Research Square

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Fisetin has Inhibitory Effects on the TLR 4/NF-κB-Mediated Inflammatory Pathway After Traumatic Brain Injury in Mice: A Potential Neuroprotective Role

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Abstract

Inflammatory response contributes to the high mortality and morbidity of traumatic brain injury (TBI). Potent anti-inflammatory effects can alleviate brain injury after TBI. Fisetin has anti-inflammatory properties in several brain injury models, but the effects of fisetin on inflammation after TBI is still unclear. Our study aimed to investigate the neuroprotective effects of fisetin against inflammation after TBI in mice.Fisetin (25 mg/kg, 50 mg/kg or 75 mg/kg) or equal volume of vehicle was given via intraperitoneal injection 30 min after TBI. The neurological severity score, brain edema and blood brain barrier (BBB) permeability were assayed after TBI. In further mechanistic studies, changes in the toll-like receptor 4 (TLR 4)/nuclear factor-κB (NF-κB) pathway and the expression of pro-inflammatory cytokines were measured. Fisetin significantly improved behavioral outcomes and reduced brain edema after TBI. These changes were associated with significant reductions in TLR 4 expression and NF-κB activity. In addition, changes in the expression of pro-inflammatory cytokines were detected 24 h after TBI. Our study provided the first evidence that fisetin exerted neuroprotective effects by inhibiting the TLR 4/NF-κB–mediated inflammatory pathway after TBI in mice.

Introduction

Traumatic brain injury (TBI), a leading cause of death in people under the age of 40, is a serious public health problem worldwide (Maas, Stocchetti, & Bullock, 2008). However, effective treatments to improve clinical outcomes are currently lacking (Hetz, Bedi, Olson, Olsen, & Cox, 2012; Roberts, Schierhout, & Alderson, 1998).The effects of TBI have been classified into primary mechanical injury to cerebral cells and secondary brain injury (Greve & Zink, 2009). Secondary injury occurs immediately after primary injury (Nelson et al., 2019) and largely determines the outcome of TBI (Blomgren & Hagberg, 2013; Schmidt, Heyde, Ertel, & Stahel, 2005). Several pathophysiological events occur during secondary brain injury (Z. Zhang et al., 2011), including an inflammatory response, apoptosis, brain edema, blood brain barrier (BBB) breakdown, and oxidative stress (Griemert et al., 2019; Ozga, Povroznik, Engler-Chiurazzi, & Vonder Haar, 2018). Among these, the crucial role of the inflammatory response has increasingly been highlighted by research (Harting, Jimenez, Adams, Mercer, & Cox, 2008; Lu et al., 2009; Morganti-Kossmann, Rancan, Stahel, & Kossmann, 2002). The inflammatory response is implicated in the development of brain edema, BBB breakdown, and secondary neuronal injury (Chang, Wu, & Kwan, 2015; Gao et al., 2015). In addition, accumulating data suggest that toll-like receptor 4 (TLR 4)/nuclear factor-κB (NF-κB)–mediated inflammatory pathways contribute to the damaging inflammatory processes in secondary brain injury following TBI, and that inhibiting TLR 4/NF-κB can reduce the severity of TBI by downregulating inflammation (Dong, Yu, Hu, Zhang, & Huang, 2011; Yu, Dong, Hu, Huang, & Zhang, 2012).

The flavonoid fisetin (3,3',4',7-tetrahydroxyflavone) (Fig. 1), which is widely distributed in fruits and vegetables (Arai et al., 2000), has a wide variety of biological activities, including anti-inflammatory, antioxidant, and neurotrophic effects (Hanneken, Lin, Johnson, & Maher, 2006; Higa et al., 2003; Maher, 2006). However, there is no data available about its side-effects. In the central nervous system (CNS), its

neuroprotective role and anti-inflammatory effects have been well documented (L. T. Zheng, Ock, Kwon, & Suk, 2008). Fisetin has been reported to mediate the inhibition of the inflammatory response and improve clinical outcomes after stroke (Gelderblom et al., 2012; C. H. Zhou et al., 2015), attenuate microglial neurotoxicity (Chuang et al., 2014) and prevent oxidative damage in hippocampal cells (Inkielewicz-Stepniak, Radomski, & Wozniak, 2012). Meanwhile, numerous studies have reported that fisetin suppresses NF-κB activation, both constitutive and that induced by various carcinogens and inflammatory stimuli (Sung, Pandey, & Aggarwal, 2007). In addition, the effects of fisetin after TBI have been reported. Zhang et al found fisetin could alleviates oxidative stress after TBI (L. Zhang, Wang, Zhou, Zhu, & Fei, 2018). But the specific effects of fisetin on inflammtion after TBI remain unknown. Therefore, the present study was conducted to investigate whether the compound could inhibit the TLR 4/NF-κB-mediated inflammatory pathway after TBI.

Methods And Materials

Animal preparation

All experimental protocols, including animal use and surgical procedures, were approved by the Animal Care and Use Committee of Ningbo First Hospital and were in conformity with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Male ICR mice (28–32 g) were purchased from the Animal Center of Ningbo University, Zhejiang, China. The mice were raised in a humidified room (25 ± 1°C, 12-h light/dark cycle) and maintained on a standard pellet diet at the Animal Center.

Animal model of TBI

The TBI model employed in our study used a weight-drop protocol as previously described (Fig.2) (Flierl et al., 2009). Mice were anesthetized with an intraperitoneal injection of chloral hydrate (1%, 4 mL/kg) and then fixed on a platform directly under the weight of the weight-drop device. A midline longitudinal scalp incision was made, and the skull was exposed. After locating the impact target area, which was the left anterior frontal area (1.5 mm lateral to the midline in the mid-coronal plane), a 200-g weight was released from a height of 2.5 cm directly onto the exposed skull. The rod was caught after each impact to prevent any rebound double strikes to the skull. The scalp wound was then sutured with standard suture material, and the mice were returned to their cage to recover from anesthesia. After these procedures, the mice were kept in cages and housed at 23 ± 1°C with free access to food and water. The sham-injured animals underwent the same procedures without being subjected to the impact of the weight drop.

Experimental groups and fisetin treatment

The mice were randomly divided into seven groups: sham, sham + fisetin (75 mg/kg), TBI, TBI + Vehicle, and TBI + fisetin (25 mg/kg, 50 mg/kg, or 75 mg/kg). Fisetin (Sigma, St. Louis, MO, USA, 98% pure), or vehicle was administered via intraperitoneal injection 30 min after TBI. The fisetin was dissolved in 15 μL of dimethyl sulfoxide and diluted immediately before injection in 150 μL of 10% aqueous cyclodextran

(Sigma, St Louis, MO, USA) to give the final concentrations. The vehicle was 15 μL of dimethyl sulfoxide in 150 μL of 10% cyclodextran.

Two sets of experiments were conducted and totally 120 mice were used. In the first set, three concentrations of fisetin (25 mg/kg, 50 mg/kg, and 75 mg/kg) were administered to assess the neuroprotective effect of fisetin after TBI. We chose these doses based on a study of ischemic stroke in mice (Gelderblom et al., 2012). The neurological severity score (NSS) and brain water content were assayed after TBI. In the second set, mice were treated with the best dose of fisetin identified in the first set for further mechanistic investigation.

