptus	1.000 ppm Nanoparticle Ozone	2.000 ppm Nanoparticle Ozone	Positive	Negative
0-15	21.45% ↓	32.72% ↓	23% ↓	28.27% ↓	22.72% ↓	27.58% ↑	3.200.000 total increase
15-30	31.70% ↓	8.10% ↓	19.54% ↓	30.76% ↓	21.56% ↓	18.91% ↑	17.18% ↑
30-50	30.71% ↓	154.11% ↑	21.42% ↓	5.55% ↑	10.62% ↓	15.15% ↑	22.66% ↑
50-65	30.92% ↓	81.01% ↓	29.09% ↓	25% ↓	24.47%↓	30.26% ↑	27.17% ↑
65-90	40.29% ↓	24.39% ↓	30.76% ↓	29.82% ↓	29.62% ↓	10.11% ↑	19.65% ↑

The increase between days 0-15 was given as a number in the negative control group. ↓: Decrease; ↑: Increase

field conditions, and a decrease of 72.41% Nosemosis spore load with 1.000 ppm nanoparticle ozone, and a decrease of 71.21% Nosemosis spore load with 2.000 ppm nanoparticle ozone was found on the day 90 compared to the day 0. The ozone value in the solutions was calculated as gr/ml and expressed as a ppm value. The preparation methods of the ozonated nano solutions are specified in the patent numbered PCT/TR2022/050177.

Discussion

Essential oils (EOs) are also used as bee diet supplements. Honeybees actively collect several phytochemicals that are also dominant components in essential oils, and many of these compounds have antibiotic activity against pathogens and parasites [27]. Honey bees fed plant-derived phytochemicals survive longer and have a high capacity to overcome infections [28]. Essential oils or single compounds isolated from them such as camphor, carvacrol, eucalyptol, menthol, thymol, and several sesquiterpenes have been used as topical medication to control *Varroa destructor* mites and as nutraceutical compounds against *Nosema* spp. spores [29]. The anti-nosemosis properties of essential oils may result from their lipophilic nature and the low molecular weight of terpenes/terpenoids. They can cause cell death or inhibit the sporulation and germination of fungi by disrupting the cell membrane structure or inhibiting chitin polymerization of the cell wall [30].

Various studies have been conducted on the antiparasitic activities of essential oils against Nosemosis. Thymol (3-hydroxy-p-cymene) is one of the most abundant active ingredients derived mainly from *Thymus vulgaris*. Thymol was used against Nosemosis by adding 0.1 g/kg sugar syrup. Fewer spore loads were found in the thymol-treated group than in the Nosemosis-positive group [31]. A study conducted treatment trials with 100 ppm thymol with the honey bees experimentally infected with *N. ceranae in vitro*. Significant differences were found between the treatment and the control group, which was a significant result (60±9 million spores/bee in the treatment group, 138±7 million spores/bee in the control group) [32]. Both studies were carried out in vitro. In a study conducted in vivo, thymol was given to each colony 6 times at 3-day intervals of 3 mL/L to 1:1 ratio sugar syrup, and an undesirable increase of *Nosema* spore load was observed in the thymol group, whereas there was a partial decrease in nettle, especially garlic [33]. Contrary to the increase mentioned in that study due to thymol usage, our study's *Nosema* spore load decreased from day 0 to day 90 *(Table 6)*.

Menthol is derived from peppermint oil and was previously used for some honey bee parasites. It has been tested with thymol to combat *Varroa in vivo* and promising results have been obtained (infestation rate reduced from 21.86% to 1.32% at the end of treatments). It has also been reported that using menthol at a dose of 2.5 mL is effective in tracheal mite infestations [34]. In our study of peppermint oil, the effectiveness of which was tested in other bee pests, it was used for the first time against Nosemosis in honey bees *in vivo*. Peppermint oil was found to be a crucial agent that suppresses *Nosema* spore load after thyme oil. According to the percent reduction test, its effectiveness was determined as 77.45%.

Eucalyptus essential oil has been studied in vitro for Varroosis [35] and Nosemosis [36]. In both, mites were reduced at 71.06% and spore burden at 23%. In a field study with eucalyptus, although a decrease was observed in the first four applications compared to the control group, an increase in Nosemosis spore load was observed in the last two applications. This increase has been described as the intestinal flora may have been damaged due to eucalyptus with the last two applications [33]. In our field study with eucalyptus essential oil, the Nosemosis spore load decreased by 76.10% at the end of the treatment. During our trials, no clinical findings, especially diarrhoea, which may occur due to damage to the intestinal flora, were not encountered.

Thyme, peppermint and eucalyptus essential oil were used by dissolving them in different volumes of PEG-400. PEG-400 was found to be inexpensive and non-toxic [37]. No adverse effects were observed in the honey bees due to using PEG-400. Various studies have been carried out on ozone. One of these studies on the medical use of ozone found that during ozone application, hydrogen peroxide, which is formed due to oxidative stress and lipid oxidation, acts as a secondary messenger. As a result of repeated ozone applications, the antioxidant system is stimulated and resistance against oxidative stress develops [38]. As seen in *Table 1*, although the temperature, humidity average, and the number of rainy days in October caused a suitable environment for the development of the *Nosema* fungal form, an increase in Nosemosis spore load was observed in the peppermint and 1.000 ppm nanoparticle ozone groups only on the 50th day in the treatment groups. The reason for this increase can be explained by the fact that the honey bee colonies in these hives are clinically weaker compared to other honey bee colonies. In addition, the negative control group was found to be positive for Nosemosis on the 15th day, and the Nosemosis load increased until the 90th day. This is because nectar, pollen, and water contaminated with *Nosema* spores are transferred to healthy hives. In the positive control group, the Nosemosis spore load increased from day 0 to day 90. This increase can be explained by the contamination of food and water sources with *Nosema* spore forms as in the negative control group. According to the results of the current study, although all agents were found to be similarly effective in reducing the burden of *Nosema* spp, the proportional effect of thyme oil was determined more effective than the other agents. Essential oils are tried by most researchers in the laboratory environment, but not under field conditions. In addition, the active ingredients with proven effectiveness in the laboratory environment have not been tested under field conditions. The reason why the active substances were given by spray is the hygienic behavior of honey bees by licking each other. With this behavior, the honey bee took the active substances given by the spray more quickly. During the study, abnormal bee death and plundering were not encountered in the colonies.

As a result, thyme, peppermint, eucalyptus essential oils, and nanoparticle ozone, which constitute our study subject, were applied to the frames by spraying in field conditions and their effectiveness values against Nosemosis were revealed. Promising results were obtained in this study and more comprehensive studies are needed in treating with natural extracts. The health of honey bee colonies and bee products can be guaranteed with these studies' results if the natural extract is applied in a suitable dose. Our field study may allow bee products that are healthier and have no drug residue problems to be offered to the public.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (M. Özüiçli) on reasonable request.

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Competing Interest

The author reports no declarations of interest.

Author Contributions

MÖ planned the study and designed the experiments, LA, AOG and AİD helped write the article and laboratory process, İK helped collect samples during fieldwork and wrote the manuscript, and YB helped with data analyses. All authors read and approved the final version of the manuscript.

Ethical Approval

Ethics committee approval is not required for this study.

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Research Article

Stereological, Embryological and Histomorphometric Studies on Embryonic Development of Cerebellum and Cerebellar Purkinje Cells in Chicks

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ABSTRACT

The cerebellum which is widely used in studies related to the motor system is responsible for maintaining balance, muscle tone and coordination. In present study, we aimed to evaluate the cerebellum at different incubation periods in terms of stereology, embryology and histomorphometry. Twenty-four Babcock White Leghorn chick embryos were used. All data regarding embryo, egg and cerebellum were measured and calculated. All histologic and histomorphometric examinations were evaluated on the preparations stained with Crossmon's trichrome stain, Kluver-Barrera stain, and silver stain. It was determined that the cerebellum weight, which was 0.0191±0.0064 g on the 10th day of incubation, increased gradually towards to hatching day. On the 10th day, it was seen that a four-layered primitive substantia grisea structure began to form. While primary foliation seen on the 10th day, secondary foliation started on the 13th day. On the 16th day, Purkinje cells forming the stratum gangliosum were arranged almost in a single row. At day 21^{th,} the general structure of the cerebellum was almost similar to that of the adult cerebellum. While the stratum moleculare and granulosum thicknesses were measured in all embryonic periods, the stratum gangliosum thickness could be measured on the 16th and hatching days. As a result, it was thought that the data obtained from this study might be a reference for studies on the cerebellum and especially on motor control disorders.

Keywords: Cerebellum, Cerebellar Purkinje cell, Chick embryo, Embryonic development

Introduction

The cerebellum, which contains more than half of all neurons in the central nervous system, has special importance among other central nervous system organs [1,2]. The cerebellum, responsible for the control and coordination of motor nerves, voluntary motor movements and declarative memory, is known as temporal regulation machine or one of the neuronal clocks [3,4]. It also plays an important role in determining the parasagittal lines of sensory perception, respiration, eye movements, nociception and gene expression [5,6]. Recent studies have moved the cerebellum to a multitasking "neuronal machine" rather than a "small brain" [6]. The cerebellum plays a regulatory role between the brain and organs. The failure of this organ to be fully formed is not life-threatening. However, studies have reported that normal motor behaviors are significantly affected [7,8].

In chickens, the cerebrum, which begins to form on the first day of incubation, is fully functional on the 7th day [9]. It has been reported that there is a narrowing between the mesencephalon and the rhombencephalon on the 4th day of incubation [10], and towards the end of the embryonic period, the cerebellum forms from the dorsolateral of the alar plates of the metencephalon [2,11]. AbidÂ and Al-Bakri [12] reported that the cerebellum surrounding the roof of the IV ventricle is a spherical shaped part of the rhombencephalon and metencephalon. It is connected to the medulla oblongata and pons with the feet called the cerebellar peduncle and is separated from these two structures via the IV ventricle [13,14].

The cerebellum consists of the right and left cerebellar hemisphere and the vermis cerebelli which connects the two hemispheres to the midline [2,7,15,16]. In mammals and birds, there are transverse grooves, called sulci cerebelli, on the outer surface of the hemispheres, and deep folds

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that defined as folia cerebelli among these grooves [12,17]. It is reported that due to its curved structure, it has a large surface area of 75% of the brain surface area [1,2]. The degree of folding of the cerebellum, which is one of the most significant differences among vertebrates, is the same in all bird species [18-20]. The vermis cerebellum consists of nine cerebellar folia, separated from each other by sulci. The cerebellum consists of anterior, posterior, and flocculo-nodular lobes and are named cerebro cerebellum, spino cerebellum, and vestibulo cerebellum, respectively [12,21]. It is also reported that the general restructuring of the cerebellum is completed shortly after hatching [10].

Nucleoli are nucleus regions where ribosomal subunits are synthesized. The regions that contain the genes that synthesize ribosomal RNA and form the nucleolus are called nucleolus organizer regions (NORs). Since these regions are argyrophilic, they are stained with silver methods, and these regions are called silver staining nucleolus organizer regions (AgNORs). NORs are indicators of cell proliferation rate according to ribosome formation and protein synthesis of cells [22-24].

Since chicken egg does not have a placental barrier, which shows an embryonic development independent of maternal effects, is accepted as one of the most suitable materials for embryotoxic and teratogenic studies [25-28].

In present study, it was aimed to evaluate the cerebellum taken from chick embryos on different days of incubation (10., 13., 16. and 21. days) in terms of stereological, embryological development and histomorphometry.

Material and Methods

Ethical Statement

Ethical approval was taken from The Ethical Committee of Health Sciences of Karamanoglu Mehmetbey University (protocol number: 2022/19).

Material

For the present study, cerebellums were obtained from 24 Babcock White Leghorn chick embryos on the 10th, 13th, 16th and 21st days of incubation considering organogenesis. According to the determined embryonic days, the randomly-selected eggs were opened from each of the groups until six live embryos were obtained for evaluation in terms of cerebellum development.

Morphometric Measurements

First, the embryo weight, pre-hatching, and initial egg weight were weighed with precision scales and the relative embryo weights (REW, %) were calculated by the following formula (Equation 1) [27].

$$REW = \frac{\text{Embryo weight}}{\text{Pre} - \text{hatching egg weight}} x100$$

Then, a dissection of the cerebellum was performed from the peripheral cerebrum tissues. Cerebellums taken from embryos according to incubation periods were weighed on precision scales and their weights were recorded. Relative cerebellum weights (RCW, %) were calculated with the following formula (Equation 2) [29].

$$RCW = \frac{\text{Cerebellum weight}}{\text{Embryo weight}} x100$$

Histological Processing of Cerebellum

Routine histological preparation procedures were performed on cerebellums fixed in 10% neutral buffered formalin solution. Using a rotating microtome, three sagittal serial sections of 5 µm thickness were taken at regular intervals from each block for histological examinations and 10 µm thickness sections were taken every 50 sections for cerebellum volume calculation [29]. The sections were oven-dried at 37°C for 24 h and were stained with Crossmon's trichrome stain [29], Kluver-Barrera stain, and silver stain [30]. After examining the stained sections with a camera-attached microscope (Leica DM-2500 attached to a DFC-320 digital camera), digital images of the necessary regions were recorded. All measurements were analyzed with an ImageJ Analysis Program [31]. For the histomorphometric measurements related to Purkinje cells, 25 Purkinje cells having nuclei with definite nucleoli were evaluated. The number of Purkinje cells per unit length (1 mm) of the ganglionic cerebellar layer was counted on digital images. In the examination of cerebellar development in different embryonic periods, cerebellar layer thicknesses and folia

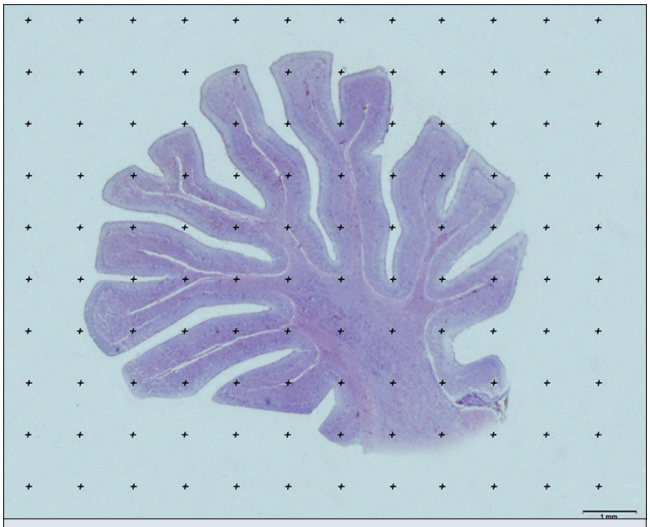

Fig 1. Application of point counting grid on histological sections of the cerebellum (area per point = 1 mm^2)

widths (in six folia) of each animal were measured from six different areas.

Volume Calculations in Cerebellum

The grid function of ImageJ was used in volume calculations and a point counting grid (d=1mm) was placed on the sagittal-section. The points on grey and white matter were counted *(Fig. 1)*. The volumes were estimated as $V = a(p) \times \Sigma p \times t$ formula. In this formula, V is the volume of the structure concerned, a(p) is the area of the one point on the grid (this value is 1 mm^2 in the study), Σp is the sum of the points on the structure of interest and t is the section thickness [29,32].

Statistical Analysis

In the present study, the analysis of the data obtained from the chick embryos was performed using the SPSS software version 21.0 statistical package program. Since the variables showed normal distribution, they were compared among groups using one-way ANOVA test. In cases where the P value was significant, pairwise post-hoc comparisons between statistically significant results were made using the Tukey test. Descriptive analyzes were given using the mean and standard deviation. A value of $P<0.05$ was accepted as the significance limit.

Results

Macroscopical Evaluation

The weights of all eggs, embryos and cerebellums used in the study are given in *Table 1*. According to the data obtained, although there was no statistical difference in both egg weights among different incubation periods, it was observed that the embryo and relative embryo weights increased gradually according to the advancing incubation days ($P<0.001$). The weight of the cerebellum, which was 0.0191±0.0064 g on the 10th day of incubation, increased gradually towards to hatching day, while the relative cerebellum weight decreased ($P<0.001$). When the measurements in *Table 1* are examined, it is seen that the cerebellum volume gradually increases during the advancing incubation periods and, it is statistically significant ($P<0.001$).

Embryonic Development of Cerebellum

The embryonic development of the cerebellum on certain days of incubation are given in *Fig. 2*. The external granular layer, rich in granular cells, was seen on the 10th day of incubation. The marginal layer that will form into the stratum moleculare in future incubation periods was just below of external granular layer. The inner cortical layer and internal granular layer were seen. The presence

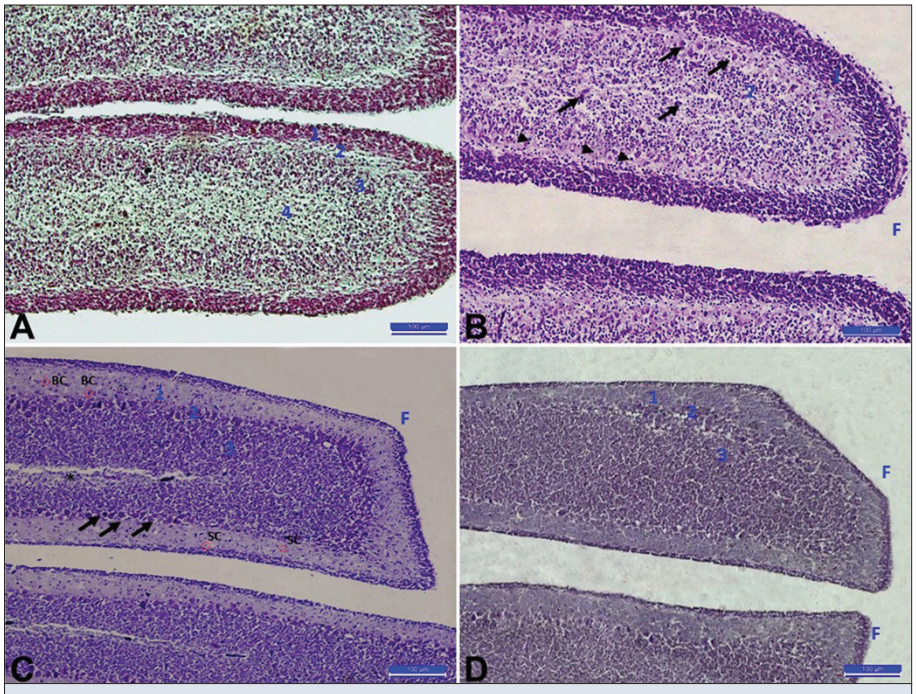

Fig 2. Embryonic development of cerebellum in different embryonic periods. **A:** The cerebellum section from a day 10 chick embryo, 1: External granular layer, 2: Marginal layer, 3: Inner cortical layer, 4: Internal granular layer, Crossmon's trichrome staining. **B:** The cerebellum section from a day 13 chick embryo, 1: Primitive stratum moleculare, 2: Primitive stratum granulosum, Arrows: Precursor Purkinje cells, Arrowheads: Granular cells, F: Foliation, Kluver-Barrera staining. **C:** The cerebellum section from a day 16 chick embryo, 1: Stratum moleculare, 2: Primitive stratum gangliosum, 3: Stratum granulosum, Star: Substantia alba, Arrows: Purkinje cells, F: Foliation, SC: Stellate cells, BC: Basket cells, Kluver- Barrera staining. **D:** The cerebellum section from a day 21 chick embryo, 1: Stratum moleculare, 2: Stratum gangliosum, 3: Stratum granulosum, F: Foliation, Crossmon's trichrome staining

of all these four layers on the 10th day showed us that the primitive substantia grisea structure began to form. During this period, precursor Purkinje cell clusters began to be found. Furthermore, the presence of deep folds that are known as folia cerebelli revealed the initiation of primary foliation. No distinguishable substantia alba structure was observed *(Fig. 2-A)*.

On the 13th day, it was noted that the thickness of the external granular layer decreased. We determined that a primitive stratum moleculare structure was formed. Precursor Purkinje cell rows, arranged in one-two rows, were seen in the inner cortical layer. The dark granular cell clusters migrating from the extra granular layer to the internal granular layer were evident. As such, we can say about a primitive stratum granulosum structure. In addition to the primary foliation, it was observed that secondary foliation started or even progressed. It was observed that the substantia alba, consisting of axons and myelinated nerve fibers, began to become prominent *(Fig. 2-B)*.

In the 16-day-old embryos, the layers forming the substantia grisea could be easily distinguished. In this period, it was observed that the stratum moleculare became prominent. It was noteworthy that Purkinje cells forming the primitive stratum gangliosum were almost arranged in a single row. The inner granular layer was filled with granular cells and was replaced by the stratum granulosum. The substantia alba was clearly visible *(Fig. 2-C)*.

Stratum gangliosum, formed by Purkinje cells with large flask-shaped bodies, central nuclei, dark nucleoli, and dendrites extending into the molecular layer, was easily seen in 21-day-old embryos. In this period, it was remarkable that the stratum gangliosum was similar to the adult cerebellum. At day 21st, the general structure of the cerebellum was almost similar to that of the adult cerebellum *(Fig. 2-D)*. Also, stellate cells were found superficially in the molecular layer. Basket cells, mostly located close to Purkinje cells, were also observed *(Fig. 2-C)*.

Histomorphological Evaluation of the Cerebellum

All histomorphometric measurements done in the cerebellum in this study are given in *Table 1*. While the stratum moleculare and stratum granulosum thicknesses of the cerebellum were measured in all embryonic periods, the thickness of the stratum gangliosum formed by the Purkinje cell row could be measured on the 16th and hatching days. As a result of measurements, it is seen that there is a statistical difference among the stratum moleculare thicknesses during the incubation. The highest value was recorded as 123.55±19.66 µm on the 21st day (P<0.001). Although there is no statistical difference in the thickness of the stratum granulosum, it is noteworthy that there is an increase in this layer thickness throughout incubation (P>0.001).

Among the incubation periods, substantia grisea and substantia alba thicknesses of the cerebellum were statistically significant (P<0.001). While the thickness of the substantia grisea increases throughout all incubation periods, it is seen that this ratio decreases gradually in the substantia alba. Ratio substantia grisea/substantia alba thickness and folia width were statistically significant, and this ratio was highest on the 16th and 21st days (P<0.001). Ratio stratum gangliosum/substantia grisea thickness did not differ statistically (P>0.001).

Purkinje cell count in a unit length (1 mm) was counted from the 13th day of incubation. The highest values were determined on the 16th day of incubation and hatching day (P<0.001). On the 13th day of incubation, the lowest transverse diameter body and nucleus of the Purkinje cell were measured as 4.44±0.47 µm and 2.22±0.41 µm, respectively, (P<0.001). Statistically, the highest nucleus area of the Purkinje cell was on the 21st day of incubation (P<0.001). While the nucleolus area of the Purkinje cell could be detected from the 13th day of incubation, the highest values were observed on the hatching day (P<0.001) *(Fig. 3)*.

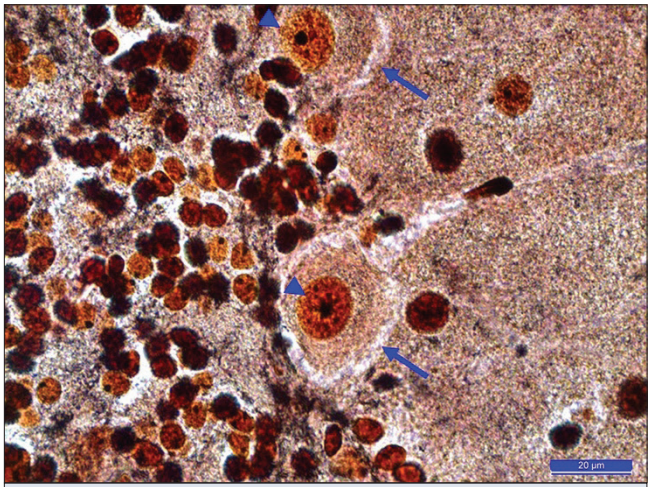

Fig 3. Purkinje cell's histological appearance, Arrows: Purkinje cells, Arrowheads: Nuclei, Silver staining method

Discussion

The cerebellum, which is considered the motor center of the brain, is important in balance and coordination of muscle movements [7,16]. Differences in the volume, morphology and histology of the cerebellum are related to the general anatomical structure and behavioral characteristics of the species [20]. Pal et al.[33] reported that the size and shape of the cerebellum depend on the type of movement, center of

Table 1. *Some morphometric values of the chick embryos according to incubation days (Mean±SD)*

Parameter	Day of Incubation (n=6)			
	10th	**13th**	**16th**	**21st**
Initial egg weight (g)	60.86±6.29	57.91±5.45	58.58±2.76	54.98±3.67
Pre-hatching egg weight (g)	56.40±6.43	53.07±5.09	50.78±3.85	48.87±3.05
Embryo weight (g)	3.06±0.15	8.88±0.57	22.74±2.71	41.17±3.58
Relative embryo weight (%)	5.56[a]	16.90[b]	44.76[c]	84.21[d]
Cerebellum weight (g)	0.0191±0.0064[a]	0.0644±0.0057[b]	0.0844±0.0103[c]	0.0932±0.0146[c]
Relative cerebellum weight (%)	0.72[a]	0.62[b]	0.37[c]	0.23[d]
Cerebellum volume (mm^3)	0.11±0.01[a]	0.31±0.03[a]	0.91±0.15[b]	1.25±0.34[c]
Stratum moleculare thickness (µm)	45.96±9.14[a]	52.69±8.31[ab]	68.48±14.30[b]	123.55±19.66[c]
Stratum gangliosum thickness (µm)	NA	NA	18.32±4.08	22.69±3.77
Stratum granulosum thickness (µm)	94.68±9.62	95.54±10.67	114.69±56.87	159.98±62.82
Substantia grisea thickness (µm)	143.13±24.19[a]	151.97±21.59[a]	199.72±72.34[a]	315.17±97.01[b]
Substantia alba thickness (µm)	NA	83.39±7.62[a]	73.11±13.45[a]	59.67±11.97[a]
Ratio substantia grisea/ substantia alba thickness	NA	1.87±0.28[a]	4.33±1.46[b]	4.74±0.51[b]
Ratio stratum gangliosum/ substantia grisea thickness	NA	NA	0.09±0.03	0.08±0.03
Width of the folia (µm)	344.49±18.63[a]	379.180±51.72[a]	441.06±164.49[ab]	619.26±176.38[b]
Mean Purkinje cell counts in a unit length	NA	NA	42.33±5.72	44.67±7.03
Mean transverse diameters of the Purkinje cell bodies (µm)	NA	4.44±0.47[a]	11.72±1.06[b]	11.79±0.79[b]
Mean transverse nucleus diameters of the Purkinje cells (µm)	NA	2.22±0.41[a]	6.49±0.47[b]	7.69±0.44[c]
Mean nucleus areas of the Purkinje cells (μm^2)	NA	10.31±0.76[a]	30.65±4.39[b]	40.16±3.74[c]
Mean NOR areas of the Purkinje cells (μm^2)	NA	1.87±0.12[a]	4.15±0.51[b]	5.42±1.43[c]

Different letters in the same row [a, b, c, d] indicate significant differences (P<0.001). NA: not available

gravity, and body posture in animals. The more complex the body movements, the more developed the cerebellum. Chicken cerebellum has been reported to be larger in size and weight than that of humans. This situation is related to the balance center and reveals the importance of the cerebellum in birds [34].

In chickens, the cerebellum begins to develop from a thick neuroepithelial layer on the roof of the IV ventricle [10]. Some researchers determined that the substantia grisea develops from the ventricular neuroepithelium and the outer granular layer [24,35]. Feirabend et al.[36] showed that mitotic activity in the outer granular layer started on the 6th embryonic day and intensified until the 18th day. In our study, we found numerous dark granular cell clumps in the outer granular layer from the 10th day of incubation. It was observed that these dark granular cells, which are important for the development of the cerebellum, migrated towards the inner granular layer. This was in line with what some researchers said [24,37]. On the 9th-10th days of incubation, some researchers reported that Purkinje cell clumps begin to appear in the inner cortical layer, which originates from the inner mantle layer. In addition, they have said that these cells take their characteristic arrangement and form the stratum gangliosum during

the incubation periods [16,24]. In present study, it was rarely encountered the inner cortical layer and its precursor Purkinje cell clusters in 10-day-old embryos. It was obvious that a primitive substantia grisea structure consisting of external granular layer, marginal layer, inner cortical layer and inner granular layer was beginning to take shape in the cerebellum *(Fig. 2-A)*.

Feirabend et al.[10] reported that foliation, which is characteristic for the embryonic development of the cerebellum, started to develop on 11th-12th days of incubation. They also found 10 primary folia and reported the secondary foliation in V-IX primary folia in a 13-day-old embryo. Akar and Sur [24] claimed the primary foliation on the 11th day. In present study, primary foliation was first seen in 10-day-old embryos, while secondary foliation was seen in 13-day-old embryos *(Fig. 1)*. Maulana et al.[4] reported that the substantia grisea was formed from the molecular and granular layers on the 7th day of incubation. Studies have shown that Purkinje cells line up in three rows and the granular layer is not yet fully organized [24,35,38]. In this study, it was observed that the primitive substantia grisea structure in 13-day-old embryos developed slightly more than on the 10th day and formed into a substantia grisea consisting of primitive stratum moleculare, primitive stratum granulosum and precursor Purkinje cell lines (two-three rows). Also, some researchers said that the substantia alba, where axons and myelinated nerve fibers are located in the medulla of the cerebellum, become evident on the 14th day of incubation [4,39,40]. On the 13th day of this study, this was consistent with the existing literature *(Fig. 2-B)*.

In chickens, four layers (external germinative layer, the molecular layer, the Purkinje layer, and the internal granular layer) of the cerebellar cortex were seen on 15th-16th incubation days [24,41]. On the 17th day of incubation, it has been reported that Purkinje cells are arranged in a single row and the molecular layer becomes clear [24,35,38,]. In the 16-day-old embryos of this study, there were three layers forming the substantia grisea (stratum moleculare, primitive stratum gangliosum, and stratum granulosum). The single-row arrangement of Purkinje cells forming the primitive stratum gangliosum began to take shape during this incubation period *(Fig. 2-C)*.

Maulana et al.[4] reported that the chicken's cerebellum layer development was completed on the 20th day of incubation and the cerebellum had a loose structure. They stated that they saw the single-row Purkinje cell arrangement between the granular and molecular layers at the hatching and that the development of the cerebellar cortex was completed [24,42]. In the present study, it was seen that the substantia grisea and substantia alba were in the known histological cerebellum structure in 21-day-old embryos. It also detected stellate cells and basket cells in the stratum moleculare, which is made up of small neurons and glial cells, as the researchers said [42-44]. Substantia alba was highly developed compared to other incubation periods *(Fig. 2-C)*.

Histologically, the layers that make up the cerebral cortex are the molecular layer, the Purkinje cell layer, and the granular layer from the outside to the inside, respectively [4,16,42,43]. In female geese aged between 10-12 months, Koral Taşçı and Bingöl [16] found the mean thickness of the molecular and granular layers as 348.53±72 µm and 184.83±48 µm, respectively. In the study of Maulana et al. [4], the molecular layer thickness obtained on the 14th day was higher than the 13th day of our study, while it was lower in terms of the granular layer. Stratum gangliosum thickness was measured from the 16th day until the hatching day, since Purkinje cells did not show a single-row array on the 10th and 13th days of incubation. On 16th-21st days, the values were found 18.32±4.08 µm and 22.69±3.77 µm, respectively. The axons of Purkinje cells reaching the stratum granulosum and being surrounded by myelin sheath in the substantia alba caused an increase in the stratum gangliosum thickness.

In our study, the substantia grisea thickness was higher on the 13th day compared to that of Maulana et al.[4] while the subtantia alba thickness was lower on the 21st day. Whereas the thickness of the substantia grisea was an increase as the incubation progressed, there was a decrease in the thickness of the substantia alba. Because the cerebellum layers were gradually developing during the advancing incubation periods. The pressure formed as a result of the shaping of the layers on the substantia alba, especially the stratum gangliosum, and it caused the substantia alba to shrink. In this study, the ratio substantia grisea/substantia alba thickness was 4.74±0.51% in 21-old-day embryos.

The Purkinje cell is one of the largest neurons of the central nervous system with its dendrites extending to the surface of the cerebellar cortex and myelinated axon extending to the granular layer [45,46]. Sur et al.[42] found the number of Purkinje cells of 1 mm length in turkeys as 16.34±1.47, in ducks 20.98±2.24, in pigeons 27.39±1.05 and in starlings 27.00±0.91. Celik et al.[47] found the number of Purkinje cells in one unit length (486 µm) of the ganglionic layer in rats as 9.38 in males and 9.53 in females. Although there is no embryological study related to this data, we found mean Purkinje cell counts in a unit length (1 mm) as 42.33±5.72 and 44.67±7.03 in 16th day of incubation and hatching day, respectively. The increase in the number of Purkinje cells indicates the functional development of the cerebellum in terms of behavioral and cognitive skills [20].

In laying hens on the hatching day, Turgay-İzzetoğlu et al.[46] nuclear diameter and nuclear area measurements of Purkinje cell are similar to those of Akar and Sur [24].

In addition to these parameters, the mean transverse diameter of the Purkinje cell was also similar with those of Akar and Sur [24]. It is thought that the reasons for the larger data in our study may be some differences such as the incubation conditions, examined Purkinje cells, the image analysis program or the embryos.

Purkinje cells, which establish morpho-functional and synaptic connections that require high metabolic activity, synthesize different amounts of protein at different developmental stages of the embryonic period [48]. NOR areas provide information about the cellular activities of cells, especially protein synthesis [49]. NORs, known as nucleolus forming regions, are associated with the nucleus area. While NOR areas obtained from this study were higher than those of Akar and Sur [24] on the hatching day, this parameter was similar with Turgay-İzzetoğlu et al.[46]. The increases in NOR area, nuclear diameter and area of Purkinje cells are thought to result from increased protein synthesis due to the development of the cerebellum during the advancing incubation days. It is also thought that incubation conditions may cause differences in Purkinje cells, image analysis program or embryos.

As conclusion, the cerebellum which is widely used in studies related to the motor system is responsible for balance, muscle tone and muscle coordination. Although the inability of this organ to fully form is not life-threatening, studies have shown that motor behaviors are significantly affected. It is thought that this study provides new data to the literature about cerebellum development by monitoring the histologic, histomorphometric and stereological developments of chick cerebellum in different incubation periods. In addition, the fact that there has not been a study that gives embryological cerebellum volume data of chicks before increases the importance of the study. Since cerebellum dysfunctions bring along motor control disorders, this organ is used especially in studies on the motor system. The data obtained from this study contribute to the studies that can be done especially on motor control disorders.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (F. Colakoglu).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

Ethical approval was taken from The Ethical Committee of Health Sciences of Karamanoglu Mehmetbey University (protocol number: 2022/19).

Author Contributions

FC: Conceptualization, methodology, investigation, writing original draft. MLS: Conceptualization, methodology, writing-review and editing.

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RESEARCH ARTICLE

First Record of Trombiculosis Due to the Chigger Mite *Kepkatrombicula desaleri* in Domestic Goats in Bulgaria and Treatment Attempts

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ABSTRACT

Trombiculosis is a parasitic skin disease caused by larvae of mites from the family Trombiculidae (chiggers). In general, larvae provoke dermatitis. *Kepkatrombicula desaleri* Methlagl, 1928 is one of species involved in the etiology of animal trombiculosis, detected in European goats. The aim of the present study was to detect the species of trombiculid mites infesting goats and to recommend appropriate means for control of provoked disease. The study included total of 141 goats from two flocks in the Kalugerovo village, Pazardzhik district, Bulgaria. All infested goats from the first flock were treated with ivermectin, and those from the second flock - with cypermethrin. The identification of larvae showed their affiliation to the *K. desaleri* Methlagl, 1928 species. In the first flock, 15 infected goats were detected (17%) vs 7 (13%) in the second flock. The mites were detected on eyelids, medial eye angles, nose and ears as orange-red papules. Neither squamae nor crusts were found in the skin adjacent to chiggers' location, and goats did not show any clinical signs. The treatment with ivermectin was ineffective, and spraying with cypermethrin resulted in reduced motility and rapid death of 100% of mites only several minutes after the treatment. The mites infected only animals with dark haircoat regardless of their age and sex.

