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Research Article

Neuroprotective Effect of Thymoquinone on Repeated Immobilization Stress-Induced Oxidative Stress in Rats

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Abstract: The present study was designed to investigate the effect of Thymoquinone (TQ) in restraint stress-induced biochemical alterations in Wistar albino rats. Restraint stress was applied for 21 days (4 h/day) and 60 min before every stress exposure the rats were treated with two doses of TQ (5 and 10 mg/kg). In serum, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Interleukin-1β (IL-1β) Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α) were estimated. Thiobarbituric Acid Reactive Substances (TBARS), reduced Glutathione (GSH), nitrite and nitrate levels and Super Oxide Dismutase (SOD), Catalase (CAT), Glutathione-S-Transferase (GST), Glutathione-Reductase (GR) and Glutathione Peroxidase (GSH-Px) activities were estimated in brain. Interleukins and enzymatic activities in serum significantly elevated by the restraint stress and were ameliorated by both the doses of TQ treatment. In brain TBARS, nitrite and nitrate levels significantly elevated due to restraint stress, the TQ treatment brings back to normal levels. Restraint stress significantly decreased in oxidative enzyme activities as the SOD, CAT, GST, GSH-Px and GR. TQ treatments significantly enhanced the activities compared to untreated stressed rats. Present results revealed that, TQ reduced the restraint stress-induced oxidative process in terms of above mentioned biochemical parameters. Thus, in view of its antioxidative nature, TQ may be developed as an effective therapeutic agent for stress-induced oxidation and CNS depression.

Keywords: CNS depression, immobilization, interleukins, oxidative stress, thymoquinone

INTRODUCTION

Immobilization stress is one of easy and suitable method to induce both psychological and physical stress resulting restricted mobility and aggression in animal model (Sahin and Gumuslu, 2007a; Akpinar *et al*., 2007). Chronic psychological stress leads to homeostasis which results biochemical and physiological changes and that follows the serious health risks such as psychiatric disorders, immunosuppressant and neurological impairments (Ya-Li *et al*., 2007; Atif *et al*., 2008). The mechanisms underlying stress-induced tissue damage are not yet fully understood. However, accumulating evidence has implied that the production of free radicals plays a critical role in this process (Madrigal *et al*., 2002; Zaidi *et al*., 2003). It also documented that, immune response is mediated by a complex network of signals and that work in bi-directional communication among the nervous, endocrine and immune systems by common receptors to neurotransmitters, hormones and cytokines (Chesnokova and Melmed, 2002). Stress known to activates the sympathetic-adrenal medulla and hypothalamic-pituitary-adrenal axis which stimulates secretion of catecholamine and glucocorticoid which are capable of modulating the immune cells and cytokine production (Haddad *et al*., 2002; Sekiyama *et al*., 2006). Among the cytokines TNF-α and Interleukin (IL)-6 belong to proinflammatory cytokines, which are pleiotropic molecules produced by a variety of peripheral cell types as well as those cells in the Central Nervous System (CNS). These mediators are often associated with host defense and, as such, the production and release of proinflammatory cytokines are increased by infectious and inflammatory stimuli. Therefore, these proinflammatory cytokines play a critical role in several components of host defense, including CNS-orchestrated events.

Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions. Oxygen radicals can attack proteins, nucleic acids and lipid membranes, thereby disrupting cellular functions and integrity. Brain tissue contains large amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical attacks (Cui *et al*., 2004). There are several studies that are related to the effects of stress on the antioxidant system and induction of lipid peroxidation in brain after immobilization-induced stress model (Akpinar *et al*., 2007, 2008; Kashif *et al*., 2004; Sahin and Gumuslu, 2007a). Furthermore, they suggested that stress increases rates of reactive nitrogen-derived species production including nitrate and nitrite which also mediates the oxidation of lipids (Matsumoto *et al*., 1999; Akpinar *et al*., 2007).

