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Neuroprotective effects of visnagin on cerebral ischemia-reperfusion injury rats and the underlying mechanisms

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Abstract. – OBJECTIVE: Cerebral ischemia-reperfusion (I/R), caused by the treatments of ischemic stroke, usually leads to brain injury. Inflammation, oxidative stress, and autophagy play pivotal roles in the pathology. Visnagin presents a protective effect on I/R injured animal models of the heart, liver, kidney, and other organs. In our research, we identified the neuroprotective effects and the underlying mechanisms of visnagin in cerebral I/R injured models.

MATERIALS AND METHODS: We constructed rat models of cerebral I/R injury and categorized them into 5 groups: sham operation group, I/R model group, and visnagin treatment I/R group (10, 30, 60 mg/kg). The neurological deficits of the rats were analyzed after 24 hours of reperfusion, then, the contents of glutathione peroxidase, malondialdehyde, superoxide dismutase catalase, caspase-3, nuclear factor kappa-B p65 unit, tumor necrosis factor-α, interleukin-1β, and interleukin6 were measured in rat models. The expressions of Bcl-2 and Bax were detected by Western blot analysis.

RESULTS: Our results suggested that the administration of visnagin alleviated the cognitive dysfunction, reduced the activities of inflammatory factors, promoted the protein expression of Bcl-2, and downregulated the expression of Bax in the I/R injured rat model.

CONCLUSIONS: Visnagin exerts a neuroprotective effect during I/R injury in rats, the underlying mechanisms may be the effect of attenuating neuroinflammation, anti-oxidative and inhibition of apoptosis.

Key Words:
Visnagin, Cerebral ischemia-reperfusion, Bcl-2/Bax signaling pathway, Antioxidative, Apoptosis.

Introduction

Approximately 700,000 people have an acute ischemic stroke each year in the United States, nearly half of them had moderate-to-severe neurologic deficits[1]. Among them, ischemic cerebrovascular disease accounts for a large proportion[2]. Even guided by modern treatments (reperfusion will help reduce cerebral ischemic injury), some irreversible damage remained, at least in function. Reperfusion after ischemic stroke induces secondary brain injury, leading to cerebral edema, brain hemorrhage, Alzheimer's disease type dementia, and neuronal death. This process is called cerebral ischemia-reperfusion (I/R) injury. The mechanisms of I/R injury are rather sophisticated, such as production of free radical, oxidative stress, inflammatory reaction, release of the excitatory neurotransmitter, disruption of intracellular calcium ion homeostasis, activation of apoptotic gene, autophagy, dysfunction of mitochondrial, the damaged blood-brain-barrier, etc[3,4].

Inflammation plays a significant role in the cerebral I/R injury process[5]. Exacerbation of inflammatory response leads to activation of toxic enzymes, activation of the apoptotic cascade, and the induction of oxidative stress. These responses are important causes of the persistent damage after brain cerebral ischemia[6]. Oxidative stress causes cellular injury and initiates a chain of deleterious responses thus leading to inflammation and cell collapse. Afterwards, several antioxidant defense mechanisms that protect against various damages and contribute to disease prevention is initiated. Nuclear factor erythroid-derived 2-like 2 (Nrf2) is one of the most important antioxidant mechanisms that protect against oxidative stresses. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and heme oxygenase (HO)-1, acting as antioxidant genes in Nrf2 signaling pathway, has previously confirmed protection effect on myocardial I/R injury *in vitro* animal models[7].

Traditional Chinese medicines have been used to treat stroke for years. Visnagin (VIS) is an ac-

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tive medicament extracted from the fruits of Ammi visnaga and has shown numerous potential therapeutic effects, for instance, antioxidant and anti-inflammatory activities[8]. It was also recently reported that VIS and khellin could prevent renal epithelial cell damage caused by oxalate in renal epithelial cells and protected against isoproterenol-induced acute myocardial injury by alleviating oxidative stress and reducing key inflammatory and apoptosis markers[9]. Another study[10] confirmed that N–isopropylacrylamide-methacrylic acid nanoparticle–encapsulated VIS protects against I/R–induced cardiac injury and dysfunction in rats through inhibition of apoptosis. However, detailed mechanisms regarding the effect of VIS on cerebral I/R injury remain to be determined. Our study aimed to assess the neuroprotective potential of VIS in cerebral I/R injured models and verify whether oxidative stress and inflammatory or apoptosis pathways involves in this neuroprotection.

