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Dietary zinc may attenuate heat-induced testicular oxidative stress in mice via up-regulation of Cu-Zn SOD

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ABSTRACT. Zinc (Zn) is important for male mammalian reproduction. In this study, we sought to clarify the role of Zn in heat-induced testicular damage in mice. Eighteen mice were divided into either control (con), heat (heat) and heat plus Zn (H+Zn) treatment groups, and fed diets containing 60 (con and heat groups) or 300 (H+Zn group) mg/kg Zn sulfate for one month. Mice in the con group were then maintained at 25°C, while mice in heat and H+Zn groups were exposed to 40°C for 2 h daily, for eight days. Mouse testes and serum from each animal were analyzed. Zinc levels in serum and testes were positively correlated to Zn feed concentrations. Mice in the heat group had higher testes index than those in the other two groups (7.22 ± 0.75, heat; 4.92 ± 0.20, con; 4.80 ± 0.30 mg/g, H+Zn; $P < 0.05$). Testicular antioxidant status showed malondialdehyde levels in heat group mice were increased compared to control mice (2.34 ± 0.15 versus 1.55 ± 0.23 nmol/mg protein; $P < 0.05$), and Cu-Zn superoxide dismutase (SOD) level differed between heat and H+Zn groups (14.04 ± 0.74 versus 18.27 ± 1.53 U/mg protein; $P < 0.05$). Testicular Cu-Zn SOD protein expression levels were significantly lower in the heat than in the control

group (0.30 ± 0.11 versus 1.22 ± 0.13; P < 0.05). These results suggest that dietary Zn may elevate the activity and protein concentration of Cu-Zn SOD, to attenuate testicular oxidative stress induced by heat exposure.

Key words: Cu-Zn superoxide dismutase; Zinc; Heat exposure; Testes; Oxidative stress; Mouse

INTRODUCTION

Temperature is one of the most important physicochemical variables, and all physiological processes within an organism occur within a limited temperature range that differs depending on the molecular and cellular mechanisms associated with each particular process (Miranda et al., 2013). In mammalian species, cooler temperatures have been shown to be essential for maintenance of an optimal environment for spermatogenesis in the testes (Hughes and Acerini, 2008). Ambient temperatures above 40°C have been shown to result in scrotal temperatures above deep body temperature (Li et al., 2013). This has been found to be unfavorable for the formation of sperm, and results in abnormal spermatogenesis (Hughes and Acerini, 2008), induction of cryptorchidism and temporary or permanent infertility (Ikeda et al., 1999; Silanikove, 2000). In order to ensure the quality of sperm, therefore, it is important to prevent whole body heat stress.

Elevated body temperature may induce metabolic changes associated with the induction of oxidative stress (Lin et al., 2006). In mammals, heat stress may enhance the formation of reactive oxygen species (ROS) and induce cellular oxidative stress (Lin et al., 2006). Oxidative stress occurs following a disturbance in the balance between the production of ROS and antioxidant defense (Celino et al., 2011). Previous studies have revealed that spermatozoa are highly sensitive to ROS-induced damage (Aitken and Clarkson, 1987). Spermatozoa and seminal plasma contain a battery of ROS scavengers, including enzymes such as superoxide dismutase (SOD), catalase, and the glutathione peroxidase/reductase system (de Lamirande et al., 1997). The SODs were a family of enzymes that very efficiently catalyze the dismutation of superoxide radical anions, combined with excess peroxide to form water and oxygen. Superoxides have the capability to initiate free radical chain oxidations, inactivating specific enzymes and leading to the production of more powerful oxidants (Okado-Matsumoto and Fridovich, 2001). Nuclear factor erythroid2-related factor 2 (Nrf2) plays an important role in inhibiting oxidative stress; it is stabilized and transferred into the nucleus to promote the transcription of antioxidant genes, via binding to the antioxidant response element (ARE) sequence (Li et al., 2014).

