Protective effects of quercetin on traumatic brain injury-induced inflammation and oxidative stress in cortex through activating Nrf2/HO-1 pathway

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Abstract

Background: Traumatic brain injury (TBI) has been a serious public health issue. Clinically, there is an urgent need for agents to ameliorate the neuroinflammation and oxidative stress induced by TBI. Our previous research has demonstrated that quercetin could protect the neurological function. However, the detailed mechanism underlying this process remains poorly understood. This research was designed to investigate the mechanisms of quercetin to protect the cortical neurons.

Methods: A modified weight-drop device was used for the TBI model. 5, 20, or 50 mg/kg quercetin was injected intraperitoneally to rats at 0.5, 12, and 24 h post-TBI. Rats were sacrificed 3 days post-injury, and their cerebral cortex was obtained from the injured side. The rats were randomly assigned into three groups of equal number: TBI and quercetin group, TBI group, and Sham group. The brain water content was calculated to estimate the brain damage induced by TBI. Immunohistochemical and Western blot assays were utilized to investigate the neurobehavioral status. Enzyme-linked immunosorbent assay and reverse transcription polymerase chain reaction were performed to evaluate the inflammatory responses. The cortical oxidative stress was measured by estimating the activities of malondialdehyde, superoxide dismutase, catalase, and glutathione-Px. Western blot was utilized to evaluate the expression of nuclear factor erythroid 2-related factor 2 (Nrf-2) and heme oxygenase 1 (HO-1).

Results: Quercetin attenuated the brain edema and microgliosis in TBI rats. Quercetin treatment attenuated cortical inflammatory responses and oxidative stress induced by TBI insults. Quercetin treatment activated the cortical Nrf2/HO-1 pathway in TBI rats.

Conclusions: Quercetin ameliorated the TBI-induced neuroinflammation and oxidative stress in the cortex through activating the Nrf2/HO-1 pathway.

Keywords: Quercetin; traumatic brain injury; neuroinflammation; oxidative stress

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Traumatic brain injury (TBI) is currently one of the main causes of disability and death among people of all ages and has become a huge public problem that threatens human health and life (1). The activation of multiple molecular pathways during the progression of TBI in damaged cerebral area and surrounding normal tissues leads to neuroinflammation, mitochondrial dysfunction, oxidative stress, calcium overload, and blood brain barrier (BBB) destruction, resulting in cerebral ischemia, edema, cytotoxic swelling, and increased intracranial pressure (2–4). Among them, neuroinflammation is critical to the removal, repair, and regeneration of debris post-TBI (5, 6). And inflammatory disorders induced by neuroinflammatory can cause acute and chronic brain damage (7, 8). It is urgent for us to identify new agents to protect cerebral tissues from neuroinflammation.

Quercetin is a flavonoid compound and a main bioactive component in traditional Chinese medicine (9). Accumulating evidence has demonstrated the multiple pharmacological actions of quercetin, including anti-inflammatory, anti-carcinoma, antiviral, antibacterial, and hypoglycemic effects (10–12). It has also been reported that quercetin treatment induces the removal of reactive oxygen species (ROS) and protects cerebral tissues from oxidative stress (13–15). Quercetin can inhibit the conjunction of platelets, white blood cells, and endothelial
cells, thus reducing inflammation (16). Previous research has found that quercetin effectively inhibits tumor necrosis factor α (TNF-α) level induced by lipopolysaccharide in the plasma (17). The anti-inflammatory mechanism of quercetin may be related to its ability to inhibit the spontaneous apoptosis of neutrophils and reduce the sensitivity of neutrophils to inflammatory factors (18).

Nuclear factor erythroid 2-related factor 2 (Nrf-2) is one of the critical anti-oxidative factors that promptly induce the production of anti-oxidant enzymes and stress-related transcription factors when cells are attacked by oxidants or electrophiles (19). The vicious circle of pro-inflammatory cytokine overexpression and ROS production is inhibited by the activation of the Nrf2/antioxidant response element (ARE) signaling pathway (20). Nrf2-mediated heme oxygenase 1 (HO-1) upregulation can inhibit the activation of nuclear factor kappa-B and the overexpression of monocyte chemotactic protein-1, thus alleviating the symptoms of multiple immune diseases (21).

