THYMOQUINONE A POTENTIAL THERAPY FOR CEREBRAL OXIDATIVE STRESS

Bassem Y. Sheikh[1], Ahmed M. Mohamadin[2]

[1]Department of Surgery, Faculty of Medicine,
[2]Department of Clinical Biochemistry,
Taibah University,
SAUDI ARABIA
askdrsheikh@gmail.com

ABSTRACT

Background:_Subarachnoid hemorrhage is a common cerebrovascular event related to different etiologies, primarily aneurysmal rupture. Cerebral vasospasm is the main complication of subarachnoid hemorrhage leading to major morbidity and mortality. Various studies demonstrated that the vasoconstriction secondary to subarachnoid hemorrhage is induced by oxidative stress. It has been previously reported that Nigella sativa (NS) oil and thymoquinone (TQ), active constituent of NS, may prevent oxidative injury in various models._

Methods: _The present study investigated the possible protective effects of thymoquinone (TQ), a compound derived from Nigella sativa with strong anti-oxidant properties, against oxidative stress in rat brains. Thirty two rats were randomly divided into four groups: A (control), B (TQ treated), C (oxidative stress induced) and D (TQ plus oxidative stress induced). Groups B, C and D received TQ (10 mg/kg body weight), PPr (8.51 mg/kg body weight) and (TQ 10 mg/kg + PPr 8.51 mg/kg) once a day orally by using intra-gastric intubation for 4 weeks, respectively._

Results: _A stat of oxidative stress was induced in the rats proved by the production of significant elevation in brain lipid peroxidation, protein oxidation, DNA oxidation and DNA fragmentation, 338%, 132%, 87% and 103%, respectively; associated with a marked decrease in brain-reduced glutathione (GSH) and vitamin C content reaching 63% and 48%, respectively. In addition, PPr administration resulted in significant reduction in the acetylcholine esterase activity (53.6%) and enzymatic antioxidant parameters of brain. TQ treated groups showed significant change in oxidative stress biochemical markers, with almost total reversal towards the control group._

Conclusions:_Thymoquinone treatment counteracted the induced oxidative stress in rats' brain tissue by reducing the levels of peroxidation, and enhancing the activities of enzymatic and non-enzymatic antioxidants. Thymoquinone is a promising agent for the management of cerebrovascular ischemic insults like those resulting from vasospasm._

Keywords:Thymoquinone, Oxidative stress, Lipid proxidation, Brain, Rat, Acute brain injury, Cerebral vasospasm, Nigella sativa

INTRODUCTION

Subarachnoid hemorrhage is a common cerebrovascular event related to different etiologies, primarily aneurysmal rupture. Cerebral vasospasm is the main complication of subarachnoid hemorrhage leading to major morbidity and mortality. Various studies demonstrated that the vasoconstriction secondary to subarachnoid hemorrhage is induced by oxidative stress.(Vecchione C) Furthermore, Endogenous overexpression of extracellelar superoxide dismutase was shown to play a direct role in the etiology of vasospasm.(McGirt MJ)

The rat brain has been shown to have a high oxidative capacity and a low antioxidant defense capacity relative to other body organs.

The brain exhibits distinct variations in cellular as well as regional distribution of antioxidant biochemical defenses (Verma and Srivastava, 2001). Thus, neural cells and/or brain regions are likely differentially respond to changes in metabolic rates associated with the generation of ROS (Hussain et al., 1995). Indeed, there is abundant evidence invoking regional sensitivity to oxidative stress that is dependent on cellular and regional redox status (Baek et al., 1999).

Oxygen free radical (OFR) enzymatic scavengers such as superoxide dismutase (SOD)and catalase (CAT); and glutathione (GSH) metabolism-regulating enzymes such as gamma glutamyltranspeptidase, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST); all may protect the cellular system from various deleterious effects of free radicals (El-Sharkawy et al., 1994; Banerjee et al., 1999; 2001).

