

Original Research Article

Shenxiong Drop Pill exerts neuroprotective effect against focal cerebral ischemia partly via regulation of the expressions of ICAM-1 and caspase-3

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Abstract

Purpose: To investigate the effect of Shenxiong Drop Pill (SXD) on cerebral infarction (CI) in rats, and the involvement of anti-inflammatory response in the process.

Methods: Rats were sacrificed at three different time points, viz, 24, 48 and 72 h after establishment of CI model. Neurological deficit score (NDS) was determined using Bederson's neurological behavioral scoring method, whereas triphenyltetrazolium chloride (TTC) staining was used to show brain injury. The integrated optical density (IOD) of Nissl bodies and caspase-3-positive nerve cells were measured with Nissl staining and SP kit, respectively. The mRNA expression of intercellular adhesion molecule 1 (ICAM-1) was determined using reverse transcription-polymerase chain reaction (RT-PCR).

Results: SXD produced neuroprotective effect at high, medium, and low doses. The infarct volumes in the high-, medium- and low-dose SXD, and cyclophosphamide groups were significantly reduced at each time point. Different doses of SXD significantly reduced the mRNA expression of ICAM-1 and the IOD of caspase-3.

Conclusion: These results indicate that SXD exerts neuroprotective effects against ischemic injury by negatively regulating ICAM-1/caspase-3 downstream of inflammatory and apoptosis pathways.

Keywords: Acute ischemic stroke, Neuroprotection, Inflammation, Middle cerebral artery occlusion, Caspase-3, Intercellular adhesion molecule 1

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INTRODUCTION

Stroke is a common disease worldwide. It is the third leading cause of death amongst human diseases, and the most common cause of disability [1]. Acute ischemic stroke, the most

common type of stroke, accounts for 90 % of strokes, and it is associated with high morbidity and mortality [2]. Rapid complete reperfusion is a regular technique used in animal models. However, in clinical experience, only a small percentage of stroke patients (approximately 10

%) achieve complete reperfusion, with timely treatment using tissue plasminogen activator (tPA) and thrombectomy. Indeed, most patients seldom achieve recanalization [3]. The main strategy used for acute stroke treatment is to find effective neuroprotective agents that protect nerve cells. Blockage of blood vessels leads to neuroinflammation which may trigger cell death. If the repair of vascular inflammation is enhanced, the adverse consequences of stroke may be reduced [4]. Apoptosis is considered to be the major contributor to neuronal loss after cerebral ischemia, and it is related to the enlargement of infarct areas [5].

A lot of research effort has been made in order to understand the implications of neuroinflammation and apoptosis in stroke [4]. Studies have been carried out on various treatments for reducing the risk of stroke via reductions in inflammation and apoptosis, and further research is still ongoing.

Shenxiang Drop Pill (SXDP) is comprised of *Astragalus* saponins, total salviolic acid, and volatile oils from *Ligusticum chuanxiong*. It has a positive effect on acute ischemic stroke, as indicated in previous studies [6,7]. Intercellular adhesion molecule-1 (ICAM-1) plays a key role in the accumulation of granulocytes after focal cerebral ischemia. Deficiency of ICAM-1 reduces impairment of microcirculation and infarct size after cerebral ischemia [8]. Caspase-3 protein is a major indicator of apoptosis. Inhibition of caspase-3 activity reduces infarct size [5]. Despite what is already known, only few studies have been conducted on the dynamics of ICAM-1 and caspase-3 in ischemic stroke.

In this study, the relationship between ICAM-1 and caspase-3 in the penumbra at 24, 48 and 72 h post-permanent ischemic stroke, was investigated. The findings may provide new treatment strategy for ischemic stroke.

