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Effect of cinnamon (*Cinnamomum zeylanicum*) bark oil on heat stress-induced changes in sperm production, testicular lipid peroxidation, testicular apoptosis, and androgenic receptor density in developing Japanese quails

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ABSTRACT

The aim of this study was to investigate the effect of cinnamon bark oil (CBO) on heat stress (HS)-induced changes in sperm production, testicular lipid peroxidation, testicular apoptosis, and androgenic receptor (AR) density in developing Japanese quails. Fifteen-day-old 90 male chicks were assigned to two main groups. The first group (45 chicks) was kept in a thermo-neutral room at 22 °C for 24 h/day. The second group (45 chicks) was kept in a room with high ambient temperature at 34 °C for 8 h/day (from 9 AM–5 PM) and at 22 °C for 16 h/day. Each of these two main groups was then divided into three subgroups (CBO groups 0, 250, 500 ppm) consisting of 15 chicks (six treatment groups in 2 × 3 factorial order). Each of subgroups was replicated for three times and each replicate included five chicks. Heat stress caused significant decreases in body weight, spermatid and testicular sperm numbers, the density of testicular Bcl-2 (antiapoptotic marker) and AR immunopositivity, and significant increases in testicular lipid peroxidation level, the density of testicular Bax (apoptotic marker) immunopositivity, and a Bax/Bcl-2 ratio along with some histopathologic damages. However, 250 and 500 ppm CBO supplementation provided significant improvements in HS-induced increased level of testicular lipid peroxidation, decreased number of spermatid and testicular sperm, decreased densities of Bcl-2 and AR immunopositivity, and some deteriorated testicular histopathologic lesions. In addition, although HS did not significantly affect the testicular glutathione level, addition of both 250 and 500 ppm CBO to diet of quails reared in both HS and thermoneutral conditions caused a significant increase when compared with quails without any consumption of CBO. In conclusion, HS-induced lipid peroxidation causes testicular damage in developing male Japanese quails and, consumption of CBO, which has antiperoxidative effect, protects their testes against HS.

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1. Introduction

The accepted thermoneutral (TN) ambient temperature ranges from 14 °C to 25 °C for poultry. The animals feel comfortable themselves in this temperature interval. When the ambient temperature exceeds the upper limit of TN zone values, heat stress (HS), which is called the deterioration of balance between the body temperature and heat thrown out from the animal's body, occurs [1]. Heat stress begins when the ambient temperature becomes greater than 27 °C and is readily apparent at greater than 30 °C [2]. Heat stress–related failures on productivity of quails range from reduced growth rate [3,4], reduced feed intake [3], reduced feed efficiency [5,6], reduced carcass weight [3,4,6], and reduced egg quality [5]. In addition, the detrimental effects of HS on reproductive features of male poultry have been reported to be decrease in testis weight [7], sperm count, and sperm motility and increase in dead sperm rate [7–9].

The physiological mechanisms that result in HS-induced reproductive failure in poultry are not completely understood, but most logically explained by increased intra-testicular temperature results from increased body temperature [7,10] and lipid peroxidation because of the accelerated metabolic rates under stress conditions [8,9]. The cell membrane of avian spermatozoa contains high amounts of polyunsaturated fatty acids (PUFAs). The high levels of PUFAs render avian spermatozoa vulnerable to lipid peroxidation, which is considered to be an important factor for male reproductive dysfunction, and therefore these cells require adequate antioxidant capacities. The precise balance between generation of reactive oxygen species (ROS) and antioxidant defense system, which is capable of protecting against free radicals and toxic products of their metabolism, is considered to be an important determinant for semen quality in avian species [11].

To overcome the HS-induced productive and reproductive failures in male and female poultry population, it has been reported that different approaches including environmental management (such as facilities design, ventilation, sprinkling, shading, and so forth), nutritional manipulation (i.e., diet formulation according to the metabolic condition of the birds), inclusion of feed additives in the diet (e.g., antioxidants, vitamins, minerals, probiotics, prebiotics, essential oils, and so forth), and water supplementation with electrolytes [12] should be used. Different antioxidants such as betaine, vitamin C, folic acid [2], selenium, and vitamin E [8,9] were used to prevent HS-induced reproductive dysfunctions in avian species. Cinnamon has been used as a spice and traditional herbal medicine for centuries. The most important volatile oils derived from cinnamon are *Cinnamomum zeylanicum* bark and leaf oils, *Cinnamomum cassia* (cassia oil), and *Cinnamomum camphora* [13]. The major compounds of cinnamon bark oil (CBO) have been reported to be cinnamaldehyde, benzyl alcohol, and eugenol. The composition of the essential oil of *C zeylanicum* is quite variable, depending on the locality of growth and different part of the plant. However, eugenol is the main component of the oil from leaf and cinnamaldehyde for the oil from bark [14]. It has been reported that CBO reduces lipid peroxidation in liver, heart, and kidney tissues of quails reared in HS [14], and dietary plant extract mixture composed of carvacrol, capsicum oleoresin,

and cinnamaldehyde affectively protects pig lymphocytes against oxidative DNA damage [15]. Besides, CBO consumption has been reported to provide marked increases in productive efficiency of quails reared in HS conditions [3] and reproductive functions in healthy [16] and intoxicated male rats [17] by decreasing the lipid peroxidation level and increasing the antioxidant activity. However, there is no evidence concerning the effect of CBO on HS-induced reproductive dysfunctions in male quails. Therefore, this study was conducted to investigate whether CBO has a protective effect on HS-induced negative changes in reproductive system of male quails by examining their spermatid and testicular sperm numbers, testicular lipid peroxidation level and antioxidant enzyme activity, testicular histologic structure, and the density of testicular apoptotic cells and androgenic receptors (ARs).

