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Salvianolate increases heat shock protein expression in a cerebral ischemia-reperfusion injury model

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Research Highlights

(1) Salvianolate is a highly concentrated form of salvianolic acid B. This study was the first to investigate heat shock protein expression after salvianolate administration.

(2) Administration of salvianolate during reperfusion after ischemia appears to attenuate brain tissue damage and inhibit neuronal apoptosis by increasing heat shock protein 22 and phosphorylated protein kinase B expression.

Abstract

Stroke remains a worldwide health problem. Salvianolate exerts a protective effect in various microcirculatory disturbance-related diseases, but studies of the mechanisms underlying its protective action have mainly focused on the myocardium, whereas little research has been carried out in brain tissue following ischemia-reperfusion. We assessed the neuroprotective effects of salvianolate in a rat model of cerebral ischemia-reperfusion injury induced using the suture method. At onset and 24 and 48 hours after reperfusion, rats were intraperitoneally injected with salvianolate (18 mg/kg) or saline. Neurological deficit scores at 72 hours showed that the neurological functions of rats that had received salvianolate were significantly better than those of the rats that had received saline. 2,3,5-Triphenyltetrazolium chloride was used to stain cerebral tissue to determine the extent of the infarct area. A significantly smaller infarct area and a significantly lower number of apoptotic cells were observed after treatment with salvianolate compared with the saline treatment. Expression of heat shock protein 22 and phosphorylated protein kinase B in ischemic brain tissue was significantly greater in rats treated with salvianolate compared with rats treated with saline. Our findings suggest that salvianolate provides neuroprotective effects against cerebral ischemia-reperfusion injury by upregulating heat shock protein 22 and phosphorylated protein kinase B expression.

Key Words

neural regeneration; traditional Chinese medicine; brain injury; salvianolic acid B; salvianolate; heat shock protein 22; protein kinase B; cerebral ischemia-reperfusion injury; apoptosis; neuroprotection; neuroregeneration

INTRODUCTION

Stroke remains a worldwide health problem. Restoration of blood flow is the most important and effective way to prevent irreversible tissue injury, but the reperfusion period itself can also lead to tissue damage^[1]. Organs can be protected against reperfusion-indu-

ced injury in two ways: ischemic preconditioning and ischemic postconditioning; the former is a series of brief mechanical occlusion-reperfusion cycles applied before ischemia, and the latter is the same technique applied after the ischemic event^[2]. In the clinic, ischemic preconditioning as a means of protection can only be used in a limited number of patients for whom it is posi-

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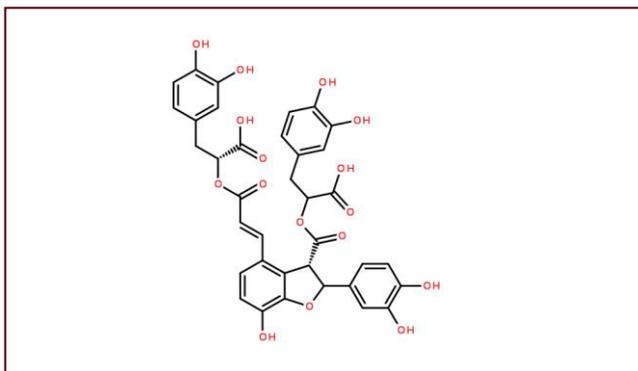
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ble to predict cerebral ischemic events, restricting the use of this technique. Postconditioning can be more widely applied, but therapeutic challenges also remain for this technique. For example, there is a high risk associated with inducing ischemia in an organ that has already suffered a prolonged and severe ischemic insult, and it may not always be possible to reach the artery leading to the ischemic area^[2]. Furthermore, anesthetics and some pharmaceuticals can be used as postconditioning stimuli to exert protective effects, defined as anesthetic or pharmacological postconditioning^[3-4].

