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Quercetin attenuates hypoxia-ischemia induced brain injury in neonatal rats by inhibiting TLR4/NF-κB signaling pathway

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ABSTRACT

Neonatal hypoxic ischemia (HI) is a kind of brain damage that occurs when an infant's brain does not receive enough oxygen and blood. The unrepairable damage leads to newborn death and short/long term brain dysfunctions. Due to the complicated causes and the variety of brain damages, there is no definitive treatment of neonatal HI. In this study, we set up a HI injury model of newborn rat and administrated Quercetin (Que) to treat rat pups before and after HI injury. We performed immunohistochemistry, quantitative PCR and immunoblot experiments to examine whether Que. has a role in attenuating brain injury after HI. We found that Que. treatment could clearly attenuate cortical cell apoptosis, as well as suppress apoptosis marker Bax, and activate anti-apoptosis marker Bcl-2. Moreover, Que. treatment decreased the number of cortical cells microgliosis and astrogliosis induced by HI injury. Furthermore, Que. treatment decreased cortical inflammation. Finally, it is suggested that Que. played a neuroprotective function on HI brain injury via inhibiting the TLR4/NF-κB signaling pathway. From these results, we conclude that Que. treatment may be a used as a therapeutic drug to prevent and decrease the newborn brain damage caused by HI.

1. Introduction

Hypoxia-Ischemia (HI) of brain damage results from a reduction in the supply of oxygen (hypoxia) and low blood flow (ischemia). This type of brain damage affects 20 of every 1000 full term newborns; the incidence rate in premature babies is 60% of all live births [1]. According to the extent of damage, newborn babies suffer different impairment in central and peripheral nerve systems, such as epilepsy, delay of neurodevelopment and motor development, cognitive dysfunction, or in the most severe condition, death [1,2]. The impairments are difficult to be identified at the time of HI occurrence, and the brain damage caused by HI can be permanent. As a result, the children and family with this condition suffer severe physical, mental and economical burdens. Due to the various causes and the severity of brain damage, there are no definitive treatments of neonatal HI besides hypothermia [2]. Therefore, actions should be taken for the prevention before HI occurrence and prompt treatments are particularly critical. The features of brain damage induced by HI injury are usually described as cortex cell apoptosis and necrosis, the increase of brain glial cells and initiation of a series of inflammation responses [3]. General treatment methods include mechanical ventilation, hyperbaric oxygen treatment, heart function maintenance and blood pressure control, general anesthesia and medications for seizure control [3–5]. The treatments described above can partly reverse brain damage and the future sequela symptoms. However, better and more effective treatment methods are needed without limitation. Recently, some drugs and compounds which possess effects to prevent and treat neonatal brain damage have been elucidated.

Quercetin (3,5,7,30,40-pentahydroxyflavone, Que) is a kind of plant flavonoids, which presents in many plants and plant-based foods, such as red wine, onions, green tea and berries. It is known as a kind of health care product because it was shown to have antioxidant and free radical scavenger effects [6], as well as acting as an anti-inflammatory and anti-blood coagulation medicine [7–10] by regulating enzymes and transcription factors in the inflammatory signaling cascade [11]. Ever since Que. was found to possess neuroprotective effect, studies of Quercetin for the treatment of Hypoxia-Ischemia on brain injury have been conducted [12]. Some studies described that Que. could protect oligodendrocyte progenitor cells from HI induced in vitro [13]; moreover, Que. treatment has been observed to attenuate perinatal hypoxia-ischemia induced myelin damage in vivo [10]. However, whether Que. has a preventive effect on neonate HI brain injury has not been clarified. In some situations, the prevention is more critical than treatment, especially in the cases of brain damage induced by HI. Thus, in this

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study we use the postnatal day 7 (P7) HI rat pup model, whose brains endured HI injury, to examine the roles of Que. on improving the recovering brain damage in vivo.

2. Materials and methods

2.1. Neonatal HI injury rat model

All animal experiments were compliant to the guidance of the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the Laboratory Animal Ethics Committee of The Second Hospital of Shandong University. All animals were housed in a central air conditioner of constant temperature under a 12 h light/dark cycle and allowed free access to food and water ad libitum. Male pups at postnatal day7 (P7) were obtained Shanghai Laboratory Animal Center (Shanghai, China). The HI injury model was performed as described [3]. Briefly, male rat pups were anesthetized with 3% isoflurane and received a left common carotid artery ligation, then were recovered for 2 h in their nest. A chamber was perfused with a humidified gas mixture of 8% oxygen and 92% nitrogen at 37.5 °C to maintain a hypoxia environment. Pups were placed in the chamber for 2.5 h and returned to their dam. The control Sham group neither received actual artery ligation nor exposure to the hypoxia conditions. The mortality rate of our rat model was < 30%. 24 h after HI injury, all rats were sacrificed for the next experiment procedure.

