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Curcumin dose-dependently improves spermatogenic disorders induced by scrotal heat stress in mice

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ABSTRACT

Approximately 20% of couples worldwide are infertile and about half of these have male infertility. Therefore, it is important to develop effective strategies to prevent male infertility. In this study, we examined the effects and regulatory mechanisms of curcumin, an active ingredient in the traditional herbal treatment derived from the dietary spice turmeric (*Curcuma longa*), on exogenous scrotal heat stress-induced testicular injuries in mice. Adult mice were orally administered three different doses of curcumin (20, 40, or 80 mg/kg/day) for 14 consecutive days and then subjected to transient scrotal heat stress at 43°C for 20 min on day 7. The testes and blood of the mice were collected on day 14. Mice exposed to heat stress showed low testicular weight, severe vacuolization of seminiferous tubules followed by loss of spermatogenic cells, and appearance of multinucleated giant cells and degenerative Leydig cells. In addition, great changes in oxidative stress (lipid peroxidation, superoxide dismutase (SOD) activity, cytoplasmic SOD, mitochondrial SOD, and phospholipid hydroperoxidase glutathione peroxidase mRNAs), apoptosis (B-cell lymphoma-extra large and caspase 3 mRNAs), heat shock reaction (heat shock transcription factor-1 and transforming growth factor- β 1 mRNAs) and androgen biosynthesis (testosterone concentration and 3 β -hydroxysteroid dehydrogenase mRNA) were observed. However, these all testicular injuries induced by the scrotal hyperthermia were significantly improved by curcumin treatment (20, 40 and 80 mg/kg) in a dose-dependent manner via its antioxidative, anti-apoptotic and androgen synthesis effects, indicating that it has the potential for prevention of male infertility.

Keywords: Heat stress; Spermatogenic disorder; Curcumin; Prevention; Regulatory mechanism

1. Introduction

In mammals, testicular temperature is a few degrees lower than that of the core body to maintain an optimum environment for spermatogenesis.¹ In addition to the scrotum, a second thermoregulatory system is located in the spermatic cord, where there is counter-current heat exchange between arterial blood and venous blood.² The pathological dilation of testicular veins and the pampiniform plexus, which is usually referred to as the varicocele, can elevate intrascrotal temperatures and is thus frequently associated with decreased pregnancy rates among infertile couples.^{3,4} Scrotal heat stress (HS) can result in disruption of the seminiferous epithelium, accumulation of lipids in Sertoli cells, local dilations of the intercellular spaces between Sertoli cell junctions, and increased apoptotic rate.⁵ Slight increases in testicular temperature can disturb spermatogenesis and ultimately cause problems in fertility such as germ cell apoptosis owing to endogenous cryptorchidism and exogenous scrotal HS.^{6,7} Testes have a variety of mechanisms that are triggered upon exposure to HS, including heat shock response, DNA repair, oxidative stress response, apoptosis and cell death.⁸ Therefore, it is important to reduce spermatogenic disorders and infertility caused by abnormal increases in testicular temperature induced by endogenous and exogenous hazards to ensure the production of healthy sperm.

Curcumin [1,7-bis (4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3, 5-dione; diferulylmethane] is the active ingredient in the traditional herbal treatment derived from the dietary spice turmeric (*Curcuma longa*). Curcumin has many beneficial activities, including antioxidant⁹, anticancer^{10,11}, anti-inflammatory¹², antidepressant¹³, antimicrobial¹⁴, antimutagenic¹⁵, and anti-human immunodeficiency virus properties¹⁶. Phenolic groups in the structure of curcumin are also essential to its ability to eliminate oxygen derived free radicals¹⁷ and superoxide radicals.¹⁸

Approximately 20 percent of modern couples are infertile worldwide, and about half of these couples have been suffered from male infertility.¹⁹ Therefore, it is important to develop effective preventive strategies for male infertility. As shown in above reports, scrotal hyperthermia can induce severe oxidative damage on spermatogenesis, but curcumin might have potent antioxidant activities in disease control of human and animals. In this study, we examined the preventive dose level and regulatory mechanism of curcumin on exogenous scrotal HS-induced testicular injuries in mice.

2. Materials and methods

2.1 Animals

Eight-week-old ICR male mice (Orient Bio, Gyeonggi, South Korea) were used in this study. The animals were housed in polycarbonate cages in a well-ventilated room maintained at $21 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ relative humidity on a 12 hour light/dark cycle. The animals were fed standard mouse chow (Samyang Ltd., Incheon, South Korea) and tap water *ad libitum* throughout the experimental period. All experiments were approved by the Chungbuk National University Animal Care Committee and carried out according to the Guide for Care and Use of Animals (Chungbuk National University Animal Care Committee, CBNUA-469-13-02).

