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Original Article

Neuroprotective effects of Shi-da-La-zhi Wan in rats with cerebral ischemia/reperfusion-induced neuronal injury

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Abstract: The present study aimed to investigate the therapeutic function of Shi-da-La-zhi Wan (SDLZ) in rat models to ameliorate cerebral ischemia/reperfusion (I/R) injury. SD rats were divided into four groups: sham operation, model, SDLZ and Ginaton groups. The cerebral I/R injury in rat models were induced through middle cerebral artery occlusion (MCAO). Zea longa's scoring scale was used to assess the neurological function of the rats. TUNEL staining assay was conducted to observe cell apoptosis in the ischemic penumbra of brain. Immunohistochemistry and western blot analysis were carried out to detect Mcl-1, caspase-3 and caspase-9 proteins expression in the ischemic penumbra of brain. We found that SDLZ significantly improved the neurological function of the rats with cerebral I/R injury, and evidently decreased the number of apoptotic cells induced by I/R in the ischemic penumbra of brain. Furthermore, SDLZ obviously suppressed caspase-3 and caspase-9 and promoted Mcl-1 protein expression levels in the ischemic penumbra of brain. Thus, the present article has provided evidence that SDLZ exerts an exciting therapeutic function in treatment of cerebral I/R injury by inhibiting neuron apoptosis in the ischemic penumbra of brain.

Keywords: Cerebral ischemia/reperfusion, Shi-da-La-zhi Wan, neurological function, apoptosis, Mcl-1, caspase-3, caspase-9

Introduction

Stroke, including haemorrhagic and ischemic stroke, is regarded as one of the most prevailing reasons for mortality and disability in adults globally. Ischemic stroke is the most common kind of stroke, which is induced by a drastic loss of blood supply to part of the brain [1]. As a complex abnormality, ischemic stroke involves multi-processes, including oxidative stress [2], calcium overload [3], increased excitotoxicity [4], formation of free radicals [5] and inhibition of protein synthesis [6]. Cerebral ischemia/reperfusion (I/R) injury mainly refers to the brain injury aggravated after recovering the blood perfusion of ischemic brain tissue, suggesting a more obvious sign of nerve damage and its morphological change [7], which usually caused a poor prognosis of patients with cerebral ischemia.

Overwhelming studies have reported that neuronal apoptosis is a frequent eventin cerebral I/R injury [8, 9]. Apoptosis is a type of cell death, involving a series of gene activation, expression and regulation [10], which is associated with poor prognosis of patients with cerebral I/R injury [11]. Cysteine-requiring aspartate directed proteases (caspases) gene family serves a critical role in the process of cell apoptosis. Mammalian caspases, functioned as initiators and effectors, are closely associated to cerebral I/R injury [12]. Among mammalian caspases, caspase-3 is one of most important apoptotic factors associated to a number of diseases, including ovarian cancer [13], lung cancer [14], gastric cancer [15] and osteosarcoma [16]. The article of Hu *et al.* reported that Senkyunolide I protected brain function against cerebral I/R injury via up-regulating p-Erk1/2, Nrf2/HO-1 and inhibiting caspase-3 [17]. Specifically, down-regulation of caspase-3 might exert a protective role on cerebral I/R injury through inhibiting neuronal apoptosis [11]. In addition, caspase-9 may also function as apoptotic factors, which can aggravate cerebral I/R injury [18].

To date, a number of therapeutic medicines have been applied in the treatment for cerebral I/R injury, including radical scavengers, excitatory amino acid antagonists, calcium channel blockers and traditional Chinese medicine (TCM) [19]. For example, Ginaton, derived from Ginkgo biloba leaves, is an effective medicine widely used for treating blood circulation disorders in cerebral tissues in clinical application [20]. Shi-da-La-zhi Wan (SDLZ), a well-known TCM, was originally first documented in *HuiHui Yao Fang*, an Islamic medical encyclopedia which was compiled in Ming Dynasty. SDLZ ismainly used for various acute/chronic cerebral diseases in China, especially ischemic stroke, and achieved good therapeutic effects in clinic. However, up to now, the neuroprotective effects and underlying mechanisms of SDLZ involved in these diseases still remain largely elusive.

