Effect of Diammonium Glycyrrhizinate in Improving Focal Cerebral Ischemia-Reperfusion Injury in Rats Through Multiple Mechanisms

Hong Wang1,*, Binbin Zhang2,*, Weiwei Dong3,*, Yuying Li4, Liwen Zhao5, and Ying Zhang1

Abstract

Objective: Acute ischemic stroke is a current major disabling and killer disease worldwide. We aimed to investigate the protective effect and mechanism of diammonium glycyrrhizinate in alleviating acute ischemic stroke.

Methods: Ninety male Sprague Dawley (SD) rats (weighing 250–300 g) were randomly allocated into three groups: sham operation group (sham group), diammonium glycyrrhizinate group (DG group) and model group (model group) each with 30 individuals. A rat model of focal CIR injury was established by reversible middle cerebral artery occlusion.

Results: Zea-Longa scores for the rats in the DG group and model group were 7-fold and 8-fold higher than those of the sham group 2 h post-surgery (2.90 ± 0.99 vs. 0.30 ± 0.53, P < .05; 2.80 ± 0.61 vs. 0.30 ± 0.53, P < .05, respectively). Three days after model establishment, the scores of DG group were 26.92% lower compared with those of the model group (1.90 ± 0.76 vs. 2.60 ± 0.62, P < .05). In addition, compared with the sham group, the number of Nissl bodies and Akt-positive cells in were 27.35% and 30.42% lower in the hippocampus of the DG group (Nissl bodies: 83.40 ± 7.01 vs. 115.60 ± 11.97, P < 0.05; Akt-positive cells: 94.70 ± 8.23 vs. 136.10 ± 10.37, P < .05) and 58.65% and 57.31% lower in the model group (Nissl bodies: 47.80 ± 4.91 vs. 115.60 ± 11.97, P < .05; Akt-positive cells: 58.10 ± 4.98 vs. 136.10 ± 10.37, P < 0.05), respectively. However, the number of Nissl bodies and Akt-positive cells in the hippocampus of DG group were 74.48% and 62.9% higher compared with the model group, respectively (Nissl bodies: 83.40 ± 7.01 vs. 47.80 ± 4, P < 0.05; Akt-positive cells: 94.70 ± 8.23 vs. 58.10 ± 4.98, P < .05). In addition, compared with the sham group, the number of caspase-3-positive cells, the expression level of p38 mitogen-activated protein kinase (p38MAPK) and the expression of matrix metalloproteinase 9 (MMP-9) were 2-fold, 34.38%, 64.78% higher in the DG group (caspase-3-positive cells: 78.70 ± 6.52 vs. 27.10 ± 3.00, P < .05; p38MAPK: 0.43 ± 0.15 vs. 0.32 ± 0.10, P < .05; MMP-9: 14.83 ± 1.18 vs. 9.00 ± 2.05, P < .05, respectively), and more than 3-fold, 1-fold and 1-fold higher in model group (caspase-3-positive cells: 121.10 ± 11.04 vs. 27.10 ± 3.00, P < .05; p38MAPK: 0.70 ± 0.12 vs. 0.32 ± 0.10, P < .05; MMP-9: 19.00 ± 1.90 vs. 9.00 ± 2.05, P < .05), respectively. However, the number of caspase-3-positive cells and the expression levels of p-38MAPK and MMP-9 were 35.01%, 38.57% and 28.12% lower in DG group compared with the model group (caspase-3-positive cells: 78.70 ± 1.90 vs. 47.80 ± 4, P < .05; p-38MAPK: 0.43 ± 0.15 vs. 0.32 ± 0.10, P < .05; MMP-9: 14.83 ± 1.18 vs. 9.00 ± 2.05, P < .05, respectively).
Background

The global incidence of stroke has been increasing steadily with World Health Organization projecting that stroke-induced deaths will reach 78 million by 2030.1,2 Ischemic stroke is expected to account for 70–80% of these deaths.3–5 Cerebral perfusion at the early stage is an effective way of improving symptoms following acute infarction. However, failure to restore brain function within a specified period after the restoration of blood supply following cerebral ischemia could result in cerebral ischemia-reperfusion (CIR) injury and may induce severe dysfunctions. Cerebral ischemia-reperfusion is thought to be associated with a complex pathophysiological process, which triggers stress-induced signal transduction and involves many complex factors related to excitotoxicity, oxidative stress, inflammation, apoptosis, and blood–brain barrier destruction.6 However, this complex pathophysiological process has not been fully elucidated, including the interactions among critical factors in each stage. For treatment purposes, there is a need to focus on essential active mediators of apoptosis and inflammation, combined with intervention on these indicators to improve cell function and survival.