Zinc (Zn) is an essential bio-element with a fundamental role in a wide range of biochemical processes. It is a major component of various proteins and an important modulator of mammalian immunity, cellular proliferation, reproduction, and nervous system function, and provides defense against free radicals (Nowak et al., 2003; Ho, 2004). Zinc has also been found to be an indispensable element in reproduction and is required for the maintenance of germ cells; progression of spermatogenesis; and, regulation of sperm motility (Zhao et al., 2011). The effects of Zn deficiency are similar in most animal species and include dermatitis, alopecia, ocular lesions, testicular atrophy, growth retardation, and anorexia (Rossi et al., 2001). Zinc deficiency in male reproduction results in gonad dysfunction, decreased testicular weight, and seminiferous tubule shrinkage (Bedwal and Bahuguna, 1994). Zinc supplementation may inhibit ROS generation, and enhance the activity of antioxidant pathways (Zhou et al., 2005). Furthermore, Zn participates in

cell membrane stabilization, metallothionein (Mt) synthesis and Cu-Zn SOD structure (Jemai et al., 2007), and, when added to the diet of healthy humans, may reduce concentrations of oxidative stress (Prasad et al., 2007). It remains unclear, however, whether Zn protects the testis from heat-induced oxidative damage. In the present study, therefore, we investigated the effects of Zn supplementation on the testicular damage of heat-induced mice.

MATERIAL AND METHODS

Animals

Male adult Institute of Cancer Research (ICR) mice, 8weeks old, were purchased from Nanjing Qinglongshan Experimental Animal Center (China, Jiangsu). Mice were provided with food and water *ad libitum* and were maintained on a 12 h light-dark cycle under controlled temperature (25°C) and relative humidity (RH) (50 ± 5%). Mice were allowed to acclimatize to the experimental setting for one week before the commencement of the study. Procedures involving live animals were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Experimental design

Eighteen mice were divided into three groups (each N = 6). Prior to heat exposure, the three groups were fed a diet with Zn supplemented in the form of $ZnSO_4$ at 60 mg/kg body weight for animals in the con and heat groups, and 300 mg/kg for mice in the H+Zn group. Mice were maintained on this diet for one month. The higher dose was selected on the basis of a previous study, in which 300 mg/kg Zn added to the diet induced minimal adverse effects associated with high dietary Zn (Simon and Taylor, 2001). After one month on the diet, mice in the heat and H+Zn groups were exposed to whole body heat treatment.

To investigate the effect of whole body heat treatment on testicular oxidative stress and antioxidant system, mice in the treatment groups were exposed to an ambient temperature of 40°C. Mice in the con group were housed under controlled conditions (25°C, 50 ± 5% RH) throughout the heat treatment period. Mice in heat group were housed under control group conditions, but additionally subjected to 40°C and 50 ± 5% RH between 12 and 2 p.m. daily, for eight consecutive days during heat treatment period. The whole body heat temperature of 40°C for 2 h was selected on the basis of previous reports of total recovery after heat treatment and feasible parameters for a chronic heat model in mice (Rossi et al., 2001; Li et al., 2013). Following heat treatment each day, feed weight (as a measure of feed intake) and body weights were of all mice were recorded.

Sample collection

After eight days of heat treatment, mice were weighed. Immediately after mice anesthetized with ether, blood samples were collected and centrifuged at 3500 *g* for 10 min. Isolated sera were stored at -80°C prior to further processing. Testes were collected, immediately placed on ice and dissected. Upon further processing, testes were weighed and the left testis returned to -80°C for subsequent biochemical measure and western blot analysis. The right testis was immersed in 4% paraformaldehyde solution for histological analysis.

Measurement of Zn levels in testis and serum

Zinc levels in testes were measured using an atomic absorption spectrometer with air-acetylene flame, following tissue digestion in nitric acid (Zhao et al., 2011). Using this assay, total Zn in tissues, including free and protein-bound forms, were measured and expressed per gram wet tissue (Cai et al., 2002). Tissue samples (approximately 0.5 g)and serum (100 µL) were incubated overnight in nitric acid (10 mL) at room temperature and then heated to 95°C for 1 h, 150°C for 1 h, and 230°C for 30 min to facilitate digestion. When liquid temperature decreased to room temperature, then ultrapure water was added to dilute the sample (final volume 25 mL). The resulting clear liquid was used to determine Zn concentrations by inductively coupled plasm optical emission spectrometry (Optima 2100DV, PerkinElmer, Waltham, MA, USA).