Thymoquinone is the main constituent of the volatile oil of Nigella sativa seeds (black seeds). The volatile oil of N. sativa was shown to contain about 24% TQ (Al-Dakhakhny, 1965) and it has been subjected to a range of pharmacological investigations. Thymoquinone was reported to inhibit eicosanoid generation in rat peritoneal leukocytes and on brain membrane lipid peroxidation (Houghton *et al*., 1995) and it has been shown to attenuate a variety of renal toxicities that are the consequence of oxygen free radical damage, such as cisplatin and doxorubicin-induced nephrotoxicity in rats and mice (Badary *et al*., 1997, 2000). Similarly TQ could protect against carbon tetrachloride-induced hepatotoxicity in mice (Mansour *et al*., 2001) and doxorubicin-induced cardio-toxicity in mice (Al-Shabana *et al*., 1998). Mahgoub (2003) reported that, TQ protects against experimental colitis in rats. With this background, present study was designed to investigate the neuroprotective effects of TQ on restraint stress-induced oxidative stress by using pro-oxidative and inflammatory biomarkers.

MATERIALS AND METHODS

Animal: Twenty-four male Wistar albino rats, roughly the same age and weighing 280-300 g were received from the Experimental Animal Care Center, King Saud University, Riyadh, Saudi Arabia. Animals were maintained under controlled conditions of temperature (22±1°C), humidity (50-55%) and light (12 h light/dark cycles) and were provided with Purina chow (Grain Silos and 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) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Immobilization of rats: Animals were randomly divided into four groups by taking six rats for each group as follows:

- Control
- The group treated with TQ
- The group exposed to immobilization stress
- The group exposed to immobilization stress and treated with TQ. TQ (5 mg/kg/day) was administered orally to the group 2 and 4 for 21 days

The immobilization stress method used in the present study was modified from earlier reports (Nadeem *et al*., 2006; Zaidi *et al*., 2003). In a review by Pare and Glavin (1986), it was concluded that placing animals in their exact size tube was a good immobilization procedure since it involves minimum pain with minimum movement including that of the tail. The rats were deprived of food and water during stress exposure (Liu *et al*., 1996).

Immobilization stress was accomplished by placing individual animals in plastic/well-ventilated tubes of their size. Animals in stress groups were exposed to immobilization stress procedure for 4 h daily for five consecutive days per week. Daily fresh tea solutions were continued even during the unstressed days. The rats were deprived of food and water during stress exposure (Liu *et al*., 1996). Body weights were recorded weekly for the entire study. At the end of each treatment period, animals were sacrificed immediately after the last stress session and blood samples were collected through cardiac puncture in heparin coated centrifuge tubes. Serum samples were separated and kept in freezer at -70°C till analysis. Immediately brain and liver tissues were excised, washed with chilled normal saline, dipped in liquid nitrogen for one minute then preserved at -70°C till analysis.

Serum analysis: In serum, AST and ALT levels were estimated by using commercially available diagnostic kits (Randox diagnostic reagents, Randox Laboratories, USA) and IL-6, IL-1β and TNF-α levels were assayed by Enzyme-Linked Immunosorbent Assay (ELISA) kits (ShangHai SenXiong Science and Technology Company, China). The estimation procedures were followed the instruction provided by the manufacturer.

Tissue parameters: Whole brain was homogenized in 50 mM phosphate buffered saline (pH 7.4) by using a glass homogenizer (Omni International, Kennesaw, GA, USA). The homogenate was centrifuged at 1000 g for 10 min at 4°C 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, MDA, GSH, nitrite and nitrate levels were estimated. In post-mitochondrial supernatant, SOD, CAT, GST, GSH-Px and GR activities were measured.

Estimation of GSH in brain: Glutathione (GSH) levels in brain were assayed according to the method described by Sedlak and Lindsay (1968). The 0.5 mL of tissue 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). Sample tubes were centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was recorded using spectrophotometer at 412 nm in one centimeter quarts cells.

