

Dietary rosemary oil alleviates heat stress-induced structural and functional damage through lipid peroxidation in the testes of growing Japanese quail



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ABSTRACT

Supplementation of natural antioxidants to diets of male poultry has been reported to be effective in reducing or completely eliminating heat stress (HS)-induced reproductive failures. In this study, the aim is to investigate whether rosemary oil (RO) has a protective effect on HS-induced damage in spermatozoa production, testicular histologic structures, apoptosis, and androgenic receptor (AR) through lipid peroxidation mechanisms in growing Japanese quail. Male chicks ($n = 90$) at 15-days of age were assigned to two groups. The first group ($n = 45$) was kept in a thermo-neutral (TN) room at 22 °C for 24 h/d. The second group ($n = 45$) was kept in a room with a greater ambient temperature of 34 °C for 8 h/d (from 9:00 AM to 5:00 PM) and 22 °C for 16 h/d. Animals in each of these two groups were randomly assigned to three subgroups (RO groups: 0, 125, 250 ppm), consisting of 15 chicks (six treatment groups in 2×3 factorial design). Each of subgroups was replicated three times with each replicate including five chicks. The HS treatment significantly reduced the testicular spermatogenic cell counts, amount of testicular Bcl-2 (anti-apoptotic marker) and amount of AR. In addition, it significantly increased testicular lipid peroxidation, Bax (apoptotic marker) immunopositive staining, and the Bax/Bcl-2 ratio in conjunction with some histopathologic damage. Dietary supplementation of RO to diets of quail where the HS treatment was imposed alleviated HS-induced almost all negative changes such as increased testicular lipid peroxidation, decreased numbers of spermatogenic cells, and decreased amounts of Bcl-2 and AR, increased ratio of Bax/Bcl-2 and some testicular histopathologic lesion. In conclusion, dietary supplementation of RO for growing male Japanese quail reared in HS environmental conditions alleviates the HS-induced structural and functional damage by providing a decrease in lipid peroxidation.

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1. Introduction

Environmental factors often affect physiological functions of each organ in the body in almost all animal species. However, avian species seem to be particularly sensitive to environmental challenges, especially heat stress (HS) because of the increased production of body heat by modern poultry genotypes due to the greater metabolic activity associated with this increased productivity (Deeb and Cahaner, 2002). Exposure to HS has a deleterious effect on fertility and is considered to be one of the significant risk factors causing infertility in males. In most homeothermic birds and mammals, including humans, testicular function is influenced by ambient temperature. Temperatures outside of the animal's thermo-neutral (TN) zone are often not optimal for testicular function and can potentially disrupt spermatogenesis (Durairajanayagam et al., 2014). Reductions have been reported for testis weight (McDaniel et al., 2004), semen volume, numbers of normal-shaped spermatozoa (Joshi et al., 1980), spermatozoa count, spermatozoa motility, and there is an increase in number of dead spermatozoa (McDaniel et al., 2004; Ebeid, 2012) as well as testicular degenerative disorders (Terim Kapakın et al., 2013; Türk et al., 2015) in different poultry species in response to HS.

The increased intra-testicular temperature resulting from increased body temperature (McDaniel et al., 2004), and in particular greater production of reactive oxygen species (ROS) leading to lipid peroxidation due to the increased metabolic activity under stress conditions (Ebeid, 2012), have been considered to be responsible mechanisms for the HS-induced reproductive failures in poultry. The plasma membrane of avian spermatozoon contains abundant polyunsaturated fatty acids (PUFAs). While PUFAs provide the fluidity that is necessary for flagellar movement and fusion-related events, it also makes the spermatozoa susceptible to lipid peroxidation. Spermatozoa need adequate antioxidant capacities because lipid peroxidation can lead male reproductive dysfunction. Therefore, avian testes need a precise oxidant/antioxidant balance for regular spermatozoa production and subsequently semen with high quality (Surai et al., 2001).

Supplementation of natural antioxidants to diets of male poultry is effective in reducing or completely eliminating HS-induced reproductive failures (Lara and Rostagno, 2013). Various antioxidants such as cinnamon bark oil (Türk et al., 2015), betaine, vitamin C, folic acid (Ezzat et al., 2011), selenium, and vitamin E (Ebeid, 2012) have been added to the diets to prevent HS-related reproductive disturbances in avian species. Rosemary (*Rosmarinus officinalis*) is a common household plant, which belongs to the family of Lamiaceae that is grown in many parts of the world. Rosemary contains active anti-oxidative substances such as phenolic diterpenes, flavonoids, phenolic acids (Ho et al., 2000), and volatile oils (Begum et al., 2013). The volatile oils consist of borneol, bornyl acetate, camphene, cineol, pinene, and camphor (Begum et al., 2013). Rosemary oil (RO) has various biological properties including great antioxidant and free radical scavenging activities (Adorjan and Buchbauer, 2010). However,

there is inconsistent information regarding the effects of rosemary on the male reproductive system, because there are studies indicating that rosemary and its different extracts have been harmful (Nusier et al., 2007; El-Din et al., 2012; Heidari-Vala et al., 2013) while other studies have found there were beneficial (Luno et al., 2014; Motlagh et al., 2014; Uyeturk et al., 2014) effects on structure and functions of spermatozoa. One study (Superchi et al., 2005) was conducted that relates to the protection of rosemary extract against spermatozoa damage caused by HS in boars, but to the best of our knowledge there is no previous research regarding the effect of RO on HS-induced reproductive disturbance in male quail. The present study was, therefore, conducted to investigate whether RO has a preventive or aggravating effect on HS-induced disturbance impacting reproductive efficiency of male quail by examining the changes in spermatogenic cell counts, testicular oxidant-antioxidant markers, testicular histologic structures, and quantity of testicular apoptotic cells and androgenic receptors (AR).

