

THYMOQUINONE ATTENUATES BRAIN INJURY VIA AN ANTI-OXIDATIVE PATHWAY IN A STATUS EPILEPTICUS RAT MODEL

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Abstract

Aim: Status epilepticus (SE) results in the generation of reactive oxygen species (ROS), which contribute to seizure-induced brain injury. It is well known that oxidative stress plays a pivotal role in status epilepticus (SE). Thymoquinone (TQ) is a bioactive monomer extracted from black cumin (*Nigella sativa*) seed oil that has anti-inflammatory, anti-cancer, and antioxidant activity in various diseases. This study evaluated the protective effects of TQ on brain injury in a lithium-pilocarpine rat model of SE and investigated the underlying mechanism related to antioxidative pathway. **Methods:** Electroencephalogram and Racine scale were used to value seizure severity. Passive-avoidance test was used to determine learning and memory function. Moreover, anti-oxidative activity of TQ was observed using Western blot and super oxide dismutase (SOD) activity assay. **Results:** Latency to SE increased in the TQ-pretreated group compared with rats in the model group, while the total power was significantly lower. Seizure severity measured on the Racine scale was significantly lower in the TQ group compared with the model group. Results of behavioral experiments suggest that TQ may also have a protective effect on learning and memory function. Investigation of the protective mechanism of TQ showed that TQ-pretreatment significantly increased the expression of Nrf2, HO-1 proteins and SOD in the hippocampus. **Conclusion:** these findings showed that TQ attenuated brain injury induced by SE via an anti-oxidative pathway.

Keywords

• brain injury • Nrf2 • oxidative stress • status epilepticus • thymoquinone • status epilepticus

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Introduction

Status epilepticus (SE) is a common, life-threatening neurologic disorder characterized by acute, prolonged epileptic seizures. The incidence rate of SE is about 0.05 to 0.1% in the general population and 1.1 to 14% in the epileptic population. SE has a poor prognosis because seizures that cannot be terminated quickly result in irreversible brain injury. It is known that multiple factors are involved in the pathogenesis of brain injury induced by SE, one of which is oxidative stress [1–2]. The generation of excess free radicals and increase in lipid peroxidation that occur during SE damage brain tissue, therefore, antioxidants might be effective for treatment of SE [3–5]. Thymoquinone (TQ), a bioactive monomer derived from black seed (*Nigella sativa*) oil, has significant antioxidant effects [6–8]. It has been shown to suppress oxidative stress

and attenuate seizure activity and lower hippocampal neuronal loss in an epilepsy model and in children with refractory seizures [9–11]. We speculate that TQ alleviates the brain injury of SE by modulating oxidative stress. It is thought that the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway plays a key defensive role against oxidative stress in epilepsy [12–14]. This study evaluated the protective effects of TQ on brain injury in a status epilepticus rat model, and the underlying mechanism related to antioxidative pathway using biochemistry techniques and behavioral and electrophysiological tests.

Materials and methods


Reagents

Pilocarpine, Lithium chloride, and TQ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and study groups

Male Sprague-Dawley (SD) rats weighing 200–230 g were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). All animal experiments were carried out following the Guidelines for Animal Experiments of the Chinese Academy of Medical Sciences and were approved by the ethics committee for animal care of Jinshan Hospital. Rats were housed in a standard animal room with three animals in each cage and 12 h of light per day. The temperature was maintained at 20±2°C, the relative humidity at 60%. Animals were randomly divided into three groups: normal control, status epilepticus model (model) and status epilepticus + thymoquinone (TQ)(n=11). Rats in the TQ group were administered TQ 10 mg/kg twice by intraperitoneal injection 24 hours and 1 hour prior to injection of pilocarpine. Rats in the model group were administered equivalent volumes of normal saline.

