

# Sperm flow cytometric parameters, antioxidant status, and testicular histomorphology in roosters fed diets supplemented with camphor

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**ABSTRACT** Genetic selection based on the high growth rate and consequently high slaughter weight in broiler chickens has caused many problems in broiler breeders. A negative correlation between growth and reproductive traits has declined semen quality and fertility in roosters. The present study aimed to evaluate the effects of camphor levels on some reproductive parameters included semen parameters, antioxidant status, and testicular development in broiler breeder roosters. Thirty-five ROSS 308 broiler breeder roosters were divided into 5 groups to receive camphor (C) levels: C0, C50, C250, C750, and C1000 ppm for 12 consecutive weeks (31–43 wk). Body weight, seminal volume, sperm concentration, and percentage of live and morphologically normal sperm were not affected by diets ( $P > 0.05$ ), however, significantly were changed by bird age over the experiment ( $P < 0.05$ ). Semen quality factor (SQF) significantly was affected by both diets and age ( $P < 0.05$ ). Mitochondrial activity, apoptotic-like

changes, and DNA fragmentation were improved in the groups fed camphor levels compared to the control group ( $P < 0.05$ ). Testes weight (left, right, and combined weights) and gonadosomatic index were increased linearly by the camphor supplementation ( $P < 0.05$ ). The serum activity of glutathione peroxidase (GP<sub>x</sub>) was not affected by treatments, however, superoxide dismutase (SOD) activity, ferric ion reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity were significantly higher in C50, C250, and C750, respectively ( $P < 0.05$ ). The lower malondialdehyde (MDA) content in the testes and liver samples was observed in C750 ( $P < 0.05$ ). Excluding the number of Sertoli cells and blood vessels, other histomorphological traits of testes showed one of the linear or quadratic responses to the camphor levels ( $P < 0.05$ ). It can be concluded that camphor as an antioxidant source may improve reproduction performance in roosters.

**Key words:** antioxidant, camphor, flow cytometry, rooster, testicular histomorphology

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## INTRODUCTION

Although the male to female ratio in broiler breeder flocks is low, males contribute to half of the genetic information. Therefore, it is crucial to consider the fertility of roosters for achieving maximum flock reproduction performance (Triques et al., 2016). Due to the presence of polyunsaturated fatty acids (PUFAs) in the sperm plasma membrane and low antioxidant capacity, avian sperm is susceptible to lipid peroxidation (Saemi et al., 2012). In some studies, compounds such as ginger (Akhlaghi et al., 2014), rosemary (Borghai-Rad et al., 2017), sage (Ommati et al., 2013), and lycopene (Mangiagalli et al., 2010) as herb additives have been used to improve

semen quality and antioxidant capacity in roosters. These reports have been proved the positive effect of antioxidant therapy on the reduction of oxidative stress in roosters' sperm (Borghai-Rad et al., 2017). However, researches addressing to evaluate other dietary sources are needed to improve reproductive performance in roosters.

Camphor with the chemical formula C<sub>10</sub>H<sub>16</sub>O is a terpenoid, naturally derived from *Cinnamomum camphora* wood. Nowadays, synthetically camphor is available for medical, sanitary, and various purposes (Shata et al., 2014). Camphor has a wide range of applications in common medicine, including contraceptive, abortion (Shata et al., 2014), and anticancer (Chen et al., 2013) effects, as well as treating inflammation diseases in particular rheumatism, sprains, respiratory diseases such as bronchitis and asthma (Lee et al., 2006). Besides, camphor has been modulating some problems such as hysteria and nervousness (Lee et al., 2006). In addition, camphor is considered in the cosmetics industry, food additives, and the synthesis of perfume (Gomes-Carneiro et al., 1998).

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## MATERIALS AND METHODS

### Chemical

Chemicals were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich Company (St. Louis, MO). All experimental procedures were approved by the Animal Welfare Committee of the Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran and were carried out in compliance with the standards of the Royan Institute Ethical Committee.

### Birds, Treatments, and Semen Collection

Thirty-five 29-wk-old Ross 308 broiler breeder roosters were individually caged (60 × 50 × 75 cm), and kept under uniform environmental conditions (15 L: 9D light photoperiod at 21–23°C ambient temperature). Roosters were fed a commercial formula based on the instruction from Ross 308 broiler breeder recommendations (Aviagen, 2016), without additional antioxidant supplementation (Table 1), and water was provided ad libitum. The roosters were randomly allotted into 5 groups (7 birds per group). At the beginning of the experiment, the roosters were fed a basic diet for 14 d, and then received 5 levels (0 (C0), 50 (C50), 250 (C250), 750 (C750), and 1,000 (C1000) ppm) of camphor. Camphor crystals (Merck) were dissolved in diet sunflower oil. According to the method of Burrows and Quinn (1937), the roosters were conditioned for 2 weeks (wk 29 and 30) using dorso-abdominal massage for semen collection.

**Table 1.** Composition of the basal diet and nutrient value.

Ingredients	Composition (%)
Corn	67.06
Soybean meal (44%)	8.55
Wheat bran	20.73
Di-calcium phosphate	1.07
CaCO <sub>3</sub>	1.09
Sunflower oil	0.35
NaHCO <sub>3</sub>	0.22
Common salt	0.25
Vitamin premix <sup>1</sup>	0.25
Trace-mineral premix <sup>2</sup>	0.25
DL-methionine	0.09
L-lysine HCl	0.09
Total	100
Calculated nutritive value	
Metabolizable energy (kcal/kg)	2,739
Crude protein (%)	12
Ca (%)	0.72
P (%)	0.36
Lysine (%)	0.53
Methionine (%)	0.24
Threonine (%)	0.29

<sup>1</sup>Supplied per kg diet: vitamin A, 12,000 IU; vitamin D3, 3,500 IU; niacin, 50 mg; vitamin E, 100 IU; vitamin K3, 5 mg; riboflavin, 12 mg; thiamin, 3.0 mg; D-pantothenic acid, 13 mg; folic acid, 2 mg; pyridoxine, 6 mg; vitamin B12, 0.03 mg, and biotin, 0.66 mg.

<sup>2</sup>Supplied per kg diet: Fe (FeSO<sub>4</sub>·H<sub>2</sub>O), 50 mg; Mn (MnSO<sub>4</sub>·H<sub>2</sub>O), 120 mg; Zn (ZnO), 110 mg; Cu (CuSO<sub>4</sub>·5H<sub>2</sub>O), 10 mg; iodine (KI), 2 mg; and Se (Na<sub>2</sub>SeO<sub>3</sub>), 0.3 mg.