2. Materials and methods

2.1. Rosemary oil and chemicals

Rosemary oil was purchased from a local store (Agromiks Food Additive Co., Izmir, Turkey). The compounds and percentages of volatile components within the RO have been reported to be 1,8 cineole (39.31%), camphor (14.69%), α -pinene (13.85%), β -pinene (9.87%), camphene (6.17%), limonene (3.17%), p-cymene (2.58%), borneol (2.33%), α -terpineol (2.28%), myrcene (2.02%), bornyl acetate (1.46%), and others (2.27%) by manufacturer. RO was kept at 4 °C until being used. The other chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

2.2. Experimental protocol and dietary regimen

The Animal Experimentations Local Ethics Committee of Firat University (Elazığ, Turkey) approved the experimental protocol of the present study. A total of 90 male Japanese quail chicks (*Coturnix coturnix japonica*) at 5-days of age were purchased from a commercial company (Deva-Yum Co., Elazığ, Turkey). After a 10-day adaptation period to experimental conditions of the Poultry Unit of Firat University, chicks were placed in wire cages in temperature-controlled rooms, and the study was initiated. The chicks were randomly divided into two groups. The first group ($n = 45$) were housed in a TN condition at 22 °C for 24 h/d. The second group ($n = 45$) were placed in a room with high ambient temperature (HS) at 34 °C for 8 h/d (from 9:00 AM to 5:00 PM) and at 22 °C for 16 h/d. The chicks in the two groups were then randomly assigned to three subgroups (RO groups: 0, 125, 250 ppm) consisting of 15 chicks (six treatment groups in 2×3 factorial design). The study with each of the subgroups was replicated three times with each replicate being with five chicks. The relative humidity of both TN and HS rooms was 60–65%. At both temperatures, chicks were fed either a basal diet (0 ppm) or the

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

Feed ingredients	% Fed	Nutritional composition	%
Maize	29.03	Dry matter	88.25
Wheat	25.00	Crude protein	23.87
Soybean meal (44 CP)	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
NaHCO ₃	0.12	Methionine ^a	0.40
Salt	0.28	Lysine ^a	1.18
DL-Methionine	0.02	ME, kcal/kg ^a	2897
Vitamin mix ^b	0.25	Total saturated fatty acid ($\sum$ SFA) ^c	13.16
Mineral mix ^d	0.10	Total monounsaturated fatty acid ($\sum$ MUFA) ^e	22.00
Additive ^f	1.00	Total polyunsaturated fatty acid ($\sum$ 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-pantotenat 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 $\sum$ SFA consist of palmitic (C16:0, 11.73%) and stearic (C18:0, 1.43%) acids.

^d Mineral premix supplied per kg; 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 $\sum$ MUFA consist of palmitoleic (C16:1 ω 7, 0.57%), oleic (C18:1 ω 9, 20.88%) and eicosanoic (C20:1 ω 9, 0.55%) acids.

^f Group rosemary oil 0 (1000 g zeolite); Group rosemary oil 125 (12.5 g rosemary oil + 987.5 g zeolite); Group rosemary oil 250 (25 g rosemary oil + 975 g zeolite).

^g $\sum$ PUFA consist of linoleic (C18:2 ω 6, 56.90%), linolenic (C18:3 ω 3, 6.26%), eicosadienoic (C20:2 ω 6, 0.22%), eicosapentaenoic (C20:5 ω 3, 0.50%), docosadienoic (C22:2, 0.38%) and docosapentaenoic (C22:5, 0.58%) acids.

basal diet supplemented with 125 or 250 ppm RO until they were 43-days of age (28 days experimental period). The RO was mixed in a carrier (zeolite), which was added to the basal diet at the rate of 1 kg per 1000 kg. Diet and fresh water were provided *ad libitum*. Light was provided continuously (24 h) throughout the experiment. Ingredients, chemical and fatty acid compositions of the basal diet are provided in Table 1.

2.3. Collection of samples

In accordance with the proposals of the Local Ethics Committee, six individuals of the 15 total animals in each group were randomly selected and decapitated (a total of 36 quail) at the end of the study (on day 43) to avoid slaughtering the excessive number of animals. Testes were gently removed from the body and weighed. Mean absolute testis weights of quail in each group were recorded. In addition, relative testis weights [gonado-somatic-index (GSI) = absolute weight of testes/final body weight $\times$ 100]. Left testis samples of each animal were used for counting of testicular spermatogenic cells including spermatogonia, spermatocytes, spermatids, and spermatozoa. Right testis samples were gently divided to two equal parts by scalpel and, one piece of tissue was fixed in Bouin's solution for histopathologic and immunohistochemical examinations. The other piece of tissue was stored at -20°C for biochemical analyses. For the biochemical analyses, testes were taken from a -20°C freezer and immediately transferred to the cold glass tubes. The testes were diluted with a nine-fold volume of PBS (pH 7.4) and minced in a glass and homogenized by a Teflon-glass homogenizer for 3 min in cold physiological saline on ice.

2.4. Measurement of testicular lipid peroxidation concentration and antioxidant activity

All analyses were performed with the aid of a spectrophotometer (2R/Ultraviolet-Visible; Shimadzu, Tokyo, Japan) by using the methods reported in the study of Türk et al. (2015). Amount of lipid peroxidation was measured according to the concentration of thiobarbituric acid reactive substances and the amount of malondialdehyde (MDA) produced was used as an index of lipid peroxidation. The MDA concentration at 532 nm was expressed as nmol/g protein. The reduced glutathione (rGSH) concentration at 412 nm was expressed as nmol/g protein. Glutathione-peroxidase (GSH-Px, EC 1.11.1.9) activity at 340 nm was expressed as IU/g protein. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide (H₂O₂) at 240 nm and was expressed as k/g protein, where *k* is the first-order rate constant. Protein concentration was also determined.

2.5. Counting of testicular spermatogenic cells

The Tunica albuginea of the left testis samples 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. A 100 μl sample of this homogenate was again diluted (1:9) with the same solution; one drop (approx. 10 μl) was taken and transferred to a counting chamber of Improved Neubauer instrument (deep 1/10 mm, field size of 0.0025 mm²; LABART, Munich, Germany), where it remained for 5 min. The spermatogonia, spermatocytes, spermatids, and spermatozoa were visually counted using a light microscope at $\times 200$ magnification. Total numbers of

Table 2

Effects of rosemary oil (RO) on testis weight, GSI, testicular spermatogenic cell counts in Japanese quail reared in a thermo-neutral (TN) environment and under heat stress (HS).