Analysis of the testicular oxidative stress parameters

Testes samples were assessed to determine parameters of oxidative stress that included levels of enzymatic antioxidants Cu-Zn SOD, glutathione peroxidase (GPX), malonaldehyde (MDA), and γ-glutamyl cysteine synthetase (γ-GCS). Frozen testicular tissue was homogenized in ice-cold NaCl solution, then centrifuged at 1700 *g* for 15 min at 4°C. Enzyme activities in sample supernatant were then analyzed using a commercial assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer instructions.

Histological evaluation of testicular tissues

Testes were fixed in 4% paraformaldehyde and embedded in paraffin. Then, 6-µm sections were affixed to slides, stained with hematoxylin-eosin (HE) and assessed for morphologic changes, using an optical microscope (BX 51, Olympus, Tokyo, Japan).

Western blot analysis of testicular protein expression

Protein was extracted from the nucleus to test the Nrf2 level in nucleus, and whole-cell levels of Cu-Zn SOD were assessed. Testicular tissues were homogenized and fractionated on 10% SDS-PAGE (w/v) gels, and proteins were transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat dried milk (w/v) for 1 h, and incubated overnight at 4°C with anti-Cu-Zn SOD (1:1000) and anti-Nrf2 (1:500) antibodies (both Abcam, MA, USA). After unbound antibodies were removed with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated at room temperature with secondary antibody for 2 h. Antigen-antibody complexes were visualized with an enhanced chemiluminiscence kit (Thermo Scientific, New York, USA). Quantitative densitometry was performed on identified bands using a computer-based measurement system (Zhao et al., 2011). Histon3 and β-actin were employed as loading controls for the nuclear and cytoplasmic protein, respectively.

Statistical analysis

Data were calculated as group means ± standard errors of the mean (SEM). Statistical analysis was performed using GraphPad Prism Version 5.0 software program (GraphPad Software,

San Diego, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Zn levels in testes and serum

Zinc levels in testes and sera were significantly increased in the 300 mg/kg dietary Zn groups, compared with the con group ($P < 0.01$; Figure 1).

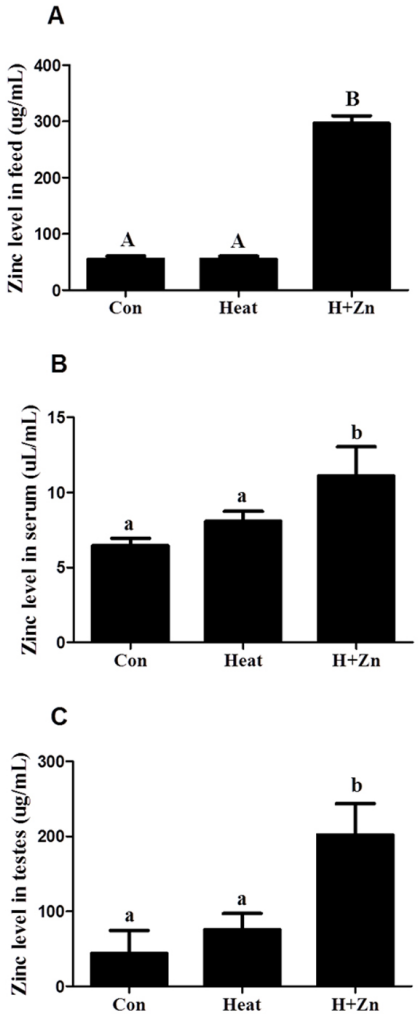

Figure 1. Zinc level in feed, serum, and testis of experimental groups. Zinc levels in feed (**A**), serum (**B**) and testis (**C**). Bars represent the mean ± SEM (N = 6). Means in the same rows with different lowercase letters are significantly different ($P < 0.05$), and uppercase letters indicate significant differences ($P < 0.01$).

Feed intake and testes index

Mean body weight gain was measured for the three groups and compared every day following heat treatment. Weight gain did not differ between all groups (P > 0.05), but H+Zn mice showed a trend of increased weight, compared to the other two groups (Table 1). Average daily feed intake in mice in the heat group was reduced compared to the control, and significantly reduced compared to the H+Zn group (P < 0.05) (Table 1). Testes indexes were obtained by dividing testes weight by body weight; this measure was significantly higher in the heat group compared to the other two groups (P < 0.05), and was reduced in the H+Zn group (P < 0.01; Table 1).

Table 1. Effect of Zn supplementation on body and testes weight, and average daily feed intake of heat-treated mice.

