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Puerarin protects brain tissue against cerebral ischemia/reperfusion injury by inhibiting the inflammatory response

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Abstract

Puerarin, a traditional Chinese medicine, exerts a powerful neuroprotective effect in cerebral ischemia/reperfusion injury, but its mechanism is unknown. Here, we established rat models of middle cerebral artery ischemia/reperfusion injury using the suture method. Puerarin (100 mg/kg) was administered intraperitoneally 30 minutes before middle cerebral artery occlusion and 8 hours after reperfusion. Twenty-four hours after reperfusion, we found that puerarin significantly improved neurological deficit, reduced infarct size and brain water content, and notably diminished the expression of Toll-like receptor-4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α in the ischemic region. These data indicate that puerarin exerts an anti-inflammatory protective effect on brain tissue with ischemia/reperfusion damage by down-regulating the expression of multiple inflammatory factors.

Key Words: *nerve regeneration; brain injury; puerarin; cerebral ischemia; reperfusion injury; rats; inflammatory reaction; Toll-like receptor-4; nuclear factor kappa B; myeloid differentiation factor 88; tumor necrosis factor-α; middle cerebral artery occlusion; neural regeneration*

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Introduction

Cerebral ischemia/reperfusion injury can trigger a strong inflammatory response (Lenardo et al, 1989; Vila et al., 2000; Liao et al., 2001). Immune and inflammatory responses are intrinsic to neuronal injury induced by cerebral ischemia and reperfusion (Danton et al., 2003; Monje et al., 2003; Hayden et al., 2004; Huang et al., 2006; Wong et al., 2008). Nuclear factor kappa B is an important transcription factor and a central mediator of the inflammatory response. It initiates the transcription of genes associated with the immune response and inflammation, including tumor necrosis factor-α, interleukin-1, interleukin-6 and inducible nitric oxide synthase, which all contribute to the adverse outcomes induced by cerebral ischemia/reperfusion injury (Lindsberg et al., 2003; Leeman et al., 2008; Lambertsen et al., 2012; Yang et al., 2013). Nuclear factor kappa B can be activated by various stimulating factors, including cytokines and bacteria (Zhou et al., 2012; Liu et al., 2014). These stimulating factors can be identified by specific receptor families such as Toll-like receptors (Schneider et al., 1999; Kumar et al., 2004; Ridder et al., 2009; Baker et al., 2011), a large class of signal transduction molecules that are involved in inherent and adaptive immunity (Aderem et al., 2000; Takeda, 2005; Akira, 2006). Toll-like receptor 4 was the first Toll-like receptor to be found in mammals, and its expression is associated with many central nervous system diseases, such as inflammatory autoimmune diseases and cerebral ischemic injury (Kerfoot et al., 2004; Cao et al., 2006; Hua et al., 2007, 2009). Stimulation of Toll-like receptor 4 triggers a signaling pathway, activating NF-kB transcription through the dependent or independent myeloid differentiation factor 88 pathway, and resulting in the expression of inflammatory response factors such as tumor necrosis factor-α and interleukin-1 (Hallenbeck et al., 2002; Janeway et al., 2002; Kaczorowski et al., 2009; Ishizuka et al., 2013).

Puerarin ($C_{21}H_{20}O_9$, relative molecular weight 416.38) is a major isoflavonoid, extracted from the traditional Chinese medicine Radix puerariae (kudzu root) (Wang et al., 2012; Chen et al., 2013), and is used to treat many conditions including ischemic cerebrovascular disease (Wang et al., 1997, 2014; Sang et al., 2001; Ding et al., 2007; Wu et al., 2007). Isoflavones in food improve the outcome of cerebral ischemic injury, reducing infarct volume and improving neurological function (Pan et al., 2005; Burguete et al., 2006; Zhang et

Table 1 Primer sequence for PCR

Gene	Primer sequence	Product size (bp)
TLR4	Forward: 5'-GCC GGA AAG TTA TTG TGG TGG T-3' Reverse: 5'-ATG GGT TTT AGG CGC AGA GTT T-3'	356
MyD88	Forward: 5'-CAA CCA GCA GAA ACA GGA GTC T-3' Reverse: 5'-ATT GGG GCA GTA GCA GAT GAA G-3'	157
NF-κB	Forward: 5'-GCG CAT CCA GAC CAA CAA TAA C-3' Reverse: 5'-GCC GAA GCT GCA TGG ACA CT-3'	425
TNF-α	Forward: 5'-GGC CAC CAC GCT CTT CTG-3' Reverse: 5'-GCC ATT GGC CAG GAG GGC-3'	546
β-Actin	Forward: 5'-GCC ATG TAC GTA GCC ATC CA-3' Reverse: 5'-GAA CCG CTC ATT GCC GAT AG-3'	375