Estimation of TBARS in brain: A TBARS assay kit (ZeptoMetrix) was used to measure the lipid

peroxidation products, Malondialdehyde (MDA) equivalents. One hundred microliters of homogenate was mixed with 2.5 mL reaction buffer (provided by the kit) and heated at 95°C for 60 min. After the mixture had cooled, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The lipid peroxidation products are expressed in terms of nmoles MDA/mg protein using molar extinction coefficient of MDA-thiobarbituric chromophore (1.56×10^5/M/cm).

Estimation of SOD activity in brain: Super Oxide Dismutase (SOD) activity in brain was assayed using the method described by Kakkar *et al.* (1984) with the aid of nitro blue tetrazolium as the indicator. One hundred mL of post-mitochondrial supernatant of brain was used to measure the SOD activity. The reagents: sodium pyrophosphate buffer 1.2 mL (0.052 M) pH 8.3, 0.1 mL phenazine methosulphate (186 μM), 0.3 mL nitro blue tetrazolium (300 μM) and 0.2 mL NADH (780 μM) were added to 0.1 mL of processed tissue sample. The sample mixture was incubated for 90 min at 30°C. Four mL of n-butanol and 1 mL of acetic acid were then added to each sample and the mixture was shaken vigorously. Samples were centrifuged at 4000 rpm for 10 min and the organic layer was withdrawn and absorbance was measured at 560 nm using a spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The SOD activity was estimated as unit/min/mg of protein.

Estimation of CAT activity in brain: The Catalase (CAT) activity was measured by the method of Aebi (1984) using hydrogen peroxide as substrate in brain 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).

Estimation of GST in brain: Glutathione-S-Transferase (GST) activity was measured by the method of Habig *et al*. (1975). The reaction mixture consisted of 0.067 mM reduced glutathione, 0.067 nm CDNB, 0.1 M phosphate buffer (pH 6.0) and 0.1 mL of post mitochondrial supernatant in a total volume of 3 mL. Absorbance was read at 340 nm for 10 min every 30 sec by an optical plate reader and the enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6×10^3/M/cm.

Estimations of GSH-Px activity: Glutathione peroxidase activity was modified from the method of Flohe and Gunzler (1984). For the enzyme reaction, 0.2 mL of the post-mitochondrial supernatant was placed into a tube and mixed with 0.4 mL reduced glutathione and the mixture was put into an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 3000 rpm, 0.48 mL of the supernatant was placed into a cuvette and 2.2 mL of 0.32 M Na_2HPO_4 and 0.32 mL of 1.0 mmol/L DTNB were added for color development. The absorbance at wavelength 412 nm was measured on spectrophotometer (LKB-Pharmacia, Mark II and Ireland) after 5 min. The enzyme activity was calculated as nmol/mg protein.

Estimations of GR activity: Glutathione Reductase (GR) activity was measured in the post-mitochondrial supernatant by the method of Carlberg and Mannervik (1985). GSSG is reduced to GSH by NADPH in the presence of GR. Enzyme activity was measured by following the decrease in absorbance (oxidation of NADPH) for 3 min spectrophotometrically at 340 nm. The activity of enzyme was expressed as nmoles NADPH oxidized/min/mg protein, using molar extinction coefficient of NADPH (6.22 · 106/M/cm).

Estimation of total RNA in brain: The method of Bregman (1983) was used to determine levels of RNA in brain. The homogenates were extracted in different concentrations of cold and hot Trichloroacetic Acid (TCA) and 95% ethanol. The final extraction with 5% TCA was incubated in water bath at 90°C for 15 min. After cool down the tubes were centrifuged at 3000 rpm for 10 min. For quantification of RNA, the extract was treated with orcinol reagent and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

Estimation of nitrite levels in brain: Nitrite levels in brain homogenate were measured by a flourometric assay defined by Misko *et al*. (1993). This assay is based on the reaction of nitrite with an acid form of 2, 3 diaminonaphthalene to form the highly fluorescent product 1- (H) -naphthotriazole. The fluorescence was measured using a fluorimeter, with the excitation wavelength set at 365 nm and the emission wavelength set at 450 nm. Nirite values are expressed as nmoL/g protein.

