RESEARCH ARTICLE

Acacetin protects against cerebral ischemia-reperfusion injury *via* the NLRP3 signaling pathway

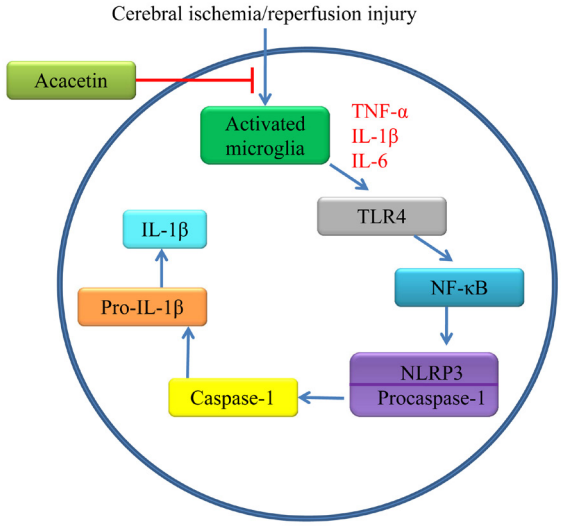
Juan Bu[1], Shen Shi[2], Hui-Qin Wang[1], Xiao-Shan Niu[3], Zong-Feng Zhao[1], Wei-Dong Wu[1], Xiao-Ling Zhang[1], Zhi Ma[3], Yan-Jun Zhang[1], Hui Zhang[1], Yi Zhu[3,*]

1 Clinical Research Center, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang Uygur Autonomous Region, China
2 Laboratory Animal Research Center, Center for Disease Control and Prevention, Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang Uygur Autonomous Region, China
3 Department of Neurology, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang Uygur Autonomous Region, China

Funding: *This study was supported by the Natural Science Foundation of Xinjiang Uygur Autonomous Region of China, No. 2016D01C120 (to JB).*

Graphical Abstract

Acacetin alleviates cerebral ischemia-reperfusion injury *via* the NLRP3 signaling pathway mediated by microglia



***Correspondence to:**
Yi Zhu, MS,
zhuyixinjiang@sina.com.

orcid:
0000-0002-1783-7101
(Yi Zhu)

doi: *10.4103/1673-5374.247465*

Received: *June 26, 2018*
Accepted: *November 1, 2018*

Abstract

Acacetin (5,7-dihydroxy-4′-methoxyflavone), a potential neuroprotective agent, has an inhibitory effect on lipopolysaccharide-induced neuroinflammatory reactions. However, whether acacetin has an effect on inflammatory corpuscle 3 (NLRP3) after cerebral ischemia-reperfusion injury has not been fully determined. This study used an improved suture method to establish a cerebral ischemia-reperfusion injury model in C57BL/6 mice. After ischemia with middle cerebral artery occlusion for 1 hour, reperfusion with intraperitoneal injection of 25 mg/kg of acacetin (acacetin group) or an equal volume of saline (0.1 mL/10 g, middle cerebral artery occlusion group) was used to investigate the effect of acacetin on cerebral ischemia-reperfusion injury. Infarct volume and neurological function scores were determined by 2,3,5-triphenyltetrazolium chloride staining and the Zea-Longa scoring method. Compared with the middle cerebral artery occlusion group, neurological function scores and cerebral infarction volumes were significantly reduced in the acacetin group. To understand the effect of acacetin on microglia-mediated inflammatory response after cerebral ischemia-reperfusion injury, immunohistochemistry for the microglia marker calcium adapter protein ionized calcium-binding adaptor molecule 1 (Iba1) was examined in the hippocampus of ischemic brain tissue. In addition, tumor necrosis factor-α, interleukin-1β, and interleukin-6 expression in ischemic brain tissue of mice was quantified by enzyme-linked immunosorbent assay. Expression of Iba1, tumor necrosis factor-α, interleukin-1β and interleukin-6 was significantly lower in the acacetin group compared with the middle cerebral artery occlusion group. Western blot assay results showed that expression of Toll-like receptor 4, nuclear factor kappa B, NLRP3, procaspase-1, caspase-1, pro-interleukin-1β, and interleukin-1β were significantly lower in the acacetin group compared with the middle cerebral artery occlusion group. Our findings indicate that acacetin has a protective effect on cerebral ischemia-reperfusion injury, and its mechanism of action is associated with inhibition of microglia-mediated inflammation and the NLRP3 signaling pathway.

Key Words: *nerve regeneration; acacetin; cerebral ischemia-reperfusion injury; microglia; NLRP3 inflammasome; inflammatory factor; infarct volume; signaling pathway; nuclear factor-κB; neuroprotection; neural regeneration*

Chinese Library Classification No. *R453; R363*

Introduction

Stroke, including ischemic and hemorrhagic forms, is characterized by high morbidity, high mortality, and high disability, resulting in a heavy burden for patients, families, and society (Demaerschalk et al., 2010; Radu et al., 2017). Globally, stroke has become the fourth most common cause of death and the most common cause of disability. Indeed, there are more than 11 million stroke patients in China, with 2.5 million new stroke cases each year, of which about 78% are ischemic (Wang et al., 2017). Although progress has been made to determine pathological mechanisms of stroke, effective conversion of this information to clinical applications has not been successful. Therefore, examining the pathogenesis of stroke continues to be of great significance to identify effective biological targets for the development of safe and effective therapeutic drugs for ischemic cerebrovascular disease.

Cerebral ischemia-reperfusion injury is the main pathophysiological mechanism of ischemic stroke (Fu et al., 2015). Rapid decline in blood flow during the early stage causes energy depletion and energy synthesis disorders, leading to depolarization of neuronal cell membranes and imbalances of ion homeostasis inside and outside the cell. This rapidly initiates a post-ischemic cascade that induces a series of biochemical events, such as excitotoxicity, oxidative stress, inflammation, edema, apoptosis, necrosis, nerve repair, and regeneration (Lo, 2009; Wang et al., 2016; Wu et al., 2017; Cruz et al., 2018). An increasing number of studies have shown that microglia-mediated inflammation plays an important role in cerebral ischemia-reperfusion injury (Iadecola and Anrather, 2011; Lee et al., 2014; Xiang et al., 2016; Xiong et al., 2016; Cruz et al., 2017). Therefore, inhibition of microglial activation and subsequent release of pro-inflammatory factors is an important strategy for the treatment of ischemic cerebrovascular disease.