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Establishment of a status epilepticus rat model

Rats were intraperitoneally injected with 1% lithium chloride (3 mg/kg) followed by 1% pilocarpine (30 mg/kg) 20 hours later. Preliminary observation confirmed that seizures would appear 15 min after pilocarpine injection. Seizure behaviors after kindling were classified using the Racine scale as Stage I, mouth and facial movements; Stage II, head nodding; Stage III, forelimb clonus; Stage IV, rearing; and Stage V, rearing and falling [15]. Rats with consecutive Stage IV seizures for 60 min was injected diazepam to terminate the seizures. Passive avoidance task were carried out after the model was established for 48 hours.

Electroencephalograms and seizure behavior

Rats were immobilized on the stereotaxic instrument after intraperitoneal anesthesia with 10% chloral hydrate. To record EEGs, stainless steel screw electrodes were placed epidurally into the bone. The recording electrode was placed 2 mm lateral and 2 mm posterior to the bregma. The reference electrode was 2 mm lateral and -2 mm posterior to the bregma, and the ground electrode was -10 mm lateral and 0 mm posterior to the bregma. Two stainless steel screws were fixed on the bone with dental acrylic. After waiting 3 days for the animals to recover, EEG signals were recorded monopolarly using an animal Bluetooth digital electroencephalograph (BT-8, Nuocheng, Shanghai, China) in freely moving rats. The latency to first seizure and total power (frequency and amplitude) were recorded. Behavioral changes were recorded by video cameras, and seizures induced by lithium chloride-pilocarpine were scored according to the Racine scale.

Passive-avoidance test

Passive-avoidance tests were performed in two identical light and dark square boxes. On day 24, the rats were initially placed in the light chamber; after 10 seconds, the door between the compartments was opened. When rats entered the dark compartment, the door was

closed and an electrical foot shock (0.1 mA/10 g body weight) was delivered through stainless steel rods for 2 sec (one training trial). Twenty-four hours after the training trial, the rats were again placed in the light compartment and the step-through latency to enter the dark compartment was measured [17].

Western blot assay

Total protein was extracted from hippocampal and cortical tissue with 1× RIPA lysis buffer (Beyotime Institute of Biotechnology, China) supplemented with 1 mM PMSF. Protein samples were run on SDS-PAGE gels and transferred to PVDF membranes. The membrane was incubated with anti-Nrf2 rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology, USA), anti-HO-1 mouse monoclonal antibody (1:500; Abcam, USA), and anti-alpha tubulin primary antibody (1:5000; Proteintech group, USA) at 4°C overnight. Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5000; Proteintech group) or goat anti-mouse (1:5000; Proteintech group) secondary antibody at room temperature for 2 h. Signals were detected by electrochemiluminescence (ECL-Plus; Merck Millipore, Darmstadt, Germany) and quantified using a Bio-Rad 2000 gel imaging system with QUANTITY ONE software (Bio-Rad Laboratories, Hercules, CA, USA).

Assay of super oxide dismutase (SOD) activity

The inhibition rate of SOD was assayed with SOD detection kit (DOJINDO, Shanghai, China) following the manufacturer's instructions and OD value was read by Bio-Rad 680 microplate reader (Bio-Rad Laboratories).

Statistical analysis

Data were expressed as means±standard deviation (SD). Mean values were compared using the paired-sample t-test or one-way analysis of variance (ANOVA). Behavioral scores were tested for significance using Kendall's rank correlation analysis. All statistical analyses were performed using SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$.

Results

Rat model of SE

In the model group, two rats died during establishment of the model. The remaining 11 rats were successfully modeled. No rats died in the TQ group.

Effect of TQ on EEG

As shown in Figure 1, the latency to SE in the TQ group after pilocarpine administration was longer than that in the model group ($p < 0.05$). In addition, the total power was significantly lower in the TQ group than in the model group ($p < 0.05$).

Effect of TQ on seizure behavior

Seizure behavior was measured by Racine scale grade. As shown in Figure 2, Grade IV, and V seizures were observed in the model group. In the TQ group, nearly all seizures were Grades II and III, only one seizure was Grade IV. Compared with the model group, the Racine grades of the TQ group were significantly lower ($p < 0.05$). The data suggest that the overall seizure behavior was milder in the TQ group.