2.2 Chemicals

Curcumin and olive oil were purchased from Sigma Chemical Company (St. Louis, MO, USA) and Junsei Chemical Company (Tokyo, Japan).

2.3 Experimental design

The best effective quantity and route of curcumin were determined by our preliminary experiments based on the previous reports^{9,13}. Curcumin was dissolved in the olive oil to give concentrations of 20, 40 and 80 mg/kg body weight. The mice were randomly divided into five experimental groups (n=20 per group), a normal control group treated with vehicle (Con), HS alone group (HS), HS plus curcumin 20 mg/kg administration group (HS+Cur20), HS plus curcumin 40 mg/kg administration group (HS+Cur40) and HS plus curcumin 80 mg/kg administration group (HS+Cur80). Starting 1 week before scrotal heat exposure, all substances were administered by intragastric intubation at 4:00 pm., and treatment was conducted once a day for consecutive 14 days. On day 7, mice were subjected to a single HS at 43°C for 20 min under intramuscular anesthesia using Zoletil (Virbac, Carros, France). For HS exposure, the lower half of the torso of each animal, including the scrotum, tail and hind legs was submerged in a water bath. After returning to their cages, the animals were placed on a warm mat (25°C) to maintain their body temperature until they had fully recovered from the anesthesia. At 7 days after heat exposure, all animals were sacrificed under inhalational diethyl ether anesthesia. Thereafter, testes and blood samples were collected and stored at -80°C until further use for biochemical and histopathological investigations.

2.4 Body and testes weight

The body weights were daily measured from the first day to the terminal day (prior to sacrifice) of the experimental period. Testicular weights of each animal were also recorded after isolating the testes from the surrounding fat.

2.5 Histopathology

Testes were removed, fixed in Bouin's solution and processed according to routine histological techniques. Paraplast-embedded tissues were sectioned to 4 μm thick, then stained with hematoxylin and eosin (H&E) for histopathological evaluation.

2.6 Lipid peroxidation

Lipid peroxidation was evaluated according to the thiobarbituric acid-reactive species (TBARS) levels. Briefly, malondialdehyde (MDA) levels in the testes were measured spectrophotometrically by determining the TBARS concentration and expressed as nmol/mg protein. Testes in each group were homogenized in chilled 10 mM phosphate buffer and then mixed thoroughly with a solution containing 8.1% sodium dodecyl sulfate, 20% acetic acid, and 0.75% 2-TBA. Next, the solution was heated at 95°C for 30 min in an oven. After cooling, the flocculent was removed by centrifugation at 3,500 g for 15 min. The absorbance of the supernatant was then measured at 532 nm using a spectrophotometer and compared to the values for the prepared 1,1,3,3-tetramethoxypropane standard curve. The protein content of the testes was measured according to the Bradford method using bovine serum albumin as the standard. Data from five independent experiments were analyzed.

2.7 Superoxide dismutase (SOD) activity

Total SOD activity was assayed using a SOD assay kit-WST (Dojindo Laboratories, Kumamoto, Japan). Briefly, the mouse testes were homogenized and protein concentrations of the supernatants were measured using the Bradford method.²⁰ The supernatants were then incubated with an assay solution containing xanthine, xanthine oxidase, and a water-soluble tetrazolium salt, WST-1. Superoxide free radicals generated from the xanthine substrate by xanthine oxidase reduced WST-1 to WST-1 diformazan, which produces a maximum absorbance at 450 nm. SOD in the testes inhibited WST-1 reduction since they catalyzed the dismutation of superoxide ions into molecular oxygen and hydrogen peroxide. The reduction of WST-1 was measured spectrophotometrically at 450 nm. SOD activity was calculated as an inhibition rate for which 1 U was defined

as a 50% decrease from the control value over a period of 30 min at 37°C. The results were presented as the specific activity calculated as the total SOD activity per testis divided by the total amount of protein per testis. Data from five independent experiments were analyzed.

