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Original Article

Quercetin induces mitochondrial biogenesis in experimental traumatic brain injury via the PGC-1α signaling pathway

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Abstract: Quercetin, a dietary flavonoid used as a food supplement, has been found to have protective effect against mitochondria damage after traumatic brain injury (TBI) in mice. However, the mechanisms underlying these effects are still not well understood. The aim of the present study was to investigate the effect of quercetin on the potential mechanism mediating these effects in the weight-drop model of TBI in male mice that were treated with quercetin or vehicle via intraperitoneal injection administration 30 min after TBI. Brain samples were collected 24 h later for analysis. Quercetin treatment upregulated the expression of PGC-1α and restored the level of cytochrome c, malondialdehyde (MDA) and superoxide dismutase (SOD). These results demonstrate that quercetin improves mitochondrial function in mice by improving the level of PGC-1α following TBI.

Keywords: Quercetin, traumatic brain injury, PGC-1α, mitochondria

Introduction

Traumatic brain injury (TBI) is the leading cause of death in young adults in most part of the western world [1]. Admittedly, the high cost of caring for these patients may compromise the entire health care system [2]. Secondary brain damage after TBI precipitates a complex, secondary pathological process which can result in a cascade of deleterious side effects far from the site of the initial injury [3, 4]. One of the major concerns of the secondary injury is swelling and the release of various chemicals that promote inflammation and cell injury or death. Among these processes, oxidative stress plays an important role in the secondary damage [5]. The excessive production of reactive oxygen species, due to oxidative stress, and the exhaustion of the endogenous antioxidant system induce the peroxidation of lipids and proteins, the oxidation of nucleic acids and DNA breakdown [6, 7]. The enzymes regarded as antioxidants include nicotinamide adenine dinucleotide phosphate (NADPH)-linked enzyme, quinone oxidoreductase-1 (NQO1) and superoxide dismutase (SOD) [8, 9]. Certainly, the degree of mitochondrial injury or dysfunction can be an important determinant of cell survival or death, as it would spread from mitochondrion to mitochondrion ultimately damaging the cell itself [10, 11]. Accordingly, mitochondrial biogenesis can enhance cellular function and promote cellular recovery from damage caused by adverse environmental and pathophysiological insults [12-14].

The peroxisome proliferator-activated receptor (PPAR) γ coactivator-1 (PGC-1) family of transcriptional coactivators is emerging as a 'molecular switches' in many metabolic pathways. PGC-1α, the first characterized PGC-1 family member, activates the mitochondrial transcription factor A (TFAM), which is responsible for transcribing nuclear encoded mitochondrial proteins including structural proteins as well as proteins involved in mitochondrial DNA (mtDNA) transcription, translation, and repair [15-18]. PGC-1α appears to play a key role in various

central nervous system diseases. For example, altered expression of PGC-1α in the brain has been reported in Parkinson's disease and Alzheimer's disease [19].

Additionally, increased mRNA and nuclear PGC-1α protein levels were detected in the neurons of the cerebral cortex after TBI [20]. Thus, these results mean that PGC-1α may play an important role in the brain after TBI.

Quercetin (Que), 3, 3', 4', 5, 7-pentahydroxyflavone, a member of the polyphenolic flavonoid family of compounds, is a naturally occurring flavonoid which has a broad spectrum of bioactive effects and can regulate mitochondrial biogenesis by reducing reactive oxygen species (ROS) production in various cell types [21, 22]. In particular, Que can impact mitochondrial biogenesis by regulating enzymes and transcription factors in the inflammatory signaling cascade [23, 24]. Previous studies have shown that Que can increase the level of PGC-1α and the mRNA of cytochrome in brain tissue [23-26].

Accordingly, it is of interest to explore whether the protective effects of Que on mitochondrial biogenesis after TBI are related to the activation of the PGC-1α pathway. For this reason, the present study was undertaken to investigate the protective effect of quercetin on TBI-induced mitochondria injury and the possible ameliorative mechanisms of Que in mice.