EXPERIMENTAL

Animals

Male adult SD rats weighing 250 - 300 g were obtained from the Animal Experimental Center of Chengdu University of TCM. The rats were raised under standard conditions before and after surgery in the Time Biology Laboratory of Chengdu University of TCM, at room temperature range of 23 - 25 °C, and were fed normally for 7 days to adapt them to the laboratory environment. Thereafter, the rats were randomly assigned to seven groups: normal control, sham-operated, model and cytoxin groups, as well as high-dose SXDP, medium-

dose SXDP, and low-dose SXDO groups. The study was approved by the Laboratory Animal Welfare and Ethics Committee of the Chengdu University of TCM, China (approval no. 2016-13). All experimental procedures involving rats were undertaken according to the principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, as published by the National Science Council, China. Fresh feed and water were provided *ad libitum* to all animal groups.

Drugs and reagents

Shenxiang Drop Pill (SXDP) was produced by the Pharmacy School of Chengdu University of TCM (Patent no. ZL200910262686, China) as a suspension at a concentration of 2 g/mL. The rats received SXDP via gavage administration of the suspension prior to MCAO surgery. The intragastric doses for the SXDP groups were determined in line with the TCM clinical dosing guidelines (1 g/kg). In this guideline, the high-dose was 20 times the clinical dose; the medium dose was ten times the clinical dose, and the low-dose was five times the clinical dose. The drug was administered intragastrically 8 days before MCAO surgery. At the same time, the cyclophosphamide group received intragastric administration of SXDP suspension at a dose of 15 mg/kg (the dose was based on the minimum dose for body surface area, and the coefficient for 200-g rat was 0.018). The control, sham-operated and MCAO groups were administered equivalent volumes of normal saline daily, in place of SXDP. The other reagents used, and their sources (in parenthesis) were: cytoxin (HC8239, BioBomei, China); caspase-3 p20 goat polyclonal IgG (sc1226, Santa Cruz, USA), TRIZOL Reagent ICAM-1 (sc-71303, Santa Cruz, USA); TTC (ST2335, BioBomei, China); hematoxylin solution (C200301, Best Sun, Zhuhai, China); eosin in alcohol solution (C200403, Best Sun, Zhuhai, China), and methylene blue trihydrate (190410, Regal Biology, Shanghai, China).

Establishment of MCAO rat model

The rats were subjected to MCAO as indicated previously [6]. The rats were anesthetized with 2 % sodium pentobarbital (F200209150; Shanghai Chemical Reagent Company, China). Then, each rat was placed in a supine position, with the limbs were fixed. The internal and external carotid arteries were gradually separated and threaded separately. The distal end of the external carotid artery was tied and threaded at the proximal end. The spare lines of the common and internal carotid arteries were gently pulled to

temporarily block blood flow. A 5-8 mm cut was made from the bifurcation of the external carotid artery, and a fishing line was inserted into the cut and into the internal carotid artery through the bifurcation of the common carotid artery, to lengths of 18 - 20 mm, and the entrance of the MCA was deemed blocked when resistance was felt. The proximal end of the external carotid artery was ligated, and the internal carotid artery suture was drawn out to stop bleeding. Then, the incision was sutured layer-by-layer.

NDS test

In accordance with Bederson method [10], the neurological deficit score was determined and recorded 24 h after the establishment of the MCAO model. The scoring criteria were as follows: 0 = no symptoms of nervous system damage; 1 = moderate forelimb flexion; 2 = leaning to the paralyzed side when walking; 3 = falling to the opposite side of the lesion; and 4 = inability to walk alone. The people who conducted the tests and analyses were blinded to the various treatments.

Sample processing

After the neurologic deficit score was established, the rats were euthanized via intraperitoneal injection of 2% pentobarbital sodium at 24, 48 and 72 h after the establishment of MCAO, and the brains of rats in each group were excised. The brain samples were divided into two parts: one part was used for the measurement of cerebral infarction volume, while the other was fixed in 4% paraformaldehyde, embedded in paraffin, and sliced for hematoxylin-eosin (H & E) staining.