Hypoxic environment and oxygen-free radicals can induce expression of many genes involved in inflammation and apoptosis which, in turn, can lead to cell damage and death.7 Akt (protein kinase B) is a serine/threonine-specific protein kinase involved in many cellular processes, including glucose metabolism, cell proliferation and apoptosis.8 Caspase-3 is a major protein in apoptosis and a terminal factor, particularly in the process of apoptotic cell death. It has, therefore, been accepted as a marker for activation of apoptosis. Inhibiting caspase-3 activation could play a role in the last step of the apoptosis cascade and block the death of apoptotic cells.9

Several lines of evidence have demonstrated that inflammatory mediators, such as p38 mitogen-activated protein kinase (p38 MAPK) and matrix metalloproteinase-9 (MMP-9), play up-regulating or destructive roles after CIR injury.10–12 The MAPK signaling pathway has a significant inflammatory effect on the process of central nervous system injury, including traumatic brain injury and transient focal cerebral ischemia. In addition, p38 MAPK has been shown to inhibit focal cerebral ischemia.12–14 On the other hand, activation of MMP-9 plays a vital role in inflammation, tumor invasion and metastasis10,15,16 with its abnormal expression known to negatively affect stroke-induced brain and ischemia-reperfusion (I/R) injuries.11,17

Diammonium glycyrrhizinate, an ammonium salt preparation of 18α-glycyrrhetinic acid, has been reported to have the ability to protect tissues from reperfusion injury through inflammatory catalysts, such as interleukin-1 (IL-1), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), and nuclear factor-kappa B (NF-κB).18 However, CIR is attributed to a complex pathophysiological process. It has been reported that glycyrrhizic acid can treat tumors, liver diseases, and enteritis by regulating and inhibiting inflammation through the Akt/caspase-3, MAPK and MMP-9 pathways.19–21 Therefore, we hypothesized that diammonium glycyrrhizinate could (i) improve apoptosis in CIR and (ii) improve brain injury in CIR by down-regulating the level of inflammatory mediators. In this study, the middle cerebral artery occlusion reperfusion (MCAOR) model was used to simulate CIR injury and observe the effect of diammonium glycyrrhizinate on the number of Nissl bodies in neurons, expression of Akt and caspase-3 proteins as well as that of inflammation-related factors (including p38 MAPK and MMP-9) in CIR and CIR brain injuries. We further investigated the neuroprotective effect and mechanism of action of diammonium glycyrrhizinate in CIR injury in rats.

Methods

Source of Reagents

Diammonium glycyrrhizinate and sodium chloride injections were procured from Zhengda Tianqing Pharmaceutical Group Co., Ltd, China. Akt/Caspase-3 monoclonal antibodies were procured from Wuhan Boster Biotechnology Co., Ltd, China, whereas immunohistochemistry and 3,3′-Diaminobenzidine (DAB) Color developing kits were procured from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, China.

Animals and Establishment of the CIR Injury Model

Rats were housed under controlled conditions: 12 h light/dark cycle, at 21°C ± 2°C and 60%–70% humidity. The rats were provided with free access to standard rodent food and water.
Approval for the experimental protocol was obtained from the local ethical committee for animal research. All animals were treated in strict accordance with the National Institutes of Health Guide for the care and Use of Laboratory Animals, and the experimental protocols were approved by the Ethics Committee of Tianjin Medical University, Tianjin, China. The utmost possible efforts were made to diminish the suffering of the experimental animals in this study.

Focal Cerebral Ischemia Model by Endovascular Suture Occlusion of the Middle Cerebral Artery in the Rat: The MCAO rat model was established using the reversible suture-oclusion method as earlier described. The rats were placed in the animal operating room and anesthetized with 5% chloral hydrate (8 mL/kg) by intraperitoneal administration. After successful anesthesia, each rat was fixed on the animal operating table, and neck hair was shaved and disinfected with iodine. A 2–3 cm long incision was made .5 cm to the left of the middle of the neck, and the subcutaneous fascia and neck muscles were separated layer by layer to expose the left common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA). The distal end of ECA was ligated, CCA and ICA distal ends were temporarily occluded using arterial clamp, a 2–3 cm incision was made at the proximal end of ECA ligation and the ECA cut off was then pulled to the same line as the ICA. After inserting the free ECA at the distal end using an imported fishing line (diameter, .23 mm) with a pretreated head end, the artery clamp at CCA and ICA was released successively. The fishing line was anterogradely inserted along the ICA to a depth of approximately 18–22 mm with the insertion action halted when resistance was encountered. At this point, the inserting line was stopped, the inserted ECA together with the fishing line were ligated followed by a suture of the neck tissue and skin, layer by layer. The fishing line was subsequently pulled out after 2 h. After disinfection, the rats were exposed to a 100 W incandescent lamp at a height of 50 cm to provide the rats with a suitable temperature environment. The model was considered successfully established when hemiplegia appeared after the rats had recovered from anesthesia. Rats in the sham operation group underwent the same operation procedure minus insertion of the suture.

Rats in DG group were intraperitoneally injected with 20 mg/kg/d Diammonium Glycyrrhizinate 2 h post-surgery. We chose this dose because it had been established as the most appropriate dose in related dose-course studies, and has subsequently been used in a number of other studies. Rats in model group were intraperitoneally injected with an equivalent amount of normal saline (10 mL/d) 2 h postsurgery, once a day for 3 days. Based on results of previous studies of Diammonium glycyrrhizinate in cerebrovascular diseases, we chose the most common time point of 3 days as the observation point.