Estimation of nitrate levels in brain: Nitrate levels were measured by the method of Bories and Bories (1995). Brain homogenate (100 μL) was suspended in 250 μL of 100 mmol/L potassium phosphate buffer (pH 7.5), 50 μL of 0.2 mmoL/L FAD and 10 μL of 12 mmoL/L β-NDPH.

Statistical analysis: All data were presented as the mean±Standard error (S.E.). The data were evaluated by a one-way ANOVA using GraphPad InStat program (version 3.06) and the differences between means were assessed using Student Newman-Keuls. The differences were considered statistically significant at $p<0.05$.

RESULTS

Enzymatic activities including AST (65.61±3.07) and ALT (34.59±1.91) increased significantly ($p<0.01$) in restraint stressed rats up to 3 consecutive weeks as compared their control levels (45.75±2.81) and (20.66±0.86) respectively. Thymoquinone (5 mg/kg) treatment significantly ($p<0.05$) decreased the increased values by the immobilization stress in all the four estimated enzymes (Fig. 1).

As shown in Fig. 2A, the serum IL-1β levels significantly ($p<0.01$) decreased after 3-weeks of immobilization stress in the vehicle-treated rat as compared to controls. Drug treated plus stress group, TQ (5 mg/kg/day) treatment daily one hour before the stress session significantly ($p<0.01$) decreased the IL-1β levels in serum compared to vehicle treated group.

Earlier reports showed an elevation in IL-6 levels by the immobilization stress. In order to investigate the effect of orally administered TQ on serum IL-6 in 3

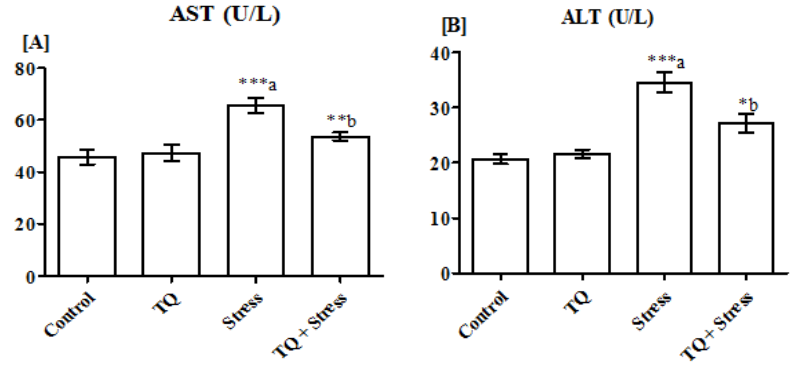

Fig. 1: Effect of TQ on liver enzymes (AST and ALT) activities in restraint stressed rats. Data were expressed as mean + SE. and Applied one-way ANOVA and student Newman-Keulsmultiple test. Sex rats were used in each group; *P < 0.05,**P < 0.01 and ***p<0.001. 'a' vs control and 'b' vs stress group