2.2. Quercetin administration

Quercetin (Que) was purchased from Sigma (St. Louis, MO, USA). We randomly separated the pups into 3 groups, Sham, HI and HI + Que. The Sham control group was given distilled water (dH_2O/0.1% Tween-80) without Quercetin. Que. was dissolved in distilled water containing 0.1% Tween-80, administered intragastrically once a day. Before the HI surgery was conducted, we administered Quercetin 40 mg/kg/day to the HI + Que. group rats for 7th day, the last administered was 2 h after the HI surgery. 24 h after HI surgery, the rats were sacrificed, the brains of rats were kept at −80 °C for further analysis. The Que. dose regimen was based on a report which demonstrated successful attenuation of brain injury in adult rat models of cerebral ischemia [10].

2.3. TUNEL assay and immunohistochemistry staining

For identification of apoptosis of DNA-strand breaks and identification of cortex cells at the very early stages of apoptosis, TUNEL assay was conducted (in Situ Cell Death Detection Kit, Roche, South San Francisco, CA, USA). Procedure was followed as per the manufacturer's protocol and referred as [3]. Neonatal brain specimens were collected at 24 h post HI injury, after which the samples were de-paraffin and rehydrated, the sections were treated in a 20 μg/ml proteinase K working solution for 10 min at 37 °C, then washed with phosphate-buffered saline (PBS) twice for 3 min. Fifty μl of TUNEL cocktail was put on test sections and negative controls were obtained by omitting the TdT enzyme. Sections were incubated in humidified chamber for 60 min at 37 °C. DAPI was used to stain all the sections for 7 min at room temperature. Sections were then dehydrated and slides were mounted with Shandon-Mount (Thermo Scientific, Waltham, MA USA), applied micro cover glasses and air-dried. Apoptotic cells were characterized by green fluorescence (signal with blue light excitation 450–500 nm) of the nucleus and nuclear membrane. For quantitation of the sections, we counted the number of positive cells in five randomly chosen fields within each slide at 400× magnification. The results were analyzed by using ImageJ software.

2.4. Immunofluorescence staining

Cortex brain section were kept at −80 °C, and immunofluorescence was performed [3,4]. Firstly, paraffin slides were made, brain tissues were post-fixed in 4% formalin solution overnight at 4 °C, dehydrated using graded ethanol and xylene, and embedded in paraffin and sectioned coronal into 4 μm slices for further use. For immunofluorescence, the paraffin slides were hydrated with 100% xylene for 6 min for 3 series tanks, with 100% ethanol for 5 min for 2 times and 95% ethanol for 5 min. After treating with suitable retrieving buffer, sections obtained at 24 h post HI injury were incubated with primary antibodies targeting the following proteins: Iba-1 (1:400), glial fibrillary acidic protein (GFAP, 1:200) for 1 h at room temperature, then rinsed with tris-buffered saline and treated with the AlexaFluor 488 anti-rabbit/goat secondary antibodies (1:1000) for 1 h at room temperature. All antibodies were purchased from Abcam. For nuclear staining, the slides were stained with DAPI for 7 min, and were sealed with a coverslip after being rinsed with PBS. All the images were captured using a fluorescence microscope. The extent of brain damage was measured by calculating the amount of surviving tissue in each section. Results were analyzed by the ImageJ software.

2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

Pups were in deep anesthesia before they were sacrificed. The brains were quickly dissected out and separated into the cortex and kept at −80 °C. Total RNA was extracted using Total RNA Mini Kit (Invitrogen, Waltham, MA USA). The concentration and quality of sample RNAs were measured by Nanodrop spectrometry, only the absorb OD 260/280 > 1.8 were used. 1 μg of total RNA was extracted from rat cortex cell. cDNA synthesis was performed using iScript reverse transcription Supermix kit (Bio-Rad, Hercules, CA). Primers are designed and referenced as follows [4]:

Bax: Forward 5′- GTT TCA TCC AGG ATC GAG CAG-3′.
Reverse 5′- CAT CTT CTT CCA GAT GGT GA −3′
Bcl-2: Forward 5′- CCT GTG GAT GAC TGA GTA CC-3′
Reverse 5′- GAG ACA GCC AGG AGA AAT CA-3′
TNF-α: Forward 5′- TACTCCCAGGTTCTCTTCAAGG-3′.
Reverse 5′- GGAGGCTGACTTTCTCCTGGTA-3′.
IL-1β: Forward 5′- CACCTCTCAAGCAGAGCACAG-3′.
Reverse 5′- GGGTTCCATGGTGAAGTCAAC-3′.
IL-6: Forward 5′- GAGTTGTGCAATGGCAATTC-3′.
Reverse 5′- ACTCCAGAAGACCAGAGCAG-3′.
β-actin: Forward 5′- AAGTCCCTCACCCTCCCAAAAG-3′.
Reverse 5′- AAGCAATGCTGTCACCTTCCC-3′.