Variables	HS			TN				Main effects of environmental conditions and feed additive on measured variables (According to the General Linear Model procedure)	
	RO (ppm)			RO (ppm)					
	0	125	250	0	125	250	s.e.	HS significance(<i>P</i> -value)	RO significance(<i>P</i> -value)
Absolute testis weight [(g), right + left/2]	2.34	3.01	3.01	2.42	2.85	3.24	0.12	0.833	0.061
Gonado–Somatic-Index (GSI)	1.32	1.61	1.67	1.35	1.62	1.72	0.06	0.985	0.068
Spermatogonium count(million/per testis)	13.83 ^b	19.66 ^a	20.40 ^a	28.66	35.50	37.50	1.75	0.0001 ^{***}	0.022 [*]
Spermatocyte count(million/per testis)	57.66 ^b	87.00 ^a	97.80 ^a	111.66	110.83	122.50	5.97	0.005 ^{**}	0.046 [*]
Spermatid count (million/per testis)	107.16 ^b	242.20 ^a	258.50 ^a	274.00	317.66	345.50	16.89	0.0001 ^{***}	0.001 ^{**}
Sperm count(million/per testis)	11.50 ^b	20.40 ^a	22.00 ^a	22.50	23.33	26.00	0.85	0.0001 ^{***}	0.0001 ^{***}

Data are expressed as mean and pooled standard errors (s.e.)

^{a,b} Mean values having different superscripts in the same row within the HS groups are different from each other.

^{*} $P < 0.05$.

^{**} $P < 0.01$.

^{***} $P < 0.001$.

spermatogonia, spermatocytes, spermatids, and spermatozoa were calculated and expressed as million per testis (Grote et al., 2008).

2.6. Histopathologic evaluation

Testis tissues were fixed in Bouin's solution for 48 h and were dehydrated transferring through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5 μ m thicknesses and stained with Mayer's hematoxylin and eosin. Seminiferous tubules (ST, $n = 25$) were randomly examined per section and, the diameters and germinal cell layer thickness (GCLT; from the basal membrane towards the lumen of the tubule) were measured using an ocular micrometer in a light microscope, and the mean size of ST and GCLT were calculated.

2.7. Immunohistochemical evaluation

The avidin–biotin–peroxidase complex procedure was used for immunohistochemical staining of testis tissues. For this procedure, the commercial immunoperoxidase kits (Ultravision Detection System, Antipolyvalent, HRP/DAB, Thermo Scientific, Cat No:TP-015-HD) were used and all procedures were done according to the manufacturer's instructions. The staining intensities of Bax, Bcl-2, and AR for immunopositive proteins were evaluated under a light microscope and recorded as a percentage based on previously described procedures (Kandi Coşkun and Çobanoğlu, 2005). Score 0: Negative stained cells; Score 1: <25% positive stained cells; Score 2: 26–50% positive stained cells;

Score 3: 51–75% positive stained cells; and Score 4: >75% positive stained cells.

2.8. Data analysis

The General Linear Model (GLM) was used to determine the main effect of HS and feed supplementation (RO) on all the variables measured in the present study. Mean differences were determined with the one-way analysis of variance (ANOVA) and *post hoc* Tukey-HSD test. Data are presented as mean and pooled standard error (s.e.). The value of $P < 0.05$ was considered as significant. All the analyses were conducted using the SPSS software program (Version 22.0; Chicago, IL, USA).

3. Results

3.1. Changes in testis weight and GSI

Changes in the values of testis weight and GSI are demonstrated in Table 2. Although the imposition of HS resulted in a numerical decrease and supplementation of diets with RO resulted in a numerical increase in the values of absolute testis weight and GSI, any changes for these variables were not statistically significant.

3.2. Changes in testicular lipid peroxidation concentration and antioxidant markers

The mean values of MDA, by-product of lipid peroxidation, and antioxidant markers are presented in Table 3.

Table 3

Effects of rosemary oil (RO) on testicular malondialdehyde (MDA), reduced glutathione (rGSH) concentrations and glutathione-peroxidase (GSH-Px) and catalase (CAT) activities in testicular tissue of Japanese quail reared in a thermo-neutral (TN) environment and under heat stress (HS).

Variables	HS			TN				Main effects of environmental conditions and feed additive on measured variables (According to the General Linear Model procedure)	
	RO (ppm)			RO (ppm)					
	0	125	250	0	125	250	s.e.	HS significance(<i>P</i> -value)	RO significance(<i>P</i> -value)
MDA (nmol/g protein)	7.71 ^a	4.90 ^b	4.04 ^b	4.00 ^A	3.98 ^A	2.80 ^B	0.31	0.001 ^{**}	0.001 ^{**}
rGSH (nmol/g protein)	2.51 ^b	9.86 ^a	10.79 ^a	3.13 ^B	9.98 ^A	10.92 ^A	0.66	0.679	0.001 ^{**}
GSH-Px (IU/g protein)	1.32	1.38	2.46	1.78	1.79	3.59	0.25	0.356	0.192
CAT (k/g protein)	18.64 ^b	52.34 ^a	64.79 ^a	23.46	42.20	44.79	4.56	0.459	0.041 [*]

Data are expressed as mean and pooled standard errors (s.e.)

^{a, b} Mean values having different superscripts in the same row within the HS groups are different from each other.

^{A, B} Mean values having different superscripts in the same row within the TN groups are different from each other.

^{*} $P < 0.05$.

^{**} $P < 0.01$.

Although the imposition of HS resulted in an increase ($P < 0.01$) in the MDA concentration in comparison with the quail reared in TN conditions, it did not affect the rGSH, GSH-Px, and CAT antioxidant markers. However, dietary supplementation of 125 and 250 ppm RO decreased ($P < 0.01$) the HS-induced increment in MDA concentration compared with the quail that had no RO dietary supplementation. Although the imposition of HS had no significant effects on the rGSH concentration, GSH-Px activity and CAT activity, RO supplementation in amounts of 125 and 250 ppm to the feed under HS conditions increased ($P < 0.01$) the rGSH concentration and increased ($P < 0.05$) CAT activity. For birds with the TN treatment, dietary supplementation of 250 ppm RO resulted in a decrease ($P < 0.01$) in MDA concentration and both amounts (125 and 250 ppm) of dietary supplementations increased ($P < 0.01$) the rGSH concentrations.