Neurological evaluation and brain water content

The neurological function of the mice was evaluated 1 d, 3 d, and 7 d after TBI using the NSS (Table 1). All mice were trained 1 d before TBI. The investigators evaluated the ability of each mouse to perform 10 different tasks that tested motor function, balance, and alertness. One point was given for failing to perform each of the tasks; thus, 0 = minimum deficit and 10 = maximum deficit (Flierl et al., 2009). All tests were performed by two investigators who were blinded to experimental group.

Brain water content was measured as previously described (Manaenko, Chen, Kammer, Zhang, & Tang, 2011; Wei et al., 2015). Briefly, mice were sacrificed and their brains were removed 24 h after TBI. The brainstem and cerebellum were removed, and the left (ipsilateral) cortical tissues were weighed immediately to obtain the wet weight. The samples were then dried at 80°C for 72 h and re-weighed to obtain the dry weight. The brain water content was calculated as a percentage using the following formula: 100 × (wet weight – dry weight)/wet weight.

Perfusion–fixation and tissue preparation

Brain tissues were harvested from mice after transcardiac perfusion with 0.9% normal saline solution (4°C) under anesthesia. The left (ipsilateral) cerebral cortex pericontusion volume was collected and immediately transferred to a -80°C freezer pending further analysis. For immunohistochemistry (IHC) and immunofluorescence (IF), the mice were perfused with 0.9% normal saline solution (4°C) followed by 4% buffered paraformaldehyde (4°C); the entire brain tissue was then immersed in 4% buffered paraformaldehyde (4°C).

BBB permeability

Evans blue (EB) extravasation was used to assay BBB permeability, as described previously (Manaenko et al., 2011). In brief, the mice were injected intraperitoneally with EB dye (2%, 4 mL/kg). They were anesthetized 3 h later and perfused transcardially with normal saline solution (4°C) to remove intravascular EB dye. After decapitation, the brains were removed and homogenized in physiological phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 15,000 g for 30 min. An equal volume of 50% trichloroacetic acid was then added to 0.5 mL of the resulting supernatant. After incubation overnight and centrifugation at 15,000 g for 30 min at 4°C, the dye concentration of the supernatant was

spectrophotometrically determined at 610 nm. The EB content was calculated as (μg of Evans blue stain)/(g of tissue).

Total protein extraction and western blot analysis

To extract total protein, the tissues were mechanically lysed in 20 mM Tris at pH 7.6, containing 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride, and 0.11 IU/mL aprotinin (all from Sigma, St. Louis, MO, USA). Homogenates were centrifuged at 14,000 g for 15 min at 4°C. The supernatant was collected and stored at -80°C pending analysis.

Equal quantities of total protein were loaded into each lane of a 10% SDS-PAGE gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked to prevent non-specific binding in 5% skimmed milk for 2 h at 25°C and incubated overnight at 4°C with primary antibodies. The primary antibodies were: rabbit anti-TLR 4 (1:200; sc-293072, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-matrix metalloproteinase-9 (MMP-9) (1:5,000; ab38898, Abcam), rabbit anti–ZO-1 (1:200; sc-10804, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti–Bcl-2 (1:200; sc-492, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti–cleaved caspase-3 (1:5,000; 9661, Cell Signaling, Beverly, MA, USA), and rabbit anti–β-actin (1:5000; AP0060, Bioworld Technology, Minneapolis, MN, USA). anti-rabbit horseradish peroxidase-conjugated IgG (1:5000, BS13278, Bioworld Technology, Minneapolis, MN, USA) for 2 h at 25°C. Bands were visualized using the enhanced chemiluminescence reagent kit (Millipore Corporation, Billerica, MA, USA), quantification was performed with ImageJ software (NIH) using optical density methods, and the data were normalized to β-actin.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

The nuclear protein of the tissue was extracted and quantified as previously described (Hang et al., 2004). EMSA was performed using a kit (Pierce Biotechnology, Rockford, IL, USA) to measure NF-κB DNA-binding activity according to the manufacturer's instructions and a previous study. A consensus oligonucleotide probe for NF-κB (5-AGT TGA GGG GAC TTT CCC AGG C-3, 3-TCA ACT CCC CTG AAA GGG TCC G-5) was end-labeled with biotin. Binding reactions were carried out for 20 min at 25°C in the presence of 50 ng/μL of poly(dI-dC) with 0.05% nonidet P-40, 5 mM MgCl2, 10 mM EDTA, and 2.5% glycerol in a 1× binding buffer using 20 fmol of biotin-end-labeled target DNA and 10 μg of nuclear protein. Assays were loaded onto native 6% polyacrylamide gels, pre-electrophoresed for 60 min in 0.5× tris-borate-EDTA, and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane in 0.5× tris-borate-EDTA at 300 mA for 30 min. Transferred DNAs were cross-linked to the membrane and detected using horseradish peroxidase-conjugated streptavidin.

Enzyme-linked immunosorbent assay (ELISA)

Total protein was assayed using a bicinchoninic acid assay kit (Pierce Biochemicals). The production of inflammatory cytokines in the brain tissue was quantified using ELISA kits specific for rat proteins,

according to the manufacturer's instructions (Diaclone Research, France). The inflammatory cytokine levels were calculated as picograms per milligram of protein.

IHC and IF staining

IHC staining was performed as described in our previous study (Li, Wu, et al., 2014). The brains were immersed in 4% buffered paraformaldehyde, embedded in paraffin, and cut into 10-μm slices. The sections were deparaffinized and incubated with 3% H2O2 in PBS for 10 min. The sections were then blocked with 5% normal fetal bovine serum in PBS for 2 h before incubation with primary antibodies overnight at 4°C. The primary antibody was rabbit anti-TLR 4 (1:200; sc-293072, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with PBS, the sections were incubated with goat anti-rabbit IgG–horseradish peroxidase (1:500, BS13278, Bioworld Technology, Minneapolis, MN, USA) at 25°C for 60 min. The sections were then stained with diaminobenzidine and counterstained with hematoxylin.

IF staining was performed by the method of our previous study (Sun et al., 2014). Brain tissues were fixed in 4% paraformaldehyde overnight and dehydrated in 20% sucrose in PBS for 2 days, followed by 30% sucrose in PBS for another 2 days. Brain sections of 6-μm thickness were blocked with 5% normal fetal bovine serum in PBS containing 0.1% Triton X-100 for 2 h at 25°C, followed by incubation with antibodies against p65 (1:200; sc-8008, Santa Cruz, CA, USA), neuronal nuclei (NeuN; MAB377X, 1:200, Millipore, USA), glial fibrillary acidic protein (1:200, 556327, BD Biosciences, USA), or ionized calcium binding adapter molecule 1 (Iba 1) (1:20, NBP2-19019, Novus Biologicals, USA), overnight at 4°C. The following day, after washing with PBS for 45 min, the sections were immunolabeled with appropriate fluorescent dye-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) in the dark for 1 h at 25°C. The sections were then washed with PBS for 45 min and counterstained with DAPI for two min. After three further washes, the sections were observed under a fluorescence microscope.