Materials and methods

Animals

Male 6-8 week old ICR mice (Experiment Animal Centre of Nanjing Medical University, Jiangsu, China) weighting 28-32 g were used in this study. The mice were housed at 23 ± 1°C with a 12 h light/dark cycle with ad libitum access to food and water. Experimental protocols were approved by the Animal Care and Use Committee of Nanjing University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH).

Model of TBI

The model of TBI used in this study is based on the Marmarou's weight-drop model previously described by Flierl et al. and our previous study [27, 28]. Briefly, mice were intraperitoneally anesthetized with chloral hydrate and then placed on a platform directly under the weight-drop device. A midline longitudinal scalp incision about 1.5 cm was made, and then, the skin and fascia were reflected to expose the skull. After locating the left anterior frontal area as the impact area, a 200 g weight was released onto the skull. Subsequently, the scalp wound was closed with standard suture material. Then, the mice were returned to the cages where they were given ad libitum access to food and water. Sham animals were subjected to the same procedures except for the brain injury by the weight drop.

Experimental design

After 1 week of acclimation, ninety-six mice were divided into four groups (n = 24 for each group), namely the sham group, TBI group, TBI + vehicle group, and TBI + Que group. The mice of the TBI + Que group were injected intraperitoneally with Que (Sigma Aldrich, Shanghai, China) at 50 mg/kg 30 minutes after TBI. Meanwhile, mice in the TBI + vehicle group received equal volumes of vehicle (DMSO + 0.9% saline) at the indicated time points. The doses used in this study were based on a previous study on the neuroprotective effects of Que in an acute hypobaric hypoxia model [26]. All the mice of each group were euthanized at 24 hours post TBI.

Tissue processing

The tissue analyzed by Western blotting was localized directly over the center of the injury site and included both contusional and penumbral tissue. Samples were immediately frozen in liquid nitrogen, and then stored at -80°C until use. For immunohistochemistry analysis, the whole brain was removed 24 h after TBI and immersed in 4% paraformaldehyde overnight.

Isolation of mitochondria

Mitochondrial and cytosolic proteins were extracted from the left cerebral cortical tissue using the Tissue Mitochondria Isolation Kit for Tissue Protocols (Beyotime Institute of Biotechnology, Shanghai, China). Briefly, fresh tissue samples were homogenized with a Polytron

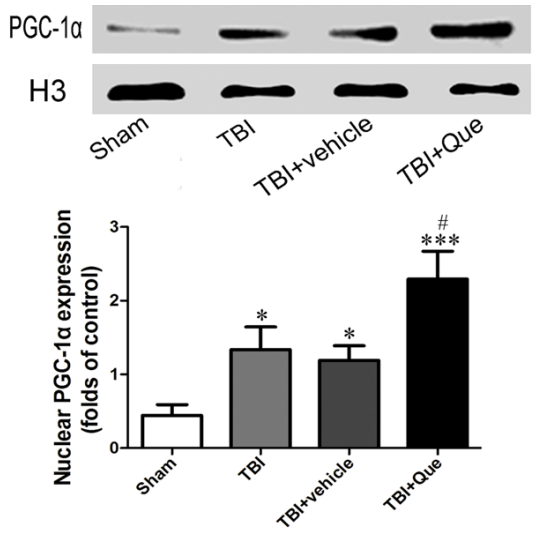

Figure 1. Nuclear PGC-1α expression was assessed 24 h after TBI by Western blot analysis. Data are presented as mean ± SEM (n = 6 per group). *P < 0.05, ***P < 0.001 vs. sham group; #P < 0.05 vs. TBI group.

grinder in ice-cold homogenization buffer and centrifuged at 1000×*g* for 5 min at 4°C to isolate the nuclear fraction. The obtained supernatants were centrifuged at 3500×*g* for 10 min at 4°C, and then the sedimented pellet was mitochondria. The supernatants were collected, and centrifuged at 12,000×*g* for 10 min at 4°C to remove sediment and obtain cytoplasmic proteins. Brain tissue was weighed, homogenized, and centrifuged at 12,000×*g* for 15 min at 4°C. The protein content of each sample was determined using a protein assay kit.

