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Pretreatment with *tert*-butylhydroquinone attenuates cerebral oxidative stress in mice after traumatic brain injury

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ABSTRACT

Background: Traumatic brain injury (TBI) is a worldwide health problem, identified as a major cause of death and disability. Increasing evidence has shown that oxidative stress plays an important role in TBI pathogenesis. The antioxidant transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), is a known mediator in protection against TBI-induced brain damage. The objective of this study was to test whether *tert*-butylhydroquinone (*t*BHQ), a novel Nrf2 activator, can protect against TBI-induced oxidative stress.

Methods: Adult male imprinting control region mice were randomly divided into three groups: (1) sham + vehicle group; (2) TBI + vehicle group; and (3) TBI + *t*BHQ group. Closed-head brain injury was applied using the Feeney weight-drop method. We accessed the neurologic outcome of mice at 24 h after TBI, and subsequently measured protein levels of Nrf2 and the NOX2 subunit of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), the concentration of malondialdehyde, superoxide dismutase activity, and brain edema.

Result: The NOX2 protein level was increased fivefold in the TBI + vehicle group, whereas pretreatment with *t*BHQ markedly attenuated the NOX2 protein expression relative to that in the TBI + vehicle group. TBI increased Nrf2 formation by 5% compared with the sham group, whereas treatment with *t*BHQ further upregulated the Nrf2 protein level by 12% compared with the sham group. The level of the oxidative damage marker malondialdehyde was reduced by 29% in the TBI + *t*BHQ group compared with the TBI + vehicle group, Moreover, pretreatment with *t*BHQ significantly increased the antioxidant enzyme superoxide dismutase activity. Administration of *t*BHQ also significantly decreased TBI-induced brain edema and neurologic deficits.

Conclusions: Pretreatment with *t*BHQ effectively attenuated markers of cerebral oxidative stress after TBI, thus supporting the testing of *t*BHQ as a potential neuroprotectant and adjunct therapy for TBI patients.

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1. Introduction

Traumatic brain injury (TBI) is a worldwide health problem, which has become the major cause of death and disability among young adults in industrialized countries. An estimated 1.7 million people suffer traumatic brain injuries each year in the United States. Approximately 25% of these injuries result in long-term disabilities, causing significant familial, social, and economic burdens [1,2]. Despite the progress in diagnosis, neuroradiology, neurosurgical care, and treatment of TBI in recent years, the options for treatment of extensive TBI remain limited. Consequently, it is of critical importance to develop more effective treatment strategies for these patients.

Although the etiology of progressive neuropathology in TBI remains unclear, increasing evidence has shown that oxidative stress plays an important role in its development. TBI upregulates pro-oxidants, such as NADPH oxidases (NOX1–5) and inducible nitric oxide synthase, leading to the production of reactive oxygen species (ROS) and reactive nitrogen species, which cause oxidative damage of DNA, proteins, and lipid in brain tissues [3,4]. It has been generally assumed that mitochondria are the major source of ROS after brain injury. However, recent work has shown that the enzyme NADPH oxidase is a major contributor to posttraumatic cellular ROS production [5,6].

tert-Butylhydroquinone (tBHQ) is widely used as a food preservative, based on its powerful antioxidant potential. It has furthermore been shown to protect the living animal and cell lines against acute toxicity and oxidative insult, presumably through the induction of the nuclear translocation of transcription factor (nuclear factor erythroid 2-related factor 2 [Nrf2]), which in turn regulates the expression of many cytoprotective proteins, including NAD(P)H: quinone oxidoreductase 1, glutathione-S-transferase, glucuronyltransferase, and heme oxygenase-1 (HO-1) [7,8]. Novel anti-inflammatory effects of tBHQ, which have been reported in an experimental TBI model, include suppression of nuclear factor κB (NF-κB) activity, increased Nrf2 levels, inhibition of the concentrations of inflammatory cytokines (interleukin [IL]-1, IL-6, and tumor necrosis factor α), and abatement of brain edema and apoptosis index [9]. However, it remains unknown if tBHQ can suppress NADPH activity or oxidative stress in TBI. To resolve this issue, we used a moderate closed-head injury model in mice, and evaluated the effect of tBHQ pretreatment on NOX2 production, malondialdehyde (MDA), and other markers of antioxidant expression. In addition, we monitored effects of this pretreatment on the neurologic outcome and brain edema caused by TBI in the same mice.

