The neuroprotective effects of Quercetin on hypoxic-ischemic brain injury in neonatal mice

Yiwen Zhang¹*, Yan Xiong², Hongbo Pang³

¹ Department of Neurology, Suining Municipal Hospital of TCM, Suining 629000, China
² Department of Endocrinology, Wenjiang People’s Hospital of Chengdu city, Chengdu 611130, China
³ Department of Neurology, Suining Central Hospital, Suining 629000, China

* Corresponding author
E-mail: 535626069@qq.com

Abstract:

Aim: To investigate the effects of quercetin on hypoxic-ischemic injury (HI) in neonatal mice and the mechanisms of action. Methods: 40 male C57BL/6J neonatal mice aging seven days were randomly divided into 4 groups: a sham with normal saline group (SOS), a sham with Quercetin group (SOQ), a hypoxia/ischemia with normal saline group (IHS), and a hypoxia/ischemia with quercetin group (IHQ). Then, 72 h after HI, mice were sacrificed to collect the brain tissues. Measuring the IL-1α and IL-6 concentrations by Elisa assay in brain. The nerve cell apoptosis of mice brain were measured by TUNEL assay. The IKK-β and NF-κB proteins expressions were measured by Immunohistochemistry (IHC) assay. Area of infarction and cerebral damage were measured by Nissl staining. Results: HIE induced neuronal death and tissue necrosis. Compared with IHS group, neuronal death and infarction area were significantly reduced in the IHQ group (P<0.05, respectively). Compared with IHS group, the IL-1α and IL-6 were significantly depressed, and IKK-β/NF-κB pathway were significantly suppressed in IHQ group (P<0.05, respectively). Conclusion: Quercetin improved HIE induced brain injury via IKK-β/NF-κB pathway.

Key words: Quercetin, HIE, IKK-β, NF-κB
Introduction

Neonatal hypoxic ischemic encephalopathy (HIE) is hypoxic ischemic brain damage caused by perinatal asphyxia. HIE has a detrimental effect on brain development and is a major cause of nervous system dysfunction in children. It often leads to neonatal abnormal behaviors and motor deficits, including mental retardation, epilepsy, cerebral palsy, learning disabilities, etc. (1). HIE is also an important cause of increased infant mortality and morbidity, and there is currently no thorough treatment (2). Studies have shown that HIE is highly destructive and hypoxia-ischemia is the main cause of brain damage in children. In the process of neonatal brain growth, inflammation caused by hypoxia-ischemia is a key factor leading to brain damage. Perinatal neuroinflammation increases the risk of neurological and neuropsychiatric diseases in childhood and adulthood. Therefore, reducing the neuropathological damage caused by inflammation can improve the function of nervous system (3). The disease not only seriously threatens the life of newborns, but also has a high disability rate. At present, there are still no effective drugs for treating the disease (4). Quercetin is an important polyphenolic substance with extensive pharmacological effects in anti-inflammation and anti-tumor (5, 6). However, neuroprotective effect of quercetin on neonatal hypoxic ischemic brain damage and its mechanism remain to be further studied. In this experiment, hypoxic ischemic neonatal mice were used as research objects for the study on the neuroprotective effect of quercetin on hypoxic ischemic brain damage in neonatal mice and its possible molecular mechanism, which provided a new research goal for clinical treatment of neonatal hypoxic ischemic encephalopathy.

Materials and Methods

Experimental animals and reagents

A total of 40 healthy 7-day-old male C57BL/6J mice (clean animals) had an average body weight of (4.00±0.50) g. In this experiment, wild-type c57BL/6J mice
were purchased from Experimental Animal Center of Nanjing University of Chinese Medicine and were fed at the Experimental Animal Center of Nanjing University of Chinese Medicine. All reagents were as follows: Quercetin (Sigma, USA); Interleukin-1α (IL-1α) and Interleukin-6 Elisa Kit (eBioscience Inc., San Diego, CA); TUNEL Kit (Sigma, USA) Rabbit Anti-Mouse IKK-β and NF-κB Monoclonal Antibodies (Abcam, UK); Nissl's Staining Kit (Shanghai Xin Fan Biotechnology Co., Ltd., China).