Quantitative RT-PCR for associated genes was performed using the SsoAdvanced Universal SYBR Green Supermix kit and run on 95 °C, 2 min; 95 °C, 5 s and 60 °C, 30 s for 40 cycles; and every 5 s the temperate was increased by 0.5 °C from the initial 65 °C to 95 °C. All above assays followed the manufacturer's protocol. Expression level of β-actin was used for housekeeping gene control. The fluorescence threshold value (CT value) was analyzed by comparative threshold cycle ($2^{-\Delta\Delta}$ct) method and normalized to the Sham group. The expressions were calculated and expressed as fold change normalized with Sham group.

2.6. Immunoblot

After 24 h of HI injury, rats were deeply anesthetized and sacrificed by decapitation. Cortex was dissected and samples were kept at −80 °C immediately following sacrifice until analysis. For sodium dodecyl sulfate–polyacrylamide gel electrophoresis, cells were lysed on ice in 1 × lysis buffer (Cell Signaling Technology, Danvers, MA) in 20 mM Tris·HCl buffer, pH 7.5, containing 150 mM NaCl,1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 and 1% Triton) with 1× Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) or 1 × phosphatase inhibitor cocktail set

II (Calbiochem, La Jolla, CA). Ten to thirty micrograms of total protein from each sample was resolved on 8% to 12% sodium dodecyl sulfate Bis-Tris. Cl polyacrylamide gel with running buffer and transferred onto 0.2 μm polyvinylidene fluoride membranes (Bio-Rad). The membranes were probed with various antibodies. For western blots, membranes were first blocked with 5% non-fat dry milk for 2 h at room temperature, then membranes were incubated with the primary antibody overnight at 4 °C with shaking, following which the membranes were washed with 3 × 5 min of tris-buffered saline and Tween-20 and incubated with appropriate horse radish peroxidase-conjugated secondary antibodies for 1.5 h at room temperature, β-actin used as loading amount control. The signals were visualized by ChemiDoxMRS' Imaging System. The bands of western blot were quantified by the Image Lab software. All experiments were repeated at least three times.

2.7. Statistical analysis

Experiment values are presented as mean ± standard deviation (SD). p-Values of *p < 0.05 and **p < 0.01 were determined as statistical significance, which was calculated by one-way analysis of variance followed by a Tukey's post hoc test.

3. Results

3.1. Quercetin attenuates cortical cell apoptosis induced by HI injury

To clarify whether Que. treatment reduces cortical cell apoptosis induced by HI injury, we performed TUNEL staining (Fig. 1). In the HI group, TUNEL-positive cells were detected after 24 h of HI injury, meanwhile, in the HI + Que. group, TUNEL-positive cells were dramatically decreased (Fig. 1A, B), compared to the Sham group ($p < 0.01$ and $p < 0.05$ respectively). This data indicates that Que. treatment has an effect in attenuating cortical cell apoptosis induced by HI brain injury.

Next, we examined pro-apoptosis protein Bax and anti-apoptosis protein Bcl-2 in cortical cell of rat HI model with quantitative RT-PCR and immunoblot (Fig. 2). As expected, HI group induced Bax significantly increased and Bck-2 decreased, which means cortical cell endured apoptosis, on the contrary, HI + Que. group attenuated this trend significantly (Fig. 2A, B). Western blot indicated the consistent result (Fig. 2C, D). The results suggest that Que. treatment relieves HI injury via inhibiting cortical cell apoptosis.

3.2. Quercetin attenuates cortical cell microgliosis induced by HI injury

The HI injury activates glial cells in the brain. Thus, we checked whether HI injury activates cortical cell microgliosis in our rat model. We used microglia marker Iba-1 to examine the expression level of Iba-1 in Sham, HI and HI + Que. groups, and results generated from each group are shown in Fig. 3. HI injury induced high level of Iba-1 expression in both immunohistochemistry and western blot, however, HI + Que. group partially abrogated these expressions (Fig. 3A, C), and the differences are evident (Fig. 3B, D). These data suggested that Que. treatment decreased the expression of microgliosis marker Iba-1.

3.3. Quercetin attenuates cortical cell astrogliosis induced by HI injury

We also tested whether Que. treatment reduces cortical cell astrogliosis by detecting GFAP, the astrogliosis marker. Our data is shown in Fig. 4. The HI + Que. group showed significantly less GFAP expression compared with it of the HI group (Fig. 4A, B). Western blot showed the consistent result as quantitative RT-PCR (Fig. 4C, D), the two groups showed great differences compared with Sham group (Fig. 4). From our results, we concluded that Que. treatment is effective in attenuating the astrogliosis at the mRNA and protein levels in HI rat model.

3.4. Quercetin treatment down-regulated cortical inflammatory factors induced by HI injury

It has been reported that Que. treatment inhibited the neuro-inflammation in animal model [7,10,14]. As brain inflammation occurs prior to the onset of a brain damage [15], it is extremely important to detect the inflammatory factors such as IL-6, IL-1β and TNF-α. Quantitative real-time RT-PCR was performed to detect the expressions of these inflammatory factors. In HI group model, all of the IL-6, IL-1β and TNF-α were up-regulated, whereas in the HI + Que. group, the inflammatory factors were down-regulated (Fig. 5A-C) following Que. administration. These data suggest that Quercetin treatment partially reversed the neuro-inflammation in the neonatal rats induced by HI injury.