Keywords: Bulgaria, chiggers, goats, mites, treatment

INTRODUCTION

Trombiculosis is a parasitic skin disease caused by larvae of mites from the family Trombiculidae with more than 1500 known species worldwide [1]. The larvae (chiggers, red bugs, and harvest mites) may infect many host species, including amphibians, birds and mammals [2]. The consequent stages of their life cycle are: egg, prelarva (deutovum), larva, protonymph (nymphochrysalis), deutonymph (nymph), tritonymph (imagochrysalis) and imago. In moderate climatic areas, the parasite has one to three generations per year [3]. After successful attachment to an appropriate host, the chiggers feed on for 3-5 days and then pursue their development as free-living arthropods. Chiggers feed mainly on partially lysed cutaneous cells and lymph and usually cause dermatitis. Feeding is realized by a feeding tube or stylostome formed by the interaction of mite saliva and host tissue [2]. The host skin reaction is strong and manifested with intense infiltration of leukocytes, neutrophils and erythrocytes in inflammatory foci. Apart from inflammatory exudate, larvae may also uptake red blood cells from foci of inflammation. In the view of [4] the swelling, epidermal hyperplasia and hyperkeratosis are not specific signs of this infestation. Some species serve as vectors of the rickettsia *Orientia tsutsugamushi*, which causes an acute infectious disease spread in southeastern Asia [5].

In Europe, trombiculosis is caused by the larvae of about 50 mite species [6], including 8 species isolated from murid rodents in Turkey [7]. Four species from this family have been described in men: *Neotrombicula autumnalis* Shaw, 1790, *Kepkatrombicula desaleri* Methlagl, 1928, *Blankaartia acuscutellaris*, Walch, 1922 and *Trombicula toldti*, Winkler, 1952 [6]. Despite that the parasites are known to infect numerous mammalian species, the reports in hoofed animals are rare [4,8,9]. In this connection, the most prevalent species is *N. autumnalis* Shaw, 1790 [10]. The morphology of *K. desaleri* larva is very similar to that of *N. autumnalis* larva [11]. The difference between the two species is the presence of elongated seta on the tibia of the last pair of legs. This fact as well as the unclear taxonomy of the group probably leads to misidentification, especially in hoofed mammals.

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Kepkatrombicula desaleri Methlagl, 1928 has been detected on goats in Europe [8,11]. It infects ungulates, whereas in humans it was registered only by the author that described it for the first time. Unlike most trombiculid mites, this species has a sucker disk in the apical part of the hypostome, allowing successful feeding on large hosts [4].

Clinical signs reported in goats are milder than those observed in dogs and humans; in massive infestation, they include scratching, rubbing and licking. Also, they are similar to those of scabies, which should be considered in the differential diagnosis list [13].

Attempts for control of the disease at a global scale have been made only in companion animals and horses. Therefore, the aim of the present study was to determine the species of trombiculid mites infesting goats from studied flocks and to recommend appropriate means for control of resulting trombiculosis.

Material and Methods

Ethical Statement

Ethics committee approval is not required for this study as it is within the scope of veterinary practice.

Animals

The study was conducted from 30 October to 19 November 2022. It was performed on two goat flocks in Kalugerovo village, Pazardzhik district: the first with 88 goats and the second - with 53 goats.

Examinations

All animals were examined with a magnifying glass for presence of skin alterations and ectoparasites. Detected parasites were collected in 70% ethanol and transported to the laboratory for identification. A DMi1 S/M 424790 Leica® microscope (Leica Microsystems CMS GmbH) equipped with a Leica MC120 HD camera was used for the microscopic exams and photographic materials. The identification was done according to descriptions of Kudryashova and Stekolnikov [11].

Treatment

All infested goats from the first flock were treated subcutaneously with ivermectin (Pandex™ 1%, Biovet), at a dose of 0.2 mg/kg body weight. The treatment results were evaluated twice: on the 1st and 3rd post treatment days. The trombiculid-positive goats from the second flock were treated with cypermethrin (Ectomin 10%, Farmavet OOD), applied by spraying at a concentration of 0.2%.

Statistical Analysis

All measurements (total body length ± SD) were calculated by means of descriptive statistics (IBM® SPSS® Statistics 26.0 software).

Results

Out of all collected 42 chiggers, 16 were with preserved integrity and were identified as larvae of the *K. desaleri* species (*Fig. 1*; *Fig. 2*; *Fig. 3*). The total body length varied from 240 to 520 µm (mean±SD 337.5±86.7 µm), depending on the feeding level. That is why, the latter is not used as an identification criterion. In contrast, the shape of the scutum and the number and arrangement of setae are indicative for identification. Our results revealed that the scutum is broad with rounded posterior margin; present of anterolateral shoulders; 48-61 dorsal idiosomal setae with anterior two rows paired; 4 sternal setae and 32-41 ventral setae; 82-103 total number of idiosomal setae.

Out of the studied 88 goats in the first flock, 15 were positive for the parasite (17%). In the second studied flock, 7 out of 53 animals (13%) harboured mites from the same species. Neither squamae nor crusts were found out on the skin surrounding the attachment site of the parasites, and goats showed no clinical signs. Infected goats were

Fig 1. *Kepkatrombicula desaleri* Methlagl, 1928 - dorsal view

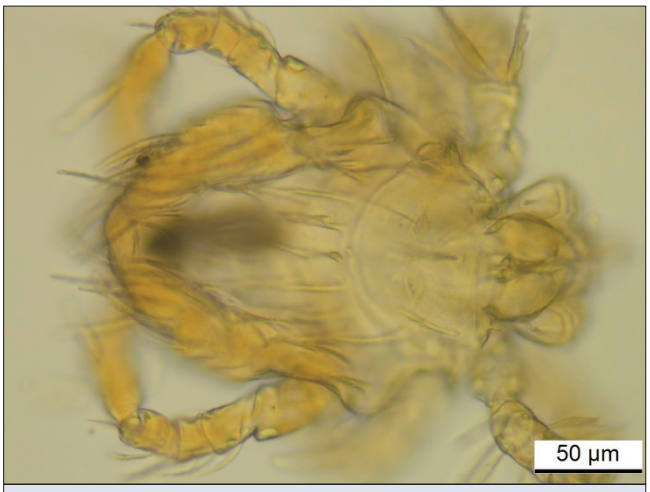

Fig 2. *Kepkatrombicula desaleri* Methlagl, 1928 - scutum

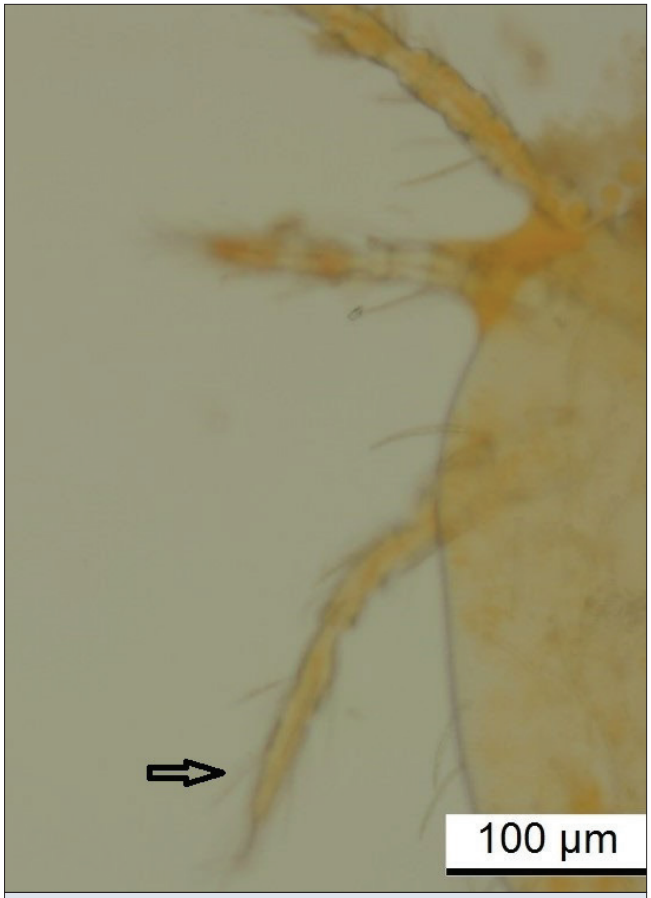

Fig 3. Elongated bristle (seta) of the tibia of the last pair of legs

from both sexes, at various ages and with different body conditions. A specific finding was that only goats with dark (black) haircoat were infested. The mean intensity of infestation was 32 mites per animal. In all goats, the mites were detected on eyelids, medial eye angles *(Fig. 4)*, nose and only in one animal on ears.

The injection of ivermectin (Pandex™ 1%, Biovet) showed no efficacy against *K. desaleri* both on the first and on the third post treatment days. The treatment with cypermethrin (Ectomin 10%, Farmavet OOD) resulted in decreased motility and rapid death of all (100%) mites only within several minutes. No mites were found on the first and the third post treatment days.

Discussion

The species affiliation of larvae detected in this study matched the data of Rehbein et al.[8] and Stekolnikov and Mumcuoglu [12]. Stekolnikov and Kar [14] identified larvae of the *Neotrombicula heptneri* Kudryashova, 1973 species on goats in Turkey, whereas according to Faccini et al.[15] caprine trombiculosis in Spain was caused by the species *Eutrombicula alfreddugesi* Oudemans, 1910 and *Eutrombicula batatas* Linnaeus, 1758. These findings made clear that goats may become infested with more

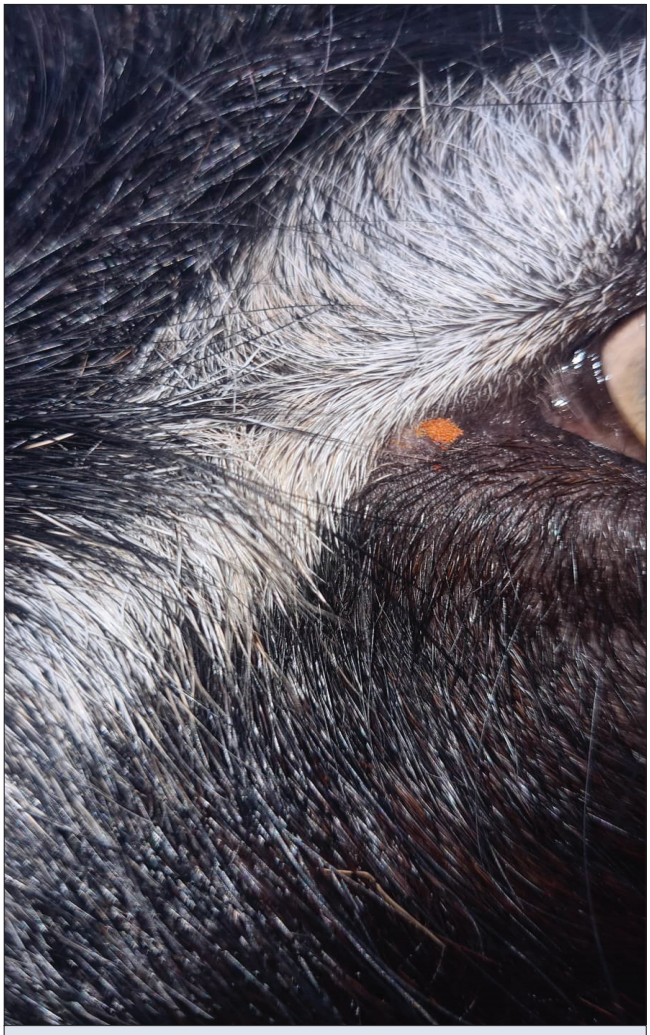

Fig 4. Localisation of *Kepkatrombicula desaleri* Methlagl, 1928 in the medial angle of the eye

than one trombiculid mite species. Additional studies are necessary to find out whether goats in Bulgaria are infested only with *K. desaleri* or other chigger species are also encountered.

In this study, the chiggers infestation was detected during the autumn, in line with data published by Gerstgraser [16] and Stekolnikov and Kar [14]. In Spain, Faccini et al.[15] reported an extensive infestation during the rainy season (March-September) which contradicts our results. This may be attributed to the different climatic conditions specific for the two studies. The same researchers also indicated humidity, rainfall and ambient temperature as factors favouring the infestation, i.e. conditions similar to those during the autumn season in our country. According to Rashmir-Raven [17], the different species from the family have various seasonal patterns.

The relatively low intensity and extensity of infestation, established in the present study, do not correspond to data of Stekolnikov and Kar [12], reporting 100% extensity

and intensity from 20 to 80 mites per animal. A possible reason for the discrepant results may be sought in factors influencing the non-parasitic (free-living) life stages of mites.

Regarding the localisation of parasites, all authors agreed that orange-red skin bumps were detected on areas sparsely covered with hair in the region of the head - eyelids, ears, nose, mouth [8,14,15]. In infestations with high intensity, mites may also colonize the back, chest, abdomen, udder and extremities [15].

According to Gerstgraser [16], goats of all age groups may be affected. This was confirmed in our study, but a more extensive survey is needed to determine any age-dependent relationships.

Clinical signs and skin alterations, as scratching, rubbing, licking, alopecia, scaly erythematous skin and papules reported by others [15] were not observed, probably because of the low intensity of invasion (n=32).

In relation to disease treatment, phenylpyrozoles and isoxazolines have shown a high efficacy against mites from this family. Fipronil, as a broad-spectrum local insecticide, resulted in removal of most chiggers within 12 h after application, whereas isoxazoline administration - for 6-8 h [18]. These drug groups are however contraindicated for use in farm animals such as goats.

Leone and Albanese [19] have used a selamectin spot-on in cats at 6 mg/kg and established 100% efficacy on the second day without recurrence of the infestation until the 60th day. Despite that ivermectin is from the same therapeutic group (macrocyclic lactones), yet applied by a different route and in a different animal species, the reported result was not observed by us. This finding is perplexing as being a strongly lipophilic compound [20], ivermectin attains a high level in the skin [21], where the parasite feeds on. Mondragón and Guzmán [22] recommended the drug for local application in men. Possibly, its inefficacy was due to the route of application (s.c.) that could hardly yield the optimum skin concentrations.

In horses, Rashmir-Raven [17] recommended permethrin, pyrethrin, cypermethrin and foxim for treatment of trombiculosis, applied through spraying or bathing. The high efficacy of cypermethrin was confirmed in our studies on goats.

The detected chigger on goats in Bulgaria, *K. desaleri,* demonstrated low intensity and extensity of infestation and was not accompanied by clinical signs. Like all periodically stationary ectoparasites, the chiggers population was strongly influenced by environmental factors. The changes in the latter may increase mite numbers with appearance of medium- to severe clinical signs reported in the literature. The efficacy of ivermectin used for treatment in the present study was poor which does not match with what has been published previously, therefore these results should be subjected to further controlled experiments. In contrast, the efficacy of cypermethrin was high (100%) and should be the treatment of choice for control and treatment of caprine trombiculosis.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (N. Nizamov) on reasonable request.

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Ethical Statement

Ethics committee approval is not required for this study as it is within the scope of veterinary practice.

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Conflict of Interest

The authors declared that there is no conflict of interests.

Author Contributions

NN: Researched the literature, and drafted manuscript; PI: Participated in laboratory analyses and revised the manuscript; HB: Conceived the work. All the authors read and approved the final manuscript.

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Research Article

Fitting Various Growth Equations to the Daily Milk Yield Data of Nili-Ravi Buffaloes and Cholistani Cows at Intake at Maintenance Levels

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ABSTRACT

This study described the daily MY in buffalo and cow under restricted feeding conditions using the five growth models (Brody, Von Bertalanffy, Logistic, Gompertz and Wood). In addition, the species-wise differences in lactation parameters were also tested. These models were fitted to the lactation data of four whole lactations (two buffaloes and two cows) using 1200 unadjusted MY records. Fitting of the model was evaluated through appropriate fitness indicators such as the adjusted R^2, Akaike's Information Criterion (AIC), Bayesian Information Criterion (BIC) and Root Means Squared Error (RMSE). The Wood's model provided the best fit of the lactation curves with logical values of parameter estimates owing to higher R^2 and lower AIC, BIC and RMSE than other equations. The Wood's model had a better fit of lactation data of cows than of buffaloes. The average estimated values for the initial MY (a), ascending phase before peak MY (b) and descending phase after peak MY (c) were 4.75, 0.238 and 0.004, and 2.56, 0.321 and 0.006 kg/day for buffaloes and cows, respectively. The magnitude of lactation parameters remained higher ($P<0.05$) in buffaloes for a, peak MY, persistency and lactation yield than in cows. The Woods' model fairly accurately described the lactation data than other equations under restricted feeding conditions, with poor fitting in buffaloes to moderate fitting in cows.

Keywords: Intake at maintenance, Growth models, Lactation curves, Species differences

Introduction

Alterations in daily milk yield (MY) are evident such that it increases from calving to the peak production and thereafter decreases smoothly until the end of lactation [1]. Knowledge of lactation curves in dairy cattle is important for decision making on herd management involving feeding and selection strategies, and it is also a key element in determining optimum strategies for insemination and replacement of dairy cows [2,3]. The data on MY has been well described in cows [1,4,5] and buffaloes [6,7] using various lactation functions [8]. Variations in the shape and form of the lactation curve arise from the factors such as genetic make-up, parity, diet, and other environmental influences [3,6,9]; the diet being one of the important factors [10,11]. The MY data described by various researchers presented data of lactating animals fed at intake at production levels [6, 11] or if there were feed restrictions, they were carried for a certain period of lactation cycle [12,13] but not for the whole length of lactation. We therefore, expected to observe different shape and form of lactation curve of lactating animals under intake at maintenance levels than intake at production levels.

To describe the milk yield, two types of models can be

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used i.e., the mechanistic ones, to describe the causative mechanism under the biology of lactation [14,15]; and the empirical ones, mainly for the quantification [6]. The selection of the model is the basic tool between the fitting biological properties of a model and its biological interpretations [16]. For instance, the mechanistic model [15] have parametric advantages for biological interpretations, but difficult to fit in when parameters showing greater standard errors and multi-collinearity. Conversely, the empirical ones can provide the acceptable solutions and adequate fits to data, but not on the biological basis. The mechanistic model proposed by Dijkstra et al.[14], may be the solution for such a gap providing fitting properties and biological interpretations [17].

The purpose of the current study was to investigate the suitability of lactation curve model in describing the daily MY data of buffaloes and cows at intake at maintenance levels. The second objective was to compare buffaloes with cows regarding differences in the magnitude of lactation parameters.

Material and Methods

Ethical Statement

All experiments were conducted according to the criteria of the University's Animal Care and Management Committee (The IUB, 2015).

Milk Yield Performance of Lactating Animals under Maintenance Feeding Regime

This study was conducted at The Department of Livestock Management, The Islamia University of Bahawalpur (29.39°N, 71.68°E), Bahawalpur, Pakistan. Four rumen-cannulated (Bar Diamond, Parma, ID, USA) animals including 2 lactating Nili-Ravi buffaloes, mean live weight (LW) = 509±43.4 kg, age = 2225±49.5 days; and 2 lactating Cholistani cows, LW = 289±29.4 kg, age = 1115±21.9 days at the start of the experiment were used for the production trial. The animals were offered a standard diet slightly above the maintenance level for meeting the requirements of milk production but not ad libitum throughout the experiment. The animals were restricted to consume dry matter at 1.80% of LW instead of 3.0% (as recommended by National Research Council, 2001), with a forage to concentrate ratio of 80:20 on dry matter basis. Ingredients and mean chemical composition of the diets are presented elsewhere [18]. The animals were confined to individual stalls, individually fed and given access to fresh clean water as per requirements. Animal were milked twice daily and MY was recorded manually by the milk man. The animals were placed in the trial from the 1st day just after parturition. Data of daily MY of 4 complete lactations with 1200 daily milk records were used which were collected between the years 2015 and 2016. Milk yield was recorded daily from day 5 to 305 after parturition and milk samples for quality analyses were collected weekly. We did not exclude data based on minimum daily MY, fat or protein content. The following formula was used for the calculation of 4% FCM of each cow: FCM = [(0.4 x kg milk) + (0.15 x kg milk x fat %)] [19]. Also, the yield of energy-corrected milk (ECM) was calculated by the following formula: ECM (kg) = kg milk x (383 x fat% + 242 x protein% + 783.2)/3140 [20]. The standard milk fat and protein contents i.e. 5.5 and 4.0%, and 4.0 and 3.73% based on data obtained using wet chemistry analyses for buffaloes and cows, respectively were used to calculate the FCM and ECM.

Model Fitting to the Lactation Data

The lactation data were cut at a standard lactation length of 305 days. No adjustments of the raw data were made i.e. outliers and out-of-range productive records were not deleted from the analyses and the models presented in *Table 1* were fitted to these data.

The models were fitted using R (ver. 4.2.1; The R Foundation for Statistical Computing, 2015), using the statement nlsfit in software package Easyreg® (easyreg:

Table 1. *Growth equations used to describe the lactation curve in buffaloes and cows*

Equation	Functional Form	Time to Peak MY	Peak MY	Persistency	Lactation Yield
Brody [21,22]	$y = a(1 - bexp^{-ct})$	---	---	---	---
Gompertz-Laird [22,23]	$y = abexp\left[\frac{b(1-e^{-ct})}{c-ct}\right]$	---	---	---	---
Logistic-Nelder [24]	$y = \frac{abc(c-b)e^{-at}}{[b+(c-b)e^{-at}]^{2}}$	---	---	---	---
Wood [5]	$y = at^{b}exp(-ct)$	b/c	$a(\frac{b}{c})^{b}exp^{-b}$	$at^{b-1}\exp(-ct)^{(b-ct)}$	$\left(\frac{a}{c}\right)^{(b+1)}(b+1)$
Von Bertalanffy [25,26]	$y = a(1 - bexp^{-ct})^{3}$	---	---	---	---

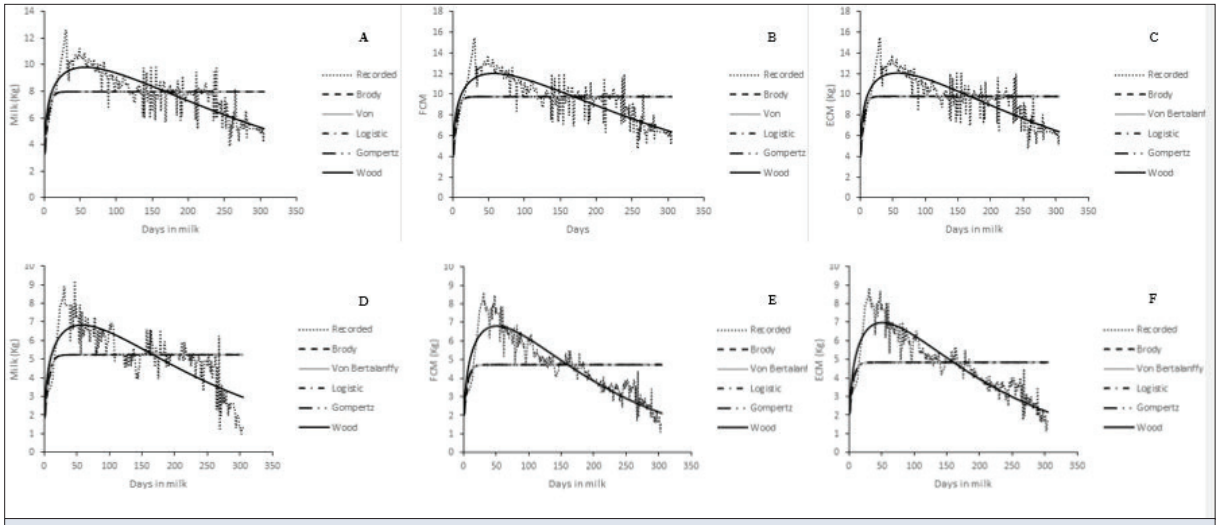

Fig 1. Fitting the growth equations on unadjusted milk, fat corrected milk (FCM) and energy corrected milk (ECM) records of buffaloes (A, B, C) and cows (D, E, F) plotted as days in milk on x-axis vs. milk yield (kg/day) on y-axis

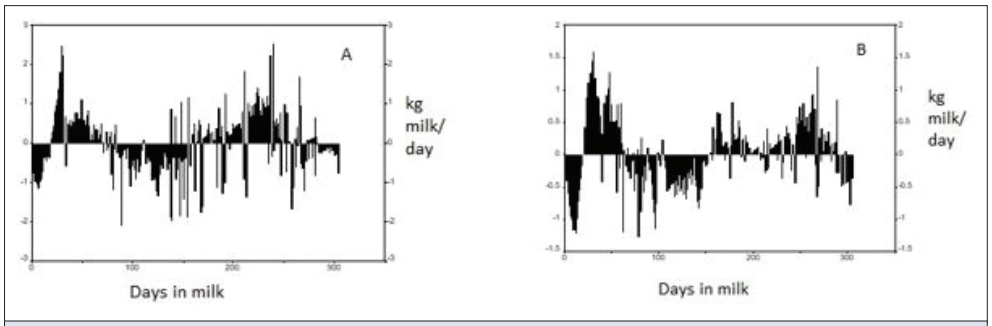

Fig 2. Plot of mean residuals after fitting the Wood's model on unadjusted milk yield records of buffaloes (A) and cows (B) (A; mean residuals = kg/day)

Easy Regression version 4.0: https://CRAN.R-project.org/package=easyreg), whereas Y denotes the MY at a given time t, a is linked to MY at the beginning of the lactation, b the ascending phase before peak MY and, c the descending phase after peak MY, and t the time from parturition. The observed and model predicted curves for all equations *(Fig. 1-A-F)* were constructed using Excel (Office 10, Microsoft Inc.) and standardized residual curves *(Fig. 2-A,B)* based only on Wood's model were constructed using TableCurve® 2D (ver. 5.0, SPSS Inc. NY).

Statistical Analyses

The statistical analyses were performed using the GLM procedure of Minitab® 16.1.1.0. The effects of species on lactation parameters were evaluated according to the model:

$$Y_{ij} = \mu + S_i + \varepsilon_{ij}$$

Where Y_{ij} is the dependent variable, μ is the overall mean, S_i is the effect of ith species and ε_{ij} is the residual error. Results were presented as least square means and were considered statistically significant when the P was ≤0.05 and the trends were considered when P was more than 0.05 but less than 0.10.

Results

The observed and model predicted goodness of fit indicators of unadjusted MY, FCM and ECM determined using various growth equations are shown in *Table 2*, *Table 3*, *Table 4*. The Wood's model provided the best fit with logical values of parameter estimates owing to the highest R^2 and the lowest Akaike Information Criteria (AIC), Bayesian Information Criteria (BIC) and Root Mean Squared Error (RMSE) than other equations. Therefore, the Wood's model was selected to calculate further lactation parameters such as time to peak MY, peak MY, lactation yield, persistency of lactation, etc. The shapes of lactation curves resulting from various growth equations are presented *(Fig. 1-A-F)*.

The observed and model predicted lactation parameter estimates of unadjusted MY, FCM and ECM determined using various growth equations are shown in *Table 2*, *Table 3*, *Table 4*. Observed MY, FCM and ECM increased from day 1 of lactation (1 DIM) to a peak a few weeks later, decreased thereafter until 305 DIM. The average MY, increased from 4.0 and 2.6 kg/day at 1 DIM to the Peak MY of 12.8 and 8.6 kg/day on 30 DIM and subsequently

Table 2. *Least square means of the model predicted and recorded lactation characteristics of unadjusted milk yield records of buffaloes and cows (The values are expressed in kg unless otherwise stated)*

Parameters	Buffalo (n=2)					Cow (n=2)					Probability	Two Sample t-test
	Milk Records (n=600)					Milk Records (n=600)						
	Brody	Von Bertalanffy	Logistic	Gompertz	Wood	Brody	Von Bertalanffy	Logistic	Gompertz	Wood	Species[a]	
A	7.97	7.97	7.98	7.97	4.75	5.23	5.24	5.24	5.24	2.56	0.035	
B	0.732	0.317	1.643	1.086	0.321	0.775	0.351	2.055	1.239	0.238	0.013	
C	0.214	0.233	0.274	0.243	0.004	0.178	0.195	0.231	0.203	0.006	0.635	
Adj R^2	0.05	0.05	0.05	0.05	0.57	0.04	0.05	0.05	0.05	0.64	0.065	
AIC	2525	2524	2523	2524	2041	2277	2276	2274	2275	1679	0.115	
BIC	2542	2542	2541	2542	2059	2295	2293	2292	2293	1696	0.115	
RMSE	1.91	1.91	1.90	1.90	1.28	1.55	1.56	1.56	1.56	0.95	0.043	
ED											<0.001	3.23
Lactation yield (modeled)					7927					3937	0.004	
Lactation yield (recorded)					2404					1421	0.022	
Peak MY (modeled)					9.81					6.84	0.017	
Peak MY (recorded)					12.80					8.61	0.013	
Time to peak MY (modeled) days					57					51	0.459	
Time to peak MY (recorded) days					30.5					31.0	0.443	
Persistency of lactation					1.67					1.11	0.025	

A = initial milk yield, B = incline in milk yield before peak, C = decline in milk yield after peak, MY = milk yield, AIC = Akiake Information Criteria, BIC = Bayesian Information Criteria, RMSE = Root Mean Squared Error, ED = Estimate of difference was calculated using 2 sample t-test; a = effect of species of the milking animal (buffalo vs. cow)

declined to 5.0 and 1.6 kg/day on 305 DIM for buffaloes and cows, respectively. The Wood's model provided the closely related lactation parameter estimates to the observed values than other equations did. The average estimated values of a, b and c were 4.75, 0.238 and 0.004, and 2.56, 0.321 and 0.006 kg/day for buffaloes and cows, respectively. The observed time to peak MY was 30 DIM for each species with 2946 and 1421 kg total MY during a 305-days lactation period for buffaloes and cows, respectively. The Wood's model under-predicted the peak MY and over-estimated the time to peak MY and lactation yield in both species.

The wood's model had a better fit of lactation data for cows than of buffaloes with higher R^2 (P=0.070), and lower AIC, BIC and RMSE (P<0.05). The magnitude of lactation parameters remained higher (P<0.05) in buffaloes for a, peak MY, persistency and lactation yield than in cows. Model predicted time to peak MY remained unchanged (P>0.05) between buffaloes and cows. The magnitude of b and c were greater in cows than in buffaloes (P<0.05). The fitness of the model to individual species milk data were also compared by standardized residual curves (*Fig. 2-AiB*), respectively. The average residuals distributed uniformly around the zero in case of cows, but distributed widely in case of buffaloes. Whereas the model slightly overestimated MY from 1 to 20 DIM for both species, followed by an under-estimation from 20 to 80 DIM in buffaloes and from 20 to 52 DIM in cows. The model again over-estimated the MY from 80 to 165 DIM in buffaloes and from 52-155 DIM in cows, followed by an under-estimation until the end of lactation. The shape and form of the curve (lactation parameter estimates) in FCM and ECM remained the same as that of unadjusted MY except that average estimated values of a, peak MY and lactation yields were improved *(Table 3, Table 4)*.

Table 3. *Least square means of the model predicted and recorded lactation characteristics of unadjusted milk yield records of buffaloes and cows (The values are expressed in kg unless otherwise stated)*

Parameters	Buffalo (n=2)					Cow (n=2)					Probability	Two Sample t-test
	Milk Records (n=600)					Milk Records (n=600)						
	Brody	Von Bertalanffy	Logistic	Gompertz	Wood	Brody	Von Bertalanffy	Logistic	Gompertz	Wood	Species[a]	
A	9.77	9.77	9.77	9.77	5.82	4.72	4.72	4.73	4.72	2.32	0.037	
B	0.732	0.317	1.643	1.086	0.367	0.704	0.304	1.581	1.042	0.238	0.019	
C	0.214	0.233	0.274	0.243	0.004	0.202	0.216	0.247	0.224	0.007	0.635	
Adj R^2	0.05	0.05	0.05	0.05	0.57	0.02	0.02	0.02	0.02	0.68	0.070	
AIC	2773	2772	2771	2772	2289	2467	2466	2466	2466	1776	0.445	
BIC	2791	2790	2789	2790	2307	2484	2484	2483	2484	1794	0.445	
RMSE	2.34	2.34	2.34	2.34	1.57	1.82	1.82	1.82	1.82	1.03	0.048	
ED											<0.001	4.91
Lactation yield (modeled)					10194					3811	0.001	
Lactation yield (recorded)					2946					1421	0.022	
Peak MY (modeled)					12.02					6.81	0.017	
Peak MY (recorded)					15.68					8.61	0.013	
Time to peak MY (modeled) days					57					51	0.349	
Time to peak MY (recorded) days					30.5					31.0	0.443	
Persistency of lactation					3.51					1.84	0.025	

A = initial milk yield, B = incline in milk yield before peak, C = decline in milk yield after peak, MY = milk yield, AIC = Akiake Information Criteria, BIC = Bayesian Information Criteria, RMSE = Root Mean Squared Error, ED = Estimate of difference was calculated using 2 sample t-test; a = effect of species of the milking animal (buffalo or cow)

Discussion

Although the number of animals included in the study seems small and there might be larger data sets than ours in studies where shape and form of lactation curves were analyzed. However, a number of 300 MY records per animal per lactation indicate that data are suitable, albeit not optimal, for this type of analysis.

Since the shape and form of lactation curve changes with the genetic make-up of animals, parity number, diet regime and other environmental factors [6,11,27], the choice of growth model to describe the lactation data of example group of lactating animals is very critical. In the present study, all equations other than the Wood's had poor fitting to the lactation data. The common understanding with the use of growth equations to lactation data is to mimic growth of mammary tissues to that of general body taking at intake at ad libitum feeding. Since the animal in the current study were fed at intake at maintenance levels, the growth of mammary glands might have not mimicked the general body growth, thereby, poor fitting of the growth equations. From the evaluation of the different equations used in the current study, it is evident that the non-linear growth equations except that of Wood had poor potential for fitting MY records of buffaloes and cows under intake at maintenance levels. It is therefore suggested that models which can account for the level of feed intake by the animal may be developed to better describe the data under restricted feeding conditions.

Aziz et al.[28] reported higher a value in Egyptian buffaloes, while b and c were consistent with our results. Contrarily, Anwar et al.[8]'s findings of all these parameters are highly

Table 4. *Least square means of the model predicted and recorded lactation characteristics of unadjusted milk yield records of buffaloes and cows (The values are expressed in kg unless otherwise stated)*

Parameters	Buffalo (n=2)					Cow (n=2)					Probability	Two Sample t-test
	Milk Records (n=600)					Milk Records (n=600)						
	Brody	Von Bertalanffy	Logistic	Gompertz	Wood	Brody	Von Bertalanffy	Logistic	Gompertz	Wood	Species[a]	
A	9.79	9.80	9.80	9.80	5.83	4.84	4.84	4.84	4.84	2.38	0.038	
B	0.732	0.317	1.643	1.086	0.367	0.704	0.304	1.581	1.042	0.238	0.020	
C	0.214	0.233	0.274	0.243	0.004	0.202	0.216	0.247	0.224	0.007	0.750	
Adj R^2	0.05	0.05	0.05	0.05	0.57	0.02	0.02	0.02	0.02	0.68	0.069	
AIC	2776	2776	2775	2776	2293	2497	2496	2495	2496	1806	0.337	
BIC	2794	2793	2793	2793	2310	2514	2514	2513	2514	1823	0.337	
RMSE	2.35	2.35	2.35	2.35	1.58	1.87	1.87	1.87	1.87	1.06	0.041	
ED											<0.001	5.0
Lactation yield (modeled)					10216					3947	0.002	
Lactation yield (recorded)					2955					1456	0.022	
Peak MY (modeled)					12.05					6.98	0.017	
Peak MY (recorded)					15.74					8.82	0.013	
Time to peak MY (modeled) days					57					51	0.449	
Time to peak MY ((recorded) days					30.5					31.0	0.443	
Persistency of lactation					6.25					4.39	0.025	

A = initial milk yield, B = incline in milk yield before peak, C = decline in milk yield after peak, MY = milk yield, AIC = Akiake Information Criteria, BIC = Bayesian Information Criteria, RMSE = Root Mean Squared Error, ED = Estimate of difference was calculated using 2 sample t-test, [a] = effect of species of the milking animal (buffalo or cow)

valued compared to this study's results. This difference in the results can be due to the parity and production conditions. Tekerli et al.[27] reported lower lactation yield, peak MY and time to peak MY, and higher persistency in lower parity animals, whereas the opposite trends for these parameters were found in higher parity animals. We found a smaller magnitude of b and c in buffaloes compared to cows. These two parameters are an indirect measure of persistency in lactation and therefore our data suggested greater persistency of lactation in buffaloes than local cows, which are similar to the findings of Khan & Chaudhry [29]. The Woods' lactation parameters b and c are directly correlated with lactation length and parity, and greater lactation length and parity are translated into sharper b and slower c in pre and post-peak lactation periods [8].