**Experimental grouping and model establishment**

Neonatal mice were randomly divided into 4 groups: sham operation + saline group (SOS); sham operation + quercetin (SOQ) group; ischemia-hypoxia + saline group (IHS) and ischemia-hypoxia + quercetin (IHQ), with 10 mice in each group. Brain tissues were removed for testing in 72 hours after model establishment.

The HIE model establishment method is as follows: Newborn mice were continuously inhaled with isoflurane and lay supine on operating table, and then limbs and heads were fixed. After routine surgical sterilization, under the dissecting microscope, an incision was made in the middle of the neck to separate the left common carotid artery; distal and proximal ligation were performed with a 6-0 sterile silk thread, and then it was cut from the middle; the 8-0 silk thread was used to suture the wound. The entire procedure was completed within 10 minutes. In 1 hour after the operations, the mice were placed in a 37°C thermostatically sealed hypoxic chamber (a mixture of 8% O₂ and 92% N₂). The hypoxia time was 75 min. And then the mice were returned to their mothers for feeding. In the sham operation group, only the left common carotid arteries of the mice were isolated without ligation, and they were not in oxygen deficiency.

Quercetin was dissolved in saline and prepared into 1.6 g/L for reserve. Intraperitoneal injection of quercetin (16 mg/kg) was performed in 24 hours and 48 hours after model establishment in the IHQ group; intraperitoneal injection of equal dose of saline was performed in saline group; in 72 hours after modeling, the mice were sacrificed, and their brain tissues were taken out for testing.
Protein extraction and Elisa detection

The mice were decollated and their brain tissues were taken; the fresh brain tissues were washed with phosphate buffer saline (PBS) and then radio immunoprecipitation assay (RIPA) lysate was added; they were shaken up slowly in ice bath, placed stably and lysed for 30 minutes; they were centrifuged at 10,000×g for 10 min, and the supernatant was aspirated. The protein was quantified (determined) by the BCA method. The determination of IL-1α and IL-6 was carried out according to the steps of the Elisa kit. The brief steps were as follows: ①200µl 1×ELISPOT (enzyme-linked immunospot assay) Diluent was added to each hole, and blocked at room temperature (22~28°C) for 1 hour; the plate was washed for 5 times; ②Dilute the standard samples; the diluted standard samples and samples were added into each hole in turn, with 100µl for each hole, overnight under 4°C; the plate was washed for 5 times; ③100 µl diluted detection antibody was added to each hole, the plates were sealed with microplate sealers, and incubated for 1 hour at room temperature; the plate was washed for 5 times; ④100 µl diluted Horseradish Peroxidase (HRP) labeled with avidin was added to each hole; the plates were sealed with microplate sealers, and incubated at room temperature for 30 min; the plate was washed for 5 times; ⑤10µl substrate was added to each hole and incubated at room temperature for 15 min; ⑥50 µl stop buffer (stop solution) was added to each hole; ⑦The optical density (D value) at a wavelength of 450 nm was detected with a microplate reader. A standard curve was established based on the D value, and the concentrations of IL-1α and IL-6 were calculated based on the standard curve.

Filling, fixing, drawing and slicing

The mice were anesthetized with chloral hydrate and sacrificed by the perfusion of saline and 4% paraformaldehyde. Their brain tissues were obtained and fixed with 4% paraformaldehyde (overnight). Their brain tissues were immersed in a 15% sucrose solution for dehydration; then it was immersed in a 30% sucrose solution for dehydration. The dehydrated brain tissues were embedded with optimum cutting temperature (OCT) compound, and then the brain tissues were sectioned (thickness:
10 μm) by a freezing microtome.

**Nissl’s staining**

The slices were placed in a 0.1% cresyl violet aqueous solution for 60 min, and then they were put in water bath under 37°C; distilled water was used to wash off floating color, and then they were transferred into 70% and 80% ethanol for 2 seconds respectively in turn; they were transferred into 95% ethanol for 2 times, in 2 seconds for each time; they were transferred into 100% ethanol for 2 times, in 1 minute for each time; they were transferred into xylene for 2 times, in 5 minutes for each time. Neutral resin was used to seal the slices, which were observed with a microscope, and taken pictures.

**Hematoxylin-eosin staining (H&E staining)**

The brain tissues were taken and fixed in 4% paraformaldehyde solution, dehydrated, paraffin-embedded, sectioned and stained with HE method. The pathological changes of the brain tissues were observed under an optical microscope.