3.5. Quercetin protects the HI induced brain injury by inhibiting toll-like receptor 4/nuclear factor kappa-B (TLR4/NF-κB) signaling pathway

To further investigate the mechanism(s) of how Quercetin treatment mitigates HI induced brain injury, we examined the possible signal

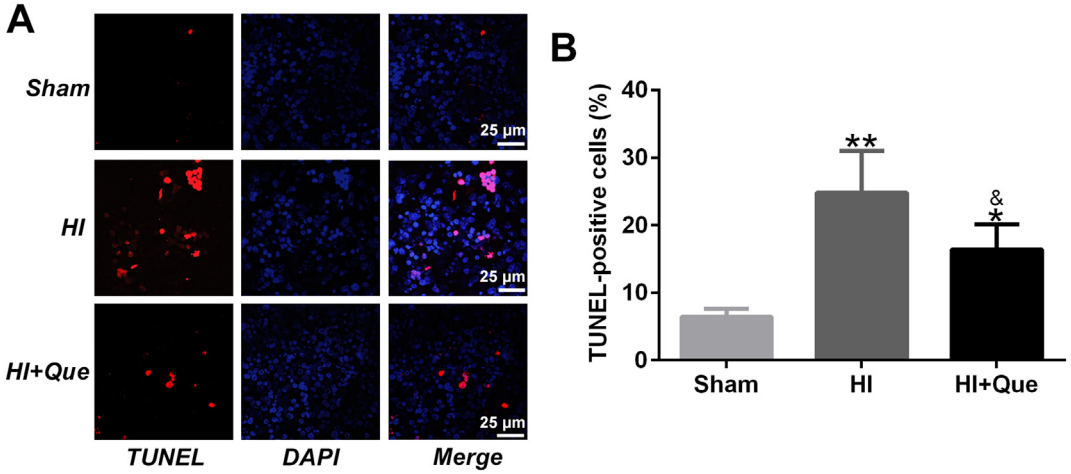

Fig. 1. Quercetin treatment attenuated cortical cell apoptosis induced by HI injury. A. Representative TUNEL-stained (red) and DAPI-stained (blue) brain sections in the cortex among Sham, HI and HI + Que. groups at 24 h post HI injury. B. Quantification of TUNEL positive cortical cells from the TUNEL staining. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 compared to Sham group, $^{\&}p < 0.05$ compared to HI group (Details are described in the method). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

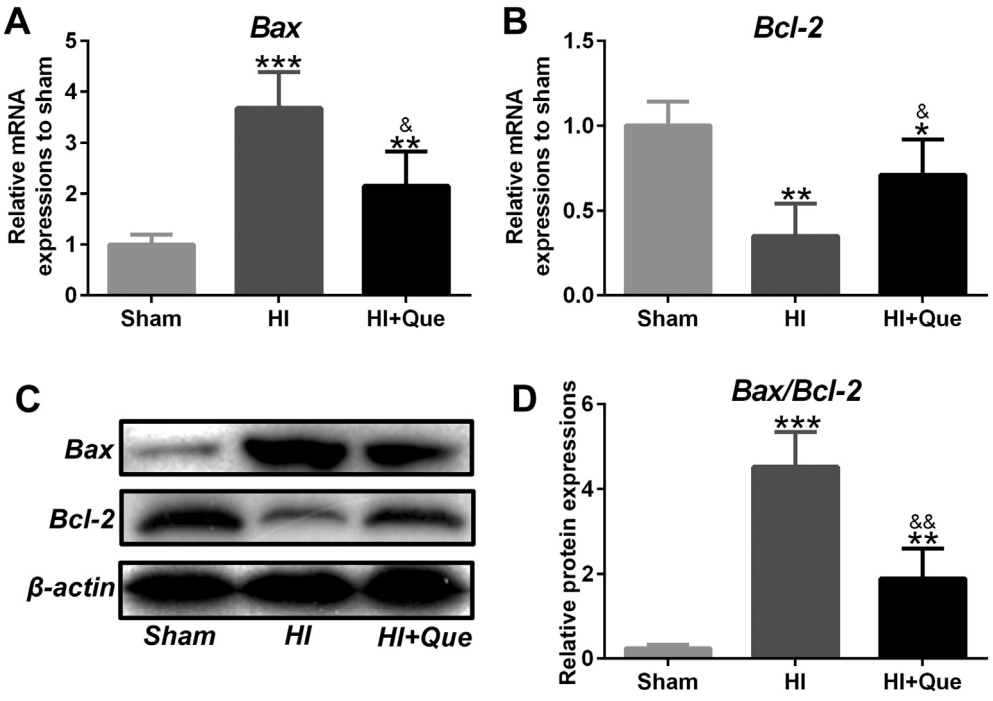

Fig. 2. Quercetin treatment attenuated cortical cell apoptosis induced by HI injury from mRNA and protein level. A, B: qRT-PCR analysis of Bax and Bcl-2, mRNA expressions in cortex of rats among different groups at 24 h post HI injury were showed. The expressions were calculated and expressed as fold change normalized with Sham group, β-actin was used as a housekeeping gene control. C. Representative of immunoblots of Bax and Bcl-2 in cortex of rats among different groups at 24 h post HI injury, β-actin was used as a protein loading control. D. Quantification of immunoblots of Bax/Bcl-2. Expressions were normalized to Sham group. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to Sham group, $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ compared to HI group (Primer sequences were shown in method part).