3.3. Changes in testicular spermatogenic cell count

The mean values of spermatogenic cell counts in the left testis samples are given in Table 2. Significant reductions were determined in the counts of all the spermatogenic cells including spermatogonia ($P < 0.001$), spermatocytes ($P < 0.01$), spermatids ($P < 0.001$), and spermatozoa ($P < 0.001$) of quail where the HS treatment was imposed. However, supplementation of both 125 and 250 ppm RO to diets of animals, in which HS was imposed, prevented the HS-induced reductions in spermatogonium ($P < 0.05$), spermatocyte ($P < 0.05$), spermatid ($P < 0.01$), and spermatozoon ($P < 0.001$) counts.

3.4. Changes in testicular histologic structure

When assessed microscopically, the testes of quail had normal histology with 0 (Fig. 1A), 125 (Fig. 1C) and 250 (Fig. 1E) ppm of RO dietary supplementation for animals housed in the TN environmental conditions. The histopathologic changes were mostly observed in the quail with no RO dietary supplementation (Fig. 1B) and histopathologic disorders were less in quail that had dietary supplementations of 125 (Fig. 1D) and 250 (Fig. 1F)

ppm RO under the HS environmental conditions. Disorganization and degeneration of germinal cells (Fig. 1B) together with dilatation of seminiferous tubules ($P < 0.001$) and reductions in GCLT ($P < 0.001$) were the most marked changes observed in the testes of quail without RO dietary supplementation and reared under the HS environmental conditions (Table 4). In addition to the histopathologic lesions, there were more immature spermatids and spermatogonia within the tubular lumen along with indications of spermatogenic cessation and fewer spermatocytes were observed in some seminiferous tubules of the quail with no RO dietary supplementation when the birds were housed under the HS environmental condition. Increases were observed in the GCLT and there was a decrease in the diameters of seminiferous tubules (DST; $P < 0.001$, Table 4) and degree of degenerations in testicular tissues of quail with both 125 (Fig. 1D) and 250 (Fig. 1F) ppm RO dietary supplementation in comparison with the quail with no RO dietary supplementation that were housed under the HS environmental condition. Supplementation of 125 and 250 ppm RO to quail diets reared in the TN environmental condition increased ($P < 0.001$, Table 4) the GCLT value as compared with quail provided no RO dietary supplementation.

3.5. Changes in numbers of testicular apoptotic and anti-apoptotic germ cells

The numbers of apoptotic germ cells as determined by Bax immunostaining of the testes of quail reared in both TN and HS environmental conditions are shown in Fig. 2. Bax immunopositive staining was more intense in the germinal cell line of the quail that had HS imposed (Fig. 2B, D, and F) compared with those housed under the TN environmental condition (Fig. 2A, C, and E). The Bax immunopositive staining of testis tissues was most intense in quail that were fed diets with no RO dietary supplementation and reared in the HS environmental condition (Fig. 2B), while there was less Bax immunopositive staining in the tissues quail with 125 (Fig. 2D) and 250 (Fig. 2F) ppm RO dietary supplementation that were managed under the HS condition. Besides, with respect to numerical value

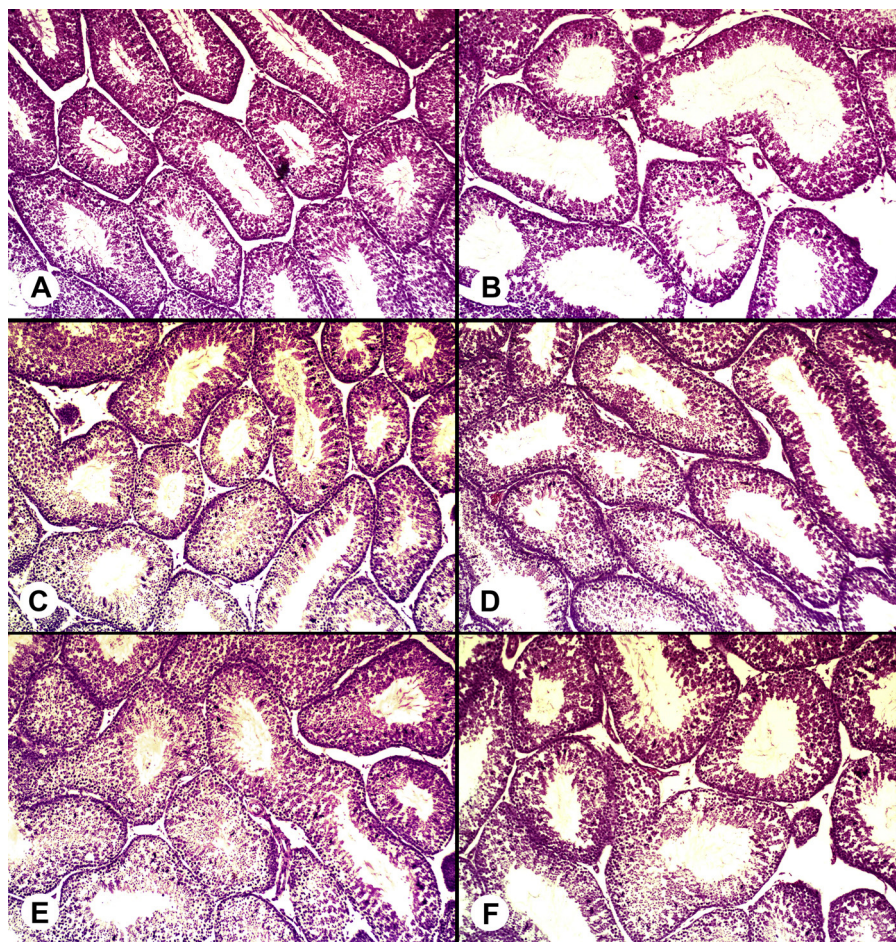


Fig. 1. Effects of rosemary oil (RO) on histopathologic structure of testes in Japanese quail reared in a thermoneutral (TN) environment and under heat stress (HS) (hematoxylin and eosin-staining, $\times 40$ magnification). Footnote for Fig. 1. Normal appearance of seminiferous tubules of testes in quail that were supplemented with 0 (A), 125 (C), and 250 (E) ppm RO in TN environmental conditions; Dilatation in the diameters of seminiferous tubules, decrease in germinal cell layer thickness, and immature spermatocytes in lumen of tubules of testes in quail with dietary supplementation of 0 ppm RO (B), and the decrease in lesions along with dilatation in quail supplemented with 125 (D) and 250 (F) ppm RO in HS environmental conditions.