2.8 Quantitative real-time PCR

Total RNA was isolated from mouse testes using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA concentrations were determined based on the UV absorbance. Next, 2 µg of total RNA were reverse-transcribed using random primers and High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out in a 20 µL reaction using SYBR Green Master Mix (Applied Biosystems) and testes cDNA (1.6 µg) as the template. Reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Gene-specific primers were designed by TIB Mol-Bio Synthesis (Berlin, Germany). Primer sets were used to amplify mouse cytoplasmic Cu/Zn SOD (SOD1), mitochondrial manganese SOD (SOD2), phospholipid hydroperoxide glutathione peroxidase (GPx4), B-cell lymphoma-extra large (Bcl-xL), caspase 3, heat shock protein factor 1 (HSF1), transforming growth factor-β1 (TGF-β1) and 3β-hydroxysteroid dehydrogenase (3β-HSD). GAPDH primers were used as an internal standard to normalize target transcript expression. Data from nine independent runs were analyzed using the comparative Ct method.²¹

2.9 Testosterone concentration

Blood was centrifuged for 14 min at 1000 g. The supernatant was then transferred to new tubes and stored at -70°C. The testosterone concentration of serum was assayed using a mouse testosterone ELISA Kit (Cusabio, Hubei, China) based on the absorbance at 450 nm.

2.10 Statistical analysis

Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was established at $P < 0.05$. All data were expressed as the means ± standard error of the mean (SEM). All analyses were conducted using SPSS for Windows software, version 10.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Body and relative testes weights

Mouse body weights in all groups increased steadily until the end of the experiment, but these changes were not significant (data not shown). Figure 1A shows the ratio of relative testes weight to that of the normal control group. The relative testes weight ($38.9 \pm 2.87\%$) in the HS group decreased significantly relative to the control group (100%). However, the testes weight ratios in all curcumin treatment groups (20, 40 and 80 mg/kg of curcumin) were significantly higher than that of the HS group, and these increases occurred in a dose-dependent manner ($49.8 \pm 6.66\%$, $68.4 \pm 5.36\%$, and $81.9 \pm 7.07\%$, respectively at $P < 0.05$).

3.2 Lipid peroxidation

Lipid peroxidation was measured based on TBARS assays of the MDA levels. As shown in Figure 1B, mouse testes exposed to HS exhibited significant increases in MDA level (14.1 ± 1.92 nmol/mg) relative to the control group (5.7 ± 1.12 nmol/mg at $P < 0.05$). However, co-treatment with curcumin (40 and 80 mg/kg) led to a remarkable decrease in the increase of MDA levels induced by HS (9.6 ± 1.02 and 6.0 ± 1.80 nmol/mg, respectively; $P < 0.05$), with levels similar to controls being observed in response to treatment with high levels of curcumin.

3.3 Histopathological changes in testes

The testes of control mice showed normal morphology with dynamic spermatogenesis (Fig. 2A). Heat stress caused severe damage to the seminiferous tubules, which showed disordered and hollow structures. The atrophic seminiferous epithelium with a large proportion of tubules showed signs of degeneration, vacuolization and disorganization. Disarray and desquamation of the spermatogenic cells, Sertoli cells and Leydig cells were also observed (Fig. 2B and C). However, administration of curcumin to heat-treated groups induced a significant improvement in histopathological findings in a dose-dependent manner (Fig. 2D-F). Moreover, the testicular structure in the HS plus curcumin 80 mg/kg treatment group was almost completely restored to normal, with well-preserved tubular morphology and many tubules containing abundant spermatocytes and spermatids (Fig. 2F).

3.4 Superoxide dismutase activity

Mouse testes exposed to HS exhibited significantly reduced SOD activity (0.70 ± 0.10 U/mg) relative to the control group (0.95 ± 0.19 U/mg at $P < 0.05$). However, curcumin treatment (20, 40 and 80 mg/kg) with HS significantly increased the SOD activity (0.85 ± 0.05 , 0.92 ± 0.07 and 0.97 ± 0.11 U/mg, respectively) relative to the HS group at $P < 0.05$ (Fig. 3A).

3.5 Gene expression patterns of antioxidant enzymes

The cytoplasmic SOD1 mRNA level in mouse testes exposed to HS was 0.85-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular SOD1 mRNA levels (1.26-fold, 1.30-fold and 1.40-fold that of control, respectively) were significantly higher than those of the HS group at $P < 0.05$ (Fig. 3B).

The mitochondrial SOD2 mRNA level in mouse testes exposed to HS was 0.37-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular SOD2 mRNA levels (0.81-fold, 0.84-fold and 0.93-fold that of the control, respectively) were significantly higher than that of the HS group at $P < 0.05$ (Fig. 3C).

The GP \times 4 mRNA level in mouse testes exposed to HS was 0.33-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular GP \times 4 mRNA levels (0.90-fold, 0.99-fold and 1.03-fold that of the control, respectively) were significantly higher than that of the HS group at $P < 0.05$ (Fig. 3D).