2. Materials and methods

2.1. Cinnamon bark oil and chemicals

Cinnamon bark oil was purchased from a local store (Agromiks Food Additive Co., İzmir, Turkey). According to the manufacturer's procedure, *C zeylanicum* barks were transported in polypropylene bags and were dried to constant weight in room temperature. Cinnamon bark oil was obtained by hydrodistillation method. The plant materials (about 100 g) were then ground into small pieces and were placed in a flask (2 L) together with double distilled water (1.5 L). The mixture was boiled for 4 hours. The extract was condensed in cooling vapor to collect the essential oil. The extracted oil was dried over anhydrous sodium sulfate. The CBO used in this study was previously analyzed by gas chromatography-mass spectrometry by our study group, and the components of CBO were reported in a study made by Şimşek et al. [14]. In that study, the components of CBO were reported to be cinnamaldehyde (88.2%), benzyl alcohol (8.1%), eugenol (1.0%), cinnamaldehyde propylene glycol acetal (0.5%), benzyl cinnamate (0.3%), benzaldehyde (0.3%), formic acid (0.2%), p-xylene (0.2%), α-terpinolene (0.1%), benzenepropanol (0.1%), α-terpineol (0.1%), and 1H-cycloprop[e] azulene (0.1%) [14]. Cinnamon bark oil was kept at 4 °C until being used. The other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Animals, experimental design, and diet regimen

The experimental protocol of this study was approved by the Animal Experimentations Local Ethics Committee of Fırat University (Elazığ, Turkey). Animal care and experimental protocols complied with the National Institutes of Health Guide for the Care and Use of Experimental Animals. Before starting work, approximately 200 male Japanese quail chicks (*Coturnix coturnix japonica*) of 5-day-old reared in a commercial company (Deva-Yum Co., Elazığ, Turkey) were weighed therein, and a total of 90 chicks with body weights being very close to each other were purchased. This selection resulted in 90 animals. These 90 animals were then brought to the Poultry Unit of Fırat University, and they were subjected to 10-days adaptation period. After adaptation period (on Day 15), animals were weighed

again and their initial body weights were recorded. The chicks were kept in wire cages in a temperature controlled rooms and assigned to two main groups. The first group (45 chicks) was kept in a TN room at 22 °C for 24 h/day. The second group (45 chicks) was kept in a room with high ambient temperature (HS) at 34 °C for 8 h/day (from 9 AM–5 PM) and at 22 °C for 16 h/day. Each of these two main groups was then divided into three subgroups (CBO groups 0, 250, 500 ppm) consisting of 15 chicks (six treatment groups in 2 × 3 factorial order). Each of subgroups was replicated for three times and each replicate included five chicks. At both temperatures, chicks were fed either a basal diet (0 ppm) or the basal diet supplemented with 250 or 500 ppm CBO until they were 43 days old (28 days experimental period). At the end of the study (on Day 43), all animals were weighed again to determine final body weight and body weight gain in each group. The CBO was mixed in a carrier (zeolite), which was then added to the basal diet at the rate of 1 kg per 1000 kg. Diet and fresh water were given *ad libitum*. Light was provided continuously (24 hours) throughout the experiment. Ingredients, chemical and fatty acid composition, of the basal diet are shown in Table 1. For the fatty acid analysis of basal diet, lipids were firstly extracted from feed by using the method described by Hara and Radin [18] and then fatty acids were analyzed in a gas chromatograph instrument (Shimadzu GC-17A) by using the method of Christie [19]. The basal diet contained 23.87% crude protein and 2897 kcal/kg of metabolizable energy.

2.3. Sample collection and homogenate preparation

At the end of the study, six individuals of 15 animals in each group were randomly selected and decapitated (a total of 36 quails) to avoid slaughtering the excessive number of animals in accordance with the proposals of our Local Ethics Committee. The selection method was based on the earlier study [14]. Testes were gently removed from the body and weighed. The absolute and relative testis weights (gonado-somatic-index [GSI] = absolute weight of testes/final body weight × 100) of 36 quails were recorded. Left testis samples were used to detect the spermatid and testicular sperm numbers. Right testis samples were gently divided into two equal pairs by scalpel, and one pair was fixed in Bouin's solution for histopathologic and immuno-histochemical examinations. The other pair of right testis samples was stored at −20 °C for the analyses of malon-dialdehyde (MDA), reduced glutathione (GSH) levels, and glutathione-peroxidase (GSH-Px) and catalase (CAT) activities. For these analyses, testes were taken from a −20 °C freezer and immediately transferred to the cold glass tubes. Then, the testes were diluted with a ninefold volume of PBS (pH 7.4) and minced in a glass and homogenized by a Teflon-glass homogenizer for 3 minutes in cold physiological saline on ice.

2.4. Determination of spermatid and testicular sperm numbers

The left testis of each male was used for the determination of spermatid and testicular sperm numbers. For

Table 1
Ingredients, chemical and fatty acid composition, of standard diet.

Feed ingredients	%	Nutritional composition	%
Maize	29.03	Dry matter	88.25
Wheat	25.00	Crude protein	23.87
Soybean meal (44 crude protein)	34.29	Crude fiber	2.55
Corn gluten	4.10	Ether extract	4.75
Vegetable oil	2.92	Ash	5.45
Di-calcium phosphate	2.02	Calcium[a]	1.00
Ground limestone	0.87	Available phosphorus[a]	0.79
NaHCO₃	0.12	Methionine[a]	0.40
Salt	0.28	Lysine[a]	1.18
DL-methionine	0.02	Metabolizable energy,[a] kcal/kg	2897
Vitamin mix[b]	0.25	Total saturated fatty acid (∑SFA)[c]	13.16
Mineral mix[d]	0.10	Total monounsaturated fatty acid (∑MUFA)[e]	22.00
Additive[f]	1.00	Total polyunsaturated fatty acid (∑PUFA)[g]	64.84

[a] Calculated.
[b] Vitamin premix supplied per 2.5 kg; vitamin A 12.000.000 IU; vitamin D3 2.000.000 IU; vitamin E 35.000 mg; vitamin K3 4.000 mg; vitamin B1 3.000 mg; vitamin B2 7.000 mg; Niacin 20.000 mg; calcium D-pantothenate 10.000 mg; vitamin B6 5.000 mg; vitamin B12 15 mg; folic acid 1.000 mg; D-biotin 45 mg; vitamin C 50.000 mg; choline chloride 125.000 mg; canthaxanthin 2.500 mg; apo carotenoic acid ester 500 mg.
[c] ∑SFA consist of palmitic (C16:0, 11.73%) and stearic (C18:0, 1.43%) acids.
[d] Mineral premix supplied per kilogram; Mn 80.000 mg; Fe 60.000 mg; Zn 60.000 mg; Cu 5.000 mg; Co 200 mg; I 1.000 mg; SE 150 mg.
[e] ∑MUFA consists of palmitoleic (C16:1 ω7, 0.57%), oleic (C18:1 ω9, 20.88%), and eicosanoic (C20:1 ω9, 0.55%) acids.
[f] Group cinnamon bark oil 0 (1000 g zeolite); group cinnamon bark oil 250 (25 g cinnamon bark oil + 975 g zeolite); group cinnamon bark oil 500 (50 g cinnamon bark oil + 950 g zeolite).
[g] ∑PUFA consists of linoleic (C18:2 ω6, 56.90%), linolenic (C18:3 ω3, 6.26%), eicosadienoic (C20:2 ω6, 0.22%), eicosapentaenoic (C20:5 ω3, 0.50%), docosadienoic (C22:2, 0.38%), and docosapentaenoic (C22:5, 0.58%) acids.