In the present study, the modeled peak MY was under-estimated than observed one in both species. These findings are consistent with those of Anwar et al.[8] and Dematawewa and Dekkers [17] who found that Wood's model under-predicted MY than the observed at farm. However, Boujenane [30] found no difference among the modeled and observed lactation yields in Holstein-Friesian dairy cows. Moreover, under-estimation was more prominent in buffaloes than in cows ($P<0.05$). In a similar way, model predicted time to peak MY was over-valued in both species, and numerically, this estimation was poorer in buffaloes than cows. Similar to our findings, other researchers also reported under-estimation of peak MY and over-estimation of the time to peak MY [31,32]. The current study findings of lactation yield in buffaloes are in agreement with the results reported by Sezer et al.[9]. However, lactation yield in buffaloes was lower than the yield determined by Khan [33], and higher than recorded by Anwar et al.[8], Khan and Chaudry [29] in Nili-Ravi buffaloes and by Şahin et al.[34] in Anatolian buffaloes. In Cholistani cows, the complete lactation yields were 1st reported by

Ashfaq et al.[35] who found greater values of lactation yield than ours. This difference in the standardized lactation yields can be attributed to factors related to genetics, feeding and individual animal variability [10]. The model predicted data showed more persistency in lactation for buffaloes ($P<0.05$) which is evident from the values of b and c.

In this experiment, Woods' model was fitted to the buffaloes and cows' complete lactation length. Preliminary assessment of goodness of fit indicators such as greater adjusted R^2 and lower AIC, BIC and RMSE in case of cows indicated a trend ($P<0.10$) of better fitting of the model to the lactation data of cows than of buffaloes. These results are similar to the findings of other researchers [8,17,28,34], indicating poor fitting of the Woods' model to the lactation data in buffaloes. On contrary, Soysal et al.[36] reported that the Woods' model had a best for lactation data in Italian origin buffaloes.

Fig. 2-A and B showed larger variations (spread between 0±2.5 kg milk/day) in the spread of standardized residuals in buffaloes than in cows (spread between 0±1.5 kg milk/day). To adequately fit the model to the data, the residuals have to oscillate on both sides showing no trend [30]. It is also apparent that the divergence of residuals was higher around the peak MY and around the end of lactation than around the mid lactation. This deviation was prominently higher in buffaloes and decreased rigorously in cows. These results are similar to Cole et al.[37]'s findings, who all reported a wider range of residual data around the two mentioned stages of lactation using the Woods' model. Inclusion of as many as possible fitness indicators in the regression model improves description of the data and aids in decision making [38,39].

It is concluded that the Woods' model has the potential to fairly accurately describe the lactation data of buffaloes and cows, under restricted feeding conditions, among all growth equations used. Even though, the model under-predicted milk yields in the beginning, around the peak and at the end of lactation than the observed one. The model showed a moderate fitting to the lactation data in cows whereas a poor fitting in buffaloes. Further, the indication of relatively smaller changes in pre- and post-peak milk yields, suggests more persistency in milk yields in buffaloes.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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Ethical Approval

All experiments were conducted according to the criteria of the University's Animal Care and Management Committee (The IUB, 2015).

Competing Interests

No potential conflict of interest was found by the authors.

Author's Contributions

ZK conducted this experiment under the supervision of SN, TNP, JAB and MNT. NS, RR and MNT conducted the statistical analyses. MÖ and TŞ helped MNT and RR in writing, reviewing and formatting the manuscript.

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Research Article

Seasonal Gene Expression Profile Responsible for Hair Follicle Development in Angora Goats

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ABSTRACT

Mammals have physiological reprogramming adaptation ability to changing seasonal light and temperature, through their biological clocks maintained by circadian rhythm, photoperiodism and thermoperiodism. Seasonal differences do not only affect vital activities of animals like migration, reproduction, and sleeping, but also cause dramatic changes in their economically important characteristics (e.g. fur quality and fattening level). Mohair is constituted of non-medullary hairs produced by secondary hair follicles in Angora goats and the effects of seasonal differences on mohair structure and related genes are still unknown. We examined the gene expression levels of *BMP-2, FGF-5, HOXC13, KAP9.2 and TGFBR2* normalized with *GAPDH* in skin biopsies taken from Angora goats (n=20) in two different follicle development stages; telogen (in February) and anagen (in June). *HOXC13* showed overexpression in anagen phase ($P<0.005$) whereas expression was undetectable in telogen phase. *BMP-2* ($P<0.005$), *FGF-5* ($P<0.005$) and *TGFBR2* ($P<0.01$) were significantly upregulated in anagen, while *KAP9.2* expression showed no difference between two phases. This is the first study on hair follicle-related genes in the Angora goat. Additionally, depending the role of HOXC13 in pathways, it suggests that its overexpression may be one of the main factors associated with the non-medullary hair structure in Angora goats.

Keywords: Anagen, Hair follicle development, Mohair goat, Secondary hair follicles, Telogen

Introduction

Goat fiber is the most luxurious fiber in the world and is divided into two major products; Cashmere and Mohair, which are obtained from unique breeds [1]. However, many goat breeds yield less-valuable goat hair that is used mainly to produce felts, carpets, and tents. Due to its silky-like structure, heat resistance, easy dyeability and unique luster with a fine texture, mohair is one of the most preferred raw materials for the textile industry [2,3]. Mohair is produced by the Angora goat that originated from the district called Angora in Anatolia (present-day Ankara, the capital city of Türkiye).

The phylogenetic studies showed that maternal and paternal origins of the Angora goats were common with the other native goat breeds and the results pointed out Türkiye as the area of domestication and breeding center [3-7]. The beauty and eye-catching features of Angora goats have been well described in written records found in Sumerian cuneiforms [2]. During the Ottoman Empire period, the Angora goat was presented as a precious gift to other kingdoms and empires. Nowadays, Angora goats have been raised foremost in Turkey, United States, New Zealand and Argentina, for mohair production [8]. Although weaving with mohair is known as one of the oldest handicrafts of Anatolia and women have played an important role in this tradition for thousands of years, breeding Angora goats and producing of mohair are becoming values being more neglected and forgotten in Türkiye [8,9].

Animals show specific seasonal adaptation features according to light and temperature changes, and these physiological mechanisms are mainly coordinated by circadian rhythm, thermoperiodism and photoperiodism [10,11]. These factors do not only affect many vital activities such as hibernation and reproduction, but also affect the traits of economic importance e.g. fiber quality, fattening and milk yield [11]. Fiber production and

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hair follicle (HF) development are mainly controlled by increased levels of melatonin hormone secretion during shortened daylight in autumn and winter [10,11]. There are two types of HF; primary hair follicles (PHF) and secondary hair follicles (SHF), the latter provides the non-medullary characteristics of Mohair [12].

There are three phases in the hair follicle development cycle: anagen (growth), catagen (regression), and telogen (quiescence) [13,14]. In goats, SHF forming the cashmere and mohair fibers remain in the active anagen phase between June and November (approximately 185 days), followed by the catagen phase between December and January (approximately 60 days), then from February to the end of May, the telogen phase proceeds (approximately 120 days) [15,16]. Differentiated gene expressions are also closely linked with hair follicle proliferation, growing and falling phases in the epidermal and mesenchymal cells. Despite the differentially expressed genes in follicle development phases that have been previously described and several genes were pointed out as key genes in the regulation of HF morphogenesis in Cashmere goats [17-19]. It has been shown that Homeobox C13 *(HOXC13)* was essential for hair shaft differentiation [20]. Bone Morphogenetic Protein-2 *(BMP-2),* Fibroblast Growth Factor-5 *(FGF-5)* and Transforming Growth Factor Beta Receptor-2 *(TGFBR2)* were associated with hair growth cycle regulation [18,19], while Keratin-Associated Protein-9.2 *(KAP9.2)* was responsible for keratinization [21]. However, there is no information on these genes for the mohair.

In the present study, it was aimed to examine the gene expression levels of *BMP-2, FGF-5, HOXC13, KAP9.2* and *TGFBR2* genes in the skin biopsies from Angora goats between two different follicle development periods to shed light on mohair development.

Material and Methods

Ethical Statement

All the procedures were carried out in accordance with the approval of the Animal Welfare Act. Ethical Committee of Ankara University, with the approval number 2014-18-137.

Sample Collection

In this study, skin biopsies were collected from 20 female Angora goats (2-3 years old), without at least 3 generations of common relatives according to their official records and best representing nationally registered breed characteristics in terms of hair, body size, and morphological characteristics. The goats were sampled from Güdül district of Ankara, which is mainly breeding area of Angora goats under the breeders condition and governmental inspection to protect breed characteristics. Before sampling hairs were trimmed and the skin surface was disinfected biopsies were taken from the right thoracic region using a 5 mm diameter sterile punch biopsy and the skin was sutured with a disposable alloy stamp after the biopsy procedure. The time schedule of the sampling has been determined considering the months with the highest and lowest annual temperatures and day lengths according of the studied geographic location [22] *(Fig. 1)*. According these parameters, telogen and anagen follicle development phases were sampled in February and June, respectively.

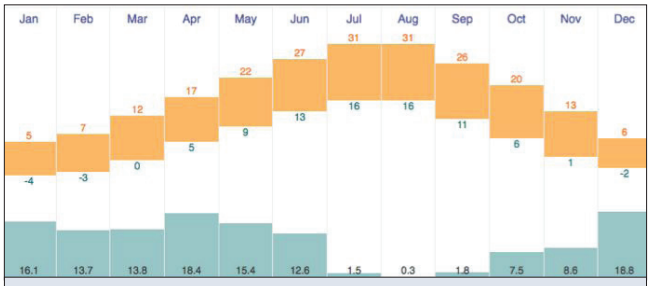

Fig 1. Annual weather averages near Ankara. The data was based on weather reports collected during 1985-2015. Orange lines represent lowest and highest temperatures (°C), green lines represent precipitation levels (mm)

Nucleic Acid Extraction and cDNA Synthesis

Skin samples (approximately 50 mg) were stored in cryovials immersed into liquid nitrogen (-196°C) to inhibit/stop RNAse activity. Tissue samples were homogenized using a pestle and mortar in liquid nitrogen and RNAs were extracted using PureZol, a monophasic combination of phenol and guanidine isothiocyanate (Biorad, USA, Cat no 7326890), according to the manufacturer's instructions. Obtained RNAs were treated with DNase (ThermoFisher, Germany, Cat no EN0525) and 1µg of total RNA was converted to cDNA in a reverse transcription (RT) reaction using the iScript cDNA Synthesis Kit (Biorad, USA, Cat no 1708891). Nucleic acids were measured by NanoDrop C2000 (ThermoFisher, Germany), visualized in Safe-View (NBS Biologicals, England, Cat no NBS-SV1) stained 1% agarose gel electrophoresis. The A260/A280 ratio of 1.8-2.1 was used to indicative assess RNA purity, while the integrity of the RNA was evaluated by 28S/18S ratio close to 2 to be indicative of intact RNA.

Histopathological Analysis

For histopathologic analyses, the frozen samples (half parts of skin biopsies) were directly transferred into cold 10% buffered formalin and fixated for 24 h. Following tissue processing procedure, tissues were dehydrated in degraded alcohol series, and cleared in xylene series, and they were embedded in paraffin (Leica TP1020). Paraffin

Table 1. *Designed oligonucleotides, amplicon length and Genebank accession numbers*

Gene	Sense Primer (5'-3') Antisense Primer (5'-3') TaqMan Probe (bold letters)	Amplicon Size, bp	Accession Number
BMP-2	ACACAGTGCGCAGCTTTCAC AAGAAGAATCGCCGGGTTGT **TCCCACTCATTTCCGGCAGTTCT**	82	NM_001287564.1
FGF-5	CCTCAGCACGTCTCTACCCA GACTTCTCCGAGGTGCGGAA **TCAAGCAATCGGAGCAGCCGGAACT**	145	XM_013964679.2
HOXC13	GCCCACCTCTGGAAGTCTCC TTGCTGGCTGCGTACTCCTT **TGCGCCCGCGCCTGTAGCTGT**	140	XM_018047656.1
KAP9.2	TGACCACCTGCTGTCAACCC CAGCTGGACCCACTGAAGGT **CCACAGCTGCTGGACCCACAGCAGGT**	70	XM_018065084.1
TGFBR2	ATCACGGCCATCTGCGAGAA GCAGACCGTCTCCAGTGTGA **CAGCCACGCAGACCTCCTCCGGC**	87	XM_018067217.1
*GAPDH**	GCATCGTGGAGGGACTTATG CAGTAGAAGCAGGGATGATGTT **ATCACTGCCACCCAGAAGACTGTG**	129	AJ431207.1

** Internal control gene*

blocks were sectioned at a thickness 6-8 µm and stained with hematoxylin-eosin [23]. Histopathologic evaluation was performed using a light microscope (Trinocular Olympus BX51 microscope attached with DP25 digital camera) for the hair follicle development phases and hair morphologic structures.

q-PCR and Measurement of Expression Levels

To analyze seasonal effects on the molecular regulation of hair follicle cycling, *BMP-2, FGF-5, HOXC13, KAP9.2* and *TGFBR2* genes were selected based on Wnt, activin/BMP, and TGFB signaling pathways according to the previous comparative transcriptomic study in skin of Cashmere goats [19]. The specific oligonucleotides and TaqMan probes were designed according to mRNA sequences of the genes in concordance with exon-exon junction regions by using Genscript [24] *(Table 1)*. *GAPDH* was used as a reference gene to normalize the gene expressions [25].

Quantitative real-time PCR (qPCR) was performed in duplicate using the CFX96 Connect real time system (Biorad, USA). A 20 µL reaction mix containing 20 ng of cDNA template, 1x SsoAdvanced Universal Probes Super mix (Biorad, USA, Cat no 1725280), 0.25 µM each primer and 0.2 µM specific Taqman probe were amplified at the following conditions, initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10 sec, annealing at 68°C for 10 sec, and extension at 72°C for 10 sec. Amplification was completed by an additional cycle at 72°C for 30 sec. To evaluate the amplification specificity of TaqMan primers, melting curves were generated in the range of 65°C to 95°C, with the temperature increasing at a rate of 0.5°C/sec. Differentially expressed gene levels were analyzed by using relative gene expression analysis with $2^{\Delta\Delta Ct}$ [26]. A Wilcoxon two-group test was performed to determine the significance of the differences between the two groups, due to the small sample size. In order to further explore the molecular mechanism of the proteins in hair follicle development, a biological pathway was evaluated from wikipathway [27].

Results

Histological Findings

The follicle development cycle was classified as anagen (growth), catagen (regression), and telogen (quiescence). Anagen follicles in the deep dermis have fully developed sebaceous glands and well-identified inner and outer root sheaths *(Fig. 2-A)*. The catagen phase frequently showed a transitional period from growth to rest periods and characterized dermal papilla condensation and thickness in basement membranes of follicle epithelia. The shrinking outer root sheath is highly degenerative and had a characteristic hyaline vitreous membrane *(Fig. 2-B)*. Well-defined distinct inner and outer root sheaths indicated the anagen *(Fig. 2-C)*. Follicles characterized by wrinkled inner root sheath appearing as an amorphous keratin mass (trichilemmal keratinization) were considered as telogen phase *(Fig. 2-D)*.

qRT-PCR Analysis and Statistics

Statistical analyses were performed on log fold-changes (ddCTs), and fold changes ($2^{\Delta\Delta Ct}$) were illustrated as bar graphic *(Fig. 3)*. To compare anagen and telogen,

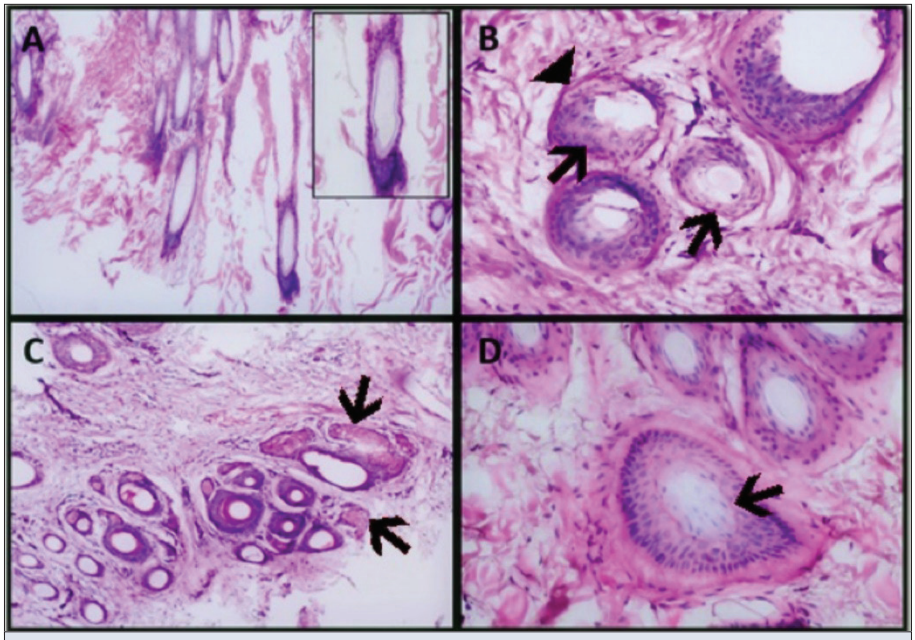

Fig 2. Classification of hair cycle stages in Angora goats using transverse hair follicle sections. Results were examined by HE staining. **A.** Deep dermis anagen (growth phase) follicles, well-defined inner and outer root sheats, **B.** Degenerative outer root sheaths *(black arrows)* and hyaline membrane *(arrowhead)* in the catagen phase, **C.** Well defined distinct inner and outer root sheaths indicate anagen. Advanced sebaceous glands *(black arrows),* **D.** Telogen phase characterized by an inner root sheath that appears as an amorphous keratin mass *(black arrow)*

descriptive statistics and ddCTs of the genes normalized to *GAPDH* were presented in *Table 2*. It was determined that *BMP-2* (P<0.005), *FGF-5* (P<0.005) and *TGFBR2* (P<0.01) were significantly upregulated in anagen, while no significant change was observed between two phases for *KAP9.2* gene expression (P>0.05). A high level of expression was determined for *HOXC13* in the anagen phase (P<0.005), whereas expression was not detected in the telogen phase. To estimate the relative quantification, Ct values were accepted as 40 for this gene in the telogen phase.

Discussion

Mohair production and intense Angora goat breeding are currently made in many countries in the world and the processed mohair products have been considered exclusively unique and their economic value is at the utmost level. Albeit Turkey has excellent preciousness as being the first goat domestication and mohair goat production site [3-7], due to improper agricultural policies and socio-economic conditions, losing its advantages in terms of qualified mohair production.

The ratio of primary/secondary follicles per mm² in Angora goat skin is between 5.3 to 9.1. The hair of the Angora goat differs from other wool fibers by their homogenous mohair production, which is relatively close to each other, due to the low amount of medullary fibers and the high number of secondary hair follicles that solely produce mohair. The cuticle, cortex, and medulla layers of mohair fiber are the same as in other fibers. The cells in

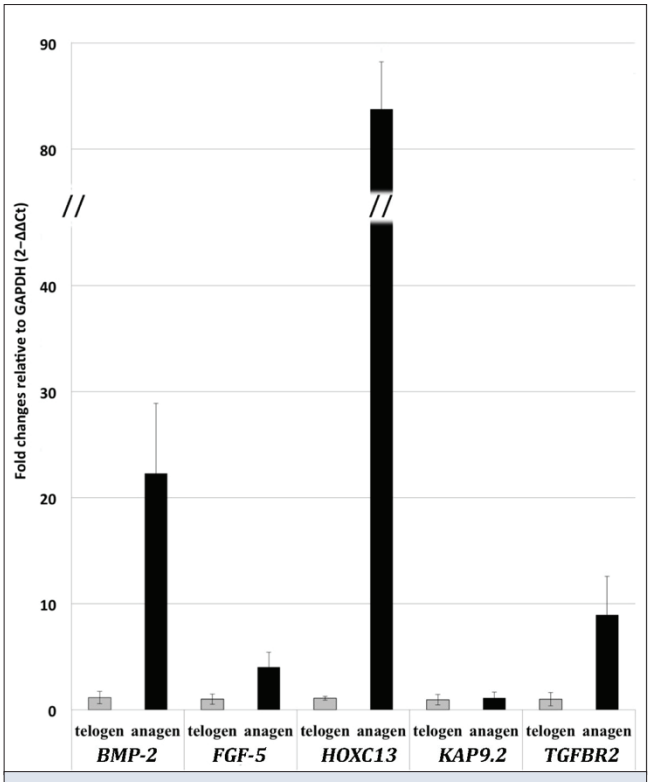

Fig 3. Bar graph presentation of fold change of *BMP-2, FGF-5, HOXC13, KAP9.2* and *TGFBR2* genes in June samples (anagen) compared to February samples (telogen). All Ct values are normalized to *GAPDH* and expressed as change from their respective controls

Table 2. *Log fold-changes (ddCTs) of the genes normalized to GAPDH in anagen compared to telogen phase. The mean ddCT values and standard deviations were estimated on two technical replicates in each condition*

ddCT Descriptive Statistics	***BMP-2***	***FGF-5***	***HOXC13***	***KAP9.2***	***TGFBR2***
Mean	4.4268	2.0423	6.3797	-0.1104	3.0485
Standart deviation	0.4020	0.5233	0.5479	1.1275	0.6171
P-value	0.0026**	0.0034**	0.0049**	0.4473	0.0051*

*Significant difference is indicated by asterix, *P<0.01; **P<0.005*

the cuticle layer are thin, wide in morphology and do not fold over each other, causing the mohair to be brighter and softer. The fact that the cortex layer has a small number of orthocortex cells in the mohair fiber enables the fiber to take dye easily. Mohair fiber has a discontinuous type of medulla. The quality of the mohair decreases when the medulla fiber ratio exceeds 4% [28]. In the present study, it was demonstrated that the hair follicles were not containing a prominent medulla typical of Angora goat mohair. Skin biopsies collected during winter months showed that telogen phase hair follicles histology, while their morphology changed to anagen phase in the summer months. In this study, skin biopsies were stored in liquid nitrogen and then they were transferred to frozen sectioning for histologic analysis. Thus, albeit the section quality was enough for the histologic evaluation of follicle structures, especially collagen fibers and epithelia were not in the desired consistency and showed some understaining features with hematoxylin eosin. It is also suggested that skin biopsies frozen in liquid nitrogen might be suitable for prolonged periods, at least 6 months, for histologic evaluation, if they are forwarded to the frozen sectioning.

Homoebox *(Hox)* genes are evolutionarily conserved transcription factors that regulate cell fate during embryonic development. In mammals *Hox* genes are clustered into four groups through a-d and genes are divided into 13 paralogous groups [29]. In the later embryonic stages in mice, *HOXC13* expression was found in all body hair follicles, in the filiform papillae of tongue epithelium and the footpad epidermis, while in the postnatal stage, expression was determined in anagen hair follicles, mainly in the matrix of the hair bulb and the precortical region of the hair shaft [30], proving its importance in hair growth cycle regulation. In Cashmere goats, though the measured expression of *HOXC13* was higher in telogen, expression was determined in both phases and even higher expression was determined in low fleece yielding Cashmere goats [21]. However other studies [20,31] found that *HOXC13* expression was higher in anagen, compared to catagen and telogen. In the presented study, in contradiction to previous findings, *HOXC13* gene expression was measured only in the anagen phase. Moreover, Tkatchenko et al.[32] speculated that overexpression of *HOXC13* inhibits hair follicle specific gene/genes. The downregulation caused by inhibition acts as negative feedback circuits. In concordance with the non-medullary SHF results of microscopy, we speculate that overexpression of *HOXC13* might lead to the same effect, for non-medullary HFs in mohair goats. The biological pathway [27] showed that *HOXC13* was related indirectly via *FOXQ1* and interestingly *FOXQ1*-null mice showed non-medullary HFs and satin hairs [33]. Non-medullary HFs resulting in silky and satin texture of mohair are important characteristics of Angora goat [22,34]. To understand the genetic mechanism behind this formation, HOXC13 and FOXQ1 proteins should be structurally investigated and those amounts should be measured in different phases of HF development in Angora goats.

Transforming Growth Factor Beta proteins (TGFBs) have vital importance in the regulation of the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis [35]. The lack of TGFB2 protein in mice results delay in HF morphogenesis and also decreases the number of HF [36]. Oshimori and Fuchs [37] also showed that conditional loss of TGFBR2 led to a prolonged telogen phase and delayed anagen initiation in TGFB2 signaling-deficient mice. Consistent with the literature, we found that *TGFBR2* was present in telogen, however, significant expression was measured in the anagen (P<0.01).

BMPs belong to the TGFB superfamily and TGFB/BMP interactions have been shown to play a central role in hair shaft growth and differentiation [18]. *BMP-2* expression is important for early embryonic development, maintaining homeostasis and cell fate in adults [38]. Also, in HF stem cells TGFB and BMP activation cause proliferating keratinocytes to transiently withdraw from the cell cycle thus changing the HF phase [18,39]. Li et al.[31] reported that *BMP-2* was highly expressed during the anagen by analyzing with time-course RNA-seq analysis on skin biopsies of Inner Mongolia cashmere goats. In the comparison of anagen and telogen phases for the expression of *BMP-2* gene, we have determined a strong upregulation in the anagen (P<0.01). Similarly, Su et al.[40] also determined an upregulation in secondary hair follicles in early anagen of Cashmere goats. Obtaining data from Angora goats is compatible with this knowledge.

FGF-5 is a signaling protein during the hair growth cycle, which inhibits hair growth by blocking papilla cell activation [41]. The *FGF-5* gene is associated with the Angora phenotype (long hair coat) in mice [42], long furred breeds of cats [43] and dogs [44], and as well as trichomegaly in humans [45]. Silencing of the *FGF-5* gene showed an increase in not only hair length but also the number of SHF in Cashmere goats [46]. Guo et al.[47] reported that one novel SNP (c.-253G>A) in the 5′-UTR of *FGF-5* resulted in a premature protein and was likely a causal variant for the long hair phenotype of cashmere goats. In the present study, *FGF-5* showed a moderate upregulation in the anagen ($P<0.01$). This result was consistent with the study of Zhang et al.[48]. Deep sequencing of *FGF-5* and revealing possible variants in Angora goats might contribute to the understanding of the angora phenotype.

According to relative gene expressions, all genes upregulated during the anagen stage except for *KAP9.2* gene. As a member of a KAP family consisting of 27 families with 100 genes, *KAP9.2* gene is responsible for keratinization [49]. Liu et al.[50] analyzed expression levels of *KAP9.2* in anagen, catagen, and late telogen in sheep and showed statistically significant differences between HF development phases. Even though studies determined the importance of *KAP9.2* expression in Cashmere goats as well [21], unlikely, our data didn't show any differences in the expression level of this gene in Angora goats between anagen and telogen phases. Wang et al.[21] indicated that lower expression of *KAP9.2* gene during anagen stages resulting higher yields in cashmere goats than that of their catagen stages. Thus, this is the clear indication of *KAP9.2* gene activity shows seasonal differences in cashmere goats. Moreover, it is also evidenced that the goat hairs in anagen stages having low *KAP9.2* gene expression yield high-quality cashmere, than that of individuals having higher *KAP9.2* gene activity in anagen stages. The fine and non-medullary structure of Angora goat's hair, has enhanced its quality and well-curling nature. Low levels of *KAP9.2* during both anagen and telogen stages in the present study can be explained by the quite different hair morphology of Angora goat breed, which has no hair medulla and fiber pigmentation. As far as the authors' knowledge, *KAP9.2* gene analysis and its correlation between the hair developmental stages in Angora goats have not been studied before, and this finding is indeed interesting as it can be considered for the breed genetic difference and may give great information on the development of Angora hair's constant quality. However, the authors believe that this novelty needs confirmation in the future.

In conclusion, the comparison of some gene expression levels in anagen and telogen phases; *FGF-5, TGFBR2* and *BMP-2* expressions are significantly up-regulated in anagen of the skin biopsies from Angora goats. Between the two phases for *KAP9.2* expression showed no difference. The *HOXC13* was strongly overexpressed in anagen and was undetectable in telogen. This is the first study on hair follicle-related genes in the Angora goats. The comparative studies including different geographical zones and further analyses based on omics technologies can improve our understanding of mohair regulation and secondary hair follicle formation.

Availability of Data and Materials

The authors declare that the data and materials are available on request from the corresponding author (B. Çınar Kul).

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Competing Interest

The authors declared that there is no competing interest.

Author Contributions

Experimental design was performed by BCK, NB and OK, material preparation and analysis were performed by BCK, NB, MYA, OSC, OO, MB, OK, results were interpreted by BCK and OK, the first draft of the manuscript was written by BCK and OK, all authors contributed to the final version of the manuscript.

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RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Analysis of a Novel *Porcine Epidemic Diarrhea Virus* Circulating in Large-scale Pig Farms in Xinjiang, China

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ABSTRACT

Porcine epidemic diarrhea (PED) is a highly contagious disease caused by *porcine epidemic diarrhea virus* (PEDV), which is characterized by severe diarrhea and vomiting in lactating piglets, resulting in serious economic losses to the pig industry worldwide. Here, a novel variant strain of PEDV (named PEDV/CH/XC/2020) was isolated from the feces of infected piglets, and subjected to genetic variation and recombination analysis based on whole genome sequencing. The results showed that PEDV/CH/XC/2020 belonged to a GIIa subtype variant strain of PEDV, with the genome of 28128 nt in length, which shared only 94% identities with the vaccine strain CV777. Furthermore, multiple amino acid mutations were occurred in the neutralizing antigenic epitopes of COE (499-638 aa) and S1D (636-789 aa) regions of spike protein. It was worth noting that genetic recombination was occurred in the 24010-26546 nt and 27000-27663 nt regions, suggesting that this isolate may arise from genetic recombination with parental strains such as GD-1, CH/ZMDZY and PEDV-CHZ and continuous mutation in epidemic process. In contrast to other mutant strains, the mutation sites $_{767}$F, $_{838}$L and $_{1060}$C of S protein are unique, which might result in the alterations of virulence and immunogenicity of PEDV/CH/XC/2020. These findings of the molecular characteristic of this novel variant strain provided new insights into the genetic variation and diversity and enriched molecular epidemiological date of PED.

Keywords: Porcine epidemic diarrhea virus, Molecular characterization, Phylogenetic analysis, Genetic recombination

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a member of the genus Coronavirus in the *Coronaviridae* family, which can cause severe diarrhea, vomiting and dehydration in lactating piglets, often with a mortality rate of 85% or more [1-3]. Since this infectious disease was first reported in UK in 1971, it has been prevalent in many countries, including Japan, Canada, France and Bulgaria [4,5]. As an emerging and re-emerging epizootic swine virus, a variant strain of PEDV was first reported in Korea in 2009 [6]. Subsequently, PEDV variant strains have become widespread in many regions in Asia, America and Europe, causing great economic losses to the global pig industry [7,8].

The genome of PEDV is a linear positive-stranded single-stranded RNA with an entire genome length of 27.000-33.000 nucleotides, which harbors seven open reading frames, with the gene arrangements of 5'UTR-ORF1a-1b-S-ORF3-E-N-S-3'UTR [9,10]. Among them, S gene encodes the spike protein, a protective antigen that induces neutralizing antibodies in the body [11-13]. Due to being located on the surface of the viral particle, the spike (S) protein is most susceptible to mutation under the immune pressure of vaccine, leading to changes in the virulence

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and immunogenicity of PEDV, thereby evading the body's immune response [14-16].

Xinjiang province is one of the major bases of the pig industry in China, with a current pig stock of over 4 million head. In recent years, the incidence of PEDV infection in piglets has been increasing despite the increasing immunization with PEDV inactivated vaccine, which has brought huge economic losses to the local pig industry. However, the genetic characterization of prevalent strains of PEDV remains unknown. The main objective of this study was to characterize the molecular characterization of PEDV epidemic strain from large-scale pig farms in Xinjiang, China, and clarify the reasons for the decreasing immunization effect of inactivated PEDV vaccine. Here, a novel variant strain of PEDV was isolated from fecal samples of piglets with diarrhea, and its whole genome was sequenced to analyze the genetic variation characteristics of this PEDV isolate. This study not only provides valuable molecular epidemiological data for the study of PEDV variation, but also provides a scientific basis for the rational use of PED vaccine in large-scale pig farms in Xinjiang.

Material and Methods

Collection of Clinical Samples

The feces of piglets with diarrhea were collected in September 2020 from a large-scale pig farm in a region of Xinjiang, China, where there was prevalent of PEDV infection. All sows in this farm were immunized with Transmissible gastroenteritis (TGE) and porcine epidemic diarrhea (PED) inactivated vaccine 40 days before farrowing. Clinical feces were collected from 8-day-old piglets with diarrhoea and kept in sterilized centrifuge tubes at 4°C for PEDV detection and isolation.

RT-PCR Detection

Primers were designed according to the conserved sequences of PEDV (GenBank accession number: AF353511.1). Then, RT-PCR was performed to detect PEDV in piglet feces using FP1 (5'-GCAGGACACATTCTTGGT-3') and RP1 (5'-AGATGAAGCATTGACTGAAC-3') primers. Briefly, total RNA was isolated from the diarrhea feces according to the instructions of RNA extraction kit (Qiagen, Germany), followed by reverse transcription using SuperScript® II Reverse Transcriptase (Invitrogen, USA) following the manufacturer's protocol. The PCR reaction mixture contains 20 µL of water, 1 µL (0.2 µmol/L) of each FP1-RP1 primer, 25 µL of 2× Premix Ex Taq (TaKaRa, Japan), and 3 µL of DNA template. After that, PCR products were detected by 1.5% agarose gel electrophoresis.

Virus Isolation

The positive feces were homogenized and mixed with saline at a volume ratio of 1:6, centrifuged at 12000 rpm for 10 min at 4°C, and then filtered through a 0.45 µm membrane. Vero-E6 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 IU/mL penicillin and 100 IU/mL streptomycin). The virus filtrate (2%) and trypsin solution were added into Vero-E6 cells, respectively. After 1 h of adsorption at 37°C, the DMEM medium was discarded and cells were cultured in DMEM medium containing trypsin (a final concentration of 10 µg/mL) for 3-4 days. The inoculated Vero-E6 cells were passaged blindly until the stable cytopathic effects (CPE) were observed.

Virus Identification

The infected cells were repeatedly freeze-thawed three times and centrifuged at 4°C, 12000 r/min for 10 min. Then, the cells were negatively stained with 2% phosphotungstic acid staining solution (pH 6.8), followed by the observation of the morphological features of viral particle under transmission electron microscopy (TEM) (Hitachi, Japan). To further identify the isolate, indirect immunofluorescence assay (IFA) was performed in infected Vero-E6 cells. Briefly, Vero-E6 cells was inoculated with the viral solution at 2% v/v, and cultured for 24 h and then fixed in 4% paraformaldehyde for 20 min. These cells were treated with Triton X-100 for 10 min, washed with PBS and then closed with 0.5% BSA at room temperature for 2 h. The anti-N protein monoclonal antibody was incubated at 37°C for 1 h, and then added to Alexa fluor 488-labeled goat anti-mouse IgG antibody (1:1000) (Sigma, USA), incubated for 1 h at 37°C, washed with PBS and the results observed under a fluorescent microscope. In parallel, normal Vero-E6 cells were used as a negative control.

Full-length Genome Sequencing

Viral particle was purified by cesium chloride (CsCl) gradient centrifugation. After that, the total viral RNA was extracted using RNA simple Total RNA Kit (TaKaRa, Japan). Then, total RNA was reverse transcribed into cDNA using PrimeScript™ RT Master MixcDNA (TaKaRa, Japan) and sent to Beijing Bio Sequencing Co Ltd. for sequencing. Then, these sequences were assembled to obtain the whole genome sequence of the isolate.

Genetic Variation and Phylogenetic Analysis

To clarify the evolutionary relationship between this isolate and other virulent strains, the sequence was compared with the reference gene sequences registered in GenBank to analyze the homology. Phylogenetic trees based on the whole genome and S gene were constructed using MEGA10.0 software *(https://www.megasoftware.net),* respectively. Meanwhile, the variant sites in spike protein encoded by S gene were analyzed using DNAMAN software.