Thymoquinone is the main constituent of the volatile oil of Nigella sativa seeds. (Fig. 1A). The volatile oil of Nigella sativa was shown to contain 24% thymoquinone (El-Dakhakhny, 1963). Thymoquinone (Fig. 1B) protects organs against oxidative damage induced by a variety of free radical generating pathologies.(Nagi and Mansour, 2000), (Nagi et al., 1999), (Sayed-Ahmed andNagi, 2007), (Hadjzadeh et al., 2008), (Tekeoglu et al., 2007), (Kanter, 2008), (Juhás et al., 2008), (Nagi and (Radad et al., 2009), (El-Abhar et al., 2003). The antitumor effects of TQ have also been reported in different tumor models. TQ administration may protects against oxidative stress in diabetic mice and lipid peroxidation during cerebral ischemia-reperfusion injury in rat hippocampus (Hosseinzadeh et al., 2007). (Al-Enazi, 2007)

GSH plays a vitally important role in cellular function; in fact the maintenance of GSH homeostasis is essential for the organism to perform its many functions. Indeed, glutathione levels can be monitored as a non-specific indicator of cellular injury, because a decrease in GSH, and subsequently increase of its oxidized form (GSSG), is indicative of an increased potential for cellular injury (Fonnum and Lock, 2004).

To the best of our knowledge, however, there is no report in the literature describing TQ protective role against oxidative stress in the brain. The present study has been designed to investigate the attenuating effect of thymoquinone (TQ) against oxidative stress in the brain in vivo. The extent of oxidative insult in brain caused by PPr and the protective effect of TQ was evaluated by measuring: 1) levels of malondialdehyde (MDA) as index of lipid peroxidation; 2) protein carbonyl content (PCC) as index of protein oxidation; 3) 8-OHdG as index of DNA oxidative damage; 4) activities of intracellular antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione S transferase (GST); 5) levels of non-enzymatic antioxidant, such as reduced glutathione (GSH) as well as vitamin C.

MATERIALS AND METHODS

Male Wister albino rats, weighing 210–225 g, were housed in metabolic cages under controlled environmental conditions (25 °C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated. Thymoquinone was dissolved in propylene glycol and administered 10 mg/kg bwt orally. Control group received the same volume of vehicle only (groundnut oil). Propoxur compound was utilized to induce a state of oxidative stress by dissolving in groundnut oil of pharmaceutical quality and administered orally, 8.51 mg/kg body weight (bwt)/day for 30 days with intra gastric tube. Thirty-two rats were classified into four groups (eight rats each) and subjected to treatment as follows:

Group I

Received 1 ml distilled water per 100 g bwt per day by oral gavage for 30 days and served as a negative control group.

Group II

Received TQ (2-isopropyl-5-methylbenzoquinone; Sigma–Aldrich, St. Louis, MO, USA) orally via intra gastric intubation in a dose of 10 mg/kg/bwt daily for 30 days.

Group III

Oxidative stress was induced by received an aqueous solution of Propoxur, ChemService, West Chester, PA, USA) by oral gavage in a dose of 8.51 mg/kg/ bwt daily for 30 days, and served as a positive control group.

Group IV

Received TQ orally in a dose of 10 mg/kg/ bwt 1 h before starting Propoxur treatment daily for 30 days.

Twenty-four hour after administration of the last dose, the animals were anaesthetized using ether and sacrificed. Brain tissues were collected, weighed accurately, and cut into small pieces and then homogenized in ice-cold homogenization buffer (10 mM KH2PO4 (pH 7.4); 20 mM EDTA; 30 mMKCl) to give 10% homogenate. The homogenate was used for the determination of brain contents of GSH, vitamin C, PCC, MDA, 8-OHdG and enzymatic activities of SOD, CAT, GSH-Px, and GST.

