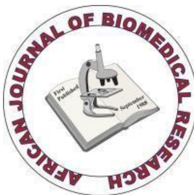

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Research Article

The Ameliorative Role of Ascorbic Acid on Hyperthermia-Induced Testicular Damage

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ABSTRACT
Introduction: The gradual decline in sperm production has become growing concern and subject of widespread debates in the last decades. Factors have been implicated as possible causes of the deterioration of the male reproductive function, including exposure to heat. Ascorbic acid (AA), an antioxidant compound, has been indicated to play protective roles in male reproductive system.
Objective: This study is aimed to evaluate the ameliorative potential of Ascorbic Acid on damages caused by elevated scrotal temperature on Testicular structure and function in rats.
Materials and methods: Forty male Sprague Dawley rats were used and divided into A-D of 10 animals each: Group A served as Control and their testes were exposed to a water bath of 20±2℃, Groups B-D served as low, medium and high doses were exposed to a water bath of 33±2℃, 43±2℃ and 53±2℃ for 60 seconds for a period of 4 weeks respectively. Upon completion, 5 rats from each group were taken and euthanized. The remaining received 100 mg/kg of Ascorbic acid for 28 days and the rats were euthanized.
Results: Heat stress has deleterious effects on male fertility as it negatively impacts seminal parameters, hormonal milieu, oxidative stress, and testicular weight, leading to reduced sperm quality, hormonal imbalance, increased oxidative damage, and testicular atrophy. However, the presence of Ascorbic acid had some ameliorative properties on these adverse effects, which is due to its anti-inflammatory and antioxidants properties.
Conclusion: From this study, the evidence suggests that ascorbic acid supplementation can ameliorate heat-induced testicular damage and holds promise as a therapeutic agent or as a dietary supplement for individuals exposed to heat stress.

Keywords: scrotal hyperthermia, Oxidative stress, Hormonal milieu, testes.

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INTRODUCTION

Heat is a detrimental environmental fortress that disrupts spermatogenesis and results in male infertility. Previous investigations have shown that heat stress reduces the motility, number, and fertilization ability of living spermatozoa (Bromfield *et al.,* 2016). Sperm production requires a temperature that is 2-4 degrees below body temperature since it is extremely sensitive to temperature variations (Wang *et al.,* 2020). The same logic explains why it is that the testicles are located in the scrotum, far from the body's heat source. When exposed to excessive heat, sperm cells typically die. Continuously being exposed to higher temperatures can reduce sperm production or result in the development of sperm cells with irregular shapes, both of which can lead to infertility (Gbotolorun *et al.,* 2017). While assessing male fertility, particular attention is paid to the testicles because they produce

sperm (Adebajo *et al.,* 2023, 2024). Some of the nutrients that are scientifically proven to affect sperm health include antioxidants, such as ascorbic acid. Clinical studies have indicated that eating antioxidant-rich foods can increase sperm health, notably sperm concentration and motility (Walke *et al.,* 2023). However, to date, there is a limited understanding of the potential ameliorative role of ascorbic acid on heat-induced testicular damage. This study aims to address this gap in knowledge by investigating the protective effects of ascorbic acid on heat-induced testicular damage using rat models.

MATERIALS AND METHODS

ETHICAL APPROVAL

Ethical approval was obtained from Research and Ethical Committee of College of Health Science, Bowen University, Iwo campus, Osun State, with a reference number BUI/COHES/ANA/017/023

Experimental Animals

A total of forty adult male Sprague-Dawley rats weighing between 130g ± 20g were used. They were obtained from the animal house of the Department of Physiology, Bowen University and left in well-ventilated plastic cages to acclimatize for a period of two weeks before the commencement of the experiment. The animals were fed with standard rat pellet feed and water *ad libitum.* The body weight of animals was recorded weekly to monitor their development.

Experimental groupings

The animals were divided based on their body weight into four groups of ten animals each. Group A served as the control group and their testes were exposed to a water bath of 20±2°C, Groups B-D served as low, medium and high doses were exposed to a water bath of 33±2°C, 43±2°C and 53±2°C for 60 seconds for a period of 4 weeks respectively. Five animals were randomly selected and euthanized. The remaining five animals left in ach group received 100 mg/kg of Ascorbic acid (AA) for 28 days and were euthanized upon completion of administration. The following parameters were analyzed. One testis was fixed in Bouin's fluid to ascertain the histology of the organ while the other was placed in sample bottles and stored at -20^{0}C to determine the oxidative stress. The cauda epididymis was collected for seminal analysis and blood was also collected for hormonal assay.