Western blot analysis

Mitochondrial, nuclear and cytosolic proteins were extracted from the cerebral cortical tissue samples and quantified as described in our previous study [29, 30]. The cerebral cortex nuclear protein samples were subjected to electrophoresis on a 10-15% sodium dodecyl sulfate-polyacrylamide gel for 45 minutes at 80 V followed by 100 minutes at 100 V and was then transferred onto nitrocellulose for 1 hour at 100 V.

The membrane was blocked with 5% skimmed milk for 2 hours at room temperature and then incubated overnight at 4°C with the primary antibodies. The antibodies used were the following: H3 (1:1000 dilutions; Cell Signaling Technology, Danvers, MA, USA), cytochrome c (1:5000 dilutions; Abcam, Cambridge, UK), Bax (1:400 dilution; (Abcam), COX IV (1:1000, Cell Signaling Technology) and PGC-1α (1:1000 dilutions; Millipore, Billerica, MA, USA). Subsequently, the membranes were incubated for 2 h with the appropriate secondary antibodies.

Immunohistochemical staining

Brain tissue samples were fixed in formalin for 24 h and embedded in paraffin. For immunohistochemical examination, the tissue sections (*4-6* μm) were incubated with an anti-PGC-1α polyclonal antibody (1:300 dilution; Millipore) overnight at 4°C, followed by a 15 minutes wash in phosphate-buffered saline (PBS). Then, the sections were incubated with horseradish peroxidase-conjugated IgG (diluted 1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. Immunolabeling was visualized as brown staining using diaminobenzidine, with haematoxylin counterstaining.

The immunohistochemical positive neurons were counted by two independent investigators who were blinded about the injured group and the sham group. Six fields were randomly selected from each section and observed using light microscopy (ECLIPSE E100, Nikon, Tokyo, Japan). Then the mean number of positive neurons in the six views was recorded as the data for each sample.

Mitochondrial SOD and MDA content

Mitochondrial malondialdehyde (MDA) content and SOD activity were measured using a spectrophotometer according to the manufacturer's instructions (Nanjing Jiancheng, Nanjing, China). Total protein concentrations were determined by the Bradford method. The content of MDA was expressed as nmol/mg protein and the activity of SOD was expressed as U/mg protein.

Statistical analysis

Data are expressed as mean ± SEM and evaluated by the ANOVA and Tukey's post hoc tests. Statistically significant difference was set at $P < 0.05$. The SPSS 17.0 software (SPSS Inc., Armonk, NY, USA) was used for the statistical analysis.