Salvia, a traditional Chinese medicine, has been widely used in the clinic to treat various microcirculatory disturbance-related diseases, such as cardiovascular disease, cerebrovascular disease, renal dysfunction, liver fibrosis, and diabetic vascular complications^[5]. Salvianolic acid B, also called salvia magnesium acetate, is a phenolic acid compound composed of three *Danshensu* units and one molecule of caffeic acid. Its molecular formula is C₃₆H₃₀O₁₆, and its chemical structure is as follows:



Salvianolic acid B promotes cell proliferation and differentiation, has anti-oxidant^[6] and anti-apoptotic functions, and contributes to the maintenance of normal cell function^[7-9]. Intervention with salvianolic acid B attenuates ischemia-reperfusion injury after myocardial infarction. Salvianolic acid B exerts strong resistance to oxidative stress owing to its free radical scavenging and lipid peroxidation inhibiting properties. Several studies have demonstrated the neuroprotective effect of salvianolic acid B *in vitro* and *in vivo*^[10-12]. It reduces oxygen-glucose deprivation/reperfusion injury in primary rat cortical neurons by decreasing reactive oxygen species, increasing the activity of Mn-superoxide dismutase, catalase and glutathione peroxidase, and enhancing cell viability^[11]. Salvianolic acid B protects the blood-brain barrier against cerebral ischemia-reperfusion injury by reducing extravasation of immunoglobulin^[13]. SMND-309, a new derivative of salvianolic acid B, can improve neurological deficit scores^[14], attenuate superoxide dismu-

tase production, decrease malondialdehyde content, and increase mitochondrial energy metabolism and respiratory chain complex activity^[15]. Thus, salvianolic acid B provides neuroprotection in permanent cerebral ischemia by maintaining mitochondrial energy production and acting as an antioxidant^[14]. Growing evidence shows the proliferation-inducing activity of salvianolic acid B, which also maintains the self-renewal capacity of neural stem/progenitor cells^[16]. All the above experiments indicate that salvianolic acid B can protect neurons or brain tissue from ischemic-reperfusion damage, but studies of the neuroprotective effects of salvianolic acid B to date have focused on its antioxidant and proliferation-inducing properties, leaving the other neuroprotective mechanisms as yet unstudied.

Heat shock proteins are a group of proteins which are expressed under high temperature and a variety of other physical, chemical and biological stressors^[17]. They have highly conserved structures and function as molecular chaperones^[18-19]. Those with a molecular weight from 12 to 43 kDa are called small heat shock proteins. Heat shock 22 kDa protein (HSP22) is widely expressed in various organs, especially the heart, stomach, muscle, cerebellum and cerebral cortex^[17]. HSP22 is thought to belong to the family of intrinsically disordered proteins and is involved in the mediation of cell signal transduction^[20]. The molecular chaperone activity of HSP22 facilitates the specific degradation of unfolded proteins^[21] and plays an important role in anti-apoptosis, protein folding and translocation, and prevents protein aggregation^[22-23]. It also can help the disaggregation and refolding of denatured proteins and promote the degradation of severely impaired proteins^[22-23]. During ischemia, hypoxia, hibernation and pressure overload injury, HSP22 can activate intracellular AMP-activated protein kinase, phosphatidylinositol 3-kinases/protein kinase B (PKB) and protein kinase C. It also protects the cell against various forms of stress injury, stimulating metabolic signaling pathways and resisting apoptosis^[24-26]. In recent years, HSP22 and other heat shock proteins have been widely studied in the field of myocardial ischemia^[27-30], but few studies have addressed its role in cerebral ischemic-reperfusion injury.

Salvianolate is a highly concentrated and purified form of salvianolic acid B. In the present study, we established rat models of ischemia-reperfusion injury *via* middle cerebral artery occlusion, and administered salvianolate as a pharmacological postconditioning treatment at the onset of reperfusion. The neuroprotective effects of salvianolate were studied by assessing neurological deficit,

infarct volume, morphological changes and cell apoptosis, as well as expression of HSP22, PKB and phosphorylated PKB in brain tissue.

RESULTS

Quantitative analysis of experimental animals

Sixty rats from the same batch were randomly divided into three groups: a sham group (sham operation), a model group (ischemic-reperfusion model + saline) and a salvianolate group (ischemic-reperfusion model + salvianolate). Longa's 5-point scale of neurological deficit^[31] was used to evaluate the success of the model; rats with a score of 0 (no neurological deficit) or 4 (severe neurological deficit with impaired consciousness) were excluded. Subarachnoid hemorrhage or death of the animal were deemed as modeling failure and these rats were also excluded from the analysis. Rats excluded after surgery were supplemented by new rats from the same batch, so that 60 animals were included in the final analysis.