Histological Procedures

Testicular tissues were processed for microscopic examination using standard tissue processing protocol and 5µm thick paraffin sections were made. Slides were stained with hematoxylin and eosin stains and photomicrographs were made at a magnification ×400 (Adebajo *et al.,* 2022)

Determination of sperm count, motility and morphology

Fluid was collected from the cauda epididymis by pipette, placed on a glass slide and examined under the microscope at ×400 to determine the sperm count, motility and morphology. The count was determined using a Heamocytometer and was conducted three times on each sample to minimize error. The motility was accessed by systematic scanning of the microscopic field and accessing the sperm encountered. Motility was classified in terms of motile and non-motile spermatozoa. To determine morphology, a smear preparation was made with formal saline and was stained with 1% eosin stain. This was allowed to stain for 20-30 minutes. 100 sperm per animal are morphologically examined at ×100 magnification.

Measurement of Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and Testosterone

The LH, FSH, and testosterone levels were determined using the enzyme-linked immunosorbent assay (ELISA) method. After blood samples were collected and processed to obtain serum, microtiter plates were coated with antibodies that selectively bind to LH, FSH, or testosterone. The serum was then added to the wells, allowing the hormones in the sample to bind to the immobilized antibodies. After incubation and washing steps to remove unbound substances, enzyme-conjugated secondary antibodies that recognize the hormones were added. After another incubation and washing step, a substrate was added, which reacts with the enzyme to generate a color change. The intensity of the color is proportional to the amount of hormone present in the sample. The absorbance of the colored solution is then measured using a spectrophotometer, and the hormone concentrations were determined by comparing the absorbance values to a standard curve generated from known hormone concentrations.

Determination of biological markers

The tissue homogenate was used for the biochemical analysis to detect the presence of Malondialdehyde (MDA), Catalase (CAT) and Superoxide dismutase (SOD). Firstly, MDA was reacted with thiobarbituric acid (TBA), resulting in the formation of a colored product that can be quantified spectrophotometrically. SOD activity was determined using an assay that utilizes the inhibition of a specific reaction. This method measures the ability of SOD to scavenge superoxide radicals. CAT activity was assessed by measuring the decomposition of hydrogen peroxide (H_2O_2) using spectrophotometric methods. The rate of H_2O_2 degradation is directly proportional to CAT activity. In these assays, appropriate controls and standard curves were used for accurate quantification of MDA, SOD, and CAT levels.

Statistics

The data obtained from all the groups were compiled and analyzed statistically using ONE WAY-ANOVA using Graph pad software version 9.5. The results of the data were expressed as mean ± SEM (standard error of mean) where $p<0.05$ was taken as significant.

RESULTS

Effect of elevated scrotal temperature and ascorbic acid on body weight in rats

A significant increase was observed in body weights of all animals in both control and treatment groups before and after administration. (Table 1).

TABLE 1: TABLE SHOWING THE BODY WEIGHT ANALYSIS OF ELEVATED SCROTAL TEMPERATURE AND ASCORBIC ACID GROUPS BEFORE AND AFTER ADMINISTRATION

ELEVATED SCROTAL TEMPERATURE			
GROUPS	Before administration (g)	After administration (g)	%Weight difference
CONTROL (23±2°C)	271.60 ± 4.21	303.00 ± 2.01*	11.56%
LOW (33±2°C)	98.40 ± 2.33	142.20 ± 1.66*	44.51%
MEDIUM (43±2°C)	109.40 ± 3.94	173.25 ± 0.86*	58.36%
HIGH (53±2ºC)	157.40 ± 3.03	189.60 ± 1.17*	20.46%
ASCORBIC ACID (ANTIOXIDANT)			
GROUPS	Before administration (g)	After administration (g)	% Weight difference
CONTROL (23±2°C)	216.40 ± 1.52	241.30 ± 2.72*	11.51%
LOW (33±2°C)	154.00 ± 2.00	241.30 ± 2.72*	17.92%
MEDIUM (43±2°C)	136.40 ± 2.84	175.00 ± 2.60*	28.30%
HIGH (53±2ºC)	170.60 ± 4.01	179.60 ± 2.61*	5.28%

Values are mean ± standard error of mean; n=5, *p<0.05 compared to control.