this purpose, *Tunica albuginea* was gently removed and the testis was minced and homogenized in 10 mL of a 0.9% NaCl solution containing 0.5% Triton X 100. Then 100 µL of this homogenate was diluted with the same solution once more at the rate of 1:9, one drop (approximately 10 µL) was taken and transferred to an Improved Neubauer counting chamber (deep 1/10 mm, field size of 0.0025 mm²; Labart, Munich, Germany) and allowed to stand for 5 minutes. The spermatid and sperm cells were visually counted with the help of a light microscope at ×200 magnification. Total spermatid and sperm numbers in the testis were calculated and expressed as millions per testis weight unit [20].

2.5. Determination of testicular lipid peroxidation and antioxidant markers

All analyses were performed with the aid of a spectrophotometer (2R/Ultraviolet-visible; Shimadzu, Tokyo, Japan). Lipid peroxidation level was measured according to the concentration of thiobarbituric acid reactive substances, and the amount of MDA produced was used as an index of

Table 2
Effects of CBO on body weight, testis weight, GSI, and spermatid and testicular sperm numbers in Japanese quails reared in TN environment and under HS.

Variables	HS CBO (ppm)			TN CBO (ppm)			Main effects of environmental conditions and feed additive on measured parameters (according to the GLM procedure)	
	0	250	500	0	250	500	HS (significance)	CBO (significance)
Initial body weight (g)	54.12 ± 0.23	54.62 ± 0.09	53.96 ± 0.17	54.73 ± 0.31	54.23 ± 0.26	55.04 ± 0.07	NS	NS
Final body weight (g)	174.00 ± 4.89	184.17 ± 5.19	182.00 ± 4.67	183.83 ± 4.71	195.83 ± 4.50	185.67 ± 3.25	*	NS
Body weight gain (g)	119.88 ± 3.12	129.55 ± 2.33	128.04 ± 3.21	129.10 ± 4.03	141.60 ± 5.86	130.63 ± 5.40	*	NS
Absolute testis weight [(g), right + left/2]	2.28 ± 0.32	2.84 ± 0.20	2.64 ± 0.10	2.93 ± 0.63	3.06 ± 0.26	2.98 ± 0.17	NS	NS
GSI	1.31 ± 0.17	1.55 ± 0.14	1.46 ± 0.07	1.58 ± 0.32	1.56 ± 0.12	1.61 ± 0.10	NS	NS
Spermatid number (millions/per testis weight unit)	132.17 ± 8.83[a]	197.17 ± 21.25[b]	208.83 ± 17.52[b]	249.00 ± 14.25	322.00 ± 48.78	307.33 ± 28.27	**	*
Sperm number (millions/per testis weight unit)	5.00 ± 0.68[A]	9.33 ± 1.17[B]	10.00 ± 0.86[B]	15.67 ± 1.86	18.17 ± 2.36	22.00 ± 2.92	**	**

*$P < 0.05$; **$P < 0.001$.
Data are expressed as the mean ± standard error of the mean.
The differences between the mean values with different superscripts ([a,b]$P < 0.05$, [A,B]$P < 0.01$) in the same row within the HS groups are statistically significant according to the ANOVA and *post hoc* Tukey honestly significant difference tests.
Abbreviations: CBO, cinnamon bark oil; GLM, general linear model; GSI, gonado-somatic-index; HS, heat stress; NS, nonsignificant ($P > 0.05$); TN, thermoneutral.

Table 3
Effects of CBO on testicular MDA, GSH levels, and GSH-Px and CAT activities in testicular tissue of Japanese quails reared in TN environment and under HS.

Variables	HS CBO (ppm)			TN CBO (ppm)			Main effects of environmental conditions and feed additive on measured parameters (according to the GLM procedure)	
	0	250	500	0	250	500	HS (significance)	CBO (significance)
MDA (nmol/g protein)	8.05 ± 0.26[a]	2.20 ± 0.08[b]	4.85 ± 0.13[c]	5.23 ± 0.25[A]	2.82 ± 0.09[B]	2.50 ± 0.21[B]	*	*
GSH (nmol/g protein)	3.13 ± 0.38[a]	10.03 ± 0.30[b]	10.90 ± 0.21[b]	2.52 ± 0.13[A]	9.24 ± 0.44[B]	11.29 ± 0.74[C]	NS	*
GSH-Px (IU/g protein)	1.39 ± 0.52	0.66 ± 0.19	2.54 ± 0.79	1.79 ± 0.65	1.02 ± 0.48	1.46 ± 0.30	NS	NS
CAT (k/g protein)	18.65 ± 3.72	37.82 ± 14.79	18.73 ± 3.82	23.46 ± 5.42	41.17 ± 17.14	73.88 ± 29.64	NS	NS