Salvianolate attenuated neurological deficit scores after cerebral ischemia-reperfusion

Focal cerebral ischemia was induced for 2 hours by occlusion of the left middle cerebral artery, followed by a 72-hour reperfusion. Neurological deficit scores were performed at the end of the reperfusion period, immediately before the rats were sacrificed. The sham group showed no neurological deficits, while deficits were observed in both the model group and the salvianolate group. Neurological deficit scores in the salvianolate group were significantly lower than in the model group (0.40 ± 0.55 vs. 1.20 ± 0.84 , $P < 0.05$).

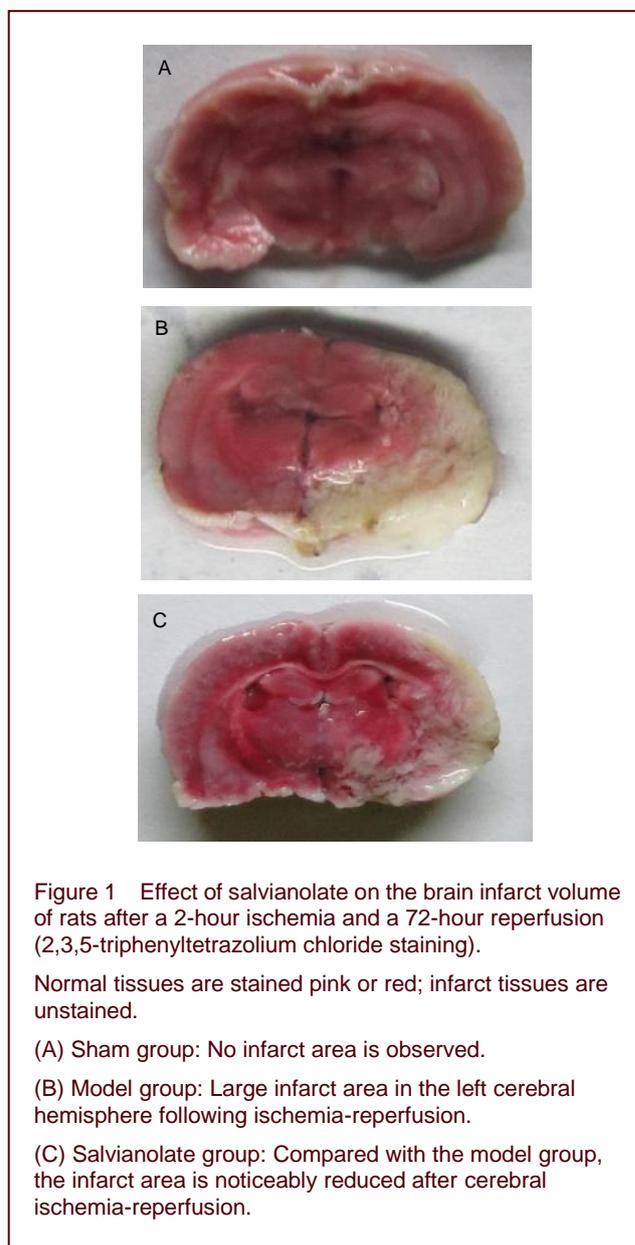
Salvianolate reduced brain infarct volume

2,3,5-Triphenyltetrazolium chloride staining showed a significant infarct area in the model and salvianolate groups after 72 hours of reperfusion (Figure 1). There was no infarct area in the sham group. The total infarct volume (%) was 41.23 ± 9.27 in the model group, and 25.41 ± 2.79 in the salvianolate group. A significantly smaller infarct area was observed after treatment with salvianolate than after saline ($P < 0.05$).

Salvianolate attenuated pathological damage in brain tissue

Hematoxylin-eosin staining showed that in the sham group, brain tissue structure was normal, with tightly-arranged nerve cells, abundant cytoplasm and clear nucleoli. The model group showed an altered brain

tissue structure; nerve cells were distributed in a sparse and irregular manner with shrunken cell bodies, nuclear condensation and fragmentation, and nuclear disappearance; these pathological changes were attenuated in the salvianolate group (Figure 2).



Salvianolate reduced cell apoptosis

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining showed apoptosis in the basal cortex regions of all three groups. Brown, TUNEL-positive cells were scattered throughout the entire ischemic penumbra but were relatively concentrated in some regions. A few TUNEL-positive cells were detected in the sham group; however, large numbers of TUNEL-positive cells were observed in the model and salvianolate groups. The number of TUNEL-positive cells was significantly lower in the salvianolate group com-

pared with the model group ($P < 0.05$; Figure 3).