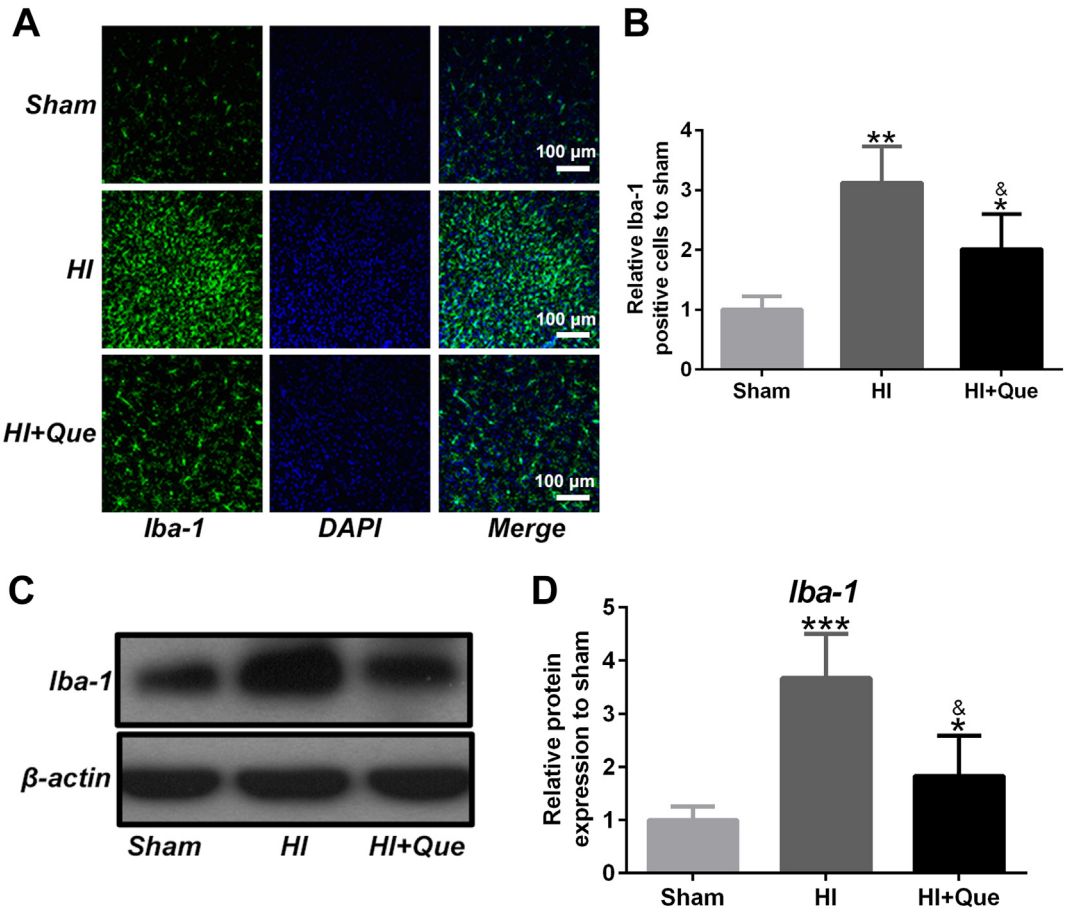

Fig. 3. Quercetin treatment decreased the number of cortical cell microgliosis induced by HI injury. A. Representative immunofluorescence staining of Iba-1 (green) and nucleus (blue) in cortex of rats among different groups at 24 h post HI injury. B. Quantification of Iba-1 positive cortical cells normalized to Sham group. C. Representative immunoblotting experiments of Iba-1 in cortex of rats among different groups at 24 h post HI injury. β-actin was used as loading control. D. Quantification of immunoblots of Iba-1. Expressions were normalized to Sham group. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to Sham group, $^{\&}p < 0.05$ compared to HI group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