Biochemical Analysis Performed Included

1)Acetylcholinesterase (AchE) assay: AchE activity was estimated by the method of Ellman et al. (1961). A total assay volume of 355 μL, consisting of 5 μL of tissue, 300 μL of chromogen/buffer (0.3mM 5,5′-dithio-bis (2-nitrobenzoic acid); final DTNB concentration in assay 0.25 mM) and 50 μL of substrate (8.45 mMacetylthiocholine iodide; final concentration in assay 1.2 mM). There is a 5-min preincubation period and the reaction is conducted at 37 °C. Specific activity of AchE was expressed as nmoles of substrates hydrolysed/min/mg protein.

2) Determination of non-enzymatic antioxidants:

GSH Assay: The level of the cellular metabolite GSH was measured according to the method of Ellman (1959). 720 μL of sample was double diluted, and 5% TCA was added to precipitate the protein content. After centrifugation (at 10,000×g for 5 min) the supernatant was taken, DTNB solution (Ellman's reagent) was added to it, and the absorbance was measured at 412 nm.

Ascorbic acid (AA) assay: Vitamin C concentration was measured by Omaye et al. (1971) method. To 0.5 ml of brain homogenate, 1.5ml of 6% TCA was added and centrifuged (3500×g, 20min). To 0.5 ml of supernatant, 0.5 ml of DNPH reagent (2% DNPH and 4% thiourea in 9N sulfuric acid) was added and incubated for 3 hours at room temperature. After incubation, 2.5 ml of 85% sulfuric acid was added and the developed color was read at 530 nm after 30 min.

3) Determination of malondialdehyde level: The tissue MDA level was determined by a method (Okhawa et al., 1979) based on the reaction with thiobarbituric acid (TBA) at 90–100 ^{o}C. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol/g protein.

4) Determination of tissue protein carbonyl content: The PC contents were determined spectrophotometrically by the method based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone (Levine et al., 1990). The results were given as nanomoles carbonyl per mg protein.

5) Determination of DNA fragmentation: Quantitative analysis of DNA was carried out by diphenylamine reaction. The percentage of fragmentation was calculated from the ratio of DNA in the supernatant to the total DNA by the method of Lin et al., 1997.

6) Determination of 8-OHdG: The brain tissue homogenate concentration of 8-OHdG was determined using the competitive enzyme-linked immunosorbent assay (ELISA) BIOXYTECH 8-OHdG-EIA diagnostic kit (Oxis Health Products, Inc., USA).

Antioxidant Enzymes:

1) Determination of superoxide dismutase activity: Brain SOD activity was determined according to the method of Sun et al.1988). The principle of the method is based on the inhibition of nitroblue-tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate and the results were expressed as U/mg protein.

2) Determination of catalase activity: Brain catalase activity was determined according to Aebi's method, 1974). The principle of the assay is based on the determination of the H_2O_2 decomposition rate at 240 nm. Results were expressed as U/mg protein.

3) Determination of glutathione peroxidase (GSH-Px) activity: Brain GSH-Px activity was measured by the method of Paglia and Valentine, 1967). The enzymatic reaction in the tube, containing: NADPH, reduced glutathione, sodium azide, and glutathione reductase, was initiated by addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer. Results were expressed as U/mg protein.

4) Determination of glutathione-S-transferase (GST) activity: The activity of GST was measured by the method of Habig et al.,1974). The reaction mixture contained suitable amount of enzyme (25 μg of protein in brain tissue homogenate), KH2PO4 buffer, EDTA, CDNB, and GSH. The reaction was carried out at 37◦C and monitored spectrophotometrically at 340 nm for 5 min. One unit of GST activity is defined as 1 μmol product formation per minute.

Protein determination: Protein concentrations were determined by the method of Lowry et al., 1951) calibrated with bovine serum albumin.

Data are expressed as mean ± S.E. The differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. A

0.05 level of probability was used as the criterion for significance. All univariate analysis of variance (ANOVA) was completed using SPSS 10.0 software (SPSS Inc., Chicago, IL).

RESULTS

The body and brain weights were significantly decreased in PPr-induced OS(Table 1). However, the pretreatment with TQ restored only the brain weight but not the body weight.