According to traditional medicine, low levels of camphor in food are aphrodisiac and stimulate the reproductive organs, however, in high doses, it shows conflicting behaviour and diminishes urinogenital irritation (Jamshidzadeh et al., 2006). Contradictory results have been shown in several studies regarding the effect of camphor on the reproductive system, which may be related to its dose-dependent effects, trial duration, and its application (Sedaghat et al., 2016). In this regard, male rats receiving 10 and 20 mg/kg of oral camphor underwent significant negative changes in their testicular tissue, which reduced spermatogenesis (Nikraves and Jalali, 2004). On the other hand, it has been reported that intraperitoneal injection of camphor in a dose of 100 mg/kg to male mice was not significantly affect the reproductive system, although it may temporarily reduce the number of spermatocytes (Leuschner, 1997). In another report, Sedaghat et al. (2016) stated that feeding male Japanese quails by camphor levels for 21 wk increased the percentage of hatched fertile eggs and mean severity of sperm penetration in the yolk inner perivitelline layer in the birds fed by the highest level of camphor (10,000 ppm). Also, Shata et al. (2014) demonstrated that intraperitoneal injection of camphor in a dose of 50 mg/kg can improve sexual performance in mice by changing seminiferous tubule maturation and enhancing testis function. Researchers showed that consuming camphor via gavage for 35 d at a dose of 30 mg/kg body weight/day reduced sperm count and increased immature sperm as a result of testicular tissue damage in mice (Morovvati et al., 2016). Some researchers have reported negative effects of camphor on concentration of sex gonadal hormones, adolescence, and reproductive organ volumes (Schlumpf et al., 2004). In this regard, the reduction of testosterone concentration by diminishing the activity of cytochrome P450 B1 and decreasing LH and FSH hormones via reducing the hormone GnRH has been shown as a result of the camphor's effect (Janjua et al., 2004; Mokhtari et al., 2007; Carou et al., 2009). Several studies have shown that 3-4-methylbenzylidene camphor (4-MBC), used to protect against ultra violet light, increases estrogenic effects in pre-mature mice and is considered an endocrine disruptors (Schlumpf et al., 2004; Carou et al., 2009).

Camphor is a major constituent in medicinal herbs such as rosemary (Brenes and Roura, 2010), *Artemisia annua* (Engeu et al., 2015), *Salvia officinalis* (Craft et al., 2017), and sagebrush (Guenther et al., 1995). These herbs, as growth-promoter and immune-stimulator, may be included in the commercial bird diets, although their levels are low. Despite the contradictory results of the effect of camphor on reproductive performance in other animals, its effect on the roosters' reproductive system-related parameters is still unclear. The current study aimed to investigate the effect of camphor levels on flow cytometric evaluation of sperm, antioxidant status, and testicular histomorphology in roosters.

## Body Weight and Seminal Characteristics

The roosters were subjected to experimental diets for 12 wk from 31 to 43 wk. During this period, semen was collected every 28 d and seminal characteristics individually were evaluated for each rooster. To assess the seminal volume, a graduated collecting tube was applied and sperm concentration was evaluated by placing a droplet of diluted semen sample (1:200 with distilled water) on the Neubauer hemocytometer (American Optical Company, New York, NY) (Ommati et al., 2013). According to the eosin-nigrosin staining method, sperm viability was evaluated, where unstained sperm was considered as live. The percentage of live sperm was assessed in duplicate by counting 250 sperm (CKX41, Olympus, Tokyo, Japan) (Fattah et al., 2017). The semen quality factor (SQF) was calculated by multiplying ejaculate semen volume (for individual bird evaluation; mL) by sperm concentration ( $\times 10^9$ /mL) in live and morphologically normal spermatozoa (%) (Liu et al., 2008). To minimize stress, once every 28 d, roosters were weighed individually 1 day after sperm collection.

## Flow Cytometry of Semen Parameters

The percentage of sperm with mitochondrial function was determined using Rhodamin-123 solution (0.01 mg/mL) and propidium iodide (PI; 1 mg/mL). Briefly, a mixture of 10  $\mu$ L Rhodamin-123 solution and 500  $\mu$ L diluted semen was incubated for 20 min at room temperature in the dark. Afterward, the samples were centrifuged at 500 *g* for 3 min and again resuspended in 500  $\mu$ L Tris buffer. Then, 10  $\mu$ L of PI (1 mg/mL) was added to the sperm suspension. For each sample, 10,000 events were recorded. Sperm cells with active mitochondria were identified by the positive signal for Rh123 and the negative signal for PI (Masoudi et al., 2016).

Apoptotic-like changes were assessed using Annexin-V (IQP, Groningen, Netherlands) described by Shahverdi et al. (2015) with a slight modification. In brief, the semen samples were washed in calcium buffer and adjusted the concentration of sperm to  $1 \times 10^6$ . Then, 10  $\mu$ L Annexin-V was added to the sperm suspension. Next, 5  $\mu$ L PI was added to the sperm suspension and incubated for 15 min at room temperature. The obtained suspension was evaluated by flow cytometer. The sperm was classified into 4 groups: 1) viable non-apoptotic cells which were negative for both Annexin V and PI ( $A^-/PI^-$ ); 2) early apoptotic cells which were positive for Annexin V but negative for PI ( $A^+/PI^-$ ); 3) late apoptotic cells which were positive for both Annexin V and PI ( $A^+/P^+$ ); and 4) necrotic cells which were negative for Annexin V but positive for PI ( $A^-/P^+$ ). The late apoptotic and necrotic cells were categorized as dead cells.

Chromatin structure assay (SCSA) was used to detect DNA damage in sperm by flow cytometry analysis (Evenson and Wixon, 2005). After sperm cells were washed with PBS was diluted to a concentration of  $3 \times 10^9$  sperm/mL. The suspension was subjected to an acid detergent solution (0.1% Triton X-100, 0.15 M

NaCl, and 0.08 M HCl, pH: 1.4), held for 40 s, and then stained with 6 mg/L purified acridine orange (AO) in a phosphate-citrate buffer. The percentage of DNA fragmentation index was calculated from the ratio between the red and total (red plus green) fluorescence intensity (Hosseinifar et al., 2015).

Flow cytometry analysis of mitochondrial activity, apoptotic-like changes, and DNA fragmentation were performed using FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with standard optics. For each sample, 10,000 events were analyzed. The fluorescent probes used in the experiment (R123, Annexin VeFITC, Acridine orange and, PI) were excited by an Argon ion 488 nm laser. Annexin V fluorescence and R123 fluorescence were detected on detector FL1 with a green filter, and PI fluorescence was detected on detector FL2 with a red filter. For DNA fragmentation, single-stranded DNA as a sign of fragmentation emits a red fluorescence detected at a 670 band pass filter (FI-3). Analysis was performed using FlowJo software (TreeStar, Inc., San Carlos, CA).

## Comb Weight and Testis Mass

Before the euthanasia by cervical dislocation, roosters were individually weighed at the end of the experiment. The comb and left and right testes of each rooster were separated and weighed. The comb weight and gonadosomatic index were expressed as the percentage of the body weight (Sun et al., 2019).

## Antioxidant Parameters of the Blood Serum

Blood samples were collected from the brachial vein of birds and the serum was separated following centrifugation at  $4,000 \times g$  for 15 min. The serum was evaluated for superoxide dismutase (SOD), and glutathione peroxidase (GP<sub>X</sub>) activities, as well as, ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays as antioxidant parameters.