TLR4: Toll-like receptor 4; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor kappa B; TNF-α: tumor necrosis factor-α.

al., 2008; Tian et al., 2013). Puerarin scavenges free radicals, increases cerebral blood flow, and exerts neuroprotective and anti-inflammatory effects in ischemia/reperfusion injury (Xu et al., 2005; Han et al., 2007; Zhao et al., 2007; Chang et al., 2009). However, the mechanism underlying these effects of puerarin in cerebral ischemia/reperfusion injury remains poorly understood. In the present study, we observed the effects of puerarin on inflammatory molecules in the Toll-like receptor 4-mediated NF-kB signaling pathway, and investigated the mechanism by which the drug exerts cerebral protection in a rat model of middle cerebral artery occlusion.

Materials and Methods

Animals and experimental groups

Thirty-six male 8-week-old Sprague-Dawley rats, weighing 250–280 g, were housed at 22–24°C with a normal light/dark cycle. The rats were allowed free access to food and water. Body temperature, heart rate, blood pressure, blood sugar, arterial blood pH, pO_2 and pCO_2 in experimental rats were monitored before artery occlusion, before reperfusion and 30 minutes after reperfusion, and no significant differences were detected between groups. Experimental protocols were conducted in accordance with the Animal Experiments Ethics Committee of Zhejiang University, China.

In the first experiment, 18 rats were equally and randomly divided into three groups: puerarin treatment, vehicle control, and sham surgery control. The vehicle and puerarin groups were subjected to middle cerebral artery occlusion for 90 minutes. Rats in the sham surgery group underwent the same surgical procedures except for occlusion of the middle cerebral artery. Thirty minutes before middle cerebral artery occlusion and 8 hours after reperfusion, rats in the puerarin group received an intraperitoneal injection of puerarin (100 mg/kg; Zhejiang CONBA Pharmaceutical Co., Ltd., Hangzhou, Zhejiang Province, China). Rats in the vehicle and sham surgery groups received an equal dose of physiological saline. Twenty-four hours after reperfusion, the neurological function was evaluated by an examiner blinded to the experimental groups. The animals were then decapitated under deep 10% chloral hydrate anesthesia (800 mg/kg intraperitoneally) and brain tissues were harvested quickly to observe the effects of puerarin on infarct size and water content.

In the second experiment, 18 rats were equally and randomly assigned into the same three groups and underwent the same surgical procedures as in the first experiment, and the mRNA and protein expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α in ischemic brain tissue was measured by real-time PCR and immunohistochemistry at 24 hours after reperfusion.

Surgical procedures

Detailed methods have been described previously (Longa et al, 1989). Briefly, rats were anesthetized with 10% chloral hydrate (400 mg/kg intraperitoneally). A midline neck incision was made and the right common carotid, external carotid and internal carotid arteries were isolated. A 4-0 nylon monofilament (Ethicon, Somerville, NJ, USA) coated with silicon resin (Xantopren; Heraeus Kulzer, Germany) was inserted into the right internal carotid artery and advanced to occlude the middle cerebral artery. Regional cerebral blood flow was measured using a laser Doppler flow meter probe (Omegaflo, Omegawave Inc., Tokyo, Japan). After 90 minutes of occlusion, the filament was withdrawn to allow reperfusion. Rectal temperature was strictly maintained at 37.0 ± 0.5°C by a thermal blanket until the animal recovered from anesthesia.

Neurological function

Neurological deficit scores were measured before the animals were sacrificed based on the following graded scoring system (Hunter et al., 2000): 0, no deficit; 1, flexion of contralateral torso and forelimb upon lifting of the whole animal by the tail; 2, circling to the contralateral side, when held by tail with feet on floor; 3, spontaneous circling to contralateral side; 4, no spontaneous motor activity.

Measurement of infarct size

After neurological deficit scores were determined, the rats were deeply anesthetized with 10% chloral hydrate (800 mg/kg intraperitoneally). The brain tissues were immediately removed and cut into 2 mm thick coronal sections. These sections were stained with 2% 2,3,5-triphenyltetrazolium chloride solution and then fixed in 4% paraformaldehyde. Each infarct area was measured using NIH Image analysis software version 1.61 (Media Cybernetics, Silver Spring, MD, USA) to calculate the infarct size.