Table 1. *Reference sequences used in genetic recombination analysis of porcine epidemic diarrhoea coronavirus strains*

Strain / Isolate	GenBank Accession No.	Country	Strain / Isolate	GenBank Accession No.	Country
CV777	AF353511.1	Belgium	CH/JX-1/2013	KF760557.2	China
SM98	GU937797.1	South Korea	JS-HZ2012	KC210147.1	China
CHYJ130330	KJ020932.1	China	JS2008	KC210146.1	China
EF185992.1 LZC	EF185992.1	China	AH2012	KC210145.1	China
CH/JX-2/2013	KJ526096.1	China	CH/ZMDZY	KC196276.1	China
CHZ	KM609209.1	China	CH/FJZZ-9/2012	KC140102.1	China
CH/S	JN547228.1	China	GD-1	JX647847.1	China
JS	KC109141.1	China	ZJCZ4	JX524137.1	China
CH/ZJCX-1/2012	KF840537.1	China	LC	JX489155.1	China
AH-M	KJ158152.1	China	CHGD-01	JX261936.1	China
CH/HNQX-3-14	KR095279.1	China	AJ1102	JX188454.1	China
JSHA2013	KR818833.1	China	GD-A	JX112709.1	China
CH/GDZHDM/1401	KR153326.1	China	GD-B	JX088695.1	China
CH/HNYF/14	KP890336.1	China	CH/FJND-3/2011	JQ282909.1	China
HLJBY	KP403802.1	China	DR13	JQ023162.1	South Korea
SC1402	KP162057.1	China	BJ-2011-1	JN825712.1	China

Recombination Analysis

The whole genome sequences of 32 strains registered in GenBank were compared with those of different regions in China *(Table 1)*, and the genetic recombination were analyzed using RDP4 software to speculate on the recombination events that occurred during its evolution.

Results

Porcine epidemic diarrhea virus was successfully detected by RT-PCR in feces of piglet with diarrhoea *(Fig. 1)*. Compared to Vero-E6 control cells, a significant CPE

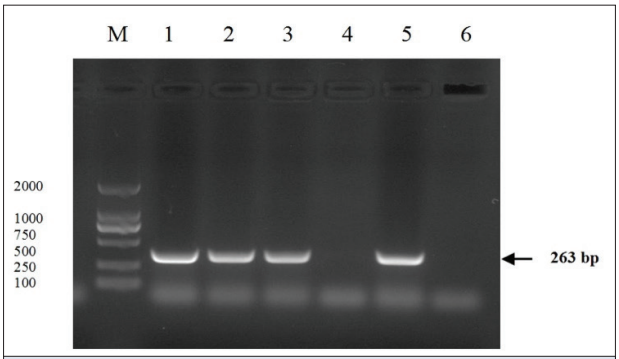

Fig 1. Molecular detection of *porcine epidemic diarrhea virus* in feces of piglet. M: Standard DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp);1: Positive control; 2, 3, 5: Positive feces detected by RT-PCR; 4: Negative feces; 6: Negative control

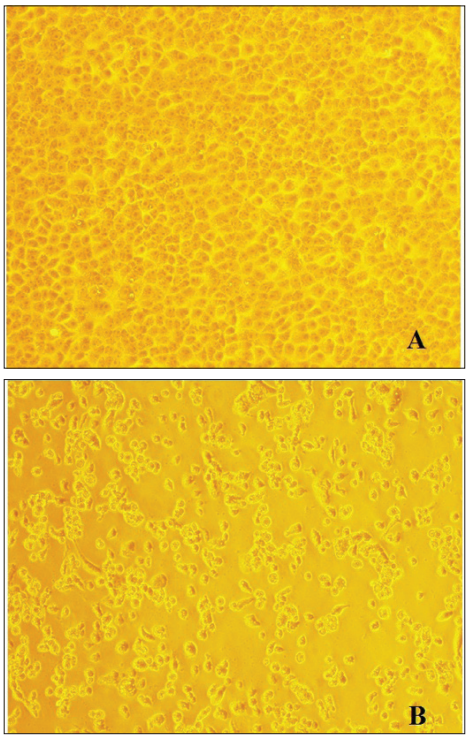

Fig 2. Cytopathic effects of PEDV CH/XC/2020 isolate infected Vero E6 cells. **A**- Mock-inoculated Vero cells culture showing normal cells; **B**- PEDV infected Vero E6 cells. PEDV CH/XC/2020 induced an obvious cytopathic effects at 36 h, the Vero E6 cells that infected by PEDV were changed to round shaped and shedding

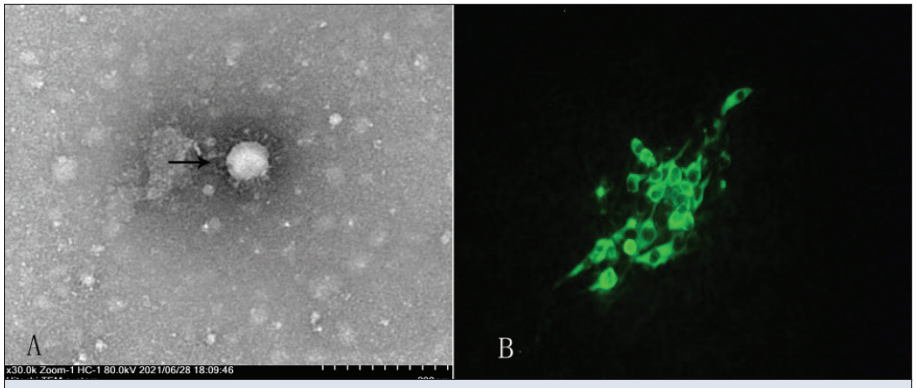

Fig 3. Identification of the isolated PEDV CH/XC/2020 strain. **A-** Transmission electron microscopic (TEM) of the supernatants of the PEDV CH/XC/2020-inoculated Vero E6 cells by using negative staining with phosphotungstic acid, typical Coronavirus particles with a corolla shape and approximately 100 nm in diameter were visible as arrow indicates; **B-** IFA detection of Vero-E6 cells infected by PEDV CH/XC/2020 isolate at 36 h, PEDV CH/XC/2020 strain in Vero E6 cells was stained using monoclonal antibody against anti-N protein and Alexa fluor 488-labelled goat anti-mouse IgG antibody, green fluorescence can be observed in infected cells

began to appear in the 6th generation. The characteristic CPE were mainly manifested by fusion of cells with each other, rounding and shedding *(Fig. 2)*. After the isolated virus was purified by etching, virus titer was measured, with $10^{5.5}$ $TCID_{50}$/mL after 20 generations of successive passages.

After negative staining, the viral particle was observed under transmission electron microscopy, which owned the typical morphological features of PEDV, with a corolla shape and approximately 100 nm in diameter *(Fig. 3-A)*. Furthermore, green fluorescence can be observed in infected cells, whereas no fluorescence was detected in control cells, indicating that a PEDV strain (namely PEDV/CH/XC/2020) was successfully isolated using Vero-E6 cells *(Fig. 3-B)*.

The whole genome of PEDV/CH/XC/2020 isolate was 28128 nt in length (GenBank accession number: OM393722), which shared 99.11% and 98.74% identities with CH/HBTS/2017 and AJ1102 strains, respectively, but only 94% identities with CV777 vaccine strain. The sequence comparisons based on S gene revealed that PEDV/CH/XC/2020 shared 99.2% identities with CH/GZZY/12/2020 (MZ161063.1) and CH/SXXY/11/2020 (MZ161017.1), but only 94.1% identities with vaccine strain CV777.

Phylogenetic analysis based on the complete genome showed that the 33 PEDV strains were mainly divided into 2 genogroups, GI (classical strain) and GII (variant strain), and each genogroup was further divided into two subgroups, GIIa and GIIb *(Fig. 4)*, among which PEDV/CH/XC/2020 isolate was located in GIIa branch. Similarly, the Phylogenetic tree based on S gene also showed that this isolate belonged to the GIIa branch of GII. However, PEDV CH/XC/2020 was genetically distant from other domestic and foreign GIIa strains *(Fig. 5)*.

The length of S gene of PEDV/CH/XC/2020 is 4158 nt, encoding 1386 amino acids. Compared with vaccine strain CV777, the isolate had multiple mutations in the neutralizing antigen epitopes of COE (499-638 aa) and S1D (636-789 aa) regions of S protein, which were mainly

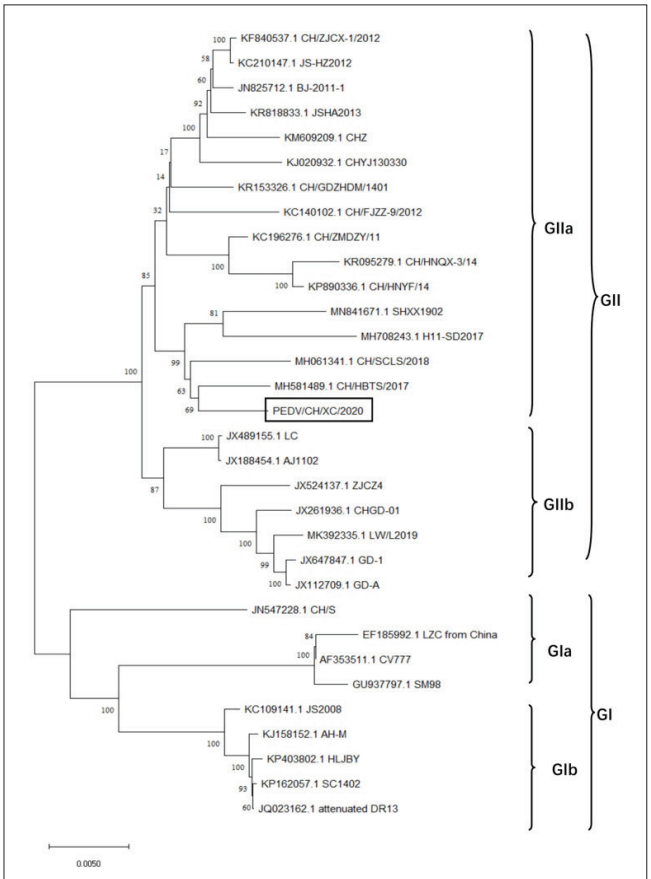

Fig 4. Phylogenetic analysis of the different epidemic strains based on the complete genome of PEDV. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA 10.0 software (1000 bootstrap replicates). Reference virus genomes from different serogroups were analysed. The PEDV CH/XC/2020 isolate was marked with black box

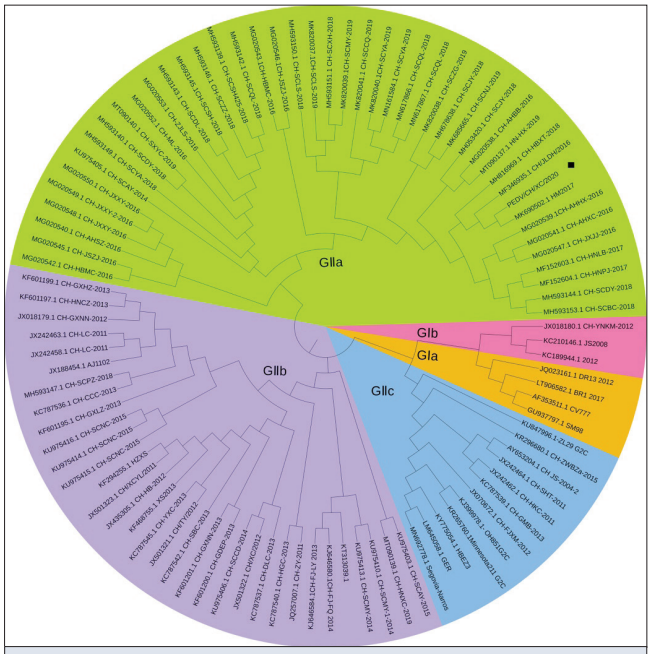

Fig 5. Phylogenetic analysis of prevalent strains in different geographical areas based on spike protein gene of PEDV. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA X program; MEGA 10.0 software (1000 bootstrap replicates). The PEDV CH/XC/2020 isolate was marked with black square

manifested in COE region as $_{526}$A→S, $_{530}$L→H, $_{532}$S→G, $_{536}$V→I, $_{558}$T→S, $_{603}$G→S, $_{614}$A→E, $_{621}$L→F, $_{640}$P→S, $_{644}$I→V, and in S1D region as $_{640}$P→S, $_{676}$I→F, $_{716}$N→D, $_{728}$N→S, $_{733}$N→S, $_{773}$S→F, and $_{775}$Y→S. There were also multiple amino acid mutations in other regions, such as $_{2}$R→K, $_{5}$I→T, $_{15}$P→S, $_{27}$QST$_{29}$→SAN, $_{55}$SMN$_{57}$→IGE, $_{58}$NQGV$_{61}$ and the insertion of $_{139}$D. These mutation sites were also present compared to strain AJ1102 and other reference strains *(Table 2)*.

Recombination analysis revealed that PEDV/CH/XC/2020 might undergo recombination events in the regions 24010-26546 nt and 27000-27663 nt *(Table 3)*, with the related parental strains GD-1, CH/ZMDZY and PEDV-CHZ *(Fig. 6)*.

Discussion

As the most mutation-prone gene in PEDV genome, spike (S) gene encodes spike protein that is primarily involved in binding to viral receptor and invading host cell *via* membrane fusion. Therefore, spike protein plays an important role in the process of infection and the induction of neutralizing antibody [17,18]. So far, only one serotype of PEDV has been identified; however, PEDV strains can

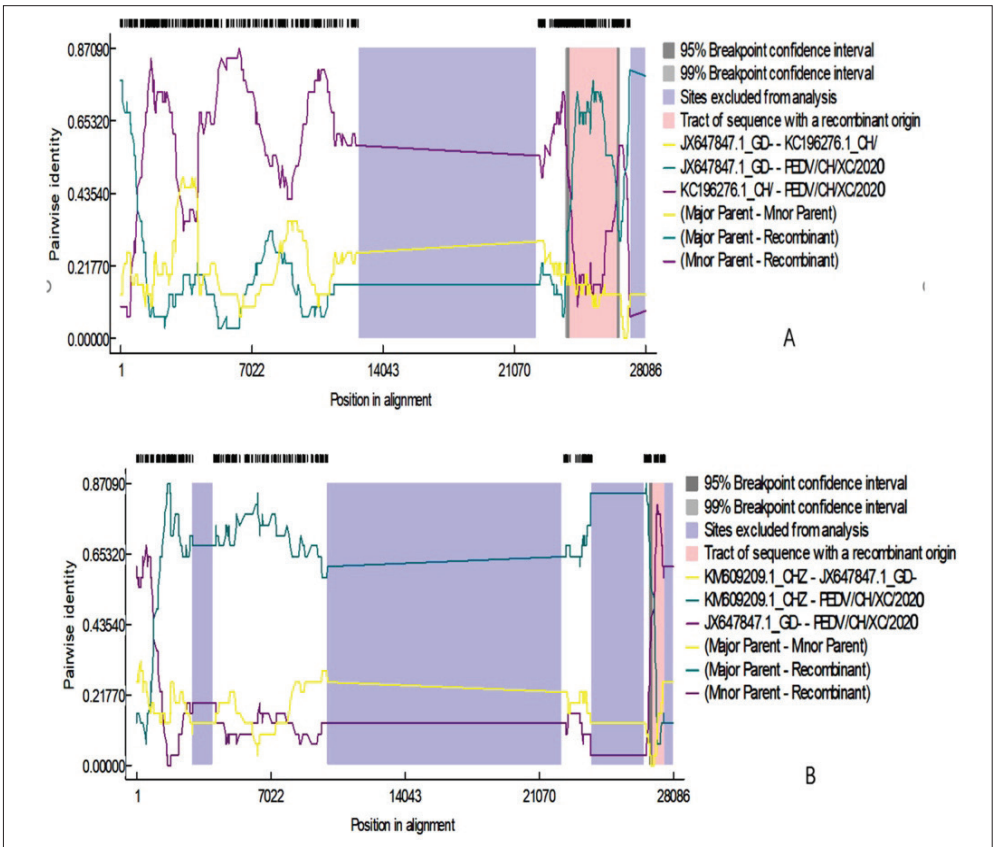

Fig 6. Analysis of potential recombination breakpoints in the genome of PEDV CH/XC/2020 isolate by RDP4 software. A- Event 1 occurred in the regions of 24010-26546 nt, the potential parent strains were GD-1 (JX647847.1) and CH/ZMDZY (KC196276.1), respectively, with the Av.P-Val of 4.035×10^{-10}; B- Event 2 occurred in the regions of 27000-27663 nt, the potential parent strains were GD-1 (JX647847.1) and PEDV-CHZ (KM609209.1), respectively, with the Av.P-Val of 2.330×10^{-6}

Table 2. *Amino acid mutations in COE (499-638 aa) and S1D (636-789 aa) regions of 25 reference strains of porcine epidemic diarrhoea coronavirus (PEDV)*

Accession no.	Variant Sites in Amino Acid Sequences																												
	505	526	529	530	532	536	545	551	558	572	603	610	614	621	639	640	641	642	643	644	676	680	716	728	733	772	773	775	783
AF353511.1	I	A	G	L	S	V	F	D	T	K	G	F	A	L	K	P	L	E	G	I	I	V	N	N	N	P	S	Y	T
CH/XC/2020	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	F	S	.
JQ023161	.	.	.	H	G	I	.	.	.	.	.	.	E	F	.	.	.	.	.	V	F	.	D	.	.	.	.	.	.
JX188454.1	T	.	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	F	D	S	S	.	.	S	.
KC210146.1	.	.	.	H	G	I	.	.	.	.	.	.	E	F	.	.	.	Q	.	V	F	.	D	.	.	.	.	S	.
MF152605.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	.	S	.	.	S	.
MF346935.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	.	S	.
MK685665.1	.	S	.	H	G	I	.	.	S	T	S	.	D	F	T	.	.	.	.	V	F	.	D	.	S	S	.	S	.
MK690502.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	.	S	.	.	S	.
MK820039.1	.	S	.	H	G	I	L	.	S	.	S	.	E	F	.	.	.	.	.	V	.	.	D	S	S	.	.	S	.
MN841671.1	.	S	.	H	G	I	.	E	S	N	S	.	E	F	.	.	.	.	.	V	F	.	D	.	S	.	.	S	.
MT090139.1	.	S	D	H	G	I	.	.	S	N	S	L	D	F	.	.	.	Q	.	V	F	.	D	S	S	.	.	S	M
MZ161017.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	.	S	.
MZ161063.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	S	.	.	.	V	F	.	D	S	S	.	.	S	.
MT263014.1	T	S	.	.	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
MT090140.1	T	S	.	R	G	I	.	.	S	.	S	.	D	F	.	.	.	G	.	V	F	.	D	.	S	.	.	S	.
MN759311.1	T	.	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	R	.	S	.
MN213154.1	T	.	.	H	G	I	.	.	.	.	S	.	E	F	.	.	.	.	.	V	F	F	D	S	.	S	.	S	.
MN114121.1	T	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	F	.	.	V	F	.	D	.	S	.	.	S	.
MK841495.1	.	S	.	H	G	I	.	E	S	.	S	.	E	F	.	.	.	V	.	V	F	I	D	.	S	L	.	S	.
MK841494.1	.	S	.	H	G	I	.	E	S	.	S	.	E	F	.	.	.	V	.	V	F	I	D	.	S	L	.	S	.
MK539948.1	T	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	F	.	.	V	F	.	D	.	S	.	.	S	.
KR296677.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
KR296673.1	T	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
KR296663.1	.	S	.	H	G	I	.	.	S	.	S	.	E	F	.	.	.	.	.	V	F	.	D	S	S	.	.	S	.
GU937797.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.

Parsimony informative sites are indicated in bold; the remaining sites are singleton sites; a period indicates no base mutation

Table 3. *Analysis of potential gene recombination events by RDP4 software*

Recombination Event no.	Methods	Major Parent	Minor Parent	Position (nt)	Recombinant Score
Event 1	RDP/GENECONV/ MaxChi	GD-1 (JX647847.1)	CH/ZMDZY (KC196276.1)	24010~26546	0.643
Event 2	RDP/GENECONV/ MaxChi	PEDV-CHZ (KM609209.1)	GD-1 (JX647847.1)	27000~27663	0.686

The recombinant score more than 60% was considered as a potential recombinant strain

be divided into two genotypes, namely Gl and GII, based on phylogenetic analysis of S gene. Among them, each genotype can be further divided into two or three subtypes, GIa and GIb, GIIa, GIIb and GIIc respectively [19,20]. In recent years, the frequent outbreaks of diarrhea in piglets caused by the variant strains of PEDV in China have resulted in massive deaths in newborn piglets, suggesting that the emerging variant strains are more pathogenic than that of classical strains [8,21,22]. Considering that vaccine strain CV777 belongs to GIa subtype, which does not provide effective protection against GIIa subtype variant, so it is urgent to develop a new vaccine against GIIa subtype variant. Here, it was revealed that PEDV/CH/XC/2020 belongs to GIIa subgroup of variant strain. This strain has multiple mutations in S gene, especially in the S1 region (1-789 aa), which is highly variable, with deletions, insertions and multiple point mutations. Moreover, some point mutations also substantively occurred in E, M and N gene. Compared to reference strains, PEDV/CH/XC/2020 possesses the characteristic mutation sites of $_{767}$F, $_{838}$L and $_{1060}$C in S gene. Furthermore, PEDV CH/XC/2020 was genetically distant from other domestic and foreign GIIa strains, suggesting that PEDV CH/XC/2020 was a new variant that is emerging in large-scale pig farms in Xinjiang, China.

Currently, it had been revealed that genetic recombination was occurring continuously in different strains of PEDV during the course of the epidemic, and that the recombination occurred not only in S gene, but also in other regions of the genome [23-25]. Antas et al.[26] showed that the end (5-400 nt) of PEDV S gene in Poland may be generated through the recombination among SeCoV MU2 (MN692770) and 1556 (MN692763) strain in Spain or DR13 (JQ23161) strain in Korea. Nefedeva et al.[27] found that two recombination events had occurred at 20476 nt (ORF1b) and 24403 nt (S) in PEDV/Belgorod/dom/2008 strain, whereas LZC (EF185992) and SLO/JH-11/2015 (KU297956) were potential parental strains. Wang et al.[28] showed that PEDV strain prevalent in China may have been generated by the recombination of parental strains USAIllinois 972013, Korea K14JB01, CHYJ130330, and CHZMDZY1. Li et al.[25] showed that the PEDV CH/HNQX-3/14 strain was produced by natural recombination of the classic strains CV777, DR13 and CH/ZMDZY/11. The present study revealed that recombination was occurred not only in S gene but also in conserved N gene of PEDV/CH/XC/2020, which displayed novel molecular characterization as compared with other PEDV epidemic strains that had been identified.

Taken together, this study for the first time revealed that the genotype GIIa variant strain was present in piglets in Xinjiang, and confirmed that this variant strain has multi-locus amino acid mutations in antigenic epitopes of S protein, which might be generated by genetic recombination of various parental strains. These findings will provide new insights into the molecular characteristics of PEDV variant strain, and facilitate the development of effective vaccines for the prevention and control of PED in veterinary practice.

Availability of Data and Materials

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Competing Interests

The authors declare no competing interests.

Author Contributions

JL Chen, LL Tian and ZY Li performed the experiment. LX Wang, Y Guo, YX Shang, YQ Sun and XT Huang contributed significantly to analysis and manuscript preparation. JL Chen and J Qiao performed the data analyses and wrote the manuscript. QL Meng, XP Cai and XZ Xia helped perform the analysis with constructive discussions.

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Research Article

Thromboelastographic Evaluation of Coagulation Profile in Dogs with Subclinical and Clinical Ehrlichiosis

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ABSTRACT

Canine monocytic ehrlichiosis (CME), a vector-borne disease of worldwide distribution, causes coagulopathy in dogs. Thromboelastography-TEG measures the efficiency of coagulation. However, there is lack of knowledge about TEG evaluation in different stages of CME. Thus, this study aimed to evaluate the coagulation status and viscoelastic properties of blood using TEG parameters in dogs with naturally-occurring CME, and their potential to discriminate between subclinical and clinical forms of the disease. The relationship between TEG parameters and C-reactive protein (CRP) was also investigated. For these purposes, 29 *E. canis*-seropositive dogs were used (12 subclinical and 17 clinical forms), and 10 healthy dogs as controls. Kaolin-activated TEG was performed in all dogs. Platelets were lower ($P<0.01$), but CRP was higher ($P<0.001$) in clinical form than in subclinical form. TEG-reaction (R) and clot-formation times (K) decreased ($P<0.01$), whereas α-angle and coagulation index (CI) increased ($P<0.01$) in both forms of CME compared to controls. The magnitude of decreases in R- and K-times and increases in α-angle and CI were higher in dogs with subclinical form compared to clinical form. CRP was correlated negatively ($P<0.05$) with TEG-Ly30. In conclusion, among TEG parameters, R-time, K-time, α-angle, and CI values may be used to differentiate subclinical form of CME from clinical form. Hypercoagulability is especially frequent in dogs with subclinical CME and may be associated with systemic inflammation.

Keywords: Coagulation, C-reactive protein, Dogs, Ehrlichiosis, Thromboelastography

Introduction

Canine monocytic ehrlichiosis (CME) is a rickettsial disease caused by *Ehrlichia canis* transmitted by the brown dog tick, *Rhipicephalus sanguineus*. The clinical symptoms of CME vary according to the host's immune response, infectious dose of the pathogen, and co-infections [1]. Ehrlichiosis can be divided into three stages based on the presence of the clinical signs: acute (early disease), subclinical (asymptomatic), and clinical (chronic) infection [2]. The most reliable parameter for the diagnosis of subclinical CME was mild thrombocytopenia without severe anemia [3]. In the chronic stage, dogs are suffering from pyrexia, jaundice, lymphadenopathy, and bleeding disorders along with thrombocytopenia [4,5].

Platelet (PLT) functions could also be altered during CME because of bone marrow suppression, increased PLT destruction and consumption, and the presence of anti-PLT antibodies [6-9]. Thrombocytopenia and PLT dysfunction in dogs with CME may lead to bleeding disorders due to primary hemostasis abnormality [6]. Thus, it seems to be essential to evaluate the coagulation status in dogs with CME regardless of the clinical stage.

Coagulation status has been evaluated by conventional screening tests such as PLT counts, prothrombin time (PT), activated partial thromboplastin time (aPTT), and d-dimer levels [10,11]. In practice, the changes in PLT indices (mean PLT volume [MPV], plateletcrit [PCT], and PLT distribution width [PDW] are also investigated to evaluate coagulation in dogs with CME [12,13]. However, these tests provide little information on the vital interaction between PLTs and the coagulation cascade [10,11,13-16].

Thromboelastography (TEG) measures the efficiency of blood coagulation from initial clot formation to fibrinolysis, thereby yielding superiority over traditional coagulation screening methods [14]. TEG evaluates clot kinetic by

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reaction (R-time) and coagulation times (K-time), clot strengthening by α-angle, PLT function by maximum amplitude (MA), and clot stability by the percentage of lysis at 30 min (Ly30) [17,18]. In a previous study [11], TEG results showed hypercoagulation and hypofibrinolysis in dogs with experimentally-induced ehrlichiosis. Currently, there is lack of information about TEG evaluation in different stages of CME in dogs. Thus, this study aimed to evaluate the coagulation status and global viscoelastic properties of blood clot formation using TEG in dogs with naturally occurring CME, and their discriminating potential between clinical forms of the disease. C-reactive protein (CRP), a non-specific inflammatory marker, was reported to play a possible pro-coagulant role in humans [19], and dogs [20]. CRP can enhance thrombogenesis [19] and inhibit fibrinolysis with the inflammatory response in humans [21]. A positive correlation between TEG-MA value and serum CRP in dogs with spirocercosis [22] and patients with poor prognosis [23] were previously reported. Thus, we also investigated the possible relationship between TEG parameters and serum CRP levels in the dogs studied.

Material and Methods

Ethical Statement

The present study was approved by the Ethics and Welfare Committee of Bursa Uludag University (Bursa, Turkey). A signed information consent form was obtained from the dog owners enrolled in the study between 2010-2016 (Decision number: 2010-06/10; Date: 24.08.2010).

Dogs and Groups

Dogs from different breeds, ages, body weights, and gender were included in the study *(Table 1)*. The dogs with or without clinical signs at admission to the clinic were screened using a combined commercial enzyme-linked immunosorbent assay (ELISA) kit for the antibody against *E. canis*, *Anaplasma marginale* and *Borrelia burgdorferi*, and antigen against *Dirofilaria immitis* (Anigen Rapid®, CaniV-4, Bionote, Korea) in Veterinary Teaching Hospital (Bursa/Türkiye). Dogs were also screened to exclude *Leishmania infantum* (Leishmania Ab Test Kits or CaniV-4 [Leish], Anigen Rapid®, Bionote, Korea). Dogs with comorbidities were not included in the study, as reported in our [8,24] and other previous studies [11].

Dogs that were found healthy based on clinical and hematobiochemical examinations along with seronegative test results were used as controls (n=10). Seropositive dogs only for *E. canis* were enrolled in the study as a subclinical form, if they did not show any clinical signs but had thrombocytopenia (n=12); and as a clinical form if they had at least one or more symptoms (loss of appetite, lethargy, depression, and/or exercise intolerance, etc.) with the presence of both thrombocytopenia and anemia (n=17) [9,11,25,26]. None of the dogs with subclinical CME were either leukopenic or neutropenic, and they had normal erythrogram values (RBC, Hct, Hgb, MCV, MCH, MCHC, and RDW) at the sampling time. Serum levels of hepato-renal injury markers were within the range of reference. Eight of the subclinical dogs had increased serum globulin concentrations (>3.7 g/dL) [11]. Additionally, all dogs included in the study did not receive any medication or vaccination at least for 10 days before being admitted to the clinic, as reported in our previous studies [8,24].

Sampling and Measurements

All dogs included in this study had initial anamnesis, physical examination, and laboratory analysis as part of the routine diagnostic procedures.

For hemogram (5-parts CBC differential with 22 parameters, VetScan® HM5, Abaxis, USA) and serum biochemistry analysis (Comprehensive profile, VetScan® VS2, Abaxis, USA), blood samples were collected from cephalic veins into the vacutainer tubes with or without anticoagulant (EDTA), respectively. Also, a total of 2 mL

Table 1. *Signalment and some clinical parameters in the dogs with subclinical and clinical ehrlichiosis and healthy dogs in the control group*

Parameter	Healthy n=10 (mean ± sd)	Subclinical Ehrlichiosis n=12 (mean ± sd)	Clinical Ehrlichiosis n=17 (mean ± sd)
Body weight (kg)	10.5±3.8^{a}	25.9±5.8^{b**}	22.4±8.8^{b*}
Gender (M/F)	5F + 5M	5F + 7M	7F + 10M
Age (months)	51.3±22.9^{a}	68.0±37.4^{a}	54.3±13.7^{a}
Temperature (°C)	37.9±0.7^{a}	38.6±0.4^{a}	38.7±0.8^{a}
Pulsation bpm	121±8^{a}	123±6^{a}	113±7^{a}
Respiration rpm	20±14^{a}	22±17^{a,b}	99±14^{b***}

M: *male,* ***F:*** *female,* ***bpm:*** *beat per minute,* ***rpm:*** *respiration per minute*
^{a,b} *Different letter in the same line represent a statistically significant change between variables*
**P<0.05 **P<0.01 *** P<0.001 compared to healthy controls*

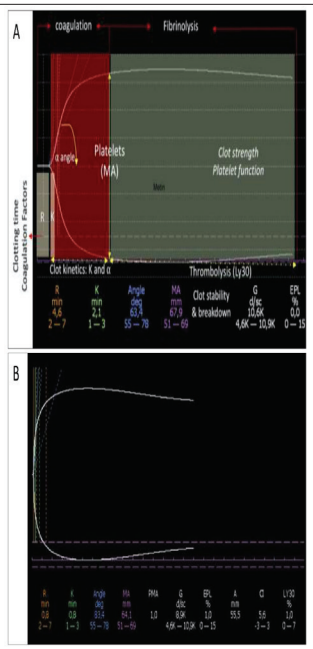

Fig 1. Two examples of thromboelastography (TEG) trace. **A-** From a healthy dog (Golden retriever, 5 years, male) with a schematic illustration of TEG trace including coagulation activation (R time and K time), clot kinetics (K time and a-angle), maximum clot stability (MA) and thrombolysis or fibrinolysis (Ly30), and **B-** From *E. canis* seropositive dog in the subclinical phase (Golden retriever, 4 years, male) showing hypercoagulable states due to shortened R- and K-time as well as increased a-angle, G value and coagulation index (CI), thereby increasing risk for intravascular coagulation (pro-thrombotic state)

blood was sampled for coagulation analysis (TEG®5000, Haemoskope, MA, USA) into vacutainer tubes with 3.2% sodium citrate (BD Vacutainer System, BD Diagnostics, NJ, USA), and were gently inverted at least 5 times to allow proper mixing, as previously described in our studies [17,18,27].

Comprehensive serum biochemistry panels included albumin, alkaline phosphatase (ALP), alanine amino-transferase (ALT), amylase, total bilirubin, blood urea nitrogen (BUN), calcium (Ca), phosphate (Phos), creatinine (Cr), glucose (Glu), sodium (Na), potassium (K), total protein (TP) and globulin. In addition, CRP in all dogs was measured to evaluate the acute phase response by the technique described in our previous study [28].

For TEG analysis, within 30 min following the blood sampling, dogs were examined for coagulation status by kaolin-activated TEG parameters *(Fig. 1, Table 2)*, namely R time and K time (clot kinetics), α-angle (clot strength), as well as parameters for PLT function (MA, projected maximum amplitude [PMA], and maximal clot strength (G value, dyn/cm^2]), clot stability (A and LY30: Percentage of lysis 30 minutes after MA, estimated potential lysis [EPL], coagulation index [CI]), as reported in our previous studies [17,18]. TEG was halted after maximal fibrin clot strength was recorded, and the G value of the clot stability was calculated from the MA value via TEG software, using the following formula: G = (5000 × MA)/(100 - MA). Coagulation status (hypercoagulable and hypocoagulable states) was characterized by TEG, as suggested [29]. The hypercoagulable and hypocoagulable states were defined by shortened reaction time (R- and/or K-time), increased α-angle, and/or MA values; whereas by prolonged R- and K- time, and/or decreased α-angle and MA values, respectively [29,30].

In this study, doxycycline (10 mg/kg, PO, q24h for 28 days) and supportive care were the treatment of choices for all *E. canis* seropositive dogs, as suggested [1,11], and they were not monitored thromboelastographically during and after the treatment.

Statistical Analysis

Statistical analysis was performed by routine descriptive statistical procedures and software (SigmaPlot® Statistical and Graphing Software, California, USA). Changes in the analytes between healthy dogs and dogs with either subclinical or clinical ehrlichiosis were assessed by One-way Analysis of Variance (ANOVA). When the normality test failed, the Mann-Whitney Rank Sum test was used. The Holm-Sidak test was used for both pairwise comparisons versus a control group. The Pearson correlation coefficient *(r)* was used for measuring a linear correlation between TEG parameters and PLT indices, and serum CRP levels. All data were expressed as mean±sd. Values of $P<0.05$ were considered significant.

Results

Signalement and Clinical Examination

A total of 39 dogs comprised of naturally infected clinical, and subclinical form of ehrlichiosis, and healthy control dogs of different breeds, ages, gender, and body weights were used in the study *(Table 1, Table 3, and Table 4)*. There was no statistical difference in age, temperature, and pulsation between the groups, but the respiratory rate increased ($P<0.01$) in dogs with clinical ehrlichiosis, compared to controls. Control dogs did not have any clinical and/or pathological findings based on the physical examination and hemato-biochemistry evaluations *(Table 1-6)*.