Acacetin (5,7-dihydroxy-4-methoxyflavone), a flavonoid with antioxidant and anti-inflammatory properties (Pan et al., 2006; Chien et al., 2011; Lin et al., 2014), reportedly exerts anti-tumor effects (Pan et al., 2006; Kim et al., 2015; Punia et al., 2017; Wu et al., 2018). Recent studies have shown that acacetin plays a protective role in the brain (Ha et al., 2012; Lin et al., 2014; Wang et al., 2015b; Kim et al., 2017), whereby it can inhibit lipopolysaccharide-induced nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (p38MAPK) inflammatory signal pathways, as well as inflammatory factors mediated by microglia, thereby reducing inflammatory damage (Ha et al., 2012). Microglia-mediated inflammatory response is an important mechanism of cerebral ischemia-reperfusion injury. Thus, we hypothesized that acacetin may protect against cerebral ischemia-reperfusion injury by inhibiting microglia-mediated inflammation.

Nucleotide-binding oligomerization domain-like receptors (NLRs), including NLRP3, NLRP1, NLRC4, and AIM2, have been increasingly studied in recent years (Yang et al., 2014). Among them, NLRP3 is the best characterized, and is widely expressed in glial and peripheral immune cells in the brain (Walsh et al., 2014; Thakkar et al., 2016). As an important pattern recognition receptor in innate immunity, NLRP3 is involved in the inflammatory response after ischemia and has attracted attention for its involvement in ischemic cerebrovascular diseases (Ma et al., 2018). Inhibition of NLRP3 can protect against cerebral ischemia-reperfusion injury. However, no previous report describes the effects of acacetin on NLRP3-mediated inflammatory response after cerebral ischemia-reperfusion injury or, accordingly, any therapeutic effects.

Herein, C57BL/6 mice were used to establish a focal ischemia-reperfusion injury model. Subsequently, the effects of acacetin on cerebral ischemia-reperfusion injury and inflammatory response were investigated. The volume of cerebral infarction, neurological function, and activation and proliferation of microglia, as well as expression of inflammatory factors and key nodes of the Toll-like receptor 4 (TLR4)/NF-κB/NLRP3 signaling pathway were detected and analyzed. This study aimed to reveal the mechanism of acacetin-mediated neuroprotection in cerebral ischemia-reperfusion injury.

Materials and Methods

Animals

A total of 72 adult male C57BL/6 mice aged 8–12 weeks and weighing 25–30 g were obtained from the Experimental Animal Research Center in Xinjiang [China, License No. SCXK (Xin) 2011-0001]. All animal experiments were conducted according to the ethical guidelines of People's Hospital of Xinjiang Uygur Autonomous Region of China (Approval No. 2015078) on March 11, 2015. All experimental procedures described herein were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Establishment of middle cerebral artery occlusion (MCAO) model

An improved suture method was used to establish the MCAO model as previously reported (Li et al., 2014, 2016; Morris et al., 2016). Briefly, mice were intraperitoneally anesthetized with 1.5% sodium pentobarbital anesthesia (Beijing Chemical Reagent, Beijing, China). Body temperature was maintained at 37.0 ± 0.5°C using a heating lamp. A silicone-coated nylon filament (0.22-mm diameter, 6023PK; Doccol, Redlands, CA, USA) was inserted into the origin of the right middle cerebral artery. Mice were subjected to 1 hour of MCAO and then the suture was withdrawn to allow 24 hours of reperfusion.

Drug administration

Mice were randomly divided into sham, MCAO, and acacetin groups (MACO + acacetin) (n = 24 per group). In the acacetin group, mice received a single intraperitoneal injection of 25 mg/kg acacetin (powder, Lot No. BCBP4411V, dissolved in normal saline; Sigma-Aldrich, St. Louis, MO, USA) after MCAO for 1 hour (Ha et al., 2012; Bu et al., 2013). An equal volume of saline (0.1 mL/10 g body weight) was injected into mice in the sham and MCAO groups. For

subsequent infarct volume measurements, immunochemical analysis, western blot assay, and enzyme-linked immunosorbent assay were employed (six mice per group).

Neurological deficit score

Neurological deficit scores were assessed according to a five-point scale 24 hours after reperfusion (Longa et al., 1989). Grade 0, no deficit; grade 1, failure to fully extend right forepaw; grade 2, circling to the left; grade 3, falling to the left; and grade 4, no spontaneous walking and with a depressed level of consciousness or death. Mice scored grade 1–3 were considered a successful model. Mice with grades 0 and 4, seizures, or cerebral hemorrhage were excluded from the study; remaining mice were assigned to each group as required. All evaluations were performed by researchers who were blinded to treatment groups.

Infarct volume measurement

Infarct volume was assessed after 24 hours of reperfusion. Mice were sacrificed and quickly decapitated to obtain brain tissues. Brains were quickly frozen at −20°C and rapidly cut into 1-mm-thick coronal sections. Brain slices were stained with 1.5% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) at 37°C for 20 minutes, then fixed in 4% paraformaldehyde (Gibco, Grand Island, NY, USA) for 2 hours. Normal brain areas were stained deep red, while infarct areas were stained white. Coronal slices were photographed and total infarct volume was calculated with Image Pro Plus 5.0 software (Media Cybernetics, Rockville, MD, USA). To adjust for edema and shrinkage, areas of ischemic lesions were calculated as contralateral hemisphere volume × measured injury volume/ipsilateral hemisphere volume, while infarct volumes were quantified (in mm^3) by multiplying summed infarct areas of sections by section thickness (Li et al., 2014; Kim et al., 2016).

Immunohistochemical analysis

Mice were intraperitoneally anesthetized with 1.5% pentobarbital sodium after 24 hours of reperfusion. The heart was exposed and transcardially perfused with cold 0.9% saline solution, followed by 4% paraformaldehyde. The brain was removed, fixed in 4% paraformaldehyde overnight, cut into 5-mm-thick coronal sections at the 2–5-mm area behind the anterior fontanelle, and then coated with poly-lysine plates. After routine dewaxing, hydration, and microwave antigen retrieval of sections, ionized calcium-binding adaptor molecule 1 (Iba1) (rabbit monoclonal antibody; ab178846, 1:500; Abcam, Cambridge, UK) was immunohistochemically stained by diaminobenzidine using an SP immunohistochemistry Kit (rabbit; Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). Instead of primary antibody, phosphate-buffered saline solution was used as the negative control. An Eclipse Ci-L microscope (Nikon, Tokyo, Japan) was used for observation. Five high magnification fields (400×) in the hippocampus on the ischemic side were randomly selected in each mouse. Image pro plus 5.0 software (Media Cybernetics) was used to count Iba1-positive cells.

Western blot assay

After 24 hours of MCAO, mice were sacrificed by cervical dislocation. Ischemic brain tissue was rapidly separated on ice, weighed, and lysed in radioimmunoprecipitation assay lysing solution (Shanghai Beyotime Biotechnology Research Institute, Shanghai, China). Protein amounts were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). After electrophoresis on a 12% sodium dodecyl sulphate-polyacrylamide gel, proteins were transferred to polyvinylidene fluoride membranes. Membranes were incubated overnight at 4°C with antibodies against TLR4 (rabbit polyclonal; 1:500, ab13556; Abcam), P65 (rabbit monoclonal; 1:1000, Cat. No. 8242; Cell Signaling Technology, Danvers, MA, USA), NLRP3 (rabbit monoclonal; 1:1000, Cat. No. 15101; Cell Signaling Technology), caspase-1 (rabbit monoclonal; 1:1000, ab179515; Abcam), mouse anti-IL-1β (mouse monoclonal; 1:1000, Cat. No. 12242; Cell Signaling Technology), and β-actin (rabbit monoclonal; 1:1000; 13E5, Cell Signaling Technology). After rinsing three times, membranes were incubated with anti-rabbit IgG horseradish peroxidase-linked or anti-mouse IgG horseradish peroxidase-linked antibodies (Cat No.7071 and 7072, respectively; 1:5000; Cell Signaling Technology) for 1 hour at room temperature. Blots were visualized using an enhanced chemiluminescence luminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) and exposed to X-ray film. The optical density of bands was determined with a Quantity-One gel image analysis system (Bio-Rad, Hercules, CA, USA). Gray values were quantified by densitometry using β-actin as a loading control. Relative expression levels were calculated using the gray value ratio of the target gene to that of β-actin.

Enzyme-linked immunosorbent assay

Brain tissue from the anterior pole to the suprachiasmatic nucleus on the surgical side was removed and ground into homogenate in phosphate-buffered saline solution (pH 7.4). The homogenate was centrifuged for 20 minutes at 12,000 × *g* and the supernatant was collected. Levels of tumor necrosis factor-α, interleukin-1 beta (IL-1β), and interleukin-6 were measured using mouse enzyme-linked immunosorbent assay kits (Boster Biotechnology, Wuhan, China) according to the manufacturer's protocol.

Statistical analysis

Graphpad Prism 5 for Windows (Graphpad, San Diego, CA, USA) was used for statistical analysis. Data are expressed as the mean ± SD and analyzed with one-way analysis of variance and Tukey's multiple comparison test. A P value < 0.05 was considered statistically significant.

Results

Effect of acacetin on infarct volume in MCAO mice

Neurological deficit score and cerebral infarct volume were measured after 24 hours of reperfusion. No neurological deficit was observed in sham-operated mice. Acacetin treatment significantly improved neurological deficit scores com-

pared with the MCAO group ($P < 0.01$; **Figure 1A**). There was no infarcted area in sham-operated mice (**Figure 1B**). Infarct volume in the acacetin group was significantly smaller compared with the MCAO group (**Figure 1C**). These results suggest that acacetin can decrease the infarct volume and improve neurological scores to exert a neuroprotective effect.

Effect of acacetin on microglial activation in the hippocampus on the ischemic side of MCAO mice

To confirm whether acacetin confers a neuroprotective effect by inhibiting microglial activation, immunohistochemical staining of Iba1, a marker of activated microglia, was performed. Resting microglia are defined as having a small amount of cytoplasm with long thin extensions, whereas activated microglia have hypertrophied cytoplasm with short thick extensions (Ma et al., 2015). In the sham group, most microglia appeared with a small amount of cytoplasm and long thin extensions (**Figure 2A**). In the MCAO group, Iba1-immunoreactive microglia showed hypertrophied cytoplasm, as well as short and thick extensions (**Figure 2A**). However, in MCAO mice with acacetin treatment, only a few Iba1-immunoreactive microglia exhibited a similar morphology to those observed in the MCAO group (**Figure 2A**). The number of Iba1-immunoreactive microglia was significantly increased in the hippocampus after cerebral ischemia ($P < 0.001$; **Figure 2B**), but was significantly reduced with acacetin treatment ($P < 0.01$; **Figure 2B**). These results demonstrate that acacetin has the ability to regulate microglial activation.

Effects of acacetin on TLR4 and NF-κB p65 expression in ischemic brain tissue of MCAO mice

To investigate the effects of acacetin on TLR4 and NF-κB, a western blot assay was performed. As shown in **Figure 3A** and **B**, expression of TLR4 and NF-κB p65 in the brain tissue of mice was significantly increased in the MCAO group compared with the sham group ($P < 0.001$). Expression of TLR4 and NF-κB p65 was significantly reduced in the acacetin group compared with the MCAO group ($P < 0.01$; **Figure 3A** and **C**). This indicates that acacetin can inhibit the expression of TLR4 and NF-κB after cerebral ischemia-reperfusion injury.