Measurement of brain water content

Water on the surface of the brain sections was blotted with filter paper and the humid weight of the paper was measured

on an electronic balance. The sections were then grilled for 48 hours at 110°C in an electrothermostatic blast oven to obtain the dry weight. Brain water content was calculated by the formula: (humid weight – dry weight) / humid weight × 100%.

Detection of mRNA expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α in ischemic brain tissue by real-time reverse transcription-PCR (RT-PCR)

RT-PCR was performed to detect the mRNA levels of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α. Total RNA was isolated from ischemic cerebral cortex using TRIzol reagent (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a high-capacity cDNA reverse-transcription kit (Takara Bio). Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α were all analyzed in the same Micro-Amp optical 96-well plate, in triplicate, using a 7900HT RT-PCR System (Takara Bio), and β-actin served as an endogenous internal control for each sample. Comparative RT-PCR assays were performed for each sample in a final reaction volume of 25 μL, containing 12.5 μL SYBR green fluorescent dye (Takara Bio), 2 μL cDNA, and 50 pmol each of the forward and reverse primers (Shanghai Biosune Biotechnology Co., Ltd., Shanghai, China). The PCR primer sequences were designed according to the Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B, tumor necrosis factor-α and β-actin gene sequences reported in GenBank (http://www.ncbi.nlm.nih.gov/genbank) (**Table 1**). Amplification was carried out at 95°C for 1 minute, followed by 40 PCR cycles of 95°C for 10 seconds, 60°C for 40 seconds, 95°C for 15 seconds, 60°C for 30 seconds, 55°C for 15 seconds, and finally 95°C for 15 seconds. The comparative Ct method was used to determine the relative expression of target genes, where expression = $2^{-\triangle\triangle Ct}$, $\triangle\triangle$ Ct= $\triangle$ $Ct_{target\ gene}$ – $\triangle$ $Ct_{calibrator}$, and $\triangle$ Ct = $Ct_{target\ gene}$ – $Ct_{control\ gene}$.

Detection of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α protein expression in the peri-infarct area of ischemic cortex by immunohistochemistry

Sections were incubated in 3% H_2O_2 to eliminate endogenous peroxidase activity. After rinsing with phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4), the sections were placed in citrate buffer (0.01 mol/L, pH 6.0) at 95–100°C for 15 minutes for antigen retrieval, and cooled to room temperature. After three rinses with PBS, the sections were blocked with 5% goat serum for 30 minutes, and were then incubated successively overnight at 4°C with primary rabbit anti-rat polyclonal antibodies (Toll-like receptor 4, 1:250; myeloid differentiation factor 88, 1:250; nuclear factor kappa B, 1:100; tumor necrosis factor-α, 1:200; all from Boster, Wuhan, Hubei Province, China). The sections were rinsed with PBS and incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:150; Boster) at 37°C for 120 minutes. The sections were rinsed again with PBS and incubated with horseradish peroxidase-labeled streptavidin at 37°C for 30 minutes. Slices were then developed with 3,3′-diaminobenzidine, counterstained with hematoxylin, and then dehydrated and mounted. Three sections from each brain, 100 μm apart, were observed, and six fields of view (200×) were selected randomly in the peri-infarct area for immunopositive cell counting under a DP70 microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data were expressed as the mean ± SD. Differences between groups were revealed by one-way analysis of variance and the Student-Newman-Keuls *post-hoc* test using SPSS 10.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Effects of puerarin on neurological function, infarct size and brain water content 24 hours after cerebral ischemia and reperfusion

Neurological function

Neurological funotion was significantly better in the puerarin treatment group than in the vehicle group 24 hours after reperfusion ($P < 0.05$; **Figure 1A**).

Infarct size

Twenty-four hours after reperfusion, no infarction was observed in the sham surgery group, while extensive infarction developed in the striatum and cortex in vehicle and puerarin groups. Compared with the vehicle group, the infarct size was significantly smaller in the puerarin treatment group ($P < 0.05$; **Figure 1B and Figure 2**).

Brain water content

Twenty-four hours after reperfusion, brain water content was significantly lower in the puerarin treatment group than in the vehicle control group ($P < 0.05$; **Figure 1C**).

Effects of puerarin on the expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α mRNA in the peri-infarct area 24 hours after cerebral ischemia and reperfusion

RT-PCR revealed that Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α mRNA expression was significantly higher in the vehicle group than in the sham surgery group ($P < 0.01$), and lower in the puerarin treatment group than in the vehicle group ($P < 0.05$; **Figure 3**).