SOD and GP<sub>X</sub> activities of the blood serum were determined by commercial kits (Teb Pazhouan Razi, Tehran, Iran, and Biorex Fars, Shiraz, Iran, respectively). SOD and GP<sub>X</sub> activities in the samples were measured from the sample's absorbance at 450 and 340 nm, respectively. Also, the activity of both enzymes was expressed as U/mg protein.

FRAP assay was measured as suggested by Benzie and Strain (1996). Briefly, FRAP solution was prepared by mixing 300 mM sodium acetate buffer, pH 3.6, 10 mM tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, and 20 mM iron (III) chloride hexahydrate in a volume ratio of 10:1:1. After adding 10  $\mu$ L of samples to 300  $\mu$ L FRAP solution, the mixture was incubated at room temperature for 3 min. The ODs of the mixture were immediately read at 593 nm. The assay was calibrated by an aqueous solution with a known FeS<sub>2</sub>O<sub>4</sub>·7H<sub>2</sub>O (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM).

Total antioxidant capacity in the blood serum was evaluated using of DPPH free radical assay (Chrzczanowicz et al., 2008). Briefly, 200  $\mu\text{L}$  of the serum was added to 200  $\mu\text{L}$  of acetonitrile, incubated for 2 min at room temperature. The samples were centrifuged at  $9,500 \times g$  for 10 min. The supernatant (deproteinized plasma) was collected. 5  $\mu\text{L}$  of 10 mM of a DPPH in methanol was added to a cuvette containing 970  $\mu\text{L}$  of mixed methanol, measuring the absorbance at 517 nm ( $A_{517}$ ) after a 3 min incubation period at 20°C. Then, 25  $\mu\text{L}$  of deproteinized plasma was added and then mixed, followed by a recording of the  $A_{517}$  decrease related to the DPPH decomposition over a 30 min incubation period. Some samples received 25  $\mu\text{L}$  of 9.50 M acetonitrile solution as a negative control. Mean plasma scavenging effect (Sc %) was calculated according to the formula:  $\text{Sc \%} = (1 - A_{517} \text{ plasma sample} / A_{517} \text{ negative control}) \times 100$ .

### Lipid Peroxidation in the Tissues

Malondialdehyde (MDA) concentration as an indicator of the lipid peroxidation in tissues was estimated by the thiobarbituric acid (TBA) reaction according to Botsoglou et al. (1994) method. Briefly, 2 g samples (testes and liver) were homogenized with 8 mL of trichloroacetic acid (50 g/L) and 5 mL of butylated hydroxytoluene in hexane (8 g/L). The mixture was centrifuged for 5 min at  $3,000 \times g$ . After removing the top hexane layer, 2.5 mL of the remaining sample was mixed with 1.5 mL of 2-thiobarbituric acid (8 g/L) and incubated at 70°C for 30 min. The iron-induced lipid oxidation method was carried out as a modification of the method of Botsoglou et al. (2002). According to the procedure, 1.5 mL of a stock solution containing 1.138 mM ferrous sulphate and 0.368 mM ascorbic acid was added to each sample and incubated at 38°C for 60 min. Subsequently, the mixture included iron-induced samples along with non-induced samples left at room temperature. The absorbance was read at 532 nm using a spectrophotometer (6320D, Jenway, UK).

### Testicular Histomorphology

To histological evaluation, left testis specimens were collected as previously described by Sun et al. (2019). Briefly, after tissue fixation in 10% neutral buffered formalin for 3 d, samples were embedded in paraffin, cut into sections of 5- $\mu\text{m}$  thickness, affixed to a microscope slide, and stained with hematoxylin and eosin. The samples were used for evaluation of the diameter of the seminiferous tubule, the thickness of seminiferous epithelium, the thickness of connective tissue, the number of seminiferous tubules, the number of Sertoli cells, the number of Leydig cells, spermatogenesis index, tubular differentiation index, and the number of blood vessels. To measure the thickness of seminiferous epithelium, the diameter of the seminiferous tubule, and the thickness of interstitial connective tissue, Image-Pro-Plus (version 6.0; Media

Cybernetics, Inc., Rockville, MD) was used at  $400 \times$  magnification. The number of active mutilated tubules inside of circles with a radius of 500  $\mu\text{m}$  was counted to the evaluation of the number of seminiferous tubules. For the estimation of the number of Sertoli cells, cells with an accumulation place of spermatids at triangular points were considered as Sertoli cells. Leydig cells were identified and counted as relatively large cells with an eccentric nucleus in close vicinity to the blood cells in the interstitium. Tubule differentiation index as the percentage of seminiferous tubules containing at least three differentiated germ cells was calculated. To spermatogenesis index determination, the ratio of the number of seminiferous tubules with spermatozooids to the empty tubules was calculated (Figure 1; Mohammadi et al., 2021).

### Statistical Analyses

Shapiro-Wilk test for normality of data and Levene's test for homogeneity of variances was applied. Repeated measurement data were analyzed with Proc mixed, while single measurement data were analyzed with Proc GLM of SAS software (SAS, 2002). All data were expressed as Least Square Means. Duncan's multiple range test was performed for the mean comparison. Linear and quadratic effects of camphor levels were tested using orthogonal polynomials. Also, the differences were defined as statistically significant when  $P < 0.05$ .

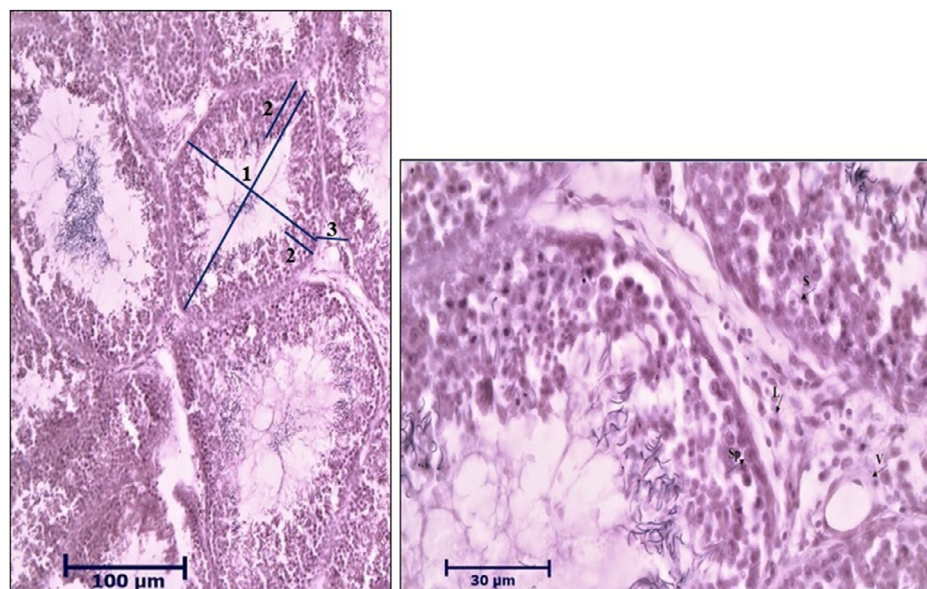
## RESULTS

### Body Weight and Seminal Characteristics

Data for the effect of camphor levels on body weight and seminal characteristics are shown in Table 2. Based on this table, excluding SQF ( $P < 0.05$ ), other parameters were not affected by experimental diets ( $P > 0.05$ ). Excluding the C50 group, SQF in roosters fed by camphor levels were significantly higher than in the control group ( $P < 0.05$ ). Also, the main effect of time on body weight and seminal attributes in roosters are presented in Table 3. All parameters in Table 3 included body weight, semen volume, sperm concentration, percentage of live and morphologically normal sperm, and SQF affected by time ( $P < 0.01$ ). Body weight and seminal volume of roosters significantly increased with rooster age over the trial duration ( $P < 0.05$ ). However, the semen concentration, percentage of live and morphologically normal sperm, and SQF decreased significantly with the increasing age of roosters.