Hemato-Biochemistry Panel

CBC values and serum biochemistry panel of all dogs (n=39) were given in detail *(Table 4, Table 5)*. As for the aim of the study, WBC, RBC, Hct, PLT count, and serum CRP levels were presented as follows: There was no statistically significant change in total WBC count between the groups. RBC was lower ($P<0.01$) in dogs with clinical CME compared to control, and Hct values in dogs with clinical CME were lower ($P<0.001$) than in

Table 2. *Coagulation panel measured by kaolin activated thromboelastography (TEG) in the dogs with subclinical and clinical ehrlichiosis and dogs in the control group.*

Parameter	Healthy n=10 (mean ± sd)	Subclinical Ehrlichiosis n=12 (mean ± sd)	Clinical Ehrlichiosis n=17 (mean ± sd)
R time (min)	5.1±1.3^{a} (2.9-7.9)	1.1±0.6$^{b\#***}$ (0.2-2.3)	2.8±1.3^{c***} (0.5-5.8)
K time (min)	2.9±1.0^{a} (1.4-5.8)	1.4±1.5^{b**} (0.8-5.4)	1.8±1.1^{b*} (0.8-4.7)
a angle (degree)	55.9±6.4^{a} (45.2-64.9)	76.7±12.0$^{b***,\#**}$ (40.1-85.2)	65.2±13.5^{c**} (29.6-83.4)
MA (mm)	56.2±6.6^{a} (44.1-66.3)	63.9±12.7^{a} (36.7-79.1)	55.4±15.3^{a} (19.8-77.6)
PMA mm	0.05±0.2^{a} (0.0-1.0)	0.6±0.4$^{b***,\#*}$ (0.0-1.0)	0.2±0.4^{a} (0.0-1.0)
G (dyn/cm^2)	6.6±1.7^{a} (4.0-9.9)	10.4±5.0^{b*} (2.9-18.9)	7.4±4.0^{ab} (1.2-17.4)
EPL %	0.2±0.7^{a} (0.0-2.8)	0.1±0.3^{a} (0.0-0.7)	0.3±0.6^{a} (0.0-2.1)
CI value	-0.7±2.1^{a} (-4.1-3.6)	4.5±2.9$^{b***,\#**}$ (-2.5-7.6)	1.7±3.4^{c*} (-7.0-6.4)
A mm	51.7±9.2^{a} (30.5-64.3)	62.1±11.5^{a} (40.3-78.4)	53.6±14.6^{a} (21.5-88.0)
Ly30 (min)	0.7±2.3^{a} 0.0-10.0	0.1±0.3^{a} (0.0-0.7)	0.3±0.6^{a} (0.0-1.7)

^{a,b,c} *between healthy dogs and subclinical and clinical ehrlichiosis (there is a significant difference between groups containing different letters in the same line);* $^{\#}$ *between subclinical and clinical ehrlichiosis*
**P<0.05.* ** *P<0.01. and* *** *P<0.001 compared to healthy controls*
R: *Reaction time,* ***K:*** *Coagulation time,* ***α-angle:*** *Alpha angle,* ***MA:*** *Maximum amplitude,* ***PMA:*** *Projected maximum amplitude,* ***G:*** *G value calculated,* ***EPL:*** *Estimated potential lysis,* ***A and LY30:*** *Percentage of lysis 30 minutes after MA,* ***CI:*** *Coagulation index*

subclinical form and in healthy controls. The mean PLT count was lower in dogs with clinical CME than that of subclinical CME (P<0.01), and control dogs (P<0.001) *(Table 3)*. Serum CRP levels in dogs with clinical CME was higher (P<0.001) than that of dogs with subclinical CME, and healthy controls *(Table 3)*.

Thromboelastography Analysis

TEG parameters including their minimum and maximum levels were presented in *Table 2*. There were statistically significant differences (P<0.05) in R time, α-angle, PMA, and CI between subclinical and clinical forms. R- and K-time decreased (P<0.05) but α-angle, PMA, G, and CI values increased (P<0.05) in dogs with subclinical CME, compared to those with clinical CME, and healthy controls. The magnitude of decrease in R- and K-time and increase in α-angle, PMA, and CI values were higher in dogs with subclinical form of CME, compared to those with clinical form.

In individual evaluation of TEG, 6 subclinical and 4 clinical dogs had higher G values from the maximum levels detected in healthy controls (9.9 dyn/cm^2). Similarly, higher CI values in 9 subclinical, and 6 clinical dogs were found compared to maximum levels measured in healthy controls.

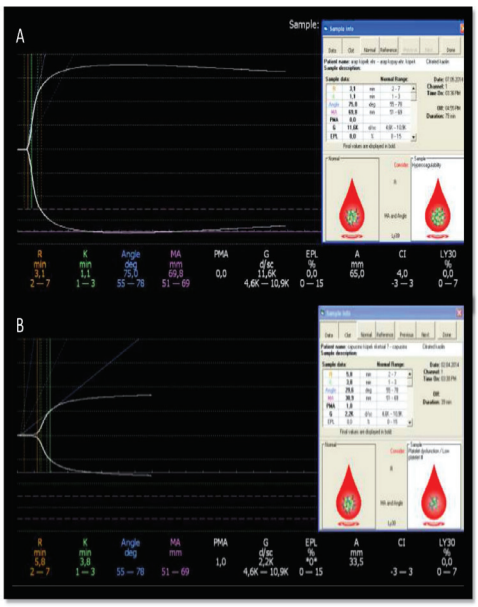

Fig 2. Thromboelastography (TEG) profiles from two dogs with clinical (chronic) ehrlichiosis. **A** shows hypercoagulable states due to shortened K time and increased maximum amplitude (MA), G value, and coagulation index (CI), **B** shows a low platelet/platelet dysfunction and/or hypofibrinogenemia characterized by low a-angle, MA, G value, and CI

Table 3. *Some hemogram parameters and serum C-reactive protein levels in the dogs with subclinical and clinical ehrlichiosis and healthy dogs in the control group*

Parameter	Healthy n=10 (mean ± sd)	Subclinical Ehrlichiosis n=12 (mean ± sd)	Clinical Ehrlichiosis n=17 (mean ± sd)
WBC (x10^3/uL)	12.2±0.6^a	12.8±7.1^a	7.8±6.6^a
RBC (x10^6/uL)	6.3±0.8^a	6.0±1.4^{ab}	4.0±0.9^{b**}
Hct (%)	41.4±7.2^a	38.4±9.0^a	25.1±6.7$^{b\#***}$
Platelet count (x10^3/uL)	257±61^a	149±22^{b**}	58.7±43$^{c\#***}$
Serum CRP (ug/mL)	15.4±12.3^a	31.9±19.8^{b**}	70.7±21.2$^{b\#***}$

WBC: *White blood cell count,* ***RBC:*** *Red blood cell count,* ***Hct:*** *Hematocrit,* ***CRP:*** *C-reactive protein*
$^{a,b\,c}$ *Different letters in the same line represent a statistically significant change between variables*
** P<0.05. **P<0.01. and *** P<0.001 compared to healthy controls. # compared to Subclinical Ehrlichiosis*

Table 4. *Complete blood cell counts in the dogs with subclinical and clinical ehrlichiosis and healthy dogs in the control group*

Parameter	Healthy n=10 (mean ± sd)	Subclinical Ehrlichiosis n=12 (mean ± sd)	Clinical Ehrlichiosis n=17 (mean ± sd)
WBC (K/uL)	12.2±0.6^a	12.8±7.1^a	7.8±6.6^a
Lymphocyte (K/uL)	3.2±0.3^a	1.7 ±1.6^{a,b}	0.7±0.5^{b**}
Monocyte (K/uL)	0.5±0.0^a	0.7 ±0.5^a	0.6±1.0^a
Neutrophile (K/uL)	8.0±0.5^a	8.1±4.0^a	4.9±3.4^a
Eosinophile (K/uL)	0.30±0.00^a	0.81±0.23^a	0.06±0.04^a
Basophile (K/uL)	0.01±0.02^a	0.04±0.03^a	0.01±0.00^a
RBC (x10^{12}/L)	6.3±0.8^a	6.0±1.4^a	4.0±0.9^{b**}
Hgb (g/dL)	13.2±2.1^a	12.3±3.3^a	7.7±2.2^{b*}
HCT (%)	41.4±7.2^a	38.4±9.0^a	25.1±6.7^{b***}
MCV (fL)	65.4±3.6^a	64.7±6.1^a	62.1±6.5^a
MCH (pg)	20.8±1.1^a	20.4±2.2^{a,b}	18.8±2.1^{b,*}
MCHC (g/dL)	31.9±0.8^a	31.4±1.7^{a,b}	29.5±3.0^{b**}
RDW (%)	15.9±0.8^a	16.4±2.9^a	15.9±6.9^a
PLT (x10^9/L)	257±61^a	149±22^{b**}	58.7±43c***
MPV (fL)	7.9±0.2^a	9.5±1.6^a	8.1±2.0^a
PCT (%)	0.22±0.05^a	0.18±0.01^{a,b**}	0.05±0.04^{b***}
PDW (fL)	29.5±0.6^a	37.2±8.7^a	32.8±9.2^a

^{a,b,c} *Different letters in the same line represent a statistically significant change between variables*
**P<0.05 ** P<0.01 *** P<0.001 compared to healthy controls*
WBC: *White blood cell count,* ***RBC:*** *Red blood cell count,* ***Hgb:*** *Hemoglobin,* ***HCT:*** *Hematocrit,* ***MCV:*** *Mean corpuscular volume,* ***MCH:*** *Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration,* ***RDW:*** *RBC distribution width,* ***PLT:*** *Platelet count,* ***MPV:*** *Mean platelet volume, PCT: Plateletcrit,* ***PDW:*** *Platelet distribution width*

In addition, the TEG analyzer showed hypercoagulable states (n=19; 11 in subclinical and 8 in clinical CME), normocoagulable states (n=18, 1 from subclinical CME, and 7 from clinical CME, and 10 from healthy controls) in dogs studied (*Fig. 1*, *Fig. 2*). Although none of the dogs did not fit into thromboelastographic definition of the hypocoagulable state, the TEG - PLT mapping assay displayed the presence of PLT dysfunction and/or hypofibrinogenemia with a low MA, α-angle, and/or CI value in two dogs with clinical CME. In addition, these two dogs had lower G and CI levels than healthy controls.

Correlations

Statistically significant correlations between TEG parameters and PLT indices and serum CRP values were as follows: Negative correlations between PLT count and α-angle (P<0.01); PDW and MA (P<0.05), G (P<0.05) and CI (P<0.01); PCT and R time, and positive correlations

Table 5. *Serum biochemistry of the dogs with subclinical and clinical ehrlichiosis and dogs in the control group*

Parameter	Healthy n=10 (mean ± sd)	Subclinical Ehrlichiosis n=12 (mean ± sd)	Clinical Ehrlichiosis n=17 (mean ± sd)
Albumin (g/dL)	2.9±0.4^{a,b}	3.1±0.7^{a}	2.1±0.5^{b*}
ALP (U/L)	57.1±36.7^{a}	60.7±32.0^{a}	161.1±96.9^{b**}
ALT (U/L)	25.8±5.5^{a}	45.6±22.5^{a}	133.7±162.3^{a}
Amylase (U/L)	468±131^{a}	696.8±407.9^{a}	805.8±278.0^{a}
Total Bilirubin (mg/dL)	0.16±0.05a	0.35±0.07^{a}	0.94±1.71^{b,**}
BUN (mg/dL)	12.0±5.6^{a}	15.6±9.8^{a}	17.7±15.8^{a}
Ca (mg/dL)	9.2±1.9^{a}	10.2±0.7^{a}	9.5±0.8^{a}
P (mg/dL)	5.5±2.4^{a}	6.1±4.5^{a}	5.3 ±1.2^{a}
Cr (mg/dL)	0.7±0.2^{a}	0.8±0.2^{a}	0.7±0.2^{a}
Glucose (mg/dL)	110±21^{a}	93±32^{a}	88±33^{a}
Na (mmol/L)	137±0^{a}	138±3^{a}	139±2^{a}
K (mmol/L)	4.2±0.1^{a}	4.7±0.2^{a}	4.5±0.1^{a}
TP (g/dL)	5.9±0.9^{a}	7.2±1.1^{a}	7.0±1.8^{a}
Globulin (g/dL)	2.2±0.7^{a}	4.0±1.1^{b**}	4.8±1.7^{b**}
CRP (ug/mL)	15.4±12.3^{a}	31.9±19.8^{b**}	70.7±21.2^{b***}

ALP: *Alkaline phosphatase,* ***ALT:*** *Alanine amino transferase,* ***BUN:*** *Blood urea nitrogen,* ***Ca:*** *Calcium,* ***P:*** *Phosphate,* ***Cr:*** *Creatinine,* ***Na:*** *Sodium,* ***K:*** *Potassium,* ***TP:*** *Total protein,* ***CRP:*** *C-reactive protein*
^{a,b} *Different letters in the same line represent a statistically significant change between variables*
**P<0.05, **P<0.01. and ***P<0.001 compared to healthy controls*

Table 6. *Pearson correlation coefficient (r) results between serum C-reactive protein (CRP) and platelet (PLT) count and TEG parameters in all dogs studied (n=39)*

TEG Parameters	PLT Count		MPV		PDW		PCT		Serum CRP	
	r	P	*r*	P	*r*	P	*r*	P	*r*	P
R time	0.189	0.33	0.025	0.89	0.321	0.09	-0.399	0.03	0.100	0.61
K time	0.146	0.45	0.171	0.386	0.519	0.004	-0.207	0.29	0.030	0.87
a-angle	-0.485	0.01	-0.213	0.27	0.049	0.80	0.475	0.01	-0.425	0.02
MA	0.159	0.42	-0.066	0.73	-0.363	0.05	-0.106	0.59	0.262	0.17
PMA	-0.084	0.66	-0.096	0.62	-0.087	0.65	0.271	0.16	-0.009	0.96
G	0.01	0.95	-0.106	0.59	-0.409	0.03	-0.014	0.94	0.133	0.49
EPL	-0.298	0.12	-0.073	0.70	0.066	0.73	0.296	0.12	-0.302	0.11
A	0.003	0.98	-0.000	0.97	-0.356	0.06	-0.026	0.89	0.113	0.56
CI	-0.112	0.57	-0.158	0.42	-0.523	0.004	0.286	0.14	0.006	0.97
LY30	-0.344	0.07	-0.086	0.66	0.046	0.81	0.470	0.01	-0.350	0.05

Underlined results show statistically significant correlation at least P<0.05 between the related parameters
TEG: *Thromboelastography,* ***PLT:*** *Platelet,* ***MPV:*** *Mean platelet volume,* ***PDW:*** *Platelet distribution width,* ***PCT:*** *Plateletcrit,* ***CRP:*** *C-reactive protein,* ***R:*** *Reaction time,* ***K:*** *Coagulation time,* ***α-angle:*** *Alpha angle,* ***MA:*** *Maximum amplitude,* ***PMA:*** *Projected maximum amplitude,* ***G:*** *G value calculated,* ***EPL:*** *Estimated potential lysis,* ***A and LY30:*** *Percentage of lysis 30 min after MA,* ***CI:*** *Coagulation index*

between PDW and K time (P<0.01), and PCT and α-angle (P<0.01), and Ly30 (P<0.01). Additionally, a negative correlation (P<0.05) between serum CRP and α-angle, and Ly30 *(Table 6);* and serum CRP level with PLT count (*r* = - 0.631, P<0.001) were determined.

Discussion

In the present study, TEG changes in *E. canis*-infected dogs, which is a disease known to cause a decrease in the number of PLTs but its effect in other aspects of coagulation is not well known. There were statistically significant differences in TEG parameters between healthy dogs, and dogs with subclinical and clinical CME. The hypercoagulable state was common in both stages, especially in the subclinical form of CME; however, PLT dysfunction and/or hypofibrinogenemia could be observed in some dogs with clinical CME.

TEG was used in this report to evaluate the coagulation, since it provides detailed information compared to traditional assays such as PT and aPTT on associations between PLTs and their effects on aggregation, clot strength, fibrin cross-linking, and the fibrinolysis process of the hemostasis [14]. Hitherto, there is no published data or study on thromboelastographic evaluation of coagulation status in dogs with naturally occurring CME. Also, the potential of TEG as a diagnostic tool to distinguish subclinical from clinical CME is almost unknown.

In this study, there were statistically significant differences ($P<0.05$) in TEG parameters (R time, α-angle, PMA, and CI) between subclinical and clinical forms. The magnitude of decreases in R- and K-time, and increases in α-angle, PMA, and CI values were higher in dogs with subclinical form compared to those of clinical form of CME. While considering TEG parameters referring to each specific step of coagulation, observed decreases in R- and K-time may be related to shortened time for the enzymatic portion of clot formation (R time) and fibrin-cross linking or clot kinetics (K time) in dogs with CME. In addition, increasing MA, PMA, α-angle, and CI were associated with increasing PLT function/aggregation (MA and PMA), and clot strength and propagation (α-angle) in dogs, especially with the subclinical form of CME. These observations showed that several distinguished TEG parameters (R time, K time, α-angle, PMA, and CI) may be used to discriminate clinical form of CME from its subclinical form in dogs.

TEG analysis has the potential to show the presence of hypercoagulation earlier than other laboratory tests. When MA increases, it means that the patient's blood is in a hypercoagulable state, and is prone to thrombosis, and *vice versa*, when the MA decreases, the patient's blood is diluted and prone to bleeding [23]. In the present study, shortened R- and K-times and high α-angle and/or MA values indicated a hypercoagulable state in 19 out of 29 dogs with CME, as reported earlier [11]. In a previous study [11], a hypercoagulable state was reported in dogs with experimentally induced ehrlichiosis, where the dogs were not divided into subclinical and clinical subgroups. Our results from the dogs with naturally occurring CME confirmed the findings of the hypercoagulation detected after experimental *E. canis* infection in dogs [11], and also, extended the current knowledge by indicating that hypercoagulable state might be more common in the subclinical form than the clinical form of the disease. Instead of more TEG parameters, only based on the TEG G value, a measurement of global clot strength, the hypercoagulable state was characterized in humans (>11 dyn/cm^2) [31], and dogs (>7.2 dyn/cm^2) [32]. Thus, the increase in TEG G value in dogs of our study supporting the hypercoagulation tendency in dogs is also in parallel to these findings [32]. Our cut-off of G value (4.0-9.9 dyn/cm^2) used in the present study seems to be higher than that of Wiinberg et al.[32], due to the possible differences between TEG activation methods used (kaolin- *vs* tissue factor-activation).

CI is used to the assessment of overall coagulation derived from other indices including R time, K time, MA, and α-angle. In the present study, the observed increase in CI value was associated with hypercoagulation, as reported in humans with trauma [33], and dogs with critical illnesses [34]. Mean TEG CI value (4.5±2.9) in dogs with subclinical CME was similar to the results (4.2±1.9) of our previous study in dogs with dilated cardiomyopathy [18] from the aspect of the presence of a hypercoagulable state.

Although the changes in TEG parameters in dogs studied did not fit the definition of a hypocoagulable state, the TEG PLT mapping showed the presence of PLT dysfunction and/or hypofibrinogenemia in two dogs with clinical CME. Observed decreases in TEG G and CI values might be suggestive of bleeding tendency (hypocoagulation) in these dogs. Also, although there was not a statistically significant difference in Ly30 value between the clinic stages of the disease, observed decreases in Ly30 may be related to a tendency for hypofibrinolysis in dogs with CME, as reported in a previous study [11]. In this study, why the dogs with clinical CME did not show bleeding signs despite severe thrombocytopenia could be explained by a common observation of hemostatic phenotype rather than a bleeding phenotype in *E. canis*-infected dogs [11].

When TEG parameters are interpreted with serum CRP improves the quality of clinical decision making, ultimately, the estimation of patient outcomes [23]. As a result of the inflammatory responses that occur after rickettsial infections, an increase in acute-phase proteins is observed as a physiological response [35,36]. In our study, a significant increase of serum CRP was observed in dogs with CME, especially in the clinical stage, indicating that CRP may be a useful indicator to detect inflammation in response to *E. canis* infection and to discriminate clinical from the subclinical stage. The higher concentrations of serum CRP in the clinical phase of ehrlichiosis in dogs could be a result of the severity of tissue damage and inflammation [35].

In the present study, the correlations showed statistically significant interactions between TEG parameters, PLT indices, and serum CRP. Circulating PLT count was correlated negatively with α-angle in dogs studied. Similarly, Zhou et al.[37] reported that despite decreased PLT count, TEG parameters of α-angle and MA increased significantly. While PLT counts decrease, PLT volume (MPV) and size (PDW) may increase to compensate for thrombocytopenia in dogs [38], thereby giving rise to a

normal coagulation profile in these patients. In parallel to this observation, in the present study, normocoagulation was defined in eight dogs with CME despite the presence of different severity of thrombocytopenia. These results showed that PLT's role in the hemostasis processes starting from clot initiation to fibrinolysis may have been more than expected, as reported in our previous study [39].

A significant negative correlation between serum CRP level and PLT count showed that thrombocytopenia was accompanied by elevated serum CRP levels, indicating that the severity of inflammation could have a role in the decrease in PLTs in this disease. There were negative correlations between serum CRP level and TEG parameter α-angle and Ly30 in the present study. This may be explained by the pro-coagulant effects of CRP because CRP was reported to be associated with the hypercoagulability of plasma and increased PLT reactivity [40]. CRP may play a key role to alter the coagulation process during CME, as reported in different inflammatory diseases in humans [20] and dogs [21].

One of the limitations of the study is that traditional tests such as PT and aPTT were not applied together with TEG analysis. However, while considering the abilities of TEG, it evaluates primary, secondary, and tertiary hemostasis. Despite the lack of additional tests evaluating coagulation status, the results of the study are thought to be inclusive and significant [11,18,27]. Also, these tests give no information on the vital interaction between PLTs and the coagulation cascade. Some patients having normal PT/aPTT values may have active bleeding because of abnormal hemostasis, therefore, PT and aPTT tests may be inadequate for coagulation monitoring [14]. This study included 29 dogs with CME, whereas, in a previous experimental study, 5 healthy beagle dogs were used to show the alteration in TEG analysis after *E. canis* inoculation [11]. Thus, the number of samples in this study seemed to be adequate for the study's aims when compared to others with smaller sample size [11]. We did not monitor the coagulation of the dogs during/after the treatment, since this was out of the scope of this study.

Currently, the indirect fluorescent antibody test (IFAT) is considered the "gold standard" method for diagnosis of CME [3]; however, cross-reactive antibodies, lack of standardization of test and trained personnel, and expensive equipment requirements were reported to limit the reliability and applicability of IFAT for this purpose [41]. Polymerase chain reaction (PCR), another test for the diagnosis of CME, is limited usage because of generally allowing detection of dogs in the acute phase of the disease [42]. In practice, combined commercial ELISA kits from different companies (Idexx Laboratories, Antech Diagnostics, and Abaxis, etc.) are commonly used to detect antibodies to *E. canis* [8] with high specificity (99-100%) and sensitivity (96.2-97.6%) in dogs [43-45]. Thus, in this study, using an in-clinic ELISA kit was thought adequate for the accurate diagnosis of CME, as performed in our [8,24] and other previous studies [11,46-50].

Our results showed that there are changes in coagulation conditions in different stages of dogs with CME. Based on the TEG values, clot stability, clot kinetics, clot strength, and fibrinolysis may vary in dogs with CME, regardless of clinical form; being some TEG parameters of potential use to distinguish subclinical from clinical ehrlichiosis in dogs. Hypercoagulability (a state of increased risk for thrombosis) is more common in dogs with CME, especially in the subclinical form, whereas PLT dysfunction/hypocoagulability (a state of increased risk for bleeding) may be seen in some cases of clinical CME. Inflammation (represented by elevated CRP) may be associated with hypercoagulation in dogs with CME. Additionally, further studies with larger sample sizes are needed to investigate the PLT dynamics in thrombocytopenic dogs with ehrlichiosis.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (M. Kocatürk).

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Competing Interests

None of the other authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Authors' Contributions

ZY and MK conceived and supervised this study, completed the main laboratory analysis, collected and analyzed data. ZY, MK, RT and JJC wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Anti-Apoptotic and Anti-Inflammatory Effects of Ginger Extract on Small Intestine Tissue in STZ-Induced Diabetic Rats

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ABSTRACT

In this study, it was aimed to investigate the effects of the ginger extract on the TNF-α, IL-1β, bax and bcl-2 expression in the small intestine (duodenum, jejunum and ileum) of rats with experimentally induced diabetes and also, it was examined the ultrastructure of the small intestine by scanning electron microscopy. Wistar albino rats were assigned to five groups as control, sham, ginger, diabetes control, ginger + diabetes. Experimental diabetes was induced by intraperitoneal injection of 50 mg/kg STZ. Ginger and diabetes + ginger group were administered ethanolic ginger extract (200 mg/kg) by oral gavage. In the diabetes control group, it was revealed that intestinal mucosa thickness increased and the villi were folded over each other in the form of a roll and there were disrupted the integrity. Also, while bax, TNF-α, and IL-1β expression increased and bcl-2 expression decreased. In the group treated with ginger, both intestinal histologies and bax, bcl-2, TNF-α, and IL-1β expressions were similar to that of the control group. It was observed that ginger has regulatory effects on inflammatory and apoptotic proteins in the small intestine in diabetes. Ginger can be evaluated in the treatment of diabetes and may provide new targets for therapeutic intervention.

Keywords: Apoptosis, Cytokine, Diabetes, Scanning electron microscopy, Small intestine

INTRODUCTION

Diabetes mellitus (DM) is a multisystemic chronic disease characterised by impaired insulin secretion and hyperglycaemia [1,2]. It was reported that DM causes many morphological and structural damages in gastrointestinal system, such as, gastro-esophageal reflux, intestinal enteropathy, neuropathy involving the gastrointestina [3], diarrhoea, vomiting, habitual constipation and faecal incontinence [4].

Ginger *(Zingiber officinale)* is known to contain more than 400 different compounds. Carbohydrates, lipids, terpenes, and phenolic compounds [5]. It also contains amino acids, protein, phytosterols, vitamins (nicotinic acid and vitamin A and minerals) [6]. Supportive and therapeutic effects are known in many gastrointestinal system disorders such as indigestion, early satiety, bloating, gastritis, ulcer, nausea, vomiting [7,8], irritable intestine syndrome [9], epigastric, pain/burning, dysphagia [10].

Tumour necrosis factor-alpha (TNF-α) is a pro inflammatory cytokine constituting 233 amino acids that secreted by the macrophage and T lymphocytes. TNF-α, in particular, has acts as a regulator of intestinal homeostasis under normal physiological conditions, but has complex regulation affected by the expression of active immune cells and other cytokines, and its dysregulation causes problems. Ultimatly, TNF-α causes inflammation, apoptosis, cytotoxicity and production of Interleukin-1β (IL-1β) [11]. IL-1β is an important mediator of the inflammatory response. It was reported IL-1β and TNF-α were effective correlated with increased complications in diabetes [12,13]. Apoptosis involves either anti-apoptotic (bcl-2) and pro-apoptotic (bax) proteins and the balance between these two groups that serve in the regulation of apoptosis and act to promote or suppress cell death. Increased glucose has been reported to increase the expression of both pro-inflammatory agents and pro-apoptotic proteins by creating a chain effect on cytokines [14,15].

For purpose of this study, the effects of diabetes in the small intestine tissue of STZ-induced diabetic rats and the healing effects of ginger extract administered by oral gavage in the small intestine of diabetic rats were investigated by histological, histomorphometric methods,

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and SEM. Anti-apoptotic and anti-inflammatory effects of ginger were also evaluated by immunohistochemical staining.

Materials and Methods

Ethical Approval

All experiments were approved by Tekirdag Namik Kemal University Ethics Committee for animal experiments (Approval no: 09.11.2022/1153).

Animals

The study was conducted using 40 female, 206±6 g, and 4-months aged Wistar albino rats were performed in Tekirdag Namik Kemal University Experimental Animal Application and Research Center. They were housed standard cages under temperature controlled room (22±2°C) and were maintained on a 12 h light/dark cycle and fed with a standard rat pellet diet and water ad libitum.

Preparation of Ethanolic Ginger Extract

Ginger fresh rhizomes were obtained from a local store and authenticated at department of Botany. Gingers were firstly washed and dried in a dark room. Dried ginger rhizomes were mechanically pulverized in a porcelain mortar. The resulting powder mixture was kept in 95% alcohol for 24 h then the mixture was filtered. This process was repeated 3 times in total. All the prepared mixtures were collected together and alcohol was removed in the low speed evaporator. Dose of 200 mg/kg/bw/day gelatinous extract was dissolved in 2% Tween 80 solution before commencement of the experiment [16].

Induction of Diabetes and Experimental Design

A single dose of 50 mg/kg STZ (St Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5), was injected intraperitoneally (i.p.) to induce diabetes. After 3 days the application, blood glucose values>250 mg/dL (Accu-Chek Instant glucometer, Roche) were considered an indicator for developing diabetes and rats were included in the diabetes control and ginger + diabetes groups [17]. The rats were divided into 5 groups including 8 animals in each one: Control group (n=8): No application was made (untreated group), sham group (n=8): Tween 80 was given to rats by oral gavage, ginger group (n=8): Fresh ginger extract (prepared daily) was given by oral gavage at the dose of 200 mg/kg, diabetes control group (n=8): This group was administered 50 mg/kg i.p. STZ, ginger + diabetes group (n=8): After diabetes was established, 200 mg/kg ginger extract was administered to this group by oral gavage.

Measurement of Fasting Blood Glucose (BG, mg/dL) and Live Body Weight (BW, g)

For determined of blood glucose levels of all animals, blood glucose were measured initial, 3rd days, and after the experimental period (on days 0, 3, and 33). Live body weights were measured daily in all groups from at the first day of the experiment (0) until the end of the experiment on 33rd days.

Histopathological and Histomorphometric Procedure

At the end of 30 days, rats were fasted for approximately 13 h before sacrificing and were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Then small intestine tissues of each rat (duodenum, jejunum, and ileum) were gently taken up for histological evaluation. Then the segments of the tissues were fixed in 10% formalin for 48 h. After fixation, the samples were processed for routine histological protocols and embedded in paraffin. The sections taken at 4-5 µm and stained with Crossman's triple staining for histological examination [18]. Villus height, and crypt depth were determined in five villi chosen randomly from six sections taken serially from the duodenum, jejunum, and ileum of each animal [19]. Differences between groups were compared statistically.

Scanning Electron Microscopy (SEM)

The tissues were washed twice in 0.1 M phosphate buffer solution (PBS). Subsequent to the washing process, the tissues fixed on 3% glutaraldehyde for 24 h. And then the tissues dehydrated through a graduated-acetone series (25%, 50%, 70%, and 100%). Two samples from each part of the intestine (inner and vertical surface) were removed and placed on stubs. FEI brand, "Quanta FEG 250" model scanning electron microscope, with technology that does not require vacuum, critical drying or coating with gold, was used. Thus, direct images were taken from identified tissues and then recorded [20].

Immunohistochemical Staining

The streptavidin biotin peroxidase complex (strepABC) method was applied in the small intestine. Sections of 4-5 µm thickness were collected on adhesive slides. The sections were processed in citrate buffer solution (pH: 6.0) for 10 min in a microwave oven at 700 watts. Then, tissues kept on hold in 3% hydrogen peroxide (H_2O_2) for 15 min. The blocking solution A was dripped to prevent the nonspecific binding by IHC Kit. Sections were incubated with bcl-2 primary antibody (ab196495, Abcam, Cambridge, MA 02139-1517 USA, 1/200 dilution), bax primary antibody (ab216494, 1/200 dilution), TNF-α primary antibody (ab220210, TNFA/11721, 1/150 dilution), and IL-1β primary antibody (ab205924, 1/300 dilution) were applied on the sections in a humid environment at the ambient temperature for 1 h. Seconder antibody and after streptavidin was dripped on the sections for 30 min. The 3,3′-Diaminobenzidine tetrahydrochloride (DAB) used as chromogen for 10 min

then Mayer's haematoxylin was used for the background staining. Rabbit serum without primer antibody served as the negative control. Sections were evaluated using research microscope (Olympus BX51, Tokyo, Japan). Evaluation of immunoreactivity of bcl-2, bax, TNF-α, and IL-1β were scored. Immunoreactive cells were categorized as having negative, mild, moderate, and intensive [17,21].

Statistical Analysis

In the study, "G. Power-3.1.9.2" program was used. The sample size was calculated at the 95% confidence level. F tests-ANOVA was used fixed effects, omnibus, one-way and compute of sample size were examined [22].

The change in weight and blood glucose values of rats in all groups was analyzed by two-way repeated measures ANOVA, followed by Bonferroni multiple comparisons test. Paired comparisons between weight and blood glucose values of matched subjects were done with paired t test. Data of villus length, and crypt depth were examined for normality distribution and variance homogeneity assumptions (Shapiro-wilk test). If normally distributed, a One-way ANOVA test was applied, and the differences between groups were analyzed with the post-hoc Tukey's test. The differences were considered significant at P<0.05, and the means and standard errors were calculated. In the study, nonparametric tests were used as the data did not provide normal assumptions. Therefore, the differences between the groups were analyzed with Kruskal Wallis and Mann Whitney U tests. Additionally, the differences were considered significant at P<0.05, and the median values (minimum-maximum) were calculated, and the table was made by means.

Results

Results of Statistical Power and Sample Size

At the end of the analysis, Number of groups=5, Output: Noncentrality parameter λ = 22.9189624, Critical F = 2.6414652, Numerator df = 4, Denominator df = 35, Actual power = 0.9639538 and the standardized effect size was found to be 0.7569 based on previous studies, and with a theoretical power of 0.95, the minimum sample size was calculated as 40, with 8 in each group *(Fig. 1)*.

Blood Glucose Levels Results (BG, mg/dL)

The statistical results showed that streptozotocin (STZ) administration increased blood glucose (BG) levels, and ginger treatment significantly decreased the BG levels in the ginger + diabetes group. Also, there was no statistically significant difference in BG levels between control, sham, and ginger groups (P>0.05) *(Table 1)*.

Body Weight Results (BW, g)

At the end of the study, a significant decrease in body weight (BW) gain was observed in the diabetes control and ginger + diabetes groups. When the rats in the ginger group were evaluated in terms of the increase in BW from the beginning to the last day of the study, it was found that there was no statistically significant increase *(Table 2)*.

