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Thymoquinone Treatment Alleviate Ovariectomy-Induced Hepatic Oxidative Damage in Rats

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ABSTRACT

The present study has designed to investigate the effect of thymoquinone (THQ) on the status of hepatic oxidative stress and antioxidant defense system following ovariectomy (OVX) in Wistar rats. Animals were randomly assigned into five groups; sham, OVX and OVX+THQ treated groups in three doses (2.5, 5 and 10 mg/kg/day) orally by gavage for eight weeks. In serum, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were estimated. In liver tissue, thiobarbituric acid reactive substances (TBARS), total glutathione (T-GSH), non-protein sulfhydryl groups (NP-SH) levels as well assuperoxide dismutase (SOD) and catalase (CAT) activities were also determined. Serum pre-oxidative markers (AST, ALT and ALP) were significantly increased in OVX rats compared to sham group. THQ inhibited these levels in a dose dependent manner. The lipid peroxidation product, TBARS, was significantly increased in OVX animals, which was inhibited by the THQ. In contrast, T-GSH and NP-SH levels were decreased in OVX rats, THQ treatments ameliorated these levels. Activities of SOD and CAT were significantly reduced in OVX group. THQ treatments significantly enhanced their activities in a dose dependent manner.The present results revealed the preventive effect of THQ on hepatic oxidative damage-induced by ovariectomy in rats.

INTRODUCTION

There is growing evidence indicating that post-menopausal state induces oxidative imbalance leading to metabolic alterations, which are involved in pathogenesis of different illnesses. Earlier scientific evidences confirmed such hypothesis by determinations of different oxidative stress parameters in postmenopausal women (Gurdol *et al.*, 1997). Recent experimental studies further confirmedthat ovariectomy (OVX) induces oxidative stress in animals (Kankofer *et al.*, 2007; Yalin *et al.*, 2012).

Puel and his colleagues (2005) showed that the formulae currently in use for the therapy of menopausal complaints have structural features characteristic for antioxidant agents (Puel *et al.*, 2005). Hormonal balance is one of the crucial factors necessary for proper functions of living organism. Any imbalance may influence the disturbances of metabolic processes. Such imbalance can be reflected by the variations in oxidative/anti-oxidative status, defined by respective parameters in hepatic tissues (Bai *et al.*, 2004; Galli *et al.*, 2011; Kara *et al.*, 2011).

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Proteins and lipids are susceptible to peroxidative damage caused by reactive oxygen species (ROS) excess. Excessive generation of intracellular ROS results in oxidative stress that exerts harmful impact on health (Bai *et al.*, 2004; Galli *et al.*, 2011; Kara *et al.*, 2011). The balance between production and neutralization of ROS is maintained by concert action of enzymatic and non-enzymaticdefense systems among them are SOD andCAT.Any disturbance in anti-oxidative/oxidative balance can be detected as well by the determination of products of peroxidative processes (Sies, 1993).

Clinically, the influence of metabolic disturbances on liver is of interest because it may play a potential role in aggravating liver diseases via generation of ROS excess.

Black seed (*Nigella sativa* L. Family: Ranunculacea) has been employed for hundreds of years as a traditional folk medicine, for treatment of numerous diseases in southern Europe and in most of the Islamic countries (Badary *et al.*, 2007). The historical tradition of black seed in medicine is significant. Moreover, it is identified as the curative black cumin in the Holy Bible, and is described as the Melanthion of Hippocrates and Dioscorides and as the Gith of Pliny (Badary *et al.*, 2007).

The composition and pharmacologically properties of black seed have been heavily investigated. Several beneficial pharmacological effects have been identified, including antihistaminergic, antihypertensive, hypoglycemic, antimicrobial, mast cell stabilizing, antitumor, galactagogue, insect repellent effects and antiinflammatory activities (Agarwal *et al.*, 1979; el Tahir *et al.*, 1993; Houghton *et al.*, 1995; Hajhashemi *et al.*, 2004; Fararh *et al.*, 2005; Badary *et al.*, 2007). The bioactive constituent of the volatile oil of black seed is Thymoquinone (THQ). It was first extracted by in 1963 by El-Dakhakhny (Badary *et al.*, 2007). THQ was reported to have anti-inflammatory, antioxidant, inducible nitric oxide synthase (iNOS) expression's inhibition and antineoplastic effects both in vitro and in vivo (Houghton *et al.*, 1995; Worthen *et al.*, 1998; El-Mahmoudy *et al.*, 2002; Badary *et al.*, 2003; Rooney & Ryan, 2005). Furthermore, THQ is reported to protect laboratory animals against chemical toxicity and to possess a strong antioxidant properties (Houghton *et al.*, 1995; Nagi & Mansour, 2000). Taken together, this prompted usto initiate this study to gain insights into the possibility of mechanism-based protection by THQ supplementation against OVX-induced initiation of hepatic injury. To the best of our knowledge, the antioxidant system and lipid peroxidation effect of THQ has not yet been studied on ovariectomized rat model.