Reaching a state of oxidative stress in Group 3 as compared with control group was evident by the following findings,: 1) decreased AchE activity by 53.6% (P < 0.001); 2) decreased GSH and vitamin C levels, 63% and 48%, respectively (P < 0.001); 3) elevated levels of MDA and PCC, 338% and 132%, respectively (P < 0.001) (Figure4); 4) increased ratio of DNA fragmentation and 8-OHdG level, 103% and 87%, respectively (P < 0.001); 5) decreased activities of SOD, CAT, GSH-Px and GST, 52, 66, 56 and 58%, respectively (P < 0.001); 6) A significant positive correlation coefficients between 8-OHdG level and MDA ($r = 0.683$; $P < 0.01$) and PCC levels ($r = 0.610$; $P < 0.01$).

In group 4, TQ was intruded 1 hour prior to the induction of the state of oxidative stress. The protective effects of TQ on the oxidative stress model group was evident by the following facts: 1) restoration of AchE activity by 74% compared to OS group (P < 0.001) (Fig 2); increased levels of GSH and vitamin C, 134% and 62%, respectively) (Fig. 3); 3) decreased levels of MDA and PCC, reaching the control group level (Figure 4); 4) decreased levels of DNA fragmentation and 8-OHdG, 55% and 63%, respectively (Fig. 5); 5) restoration of activities of SOD, CAT, GSH-Px and GST, reaching the control group level (Figure 6); 6) a significant negative correlation coefficients between GSH level and 8-OHdG ($r = –0.712$; $P < 0.01$) and MDA ($r = –0.823$; $P < 0.01$) (Fig. 8a-b).

DISCUSSION

Scientific evidence suggests that oxidative stress is involved in the pathogenesis of several neurological diseases including acute stroke and cerebral vasospasm following subarachnoid hemorrhage (SAH).(Ayer R.E) (Jurcau A). Ischemic brain insult initiates metabolic events that involve the generation of oxygen free radicals. These free radicals and related reactive chemical species are responsible for much of the damage that occurs after brain ischemia.(Love S)(Jurcau A)

Free radicals may be generated following SAH secondary to disrupted mitochondrial respiration and extracellular hemoglobin with up regulation of free radical producing enzymes. This occurs in addition to inhibition of the intrinsic protective antioxidant systems such as superoxide dismutase and glutathione peroxidase.(Ayer R.E)

The brain consumes a large quantity of oxygen, making it particularly susceptible to oxidative stress.

Experiments have linked free radicals to the apoptosis of neurons and endothelial cells, blood brain barrier breakdown and the altered contractile response of cerebral vessels following SAH. Antioxidant therapy has provided neuroprotection and antispasmotic effects in experimental SAH and some therapies have demonstrated improved outcomes in clinical trials. These studies have laid a foundation for the use of antioxidants in the treatment of aneurismal SAH.

Laboratory evidences showed that reactive oxygen species such as peroxide, superoxide and peroxynitrite are likely involved in the etiology of cerebral vasospasm after SAH.(Pyne-Geithman G)(Sasaki T)(Saito A)(Morgan CJ)

Oxygen radicals are known to damage cellular lipids, proteins, and nucleic acids and to initiate cell signaling pathways after cerebral ischemia. (Saito A)

The reactive oxygen species in cerebrospinal fluid from SAH patients are thought to come from three sources: (1) oxyhemoglobin, (2) immune cells and (3) aberrant activity of nitric oxide synthase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. These reactive oxygen species are thought to elicit smooth muscle contraction by peroxidation of membrane lipids both in the vascular smooth muscle and the endothelial cells.

Studies suggested that TQ may have protective effects on lipid peroxidation process during brain ischemia in rats.

In the present study, a state of oxidative stress was induced in the experiments' rat brain, proved by disturbing the status of the different antioxidant indices including: lipid peroxidation, protein carbonylation, DNA oxidative damage and intracellular antioxidant power. All the induced antioxidant indices were reversible / preventable by the administration of thymoquinone.