Effect of elevated scrotal temperature and ascorbic acid on testicular weight

When treatments groups of ascorbic acid group were compared to treatments groups of the elevated scrotal temperature group, a slight decrease was observed in both medium and low doses, however, a slight increase was observed in the high dose group. (Table 2)

TABLE 2: TABLE SHOWING THE ORGAN WEIGHT ANALYSIS OF THE ELEVATED SCROTAL TEMPERATURE AND ASCORBIC ACID GROUPS.

AVERAGE ORGAN (TESTES) WEIGHT		
GROUP	ELEVATED SCROTAL TEMPERATURE	ASCORBIC ACID
CONTROL (23±2°C)	1.33 ± 0.01	0.89 ± 0.01
LOW (33±2°C)	1.04 ± 0.01	0.97 ± 0.01
MEDIUM (43±2°C)	1.24 ± 0.01	1.00 ± 0.01
HIGH (53±2ºC)	0.96 ± 0.01	1.08 ± 0.01

Values are expressed on mean ± standard mean (SEM). ^{a}p<0.05 significant compared to control; ^{b}p<0.05 significant compared with low dose, ^{c}p<0.05 significant compared to medium dose, *p<0.05 significant compared to scrotal temperature (treatment group) (ANOVA test).

Effect of elevated scrotal temperature and ascorbic acid on seminal parameters

Increase in scrotal temperature led to a significant decrease in sperm count, motility and morphology values when compared to control while administration of ascorbic acid led to a significant increase in sperm count, motility and morphology values. When treatment groups for sperm count, motility and morphology values in the antioxidant group and elevated scrotal temperature group were compared, a significant increase in values was observed. (Table 3)

TABLE 3: TABLE SHOWING THE SEMINAL ANALYSIS OF THE ELEVATED SCROTAL TEMPERATURE AND ASCORBIC ACID GROUPS.

ELEVATED SCROTAL TEMPERATURE			
GROUPS	MOTILITY	COUNT	MORPHOLOGY
CONTROL (23±2°C)	93.20 ± 2.24	87.16 ± 2.04	93.34 ± 0.28
LOW (33±2°C)	47.22 ± 0.69^{a}	52.96 ± 1.08^{a}	51.30 ± 0.71^{a}
MEDIUM (43±2°C)	33.00 ± 0.82^{ab}	39.46 ± 0.71^{ab}	43.90 ± 0.30^{ab}
HIGH (53±2ºC)	23.60 ± 1.52^{abc}	21.74 ± 0.26^{abc}	30.52 ± 0.61^{abc}
ASCORBIC ACID (ANTIOXIDANT)			
GROUPS	MOTILITY	COUNT	MORPHOLOGY
CONTROL (23±2°C)	90.22 ± 3.34	94.00 ± 1.16	93.60 ± 1.11
LOW (33±2°C)	56.56 ± 0.52^{a*}	62.78 ± 0.40^{a*}	59.24 ± 0.18^{a*}
MEDIUM (43±2°C)	49.36 ± 0.26^{ab*}	50.22 ± 0.53^{ab*}	55.98 ± 0.45^{ab*}
HIGH (53±2ºC)	39.38 ± 0.47^{abc*}	35.02 ± 0.17^{abc*}	38.18 ± 0.15^{abc*}

Values are expressed on mean ± standard mean (SEM). [a]p<0.05 significant compared to control; [b]p<0.05 significant compared with low dose, [c]p<0.05 significant compared to medium dose, *p<0.05 significant compared to scrotal temperature (treatment group) (ANOVA test)

Effect of elevated scrotal temperature and ascorbic acid on hormonal levels

Exposure to elevated scrotal temperature led to a significant decrease in FSH and Testosterone levels and an increase in LH levels. After administration of ascorbic acid, there a significant increase in FSH and Testosterone levels and a slight increase in LH levels. (Table 4)

TABLE 4: TABLE SHOWING THE HORMONAL MILIEU ANALYSIS OF THE ELEVATED SCROTAL TEMPERATURE AND ASCORBIC ACID GROUPS.