3.6 Expression patterns of apoptosis-related genes

The Bcl-xL mRNA level in mouse testes exposed to HS was 0.31-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular Bcl-xL mRNA levels (0.69-fold, 0.71-fold and 0.76-fold that of control, respectively) were significantly greater than that of the HS group at $P < 0.05$ (Fig. 4A).

The caspase 3 mRNA level in mouse testes exposed to HS was 2.70-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular caspase 3 mRNA levels (1.76-fold, 1.52-fold and 1.51-fold that of the control, respectively) were significantly lower than that of the HS group at $P < 0.05$ (Fig. 4B).

3.7 Expression of HSF-1 mRNA

The HSF-1 mRNA level in mouse testes exposed to HS was 0.35-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular HSF-1 mRNA levels (1.09-fold, 1.29-fold and 1.45-fold that of control, respectively) were significantly higher than that of the HS group at $P < 0.05$ (Fig. 4C).

3.8 Expression of TGF- β 1 mRNA

The TGF- β 1 mRNA level in mouse testes exposed to HS was 2.97-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, the testicular TGF- β 1 mRNA levels (2.25-fold, 2.16-fold and 1.95-fold that of control, respectively) were significantly lower than that of the HS group at $P < 0.05$ (Fig. 4D).

3.9 Expression patterns of 3 β -HSD mRNA and testosterone concentrations

To evaluate whether Leydig cells act normally in response to exogenous HS exposure, the expression of 3 β -HSD mRNA was examined in the testes after HS with or without treatment of curcumin using a type VI isoform of 3 β -HSD. The 3 β -HSD mRNA level in mouse testes exposed to HS was 0.33-fold that of the control group (1-fold). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, 3 β -HSD mRNA levels (1.47-fold, 1.51-fold and 1.61-fold that of the control, respectively) were significantly greater than that of the HS group at $P < 0.05$ (Fig. 5A).

The testosterone concentration in the serum of the HS group was significantly reduced (0.07 ± 0.01 ng/ml) relative to the control group (0.3 ± 0.07 ng/ml). However, when mice were treated with curcumin (20, 40 and 80 mg/kg) and HS, testosterone concentrations were significantly recovered to 0.2 ± 0.06 , 0.2 ± 0.12 , and 0.3 ± 0.08 ng/ml, respectively ($P < 0.05$; Fig. 5B).

4. Discussion

People are commonly exposed to local testicular HS, which affects sperm count, motility and morphology and results in male infertility.²² Therefore, preventive and therapeutic agents from dietary materials are needed to reduce HS-induced reproductive toxicity. In the present study, we measured the preventive and therapeutic dose levels and regulatory mechanism of orally administered curcumin against HS-induced testicular injuries in mice.

The treatment of curcumin (20, 40 and 80 mg/kg) dose-dependently improved the spermatogenic disorders induced by scrotal HS in mice.

Heat stress causes desquamation of germ cells from seminiferous tubules, as well as degeneration of spermatogenic cells and reduction in testicular weight.⁶ In the present study, scrotal HS led to a decrease in testes weight and considerably smaller testes, but the reduced testicular weight due to HS was significantly recovered by administration of curcumin (20, 40 and 80 mg/kg), and this recovery occurred in a dose-dependent manner. Furthermore, some nuclear condensation of spermatogenic cells within the seminiferous epithelium, disordered and hollow seminiferous tubules, disturbance of the cellular arrangement in the seminiferous tubules and disappearance of spermatocytes and spermatids were observed in the testes of the HS group after scrotal hyperthermia (43°C, 20 min). However, pre- and post-treatment of curcumin with HS induced repair morphology patterns, as indicated by increased spermatocytes and spermatids that occurred in a dose-dependent manner, with almost full recovery occurring following treatment with 80 mg/kg curcumin. These findings indicate that curcumin can prevent the spermatogenic cell death induced by scrotal hyperthermia.

In the present study, mouse scrotal HS increased lipid peroxidation and decreased SOD activity in the testes. However, when mice were pre- and concurrently treated with HS and curcumin, these oxidative stress responses and decreased antioxidant enzyme levels were recovered to the control levels. A previous study revealed that curcumin has the ability to inhibit superoxide anion and hydroxyl radical generation by preventing oxidation.¹⁷ Therefore, curcumin might improve the SOD status and scavenge the reactive oxygen species generated by HS in the testes.