Effect of acacetin on NLRP3 inflammatory signaling pathway in ischemic brain tissue of MCAO mice

After 24-hour ischemia-reperfusion, expression of NLRP3, procaspase-1, caspase-1, proIL-1β, and IL-1β in brain tissues

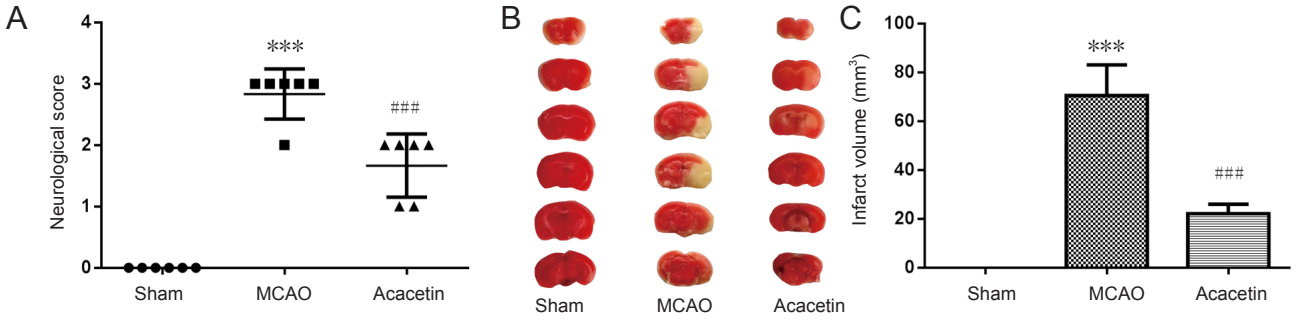


Figure 1 Neuroprotective effects of acacetin on middle cerebral artery occlusion (MCAO) mice.
Mice received 1 hour of MCAO followed by 24 hours of reperfusion or sham operation. Mice were divided into sham, MCAO, and acacetin groups. (A) Neurological deficit scores. (B) Brain coronal sections were stained with 2,3,5-triphenyltetrazolium chloride. The ischemic area remained white, while the intact area was stained red. (C) Analysis of brain infarct volumes. Data are expressed as the mean ± SD ($n = 6$; one-way analysis of variance followed by Tukey's multiple comparison test). ***$P < 0.001$, *vs.* sham group; ###$P < 0.001$, *vs.* MCAO group.

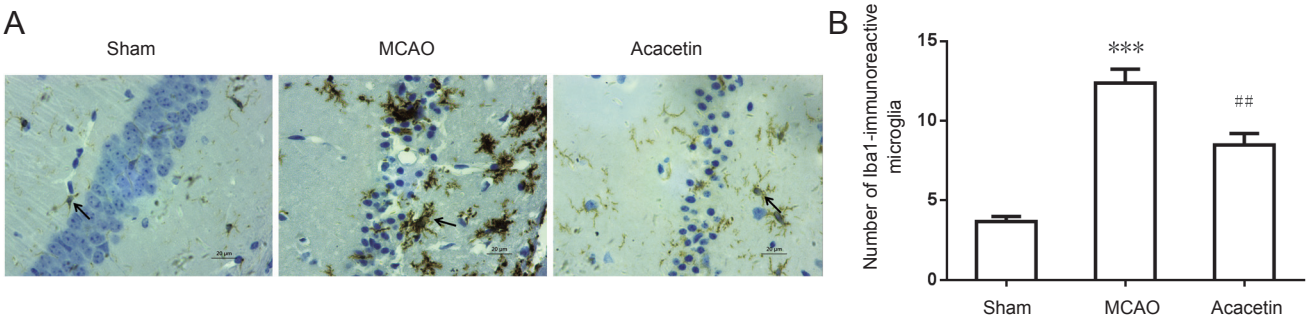


Figure 2 Acacetin decreases microglial activation in ischemic hippocampus after cerebral ischemia-reperfusion injury.
Mice received 1 hour of middle cerebral artery occlusion (MCAO) followed by 24 hours of reperfusion or sham operation. Mice were divided into sham, MCAO, and acacetin groups (25 mg/kg acacetin). (A) Immunohistochemical staining for Iba1 in ischemic hippocampus (400×). Eclipse Ci-L microscope (Nikon) was used for observation. Iba1-immunoreactive microglia were observed as brown-stained nuclei (arrows). Scale bars: 20 μm. (B) Number of Iba1-immunoreactive microglia. Data are expressed as the mean ± SD ($n = 6$; one-way analysis of variance followed by Tukey's multiple comparison test). ***$P < 0.001$, *vs.* sham group; ##$P < 0.01$, *vs.* MCAO group.

was measured by western blot assay (**Figure 4A**). Levels of these proteins in the MCAO group were significantly higher compared with the sham group ($P < 0.001$; **Figure 4B–F**). However, levels were much lower in the acacetin group compared with the MCAO group ($P < 0.01$). These results indicate that acacetin can exert neuroprotective effects by downregulating expression of inflammatory factors *via* the NLRP3 pathway.

Effect of acacetin on secretion of inflammatory factors in ischemic brain tissue of MCAO mice

Enzyme-linked immunosorbent assay results showed that levels of tumor necrosis factor-α (**Figure 5A**), IL-1β (**Figure 5B**), and IL-6 (**Figure 5C**) were significantly higher in the MCAO group compared with the sham group. However, these levels were reduced in the acacetin group compared with the MCAO group ($P < 0.05$). This indicates that acace-

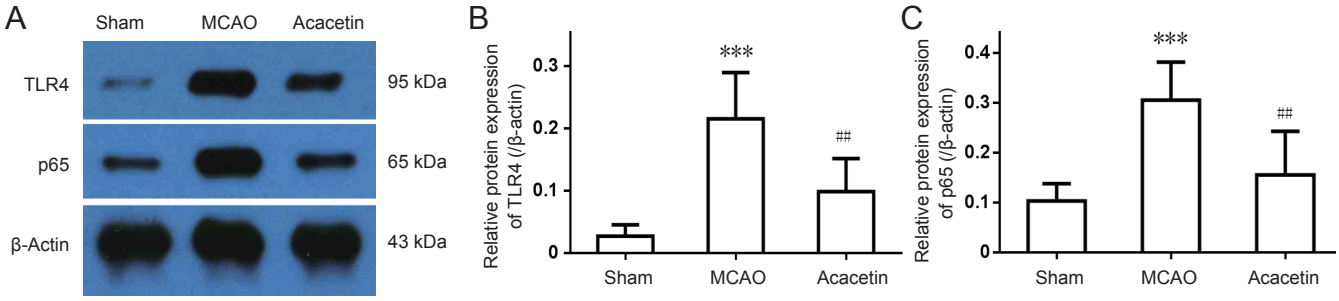


Figure 3 Acacetin inhibits expression of Toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF-κB)/p65 in ischemic brain tissues after cerebral ischemia-reperfusion injury.

Mice received 1 hour of middle cerebral artery occlusion (MCAO) followed by 24 hours of reperfusion or sham operation. Mice were divided into sham, MCAO, and acacetin groups. Relative protein expression of TLR4 and p65 was determined by western blot assay. Protein levels were normalized to β-actin. (A) Representative western blot assay results. (B, C) Quantification of TLR4 and p65 expression, respectively. The gray value of each protein was used for quantification. Data are expressed as the mean ± SD ($n = 6$; one-way analysis of variance followed by Tukey's multiple comparison test). ***$P < 0.001$, *vs.* sham group; ##$P < 0.01$, *vs.* MCAO group.

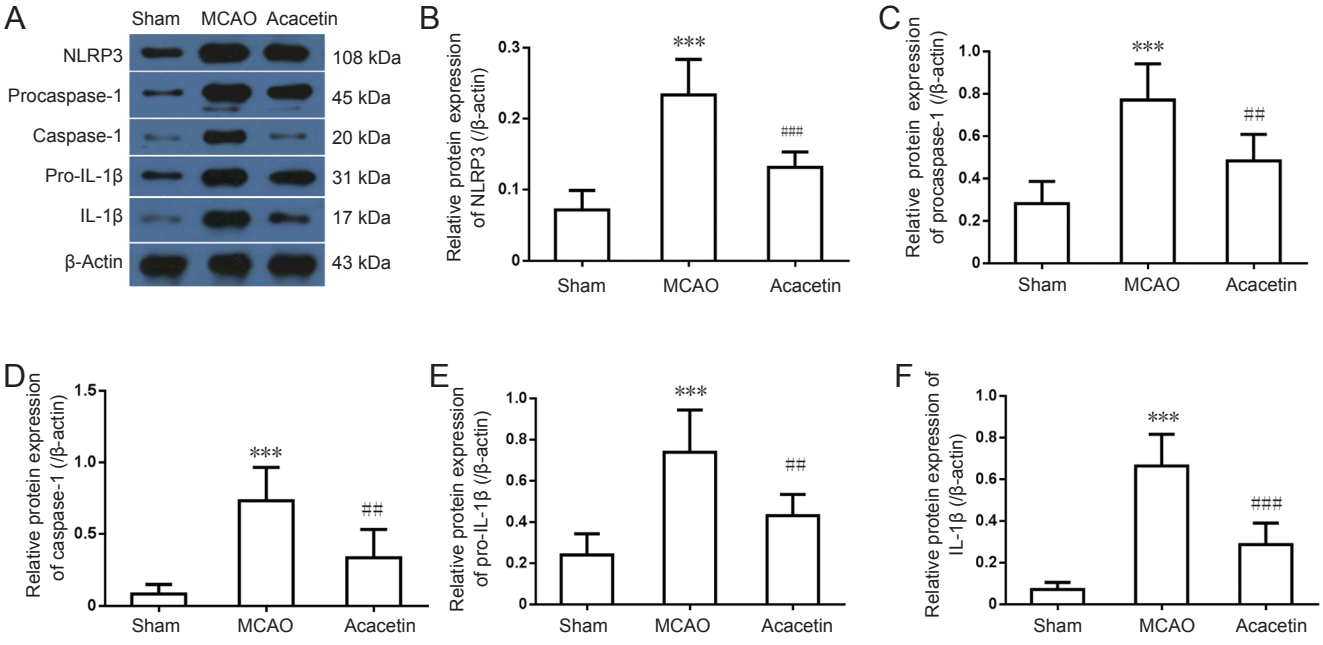


Figure 4 Acacetin suppresses activation of the NOD-like receptor pyrin domain containing three (NLRP3) inflammasome in ischemic brain tissues after cerebral ischemia-reperfusion injury.

Mice received 1 hour of middle cerebral artery occlusion (MCAO) followed by 24 hours of reperfusion or sham operation. Mice were divided into sham, MCAO, and acacetin groups. Relative protein expression of NLRP3, procaspase-1, caspase-1, pro-interleukin-1 beta (IL-1β), and IL-1β were determined by western blot assay. (A) Representative western blot assay results. Protein levels were normalized to β-actin. (B–F) Quantification of NLRP3, procaspase-1, caspase-1, pro-IL-1β, and IL-1β expression. The gray value of each protein was used for quantification. Data are expressed as the mean ± SD ($n = 6$; one-way analysis of variance followed by Tukey's multiple comparison test). ***$P < 0.001$, *vs.* sham group; ##$P < 0.01$, ###$P < 0.001$, *vs.* MCAO group.

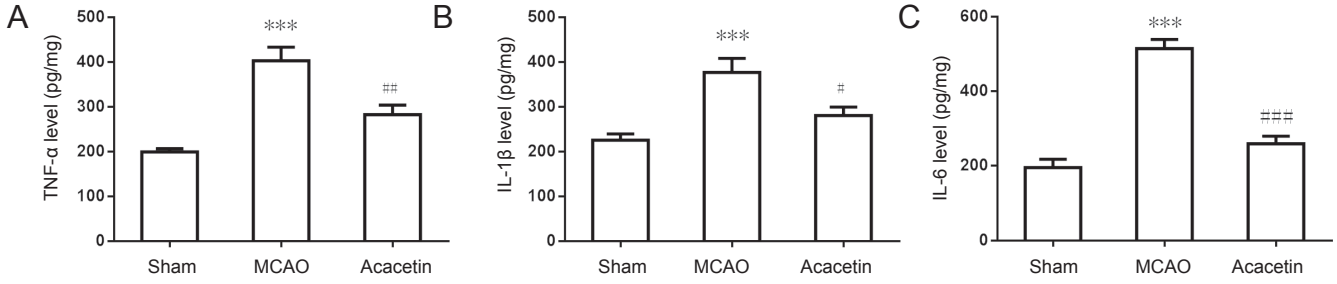


Figure 5 Acacetin inhibits release of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) in ischemic brain tissues after cerebral ischemia-reperfusion injury.

Mice received 1 hour of middle cerebral artery occlusion (MCAO) followed by 24 hours of reperfusion or sham operation. Mice were divided into sham, MCAO, and acacetin groups. Levels of (A) TNF-α, (B) IL-1β, and (C) IL-6 were determined by enzyme-linked immunosorbent assay after 24 hours of reperfusion. Data are expressed as the mean ± SD (n = 6; one-way analysis of variance followed by Tukey's multiple comparison test). ***P < 0.001, *vs*. sham group; #P < 0.05, ##P < 0.01, ### P < 0.001, *vs*. MCAO group.

tin can decrease the release of inflammatory factors tumor necrosis factor-α, IL-1β, and IL-6.