	Treatment group		
	Con	Heat	H+Zn
Body weight (g)	43.15 ± 1.16	38.19 ± 0.91	37.02 ± 1.46
Average daily weight gain (g)	0.13 ± 0.12	0.10 ± 0.15	0.23 ± 0.12
Average daily feed intake (g)	$4.29 \pm 0.21^{a\,b}$	3.81 ± 0.18^{a}	4.56 ± 0.14^{b}
Testes weight (mg)	211.43 ± 0.83^{AB}	275.73 ± 0.61^{A}	177.70 ± 0.92^{B}
Testes weight/body weight (mg/g)	4.90 ± 0.20^{a}	7.22 ± 0.75^{Ab}	4.80 ± 0.30^{B}

Values are expressed as group mean ± SEM (N = 6). Different lowercase letters indicate significant differences between groups (P < 0.05); uppercase letters indicate significant differences (P < 0.01).

Oxidative stress parameter in the testes

Levels of MDA were higher in mice in the heat group, compared to those in the other two groups (P < 0.05; Figure 2A). The H+Zn group showed increased Cu-Zn SOD activity in the testes, but decreased γ-GCS activity, compared to the heat group (both P < 0.05; Figure 2B and D). There was no difference in GPX activity between the groups (P > 0.05; Figure 2C).

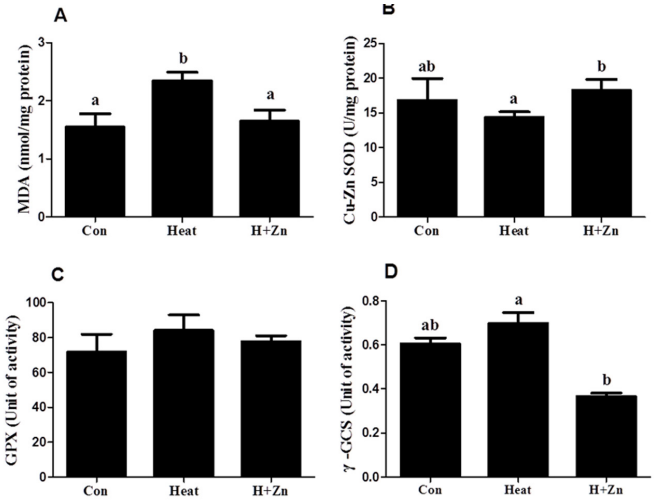

Figure 2. Effects of zinc on the oxidative stress parameters in the testes of the heat-treated mice. **A.** Malondialdehyde, MDA; **B.** Cu-Zn superoxide dismutase, Cu-Zn SOD; **C.** glutathione peroxidase, GPX and **D.** γ-glutamyl cysteine synthetase, γ-GCS. Bars represent the mean ± SEM (N = 6). Different lowercase letters indicate significant differences between groups (P < 0.05).

Histological evaluation of testes

There were obviously different degrees of pathological changes in the testes of mice from the different groups. For the con group, integrated seminiferous ducts and normal spermatogenic cells were observed (Figure 3A). There were obvious condensation of germ cell nuclei and lost germ cells in the seminiferous epithelium, suggesting that germ cells were undergoing apoptosis (Figure 3B). Testicular structure was relatively intact in mice in the H+Zn group as compared to the heat group. However, multinucleated giant cells and single germ cells were still clearly observed in testicular lumen, implying that germ cells were undergoing necrosis (Figure 3C).

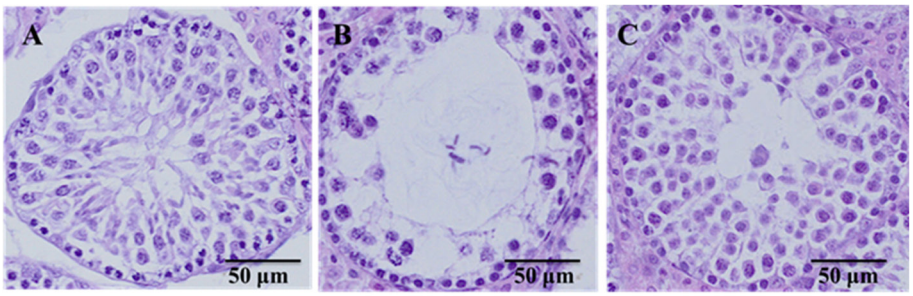

Figure 3. Hematoxylin-eosin staining of mouse testes. Control (con; **A**) and heat (heat; **B**) and heat plus zinc group (H+Zn; **C**) treatment groups. Bar = 50 μm. For each experimental group, representative images from five mice are shown.