Ninety male Sprague Dawley rats (weight 250–300 g, provided by Experimental Animal Center of Chinese Academy of Military Medical Sciences) were randomly divided into sham operation group (sham group), diammonium glycyrrhizinate group (MCAO + DG, DG group) and model group (MCAO + saline, model group), with 30 rats in each. Three days after model establishment, before this procedure, rats were anesthetized by intraperitoneal injection of 2% chloral hydrate (3 mL/kg). Then, under deep anesthesia, whole brains were collected from the rats by craniotomy and fixed by perfusion with 4% paraformaldehyde. One block of tissue was taken in the coronal position along the direction of the optic chiasma, 2–3 mm away from the anterior and posterior areas at the location of the coronal plane, which was fixed with 4% paraformaldehyde for 24 h. After dehydration with different (gradient) ethanol concentrations and vitrification with dimethylbenzene, tissues were embedded in paraffin and preserved. The animal carcasses were put in special carcass bags and stored in carcass refrigerators or freezers until incineration.

Observation Indicators

Neurological Score. The neural function of rats was evaluated using Zea-Longa neurological scores before modeling, and 2 h and 3 d after model establishment. The scores were as follows: 0 point for normal performance and lack of neurological deficit symptoms; 1 point for failure to fully extend the right forepaws; 2 points for rotation on the right side when walking; 3 points for falling to the right when walking; 4 points for inability to walk spontaneously and with a reduced level of consciousness.

Nissl Staining. Prior to this procedure, rats were anesthetized by intraperitoneal injection with 2% chloral hydrate (3 mL/kg). Then, the brain was harvested under deep anesthesia. The embedded paraffin blocks were sectioned continuously at a thickness of 5 μm (Leica RM2016, Wetzlar, Germany). Sections were subsequently dewaxed with dimethylbenzene, dehydrated in anhydrous alcohol for 2 h, immersed in cresyl violet solution for 1 h, rapidly differentiated with 95% alcohol and then dehydrated in anhydrous alcohol. They were then vetrified with dimethylbenzene and finally sealed in neutral gum. Three visual fields (∼100) were selected from each section to count the number of Nissl bodies under a light microscope (Nikon, Eclipse E600POL, NIKON, Co., Tokyo, Japan).

Immunohistochemical Staining. After routine treatment such as dewaxing of the paraffin-embedded sections of the basilar artery, the operations were performed as per the instructions of the immunohistochemistry kit, including developing by DAB, hematoxylin re-staining, conventional dehydration, vitrification, and sealing. Three visual fields (∼400) were selected from each section for counting the number of Akt and caspase-3-positive cells under the microscope.
Detection of p-38MAPK expression using western blotting. Total protein was isolated from hippocampal tissues of rats (Eppendorf 5804 R Low Temperature High Speed Centrifuge, Eppendorf Co., Germany). After denaturation on 10% polyacrylamide gel electrophoresis, the protein was transferred to a nitrocellulose membrane then sealed in phosphate-buffered saline for 1 h at room temperature. Primary (rabbit anti-mouse p-38MAPK, 1:250 dilution) and secondary (horseradish peroxidase-labeled goat anti-rabbit IgG, 1:1,000 dilution) antibodies were successively added and incubated for 1 h at room temperature followed by substrate luminescence and developed for 5 min. The gray value of protein bands was determined using gel automatic analysis and imaging software (Image J Software Analysis).

Detection of MMP-9 Expression by Immunohistochemistry. After routine treatment such as dewaxing of paraffin-embedded sections of brain tissues, the operation was conducted as per the instructions of the immunohistochemistry kit, including development using DAB, hematoxylin re-staining, conventional dehydration, vitrification, and sealing. Five visual fields (×400) were selected from each section for observation under the light microscope and counting the number of positive cells.

Statistical Analysis. All data were analyzed using SPSS version 17.0. software. Variables were analyzed using one-way analysis of variance (ANOVA) and expressed as means ± standard deviations. A Bonferroni’s t-test followed by the post hoc was use for multivariate analysis. Significant differences were determined at P < .05.

Results
Changes in the Neurological Score of Rats
The Zea-Longa scores for the rats were 0 before surgery. Compared with the sham group, the scores were more than 8-fold and 7-fold higher in DG group and model group (2.90 ± .99 vs .30 ± .53, P < .05, 2.80 ± .61 vs .30 ± .53, P < .05), respectively. The neurological score of DG group was 26.92% lower than that of the model group 3 d after modeling (1.90 ± .76 vs 2.60 ± .62, P < .05) [Table 1].

Changes in the Number of Nissl Bodies in Neurons
In DG and model groups, the number of Nissl bodies in neurons of the hippocampus and bilayer parietal cortex was 27.85% and 58.65% lower compared with sham group (83.40 ± 7.01 vs 115.60 ± 11.97, 47.80 ± 4.91 vs 115.60 ± 11.97, P < .05), respectively. On the other hand, Nissl bodies in the DG group was 74.48% higher compared with the model group (83.40 ± 7.01 vs 47.80 ± 4.91, P < .05), [Table 2 and Figure 1].

Changes in Positive Cell Number and Protein Expression of Akt- and Caspase-3
The number of Akt-positive cells in the hippocampus of rats was 30.42% and 57.31% lower in the DG group and model group compared with sham group (94.70 ± 8.23 vs 136.10 ± 10.37, 58.10 ± 4.98 vs 136.10 ± 10.37, P < .05), respectively. Conversely, the number of Akt-positive cells in the DG group were 62.99% higher than those in model group (94.70 ± 8.23 vs 58.10 ± 4.98, P < .05). The number of caspase-3-positive cells in the hippocampus of rats in the DG group and model group were nearly twice and more than 3 times those recorded in sham group (78.70 ± 6.52 vs 27.10 ± 3.00, 121.10 ± 11.04 vs 27.10 ± 3.00, P < .05). However, the number of caspase-3-positive cells in the hippocampus of rats in DG group was 35.01% lower than those in model group (78.70 ± 6.52 vs 121.10 ± 11.04, P < .05) [Table 2 and Figure 2 and 3].

Comparison of p-38MAPK Expression Across Groups
The p-38MAPK expression values were .32, .43 and .70 in the sham group, DG group and model group, respectively.

Table 1. The Zea-Longa neurological scores in each group.

| Group                  | n  | Before modeling | 2 h after modeling | 3 d after modeling |
|------------------------|----|-----------------|--------------------|--------------------|
| Sham operation group   | 30 | .00 ± .00       | .30 ± .53          | .00 ± .00          |
| Diammonium glycyrrhizinate group | 30 | .00 ± .00       | 2.90 ± .99#        | 1.90 ± .76#        |
| Model group            | 30 | .00 ± .00       | 2.80 ± .61*        | 2.60 ± .62*        |

Notes: *compared with sham operation group, P < .05; #: compared with model group, P < .05.

Table 2. Effects of diammonium glycyrrhizinate on hippocampal damage in rats with CIR.

| Group                  | Number of Nissl bodies | Number of Akt-positive cells | Number of Caspase-3-positive cells |
|------------------------|------------------------|-----------------------------|-----------------------------------|
| Sham group             | 115.60 ± 11.97         | 136.10 ± 10.37              | 27.10 ± 3.00                      |
| DG group               | 83.40 ± 7.01*#         | 94.70 ± 8.23*#              | 78.70 ± 6.52*#                   |
| Model group            | 47.80 ± 4.91*          | 58.10 ± 4.98*               | 121.10 ± 11.04*                  |

Notes: *compared with sham group, P < .05; #: compared with model group, P < .05.
Figure 1. Nissl staining of the hippocampus after CIR injury in rats (×100) A. Sham operation group: clear outlines of neurons and filling of dark blue Nissl bodies in the cytoplasm; B. DG group: slight decrease in the number of Nissl bodies and dark staining of the cytoplasm; C. model group: fuzzy outline of neurons, autolysis in partial cytoplasm, disappearance of nucleus fragmentation and apparent reduction in Nissl body content.

Figure 2. Immunohistochemical staining of Akt in the hippocampus after CIR injury in rats (×400) A. Sham operation group: highly expression of Akt-positive cells (brown) in the cytoplasm with the presence of regular arrangement; B. DG group: expression of some Akt-positive cells (brownish yellow) and slightly disordered arrangement; C. model group: expression of only a small amount of Akt-positive cells (brownish yellow) with reduced number of cells.

Figure 3. Immunohistochemical staining of caspase-3 in the hippocampus after CIR injury in rats (×400) A. Sham operation group: purple or light blue staining of the nucleus, and low expression of only a small amount of caspase-3-positive cells (light brownish yellow); B. DG group: expression in some cells (brownish yellow), which were caspase-3-positive cells; C. model group: expression in most cytoplasm (brownish yellow), which was high expression of caspase-3-positive cells.
The p-38MAPK expression was 34.38% and more than 1-fold higher in DG group and model group than that in the sham group (.43 ± .15 vs .32 ± .10, .70 ± .12 vs .32 ± .10, P < .05), respectively. Compared with the model group, p-38MAPK expression was 38.57% lower in DG group (.43 ± .15 vs .70 ± .12, P < .05) [Table 3, Figure 4].

Group A: sham group; group B: DG group; group C: model group. The expression of p-38MAPK in group B was higher than in group A (P < .05). The expression of p-38MAPK in group B was significantly lower than in group C (P < .05).

Comparison of MMP-9 Expression Across Groups

MMP-9 expression values were 9.00, 14.83, and 19.00 in the sham group, DG group and model group, respectively. The MMP-9 expression was 64.78% and 100% higher in the DG group and model group than that in the sham group (14.83 ± 1.18 vs 9.00 ± 2.05, 19.00 ± 1.90 vs 9.00 ± 2.05, P < .05), respectively. Compared with the model group, MMP-9 expression was 28.12% lower in DG group (14.83 ± 1.18 vs 19.00 ± 1.90, P < .05) [Table 3 and Figure 5].

Table 3. Effects of diammonium glycyrrhizinate on hippocampal damage in rats with CIR.

| Group                     | p-38MAPK protein content | Number of MMP-9-positive cells |
|---------------------------|--------------------------|-------------------------------|
| Sham operation group      | .32 ± .10                | 9.00 ± 2.05                   |
| Diammonium glycyrrhizinate group | .43 ± .15*            | 14.83 ± 1.18*                 |
| Model group               | .70 ± .12                | 19.00 ± 1.90                  |

Notes: * compared with sham operation group, P < .05; #: compared with model group, P < .05.

Figure 4. Western blot bands showing the brightness of p-38MAPK protein in each group.

Figure 5. Expression of MMP-9-positive cells in brain tissues of rats in each group (immunohistochemistry, × 400) A: sham group; B: DG group; C: model group. The expression of MMP-9 in B was higher than that in A (P < .05). The expression of MMP-9 in B was significantly lower than that in C (P < .05).
**Discussion**

The mechanism of CIR injury is complex and its cause remains unknown. Multiple cytokines and inflammatory mediators at the injury site may cause edema, inflammatory reaction, free radical accumulation, and cell ischemic necrosis and apoptosis.

Currently, there is no specific drug for the treatment of CIR injury. Recently, clinicians have raised concerns about the use of diammonium glycyrrhizinate for focal ischemic injury. The chemical name of diammonium glycyrrhizinate is 20β-carboxyl-11-oxo-n-oleanolane-12-alkene-3β-2-O-β-D-glucopyranosylaldosyl-a-D-glucopyranosylaldol-diammonium salt, with a molecular formula of C42H68N2O16 and molecular weight of 857.01. Previous studies have shown that diammonium glycyrrhizinate has anti-inflammatory effects, and can act as an immune regulator, as well as an anti-allergy and anti-apoptosis agent and maintains the cell membrane integrity. It has also been found to confer myocardial and renal tissues ischemic refocus injury protection. Furthermore, it has been reported to prevent non-alcoholic fatty liver disease in mice. In addition, it can prevent acute renal injury caused by sepsis by inhibiting apoptosis and inflammation. Hou et al. found that diammonium glycyrrhizinate could inhibit inflammatory response after CIR injury but did not elucidate the specific mechanisms of action. In our study, a CIR injury model was established in rats to investigate the potential mechanism of diammonium glycyrrhizinate action in preventing CIR injury. A dose of 20 mg/kg/d of Diammonium glycyrrhizinate, which had been previously established as the most appropriate dose was chosen as the experimental dose. Besides, different doses have been investigated in other studies. In one study, two doses of diammonium glycyrrhizinate, 15 mg/kg and 30 mg/kg, were found to have some effect in improving cerebral ischemia-reperfusion injury, suggesting a tendency of higher doses to have better improvement effect, but no statistical difference was mentioned. Other glycyrrhizinate doses has been studied in organ ischemia-reperfusion injury at various doses, including 10 mg/kg and 50 mg/kg for myocardial ischemia-reperfusion, 50 mg/kg for hepatic ischemia-reperfusion injury and 10 mg/kg for spinal cord ischemia-reperfusion injury.

Findings of this study showed that the neurological score of the model group was significantly higher than that of sham group 2 h post-surgery, indicating the successful establishment of the middle cerebral artery occlusion reperfusion (MCAOR) model. Moreover, the neurological score of DG group decreased significantly 3 d after modeling, suggesting that diammonium glycyrrhizinate could improve the neurological function of rats with CIR injury.

CIR injury is often associated with overexpression of several genes, most of which are related to apoptosis. Nissl bodies are composed of well-developed rough endoplasmic reticulum and free ribosomes, and mainly synthesize structural proteins and enzymes needed for the renewal of organelles. In the case of damage to neurons, Nissl bodies gradually decompose and disappear, suggesting that a decrease in Nissl bodies could signify the death of neurons. In our study, a clear outline of neurons was observed in the sham operation group after Nissl staining of the rat cortex. The cytoplasm was homogeneous and Nissl bodies were evenly distributed appearing as dark blue granules. In the model group, there was a fuzzy outline of neurons, irregular distribution of cytoplasm, autolysis in partial cytoplasm, the disappearance of Nissl bodies after Nissl staining. Therefore, the damage of Nissl bodies could also partially indicate damage to neurons after I/R injury. However, in DG group, the number of neurons and Nissl bodies significantly increased, suggesting that diammonium glycyrrhizinate could protect the survival of neurons.

Serine/threonine kinase (Akt), also known as protein kinases B, is an inhibitor and activator of a substrate that can concurrently trigger multiple downstream functions. It is, therefore, considered a central regulator in various signal complexes. Akt is mainly responsible for the transmission of biological information initiated by phosphatidylinositol 3-kinase (PI3K). It is, therefore, the main downstream target molecule of PI3K, which functions primarily in cell cycle regulation, initiation of apoptosis, angiogenesis, telomerase activity, and cell invasion. Akt has been shown to play a crucial role in the protection against apoptosis in CIR injury. Apart from inhibiting pro-apoptotic factors, Akt can also activate the transcription of anti-apoptotic genes. After activation, Akt directly promotes the phosphorylation and inactivation of caspase-3 and inhibits apoptosis. Caspase-mediated apoptosis is initiated by the release of cytochrome C from mitochondria and the activation of apoptotic complexes, which in turn activate caspase-3. Activated caspase is a proteolytic enzyme that can modify vital homeostasis and repair proteins. Particularly, caspase-3 may play a key role in ischemia-induced apoptosis. The results of our study showed that the application of diammonium glycyrrhizinate in the early stages after CIR injury could improve the neural function of rats, increase the expression levels of Akt protein and reduce the levels of caspase-3 protein. These findings suggested that diammonium glycyrrhizinate could inhibit caspase-3 expression, reduce neuron apoptosis and improve the neural function of rats with CIR injury by activating the Akt signaling pathway.