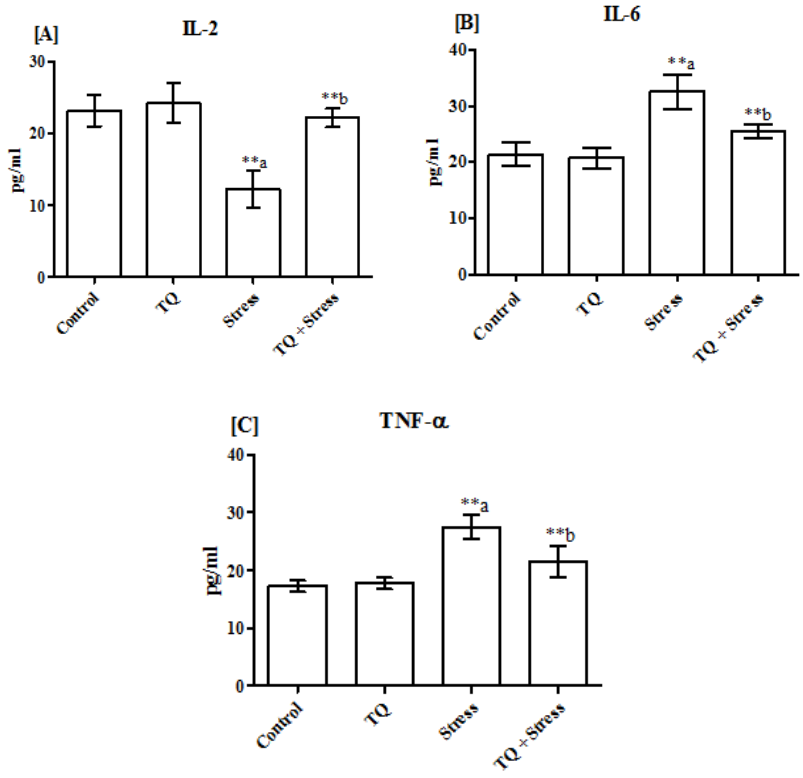

Fig. 2: Effect of TQ on serum interleukin (IL) -2, interleukin (IL) -6 and tumor necrosis factor-α (TNF- α) in restraint stress rats Data were expressed as Mean±S.E. and applied one-way ANOVA and Student-Newman-Keuls multiple comparisons test; Six rats were used in each group; *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$ 'a' vs control and 'b' vs stress group

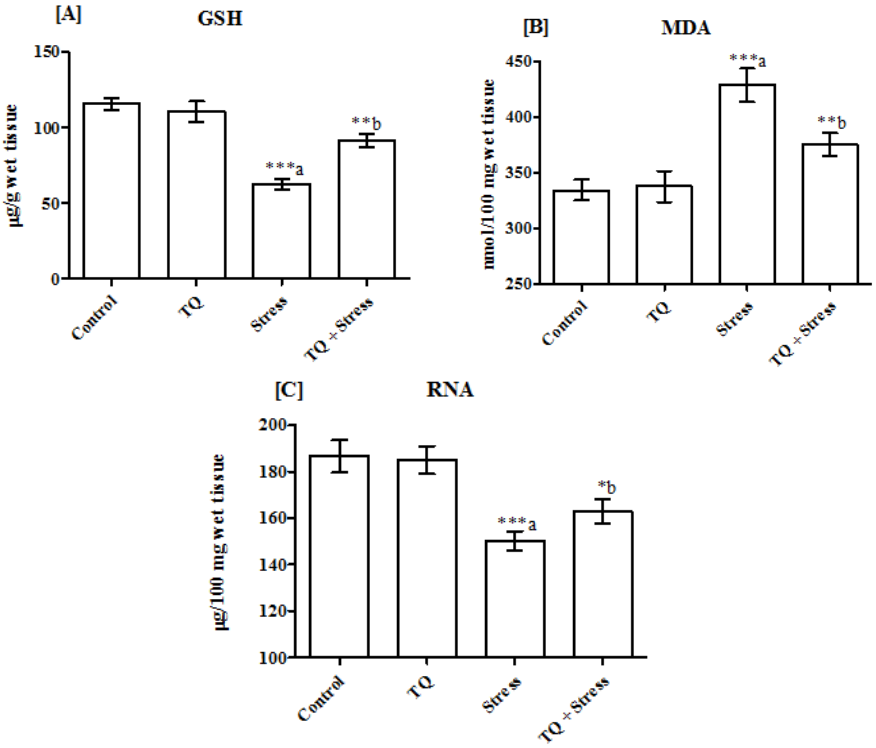

Fig. 3: Effect of TQ on reduced glutathione (GSH), TBRARS (MDA) and RNA levels in brain of restraint stress rats
Data were expressed as mean±S.E. and applied one-way ANOVA and Student-Newman-Keuls multiple comparisons test; Six rats were used in each group; *: p<0.05; **: p<0.01; ***: p<0.001 'a' vs control and 'b' vs stress group