ELEVATED SCROTAL TEMPERATURE			
GROUPS	FSH (mlU m/L)	LH (mlU m/L)	Testosterone (ng m/L)
CONTROL (23±2°C)	1.63 ± 0.04	10.89 ± 0.03	5.30 ± 0.07
LOW (33±2°C)	1.16 ± 0.01[a]	17.91 ± 0.02[a]	3.80 ± 0.05[a]
MEDIUM (43±2°C)	0.74 ± 0.01[ab]	21.12 ± 0.17[ab]	3.37 ± 0.02[ab]
HIGH (53±2°C)	0.33 ± 0.01[abc]	23.83 ± 0.17[abc]	3.11 ± 0.04[abc]
ASCORBIC ACID (ANTIOXIDANT)			
GROUPS	FSH (mlU m/L)	LH (mlU m/L)	Testosterone (ng m/L)
CONTROL (23±2°C)	1.35 ± 0.01	11.27 ± 0.15	5.52 ± 0.05
LOW (33±2°C)	1.24 ± 0.01[a*]	22.13 ± 0.10[a]	4.16 ± 0.02[a*]
MEDIUM (43±2°C)	0.95 ± 0.01[ab*]	23.35 ± 0.19[ab]	3.93 ± 0.03[ab*]
HIGH (53±2°C)	0.59 ± 0.01[abc*]	27.59 ± 0.17[abc]	3.41 ± 0.12[abc*]

Values are expressed on mean ± standard mean (SEM). [a]p<0.05 significant compared to control; [b]p<0.05 significant compared with low dose, [c]p<0.05 significant compared to medium dose, *p<0.05 significant compared to scrotal temperature (treatment group) (ANOVA test)

Effect of elevated scrotal temperature and ascorbic acid on oxidative stress markers

Elevated scrotal temperature caused an increase in Malondialdehyde (MDA) and Superoxide dismutase (SOD) levels and a decrease in Catalase (CAT) values in a dose-dependent manner. Administration of ascorbic acid led to a decrease in MDA and a statistically significant increase in SOD and CAT levels when the treated groups were compared in both elevated scrotal temperature and ascorbic acid groups. (Table 5).

TABLE 5: TABLE SHOWING THE OXIDATIVE STRESS ANALYSIS OF THE ELEVATED SCROTAL TEMPERATURE AND ASCORBIC ACID GROUPS.

ELEVATED SCROTAL TEMPERATURE			
GROUPS	MDA (nmol/mg)	SOD (min/mg)	CAT (nmol/mg)
CONTROL (23±2°C)	268.2 ± 1.08	2.07 ± 2.85	562.3 ± 2.44
LOW (33±2°C)	360.9 ± 0.29[a]	3.20 ± 3.61[a]	511.1 ± 34.64[a]
MEDIUM (43±2°C)	424.8 ± 1.51[ab]	3.97 ± 0.98[ab]	527.6 ± 29.40[ab]
HIGH (53±2°C)	696.5 ± 1.78[abc]	3.89 ± 0.45[ab]	473.3 ± 45.89[abc]
ASCORBIC ACID (ANTIOXIDANT)			
GROUPS	MDA (nmol/mg)	SOD (min/mg)	CAT (nmol/mg)
CONTROL (23±2°C)	268.1 ± 1.12	2.07 ± 0.40	561.1 ± 1.59
LOW (33±2°C)	330.6 ± 5.57[a*]	3.96 ± 0.87[a*]	530.7 ± 3.92*
MEDIUM (43±2°C)	381.9 ± 1.12[ab*]	4.30 ± 1.32[ab*]	557.2 ± 2.21*
HIGH (53±2°C)	497.2 ± 4.74[abc*]	5.21 ± 1.50[abc*]	676.6 ± 3.30[abc]

Values are expressed on mean ± standard mean (SEM). [a]p<0.05 significant compared to control; [b]p<0.05 significant compared with low dose, [c]p<0.05 significant compared to medium dose, *p<0.05 significant compared to scrotal temperature (treatment group) (ANOVA test).