Table 4

Effects of rosemary oil (RO) on some testicular histopathologic and immunohistochemical measurements in Japanese quail reared in a thermo-neutral (TN) environment and under heat stress (HS).

Variables	HS			TN				Main effects of environmental conditions and feed additive on measured variables (According to the General Linear Model procedure)	
	RO (ppm)			RO (ppm)					
	0	125	250	0	125	250	s.e.	HS significance(<i>P</i> -value)	RO significance(<i>P</i> -value)
DST (μm)	246.80 ^a	229.30 ^b	227.70 ^b	206.20	209.50	208.88	1.68	0.0001 ^{***}	0.0001 ^{***}
GCLT (μm)	52.10 ^c	58.20 ^b	63.80 ^a	59.80 ^C	64.70 ^B	74.00 ^A	0.75	0.0001 ^{***}	0.0001 ^{***}
AR positivity (0–4)	1.16 ^b	1.26 ^b	1.47 ^a	1.66 ^B	1.72 ^B	2.00 ^A	0.03	0.0001 ^{***}	0.0001 ^{***}
Bax positivity (0–4)	1.66	1.50	1.33	0.66	0.50	0.50	0.04	0.0001 ^{***}	0.505
Bcl-2 positivity (0–4)	0.50 ^b	0.66 ^{ab}	0.87 ^a	1.00 ^B	1.33 ^A	1.33 ^A	0.03	0.0001 ^{***}	0.0001 ^{***}
Bax/Bcl-2 ratio	3.32 ^b	2.27 ^{ab}	1.53 ^a	0.66 ^B	0.38 ^A	0.38 ^A	0.05	0.0001 ^{***}	0.0001 ^{***}

Data are expressed as mean and pooled standard errors (s.e.)

DST: Diameter of seminiferous tubules, GCST: Germinal cell layer thickness, AR: Androgenic receptor.

^{a, b, c} Mean values having different superscripts in the same row within the HS groups are different from each other.

^{A, B, C} Mean values having different superscripts in the same row within the TN groups are different from each other.

^{***} $P < 0.001$.

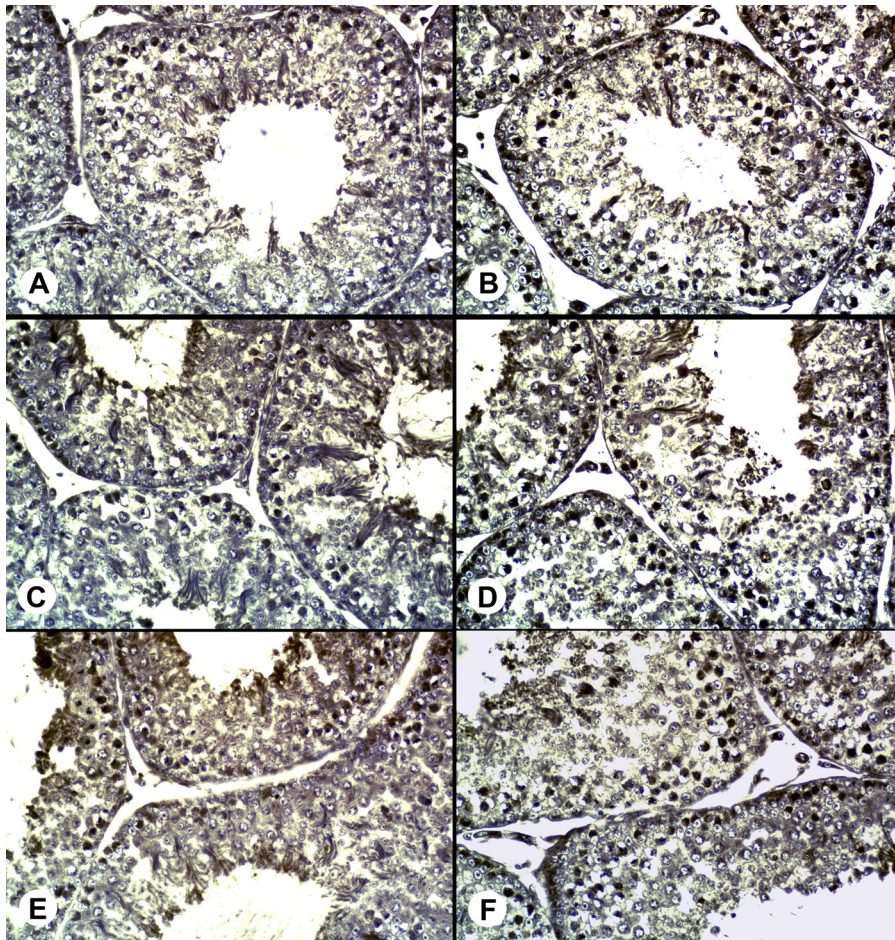


Fig. 2. Effects of rosemary oil (RO) on apoptotic germ cell intensity, demonstrated by Bax immunopositive staining in the testes of Japanese quail reared in a thermoneutral (TN) environment and under heat stress (HS) (Mayer's hematoxylin, $\times 200$ magnification). Footnote for Fig. 2. Bax immunopositive staining (at specific ratios) in the spermatogenic cells of quail that were supplemented with 0 (A), 125 (C), and 250 (E) ppm RO in TN conditions; There was more Bax immunopositive staining in the spermatogenic cells of quail that were supplemented with 0 ppm RO (B), and the slight but an insignificant decrease in the staining intensity of spermatogenic cells of quail that were supplemented with 1250 (D) and 2500 (F) ppm RO in HS environmental conditions.

of Bax immunopositive germinal cell staining intensity, although the imposition of the HS environmental condition resulted in an increase ($P < 0.001$) when compared to the cells from the TN group, dietary supplementation of both 125 and 250 ppm RO to the diets of quail where imposition of HS occurred did not result in decreases in germinal cells stained with Bax immunopositive staining when compared with the group with no RO dietary supplementation that was housed under the HS environmental condition (Table 4).