SOD plays an important antioxidant enzyme role in the testes.²³ Recent studies revealed that high levels of Zn and SOD1 protect spermatogonia from oxidative stress²⁴ and GPx4 reduces hydrogen peroxide to H₂O via glutathione oxidation²⁵, which is mainly expressed in spermatids and spermatozoa.²⁶ In the current study, HS led to a significant decrease in SOD1, SOD2 and GPx4 gene expressions and SOD activity in mouse testes, but their reduced expression levels were restored by co-treatment with curcumin in a dose-dependent manner. These results indicate that curcumin could protect the spermatogenic cells from exogenous HS via activation of the representative antioxidative enzymes SOD and GPx4.

Previous studies have confirmed that heat exposure by immersing the scrotum in a hot water bath at 43°C for 20 min results in germ cell apoptosis in the testes.^{7,27} In the current study, Bcl-xL, one of the anti-apoptotic proteins, decreased remarkably and caspase 3, a marker of cell apoptosis, increased significantly in the testes

following HS. However, this apoptotic damage induced by heat exposure was prevented by co-treatment with curcumin in a dose-dependent manner. These results indicate that curcumin protects the testes via an anti-apoptotic effect.

HSF1, which is known to be a major regulator of stress-inducible response, is characterized by rapid and transiently increased expression of genes encoding heat shock proteins.²⁸ HSF1 exerts two opposite roles in testes during HS: anti-apoptotic in mitotic spermatogonia²⁹ and proapoptotic in meiotic male germ cells.³⁰ In the present study, HS mainly destructed spermiogenic cells and further significantly decreased HSF1 gene expression in the testes, but this reduced level was increased by cotreatment with curcumin in a dose-dependent manner. These findings indicate that curcumin may exert anti-apoptotic function against HS-induced spermiogenic damage via modulation of HSF1 gene expression.

TGF- β 1 is a member of the transforming growth factor beta superfamily that is a secreted protein involved in many cellular functions, including control of cell growth, proliferation, differentiation and apoptosis.³¹ TGF- β 1 is observed in round spermatids and Leydig cells of matured mouse testes over 50 days of age.³² The levels of TGF- β 1 were shown to increase greatly in response to rat testicular damage induced by streptozotocin.³³ In the current study, HS significantly increased the gene expression of TGF- β 1 in the testes, but curcumin significantly reduced the mRNA level of TGF- β 1 overexpressed by HS. These results suggest that curcumin may protect the testes from heat-induced damage via regulation of TGF- β 1 gene expression; however, additional studies are needed to confirm this.

Testosterone is primarily secreted by interstitial Leydig cells in the testes, although smaller amounts are produced by the adrenal gland in both sexes.³⁴ Leydig cells, like all other steroid-producing cells, synthesize steroid hormones from cholesterol.³⁵ When cholesterol is delivered to the inner mitochondrial membrane, it is converted to pregnenolone by cytochrome P450 cholesterol side-chain cleavage enzyme. Pregnenolone is further metabolized to progesterone by mitochondrial or microsomal 3 β -HSD.³⁶ The synthesis of testosterone in LCs is dependent on the expression of highly regulated genes such as 3 β -HSD and 17 β -HSD.³⁷ In the present study, the 3 β -HSD mRNA level was significantly decreased by HS in the testes, but co-treatment with curcumin significantly increased the 3 β -HSD mRNA level relative to the HS group. Furthermore, curcumin significantly recovered the serological testosterone levels reduced by HS in a dose-dependent manner. These results indicate that curcumin could repair the Leydig cells damaged by HS via modulation of the 3 β -HSD gene and consequently promote androgen biosynthesis for spermatogenesis.

In conclusion, the results of the present study indicate that testicular dysfunction induced by scrotal hyperthermia could be ameliorated by curcumin treatment (20, 40 and 80 mg/kg) in a dose-dependent manner via its antioxidative, anti-apoptotic and androgen synthesis effects, suggesting that it has the potential for prevention of male infertility.

Abbreviations

Bax	BCL2-associated X protein
Bcl-xL	B-cell lymphoma-extra large
GPx4	Phospholipid hydroperoxide glutathione peroxidase
HSF1	Heat shock transcription factor 1
HS	Heat stress
MDA	Malondialdehyde
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
3 β -HSD	Three beta-hydroxysteroid dehydrogenase
TGF- β 1	Transforming growth factor-beta 1

Acknowledgements

This research was supported by Basic Science Research Program (2013R1A2A2A03016519 and 2014R1A1A3052735) and the Priority Research Centers Program (2015R1A6A1A04020885) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology.