[a,b,c]The differences between the mean values with different superscripts ($P < 0.001$) in the same row within the HS groups are statistically significant according to the ANOVA and *post hoc* Tukey honestly significant difference tests.
[A,B,C]The differences between the mean values with different superscripts ($P < 0.001$) in the same row within the TN groups are statistically significant according to the ANOVA and *post hoc* Tukey honestly significant difference tests.
*$P < 0.001$.
Data are expressed as the mean ± standard error of the mean.
Abbreviations: CAT, catalase; CBO, cinnamon bark oil; GLM, general linear model; GSH, reduced glutathione; GSH-Px, glutathione-peroxidase; HS, heat stress; MDA, malondialdehyde; NS, nonsignificant ($P > 0.05$); TN, thermoneutral.
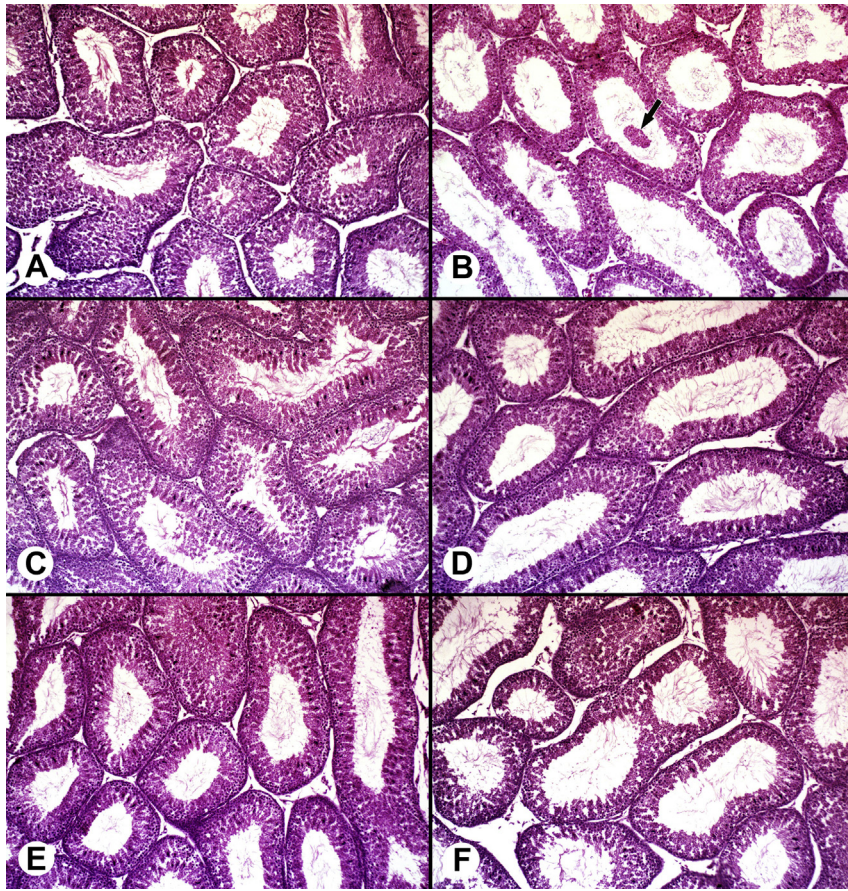
Fig. 1. Effects of cinnamon bark oil (CBO) on histopathologic structures of testes in Japanese quails reared in thermoneutral (TN) environment and under heat stress (HS) (hematoxylin and eosin-staining, ×40 magnification). Normal appearance of seminiferous tubules of testes in quails that consumed 0 (A), 250 (C), and 500 (E) ppm CBO in TN conditions. Dilatation in the diameters of seminiferous tubules, decrease in germinal cell layer thickness, and immature spermatocytes in lumen of tubules (arrow) of testes in quails that consumed 0 ppm CBO (B), and the decrease in lesions along with dilatation in quails that consumed 250 (D) and 500 (F) ppm CBO in HS conditions.

lipid peroxidation. The MDA level at 532 nm was expressed as nanomoles per gram of protein [21]. GSH level was measured using the method described by Sedlak and Lindsay [22]. The level of GSH at 412 nm was expressed as nanomoles per gram of protein. Glutathione-peroxidase (GSH-Px, EC 1.11.1.9) activity was determined according to the method described by Lawrence and Burk [23]. The GSH-Px activity at 340 nm was expressed as international units per gram of protein. Catalase (EC 1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm and was expressed as k per gram of protein, k is the first-order rate constant [24]. Protein concentration was determined using the method of Lowry et al. [25].

2.6. Histopathologic examination

Testis tissues were fixed in Bouin's solution for 48 hours; they were dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5-μm thicknesses, and stained with Mayer's hematoxylin and eosin. Twenty-five seminiferous tubules were randomly examined per section, and their diameters and germinal cell layer thicknesses (GCLT; from the basal membrane toward the lumen of the tubule) were measured using an ocular micrometer in a light microscope and, the diameter of seminiferous tubules (DST) and GCLT were calculated.

2.7. Determination of Bax (apoptotic marker), Bcl-2 (antiapoptotic marker), and AR immunopositivities by immunohistochemical examination

Immunohistochemical staining was performed with the avidin-biotin-peroxidase complex procedure, using commercially available immunoperoxidase kits (Ultravision Detection System, Antipolyvalent, *horseradish peroxidase/* diaminobenzidine; Thermo Scientific, Cat No. TP-015-HD). For immunoperoxidase staining, deparaffinized tissue sections were placed in citrate buffer solution (10 mM citric acid, pH 6.0) and were kept in a microwave for 20 minutes for antigen retrieval stage. The sections were incubated in 70% methanol with 3% H_2O_2 for 10 minutes to inhibit endogenous peroxidase activity and were then washed three times in PBS.

Table 4
Effects of CBO on some testicular histopathologic and immunohistochemical measurements in Japanese quails reared in TN environment and under HS.

Variables	HS CBO (ppm)			TN CBO (ppm)			Main effects of environmental conditions and feed additive on measured parameters (according to the GLM procedure)	
	0	250	500	0	250	500	HS (significance)	CBO (significance)
DST (μm)	246.90 ± 1.87	241.90 ± 1.50	231.90 ± 1.37	206.20 ± 1.96	208.40 ± 1.35	209.80 ± 1.60	*	NS
GCLT (μm)	47.70 ± 0.63[a]	52.10 ± 0.65[b]	53.20 ± 0.50[b]	58.50 ± 0.81[A]	61.80 ± 0.50[B]	64.70 ± 1.17[C]	*	*
AR positivity (0–4)	1.17 ± 0.06[a]	1.33 ± 0.06[ab]	1.50 ± 0.08[b]	1.67 ± 0.08[A]	2.00 ± 0.09[B]	1.67 ± 0.08[A]	*	*
Bax positivity (0–4)	1.67 ± 0.07	1.50 ± 0.08	1.33 ± 0.07	0.50 ± 0.08	0.50 ± 0.08	0.67 ± 0.07	*	NS
Bcl-2 positivity (0–4)	0.50 ± 0.08[a]	0.67 ± 0.07[ab]	0.83 ± 0.06[b]	1.00 ± 0.00[A]	1.33 ± 0.07[B]	1.33 ± 0.07[B]	*	*
Bax/Bcl-2 ratio	3.34 ± 0.04[a]	2.24 ± 0.10[b]	1.60 ± 0.09[b]	0.50 ± 0.03	0.38 ± 0.08	0.50 ± 0.06	*	*

[a,b] The differences between the mean values with different superscripts (P < 0.05) in the same row within the HS groups are statistically significant according to the ANOVA and *post hoc* Tukey honestly significant difference tests.