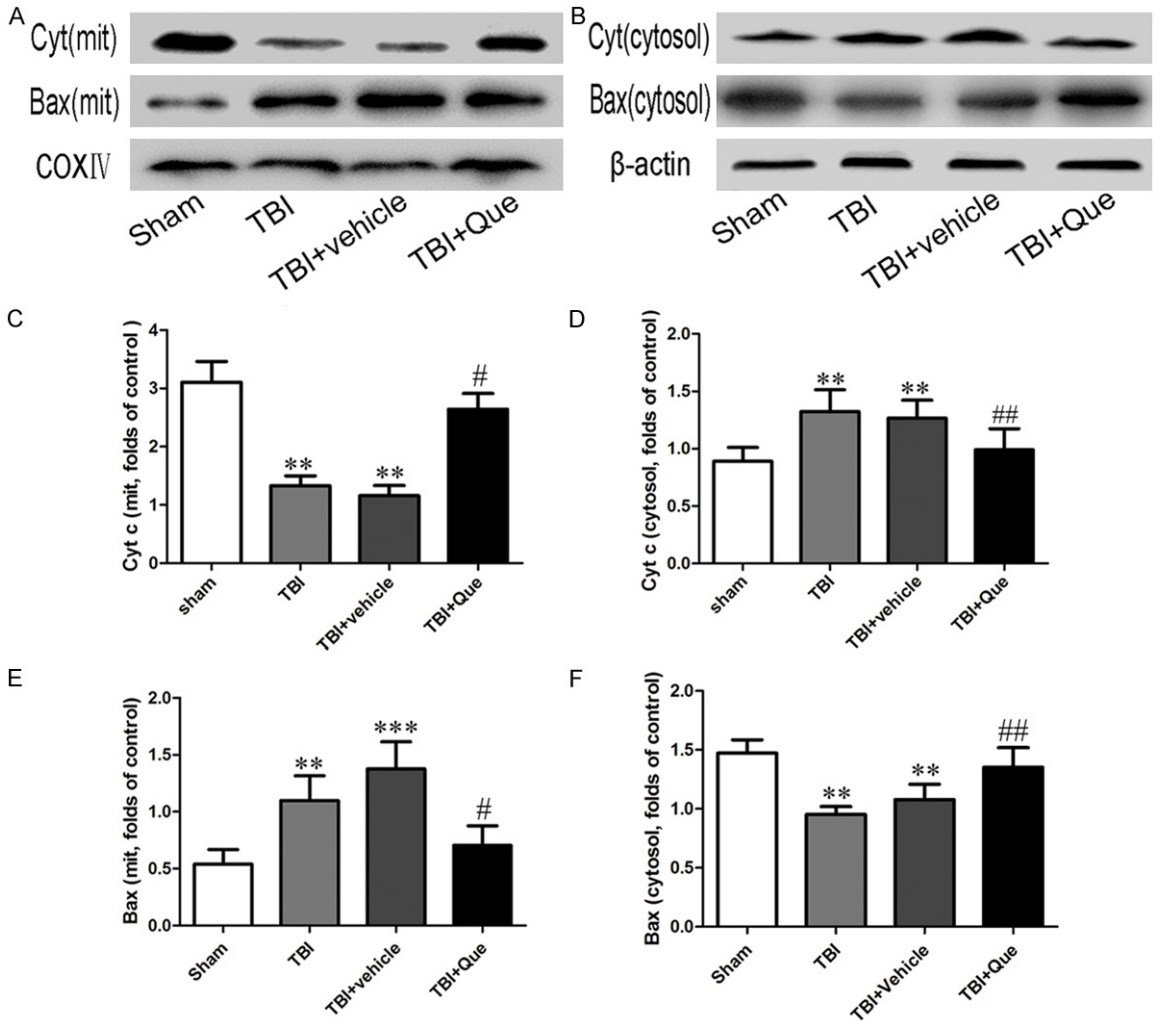

Figure 2. Effect of Que on pro-apoptotic protein expression was assessed following TBI. (A, B) The expression of Bax and cytochrome c in the ipsilateral cortex was evaluated by Western blotting 24 h after injury. Representative blots show the relative expression of (C, E) mitochondrial and (D, F) cytosolic Bax and cytochrome c. Expression was normalized tothe level of COX IV or β-actin. Data represent the mean ± SEM ((n = 6 per group)). **P < 0.01, ***P < 0.001 vs. sham group; #P < 0.05, ##P < 0.05 vs. TBI.

Results

Western blot analysis

The results indicated that compared with the sham group, both TBI and administration of Que were inductors of PGC-1α nuclear translocation (**Figure 1**). In addition, treatment with 50 mg/kg of Que resulted in marked increased expression of PGC-1α in the nucleus of the cerebral cortex neurons compared to the TBI group and TBI + vehicle group (**Figure 1**). Moreover, compared with the vehicle-treated group, the Que-treated group showed significantly increased nuclear localization of PGC-1α. Meanwhile, the expression of PGC-1α in the TBI group and TBI + vehicle group was only negligibly increased, and did not reach the expression levels of the TBI +Que group. These observations suggest that Que increases the expression of PGC-1α at the nuclear protein level.