Testicular expression of Cu-Zn SOD and Nrf2

A band for Cu-Zn SOD protein was detected for all groups, that was obviously shallower in the heat group than the others (Figure 4). The Cu-Zn SOD protein level in the heat group was significant lower than the other two groups ($P < 0.05$). There was no significant difference in Cu-Zn SOD protein level between the con group and the H+Zn group.

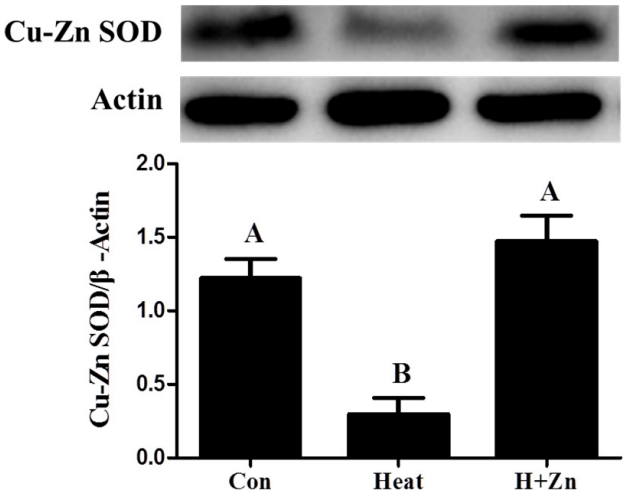

Figure 4. Cu-Zn SOD protein change in the testes of heat-treated mice. Western blotting. Different uppercase letters indicate significant differences between groups ($P < 0.01$).

We investigated whether changes occurred in the intra-nuclear levels of protein Nrf2, which is a key regulator in the cellular defense system against oxidative stress (Shen et al., 2014). Levels of Nrf2 expression in the heat group were increased compared to the control group, while levels of intra-nuclear Nrf2 in the H+Zn group were lower, compared to the heat group ($P < 0.05$; Figure 5). This finding suggests that heat treatment promotes Nrf2 expression in the nucleus, and that increased dietary Zn could decrease this state.

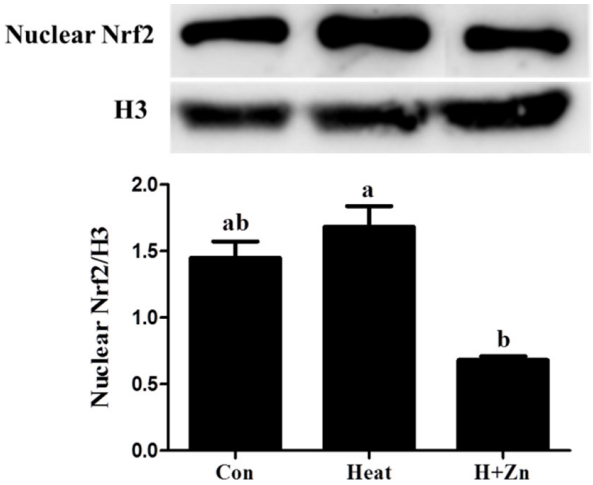

Figure 5. Nrf2 protein change in the testes of heat-treated mice. Western blotting. Different lowercase letters indicate significant differences between groups ($P < 0.05$).

DISCUSSION

In the present study, heat exposure caused testicular oxidative stress in mice. We observed that dietary Zn supplementation up-regulated testicular Cu-Zn SOD levels, which may remit this oxidative damage to some degree.

It has been previously demonstrated that low Zn levels in diets result in reduced levels of Zn in serum, urine, sweat and hair (Paknahad et al., 2007), indicating that dietary Zn accumulates in the tissues of animals. The uptake and accumulation of Zn has also been shown to result in mitochondrial release of cytochrome c, which initiates the caspase cascade that leads to apoptosis (Feng et al., 2008); it has therefore been suggested that dietary Zn may prevent cellular apoptosis. In the current study, we observed a positive correlation between Zn levels in testes and sera, and levels in feed, indicating that dietary Zn accumulates in mouse tissues and serum.

