Full Length Research Paper

Effects of vitamin E and thymoquinone on physiological and histological characteristics of heat-stressed male mice

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Accepted 1 November, 2011

Previous studies have shown that heat stress can lead to tissue damage and multiple organ dysfunctions. The present study elucidates the negative effects of heat stress and the possible protective effects of vitamin E or thymoquinone against the physiological and histological consequences of heat stress. Forty male mice were distributed into four groups as follows: group I was a control group that was orally supplemented with distilled water; group II was subjected to heat stress (HS) (at a humidity of 50 to 55% and a temperature of 42°C) for 75 days; group III was subjected to heat stress and was orally supplemented with vitamin E (20 IU/kg body weight/day for 75 days); and group IV was subjected to heat stress and was orally supplemented with thymoquinone (TQ) (5 mg/kg body weight/day for 75 days). We found that the leucocyte count, Hb, and alanine aminotransferase (ALT) were significantly decreased in the HS-treated group. In contrast, the free radical (FR) levels were significantly elevated. Moreover, histopathological and ultrastructural studies of the HS-treated group revealed dilatation of the hepatic sinusoids, interstitial hemorrhage, hepatocytes that were infiltrated with fat droplets in the liver, hemorrhage enlargement of the mitochondria and dilatation of the renal tubules. Notably, supplementation with either TQ or vitamin E completely reversed the biochemical, histopathological and ultrastructural changes that were induced by heat stress yielding levels that were similar to the control values. Taken together, our data revealed the benefits of vitamin E or TQ supplementation as a means to ameliorate the negative effects of heat stress.

Key words: Free radicals, heat stress, kidney, liver, thymoquinone, vitamin E.

INTRODUCTION

It has been observed that the temperature and moisture of the air are two major environmental factors that control the heat stress of livestock (Bouraoui et al., 2002; St-Pierre et al., 2003). Heat stress can negatively affect animal performance. Increase in body temperature and respiration rate are the most important signs of heat stress in farm animals. The increase in body temperature is associated with a marked reduction in food intake (Rhoads et al., 2009), the redistribution of blood flow and changes in endocrine functions (Khodaei-Motlagh et al., 2011), which can negatively affect the productive and reproductive performance of the animals. The blood electrolyte balance is also altered during heat stress (Borges et al., 2004). A previous study demonstrated that heat stress can increase lipid peroxidation, which was associated with the production of a large number of free radicals that can initiate the peroxidation of polyunsaturated fatty acids (Altan et al., 2003). Additionally, environmental stress has been shown to cause an increase in the oxidative stress and an imbalance in the antioxidant status (Sahin et al., 2001).

Several strategies have been recommended to ameliorate the negative effects of a high environmental...
temperature. The effects of heat stress can be noticeably ameliorated by acclimation (Yalcin et al., 2001), fortification with trace elements (Nollet et al., 2008) and dietary supplementation with vitamins (Al-Enazi, 2007).

Vitamin E has been reported to be an excellent biological chain-breaking antioxidant that protects cells and tissues from the lipoperoxidative damage induced by free radicals (Bou et al., 2004). Previous studies have indicated that vitamin E as an additive in feed can reduce the complications of heat stress (Ajakaiye et al., 2010). Moreover, supplementation with various levels of vitamin E has been shown to improve growth performance and the immune responses of broilers under heat stress (Niu et al., 2009). Previous laboratory studies have revealed that enzymes and vitamins can effectively increase body weight gains and improve the hemato-biochemical profiles in broiler birds (Ahmed et al., 2007). The addition of vitamin E is one method that is recommended to possibly eliminate the undesirable effects of heat stress (Halici et al., 2011).