H & E staining

Deparaffinized sections were left in hematoxylin solution for 12 min, washed with domestic water for 5 min, and differentiated with 1 % hydrochloric acid solution for 30 sec. Then, the sections were washed with domestic water for 5 min, blued with 1 % ammonia water for 10 sec, rinsed again with domestic water for 20 min, and placed in 95 % alcohol for 3 min. Then, the sections were fixed in 1 % eosin in alcohol for 5 min. Thereafter, the sections were dehydrated in alcohol gradient (95 % alcohol for 2 min, anhydrous alcohol I for 2 min, and anhydrous

alcohol II for 2 min), followed by clearing in xylene I for 5 min, and in xylene II for 5 min. The transparent sections were mounted on glass slides using neutral gum, and covered with cover slips prior to drying in a 37 °C oven.

Determination of cerebral infarction volume

Triphenyltetrazolium chloride (TTC) staining was used for measurement of cerebral infarction volume. The rat brain was frozen at -20 °C for 15 min and sectioned into 2-mm slices which were placed in 2 % TTC solution at 37 °C for 20 min, and fixed with 4 % paraformaldehyde for 24 h. The principle of this test is that TTC chemically reacts with mitochondrial respiratory enzymes to produce a bright red color that contrasts clearly with the color of the infarct. The Image-pro Plus 6 software was used to analyze digital images of the TTC-stained brain slices.

NISSL staining of rat brain sections

Brain sections were used for Nissl staining with methylene blue trihydrate. The images were taken using Olympus image system (IX-B50, Olympus Co. Ltd., Japan), while Image-pro Plus 6 software was used for semi-quantitative analysis of the Nissl staining results at a magnification of 200. The IOD of Nissl body staining was measured in unit visual field in the normal, sham-operated and model groups; in the different SXDP-dose groups, and in the cyclophosphamide group. Five IOD values that expressed the strongest visual field for each specimen, were used as the measured values of each sample.

Expression of ICAM-1 mRNA

Real time-PCR was used to determine the expression of ICAM-1 mRNA. Total RNA was extracted from the brain tissue using TRIzol single-step method, and the RNA was preserved at -80 °C. After the extraction of total RNA from brain tissue sections, agarose gel electrophoresis was used to purify it, and the RNA was visualized under UV light. The primers for ICAM-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by PCR primer design and synthesis. The RT-PCR was performed, with GAPDH as the internal reference gene. The sequences of the primers used are shown in Table 1.

Table 1: Primer sequences used for RT-PCR

| Gene | Upstream primer sequence 5'-3' | Downstream primer sequence 5'-3' | Length (bp) |
|--------|--------------------------------|----------------------------------|-------------|
| ICAM-1 | 5'-GGGTTGGACTAACTGGATGA-3' | 5'-GGATCGAGCTCCACTCGCTC-3' | 181 |
| GAPDH | 5'-CGGAGTCAACGGATTTGGTCGTAT-3' | 5'-AGCCTTCTCCATGGTGAAGAC-3' | 306 |

The DNA content was density-scanned using the gel imaging analysis system, and the area under the corresponding GAPDH scan curve was standardized to represent the relative expressions of ICAM-1 mRNA. Total RNA (1 ug) was used in a 50 uL reaction system. The PCR conditions were as follows: cDNA synthesis and pre-denaturation involved one cycle of reverse transcription at 50°C for 30 min, and at 94°C for 2 min. The PCR amplification was carried out in 29 cycles, including denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, elongation at 72 °C for 30 sec, and a final extension step at 72 °C for 10 min. The ICAM-1 can amplify a cDNA fragment with a length of 181 bp, while GAPDH can amplify a cDNA fragment with a length of 306 bp.