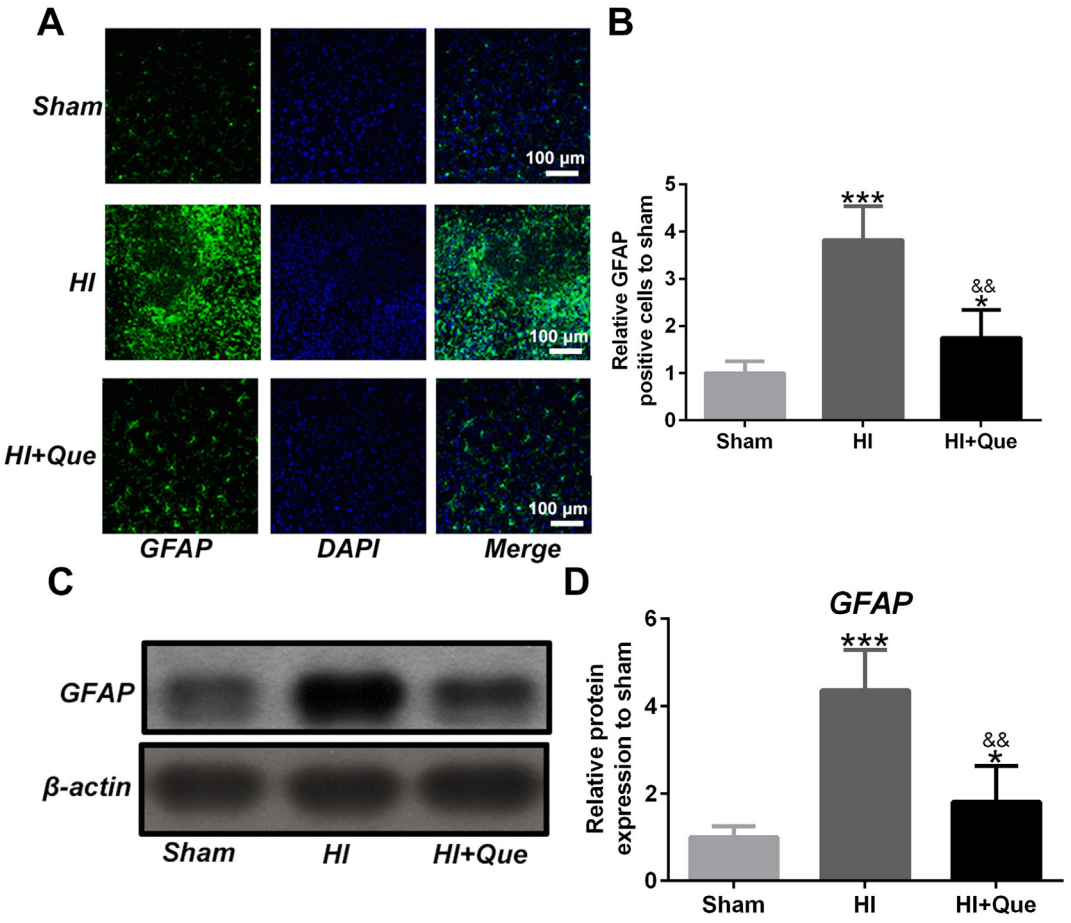

Fig. 4. Quercetin treatment decreased the number of cortical cell astrogliosis induced by HI injury. A. Representative immunofluroscence staining of GFAP (green) and nucleus (blue) in cortex of rats among different groups at 24 h post HI injury. B. Quantification of GFAP positive cortical cells normalized to Sham group. C. Representative western blots of GFAP in cortex of rats among different groups at 24 h post HI injury. β-actin was used as loading control. D. Quantification of immunoblots of GFAP. Expressions were normalized to Sham group. Data are presented as mean ± SD. *$p < 0.05$ and ***$p < 0.001$ compared to Sham group, $^{\&\&}p < 0.01$ compared to HI group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pathway of Que. acting at neonatal rat brain. On the basis of the findings of previous studies which observed the TLR4/NF-κB signaling pathway being partly responsible to Quercetin's effects [16], we tested the expressions of the representative factors in this pathway, including TLR4, p-p65 and p-p-IκBα. Results are shown in Fig. 6. Among those, western blot of TLR4, p-p65 and p-p-IκBα expression showed an up-regulation of approximately 2–3 folds in the HI group compared with the Sham group. On the other hand, Que. partially reversed this up-regulation, and the total amount of those proteins (p-65, p-IκBα) remained with no obvious change (Fig. 5A-C). Our data suggested that Quercetin reversed HI induced brain injury by inhibiting TLR4/NF-κB signaling pathway.