Additionally, after TBI, the levels of mitochondrial and cytosolic Bax protein were increased and decreased, respectively, compared with the sham and sham + vehicle groups (**Figure 2**), while mitochondrial and cytosolic cytochrome *c* levels were decreased and increased, respectively, compared with sham-operated animals

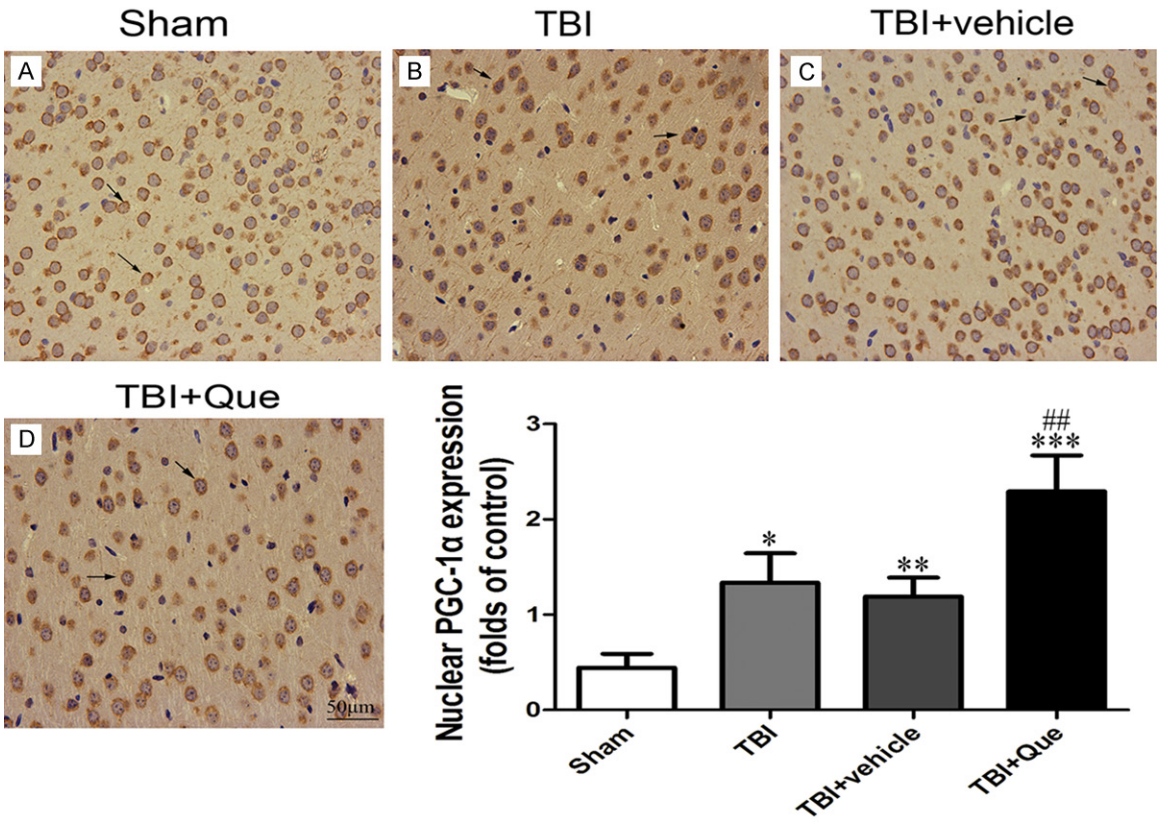

Figure 3. Immunohistochemical staining of nuclear PGC-1α (average of six high power fields). A. Sham group. B. TBI group. C. TBI+vehicle. D. TBI+Que. Bars: 50 μm, Data represent the mean ± SEM (n = 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. sham group, #P < 0.05 vs. TBI. Black arrows: nuclear PGC-1α positive neurons.

(**Figure 2**). These effects were reversed in Que-treated TBI mice, in which mitochondrial translocation of Bax and subsequent cytosolic release of cytochrome *c* were inhibited.