Herein, we speculated that whether SDLZ exerted a neuroprotective effect in treatment for cerebral I/R injury in rat models. Our results might provide a promising therapeutic strategy for further application of SDLZ in patients with cerebral I/R injury in the near future.

Materials and methods

Experimental animals and drugs

Adult male Sprague-Dawley (SD) rats (250-320 g, n=156) were purchased from the experimental animal center of Ningxia Medical University (Yinchuan, China). All rats were kept in one room with an alternating 12-h light-dark cycle, a constant temperature and humidity and free access to water and food. All animal protocols and procedures in this research were reviewed and approved by the Ethical Committee for Animal Experiments of Ningxia Medical University.

Shi-da-La-zhi Wan (SDLZ) weighs 211 g, which comprises bupleurum (31 g), black myrobalan (31 g), aloe (62 g), corydalis (6 g), acoruscalamus (6 g), *Fan* salt (6 g), citrullus colocynthis schrad (9 g), pine mushroom (9 g), benzoin (9 g), ferulic (9 g), ginger (3 g), chavica roxburghii (3 g), pepper (3 g), white mustard seed (3 g), rue (3 g), croton (3 g) and sugar (15 g). All these components were soaked in water (w/v 1:10) for 60 min, then decocted on a high fire. After boiling (benzoin and ferulic were later added), the mixture were continuously decocted on a low fire for 30 min. Decoction was thus acquired. Medicinal materials of SDLZ were provided by the Department of Pharmacy, Second Affiliated Hospital of Ningxia Medical University.

Ginaton was obtained From Dr. Willmar Schwabe GmbH & Co. KG (40 mg/Tab). According to an equivalent dose conversion formula based on body surface area (70 kg humans and 400 g rats): 2.857 mg/kg/d × 70 kg × [0.09 × (0.4)2/3/0.1 × (70)2/3]=5.76 mg/400 g/d. The gastric volume of rat is 1 ml/100 g, the final drugs concentrations were 1.44 mg/ml (5.76 mg/4 ml).

Drug administration protocol

All rats were randomly divided into fourgroups, including sham operation group, model group, SDLZ group and ginaton group (n=39 rats/group). Then, sham operation group, model group, SDLZ group and ginaton group were divided into three subgroups according to the indicated time point (12 h, 24 h, 72 h), respectively. Sham operation group and model group were given equal volume of normal saline (Sinopharm Chemical Reagent Co., Ltd), whereas SDLZ group and ginaton group were delivered SDLZ or ginaton intragastrically (1.44 mg/ml, two times daily) two days prior to modeling until sacrifice.

Cerebral ischemia/reperfusion model

As previously described [21], the cerebral I/R injury in rat models were induced by middle cerebral artery occlusion (MCAO). Briefly, the rats were anesthetized by 10% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd; 0.3 ml/100 g b.w., i.p.). The internal carotidartery (ICA), left common carotidartery (CCA) and external carotidartery (ECA) were carefully exposed by a midline cervical incision. The monofilament with a silicone (16-20 mm) was inserted into the CCA and advanced into the ICA. Reperfusion was achieved after removal of the monofilament 1.5 h after occlusion. A successful cerebral I/R injury rat model was considered as a reduction in cerebral blood flow to <20-30% of baseline after occlusion and >70% after reperfusion. Sham operating rats were subjected to the same surgical procedure without the monofilament insertion.

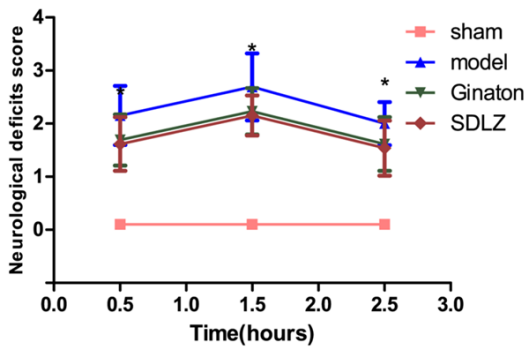

Figure 1. Effects of SDLZ on neurological scores at 12 h, 24 h and 72 h after reperfusion. Data are presented as the mean ± SD (standard deviation), n=39/group. *P<0.05.