Caspase-3 staining of rat brain tissue sections

The SP method was used to display caspase-3. The sections were deparaffinized for 15 min, and rinsed twice in distilled water and 0.02 M PBS. Then, the sections were cooled at room temperature for 15–20 min and rinsed three times in 0.02 M PBS. Ten percent normal goat serum (diluted in 1:20 PBS) was used to mount the slides, which were incubated for 20 min in a humidified cabinet at 37 °C. Filter paper was used to remove the blocking agent, followed by addition of 50 µL goat anti-rat polyclonal antibody (1:100 dilution) and incubation at 37 °C for 60 min. Thereafter, the slides were rinsed thrice with 0.02 M PBS, after which they were incubated with the secondary antibody i.e., biotin-labeled rabbit anti-goat IgG, for 30 min in a humid box at 37 °C, and rinsed three times with 0.02 M PBS (each rinse for 5 min). Second-generation horseradish enzyme-labeled streptavidin was added, and the slides were incubated for 30 min in a humid chamber at 37 °C, and rinsed three times with 0.02 M PBS. The samples were then stained with DAB, counterstained with hematoxylin, dehydrated, permeabilized, fixed and observed under a light microscope. The images were taken using Olympus image system (IX-B50, Olympus Co. Ltd., Japan), while Image-pro Plus 6 software was used for semi-quantitative analysis of the caspase-3 staining results, at a magnification of 200.

The IOD of caspase-3 staining was measured in unit visual field in the normal, sham, model and cyclophosphamide groups, as well as in the different SXDP-dose groups. Five fields with the strongest expression were selected from each specimen section, and the IOD of caspase-3-positive nerve cells in each field was measured. The IOD value of positive cells in five fields of

each specimen was taken as the measured value of the specimen.

Statistical analysis

The data are presented as mean \pm SD, and were statistically analyzed using SPSS 22.0 software. Differences between and amongst the groups were analyzed using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of SXDP on histopathology of brain tissue

The neurons in the normal group and the sham operation group had clear cellular outlines, and they were arranged closely, with intact cellular morphologies. In the model group, varying sizes liquefaction necrotic areas were seen in the cerebral cortex, with neurons disappearing in the areas of necrosis. Moreover, pyknotic nuclei were visible at the edge of some infarcted areas. Edematous cells, fuzzy cell outlines, severe cell deformation, and varying degrees of inflammatory cell infiltration were also observed. However, these abnormalities were markedly alleviated in the SXDP and cytoxin groups (Figure 1).

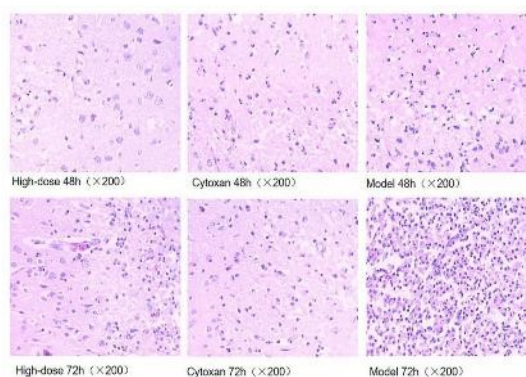


Figure 1: Effect of SXDP pretreatment on cerebral cortical histopathology in rats, as determined using H & E staining

SXDP pretreatment reduced the area of cerebral infarction

The brain tissue specimens of the normal and sham operation groups showed no infarcts. However, as shown in Figure 2, compared with the model group, the infarct volumes at each time point at 24, 48 and 72 h in the high-dose SXDP, medium-dose SXDP, low-dose SXDP and cytoxin groups, were significantly reduced ($p <$

0.05). Moreover, the cerebral infarction volume at 48 and 72 h were markedly higher than that at 24 h ($p < 0.05$). The treatment with SXDP reduced cerebral infarction volume in a dose-dependent fashion ($p < 0.05$). The results for the cyclophosphamide group and SXDP high-dose group were similar ($p > 0.05$) (Figure 2).

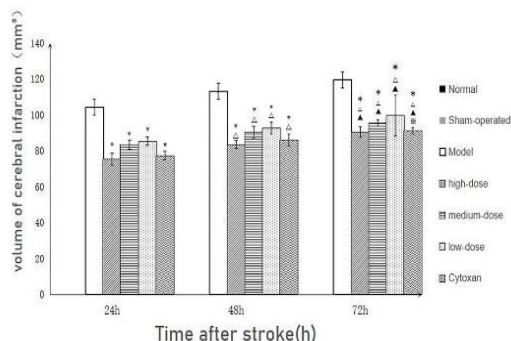


Figure 2: Effect of SXDP pretreatment on cerebral infarction volume of each group. Results are expressed as mean \pm SD. * $P < 0.05$ vs. model group at same time after stroke; $\Delta P < 0.05$ vs. corresponding group at 24 h; $\blacktriangle p < 0.05$ vs. corresponding group at 48 h; $\ast p > 0.05$, vs. high-dose at the same time point after stroke

SXDP pretreatment increased expression of Nissl body

The integral optical density of Nissl bodies differed significantly between the normal group and the sham-operated group ($p > 0.05$). The integral optical density of Nissl body in the model group was significantly lower than those of the normal and the sham-operated groups ($p < 0.05$). After infarction, the integral optical density of Nissl body gradually decreased with time, when compared with values at 24, 48 and 72 h ($p < 0.05$). Compared with the normal group and the sham-operated group, the integral optical density of Nissl body in the high-dose SXDP, medium-dose SXDP, low-dose SXDP, and cytoxin groups were significantly lower ($p < 0.05$). In contrast, treatment with SXDP resulted in dose-dependent increases in the expressions of Nissl body. However, the efficacies of the cytoxin group and SXDP high-dose group were comparable ($p > 0.05$; Figure 3).

SXDP pretreatment reduced mRNA expression of ICAM-1

The mRNA expression levels of ICAM-1 in the normal and sham-operated groups were comparable and very weak ($p > 0.05$). In contrast, the expression of ICAM1 mRNA in the model group increased significantly, peaked at 24 h, and decreased at 48 and 72 h, when compared with the normal and sham-operated

groups ($p < 0.05$). However, treatment with cytoxin and different doses of SXDP led to significant reductions in the expression of ICAM-1 mRNA ($p < 0.05$). A dose-dependent effect was observed with DXDP on the expression of ICAM-1 mRNA, but not with Cytoxin (Figure 4).

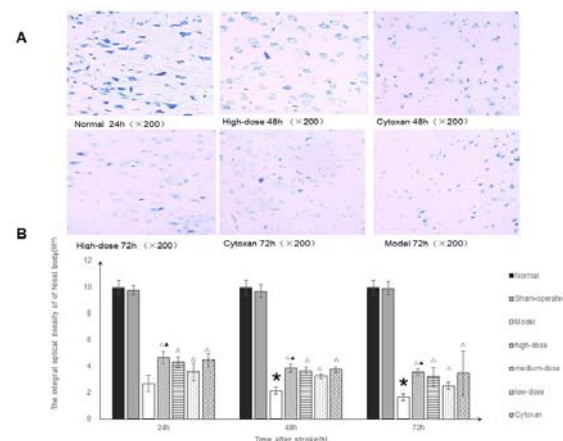


Figure 3: Effect of SXDP pretreatment on the integral optical density of Nissl bodies in each group. Values are expressed as mean \pm SD. * $P < 0.05$, vs. model at 24 h; $\Delta p < 0.05$, vs. model group at the same time point after stroke; $\blacktriangle p > 0.05$, vs. cytoxin at the same time point after stroke