Effect of TQ on cognitive function

The step-through test was used to estimate the effect of TQ on cognitive function. As shown in Figure 3, post-SE latent period in model group rats was significantly shorter than that observed pre-SE, thus indicating that SE impaired learning and memory. Compared with the rats in model group, the post-SE escape latency in TQ group rats was significantly longer ($p < 0.05$). These results indicated the improvement of spatial learning and memory in TQ group.

Antioxidative effect of TQ

We assayed Nrf2, HO-1, and SOD levels in the hippocampus and cortex to determine whether TQ inhibited the oxidative stress induced by SE. Western blots showed that the levels of Nrf2 and HO-1 protein increased significantly in the TQ group compared with the model group ($p < 0.05$) (Figure 4A,B). SOD inhibition rate was higher in the TQ group than that in the model group ($p < 0.05$) (Figure 4C).

Discussion

The International League Against Epilepsy (ILAE) defines SE as a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms that lead to abnormally prolonged seizures. It is obvious that SE can

lead to long-term consequences, including neuronal death and neuronal injury [18]. The brain injury induced by SE is unavoidable. It has been reported that a single SE seizure can reduce cognitive function of patients by 8 to 26% [19]. Clinical studies have demonstrated that SE patients present neurological deficits associated with seizures that correspond to

imaging changes, such as high T2WI signals, and brain atrophy [20]. The existence of pathological changes of hippocampus, limbic system, cortex and cerebellar tissue have been confirmed by studies in both animal models and SE patients [21,22]. SE can induce increased oxygen consumption, apnea, and vasomotor dysfunction, which lead to cerebral hypoxia. Subsequent to brain injury, other pathological events such as excitatory neurotoxicity, inflammation, and changes in the microenvironment of glial cells and neurons contribute to the brain injury caused by SE [23–25].

Recent evidence shows that oxidative stress plays a key role in SE [26,27]. Under normal circumstances, brain tissue continuously produces ROS, such as NO, hydrogen peroxide, and superoxide. At the same time, many antioxidant enzymes are released in brain tissue, including HO-1, GSH and SOD. To protect brain tissue from damage by ROS, a delicate balance is maintained. When ROS increase rapidly, the balance is disturbed, and oxidative damage occurred [28]. In animal models, SE can lead to increased oxidative stress in the brain [26]. Oxygen free radicals are produced by the abnormal metabolism in the brain as a consequence of SE, and further aggravate brain injury. It is well known that mitochondrial damage is a key step in the pathophysiological increase of excitatory neurotransmitter synthesis and promotion of neuronal apoptosis in the brain [27]. A number of studies have indicated that inhibiting oxidative stress has a protective effect on the brain injury induced by SE [29,30].

TQ is a bioactive monomer isolated from the oil of black cumim (*Nigella sativa*) seed, and is traditionally used for treating hypertension, asthma, dysentery and eczema [31]. TQ has multiple bioactive properties, including anticancer, anti-inflammatory and antioxidant effects [6–8]. Recently, it was shown that TQ is potent mitochondria-targeted antioxidants effective in treating a large number of age-related pathologies [32, 33]. Previous studies in a temporal lobe epilepsy model epilepsy have shown that TQ reduced seizures and protected hippocampal neurons against apoptosis [9,10]. Other studies have evaluated TQ for treatment