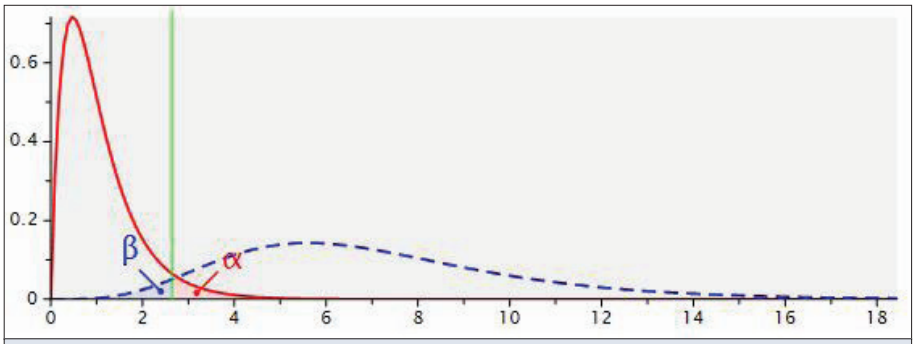

Fig 1. Results of statistical power and sample size

Table 1. *Comparison of blood glucose (mg/dL) change among all groups*

Groups	Initial Blood Glucose (0thDay) (M ± SD)	Diabetic Blood Glucose (3th Day) (M ± SD)	Final Blood Glucose (33th Day) (M ± SD)
Control	78.50±2.29	77.00±2.29	82.50±4.33
Sham	77.75±2.50	80.75±3.00	84.38±3.01[b]
Ginger	88.50±3.40	84.88±1.78	88.00±3.26
Diabetes control	78.25±3.12	270.00±5.05[a]	438.63±21.25[bc]
Ginger + Diabetes	76.75±2.26	261.88±4.46[a]	262.00±22.25[bc]

M: mean, SD: standart deviation. Different superscripts (a,b,c) indicate significant differences between groups. The differences were considered significant at P<0.05

Table 2. *Statistical evaluation of rats body weight changes between all groups (g)*

Groups	Initial Body Weight (0th day) (M ± SD)	Diabetic Body Weight (3th days) (M ± SD)	Final Body Weight (33th days) (M ± SD)
Control	206.00±6.01	229.25±5.07[a]	241.75±5.58[b]
Sham	202.75±4.17	225.50±11.85[a]	243.88±9.85[b]
Ginger	214.50±5.86	232.00±6.48[a]	225.38±6.36
Diabetes control	227.50±2.85	222.25±2.90[a]	170.88±0.72[bc]
Ginger + Diabetes	236.88±9.51	215.25±6.79	209.25±6.82

M: mean, SD: standart deviation. Different superscripts (a,b,c) indicate significant differences between groups. The differences were considered significant at P<0.05

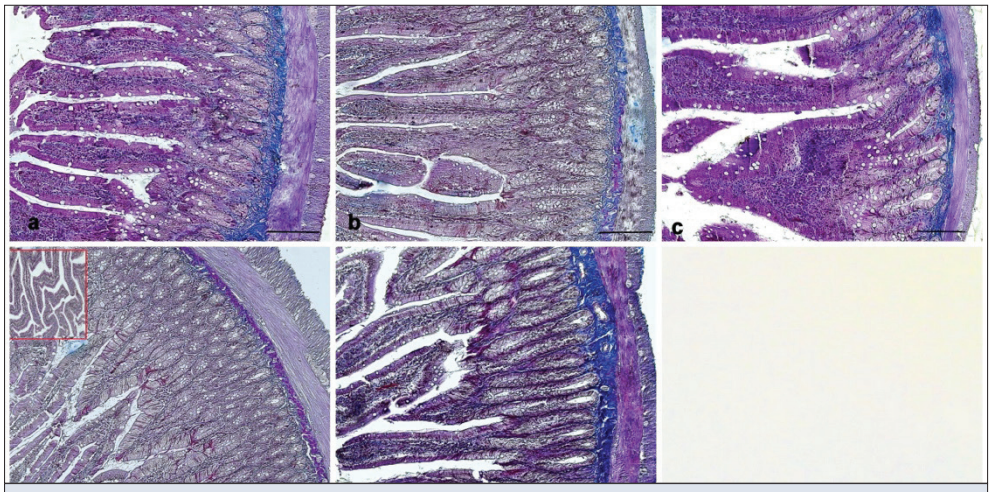

Fig 2. Microphotography of rat duodenum. Control (a), sham group (b), ginger group (c), diabetes control group, longer and irregularly curved villi (small picture) (d), Ginger + diabetes group (e). Crossman's triple staining, bars=200 µm

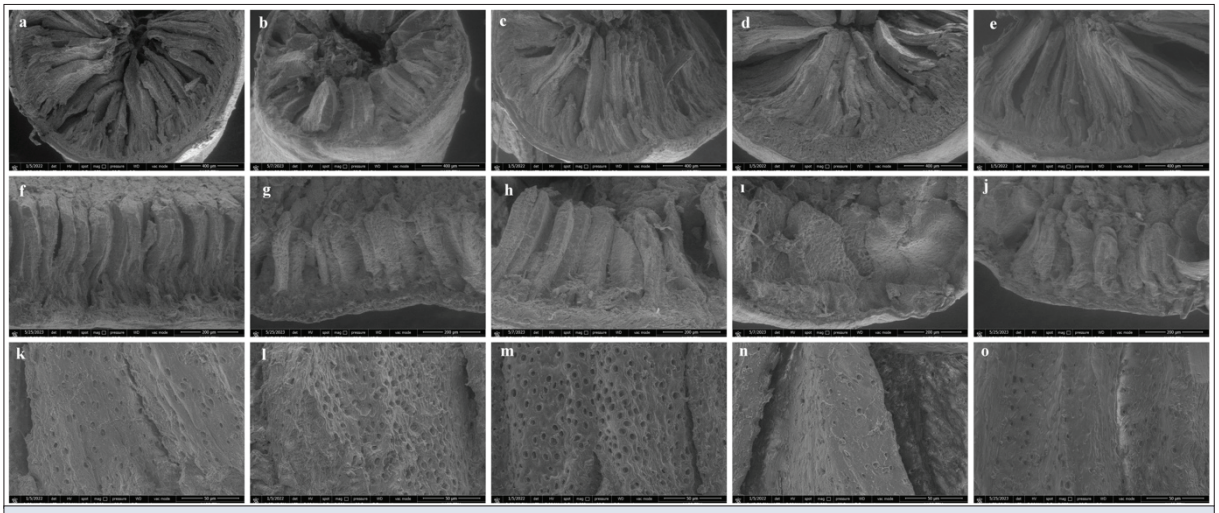

Fig 3. SEM microphotography of rats duodenum, jejunum and ileum. General view of in duodenum of control (a), sham (b), ginger (c), diabetes control (d), ginger + diabetes group (e). Structure of villi in jejunum of control (f), sham (g), ginger (h), diabetes control (i), ginger + diabetes group (j). Goblet cells in ileum of control (k), sham (l), ginger (m), diabetes control (n), ginger + diabetes group (o)

Histopathological and Histomorphometry Result

Normal histological findings were found in the duodenum *(Fig. 2-a,b,c,d,e)*, jejunum and ileum of small intestines of the control, sham and ginger groups. In the diabetes control group, the villi covering the lumen were dense. The Villus height could not be measured in the duodenum due to distortion in the villi *(Fig. 2-d)*. Like as the villus height, crypts depth, the thickness of tunica muscularis and tunica serosa layers increased and this increase was found to be significant in the diabetes control group. *(Table 3)*. In the ginger + diabetes group,

Table 3. Comparison of villus height, crypt depth, thickness of tunica muscularis layer, and thickness of tunica serosa layer among groups

Measurement (µm)	C M+SD	SH M+SD	G M+SD	DC M+SD	G+D M+SD
VH (jejunum)	231.0 (180.6-330.5)	234.7 (134.5-346.8)	229.7 (178.9-307.1)	400.1 [ac] (233.6-578.9)	227.0[b] (166.2-343.8)
VH (ileum)	113.3±1.6	111.2±2.0	108.9±0.9[e]	274.2±7.9[ac]	108.5±1.1 [b]
CD (duodenum)	31.2±0.8	34.0±0.80	33.6±0.8	57.4±1.7 [ac]	35.8±1.1 [b]
CD (jejunum)	25.8 (16.2-41.9)	26.2 (15.2-46.4)	29.30[e] (17.2-40.7)	42.65 [ac] (21.1-88.4)	26.45 [b] (15.1-50.5)
CD (ileum)	20.10 (10.1-34.2)	19.25 (10.1-35.1)	19.65 (10.4-32.4)	36.00[ac] (18.8-79.5)	19.75 [b] (11.2-38.1)
TTM (duodenum)	37.75 (28.6-49.6)	38.95 (35.8-48.2)	40.65 (34.5-43,2)	98.55[ac] (66.1-117.5)	74.70[bd] (54.3-91.7)
TTM (jejunum)	45.10 (34.4-59.5)	36.30 (31.5-53,6)	38.30 (31.5-52.6)	74.90[ac] (55.4-99.1)	61.80[bd] (51.3-81.7)
TTM (ileum)	38.15 (11.4-55.4)	36.45 (20.1-48.4)	30.40 (15.3-43.2)	60.90[ac] (25.5-87.4)	43.30[bd] (26.8-54.2)
TTS (duodenum)	25.00 (17.9-31.2)	25.65 (17.2-33.2)	26.80 (17.8-33.2)	68.50[ac] (49.5-107.1)	50,65[bd] (37.8-57.3)
TTS (jejunum)	24.70 (17.8-31.4)	25.50 (14.60-37.50)	23.45 (16.40-36.20)	63.30[ac] (42.60-98.40)	47.10[bd] (24.30-58.90)
TTS (ileum)	24.65 (19.6-33.8)	26.55 (18.6-30.4)	25.00 (17.6-32.5)	68.10[ac] (48.7-95.8)	37.90[bd] (32.6-48.7)

C: control, SH: sham, G: ginger, DC: diabetes control, G+D: ginger + diabetes. TTM: thickness of tunica muscularis layer, TTS: thickness of tunica serosa layer, VH: villus height, CD: crypt depth, M: mean, SD: standart deviation. a,b,c,d,e Values within a row with different superscripts differ significantly at P<0.05. a: Diabetes group versus control group, b: ginger + diabetes group versus diabetes group, c: diabetes group versus sham group, d: ginger + diabetes group versus ginger group, e: ginger group versus control group

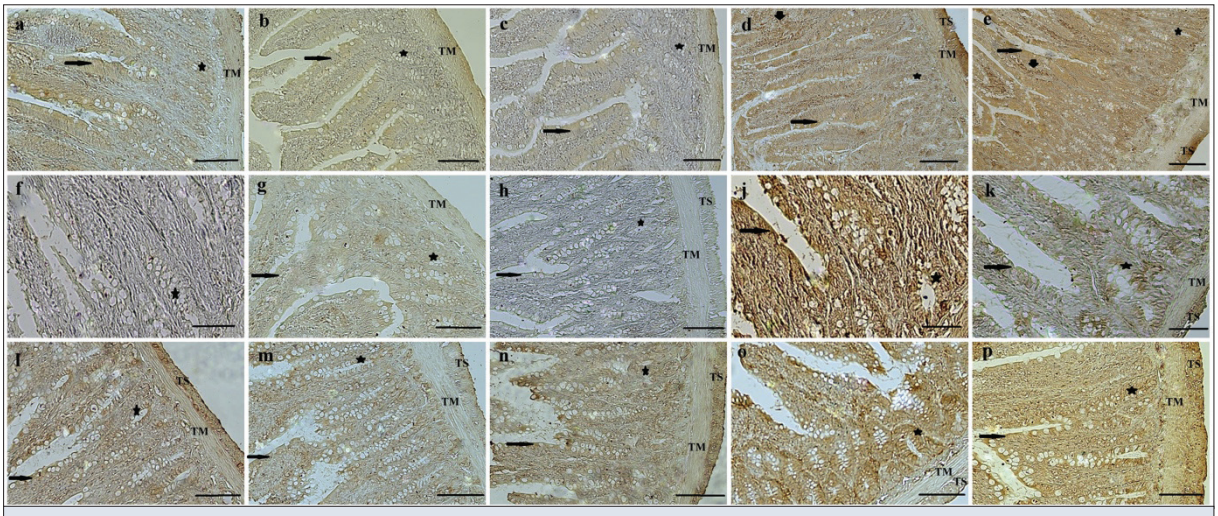

Fig 4. Bax expression in duodenum (a, b, c, d, e), jejunum (f, g, h, j, k) and ileum (l, m, n, o, p) of rats. Control group (a, f, l), sham group (b, g, m), ginger group (c, h, n), diabetes control group (d, j, o), ginger + diabetes group (e, k, p). Villus epithelial cells *(arrow)*, crypt epithelial cells *(star)*, connective tissue cells *(thick arrow)*, tunica muscularis (TM), Tunica serosa (TS). Immunohistochemical staining, bars=200 µm

it was noticed that the villi showed a more regular and distinguishable arrangement with each other. There was also a noticeable reduction in the appearance of the folds seen in the diabetes control group. This group showed similar characteristics to control, sham and ginger group in microscopic examinations *(Fig. 2-e)*.

SEM Results

When the general structures of all groups were compared, the control, sham, ginger and ginger + diabetes groups were found to have normal histological findings *(Fig. 3-a,b,c,e)*. In the diabetes control group, the arrangement of the villi was not regular. In addition, an increase was noted in the tunica mucosa, tunica muscularis and tunica serosa layers *(Fig. 3d)*. It was observed that the villi of the duodenum were leaf-shaped, the ends of the jejunum had a blunt finger appearance and the ileum had a tongue-shaped appearance *(Fig. 3, Fig. 4, Fig. 5)*. In the control,

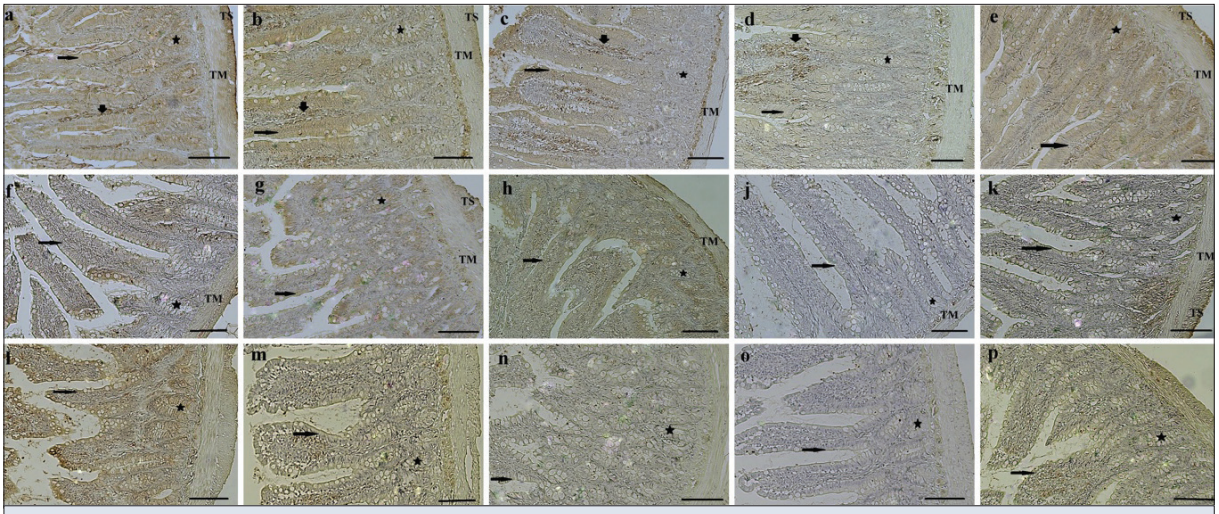

Fig 5. Bcl-2 expression in duodenum (a, b, c, d, e), jejunum (f, g, h, j, k) and ileum (l, m, n, o, p) of rats. Control group (a, f, l), sham group (b, g, m), ginger group (c, h, n), diabetes control group (d, j, o), ginger + diabetes group (e, k, p). Villus epithelial cells *(arrow),* crypt epithelial cells *(star),* connective tissue cells *(thick arrow),* tunica muscularis (TM), Tunica serosa (TS). Immunohistochemical staining, bars=200 µm

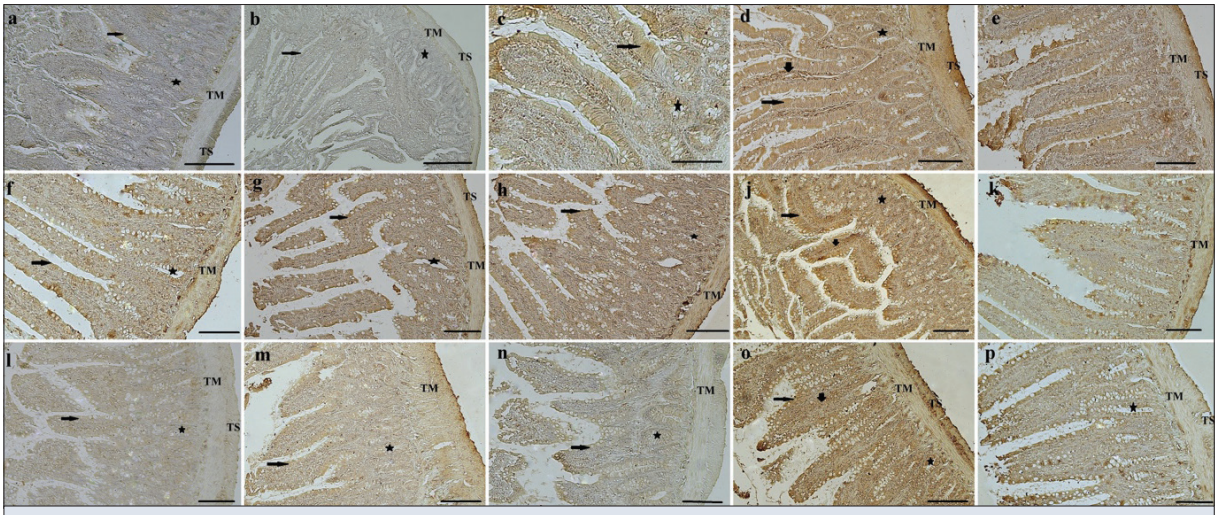

Fig 6. TNF-α expression in duodenum (a, b, c, d, e), jejunum (f, g, h, j, k) and ileum (l, m, n, o, p) of rats. Control group (a, f, l), sham group (b, g, m), ginger group (c, h, n), diabetes control group (d, j, o), ginger + diabetes group (e, k, p). Villus epithelial cells *(arrow),* crypt epithelial cells *(star),* connective tissue cells *(thick arrow),* tunica muscularis (TM), Tunica serosa (TS). Immunohistochemical staining, bars=200 µm

sham, ginger, and ginger +diabetes groups, the villi were arranged parallel and regularly *(Fig. 3-f,g,h,j)*. In the diabetes control group, the villi lay on top of each other and irregular spaces were formed between them *(Fig. 3-i)*.

While the shape of the goblet cells was normal structure in control, sham, ginger group *(Fig. 3-k,l,m,o),* the shape of the goblet cells was contracted and their diameters were decreased in diabetes control group *(Fig. 3-n)*. Ginger + diabetes group was similar to control group *(Fig. 3-o)*.

Immunohistochemical Results

While mild bax expression was observed in villus epithelial cells, crypt epithelial cells, and tunica muscularis and moderate bax expression was observed in tunica serosa of duodenum, jejunum and ileum of control, sham and ginger groups *(Fig. 4-a,b,c,f,g,h,l,m,n)*, intensive expression was detected in the diabetes control group *(Fig. 4-d,j,o)*. In the ginger + diabetes group, moderate intensity expression was detected in villus and crypt epithelial cells, and tunica muscularis *(Fig. 4-e,k,p)*. Also, intensive expression was detected in connective tissue cells *(Fig. 4-e)*.

Moderate bcl-2 expression was observed in villus and crypt epithelial cells, connective tissue cells, tunica serosa and mild expression was determined tunica muscularis in duodenum *(Fig. 5-a,b,c,e)* and mild bcl-2 expression was observed in villus and crypt epithelial cells in jejunum and ileum of control, sham, ginger and ginger + diabetes groups *(Fig. 5-f,g,h,k,l,m,n,p)*. In the diabetes control group, only moderate expression was noted some of connective tissue cells in duodenum *(Fig. 5-d)*. It was not determined bcl-2 expression in jejunum and ileum *(Fig. 5-k,p)*.

Mild TNF-α expression was determined in the duodenum and ileum of control and sham groups *(Fig. 6-a,b,l,m)*, and

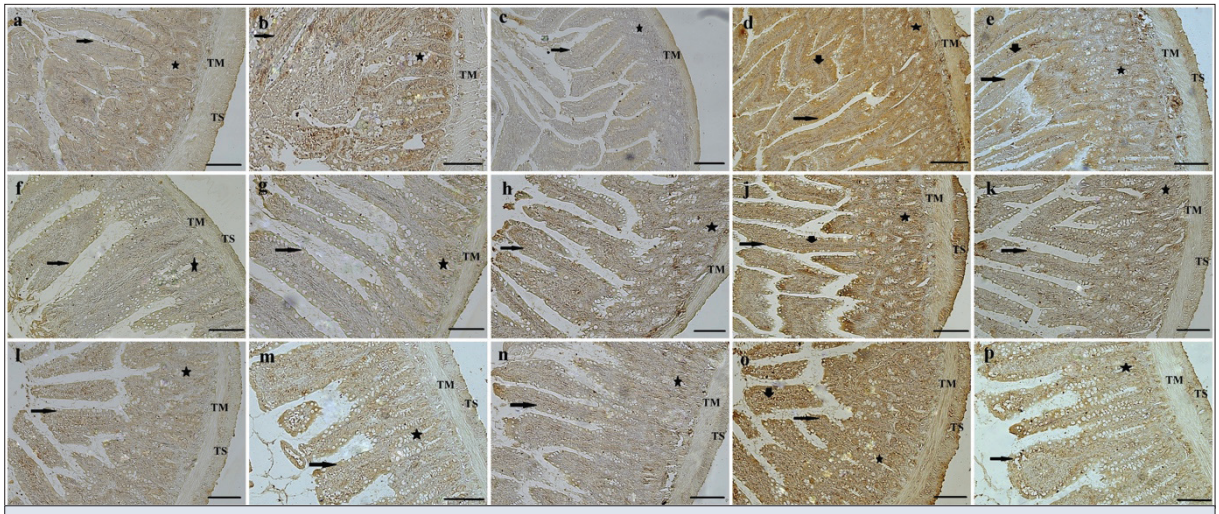

Fig 7. IL-1β expression in duodenum (a, b, c, d, e), jejunum (f, g, h, j, k) and ileum (l, m, n, o, p) of rats. Control group (a, f, l), sham group (b, g, m), ginger group (c, h, n), diabetes control group (d, j, o), ginger + diabetes group (e, k, p). Villus epithelial cells *(arrow),* crypt epithelial cells *(star),* connective tissue cells *(thick arrow),* tunica muscularis (TM), Tunica serosa (TS). Immunohistochemical staining, bars=200 µm

moderate TNF-α expression was noted in in ginger and ginger +diabetes groups *(Fig. 6-c,h,n,e,j,p)* were detected in the villus and crypt epithelial cells and tunica muscularis of the duodenum, jejunum, ileum. Intensive expression was detected in the villus and crypt epithelial cells, tunica muscularis and tunica serosa *(Fig. 6-d,j,o).*

IL-1β expression was mild reaction in the villus and crypt epithelial cells, tunica muscularis, connective tissue cells and tunica serosa layers of all groups except diabetes control group *(Fig. 7-a,b,c,e,f,g,h,k,l,m,n,p).* IL-1β expression was found to be increased in the cells of all layers in the diabetes control group and intensive expression was found in the villus, crypt epithelial cells, connective tissue cells, tunica serosa and moderate expression was found in tunica muscularis *(Fig. 7-d,j,o).*

Discussion

Diabetes is one of the fastest growing diseases worldwide [23]. Many studies reported that ginger extract is responsible for hypoglycaemic activity [16,24,25]. Daily et al.[26] examined several clinical experiments published in 2013-2014 and made a meta-analysis to clarify the evidence for using ginger to decrease blood glucose, and they reported ginger supplementation significantly lowers fasting blood glucose and HbA1c levels. This study was observed that fasting blood glucose increased in the diabetes control group. When it was compared to the diabetes control group with the ginger + diabetic group, it was measured a significant difference between the two groups.

It was reported that body weight decreases in diabetic rats and the loss of weight may be due to excessive degradation of tissue proteins, and protein losing due to unavailability of carbohydrates as an energy source and catabolism of fats. But they indicated that when diabetic rats were fed with ginger for 30 days, their body weight improved. They added that these results may be connected with the fact that ginger contains many bioactive and pharmacological compounds [16]. This study was indicated that a significant decrease in body weight in the diabetes control group and the body weight increased when it was treated with ginger extract for 30 days.

Small intestine morphology can provide information about intestine health. These include hyperplasia and hypertrophy of epithelial cells, increasing crypt depth, villus height and smooth muscle cells [5,27-30]. In studies, light microscopic examination showed that the small intestine tissue of the diabetes group was thicker, the villi and crypts were longer and had deeper volume and surface area compared to the control group [31-33]. Another study was reported that the villus height and crypt depth increased in hyperglycaemia the main reason might be due to a measure taken by the organism against nutrient deficiency and restriction as a result of inadequate nutrition of tissues in the absence of glucose [34]. In this study, it was found that the thickness of tunica mucosa, tunica muscularis, tunica serosa layers, villus height and crypt depth increased in the diabetes control group similar to the previous studies [28-30]. In the group treated with ginger, a regression in the thickness of the mentioned layers was detected. In the study, ginger was found to have an ameliorating effect on the findings detected in the small intestinal mucosa of diabetes.

In a study in which electron microscopic examination was performed, it was reported that the shape of jejunal and ileal villi in diabetic mice was found to be variable; many were twisted, some were conical and others collapsed laterally onto neighbouring villi. Diabetes was reported to increase the adverse effects towards the end of the small

intestine [35]. In the study, it was observed that the villi in the duodenum, jejunum and ileum in the diabetes control group were more elongated, curved and adhered to each other in a wrapped appearance compared to the control group.

One of the main opinion in DM is the view that diabetes causes disruption of intestinal homeostasis, i.e., the balance between cell proliferation and death. Chen et al.[33] found that the expression of TNF-α, IL-1 and IL-6, and bax increased markedly with increasing hyperglycaemia, bcl-2 expression decreased in cell culture. In the present study, the release of bax expression increased while bcl-2 expression decreased in the diabetes group. It has been reported that increased of TNF-α expression activate bax, which cause apoptosis [13]. In the study, increased TNF-α, IL-1β expressions were observed in the diabetes control group. The increase in the expression of bax, one of the pro-apoptotic proteins, together with both cytokines suggests that cytokines have a chain effect with each other and TNF-α has the mentioned inducing effect. In a study was reported that IL-6 levels were higher in diabetics than in non-diabetics. In the 4-year study, they drew attention to the fact that women who did not have diabetes but later developed diabetes had high levels of IL-6 before they developed diabetes. As a result of the study, cytokines may be higher in individuals at risk and inflammatory markers may help early diagnosis of this disease [36].

In conclusion, the study evaluated the effects of diabetes both microscopically and histomorphometrically as well as apoptotic and inflammatory effects. In addition, the ameliorative effects of 30-day 200 mg/kg ginger extract against the adverse effects of diabetes were also investigated. At the end of the study, the villus height, crypt depth, tunica mucosa, tunica muscularis and tunica serosa layers were increased in the diabetes control group. Although diabetes means that organs and/or tissues are deprived of glucose, which is the main source of energy, the fact that it leads to a proliferative result contrary to what is known suggests that there may be different mechanisms. To this end, more extensive studies including food intake, microbiota and hormones are needed. In addition, it was determined that diabetes caused an increase in inflammation, increased TNF-α, IL-1β expression, triggered apoptosis, increased bax release and decreased bcl-2 release. It was observed that ginger extract administered orally decreased and/or eliminated the mentioned negative effects. This study may be a reference for other studies on this subject.

Availability of Data and Materials

The findings of the current study are available from the corresponding author (B. Bakir) upon reasonable request.

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RESEARCH ARTICLE

Insulin and Bull Sperm Interactions During Cryopreservation

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ABSTRACT

The aim of the present study was to evaluate the effects of insulin supplemented extender on bull semen at a post-thaw stage. Semen samples were collected four times from a Zavot bull which is endangered species in Türkiye. Semen samples were diluted with Tris based extender supplemented with 10 IU or 15 IU of insulin and control. Motility and plasma membrane integrity were evaluated by a phase-contrast microscope. Acrosome integrity (Fitc-Peanut Agglutinin), mitochondrial membrane potential (Rhodamine 123), nitric oxide level (4,5-Diaminofluorescein-2/diacetate) were evaluated with dual staining propidium iodide using flow cytometry. Motility and plasma membrane functional integrity were better preserved compared to control group ($P<0.05$). Acrosome integrity results were statistically similar between control and 10 IU insulin groups ($P>0.05$), but increased insulin negatively affected acrosome integrity ($p<0.05$). Mitochondrial membrane potential was found to be higher when compared all cells in insulin containing groups compared to control ($P<0.05$). However, results were found similar in all groups with PI negative/mitochondrial membrane potential positive ($P>0.05$). There were not any significant differences among groups in terms of nitric oxide level ($P>0.05$). In conclusion, it was thought that insulin involved in energy metabolism and improved preservation of bull semen.

Keywords: Zavot, Bull semen, Insulin, Cryopreservation

INTRODUCTION

Embryos and germ cells are frozen and stored in order to ensure the continuance of fertility in all living species. The storage of genetic resources for this reason may avert future fertility declines and, perhaps, the extinction of a race or species [1]. Accordingly, the preservation of gamete cells in endangered species, such as the Zavot cow, which is endangered in Türkiye, is of particular relevance [1-3].

In many cases, frozen sperm is used in assisted reproduction. Many investigations are conducted on the composition and effects of semen extenders in an effort to lessen the deleterious effects of freezing and thawing on semen and so boost its reproductive potential [4-6]. In these research, fertility-related characteristics like as motility and acrosome integrity are evaluated [7].

Insulin enhances sperm motility through participating in glucose and lipid metabolism, and it also affects the plasma membrane and acrosome of sperm [9,10]. It has been shown that insulin given to several species' sperm extenders improves sperm motility and reduces the negative effects of freezing phases [9,11,12]. The purpose of the current study is to explore the effects of freezing bull sperm and insulin at post-thaw stage.

MATERIAL AND METHODS

Ethical Approval

Animal Experiments Local Ethics Committee of Kafkas University (Approval number: KAÜ-HADYEK/2021-194) have approved all issues concerning the experimental setups and evaluation techniques.

Animals

A Zavot bull of the 3 years old was maintained at Prof. Dr. Ali Riza Aksoy Education, Research and Application Farm at Faculty of Veterinary Medicine, Kafkas University, Kars, Türkiye.

Chemicals

4,5-Diaminofluorescein-2/diacetate (DAF-2/DA) was

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from Genaxxon Bioscience (Biberach, Germany). Other chemicals used in the study were obtained from Merck (Merck, Darmstadt, Germany) and Sigma (Sigma, USA) unless those mentioned.

Experimental Design

The study was constructed with a total of three groups: those with 10 IU and 15 IU of insulin added to the extenders, as well as the control group. 10 IU (I-10) and 15 IU (I-15) insulin lispro (Humalog, Lilly, Italy) added on 3 mL of extenders. Extenders consisted of 223.7 mmol/L Tris, 55.5 mmol/L fructose, 66.6 mmol/L citric acid, 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA (Merck), 4 g/L penicillin G, 3 g/L dihydrostreptomycin, 20% egg yolk (v/v) in distilled water.

Semen collection was performed four times every other day using artificial vagina. After the collection, the ejaculates were transferred to a water bath (37°C). Rapid wave motion and motility was evaluated by a phase-contrast microscope (Nikon Eclipse-E400, Tokyo, Japan) with a warm slide (37°C). Ejaculates with > 70% motility and > $1.5x10^9$ spermatozoon/mL were chosen for cryopreservation.

Each of the groups was diluted to $25x10^6$ sperm/mL with related extender. The groups were then cooled to 5°C within 1 h. Following equilibration, the straws were subjected to cryopreservation by exposure to liquid nitrogen vapor at a distance of 4 cm above the surface of the liquid nitrogen, which maintained a temperature of -120°C, for a duration of 15 min. Subsequently, the straws were immersed in liquid nitrogen for preservation. The cryopreserved semen contained within straws underwent a thawing process at a temperature of 37°C for a duration of 30 sec within a water bath, in order to facilitate subsequent evaluation following the thawing process [7,13].

Semen Analysis

The motility of sperm was evaluated subjectively using a 400x phase-contrast microscope with a 37°C warm slide [26]. The functional integrity of the sperm membrane was assessed through the use of Hypo osmotic swelling test (HOST) [14], which involved the incubation of 10 µL of semen with 100 µL of 100 mOsm hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min. The evaluation was based on the observation of curled and swollen tails. Following incubation, a volume of 20 µL of the aforementioned mixture was applied onto a heated slide and subsequently covered with a slip. Two hundred sperm cells were examined using a phase-contrast microscope at a magnification of 1000x. Spermatozoa exhibiting morphological anomalies such as tail swelling or coiling were documented.

Flow Cytometric Analysis

Analysis was performed using Attune NxT Acoustic Focusing Cytometer (Invitrogen, USA). Fluorescence was measured at 480 nm excitation (10 nm excitation bandwidth) and 530/30 nm filter (BL-1) and 695/40 nm filter (BL-3) emission connected to Attune NxT software v2.7 (Thermo Fisher). After gating the cell population using forward and side scatter light signals, the average fluorescence intensity of the sperm cells under analysis was measured. There were 10.000 sperm cells in the per assay.

The fluorescein isothiocyanate–conjugated peanut agglutinin (PNA)/propidium iodide (PI) dual-staining technique was used to evaluate acrosome integrity. Rhodamine 123/PI was used to evaluate mitochondrial membrane potential. DAF-2/DA / PI was used to evaluate nitric oxide level in live cells. All flow cytometric analysis performed as previously described by Gürler et al.[15].

Statistical Analysis

IBM SPSS version 28 was used to do statistical analysis. The Shapiro Wilk test was used to assess for normality. The data were shown as mean ± standard error. The statistical significance of the differences between subdivided groups was determined using one-way ANOVA followed by Tukey. The Kruskal-Wallis test was used to examine data having a non-normal distribution. P values less than 0.05 were deemed statistically significant.

Results

Motility, plasma membrane functional integrity, acrosome integrity, mitochondrial membrane potential, and nitric oxide concentration are detailed in *Table 1*.

The post-thaw motility was found to be 47.08, 55.00 and 52.00 in control, I-10 and I-15 groups, respectively. Motility was better preserved in groups containing insulin ($P<0.05$). It was observed that the semen dilutions with extender containing insulin protected the plasma membrane integrity better than the control group ($P<0.05$). In living cells, the overall acrosome integrity and the acrosome integrity ratio were equivalent in the control and I-10 groups ($P>0.05$), but considerably lower in the I-15 group ($P<0.05$). By analyzing all cells, it was shown that mitochondrial membrane potential was higher in insulin-containing groups ($P<0.05$). All groups exhibited a similar ratio of mitochondrial membrane potential in living cells ($P>0.05$). Nitric oxide levels in living cells did not differ significantly among the groups ($P>0.05$).

Discussion

Motility of spermatozoa is one of the most critical measures in evaluating male reproductive ability [16].

Table 1. *Effects of insulin on spermatological parameters*

Groups	Measurements						
	Motility (%)	**HOST (%)**	**A (%)**	**A-P (%)**	**M (%)**	**M-P (%)**	**NO (%)**
Control	47.08±1.30[a]	73.25±1.30[a]	75.12±1.27[a]	63.06±1.89[a]	71.84±2.89[a]	37.84±1.64	41.72±1.68
I-10	55.00±1.51[b]	79.00±0.69[b]	74.35±1.47[a]	60.09±1.43[a]	81.82±2.29[b]	38.21±2.25	37.43±1.74
I-15	52.00±1.14[b]	77.67±0.87[b]	70.33±1.30[b]	53.08±1.73[b]	85.27±1.29[b]	38.44±1.79	38.07±1.64

[a,b] *Values with different superscripts in the same column for each times are significantly different (P<0.05),* ***HOST:*** *Plasma Membrane Functional Integrity,* ***A:*** *Total Acrosome Integrity,* ***A-P:*** *Acrosome integrity with Intact Plasma Membrane,* ***M:*** *Total Mitochondrial Membrane Potential,* ***M-P:*** *Mitochondrial Membrane Potential with Intact Plasma Membrane,* ***NO:*** *Nitric Oxide Positive with Intact Plasma Membrane*

Diabetes mellitus type-1 has been observed to negatively impact sperm motility in males [11]. Furthermore, glucose-free media was found to suppress the acrosome response of human spermatozoa [8]. Spermatozoa generate metabolic activity depending on an insulin-dependent signaling mechanism [22]. As a matter of fact, with the discovery of insulin receptors in spermatozoa in recent years, the complex mechanism of how spermatozoa can metabolize sugars, amino acids and fatty acids has been shed light on. In the current investigation, motility was shown to increase considerably in the groups containing insulin. It is believed that the reduction in unfavorable effects of the freeze-thaw process is related to insulin's ability to boost the use of energy sources. Sugars added to sperm extenders create osmotic pressure and inhibit the development of ice crystals during freezing. And, the sperm metabolism is slowed by the chilling procedure before the freezing process [17,18]. The interesting aspect of our study is that spermatozoa's potential absorption of sugar sources may increase metabolic activities and the effectiveness of cooling and freezing processes.

There is a correlation between sperm membrane functional integrity and motility, according to studies [7,13,14]. Fertilization, the most fundamental success criterion of sperm freezing, requires that the functional integrity of the sperm membrane be maintained throughout the freezing and thawing processes. The hypo-osmotic swelling test is used to evaluate the plasma membrane integrity; it examines the swelling and curling of spermatozoa with an active biochemical structure in an environment containing water. Researchers prefer this test because it is reliable and affordable [7,16,25]. The integrity of the plasma membrane was better preserved in the insulin-containing groups, as evidenced by our study, which is consistent with the motility results.

In order for sperm freezing to be successful, it is crucial to maintain the integrity of the acrosome, which plays a crucial role in the fertilization [16]. In our investigation, 10 IU of insulin resulted in acrosome integrity comparable to that of the control group, but a high dose had a deleterious effect on acrosome integrity. According to research on human spermatozoa, spermatozoa have insulin receptors in the acrosome area and trigger the acrosome reaction [10,11]. In light of this knowledge, it is believed that raising the insulin dosage in the trial at hand increases the acrosome reaction.

A positive correlation exists between elevated mitochondrial membrane potential and increased motility [19]. However, mitochondrial membrane potential decreases in time after sperm retrieval, especially in humans, and it has been stated that mitochondrial membrane potential can be used to predict motility in humans [23]. In our research, insulin groups had a high percentage of total mitochondrial membrane potential. This is consistent with the increased motility seen in the insulin-treated groups and the function of insulin in glucose and lipid metabolism. The mitochondrial membrane potential data of live spermatozoa, however, did not reveal any significant differences.

It is also a known fact that nitric oxide plays a critical role in male fertility. Nitric oxide can regulate spermatogenesis, sperm maturation and motility, and the apoptosis process in abnormal germ cells, as well as decrease sperm motility and cause sperm toxicity and infertility [24]. According to the research conducted by Lampiao and Du Plessis [11], insulin promotes an increase in the concentration of nitric oxide in human sperm. Moreover, it has been reported that L-arginine stimulates the nitric oxide production [20]. Hoshiyama et al.[21] reported in their work on Human Glomerular Endothelial Cells that an increased level of glucose in the medium inhibits nitric oxide production, and the addition of L-arginine to the medium weakens this impact of glucose. With this information, when the presence of L-arginine in the diluent content used by Lampiao and Du Plessis is evaluated, it is thought that the increase in the amount of nitric oxide in their study might be caused by L-arginine. Furthermore, insulin added to human sperm extender has an antioxidant effect and decreases the number of reactive oxygen species, according to another research [12]. In the presented

investigation, it was shown that the addition of insulin had no influence on the level of nitric oxide. On the other hand, a value below that of the control group was discovered. The plausible causes for this situation, as posited by the authors, include a higher concentration of carbohydrates in our investigation compared to the study conducted by Lampiao and Du Plessis [11], the L-arginine content in Ham's F10 medium as observed in the study by Lampiao and Du Plessis, differences in the content of the diluent, and variations in species.