Effects of puerarin on the expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α protein in the peri-infarct area 24 hours after cerebral ischemia and reperfusion

Immunohistochemistry showed minimal expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α protein in the sham

surgery group. Expression of all four proteins was notably increased in the vehicle and puerarin groups, but significantly less in the puerarin treatment group than in the vehicle control group ($P < 0.05$; **Figure 4**).

Discussion

Huang et al. (2006) found that inflammatory cell adhesion molecules, chemokines and cytokines play a central role in cerebral ischemia/reperfusion injury in animal models of middle cerebral artery occlusion. Investigations into the pathophysiological mechanisms of cerebral ischemia/reperfusion injury are necessary to find new and more effective drugs for stroke. Puerarin, a major isoflavonoid derived from the Chinese herb Radix puerariae, reduces cerebral ischemia/reperfusion injury induced by middle cerebral artery occlusion in rats (Pan et al., 2005; Zhang et al., 2008), but the mechanism is unknown.

In the present study, we investigated the effects of puerarin on the expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α in the ischemic region after middle cerebral artery occlusion in rats. We found that its mechanism may involve inhibition of the Toll-like receptor 4-mediated and myeloid differentiation factor 88-dependent signaling pathway, which would in turn suppress the inflammatory reaction, improve neurological deficit levels, and reduce infarct size and cerebral edema.

Results from the present study confirmed that puerarin diminished Toll-like receptor 4 expression in brain tissue with ischemia/reperfusion injury. Toll-like receptors are a large family of transmembrane proteins, able to identify a number of microbial products and pathogen-associated molecular patterns. Upon activation, Toll-like receptors trigger inherent and adaptive immune responses through cytokines, interferons, chemokines, and cell surface receptor molecules (Longa et al., 1989; Frangogiannis et al., 1998; Minami et al., 2005, 2006; Frangogiannis, 2007). Several studies have indicated that Toll-like receptors play a key role in cerebral ischemia/reperfusion injury (Arumugam et al., 2009; Hamanaka et al., 2011; Kong et al., 2011; Wang et al., 2011). Here, Toll-like receptor 4 expression was notably greater in the peri-infarct area, providing further evidence in support of its role in the pathophysiology of ischemia/reperfusion injury (Tang et al., 2007; Zhang et al., 2013). Activation of Toll-like receptors is involved in adverse reactions during cerebral ischemia (Yang et al., 2008; Hyakkoku et al., 2010). Toll-like receptor 4 activation can also lead to a decrease in nuclear factor kappa B and tumor necrosis factor-α expression, indicating that the activation of the Toll-like receptor 4-mediated myeloid differentiation factor 88-dependent signaling pathway is associated with an increased expression of nuclear factor kappa B and tumor necrosis factor-α after cerebral ischemia (Gosselin et al., 2008; Gao et al., 2009).

Toll-like receptor 4 can be activated by endogenous and exogenous factors after cerebral ischemia/reperfusion injury, triggering signal transduction pathways, activating and transcribing nuclear factor kappa B through the dependent or independent myeloid differentiation factor 88 pathways, and resulting in the expression of tumor necrosis factor-α, interleukin-1 and interleukin-6 that are associated with inflammatory responses (Yang et al., 2008; Arumugam et al., 2009; Backer et al., 2011). Studies addressing lipopolysaccharide-stimulated microglia confirmed that isoflavones inhibited the expression of inflammation-related cytokines by blocking nuclear translocation of nuclear factor kappa B. Isoflavones may also exert therapeutic effects in some neurodegenerative diseases and ischemic brain injury (Park et al., 2007; Singh et al., 2013; Jeong et al., 2014). The nuclear factor kappa B signaling pathway provides many possible targets for intervention during cerebral ischemia/reperfusion injury (Leeman et al., 2008). Inhibition of the inflammatory response in the early stage of ischemia/reperfusion injury is an attractive therapeutic strategy (Caso et al., 2007; Pradillo et al., 2009). The results of our study indicate that puerarin attenuates the transcription of nuclear factor kappa B through myeloid differentiation factor 88 dependent pathways, since the expression of both myeloid differentiation factor 88 and nuclear factor kappa B expression was reduced by puerin.