Statistical analysis

All results of the summary statistical analysis are presented as mean ± SEM and were analyzed using SPSS 17.0 and GrahPad Prism 5.0 software. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. The threshold of statistical significance was set at $p < 0.05$.

Results

Fisetin improved neurological function and reduced brain water content after TBI

We first used the NSS scores to evaluate whether fisetin was neuroprotective after TBI. As shown in Fig. 3A, the NSS score of TBI mice was significantly higher than that of sham mice 1 d and 3 d after TBI ($p < 0.001$). Treatment with 50 and 75 mg/kg fisetin dramatically ameliorated losses of neurological function ($p < 0.01$, $p < 0.05$); however, 25 mg/kg fisetin did not show significant effects ($p > 0.05$). It is worth noting that a larger dose (75 mg/kg) of fisetin did not show better effects than did 50 mg/kg ($p >$

0.05). Seven days after TBI, we observed no significant differences among groups. In addition, there is no significant difference of NSS score between sham group and sham + fisetin group.

According to a previous study, brain water content measured 24 h after TBI is an indicator of brain edema (Dogan et al., 1997). In our study, brain water content 24 h after TBI was greater than that in the sham group (Fig. 3B, $p < 0.01$). Fisetin at doses of 50 g/kg and 75 g/kg significantly reduced the brain water content relative to untreated, impacted groups ($p < 0.05$), but 25 g/kg fisetin had no significant effect ($p > 0.05$). Consistent with the NSS scores, 75 g/kg fisetin did not have a greater effect than 50 g/kg ($p > 0.05$). Therefore, we selected 50 g/kg for subsequent experiments. In addition, there is no significant difference of brain water content between sham group and sham + fisetin group.

Fisetin decreased TLR 4 expression at 24 h after TBI

In further mechanistic studies, we examined the expression of TLR 4. Western blotting (Fig. 4A) showed that the expression of TLR 4 in the TBI group was significantly higher than that in the sham group ($p < 0.05$). However, fisetin treatment completely blocked this upregulation of TLR 4 ($p < 0.05$). IHC against TLR 4 showed that the number of TLR 4-positive cells increased after TBI and that this upregulation was reduced after fisetin treatment (Fig. 4B; $p < 0.001$ and $p < 0.01$, respectively).

Fisetin inhibited the activity of NF-κB at 24 h after TBI

EMSA was conducted to investigate NF-κB activity (Fig. 5A). NF-κB activity was higher after TBI compared with that in the sham group and this increase was inhibited by fisetin treatment. In addition, to further illustrate NF-κB activity, IF staining was performed (Fig. 5B). Compared with the sham group, an obvious translocation of p65 from the cytoplasm to the nucleus was observed in the TBI group. However, a lower proportion of nuclei positive for p65 was detected after fisetin administration.

Fisetin decreased the production of inflammatory cytokines at 24 h after TBI

To study the effects of fisetin on the inflammatory response, inflammatory cytokines (TNF-α, IL-6, and IL-1β) were assessed using ELISA. As shown in Fig. 6, the concentrations of cytokines were low in the sham group and significantly increased after TBI ($p < 0.05$). However, these upregulations were obviously decreased with fisetin administration ($p < 0.05$).

Brain edema was ameliorated after fisetin treatment at 24 h after TBI

To investigate the effects of fisetin on brain edema after TBI, BBB permeability and the levels of MMP-9 and ZO-1 were determined. The increases in MMP-9 expression and ZO-1 degradation observed after TBI ($p < 0.01$) were reversed by fisetin administration (Fig. 7A, B; $p < 0.05$). In addition, BBB permeability increased after TBI ($p < 0.01$; Fig. 7C) and this increase was ameliorated after fisetin treatment ($p < 0.05$).

Discussion

The present study was the first investigation of whether fisetin could provide neuroprotective effects after TBI and the possible role of the TLR 4/NF-κB-mediated inflammatory pathway in the neuroprotective mechanism. The major findings were as follows: Fisetin could provide neuroprotection after TBI and it's associated with its effects on inhibiting inflammatory response through TLR 4/NF-κB-mediated inflammatory pathway. These findings provide vital evidence that fisetin plays a neuroprotective role after TBI, and its beneficial effects were possibly associated with inhibition of the TLR 4/NF-κB-mediated inflammatory pathway. This pathway might be a new target for the clinical treatment of TBI.

When the homeostasis of the CNS is controlled and well balanced, inflammatory responses mainly contribute to the repair of damaged tissues (Xian et al., 2019). However, excessive inflammation damages the surrounding tissues and is involved in the progression of several CNS diseases (Lucas, Rothwell, & Gibson, 2006; Z. V. Zheng et al., 2020). Numerous studies have demonstrated that early inflammation contributes to brain injury and results in neurological deficits (Corps, Roth, & McGavern, 2015; Lim et al., 2020). After TBI, inflammatory cells respond quickly to brain injury and initiate a complex series of inflammatory cascades. It has been reported that the TLR 4/NF-κB-mediated inflammatory pathway plays a substantial role in the induction of inflammation after TBI (Downes & Crack, 2010; Racke & Drew, 2009). As the first mammalian TLR recognized, TLR 4 is a key factor in secondary brain injury and plays a key role in initiating a complex series of inflammatory responses after TBI (M. L. Zhou et al., 2007; Zhu et al., 2014). Moreover, NF-κB, which is downstream from TLR 4, is a transcription factor associated with innate immunity and inflammation (Harari & Liao, 2010; Xiang et al., 2019). The upregulation of NF-κB DNA-binding activity leads to increased production of cytokines and chemokines, and some of these inflammatory mediators also activate NF-κB, creating a positive feedback loop to amplify inflammation (Monaco & Paleolog, 2004). In addition, the activation of NF-κB and the upregulation of inflammatory mediators contribute to brain edema and neuron apoptosis (Ridder & Schwaninger, 2009). Previous studies have indicated that inhibiting the TLR 4/NF-κB-mediated inflammatory pathway could provide protection against brain injury (Gao et al., 2015; You et al., 2013). Numerous studies have demonstrated that fisetin exerts anti-inflammatory effects by inhibiting NF-κB (Murtaza, Adhami, Hafeez, Saleem, & Mukhtar, 2009; Sung et al., 2007). Therefore, we hypothesized that fisetin would provide neuroprotection after TBI by inhibiting the TLR 4/NF-κB-mediated inflammatory pathway. In this study, we found that fisetin treatment reduced the expression of TLR 4 and the activation of NF-κB that follows TBI. Moreover, fisetin reduced the production of inflammatory cytokines after TBI. These data suggest that fisetin inhibits the TLR 4/NF-κB-mediated inflammatory pathway after TBI.