In conclusion, it was found that insulin had positive effects on motility, plasma membrane integrity and mitochondrial membrane potential, but increased dose had a negative effect on acrosome. Overall results have been shown that 10 IU insulin facilitates the effective freezing of bull sperm and may have positive impacts on potential fertility.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

Animal Experiments Local Ethics Committee of Kafkas University (Approval number: KAÜ-HADYEK/2021-194) have approved all issues concerning the experimental setups and evaluation techniques.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

NTO designed study and wrote the manuscript. NTO analyzed post-thaw motility and flow cytometry results. TG performed HOST. CK and MCD provided the bull. SY and YÖ performed semen analysis at semen collection. NTO, MCK and OS performed cryopreservation process. NTO performed the statistical analysis.

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Research Article

Effect of Coneflower, Neem, and Thyme Extracts on Growth Performance, Blood Chemistry, Immunity and Intestinal Microbial Population of Broilers

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ABSTRACT

This study investigated the effects of herbal extracts on growth performance, organ development, immunity parameters and intestinal microbial population of broilers. A total of 840 (one day old, initial weight 45±2 g) broiler chicks were divided into 5 groups with 6 replicates having 28 chicks in each. The control group (G1) fed with basal diet, and the antibiotic group (G2) was added with 15 mg/kg virginiamycin, while herbal extracts were given coneflower 20 mL/L (G3), neem 20 mL/L (G4), and thyme 20 mL/L (G5), respectively, in drinking water. The results showed that G3 presented significant results ($P<0.05$) on parameters like feed intake, body weight, weight of bursa of fabricius, weight of the small intestine, blood parameters as compared to the G2 group. As for as intestinal microbiota population concerned G5 decreased the *Escherichia coli (E. coli)* contents while, G4 increased the *Lactobacillus* contents. In conclusion, coneflower improved feed conversion ratio compared to virginiamycin in broilers while neem and thyme were the most effective in the enhancement of immune parameters, serum chemistry and *E. coli* contents.

Keywords: Herbal Medicine, Broiler, Immunity, Intestinal microbe

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Introduction

In recent decades, antibiotics have received immense attention from scientists owing to their effectiveness against several diseases. However, these have been found to have various side effects like toxicity, drug residual problem and drug resistance. Thus, scientists are trying to find alternative protocols. For this, the use of additives and natural therapies like medicinal plants rich in anti-oxidents have become best choice to control the different poultry diseases and improve the weight gain and feed conversion ratio (FCR) [1,2]. Among many additives, herbal extracts have been used to replace the generation of antibiotics, which can promote animal health in intensive poultry production [3,4]. Herbal medicine, extracts or compounds can promote animal growth and improve the immune system of the animal which helps to fight against various diseases [5-8].

The main effects of herbal extracts on animals health include to promote appetite, increased feed intake, secretion of endogenous digestive enzymes, activation of the immune system, antibacterial, antiinflammatory and antioxidant properties [9,10]. Like other compounds medicinal plants are also rich in flavonoids, glucosinolates and their intermediates affect the physical structure and chemical properties of the digestive tract [11]. There are many research reports on herbal medicines to cure human and animal diseases [12]. For example, the volatile oil extracted from Incense has antibacterial and antiviral effects [13,14]. Similarly, allicin is widespread on the earth and has many biological functions, such as lowering serum cholesterol, inhibiting bacterial growth and reducing oxidative stress [15,16]. In animals, the effect of applied herbal medicine is not obvious, but it can significantly improve nutrient digestibility and the reduction of *E.coli* bacteria population in intestine and also reduce the number of *clostridia* [17]. The use of feed antibiotics was once considered for the livestock industry in the 20th century and the revolution in feed industry has promoted rapid economic development [18]. However, with the long-term and large-scale abuse of antibiotics residues in the product directly threaten the animal and human health[19]. Antibiotics are helpful to screen resistant strains, leading to identify drug resistance problem which is ultimately threatening to poultry industry all over the world [20]. After the emergence of these problems, countries around the world quickly introduced relevant policies or alternatives to antibiotics. Switzerland banned the use of feed antibiotics in 1992 while, South Korea completely banned addition of antibiotics in animal feed in 2011. Even if there is no prohibition on the use of antibiotics as growth promoters, countries such as Japan and the United States have also legislated to strictly restrict the use and stipulated withdrawal period. Similarly, European countries have also implemented the newly revised feed reforms in May 2012 and feed additives management regulations with the prohibition of antibiotics for growth-enhancing purposes [21,22]. Thus, the choice of alternative especially the use of plants and their herbal extracts has been highly appreciated with no side effects or harmful residues in poultry.

Virginiamycin is an antibiotic produced through mutation of *Streptococcus virginiae* which is found to be effective against gram-positive bacteria and resistance development found minimal also againt gram-negative bacteria like *Enterobacteriaceae*. It inhibits *Enterobacteriaceae* growth, improves digestive system and tissue residues are rare [23]. Supplementing broiler diets with virginiamycin enhanced energy metabolism, growth and FCR. When virginiamycin was added to diets containing insufficient levels of protein, broilers metabolised protein more effectively [24]. It is also discovered that pullets given diets containing virginiamycin improved weight gain [25].

Latest studies have investigated the addition of herbals extract as replacement to antibiotics as growth promoters [26,27]. These herbal extracts have unique mechanism of action based on the alteration of intestinal microbiota, enhancement of enzyme secretion, improvement of immune response, morpho-histological maintenance of the gastrointestinal tract and anti-oxidant activity [28,29]. Several research studies have demonstrated their *in vitro* effect against many pathogens, with antimicrobial, antifungal and anthelmintic activity in addition to antioxidant effects [30,31]. Furthermore, some studies showed the the positive effects of neem and thyme leaf extracts on growth, FCR, intestine microflora, body weight and immune system of birds [32,33]. Therefore, on the basis of importance of above mentioned medicinal plants, the current study was planned to evaluate the effects of three commercial herbal extracts including coneflower, neem, and thyme as a substitute to antibiotics on growth performance, blood chemistry, immune system and intestinal microflora in broiler chickens. Moreover, the antidiabetic potential of these herbal extracts was also studied.

Material and Methods

Ethical Statement

The research was conducted with approval from ethical committee of MNS University of Agriculture Multan under the Ref. No. 144/IPFP-II (Batch-I) /SRGP/NAHE/HEC/2020/93.

Plant Material

Herbal extracts of three plants including coneflower,

neem, and thyme were purchased from Multan Herbal Pharma® Company.

Experimental Design

A total of 840 (one-day-old, intial weight 45±2 g) commercial cob broilers of Jadeed Group® were purchased and randomly divided into 5 groups, each group was further divided into 6 replicates and each replicate contained 28 birds. The G1 served as Normal control group while G2 served as as antibiotic treated Positive control group (+ Control) and other three groups G3, G4 and G5 were treated with herbal extracts of coneflower, neem, and thyme. Positive control group was treated with virginiamycin at 15 mg/kg with a basal diet, and G3, G4, and G5 were given coneflower at 20 mL/L, neem at 20 mL/L, and thyme at 20 mL/L, respectively, in a drinking water. During the experiment, simple water and commercial feeds (starter, grower, and finisher ration) were offered to the birds. The experiment was conducted in a closed system farm with a 21 hours light and 3 hours dark cycle. *Table 1* shows the diet composition and nutritional level in feed.

Growth Performance

During the experiment, body weight was measured on weekly basis until 42 days and daily feed intake was measured.

Immune Performance and Relative Organ Weight

The immune organ index was calculated through the following formula:

Immune organ index (mg/g) = immune organ weight/live weight

At the end of experiment, 3 birds from each replicate were randomly selected for slaughtering. Carcasses and different organs weight were calculated including abdominal fat, liver, pancreas, proventriculus, gizzard, heart and small intestine. On the 28th, 35th, and 42nd days of age, the thymus, spleen, and bursa of fabricius were taken for organ weight.

Hematological Parameters and Intestinal Bacterial Population

At the end of the experiment, 5 mL blood samples were taken from 6 birds and stored in an ETDA coated tube for haemoglobin concentration (Hb) and packed cell volume (PCV) analysis [4]. Hb and PCV were determined using Blood Chemistry Analyzer (Sysmax KX-21). Other blood parameters such as red blood cells (RBC) and white

Table 1. *The composition and nutritional level of the basal diet for broilers at each stage*

Raw Material Composition	**1～21 d**	**22～35 d**	**36～42 d**
Corn (%)	58.5	65.00	61.50
Soybean meal (%)	28.00	20.00	16.50
Wheat (%)	4.40	6.50	14.00
Fish meal (%)	4.60	2.80	2.80
Canola meal (%)	1.80	1.90	3.30
Stone powder (%)	0.90	0.95	0.93
Soybean oil (%)	0.90	1.90	—
Salt (%)	0.21	0.25	0.25
DL-methionine (%)	0.10	0.10	0.13
L-lysine (%)	0.09	0.10	0.09
Compound multi-ore (%)	0.25	0.25	0.25
Nutritional Level			
Metabolizable energy (Mcal/kg)	2.85	2.95	3.05
Crude protein (%)	21.50	19.50	17.50
Calcium (%)	1.00	0.95	0.90
Available phosphorus (%)	0.50	0.45	0.42
Lysine (%)	1.20	1.15	1.00
Methionine (%)	0.55	0.48	0.45
Methionine + cysteine (%)	0.95	0.85	0.75
Sodium (%)	0.18	0.16	0.14

blood cells (WBC) were also calculated. Meanwhile, diabetes parameters such as cholesterol level and blood glucose were also calculated following the method of [4]. A compound microscope was used to obtain differential leukocyte count of blood samples. The contents of *Escherichia coli* and *Lactobacillus* in the ileum were also determined by agar plate method.

Statistical Analysis

Using SPSS Software, one-way analysis of variance was done to test the data, and the Tukey test was performed for comparison of the means. In comparison, P≤0.05 was used for significant difference.

Results

The results on growth performance and organ weight of herbal extracts on broilers are given in *Table 2*. The results showed that the addition of herbal extracts to the diet affected the daily feed intake, feed conversion ratio, body weight and organ weight (%) of broilers. G3 showed significantly higher results (P≤0.05) as compared to G2 group.

The results of the Immune Index and Performance of herbal extracts are given in *Table 3*. The results showed that the addition of herbal extracts to the diet affected the Thymus, Spleen and Bursa weight of broilers. G3 showed the results which were significantly higher (P≤0.05) as compared to control group G2.

The results of herbal extracts on blood chemistry are shown in *Table 4*. The results showed that the addition of herbal extracts positively affected on blood sugar leval, packed cell volume, blood cells, hemoglobin and serum chemistry of broiler. G3 showed better results than G4 and

Table 2. *Effect of Coneflower, Neem and Thyme extracts supplementation on growth performance and carcass characteristics of broilers*

Parameters	Control (G1)	Virginiamycin (G2)	Coneflower (G3)	Neem (G4)	Thyme (G5)
Initial body weight (g)	188±1.250	191±2.123	189±1.740	185±2.175	186±2.132
Final body weight (g)	1826±1.433^{c}	1924±2.121^{ab}	2024±2.145^{a}	1981±1.631^{b}	1962±1.231^{ab}
Weight gain (g)	1635±2.092^{b}	1760±2.030^{ab}	1878±1.241^{a}	1795±1.302^{ab}	1775±1.432^{ab}
Feed conversion ratio	1.85±0.302^{b}	1.69±0.241^{a}	1.74±0.213^{a}	1.87±1.012^{b}	1.78±1.081^{b}
Feed intake (g)	3389±1.451^{c}	3331±2.456^{a}	3467±1.922^{a}	3421±1.132^{b}	3424±2.383^{b}
Dressing (%)	65.81±1.425^{c}	66.31±2.^{21ab}	67.69±2.901^{ab}	65.01±1.892^{c}	66.76±2.931^{ab}
Liver (g/100 g body weight)	2.41±1.751^{b}	2.49±1.021^{a}	2.89±1.012^{a}	2.79±1.081^{ab}	2.39±1.021^{c}
Heart (g/100 g body weight)	0.44±1.009^{c}	0.56±1.021^{ab}	0.57±0.518^{ab}	0.58±0.065^{ab}	0.59±0.069^{b}
Abdominal fat (g/100 g body weight)	3.21±0.731^{b}	3.09±0.531^{a}	3.19±0.077^{a}	3.10±0.312^{ab}	2.89±0.941^{b}
Breast (g/100 g body weight)	20.98±1.075^{c}	22.96±0.921^{b}	24.31±1.025^{b}	22.08±1.028^{ab}	21.38±1.213^{b}
Thigh (g/100 g body weight)	19.98±1.061	20.98±1.511	21.89±0.189	18.97±1.031	19.89±1.0251
Gizzard (g/100 g body weight)	1.51±0.415^{c}	1.61±0.221^{b}	1.68±0.074^{a}	1.69±0.314^{a}	1.58±0.104^{ab}

^{abc} *means with different superscripts in a row are significantly different (P<0.05)*

Table 3. *Effect of Coneflower, Neem and Thyme extracts supplementation on immune performance of broilers*

Parameters		Control G1	Virginiamycin G2	Coneflower (G3)	Neem (G4)	Thyme (G5)
Days	Organs					
(7-14 Days) Week 1	Thymus	0.221±0.107^{c}	0.269±0.243^{ab}	0.259±0.102^{ab}	0.269±0.097^{ab}	0.261±0.215^{b}
	Spleen	0.112±0.129^{c}	0.141+0.078^{b}	0.169±0.092^{b}	0.156+0.079^{ab}	0.151+0.068^{b}
	Bursa	0.149±0.081^{bc}	0.161±0.089^{ab}	0.185±0.096^{ab}	0.157±0.086^{b}	0.173±0.046^{ab}
(15-21 Days) Week 2	Thymus	0.599±0.125^{c}	0.609±0.206^{ab}	0.641±0.276^{ab}	0.624±0.198^{ab}	0.632±0.356^{ab}
	Spleen	0.239±0.098^{c}	0.253±0.059^{a}	0.291±0.056^{a}	0.266±0.079^{ab}	0.271±0.083^{ab}
	Bursa	0.448±0.076^{c}	0.469±0.214^{ab}	0.498±0.201^{ab}	0.472±0.247^{ab}	0.479±0.524^{ab}
(22-28 Days) Week 3	Thymus	1.039±0.461^{c}	1.052±0.151^{a}	1.190±0.283^{a}	1.172±0.149^{b}	1.68±0.098^{ab}
	Spleen	0.441±0.215^{c}	0.489±0.212^{ab}	0.519±0.142^{ab}	0.508±0.214^{b}	0.491±0.134^{ab}
	Bursa	0.719±0.182^{bc}	0.726±0.218^{a}	0.768±0.412^{a}	0.762±0.241^{b}	0.761±0.316^{ab}
(29-35 Days) Week 4	Thymus	1.834±0.922^{c}	2.042±0.461^{ab}	2.214±0.204^{ab}	2.195±0.704^{a}	2.185±0.682^{ab}
	Spleen	0.649±0.203^{c}	0.741±0.184^{a}	0.780±0.215^{a}	0.772±0.161^{b}	0.769±0.189^{a}
	Bursa	0.321±0.210^{bc}	0.374±0.182^{a}	0.424±0.950^{a}	0.415±0.213^{b}	0.408±0.271^{ab}

^{abc} *means with different superscripts in a row are significantly different (P<0.05)*

Table 4. Effect of Coneflower, Neem and Thyme extracts supplementation on broilers blood chemistry

Parameters	Control (G1)	Virginiamycin (G2)	Coneflower (G3)	Neem (G4)	Thyme (G5)
Cholesterol (mg/dL)	134.12±0.201^{c}	129.91±0.082^{a}	120.67± 0.842^{a}	119.00±0.715^{a}	126.00±0.961^{ab}
Blood sugar (mg/dL)	212.74±0.981^{c}	201.41±0.120^{b}	189.41±0.197^{b}	196.42±1.40^{ab}	201.02±0.270^{b}
Packed cell volume (%)	25.98±0.416	26.83±2.091	28.94±2.913	26.93±2.191	27.92±6.021
White blood cell (%)	203.42±0.532	209.45±0.294	229.00±0.963	217.15±1.510	219.41±0.214
Red blood cell (%)	2.09±0.098^{c}	2.15±1.721^{b}	2.31±2.015^{a}	2.21±1.352^{ab}	2.24±2.104^{a}
Hemoglobin (g/dL)	9.49±0.561	9.79±0.691	10.35±0.582	9.97±1.141	10.26±0.516
Triglyceride mmol/L	93.9±24.7^{bc}	98.8±10.4^{a}	100.7±38.7^{a}	67.4±18.0^{c}	93.5 ± 35.5^{ab}
HDL (mg/100 mL)	36.00±0.50	39.00±1.87	38.00±1.67	40.00±1.22	38.00±1.14
LDL (mg/100 mL)	94.00±5.60^{bc}	87.75±3.10^{a}	86.75±2.22^{a}	85.00±1.83^{b}	89.50±1.91^{bc}
Blood lipoprotein mmol/L	3.04±0.05^{b}	2.96±0.04 ^{b}	3.31±0.10^{ab}	3.22±0.12^{ab}	3.30±0.10 ^{ab}

^{abc} *means with different superscripts in a row are significantly different (P<0.05)*

Table 5. Effect of coneflower, neem and thyme extracts supplementation on intestinal microbial population of broilers

Bacteria	Control (G1)	Virginiamycin (G2)	Coneflower (G3)	Neem (G4)	Thyme (G5)
E. coli	7.10^{a}	4.42^{b}	4.82^{b}	5.20^{ab}	4.32^{b}
Lactobacillus	4.52^{c}	4.92^{b}	6.20^{a}	5.37^{ab}	5.82^{ab}

^{a,b,c} *with in row, value with a similar superscript were not different with probability P≥0.05*

G5 groups which were significantly higher (P≤0.05).

Table 5 shows the results of the intestinal microbiota population. The results showed that the population of *E. coli* was decreased in all treatment groups except the control group suggesting the reduction of spoilage microorganisms which were significantly higher (P≤0.05) in the antibiotic group followed by G5 and G4 when compared with the control. Moreover, the concentration of *Lactobacillus* was increased in all treatment groups except control groups. The highest concentration was found in G3 suggesting improvement of probiotics bacterial community which ultimately enhanced the immune parameters.

Discussion

The screening of natural plants and their derivatives for health-promoting activities has been a key priority owing to their least side effect and long-term benefits [34,35]. This study found that the coneflower group significantly improved the body weight gain of broilers during the whole trial period. Thyme and vigemycin had no significant effect on the daily weight gain of broilers. Some other studies reported that the supplementation of herbs like thyme extract improved the immunity and FCR of chickens [36]. The supplementation of 2% thyme extract in drinking water or feed of poultry has already been reported to have positive effects [37,38]. In this study improved growth performance and better carcass results of coneflower and neem treated group were also observed. Previous studies reported that supplementation of herbal extract showed improvement in weight of digestive tract. Similarly, addition of 2% thyme powder to broiler diets showed significant effect on relative weight of digestive tract and have no residual effect on poultry meat [39]. Likewise, another study showed that supplementation of herbs increased body weight, breast muscle weight and improved feed conversion ratio [40].

Blood glucose and cholesterol levels are the indicators of diabetes and generally low blood glucose levels along with low cholesterol are assumed for a healthy life in animals including poultry. High blood sugar influences hyperglycemia, thus should be controlled in order to control diabetes. In this study, it is observed that the supplementation of coneflower and neem lowered the blood glucose level which indicates the antidiabetic potential of these herbs. This might be attributable to the fact that extracts contain more phenolic compounds, resulting reduction in blood glucose and cholesterol levels and also help to boost the immune status and growth performance of the birds [41]. Supplementation of Chinese herbs in drinking water improved the weight of the thymus, spleen and bursa which represent better immunity in birds and also improved the production

of antibodies and immune organs [42]. According to a study, any kind of diseases in the body will reduce the growth of broilers and FCR [43]. Study showed that plant extracts improved immune response by activating cytokines, interleukin-1/6, interferon-α which are the earliest mediator secreted by the body used to respond to antigens [44]. The findings of the another study showed that RBC, WBC and hemoglobin levels were increased with the addition of herbal extracts which indicates the improvement of the blood and immunity parameters [45]. The results of this study indicated that herbal extracts (having polyphenols and antioxidants) have the potential to provoke erythropoietic release from the kidney, which acts as a hormonal regulator in order to produce RBC. Another study showed that herbal extracts raised the WBC and RBC levels, which have the ability to raise the oxygen-carrying capability and the transportation of nutrients [46].

The results of this study found that the addition of coneflower extract to the diet significantly reduced low-density lipoprotein content and significantly increased high-density lipoprotein protein content. Another study showed that allicin treated group reduced the serum triglycerides, cholesterol, and low-density lipoprotein content [47]. The inhibitory mechanism of allicin on bacteria is still unknown, but it can be concluded that the chemical components in allicin can affect the bacterial cell membrane and its cell permeability [48,49]. This study found that thyme extract increased the content of *Lactobacillus* in intestinal contents and it may be due to the oligosaccharide content, because oligosaccharides can increase the production of lactic acid, promote the reproduction of beneficial bacteria in the intestine and reduce the generation of gram-negative bacteria. The supplementation of chinese herbs in drinking water enhanced the weight of the thymus, spleen, and bursa weight, which represents better immunity in birds and also showed better growth performance [50].

The study provides meaningful insights regarding the immune enhancing potential and the growth promoting activities of herbal extracts to be used as an alternative to antibiotics.

Availability of Data and Materials

Research and supporting data will be available from the author (A. Rehman) on request

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Competing Interest

The author declared that there is no conflict of interest

Ethical Statement

The research is conducted by the approval from ethical committee of MNS University of Agriculture Multan under the Ref. No. 144/IPFP-II (Batch-I) /SRGP/NAHE/HEC/2020/93.

Author Contributions

AR conceived and designed the experiments; KH, AZ, MAZF, AA analyzed the data, drafted and the manuscript; WMSM, RZA and MUW, ZR, JAK, MAR, MN done additional changes in manuscript ;All authors read and approved the final manuscript.

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RESEARCH ARTICLE

The Effect of Egg Weight on Egg External Quality Characteristics and Hatching Performance in Pekin Ducks

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ABSTRACT

This study aimed to investigate the effect of different egg weights on egg external quality characteristics and hatching performance in Pekin ducks. A total of 800 eggs were divided into three weight categories: "light" (Lo; <76 g), "medium" (Me; 76-82 g), and "heavy" (Hi; >82 g). Parameters indicating egg external quality characteristics were calculated. Eggs were incubated and on the 25th day, transferred to the incubator for hatching. The mean egg weight for light, medium, and heavy categories were 70.25 g, 78.47 g, and 85.17 g, respectively (P<0.001). As egg weight increased, there was a significant decrease in shell thickness (P<0.001). The effect of different egg sizes on fertility was insignificant. The hatchability of fertile eggs was higher in the light and medium categories compared to the heavy category (P<0.05). Egg weight had a significant effect on early embryonic mortality rate (P<0.05) and chick hatch weight (P<0.001). In conclusion, different egg weights have varying effects on egg external quality parameters in Pekin ducks. Although heavy eggs produce chicks with higher weights, lighter or medium-weight eggs may be more advisable for incubation due to their better hatchability.

Keywords: Chick, Fertility, Shell, Incubation, Pekin duck, Egg weight

INTRODUCTION

Ducks are widely used as a source of food across the globe, especially in Europe and Asia, where their meat and eggs are frequently consumed. Compared to chicken meat, duck meat is considered to be tastier and more nutritious. Additionally, duck eggs are bigger and more nutrient-rich than chicken eggs. Duck meat is an important source of food for countries that are grappling with poverty and issues surrounding food security [1-3]. As the world's population continues to grow, the demand for protein is increasing. White meat is becoming more popular as a cheaper source of protein compared to red meat, resulting in an increase in both chicken meat and alternative poultry meat production. Duck breeding, with its short production period, is one of the alternatives to meet the demand for poultry meat in the industry [4,5]. Overall, integrated duck farming is a sustainable and profitable practice that has been an important part of traditional agriculture in Asia for centuries. With the increasing demand for protein and the need for sustainable agriculture practices, integrated duck farming could be a valuable model for other regions to consider [1,6].

According to the Turkish Statistical Institute, duck breeding in Türkiye has increased by 21.23% in the past decade. The total number of ducks in Türkiye is 432,457 as of 2022. Compared to other poultry types, ducks represent only 0.1% of the total poultry population [7].

Pekin ducks are a popular breed for meat production due to their fast growth rate, feed efficiency, and lower disease risk. They are easy to care for and feed, making them suitable for small-scale farmers and newcomers. Pekin ducks' tender meat and rich flavor make them sought after in high-end restaurants, offering farmers access to valuable markets. Overall, Pekin ducks contribute to job opportunities, economic growth, and increased production efficiency and profitability in the poultry industry [4,8]. Fertile egg supply, proper storage of hatching eggs, and optimal incubation conditions are crucial for the economic success of duck farming. Numerous studies have focused on these factors, including the appropriate

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weight and storage duration for hatching eggs, and the ideal hatching conditions for Pekin ducks [1,2,6].

Various factors, both genetic and non-genetic, can influence both fertility and hatching yield. These may include the farmer's management practices, the quality of the eggs, and the methods used during incubation [8–10] Fertility can be influenced by factors such as parent quality, male-to-female ratio, temperature, storage time, and housing systems. In Pekin ducks, the storage time of eggs before incubation can affect fertility, hatching yield, and early embryonic mortality [11,12]. Storing duck eggs for more than six days can decrease fertility [13]. Different external features such as weight, index (width/length), shell thickness, number of pores, surface quality, and resistance to breakage are commonly measured in studies [14-16]. The hatchability of low weight eggs can be higher than that of high weight eggs in better ratios [11,17]. In heavy eggs nutrient and energy reserves are greater [14]. Light weight eggs can have shorter incubation periods [18], and egg size can affect hatchability [11]. Proper incubation conditions such as humidity, temperature, egg turning, and ventilation are crucial for achieving high hatch rates in duck eggs. Providing suitable conditions for incubating duck eggs can result in a high hatch rate and increased production, but this balance is very delicate and subject to dynamic changes [10].

The aim of this study is to determine the effect of egg weight on egg external quality characteristics and hatchability performance, in Pekin ducks raised in the province of Kars, Türkiye.

Material and Methods

Ethical Statement

The care and use of the ducks in this study comply with the laws and regulations of Türkiye. Additionally, this study was conducted after obtaining approval from the Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK/2020-180), Kars, Türkiye.

Location

Kars province is located at coordinates 40°36'18"N and 43°5'48"E, at an altitude of 1760 meters above sea level. Kars province is located in the eastern region of Türkiye and shares a border with Armenia.

Hatching Egg Collection

The incubation eggs used in this study were obtained from a breeding flock that was 33-35 weeks old and had an average weight of 3.2-3.8 kg. The breeding flock was raised using standard industry practices in a poultry production unit. A total of 800 incubation eggs were included in the study. Prior to incubation, the eggs were stored at 17-19°C and 70% relative humidity for 3-7 days.

External Quality Characteristics of Eggs

The egg weights (EW) were weighed with a precision of ± 0.1 g immediately before placing them in the incubator. Then, the eggs were classified into three categories: "light" (<76 g), "medium" (76-82 g), and "heavy" (>82 g), by modifying the classification made by Ipek and Sozcu [15]. The length (L) and width (W) of the eggs were measured with a digital caliper with a precision of 0.01 mm. Geometric mean diameter (Dg), surface area (S), volume (V), shape index (SI), sphericity (Sp), elongation (E), and specific gravity (SG) were calculated using the following formulas [16,19,20]:

$D_g\,(mm) = (LW^2)^{1/3}$

$S\,(mm^2) = \pi D_g^{\,2}$

$V\,(mm^3) = \pi/6\,(LW^2)$

$SI\,(\%) = (W/L) \times 100$

$Sp\,(mm) = [(LW^2)^{1/3}/L] \times 100$

$E\,(mm) = L/W$

$SG\,(g/cm^3) = (EW/V)$

Shell weight (SW) was weighed with a precision of 0.1 g, and shell thickness (ST) was measured with an electronic digital micrometer with a precision of 0.001 mm from three different points (sharp end, blunt end, and equator) of each eggshell and their average was taken. Shell density (SD), shell volume (SV), shell specific gravity (SSG), and shell ratio (SR), pore number (PN) were calculated using the following formulas [19].

$SD\,(g/cm) = (SW/S \times ST)$

$SV\,(cm^3) = ST \times S$

$SSG\,(g/cm^3) = SW/SV$

$SR\,(g) = (SW/W) \times 100$

$PN = 304 \times W^{0.767}$

Incubation and Hatching

The eggs were incubated at 37.5°C and 62% humidity, with turning for the first 25 days. Starting from the 8th day of incubation, water was sprayed in the hatcher until the hatch. On the 25th day of incubation, all eggs were transferred to a hatcher that operated at 37.0°C and 72% relative humidity. The healthy chicks hatched on the 28th day of incubation were recorded.

The number of unhatched eggs was counted, opened, and macroscopically evaluated to determine fertility and the stage of embryonic death (early and late). The percentage of embryonic death was categorized as early and late. Fertility was calculated as the ratio of fertile eggs to total eggs. Sticky and dead embryos at the end of incubation

were counted as dead in shell. The parameters obtained at the end of incubation were calculated using formulas reported in previous studies [15,21-23]. Hatching weight was determined by weighing each individual chick hatched from the eggs.

Statistical Analysis

This study utilized the SPSS software (version 26.0, Chicago, IL, USA) for statistical analysis. The Kolmogorov-Smirnov test was used to evaluate the distribution of the groups created based on egg sizes. Parametric tests were used since the data had a normal distribution. One-way ANOVA was used for multiple comparisons, while the Tukey HSD test was used for pairwise comparisons. Chi-Square Tests or Fisher's Exact Test were used to compare parameters with frequency data based on egg weights. Pearson correlation coefficients were calculated to determine the relationship between variables. Mean and standard error of mean (SEM) were used to present the results. Statistical significance was considered at a P-value of <0.05.

Results

The external quality characteristics of eggs classified as light, medium, and heavy are given in *Table 1*. The average weight of eggs in the light, medium, and heavy groups were 70.25 g, 78.47 g, and 85.17 g, respectively, and the differences were statistically significant (P<0.001). The external quality characteristics of the eggs were also found to be significantly different among the groups (P<0.001). Specifically, the shape index was lower in the light and medium groups compared to the heavy group, with a statistically significant difference (P<0.001).

The shell characteristics of eggs with different weights are given in *Table 2*. The shell weight, shell density, shell

Table 1. *External quality characteristics of eggs of different weights*

Parameters	Light	Medium	Heavy	Total
n	445	205	150	800
Weight (g)	70.25±0.16^{c}	78.47±0.13^{b}	85.17±0.20^{a}	75.15±0.23
Width (mm)	45.27±0.11^{c}	46.61±0.06^{b}	48.29±0.07^{a}	46.18±0.08
Length (mm)	62.44±0.12^{b}	63.24±0.15^{a}	63.64±0.18^{a}	62.87±0.08
Geometric diameter (mm)	50.36±0.07^{c}	51.59±0.06^{b}	52.94±0.07^{a}	51.16±0.06
Surface area (cm^{2})	79.74±0.23^{c}	83.63±0.19^{b}	88.06±0.24^{a}	82.30±0.18
Volume (cm^{3})	67.05±0.32^{c}	71.95±0.25^{b}	77.73±0.31^{a}	70.31±0.25
Shape index (%)	72.70±0.32^{b}	73.79±0.21^{b}	75.98±0.26^{a}	73.59±0.20
Elongation	1.38±0.004^{a}	1.36±0.004^{b}	1.32±0.005^{c}	1.36±0.003
Sphericity (%)	80.79±0.22^{c}	81.64±0.15^{b}	83.25±0.19^{a}	81.47±0.14
Specific gravity (g/cm^{3})	1.05±0.003^{b}	1.09±0.004^{a}	1.10±0.003^{a}	1.07±0.002

$^{a-c}$ Different letters in the same line are statistically different (P<0.001)

Table 2. *Shell characteristics of eggs of different weights*

Parameters	Light	Medium	Heavy	Total
n	445	205	150	800
Shell weight (g)	5.95±0.02^{c}	6.73±0.01^{b}	7.38±0.02^{a}	6.42±0.02
Shell thickness (mm)	0.388±0.003^{a}	0.375±0.005^{b}	0.358±0.008^{c}	0.379±0.005
Shell density (g/cm^{3})	2.90±0.01^{b}	2.99±0.01^{a}	2.98±0.01^{a}	2.94±0.01
Shell volume (cm^{3})	30.92±0.09	31.22±0.08	31.28±0.11	31.07±0.06
Shell specific gravity (g/cm^{3})	0.19±0.001^{c}	0.21±0.001^{b}	0.23±0.001^{a}	0.21±0.001
Shell ratio (g)	8.47±0.002^{c}	8.56±0.003^{b}	8.65±0.003^{a}	8.53±0.003
Number of pores	7794.1±13.59	8482.1±10.31^{b}	9029.6±16.48^{a}	8202.1±19.37

$^{a-c}$ Different letters in the same line are statistically different (P<0.001)

Table 3. *Hatchability results according to egg weights*

Parameters	Light	Medium	Heavy	P	Total
n	445	205	150	-	800
Fertility (%)	82.9	83.4	81.3	NS	82.8
Hatchability of fertile eggs (%)	88.1[a]	89.5[a]	79.5[b]	*	86.9
Hatchability of total eggs (%)	73.0[a]	74.6[a]	64.7[b]	*	71.9
Early embryonic death (%)	1.9[b]	2.9[ab]	6.6[a]	*	3.0
Late embryonic death (%)	6.2	4.6	7.3	NS	6.0
Dead in shell (%)	3.5	2.9	6.6	NS	3.9
EED / Total death (%)	15.9	27.8	32.0	NS	23.0
LED / Total death (%)	54.5	44.4	36.0	NS	47.1
Dead in shell / Total deaths (%)	29.5	27.8	32.0	NS	29.9
Chick hatching weight (g)	43.61 ± 3.95[c]	47.22 ± 4.78[b]	52.12 ± 4.35[a]	***	46.01 ± 5.29
Chick / Egg weight (%)	62.00 ± 5.49	61.14 ± 6.62	62.14 ± 5.47	NS	61.79 ± 5.81

[a-c] Different letters in the same line are statistically different. *: P≤0.05, ***: P<0.001, NS: Not significant, EED: Early embryonic death, LED: Late embryonic death

Table 4. *Pearson correlation coefficients between external egg quality, shell characteristics, and hatching weight*

Parameters	Shell weight (g)	Shell thickness (mm)	Shell density (g/cm³)	Shell volume (cm³)	Shell specific gravity (g/cm³)	Shell ratio (g)	Number of pores	Hatch weight (g)	Chick / Egg (%)
Weight (g)	0.940**	-0.760**	0.492**	0.285**	0.872**	0.908**	0.794**	0.586**	-0.173**
Width (mm)	0.561**	-0.443**	-0.202**	0.771**	0.247**	0.558**	0.457**	0.279**	-0.096*
Length (mm)	0.261**	-0.158**	-0.032	0.209**	0.106**	0.263**	0.192**	0.215**	0.028
Geometric diameter (mm)	0.704**	-0.533**	-0.166**	0.818**	0.337**	0.702**	0.564**	0.405**	-0.090*
Surface area (cm²)	0.681**	-0.518**	-0.191**	0.830**	0.312**	0.678**	0.546**	0.387**	-0.087*
Volume (cm³)	0.653**	-0.499**	-0.217**	0.841**	0.284**	0.650**	0.524**	0.366**	-0.083*
Shape index (%)	0.210**	-0.194**	-0.192**	0.486**	0.053	0.208**	0.183**	0.064	-0.079
Elongation	-0.315**	0.273**	0.006	-0.325**	-0.220**	-0.310**	-0.269**	-0.130**	0.093*
Sphericity (%)	0.233**	-0.211**	-0.159**	0.461**	0.085*	0.229**	0.201**	0.076	-0.082*
Specific gravity (g/cm³)	0.358**	-0.269**	0.871**	-0.667**	0.680**	0.363**	0.274**	0.147**	-0.109**
Hatching weight (g)	0.501**	-0.535**	0.171**	0.113**	0.521**	0.583**	0.578**	-	0.694**

* Correlation is significant at the 0.05 level (2-tailed). **: Correlation is significant at the 0.01 level (2-tailed)

specific gravity, and pore count increased proportionally with egg weight (P<0.001). As egg weight increased, the shell thickness decreased (P<0.001). There was no statistical difference between the groups in terms of shell volume. The shell ratio was determined as 8.47 g, 8.56 g, and 8.65 g for light, medium, and heavy eggs, respectively (P<0.001).