EFFECT OF ELEVATED SCROTAL TEMPERATURE AND ASCORBIC ACID ON TESTICULAR CYTOARCHITECTURE

Effect of elevated scrotal temperature on testicular cytoarchitecture

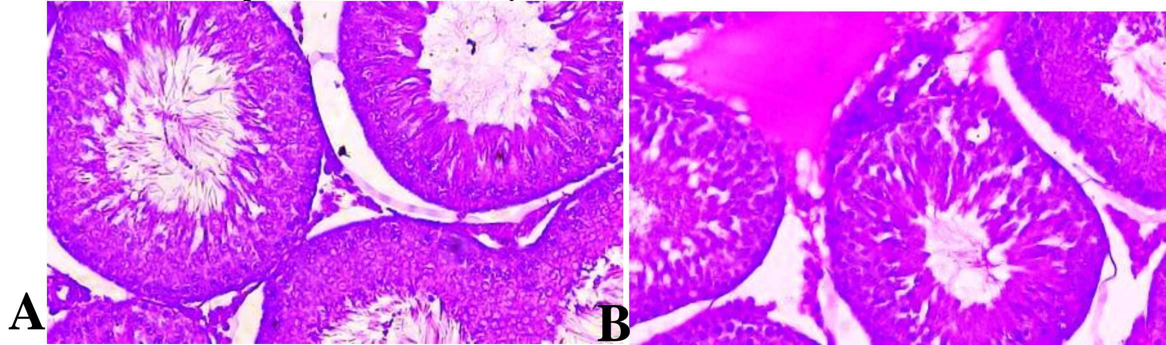

Figure 1 Figure 2

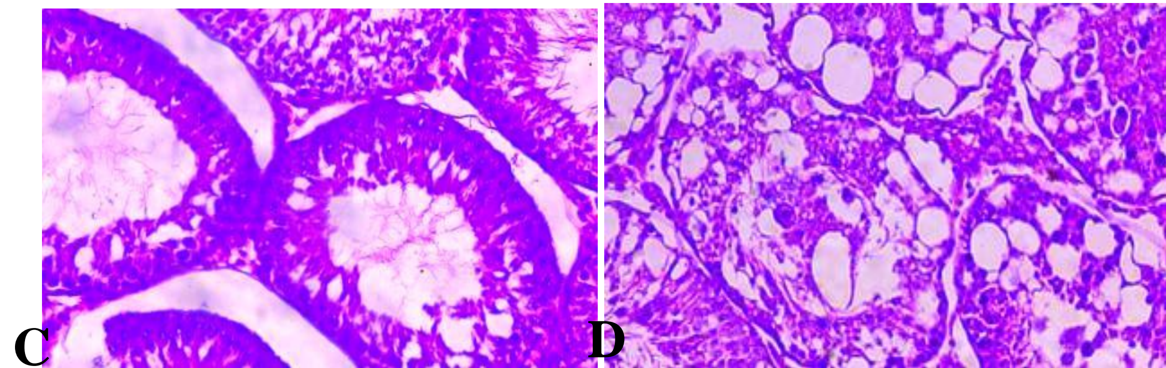

Figure 3 Figure 4

Figure 1: Section of control ((23±2°C) showing normal seminiferous tubules, lumen containing spermatozoa and completely developed germinal cells. The interstitial spaces show normal Leydig cells; Figure 2: Section of low ((33±2°C) showing lumen containing spermatozoa, normal and completely developed germinal cells. The interstitial spaces show normal Leydig cells; Figure 3: Section of medium (43±2°C) showing some degenerated seminiferous tubules exhibiting maturation and lumen devoid of spermatozoa; Figure 4: Section of high (53±2°C) showing poor testicular architecture with several atrophic seminiferous tubules with ceasation of germ cells development and vacuolation with the interstitial spaces show leydig cells hyperplasia.