Anti-apoptotic germ cell Bcl-2 immunopositive staining intensity results are shown in Fig. 3 for the testes of quail reared under both TN and HS conditions. With both HS (Fig. 3B,D,F) and TN environments (Fig. 4A, C, and E), the Bcl-2 immunopositive staining intensity of testes of quail provided both 125 (Fig. 3C) and 250 (Fig. 3E) ppm of RO was more pronounced than for quail with no RO dietary supplementation (Fig. 3A). With respect to numerical value of Bcl-2 immunopositive staining intensity, HS caused a decrease ($P < 0.001$) when compared with the TN group.

However, dietary supplementation of 250 ppm RO to quail with HS imposition resulted in an increase ($P < 0.001$) when compared to the group with no RO dietary supplementation that was reared in HS conditions. Similar increases were observed in quail with both 125 ($P < 0.01$) and 250 ($P < 0.01$) ppm RO dietary supplementation as compared to animals with no RO supplementation that were housed under the TN condition. While HS increased ($P < 0.001$) the Bax/Bcl-2 ratio, dietary supplementation of 250 ppm RO in quail imposed with HS decreased ($P < 0.001$) Bax/Bcl-2 ratio (Table 4).

3.6. Changes in testicular AR

The amount of AR immunopositive staining was greater in round and elongated spermatids than spermatogonia, primary and secondary spermatocytes, Sertoli and Leydig cells in the testes of all groups of quail reared in both TN and HS conditions. While there was some AR immunopositive staining in the testes of quail with

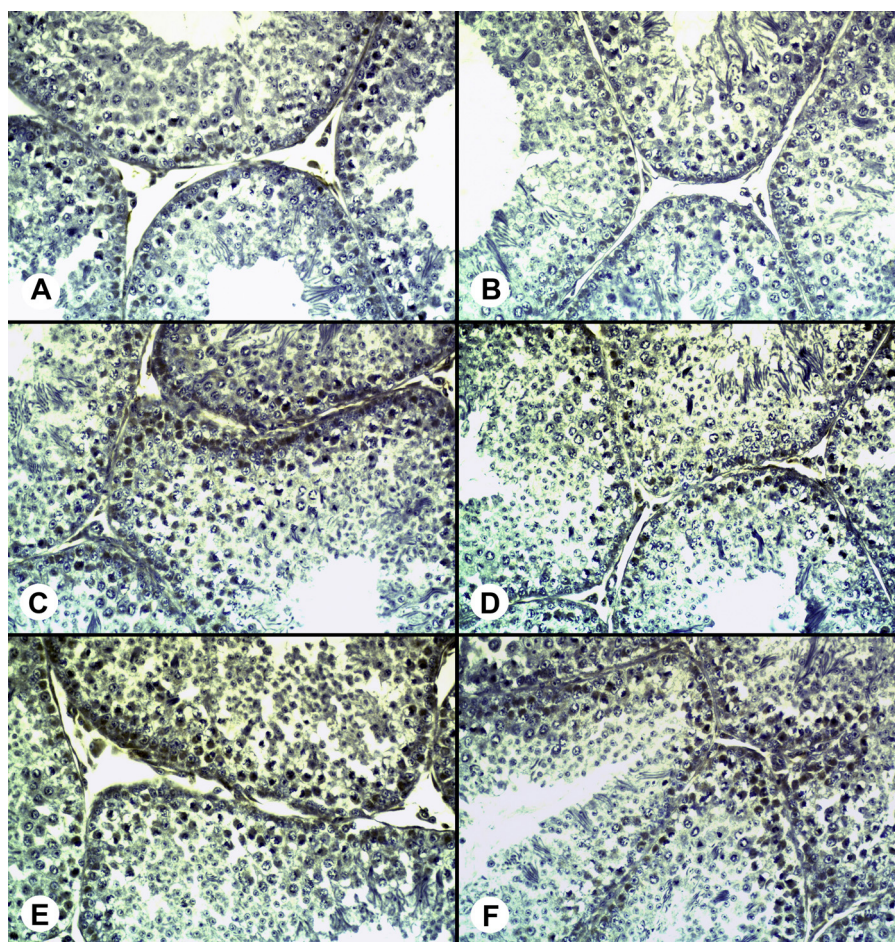


Fig. 3. Effects of rosemary oil (RO) on anti-apoptotic germ cell intensity, demonstrated by Bcl-2 immunopositive staining, in the testes of Japanese quail reared in a thermoneutral (TN) environment and under heat stress (HS) (Mayer's hematoxylin, $\times 200$ magnification). Footnote for Fig. 3. Normal Bcl-2 immunopositive staining in the spermatogenic cells of quail that were supplemented with no additive (A), and the greater Bcl-2 immunopositive staining in the spermatogenic cells of quail supplemented with 125 (C) and 2500 (E) ppm RO in TN environmental conditions; Bcl-2 immunopositive staining (at specific ratios) in the spermatogenic cells of quail supplemented with 0 ppm RO (B) and the increase in staining intensity of spermatogenic cells of quail supplemented with 125 (D) and 250 (F) ppm RO in HS environmental conditions.

no RO dietary supplementation that were reared in HS environment (Fig. 4B, Table 4), greater staining was observed in the testes of quail with 250 ppm RO dietary supplementation and reared in TN environment (Fig. 4E, Table 4). In germinal cells of quail raised in TN conditions, the intensity of AR immunopositive staining was greater in the RO-treated groups (Fig. 4C, and E and Table 4) particularly those with dietary supplementation of 250 ppm RO as compared with the non-supplemented group (Fig. 4A, Table 4). Although both amounts of RO supplementation (Fig. 4D and F) to diets of birds reared under HS imposed conditions resulted in an increase in the intensity of AR immunopositive staining in comparison with the quail with no dietary supplementation (Fig. 4B) that were housed in the HS imposed condition, the increase observed with 250 ppm supplementation in the RO-treated group was the only value of statistical significance (Table 4).