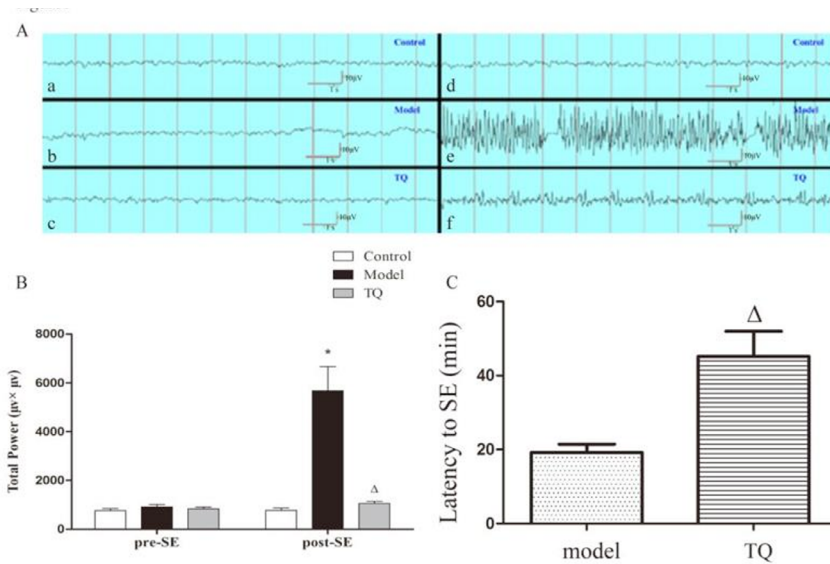


Figure 1. Total power was significantly lower in the TQ group after pilocarpine administration than in the model group (A, B). Pre-SE EEG of control group (a), model group (b), and TQ group (c) rats; post-SE EEG of control group (d), model group (e), and TQ group (f) rats. The latency to SE in the TQ group was longer than that in model group. *model group vs. control group, $p < 0.05$, Δ TQ group vs. model group, $p < 0.05$.

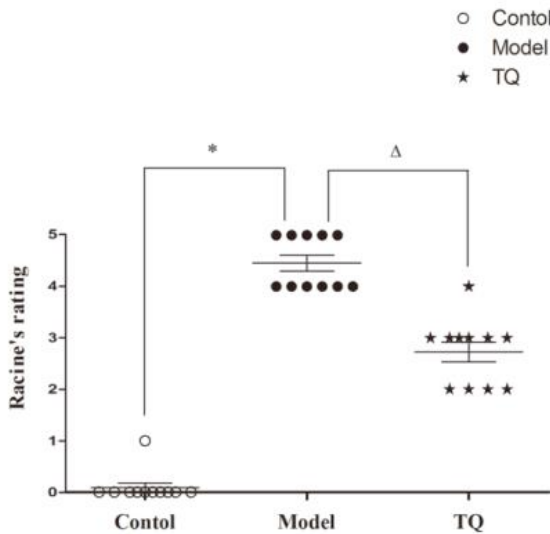


Figure 2. Effect of TQ on the epileptic behavior of status epileptic rats. *model group vs. control group, $p < 0.05$, Δ TQ group vs. model group, $p < 0.05$.

of refractory epilepsy in children [11]. We thus speculated that TQ has a protective effect on SE. We found that TQ had an anticonvulsant effect as measured by better representation of EEG, and a decrease in seizure severity. In addition, results of the behavioral experiments suggest that TQ may also have a protective effect on learning and memory function. In our study, we used passive-avoidance testing to evaluate the learning and memory function in a state of fear.

The protective mechanism of TQ was evaluated in rats with pilocarpine-induced SE. We also assayed the expression of antioxidants (Nrf2 HO-1 and SOD) in brain tissue. The expression level of proteins is difference between cortex tissue and hippocampus tissue, so we detected the expression in both cortex tissue and hippocampus tissue. The results showed that Nrf2 and HO-1 proteins and SOD were highly expressed in TQ group rats, and indicate that TQ may reduce the brain injuries caused by SE by Nrf2-mediated activation of antioxidant defense. Nrf2 is a key transcription factor involved in antioxidant response, and can thus protect cells from toxic substances and pathogens [34]. Nrf2 belongs to a subset of basic leucine zipper genes. Under normal conditions, Nrf2 is tethered in the cytoplasm by the Keap1 protein. Activated Nrf2 translocates into the nucleus, where it binds to a small Maf protein and activates the transcription of target genes, such as heme oxygenase-1 (HO-1), NADPH: quinone oxidoreductase 1 and SOD. HO-1 is a rate-limiting enzyme for degrading heme into biliverdin, carbon monoxide (CO), and free iron [35]. As antioxidant enzymes, HO-1 and SOD can protect against oxidative stress. The crucial role of Nrf2 and its downstream products in the antioxidant defense has been demonstrated in various diseases [13,36].