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Figure captions

Fig. 1 Relative testes weight ratios (A) and protective effects of curcumin (Cur) against oxidative damage induced by heat stress (HS) in mouse testes (B). Adult mice were administered 3 different doses of Cur (20, 40, or 80 mg/kg/day) orally for 14 consecutive days. During treatment, mice were subjected to transient scrotal HS at 43°C for 20 min on day 7, and their testes were collected on day 14. The testes weight in the HS group decreased significantly relative to the control group, but the testes ratios in all Cur cotreatment groups were significantly higher than that of the HS group, and these increases were dose-dependent (A). Lipid peroxidation was evaluated by malondialdehyde (MDA) concentrations (B). Mouse testes exposed to HS alone exhibited a significant increase in MDA level relative to the control group. However, co-treatments with Cur led to a remarkable reduction in this MDA level in a dose-dependent manner, with the highest dose resulting in almost normal control levels. The results are the means \pm SEM (n = 5). Significant differences among each treatment group vs. normal control (Con; a) or HS (b) group as evaluated by one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$.

Fig. 2 Representative histopathological findings of mouse testes subjected to heat stress (HS) with or without curcumin (20, 40, and 80 mg/kg; D-F) administration. The control group showed normal testicular morphology and spermatogenesis (A). In the HS group, the arrangement of cells was disturbed in the seminiferous tubules, which were much smaller in diameter than in the normal control group. The epithelium with a large proportion of tubules showed signs of degeneration, vacuolization and disorganization. Additionally, disarray and desquamation of spermatogenic cells characterized the extent of HS injury (B and C). However, curcumin administration (D-F) to heat-treated groups induced a significant improvement in a dose-dependent manner relative to the HS group. H&E staining (Bar: 100 μ m).

Fig. 3 Antioxidative effects of curcumin (Cur; 20, 40 and 80 mg/kg) against heat stress (HS)-induced oxidative stress. (A) Superoxide dismutase (SOD) activity levels. (B-D) Quantitative real-time PCR analyses of antioxidant enzyme mRNA levels in mouse testes induced by HS in the presence or absence of curcumin. (B) cytoplasmic superoxide dismutase (SOD1), (C) mitochondrial SOD (SOD2), (D) phospholipid hydroperoxide

glutathione peroxidase (GPx4). Results are the means \pm SEM (n=5). a; vs. normal control (Con) and b; vs. HS at $P < 0.05$. GAPDH was used as an internal standard to normalize target transcript expression.

Fig. 4 Quantitative real-time PCR analyses of anti-apoptotic B-cell lymphoma-extra large (Bcl-xL; A), apoptotic caspase 3 (B), heat shock protein factor 1 (HSF1; C), and transforming growth factor- β 1 (TGF- β 1; D) in mouse testes exposed to heat stress (HS) in the presence or absence of curcumin (Cur: 20, 40 and 80 mg/kg). Results are the means \pm SEM (n=5). a; vs. normal control (Con) and b; vs. HS at $P < 0.05$. GAPDH was used as an internal standard to normalize target transcript expression.

Fig. 5 Testicular 3 β -hydroxysteroid dehydrogenase (3 β -HSD; A) mRNA and serological testosterone concentration (B) levels in mice after heat stress (HS) with or without treatment of curcumin (Cur: 20, 40 and 80 mg/kg). Testosterone concentration and 3 β -HSD mRNA level were significantly decreased in the HS group. However, curcumin supplementation significantly recovered these decreases to levels similar to the control group. Results are the means \pm SEM (n=5). a; vs. normal control (Con) and b; vs. HS at $P < 0.05$. GAPDH was used as an internal standard to normalize target transcript expression.

Table 1 Primers used for quantitative real-time PCR analysis

Gene	Accession no.	Primer (5'-3')	
		Forward	Reverse
GAPDH	NM_008084	CGTGCCGCCTGGAGAAACC	TGGAAGAGTGGGAGTTGCTGTTG
GPx4	NM_008162	TAAGAACGGCTGCGTGGT	GTAGGGGCACACACTTGTAGG
SOD1	NM_011434	TGCGTGCTGAAGGGCGAC	GTCCTGACAACACAACCTGGTTC
SOD2	NM_013671	GGAGCAAGGTCGCTTACAGA	GTGCTCCCACACGTCAATC
Bcl-xL	NM_009743	TGACCACCTAGAGCCTTGGGA	TGTTCCCGTAGAGATCCACAA
Caspase 3	NM_009810	AAAGCCGAAACTCTTCATCAT	GTCCCACTGTCTGTCTCA
TGF β 1	NM_011577	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
HSF1	NM_008296	ACTCTGGTCCGTGTCAAGCAA	GGATGGAGTCAATGAAGGCAGTT
3 β -HSD	NM_153193	GACTGCTGACACACCACAC	GGGAGTGAGGTAACTTAATGTACG