2. Materials and methods

2.1. Animals

Imprinting control region mice were originally purchased from Animal Center of Nanjing Medical University, Nanjing, China, and were subsequently bred and raised in our laboratory. A total of 80 male mice weighing 28–32 g were housed five per cage under a constant 12h light–dark cycle. Food and water were supplied *ad libitum*. All experiments conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Treatment

A total of 50 mg of tBHQ (Sigma-Aldrich Co, St Louis, MO) was dissolved in dimethyl sulfoxide, to a final concentration of 5 mg/mL in 1% DMSO-saline and intraperitoneally (i.p.) injected at a dose of 50 mg/kg, divided into three injections at intervals of 8 h before TBI. This dose and route of tBHQ administration has been used in analogous animal models described elsewhere [2]. All mice were randomly assigned to the following groups: sham + vehicle (DMSO/saline), TBI + vehicle (DMSO/saline), and TBI + tBHQ. These groups were allocated either to (1) biochemical analyses or (2) to edema and neurologic examinations, giving final groups sizes of five animals because of mortality.

2.3. Closed-head TBI injury

We used the weight-drop model with minor modifications to induce moderate closed-head TBI injury, as described previously [10,11]. After anesthesia with pentobarbital sodium (50 mg/kg i.p.; Sigma, St Louis, MO), mice were fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and the scalp was shaved and cleaned with iodophor. A midline incision was performed to expose the skull between bregma and lambda suture lines (2.0–2.5 cm), and the left lateral aspect of the skull was exposed by retracting the skin and surrounding soft tissue. Focal brain trauma was induced by dropping a 200-g steel weight with a flat end from a height of 3 cm onto the left lateral skull. Then the scalp was sutured. The free-falling weight produced a moderate contusion injury of the left parietal cortex. Sham-injured animals served as control subjects; these animals underwent scalp incision with the exception that no injury was induced. As in other studies of this type, 20%–25% of the mice died in the first seconds after the trauma, but there was no delayed mortality or prostration in the surviving mice [12]. During the surgery, rectal temperature was monitored and maintained between 36.5°C and 37.5°C with heating pads and lamps. The animals were returned to their quarters after recovery from anesthesia, and were killed at 24 h after sham or TBI.

2.4. Western blot analysis

After induction of anesthesia with pentobarbital sodium (50 mg/kg, i.p.), mice were decapitated and the left brains were rapidly dissected within 5 min, frozen, and stored at −80°C until the day of analysis ($N = 5$). The brain tissues were then thawed and mechanically lysed in the radioimmunoprecipitation assay buffer supplemented with protease inhibitors [13]. Homogenates were centrifuged at 12,000*g* for 15 min at 4°C, and supernatants were collected. The protein concentration was determined using the bicinchoninic acid method. Equal portions of protein preparations were run on 10% sodium dodecyl sulfate–polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes (ATTO Bio Instrument, Tokyo, Japan). The membranes were blocked in blocking buffer (5% skimmed milk in TBS containing 0.05% Tween 20) for 1–2 h, and then incubated with primary antibodies against NOX2 (1:5000;

Abcam Inc., Cambridge, MA, USA) and β-actin (1:3000; Bioworld Technology, Inc., Louis Park, MN, USA) overnight at 4°C with slow rocking. The next day, the membranes were blotted with horseradish peroxidase (HRP)–conjugated secondary antibody (1:5000; Bioworld) for 2 h, and immunoreactive bands were detected by a chemiluminescent reaction (ECL kit; Amersham Pharmacia Biotech UK Ltd) on exposure to an x-ray film. Quantification of band density was performed using the UN-Scan-It 6.1 software (Silk Scientific, Orem, UT), with normalization to β-actin and expressed as a percentage of levels in the sham group.

