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Research Article

Tocopherol Alleviates Oxidative Stress and Increases Androgen Receptors in Mice Testicular Tissues under Forced Swimming in Warm Water

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The purpose of this study was to investigate the effect of tocopherol supplementation and swimming in warm water on the spermatogenesis, expression of androgen receptors (ARs), and oxidative stress markers in mouse testis. Adult male mice were divided into seven groups: (A) control, (B) solvent, (C) vitamin E (VE), (D) swimming at 23°C water, (E) swimming at 23°C water and receiving VE, (F) swimming at 35°C water, and (G) swimming at 35°C water and receiving VE. Hormones were measured using ELISA. Spermatogenesis was examined through histopathology. The ARs were studied by immunohistochemistry. The testis oxidative stress markers were assessed using colorimetric assays. The distribution of VE in testis tissue was also evaluated using high-performance liquid chromatography (HPLC). Swimming of mice at 23°C was safe for sperm development. Swimming at 35°C resulted in lower testosterone, LH, sperm parameters, sperm differentiation, AR expression, SOD activity, and increased MDA in the testis, compared to control ($P < 0.05$). The abovementioned parameters were significantly altered by VE, in comparison to the animals that swam in warm water at 35°C ($P < 0.05$). The results of HPLC showed that tissue distribution of VE was significantly higher in group G, compared to group E ($P < 0.05$). Swimming in water at 23°C is conducive to sperm development, whereas swimming at 35°C impairs sperm production. In conclusion, VE counteracts the negative effects of swimming at 35°C water on sperm development by altering the hypothalamic–pituitary–gonadal axis, distribution of VE, AR expression, and lipid peroxidation in testicular tissue.

1. Introduction

Male infertility accounts for 50% of all infertile individuals. After cancer and cardiovascular diseases, infertility is the most frequent disease that leads to social problems, stress, and disappointment [1]. Spermatogenesis is a temperature-dependent process and is reduced by 14% with a 1°C increment in testicular temperature [2] and a 40% reduction in sperm output [3]. Heat stress negatively affects semen volume, and sperm parameters, and increases sperm DNA fragmentation and apoptosis [2]. Heat stress impairs both human [4, 5] and animal fertility [6]. Hyperthermia through increasing apoptosis of spermatids and spermatocytes causes spermatogenesis failure [7]. Factors such as increased testicular temperature, environmental and occupational factors, increased global temperature, and varicocele have negative or harmful effects on fertility [2].

Reactive oxygen species (ROS) are abundantly produced in the male reproductive tract. In the testis, both the amount of unsaturated fatty acids and the germ cell proliferation speed are high; therefore, testis can be considered a perfect environment for the production of ROS [8]. Lifestyle choices like smoking [9, 10], severe exercise, obesity [11], and heat stresses [7] can lead to an increase in ROS levels in the male

reproductive system. Following the increase in ROS, apoptotic pathways are activated in the testis and affect spermatogenesis and sperm. Therefore, the membrane potential of mitochondria is reduced. Sperm DNA damage and lipid oxidation cause sperm apoptosis, which decreases sperm count, motility, and viability [12].

Testosterone through binding to its androgen receptors (ARs) [1] affects male fertility. AR can be found throughout the male reproductive system and is stimulated by either testosterone or dihydrotestosterone. AR is essential for male fertility, which involves sexual differentiation, puberty induction, spermatogenesis, and meiosis completion [13]. Defects in AR may result in male infertility [14]. In testis, AR mostly is expressed in somatic cells including Sertoli, Leydig, and myoid cells and it also is found in spermatids [14]. Infertile men with a lower sperm quality have lesser AR in their sperm [13]. In infertile men, AR positively correlates with sperm parameters and acrosome reaction [13]. *In vitro* study in monkey's Sertoli cells showed hyperthermia reduces AR expression in Sertoli cells [15].

Alpha-tocopherol is the most common form of vitamin E (VE) in tissues and possesses a powerful antioxidant property [16]. VE prevents spermatogenesis from damage in rats exposed to long whole-body hyperthermia (41°C, 60 min) in an incubator [17]. Selenium and VE have been shown in rats to protect the testis and sperms from apoptosis and ROS, induced by scrotal hyperthermia (42°C, 30 min) [18].

The purpose of this study was to investigate the effect of tocopherol supplementation and swimming in warm water on the spermatogenesis, AR expression, and oxidative stress markers in mouse testis.

2. Materials and Methods

2.1. Animals. A total of 42 adult Swiss-type male mice, aged 8–10 weeks and weighing 20–25 g, were obtained from the Pharmacology Faculty, Guilan University of Medical Sciences, Guilan, Iran. The animals were housed in standard lighting conditions (12 hr light–dark cycles) at 25 ± 2°C with free access to water and food. After adaptation for at least 1 week in the animal room, the study was started. Animals were maintained and handled according to the protocols, approved by the Animal Care and Use Committee of Guilan University of Medical Sciences (IR.RUMS.AEC.1400.003). Animals were divided randomly into seven groups ($n = 6$ per group).

Group A: Control, without swimming; Group B: solvent (sesame oil), without swimming, received VE solvent intraperitoneally; Group C: VE, without swimming, received VE intraperitoneally; Group D: swimming in water at 23°C without receiving VE; Group E: swimming in water at 23°C with receiving VE; Group F: swimming in warm water at 35°C without receiving VE; and Group G: swimming in warm water at 35°C with receiving VE.