Our previous study has demonstrated that quercetin treatment protects the cortical neurons against TBI in a rat model (22). However, the detailed mechanisms underlying this protection process remains unclear. In this research, we demonstrated that quercetin treatment activated the Nrf2/HO-1 signaling to ameliorate the inflammation and oxidative stress in the cortex of TBI rats, thus attenuating their brain edema and microgliosis. We believed that our research could provide new evidence supporting the clinical application of quercetin.

**Methods**

**Animal**

Animal studies were approved by the ethics committee of Cangzhou Central Hospital. Sprague-Dawley rats weighing between 260 and 330 g (5–8 weeks) were purchased from Cyagen Biosciences Inc. (Suzhou, China). Rats were anesthetized by the administration of chloral hydrate (15%) at 350 mg/kg. The middle of the rat’s head was shaved. Iodine and alcohol were used to wipe and disinfect the surgical area. The scalp of the rat was incised slightly to the right along the middle of the head for about 2 cm. The soft tissue and periosteum were bluntly separated to expose the skull. A skull drill was used to open a circular bone window with a diameter of 4 mm. Its edge was at 2 mm in front of the lambda line and 2 mm next to the midline of the skull. The dura mater remained intact. A metal weight of 40 g falling vertically from a height of 25 cm was used to hit the cylinder placed on the dura mater. The traumatic impact force was 1,000 g/cm, which caused a contusion and laceration of the right parietal lobe. The injury area was 4 × 4 mm. Then, bone wax was used to close the bone window, and the scalp was sutured. The rats were sacrificed 3 days after TBI modeling. The cerebral cortex tissue on the injured side was obtained and analyzed experimentally. Rats were randomly divided into three groups: Sham group (Sham); TBI group (TBI); and TBI + quercetin group (Que). Rats in the TBI group received the TBI surgery. The rats in the Sham group only received the opening of the skull in the corresponding location of the right parietal lobe without brain injury. Rats in the Que group received the TBI surgery and the administration of quercetin (Q4951, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). 0.9% saline solution (AM9624, Thermo Fisher Labsystems, Waltham, USA) was used to dissolve quercetin. 5, 20, or 50 mg/kg quercetin was injected intraperitoneally to rats at 0.5, 12, and 24 h post-TBI. In the modified neurological severity score (mNSS) test, 8 rats in each group were used. These rats were used to measure the brain water content. Afterward, 50 mg/kg quercetin was used. In the immunofluorescence staining of ionized calcium binding adapter molecule-1 (Iba-1) experiments, 6 rats in each group were used. To conduct the enzyme-linked immunosorbent assay (ELISA), quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, and oxidative biomarker measurement, 10 rats in each group were used.

**mNSS test**

The mNSS test is an established method. Rats were subjected to exercise (muscular state and abnormal action), sensation (visual, tactile, and balance), and reflex examinations and assigned a mNSS score that was recorded when a task was failed to be completed or when the corresponding reflex was lost. The mNSS score was graded on a scale of 0–18, where a total score of 18 points indicated severe neurological deficits, a score of 0 indicated normal performance, 13–18 points indicated severe injury, 7–12 indicated mean-moderate injury, and 1–6 indicated mild injury. Neurological function was measured at different time points by investigators who were blind to group assignment. The detailed information of mNSS was as follows:

1) Tail lifting test: the rats were suspended with the tail lifted for one meter, and the deviation and buckling of the head and fore- and hindlimbs were observed. Normal rats had no or ≤10° angle between the head and body vertical axis within a short period of time, and their limbs extended to the ground were scored as 0 points, for example, forelimb flexion, hind limb flexion, and head deviated from the vertical axis >10 within 30 s. 1 point will be scored for exceptions.
2) Walking test: the rats were placed on a large soft mat, and the rats were observed to walk freely. The rats were scored 0–3 for normal walking, unable to walk in
a straight line, turning in a circle to the paraplegic side, and dumping to the paraplegic side.