MATERIALS AND METHODS

Animals

Thirty female Wistar albino rats, roughly the same age of 8-10 weeks and weighing 250-280 gwere received from the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia). They were maintained under controlled conditions of temperature (22±1ºC), humidity (50-55%) and light (12 h light/dark cycles) and were provided with Purina chow (Grain Silos & Flour Mills Organization, Riyadh, Saudi Arabia) and water *ad libitum*. All procedures including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996, USA) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Materials

Thymoquinone (THQ) was purchased from Sigma Chemical Co., (St. Louis, MO). All other chemicals used were of the highest analytical grade.

Study design

Twenty four animals were subjected to a bilateral ovariectomy operation (OVX) as follow: Under ether anesthesia, a longitudinal incision was made inferior to the rib cage on the dorsolateral body wall and the ovaries were exteriorized, ligated and excised (Kaczmarczyk-Sedlak *et al.*, 2009). The reaming six rats were subjected to sham operation using the same procedure except for the ligation and excision steps. Topical antibiotic (fusidic acid) was administrated twice weekly to eliminate the risk of postoperative infection. Rats were then divided into five groups six animals in each as follows: Group-1: Sham (Vehicle), Group-2: OVX (Vehicle), Group-3: OVX + THQ (2.50 mg/kg/day), Group-4: OVX + THQ (5mg/kg/day) and Group-5: OVX + THQ (10mg/kg/day) Two weeks after the ovariectomy and sham operations treatment with THQ was started. THQ was suspended in 10% CMC solution and administered orally (gavage) for 8 consecutive weeks. Sham and OVX groups were served as vehicle groups during the treatments period. Weekly body weight of each rat was recorded and the general health and behavior of animalsin each group were monitored during the entire study. Finally, animals were sacrificed under ether anesthesia, blood samples were obtained by cardiacpuncture and left for 30 min to coagulate then centrifuged at 4000 RPM. Serum samples were separated and stored at -70˚C till analysis. Whole liver was removed from each rat, weighed and calculated its ratio with body weight (liver g/100 g body weight) then preserved in -70 ˚C till analysis.

Serum Biochemical parameters

In serum, the activity of ALT, AST, ALP levels were estimated by using commercially available kits (RANDOX Laboratories Ltd.,Diamond Road, Crumlin, Co., Antrim UK).

Liver tissue preparation

Liver samples were homogenized in 50 mM phosphate buffered saline (pH 7.4) by using a glass homogenizer (Omni International, Kennesaw, GA, USA). Half of the homogenates were centrifuged at 1000 g for 10 min at 4ºC to separate nuclei and unbroken cells. The pellet was discarded and a portion of supernatant was again centrifuged at 12000 g for 20 min to obtain post-mitochondrial supernatant. In homogenate, TBARS, T-GSH and NP-SH levels were estimated. In post-mitochondrial supernatant, SOD and CAT activities were measured.

Estimation of TBARS levels in liver

A thiobarbituric acid reactive substances (TBARS) assay kit (ZeptoMetrix) was used to measure the lipid peroxidation products, malondialdehyde (MDA) equivalents. One hundred microliters of homogenatewas mixed with 2.5 ml reactionbuffer (provided by the kit) and heated at 95 °C for 60 min. After themixture had cooled, the absorbance of the supernatant was measuredat 532 nm using a spectrophotometer. The lipid peroxidation productsare expressed in terms of nmolesMDA/mg protein using molar extinction coefficient of MDA-thiobarbituricchromophore (1.56×10^5/M/cm).

Estimations of T-GSH and NP-SH levels in liver:

The levels of T-GSH and NP-SH were measured using the method described by Sedlak and Lindsay (1968). Homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature

for 15 min. For NP-SH estimation homogenates were deproteinized 50% trichloroacetic acid, centrifuged and supernatants were mixed with 100 µL of DTNB solution. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in one centimeter quarts cells.