**TUNEL staining**

The brain tissues were fixed and dehydrated, and then they were paraffin-embedded and sliced; after the slices were dewaxed, they were stained according to the instructions of TUNEL staining kit, and sealed (mounted) with neutral balsam. After staining, if the cells were browned, that was Tunel positive cells, which meant that the cells were apoptotic.

**Immunohistochemical staining (IHC)**

The brain tissues were fixed for 24 hours, dehydrated and sectioned (5 μm/slice). After microwave reparation for slices, they were incubated with IKK-β or NF-κB primary antibodies and corresponding secondary antibodies respectively, and they were mounted with neutral balsam. After staining, if the immune cells were stained brown, they were positive cells. The integrated option density (IOD) values of stained positive cells were measured using the B-I2000 medical image analysis system.

**Statistical methods**

GraphPad Prism6.0 statistical software was used for data analysis. Each set of data
was represented by Mean ± SD. One-way ANOVA (Analysis of Variance) was used for data analysis. \( p<0.05 \) was considered that the differences were statistically significant.

Results

H&E staining results for brain tissues

As shown in Fig 1, in the SOS and SOQ groups, neonatal rat brains were clearly structured and regular in morphology, and the size, shape, number and arrangement of glial cells were normal and regular, and the neuronal cells were arranged in an orderly manner, with clear nucleolus and normal morphology, without special lesions. In the IHS group, the structures of the right brain tissues were destroyed, the neurons were arranged in disorder, the number of the neurons was decreased, some neurons were swollen, degenerated and necrotic, and inflammatory cells were infiltrated and glial cells were proliferated. The brain tissue edema was obvious, and there were many degenerative and necrotic nerve cells. In the IHQ group, the structures of the brain tissues were almost normal, degenerative and necrotic nerve cells were significantly reduced, and pathological damage was significantly improved.
Figure 1. H&E staining of brain tissues (×100)

SOS: sham operation + saline group; SOQ: sham operation + quercetin; IHS: ischemia-hypoxia + saline group; IHQ: ischemia hypoxia + quercetin group

Apoptosis in each group

According to TdT-mediated dUTP Nick-End Labeling (TUNEL) assay, there was no significant difference in the apoptosis rate of brain tissue between SOS group and SOQ group ($p>0.05$). The apoptosis rates of brain tissues in IHS group were significantly higher than those in SOS group ($p<0.001$). However, after the intervention of quercetin, the apoptosis rates of brain tissues in IHQ group were significantly lower than those in IHS group ($p<0.01$). Detailed results were shown in Figure 2.
**Results of Nissl’s staining**

In the SOS group and the SOQ group, the structures of brain tissues were clear, the cell outlines were normal, the cell nucleuses were in the middle, the nucleoli were clear, the Nissl bodies were evenly distributed around the nucleuses, and the Nissl bodies were bigger in sizes and were more in number. IHS group: the necrocytosis, nucleus pyknosis, nuclear fragmentation and cell lysis were evident in the hippocampus of the ischemic side, and the number of Nissl bodies was significantly reduced or even disappeared. IHQ group: the necrocytosis, nucleus pyknosis, nuclear fragmentation, cell lysis and disappearance were in the hippocampus of the ischemic side, and the number of Nissl bodies was reduced. Detailed results were shown in Figure 3.
Figure 3. The Nissl’s staining of difference groups (×200)

SOS: sham operation + saline group; SOQ: sham operation + quercetin; IHS: ischemia-hypoxia + saline group; IHQ: ischemia hypoxia + quercetin group

Results of Elisa assay

Compared with the circumstances in SOS group, ischemia and hypoxia in IHS group could increase the release of IL-1α and IL-6 in brain tissue. It was found that quercetin could inhibit the release of inflammatory factor IL-1α and IL-6 induced by ischemia and hypoxia, and it was also found that the intervention of quercetin did not affect the release of IL-1α and IL-6 in brain tissue. Detailed data was shown in Table 1.