3) Sensory tests
(a) Visual experiment: the animal was held in hand with its front legs dangling, from 10 cm above the desktop to the desktop slowly tilted 45° near (this time table was located in the front or side) the rats, where normal rats reacted with forelimbs instantly grasping to the desktop (0 point), rats with physical injury showed response latency (1 point).
(b) Tactile experiment: the head of the rat was raised to 45°, and the front paws were suspended. At this time, the rats could neither see nor touch the desktop with their whiskers. The rats could touch the desktop with the back of their front paws, and the depth of stimulation was only up to the skin and hair.
(c) Proprioception-sensing experiment: rats were placed on the table, with their heads facing the edge of the table, and gently pushed from behind to the edge of the table. Under normal circumstances, the rats would grasp the edge of the table, and the limbs on the affected side would fall off. When the rats were pushed to the edge of the table, the position of the front and hind limbs of the rats on the affected side was observed. Normal rats could grasp the edge of the table, while the front and hind limbs of the rats on the affected side could not grasp the edge of the table.

4) Balance beam test: the square wooden bar with the length of 170 cm and the width of 2 cm was placed flat at a height of 70 cm from the ground, and the balance of the rats was observed on the balance beam.

5) Loss of reflex and abnormal movement: auricle reflex: the rats were placed on the table and touched the external auditory canal with their hands, normal rats had shaking of their head (0 points), and normal rats had no shaking of their head (1 point); cornal reflex: blinking (0 min) when using cotton filament to light the antennal membrane, and abnormal (1 min) if there was no blinking reaction; panic reflex: if the rats were placed on the table and the cardboard was near the auricle and could make noises rapidly, the normal rats could show escape motion (0 points), and the normal rats could show no escape motion (1 point); epilepsy, myoclonus, and dystonia abnormalities appeared in any of the three symptoms can be considered abnormal (1 point), and the three were not normal (0 points) (23).

**Brain edema measurement**
The water content in the brain tissue was determined using the wet-dry method by weighing fresh brain tissue and completely dried brain tissue and comparing the difference (22). The wet-dry weight method was used to evaluate brain edema in rats 3 days after TBI insult. The rat was over anesthetized and directly decapitated. The brain was taken, and the olfactory bulb, cerebellum, and lower brain stem were removed. The left and right cerebral hemispheres were separated, and the wet weight was immediately weighed. The rat brain was then placed in an electric oven at 110°C for 24 h to dry. The dry weight of brain tissue was then weighed. Brain water content (%) = (wet weight-dry weight) / wet weight × 100%.

**ELISA**
ELISA was performed to demonstrate the cytokine levels in the serum of rats according to the manufacturer’s protocol. The serum inflammatory factors including TNF-α, inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), and IL-1β were measured by commercially available kits (Life Technologies, Carlsbad, CA, USA).

**qRT-PCR**
The brain tissue of each rat was thoroughly ground mechanically and mixed with 1 mL Trizol (15596018, Life Technologies, Carlsbad, CA, USA) in a centrifuge tube. Chloroform and isopropanol (401570, Life Technologies, Carlsbad, CA, USA) were used to extract RNA from tissues. The FastKing cDNA reverse transcription kit (kr116, TIANGEN Biotech, Beijing, China) was used to remove genomic DNA from RNA and reverse transcribe RNA into cDNA. The SuperReal PreMix Plus (FP205, TIANGEN Biotech, Beijing, China) kit was used to perform qRT-PCR, in which SYBR green (FP207-02, TIANGEN Biotech, Beijing, China) was used as the fluorescent signal. The mixture configured according to its instructions was placed in a Roche fluorescent quantitative PCR machine, and the LightCycler480 software is used to analyze the data with a built-in algorithm. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a negative control. The calculation method of 2^−ΔΔCt was used during the analysis. The average of the sham group was regarded as 1 (GAPDH).