Evaluation of neurological function

For sham operation group, model group, SDLZ group and ginaton group, neurological function was evaluated in each subgroup (12 h, 24 h, 72 h) after reperfusion. Neurological function was assessed by Zea longa's scoring, a five-point scale as previously described [21]: 0, no neurological deficit (normal); 1, mild transient focal neurological deficit (failure to lift forepaw fully); 2, moderate transient focal neurological deficit (circling to the left); 3, severe transient focal neurological deficit (falling to the left); and 4, very severe transient focal neurological deficit (failure to walk spontaneously, depressed level of consciousness). Rats scored "0" were excluded.

Samples preparation

At 12 h, 24 h, 72 h after reperfusion, the rats were anesthetized with 10% chloral hydrate, and their brains were rapidly excised and frozen for 30 min. For immunohistochemistry and TUNEL staining, the frozen brains were sectioned into 2-mm thick tissue sections and fixed with 4% paraformaldehyde. For western blotting analysis, 4 mm^3 brain tissues were collected, placed in vials and stored in liquid nitrogen.

Immunohistochemistry

2-mm thick tissue sections were fixed with 4% paraformaldehyde, embedded in paraffin after dehydration followed by antigen retrieval in 0.01% citrate buffer. The sections were incubated with mouse anti-rat caspase-3 antibody (1:50 dilution; Nanjing Key GEN Bio TECH Corp., Ltd, China) overnight at 4°C and PBS instead of primary antibody as a negative control. The sections were incubated with Biotin-labeled goat anti-mouse IgG for 30 min at 37°C. The sections were observed under a microscope. In the present study, we set four grades to assess the staining intensity: negative, 0; weak, 1; moderate, 2; and strong, 3. The percentage of positive cells in the sections was divided into 5 levels: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The final scores were calculated by multiplying the above two scores. Experimental procedures of caspase-9 and Mcl-1 were similar with caspase-3.

TUNEL staining

TUNEL staining was performed with an in situ Cell Death Detecting Kit (Roche Diagnostics GmbH, Germany) to investigate DNA fragmentation correlated to apoptosis. Briefly, 2-mm thick tissue sections were dewaxed, rehydrated, incubated in 20 μg/ml proteinase K (15 min, 37°C). Then, slides were washed with PBS (5 min × 3) and incubated in TUNEL reaction mixture in a dark and humidified atmosphere (1 h, 37°C). After washing with PBS (5 min × 3), sections were blotted up and incubated with Converter-POD solution (0.5 h, 37°C). The stained positive cells were counted through five randomly selected regions under a fluorescence microscope (Olympus/BX51, Tokyo, Japan).

Western blotting analysis

Total protein was extracted from the cerebral tissues and concentrations were determined by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amount ofprotein was electrophoresed on SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk for 2 h, the membrane was incubated with mouse anti-caspase-3 primary antibody (1:500 dilution; Nanjing KeyGEN Bio-TECH Corp., Ltd, China) overnight at 4°C followed by incubation with goat anti-mouse secondary antibody for 2 h. The membrane was washed by TBST (10 min × 3), stained using an ECL detection system (Amersham, Little Chalfont, UK) and finally imaged by BOX chemiXR5. Gray values of the bands were measured by Gel-Pro32 Software. Experimental procedures of caspase-9 and Mcl-1 were similar with caspase-3. The protein levels were normalized to β-actin expression.