### Flow Cytometric Analysis

Figure 2 presents the effect of camphor levels on the percentage of mitochondria activity of sperm. Although on d 0 of the experiment, the mitochondrial activity of experimental rooster sperm was not different ( $P > 0.05$ ), on d 28, 56, and 84 of the experiment there was a



**Figure 1.** A sample of photomicrograph of testicular section of broiler breeder roosters were stained with hematoxylin and eosin. (A) Seminiferous tubules diameter (1), seminiferous epithelium thickness (2), interstitial connective tissue (3). (B) Sertoli cell (S), Leydig cell (L), blood vessel (V), spermatogonium cell (Sp).

significant difference between groups ( $P < 0.05$ ). According to [Figure 2](#), on d 28, roosters fed by 50 ppm had higher mitochondria activity (84.50%) compared to the C750 and C1000 groups (81.25 and 79.68%, respectively;  $P < 0.05$ ). Interestingly, the mitochondria activity in the C750 group on d 56 was significantly higher (81.50%) than in the C50 and C1000 groups (76.33 and 76.83%, respectively;  $P < 0.05$ ). In addition, on d 84, the highest mitochondria activity was observed in the C250 and C750 groups (78.00 and 76.6%, respectively;  $P < 0.05$ ).

The effects of experimental diets on the percentage of live sperm of roosters are shown in [Figure 3](#). On d 0 and 56 of the experiment, no significant difference was observed for the percentage of live sperm in different camphor levels ( $P > 0.05$ ). Although on d 28 the lowest percentage of live sperm was found in C750 group (75.25%), on d 84 the percentage of live sperm in this group was significantly higher (85.60 %) than the other groups ( $P < 0.05$ ).

As shown in [Figure 4](#), on d 28, 56, and 84 percentage of apoptotic sperm in different camphor levels was

**Table 2.** Body weight and seminal attributes in roosters fed diets containing camphor level.

Trait	Diet <sup>1</sup>					Pooled SEM	P-Value			Effect (P)	
	C0	C50	C250	C750	C1000		Diet	Time	Diet × Time	Linear	Quadratic
Body weight (kg)	4.39	4.29	4.37	4.43	4.22	0.07	0.68	<0.0001	0.11	0.11	0.001
Seminal volume (mL/bird)	0.50	0.61	0.49	0.54	0.57	0.10	0.57	0.0009	0.62	0.59	0.36
Sperm concentration ( $\times 10^9$ cells/mL)	3.27	3.29	3.33	3.34	3.26	0.18	0.96	0.0025	0.99	0.96	0.39
Live and morphologically normal sperm (%)	88.15	88.66	88.80	88.91	88.07	1.75	0.94	<0.0001	0.19	0.82	0.06
SQF <sup>2</sup>	146.21 <sup>b</sup>	169.17 <sup>a</sup>	147.77 <sup>b</sup>	158.03 <sup>ab</sup>	165.19 <sup>a</sup>	10.27	0.04	<0.0001	0.001	0.15	0.22

<sup>1</sup>The birds received diets containing 0 (C0), 50 (C50), 250 (C250), 750 (C750) or 1,000 (C1000) ppm of camphor (C) for 84 d (31–42 wk of age). The values represent the means of records obtained from the consecutive every 28 d once measurements.

<sup>2</sup>SQF (semen quality factor) = seminal volume (mL) × sperm concentration ( $\times 10^9$ /mL) × live and morphologically normal spermatozoa (%).

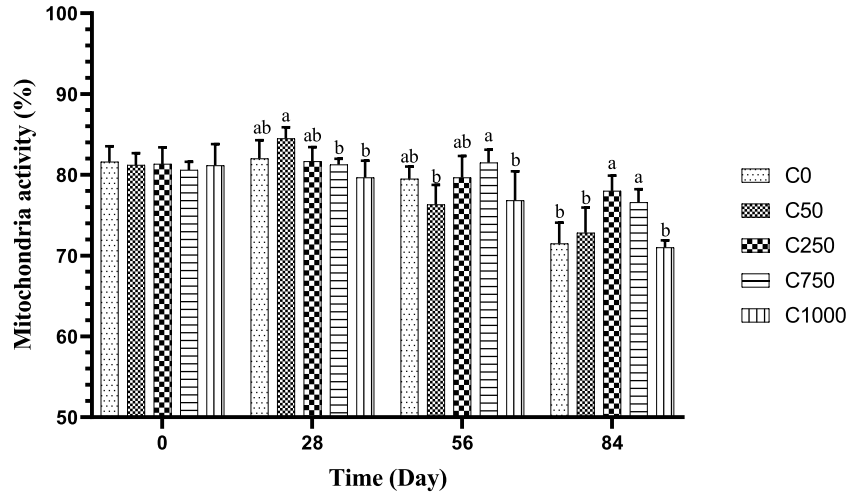
<sup>a-c</sup>Values within the same row followed by different superscript letters differ significantly ( $P \leq 0.05$ ).

**Table 3.** The effect of time on body weight and seminal attributes in roosters.

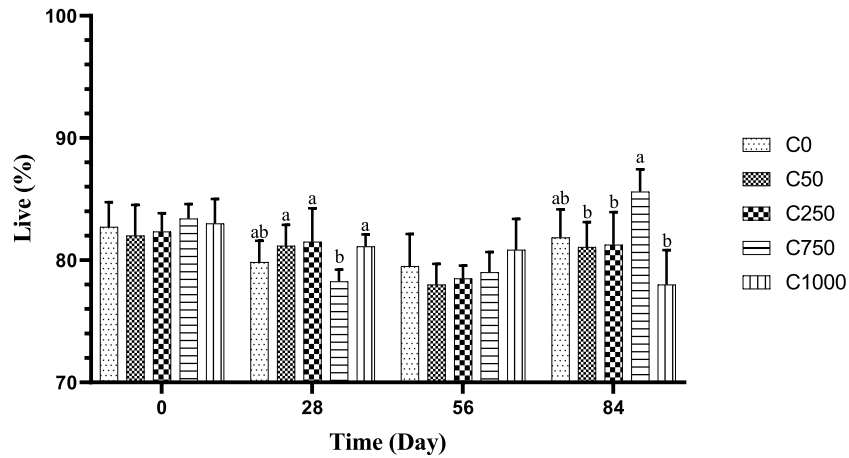
Trait	Time (day)				Pooled SEM	P-value
	0	28	56	84		
Body weight (kg)	4.18 <sup>c</sup>	4.28 <sup>bc</sup>	4.39 <sup>ab</sup>	4.50 <sup>a</sup>	0.04	<0.0001
Seminal volume (mL/bird)	0.42 <sup>b</sup>	0.63 <sup>a</sup>	0.53 <sup>ab</sup>	0.56 <sup>a</sup>	0.006	0.0009
Sperm concentration ( $\times 10^9$ cells/mL)	3.51 <sup>a</sup>	3.67 <sup>ab</sup>	3.20 <sup>b</sup>	3.12 <sup>b</sup>	0.03	0.0025
Live and morphologically normal sperm (%)	89.84 <sup>a</sup>	88.70 <sup>ab</sup>	88.43 <sup>b</sup>	87.10 <sup>c</sup>	0.56	<0.0001
SQF <sup>1</sup>	133.88 <sup>c</sup>	188.96 <sup>a</sup>	155.74 <sup>b</sup>	150.50 <sup>bc</sup>	10.27	<0.0001