2.5. *Measurement of SOD activity and MDA level*

To investigate the oxidative damage during TBI, superoxide dismutase (SOD) activity and brain concentrations of MDA, a presumptive biomarker of oxidant-mediated lipid peroxidation, were measured using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions. In brief, at 24 h after TBI, the left brain hemispheres of mice ($N = 5$) were removed and homogenized in nine volumes of grams per liter ice-cold saline for 10 min using a Dounce Tissue Grinder (Kimble and Kontes, Vineland, NJ). The samples were centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was used for the measurement of SOD activity and MDA content. The results were expressed as micrograms of protein and nanomoles per gram of protein, respectively, relative to prepared standards.

2.6. *Enzyme-linked immunosorbent assay analysis*

Nrf2 concentrations of traumatically injured left brain hemispheres ($N = 5$) were quantified using an enzyme-linked immunosorbent assay specific for mice, according to the manufacture's instructions (Active Motif, Carlsbad, CA). In brief, at 24 h after TBI, left brain hemispheres were removed and homogenized in the radioimmunoprecipitation assay buffer and a cocktail of protease inhibitors. Then the samples were centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was collected and used for the measurement of Nrf2 content, expressed as picograms per milliliter of protein relative to standards.

2.7. *Neurologic outcome*

The neurologic outcome was assessed in mice ($N = 5$) at 24 h after TBI by an independent researcher who was blind to the treatment group, using a scoring system as reported by Garcia *et al.* with slight modifications [14,15]. Sensorimotor testing was graded on a scale of 0–3 each on spontaneous activity, symmetry of walking, symmetry of movements, symmetry of forelimbs, climbing ability, vibrissae response, and side stroking response. Neurologic deficit scores were assigned as follows: 0, complete deficit; 1, definite deficit with some residual function; 2, decreased response or mild deficit; and 3, no evidence of deficit. Thus, the minimum neurologic score was 3 and the maximum score was 18.

2.8. *Evaluation of brain edema*

Brain water content, a sensitive measure of cerebral edema, was determined using the wet weight (WW) to dry weight (DW) ratio method, as previously described [16], in the same mice investigated for the neurologic outcome. In brief, after the induction of pentobarbital anesthesia at 24 h after TBI, groups of mice ($N = 5$) were decapitated, and their left brain hemispheres were rapidly removed and weighed to obtain WW. The tissue was then dried at 70°C for 72 h and weighted again to obtain DW. The percentage of tissue water content was calculated as $(WW - DW)/WW \times 100\%$.

2.9. *Statistical analysis*

All data were expressed as the mean ± standard deviation. The measurements were subjected to a one-way analysis of variance, and the differences between experimental groups were determined by the Fisher least significant difference posttest. Significance was assigned at $P < 0.05$.

3. Results

3.1. *Effect of treatment with tBHQ on NOX2 protein level*

Figure 1 shows the low cerebral NOX2 protein level in the sham + vehicle group. At 24 h after TBI, the NOX2 protein level was increased by fivefold in the TBI + vehicle group compared