In summary, our study provides new evidence for the mechanism of the protective effect of puerarin on focal cerebral ischemia/reperfusion injury. The effect was associated with a lower expression of Toll-like receptor 4, myeloid differentiation factor 88, nuclear factor kappa B and tumor necrosis factor-α in the acute phase of cerebral ischemia/reperfusion injury. An anti-inflammatory effect *via* inhibition of the Toll-like receptor 4-myeloid differentiation factor 88-nuclear factor kappa B pathway may underlie the neuroprotective effect of puerarin in cerebral ischemia/reperfusion injury.

Author contributions: *Shen H and Zhou F designed the study. Wang L and Liu PP obtained the data. Hu WW and Zhu XD analyzed the data. Yao YY and Wang L wrote the report. Zhou F critically revised the report. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*

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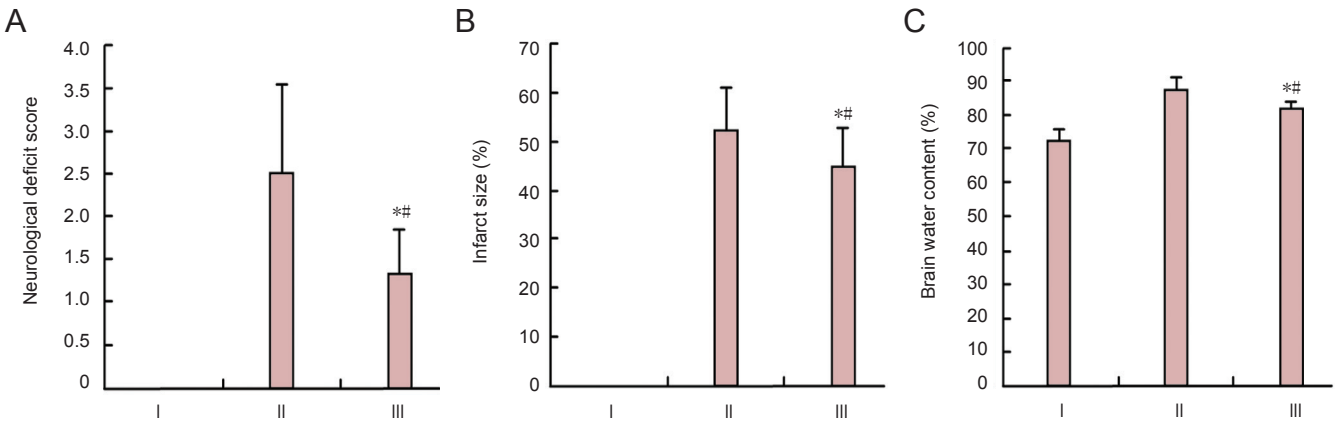

Figure 1 Effects of puerarin on neurological deficit score, infarct size and brain water content 24 hours after cerebral ischemia and reperfusion.
$*P < 0.05$, *vs.* vehicle group; $\#P < 0.01$, *vs.* sham surgery group. Data are expressed as the mean ± SD ($n = 6$), and one-way analysis of variance and the Student-Newman-Keuls test were used. I: Sham surgery group; II: Behicle group; III: Puerarin treatment group.

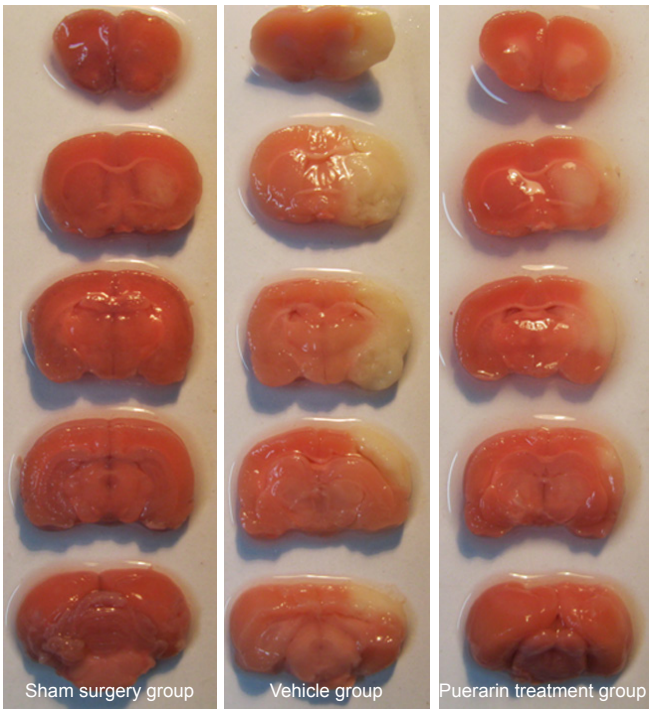

Figure 2 Ischemic regions of representative rats from each group 24 hours after reperfusion (2% 2,3,5-triphenyltetrazolium chloride staining).
White area indicates infarcted brain tissue.