<sup>1</sup>SQF (semen quality factor) = seminal volume × sperm concentration × live and morphologically normal spermatozoa.

<sup>a-c</sup>Values within the same row followed by different superscript letters differ significantly ( $P \leq 0.05$ ).



**Figure 2.** Mitochondria activity of roosters' sperm fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE.

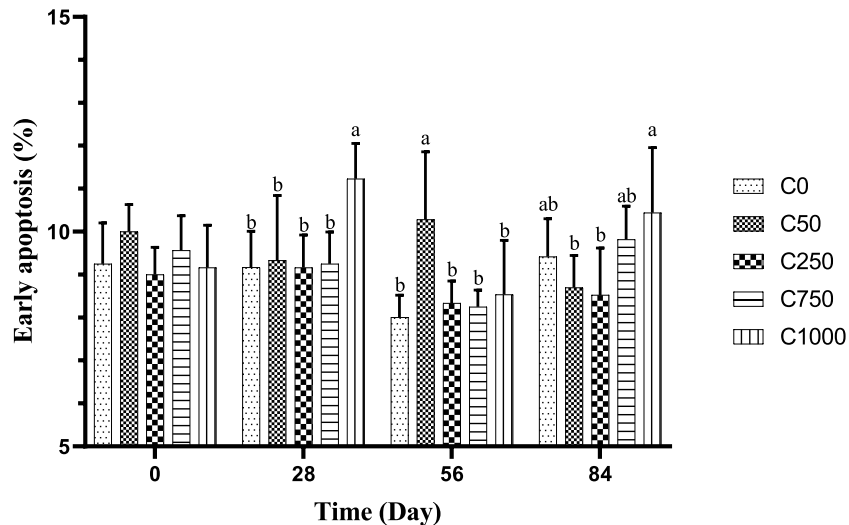


**Figure 3.** Percentage of live sperm of roosters fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE.

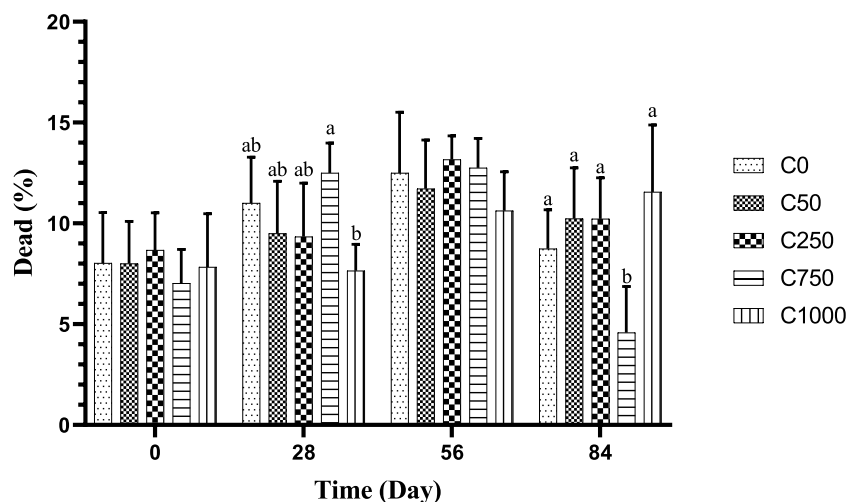
affected ( $P < 0.05$ ). On d 28 and 56, there was higher early apoptotic sperm in C1000 and C50 groups (11.23 and 10.28%, respectively) ( $P < 0.05$ ). On d 84, early apoptotic in the C50 and C250 groups was significantly

lower (8.70 and 8.53%, respectively) than in the C1000 group (10.43%;  $P < 0.05$ ).

Although on d 0 and 56, the percentage of dead sperm was not significantly affected by the different diets, this



**Figure 4.** Percentage of apoptotic sperm of roosters fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE.



**Figure 5.** Percentage of dead sperm of roosters fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE.

parameter by feeding camphor levels was changed significantly on d 28 and 84 (Figure 5;  $P < 0.05$ ). Although on d 28, the C750 group had a higher (12.5 %) percentage of dead sperm compared to the C1000 group (7.65%), on d 86, the lowest percentage of dead sperm was observed in the C750 group (4.58%;  $P < 0.05$ ).

DNA fragmentation of sperm was affected by experimental diets on d 28 and 84 (Figure 6). On d 28, the percentage of DNA fragmentation of sperm was lower in roosters receiving 50 and 750 ppm camphor (5.82 and 5.60%, respectively) when compared to those in the C1000 group (7.90%;  $P < 0.05$ ). However, on d 84, the percentage of DNA fragmentation in C50 was higher (13.50%) than in the other groups ( $P < 0.05$ ).

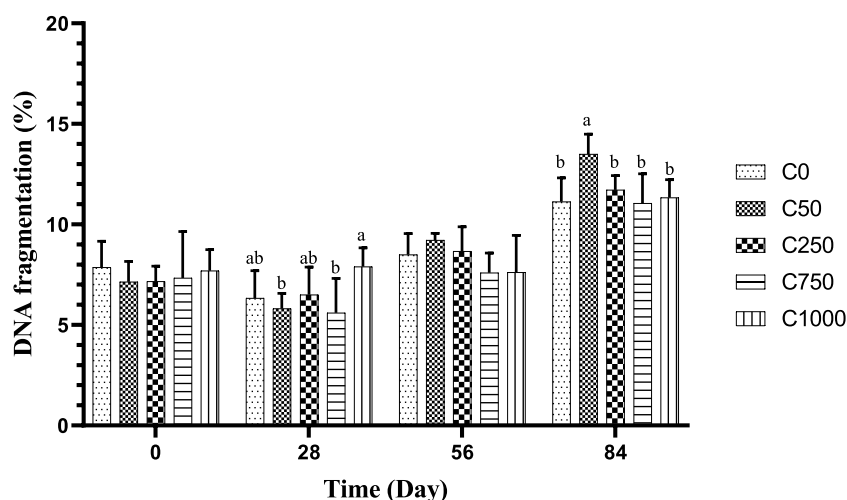
### Comb Weight and Testis Mass

Data for the effect of camphor levels on comb weight and testis mass are shown in Table 4. Based on the data,

roosters' comb weight was not affected by treatments ( $P > 0.05$ ). Combined testis weight ( $P < 0.0001$ ), left and right testis weights ( $P < 0.0001$ ), and gonadosomatic index ( $P = 0.02$ ) increased linearly as camphor was added to the diets.