Effect of ascorbic acid on testicular cytoarchitecture

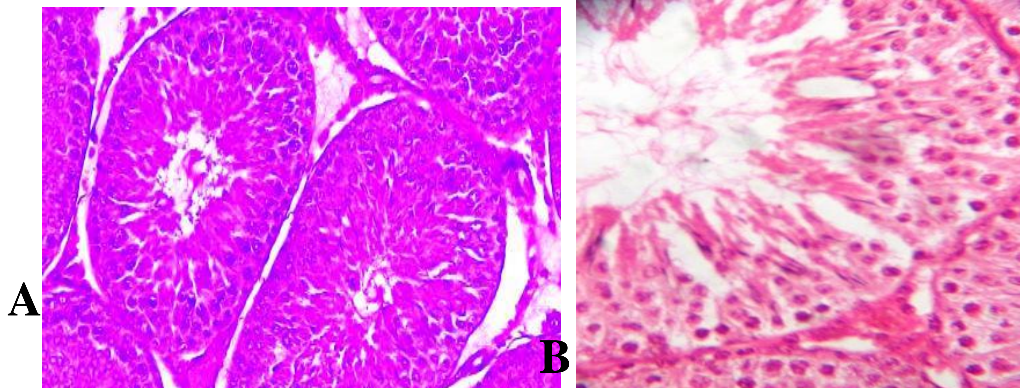

Figure 5 Figure 6

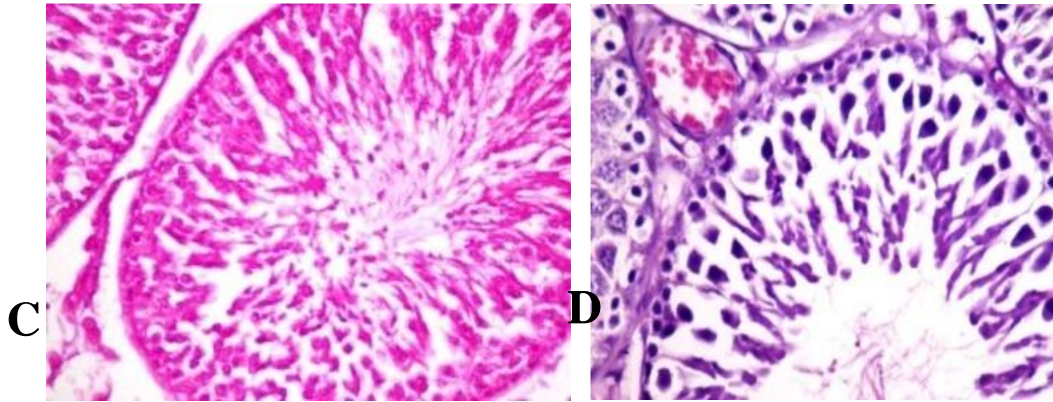

Figure 7 Figure 8

Figure 5: Section of control (23±2°C) showing normal seminiferous tubules showing lumen containing spermatozoa, normal and completely developed germinal cells and the interstitial spaces show normal Leydig cells; Figure 6: Section of low (33±2°C) showing normal seminiferous tubules containing normal maturing germinal cells layer, the spermatogonia cells and sertoli cells appear normal; Figure 7: Section of medium (43±2°C) showing normal testicular architecture with normal seminiferous tubules and normal maturation stages with presence of spermatozoa within their lumen and the interstitial spaces show normal leydig cells; Figure 8: Section of high (53±2°C) showing normal testicular architecture with normal seminiferous tubules and normal maturation stages with presence of spermatozoa within their lumen

DISCUSSION

The rise of male-factor infertility associated with heat-induced testicular damage is a recognized issue supported by scientific evidence (Aldahhan *et al.,* 2021). Numerous studies have explored the impact of heat stress on testicular function and male fertility. Environmental factors, such as hot climates and occupational heat exposure, have been associated with increased risks of male infertility. Research has shown that prolonged exposure to high temperatures, such as those experienced in hot climates, can negatively affect sperm quality and decrease sperm production (Hoang-Thi *et al.,* 2022). Heat stress is known to have detrimental effects on testicular function and sperm production. The testes require a slightly lower temperature than the rest of the body for proper spermatogenesis. Prolonged exposure to high temperatures can disrupt this delicate balance, leading to testicular damage and impaired sperm production (Shahat *et al.,* 2020). Heat-induced testicular damage can manifest as reduced sperm count, poor sperm motility, and abnormal sperm morphology, all of which are factors to male-factor infertility.

The protective effects of ascorbic acid against oxidative stress, inflammation, and hormonal imbalances have been elucidated. This study showed that elevated scrotal temperature negatively impacts seminal parameters. It is associated with decreased sperm concentration, motility, and morphology which can be associated with cessation of spermatogenesis (Kulaksiz *et al.,* 2022). A lower sperm count reduces the chances of successful fertilization as the number of sperm available for reaching the egg decreases (Pizzari *et al.,* 2008). Normal morphology is essential for sperm to penetrate and fertilize the egg effectively (Ombelet *et al.,* 1995). Poor sperm motility hampers the sperm's ability to reach the egg, decreasing the chances of successful fertilization (Pitnick *et al.,* 2020). Ascorbic acid, a potent antioxidant, has been studied for its potential role in improving seminal parameters and mitigating the harmful effects of oxidative stress on sperm (Ahmad *et al.,* 2017). An improvement was observed in sperm concentration, motility, and morphology after ascorbic acid supplementation. These findings suggest that ascorbic acid has a positive impact on seminal parameters.