Table 1. The IL-1α and IL-6 levels in the brain of newborn mice (n=10, Mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-1α</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS</td>
<td>126.54±2.66</td>
<td>27.84±0.92</td>
</tr>
<tr>
<td>SOQ</td>
<td>125.15±3.26</td>
<td>27.69±0.95</td>
</tr>
<tr>
<td></td>
<td>IHS</td>
<td>SOQ</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>IHS</td>
<td>159.73±1.46</td>
<td>42.48±0.19</td>
</tr>
<tr>
<td>IHQ</td>
<td>128.49±1.77</td>
<td>33.95±0.53</td>
</tr>
</tbody>
</table>

SOS: sham operation + saline group; SOQ: sham operation + quercetin; IHS: ischemia-hypoxia + saline group; IHQ: ischemia hypoxia + quercetin group

#: P<0.05 vs. WS group; *: P<0.05 vs. WT group

### The expressions of IKK-β and NF-κB in brain tissues were detected by IHC

According to the results of IHC detection, there were no significant differences in the expressions of IKK-β and NF-κB proteins in the brain tissues between SOS group and SOQ group (p>0.05, respectively). This result indicated that quercetin in the brain tissues of newborn mice had no effect on the expressions of IKK-β and NF-κB proteins. Compared with SOS group, the expressions of IKK-β and NF-κB proteins were significantly increased in IHS group (p<0.001, respectively); After quercetin intervention, compared with IHS group, the expressions of IKK-β and NF-κB proteins in the brain tissues of IHQ group were significantly inhibited (p<0.01, respectively). Detailed data was shown in Figure 4.

![Figure 4. The relative proteins expressions of difference groups by IHC assay (×200) A. The IKK-β protein expression of difference groups](image-url)
ischemia-hypoxia + saline group; IHQ: ischemia hypoxia + quercetin group

###:P<0.001 vs. SOS group; **:P<0.01 vs. IHS group

B. The NF-κB protein expression of difference groups

SOS: sham operation + saline group; SOQ: sham operation + quercetin; IHS: ischemia-hypoxia + saline group; IHQ: ischemia hypoxia + quercetin group

###:P<0.001 vs. SOS group; **:P<0.01 vs. IHS group

Discussion

Neonatal hypoxic ischemic encephalopathy is a serious complication belonging to neonatal asphyxia, which poses a serious threat to the life and health of newborns. At present, the pathogenesis of HIE is not yet clear and there is still no effective treatment (7). Many studies have shown that neuronal death is considered to be the main pathological change of hypoxic ischemic brain damage (8). The immuno-inflammatory responses in ischemic hypoxic brain tissues are associated with neuronal death (9, 10).

Quercetin is a natural flavonoid compound found in the flowers, leaves and fruits of various plants. It has antioxidant, anti-inflammatory, anti-adhesion, anti-thrombotic, antiviral, antineoplastic, hypoglycemic, hypolipidemic and hypotensive effects as well as immune regulation (11, 12). Quercetin protects against hypoxic ischemic brain damage by reducing the inflammatory response, for which it is still unclear at present.

In this experiment, an ischemic hypoxic brain damage model was established by blocking the common carotid artery combined with the inhalation of mixed gas with 8% O₂. The results of Nissl’s staining showed that the apoptosis rates of brain tissues in the IHQ group was significantly lower than those in the IHS group. So the differences were statistically significant. The necrocytosis in the hippocampal region of the ischemic side, karyopyknosis, nuclear fragmentation, significant cell lysis and disappearance, and the number of Nissl's bodies in the IHS group were significantly lower than those in the IHQ group. It shows that progesterone can still play a
neuroprotective role in hypoxic ischemic brain damage of neonatal mice. The number of neuronal deaths decreased significantly after treatment with quercetin; it indicates that the pathological changes of ischemic hypoxic brain damage in neonatal mice are closely related to neuronal death, so quercetin can alleviate the neuronal death caused by ischemia and hypoxia.