### Antioxidant Parameters

As illustrated in Table 5, SOD activity, FRAP, and DPPH values were significantly affected by camphor supplementation ( $P < 0.05$ ). SOD activity was significantly increased in C50 birds (100.38 U/mg protein) than in the other groups ( $P < 0.05$ ). Diets supplemented with camphor quadratically was increased FRAP values, with the highest values was observed at C250 compared to C1000 as the lowest value (1030.98 vs. 989.35  $\mu\text{M}$ ) ( $P < 0.05$ ). Also, the highest values for neutralization of DPPH radical were recorded for roosters fed diets supplemented with 750 ppm camphor (C750) (22.50%;  $P < 0.05$ ).



**Figure 6.** Percentage of DNA fragmentation roosters' sperm fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE.

**Table 4.** Testes weight and comb weight in roosters fed diets containing camphor levels.

Trait	Diet <sup>1</sup>					Pooled SEM	P-value	Effect (P)	
	C0	C50	C250	C750	C1000			Linear	Quadratic
Left testis weight (g)	18.17 <sup>b</sup>	20.11 <sup>b</sup>	18.15 <sup>b</sup>	25.03 <sup>a</sup>	20.56 <sup>b</sup>	0.76	<0.0001	<0.0001	0.02
Right testis weight (g)	16.18 <sup>b</sup>	18.11 <sup>b</sup>	16.14 <sup>b</sup>	23.04 <sup>a</sup>	18.56 <sup>b</sup>	0.75	<0.0001	<0.0001	0.02
Combined testis weight (g) <sup>2</sup>	34.35 <sup>b</sup>	38.22 <sup>b</sup>	34.29 <sup>b</sup>	48.07 <sup>a</sup>	39.12 <sup>ab</sup>	1.51	<0.0001	0.0005	0.06
Gonadosomatic index (%) <sup>2</sup>	0.80 <sup>b</sup>	1.00 <sup>ab</sup>	0.81 <sup>b</sup>	1.16 <sup>a</sup>	0.94 <sup>ab</sup>	0.06	0.001	0.02	0.16
Comb weight (g)	42.41	44.48	39.34	37.76	39.66	2.05	0.20	0.08	0.17

<sup>1</sup>The birds received diets containing 0 (C0), 50 (C50), 250 (C250), 750 (C750) or 1,000 (C1000) ppm of camphor (C) for 84 d (31–42 wk of age).

<sup>2</sup>Combined testes weight = left testis weight + right testis weight, Gonadosomatic index = (combined testis weight/BW) × 100.

<sup>a-c</sup>Values within the same row followed by different superscript letters differ significantly ( $P \leq 0.05$ ).

**Table 5.** Antioxidant parameters in the blood serum of roosters fed diets containing camphor levels (0–84 d).

Trait	Diet <sup>1</sup>					Pooled SEM	P-value	Effect (P)	
	C0	C50	C250	C750	C1000			Linear	Quadratic
SOD (U/mg protein)	92.27 <sup>b</sup>	100.38 <sup>a</sup>	90.20 <sup>b</sup>	87.29 <sup>b</sup>	88.93 <sup>b</sup>	1.22	0.0002	0.0002	0.03
GP <sub>x</sub> (U/mg protein)	77.64	84.22	76.73	82.72	76.75	5.27	0.76	0.83	0.79
FRAP ( $\mu$ M)	997.51 <sup>bc</sup>	1018.60 <sup>ab</sup>	1030.98 <sup>a</sup>	1001.83 <sup>bc</sup>	989.35 <sup>c</sup>	7.94	0.02	0.02	0.01
DPPH (%)	19.77 <sup>b</sup>	18.82 <sup>b</sup>	18.53 <sup>b</sup>	22.25 <sup>a</sup>	18.25 <sup>b</sup>	0.31	<0.0001	0.11	0.0009

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity; FRAP, ferric reducing antioxidant power; GP<sub>x</sub>, glutathione peroxidase activity; SOD, superoxide dismutase activity.

<sup>1</sup>The birds received diets containing 0 (C0), 50 (C50), 250 (C250), 750 (C750) or 1,000 (C1000) ppm of camphor (C) for 84 d (31–42 wk of age).

<sup>a-c</sup>Values within the same row followed by different superscript letters differ significantly ( $P \leq 0.05$ ).

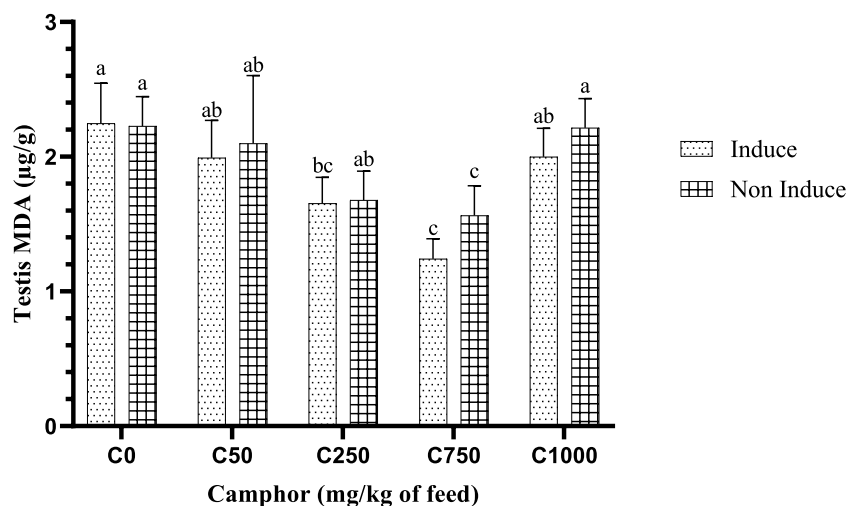
## Lipid Peroxidation

The results of MDA content of testis and liver samples as lipid peroxidation index are shown in Figures 7 and 8, respectively. The amount of MDA content in testes and liver of C750 with a similar trend inoxidation-induced and non-induced conditions was significantly lower than other groups ( $P < 0.05$ ).