Estimations of SOD activity in liver

The activity of SOD in liver was estimated using the method described by Kono (1978) with the add of nitrobluetetrazolium as the indicator. Superoxide anions are generated by the oxidation of hydroxylamine hydrochloride. The reduction of nitrobluetetrazolium to blue formazon mediated by superoxide anions was measured 560 nm under aerobic conditions. Addition of superoxide dismutase inhibits the reduction of nitrobluetetrazolium and the extent of inhibition is taken as a measure of enzyme activity. The SOD activity was expressed as units/mg protein.

Estimation of CAT activity in liver

The CAT activity was measured by the method of Aebi (1974) using hydrogen peroxide as substrate in post-mitochondrial supernatant. The hydrogen peroxide decomposition by catalase was monitored spectrophotometrically (LKB-Pharmacia, Mark II, Ireland) by following the decrease in absorbance at 240 nm. The activity of enzyme was expressed as units of decomposed/min/mg proteins by using molar extinction coefficient of H_2O_2 (71/M/cm).

Statistical analysis

All data were presented as the mean ± Standard Deviation (SD). The data were evaluated by a one-way ANOVA using GraphPad Prism program and the differences between means were assessed using Student Newman-Keuls. The differences were considered statistically significant at $P<0.05$.

RESULTS

Mean body weights were significantly ($P<0.001$) increased in ovariectomized rats compared to sham group of animals. THQ treatment with three doses (2.5, 5 and 10 mg/kg/day, orally) to ovariectomized rats for eight consecutive weeks could not alter the body weights significantly ($P>0.05$) when compared to OVX group.

The mean ration between liver and body weights were significantly ($P<0.01$) decreased in OVX rats as compared to sham group. However, the decreased ratio of liver and body weights were significantly enhanced after THQ treatment to OVX rats in relative dose dependent manner (Fig. 1).

Serum hepatic enzymes were significantly increased in OVX rats compared to sham operated animals. Higher doses (5 and 10 mg/kg/day) of treatments with THQ to OVX rats significantly inhibited the serum AST and ALT levels compared to untreated OVX rats. However, THQ treatment dose dependently inhibited the levels of ALP in serum when compared OVX group (Fig. 2).

In hepatic cells, oxidative product, MDA levels increased significantly ($P<0.001$) in OVX rats compared to sham group of animals. The liver levels of T-GSH and NP-SH were significantly ($P<0.001$) inhibited in OVX rats compared to sham group. These changes in oxidative markers were significantly and dose dependently protected by the THQ treatment for eight consecutive weeks (Fig. 3).

The enzymatic SOD and CAT activities found significantly ($P<0.001$) inhibited in hepatic cells of OVX rats when compared to sham operated animals. In THQ treated rats, SOD activity was increased dose dependent manner compared to OVX group. CAT enzymatic activity was found to increase in higher THQ treated animals (Fig. 4).