4. Discussion

Hypoxia-Ischemia (HI) is a severe condition of brain dysfunction

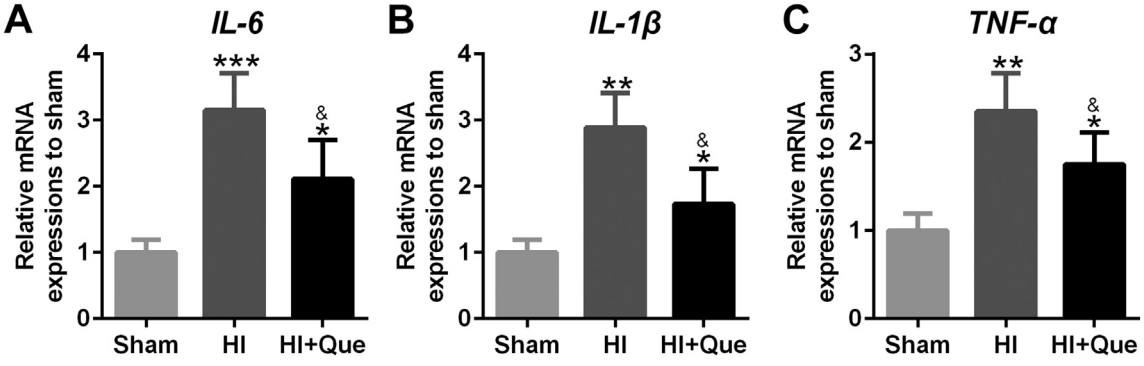

Fig. 5. Quercetin treatment suppressed cortical inflammatory factors induced by HI injury. qRT-PCR analysis of IL-6 (A), IL-1β (B) and TNF-α (C) mRNA expressions in cortex of rats among different groups at 24 h post HI injury. Relative expression pattern was analyzed by comparative threshold cycle (2-ΔΔct) method and normalized to Sham group. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to Sham group, $^{\&}p < 0.05$ compared to HI group.

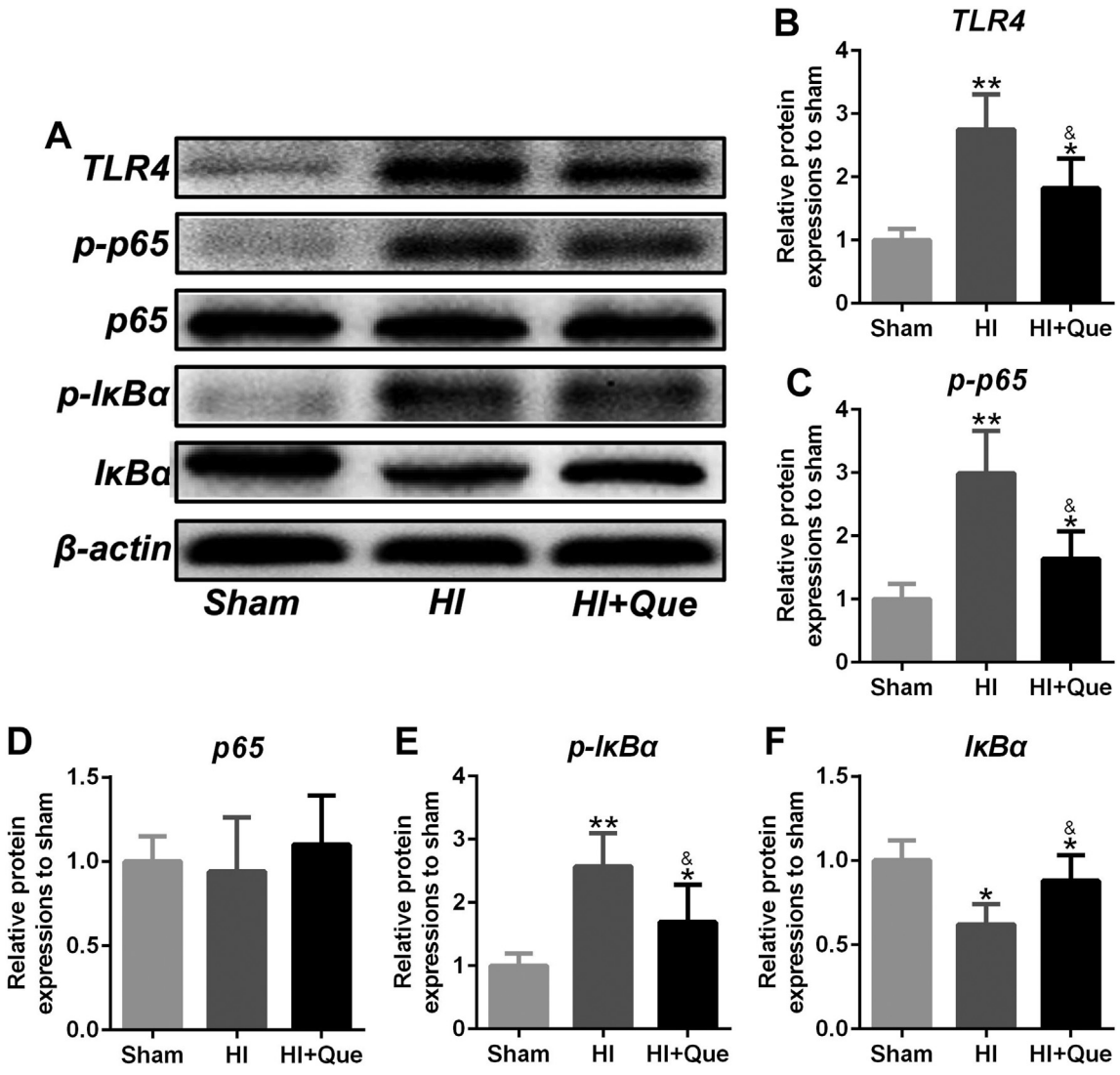

Fig. 6. Quercetin decreased TLR4 in HI induced brain injury by inhibiting NF-κB signaling pathway. A. Representative immunoblots of TLR4, p-p65, p65, p-IκBα and IκBα in cortex of rats among different groups at 24 h post HI injury. β-actin was used as loading control. B to F. Quantification of immunoblots of TLR4, p-p65, p65, p-IκBα and IκBα. Expressions were normalized to Sham group. Data are presented as mean ± SD. *$p < 0.05$ and **$p < 0.01$ compared to Sham group, $^{\&}p < 0.05$ compared to HI group.

which leads to neonatal mortality and morbidity. The standard treatment of newborn HI injury is therapeutic hypothermia [17]. Adjunctive neuroprotective treatments includes Erythropoietin (Epo) [18], Melatonin [4,19], Metforminutes [3], stem cell therapy [20] etc. [14], which have been proven to partly reduce HI brain damage. However, more effective treatments are needed.