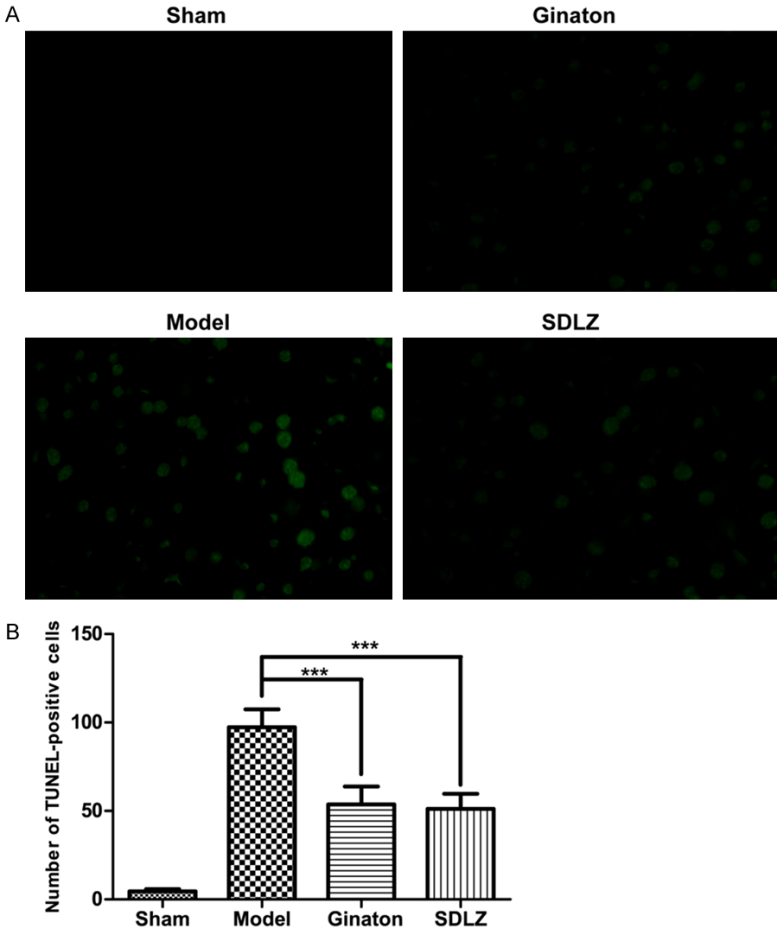

Figure 2. The influence of SDLZ on neuron apoptosis at 24 h after reperfusion. A. Representative photo micrographs of immunofluorescence labeling with TUNEL (green) staining (Magnification: 10 × 40). Cell with green staining was indicated as TUNEL positive cell. B. The number of TUNEL-positive cells in each group at 24 h after reperfusion. Data are presented as the mean ± SD (standard deviation), n=39/group. ***P<0.001.

Statistical analysis

All statistical analysis in this study was performed using SPSS 17.0 statistical software (Chicago, USA) and Graph PAD prism software 5.0 (GraphPad Software, Inc., US). Data were presented as mean ± standard deviation (SD). Data from all experiments were compared using a one-way analysis of variance (ANOVA) and unpaired Student's *t*-tests. P<0.05 was considered statistically significant.

Results

SDLZ alleviates the neurological deficits after cerebral I/R injury

The impairment of neurological function and the function of SDLZ on cerebral I/R injury were assessed by the neurological deficits score. The results revealed that neurological function injury was aggravated gradually from 12 h to 24 h after reperfusion, and was obviously alleviated at 72 h. Compared with model group, Ginaton group and SDLZ group had significantly lower neurological scores at 12 h, 24 h, and 72 h after reperfusion (all P<0.05; **Figure 1**). Accordingly, SDLZ could alleviate the neurological impairment after cerebral I/R injury.

SDLZ inhibits neuronal cell apoptosis

Cell apoptosis was detected by TUNEL staining at 24 h after reperfusion. The results of TUNEL staining (**Figure 2**) showed that the number of TUNEL positive cells significantly increased in model group compared with sham group. After the treatment of SDLZ, the number of TUNEL positive cells evidently reduced and only a few apoptotic cells existed in the SDLZ group. These results indicated that SDLZ could inhibit neuronal cell apoptosis after cerebral I/R injury.