Overall, our results indicate that TQ has a protective effect on brain injury induced by pilocarpine. The mechanism may be mediated by modulation of an antioxidative pathway. The study provides experimental evidence to support further evaluation of TQ as a potential therapeutic drug for treatment of SE.

Competing interests

The authors have no competing interests to declare.

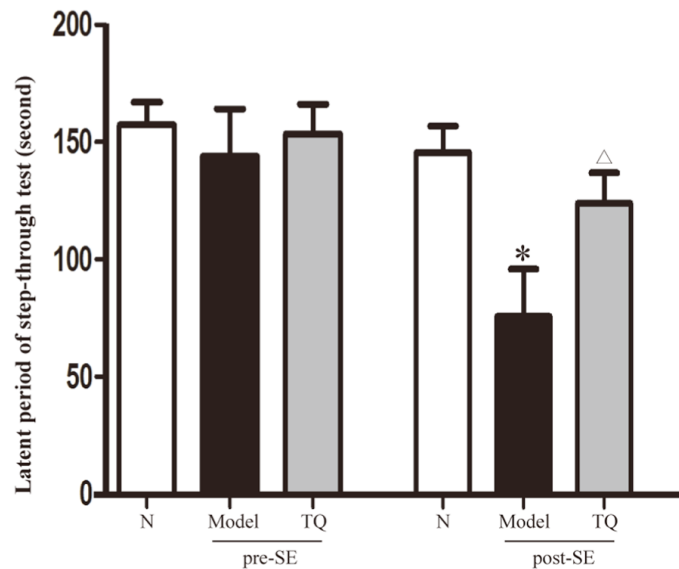


Figure 3. Effect of TQ on the epileptic behavior of SE rats. Latent period of the step-through test. *model group vs. control group, $p < 0.05$ Δ TQ group vs. model group, $p < 0.05$.

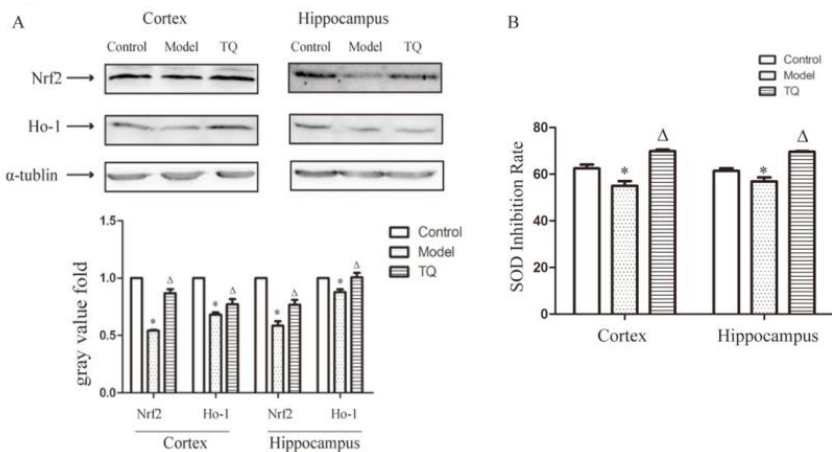


Figure 4. TQ increased Nrf2, HO-1 and SOD inhibition rate in the cortex and hippocampus of SE rats. (A) Protein extracted from hippocampal and cortical tissues was assayed by western blotting. Nrf2, HO-1 expression in SE rat brains was significantly lower than in control group rats. TQ significantly downregulated the expression of Nrf2 and HO-1. (B) The inhibition rate of SOD. *Model group vs. control group; Δ TQ group vs. model group, $p < 0.05$.

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Authors' contributions

Y Shao performed the western blot assays, generated the figures, and wrote the manuscript. Y Huang and B Li performed the

electrophysiological procedures. S Hu, Q Luo and Y Xie carried out the behavioral tests. Y Chen contributed to the development of the project and the design and performance of experiments. All authors have read and approved the final manuscript.

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