The BBB is an anatomical micro-structural unit that separates the CNS from the peripheral blood circulation (Li, Gao, et al., 2014; C. Zhang et al., 2020). Many factors, such as angiogenic factors, excitotoxicity, and inflammatory responses, can lead to BBB breakdown (Alves, 2014). Numerous studies indicate that MMP-9, a downstream mediator of TLR 4/NF-κB signaling, degrades the inter-endothelial tight-junction and basal-lamina proteins of the BBB (Feiler, Plesnila, Thal, Zausinger, & Scholler, 2011). Upregulation of MMP-9 mediates BBB disruption and inhibition of MMP-9 reduces BBB permeability and brain edema after TBI (Shi et al., 2015). In the present study, we found that fisetin inhibited the increase in MMP-9 that normally occurs after TBI and concomitantly restored levels of ZO-1 (one of the tight-junction

proteins). Meanwhile, BBB permeability, which we determined as the amount of extravasated EB dye, was reduced by fisetin administration. These data suggest that fisetin contributes to BBB protection.

However, this study has several limitations. First, our data demonstrated beneficial effects of fisetin administration after TBI due to inhibition of the TLR 4/NF-κB-mediated inflammatory pathway. However, we did not investigate the effects of fisetin on oxidative stress (Kang et al., 2014; Piao et al., 2013), which also plays an important role after TBI (Rodriguez-Rodriguez, Egea-Guerrero, Murillo-Cabezas, & Carrillo-Vico, 2014). Second, we selected the dose and time window of fisetin treatment based on a previous study on ischemic stroke (Gelderblom et al., 2012); further studies to determine the optimum dose and time window of fisetin administration after TBI are warranted.

Abbreviations

BBB, blood brain barrier; CNS, central nervous system; EB, Evans blue; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; IF, immunofluorescence; IHC, immunohistochemistry; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-κB; NSS, neurological severity score; PBS, phosphate-buffered saline; SEM, standard error of the mean; TBI, traumatic brain injury; TLR 4, toll-like receptor 4; TUNEL, terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick end-labeling; ZO-1, one of the tight-junction proteins

Conclusion

To the best of our knowledge, this study is the first to demonstrate that fisetin provides neuroprotection after TBI in mice. Moreover, as shown in Fig. 8, our results imply that fisetin exerts its neuroprotective effects by inhibiting the TLR 4/NF-κB-mediated inflammatory pathway.

Declarations

Authors declarations:

Animal Ethics are approve and consent to participate.

Consent for publication: Yes.

Availability of data and materials: Yes.

Declaration of competing interests: None.

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Acknowledgments and authors' contributions:

We thank all the members. Chenhui Zhou designed and performed the studies, data analysis, and wrote the manuscript. ChenHui Zhou and Sheng Nie were responsible for animal model. Sheng Nie contributed to evaluate neurologic function and brain water content. Zhepei Wang was responsible for the western blot and EMSA. Yiting Wang contributed ro test BBB permeability and ELISA. Jingmi Wu was responsible for IHC and IF staining. Xiang Gao contributed to the Nissl and TUNEL staining. Chenhui Zhou and Yi Huang contributed to the design and analysis of the study and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Neurological severity scoring

Items	Description	Points	
		Success/Failure	
Exit circle	Ability and initiative to exit a circle of 30 cm diameter (time limit: 3 min)	0	1
Mono-/hemiparesis	Paresis of upper and/or lower limb of contralateral side	0	1
Straight walk	Alertness, initiative,and motor ability to walk straight, when placed on the floor	0	1
Startle reflex	Innate reflex(flinching in response to a loud hand clap)	0	1
Seeking behavior	Physiological behavior as a sign of "interest" in the environment	0	1
Beam balancing	Ability to balance on a beam 7 mm in width for at least 10 s	0	1
Round stick balancing	Ability to balance on a round stick 5 mm in diameter for at least 10 s	0	1
Beam walk: 3 cm	Ability to cross a beam(length ×width, 30 ×3 cm)	0	1
Beam walk: 2 cm	Same task but with increased difficulty (beam width=2 cm)	0	1
Beam walk: 1 cm	Same task but with increased difficulty(beam width=1 cm)	0	1
Maximum score			10

Figures

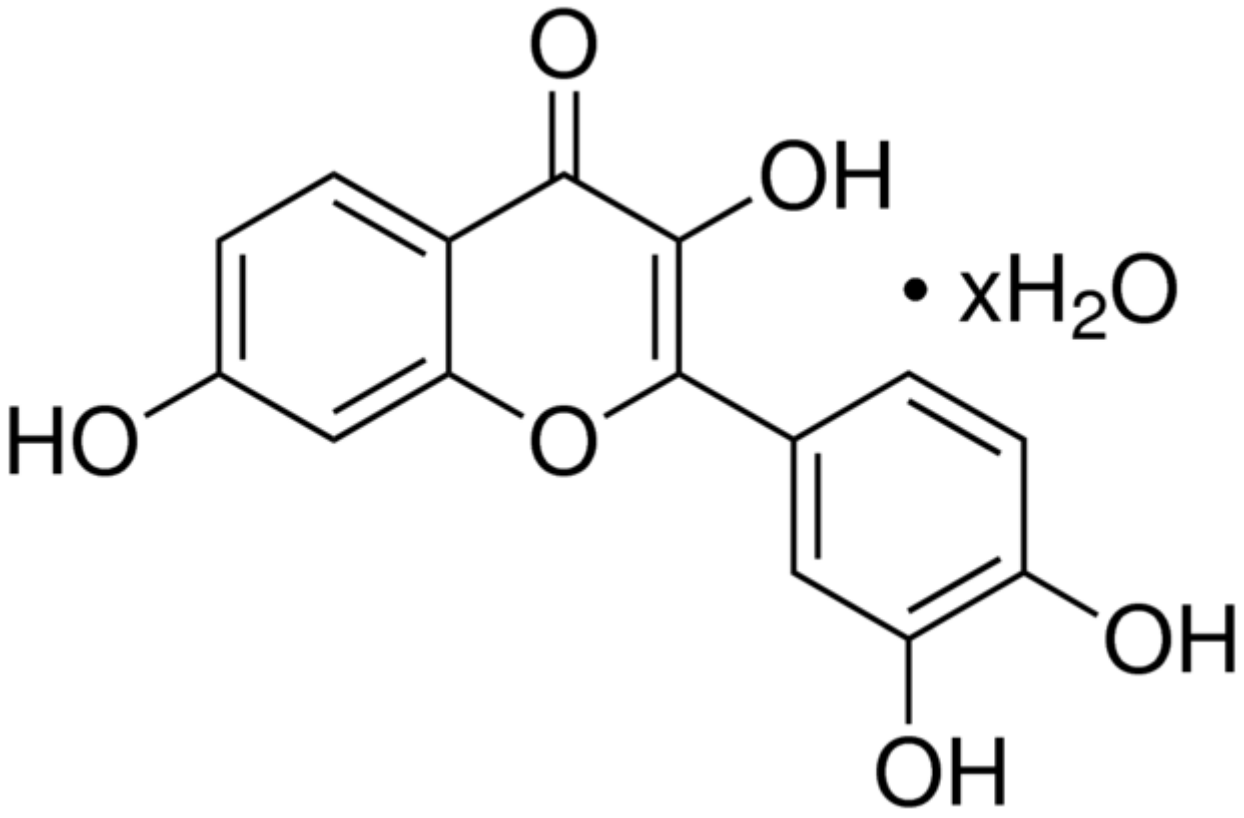

Figure 1

Chemical structure of fisetin (3,3',4',7-tetrahydroxyflavone)