Discussion

Results of the present study showed that acacetin effectively reduced the volume of cerebral infarction after cerebral ischemia-reperfusion injury, thereby improving neurological deficits. Acacetin treatment could reduce the number of Iba1-immunoreactive cells; inhibit expression of TLR4, NF-κB, NLRP3, procaspase-1, caspase-1, pro-IL-1β, and IL-1β; and decrease release of inflammatory factors tumor necrosis factor-α, IL-1β, and IL-6 in the brain tissues of MCAO mice. These results provide reliable evidence that acacetin can be used as a neuroprotective drug for treating ischemic brain injury.

The effect of acacetin on cerebral ischemia has only been reported by Ha et al. (2012), who found that acacetin could reduce neuronal cell death in an animal model of bilateral common carotid artery occlusion. Our previous study investigated the dose-dependent effect of acacetin on cerebral ischemia-reperfusion injury and found that acacetin improved the survival rate of SH-SY5Y cells after oxygen-glucose deprivation, decreased rates of apoptotic cells, reduced infarct volume, and improved the neurological deficit score of ischemia-reperfusion mice (Bu et al., 2013). The present study further confirmed the protective effect of acacetin on cerebral ischemia-reperfusion injury.

Microglia are activated after cerebral ischemia-reperfusion injury, whereby they transform into a ramified amoeboid morphology (Chen et al., 2011; Wang and Cao, 2013; Xiong et al., 2016; Wang and Wang, 2018). Ha et al. (2012) found that acacetin inhibited microglial activation. Consistently, in the present study, Iba1-immunoreactive microglia had the following phenotypes in the sham group: tan-colored, small cell volume, low cellular quantity, and ramified slender enations. In the MCAO group, an increased number of Iba1-immunoreactivecells was observed to be enlarged and strongly stained, with amoeboid morphologies and an increased number of shortened enations. However, in the acacetin group, Iba1 staining was reduced and cells were smaller with fewer slender enations. These results indicate that microglia are activated after cerebral ischemia-reperfusion injury, and acacetin inhibits this activation, thus protecting neurons.

Previous studies have shown that TLR4 plays an essential role in cerebral ischemia-reperfusion injury (Liao and Tsai, 2016; Ji et al., 2017; Zheng et al., 2017). TLR4 is widely expressed in the brain, especially in microglia and endothelial cells (Wang et al., 2011, 2015a; Shukla et al., 2017). Once activated, TLR4 activates NF-κB and MAPK to promote the release of pro-inflammatory factors (such as tumor necrosis factor-α and IL-1β), thus inducing the innate immune response (Yenari et al., 2010; Zhao et al., 2014). Knockout of TLR4 in mice inhibits microglia proliferation and NF-κB activation, thus reducing infarct volumes after ischemia-reperfusion to produce a neuroprotective effect in ischemic brain injury models (Hyakkoku et al., 2010). Baicalin has been shown to play a neuroprotective role by reducing cerebral infarct volume, decreasing TLR2/4 and NF-κB expression, and attenuating tumor necrosis factor-α and IL-1β serum contents (Tu et al., 2011) after cerebral ischemia-reperfusion injury. This study found that acacetin could also inhibit the activation of TLR4 and NF-κB, and reduce the release of inflammatory cytokines (tumor necrosis factor-α, IL-1β and IL-6) in ischemic brain tissues of mice after cerebral ischemia-reperfusion injury.

The role of NLRP3 in the development of cerebral ischemia-reperfusion injury has gained prominence (Gao et al., 2017; Ye et al., 2017). Yang et al. (2014) found that NLRP3 deficiency ameliorated ischemic cerebral injury in mice by reducing infarct volume and blood-brain barrier damage. Yu et al. (2017) found that sulforaphane exerted a neuroprotective effect against cerebral ischemia-reperfusion injury by inhibiting NLRP3 inflammasome activation. The present study also demonstrated that the NLRP3 inflammasome might be a beneficial target for pharmacological inhibition in cerebral ischemia-reperfusion injury, as NLRP3 was evidently enhanced in ischemic brain tissue after cerebral ischemia-reperfusion injury, and acacetin obviously reduced cerebral ischemia-reperfusion injury-induced NLRP3 expression.

A priming signal ensures adequate gene/protein expression of NLRP3, pro-IL-1β, and pro-IL-18 for efficient

inflammasome formation. The transcription of NLRP3 inflammasome components (*i.e.*, NLRP3) activates the TLR/NF-κB signaling pathway (Elliott and Sutterwala, 2015; Song et al., 2017). In this study, expression of NLRP3-related signaling genes, including TLR4, NF-κB, NLRP3, procaspase-1, caspase-1, proIL-1β. and IL-1β, were dramatically upregulated in the brain tissues of MCAO mice, and reduced by treatment with acacetin. In future studies, the long-term effects of acacetin should be investigated.

This study has some limitations. First, acacetin was given immediately after reperfusion. As such, the timing of administration was not investigated in detail, which may further limit the clinical application of acacetin. Second, we only investigated the short-term effect of acacetin. Thus, further studies are warranted.

In summary, acacetin plays a neuroprotective role by reducing infarct volumes and improving neurological function. The mechanism may be related to its inhibitory effect on activated microglia, thereby decreasing the expression of TLR4/NF-κB/NLRP3 and subsequent secretion of tumor necrosis factor-α, IL-1β. and IL-6. This study preliminarily determined mechanisms by which acacetin protects against cerebral ischemia-reperfusion injury and inhibits neuronal inflammation, thus providing experimental evidence for the development of acacetin as a stroke therapeutic in the future.

Author contributions: *Manuscript drafting: JB; data analysis: ZFZ, HZ, and XLZ; experiment implementation: JB, SS, HQW, XSN, ZM, WDW, and YJZ; study design and manuscript revision: YZ. All authors contributed to this study and approved the final manuscript.*
Conflicts of interest: *The authors declare that there are no conflicts of interest associated with this manuscript.*
Financial support: *This study was supported by the Natural Science Foundation of Xinjiang Uygur Autonomous Region, China, No. 2016D01C120 (to JB). The funding source had no role in study conception and design, data analysis or interpretation, paper writing or deciding to submit this paper for publication.*
Institutional review board statement: *All animal experiments were approved by the Animal Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region of China (No. 2015078) on March 11, 2015. All experimental procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.*
Copyright license agreement: *The Copyright License Agreement has been signed by all authors before publication.*
Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewer: *Clarissa Cavarsan, Brigham Young University, USA.*
Additional file: *Open peer review report 1.*

References

Bu J, Hu G, Dang H, Li H, Ding J, Sha L, Wang Z (2013) Neuroprotective effects of acacetin in oxygen-glucose deprivation in SH-SY5Y cells and cerebral ischemia/reperfusion in mice. Int J Cerebrovasc Dis 21:47-50.