Mice were subjected to forced swimming in a pool (length 80 cm, width 60 cm, and depth 40 cm) containing tap water for 4 min, 5 days/week, and for 5 weeks. Water temperature was maintained constant at 23–24°C and 35–36°C. Animals were considered to be exhausted when they could not swim to the surface and breathe within 7 s and were taken out of the water [19]. Because of the 35-day spermatogenesis process in mice, the experiment was chosen to last for 5 weeks [9, 10]. VE (alpha-tocopherol acetate, Osve-Iran) 100 mg/kg/Bw was dissolved in sesame oil and administered intraperitoneally, 60 min preswimming. For injection, the volume of VE and sesame oil were equal. The animals were anesthetized by injecting ketamine and xylazine simultaneously after the experiments concluded. The inferior vena cava was used to collect blood for hormonal analysis. Testicles were removed from the abdomen. To carry out histopathological studies, the right testis was submerged in 10% neutral buffered formalin and fixed in Bouin's solution. A part of the left testis was homogenized for evaluation of oxidative marker assay and other parts were used to study the distribution of VE. Epididymis were removed and the caudal part was separated by a knife and used for the study of sperm parameters.

2.2. Hormone Measurement. The serum was separated and transferred to −80°C for further biochemical assays. The determination of serum concentrations of testosterone (T) and luteinizing hormone (LH) in each subject was done using an ELISA kit (DKO002) and (DKO009) from DiaMetra (Italy). All procedures were performed as instructed in the kits.

2.3. Histology Procedures. After fixation, testicular tissues were dehydrated and embedded in paraffin for histological examinations. Samples were sectioned in 5 μm thicknesses and stained with hematoxylin and eosin. Three slices were prepared from each specimen, and at least five fields in each slide were processed for histological assessment. All tissues were evaluated using a light microscope (Olympus, Japan).

2.4. Spermatogenesis Evaluation. Quality of spermatogenesis was done using the Johnsen score, and maturity was scored from 1 to 10, as previously described [9, 10]. For evaluation of spermatogenesis quantity, 20 seminiferous tubules in the transverse section at stages VII and VIII of spermatogenesis were chosen in each animal. Counting of germ cells, including spermatogonia, pachytene primary spermatocytes, and round spermatids, was done in areas of 2,500 μm^2 and with 400x magnification. Similarly, Leydig cells and Sertoli cells were counted. All counting was performed based on the morphological criteria.

2.5. Evaluation of Androgen Receptors. Immunohistochemistry was performed for the evaluation of AR. A rabbit monoclonal antibody directed against the mouse AR (501-2721) from Zytomed Systems (Germany) was used for the reaction. Staining was carried out according to kit instructions. The numbers of AR-positive brown cells were counted in an area of 2,500 μm^2 with 400x magnification. The numbers of AR of the germ cells, Sertoli, and Leydig cells were calculated from the numbers of AR-positive cells in at least five microscopic areas of 2,500 μm^2 for each cell. Then, the mean was calculated.

2.6. Oxidative Markers and Antioxidant Enzymes. Testicular samples were transferred into liquid nitrogen. Testicular tissues were weighted and homogenized in 1 mL ice-cold phosphate-buffered saline (PBS), then centrifuged at 13,000x g for 5 min at

4°C. The supernatant was collected and transferred to −80°C until further analysis. Malondialdehyde (MDA) activity was measured for the lipid peroxidation study. A colorimetric kit (TPR-MDA) from Teb Pazhohan Razi (Iran) was utilized for measuring. Absorbance was recorded at the wavelength of 545 nm. The activity of catalase (CAT) [20] and superoxide dismutase (SOD) was measured for the study of the antioxidant status of homogenized testicular tissue, using the colorimetric kits (TPR-CAT) and (TPR-SOD) from Teb Pazhohan Razi (Iran), at 450 nm. The limit of detection (LOD) for MDA, SOD, and CAT was 1 U/mL, 0.1 μm, and 0.5 U/mL, respectively. The Bradford method was utilized to measure the total protein content of the supernatants [21].

2.7. Analysis of Vitamin E Distribution in Testis Tissue. High-performance liquid chromatography (HPLC) method was used to investigate the concentration of VE in testicular tissue in different groups.

Chromatographic conditions were obtained from Rupérez et al. [22] with some modifications. The analysis was performed by an HPLC system, consisting of a Wellchrom K-1001 HPLC pump from Knauer, coupled with a fluorescence detector (RF-10A XL Shimadzu). The chromatographic separation was performed on the PerfectSil C18 analytical column (250 × 4.6 mm, 5 μm) at 35°C. The mobile phase was methanol: Water (95 : 5) with a flow rate of 1 mL/min and the injection volume was 100 μL. The excitation and emission wavelengths of the detector were set at 295 and 350 nm, respectively.

For sample preparation, about 0.2 g of tissue was removed from the animal and carefully weighed and homogenized in 40 mL of methanol : water (1 : 1) solution. VE was extracted twice with n-hexane solvent and after complete evaporation of the solvent, it was reconstituted in 500 μL of methanol, and 100 μL was injected into the HPLC column. Calibration standards of VE were freshly prepared by spiking appropriate amounts of working solutions in pooled drug-free tissue samples at concentrations of 10, 20, 50, 75, and 100 μg/mL, and treated sample preparation steps were done exactly as described above. The lower LOD and limit of quantification (LOQ) were 0.978 and 3.14 μg/mL, respectively [22, 23].