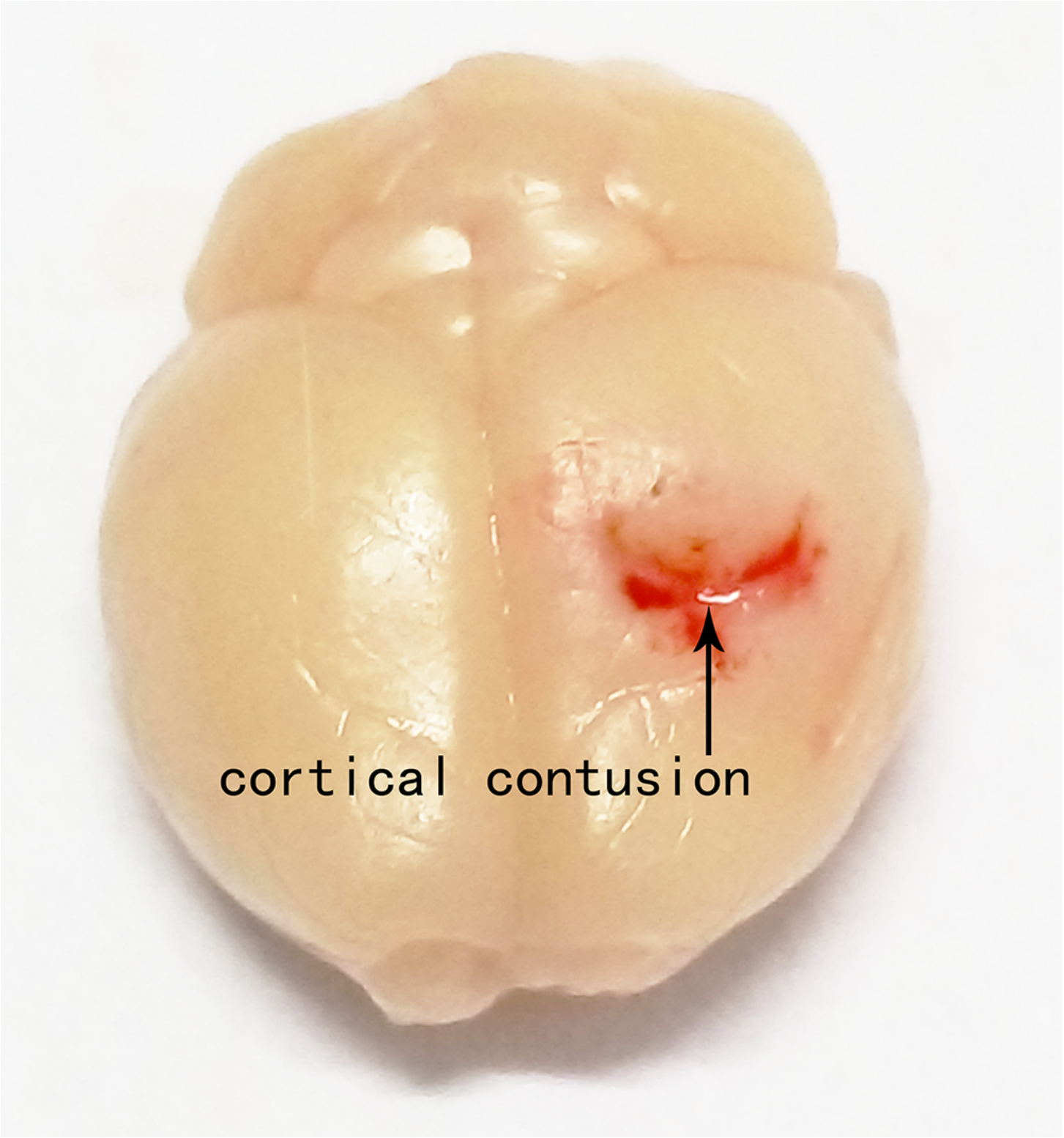

Figure 2

Experimental traumatic brain injury (TBI) mouse model Schematic of the contused cortical area induced by TBI.