The mitogen-activated protein kinase (MAPK) signaling pathway is composed of 3 most marked characteristic subgroups, namely, extracellular signal-regulated kinase, p38 MAPK, and c-Jun N-terminal kinase. Activation of p38 MAPK signal is vital in I/R-induced apoptosis and inflammatory response, which can lead to the production of pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and MMP-9 is also a member of the family.
Expression of MMP-9 is regulated by MAPKs. Matrix metalloproteinase 9 (MMP-9) activation plays a crucial role in inflammation, tumor invasion, and metastasis.\textsuperscript{10,15} It has been reported that abnormal expression of MMP-9 may have a deleterious action in stroke-induced brain injury and I/R injury.\textsuperscript{11,17} The expression of the MAPK inflammatory pathway plays a significant role in the inflammatory response of cells after CIR injury, which can be activated by various extracellular signals, causing inflammatory cascade reaction, thus leading to a series of pathological processes, such as blood-brain barrier damage and apoptosis.\textsuperscript{12} Glycyrrhizin has a similar steroid parent ring structure and pharmacological effect with diammonium glycyrrhizinate (its ammonium salt) and can reduce myocardial I/R injury by regulating the expression of MAPK, thus reducing oxidative stress, inducible nitric oxide synthase (iNOS) and inflammatory response.\textsuperscript{52} In our study, results from the western blot analysis and immunohistochemical staining showed that the expression levels of p-38MAPK and MMP-9 in the DG group were significantly lower than that in the model group, indicating that diammonium glycyrrhizinate could inhibit inflammatory response by blocking p-38MAPK signal transduction pathway and reducing the expression of inflammatory factor MMP-9. This could lead to improvement in the inflammatory response caused by I/R injury and therefore play a protective effect. However, there is still a need to further investigate the mechanisms involved in other related signaling pathways.

There are a few limitations to this study. Considering biodiversity, diammonium glycyrrhizinate may differ from humans in absorption rate, drug distribution, and pharmacokinetics in rats, future studies should evaluate the clinical protective effect of diammonium glycyrrhizinate against ischemic stroke. Certainly, it is better to study diammonium glycyrrhizinate as a potential therapeutic agent for ischemic stroke at different doses, administration intervals and mechanisms of action.

**Conclusions**

In conclusion, our study showed that diammonium glycyrrhizinate at 20 mg/kg/day had a protective effect on cerebral ischemia-reperfusion injury in rats by promoting formation of Nissl bodies and increasing protein expression of Akt while decreasing that of caspase-3, p38 MAPK, and MMP-9, either directly or indirectly, by inhibiting apoptosis and reducing neuroinflammation. All these mechanisms resulted in improved overall neurological function.

**Authors’ Contributions**

Liwen Zhao, Ying Zhang designed the study; Liwen Zhao conducted the literature search, collected, interpreted the data. Yuying Li analyzed and researched the data; Hong Wang, Binbin Zhang drafted the manuscript. Weiwei Dong, Hong Wang, Ying Zhang and Binbin Zhang literature search, analysis and interpretation of data and wrote the manuscript; Hong Wang, Ying Zhang revised the manuscript; all authors have read and approved the final version of this manuscript.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Tianjin Key Medical Discipline(Specialty) Construction Project; Tianjin Health Commission key field of Traditional Chinese medicine science and technology project (20170009).

**Ethics Approval**

All animals were treated in strict accordance with the National Institutes of Health Guide for the care and Use of Laboratory Animals, and the experimental protocols were approved by Ethics Committee of Tianjin Medical University, Tianjin, china.

**Data Availability**

The data generated and analyzed in the present study are available from the corresponding author upon reasonable request.

**ORCID iD**

Hong Wang  \(\text{https://orcid.org/0000-0003-0279-8530}\)

**References**

1. Benjamin EJ, Blaha MJ, Chiue SE, et al. Heart disease and stroke statistics—2017 update: A report from the American heart association. Circulation. 2017;135(10):e146-e603.
2. Strong K, Mathers C, Bonita R. Preventing stroke: Saving lives around the world. Lancet Neurol. 2007;6(2):182-187.
3. Maaijwee NA, Rutten-Jacobs LC, Schaapsmeerders P, van Dijk EJ, de Leeuw FE. Ischaemic stroke in young adults: Risk factors and long-term consequences. Nat Rev Neurol. 2014;10(6):315-325.
4. Krishnamurthi RV, Moran AE, Feigin VL, Barker-Collo S, Norrving B, Mensah GA. Stroke prevalence, mortality and disability-adjusted life years in adults aged 20-64 years in 1990-2013: Data from the global burden of disease 2013 study. Neuroepidemiology. 2015;45(3):190-202.
5. Meschia JF, Brott T. Ischaemic stroke. Eur J Neurol. 2018;25(1):35-40.
6. Brouns R, De Deyn PP. The complexity of neurobiological processes in acute ischemic stroke. Clin Neurol Neurosurg. 2009;111(6):483-495.
7. Lopez Nebina F, Toledo AH, Toledo-Pereyra LH. Molecular biology of apoptosis in ischemia and reperfusion. J Invest Surg. 2009;18(6):335-350.
8. Mullonkal CJ, Toledo-Pereyra LH. Akt in ischemia and reperfusion. J Invest Surg. 2009;20(3):195-203.
9. Chen J, Nagayama T, Jin K, Setlter RA, Zhu RL, Graham SH. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci. 1998;18(13):4914-4928.

10. Ruhul AA, Senga T, Oo ML, Thant AA, Hamaguchi M. Secretion of matrix metalloproteinase-9 by the proinflammatory cytokine, IL-1beta: A role for the dual signalling pathways, Akt and Erk. Gene Cell. 2003;8(6):515-523.