Several classes of antioxidant dietary compounds have been suggested to confer health benefits. Data have shown that the consumption of these products can lead to a decrease in various pro-inflammatory and/or oxidative stress biomarkers (Vouldoukis et al., 2004). Thymoquinone (TQ) is an abundant component of black seed oil extract. Its beneficial effects are related to its anti-oxidant, anti-infective, anti-tumor and anti-inflammatory properties (Ragheb et al., 2009). A few studies have revealed that the oral administration of TQ can protect several organs against the oxidative damage induced by a variety of free radical-generating agents (Nagi and Mansour, 2000), carbon tetrachloride-evoked hepatotoxicity (Mansour, 2000) and renal damage induced by ifosfamide (Badary, 1999). Recently, it has been reported that *Nigella sativa* exhibits a significant level of anti-stress activity in albino rats (Roshan et al., 2010). Although, numerous studies have reported that TQ can protect several organs against oxidative damage, few data are available concerning its effect on heat stress. To further elucidate the potential benefits of TQ and vitamin E, the present study was designed to investigate the impact of dietary supplementation of vitamin E and TQ on heat stress-induced hematological and biochemical changes, as well as the histopathological and ultrastructural changes, of the liver and kidney.

**MATERIALS AND METHODS**

**Experimental animals and housing**

Forty Swiss albino, sexually mature 12-week-old male mice (weight: 25 to 30 g each) were obtained from the Central Animal House of the Faculty of Pharmacy at King Saud University. All the animal procedures were in accordance with the standards that have been set forth in the guidelines for the care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol. The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. All of the animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. The animals were maintained under standard laboratory conditions (23°C temperature, 60 to 70% relative humidity and a 12-h light/dark cycle) and were fed a diet of standard commercial pellets and water *ad libitum*.

**Experimental design**

The animals were distributed into four experimental groups (10 mice per group). Group I was a control group that was orally supplemented with distilled water for 75 days. Group II was the heat stress group (HS), in which the animals were maintained twice a week in a well-maintained (50 to 55% humidity and 42°C temperature) incubator for 10 min for 75 days as previously described (Al-Enazi, 2007). The mice of group III were also subjected to these heat stress conditions and were orally supplemented with vitamin E (20 IU/kg body weight/day) (Yasunaga et al., 1982) for 75 days by oral gavage. The group IV animals were subjected to heat stress and were orally supplemented with TQ (5 mg/kg body weight/day for 75 days by oral gavage) (Hosseinzadeh and Parvardeh, 2004).

**Collection of the blood and tissue samples**

At the end of the experimental period, the mice of the control and treatment groups were killed and two blood samples were immediately collected. The first sample was collected in a heparinized tube (2.25 µl heparin/5 ml blood) for the blood profile. The second sample was collected in a non-heparinized tube and was centrifuged for 10 min at 3000 rpm to separate the serum, which was then stored at -80°C and was later used to measure the free radicals and other biochemical parameters. Following dissection of the animals, the liver and kidneys were removed, wiped with filter paper and fixed in 10% neutral buffered formalin fixative for histopathological examination.

**Preparation of TQ and Vitamin E**

TQ was obtained from Sigma Chemical Co., St. Louis, MO, USA. It was dissolved by an initial addition of dimethyl sulfoxide (DMSO), followed by normal saline (the final concentration of DMSO was <0.5%). The solution was administered at a dose of 5 mg/kg body weight once daily via intra-gastric intubation for 75 days. Vitamin E was purchased from Sigma Chemical Co. (St Louis, MO, USA) and was reconstituted in PEG-60. The solution was administered at a dose of 20 IU/kg body weight per day for 75 days by oral gavage.

**Cell blood count (CBC)**

At the end of the experimental period and on the day after the overnight fasting period, blood was drawn from the abdominal aorta and was used to assay the hemoglobin (Hb) content, red blood cell (RBC) count and white blood cell (WBC) count using an automatic analyzer (ABC Vet, Horiba ABX, Montpellier, France) according to the manufacturer’s instructions.

**Quantifications of the blood biochemical parameters**

The animals were anesthetized with pentobarbital (60 mg/kg body weight once daily via intra-gastric intubation for 75 days). The solution was administered at a dose of 20 IU/kg body weight per day for 75 days by oral gavage.
Histopathology
The liver and kidneys were immediately preserved in 20% formalin following their removal from the animal.