weeks immobilization stress-induced rat. As shown in Fig. 2-B, the serum IL-6 level significantly ($p<0.01$) increased after immobilization stress of the vehicle-treated rats. Daily treatment with TQ (5 mg/kg/day) effectively ($p<0.01$) attenuated the increase in serum IL-6 level (Fig. 2B). Serum TNF-α levels in vehicle-treated immobilization stress rats significantly ($p<0.01$) increased compared to unstressed control rats. Daily pretreatment with TQ (5 mg/kg) significantly decreased the increased serum TNF-α values in immobilization stress rats (Fig. 2C).

Three weeks immobilization stress caused significant ($p<0.001$) decrease in brain GSH concentrations compared to control vehicle rats. Daily treatment with TQ (5 mg/kg) before the stress session significantly ($p<0.01$) increased the GSH levels in brain while compared to the animals in immobilization stress vehicle treated group (Fig. 3A). Malondialdehyde (MDA) estimated as TBARS in brain found significant ($p<0.001$) decrease in immobilization stress-induced vehicle treated rats when compared to normal control rats. Pretreatment with TQ (5 mg/kg) significantly ($p<0.01$) increased the decreased levels of MDA in brain (Fig. 3B). Similar decrease was found in brain RNA levels after 3 weeks immobilization stress and this inhibition was significantly protected with TQ (5 mg/kg/day) pretreatment (Fig. 3C).

In brain, enzymatic activities including SOD, CAT and GST were significantly decreased in immobilization stress vehicle treated rats. The decreased enzymatic activities were found significantly ameliorated by the TQ treatment (Fig. 4A-C).

In the immobilization stress group there was a significant ($p<0.01$) increase in the brain nitrite and nitrate levels as compared to control group. TQ treatment to stressed rats decreased these levels significantly as compared to their respective untreated stressed rats (Fig. 5).

DISCUSSION

It has been documented that exposure to any stress condition can stimulate several pathways leading to enhanced production of free radicals and that known to be generated a drop producing lipid peroxidation and protein oxidation and that followed DNA damage and cell death which contribute to the happening of pathological conditions (Olivenza *et al*., 2000). Stress may also impair antioxidant defenses, leading to oxidative damage by changing balance between oxidant and antioxidant factors (Atif *et al*., 2008; Muqbil and Banu, 2006). One of the reasons for the stress-induced enhancement of free radicals may be the elevation of nitric oxide production (Matsumoto *et al*., 1999; Akpinar *et al*., 2008).