11. Fujimura M, Gasche Y, Morita-Fujimura Y, Massengale J, Kawase M, Chan PH. Early appearance of activated matrix metalloproteinase-9 and blood-brain barrier disruption in mice after focal cerebral ischemia and reperfusion. Brain Res. 1999;842(1):92-100.

12. Qiao H, Zhang X, Zhu C, Dong L, Wang L, Zhang X. Luteolin downregulates TLR4, TLR5, NF-kB and p-p38MAPK expression, upregulates the p-ERK expression, and protects rat brains against focal ischemia. Brain Res. 2012;1448:71-81.

13. Kochanek PM, Hallenbeck JM. Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. Stroke. 1992;23(9):1373-1379.

14. Wong CH, Crack PJ. Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury. Curr Med Chem. 2008;15(1):1-14.

15. Ikram MASSBJ. Genomewide association studies of stroke. J Vasc Surg. 2009;467(50):1718-1728.

16. Zhou J, Du T, Li B, Rong Y, Verkrhatsky A, Peng L. Crosstalk between MAPK/ERK and PI3K/akt signal pathways during brain ischemia/reperfusion. Asn Neuro. 2015;7(5):1759091415602463.

17. Justicia C, Panes J, Sole S, Cervera A, Deulofeu R, Chamorro A. Neutrophil infiltration increases matrix metalloproteinase-9 in the ischemic brain after occlusion/reperfusion of the middle cerebral artery in rats. J Cereb Blood Flow Metab. 2003;23(12):1430-1440.

18. Hou S, Li Y, Zhu X, Wang Z, Wang X, Xu Y. Ameliorative effects of diammonium glycyrrhizinate on inflammation in focal cerebral ischemic-reperfusion injury. Brain Res. 2012;1447:20-27.

19. Abe K, Ikeda T, Wake K, Sato T, Sato T, Inoue H. Glycyrrhizin prevents of lipopolysaccharide/D-galactosamine-induced liver injury through down-regulation of matrix metalloproteinase-9 in mice. J Pharm Pharmacol. 2008;60(1):91-97.

20. Cai Y, Zhao B, Liang Q, Zhang Y, Cai J, Li G. The selective effect of glycyrrhizin and glycyrrhetinic acid on topoisomerase Ialpha and apoptosis in combination with etoposide on triple negative breast cancer MDA-MB-231 cells. Eur J Pharmacol. 2017;809:87-97.

21. Wang YM, Du GQ. Glycyrrhizic acid prevents enteritis through reduction of NFkappaB p65 and p38MAPK expression in rat. Mol Med Rep. 2016;13(4):3639-3646.

22. Hou SZ, Li Y, Zhu XL, Wang ZY, Wang X, Xu Y. Ameliorative effects of diammonium glycyrrhizinate on inflammation in focal cerebral ischemic-reperfusion injury. Brain Res. 2012;1447:20-27.

23. Han L, Wang R, Wu B, Gu Y, Yuan Y. Effect of diammonium glycyrrhizinate on pharmacokinetics of omeprazole by regulating cytochrome P450 enzymes and plasma protein binding rate. Xenobiotica. 2019;49(8):975-980.

24. Sathyamoorthy Y, Kaliappan K, Nambi P, Radhakrishnan R. Glycyrrhizic acid renders robust neuroprotection in rodent model of vascular dementia by controlling oxidative stress and curtailing cytochrome-c release. Nutr Neurosci. 2020;23(12):955-970.

25. Wang B, Lian YJ, Dong X, Peng W, Liu LL, Su WJ. Glycyrrhizic acid ameliorates the kynurenine pathway in association with its antidepressant effect. Behav Brain Res. 2018;353:250-257.

26. Guo J, Yang CX, Yang JJ, Yao Y. Glycyrrhizic acid ameliorates cognitive impairment in a rat model of vascular dementia associated with oxidative damage and inhibition of voltage-gated sodium channels. CNS Neurol Disord: Drug Targets. 2016;15(8):1001-1008.

27. Li Y, Yao N, Zhang T, Guo F, Niu X, Wu Z. Ability of post-treatment glycyrrhizic acid to mitigate cerebral ischemia/reperfusion injury in diabetic mice. Med Sci Mon Int Med J Exp Clin Res. 2020;26:e926551.

28. Li Y, Sun F, Jing Z, Wang X, Hua X, Wan L. Glycyrrhizic acid exerts anti-inflammatory effect to improve cerebral vasospasm secondary to subarachnoid hemorrhage in a rat model. Neurol Res. 2017;39(8):727-732.

29. Li Y, Yao N, Zhang T, Guo F, Niu X, Wu Z. Ability of post-treatment Glycyrrhizic acid to mitigate cerebral ischemia/reperfusion injury in diabetic mice. Med Sci Mon Int Med J Exp Clin Res. 2020;26:e926551.

30. Liu B, Yin G, Ding L, Ma Y. Research on the influence of diammonium glycyrrhizinate on the expression of NF-kappaB and neuron apoptosis after spinal cord ischemia-reperfusion injury in rats. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2008;22(12):1466-1469.

31. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke. 1989;20(1):84-91.

32. Chang-lin ZMZY. Glycyrrhizin protects rat heart against ischaemia- reperfusion injury through blockade of HMGB1-dependent phospho-JNK/Bax pathway. Chinese Pharmacological Journal: English version. 2012;33(12):1477-1487.