Furthermore, these tissue injuries caused oxidative stress status as evidenced with decreased enzymatic antioxidants; SOD, CAT, GSH-Px and GST demonstrated the severity of PPr-induced brain oxidative stress.

The antioxidant and neuroprotective potential of several therapies has been investigated in experimental models.(Imai H) (Warner DS)

TQ, a main active constituent in Nigella sativa seed, ameliorated oxidative injury in the brain tissues.

These findings are also consistent with recent reports which demonstrate that carbofuran, a carbamate pesticide-induced oxidative stress is attenuated by antioxidant vitamins such as vitamins A, C and E (Slotkin et al., 2007; Verma et al., 2007; Yu et al., 2008), lending further support to the role of ROS in mediating PPr neurotoxicity.

Maintenance of normal cellular functions in the presence of oxygen largely depends on the efficiency of the defense mechanisms against reactive oxygen species (ROS)-mediated oxidative stress. Glutathione is considered to be the first line of cellular defense against mediated oxidative damage. GSH functions by scavenging free radicals and consequently convert to its oxidized form, glutathione disulfide (GSSG). In cerebral tissue, GSH plays an important function probably by neutralizing ROS non-enzymatically (Everett and Gronberg, 1971). The brain contains large amount of polyunsaturated fatty acids and consumes 20% of the body's oxygen (Hill et al., 1984; Pajovic et al., 2003). It has relatively poor antioxidant defense (Carbonell and Rama, 2007).

In the present study, oxidative damage was induced by PPr compound producing a higher degree of lipid peroxidation as well as protein carbonylation and DNA oxidative damage in the brain tissue of the experimental animals. Moreover, PPr exposure stimulates the DNA fragmentation ratio, which is considered to be the potent marker of oxidative stress in brain tissues. Pretreatment with thymoquinone followed by PPr exposure prevented the increase in the extent of lipid peroxidation, protein carbonylation as well as DNA oxidation.

The present findings show that thymoquinone treatment attenuated lipid peroxidation in the rat brain. This was proved by the decreased MDA level, accompanied by increased GSH and vitamin C content and enhanced activities of CAT, SOD, GSH-Px and GST enzymes. These results could be attributed to the potential antioxidant effect of thymoquinone (Sayed-Ahmed and Nagi, 2007). Others have also shown protective effects of TQ against carcinogens via induction of quinonereductase and glutathione-S-transferase enzymes in mice liver. (Nagi and Almakki 2009). Moreover, these findings are consistent with those of Hosseinzadeh et al., (2007) who demonstrated that TQ attenuates lipid peroxidation and oxidative stress during global cerebral ischemia-reperfusion injury in rat hippocampus. Recently, Kanter (2008) reported that thymoquinone supplementation prevents hippocampal neurodegeneration after chronic toluene exposure in rats.

It is well known that endogenous antioxidant enzymes are responsible for preventing and neutralizing the free radical-induced oxidative damage. The antioxidant enzymes, such as catalase, SOD, GSH-Px, GR and GST, constitute a major supportive group of defense against free radicals (Sung et al., 2000). The results of our study show that oxidative stress model may be induced by PPr compound, evident by impairment in enzymatic (SOD, CAT, GSH-Px, and GST) and non-enzymatic (GSH and vitamin C). Administration of TQ restored the activities of SOD, CAT, GSH-Px, AchE and decreased the levels of LPO, PPC and 8-OHdG in the brain to normal levels.

CONCLUSION

A status of oxidative stress was induced by PPr compound and served as oxidative stress model in all study animals. The present findings show that thymoquinone treatment can attenuate oxidative damage in the rat brain via up-regulating the activities of enzymatic antioxidants (CAT, SOD, GSH-Px and GST) and by replenishing non-enzymatic antioxidants (GSH and vitamin C).