Table 3 displays the hatchability results of Pekin ducks categorized by different egg weights. The fertility of eggs was not significantly affected by their weight. Nevertheless,

the hatchability rate was higher in the light and medium weight categories compared to the heavy weight group (P<0.05). The overall hatchability rate of light, medium, and heavy eggs was 73.0%, 74.6%, and 64.7%, respectively (P=0.05). Early embryonic mortality rate was affected by egg weight (P<0.05), but late embryonic mortality or dead in shell was not. The light eggs had a significantly lower rate of early embryonic mortality (1.9%) than the heavy eggs (6.6%) (P<0.05). The hatching weight of the chicks was also affected by the egg weight, and the average hatching weight obtained from light, medium and heavy eggs was 43.61±3.95 g, 47.22±4.78 g and 52.12 ± 4.35 g, respectively (P<0.001).

Table 4 shows the Pearson correlation coefficients between external quality and shell characteristics of eggs and hatch weight in Pekin ducks. The results revealed that egg weight had a strong positive correlation with shell weight (r = 0.940, P<0.01), but a strong negative correlation with shell thickness (r = -0.760, P<0.01). Additionally, there was a strong positive correlation between hatch weight and egg weight (r = 0.586, P<0.01), while a strong negative correlation was found between hatch weight and shell thickness (r = -0.535, P<0.01).

Discussion

The proportion of the goose population in Kars province's poultry is significant and ranks first. In terms of poultry population by species, Kars province's ranking is as follows: Chicken, goose, turkey, and duck [24,25]. However, due to the increasing demand for poultry meat, there is a growing trend towards different poultry species. One of the most important indicators of this is the increasing popularity of duck farming as an alternative to goose farming [4]. During the literature review, no study investigating the influence of various egg sizes on the external quality and shell characteristics of eggs as well as hatchability performance in Pekin ducks raised in Kars and its surrounding areas was found.

In a study conducted on Pekin ducks, the average weights of light, medium, and heavy eggs were determined as 70.6, 78.6, and 86.4 g, respectively [15]. In another study, the average weights of light, medium, and heavy eggs in Pekin ducks were determined as 76.64, 81.08, and 85.93 g, respectively [26]. In our study, the average weights of light, medium, and heavy eggs were 70.25, 78.47, and 85.17 g, respectively. The weight averages obtained from the classified eggs in the studies are similar to our study. The average width and length of our eggs were 46.18 and 62.87 mm, respectively, and the obtained data are close to the sizes of Pekin duck eggs observed by Galic et al. [16], and Balkan and Biricik [27].

In our study, the mean values for geometric diameter, surface area, volume, shape index, elongation, and specific gravity were 51.16 mm, 82.30 cm^2, 70.31 cm^3, 73.59%, 1.36, and 1.07 g/cm^3, respectively. The geometric diameter of Pekin ducks was reported as 50.41 mm [16], the surface area as 79.86-81.23 cm^2 [16,27,28], the volume as 66.38-70.19 cm^3 [27,28], the shape index as 69.69-75% [16,28-30], the elongation as 1.40-1.43 [27,28], and the specific gravity as 1.01-1.06 g/cm^3 [14,29]. The reported egg quality characteristics in previous studies were found to be close to the values obtained in our study. However, different values may be determined by using eggs with different weights or sizes in these studies. Since most studies did not classify eggs according to their weight in Pekin ducks, the general averages of our study were compared with the data from previous studies. In a study conducted on Pekin ducks with different egg weights [15], the shape index of light, medium, and heavy eggs was 72.0%, 73.6%, and 74.4%, respectively, and there was no statistically significant difference. In our study, the shape index of light, medium, and heavy eggs was 72.70%, 73.79%, and 75.98%, respectively, and there was a significant statistical difference (P<0.001). In the study conducted by İpek and Sözcü [15], the weight of eggs did not have a significant effect on the shape index, but in our study, despite finding similar values, the weight of eggs had a statistically significant effect on the shape index. Heavy eggs had a higher shape index than both light and medium eggs (P<0.001). Egg length in our study may be one of the most important factors affecting the shape index. The length of heavy eggs was close to that of the other groups. Therefore, the shape index may have been higher in the heavy group.

Shell thickness in ducks can vary between 0.36-0.42 mm on average [2]. In Pekin ducks, the shell thickness and weight are between 0.34-0.51 mm and 6.03-9.97 g, respectively [16,27-29,31]. Moreover, in Pekin ducks, the shell thickness of light, medium and heavy eggs were determined as 39.3, 38.8 and 37.9 µm, respectively [17]. In our study, shell weight increased parallel to egg weight (r = 0.940, P<0.01). Shell weight was 5.95, 6.73, and 7.38 g (general average 6.42 g) for light, medium, and heavy eggs, respectively, and there was a significant difference (P<0.001). Shell thickness decreased as egg weight increased. The strongest indicator of this was the strong negative correlation between shell thickness and egg weight (r = -0.760, P<0.01). In one study, shell weight increased numerically with increasing egg weight [15]. In a study conducted on Pekin ducks [17], there was a statistically significant difference in shell thickness among groups created based on egg weight. In another study, the egg weight increases, the shell thickness decreases in a similar way [15]. A thicker eggshell for incubation eggs prevents higher dehydration during incubation [32,33]. In Pekin ducks, as the egg weight increased, the egg breaking strength and eggshell thickness decreased. The egg breaking

force was found to be the highest in the light egg group, and the thinnest eggshell was observed in the heavy egg group [15]. The thickness of the eggshell may decrease in heavier eggs as the breeding age increases [17]. The shell density of Pekin ducks has been determined as 3.17 g/cm^3 [16] and the shell ratio as 8.5-13% [15,28] in previous studies. However, in our study, the shell density was lower than the reported study, and the shell ratio was within the range reported in previous studies. These findings contribute to our understanding of the intricate relationship between egg weight and eggshell characteristics in Pekin ducks. Further investigations should be conducted to explore the underlying mechanisms behind these observations and to develop strategies for optimizing egg quality and hatching performance in duck farming practices.

Different factors such as fertility, breeder quality, male-to-female ratio, temperature, storage time, and housing systems can affect egg production and quality in duck breeds. Moreover, ovulation, egg formation and development stages, and sperm quality are also crucial for fertility. Although there may be variations among duck breeds, these factors can still have an impact [11,34-36]. Hatchability and early embryo mortality of Pekin duck eggs are significantly affected by pre-incubation storage time. Moreover, there may be a decrease in fertility of duck eggs that are stored for more than six days from laying to incubation [10,11]. Embryonic deaths are more common in the first and last third of incubation. The survival of the embryo is not only dependent on pre-incubation and incubation environmental conditions. In particular, factors related to the genotype of the parents can have a positive or negative impact on the life of the duckling inside the egg. Chromosomal abnormalities and lethal genes acquired from the mother and father can cause high rates of early embryonic deaths [11,37]. Fertility rate in Pekin ducks can vary between 80.96% and 95.4% [8,31,38]. In this study, the overall fertility rate was 82.5%, which was within the range reported in previous studies. There was no statistically significant difference in terms of fertility rate, late and total embryonic mortality rate among light, medium, and heavy eggs. The hatchability of fertile eggs in the light and medium groups was higher than that in the heavy group ($P<0.05$). The hatchability of total eggs was lowest in the heavy eggs ($P=0.05$). Furthermore, statistically higher embryonic mortality was observed in the heavy eggs compared to the light eggs ($P<0.05$). Similar to our study, İpek and Sözcü [15] found that different egg weights did not have a significant effect on fertility. They also observed that in eggs classified as heavy, the hatching rate of fertile eggs, the hatching rate of total eggs, and the early embryonic mortality rate were lower than those of light and medium eggs. Another study on Pekin ducks found that different egg weights did not have a significant effect on fertility, early or late embryonic mortality rate, or hatchability of fertile or total eggs [17].

There is a strong positive relationship between egg weight and the weight of the chick that hatches from it. The percentage of chick weight relative to egg weight is fairly consistent across species [39]. Our study showed that hatching weight of chicks varied between 43.61 and 52.12 g, and there was a statistically significant difference among the groups ($P<0.001$). As the egg weight increased, the hatching weight of the chick also increased and a strong positive correlation was determined ($r = 0.586$, $P<0.01$). However, different egg weights did not affect the chick/egg weight ratio. In a study conducted in Pekin ducks, different weights of eggs have been found to affect the hatching weight of the chicks [17]. Similar to our study, in Pekin ducks, the hatching weight of chicks ranged from 42.8 to 54.9 g, and different egg sizes had an effect on hatching weight. However, there was no difference between groups in terms of chick/egg weight ratio [15]. There are many factors that affect chick hatching weight. Egg size and the age of the breeder are among the most influential factors. As the breeder's age increases, the egg size obtained can also vary. In this context, in a study conducted on Pekin ducks, the hatching weights of eggs obtained from breeders of different ages were statistically different. Age had a statistically significant effect on egg weight [31]. In our study, we also used breeders at 33-35 weeks of age, which limited the use of a large number of heavy eggs. However, the classification of eggs according to weight directly affected the chick hatching weights.

In conclusion, there are significant statistical differences in many external quality characteristics of eggs of different sizes in Pekin ducks raised in Kars and its surrounding areas. As egg weight increases, shell thickness decreases. In addition, different weights of eggs did not affect fertility, but fertilized and total egg hatchability had the worst results in heavy eggs. One of the most important handicaps of heavy eggs is the occurrence of early embryonic mortality. Despite this, the hatching weight of chicks from the heavy egg group was higher than that of other groups. Considering the hatchability, light or medium weight eggs can be selected for incubation.

Availability of Data and Materials

Datasets analyzed during the current study are available in the author (B. Boğa Kuru) on reasonable request.

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Ethical Approval

This study was conducted after obtaining approval from the Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK/2020-180), Kars, Türkiye.

Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

BBK and TK: Study design, data collection, draft writing. MMC and SAI: Contribution to study design, pre-editing of manuscript. BBK, TK, and SAI: Statistical analysis, article editing. BBK and TK: Article writing and editing, supervision. All authors have read, reviewed, and approved the final draft.

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SHORT COMMUNICATION

Lipoteichoic Acid Disrupts Mammary Epithelial Barrier Integrity by Altering Expression of Occludin and Zonula Occluden (ZO)-1

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ABSTRACT

This study aimed to investigate the effect of the cell wall component lipoteichoic acid (LTA) of *Staphylococcus aureus* on mammary alveolar tight junctions (TJs) proteins including claudin-1, occludin and zonula occluden (ZO)-1. Primary bovine mammary epithelial cells were grown on Transwell inserts for 108 hours. The integrity and tightness of the growing epithelial cell layer were evaluated by measuring transepithelial electrical resistance. The permeability of bovine mammary epithelial cells (BMECs) was assessed by measuring horseradish peroxidase transmission. The mRNA levels of BMECs TJ components were measured with quantitative real-time polymerase chain reaction. In LTA-induced mastitis of mouse model, the protein expression of TJs (claudin-1, occludin and ZO-1) were determined by western blot analysis. Our results showed that LTA increased barrier permeability of BMECs. Treatment with LTA decreased mRNA and protein levels of occludin and ZO-1 in vivo and in vitro, however, the expression of claudin-1 did not change. The results suggested that disruption of mammary epithelial barrier integrity is caused by the alteration of occludin and ZO-1.

Keywords: Lipoteichoic acid, Mammary, Tight junctions

INTRODUCTION

Cow mastitis is an inflammation of the mammary gland commonly caused by bacterial infection [1]. *Staphylococcus aureus* is one of the predominant pathogens of clinical and subclinical bovine mastitis [2]. Chronic mastitis caused by *S. aureus* leads to reduced milk quantity and quality, and even leads to culling of cows, resulting in serious economic losses [3]. The major immunogenic cell wall component, lipoteichoic acid (LTA), deriving from *S. aureus*, are commonly infused in the bovine mammary gland to elicit experimental mastitis [2].

The blood-milk barrier is mainly composed of mammary epithelial cells. The blood-milk barrier has several functions, such as resisting pathogen invasion and preventing leakage between milk and blood components [4]. Disruption of the blood-milk barrier leads to the aggravation of bacterial infections and the development of inflammation [5]. Tight junctions (TJs) are important for the establishment of the blood-milk barrier [6]. TJs are made of multiprotein complexes such as claudins, occludin, zonula occludens (ZO) and junctional adhesion molecules (JAMs) [7]. The tight junctions act as a barrier to prevent leakage of milk and blood components [8]. TJs function as a barrier to control the paracellular passage and selectively allow solutes and water as well as some microorganisms to pass through. Some bacterial pathogens and toxins can break barrier function by disruption of TJs, which leads to facilitate bacterial entry and spread [9].

Mastitis is able to disrupt the tight junction barrier and it has been reported that LPS disrupts the blood-milk barrier by regulating the expression of claudins and occludin proteins [10,11]. LTA has been reported in the literature to affect bovine mammary epithelial barrier integrity [12]. However, the effect of LTA-induced mastitis on mammary tight junction proteins remains unclear. In this study, we determined permeability of bovine mammary epithelial cells (BMECs) and the differential expression of genes of claudin-1, occludin and ZO-1 in BMECs stimulated with purified LTA. We injected LTA into the mouse mammary glands to induce experimental mastitis and investigated TJ proteins of claudin-1, occludin and ZO-1.

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Materials and Methods

Ethical Approval

All the procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Inner Mongolia Agricultural University (IMAU) (License No. SYXK, Inner Mongolia, 2014–0008) with adherence to IMAU guidelines.

Primary BMEC Culture and Stimulation

BMECs were isolated from lactating cows as previously described, with slight modifications [13]. The mammary tissue pieces were washed twice with phosphate buffered saline before digestion. The tissue samples were minced using surgical scissors. Minced samples were incubated in aseptic Hank's balanced salt solution for 1 h at 37°C. The prepared tissue was digested with collagenase I (Sigma-Aldrich, St. Louis, MO) for 3 h at 37°C. The digest was filtered through a nylon mesh (BD Bioscience, 100 µm) and the filtrate centrifuged for 10 min/1.250 rpm. The cell inoculum was transferred into 25 cm^2 cell culture flasks (Corning, Corning, NY, USA) and cultured in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (DMEM/F12; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, USA), and 100 U/mL penicillin and streptomycin (Invitrogen). Fibroblasts-free cultures were obtained by differential trypsinization. BMECs were identified as previously described [14]. For stimulation of BMECs with LTA, the medium was removed, and 0, 0.01, 0.1, 1.0, 10 µg/mL LTA in stimulation medium were added at the desired concentration in 1 mL of stimulation medium. After incubation for 24 h, cell culture supernatant was removed and BMECs were harvested for RNA extraction.

Measurement of Transepithelial/Transendothelial Electrical Resistance

Cells were seeded on the upper surface of polycarbonate membrane with 3 µm pores in Transwell inserts in 12-well cell culture plates (Corning). Cells were grown in 1.5 and 0.5 mL of growth medium on the basolateral and apical sides, respectively, in the presence of 5% CO_2 at 37°C. Medium was changed every 48 h, Cells were seeded in Transwell plates and recorded as 0 h. Transepithelial/transendothelial electrical resistance (TEER) was measured at 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h and 108 h using an EVOMX ohmmeter with a STX2 Chopstickelectrode set (World Precision Instruments, Sarasota, Fla., USA). TEER values ($\Omega \times cm^2$) were obtained by subtracting the resistance of blank filters without cells from the resistance of filters with cells. The results were then multiplied by the area of the membrane from the filter insert.

HRP Flux

Horseradish peroxidase (HRP, Sigma-Aldrich) transmissivity was measured in order to determine the *in vitro* permeability of BMECs. At 24 h after LTA exposure, the culture medium was replaced with DMEM/F12 without phenol red. In order to maintain the culture supernatant level, 0.5 mL of medium containing 50 ng HRP was added into the insert, and 1 mL of medium was added into the well. A total of 50 µL of medium was collected from each well at 1 h, which was replaced with 50 µL of fresh medium after each collection in order to maintain the liquid level on both sides. The collected samples were stored at 4°C until processing, while 50 µL of peroxidase substrate containing tetramethyl benzidine and hydrogen peroxide was added to each sample and incubated for 10 min. The reaction was terminated by adding 50 µL of 1 M sulphuric acid. The optical density was measured at 450 nm, and the HRP transmissivity was assayed from the standard curve according to the following equation: $P_{HRP\%} = [(C_{HRPo} \times V_o / C_{HRPi} \times V_i) \times 100\%]$, where C_{HRPo} is the HRP concentration in the well, C_{HRPi} is the HRP concentration in the insert, V_o is the medium volume in the well, and V_i is the medium volume in the insert.

Reverse Transcription and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from treated BMECs using TRIzol reagent (Invitrogen). Total RNA was reverse transcribed with the PrimeScript RT reagent kit (Takara Bio, Dalian, Japan) according to the manufacturer's instructions. The sequences of primers were as follows:
claudin-1: F:AAGACGACGAGGCACAGAAGA
and R:GAAGGTGCTGGCTTGGGATAG;
occludin: F:CAGCAGCAGTGGTAACTTGGA
and R:CCGGTCGTGTAGTCTGTTTCAT;
ZO-1: F:GCGAAATGAGAAACAAGCACC
and R:ATGAGTTGAGTTGGGCAGGAC;
GAPDH: F:GTTTGTGATGGGCGTGAACC and
R:CAGTCTTCTGGGTGGCAGTGAT.

Quantitative RT-PCR was performed with a SYBY Green Master Mix (Takara Bio, Dalian, Japan) using Mx3000P Real-Time QPCR System (Stratagene, USA). PCR cycles consisted of an initial denaturation step at 95°C for 30 s, followed by 95°C for 5 s, and 60°C for 30 s. All values were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as the change relative to GAPDH mRNA expression.

Animals

Our experimental protocols were conducted in accordance with the NIH guidelines outlined in the Guide for the Care and Use of Laboratory Animals and were approved by a local ethics committee. Pregnant BALB/c mice (10 to 12 weeks old, 25 to 30 g weight) were purchased from Changchun Institute of Biological Products. After parturition, the lactating mouse was kept with suckling neonatal pups. LTA that originated from *S. aureus* (InvivoGen, San Diego, CA, USA) was solubilized

in physiological saline. Doses of 0, 0.01, 0.1, 1.0 and 10 μg were injected into the fourth inguinal mammary gland via teat canal on day 7 of lactation under anesthesia with pentobarbital. No symptoms were observed in the mice. 24 h after LTA injection, the mice were decapitated, and the mammary glands were collected.

Western Blot Analysis

Frozen mammary glands (0.1 g) were homogenized and lysed in RIPA buffer (Beyotime, China) supplemented with protease inhibitor cocktail (Sigma). The homogenized lysate was centrifuged at 12.000xg at 4°C for 15min, and the supernatants were collected and determined with BCA protein assay kits (Beyotime). Equal volumes of protein (30 μg) were loaded onto SDS-PAGE gels and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, MA, USA) using a Mini Trans-Blot cell (Bio-Rad, CA, USA). The membranes were blocked for 2 h with 5% milk in TBST (0.1% Tween-20 in tris-buffered saline) at room temperature and incubated overnight at 4°C with primary antibodies against claudin-1 (1:200 dilution, santa cruz), occludin (1:200 dilution, santa cruz), ZO-1(1:200 dilution, santa cruz) and GAPDH (1:10000 dilution, abcam). The membranes were further incubated with horseradish peroxidase-(HRP-) conjugated anti-rabbit or anti-mouse IgG for 1 h at room temperature and finally developed with an electrochemiluminescence system (ECL; Solarbio, Beijing, China). To quantify relative protein expression levels, the intensity of specific protein bands was quantified using Quantity One software and then normalized normalized to β-actin for each lane.

Statistical Analysis

Standard statistical analysis was completed using SPSS16.0 (SPSS, Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA). $P<0.05$ was considered significant.

Results

TEER of BMECs Monolayer

Before the transepithelial permeability study, TEER measurement was used to assess the tightness of the BMECs monolayer. The TEER of the cells gradually increased in a time-dependent manner *(Fig. 1)*. TEER increased from 24 h (6.8±1.0Ω•cm^2) to 108 h (260.3±13.00Ω•cm^2). After seeding, an obvious increase in the TEER value could be observed from 24 h to 96 h. The TEER reached a plateau after 96 h.

LTA Increased Barrier Permeability of BMECs

The permeability of the BMECs monolayer was assessed by Horseradish Peroxidase (HRP) flux assay after LTA treatment. As shown in *Fig. 2*, compared to the untreated group, the HRP permeability significantly increased at 24 h after treatment with 0.1, 1.0 and 10 μg/mL LTA ($P<0.05$). When LTA concentration was 0.01 μg/mL, HRP permeability was unchanged.

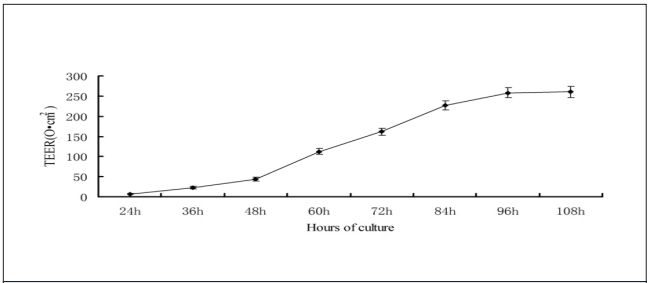

Fig 1. TEER values of BMECs monolayers on various hours in cultures. Each value represents the mean±S.D. (n=3)

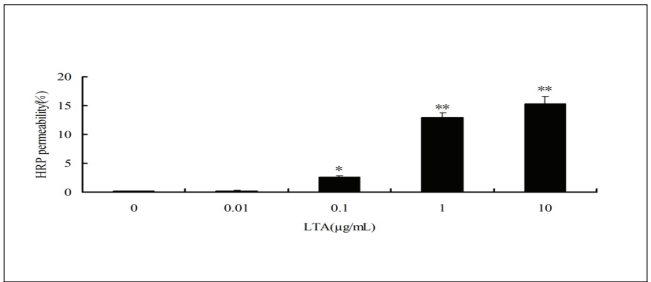

Fig 2. HRP permeability increased in BMECs monolayers challenged with LTA. Each data represents the mean±S.D. (n=5). *P<0.05, **P<0.01, compared with corresponding sham-treated controls

LTA Decreased Occludin and ZO-1 mRNA in BMECs

To examine the effects of LTA on the TJ protein expression levels, BMECs were exposed to different concentrations of LTA (0, 0.01, 0.1, 1.0 and 10 μg/mL) for 24 h, LTA treatment significantly decreased occludin and ZO-1 mRNA expression at concentrations of 0.1, 1.0 and 10 μg/mL ($P<0.05$). 0.01 μg/mL LTA had no effect on the expression of occludin and ZO-1 mRNA in BMECs. However, LTA treatment had no effect on expression of claudin-1 *(Fig. 3)*.

LTA Decreased Occludin and ZO-1 Protein Expression in Mouse Mammary Glands

We further investigated whether LTA exerted a prohibitive effect by regulating expression of TJ proteins in mouse mammary glands. Using immunoblotting, 0.01 μg LTA treatment had no effect on the expression of occludin and ZO-1 protein. LTA treatment with 0.1, 1.0 and 10 μg significantly decreased occludin and ZO-1 protein expression in mouse mammary glands ($P<0.05$). However, claudin-1 showed no change 24 h after LTA injection *(Fig. 4)*.

Discussion

TEER is a method to measure the integrity of TJs in cell

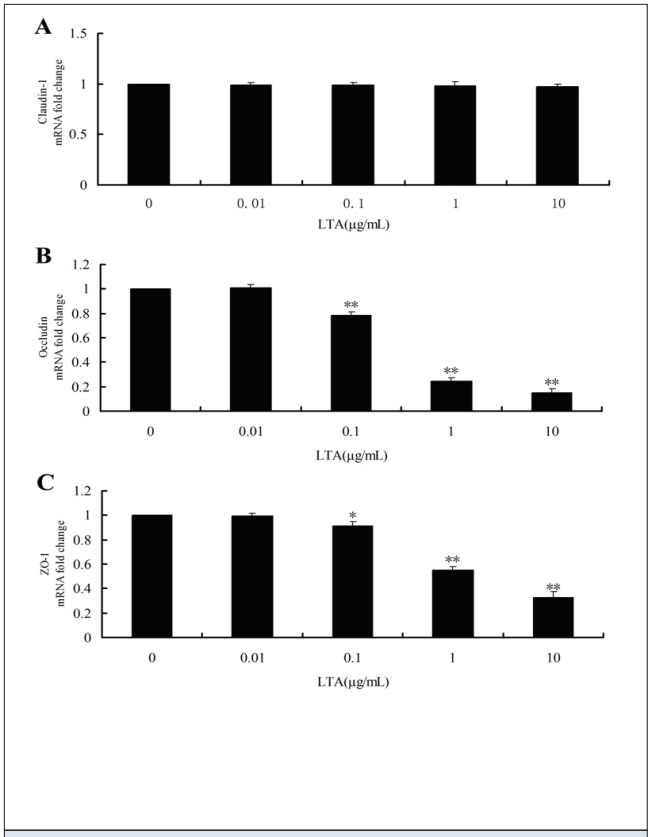

Fig 3. Effect of LTA on mRNA expression of TJ proteins in BMECs.Real-time PCR was performed to determine mRNA expression of (A) claudin-1, (B) occludin and (C) ZO-1 at 24 h after LTA challenge, respectively. Data represent means ± SD (n = 3) and were analyzed using one-way ANOVA followed by the Duncan test for multiple comparisons. *P<0.05, **P<0.01, compared with corresponding sham-treated controls

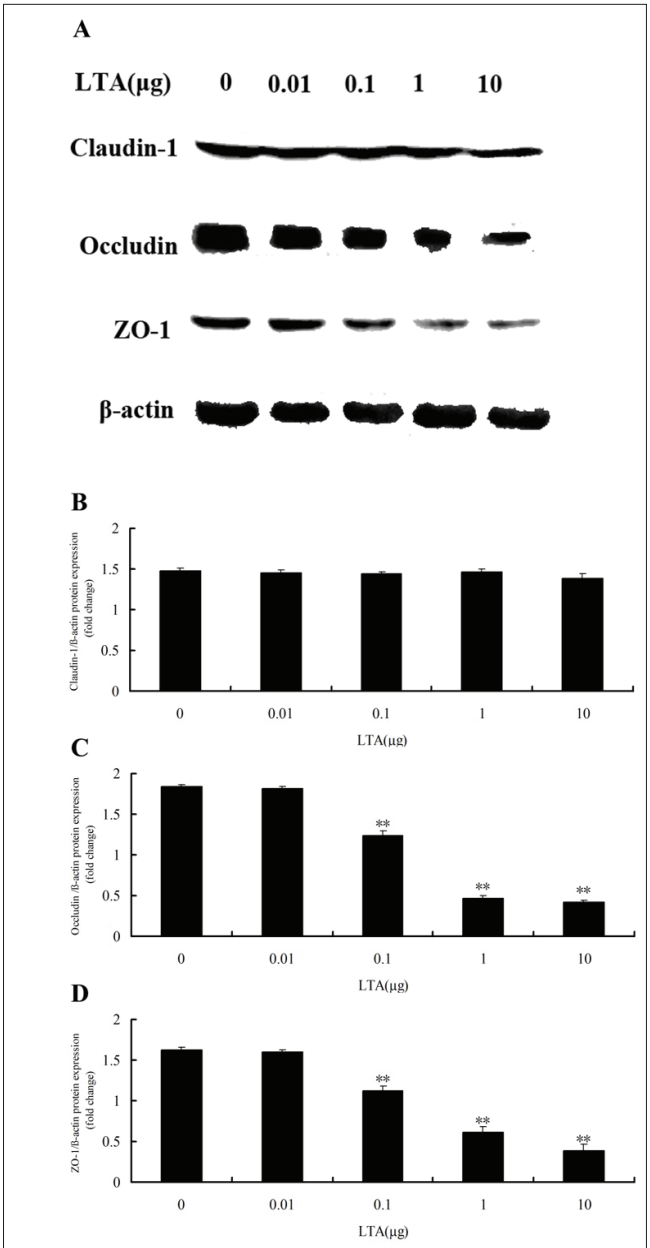

Fig 4. Effects of LTA on protein expression of TJ proteins challenged mouse mammary glands. (A) Results of a western blot analysis of claudin-1, occludin, ZO-1 and β-actin in the mammary glands 24 h after LTA injection. The relative expression levels of (B) claudin-1, (C) occludin and (D) ZO-1 were analyzed by densitometry. β-actin was used as a normalization control. Data represent mean means ± SD (n = 3). *P<0.05, **P<0.01, compared with corresponding sham-treated controls

culture models of endothelial and epithelial monolayers and reflects the integrity of the cellular barriers [15]. It has been demonstrated that primary bovine mammary epithelial cells cultured on Transwell inserts were grown to confluence by measurement of TEER [16]. In our study, continuous enhancement of TEER values indicated that cultured epithelial cells were able to form a tight barrier. In this experiment, the TEER value of primary BMECs reached a plateau at 96 hours, which was similar to the result that the TEER value of bovine mammary alveolar cell line (MAC-T) almost reached a plateau at 4 days [17].

HRP has been used to study macromolecule diffusion across epithelial/endothelial monolayers by tracking supernatant HRP activity [15]. The challenge of BMECs with 0.1, 1.0 and 10 μg/mL LTA resulted in a significant increase of BMECs permeability. Another study demonstrated that LPS or LTA can increase BMECs permeability by diffusion of Lucifer yellow [12]. In accordance with *in vitro* study, *in vivo* study showed that intramammary administration of LPS or LTA could cause impairment in the permeability of the blood-milk barrier leading to transfer of blood and milk components [18]. It has been reported that BMECs were treated with LPS of 10 μg/mL, and mastitis was induced by LPS of 10 μg in mice [19,20]. In this study, the doses of 0, 0.01, 0.1, 1.0, 10 μg/mL LTA were selected to treat BMECs according to LPS, and the doses of 0, 0.01, 0.1, 1.0 and 10 μg were used to induce mastitis in mice.

A decrease in the TJ protein mRNA and protein levels has been shown to damage the barrier's architecture, resulting in an increased leaking of the barrier [21]. LTA is recognized by TLR2 as a pathogen-associated molecular pattern and

induces inflammation and inflammatory cytokines via TLR2/NF-κB [22]. Therefore, we infer that LTA induced mastitis causes BMECs increased permeability is likely to be due to the decrease of TJ protein expression. This study showed that LTA decreased mRNA levels of TJ proteins occludin and ZO-1 but not claudin-1 in BMECs. It has been demonstrated that LPS or LTA disrupted epithelial/endothelial tight junction *in vitro* by suppressing expression of TJ proteins [23]. Different from LTA, the transcription levels and protein expression levels of occludin, ZO-1 and claudin-1 in BMECs treated with LPS were decreased [20]. We speculate that this difference may be related to the difference in the levels of inflammatory cytokines induced by LTA and LPS in BMECs.

The TJ proteins ZO-1 and occludin are two key proteins that maintain the integrity of epithelial cell TJ in mammary glands [24]. To assess the *in vivo* effects of LTA, we also developed an *in vivo* mouse mammary model of LTA modulation of expression levels of claudin-1, occludin and ZO-1. Our results demonstrate that LTA (0.1 μg, 1.0 μg, and 10 μg) obviously decreases occludin and ZO-1 protein levels. However, in our study LTA treatment had no effect on protein expression of claudin-1. Recently, it has been reported that LPS-induced mastitis in mice reduces the protein expression of occludin, ZO-1 and claudin-1 [19]. We hypothesized that LPS-induced mastitis may result from more intense production of inflammatory cytokines than LTA.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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Competing Interests

The author declared that there is no competing interests.

Ethical Approval

All the procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Inner Mongolia Agricultural University (IMAU) (License No. SYXK, Inner Mongolia, 2014-0008) with adherence to IMAU guidelines.

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ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's publication policy and ethical principles include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

The ethical statement of Kafkas Universitesi Veteriner Fakultesi Dergisi is based on the principles indicated in the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/Code_of_conduct_for_journal_editors_Mar11.pdf) and "COPE Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/u2/Best_Practice.pdf).

GENERAL ETHICAL PRINCIPLES

• Objectivity and Independence

Editor-in-chief, editors, associate editors, and referees conduct the evaluation process of the manuscript sent to the journal objectively and in coordination within the framework of ethical principles. Editorial decisions are independent, and internal or external factors cannot influence these decisions. In accordance with the principle of impartiality, academics working in our institution are not deemed eligible to work as a section editor in Kafkas Universitesi Veteriner Fakultesi Dergisi, in order not to be effective in the evaluation of articles due to conflict of interest.

• Privacy

The content of the articles and the personal information of the authors such as name, e-mail address, and telephone numbers that are sent to Kafkas Universitesi Veteriner Fakultesi Dergisi are used only for the scientific purposes of the journal and not for other purposes, and cannot be shared with third parties. Article evaluation processes are also carried out confidentially.

• Authorship and Authors Rights

The authors of the manuscripts sent to Kafkas Universitesi Veteriner Fakultesi Dergisi must have contributed significantly to the design, execution or interpretation of the study. For example, in view of the research and publication ethics as well as authors rights, it is not acceptable to include those as authors who do not actively contribute to the research but just only help in writing or data collection processes, which may not require any scientific knowledge. All the authors in a publication should be in agreement of the names and the orders of the authors in the manuscript.

The competence of the authors to the subject of the study is evaluated by the editor within the framework of deontological rules and the professional fields of each author.

The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

• Originality of Research Findings

The authors should declare that the article they presented contained the original research results, that the study data were analyzed correctly, and that they were prepared for publication using adequate and appropriate references, in the "cover letter" section of the on-line system at the submission stage. Using expressions such as "it is the first study done", "there has been no previous study on this subject" and "there is a limited number of studies" to add originality and importance to the article is not acceptable and may cause prevention of the scientific evaluation of the article by the editor.

• Similarity

Articles submitted to the journal are subjected to similarity analysis using appropriate software (iThenticate by CrossCheck) at the beginning and at every required stage. If unethical similarities are detected regardless of the rate of similarity, this situation is reported to the authors and corrections are requested or articles containing excessive similarities are rejected at the first evaluation stage without being evaluated.

• Plagiarism/Self-Plagiarism, Duplicate Publication

Kafkas Universitesi Veteriner Fakültesi Dergisi applies publication ethics and verifies the originality of content submitted before publication and checks all submitted manuscripts for plagiarism/self-plagiarism, similarity and duplication. All submitted manuscripts are meticilously screened by a similarity detection software (iThenticate by CrossCheck). Papers previously presented at scientific meetings and published only as an "abstract" should be indicated in the Title Page file as stated in the "Guidance for Authors". Authors do not have the right to use entire paragraphs from their previous publications into a new submission. These actions are also considered as a plagiarism. In any case, the manuscript should be original in terms of scientific contents and writing. In the event of alleged or suspected research misconduct, the Editorial Board will follow and act in accordance with "COPE Guidelines".

• Multi-part Publication (Piecemeal Publication)

Some authors may tend to divide study data into two or more articles and publish the results in different journals also having different authors names and orders. In principal, Kafkas Universitesi Veteriner Fakultesi Dergisi is against multi-part publication. When necessary, the ethical committee approval information of the study, project information, congress presentations, etc. are checked and such situations that will create an ethical problem are identified and reported to the authors.

Authors may think that their work should be published in multi parts that complement each other. For this, each part of the article should be titled "Part-I", "Part-II" and submitted to the journal "simultaneously". This issue can be evaluated by the editor-in-chief/subject editors/ referees who may suggest that the article can be published in parts or as a whole. In addition, rejection of a submission presented in parts means that all parts will be rejected.

• Animal Rights and Ethics

The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

• Conflicts of Interest/Competing Interests

The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

• Copyright

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The authors must fill in the "Copyright Agreement Form" and sign it with a wet signature. Authors who submit articles from abroad should scan the signed form and send it to the editor via the system or by e-mail. Original forms that are wet signed for articles sent domestically should be submitted to the journal via mail or cargo. The works of the authors who do not submit the Copyright Agreement Form on time are not published.

• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/ subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

OPEN ACCESS STATEMENT

Kafkas Universitesi Veteriner Fakultesi Dergisi is an open access publication. The journal's publication model is based on Budapest Open Access Initiative (BOAI) declaration. Articles published in Kafkas Universitesi Veteriner Fakultesi Dergisi are available online, free of charge at https://vetdergikafkas.org/archive.php.

Except for commercial purposes, users are allowed to read, download, copy, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. The open access articles in the journal are licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) licence.

ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• **Preliminary Evaluation Process**

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• **Peer-review Process**

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• **Publication Process of an Article**

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

6- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university

8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at http://vetdergi.kafkas.edu.tr/

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Availability of Data and Materials

- Acknowledgements

- Funding Support

- Competing Interests

- Authors' Contributions

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided