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ORIGINAL PAPER

Protective Effect of Shikonin in Experimental Ischemic Stroke: Attenuated TLR4, p-p38MAPK, NF-κB, TNF-α and MMP-9 Expression, Up-Regulated Claudin-5 Expression, Ameliorated BBB Permeability

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Abstract Inflammatory damage plays an important role in cerebral ischemic pathogenesis and represents a new target for treatment of stroke. Shikonin has gained attention for its prominent anti-inflammatory property, but up to now little is known about shikonin treatment in acute ischemic stroke. The aim of this study was to evaluate the potential neuroprotective role of shikonin in cerebral ischemic injury, and investigate whether shikonin modulated inflammatory responses after stroke. Focal cerebral ischemia in male ICR mice was induced by transient middle cerebral artery occlusion. Shikonin (10 and 25 mg/kg) was administered by gavage once a day for 3 days before surgery and another dosage after operation. Neurological deficit, infarct volume, brain edema, blood–brain barrier (BBB) dysfunction, and inflammatory mediators were evaluated at 24 and 72 h after stroke. Compared with vehicle group, 25 mg/kg shikonin significantly improved neurological deficit, decreased infarct volume and edema both at 24 and 72 h after transient ischemic stroke, our data also showed that shikonin inhibited the pro-inflammatory mediators, including TLR4, TNF-α, NF-κB, and phosphorylation of p38MAPK in ischemic cortex. In addition, shikonin effectively alleviated brain leakage of Evans blue, up-regulated claudin-5 expression, and inhibited the over-expressed MMP-9 in ischemic brain. These results suggested that shikonin effectively protected brain against ischemic damage by regulating inflammatory responses and ameliorating BBB permeability.

Keywords Shikonin · Cerebral ischemia · Neuroprotection · Inflammation

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Introduction

Ischemic stroke is one of the leading causes of death and disability in adults worldwide and its incidence increases with the growing of aging population [1]. Recanalization following ischemia is the most effective method for treatment of acute cerebral infarct and correction of hypoxia, but paradoxically causes severe cerebral ischemia/reperfusion injury in local region [2]. Inflammatory damage has been confirmed to play an important role in cerebral ischemia/reperfusion pathogenesis. Excessive inflammatory responses result in blood–brain barrier (BBB) disruption and contribute to deterioration and progression of ischemic injury after stroke.

Toll-like receptors (TLRs) are highly conserved members of the interleukin-1 receptor superfamily that respond to microbial signature motifs, leading to the activation of

innate immune responses [3]. Toll-like receptor-4 (TLR4) is the first recognized mammalian TLRs that enable the recognition of conserved structural motifs in a broad array of pathogens. It is believed that injured tissue and necrotic cells release endogenous activators [4]. These activators can combine with TLR4 in the cell membrane. Through TLR4 intrinsic activator, the former can combine with differentiation-14 (CD14) and anchor on cell surface and result in the recruitment of myeloid differentiation protein-88 (MyD88) [5]. In this pathway, TLR-MyD88 association activates interleukin receptor associated kinase which further leads to the activation of the NF-κB and mitogen activated protein kinase (MAPK) cascades [6–9], both of which are essential for the expression of inflammatory mediators such as tumour-necrosis factor-α (TNF-α) and interleukin-1 (IL-1) [4, 10]. Reducing the over-expression of pro-inflammatory mediators is regarded as one of major ways to alleviate ischemic injury in brain parenchyma after stroke.

Despite considerable advances in understanding of the pathogenesis of brain ischemia, there are few therapeutic strategies available for minimizing brain damage and improving functional recovery after stroke. Shikonin, one of the major naphthoquinone pigment extracted from a traditional herbal medicine Lithospermum erythrorhizon, has been shown to possess a wide range of biological effects, such as anti-inflammatory, antithrombotic and anticancer activities [11, 12]. In studies of the anti-inflammatory potential of shikonin, it has been shown to be effective in inhibiting thermal edema, arthritis, and atherosclerosis [13–15]. Treatment with shikonin also has been found to be protective against UVB-induced inflammation in cultured human epidermal keratinocytes [16]. A recent study found that shikonin attenuated microglial inflammatory responses by inhibition of NF-κB in primary microglial cells culture [17]. However, there is a paucity of literature about the role of shikonin in the brain parenchymatous tissue injury after acute cerebral ischemia. The aim of the present study is to investigate the potential neuroprotective role of shikonin in ischemic stroke and explore the underlying mechanisms.

Materials and Methods

Ethical Approval

All efforts were made to minimize animal suffering. All experimental procedures were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the institutional care of experimental animals committee.

Experimental Animals

Adult male ICR mice (28–30 g) were purchased from Vital River Laboratories (Beijing, China). The protocol was approved by the institutional animal care and use committee and the local experimental ethics committee. All mice were allowed free access to food and water under controlled conditions (12/12 h light/dark with humidity of 60 ± 5 %, 22 ± 3 °C).

Cerebral Ischemia/Reperfusion Model

Transient middle cerebral artery occlusion surgical procedures were performed on mice by the intraluminal suture method originally described by Longa et al. (1989) and our previous study. Transient focal cerebral ischemia was induced by right middle cerebral artery occlusion (MCAO). Mice were anesthetized with 10 % hydral (350 mg/kg). Body temperature was monitored and maintained at 36.5–37.5 °C. Briefly, after midline skin incision, the right common carotid artery, external carotid artery (ECA), and internal carotidartery (ICA) were exposed. Then a 6-0 nylon monofilament with a rounded tip was inserted into the right ICA through the broken end of the ECA to block the origin of MCA. The regional cerebral blood flow (CBF) was measured to less than 20 % baseline during anesthesia. The regional CBF of the right cortex were monitored using a blood flow monitor (moor-VMS-LDF, Moor Instruments Ltd, UK) with a fiber optic probe, adhered onto the skull surface of core area supplied by the MCA (3 mm lateral and 2 mm posterior from the bregma). Cerebral ischemia through the intraluminal suture was maintained for 60 min, followed by removal and reperfusion. Twenty-four hours and seventy-two hours after transient MCAO, neurological deficit scores were assessed, and then the brains were obtained by decapitation. Mice in the sham operated group underwent the same surgical procedures without inserting a filament.

Drug Administration and Groups

Shikonin (Santa Cruz Biotechnology) with purity of 98 %, was dissolved in dimethyl sulfoxide (DMSO) to prepare concentrations of 10 and 25 mg/ml. Shikonin was administered by gavage once a day for 3 days before surgery and another dosage after operation. 120 mice were randomly divided into four groups (30 mice in each group). Sham operated group: mice received equal volume PBS including 1 % DMSO (Sham); Vehicle group: mice received transient MCAO and treated with equal volume PBS including 1 % DMSO (Vehicle); low dose group: mice received transient MCAO and treated with shikonin at dose of 10 mg/kg (Shi-L); high dose group: mice

received transient MCAO and treated with shikonin at dose of 25 mg/kg (Shi-H).

Analysis of Neurological Deficit Scores

A neurological test was administered by blinded to the experimental groups at 24 and 72 h postischemia. A modified five point scale system was used, 0: normal spontaneous movements; 1: left front leg was flexed but no circling clockwise; 2: circling clockwise; 3: spin clockwise longitudinally; and 4: unconsciousness and no response to noxious stimulus.

Determination of Infarct Volume

At 24 and 72 h after transient MCAO, mice were reanesthetized with chloral hydrate. The fresh brains were dissected and cut into 4 coronal slices, the slices were incubated in 2 % 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37 °C, and followed by immersion-fixation in 4 % paraformaldehyde. The stained cerebral sections were photographed, ipsilateral and contralateral hemispheric volumes and infarct volumes were quantified using Image Pro-Plus 5.1 analysis system. The lesion volumes were calculated as a percentage of the contralateral hemisphere volume to compensate for the effect of brain edema using following formula described by Tatlisumak et al. (1998): {[total infarct volume−(the volume of intact ipsilateral hemisphere−the volume of intact contralateral hemisphere)]/contralateral hemisphere volume} × 100 %.

Measurement of Brain Water Content

Brain water content was measured using the standard wet–dry method described by Hatashita et al. (1988) and our previous study [9]. Mice were reanesthetized and the brains were removed quickly 24 and 72 h after transient MCAO. A coronal brain slice (about 3 mm thick) 4 mm from the frontal pole was cut and the slice was divided into the ipsilateral and contralateral hemispheres. Brain samples were immediately weighed on an electronic balance to obtain wet weight. Then brain samples were dried in an oven at 100 °C for 24 h to obtain the dry weight. Brain water content was calculated as (wet weight–dry weight) × 100/wet weight

Immunohistochemistry

Brains were removed quickly at 24 and 72 h after transient MCAO, immersed with 4 % paraformaldehyde in PBS for 24 h at 4 °C. Brain sections (5 μm thick) were blocked in 3 % H_2O_2, 3 % normal goat serum, and incubated with TLR4, TNF-α and NF-κB P65 rabbit polyclonal antibody (1:100, Santa Cruz Biotechnology), MMP-9 rabbit polyclonal antibody (1:150, Abcam Biotechnology), p-p38MAPK rabbit polyclonal antibody (1:100, Cell Signaling Technology) in 0.01 mol/L phosphate-buffered saline over night. The secondary antibodies, secondary biotinylated conjugates and diaminobezidine were from the Vect ABC kit (Zhong shan Biology Technology Company, China). Five visual fields of ischemic region of the infarct were selected and the immunoreactive cells were counted under a 400 × light microscope.

Real-Time Reverse Transcription–Quantitative PCR (RT-qPCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze the levels of TLR4, NF-κB, p38MAPK, TNF-α, MMP-9 and claudin-5 at 24 and 72 h after transient MCAO. Mice were anesthetized and the brains were removed and frozen in liquid nitrogen. Total RNA was extracted from the ischemic cortex in ipsilateral hemisphere using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Only samples with an OD260/OD280 value >1.8 were considered appropriate for use. Reverse transcription was performed with 2 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas International Inc, Burlington, Canada). Forward and reverse primers were5′-GGGAACAAACAGCCTGAGACA-3′ and 5′-ATAAGA GATGTCAAGGTAAAGTAGC -3′ for TLR4, 5′-CAAT GGCTACACAGGACCAGGAACA-3′ and 5′-GGATTCG CTGGCTAATGGCTTGCTC -3′ for NF-κB, 5′-GTCGTA GCAAACCACCAAGTG-3′ and 5′-CAGATTTGTGTTG TGGTCCTTC -3′ for TNF-α, 5′-CACGGCAACGGAGA AGGCAAAC-3′ and 5′-CCTGGTCATAGTTGGCTGTG GTGGC A-3′ for MMP-9, 5′-ACCGTTTCAGTCCATCA TTCA-3′ and 5′-CAGACCAATCA CATTTTCGTGT -3′ for p38MAPK, 5′-GGCGATTACGACAAGAAGAACT-3′ and 5′-TAGTGATGGTCAACGGACTCTG-3′ for claudin-5, 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-GCTTCA CCACCTTCTTGATGTC-3′ for GAPDH. Amplification and detection were carried out with the ABI 7,500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using UltraSYBR Mixture (with Rox) (ConWin biotech. Co, Beijing, China) under the following conditions: 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s. Results for each sample were collected at least three times. All the threshold cycle values of target genes were normalized relative to that of the GAPDH, and relative expression ratios were calculated via the $2^{-\Delta\Delta}$ct method.

Western Blot

Protein extraction for TLR4, p-p38MAPK, p38MAPK, NF-κB, TNF-α, MMP-9 and claudin-5 was performed as follows. The tissue was homogenized in ice-cold lysis buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCL, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L EGTA) for 15 min. After adding NP-40, it centrifuged 10,000 rpm at 4 °C for 3 min. The pellets were homogenized in ice-cold lysis buffer (20 mmol/L HEPES, pH 7.9, 400 mmol/L NaCL, 1 mmol/L EDTA, 0.1 mmol/L EGTA) for 15 min. Then centrifuged 12,000 rpm at 4 °C for 10 min, collected the supernatant and added PMSF to the final concentration 1 mmol/L as the nuclear protein for NF-κB. Preparation of total protein for TLR4, p-p38MAPK, p38MAPK, TNF-α, MMP-9 and claudin-5 extracts from the ipsilateral–cerebral cortex of mice sacrificed immediately at 24 and 72 h after transient MCAO. The protein extracts were prepared according to the manufacturer's instructions (Applygen Technologies Inc, Beijing). Then the protein concentration of the supernatant was determined using a BCA Protein Assay reagent kit (Novagen, Madison, WI, USA). 50 μg of protein was separated by SDS/PAGE, transferred 2 h on to PVDF membranes, and the nonspecific binding of antibodies was blocked with 5 % nonfat dried milk in PBS. Membranes were then probed with polyclonal rabbit anti- p-p38MAPK antibody, polyclonal rabbit anti- p38MAPK antibody (1:500, Abcam Biotechnology) and polyclonal rabbit anti-NF-κB P65, TLR4, TNF-α, claudin-5 and β-actin antibody (1:100, Santa Cruz Biotechnology), polyclonal rabbit anti-MMP9 antibody (1:500, Abcam Biotechnology) overnight at 4 °C. After four washes with TPBS, IRDye® 800-conjugated goat antirabbit second antibody (1:3,000, Rockland, Gilbertsville, PA, USA) was incubated with membranes for 1 h at room temperature. The relative density of bands was analyzed on an Odyssey infrared scanner (LI-COR Bioscience). The densitometric values were normalized with respect to the values of actin immunoreactivity to correct for any loading and transfer difference between samples.

Evaluation of BBB Damage

The permeability and integrity BBB was assessed by leakage of Evans blue from microvessels after intravenous injection. Evans blue solution (2 % in saline, 4 mL/kg) was intravenously administrated via the tail vein at 22 h after artery occlusion. Mice were then perfused transcardially with saline 24 h after MCAO to clear the blood and intravascular Evans blue remaining in the vascular system. After decapitation, brains were removed and divided into ipsilateral and contralateral hemispheres. Each hemisphere was weighed and then homogenized in 50 % trichloroacetic acid solution. Following centrifugation at 10,000 *g* for 10 min, supernatant fluids were diluted with ethanol (1:3) and fluorescence was measured at 620 nm to determine the amount of Evans blue using a spectrophotometer. The tissue content of Evans blue was quantified from a standard curve derived from known amounts of the dye. Data are expressed as micrograms per milligram of brain tissue.

Confocal Microscope

Mice were transcardially perfused with saline quickly followed by 4 % paraformaldehyde in PBS. Fixed frozen cerebral sections (30 μm thick) were blocked with 10 % horse serum for half an hour and then incubated in the primary antibodies, rabbit anti-NF-κB P65 (1:100, Santa Cruz Biotechnology) together with mouse anti-SMI 32 (1:5,000, Sternberger Monoclonal Incorporation, Lutherville, MD, USA) for 2 h at 4 °C. After 3 washes by PBS, these were correspondingly incubated with Hoechst, FITC-conjugated secondary antibody and TRITC-conjugated secondary antibody (1:100, Zhong shan Biology Technology Company, China) at 37 °C for 1 h. Immunofluorescence was visualized using a Laser Scanning Confocal Microscope (Olympus FV10-ASW, Japan).

Statistical Analysis

All statistical results were expressed as mean ± SEM. Neurological deficit assessment was tested with One-way ANOVA–Tukey's multiple comparison test. Other data were analyzed with ANOVA and followed by Student–Newman–Keuls test. The significance level was set at $P < 0.05$.

Results

Shikonin Improved Neurological Deficit After Cerebral Ischemia

Neurological deficits were examined and scored on a 5 point scale at 24 and 72 h after MCAO. Compared with Vehicle group, the neurological deficit scores in Shi-H group were significantly reduced ($P < 0.05$). Although low does of shikonin reduced the neurological deficit scores, no statistically significant difference was observed between Shi-L group and Vehicle group ($P > 0.05$) (Fig. 1a).

Shikonin Reduced Brain Water Content After Cerebral Ischemia

Brain water content of both hemispheres was measured at 24 and 72 h after stroke. In Sham group, brain water content in ipsilateral hemisphere was 74.0 ± 3.9 %. There

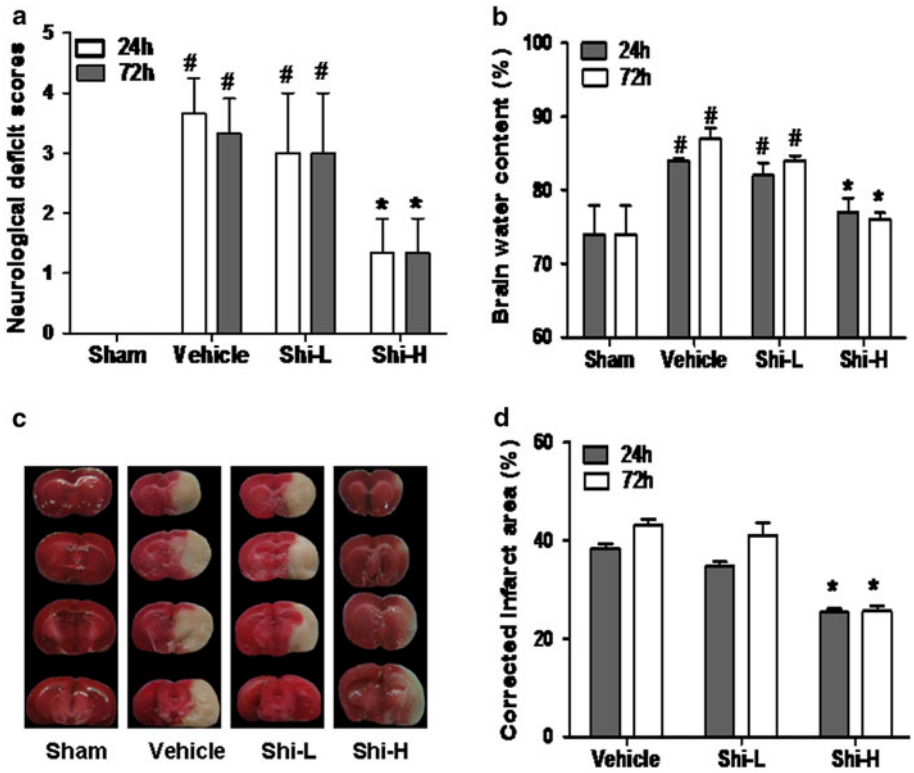

Fig. 1 The neuroprotective effect of shikonin treatment in ischemic stroke. **a** Effect of shikonin on behavior test in ischemic stroke. High dose of shikonin (25 mg/kg, Shi-H) but not low dose (10 mg/kg, Shi-L) significantly improved neurological deficits at 24 and 72 h after ischemic stroke.$^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group. **b**, **d** Effect of shikonin on brain edema and infarct volume in ischemic stroke. High dose of shikonin but not low dose significantly decreased brain edema and infarct size at 24 and 72 h after ischemic stroke.$^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group. **c** Representative images of TTC staining at 72 h after stroke

was no change in brain water content of both hemispheres in Sham group over time after surgery. Cerebral ischemia induced a significant increase in ipsilateral brain water content in the Vehicle group compared with that of Sham group both at 24 and 72 h after MCAO ($P < 0.05$). However, compared with vehicle-treatment, 25 mg/kg shikonin but not 10 mg/kg significantly decreased ipsilateral brain water content at 24 and 72 h after MCAO (Shi-H versus vehicle at 24 h: 77.0 ± 1.9 versus 84.0 ± 0.3 %; Shi-H versus vehicle at 72 h: 76.0 ± 0.9 versus 87.0 ± 1.4 %; $P < 0.05$) (Shi-L versus vehicle at 24 h: 82.2 ± 1.7 versus 84.0 ± 0.3 %; Shi-L versus vehicle at 72 h: 84.0 ± 0.7 versus 87.0 ± 1.4 %; $P > 0.05$). While the brain water content in contralateral hemisphere did not show any difference among these groups ($P > 0.05$) (Fig. 1b).

Shikonin Reduced Infarct Volume After Cerebral Ischemia

Cerebral infarction was detected by TTC staining and was shown in Fig. 1c, d. No infarction was observed in Sham group, while extensive lesion was developed in both ipsilateral striatum and cortex in Vehicle group. In Shi-H group, infarct size was significantly reduced compared with that of Vehicle group both at 24 and 72 h after MCAO ($P < 0.05$). While low dose of shikonin did not display significant neuroprotective effect on lesion volume at 24 and 72 h after stroke (Fig. 1d).

Shikonin Suppressed the Expression of Pro-Inflammatory Mediators After Cerebral Ischemia

Previous studies have demonstrated that TLR4, TNF-α, p38MAPK, and NF-κB are important pro-inflammatory mediators involved in ischemic brain injury. Then we sought to investigate whether shikonin treatment influenced the expression of pro-inflammatory mediator in ischemic brain after MCAO. TLR4, NF-κB, TNF-α, and p38MAPK were examined respectively with immunohistochemistry, Western blot and RT-qPCR at 24 and 72 h after stroke. The result of immunohistochemistry showed that few cells in the cortex of Sham group were reactive with primary antibodies of TLR4, NF-κB, TNF-α and phosphorylated p38MAPK (p-p38MAPK). However, the number of positive cells of TLR4, p-p38MAPK, NF-κB and TNF-α were significantly increased in the ischemic cortex of Vehicle group. Compared with vehicle-treatment, 25 mg/kg shikonin but not 10 mg/kg significantly decreased the number of positive cells of TLR4, p-p38MAPK, NF-κB and TNF-α in ischemic cortex both at 24 and 72 h after MCAO ($P < 0.05$; Fig. 2a, c, d).

NF-κB is an important nuclear transcription factor and initiates targeted genes transcription in pathophysiological conditions. When cells became injured or underwent necrosis, NF-κB, which normally resides in cytosol, translocated to the nuclei to activate downstream pro-inflammatory responses. By colocalization determination,

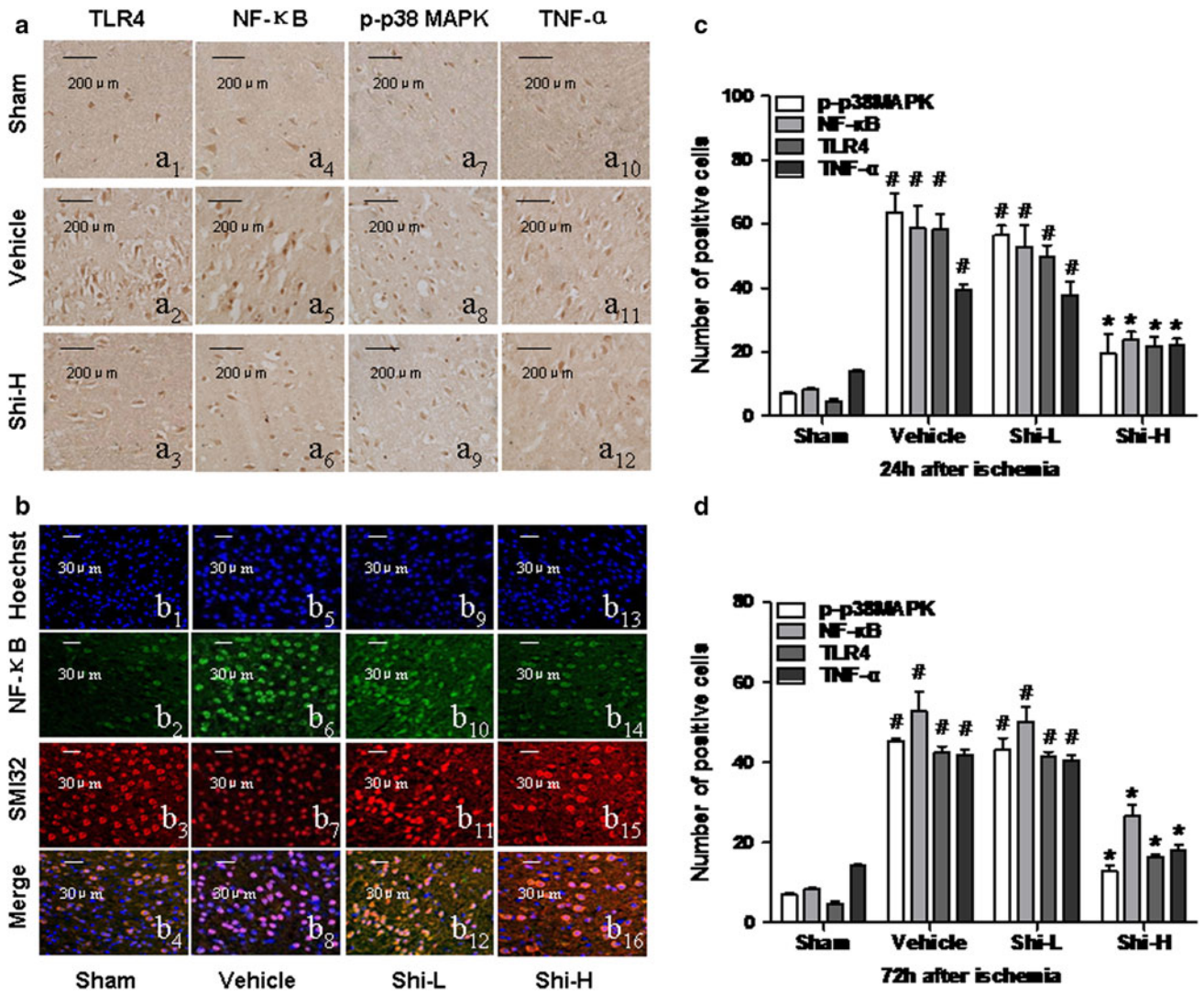

Fig. 2 Effect of shikonin on immunoreactivity of TLR4, p-p38MAPK, NF-κB, and TNF-α in ischemic stroke. **a** Representative images for immunoreactive staining of TLR4 (a_1-a_3), NF-κB (a_4-a_6), p-p38MAPK (a_7-a_9) and TNF-α (a_{10}-a_{12}) in different groups. *Scale bar*, 200 μm. Magnifications of the microphotograph, 400×. **b** Confocal images showed the influence of shikonin in NF-κB translocation in ischemic neurons after stroke. SMI-32 was used to label neurons specifically. NF-κB constitutively expressed in cytoplasm in neurons in Sham cortex (b_1–b_4), and translocated into nuclei after ischemia (b_5–b_8). High dose of shikonin (25 mg/kg, Shi-H) (b_{13}–b_{16}) but not low dose (10 mg/kg, Shi-L) (b_9–b_{12}) significantly decreased the number of nuclei positive NF-κB. (**c**, **d**). Quantification of the number of immunoreactive cells in different groups. High dose of shikonin significantly decreased the number of immunoreactive cells of TLR4, phosphor-p38MAPK, nuclei NF-κB, and TNF-α in ischemic cortex both at 24 h (**c**) and 72 (**d**) after cerebral ischemia. $^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group

we found that NF-κB reactivity mainly detected in cytoplasm in the brain of sham group. However, the number of positive nuclei of NF-κB increased in ischemic brain after MCAO. In Shi-H group, the positive nuclei of NF-κB were significantly reduced and lots of cells labeled by NF-κB were stained only in cytoplasm ($P < 0.05$; Fig. 2b). However, there were no significant differences about the positive nuclei of NF-κB between Vehicle group and Shi-L group.

Another parallel set of samples treated with low and high doses of shikonin were also used to evaluate the mRNA expression of TLR4, NF-κB, p38MAPK and TNF-α by RT-qPCR at 24 and 72 h after MCAO. In agreement with the results of immunohistochemistry, the mRNA expression of TLR4, NF-κB, p38MAPK and TNF-α in ischemic cortex were up-regulated in Vehicle group compared with Sham group ($P < 0.05$). The over-expression of those factors induced by ischemia injury was significantly decreased in Shi-H group ($P < 0.05$). Whereas Shi-L group did not display changes of TLR4, p38MAPK, NF-κB and TNF-α expression compared with Vehicle group (Fig. 3a, b).

We further analyzed the protein levels of NF-κB, TLR4, p38MAPK and TNF-α in ischemic cortex by western blot (Fig. 4a–d). As shown in Fig. 4, the level of NF-κB protein

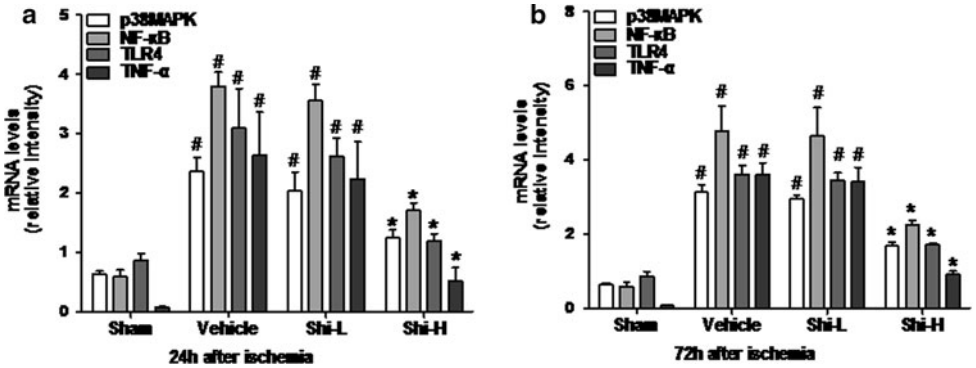

Fig. 3 Effect of shikonin on mRNA expression of TLR4, p38MAPK, NF-κB and TNF-α at 24 h (**a**) and 72 h (**b**) after ischemic stroke. High dose of shikonin (25 mg/kg, Shi-H) significantly decreased mRNA expression of TLR4, p38MAPK, NF-κB, and TNF-α both at 24 and 72 h after stroke. $^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group

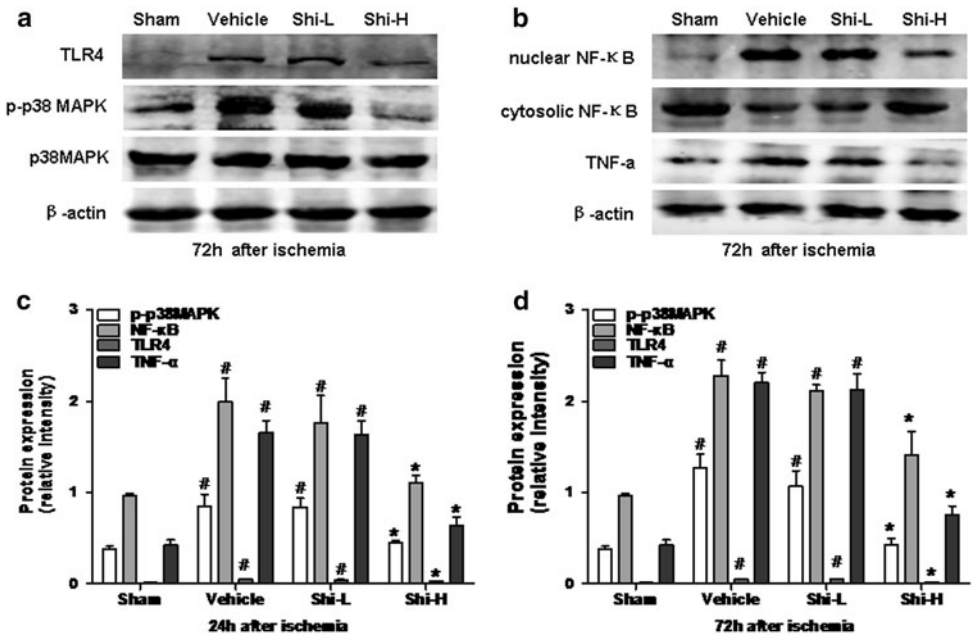

Fig. 4 Effect of shikonin on protein expression of TLR4, p38MAPK, NF-κB and TNF-α in ischemic stroke. **a**, **b** Representative photographs of western blot for TLR4, p-p38MAPK, NF-κB and TNF-α at 72 h after ischemia. **c**, **d** Quantification of protein level of TLR4, p-p38MAPK, and TNF-α in different groups. The expression of TLR4, p-p38MAPK, and TNF-α was down-regulated in Shi-H group at 24 h (**c**) and 72 h (**d**) after stroke. In addition, the nuclei translocation of NF-κB was also inhibited (**c**, **d**) with a decreased nuclei level in Shi-H group (**b**). $^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group

was high in cytosolic fractions but lower in nuclear extracts in cortical extracts of Sham group. In contrast, NF-κB protein in cerebral cortex of Vehicle group was significantly enhanced in nuclear fraction and concurrently decreased in cytosol at 24 and 72 h after ischemia, indicating the translocation of NF-κB subunits from cytosol to nucleus after ischemia. However, high dose of shikonin inhibited the nuclei translocation of NF-κB, and resulted in a significant decrease in nuclear NF-κB protein, suggesting that high dose of shikonin effectively decrease the activity of NF-κB-mediated signaling. In addition, the expression of TLR4, p-p38MAPK and TNF-α proteins was also decreased by high dose of shikonin ($P < 0.05$). But there were no significant differences in the protein levels of TLR4, NF-κB, p-p38MAPK and TNF-α between Vehicle group and Shi-L group.

Shikonin Protected the Structure and Function of BBB After Cerebral Ischemia

The permeability and integrity of BBB in ischemic hemisphere was assessed by examining the leakage of Evans blue, the expression of MMP-9 and claudin-5 after transient MCAO. At 24 h after MCAO, considerable leakages of Evans blue into the ischemic brain was observed, suggesting that the BBB had been disrupted in the ischemic hemisphere after MCAO. Compared with Vehicle group, Evans blue evasion of brain tissues was significantly decreased in Shi-H group but not in Shi-L at 24 h after MCAO ($P < 0.05$; Fig. 5a, b). Moreover, the over-expressed MMP-9 induced by ischemic injury in ischemic cortex was also significantly decreased in Shi-H group when it was examined with immunohistochemistry,

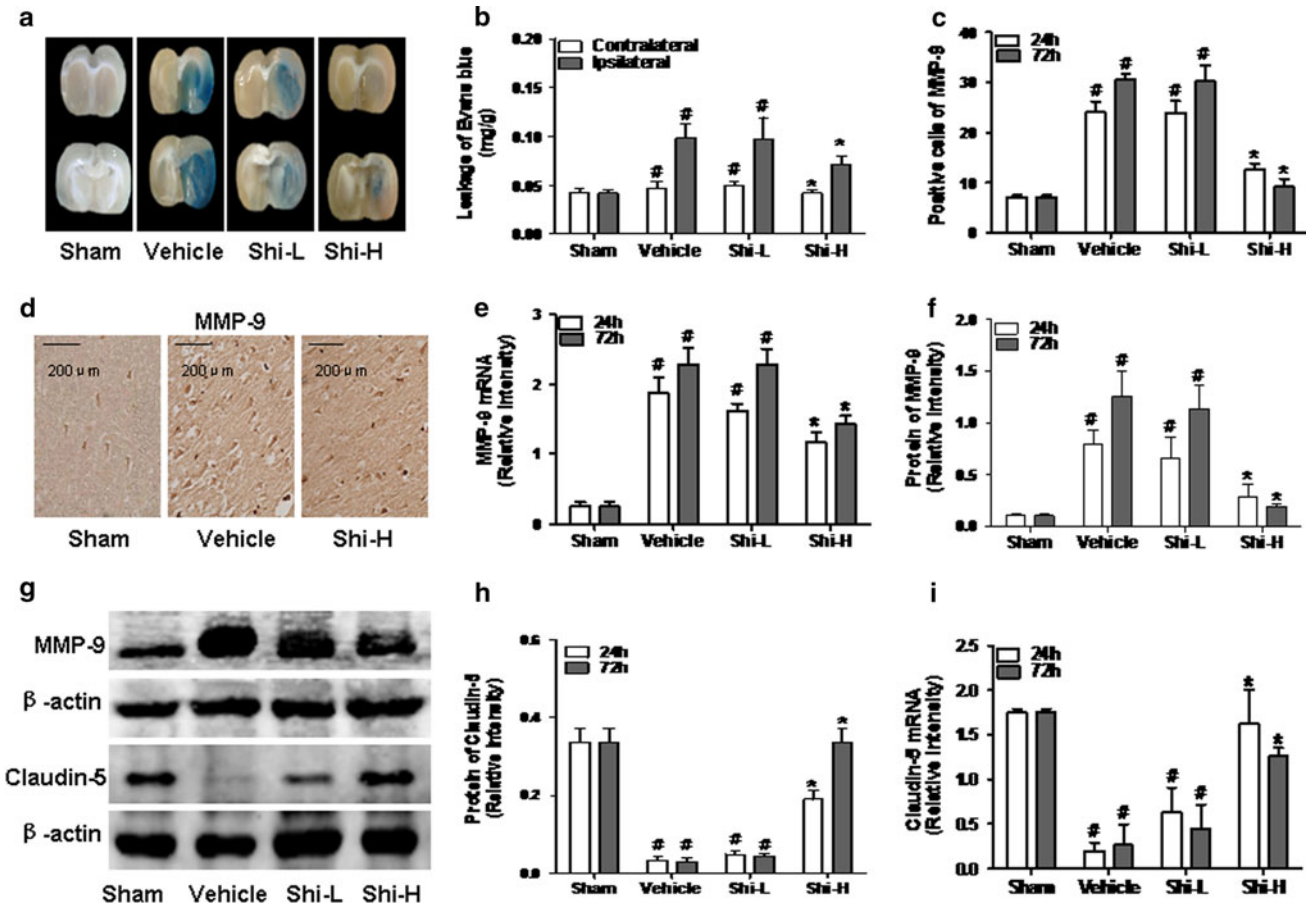

Fig. 5 Effect of shikonin on permeability and integrity of BBB in ischemic stroke. **a** Representative images for Evans blue evasion after stroke. **b** Quantification of Evans blue content in ischemic brain. Considerable leak of Evans blue into the ischemic brain after MCAO, Evans blue evasion of brain tissues was significantly decreased in Shi-H group. $^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group. **c–g** Effect of shikonin on the expression of MMP-9 in ischemic cortex after stroke. Compared with Sham group, the expression of MMP-9 was significantly increased in Vehicle group and Shi-L group at 24 and 72 h after stroke. Compared with Vehicle group, the expression of MMP-9 was dramatically decreased at 24 and 72 h in Shi-H group. $^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group. (**g–i**) Effect of shikonin on the expression claudin-5 in ischemic cortex after stroke. *Bar graph* illusitrating up-regulation of protein **h** and mRNA expression **i** of claudin-5 in Shi-H group at 24 and 72 h after stroke. $^{\#}P < 0.05$ versus Sham group. $^{*}P < 0.05$ versus Vehicle group