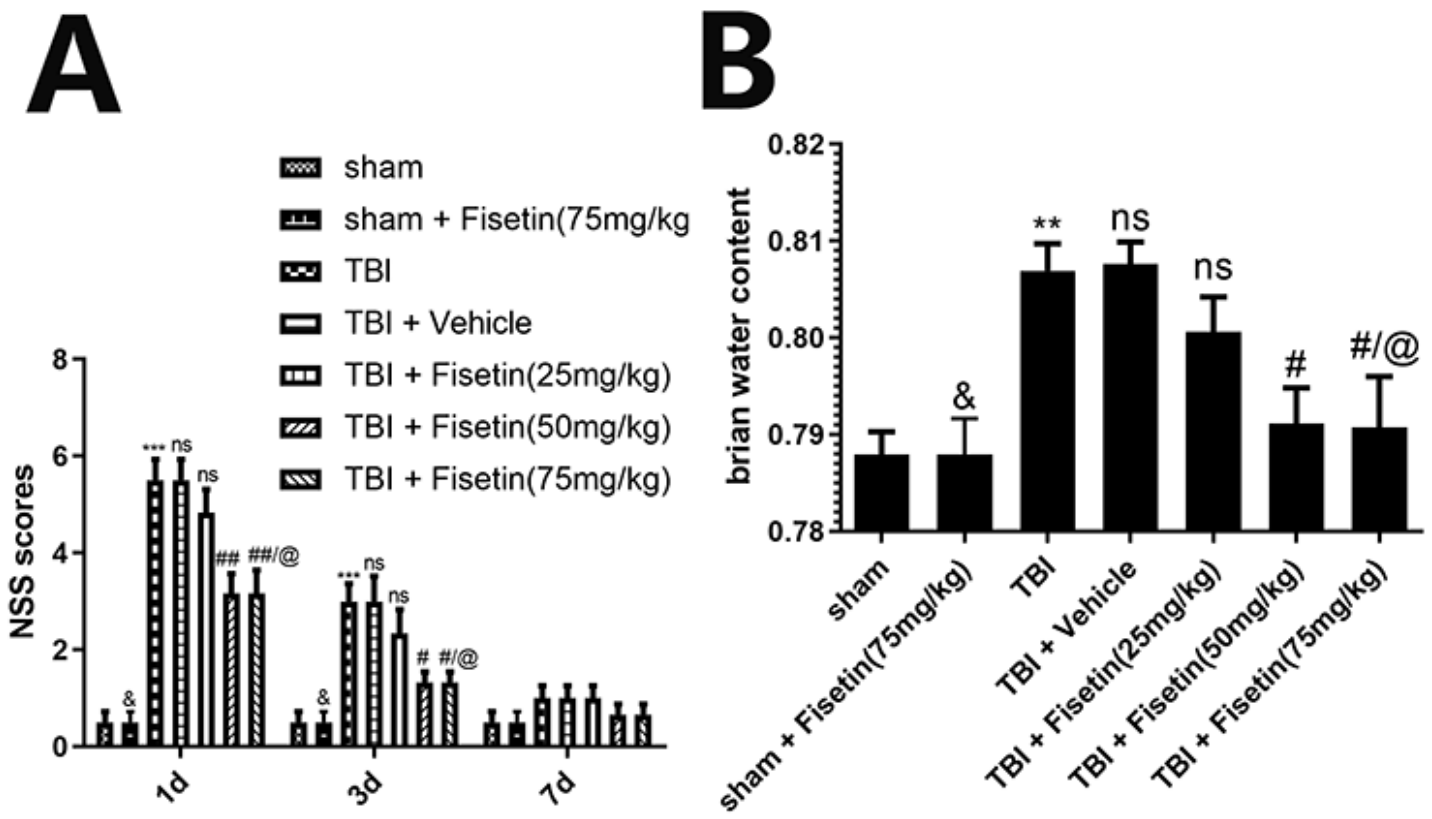

Figure 3

Neurological evaluation and brain water content after TBI Shown is the neurological evaluation score (NSS) examined at 1 d, 3 d, and 7 d after TBI and the brain water content evaluated 1 d after TBI. (A) Both 50 mg/kg and 75 mg/kg of fisetin obviously improve neurological function at 1 d and 3 d after TBI; however, 25 mg/kg of fisetin has no significant effects. A larger dose (75 mg/kg) of fisetin does not exhibit greater effects than does a 50-mg/kg dose. No significant differences among groups are observed 7 d after TBI. (B) Brain water content increases after TBI. While 50 mg/kg and 75 mg/kg of fisetin significantly reduce brain edema, 25 mg/kg of fisetin has no significant effects. However, 75 mg/kg of fisetin does not exhibit better neuroprotection than does 50 mg/kg. No significant differences are observed between the TBI group and the TBI + Vehicle group. In addition, there ws no difference between the sham group and sham + fisetin (75 mg/kg) group. Data are expressed as mean ± SEM; n = 6 for each group. &p > 0.05 versus the sham group, **p < 0.01 and ***p < 0.001 versus the sham group; nsp > 0.05 versus the TBI group; #p < 0.05 and ##p < 0.01 versus the TBI group; and @p > 0.05 versus the TBI + fisetin (50 mg/kg) group.

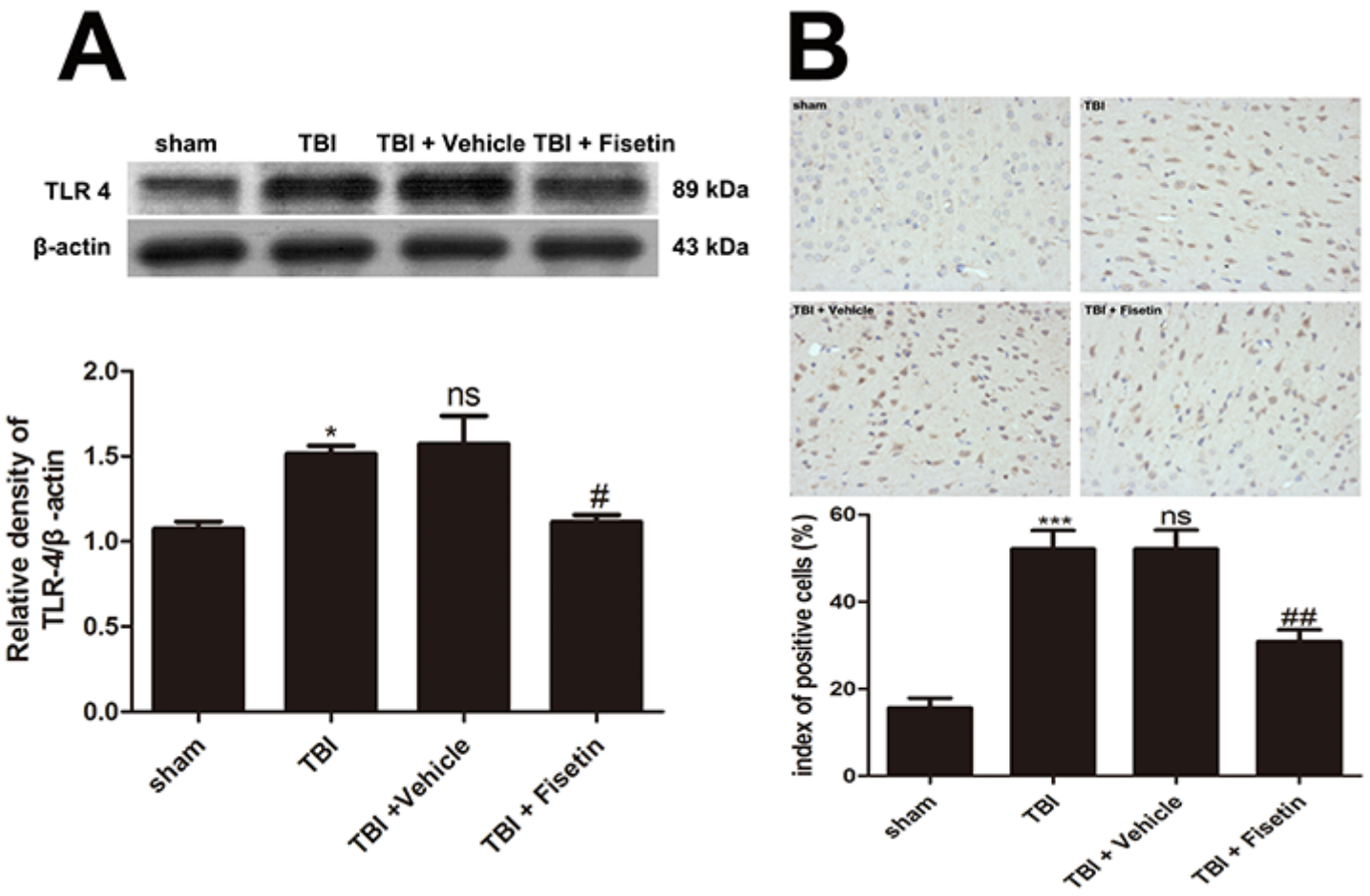

Figure 4

Representative western blot analysis and immunohistochemistry (IHC) of TLR 4 at 24 h after TBI (A) The expression of TLR 4 is significantly increased in the TBI and TBI + Vehicle groups compared with that in the sham group. After TBI and treatment with fisetin, the expression of TLR 4 is significantly decreased relative to the untreated TBI groups. (B) TLR 4 immunoactivity largely increases after TBI and decreases after TBI and treatment with fisetin relative to the untreated TBI group. No difference is detected between the TBI group and the TBI + Vehicle group. Data are expressed as mean ± SEM; n = 6 for each group. *p < 0.05 and ***p < 0.001 versus the sham group; nsp > 0.05 versus the TBI group; #p < 0.05 and ##p < 0.01 versus the TBI group