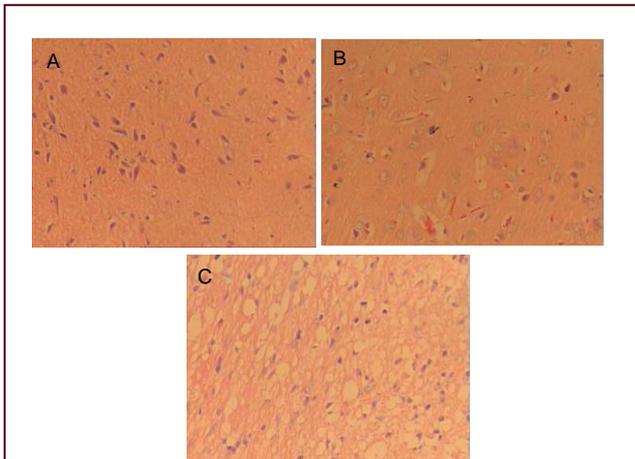


Figure 2 Effect of salvianolate on histopathological changes in the injured brain tissue of rats after a 2-hour ischemia and a 72-hour reperfusion (hematoxylin-eosin staining, light microscope, $\times 400$).

(A) Sham group: Normal structure, no pathological changes observed.

(B) Model group: Destruction of neurons, nuclear condensation and fragmentation.

(C) Salvianolate group: Fewer pathological changes than observed in the model group.

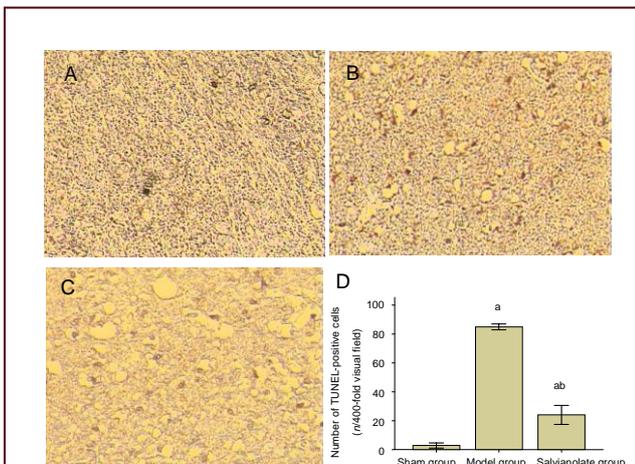


Figure 3 Effect of salvianolate on cell apoptosis in the injured brain tissue of rats with cerebral ischemia-reperfusion injury.

(A–C) Brown cells are terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells undergoing apoptosis (light microscope, $\times 400$).

(A) Sham group: Very few TUNEL-positive cells are observed. (B) Model group: Apoptosis is noticeably increased. (C) Salvianolate group: The number of TUNEL-positive cells is lower after treatment with salvianolate than in the model group.

(D) Quantification of cell apoptosis in the three groups (mean \pm SD; $n = 5$). ^a $P < 0.05$, vs. sham group; ^b $P < 0.05$, vs. model group (one-way analysis of variance with least significant difference t -test).

Effect of salvianolate on HSP22, PKB and phosphorylated PKB expression in rats with cerebral ischemia-reperfusion injury

Western blot analysis was used to determine levels of HSP22, PKB and phosphorylated PKB expression in ischemic cortex. The expression of HSP22 and phosphorylated PKB was significantly greater in the model and salvianolate groups than in the sham group ($P < 0.05$); the salvianolate group showed the greatest expression of these proteins ($P < 0.05$ compared with the model group). There were no differences in PKB expression in ischemic cortex among the three groups ($P > 0.05$; Figure 4, Table 1).

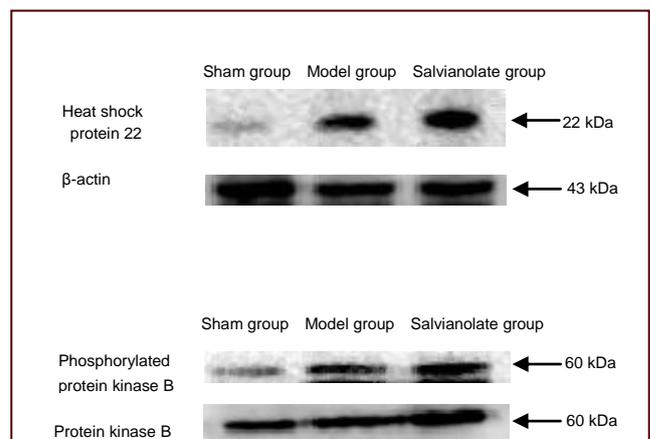


Figure 4 Effect of salvianolate on heat shock protein 22, protein kinase B, and phosphorylated protein kinase B expression in ischemic cortex of cerebral ischemia-reperfusion rats.