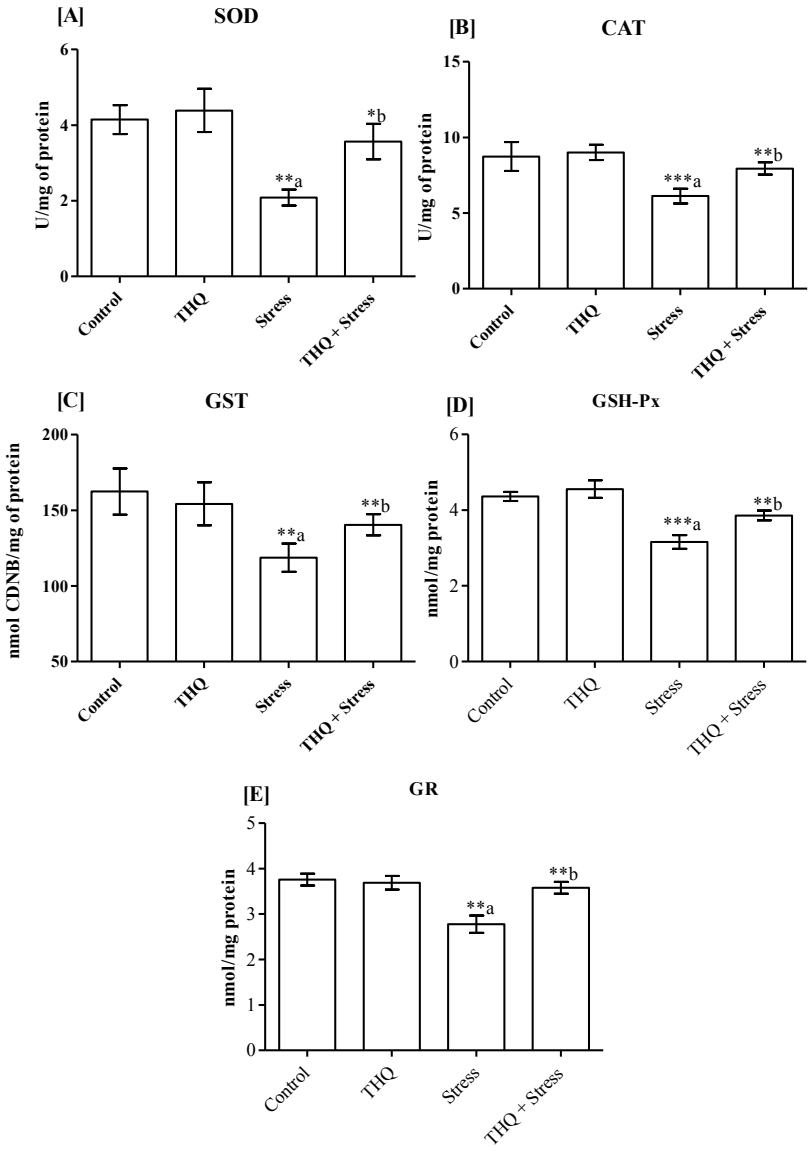

Fig. 4: Effect of TQ on brain pro-oxidative enzymatic activities (SOD, CAT, GST and GSH-Px) in restraint stressed rats. Data were expressed as mean + SE. and Applied one-way ANOVA and student Newman-Keulsmultiple test. Sex rats were used in each group; *p<0.05, **p<0.01 and ***p<0.001. 'a' vs control and 'b' vs stress group

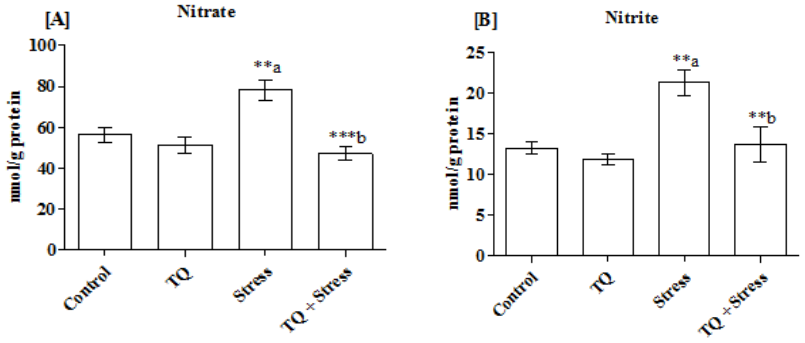

Fig. 5: Effect of TQ on nitrite and nitrite levels in brain of restraint stress rats Data were expressed as mean±S.E. and applied one-way ANOVA and Student-Newman-Keuls multiple comparisons test; Six rats were used in each group; *: p<0.05; **: p<0.01; ***: p<0.001 'a' vs control and 'b' vs stress group

Enzymes such as AST, ALT, ACP and ALP are considered to be the biochemical markers for assessing oxidative function. Increased permeability of cells and necrosis are usually characterized by rise in these marker enzymes (Al-Athar, 2004). In the present study, all such markers in serum were significantly increased after 3-weeks immobilization stress. Similar results were found earlier in rats followed by 3 h/day restraint stress for ten days (Muqbil and Banu, 2006). Present data showed that the daily treatment with TQ to restraint rats significantly decreased these enzyme levels compared to vehicle treated stressed rats. Present results are in agreement with Alsaif (2007) showed TQ protection against ethanol-induced oxidative stress in rats by decreasing the leakage of pro-oxidative enzymes.