[A,B] The differences between the mean values with different superscripts (P < 0.05) in the same row within the TN groups are statistically significant according to the ANOVA and *post hoc* Tukey honestly significant difference tests.

*P < 0.001.

Data are expressed as the mean ± standard error of the mean.

Abbreviations: AR, androgenic receptor; CBO, cinnamon bark oil; DST, diameter of seminiferous tubules; GCLT, germinal cell layer thickness; GLM, general linear model; HS, heat stress; NS, nonsignificant (P > 0.05); TN, thermoneutral.

The sections were treated with blocking solution for 10 minutes. After draining the blocking serum, the sections were incubated with primary antibodies, the polyclonal rabbit anti-AR Cat No. sc-816 (Santa Cruz), the polyclonal rabbit anti-Bax Cat No. SAB4502549 (Sigma), and the polyclonal rabbit anti Bcl-2 Cat No. SAB4500005 (Sigma) diluted to 1:100, 1:200 and 1:400, respectively in PBS at 4 °C overnight in a humidified chamber. After three times washing in PBS, the sections were incubated with biotinylated anti-goat polyvalent secondary antibody for 10 minutes. Then the sections were washed three times in PBS and treated with the peroxidase-conjugated streptavidin. After another PBS bath, the sections were incubated with 3,3-diaminobenzidine. After the change in color, sections were washed with tap water and then counterstained with Mayer's hematoxylin. Stained tissues were covered with immune-mount and then 10 different fields were examined in each sample. The staining intensities of Bax, Bcl-2, and AR immunopositivities were evaluated under a light microscope and scored as a percentage given subsequently [26].

Score 0: negative stained cells.
Score 1: less than 25% positive stained cells.
Score 2: 26% to 50% positive stained cells.
Score 3: 51% to 75% positive stained cells.
Score 4: greater than 75% positive stained cells.

2.8. Statistical analysis

Data are presented as the mean ± standard error of the mean. The degree of significance was set at P < 0.05. General linear model was used to determine the main effect of high ambient temperature (HS) and feed additive (CBO) on all the parameters measured in this study. Mean differences were determined with one-way ANOVA and *post hoc* Tukey honestly significant difference test. All the analyses were carried out using the Statistical Package for the Social Sciences (SPSS) software program (Version 22.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Effects of HS and CBO on body and testis weights

Table 2 reports the changes in the weight of body and testis in response to HS and feed additive. Heat stress significantly reduced the final body weight and body weight gain in quails (P < 0.05). However, CBO addition at different doses had no significant effect on these parameters of quails reared in high temperature and TN conditions. Heat stress and CBO did not affect the testis weight and GSI.

3.2. Effects of HS and CBO on spermatid and testicular sperm numbers

The mean values of spermatid and testicular sperm numbers are presented in Table 2. Exposure to HS significantly reduced both spermatid (P < 0.001) and testicular sperm (P < 0.001) numbers. However, consumption of feed including 250 and 500 ppm CBO by animals under
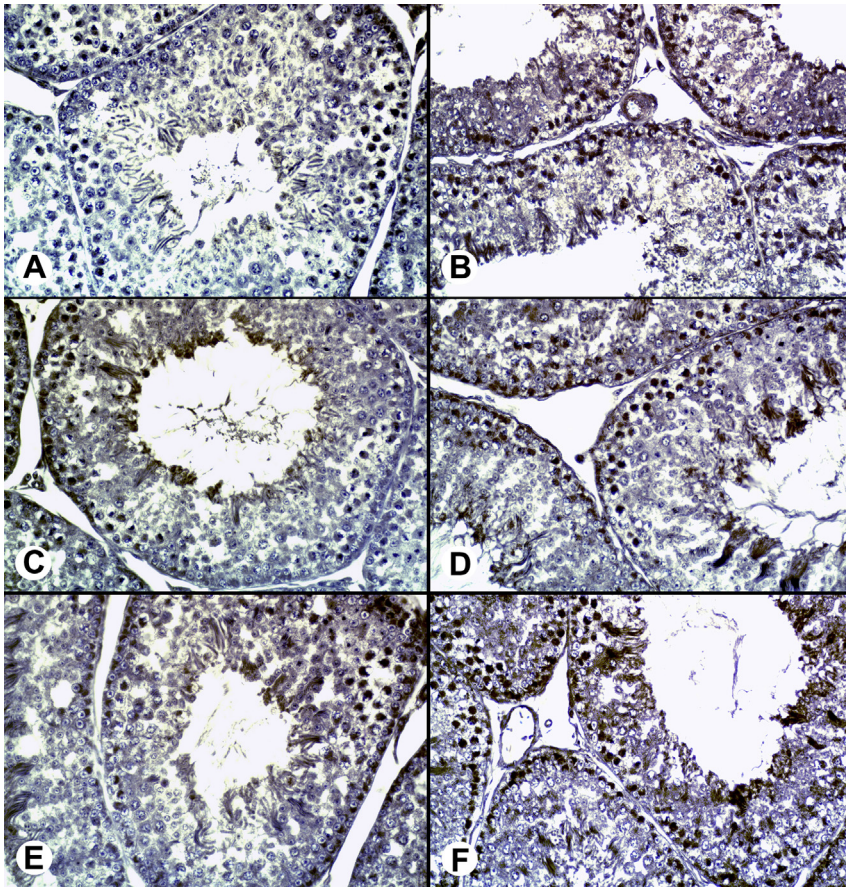
Fig. 2. Effects of cinnamon bark oil (CBO) on apoptotic cell density, demonstrated by Bax immunopositivity, in the testes of Japanese quails reared in thermoneutral (TN) environment and under heat stress (HS) (Mayer's hematoxylin, ×200 magnification). The little or no Bax immunopositive staining in the seminiferous tubules of testes in quails that consumed 0 (A), 250 (C), and 500 (E) ppm CBO in TN conditions. The intensive Bax immunopositive staining (brownish appearance) in the seminiferous tubules of testes in quails that consumed 0 ppm CBO (B), and the decrease in the intensity of staining in quails that consumed 250 (D) and 500 (F) ppm CBO in HS conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

HS partially prevented the HS-induced reductions in spermatid ($P < 0.05$) and testicular sperm ($P < 0.01$) numbers.