Tissue processing
The liver and kidney tissues were placed in 10% formalin (diluted to 10% with normal saline) for 1 h to reverse the shrinkage that had been caused by the high concentration of formalin. The tissues were dehydrated in an ascending grade of isopropyl alcohol by immersion in 80% isopropanol overnight and in 100% isopropyl alcohol for 1 h. The dehydrated tissues were cleared in two changes of xylene for 1 h each. The wax-impregnated tissues were embedded in paraffin blocks using wax of the same grade. The paraffin blocks were mounted and cut with a rotary microtome at a 3-micron thickness. The sections were floated in a tissue floatation bath at 40°C, and were placed on glass slides that were smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60°C for 5 min, and were allowed to cool.

Tissue staining
The sections were deparaffinized by immersion in xylene for 10 min in a horizontal staining jar. The deparaffinized sections were washed in 100% isopropyl alcohol, and were stained in Ehrlich's hematoxylin for 8 min in a horizontal staining jar. After staining in the hematoxylin, the sections were washed in tap water, and were dipped in acid alcohol to remove the excess stain (8.3% HCl in 70% alcohol). The sections were then placed in running tap water for 10 min to promote bluing (slow alkalinization). The sections were counter-stained in 1% aqueous eosin (1 g in 100 ml tap water) for 1 min, and the excess stain was washed in tap water before the sections were dried. The complete dehydration of the stained sections was achieved by placing the sections in an incubator at 60°C for 5 min. When the sections were cooled, they were mounted in diplax (DPX) mount, which has the optical index of glass (the sections were wetted in xylene and inverted onto the mount and then placed on the cover slip). The architecture was observed using a low-power objective under a microscope.

Electron microscopy studies
Small pieces of the liver and kidney tissues were fixed in 2.5% glutaraldehyde for 24 h. The small pieces were washed with phosphate buffer (0.1 M, pH 7.4). Following the fixation, they were placed in 1% osmium tetroxide, which was buffered to pH 7.4 with 0.1 M phosphate buffer, at 4°C for 1 to 2 h; they were then washed in phosphate buffer to remove the excess fixative. The samples were dehydrated using ascending grades of ethanol, which was followed by clearing in propylene oxide. The specimens were embedded in araldite. The capsules were polymerized at 60°C. Ultrathin sections (100 nm) were prepared using an ultramicrotome and were placed onto uncoated copper grids. Following double staining with uranyl acetate and lead citrate, the sections were examined and photographed using a JEOL 100 Cx transmission electron microscope, Japan (Bancroft and Stevens, 1996).

Statistical analysis
The data are expressed as the means ± SEM (standard error of mean). The statistical differences between the groups were analyzed using one-way analysis of variance (for more than two groups), followed by Tukey's post-test, using the SPSS software version 17. The differences were considered to be statistically significant at P < 0.05.

RESULTS
Effect of oral administration of vitamin E or TQ on the cell blood count (CBC) during heat stress
We monitored the hematological parameters and leukocyte counts in each of the animal groups throughout the experimental period. We first observed a significant decrease in the levels of the total leucocyte counts and the Hb content in the HS-treated group relative to the control group (P < 0.05). In contrast, a significant increase in the WBC count was observed in the vitamin E and TQ-treated groups as compared to the HS group (P < 0.05 and *P < 0.05, respectively) (Table 1). Nevertheless, the administration of vitamin E or TQ did not affect the levels of RBCs, platelets, mean corpuscular volume (MCV) or hematocrit.