## Testicular Histomorphology

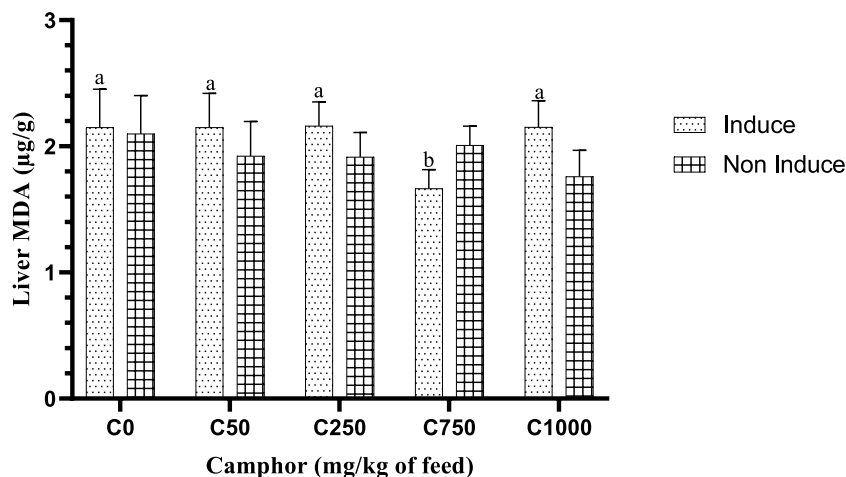
Testicular histomorphology data for roosters fed camphor levels are presented in Table 6. Linear (interstitial connective tissue, the number of seminiferous tubules, spermatogenesis index, and tubular differentiation index) and quadratic (the diameter of seminiferous

tubules, the seminiferous epithelium thickness, interstitial connective tissue, the number of seminiferous tubules, the number of Leydig cells, spermatogenesis index, and tubular differentiation index) trends were observed by camphor levels. Excluding the number of Sertoli cells and the number of blood vessels ( $P > 0.05$ ), other parameters were significantly affected by the treatments ( $P < 0.05$ ). The seminiferous tubules diameter and the seminiferous epithelium thickness were significantly higher in the testes of C750 roosters (262.75 and 65.67  $\mu$ m, respectively) than the other groups ( $P < 0.05$ ). Improvements in interstitial connective tissue, the number of seminiferous tubules, the number of Leydig cells, and spermatogenesis index were observed significantly in roosters fed by moderate levels of camphor (C250 and C750;  $P < 0.05$ ).



**Figure 7.** Accumulation of MDA in roosters' testis fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE. Abbreviation: MDA, malondialdehyde.





**Figure 8.** Accumulation of MDA in roosters' liver fed diets containing camphor levels. Different letters (a, b) above the bars indicate significant differences ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE. Abbreviation: MDA, malondialdehyde.

## DISCUSSION

Although several studies have examined changes in the reproductive system of mice (Jamshidzadeh et al., 2006; Shata et al., 2014) and Japanese quail (Sedaghat et al., 2016) after camphor administration, its effect on the reproductive characteristics in broiler breeder roosters, including semen quality and testicular morphological changes, has not been investigated. Having considered the negative effect of the highest level of camphor (1,000 ppm) on some parameters, in general, diets supplemented with camphor have improved semen characteristics, antioxidant status, lipid peroxidation of the testis, and liver tissues, as well as testicular morphology.

In the current study, the body weight and seminal attributes were affected by time. During the experiment with weight gain, semen characteristics included sperm concentration, and the percentage of live and morphologically normal sperm significantly were decreased. In this regard, with the aging of male broiler breeders, factors such as a change in hormonal concentration, testicular development, physical body composition, and activity could reduce the reproductive performance (Sarabia Fragoso et al., 2013). The factors that reduce the semen quality with age included 1) membrane destabilization originating due to peroxidative damage of

PUFAs; 2) changes in semen biochemical characteristics imputable to aging such as protein levels, activities of acid phosphatase, aspartate aminotransaminase, amidase, and antiproteinase (Iaffaldano et al., 2008); and 3) decline antioxidant capacity (Lewis et al., 1995). It has also been reported that in aged roosters a decrease in live sperm concentration despite the active seminiferous tubules and an increase in morphological malformations of Sertoli and Leydig cells are conceivable (Weil et al., 1999).

Results of the effect of camphor levels on semen characteristics in roosters in the present study indicate that although seminal volume, sperm concentration, and live and morphologically normal sperm were not affected by treatments, SQF increased in camphor receiving groups. Also, semen flow cytometric analysis of the tested roosters showed a positive effect of camphor, especially level 750 ppm, on semen quality. Contrary to our results, it has been reported that camphor reduces the body's detoxifying capacity by binding to glucuronic acid (as a detoxifier and transporter of some hormones) and inhibiting its activity (Rabl et al., 1997). In this regard, the increase in the number of degenerated seminiferous tubules and the decrease of mature sperm have been attributed to the increase in toxins released through camphor metabolism or the failure of the body's

**Table 6.** Testicular histomorphology in roosters fed diets containing camphor levels.

Trait	Diet <sup>1</sup>					Pooled SEM	P-value	Effect (P)	
	C0	C50	C250	C750	C1000			Linear	Quadratic
Seminiferous tubules diameter ( $\mu\text{m}$ )	242.98 <sup>b</sup>	247.04 <sup>b</sup>	244.55 <sup>b</sup>	262.75 <sup>a</sup>	239.54 <sup>b</sup>	3.96	0.0005	0.39	0.002
Seminiferous epithelium thickness ( $\mu\text{m}$ )	58.68 <sup>bc</sup>	58.22 <sup>bc</sup>	60.79 <sup>b</sup>	65.67 <sup>a</sup>	54.95 <sup>c</sup>	1.03	<0.0001	0.99	<0.0001
Interstitial connective tissue ( $\mu\text{m}$ )	15.36 <sup>b</sup>	13.71 <sup>c</sup>	13.59 <sup>c</sup>	12.27 <sup>c</sup>	17.04 <sup>a</sup>	0.39	<0.0001	0.02	<0.0001
Number of seminiferous tubules (n)	14.39 <sup>c</sup>	14.03 <sup>c</sup>	17.39 <sup>ab</sup>	18.08 <sup>a</sup>	15.78 <sup>bc</sup>	0.50	<0.0001	0.0001	<0.0001
Number of Sertoli cells (n)	27.80	27.07	26.40	23.87	25.87	1.71	0.19	0.08	0.30
Number of Leydig cells (n)	27.47 <sup>c</sup>	29.00 <sup>bc</sup>	34.27 <sup>a</sup>	32.06 <sup>ab</sup>	27.73 <sup>c</sup>	0.94	<0.0001	0.92	<0.0001
Spermatogenesis index (%)	70.25 <sup>b</sup>	70.00 <sup>b</sup>	77.25 <sup>a</sup>	75.20 <sup>a</sup>	61.59 <sup>c</sup>	1.11	<0.0001	<0.0001	<0.0001
Tubular differentiation index (%)	62.76 <sup>b</sup>	59.57 <sup>c</sup>	69.90 <sup>a</sup>	65.33 <sup>b</sup>	57.10 <sup>c</sup>	1.09	<0.0001	0.01	<0.0001
Blood vessels (n)	2.34	2.48	2.28	2.14	2.17	0.15	0.51	0.11	0.64

<sup>1</sup>The birds received diets containing 0 (C0), 50 (C50), 250 (C250), 750 (C750) or 1,000 (C1000) ppm of camphor (C) for 84 d (31–42 wk of age).