**Western blot**
Fresh tissue samples were homogenized with a Polytron grinder in an ice-cold homogenization buffer and centrifuged at 1,000 × g for 5 min at 4°C to isolate the nuclear fraction. The brain tissue and nuclear fraction were thoroughly ground mechanically and mixed with 200 μL radioimmunoprecipitation assay buffer. 200 μL of 2× loading buffer was added to the mixture. The mixture was placed in a metal bath at 100°C to denature the protein. A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to separate the proteins in an electrophoresis apparatus with a constant voltage of 140 V for 120 min. The protein on SDS-PAGE was then transferred to a polyvinylidene difluoride membrane (Real-Times Biotechnology Co. Ltd, Beijing, China) in a transfer tank with a constant current of 300 A. The
membrane was co-incubated with anti-Iba-1 (ab178846), anti-Nrf2 (ab1808Y), anti-HO-1 (ab189491), anti-Histon H3 (ab1791), and anti-β-actin (ab8226) (1:1,000, Abcam, Beijing, China) at 37°C for 1 h and washed by PBS for 3 times. Then, the goat anti-rabbit secondary antibody (ab150077, 1:10,000, Abcam, Beijing, China) was incubated with the membrane at 37°C for 1 h. An AEC Peroxidase Substrate Kit (Solarbiotech, Shanghai, China) was used for blot imaging.

Oxidative biomarker measurement
The biomarkers of oxidative stress in brain tissues including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione-Px (GSH-Px) were evaluated by the Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric, ab118970, Abcam), OxiSelectTM Superoxide Dismutase Activity Assay Kit (STA-340, Cell Biolabs, Inc., San Diego, CA, USA), OxiSelectTM Catalase Activity Assay Kit (STA-345, Fluorometric, Cell Biolabs, Inc., San Diego, CA, USA), and Glutathione Peroxidase Cellular Activity Assay Kit (CGP1, Sigma-Aldrich, Saint Louis, MO, USA).

Statistical analysis
The categorized variables were shown as frequency or percentage. And mean and standard deviation were utilized to demonstrate the continuous variables. One-way ANOVA followed by Dunn’s multiple comparisons test was performed for the statistical analysis in this research. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered as the significant difference. Graphpad Prism 10.0 was used for the plotting and analysis of the data in this research.

Results

Effects of quercetin on mNSS and brain water content in rat brains
To demonstrate the neuroprotective effects of quercetin on TBI rats, the mNSS test was performed at 3 days post-TBI. Quercetin was administered intraperitoneally at 0.5, 12, and 24 h post-TBI. As shown in Fig. 1A, different concentrations of quercetin treatment decreased mNSS, indicating that quercetin treatment ameliorated the brain damage caused by TBI. Cerebral edema is defined as increased brain volume caused by increased water content in the brain, which is a typical reflection of TBI-induced cerebral damage. 5, 20, and 50 mg/kg quercetin treatment significantly decreased brain water content in TBI rats (Fig. 1B). In detail, 50 mg/kg quercetin treatment reduced mNSS and brain water content of TBI rats more effectively than 5 and 20 mg/kg quercetin treatments. Therefore, we chose 50 mg/kg quercetin to treat TBI rats in the following study.

Quercetin treatment attenuated cortical cell microgliosis induced by TBI
The activation of microglia is closely related to the neuro-dysfunction induced by TBI. To demonstrate the impact of quercetin treatment on the cortical cell microgliosis during the progression of TBI, immunohistochemical staining was performed. The cerebral cortex tissues on the injured side were obtained 3 days post-injury. The protein levels of Iba-1 in the brain tissues were increased post-TBI and decreased by the treatment of quercetin (Fig. 2A and C). Correspondingly, the number of Iba-1 positive cells in the brain of TBI rats was also decreased markedly with the treatment of quercetin (Fig. 2B).