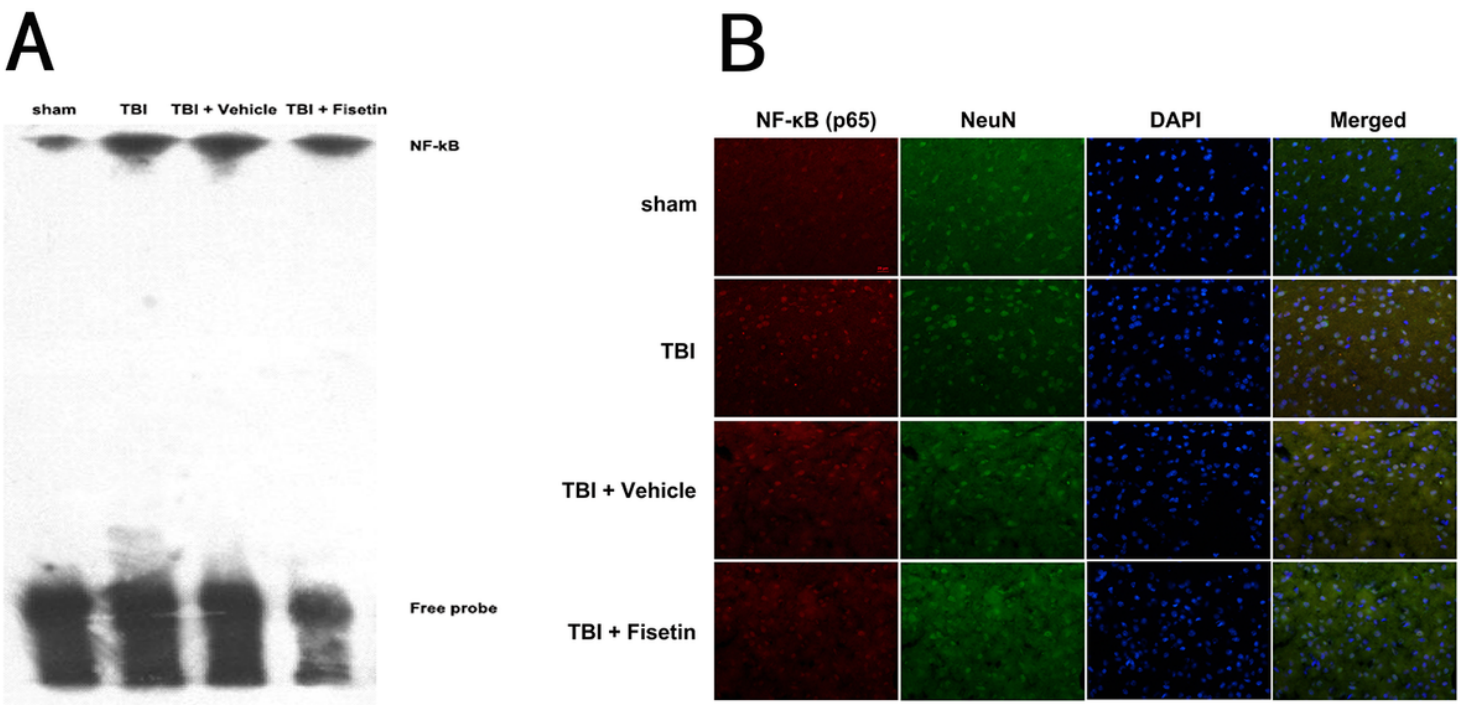

Figure 5

Representative electrophoretic mobility shift assay (EMSA) and immunofluorescence (IF) staining of NF-κB at 24 h after TBI (A) Based on EMSA, NF-κB binding activity was enhanced in the TBI and TBI + Vehicle groups compared with that in the sham group and reduced by fisetin administration after TBI relative to untreated TBI groups. N = 6 for each group. (B) NF-κB/NeuN double staining in the peri-contusive cortex. A higher proportion of NF-κB was found within nuclei in the TBI and TBI + Vehicle groups compared with that in the sham group. With treatment with fisetin after TBI, the proportion of NF-κB within nuclei is significantly decreased relative to untreated TBI. N = 6 for each group.

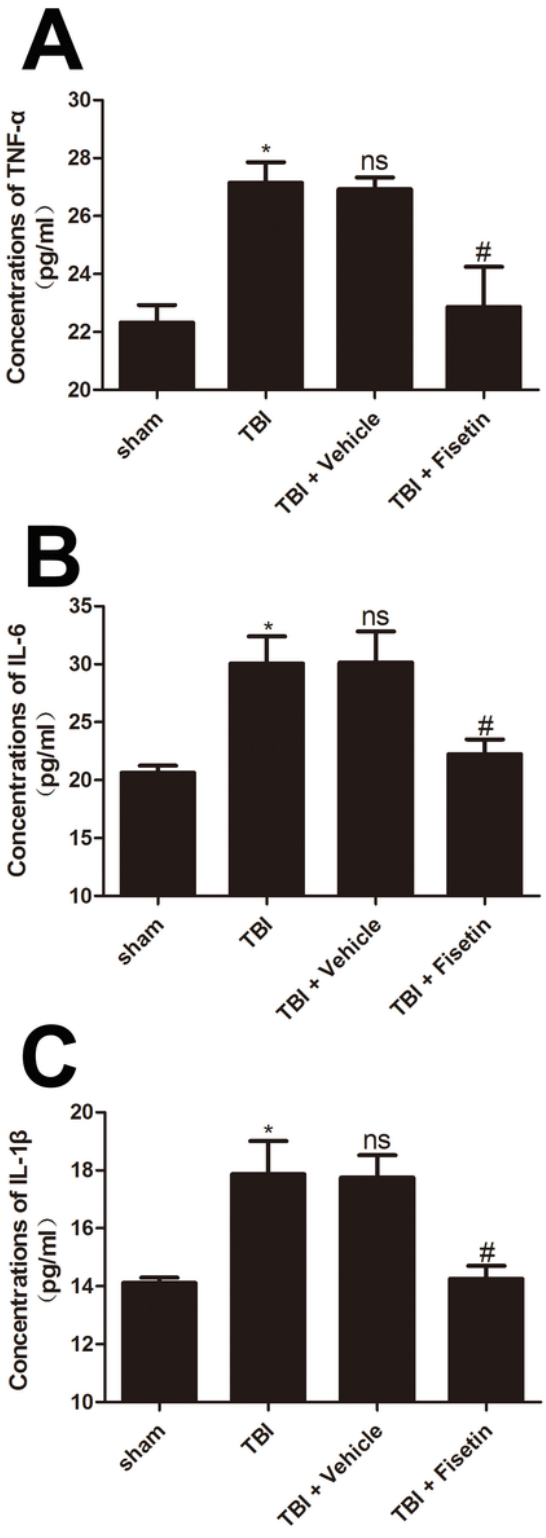

Figure 6

Results of ELISA analysis of TNF-α, IL-6, and IL-1β at 24 h after TBI Cytokine concentrations are largely increased in the TBI group compared to those in the sham group and are decreased relative to untreated TBI when fisetin treatment follows TBI. No significant difference is observed between the TBI group and the TBI + Vehicle group. Data are expressed as mean ± SEM; n = 6 for each group. *p < 0.05 versus the sham group; nsp > 0.05 versus the TBI group; #p < 0.05 versus the TBI group.