Effect of oral administration of vitamin E or TQ on the blood biochemical parameters during the heat stress period (75 days)
We also monitored the hepatic dysfunction that occurred due to heat stress by measuring the AST and ALT liver enzymes. As shown in Figure 1B and C, the level of ALT decreased significantly in the HS-treated group as compared to the control mice (P < 0.05), and the AST decreased (although this difference was not significant) during the heat stress period. FR is a marker of oxidative lipid damage and lipid peroxidation occurrence. The FR levels significantly increased in the HS-treated group relative to the control group (P < 0.05) (Figure 1A). The elevated levels of FR induced by heat stress decreased significantly in the vitamin E- and TQ-treated groups as compared to the HS group (*P < 0.05 and **P < 0.05, respectively). The serum creatinine levels were measured, because creatinine has been identified as a fairly reliable indicator of kidney function; they were found to be significantly increased in the TQ-treated group relative to the control (P < 0.05) and HS-treated groups (**P < 0.05) (Figure 1D).
Table 1. Effects of vitamin E and thymoquinone (TQ) supplementation on the blood profile during heat stress.

| Parameters                  | Control      | HS           | Vitamin E    | TQ           |
|-----------------------------|--------------|--------------|--------------|--------------|
| WBC count (x 10^3/mm^3)     | 8.57 ± 0.26  | 2.87 ± 0.32* | 8.30 ± 0.53* | 8.27 ± 0.15* |
| RBC count (x 10^6/mm^3)     | 9.79 ±0.42   | 9.15 ± 0.32  | 8.72 ± 0.12  | 8.76 ± 0.20  |
| Hb (g/dl)                   | 16.58 ± 0.83 | 13.26 ± 0.45*| 14.04 ± 0.27 | 14.93 ± 0.40 |
| Platelets (x 10^3/mm^3)     | 506.40 ±39.31| 699.20 ± 93.41| 558.20 ± 69.38| 676.80 ± 91.50|
| Hematocrit value (%)        | 49.76 ±2.50  | 45.94 ± 1.38 | 42.14 ± 0.80 | 44.80 ± 1.20 |
| MCV (µm^3)                  | 51.60 ± 0.75 | 50.20 ± 0.49 | 48.40 ± 1.03 | 51.40 ± 0.60 |

Blood parameters were measured in the four groups of mice, and the results are presented as the means ± SEM. *P < 0.05, HS versus the control; #P < 0.05, vitamin E versus HS; and +P < 0.05, TQ versus HS (ANOVA with Tukey’s post-test).

Figure 1. Serum analysis values of male mice in the control and experimental groups. Blood biochemical parameters were measured in the four groups of mice, and the results are presented as the means ± SEM. *P < 0.05, HS versus the control; #P < 0.05, vitamin E versus HS; and +P < 0.05, TQ versus HS.
Vitamin E or TQ treatment reverses heat stress-mediated liver histological changes.

To further investigate the liver damage caused by heat stress and the possible healing effect of vitamin E and TQ on hepatic injury, we examined the histopathological changes that could be observed in the liver sections. As shown in Figure 2A, the untreated control mice presented a normal hepatic lobule structure and a normal central vein. The hepatocytes contained a rich acidophilic cytoplasm and a round nucleus. The liver sections of the animals that were subjected to heat stress presented a dilatation in the hepatic sinusoids and an interstitial hemorrhage (Figure 2B). The vitamin E-supplemented mice presented a decongested central vein that contained small amounts vacuolated cytoplasm in the hepatocytes during the heat stress condition (Figure 2C). Most of the histological changes that occurred due to the
heat stress were modulated by TQ supplementation, which resulted in a liver that showed a normal, de-congested central vein that was surrounded by a healthy hepatic lobule structure (Figure 2 D).