2.8. Epididymis Sperm Parameters. The caudal part of the epididymis was transferred into a prewarmed Petri dish and moved in an incubator at 5% CO_2 for 30 min. Sperm parameters including count (10^6 spermatozoa per milliliter), percent of motile sperms, and abnormal sperms were evaluated, as previously described [9, 10].

2.9. Statistical Analysis. Statistical Package for Social Studies (SPSS), version 20, was used for the data analysis. We reported the continuous variables as mean ± SE. One-way analysis of variance (ANOVA) was used to test the difference between the means of several groups with the Tukey test as a post hoc test. All data were considered significant with $P < 0.05$.

3. Results

3.1. Hormonal Study. The concentrations of testosterone or LH were not affected in groups B, C, D, and E, compared to control. Compared to other groups, swimming in warm water at 35°C for a short time decreased testosterone and LH levels ($P = 0.001$) significantly. However, the supplementation of VE to mice swimming in warm water at 35°C (group G,) improved testosterone and LH levels ($P = 0.001$) (Table 1).

3.2. Histopathology and Spermatogenesis Process. Based on observations using light microscopy, the spermatogenesis was active in controls, vehicle, and VE groups (groups A, B, and C). All types of germ cells were observed in seminiferous tubules along with Sertoli cells. Interstitial tissue was filled with loose connective tissue cells, small blood vessels, and groups of large and acidophilic cells of Leydig cells. Germinal epithelial thickness was observed and there were no vacuoles in the epithelium or cell sloughing in the lumen. Similar histological criteria were observed in groups D and E. In group F, where mice were swimming in warm water at 35°C, seminiferous tubule diameters were reduced and the numbers of various types of germ cells were reduced significantly, in comparison with the other groups. In contrast to groups A, B, C, D, and E, swimming in warm water at 35°C caused a significant decrease in the numbers of spermatogonia, pachytene primary spermatocytes, round spermatids, and elongated spermatids. ($P < 0.05$). Supplementation of VE of animals in group G modulated these changes through a partial increase of germinal epithelial thickness, and increasing numbers of germ cells, in comparison to group F. Compared to group F, group G had an increase in germ cell count after VE supplementation. The number of spermatogonia cells was not affected by pretreatment with VE. The number of Sertoli cells did not change when swimming in group D or group F compared to the other groups. The number of Sertoli cells almost stayed constant in all experimental groups.

Johnsen scores were high in seminiferous tubules of animals in groups A–E, and there were no significant differences between the abovementioned groups. Swimming at 35°C for a short time negatively affected the maturity of seminiferous tubules and reduced the Johnsen score ($P = 0.001$). Johnsen's score in group G was significantly improved by supplementing VE, compared to group F ($P = 0.001$).

Between groups A, B, C, D, and E, the mean of Leydig cells did not differ. The Leydig cell count was negatively affected by swimming in warm water at 35°C when compared to groups A, B, C, D, and E ($P = 0.001$). Supplementation of VE in group G significantly increased Leydig cell numbers, in comparison to group F ($P = 0.004$) (Figure 1 and Table 1).

3.3. Expression of Androgen Receptors in Germ and Somatic Cells. The AR expression in germ cells in mouse testes was rarely seen. We did not find significant statistical differences in the AR expression in germ cells between different groups. Sertoli cells with their teardrop-like nucleus mostly reacted as moderate to strong with AR immunostaining in the germinal epithelium. However, the immunoreaction in Leydig cells was also positive. Swimming in group D or supplementation with VE did not affect the AR expression of Sertoli cells or Leydig cells, in comparison to the control.

Swimming in warm water at 35°C caused a significant reduction in the AR expression in both Sertoli ($P = 0.001$) and Leydig cells ($P = 0.001$) when compared with groups of

Table 1: Effect of swimming and supplementation of vitamin E on mouse testis histology, sperm parameters, and hormones.

Variables	Groups						
	(A) Control	(B) Solvent	(C) VE	(D) Swimming at 23°C	(E) Swimming at 23°C+ VE	(F) Swimming at 35°C	(G) Swimming at 35°C + VE
Johnsen score	9.64 ± 0.09^{b}	9.45 ± 0.07^{b}	9.58 ± 0.04^{b}	9.37 ± 0.08^{b}	9.54 ± 0.05^{b}	7.47 ± 0.18^{a}	9.18 ± 0.13^{b}
Spermatogonia (n/f)	8.54 ± 0.25^{b}	7.38 ± 0.38^{b}	8.13 ± 0.32^{b}	7.88 ± 0.23^{b}	7.92 ± 0.23^{b}	5.56 ± 0.33^{a}	6.89 ± 0.50
pachytene spermatocytes (n/f)	25.48 ± 1.30^{b}	25.54 ± 0.49^{b}	26.54 ± 0.74^{b}	26.99 ± 0.99^{b}	27.58 ± 1.02^{b}	19.24 ± 1.21^{a}	24.12 ± 0.68^{b}
Round spermatids (n/f)	58.25 ± 3.01^{b}	60.14 ± 2.15^{b}	61.0 ± 1.30^{b}	56.80 ± 2.99^{b}	65.0 ± 3.39^{b}	44.80 ± 1.80^{a}	55.20 ± 2.38^{b}
Sertoli cells (n/f)	11.30 ± 0.72	10.40 ± 1.04	9.40 ± 0.76	9.90 ± 0.51	9.80 ± 0.37	9.07 ± 0.5	9.79 ± 0.52
Leydig cells (n/f)	9.61 ± 0.94	9.24 ± 0.34	10.0 ± 0.38	10.30 ± 0.26	10.15 ± 0.45^{b}	6.76 ± 0.36^{a}	9.37 ± 0.31^{b}
Sperm count ($\times 10^{6}$)/mL	49.50 ± 2.72^{b}	48.86 ± 1.52^{b}	48.75 ± 2.06^{b}	40.0 ± 1.28^{b}	49.38 ± 1.12^{b}	28.79 ± 2.18^{a}	37.75 ± 2.97^{ab}
Sperm motility (%)	67.35 ± 1.8^{b}	64.25 ± 1.4^{b}	66.75 ± 1.1^{b}	64.13 ± 1.5^{b}	64.88 ± 1.69^{b}	37.88 ± 3.46^{a}	46.73 ± 1.3^{ab}
Abnormal sperm (%)	24.0 ± 1.47^{b}	27.0 ± 1.83^{b}	25.25 ± 1.65^{b}	28.50 ± 1.36^{b}	25.75 ± 1.21^{b}	58.5 ± 2.41^{a}	31.25 ± 1.63^{b}
Total serum testosterone (ng/mL)	4.33 ± 0.23^{b}	3.87 ± 0.16^{b}	3.83 ± 0.16^{b}	4.03 ± 0.13^{b}	4.45 ± 0.18^{b}	1.82 ± 0.09^{a}	3.5 ± 0.17^{ab}
Serum LH (IU/L)	9.56 ± 0.23^{b}	8.79 ± 0.56^{b}	8.88 ± 0.51^{b}	9.25 ± 0.27^{b}	9.45 ± 0.23^{b}	5.15 ± 0.27^{a}	6.69 ± 0.41^{ab}