<sup>a-c</sup>Values within the same row followed by different superscript letters differ significantly ( $P \leq 0.05$ ).

detoxification system (Rabl et al., 1997). The cell membranes of avian sperm contain higher levels of PUFAs than mammalian sperm, thereby reactive oxygen species (ROS) easily attack PUFA in the cell membrane and lead to sperm lipid peroxidation (Fattah et al., 2017). Also, the imbalance between ROS accumulation and antioxidant defense in the testis can be reduced semen quality via a reduction in sperm motility and sperm DNA damage (Alahmar, 2019). It has been reported that seed and peel extract of *Cinnamomum camphora* (as camphor tree) with antioxidant properties can scavenge the free radicals (Liu et al., 2018). The positive effects of herbal antioxidants containing camphor such as rosemary (Borghei-Rad et al., 2017; Teymouri Zadeh et al., 2020) and sage (Ommati et al., 2013) on rooster sperm quality have been documented in previous studies. Also, reduction of ROS' negative effects on sperm quality characteristics including mitochondrial activity, apoptosis status, and DNA fragmentation using antioxidant compounds such as ellagic acid (Najafi et al., 2019), vitamin E (Moghbeli et al., 2016), melatonin (Mehaisen et al., 2020), and L-carnitine (Fattah et al., 2017) during cryopreservation of rooster semen has been reported. In the present study, not only high mitochondrial activity was observed in roosters fed moderate levels of camphor, but also apoptosis was prevented. Mitochondria are not only the powerhouse of the cell, but it is also a key to apoptosis regulation (Castedo et al., 2002).

According to Surai et al. (2001), SOD, GP<sub>X</sub>, and metal-binding proteins are known as the first barrier to ROS formation. In contrast to our results, it has been reported that there was a positive relationship between SOD and GP<sub>X</sub> activities and lipid peroxidation (Partyka et al., 2012; Shanmugam and Rao, 2013). Cerolini et al. (2001) demonstrated that higher sperm viability was accompanied by higher SOD activity in boar semen after the freezing-thawing process, which partially confirms our finding. DPPH and FRAP assays are 2 complementary tests for the evaluation of antioxidant status in the fluids and tissues (Kumar et al., 2019). The DPPH assay is based on electron and H atom transfer, while the FRAP assay is based on an electron transfer reaction (Huang et al., 2005). With DPPH being a stable, the synthetic free radical remains intact in water, methanol, or ethanol (Aksoy et al., 2013) and the free radical scavenging activities of a solution depend on the capacity of antioxidant compounds to lose hydrogen and the structural conformation of these components. Thus, the DPPH free radical can easily receive an electron or hydrogen from antioxidant molecules to become a stable molecule (Soare et al., 1997). Lower DPPH scavenging and higher MDA content in sub-fertile men have been reported in human studies (Subramanian et al., 2018). It seems that antioxidant sources such as phytochemicals in the free radical scavenging process through reducing the rate of free radical chain initiation for example, either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and iron, may prevent oxidation of biological molecules (Kalia et al., 2018). High serum

FRAP values indicate that non-enzymatic antioxidants such as glutathione, uric acid, lipoic acid, ascorbic acid, and ubiquinol are high (Shanmugam et al., 2015), confirming the high antioxidant capacity of camphor-fed roosters in the present study.

Also, MDA content in testes and liver was evaluated to assess the degree of peroxidative damage and we found that the use of camphor in rooster diets reduced the accumulation of MDA in tissues. In agreement with the present study, Sedaghat et al. (2016) reported that Japanese quail fed camphor at 750 ppm had the lowest testes MDA concentration. The association between MDA concentration in tissues and the activity of cytotoxic, genotoxic, and oxidative stress-inducing products has been reported. Shata et al. (2014) reported a decrease in the serum MDA content in male rats receiving camphor, which is in concert with our study. MDA is one of the primary by-products of lipid peroxidation degradation induced by ROS (Ye et al., 2021). In another study, the use of vitamin E and selenium in the diet as an antioxidant source reduced the concentration of MDA in the testes and liver of roosters (Surai et al., 1998). Therefore, in the present study, camphor may improve the semen quality by increasing its antioxidant capacity and inhibiting ROS (Borghei-Rad et al., 2017).

Testes, as the main location of the male reproductive system, and its size are important parameters for assessing the reproductive status. Sperm production and testicular health depend on its size. According to previous reports, the right testis is about 10% smaller than the left side (Sun et al., 2019). The testicular asymmetry phenomenon (Abdul-Rahman et al., 2018) was also observed in our study. Similar to our findings, Talebi et al. (2018) reported that roosters with higher testes weight were better at sperm quality compared to other roosters. Also, quantification of the seminiferous epithelium, particularly its diameter and thickness is known as a direct method of evaluating the male reproductive system (Sun et al., 2019). For example, Sarabia Fragoso et al. (2013) reported that 44% of the testes' weight of heavy roosters was decreased with a significant reduction in the diameter of the seminiferous tubules between 36 and 55 wk.

In the present study, we found a good relationship between testicular histomorphometric and flow cytometric (as semen quality assessment parameters) evaluations which are in concert with Talebi et al. (2018). Several investigators reported that antioxidant compounds improved the testicular tissue structure in birds (Nasirikhah et al., 2019; Mohammadi et al., 2021). It is stated that the use of dietary guanidine acetic acid as an antioxidant source improved the histologic changes in rooster testes compared to the control roosters. These changes were included the increase in seminiferous tubules diameter, seminiferous epithelium thickness, spermatogonia count, and Leydig cells count (Nasirikhah et al., 2019). Mohammadi et al. (2021) demonstrated that dietary supplementation of L-carnitine improved the number of seminiferous, the number of Sertoli cells, and tubular differentiation index in young

rooster's testes. As a result of more oxidative stress due to increased ROS accumulation in testicular tissue, major changes in the seminiferous tubules structure occur along with a decrease in the number of spermatogonia cells (Sarabia Fragoso et al., 2013). Also, increased ROS production in the testicular tissue along with apoptosis of spermatogonia and Sertoli cells lead to a decrease in the semen quality of roosters (Nasirikhah et al., 2019). It is probable that camphor improves antioxidant capacity and provides an accessible cellular energy source, ameliorating Leydig, Sertoli, and spermatogonia cells degradation. Contrary to our results, the negative effect of camphor on the testicular structure of mice has been reported. Nikravesh and Jalali, (2004) demonstrated that the internal diameter of seminiferous tubules and the number of released sexual cells were decreased in mice receiving camphor. The effect of the highest level of camphor (C1000) on some of the studied parameters cannot be overlooked. Similar to our study, the use of a high concentration of antioxidant compounds such as ellagic acid (Najafi et al., 2019) and guanidine acetic acid (Nasirikhah et al., 2019) had a rebound effect on the semen quality and testes structure.

In general, the present experiment provides substantial evidence that, contrary to traditional beliefs, camphor can improve roosters' semen quality by increasing mitochondrial activity and reducing apoptotic changes, and also by the increase testicular weight to help improve the testes' histomorphometric properties. In summary, considering that reproductive function decreases in male broiler breeders by aging, adding compounds with antioxidant properties can maintain male reproductive health. The present study suggests that camphor as a potent antioxidant can be used in rooster's diet for improving the reproductive performance of roosters.

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## DISCLOSURES

The authors declare no conflicts of interest.

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