4. Discussion

4.1. Detrimental effects of HS

Small amounts of ROS have an important physiological role in modulating gene and protein activities, necessary for spermatozoa proliferation, differentiation, maturation and fertilizing ability. However, the pathological effects of ROS occur when these compounds are produced in excess of the typical physiological amounts. The cell membrane phospholipids are very susceptible to the detrimental impacts of ROS that initiate and enhance lipid peroxidation (Sharma et al., 2012). Avian spermatozoa have large amounts of PUFAs that increase susceptibility to the detrimental actions of ROS and lipid peroxidation. Enzymatic [superoxide dismutase (SOD), GSH-Px and CAT] and non-enzymatic (vitamins A, C, E, GSH, selenium, etc.) endogenous antioxidants protect the integrity of

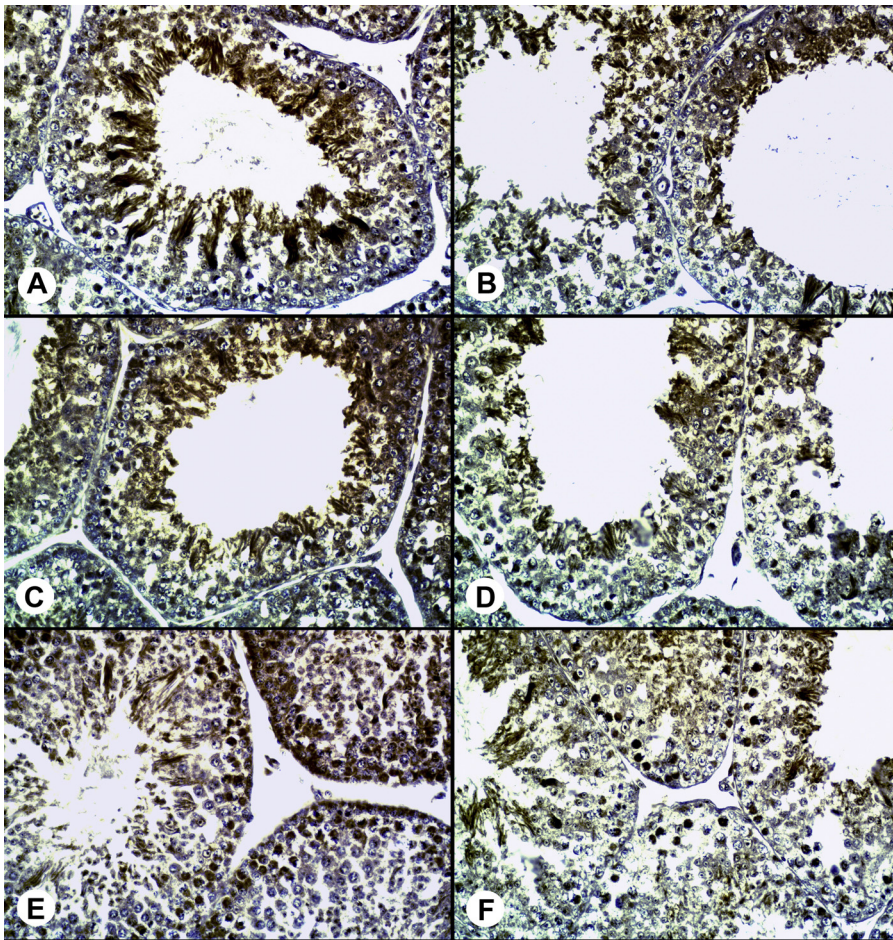


Fig. 4. Effects of rosemary oil (RO) on the intensity of androgenic receptor (AR) immunopositive staining in the testes of Japanese quail reared in a thermoneutral (TN) environment and under heat stress (HS) (Mayer's hematoxylin, $\times 200$ magnification). Footnote for Fig. 4. Normal AR immunopositive staining in the spermatogenic cells of quail that were supplemented with 0 (A) and 125 (C) ppm RO and, the greater AR immunopositive staining in the spermatogenic cells of quail supplemented with 250 (E) ppm RO in TN environmental conditions; The lesser AR immunopositive staining in the spermatogenic cells of quail supplemented with 0 (B) and 125 (D) ppm RO and, the increase in the staining intensity of spermatogenic cells of quail that were supplemented with 250 (F) ppm RO in HS environmental conditions.

unsaturated chemical bonds of membrane phospholipids against lipid peroxidation by decreasing the detrimental impacts of ROS. When the oxidant-antioxidant system is not optimal in its equilibrium as a result of increased production of oxidants, there are impairments in testicular histologic structure, spermatogenesis and spermatozoa functions due to the increased lipid peroxidation and there is an ensuing reduction in fertility (Surai et al., 2001). In the present study, HS increased testicular MDA concentrations, a by-product of lipid peroxidation, in comparison to what occurred with the TN conditions, which is in agreement with findings from a previous study (Türk et al., 2015). The HS had no effect on testicular tissue antioxidant markers including rGSH, GSH-Px, and CAT in the present study. This result is consistent with the findings of Türk et al. (2015) but not those of Altan et al. (2003) where there were increases in blood SOD, GSH-Px and CAT activities of broiler chickens housed in HS conditions. The reason for this inconsistency in findings may be the use of different species of animals and different sample

assessments between the present and previous studies. Reductions in spermatozoa count, spermatozoa motility, and increased numbers of dead spermatozoa of chickens (McDaniel et al., 2004; Ebeid, 2012) have been reported to be detrimental effects of HS on semen quality. In addition, in the previous study (Türk et al., 2015), HS reduced both spermatid and testicular spermatozoa counts. Consistent with these findings in the previous study was the significant reductions in the numbers of all spermatogenic cells including spermatogonium, spermatocyte, spermatid, and spermatozoon in the testicular tissue of the quail housed under HS conditions. The reason for the increase in testicular lipid peroxidation and decrease in spermatogenic cell numbers observed in the present study may be the increased concentration of ROS that developed in response to HS.