All data are expressed as mean ± standard error (SE). (n/f), number per field 2,500 μm^2. [a,b]Indicate significant differences within rows ($P < 0.05$).

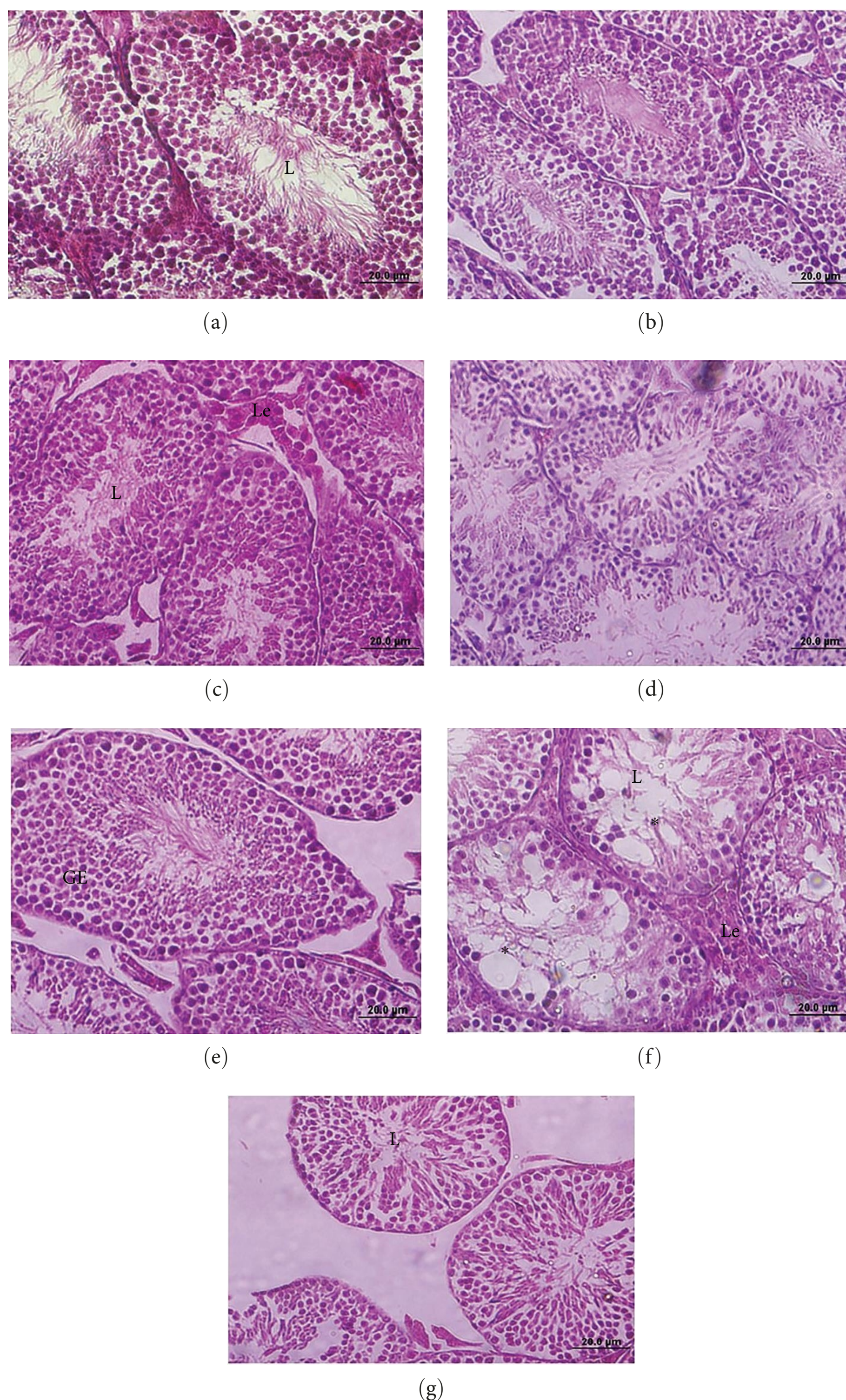

Figure 1: Light microscopic picture of the testicular tissue of a mouse: (a) control, (b) solvent, (c) VE, (d) swimming at 23°C, (e) swimming at 23°C + VE, (f) swimming at 35°C, and (g) swimming at 35°C + VE. L stands for the lumen, GE for germinal epithelium, Le for Leydig cells, and * is a representation of vacuoles. The seminiferous tubules in group F tend to have larger lumen due to a thinner germinal epithelium and the presence of vacuoles inside its epithelium. Supplementation with vitamin E protected the seminiferous tubules in group G compared to group F. 400x. Hematoxylin and eosin.

A, B, C, D, and E. The supplementation of VE in (Group G) could increase the mean of AR in both Sertoli ($P = 0.03$) and Leydig cells ($P = 0.001$), in comparison to group F (Figures 2 and 3).

3.4. Oxidative Markers and Antioxidant Enzymes. We assessed the effects of VE on alterations of the oxidant–antioxidant status by measuring the activities of oxidant MDA and antioxidants SOD and CAT. The results showed the activities of SOD in the testicular tissue of the mice in group F were significantly decreased, compared with those in groups A–E ($P = 0.01$). There were no significant statistical differences in CAT activity in testis tissue homogenate between different groups. MDA accumulation was increased in testicular tissue following swimming in warm water (group F), in comparison to control. Pretreatment with VE as an antioxidant increased the SOD activity and ameliorated the accumulation of MDA in testis tissue in group G, in comparison with those in group F ($P = 0.001$) (Figure 4).

3.5. Distribution of Vitamin E in Testis Tissue. In animals receiving VE, including groups C, E, and G, the amount of VE measured in rat testis tissue was 14.10 ± 1.70, 22.12 ± 1.39, and 28.44 ± 0.83 μg/g of tissue, respectively. The results of the tissue distribution of VE using the HPLC method showed that the distribution of this compound in group G (swimming at 35°C with receiving VE) was significantly higher than the group C ($P = 0.001$) and E ($P = 0.003$), and the distribution in group E was significantly higher than the group C ($P = 0.004$). It should be noted that in other groups that did not receive VE including A, B, D, and F, VE levels were lower than the LOD of HPLC.

3.6. Epididymis Sperm Parameters. Swimming in moderately warm water (group D) had no significant effect on sperm count, motility, and abnormal sperms, in comparison with control, vehicle, or VE. Sperm count and motility were reduced in animals in group F, in comparison with animals in groups A–E ($P < 0.05$). While pretreatment with VE elevated both sperm count and motility, in comparison with animals in group F ($P < 0.05$). Abnormal sperm morphology was increased in animals in group F, in comparison with groups A–E ($P < 0.05$). Supplementation with VE in group G ameliorated the percent of abnormal sperm morphology, in comparison with group F ($P < 0.05$) (Table 1).

4. Discussion