One of the properties of neonatal brain damage is that cortex cells endure apoptosis and necrosis after HI, the broken DNA strands can be detected by TUNEL assay (Fig. 1). Our results showed Que. treatment greatly attenuated the DNA-strands break caused by HI brain injury. Moreover, when we examined the Bax/Bcl-2, the apoptosis/anti-apoptosis markers in rat cortex cells, the expressions of Bax were dramatically down-regulated and Bcl-2 up-regulated and vice versa (Fig. 2). Our results clearly indicated that Que. has anti-apoptosis effect towards cortex cells of HI damaged rats. The anti-apoptosis effect of Que. has been described by some research groups such as Lee et al. [9], who found that Que. reduced oxidative/nitrative damage of DNA and protected the nerve cells. However, the mechanism of action of the anti-apoptosis function of Que. needs to be further studied.

The other feature of brain damage by HI is the activation of microgliosis and astrogliosis in cortex [2,10]. The activated glial cell releases numerous inflammatory and neurotoxic factors, such as tumor necrosis factor-α and interleukin-6 [21]. Our experiments were consistent with this concept of brain injury and showed an increase in the expression of microgliosis and astrogliosis markers Iba-1 and GFAP caused by HI injury (Figs. 3 and 4). The high expressions of activated microgliosis and astrogliosis were significantly attenuated by Que. treatment, those data elucidated that Quercetin can partly decrease the activated glial cell numbers and protect brain cortex cells from further damage [22].

Neonatal hypoxia-ischemia (HI) is one of the major causes of death and/or lifelong central nerve system dysfunction [21]. The lack of oxygen with low flowing of blood triggers complex immune responses. According to recent researches, inflammation takes the most contribution for neonatal brain damage. Both activated intrinsic glia cells and infiltrating cells produce several pro-inflammatory factors included cytokines and chemokines, such as IL-6, IL-1β and TNF-α. Quercetin was described to against inflammation reactions [21]. In our study, we examined the expression of these inflammation factors in rat cortex after 24 h HI injury in Sham, HI and HI + Que. groups, our data clearly indicated those factors were significantly increased in HI group. Meanwhile, Que. reduced the expression levels (Fig. 5A, B and C),

which shows that it has the effect of anti-inflammation via down-regulation of the expressions of inflammatory factors, and to some extent, protect the neonatal brain from damage.

Researchers claimed the TLR4-mediated NF-κB signaling pathway plays a vital role in the initiation of cerebral inflammation in CNS diseases [4,16]. Thus, we examined the key protein expressions in this pathway to ascertain the mechanism of the neuroprotective effect of Quercetin. As shown in Fig. 6, Que. treatment significantly decreased the phosphorylation of TLR4 and downstream signals of p65 and p-IκBα and the total protein levels remained constant. Our experiment confirmed that Quercetin took the neuroprotective effect via suppressing the TLR4-mediated NF-κB pathway.

The mechanism(s) of the neuroprotective effect of Quercetin are complex and cannot be attributed to only a single signal pathway. Quercetin related pathways were studied by several groups in vitro and in vivo. Wang et al. [23] studied the effects of isoquercetin, to primary cultured cells in a stroke model of rat hippocampal neurons and hippocampal CA1 region, they described that the isoquercetin compound had anti-inflammation and anti- oxidative functions, potentially via suppressing not only TLR4-mediated NF-κB but caspase-1, phosphorylation of ERK1/2, JNK1/2, and p38 mitogen-activated protein kinase (MAPK), as well as TNF-α), IL-1β, and IL-6. Furthermore, JAK2/STAT3 signaling pathway [24], PI3K/AKT pathway [25] and the Nrf2-dependent HO-1 pathway activation were observed as well [26]. The HI damage of neonatal brain was a complex procedure which involves numerous mechanisms. Recently, the anti-inflammation and anti-oxidative functions of Quercetin were elucidated by more and more researchers, and other mechanisms functions of Quercetin will be further studied.

5. Conclusion

Our study suggested that Quercetin, a flavonoids compound, takes neuroprotective effects including anti-apoptosis, reduction of brain glial cells and anti-inflammation in HI brain injured newborn rats, possibly through suppression of the TLR4-mediated NF-κB pathway. Our results may provide new insights for prevention and treatment of HI brain injury.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgement

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