Loss of spermatogenic cells, germ cell degeneration in seminiferous tubules, cessation of spermatogenesis in broilers (Terim Kapakin et al., 2013) and quail (Türk et al., 2015) as well as an increase in the DST, decrease in the GCLT

along with germ cell disorganization in quail (Türk et al., 2015) have been reported to be HS-related outcomes along with an increase in some testicular histopathologic lesions. Similar histopathologic lesions including the increase in the DST, reduction in the GCLT, disorganization, and degeneration in germinal cells, cessation of spermatogenesis, existence of immature spermatids and spermatogonia in the lumen of some seminiferous tubules were detected in the testes of quail where HS was imposed on the birds in the present study. Apoptosis is programmed cell death and is a biological process that protects tissue by eliminating the cells with abnormal function, thereby preventing the increase in numbers of aberrant cells. All types of spermatogenic cells undergo apoptosis, suggesting that programmed cell death may be an important regulator of spermatogenesis (Sharma et al., 2012). When the production of ROS is greater than optimal leading to lipid peroxidation, apoptosis of germ cells can be enhanced (Maheshwari et al., 2009). The actions when there is an elevated amount of anti-apoptotic (Bcl-2) protein, there is an extended survival of cells and increases of pro-apoptotic (Bax) gene expression that in turn leads to an acceleration of cell death in the tissues (Sinha Hikim and Swerdloff, 1999). In the present study, HS caused significant increases in testicular Bax immunostaining and in Bax/Bcl-2 ratio, and significant decreases in testicular Bcl-2 immunostaining in quail as compared with findings in birds under TN conditions. HS-induced lipid peroxidation causes increased testicular apoptosis in a previous study (Türk et al., 2015) in developing quail, which is consistent with the findings of the present study. Androgens and the AR have important roles in male spermatogenesis and fertility. The actions of androgens are mediated by the AR (Wang et al., 2009). In an *in vitro* study, the numbers of AR in monkey Sertoli cells were reduced after HS (Chen et al., 2008). Consistent with the findings of a previous study (Türk et al., 2015), there was a significant reduction in AR immunopositive staining in testicular tissue of quail where HS was imposed in the present study. The HS-induced increase in lipid peroxidation may lead to impairments in testicular histologic structure, increases in testicular apoptotic germ cells, as well as decreases in numbers of AR. Additionally, the relaxation of peritubular myoid cells in response to HS (Türk et al., 2015) may be the reason for the increased DST observed in the present study.

4.2. Alleviating effects of RO

Natural antioxidant products are increasingly used to treat various pathologic conditions relating to oxidative stress and the resulting pathogenesis (Raskovic et al., 2014). Antioxidants are added to the diet as a feed supplementation for improving performance, productivity and reproductive outcomes in the poultry industry. Rosemary contains active anti-oxidative substances such as phenolic diterpenes, flavonoids, phenolic acids (Ho et al., 2000) and volatile oils (Begum et al., 2013). The RO has been used previously as a preservative in the human food industry due to its antioxidant and antimicrobial activities (Raskovic et al.,

2014). Different amounts of supplementation of RO prevents HS-induced decreases in growth performance and carcass traits of Japanese quail (Çiftçi et al., 2013) and, also improves growth performance, egg traits, egg oxidative stability, and meat quality of Pharaoh quail reared in TN environmental conditions (Yesilbag et al., 2013). Different constituents (terpenoids, flavonoids, phenolic acids, volatile oils) of rosemary have been reported to function by decreasing oxidative stress in different tissues of mammals that is induced by different chemicals (Tanyıldızı et al., 2009; Singh et al., 2012; Raskovic et al., 2014) or that is produced as a result of exposure to electromagnetic field (Hajhosseini et al., 2013). RO (Melusova et al., 2014), rosmarinic acid (Hajhosseini et al., 2013), and terpenes (Singh et al., 2012) have DNA protective and anti-apoptotic features. In addition, Lin (2014) demonstrated that triterpenes from *Alisma orientalis* function as an AR receptor agonist. However, there are inconsistencies in findings between the studies concerning the effects of rosemary on the reproductive system of male mammals. While findings in some studies indicate that rosemary has a contraceptive (Nusier et al., 2007) and deteriorating effects (El-Din et al., 2012; Heidari-Vala et al., 2013) on testicular tissues, findings in other studies indicate that rosemary and its different extracts are able to prevent both functional spermatozoa damage that is induced by cryopreservation (Luno et al., 2014; Motlagh et al., 2014) and various toxicant-induced testicular, hormonal and spermatozoa damage (Hozayen et al., 2014; Uyeturk et al., 2014). In addition, Superchi et al. (2005) suggested that the antioxidant activity of rosemary extract limits the negative effects of temperatures that induce HS on reproductive efficiency of boars. To the best of our knowledge, there has been no evidence regarding the effect of RO on HS-induced damage as related to reproductive variables including spermatozoa production, testicular oxidant-antioxidant balance, testicular histopathologic structures, and incidence of testicular apoptotic cell formation and number of AR in quail. Therefore, this is the first report evaluating the protection of RO on HS-induced testicular damage in Japanese quail. In the present study, supplementation of RO to quail diets provided for significant reductions in the HS-induced increments in testicular lipid peroxidation, testicular histopathologic lesions, and Bax/Bcl-2 ratio, and decreased the detrimental effects of HS on spermatogenic cell counts, GCLT, amounts of Bcl-2 and AR in the reproductive tissues that were assessed. While HS had no significant effects on the antioxidant markers, both amounts of RO dietary supplementation increased the rGSH concentrations and CAT activity of quail under HS imposition. With the TN environmental condition, 250 ppm RO supplementation resulted in a decrease in MDA concentration and an increase in AR. Both amounts of RO supplementation increased the rGSH concentration, GCLT, and amount of Bcl-2 and also decreased the Bax/Bcl-2 ratio. The possible reason for the improvements observed in the testes of quail reared in both HS and TN environmental conditions in the present study in birds where diets were supplemented with RO is that RO has potent antioxidant and radical scavenging activities.

5. Conclusion

The results of the present study clearly suggest that RO addition to diets of growing male quail housed under HS environmental conditions reduced the HS-induced damage in the testes and spermatogenic cells. This positive effect of RO may be attributed to its anti-peroxidative activity.

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