The results of this study demonstrated animals who were swimming in water at 23°C had no problem related to their reproductive factors, including sperm parameters, hormones, and maturity of spermatogenesis. Their testicular tissue did not show increased markers of oxidative stresses. Therefore, it seems regular swimming for a short time in the water at 23°C is conducive to spermatogenesis. Regular exercise has a positive impact on body health. Swimming and other aquatic activities are among the most popular ways to meet physical activity recommendations, both for the aerobic and strength/balance aspects. The many benefits of exercising in an aquatic environment include reduced weight-bearing stress, a humid environment, and decreased heat load [24]. This study revealed swimming in warm water at 35°C had a negative impact on serum testosterone, sperm parameters, and spermatogenesis. It also decreased the expression of ARs in somatic cells, reduced SOD activity, and increased the accumulation of MDA in the testis.

Therefore, it seems that in this study hyperthermia itself, not swimming has resulted in these negative effects on testis. Normal scrotal hypothermia can be maintained by the testicular thermoregulation system in men during normal, healthy environmental conditions [25]. Chronic thermodysregulation can occur in men who are repeatedly exposed to high temperatures, such as workers exposed to high temperatures. Consequently, it causes significant modifications to sperm characteristics like motility and morphology [26]. Hyperthermia is associated with the accumulation of oxygen-free radicals in the body, resulting in oxidative stress [27]. Damage to cell components such as DNA, lipids, and proteins and then cell death can be caused by oxidative stress and oxygen free radicals. Lipid peroxidation levels increased oxygen free radicals, and oxidative stress levels are key factors in determining the extent of DNA fragmentation and apoptotic cell death [28]. According to research, ROS leads to electron leakage from spermatozoa that are actively respiring, and this is caused by intracellular redox activities. The production of ROS is significantly increased in the semen when dysfunctional sperm are present. This has an impact on its mitochondrial function and subsequently on sperm function, such as motility. Impaired sperm motility, concentration, and morphology can be linked to oxidative stress and excess production of ROS. A person's potential to produce viable sperm is greatly influenced by these parameters [29]. Thermal stress-induced accumulation of oxidative free radicals may lead to the destruction of the cell membrane resulting in a decline in sperm quality [17, 29]. Our results regarding the effects of hyperthermia on sperm parameters were consistent with previous studies conducted in rats [17, 30, 31]. Increased MDA and decreased SOD correlate with a marked decrease in the seminiferous tubular diameter, abnormal spermatogenesis, and maturation arrest [17, 29]. In this study, the Johnson score, which shows the maturation of seminiferous tubules, was reduced in animals that swam in water at 35°C. This decrease may be due to an increase in MDA in testicular tissue, a decrease in SOD, and abnormalities in LH and testosterone levels [30, 32]. Similar to our study swimming of rats in warm water at 41°C for 1 hr daily for 2 weeks resulted in a decrease of serum testosterone, an increase in serum stress markers, and a decrease in the Johnson score [17].