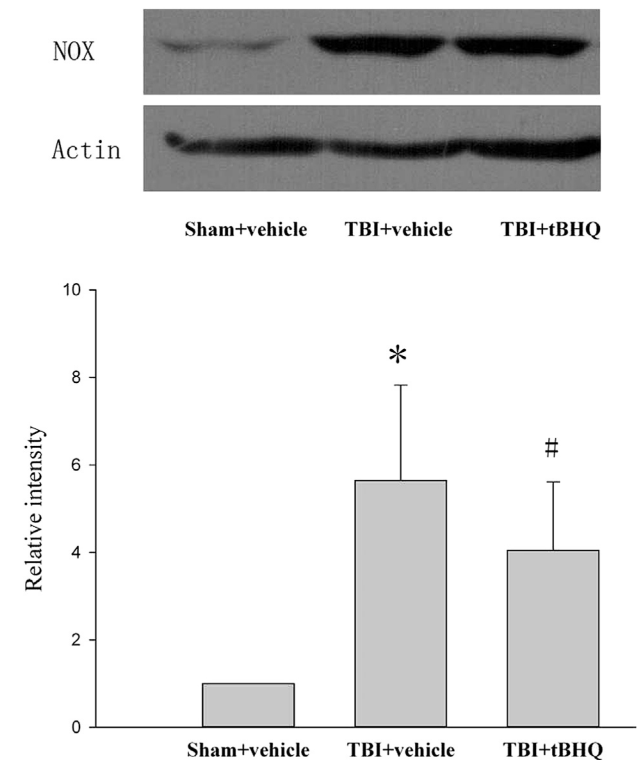

Fig. 1 – Effect of tBHQ on the NOX2 protein level ($n = 5$ per group) at 24 h after TBI. Western blot analysis of NOX2 protein, in the upper portion of the figure, indicates that NOX2 protein levels were significantly increased after TBI, and tBHQ treatment markedly decreased the NOX2 level in the TBI-injured brain. *$P < 0.05$ *versus* the sham + vehicle group. #$P < 0.05$ *versus* the TBI + vehicle group.

with the control group ($P < 0.05$), whereas pretreatment with tBHQ administration markedly attenuated the NOX2 protein relative to that in the TBI + vehicle group ($P < 0.05$).

3.2. Effect of treatment with tBHQ on Nrf2 protein level

As shown in Figure 2, TBI resulted in 5% enhanced formation of Nrf2 relative to the sham group ($P < 0.05$), whereas pretreatment with tBHQ further upregulated the Nrf2 protein level after TBI by 12% compared with the sham group ($P < 0.05$).

3.3. Effect of treatment with tBHQ on TBI-induced MDA levels and SOD activity

As shown in Figure 3, TBI significantly increased the tissue MDA level ($P < 0.05$) and significantly decreased the tissue SOD activity when compared with the sham group. Pretreatment with tBHQ showed a protective effect by markedly decreasing the MDA concentration and also increasing the antioxidant enzyme SOD activity evoked by TBI compared with the TBI group.

3.4. Effect of treatment with tBHQ on brain edema

As shown in Figure 4, the water content was low in the sham + vehicle mice group. Relative to this group, TBI resulted in brain edema, which was significantly reduced by pretreatment with tBHQ ($P < 0.05$).

3.5. Effect of treatment with tBHQ on neurologic outcome

TBI caused a significant deficit in the neurologic outcome at 24 h (13.4 ± 1.14), whereas the neurologic outcome score of the sham + vehicle group was 17.4 ± 0.89. There were statistical differences between the sham + vehicle and TBI + vehicle groups. The neurologic scores for the TBI + tBHQ group (15.4 ± 1.14) were markedly increased when compared with the TBI + vehicle group ($P < 0.05$). There were no significant differences between the sham + vehicle and TBI + tBHQ groups ($P > 0.05$).

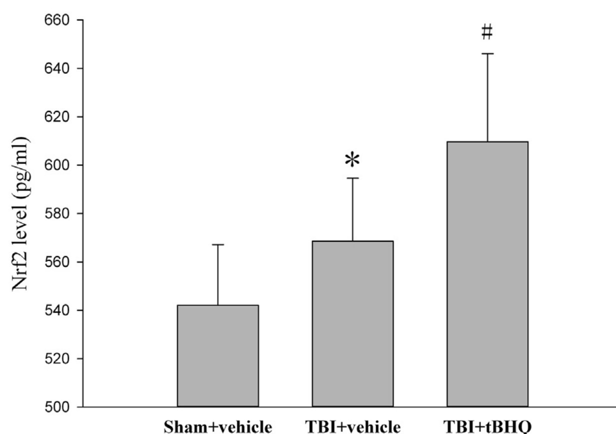

Fig. 2 – Effect of tBHQ on the Nrf2 protein level (*n* = 5 per group) at 24 h after TBI. Concentrations of Nrf2 were significantly increased after TBI and tBHQ treatment markedly upregulated the Nrf2 level in the TBI-injured brain. *P < 0.05 *versus* the sham + vehicle group. #P < 0.05 *versus* the TBI + vehicle group.