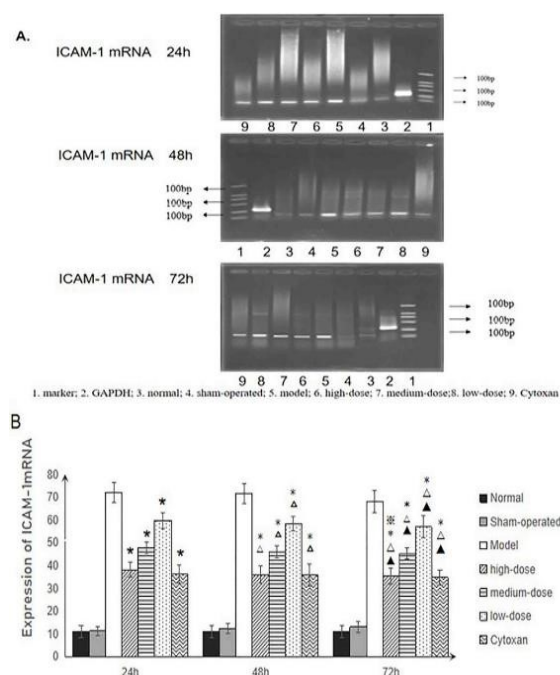


Figure 4: Effect of SXDP pretreatment on ICAM-1 mRNA expression level in each group. Values are expressed as mean \pm SD. * $P < 0.05$, vs. model group at the same time point after stroke; $\Delta p < 0.05$, vs. model group at 24 h; $\blacktriangle p < 0.05$, vs. model group at 48 h; $\ast p > 0.05$, vs. cytoxin group

SXDP pretreatment significantly inhibited the activation and expression of caspase-3

Figure 5 shows that the expression levels of caspase-3 in the normal and sham-operated groups were comparable and very weak. However, after blocking the middle cerebral artery, caspase-3 was activated. The IOD of nerve cell caspase-3 was increased significantly in the model group at 24 h, and it was gradually increased at 48 and 72 h, with significant differences amongst values at the various time points ($p < 0.05$). However, after drug intervention, the IOD of caspase-3 was significantly reduced, relative to the model group ($p < 0.05$). There was no significant difference in the IOD of caspase-3 between the cytoxin group and the high-dose CXDP group ($p > 0.05$).

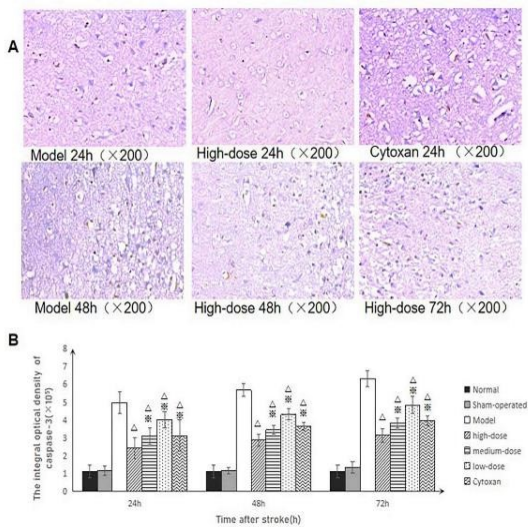


Figure 5: Effect of SXDP pretreatment on the expression levels of caspase-3 in each group. Values are expressed as mean \pm SD. $\Delta P < 0.05$, vs. model group at the same time point after stroke; $\times p < 0.05$, vs. high-dose SXDP at the same time point after stroke

DISCUSSION

Cerebral ischemia injury triggers inflammatory cascade reactions which cause the entry of peripheral immune cells into the brain tissue, thereby aggravating inflammation and cell apoptosis post-ischemia. Studies have been carried out on various treatments for reducing the risk of stroke via reduction of inflammation and apoptosis, and further research is still in progress. Leukocytes have been shown to play important roles in activation of the ischemic inflammatory cascade.

Secondary neurovascular damage to the

vascular endothelial cell adhesion molecules in the brain is a key factor in the recruitment of leukocytes to inflammation sites. The most extensively studied adhesion molecule is ICAM-1. Leukocytes adhere closely to endothelial cells in ischemic stroke through ICAM-1. This enhances the penetration of the blood brain barrier (BBB) by immune cells, thereby aggravating brain tissue damage which leads to cerebral edema and ischemic penumbra infarction [12]. Studies have shown that ICAM-1 expression is markedly increased by hypoxia within 4 h after onset of ischemia [13]. This is thought to facilitate the binding of ligands and activation of leukocytes, thereby enhancing their entry into the CNS in an inflammatory environment. Thus, ICAM-1 may be a very important effector in the regulation of the infiltration of leukocytes through the BBB in ischemic stroke. Several research groups have studied the use of ICAM-1 for preventing the recruitment of immune cells and for minimizing secondary inflammatory responses in stroke.