Physical stress activates the HPG axis, which causes an increase in corticosteroid production, leading to a reduction of LH secretion in pituitary or LH receptors in testicular Leydig cells [20, 33]. Additionally, physical stress increases oxidative stress that affects testicular blood vessel contraction, reduces testicular circulation, and results in a reduction of testosterone production by Leydig cells [34]. Testicular circulation is reduced by endurance exercise, which in turn reduces testosterone levels [35]. Another possible explanation for the decrease in testosterone following warm water

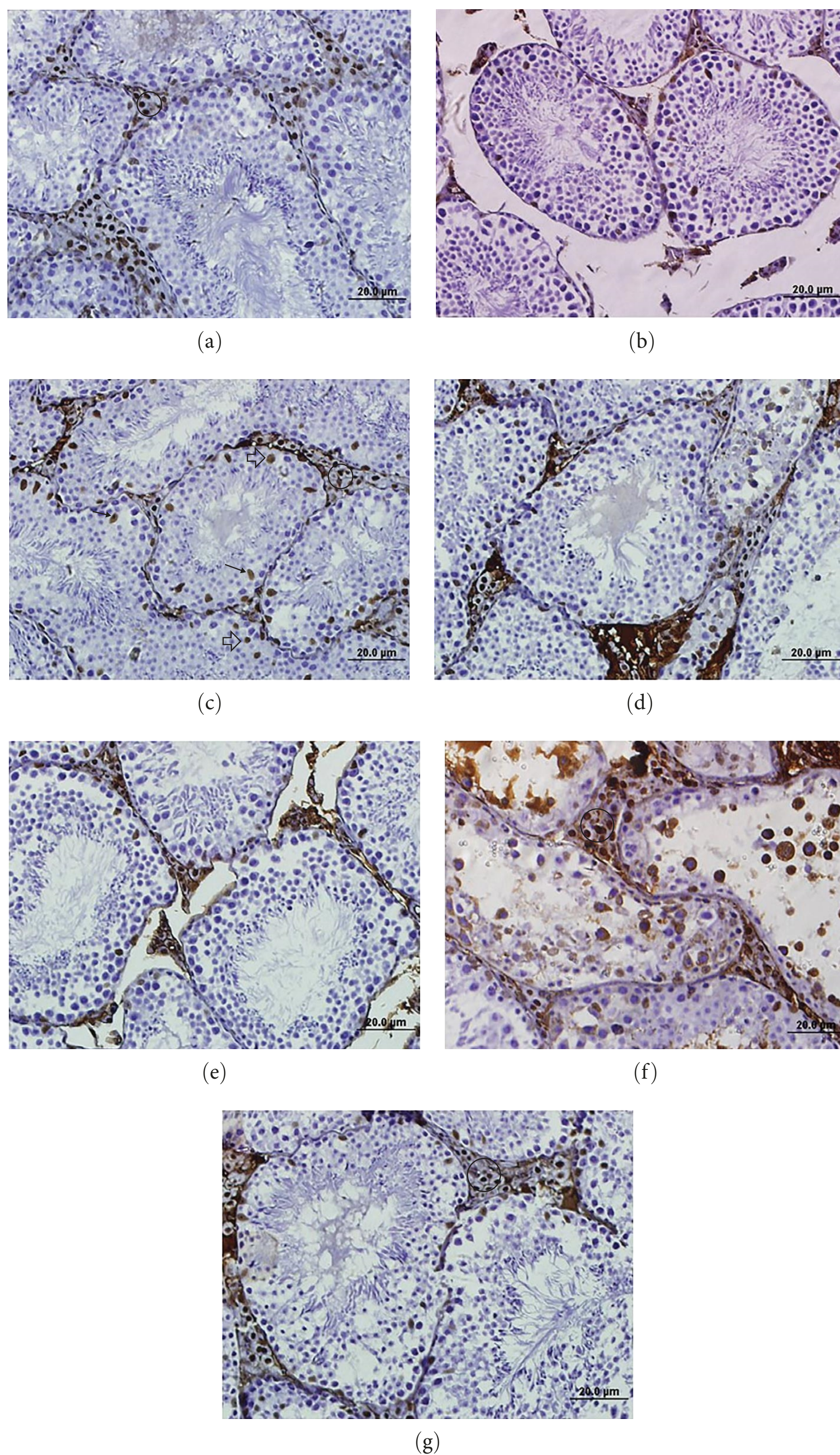

Figure 2: An image showing the immunostaining of mouse testis. Androgen receptor reaction is present in brown cells. (a) Control, (b) solvent (c) VE, (d) swimming at 23°C, (e) swimming at 23°C + VE, (f) swimming at 35°C, and (g) swimming at 35°C + VE. Sertoli cells have a teardrop-like shape as indicated by thin arrows. Germ cells with a round nucleus are depicted in short arrows, and circles depict Leydig cells found in interstitial tissues. 400x.