SDLZ enhances Mcl-1 expression and represses caspase-3 and caspase-9 expression

Generally, in the processes of cerebral I/R injury, a series of pro-apoptotic factors and anti-apoptotic factors are activated and inhibited, respectively. To elucidate whether the SDLZ could up-regulate anti-apoptotic factor (Mcl-1) expression and down-regulate pro-apoptotic factors (caspase-3, caspase-9) expression, the expression levels of these proteins were detected by western blotting and immunohistochemistry. As illustrated in **Figure 3**, the results of immunohistochemistry indicated that the levels of caspase-3 and caspase-9 in SDLZ group

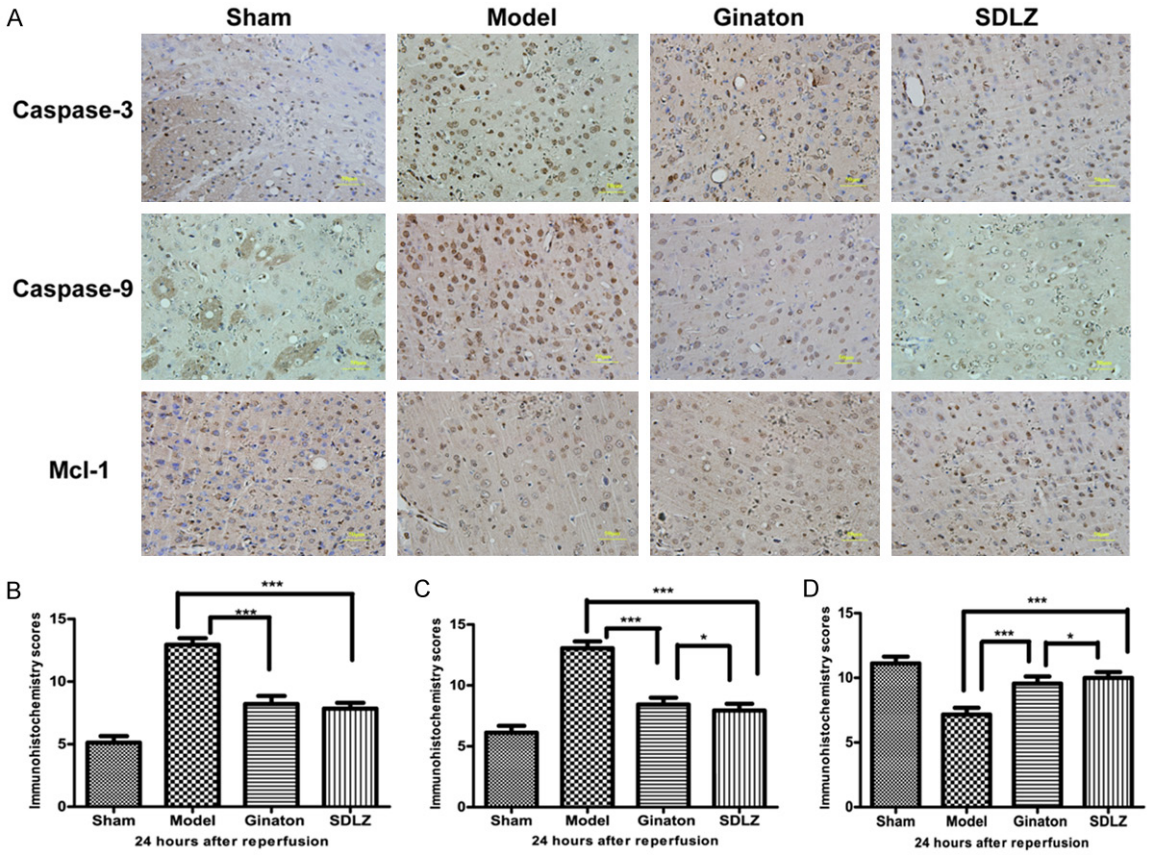

Figure 3. SDLZ up-regulated Mcl-1 expression and down-regulated caspase-3 and caspase-9 expression (immunohistochemistry). A. Representative pictures of immunohistochemistry (Magnification: 10n10). B. Immunohistochemistry scores of caspase-3. C. Immunohistochemistry scores of caspase-9. D. Immunohistochemistry scores of Mcl-1. Data are presented as the mean ± SD (standard deviation), n=39/group. **P*<0.05, ****P*<0.001.