Western blot and RT-qPCR at 24 and 72 h after stroke (Fig. 5c–g). In addition, treatment with shikonin had a beneficial role in the expression of tight junction protein claudin-5 after ischemia. In consistent with previous study, ischemic injury induced a greatly decreased expression of claudin-5 which suggested the presence of disruption of BBB integrity and function after ischemic stroke. In this study, the expression of claudin-5 was significantly increased in Shi-H group at gene and protein levels at 24 and 72 h ($P < 0.05$; Fig. 5g–i) after ischemia. Although low dose of shikonin displayed some effects on inhibiting Evans blue evasion and preserving claudin-5 expression in ischemic brain, no statistically significant differences were observed between Vehicle group and Shi-L group.

Discussion

Cerebral ischemia and local cell debris have a strong cytotoxic effect in the brain parenchyma after MCAO [18–20]. The cytotoxic response occurs within minutes from the onset of cerebral ischemia and encompasses pro-inflammatory responses, oxidative stress, neurologic damage and cell death [13]. So inhibition of inflammatory responses at the early stage of ischemia provided an attractive therapeutic strategy [11, 20].

It is well known that TLR-MyD88 association activates NF-κB and MAPK cascades, both of which are essential for the expression of activation-induced inflammatory mediators [8]. Existing data have demonstrated that TLR4-deficient mice are protected against ischemic brain damage

[21]. In this study, we observed that the expression of TLR4 was up-regulated both at 24 and 72 h after transient ischemia. These also provided the evidence that TLR4 played an important role at very early stage after transient ischemia. Intervention of the regulation of TLR4 may bring great benefits to transient cerebral ischemia. Previous study has reported that elevated NF-κB contributes to ischemia induced neurological injury [18]. Treatment with synthetic NF-κB inhibitors has been shown to reduce infarct size in permanent ischemia [22]. MAPK is an important intracellular signal transduction system, which is composed of extracellular signal-regulated protein kinase (ERK), stress-activated protein kinases (SAPKs) or c-jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38MAPK) [23, 24]. In regard to p38 MAPK, data agree on protection by p38 inhibition against focal ischemia [4, 25]. Our study showed that shikonin significantly suppressed the up-regulation of TLR4, NF-κB, p38 MAPK and TNF-α after transient cerebral ischemia. Moreover, the expression of NF-κB in nucleus was reduced by shikonin, and its expression in cytoplasm was enhanced. It can be concluded that shikonin may hamper NF-κB translocation from cytoplasm to nucleus in the cortical peri-infarct regions. Shikonin may play an important role in inhibition of inflammation after ischemia.

BBB separates circulating blood from cerebrospinal fluid in the central nervous system and has a low permeability [26]. Disruption of the BBB following brain injury results in the development of vasogenic brain edema, a most life threatening event after such events as stroke. So preservation of the BBB is a common goal among neuroprotective therapies [18]. Previous study has described the permeability and integrity of BBB could be assessed by leakage of Evans blue from microvessels after intravenous injection. In this study, considerable leakages of Evans blue was observed, revealing that the BBB had been disrupted in the ischemic hemisphere after MCAO. And we have also demonstrated shikonin significantly decreased Evans blue evasion after MCAO, suggesting a beneficial role of shikonin in improving permeability and component degradation of BBB after ischemia.

Tight junctions are well developed and play a central role in establishing BBB. Claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells and plays a key role in the appearance of barrier properties of brain capillary endothelial cells. It has been demonstrated that disruption of claudin-5 alone is enough to cause functional changes of BBB [27]. Data also has shown that activation of matrix metalloproteinase-9 (MMP-9), which is well known to play important roles in inflammation, tumor invasion and metastasis as a key orchestrator of BBB disruption [14, 28], opens the BBB by degrading claudin-5 and increases BBB permeability after stroke [14]. And an abnormal expression of MMP-9 has been shown to play a deleterious role in brain injury in both animal models of cerebral ischemia and human stroke. In view of the fundamental role of MMP-9 and claudin-5 in regulating BBB permeability and sustaining integrity of cerebrovascular endothelial cells [19], we assessed whether shikonin exert an impact on MMP-9 and claudin-5. In our study, we observed that shikonin significantly suppressed the expression of MMP-9 and increased claudin-5 after transient cerebral ischemia. It suggested that shikonin may ameliorate disruption of BBB by inducing claudin-5 and suppressing MMP-9 after ischemia. This is also the first report that claudin-5 was demonstrated to be up-regulated by shikonin in cerebral ischemia.

Shikonin has been demonstrated to possess multiple pharmacological properties, such as antioxidant [12], anti-inflammatory [29], anti-thrombotic [30], antitumor and anti-atherosclerosis [31, 32]. In this study, shikonin decreased the infarct size, neurological scores and brain edema in vivo after ischemic stroke, providing direct evidence that shikonin has a significant neuroprotective effect in cerebral ischemia. Furthermore, it also showed a significant effect on anti-inflammation and improvement of BBB permeability, with decreased degree of TLR4, p-p38MAPK, NF-κB, TNF-α, Evans blue evasion, MMP-9 and increased amount of claudin-5 in shikonin-treated brain. These data show that shikonin is a promising neuroprotective intervention after ischemia and more study is needed for clinical use in the future.

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