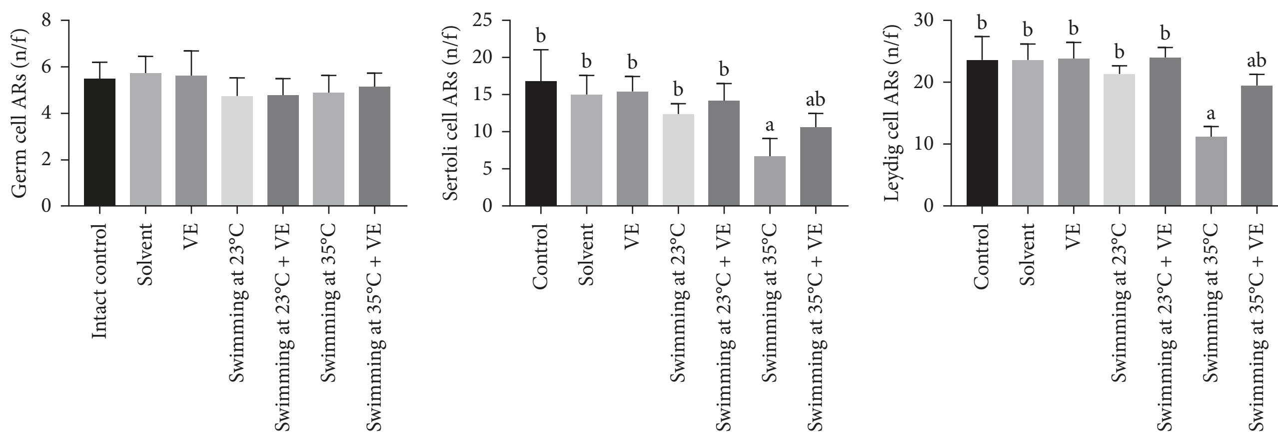

Figure 3: Numbers of androgen receptors (ARs) in different cells of Sertoli, Leydig, and germ in mouse testis. All data are expressed as mean ± SE. (n/f), number per field 2,500 μm^2. “a and b” indicates significant difference between groups ($P < 0.05$).

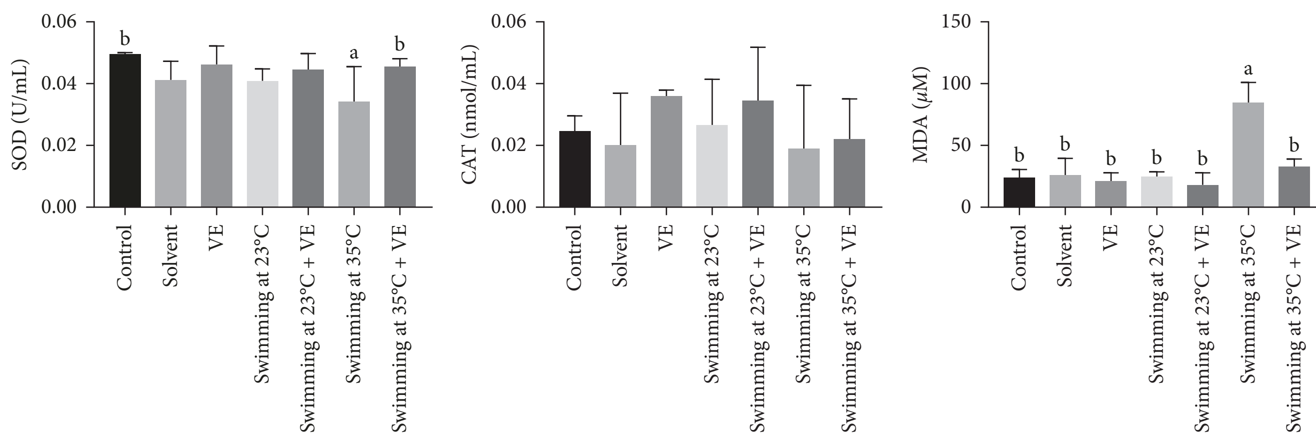

Figure 4: Stress oxidative markers in mouse testicular tissue homogenate. All data are expressed as mean ± SE. “a and b” are significant data with a $P < 0.05$. “a, b” indicates significant difference between groups ($P < 0.05$).

swimming could be the decrease in the activity of steroidogenesis enzymes in Leydig cells [36, 37], disruption in lipid metabolisms [6], and elevation of lipid peroxidation in testicular tissue. Our study showed that these are leading to the elevation of MDA and reduction of SOD, which may confirm that other mechanisms are involved. Oxidative stress itself can suppress steroidogenesis enzyme activity in Leydig cells and therefore reduces the secretion of testosterone. Although testicular MDA and SOD activity was altered in different groups, CAT activity in testicular homogenate did not differ significantly between animals in this study, which could be due to the low CAT activity in testicular tissue and sperms [38]. CAT likely operates inside peroxisomes instead of the cytosol [38]. However, the exact mechanism is unknown.

Our results showed that pretreatment of swimmers' mice with VE significantly protects spermatogenesis, and sperm parameters, and increases testosterone levels. The antioxidant properties of VE are powerful. VE supplementation modulated spermatogenesis and sperm parameters through the effect on the hypothalamic–pituitary–gonadal (HPG) axis and testosterone secretion, improvement of ARs in somatic cells of Sertoli and Leydig cells, and improvement of SOD activity in testis.