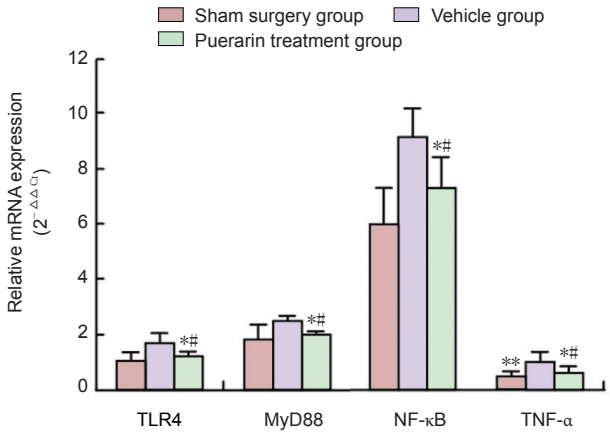

Figure 3 Effects of puerarin on TLR4, MyD88, NF-κB and TNF-α mRNA expression in the peri-infarct area of rats at 24 hours after cerebral ischemia and reperfusion.
$*P < 0.05$, *vs.* vehicle group; $\#P < 0.05$, *vs.* sham surgery group. Data are expressed as the mean ± SD ($n = 6$), and one-way analysis of variance and the Student-Newman-Keuls test were used. TLR4: Toll-like receptor-4; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor kappa B; TNF: tumor necrosis factor.

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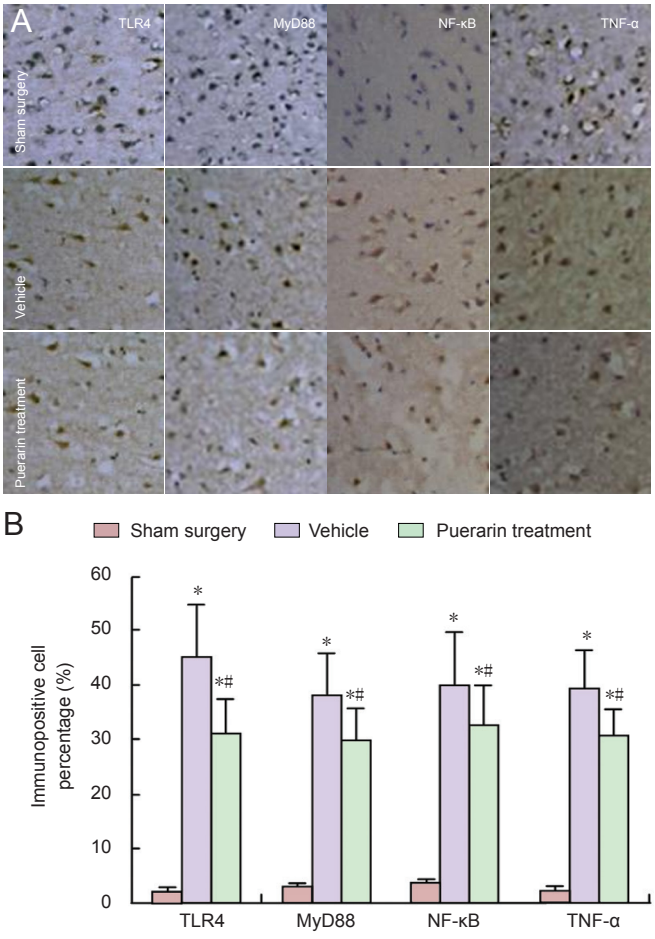

Figure 4 Effects of puerarin on the expression of TLR4, MyD88, NF-κB and TNF-α protein in the peri-infarct area 24 hours after cerebral ischemia and reperfusion (immunohistochemical staining).
(A) Photomicrographs of TLR4, Myd88, NF-κB and TNF-α-immunoreactive cells (brown grains in cytoplasm, × 200). Few positive cells were found in the sham surgery group. (B) Percentage of all counted cells that were immunopositive for TLR4, Myd88, NF-κB or TNF-α. $*P < 0.05$, *vs.* sham surgery group; $\#P < 0.05$, *vs.* vehicle group. Data are expressed as the mean ± SD ($n = 6$), and one-way analysis of variance and the Student-Newman-Keuls test were used. TLR4: Toll-like receptor-4; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor kappa B; TNF: tumor necrosis factor.

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