Immunohistochemical staining

The immunohistochemical analysis revealed that TBI increased the expression of PGC-1α in the nucleus, and Que enhanced PGC-1α localization to the nucleus, as shown in **Figure 3**. These findings indicated that Que may promote PGC-1α translocation from the cytoplasm to the nucleus, thereby leading to elevated binding ability to the downstream genes and consequently their increased transcription. In sham samples of cerebral cortex, a few PGC-1α immunostained neurons were observed, as shown in **Figure 3A**. Clearly, in the TBI group and TBI + vehicle group, the number of PGC-1α positive neurons was increased compared with the sham group, whereas it was reduced in the TBI + Que group (**Figure 3**).

Mitochondrial SOD and malondialdehyde (MDA) content

Our experimental analysis showed that mitochondrial MDA was elevated in the TBI group and TBI + vehicle group, while administration of Que significantly reduced the generation of mitochondrial MDA (**Figure 4**). In contrast, mitochondrial SOD was decreased after TBI, while Que significantly upregulated the activity of SOD.

Discussion

Mitochondria play an important role in the injured brain by activating signaling pathways via ROS production or by inducing mitochondria-dependent apoptosis [31]. A number of studies have shown that mitochondrial function after TBI in humans is limited. Factors that both inhibit and promote neuronal apoptosis appear to work by influencing mitochondrial cytochrome *c* release, while pathways that promote

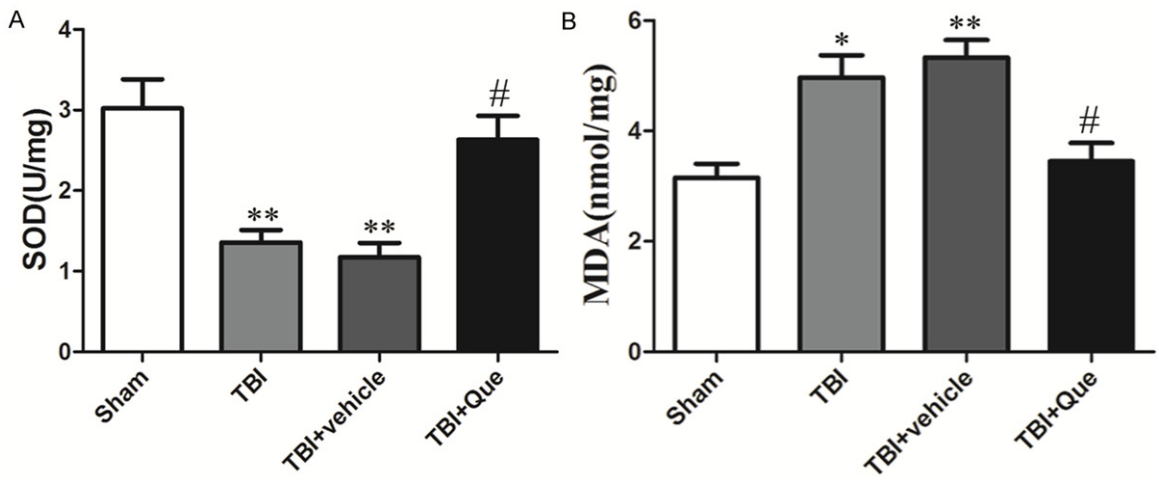

Figure 4. Que attenuated Mitochondrial oxidative stress caused by TBI. A. Measurements of malondialdehyde (MDA) levels (n = 6 per group). B. The activities of superoxide dismutase (SOD). Data represent the mean ± SEM. Numbers of animals: n = 6 per group; *P < 0.05, **P < 0.01 vs. sham group; #P < 0.05 vs. TBI.

necrotic cell death, such as excitotoxicity and oxidative stress, have profound influences on mitochondrial function. Certainly, with the growing evidence for mitochondrial participation in traumatic neuronal injury, neuroprotective approaches must include strategies aimed to limit and reverse mitochondrial dysfunction.

Accordingly, the present study investigated the protective effects of Que in an animal model of TBI. Indeed, it has been demonstrated that, following TBI, Que promoted maintenance of GSH levels, prevented the elevation of myeloperoxidase activity, and promoted better maintenance of axonal function as reported by CAPAs [32]. Actually, there are a number of studies which show that Que protects mitochondrial structure and mtDNA copy number, suggesting that Que influences mitochondrial structure, function and biogenesis [33]. Additionally, increased mitochondrial biogenesis increases oxidative phosphorylation by facilitating transcription, translation, and replication [34]. Although the exact mechanisms to explain these observations are unknown, they are likely multifaceted and related to mitochondrial response to the PGC-1α signaling pathway after TBI. To our knowledge, this is the first study evaluating the effects of Que in the regulation of the mitochondrial function in a TBI model.