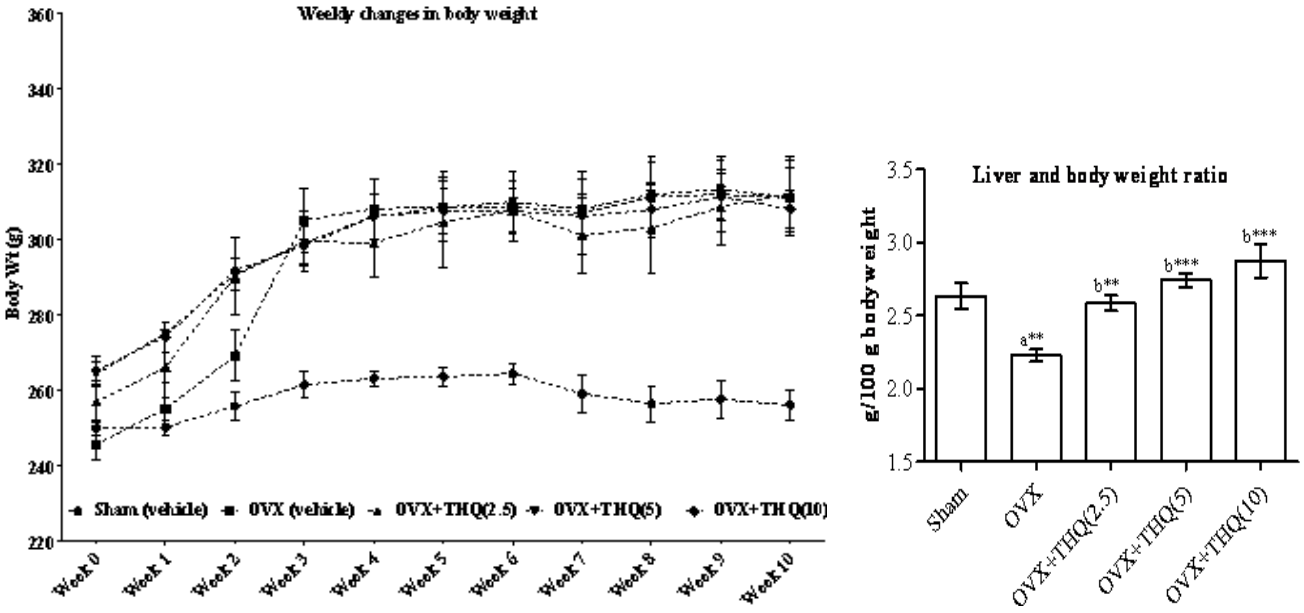

Fig. 1: Effect of THQ on body weight and liver/body weight ratio in sham operated and OVX rats. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. '[a]' OVX groups were compared with sham (vehicle). '[b]' THQ treated groups were compared with OVX (vehicle). The significance levels showed as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Six rats were used in each group; the data were expressed as Mean±SD.

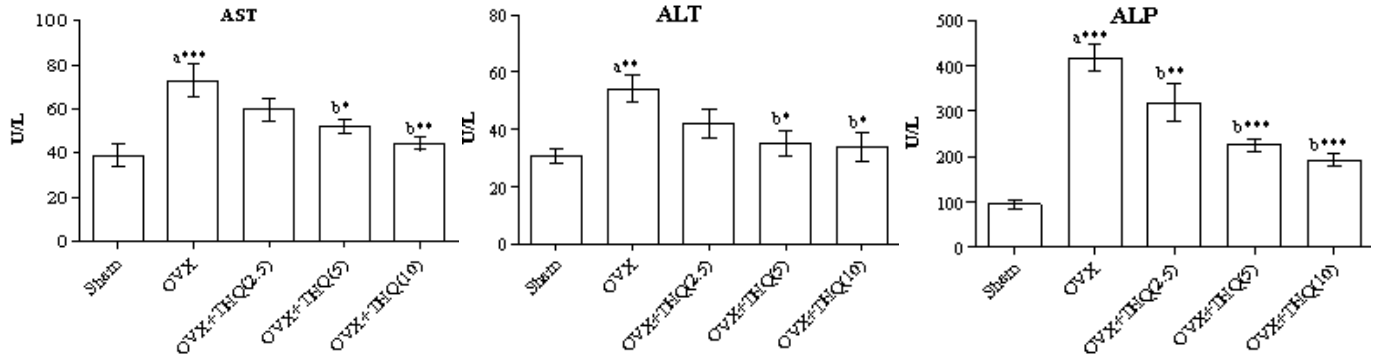

Fig. 2: Effect of THQ on serum AST, ALT and ALP levels in sham operated and OVX rats. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' OVX groups were compared with sham (vehicle). 'b' THQ treated groups were compared with OVX (vehicle). The significance levels showed as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Six rats were used in each group; the data were expressed as Mean±SD.