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Table 1. Body and brain weights in control and experimental groups

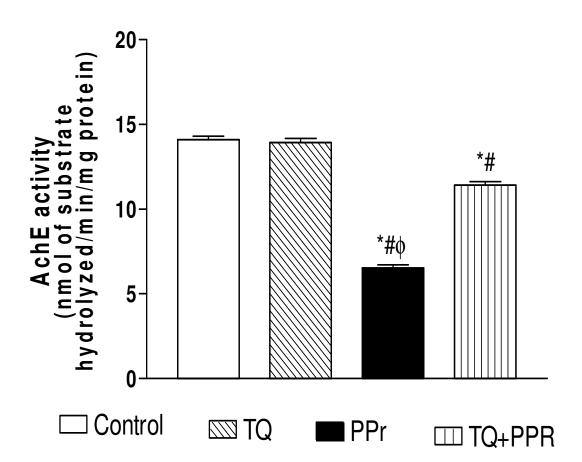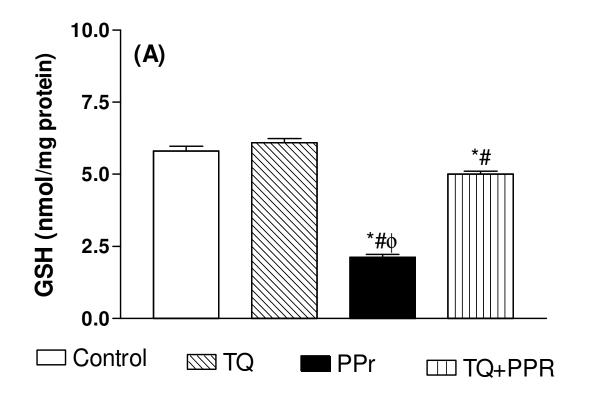|	Control (n = 8)	TQ (n = 8)	PPr (n = 8)	TQ + PPr (n = 8)
Body weight (g)	190 ± 3.5	195 ± 4.0	142 ± 3.5*	169 ± 5.0*#
Brain weight (mg)	2.07 ± 0.03	1.98 ± 0.02	1.81 ± 0.04*	1.91 ± 0.02*

Values are expressed as means ± SE; n = 8 for each treatment group.

TQ, Thymoquinone; PPr, propoxur.
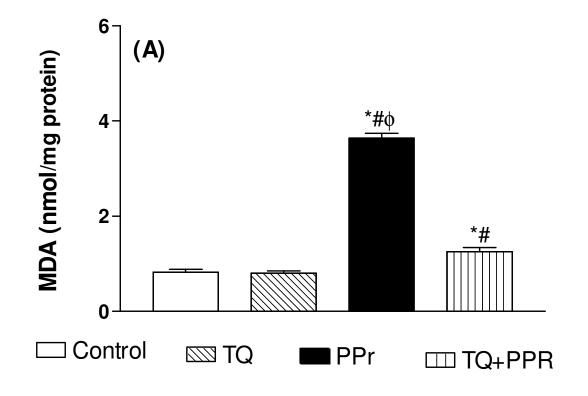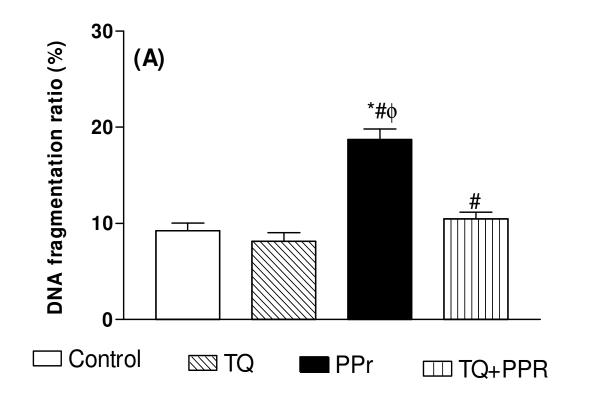
*P <0.01 compared with the control group.

#P < 0.01 compared with the PPr-treated group.

FIGURES

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