**Histopathological appearance of the kidney after vitamin E or TQ supplementation during the heat stress period (75 days)**

We investigated the histopathological changes that occurred in the kidney to determine the possible effects of vitamin E and TQ on heat stress. As shown in Figure 3A, the untreated control mice presented normal proximal and distal convoluted tubules and glomerulus in the cortex region. The kidney of the male, heat-stressed mice exhibited interstitial hemorrhage, scattered renal tubules and atrophy in the glomeruli (Figure 3B). The occurrence of the renal lesions was reduced by the administration of vitamin E to the heat-stressed mice; these mice displayed renal tubules that contained homogenous cytoplasm and minimal hemorrhage in the glomerulus (Figure 3C). The TQ-supplemented group exhibited the characteristics of recovery, including a slight hemorrhage in the renal

![Figure 3](image_url)
Figure 4. Effects of the vitamin E and thymoquinone (TQ) supplementation during the heat stress period on the liver ultrastructure. Liver of a control mouse, showing the normal structure of a hepatocyte, including its nucleus (N), mitochondria (m) and rough endoplasmic reticulum (r) (A). Liver section of a heat-stressed male mouse, showing dilatation of the hepatic sinusoids (star) and hepatocytes that are infiltrated with large droplets of fat (arrows) (B). Liver section of a male mouse that was supplemented with vitamin E during the heat stress period (75 days), showing an increased amount of rough endoplasmic reticulum (r) and a lack of fat droplets (C). Liver of a mouse that was supplemented with TQ during the heat stress condition (75 days), showing a normal structure of the hepatocytes, including the mitochondria (m) and the rough endoplasmic reticulum (r). Note the reduced number of fat droplets (arrows) (×10,000 magnification) (D).

**Figure 3D.**

Effect of vitamin E or TQ supplementation on the liver ultrastructure during the heat stress period (75 days)

Based on the electron microscopic observations, the livers of the control mice were visually normal and revealed the normal structure of a hepatocyte, including a nucleus, mitochondria and rough endoplasmic reticulum (Figure 4A). The ultrastructure features of the heat-stressed male mice revealed dilatation of the hepatic sinusoids and the presence of hepatocytes that were infiltrated with large droplets of fat (Figure 4B). Electron microscopy of the livers from the male mice that were supplemented with vitamin E during the heat stress period (75 days) revealed increased levels of rough tubules and glomerulus, during the heat stress period (Figure 3D).
Figure 5. The impact of vitamin E and thymoquinone (TQ) supplementation during the heat stress period on kidney ultrastructure. Kidney of a control mouse, showing the normal structure of the proximal convoluted tubule, including the nucleus (N), mitochondria (m) and microvilli (star) (A). Kidney section of a heat-stressed male mouse, showing enlargement of the mitochondria (m) and dilatation of the renal tubules (star) (B). Kidney from a male mouse that was supplemented with vitamin E during the heat stress period (75 days), showing increased numbers of mitochondria (m) in the renal convoluted tubules (C). Kidney of a mouse that was supplemented with TQ during the heat stress condition (75 days), showing a normal appearance of the proximal convoluted tubule, including a normal nucleus (N) and normal microvilli in the apical region (mv) (×10,000 magnification) (D).

endoplasmic reticulum (r) and the absence of fat droplets (Figure 4C). Electron microscopy revealed another interesting ultrastructural feature in the livers of the mice that were supplemented with TQ during the heat stress condition (75 days); these livers showed a normal structure of the hepatocytes, which included the mitochondria and the rough endoplasmic reticulum, but contained few fat droplets (Figure 4 D).

Effect of vitamin E or TQ supplementation on the kidney ultrastructure during the heat stress period (75 days)

To investigate kidney damage as a result of heat stress and to determine the possible modulatory effects of vitamin E and TQ on renal injury, we investigated the ultrastructural changes of the kidney. Electron microscopy of the untreated control mice revealed a normal structure of the proximal convoluted tubule, which included the nucleus, mitochondria and microvilli (Figure 5A). However, the kidney section of the heat-stressed male mice revealed enlargement of the mitochondria and dilatation of the renal tubules (Figure 5B). Electron microscopy of kidneys from the male mice that were supplemented with vitamin E during the heat stress period (75 days) revealed increased numbers of mitochondria in the renal convoluted tubules (Figure 5C). The kidneys of the mice that were supplemented with TQ during the heat stress condition (75 days) showed surprising ultrastructure features that included a normal proximal convoluted tubule, or in other words, a normal nucleus and normal microvilli in the apical region (Figure 5D).
DISCUSSION