Previously studies have indicated that high ambient temperatures lead to slow growth, feed intake, reproductive performance and decreased weight gain in animals (Spencer et al., 2003; Song et al., 2012). Supplemental Zn in the diet of nursery pigs has been shown to improve pig growth performance (Case and Carlson, 2002). Japanese quail fed with dietary Zn showed an attenuated effect on the heat stress-induced poor egg production (Sahin and Kucuk, 2003). In the current study, average daily intake was reduced in the heat group, compared to control and H+Zn groups, in accordance with previous findings. Research has demonstrated that summer heat stress may lead to a small increase in testicular growth in ram lambs (Rasooli et al., 2010). Such an effect was also observed in our study, in which an improved testes index was seen in the

heat group, which could be decreased by Zn supplement; this indicates that heat treatment may cause testicular swelling that can be alleviated by supplementation with Zn in feed. Moreover, Zn supplementation appeared to reduce the incidence of heat-induced morphological abnormalities, such as apoptosis of Leydig cells. This suggests that supplemental dietary Zn attenuated heat-induced damage in the testes.

Oxidative stress is caused by numerous factors including elevated temperature, and has been shown to be widely involved in apoptosis and germ cell death, and in the pathophysiology of male infertility (Paul et al., 2009; Shiraishi et al., 2010). Heat treatment induces oxidative stress in testes, mainly through mitochondria-derived ROS and lipid peroxidation of the cellular membrane (Li et al., 2013). Increased oxidative stress is characterized by elevated MDA levels, considered to be an indicator of oxidative stress (Li et al., 2013). In our study, we demonstrated that chronic heat stress increased testicular MDA levels in mice, and Zn supplemented in feed decreased the effect of heat on MDA levels. This suggests that dietary Zn may attenuate testicular oxidative stress in heat-exposed mice.

As a primary antioxidant enzyme, SOD plays an important role in testicular tissues. High levels of Cu-Zn SOD and Zn increase spermatogonia resistance to ROS and reduce oxidative stress (Ishii et al., 2005). Activity levels of the enzyme Cu-Zn SOD have been shown to be high in testis of eels (Celino et al., 2011). Furthermore, decreased protein levels of Cu-Zn SOD may increase ROS levels, resulting in lipid peroxidation in the liver, brain, and testes, as well as other deleterious effects on cell structure. Over-expression of Cu-Zn SOD has been shown to result in extended life span (Koksal et al., 2000; Ahlemeyer et al., 2001; Orr and Sohal, 2003; Celino et al., 2011). At the same time, Zn (II) could lead to increased Cu-Zn SOD activity (Mei et al., 2013). In the current study, the antioxidant index and protein levels of Cu-Zn SOD in testes were significantly reduced in heat stressed mice, while mice that were heat-treated but received supplemental dietary Zn had levels that were comparable to control mice, suggesting that dietary Zn improves Cu-Zn SOD levels to remit heat-induced oxidative damage in the testes of mice.

Nrf2 is a key regulator in the cellular defense system against oxidative stress (Shen et al., 2014). The presence of excess ROS and Zn could stimulate the Nrf2-antioxidative signaling pathway, thus prevent oxidative stress (Kobayashi et al., 2004; Li et al., 2014). γ-GCS has been found to be a rate-limiting enzyme involved in the synthesis of glutathione that is up-regulated by Nrf2 levels (Jeyapaul and Jaiswal, 2000). In the current study, we observed that there were obvious decrease in Nrf2 protein level and γ-GCS activity in testis in the H+Zn group. Considering that there is no significant difference in MDA level between the con and the H+Zn group, pretreatment of dietary Zn could maintain the oxidation-reduction equilibrium, and therefore the Nrf2-antioxidative signaling pathway was stabilized under the basal line level in the mouse testis during heat stress.

In summary, we demonstrated that dietary Zn pretreatment for one month could ameliorate heat-induced testicular injury in mice. Dietary Zn supplementation increased the Cu-Zn SOD level, decreased the MDA level, and maintained the Nrf2 protein expression under low level in the testes of mice, implying that it might via improving the Cu-Zn SOD level to resist heat-induced oxidative stress when mice were exposed to high ambient temperature.

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