PGC-1α is a multi-functional protein that, as a co-activator to many transcription factors, activates many nuclear receptors and functions. Actually, PGC-1α plays an important role in various central nervous system diseases. For instance, it was found that the nuclear PGC-1α protein level was significantly increased after TBI. In addition, increased PGC-1α immunostaining was detected in the neurons of the cerebral cortex after TBI.

These results suggest that PGC-1α may play an important role in the brain after TBI.

Consistent with this suggestion, a previous study has demonstrated that the level of PGC-1α protein in the nuclear fraction increased promptly after transient hypoxia in mice [35]. Likewise, in this study, Que was found to enhance the levels of nuclear PGC-1α protein after TBI. PGC-1α is a transcription factor acting on nuclear genes that encode proteins that are necessary for the mitochondrial respiratory chain. The mitochondrial respiratory chain consists of four membrane-bound complexes (Complex I-IV) and is involved in ATP synthesis [36, 37]. Our results also showed increased expression of PGC-1-related transcription factors was associated with mitochondrial biogenesis in cells treated with Que. It has previously been demonstrated that increase in cytochrome *c* concentration typically occur in conjunction with similar increase in other mitochondrial enzymes of the electron transport chain and enzymes of the tricarboxylic acid cycle, which leads to an overall increase in mitochondrial capacity [38, 39]. Accordingly,

previous reports of Que-induced increase in mitochondrial biogenesis are consistent with the increase in cytochrome *c* protein expression observed in our study.

Bax, a known apoptotic protein localized in the mitochondrial outer membrane, inhibits apoptosis by preventing cytochrome c release through the inhibition of the mitochondrial voltage-dependent anion channel [40], the shifting of the cellular oxidation reduction potential [41], or the prevention of mitochondrial superoxide radical production [41, 42]. A study of the role of Bax on cytochrome c release after cerebral ischemia and reperfusion revealed the signaling link of Bax with oxidative stress in mitochondrial cytochrome c-dependent apoptosis [43]. Our study showed that the translocation of Bax to the mitochondrial membrane and cytochrome c release into the cytosol were increased by TBI, indicating that the mitochondrial apoptotic pathway was activated. Additionally, our results indicated that the translocation of Bax to the mitochondrial membrane and the cytochrome c release into the cytosol were increased by TBI, suggesting that the mitochondrial apoptotic pathway was activated.

Several prooxidant and antioxidant enzymes are known to participate in oxidative stress-induced signaling and injury in cerebral ischemia. For instance, SOD is a well-known intracellular antioxidant enzyme, which catalyzes the reactions of reduced glutathione. Additionally, overexpression of SOD has been shown to prevent neuronal apoptosis and reduce ischemic brain injury by suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction [44].

Lipid peroxidation is typically determined by measuring the MDA content of brain tissue [45]. In a previous study, MDA levels were found to begin to increase immediately after injury, and remain elevated 24 later [46]. Accordingly, we also observed, over the same period of time, the level of MDA as an indicator of the end-products of lipid peroxidation. In the present study, Que treatment after TBI reduced the MDA level and increased SOD activity compared to the TBI and TBI + vehicle treated mice, suggesting that Que exerts antioxidant effects by protecting mitochondria.

In summary, the present study demonstrated that Que attenuated the oxidative insult of mitochondria by enhancing the expressions and activities of antioxidant enzymes in a TBI model. Furthermore, we found that the administration of Que led to the activation of the PGC-1α pathway. Thus, the preventive effects of Que against the decline of antioxidant enzyme activities after TBI may be attributed to its influence on the regulation of the PGC-1α pathway.

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Disclosure of conflict of interest

None.

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