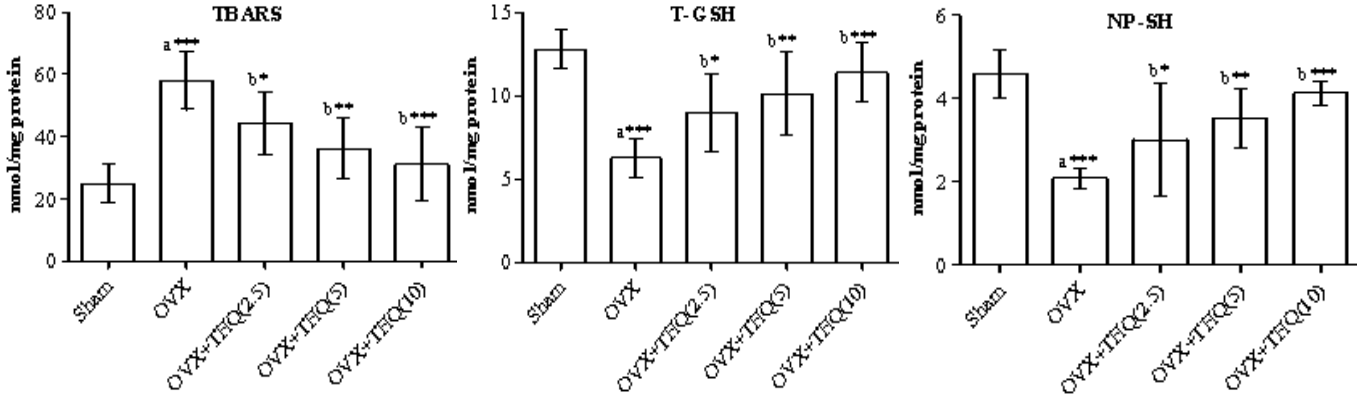

Fig. 3: Effect of THQ on TBARS, T-GSH and NP-SH levels in liver of sham operated and OVX rats.One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' OVX groups were compared with sham (vehicle). 'b' THQ treated groups were compared with OVX (vehicle). The significance levels showed as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Six rats were used in each group; the data were expressed as Mean±SD.

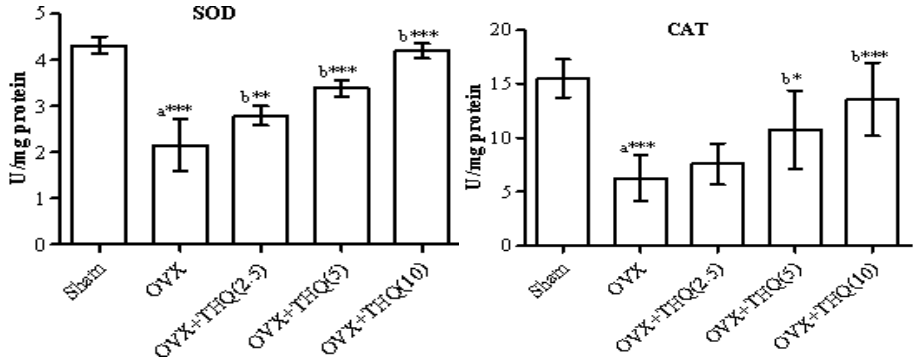

Fig. 4: Effect of THQ on enzymatic SOD and CAT activities in liver of sham operated and ovariectomyzed rats. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' OVX groups were compared with sham (vehicle). 'b' THQ treated groups were compared with OVX (vehicle). The significance levels showed as $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Six rats were used in each group; the data were expressed as Mean±SD.

DISCUSSION

In present research study, we explored the effect of THQ on OVX-induced hepatic oxidative stress in rat model. The results clearly revealed the beneficial effects of THQ in the prevention of oxidative stress induced by ovariectomy. Mean body weights were significantly increased in OVX rats compared to sham group. These results are in agreement with earlier reports and it believed that the increased in body weights are due to hormonal deficiency (Choi, 2009; Hertrampf *et al.*, 2009; Nian *et al.*, 2009). It is well known that, female hormone like estrogen deficiency occurs in menopausal women and that links to weight increase particularly epididymal fat. This process is believed to be through interference with the leptin, a hormone produced fat cells that are known to play a major role in regulation of body weight and appetite (Gao & Horvath, 2008). Brown et al., (2010), documented that, estrogen supplementation reduces food intake and decrease body weight gain after menopause, an effect that has been suggested to be regulated by estrogen receptor alpha (ER-α)(Brown *et al.*, 2010). The effect of THQ on body weight gain due to estrogen deficiency is not well studied. In present study,