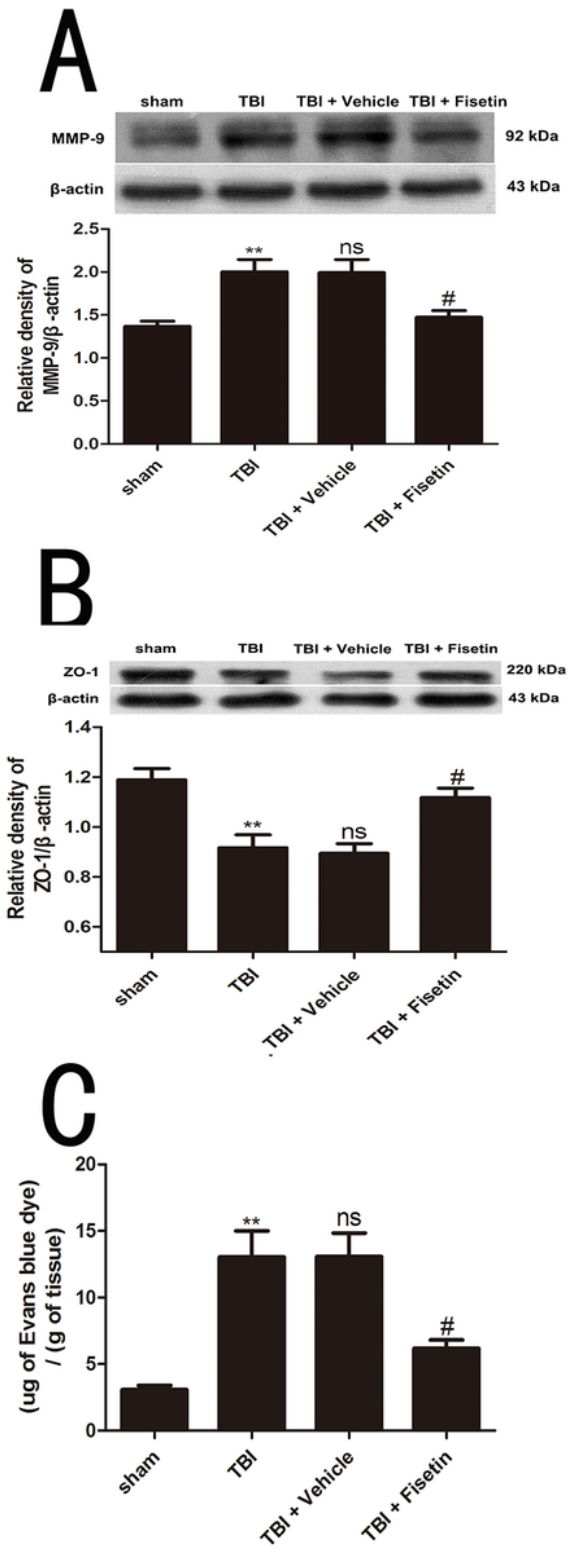

Figure 7

Representative western blot analysis of MMP-9 and ZO-1, and BBB permeability at 24 h after TBI (A) The expression of MMP-9 increases after TBI relative to sham-operated mice and decreases relative to untreated TBI when TBI is followed by fisetin administration. (B) The level of ZO-1 decreases after TBI and is ameliorated by subsequent fisetin administration. (C) BBB permeability increases after TBI and is reduced by subsequent fisetin administration. No difference is detected between the TBI group and the

TBI + Vehicle group. Data are expressed as mean ± SEM; n = 6 for each group. **p < 0.01 versus the sham group; nsp > 0.05 versus the TBI group; #p < 0.05 versus the TBI group.

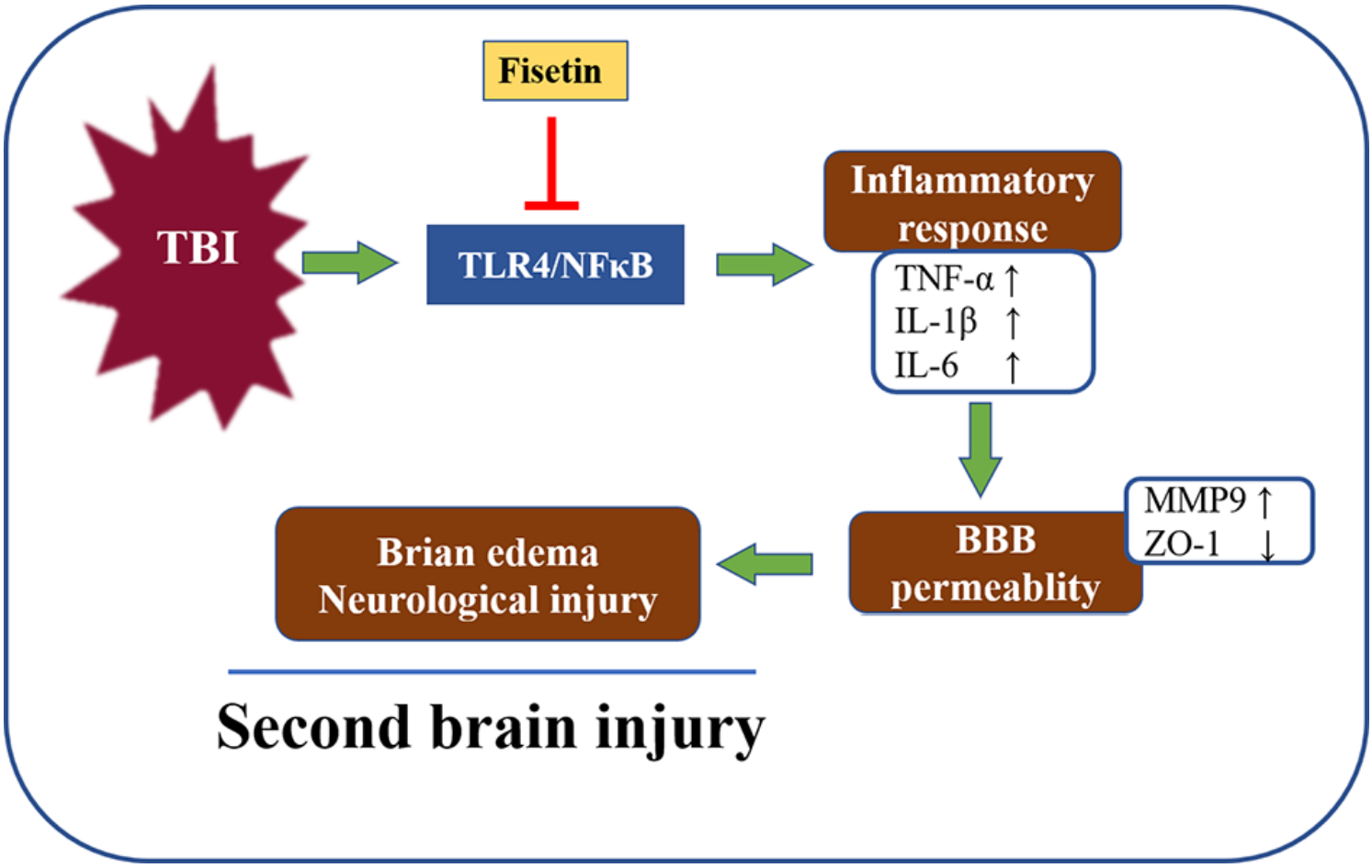

Figure 8

The machanisms of fisetin ameliorated second brian injury after TBI.