was dramatically reduced than that in model group at 24 h after reperfusion (all *P*<0.001). Moreover, the levels of Mcl-1 in SDLZ group was significantly increased than that in model group (all *P*<0.001). SDLZ had a better effect to decrease the expression of caspase-9 and increase Mcl-1 expression than Ginaton (all *P*<0.05). As showed in **Figure 4**, the results of WB indicated that expression of caspase-3, caspase-9 increased from 12 h to 24 h, and decreased at 72 h after reperfusion. In contrast, expression of Mcl-1 decreased from 12 h to 24 h, whereas had increased at 72 h after reperfusion. Compared with sham group, the expression of caspase-3 and caspase-9 greatly increased, whereas Mcl-1 expression significantly decreased in the other groups (all *P*< 0.001). Moreover, compared with model group, the expression of caspase-3, caspase-9 in SDLZ group significantly reduced, whereas Mcl-1 markedly increased at 24 h, and 72 h after reperfusion (all *P*<0.001). At 24 h and 72 h after reperfusion, SDLZ had a better effect to suppress the expression of caspase-3 and caspase-9 and promote Mcl-1 expression than Ginaton (all *P*<0.05). These results demonstrated that SDLZ could alleviate the severity of cerebral I/R injury via up-regulating Mcl-1 expression and down-regulating caspase-3 and caspase-9 expression.

Discussion

To our knowledge, this might be the first research to show the neuroprotective effect of Shi-da-La-zhi Wan (SDLZ), a well-known TCM in China, in MCAO rat models. The findings revealed that SDLZ could alleviate the neurological deficits after cerebral I/R injury in rat models, indicating a relatively better therapeutic function in comparison to Ginaton for cerebral I/R injury. Apoptosis is a crucial cellular