Oxidative stress, which is a heat stress-induced response, has long been believed to play a role in liver and kidney damage. Although, oxidative stress has been suggested to be an important factor in tissue damage, the importance of oxidative stress has recently become more widely appreciated. Antioxidants are free radical scavengers that protect the body’s defense system against heat stress-produced free radicals and can stabilize health status. Here, we investigated the effects of vitamin E and TQ on the hematological, biochemical, structural and ultrastructural changes that were caused by heat stress. First, we assessed the impact of vitamin E and TQ supplementation on the heat stress-induced changes of the complete blood profile. We observed significant decrease in the levels of the total leucocyte counts and Hb content in the HS-treated group. Similar observations have been made (Mashaly et al., 2003). Both the vitamin E- and TQ- treated groups revealed significant increases in the WBC count during heat stress. Our results agree with the results of Ajakaiye et al. (2010), who attributed the increase in the WBC count to antioxidant activity of vitamin E; similarly, TQ could increase the WBC count, depending on the role of TQ in free radical scavenging (Hadjzadeh et al., 2008). In this study, ALT was significantly decreased in the HS-treated group, but AST levels showed a decrease during the heat stress period that was not significant. These results are similar to those of Okab et al. (2008), who investigated the effects of environmental temperatures on some physiological and biochemical parameters of male New-Zealand rabbits. Heat stress increased the free radicals, which were lowered by vitamin E and TQ supplementation. Sahin et al. (2001) investigated the protective effect of vitamin E against heat stress-induced lipid peroxidation, and they reported a similar result. TQ has been reported to prevent oxidative injuries across several in vitro and in vivo studies (Daba and Abdel-Rahman, 1998; Mansour et al., 2001; Yaman and Balikci, 2010), and these results may be ascribed to its ability to scavenge free radicals. The negative effect of heat stress was further demonstrated by the histopathological and ultrastructural changes that were observed in liver and kidney tissues. Histopathological examination of the liver tissues of animals that were exposed to heat stress revealed dilatation of the hepatic sinusoids and an interstitial hemorrhage, whereas the ultrastructural features revealed dilatation of the hepatic sinusoids and infiltration of the hepatocytes with large droplets of fat. These results are in accordance with the histopathological lesions that were observed in the livers of broilers that were subjected to heat stress (Aengwanich and Simaraks, 2004); the authors of that study reported that the excess lipids in the hepatocytes indicated the occurrence of a sublethal injury. Most of the liver histological changes were reversed by TQ supplementation. Similar observations have been made by Sayed-Ahmed et al. (2010), who attributed these improvements to TQ’s ability to decrease oxidative stress and to preserve both the activity and mRNA expression of antioxidant enzymes. The livers of mice that had been supplemented with vitamin E during the heat stress period (75 days) revealed an increased amount of rough endoplasmic reticulum and a lack of fat droplets; this result can be attributed to the role of vitamin E in improving the antioxidant defense system (Kirimioglu et al., 2006). Histopathological examination of the kidney tissues of mice that were exposed to heat stress revealed interstitial hemorrhage, scattered renal tubules and atrophy in the glomeruli, whereas electron microscopy revealed enlargement of the mitochondria and dilatation of the renal tubules. These changes are similar to the effects of heat stress on the renal tubules of the broilers in a study by Aengwanich and Simaraks (2004), who reported that heat stress caused degeneration and necrosis of the renal tubules, followed by renal failure. The supplementation of TQ and vitamin E during heat stress facilitated the recovery of the structure and ultrastructure of the kidney.

Conclusion

Conclusively, the present data expand our knowledge regarding the negative effects of heat stress and the role of TQ or vitamin E supplementation in ameliorating the undesired heat stress-induced effects and the underlying mechanisms. These data suggest that TQ or vitamin E may represent a promising drug candidate to protect against heat stress.

ACKNOWLEDGEMENT

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group no: RGP- VPP -078.

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