Chen H, Kim GS, Okami N, Narasimhan P, Chan PH (2011) NADPH oxidase is involved in post-ischemic brain inflammation. Neurobiol Dis 42:341-348.

Chien ST, Lin SS, Wang CK, Lee YB, Chen KS, Fong Y, Shih YW (2011) Acacetin inhibits the invasion and migration of human non-small cell lung cancer A549 cells by suppressing the p38alpha MAPK signaling pathway. Mol Cell Biochem 350:135-148.

Cruz SA, Hari A, Qin Z, Couture P, Huang H, Lagace DC, Stewart AFR, Chen HH (2017) Loss of IRF2BP2 in microglia increases inflammation and functional deficits after focal ischemic brain injury. Front Cell Neurosci 11:201.

Cruz Y, García EE, Gálvez JV, Arias-Santiago SV, Carvajal HG, Silva-García R, Bonilla-Jaime H, Rojas-Castañeda J, Ibarra A (2018) Release of interleukin-10 and neurotrophic factors in the choroid plexus: possible inductors of neurogenesis following copolymer-1 immunization after cerebral ischemia. Neural Regen Res 13:1743-1752.

Demaerschalk BM, Hwang HM, Leung G (2010) Cost analysis review of stroke centers, telestroke, and rt-PA. Am J Manag Care 16:537-544.

Elliott EI, Sutterwala FS (2015) Initiation and perpetuation of NLRP3 inflammasome activation and assembly. Immunol Rev 265:35-52.

Fu Y, Liu Q, Anrather J, Shi FD (2015) Immune interventions in stroke. Nat Rev Neurol 11:524-535.

Gao L, Dong Q, Song Z, Shen F, Shi J, Li Y (2017) NLRP3 inflammasome: a promising target in ischemic stroke. Inflamm Res 66:17-24.

Ha SK, Moon E, Lee P, Ryu JH, Oh MS, Kim SY (2012) Acacetin attenuates neuroinflammation via regulation the response to LPS stimuli in vitro and in vivo. Neurochem Res 37:1560-1567.

Hyakkoku K, Hamanaka J, Tsuruma K, Shimazawa M, Tanaka H, Uematsu S, Akira S, Inagaki N, Nagai H, Hara H (2010) Toll-like receptor 4 (TLR4), but not TLR3 or TLR9, knock-out mice have neuroprotective effects against focal cerebral ischemia. Neuroscience 171:258-267.

Iadecola C, Anrather J (2011) The immunology of stroke: from mechanisms to translation. Nat Med 17:796-808.

Ji J, Xiang P, Li T, Lan L, Xu X, Lu G, Ji H, Zhang Y, Li Y (2017) NOSH-NBP, a novel nitric oxide and hydrogen sulfide- releasing hybrid, attenuates ischemic stroke-induced neuroinflammatory injury by modulating microglia polarization. Front Cell Neurosci 11:154.

Kim CD, Cha JD, Li S, Cha IH (2015) The mechanism of acacetin-induced apoptosis on oral squamous cell carcinoma. Arch Oral Biol 60:1283-1298.

Kim SM, Park YJ, Shin MS, Kim HR, Kim MJ, Lee SH, Yun SP, Kwon SH (2017) Acacetin inhibits neuronal cell death induced by 6-hydroxydopamine in cellular Parkinson's disease model. Bioorg Med Chem Lett 27:5207-5212.

Kim SW, Lee HK, Kim ID, Lee H, Luo L, Park JY, Yoon SH, Lee JK (2016) Robust neuroprotective effects of 2-((2-oxopropanoyl) oxy)-4-(trifluoromethyl)benzoic acid (OPTBA), a HTB/pyruvate ester, in the postischemic rat brain. Sci Rep 6:31843.

Lee Y, Lee SR, Choi SS, Yeo HG (2014) Therapeutically targeting neuroinflammation and microglia after acute ischemic stroke. 2014:297241.

Li P, Mao L, Liu X, Gan Y, Zheng J, Thomson AW, Gao Y, Chen J, Hu X (2014) Essential role of program death 1-ligand 1 in regulatory T-cell-afforded protection against blood-brain barrier damage after stroke. Stroke 45:857-864.

Li YH, Fu HL, Tian ML, Wang YQ, Chen W, Cai LL, Zhou XH, Yuan HB (2016) Neuron-derived FGF10 ameliorates cerebral ischemia injury via inhibiting NF-kappaB-dependent neuroinflammation and activating PI3K/Akt survival signaling pathway in mice. Sci Rep 6:19869.

Liao WY, Tsai TH (2016) Neuroprotective effect of paeonol mediates anti-inflammation via suppressing Toll-like receptor 2 and Toll-like receptor 4 signaling pathways in cerebral ischemia-reperfusion injured rats. 2016:3704647.

Lin TY, Huang WJ, Wu CC, Lu CW, Wang SJ (2014) Acacetin inhibits glutamate release and prevents kainic acid-induced neurotoxicity in rats. PLoS One 9:e88644.