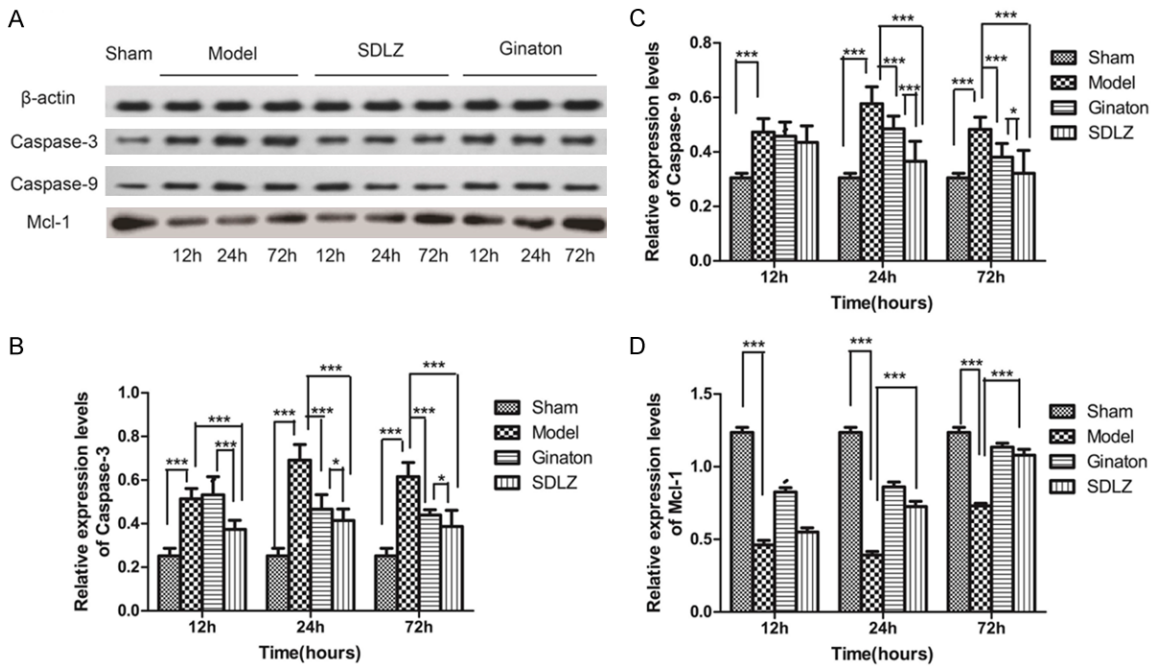

Figure 4. SDLZ promotes expression of Mcl-1 and inhibits expression of caspase-3 and caspase-9 after reperfusion. A. Representative bands of western blot results. B. Caspase-3 expression levels. C. Caspase-9 expression levels. D. Mcl-1 expression levels. Data are presented as the mean ± SD (standard deviation), n=39/group. **P*<0.05, ****P*<0.001.

event which might eventually lead to cell death after cerebral I/R injury [22]. To investigate the anti-apoptotic function of SDLZ, we observed that SDLZ inhibited neuronal cell apoptosis and exerted a neuroprotective effect after cerebral I/R injury. Our results indicated that SDLZ might suppress neuronal apoptosis in cellular level after cerebral I/R injury, consistent to our previous speculation.

Cerebral I/R injury often results in various pathological changes such as disruption of the blood-brain barrier and successive brain edema [23]. Owing to the climbing morbidity of ischemic stroke, the demands for drugs with better therapeutic effectiveness become urgent. To date, quite a few neuroprotective agents with moderate efficacy in stroke management have been already reported [24, 25]. However, most of the treatments were regarded a bit unsatisfactory for few neuroprotective agents behave well in the clinical treatment of patients [26]. Recent attention has focused on a series of natural products in the treatment against cerebral I/R injury [27-29].

The molecular mechanisms of apoptosis are quite complicated and associated with various regulation networks [30, 31]. Some articles suggested that caspase-9 and caspase-3 might play a critical role in mediating hippocampal cell death after transient cerebral ischemia [32, 33]. Our present findings indicated that SDLZ alleviated the severity of cerebral I/R injury via up-regulating Mcl-1 expression and down-regulating caspase-3 and caspase-9 expression in the ischemic penumbra of brain. Other anti-apoptotic factors and signaling pathways might contribute to the recovery of cerebral I/R injury as well. Kong *et al.* revealed the function of Chemokine-like factor 1 (CKLF1) and found that the expression of CKLF1 elevated after focal cerebral ischemia [34]; Feng *et al.* investigated the expression of poly ADP-ribose polymerase and apoptosis-inducing factor in the hippocampal CA1 region and uncovered them greatly up-regulated in the ischemia-reperfusion group than the sham-surgery group [35]. The underlying interactions between SDLZ and these downstream targets need to be further analyzed and verified.

TCM, which frequently used in Asian countries, including China, Japan and Korea for treatment of a wide range of human diseases, might pro-

vide a big opportunity for cerebral I/R injury treatment. The treatment for cerebrovascular diseases with compound TCM preparation could be originally found in the *Han* Dynasty in China [36]. Recent studies have tested a variety of well-known TCM, including Osthole [37]; Ginsenoside [38]; Angong Niuhuang Wan [39]; salidroside [40]; Resveratrol [41], most of which has been extensively used for several decades in clinic as a promising therapeutic strategy for I/R-associated cerebral diseases. Further clinical and experimental research is needed to understand their underlying effects.

In conclusion, our study focused on a novel TCM SDLZ, which might contribute to alleviate the neurological deficits after cerebral I/R injury, possibly via promoting Mcl-1 expression and suppressing caspase-3 and caspase-9 expression in the ischemic penumbra of brain. Our study might shed light on future research in both drug and mechanism of cerebral I/R injury.

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Disclosure of conflict of interest

None.

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