The development and maintenance of spermatogenesis is dependent on the pituitary gonadotropins; Follicle stimulating hormone, and Luteinizing hormone (Oduwole *et al.,* 2021). Testosterone is required for processes that are critical for spermatogenesis including supporting the completion of meiosis, the adhesion of elongated spermatids to Sertoli cells and the release of sperm (Wang *et al.,* 2022). In the study, scrotal hyperthermia has been proven to significantly reduce the production of FSH, LH and Testosterone. Low levels of these hormones can be at least partly responsible for low sperm counts, which is the biggest cause of infertility in men (Leslie *et al.,* 2024). The regulation of hormonal imbalances by ascorbic acid is another significant aspect of its ameliorative role in heat-induced testicular damage. Studies have shown that ascorbic acid supplementation can restore the levels of reproductive hormones such as testosterone and LH, which are essential for normal testicular function and spermatogenesis (Behairy *et al.,* 2020; Adelakun *et al.,* 2021). This hormonal regulation contributes to the preservation of testicular health and fertility under heat stress conditions. Furthermore, ascorbic acid has been found to regulate hormonal imbalances induced by heat stress. It can modulate the levels of reproductive hormones, such as testosterone and luteinizing hormone (LH), which are essential for normal testicular function and spermatogenesis.

The reduced testicular weight observed after comparing treatment groups to control group in the animals that were subjected to scrotal hyperthermia could be attributed to the fact that higher scrotal temperatures are associated with reduced sperm production and decreased testicular volume. Decreased testicular volume suggests a potential decrease in testicular weight (Jacobsen *et al.,* 2020). Ascorbic acid is involved in the synthesis of collagen, a protein that provides structural support

to various tissues (Boo, 2022). Collagen helps maintain the integrity and elasticity of testicular tissue (Bashiri *et al.,* 2021). Ascorbic acid enhances the activity of antioxidant enzymes, including SOD and CAT which play a crucial role in neutralizing reactive oxygen species and maintaining redox homeostasis within the testes (Sahoo and Chainy, 2023; Sengupta *et al.,* 2024). Elevated scrotal temperatures are associated with oxidative stress in the testes and a reduction in SOD and CAT activities (Gao *et al.,* 2022). Consistent with these findings, direct exposure of spermatogenic cells to elevated temperatures were found to induce high rates of apoptosis via mechanisms that were associated with elevated levels of H_2O_2 generation. In this study, an increase in SOD and CAT activity was observed in the animals that were treated with ascorbic acid which shows that it plays a role in improving oxidative stress in the testes. Also, a significant reduction in MDA activity was observed in the antioxidant treated animals which shows that ascorbic acid helps neutralize reactive oxygen species (ROS) and protect cells from oxidative damage.

Histopathological findings from the animals in the elevated scrotal temperature groups showed poor testicular architecture with several atrophic seminiferous tubules with vacuolation and maturation arrest. The animals in the antioxidant treated group showed normal seminiferous tubules showing lumen containing spermatozoa, normal and completely developed germinal cells and the interstitial spaces showed normal Leydig cells. This provides evidence of possible amelioration. In addition, ascorbic acid exhibits anti-inflammatory properties, reducing the production of pro-inflammatory cytokines and preventing the infiltration of immune cells into the testicular tissues Dutta *et al.,* 2022). This anti-inflammatory effect helps in minimizing tissue damage and promoting the recovery of the testes from heat stress (Zheng *et al.,* 2022).

CONCLUSION

This study provides evidence that ascorbic acid supplementation can reduce or lessen the effects of heat on testicles by reducing oxidative stress, boosting antioxidant defense systems, decreasing inflammation, and controlling hormonal imbalances. Considering the detrimental effects of heat stress on male fertility and the potential of ascorbic acid to mitigate these effects, it holds promise as a therapeutic agent or as a dietary supplement for individuals exposed to heat stress or those experiencing testicular dysfunction. Nevertheless, consulting with healthcare professionals is recommended before initiating any supplementation regimen to ensure individualized and appropriate usage.

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