A large number of studies have shown that inflammatory responses play a dual role in aggravating injury and promoting repair in central nervous system injury (13, 14). As part of the inflammatory response to brain injury, cytokines including interleukins, interferons, growth factors, tumor necrosis factors and chemokines are mainly secreted by immune cells to cause anti-inflammatory or pro-inflammatory responses (15, 16). In the family of cytokines associated with brain damage that have been studied, most of them are proinflammatory cytokines, including IL-1, IL-2, IL-6, IL-8, tumor necrosis factor-α (TNF-α), etc., which have adverse effects on hypoxic ischemic brain damage; some of them play neuroprotective effects through anti-inflammatory and anti-apoptotic effects, including receptor antagonists IL-4, IL-10 and IL-1, TNF-α binding protein and soluble TNF-α receptors; herein, the study on IL-1 and IL-6 has attracted much attention (17, 18). IL-1 is mainly produced by mononuclear macrophages and activated lymphocytes, and it can also be produced by neurons, astrocytes, oligodendrocytes and epithelial cells (19, 20). IL-1 in brain tissue is mainly produced by astrocytes and microglia cells (21); neurogenic IL-1 is mainly related to the transmission of surrounding information; and glial derived IL-1 is mainly involved in the process of neural trauma and repair (22). IL-1, as a pro-inflammatory mediator, its pro-inflammatory effect involves in intracephalic inflammatory response by mainly activating intracephalic microglia cells to make them release cytokines, free radicals, etc.; and endothelial cells are also stimulated to make them express leukocyte adhesion molecules, and the leukocytes accumulate around damaged brain tissues, thereby brain damage is aggravated. The experimental results of Liu et al. (23) and Wang et al. (24) suggested that the expression levels of IL-1β, IL-1 Ra, IL-1R and IL-1 RII were significantly increased in acute brain
damage. The study of Luheshi et al. (25) showed that IL-1α protein could be detected in microglia cells as early as in 4 hours after cerebral ischemia-reperfusion. Boutin et al. (26) found that the infarct sizes of IL-1α/β knockout (KO) mice was 70% lower than those of normal mice when they used IL-1α/β knockout (KO) mice as ischemia models. The above experiments suggest that IL-1 is an initial aberrant expression of the inflammatory response and has an aggravating effect on acute brain damage. IL-6 belongs to a pro-inflammatory cytokine, which can be produced by monocytes, B cells, T cells, fibroblasts and endothelial cells. In the central nervous system, it can also be produced by astrocytes, microglia cells and neurons (27). It is a pro-inflammatory medium (media); and its possible mechanism is to regulate mature neutrophils (PMN), and it play an important role in acute brain damage; the experimental results of Mullen et al. (28) also show that IL-6 can promote the oxidation of neutrophils and delay its apoptosis, and then the number of PMN on inflammation site and in circulation increases, finally neuronal damage is aggravated; at the same time, IL-6 is also a neurotrophic factor, which plays a neuroprotective role in promoting neuronal repair and anti-apoptosis (29). The results of this study showed that the expressions of IL-1α and IL-6 in IHS group (HIE model group) were significantly higher than those in SOS group (normal control group), which suggested that ischemia and hypoxia could increase the release of IL-1α and IL-6 in brain tissue. But quercetin can inhibit the release of inflammatory cytokines IL-1α and IL-6 induced by ischemia and hypoxia. Many studies have confirmed that the activation of IKK-β/NF-κB signaling pathway is the main cause of massive release of IL-1α and IL-6 (30-32). In order to investigate the mechanism of quercetin in improving nerve injury induced by ischemia-hypoxia, we carried out further study on related inflammatory pathways. The results of this study showed that the expression levels of IKK-β and NF-κB in the IHS group (HIE model group) were significantly higher than those in the SOS group (normal control group), which suggested that ischemia-hypoxia could make IKK-β/NF-κB signaling pathway activated. But quercetin can inhibit the activation of IKK-β/NF-κB signaling pathway induced by
ischemia-hypoxia.

In conclusion, ischemia-hypoxia can lead to the activation of inflammatory signaling pathways in the brain tissues of neonatal mice to promote the release of inflammatory factors, brain tissue damage and neuronal death. Quercetin may inhibit the release of inflammatory cytokines to achieve the protective effect on hypoxic-ischemic brain tissues in neonatal mice by inhibiting the activation of inflammatory signaling pathways in hypoxic-ischemic brain tissues.

References:
9. Umekawa T, Osman AM, Han W, et al: Resident microglia, rather than blood-derived macrophages, contribute to the earlier and more pronounced inflammatory reaction in the
immature compared with the adult hippocampus after hypoxia-ischemia. Glia 63:2220-2230, 2015.


www.advbiomed.org