Quercetin treatment attenuated cortical inflammatory responses induced by TBI
To investigate the therapeutic effect of quercetin on the inflammatory responses in cerebral cortex tissues on the injured side induced by TBI, ELISA and qRT-PCR were performed. As shown in Fig. 3A, B, C, and D, TBI upregulated serum cytokines, including TNF-α, iNOS, IL-1β, and IL-6. On the contrary, 50 mg/kg quercetin treatment significantly downregulated these serum cytokine levels. The inflammatory response in the brain tissues of

Fig. 1. Effects of quercetin on mNSS and brain water in rat brains. Quercetin was administered intraperitoneally at 0.5-, 12-, and 24-h post-TBI. Three days after TBI insult, mNSS (A) and brain water (B) were compared. Data are presented as mean ± standard error with all data points showing. n = 8 in each group. *P < 0.05, **P < 0.01, ***P < 0.001.
TBI rats was evaluated by qRT-PCR, where the relative mRNA levels of TNF-α, iNOS, IL-1β, and IL-6 in the brain tissues of TBI rats were all decreased by the treatment of quercetin (Fig. 3E, F, G, and H).

**Quercetin treatment attenuated cortical oxidative stress induced by TBI**

The accumulation of ROS and increased oxidative stress in cerebral cortex tissues on the injured side of rats induced by TBI can cause the neuroinflammation and neurodegeneration. To demonstrate the impact of quercetin on the oxidative stress in the brain tissues of TBI rats, activities of MDA, CAT, GSH-Px, and SOD were measured. As shown in Fig. 4A, TBI upregulated MDA activity compared to the Sham group, which could be decreased by quercetin treatment in TBI rats. Congruously, 50 mg/kg quercetin treatment at 0.5, 12, and 24 h post-TBI significantly upregulated the activities of CAT, GSH-Px, and SOD in cerebral tissues of TBI rats (Fig. 4B–D).

**Quercetin treatment activated cortical Nrf2/HO-1 pathway in TBI rats**

To demonstrate the mechanism underlying the protective effect of quercetin against the TBI-induced brain damage in cerebral cortex tissues, Western blot was performed. As shown in Fig. 5A and B, 50 mg/kg quercetin treatment upregulated both the nuclear and total Nrf2 protein levels in the brain tissues of TBI rats. Correspondingly, the HO-1 protein level was also increased in the brain tissues of TBI rats in the Que group compared to the TBI group (Fig. 5C).

**Discussion**

TBI is a global issue that severely affects public health and social economy, and it is estimated that about more than 50,000 people die from, while about 530,000 people are disabled by TBI in the United States each year (24, 25). The brain damage caused by TBI is time-dependent, and its pathophysiological process can be divided into three major stages, which sometimes overlap with each other (26). The early stage of TBI usually occurs within 24 h after injury and mainly includes a series of energy metabolism disorders such as ischemic cascade caused by decreased cerebral blood flow, calcium overload, and mitochondrial dysfunction (27). The intermediate stage of TBI occurs within a few days after brain trauma (28). The occurrence and development of neuroinflammation further lead to vascular damage and destruction of the BBB, causing the formation of cerebral edema. The final stage of TBI occurs within a few weeks or even months after the trauma, and this stage is related to the adverse neurological outcome of TBI patients (26). The deterioration of neural function leads to convulsions and seizures (26).

Neuroinflammation includes the release of endogenous harmful substances and the activation of the innate immune system. The activation of microglia caused by TBI can also promote the release of cytokines and activate other immune cells, including neutrophils, monocytes, macrophages, etc., which initiate an inflammatory cascade at the injury site (29). TBI caused the release of many substances from multiple immune cells, including anti-inflammatory factors such as transforming growth factor-β, neurotrophic factors, IL-4, and IL-13, as well as pro-inflammatory factors such as IL-10, chemokine (C-X-C motif) ligand 1, IL-1, IL-6, TNF-α, etc. Although neuroinflammation plays a key role in the tissue recovery of TBI, the immune response regulation disorder that occurs during this process can also lead to secondary brain damage. Immune disorders caused by neuritis can increase the permeability of the BBB, leading to the further development of brain inflammation and even systemic inflammatory response syndrome (30). Thus, it is urgent for us to identify new agents to ameliorate the inflammation induced by TBI and protect the neuro-functions.
In fact, in our previous article, we have discovered the protective effect of quercetin on the brain tissues of TBI rats. We have previously demonstrated that 50 mg/kg quercetin treatment could inhibit the apoptosis of brain cells and protect the function of them, thereby ameliorating the motor deficit and brain edema caused by TBI in rats (22). In this previous study, we proposed that quercetin treatment could reduce the apoptosis of rat cortical neurons caused by TBI. The results of the present study demonstrated that post-TBI administration of quercetin may attenuate brain edema, in addition to improving motor function in rats. Additionally, quercetin caused a marked inhibition of extracellular signal-regulated kinase 1/2 phosphorylation and activated Akt phosphorylation, which may result in attenuation of neuronal apoptosis. In this study, however, we mainly explored the effects of quercetin treatment on oxidative stress and inflammation in damaged brain tissues of rats. In this study, we revealed the regulatory effect of quercetin treatment on the Nrf2/HO-1 signaling pathway in the brain tissue of TBI rats. We demonstrated that the treatment of quercetin induced high expression of Nrf2 and its downstream gene HO-1 in the brain tissues of TBI rats. The activation of Nrf2 and HO-1 could further promote the expression and activation of their downstream anti-inflammatory and anti-oxidant factors, thus inhibiting neuroinflammation and protecting the functions of the nervous system.