Apoptosis is a homeostatic mechanism that controls the population of cells. It also plays an important role after brain damage caused by ischemia and hypoxia. Apoptosis induced by ischemic stroke occurs mainly in neurons. In animal models of ischemic cerebral infarction, caspase-3 has been identified as a key mediator of apoptosis. Research has demonstrated the upregulation of caspase-3 in rodent and human brains after focal ischemic attack [14,15]. Moreover, the inhibition of apoptosis is an important method for preventing brain damage during ischemia [16,17]. It has been reported that the combination of N-acetyl-seryl-aspartyl-lysyl-proline AcSDKP) and tPA significantly reduced infarct size in cerebral tissues, decreased adverse neurological outcomes, and inhibited tPA-induced leakage of aged endothelial cells, thereby down-regulating the expression of ICAM-1 [18].

In the present study, RT-PCR and immunohistochemical staining were used to determine the effect of SXDP on cell apoptosis and inflammation. Moreover, the expression levels of the major indicators i.e., cleaved caspase-3 protein and ICAM-1 mRNA were measured. The results showed that ischemic infarction was significantly reduced by SXDP in the stroke model of MCAO rats, and the neurological outcomes of the rats were markedly improved. The IOD of nerve cell caspase-3 was significantly increased in the model group at 24 h, and was slightly increased at 48 and 72 h. The effect of high-dose SXDP was similar to that of cyclophosphamide. The inhibitory effect of high-

dose SXDP on caspase-3 expression was stronger than that of cyclophosphamide. The mRNA expression of ICAM-1 in the model group increased significantly, peaked at 24 h, and then decreased slightly at 48 and 72 h.

However, SXDP decreased the expression levels of caspase-3 mRNA and ICAM-1 mRNA. These results show that SXDP exerted a neuroprotective effect against cerebral ischemia partly through targeted downregulation of mRNA expressions of caspase-3 and ICAM-1. These findings may provide a new therapeutic strategy for cerebral ischemia.

Many natural compounds have been shown to be safe and beneficial for treatment of stroke. It has been reported that salvianolic acid D (SalD) alleviated neurological impairment, decreased cerebral infarction, and inhibited the expressions of Bax, cytochrome c and reduced caspase-3 and caspase-9 [19]. These results also indicate that there were temporal differences in the activation status of ICAM-1 and caspase-3: the expression of caspase-3 was increased later than that of ICAM-1. This suggests that therapeutic targeting of these pathways should consider their unique temporal dynamics.

It is possible that inflammation plays a different role in ischemic stroke. The dual and opposing effects of inflammatory damage and repair complicate attempts at targeting inflammatory signals in stroke patients [20]. It appears that beneficial or harmful effects of the neuroinflammatory mechanism depend on the post-cerebral ischemia period. At the early stage of ischemic stroke, inflammation may cause ischemic damage to brain tissues, while in the later stage, inflammation may be a neuroprotective effect which promotes angiogenesis and recovery, as well as plasticity of brain nerves through repair [21]. Hence, a better characterization of time-dependent effects of neuroinflammation may contribute to the development of neuroprotective strategies after stroke.

CONCLUSION

These findings indicate that SXDP negatively regulates the downstream inflammation and apoptosis pathways of ICAM-1/caspase-3, inhibits inflammatory response in the early stage of cerebral infarction in MCAO model rats, and reduces inflammatory damage after cerebral ischemia. However, the molecular pathway involved in the neuroprotective effect of SXDP requires further investigation.

DECLARATIONS

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the study reported in this manuscript.

Contributions of authors

We declare that this work was done by the authors named in this manuscript, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shuo-guo Jin, Zeran Chen and Yang Zhang, as first co-authors, contributed equally to this work. All three of them conducted the experimental aspect of the research, performed statistical analysis, and prepared the initial draft of the manuscript. Fang Yang, Xin-xia Zhang and Wei-yin Chen conceived and designed the study. Zeran Chen and Ningjing Ran collected and analyzed the data. Shuoguo Jin, Ze-ran Chen and Yang Zhang wrote the manuscript. Xin-xia Zhang supervised the project. Mengyuan Huang and Meng Hou helped to complete the statistics form.

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