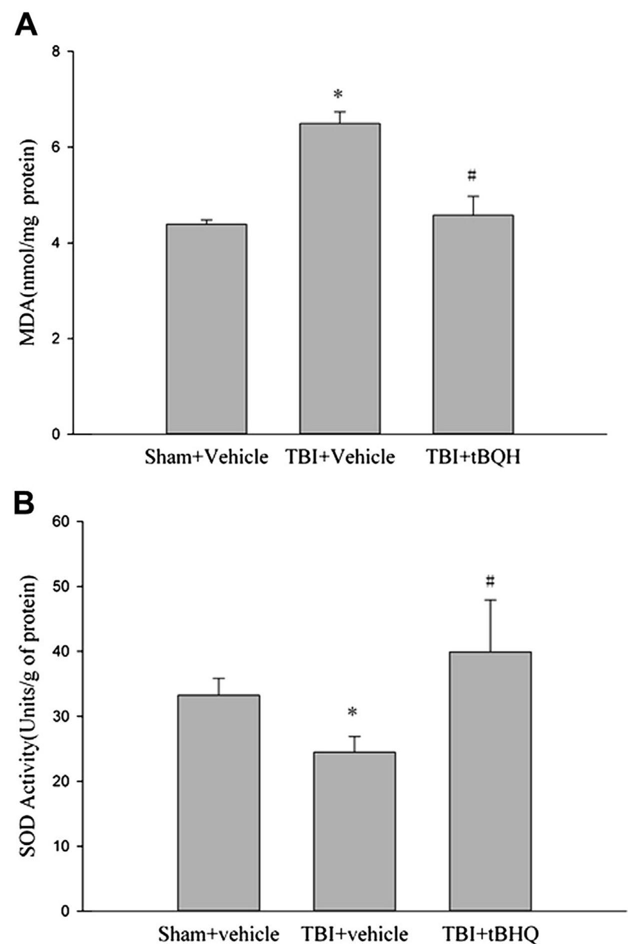

Fig. 3 – Effect of tBHQ on TBI-induced (A) MDA levels (B) and SOD activity (*n* = 5 per group) at 24 h after TBI. MDA levels were significantly increased after TBI, and tBHQ treatment markedly suppressed TBI-induced MDA production. After TBI, SOD activity was reduced when compared with the sham + vehicle group, and was restored by pretreatment with tBHQ. *P < 0.05 *versus* the sham + vehicle group. #P < 0.05 *versus* the TBI + vehicle group.

4. Discussion

There is increasing evidence that oxidative stress plays a key role in the pathogenesis of TBI, which suggests novel avenues for intervention based on antioxidative treatments. The present study was designed to elucidate the potential molecular mechanisms and antioxidant effects of tBHQ, which is used as a food-additive antioxidant for human. To this end, we tested in the closed TBI model the protective effects of tBHQ on TBI-induced oxidative stress. In support of our hypothesis, we found that (1) treatment with tBHQ suppressed the increased

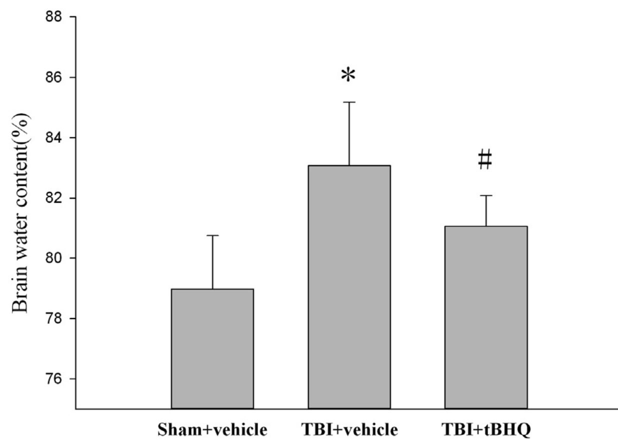

Fig. 4 – Effect of *t*BHQ on brain edema (*n* = 5 per group) at 24 h after TBI. Brain edema was significantly increased after TBI, and *t*BHQ treatment markedly attenuated TBI-induced brain edema in mice. *$P < 0.05$ *versus* the sham + vehicle group. #$P < 0.05$ *versus* the TBI + vehicle group.