**Fig. 3.** Quercetin treatment attenuated cortical inflammatory responses induced by TBI insult. Quercetin was injected intraperitoneally to rats at 0.5, 12, and 24 h post-TBI at a dosage of 50 mg/kg. Three days after TBI insult, serum concentrations of TNF-α (A), iNOS (B), IL-1β (C), and IL-6 (D) were measured by ELISA. The mRNA levels of TNF-α (E), iNOS (F), IL-1β (G), and IL-6 (H) in the injured cerebral cortex were measure by qRT-PCR. Data are presented as mean ± standard error. n = 10 in each group. Data are presented as mean ± standard error, n = 10 (**P < 0.01, and *P < 0.05).
In particular, the anti-inflammatory and anti-oxidant capacity of quercetin has become a research hotspot. For instance, Yang et al. showed that quercetin treatment could alleviate the systemic inflammatory response and oxidative stress in db/db mice, thereby slowing down the development of non-alcoholic fatty liver (31). Roslan et al. reported that quercetin could ameliorate oxidative stress, inflammation, and apoptosis of the heart of adult male diabetic rats induced by streptozotocin-nicotinamide (32). Luo et al. demonstrated that quercetin relieved the oxidative stress induced by nicotinamide adenine dinucleotide phosphate oxidase by activating HO-1 in macrophages (33). Similar to previous research, we also identified the ameliorating function of quercetin treatment on the TBI-caused inflammation and stress in this study. We reported that quercetin could not only reduce the inflammatory response in the injured area but also reduce the levels of various inflammatory factors in the circulatory system of rats. At the same time, quercetin treatment could also effectively reduce the oxidative pressure caused by tissue damage to brain cells, thereby inhibiting apoptosis and protecting the neuro-function of TBI rats.

However, there are still some shortcomings in our study. First, due to limited resource and time, the number of rats used in animal experiments was relatively small, which might affect the accuracy of the final conclusion. Second, by comparing the ameliorating effects of quercetin on hydrocephalus and neurological impairment in rats caused by TBI, we chose a concentration of 50 mg/ml quercetin to treat rats and conducted our main research. Excessively high concentrations of quercetin might bring some long-term, unknown side effects to the survival of rats, although we have not clearly observed them yet. In addition, we only observed that quercetin treatment could promote the expression of Nrf2 and its downstream HO-1, while the downstream factors of Nrf2 and HO-1 involved in this process were still unclear. We will continue to pay attention to these issues in our future research.

**Conclusion**
In conclusion, we hereby report that quercetin treatment ameliorates the TBI induced neuroinflammation and oxidative stress in rat cortex through activating the Nrf2/HO-1 pathway. We demonstrate that 50 mg/kg quercetin treatment attenuates the brain edema and protected motor functions of rat post-TBI. Quercetin treatment...
activates the cortical Nrf2/HO-1 pathway and thus attenuates cortical inflammatory responses and oxidative stress induced by TBI. We believe that our research could provide new evidence supporting the clinical application of quercetin.

Conflicts of interest and funding
The authors declare no conflict of interest.

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References


Effects of quercetin on traumatic brain injury


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