33. Lau A, Wang S, Liu W, Haig A, Zhang Z, Jevnikar AM. Glycyrrhizic acid ameliorates HMGB1-mediated cell death and inflammation after renal ischemia reperfusion injury. Am J Nephrol. 2014;40(1):84-95.

34. Li Y, Liu T, Yan C, Xie R, Guo Z, Wang S. Diammonium glycyrrhizinate protects against nonalcoholic fatty liver disease in mice through modulation of gut microbiota and restoration of intestinal barrier. Mol Pharm. 2018;15(9):3860-3870.

35. Zhao H, Liu Z, Shen H, Jin S, Zhang S. Glycyrrhizic acid pretreatment prevents sepsis-induced acute kidney injury via suppressing inflammation, apoptosis and oxidative stress. Eur J Pharmacol. 2016;781:92-99.
36. Yan S, Fang C, Cao L, Wang L, Du J, Sun Y. Protective effect of glycyrrhizic acid on cerebral ischemia/reperfusion injury via inhibiting HMGB1-mediated TLR4/NF-kappaB pathway. *Bio-technol Appl Biochem*. 2019;66(6):1024-1030.

37. Lai T, Shen Y, Chen C, Huang B, Deng T, Zhao Z. Glycyrrhizic acid ameliorates myocardial ischemia-reperfusion injury in rats through inhibiting endoplasmic reticulum stress. *Eur J Pharmacol*. 2021;908:174353.

38. Zhai CL, Zhang MQ, Zhang Y, Xu HX, Wang JM, An GP. Glycyrrhizin protects rat heart against ischemia-reperfusion injury through blockade of HMGB1-dependent phospho-JNK/Bax pathway. *Acta Pharmacol Sin*. 2012;33(12):1477-1487.

39. Gao Y, Hao J, Zhang H, Qian G, Jiang R, Hu J. Protective effect of the combinations of glycyrrhizic, ferulic and cinnamic acid pretreatment on myocardial ischemia-reperfusion injury in rats. *Exp Ther Med*. 2015;9(2):435-445.

40. Hua S, Ma M, Fei X, Zhang Y, Gong F, Fang M. Glycyrrhizin attenuates hepatic ischemia-reperfusion injury by suppressing HMGB1-dependent GSDMD-mediated kupffer cells pyroptosis. *Int Immunopharmacol*. 2019;68:145-155.

41. Ni B, Cao Z, Liu Y. Glycyrrhizin protects spinal cord and reduces inflammation in spinal cord ischemia-reperfusion injury. *Int J Neurosci*. 2013;123(11):745-751.

42. Zhu Y, Liu F, Zou X, Torbey M. Comparison of unbiased estimation of neuronal number in the rat hippocampus with different staining methods. *J Neurosci Meth*. 2015;254:73-79.

43. Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol*. 2001;21(3):893-901.

44. Duan P, Wang J, Li Y, Wei S, Su F, Zhang S. Opening of mitoKATP improves cardiac function and inhibits apoptosis via the AKT-Foxo1 signaling pathway in diabetic cardiomyopathy. *Int J Mol Med*. 2018;42(5):2709-2719.

45. Li R, Li J, Sang D, Lan Q. Phosphorylation of AKT induced by phosphorylated Hsp27 confers the apoptosis-resistance in t-AUCB-treated glioblastoma cells in vitro. *J Neuro Oncol*. 2015;121(1):83-89.

46. Vivanco I, Sawyer CL. The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. *Nat Rev Cancer*. 2002;2(7):489-501.

47. Green DR, Reed JC. Mitochondria and apoptosis. *Science*. 1998;281(5381):1309-1312.

48. Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci*. 1998;18(10):3659-3668.

49. Meldrum KK, Meldrum DR, Hile KL, Yerkes EB, Ayala A, Cain MP. p38 MAPK mediates renal tubular cell TNF-alpha production and TNF-alpha-dependent apoptosis during simulated ischemia. *Am J Physiol Cell Physiol*. 2001;281(2):C563-C570.

50. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res*. 2005;15(1):11-18.

51. Cai X, Wang X, Li J, Chen S. Protective effect of glycyrrhizin on myocardial ischemia/reperfusion injury-induced oxidative stress, inducible nitric oxide synthase and inflammatory reactions through high-mobility group box 1 and mitogen-activated protein kinase expression. *Exp Ther Med*. 2017;14(2):1219-1226.

**Appendix**

**Abbreviations**

- CIR: cerebral ischemia-reperfusion
- SD: Sprague Dawley
- MAPK: mitogen-activated protein kinase
- MMP-9: matrix metallopeptidase 9
- WHO: World Health Organization
- I/R: ischemia-reperfusion
- IL-1: interleukin-1
- iNOS: inducible nitric oxide synthase
- COX-2: cyclooxygenase-2
- TNF-α: tumor necrosis factor-α
- NF-κB: nuclear factor-kappa B
- MCAOR: the middle cerebral artery occlusion reperfusion
- DAB: 3,3′-Diaminobenzidine
- CCA: common carotid artery
- ICA: internal carotid artery
- ECA: external carotid artery
- ANOVA: one-way analysis of variance
- Akt: Serine/threonine kinase
- PKB: protein kinases B
- PI3K: phosphatidylinositol 3-kinase
- ERK: extracellular signal-regulated kinase.

---

**Dose-Response: An International Journal**