THQ treatment to OVX rats for 8 weeks could not alter the body weight increased. However, the ratio between liver and body weights were significantly and dose dependently increased after THQ treatment to OVX rats indicating a hepato-protective properties. Data presented in Figure 2 demonstrate that OVX increased serum indices of liver function including AST, ALT and ALP. It is well known that the elevation of AST and ALT activities is repeatedly credited to hepatocellular damage (Al-Majed *et al.*, 2006). Also, the increase in ALP reflects the pathological alteration in biliary flow (Bulle *et al.*, 1990). In the current study, this observed increase in serum indices of liver function by OVX could be a secondary event following OVX-induced lipid peroxidation of hepatocyte membranes with the consequent increase in the leakage of AST, ALT and ALP from liver tissues. Interestingly, eight week treatment of THQ prevented the increase in hepatic enzymes, suggesting that THQ may have potential protective effect against OVX-induced hepatotoxicity. This effect could be due to stabilization of hepatocyte membranes by THQ with the consequent decrease in the leakage of liver enzymes. Bilateral ovariectomy operation can stimulate oxidative stress and generation of ROS via hormonal disturbance. Moreover, the contribution of oxidative stress during development of hepatic injury and promotion of liver damage has been well confirmed (Bulle *et al.*, 1990). ROS generation can be detoxified by endogenous antioxidants, which will lead to depletion of their cellular stores (Candelario-Jalil *et al.*, 2001). The most prevalent and vital intracellular antioxidants is protein and non-protein containing thiol groups (T-GSH and NP-SH). They have a crucial function as a free radical scavenger. Furthermore, ROS are known to induce tissue damage by attacking lipid membranes, causing induction of lipid peroxidation process. Data from our study revealed that OVX significantly increased the lipid peroxidation product, TBARS, and decreased T-GSH as well as NP-SH levels in liver tissues, suggesting that ROS induced by OVX,can play an important role in OVX-induced initiation of hepatic injury. In addition, endogenous antioxidant enzymes such as SOD and CAT play an important role in the process of free radical detoxification and for recycling of endogenous thiol containing molecules. Activities of these enzymes were diminished in OVX treated groups which in agreement with other studies (Kankofer *et al.*, 2007). Therefore, quenching lipid peroxidation and enhancing endogenous enzymatic and non-enzymatic antioxidant status by antioxidant compounds represent an effective strategy to prevent OVX-induced hepatic damage. Thymoquinone is the main constituents of the volatile oil from Nigella sativa seeds. THQ showed protective propertiesagainst chemical toxicity and strong antioxidant properties(Houghton *et al.*, 1995; Nagi & Mansour, 2000). In this regard, earlier studies have reported that THQ prevent oxidative damage in different tissues induced by a variety of free radical generating agents including doxorubicin induced cardiotoxicity, carbon tetrachloride evoked hepatotoxicity, nephropathy produced by cisplatin, autoimmune as well as allergic encephalomyelitis and gastric mucosal injury induced by ischemia reperfusion (Nagi *et al.*, 1999; Nagi & Mansour, 2000; El-Abhar *et al.*, 2003; Mohamed *et al.*, 2005). Similarly in the current work, THQ treatment for eight weeks significantly attenuated oxidative stress biomarkers. THQ dose dependently diminished the elevated levels of TBARS and inhibited the reduced levels of T-GSH and NP-SH. The marked anti-oxidative stress effect of THQ is suggested to be through inhibition of lipid peroxidation (Nagi *et al.*, 1999). Furthermore, THQ can function as a scavenger of superoxide, hydroxyl radical and singlet molecular oxygen (Kruk *et al.*, 2000; Badary *et al.*, 2003). THQ was reported also to decrease ROS production indirectly and to inhibit NO production (Al-Majed *et al.*, 2006). Moreover, THQ was reported to inhibit oxidative brain membrane lipid peroxidation (Houghton *et al.*, 1995). THQ was also reported to inhibit in vitro non-enzymatic lipid peroxidation in mouse liver (Badary *et al.*, 2000). THQ also showed a neuro-protective effect against ischemia-induced brain injury when administered orally to rats. Author in this study suggested that this protection to be through THQ ability to inhibit oxidative stress (Al-Majed *et al.*, 2006).On the other hand, quinine reductase is an enzyme that catalyses the transformation of quinones to hydroquinones by reduction of two electrons, which reduces electrons participation and generation of ROS (Lind *et al.*, 1982; Brunmark *et al.*, 1988). THQ as a substrate for quinonereductase (Nagi *et al.*, 1999) can significantly induce its activity in hepatic tissues (Badary & Gamal El-Din, 2001). Therefore, it may be suggested that up-regulation of quinine reductase in hepatic cells is a possible cellular hepatoprotective mechanism of THQ. Furthermore, treatment of rats with THQ resulted in elevation of hepatic SOD and CAT activates, which my overcome oxidative stress induced during OVX consequences.

CONCLUSION

In summary, oral supplementation of THQ prevented from OVX-induced alterations in hepatic oxidative biomarkers. The protective effects may be due to the reduction of oxidative stress and lipid peroxidation. These observations suggest that THQ may be a clinically viable agent against postmenopausal associated conditions where cellular damage is a consequence of oxidative stress.

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