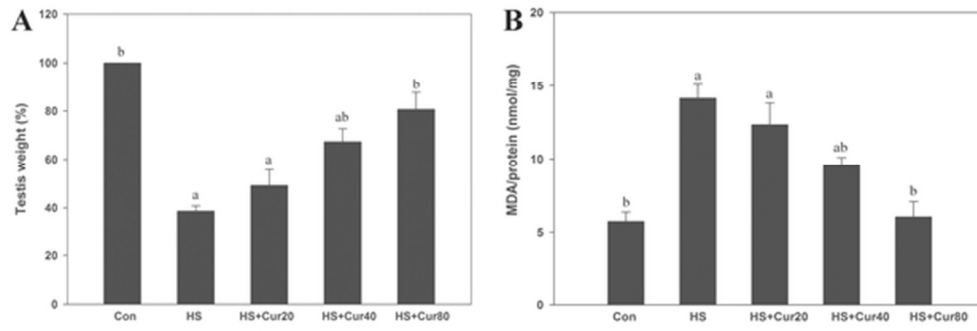
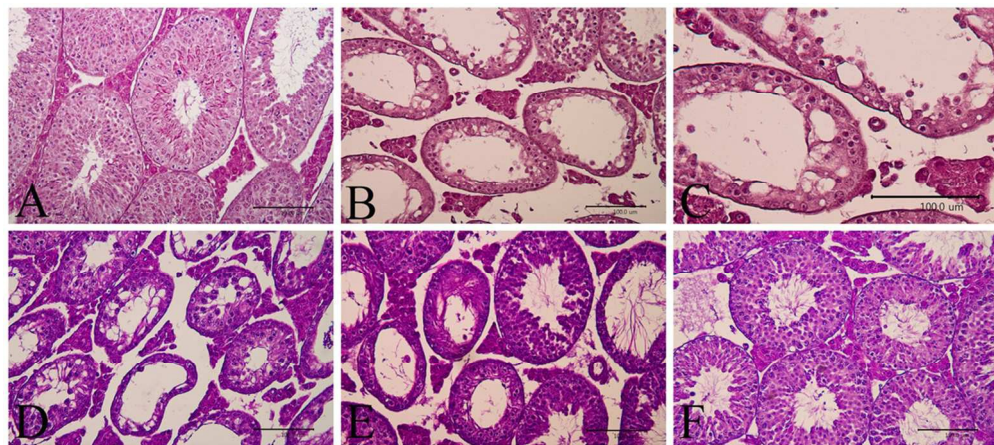


Fig 1
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97x43mm (300 x 300 DPI)