3.3. Effects of HS and CBO on testicular lipid peroxidation and antioxidant markers

The mean values of lipid peroxidation level (MDA) and antioxidant markers are given in Table 3. Heat stress significantly increased the MDA level of quails ($P < 0.001$) when compared with the quails reared in TN conditions. Consumption of 250 and 500 ppm CBO by quails exposed to HS provided a significant decrease in MDA level ($P < 0.001$) compared with quails with no additive. Although exposure to HS did not cause any significant effect on GSH level, CBO addition at the doses of 250 and 500 ppm to diet of quails under HS significantly increased the GSH level ($P < 0.001$). On the other hand, both doses of CBO provided a significant reduction in MDA level ($P < 0.001$) and a significant increment in GSH level ($P < 0.001$) of quails reared in TN conditions in comparison with the quails with no additive. However, no significant effect of CBO consumption was

observed on GSH-Px and CAT activities of animals reared in both HS and TN conditions.

3.4. Effects of HS and CBO on testicular structure

No histopathologic lesions were observed in testicular tissues of quails consumed 0 (Fig. 1A), 250 (Fig. 1C), and 500 (Fig. 1E) ppm CBO in TN condition. The histopathologic changes were detected most notably in the quails given no additive (Fig. 1B) and milder in quails that consumed both 250 (Fig. 1D) and 500 (Fig. 1F) ppm CBO under HS condition. The significant increase in DST ($P < 0.001$) because of the dilatation and the significant decrease in GCLT ($P < 0.001$; Table 4) along with disorganization and degeneration in germinal cells were the most obvious histopathologic changes in the quails given no additive under HS condition when compared with quails given no additive in TN condition. In addition, spermatogenic arrest and few spermatocytes were observed in some seminiferous tubules. Also poured immature spermatids and spermatogonia were observed in the lumen of some
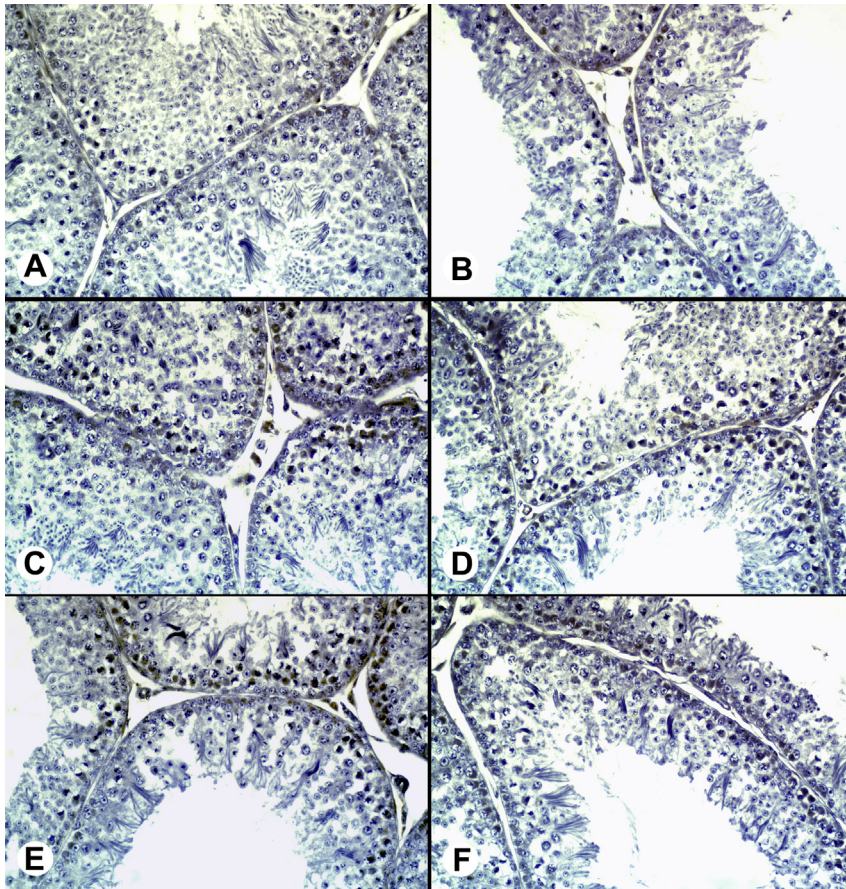
Fig. 3. Effects of cinnamon bark oil (CBO) on antiapoptotic cell density, demonstrated by Bcl-2 immunopositivity, in the testes of Japanese quails reared in thermoneutral (TN) environment and under heat stress (HS) (Mayer's hematoxylin, ×200 magnification). The intensive Bcl-2 immunopositive staining (brownish appearance) in the seminiferous tubules of testes in quails that consumed 0 (A), 250 (C), and 500 (E) ppm CBO in TN conditions. The little or no Bcl-2 immunopositive staining in the seminiferous tubules of testes in quails that consumed 0 ppm CBO (B), and the increase in the intensity of staining in quails that consumed 250 (D) and 500 (F) ppm CBO in HS conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

seminiferous tubules of quails given no additive under HS condition. However, the significant increase in GCLT (P < 0.05; Table 4) and the decrease in the severity of degenerations were observed in the quails that consumed both 250 and 500 ppm CBO in comparison with the quails given no additive under HS condition.

3.5. Effects of HS and CBO on Bax (apoptotic marker), Bcl-2 (antiapoptotic marker), and AR immunopositivities

Figure 2 illustrates apoptotic cell density, demonstrated by Bax immunostaining, in the testes of quails reared in both TN and HS conditions. The density of Bax immunostaining in germinal cell line was more severe in groups exposed to HS (Fig. 2B, D, and F) than groups reared in TN condition (Fig. 2A, C, and E). Bax immunopositivity was noticed most significantly in the group given no additive in HS condition (Fig. 2B), and as milder in groups that consumed both 250 (Fig. 2D) and 500 (Fig. 2F) ppm CBO under HS condition. Although HS caused a significant increase in the density of Bax immunopositivity (P < 0.001) compared with TN groups,

consumption of both 250 and 500 ppm CBO by quails exposed to HS did not provide significant decreases when compared with group given no additive under HS condition (Table 4).