Western blot assay shows that heat shock protein 22 and phosphorylated protein kinase B protein levels are both increased after a 2-hour ischemia and a 72-hour reperfusion. Salvianolate treatment increases the levels of heat shock protein 22 and phosphorylated protein kinase B protein.

There is no difference in the total level of protein kinase B protein among the three groups.

Table 1 Quantification of heat shock protein 22, protein kinase B, and phosphorylated protein kinase B expression detected by western blot assay in ischemic cortex of cerebral ischemia-reperfusion rats (expressed as absorbance ratio to β -actin)

Group	Heat shock protein 22	Protein kinase B	Phosphorylated protein kinase B
Sham	0.49 \pm 0.04	0.46 \pm 0.01	0.38 \pm 0.01
Model	0.92 \pm 0.03 ^a	0.47 \pm 0.04	1.06 \pm 0.21 ^a
Salvianolate	1.25 \pm 0.11 ^{ab}	0.49 \pm 0.02	2.00 \pm 0.28 ^{ab}

Data are expressed as mean \pm SD; $n = 5$ rats per group. ^a $P < 0.05$, vs. sham group; ^b $P < 0.05$, vs. model group (one-way analysis of variance with least significant difference t -test).

DISCUSSION

Ischemic postconditioning, consisting of brief cycles of mechanical occlusion and reperfusion performed after an ischemic event^[2], was first carried out in the field of myocardial ischemia but has recently been shown to protect against cerebral ischemia^[32].

Unlike preconditioning, which is restricted to a specific cohort of patients, ischemic postconditioning can be widely applied in the clinic, but some challenges still remain with this technique. Drugs that can imitate a brief ischemia, or that share common mechanisms with postconditioning, may be a preferable substitute for ischemic postconditioning^[2]. In recent years, the protective effects of pharmacological postconditioning have been widely researched.

Salvianolate injections contain over 80% magnesium l-thiospermate B, the main form of salvianolic acid B. Salvianolic acid B is widely used in the clinic after myocardial infarction. The protective effect of salvianolic acid B during reperfusion of ischemic myocardium has been confirmed by many studies. Salvianolic acid B improves blood flow, reduces oxidative injury, improves blood vessel endothelium function, and inhibits the development of coronary disease. Administration of the compound to mice after myocardial ischemia and reperfusion significantly reduces infarct volume, attenuates heart hypertrophy and left ventricular fibrosis^[33], and decreases serum cardiac troponin T and creatinine kinase MB^[34]. Treatment with salvianolic acid B in acute myocardial infarction may inhibit platelet aggregation, enhance angiogenesis, downregulate expression of adhesion molecules on leucocytes and inhibit multiple cytokines^[33]. It also affects cardiac fibroblast migration, improves collagen and cytokine secretion, and prevents ventricular muscle from remodeling^[35]. Salvianolic acid B protects the heart from ischemia-reperfusion injury through many different pathways. Now, the neuroprotective effect of salvianolic acid B after stroke is drawing increasing attention.

In the present study, focal cerebral ischemia was induced in rats by occluding the middle cerebral artery. The rats then received salvianolate during reperfusion, as a form of pharmacological postconditioning. Salvianolate exhibited neuroprotective activity, attenuating the infarct volume compared with rats in the model group that received saline during reperfusion. These results suggest that intervention with salvianolate after stroke will reduce the infarct area and limit brain tissue damage.

Neuronal degeneration and necrosis are the two major pathophysiological changes that occur following cerebral ischemia. Hematoxylin-eosin staining was used to observe neuronal degeneration and necrosis in the brain. The group that received salvianolate showed improved cell morphology and pathological changes compared with the model group; specifically, a significantly lower level of neuronal degeneration and necrosis was observed. Behavioral disturbance deficits are correlated to neuronal degeneration and necrosis^[14]; the present data support this observation, with neurological deficit scores attenuated in the salvianolate group compared with the model group, suggesting that salvianolate improves the recovery of motor function in rats after stroke.

To study the mechanisms of this neuroprotective action of salvianolate against cerebral ischemia-reperfusion injury, TUNEL staining was used to examine apoptotic cells. TUNEL-positive cells were abundant in the model group but scarce in the sham group. Fewer TUNEL-positive cells were detected in the salvianolate group than in the model group, indicating that treatment with salvianolate reduces apoptosis and promotes cell survival. These TUNEL staining data provide direct evidence that the neuroprotective effect of salvianolate is related to its inhibition of apoptosis.

Apoptosis is an important pathway for delayed death and plays a major role in brain ischemia-reperfusion injury. Ischemia leads to neuronal necrosis and apoptosis, and reperfusion further increases the damage. Reperfusion injury comprises impaired blood flow, endothelial and microvascular dysfunction, cellular necrosis and apoptosis^[36]. The phosphatidylinositol-3 kinase-PKB signaling pathway is an important intracellular signal transduction and cell survival pathway. PKB phosphorylation plays a key role in the activation of the phosphatidylinositol-3 kinase-PKB signaling pathway and is required for the anti-apoptotic action of this pathway. Phosphorylated PKB is transported to the cytoplasm or nucleus. After transportation, the privileged sites of substrate proteins are phosphorylated on serine/threonine residues to mediate the regulation of apoptotic proteins or protein kinases and directly inhibit cell apoptosis. Inhibition of apoptosis reduces reperfusion injuries, and maintains and promotes the recovery of cell function. Our data indicate that the level of phosphorylated PKB protein increased after ischemia-reperfusion injury. The highest expression of phosphorylated PKB among the three experimental groups was observed in the salvianolate group. The total expression of PKB protein did not differ between the three groups. Therefore, salvianolate inter-

vention activates PKB pathways and upregulates the expression of phosphorylated PKB, which can in turn directly phosphorylate the pro-apoptotic proteins Bad and Bax, activate the anti-apoptotic protein Bcl-2, inhibit glycogen synthase kinase-3 β phosphorylation, upregulate the level of nuclear factor- κ B and cAMP response element-binding protein, and prevent release of pro-apoptotic proteins from mitochondria in order to inhibit cell apoptosis, thus promoting cell survival^[28].

Ischemia-reperfusion causes extensive damage to the cytoskeleton and leads to mitochondrial swelling and uncoupling of oxidative phosphorylation. General protein synthesis is inhibited after such serious stresses, but heat shock proteins are efficiently translated and synthesized. Heat shock proteins are highly conserved molecules which are activated under environmental stresses, nonstress conditions, pathophysiology, and disease states in cells and tissues, such as ischemia-reperfusion. HSP22 is widely distributed in the muscles, heart and brain^[37]. In recent years, heat shock proteins, especially HSP22, have been widely studied in the field of cardiac ischemia-reperfusion. HSP22 may find aberrant proteins after ischemia-reperfusion damage, refold these denatured proteins and restore their function, limiting detrimental peptide interaction. Alternatively, irreparably damaged proteins and toxic metabolites can be transported to the right intracellular location and degraded. Mitochondria are extremely important in cellular energy metabolism and cell apoptosis in ischemia-reperfusion injury. Under ischemia-reperfusion stress, mitochondria are depolarized and then schizolysed, activating mitochondrial permeability transition pore and apoptosis. HSP22 can reduce the activity of the mitochondrial respiratory chain and the content of reactive oxygen species. It can also maintain mitochondrial integrity and function, reduce damage caused by hypoxia and inhibit the opening of mitochondrial permeability transition pore^[38]. HSP22 directly interacts and binds to both PKB and AMP-activated protein kinase, and promotes their translocation into a nuclear multiprotein complex, which stimulates the survival mechanisms in the cytosol and nucleus, including activation of anti-apoptotic factors (protein kinase C, inducible nitric oxide synthase and Bcl-2), inhibition of pro-apoptotic factors (glycogen synthase kinase-3 β , Bad and FOXO) and inhibition of the release of apoptotic cascade substances, thus suppressing cell apoptosis. Therefore, survival pathway activation induced by HSP22 triggers the anti-apoptotic response to ischemia-reperfusion^[29].

Depre *et al*^[25] found that upregulation of HSP22 expres-

sion accompanied ischemia-reperfusion. Studies in the HSP22 transgenic mouse have confirmed that the protective effects of HSP22 on myocardial ischemia-reperfusion injury are equivalent to ischemic preconditioning^[28]. The protective effect of HSP22 on myocardium has been confirmed by many experiments. However, there are very few studies examining HSP22 expression during cerebral ischemia-reperfusion. The present experiment modeled rats with middle cerebral artery occlusion, and detected HSP22 protein expression under different conditions. In the model group, HSP22 expression was higher than in the sham group. The expression of HSP22 was low under normal conditions and increased markedly under ischemia-reperfusion stress, which stimulates the body to self-protect. This then upregulates HSP22 expression to protect tissue from oxidative damage and apoptosis. After salvianolate intervention, the level of HSP22 protein is much higher than in the model group. Salvianolate increases the expression of HSP22 in ischemic brain tissue; this upregulation may be one of the mechanisms by which salvianolate protects against cerebral ischemia-reperfusion injuries.

In summary, salvianolate can significantly reduce the area of cerebral infarction, neurological impairment score and number of apoptotic cells in rats undergoing cerebral ischemia-reperfusion. It can also improve motor function, promote the recovery of neural function and protect against cerebral ischemia-reperfusion injury by increasing HSP22 expression and PKB activity; this, in turn, prevents protein aggregation, maintains mitochondrial integrity, and inhibits cell apoptosis. Salvianolate shows promise as a drug intervention for ischemic postconditioning.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed at the Experimental Animal Center of Central South University, China from January to March 2012. The molecular biology experiment was performed at the Nephrology Laboratory in the Second Xiangya Hospital, Central South University, China from April to October 2012.

Materials

Animals

Sixty adult male Sprague-Dawley rats, of specific pa-

thogen free grade, weighing 240–260 g, were obtained from the Experimental Animal Center of Central South University, China (license No. SYXK (Xiang) 2011-0001). All procedures conformed to the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[39].

Drug

Salviaolate injections were brought from Green Valley Corp, Shanghai, China (Zhunzi Z20050248, containing magnesium lithospermate B 80%).

Methods

Focal cerebral ischemia-reperfusion model

Focal cerebral ischemia was induced by the middle cerebral artery occlusion procedure as described previously^[40]. Briefly, rats were anesthetized with 10% chloral hydrate. The right common carotid artery, external carotid artery and internal carotid artery were carefully isolated. Microvascular aneurysm clips were applied to the right common carotid artery bifurcation and external carotid artery. A monofilament (Sunbio Corp., Beijing, China) was introduced into the arteriotomy hole of the common carotid artery, and was fed into the internal carotid artery until a mild resistance was felt. Focal cerebral ischemia began. The length of the suture from the common carotid artery bifurcation to the middle cerebral artery was 18–22 mm, according to the weight of experimental rats. The end portion of suture was fixed in the skin. After 2 hours of ischemia, the filament was carefully removed and reperfusion was started. The sham group received all of the surgical procedures except occlusion of the middle cerebral artery. Animal body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ during the whole period of ischemia.

Drug intervention

Salviaolate (18 mg/kg) was dissolved in 5 mL of 9% physiological saline. The salviaolate group received the drug *via* intraperitoneal injection at the onset of reperfusion and at 24 and 48 hours after reperfusion. The model group and sham group received an equivalent volume of saline intraperitoneally.

Neurological deficit score

At the end of the 72-hour reperfusion, the neurological deficit score was performed by an investigator who was blind to the grouping, according to Longa's method^[31]: a score of 0 indicated no observable neurological deficit; 1 point, a mild focal neurological deficit (failure to extend left paw fully); 2 points, a moderate focal neurological deficit (circling to the left); 3 points, a severe focal deficit

(falling to the left); 4 points, no spontaneous walking, consciousness impaired.

Evaluation of infarct volume

Brain slices were stained with 2,3,5-triphenyltetrazolium chloride^[41] to identify the ischemic infarct area. After 72 hours of reperfusion, rats were deeply anesthetized with 10% chloral hydrate and decapitated. Their brains were quickly removed and frozen, then five sections of 2 mm thickness were cut coronally (the frontal pole, occipital pole, cerebellum and lower brainstem were discarded). The slices were incubated in 2,3,5-triphenyltetrazolium chloride solution (Beyotime Institute of Biotechnology, Shanghai, China) for 30 minutes at 37°C in darkness, then removed and placed into 10% paraformaldehyde overnight. Normal tissue was stained red or pink and infarcted tissue remained unstained. Each slice was photographed and the infarct areas were measured using Image J software (National Institutes of Health, Bethesda, Maryland, USA). To avoid the effects of edema on infarction size, the noninfarcted area was calculated from the contralateral side. The area of infarction was presented as a percentage of normal hemisphere area, calculated according to the formula: $\text{infarction area}/\text{contralateral hemisphere area} \times 100\%$.