Proinflammatory cytokines (IL-1, IL-6 and TNF-α) are known as prominent venerable for stress (Alsaif, 2007; Ya-Li *et al*., 2007), which may be manifested by a cytokinemia. In the present study, immobilization stress raised both serum proinflammatory cytokines, IL-6 and TNF-α levels significantly this confirmed that local inflammatory changes and release of these cytokines did occur in our experimental model. Daily TQ treatment before the immobilization session showed significantly inhibition in elevated serum IL-6 and TNF-α level. These results are in agreement with an earlier report of Alsaif (2007) that, TQ protected the proinflammatory cytokine's (IL-6 and TNF-α) increase induced by ethanol administration in rats. El-Mahmoudy *et al*. (2005) also reported that TQ normalized the elevated levels of the IL-1β and TNF-α in diabetic rats and cultured macrophages.

Brain is a more vulnerable to oxidative stress because of its high sensitivity to stress-induced degenerative conditions. Manoli *et al*. (2000) and Baek *et al*. (1999) documented in their studies that the vulnerability to oxidative stress in the brain is region specific. However, there are several studies, which investigate the stress-induced oxidative modifications in whole brain (Kaushik and Kaur, 2003; Madrigal *et al*., 2001; Sahin and Gumuslu, 2007b). In the present study we used whole brain because of several parameters have measured. It is well established that, stress differentially affects the activity of central dopaminergic and serotonergic neurons (Torres *et al*., 2002) and it stimulates the sympathoadrenal system which activates the catecholamine biosynthetic enzymes (Nankova *et al*., 1994). In present study, restraint stress significantly induced lipid peroxidation as shown elevated MDA levels and depleted GSH in brain. Lipid peroxidation may enhance due to depletion of intracellular GSH content which is considered as a first line of defense as an endogenous non-enzymatic antioxidant. We found that TQ pre-treatment potently reduced immobilization stress-induced lipid peroxidation levels in brain. The mechanistic approach of protection against oxidative stress is mediated through the augmentation of a number of cellular antioxidants such as SOD, CAT and GSH with the supplementation of TQ (Alsaif, 2007). Furthermore, restraint stress decreases the activities of various free radical scavenging/metabolizing enzymes (Radak *et al*., 2001). In the present study, activities of SOD, GST and CAT were found to be decreased while the level of TBARS was increased in response to immobilization stress, which is an indicative of lipid peroxidation. The reactive oxygen species may propagate the initial attack on lipid rich membranes of the brain to cause lipid peroxidation (Floyd and Carney, 1992). The increased lipid peroxidation may also be due to significant depletion of GSH concentration in brain, which acts as one of the guarding factors against oxidative stress (Zaidi and Banu, 2004) and that depletion might be result of decreased activities of SOD, GST and CAT known as free radical scavenging enzymes. Immobilization-induced stress has been shown to cause antioxidant defense changes in plasma (Al-Qirim *et al*., 2002) also in brain (Zaidi and Banu, 2004).

Thymoquinone treatment was able to confer protection against brain glutathione depletion. In this model, treatment with TQ was expected to protect the rat brain against oxidative damage, revealed as normalization of the inhibited antioxidant enzymatic systems. Indeed, TQ proved beneficial in restoring declined SOD, GST and CAT due to immobilization stress. In summary, oral administration of TQ protected rats from immobilization stress-induced oxidative process in brain. Over observations suggest that TQ may be a clinically viable protective agent against a variety of conditions where cellular damage is a consequence of oxidative stress.

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