Figure 3 illustrates antiapoptotic cell density, demonstrated by Bcl-2 immunostaining, in the testes of quails reared in both TN and HS conditions. Heat stress (Fig. 3B, D, and F) caused a significant decrease in the density of Bcl-2 immunopositivity (P < 0.001) compared with TN groups (Fig. 3A, C, and E, Table 4). Consumption of both 250 and 500 ppm CBO by quails exposed to HS provided a significant increase in the density of Bcl-2 immunopositivity (P < 0.05) when compared with group given no additive under HS condition. Similar increase was observed in quails that consumed both 250 (P < 0.05) and 500 (P < 0.05) ppm CBO compared with animals with no additive in TN condition (Table 4). With respect to a Bax/Bcl-2 ratio, when HS caused a significant increase (P < 0.001), however, consumption of 250 and 500 ppm CBO by quails exposed to HS significantly decreased (P < 0.05) the increment in this ratio (Table 4).

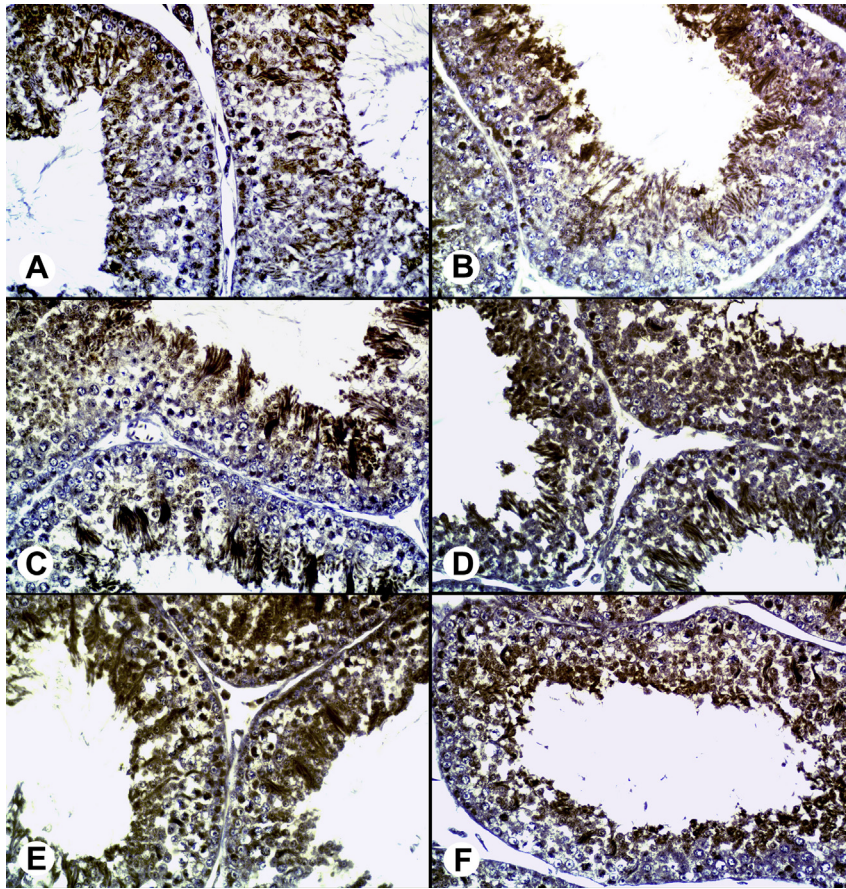The AR immunopositivity was generally determined in round and elongated spermatids rather than other

Fig. 4. Effects of cinnamon bark oil (CBO) on the density of androgenic receptor (AR) immunopositivity in the testes of Japanese quails reared in thermoneutral (TN) environment and under heat stress (HS) (Mayer's hematoxylin, ×200 magnification). The intensive AR immunopositive staining (brownish appearance) in the seminiferous tubules of testes in quails that consumed 0 (A), 250 (C), and 500 (E) ppm CBO in TN conditions. The lesser AR immunopositive staining in the seminiferous tubules of testes in quails that consumed 0 ppm CBO (B), and the increase in the intensity of staining in quails that consumed 250 (D) and 500 (F) ppm CBO in HS conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

spermatogenic cells including spermatogonia, primary, and secondary spermatocytes as well as Sertoli and Leydig cells in all group quails reared in both TN and HS conditions. No marked change was observed in the density of AR immunopositivity in quails that consumed 0 (Fig. 4A), 250 (Fig. 4C), and 500 (Fig. 4E) ppm CBO in TN condition. However, HS caused a significant decrease in the density of AR positive spermatogenic cells (from spermatogonia to elongated spermatids, $P < 0.001$) when compared with TN groups. The minimum and maximum densities of AR immunopositivity were observed in quails given no additive under HS condition (Fig. 4B) and in quails that consumed 250 ppm CBO in TN condition (Fig. 4C), respectively. The administration of both 250 (Fig. 4D) and 500 (Fig. 4F) ppm CBO to quails exposed to HS significantly increased the density of AR immunopositivity ($P < 0.05$) compared with the quails given no additive (Fig. 4B) under HS condition (Table 4).

4. Discussion

In this study, HS caused a significant reduction in the final body weight and body weight gain as confirmed by previous studies [3–6]. The possible reason for this reduction is that high ambient temperature decreases the feed intake and feed efficiency [3,4] of quails because of the increased body temperature [7]. However, no significant effect of HS was observed in the testis weight and values of GSI in this study. This finding is not confirmed by the results of McDaniel et al. [7], who found an increase in testis weight in roosters exposed to HS. The use of different species of animals may be the possible reason for this contradiction in the studies.

Heat stress causes increased lipid peroxidation [5] and changes in the concentrations of antioxidant enzymes (superoxide dismutase, GSH-Px, and CAT) [27], vitamins (A, C, and E), and minerals (selenium and zinc) in poultry [28]. Antioxidant enzymes, vitamins, and minerals protect the integrity of unsaturated bonds of membrane phospholipids by extinguishing free radical attacks capable of initiating and propagating lipid oxidation [29]. Although HS significantly increased the testicular MDA level, it did not affect the GSH level and GSH-Px and CAT activities of testicular tissues of quails when compared with the quails reared in TN conditions in this study. In terms of increased

MDA, our findings are confirmed by the results of previous studies [5,28]. However, the findings found in this study with respect to antioxidant markers are not in agreement with the results of Altan et al. [27] who revealed that exposing broiler chickens to HS resulted in a significant increase in blood superoxide dismutase and GSH-Px and CAT activities because increased antioxidant enzyme activities have been considered as a protective response against HS-induced lipid peroxidation. The possible reasons for this are that different species of animals and different samples were used in the studies. The HS-induced oxidative damage in testes may be depending on the increased free radicals in testicular tissues of quails in the present study. In addition, excessive generation of lipid peroxides and ROS by poured immature spermatids and spermatogonia [30], as evidenced in some seminiferous tubules of testes of quails reared in HS condition in this study, may be responsible for the increased lipid peroxidation.