Lo EH (2009) T time in the brain. Nat Med 15:844-846.
Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84-91.
Ma C, Liu S, Zhang S, Xu T, Yu X, Gao Y, Zhai C, Li C, Lei C, Fan S, Chen Y, Tian H, Wang Q, Cheng F, Wang X (2018) Evidence and perspective for the role of the NLRP3 inflammasome signaling pathway in ischemic stroke and its therapeutic potential (Review). Int J Mol Med doi: 10.3892/ijmm.2018.3911.
Ma Y, Bu J, Dang H, Sha J, Jing Y, Shan-jiang AI, Li H, Zhu Y (2015) Inhibition of adenosine monophosphate-activated protein kinase reduces glial cell-mediated inflammation and induces the expression of Cx43 in astroglias after cerebral ischemia. Brain Res 1605:1-11.
Morris GP, Wright AL, Tan RP, Gladbach A, Ittner LM, Vissel B (2016) A comparative study of variables influencing ischemic injury in the longa and koizumi methods of intraluminal filament middle cerebral artery occlusion in mice. PLoS One 11:e0148503.
Pan MH, Lai CS, Wang YJ, Ho CT (2006) Acacetin suppressed LPS-induced up-expression of iNOS and COX-2 in murine macrophages and TPA-induced tumor promotion in mice. Biochem Pharmacol 72:1293-1303.
Punia R, Raina K, Agarwal R, Singh RP (2017) Acacetin enhances the therapeutic efficacy of doxorubicin in non-small-cell lung carcinoma cells. PLoS One 12:e0182870.
Radu RA, Terecoasa EO, Bajenaru OA, Tiu C (2017) Etiologic classification of ischemic stroke: Where do we stand? Clin Neurol Neurosurg 159:93-106.
Shukla V, Shakya AK, Perez-Pinzon MA, Dave KR (2017) Cerebral ischemic damage in diabetes: an inflammatory perspective. J Neuroinflammation 14:21.
Song L, Pei L, Yao S, Wu Y, Shang Y (2017) NLRP3 inflammasome in neurological diseases, from functions to therapies. Front Cell Neurosci 11:63.
Thakkar R, Wang R, Sareddy G, Wang J, Thiruvaiyaru D, Vadlamudi R, Zhang Q (2016) NLRP3 inflammasome activation in the brain after global cerebral ischemia and regulation by 17beta-estradiol. Oxid Med Cell Longev 2016:8309031.
Tu XK, Yang WZ, Shi SS, Chen Y, Wang CH, Chen CM, Chen Z (2011) Baicalin inhibits TLR2/4 signaling pathway in rat brain following permanent cerebral ischemia. Inflammation 34:463-470.
Walsh JG, Muruve DA, Power C (2014) Inflammasomes in the CNS. Nat Rev Neurosci 15:84-97.
Wang M, Cao BZ (2013) Glial cell responses and matrix metalloproteinase-9 expression in the brain of chronic vascular dementia rat models. Zhongguo Zuzhi Gongcheng Yanjiu 17:1212-1219.
Wang P, He Y, Li D, Han R, Liu G, Kong D, Hao J (2016) Class I PI3K inhibitor ZSTK474 mediates a shift in microglial/macrophage phenotype and inhibits inflammatory response in mice with cerebral ischemia/reperfusion injury. J Neuroinflammation 13:192.
Wang PF, Xiong XY, Chen J, Wang YC, Duan W, Yang QW (2015a) Function and mechanism of toll-like receptors in cerebral ischemic tolerance: from preconditioning to treatment. J Neuroinflammation 12:80.
Wang W, Jiang B, Sun H, Ru X, Sun D, Wang L, Wang L, Jiang Y, Li Y, Wang Y, Chen Z, Wu S, Zhang Y, Wang D, Wang Y, Feigin VL (2017) Prevalence, incidence, and mortality of stroke in China: results from a nationwide population-based survey of 480 687 adults. Circulation 135:759-771.
Wang X, Perumalsamy H, Kwon HW, Na YE, Ahn YJ (2015b) Effects and possible mechanisms of action of acacetin on the behavior and eye morphology of drosophila models of Alzheimer's disease. Sci Rep 5:16127.
Wang XH, Wang TL (2018) MicroRNAs of microglia: wrestling with central nervous system disease. Neural Regen Res 13:2067-2072.
Wang YC, Lin S, Yang QW (2011) Toll-like receptors in cerebral ischemic inflammatory injury. J Neuroinflammation 8:134.
Wu R, Li X, Xu P, Huang L, Cheng J, Huang X, Jiang J, Wu LJ, Tang Y (2017) TREM2 protects against cerebral ischemia/reperfusion injury. Mol Brain 10:20.
Wu WY, Li YD, Cui YK, Wu C, Hong YX, Li G, Wu Y, Jie LJ, Wang Y, Li GR (2018) The natural flavone acacetin confers cardiomyocyte protection against hypoxia/reoxygenation injury via AMPK-mediated activation of Nrf2 signaling pathway. Front Pharmacol 9:497.
Xiang Y, Zhao H, Wang J, Zhang L, Liu A, Chen Y (2016) Inflammatory mechanisms involved in brain injury following cardiac arrest and cardiopulmonary resuscitation. Biomed Rep 5:11-17.
Xiong XY, Liu L, Yang QW (2016) Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke. Prog Neurobiol 142:23-44.
Yang F, Wang Z, Wei X, Han H, Meng X, Zhang Y, Shi W, Li F, Xin T, Pang Q, Yi F (2014) NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke. J Cereb Blood Flow Metab 34:660-667.
Ye X, Shen T, Hu J, Zhang L, Zhang Y, Bao L, Cui C, Jin G, Zan K, Zhang Z, Yang X, Shi H, Zu J, Yu M, Song C, Wang Y, Qi S, Cui G (2017) Purinergic 2X7 receptor/NLRP3 pathway triggers neuronal apoptosis after ischemic stroke in the mouse. Exp Neurol 292:46-55.
Yenari MA, Kauppinen TM, Swanson RA (2010) Microglial activation in stroke: therapeutic targets. Neurotherapeutics 7:378-391.
Yu C, He Q, Zheng J, Li LY, Hou YH, Song FZ (2017) Sulforaphane improves outcomes and slows cerebral ischemic/reperfusion injury via inhibition of NLRP3 inflammasome activation in rats. Int Immunopharmacol 45:74-78.
Zhao H, Perez JS, Lu K, George AJ, Ma D (2014) Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury. Am J Physiol Renal Physiol 306:F801-F811.
Zheng Y, Bu J, Yu L, Chen J, Liu H (2017) Nobiletin improves propofol-induced neuroprotection via regulating Akt/mTOR and TLR 4/NF-kappaB signaling in ischemic brain injury in rats. Biomed Pharmacother 91:494-503.

P-Reviewer: Cavarsan C; C-Editor: Zhao M; S-Editors: Wang J, Li CH; L-Editors: Deusen AV, Frenchman B, Qiu Y, Song LP; T-Editor: Liu XL