activity of NADPH oxidase (NOX2), which is known to be the major producer of ROS in TBI; (2) Nrf2 activity was significantly activated after TBI and could be further induced on pretreatment with *t*BHQ; (3) pretreatment with *t*BHQ attenuated TBI-induced increases in the MDA level, a biomarker of oxidative stress, and increased the activity of SOD level, suggesting a rebalancing of the oxidative-antioxidant system; and (4) after *t*BHQ administration, brain edema and neurologic deficit evoked by TBI were markedly ameliorated. All findings consistently suggest that the induction of Nrf2 activity by *t*BHQ can attenuate the cerebral oxidative stress and reduce brain damage after TBI.

Oxidative stress is increasingly being recognized as contributing negatively to the neurologic outcome after brain injury. The enzyme NADPH oxidase, an important source of superoxide, is a membrane protein composed of several subunits, among which are the NOX and phox subunits. There are at least five different isoforms of NOX in living cells, designated as NOX1–5 [17]. Antioxidative therapies using apocynin or genetic deletion of certain NADPH oxidase isoforms have shown promising protective effects in experimental TBI models, suppressing both increased NADPH activity and cerebral inflammatory sequelae of this injury [18,19]. In recent years, the central role of NOX2 in oxidative stress has been well established *in vitro* and *in vivo*. NOX2 level in the cerebral cortex and hippocampus has been reported to increase rapidly after controlled cortical mechanical injury in male mice, with an early peak at 1 h, followed by a secondary peak from 24–96 h after TBI [19]. Using mice genetically deficient in this enzyme ($NOX2^{-/-}$), the number of tunnel-positive cells in the vicinity of the contusion and the amount of O_2^- and peroxynitrite metabolites were less than that in wild-type mice with similar injuries [20]. A previous study also demonstrated that NOX2 is a source for ROS generation in a transient cerebral ischemia mouse model. When apocynin, a selective inhibitor NOX2 activity, was administrated i.p. 0.5 h before ischemia, the total infarct volume, neurologic impairment, and mortality were alleviated [21]. In the present work, we found the NOX2 protein level to be greatly elevated in a TBI mouse model, but suppressed to basal levels by *t*BHQ pretreatment. This finding provides evidence that NOX2 plays an important role on the secondary brain injury after TBI, predicting that the suppression of the NOX2 level may bring great benefit to patients with TBI-induced brain damage.

An increase in the brain MDA level is a marker of the occurrence of lipid peroxidation traumatized tissue. A number of experimental and clinical studies have suggested that the MDA level increases significantly in the injured hemisphere after TBI, and ultimately contributes to neuronal death and neurologic deficits [22–24]. On the contrary, SOD represents the first line of defense against oxidative stress, by catalyzing the dismutase reaction of superoxide anion to hydrogen peroxide, and it represents the antioxidative capacity of the tissue. In this study, we showed that the redox balance is shifted toward a more oxidative condition after TBI, whereas pretreatment with *t*BHQ before TBI significantly increased the impaired SOD activity, whereas decreased the MDA level, indicating a rebalancing of the oxidative-antioxidant system.