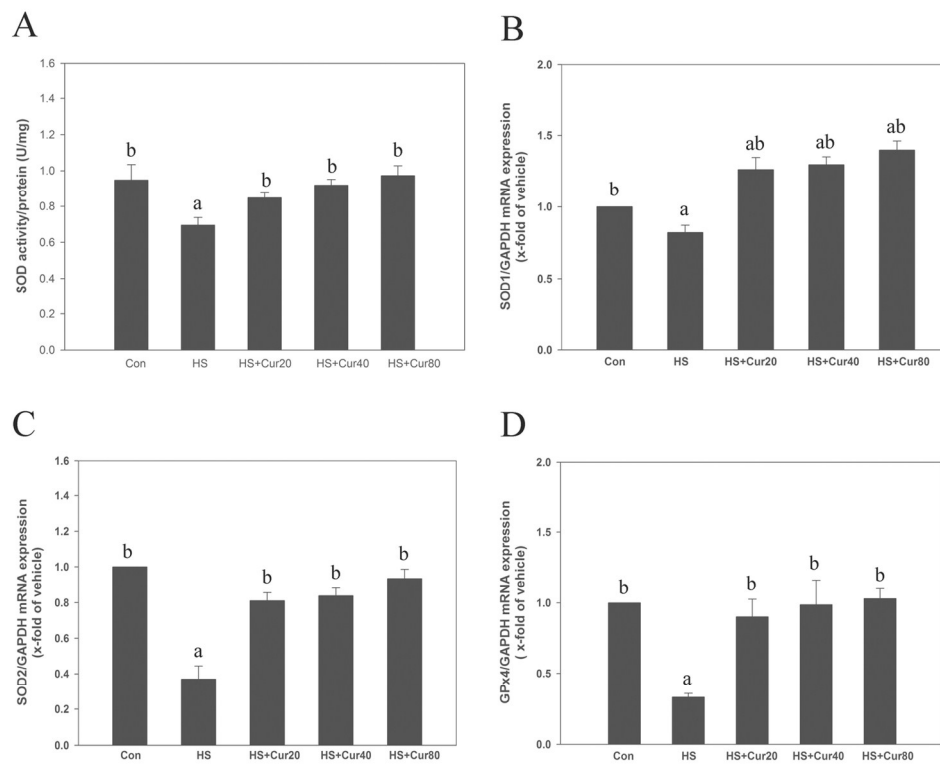


Fig 3
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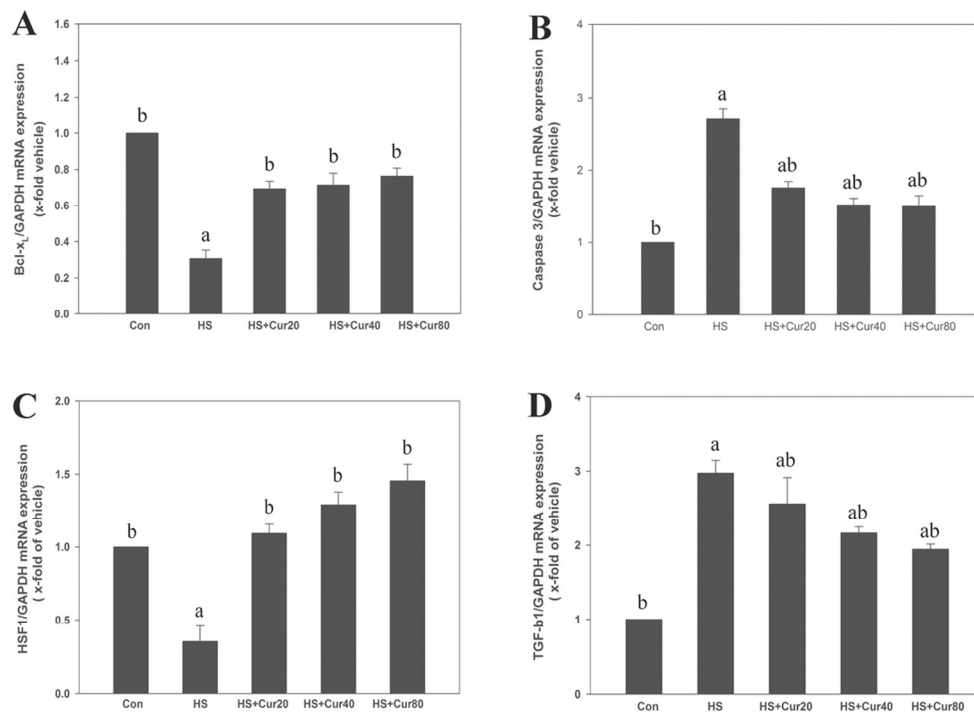


Fig 4
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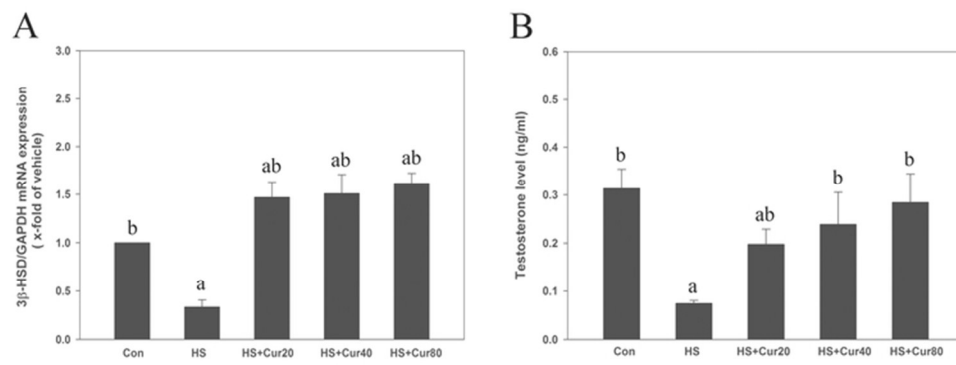


Fig 5
66x25mm (300 x 300 DPI)

Curcumin significantly improves testicular injuries (B&C) induced by scrotal heat stress in a dose-dependent manner (20, 40, and 80 mg/kg; D-F, respectively).