The distribution of VE in testicular tissue was detectable in animals receiving VE, specifically in group G. Exercise results in an increase in cardiac rate, output, and blood circulation into the tissues [39]. In this study, the distribution of VE in testicular tissues of mice in group G (swimming at 35°C + VE) was higher than in group E (swimming at 23°C + VE). Maybe in this study, testicular blood flow and hypoxia are increased in mice in group G. Following these changes probably as a compensation process, the distribution of VE is increased in testes. However, the exact mechanism related to more distribution of VE in group G is unknown and needs further studies.

A study on bulls showed the ambient temperature being raised from 5 to 35°C led to an increase in both testicular temperatures and blood flow [40]. Testicular blood flow increased in rams after increasing testicular temperature from 33 to 40°C. Additionally, it tends to enhance oxygen delivery and uptake, without any signs of hypoxia or anaerobic metabolism [41]. According to these previous studies, testicular hyperthermia is not necessarily the cause of testicular hypoxia [40]. The higher distribution of VE in group G could be caused by increased ROS production in testes tissues after being subjected to warm water at 35°C.

Short-term increase in VE in plasma occurs due to exercise. Nevertheless, it gradually diminishes in plasma due to movement inside the tissues like testis that generate more free radicals [42]. It is evident that the testes have high levels of germ cell proliferation and metabolism due to the presence of unsaturated fatty acids. Therefore, testes are the ideal environment to react with ROS [8]. The enhancement of both sperm parameters, spermatogenesis maturity, and testicular antioxidant status was observed with VE supplementation in this study, particularly in animals that were exposed to the forced swimming in warm water. VE, which is a fat-soluble compound, oxidizes instead of polyunsaturated fatty acids in the sperm membrane, which protects it from lipid peroxidation due to oxidative stress [43]. The performance of sperm is enhanced by VE, which reduces lipid peroxidation [44]. During various motility processes, the formation of oxygen-free radicals in the sperm membrane is prevented by VE [45]. In this study, both sperm parameters and spermatogenesis maturity and testicular antioxidant status were enhanced by VE supplementation.

VE's protective effects on spermatogenesis and sperm parameters may be due to the direct stimulation of steroidogenic enzymes in the testis. Gonadotropin synthesis and secretion are both regulated by VE. Additionally, it is a non-enzymatic powerful antioxidant that inhibits free radicals and lipid peroxidation and therefore increases antioxidants [37]. Similar to our study, the protective effects of VE against oxidative stress, following exercise are reported in humans [46], rats [37, 47–50], and mice [51]. The number of Leydig cells decreased in animals after swimming in warm water, as per our findings. Likely, heat stress in rats [52], pigs [53], and ram [54] reduces the number of Leydig cells.

In vitro studies have shown that Sertoli cell secretion modifies the Leydig cell's function [55]. It is known that androgens inhibit the secretion of anti-Müllerian hormone (AMH) by Sertoli cells [55]. AR in humans is found in Sertoli, Leydig, and peritubular myoid cells, round and elongated spermatids [56]. A previous study conducted in mice showed that lack of AR in Sertoli cells caused a reduction of testosterone secretion and impairment of spermatogenesis [57]. *In vivo* studies in S-AR-/y mice, lacking the AR in Sertoli cells, have shown that leads to an increase in the secretion of AMH, affecting the synthesis and secretion of testosterone by Leydig cells [57]. In this study, the numbers of AR in Leydig cells and Sertoli cells were reduced in animals submitted for swimming in warm water while the addition of VE in the latter group increased the number of AR. It may be possible that the level of AMH has increased in the last group although we did not measure it. Our immunohistochemical results showed no AR expression in round or elongated spermatids, which could be due to interspecies differences. Reduction of AR in Leydig cells resulted in hypotestosteronemia and reduced numbers of round spermatids [58], which was also confirmed by our findings. Human studies have shown that there is a relationship between the expression of AR in sperm and sperm parameters in infertile men [13].

Hyperthermia in monkeys led to a decrease in AR expression in Sertoli cells, both in testicular tissue and cultured Sertoli cells [15]. In contrary to our study, hyperthermia in boar testes increased the expression of AR [59]. Species differences and a local form of hyperthermia could be the reason for this contradiction. However, hyperthermia in our study was induced through swimming in warm water.

Based on our findings, pretreatment with VE in animals submitted to swimming in warm water (group G) could alter the AR expression and increase them both in Sertoli and Leydig cells. Our results show that VE can alter the transcription and posttranscription signaling cascade of the AR gene [60, 61]. The exact mechanisms that VE increased AR in animals subjected to swimming in warm water is unknown and further study is needed to confirm that. In a rat model of dehydroepiandrosterone-induced polycystic ovary, VE modulates the AR gene expression [62].

In conclusion, the results of this study showed swimming in water at 23°C is conducive to sperm development, whereas swimming at 35°C impairs spermatogenesis. In nut shell, VE counteracts the negative effects of swimming at 35°C water on sperm development by altering the HPG axis, distribution of VE, AR expression, and lipid peroxidation in testicular tissue.

Abbreviations

VE: Vitamin E
ELISA: Enzyme-linked immunosorbent assay
HPLC: High-performance liquid chromatography
LH: Luteinizing hormone
FSH: Follicle-stimulating hormone
ARs: Androgen receptors
SOD: Superoxide dismutase
MDA: Malondialdehyde
HPG: Hypothalamic–pituitary–gonadal
ROS: Reactive oxygen species
AMH: Anti-Müllerian hormone.

Data Availability

Under reasonable institutional conditions, the author responsible is able to provide the data that support the findings of this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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