Observation of pathological tissue using hematoxylin-eosin staining

Pathological changes in the brain after ischemia-reperfusion were examined using hematoxylin and eosin. Paraffin-embedded sections (5 μm) of the infarction area were baked in the oven at 60°C for 30 minutes before dewaxing in dimethylbenzene. The sections were hydrated through a series of alcohols (100%, 95%, 85%, 75%; 2–4 minutes per stage), washed in distilled water, dyed with hematoxylin for 5 minutes and rinsed with distilled water till the water turned clear, then dipped in 1% hydrochloric acid for 10–20 seconds. Sections were stained with eosin for 5 minutes after being rinsed in distilled water for 10 minutes, dehydrated through a second series of graded alcohols (75%, 85%, 95%, 100%), placed into dimethylbenzene for 8 minutes, and mounted with neutral gum and dried at 37°C . Sections were observed under a light microscope at $400 \times$ magnification.

Detection of cell apoptosis

Apoptotic cells were detected by TUNEL staining. Dewaxed sections were incubated with proteinase K (ST533, Beyotime) at 37°C for 15–30 minutes to permeabilize them, then rinsed in PBS. Sections were placed in 3% H_2O_2 for 10 minutes at room temperature

and washed in PBS. Following incubation with 50 μ L labeling buffer per sample for 1 hour at 37°C in a humidified chamber, and a PBS wash, the labeling reaction was performed using 0.2 mL labeling reaction termination liquid per sample, and incubated for 10 minutes at room temperature followed by another PBS wash. Slices were incubated with streptavidin-horseradish peroxidase solution (C1098, Beyotime) for 30 minutes at room temperature in a humidified chamber. After another PBS wash, the sections were placed in diaminobenzidine solution (C1098, Beyotime) and washed again with PBS before being dehydrated in alcohol and mounted. TUNEL was conducted using the Colorimetric TUNEL Apoptosis Assay Kit (C1098, Beyotime) and according to the manufacturer's instructions. Slices were observed under high magnification and positively-stained cells were counted in eight areas then averaged to get a result for that animal.

Western blot analysis

Brain tissues (ischemic cortex) were resuspended in cell lysis buffer mixed with protease inhibitor cocktail for 30 minutes in ice. The solution was centrifuged at 12 000 \times g for 10 minutes at 4°C. The supernatant was extracted and incubated with sodium dodecylsulfate polyacrylamide gel electrophoresis sample loading buffer (P0015, Beyotime) at 95°C for 5–10 minutes. Protein content was detected using the bovine serum albumin protein assay kit (P0010, Beyotime) according to the manufacturer's instructions. A total of 20 μ g protein was electrophoresed on 10% sodium dodecylsulfate polyacrylamide electrophoresis gel and transferred onto polyvinylidene fluoride membranes using wet-phase inversion. The membranes were blocked with 5% calf serum albumin in PBS containing 0.1% Tween 20 (PBS-Tween) for 1 hour at room temperature. Following three 10-minute washes in PBS-Tween, the membranes were incubated with rabbit anti-rat monoclonal antibodies to HSP22 (1:800; Cell Signaling Technology Inc, Beverly, Massachusetts, USA), PKB (1:800; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated PKB (1:900; Santa Cruz Biotechnology) and β -actin (1:1 000; ZSGB-BIO Corp, Beijing, China) overnight at 4°C and washed three times for 10 minutes with PBS. The membranes were incubated with a peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H + L) (1:5 000; ZSGB-BIO Corp) for 2 hours at room temperature, visualised using ECL reagent (Amersham Biosciences, Little Chalfont, UK) and the gels were photographed with a Kodak 4000MM imager (Rochester, New York, NJ, USA). The relative absorbance of the bands was analyzed by Quantity-One image analysis

software (Bio-Rad Corp, Hercules, CA, USA).

Statistical analysis

The statistical analysis of these experimental data was performed using SPSS 18.0 software (SPSS, Chicago, IL, USA) and all data are presented as mean \pm SD. One-way analysis of variance was used to compare multiple groups, with least significant difference *t*-test. A value of *P* < 0.05 was considered statistically significant.

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