To defend against exogenous toxins or injury, cells possess a large number of cytoprotective and detoxifying enzymes, the expression of which is rapidly increased in response to insult [9]. Nrf2 is a key transcription factor that regulates antioxidant genes as an adaptive response to oxidative stress or pharmacologic stimuli. Thus, Nrf2 knockout mice have more NF-κB activation, and the production of the inflammatory cytokines tumor necrosis factor α, IL-1, and IL-6 was higher, whereas the number of apoptotic neurons after TBI was greater compared with wild-type mice [25]. It also has been reported that the activation of Nrf2 by sulforaphane can reduce neuronal death, contusion volume, and neurologic dysfunction after TBI [26]. Various lines of evidence concur that Nrf2 has an important role in mediating secondary brain injury. The Nrf2 activator *t*BHQ is a metabolite of the antioxidant butylated hydroxyanisole, which can cross the blood–-brain barrier, and has demonstrated neuroprotective effects in several models of disease [2]. Jin *et al.* reported that the induction of Nrf2 activity by *t*BHQ markedly decreased NF-κB activation and inflammatory cytokine production in the injured brain and significantly attenuated TBI-induced cortical apoptosis [9]. Despite *t*BHQ being an antioxidant, *t*BHQ treatment of cultures from Nrf2 knockout animals produced no neuroprotection against oxidative stress. By reintroducing Nrf2 *via* infection with a replication-deficient adenovirus (ad), both the genetic response and neuroprotection were rescued, thus revealing that the antioxidant property of *t*BHQ is secondary to the Nrf2 effect. At the same time, it was found that *t*BHQ administration protected neurons against oxidative injury, and that most of this effect was mediated by astrocytes rather than neurons. In astrocytes, activation of Nrf2 was induced by pretreatment with *t*BHQ, and may thus provide a means of amplifying the existing metabolic, soluble, and physical coupling between astrocytes and neurons so as to boost synaptic function and provide protection against oxidative injury [27]. Consistent with that finding, the present study demonstrates that pretreatment with *t*BHQ can significantly increase Nrf2 tissue levels and bring great benefit to the TBI-induced brain damage model.

In addition to the redox changes described previously, we found that pretreatment with *t*BHQ attenuated TBI-induced edema, and at the same time also improving the neurologic score and relieving neurologic deficits in injured mice. A previous report has demonstrated the neuroprotective role of Nrf2 in reducing brain edema and apoptotic cell death, which together are major aspects of the secondary brain injury after TBI [28]. Our finding extends previous results to show that the induction of Nrf2 activity by *t*BHQ could reduce cerebral edema and improve the neurologic outcome in a TBI model.

Although the precise mechanism underlying the anti-oxidative stress action of *t*BHQ is still unclear, several lines of evidence indicate that Nrf2/HO-1 and NOX2 signaling pathways may contribute to this process. TBI significantly enhanced the activation of Nrf2 and the induction of Nrf2-mediated downstream antioxidant enzymes, such as HO-1 in the mouse brain [26]. There is also direct evidence that HO-1 can modulate NOX2 activity. Treatment with hemin, an HO-1 inducer, can increase HO-1 and Nrf2 levels, whereas decrease the NOX2 activity compared with a sham group [29]. Pretreatment with *t*BHQ can increase Nrf2 and HO-1 levels, which together may confer an adaptive neuroprotective response to oxidative insults mediated by decreased NOX2 activation. Moreover, it was also observed that Nrf2 reduced ROS levels and affected the redox-sensitive NF-κB signaling pathway involved in neuroinflammation [30]. Therefore, the decreased ROS level may exert feedback control on the NOX2 protein level. Taking the above findings together, it may be proposed that *t*BHQ augments the cellular antioxidative system *via* the upregulation of Nrf2 and inactivation of the NOX2 signaling pathway, resulting in decreased oxidative stress.

Several limitations of the present study should be considered when interpreting our results. The number of animals in each group was small, such that statistical power may have been compromised given the inherent variability in brain injury resulting from the weight-drop model. In addition, a "pretreatment" study is not really clinically applicable. It remains to be established if *t*BHQ is useful as a therapeutic when administered after TBI. Moreover, our study design reveals associations, but is not indicative of causality, which might entail additional arms such as an Nrf2 knockout group, or use of direct Nrf2 blockers. We omitted a control group with sham + *t*BHQ for comparing to not only the sham + vehicle group but also the TBI + *t*BHQ group. This is potentially important in our pretreatment study, as the effects of pre-injury administration of the drug alone were not studied.

5. Conclusions

Our results show that *t*BHQ protects against TBI-induced oxidative stress *via* the upregulation of Nrf2 and inactivation of the NOX2 signaling pathway. Pretreatment with *t*BHQ attenuates MDA levels, increases SOD activity, reduces brain edema, and improves the neurologic outcome in TBI mice. Taken together, our data suggest that the administration of *t*BHQ may be a viable neuroprotectant and a potential adjuvant therapy for patients with TBI. It remains to be established if *t*BHQ administration after TBI is likewise effective in reducing the development of secondary damage.

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The authors have no conflict of interest.

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