Avian spermatozoa are characterized by high proportions of PUFAs, which are associated with increased susceptibility to ROS and lipid peroxidation. The balance between ROS and antioxidants in avian semen is a fundamental determinant for membrane integrity, sperm viability, and fertilizing ability. Although ROS are involved in many physiological functions of spermatozoa, their excessive production may result in oxidative stress leading to damaged sperm functions and production. [31]. The detrimental effects of HS on sperm parameters in poultry have been reported to be decreases in sperm count, sperm motility, and increases in dead sperm rate [7–9]. In the present study, exposure to HS significantly reduced both spermatid and testicular sperm numbers, which are in agreement with the previous reports [7–9]. Increased lipid peroxidation, as evidenced by increased MDA level, may be responsible for the decreased spermatid and testicular sperm numbers observed in this study.

Although numerous studies have been conducted to determine the effect of HS on productivity and sperm quality, there are a limited number of studies concerning the effect of HS on testis structure and apoptotic changes in poultry species. Besides, there is no evidence related to the effect of HS on distribution and density of testicular AR in quails. Therefore, the findings obtained in this study are the first results regarding the effect of HS on testicular AR density in quails. Heat stress has been reported to cause the loss of spermatogenic cells and degenerative alterations in testis of broilers [32], the vacuolization of germinal epithelium, multinucleated giant cell formations in mice [33], the degeneration in seminiferous tubules, and the spermatogenic arrest in developing lambs [34] and adult llamas [35]. In this study, HS caused severe histopathologic lesions in the testes of quails such as increase in DST, decrease in GCLT along with disorganization and degeneration in germinal cells, spermatogenic arrest, poured immature spermatids, and spermatogonia in the lumen of some seminiferous tubules. The Bax (apoptotic) and Bcl-2 (antiapoptotic) proteins exist in the culmination of apoptosis after the onset of cellular stress. The ratio of these molecules has been implicated to be a critical determinant of cell fate, such that increased Bcl-2 favors extended

survival of cells and increasing levels of Bax expression accelerate cell death [36]. Apoptosis is also an indicator of DNA damage in the cells including testicular germ cells, and an increase in free radicals results in increased testicular apoptotic germ cell [37]. In this study, HS caused significant increases in the density of testicular Bax immunopositivity and Bax/Bcl-2 ratio and significant decreases in the density of testicular Bcl-2 immunopositivity of quails compared with TN conditions. It has been reported that HS-induced overproduction of free radicals causes sperm DNA damage and testicular apoptosis [38], which is in agreement with our findings. Androgens and the AR play important roles in male spermatogenesis and fertility. The actions of androgens are mediated by the AR [39]. Androgenic receptor has been reported to decrease dramatically in monkey Sertoli cells in vitro after HS [40]. Similarly, a significant decrease was observed in the density of AR immunopositivity in testicular tissues of quails exposed to HS in the present study. Increased lipid peroxidation induced by HS may possibly cause the testicular histopathologic damages and increase in the density of testicular Bax immunopositivity, as well as decrease in the densities of testicular Bcl-2 and AR immunopositivities. Peritubular myoid cells, surrounding the seminiferous tubules [41], are smooth muscle cells [42]. It has been reported that cyclic guanosine monophosphate-mediated phosphorylation of heat shock proteins [43], of which levels may increase or decrease under HS-induced oxidative stress [29,44], may cause smooth muscle relaxation [43]. One possible reason for the increased DST observed in this study after HS may be the relaxation of peritubular myoid cells.

Cinnamon primarily contains volatile oils and other derivatives, such as cinnamaldehyde, cinnamic acid, and cinnamate. The most important volatile oils derived from cinnamon are C zeylanicum bark and leaf oils, C cassia (cassia oil), and C camphora [13]. The major component of CBO used in this study was cinnamaldehyde (88.2%) [14]. It has been reported that ingestion of plant extract including cinnamaldehyde affectively protects pig lymphocytes against oxidative DNA damage [15]. Besides, dietary cinnamate has been reported to suppress lipid peroxidation via the enhancement of hepatic antioxidant enzyme activities [45]. The antioxidant and free radical scavenging activity of bark oil extracted from C zeylanicum have been reported in different experimental studies [16,17,46]. In addition, C zeylanicum consumption has been reported to improve significantly the sperm quality, reproductive organ weights [16], LH, FSH, and testosterone concentrations [47] in healthy rats and mice, and also improve sperm and reproductive organ damages in diabetic [48] and CCl4-treated [17] rats. Although CBO consumption has been reported to provide marked increase in productive efficiency [3] and to decrease significantly lipid peroxidation levels in liver, heart, and kidney tissues [14] of quails reared under HS, there has been no evidence about the protective effect of CBO on HS-induced damages in sperm production, testicular oxidant-antioxidant balance, testicular histopathologic structure, and the densities of testicular Bax, Bcl-2, and AR immunopositivities in avian species so far. Therefore, this is the first report regarding the protectiveness of

CBO on HS-induced testicular damage in Japanese quails. In this study, CBO supplementation to diet of quails significantly decreased the HS-induced increments in testicular lipid peroxidation levels and testicular histopathologic lesions and significantly increased the HS-induced reductions in spermatid and testicular sperm numbers, GCLT, antiapoptotic Bcl-2 immunopositivity, and AR immunopositivity. In addition, CBO consumption by quails reared in TN conditions provided significant increases in testicular GSH level, testicular GCLT, Bcl-2, and AR immunopositivities when compared with quails reared in TN conditions but without consuming CBO. The possible reason for the aforementioned improvements observed in the testes of quails reared in both HS and TN conditions after CBO consumption is that CBO, in particular its potential active molecule cinnamaldehyde, has potent antioxidant and radical scavenging activity.

4.1. Conclusions

The findings of the present study clearly suggest that CBO has protective effect on HS-induced testicular damage in developing Japanese quails. This protective effect of CBO seems to be closely involved with the scavenging free radicals and suppressing lipid peroxidation.

Competing Interests

The authors declare that there are no conflicts of interest.

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