Materials and Methods

Animals and Reagents

A total of 50 adult male SD rats (230-290 g) were purchased from the Laboratory Animal Center of Anhui Medical University (Hefei, Anhui, China). We used the following reagents: VIS was purchased from SigmaAldrich (St. Louis, MO, USA). GSH-Px, malondialdehyde (MDA), SOD and CAT commercial kits were purchased from Beyotime Institute of Biotechnology (Nanjing, Jiangsu, China). Caspase-3, nuclear factor-kappa B (NF-κB) p65 unit, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL6) commercial kits were obtained from Zhejiang Tianhang Biotechnology (Hangzhou, Zhejiang, China). Other reagents grade was all analytical grades. Any rats reaching humane endpoints were euthanized with inhalation of CO_2 (air displacement rate of 30% per minute). Humane endpoints were defined as hypothermia, abnormal respiration, and ataxia. The evaluations of the death were the absence of spontaneous breathing for 2-3 min and no blink reflex as well as the cessation of heartbeat. This was approved by the Medical Ethics Committee of the Forth Affiliated Hospital of Anhui Medical University (Hefei, China).

Establishment of Animal Model and Experimental Design

Animal experiments were conducted from March 2020 to April 2020. The middle cerebral artery occlusion (MCAO) I/R injury was induced according to previous studies[11]. In brief, rats were treated with drugs for 20 days before surgery. Then, anesthetized with chloral hydrate (400 mg/kg, i.p.), a rectal thermometer was inserted to monitor body temperature (37 ± 0.5°C). A neck midline incision was performed, then, both common carotid arteries (CCA) were separated from surrounding muscles and adjacent tissues. To establish cerebral ischemia, both CCA were blocked with a 3-0 monofilament suture for 15 min. Then, reperfusion was induced by removing the monofilament from the arteries.

SD rats were randomly allocated into 5 groups (n =10 each group): i) sham group (Sham), which underwent sham surgery and not ligated (treated with physiological saline 3 ml/kg/day, i.p.); ii) model group (Model), which experienced the ligation of both CCA for 20 min followed by removing of the monofilament for reperfusion (also administered with physiological saline 3 ml/kg/day, i.p.); iii) low dose group (LOW), in which I/R injured rats were treated with 10 mg/kg visnagin (i.p.) once a day; iv) moderate dose group (MOD), in which I/R injured rats were treated with 30 mg/kg/day visnagin (i.p.); v) high dose group (HIG), in which I/R injured rats were treated with 60 mg/kg/day visnagin (i.p.). Half rats of each group were sacrificed 24 hours after the reperfusion, the other animals were assigned to Morris water maze (MWM) test and euthanized at the end of research.

Evaluation of Spatial Learning and Memory

Following I/R injury, the cognitive function was evaluated using MWM test[12]. As previous study described, the MWM consisted of a black circular pool (150 cm in diameter and 50 cm in height) and a small 100 cm^2 platform submerged 1.5 cm below the water surface. Pool was divided into four quadrants. The trajectory of the rats and the analysis of the relative data extraction were applied by an automated water maze system. Before behavioral test, all animals were trained for four consecutive days. From the 5th day through the 9th day, the platform was hidden, and each SD rat was subjected to four trials in less than 5-min. The time spent (escape latency) and the distance traveled (mean path length) to reach the target quadrant were averaged to obtain each rat's visible task score. A probe trial was performed in the ending of the test. The hidden platform was removed, and the time elapsed in the target quadrant was recorded and compared. The MWM test was performed by experimenters who were blind to each experimental group.

Evaluation of Antioxidative Effects

Twenty-four hours after the reperfusion, rats were sacrificed *via* CO_2 inhalation (as mentioned above). The residual blood samples on the surface of hemisphere were collected. After centrifuged at 15,000 g for 10 min at 4°C, the supernatant was taken for use. The hemisphere was immediately weighed. Brain tissues were homogenized, proteins were quantified using bicinchoninic acid assay (BCA) method and storage at -20°C. Oxidative markers, including MDA, SOD, GSH-Px and CAT were detected according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China). The concentrations were analyzed by Image-Pro plus software.

Evaluation of Inflammatory Effects

Twenty-four hours after reperfusion, rats were sacrificed and brain tissues were homogenized, proteins were quantified using BCA method and kept at -20°C. NF-κB P65 unit, TNF-α, IL-1β and IL-6 were examined by Enzyme-Linked Immunosorbent Assay (ELISA), according to the manufacturer's instructions.

Western Blot Analysis

Proteins were extracted and quantified. Equal amounts of total protein per sample were loaded to 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in phosphatebuffered saline (PBS) for 30 minutes at room temperature. Then, the membranes were incubated with primary anti-Bcl-2 (sc-492; 1/1200; Santa Cruz Biotechnology, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bax (sc-493; 1/1200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (bsm-33033M; 1/1000, Bioss Inc, Beijing, China), at 4°C overnight. After that, they were washed with PBS for 3 times, then incubated with horseradish peroxidase-conjugated secondary goat antimouse antibody (sc2075; 1/5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat antirabbit antibody (sc45101; 1/5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours. The protein signal was detected by an enhanced chemiluminescence kit. The values were normalized to the GAPDH content and exhibited as relative intensity.

Evaluation of Anti-Apoptosis Effects

Caspase3 is a critical molecule in cellular apoptosis, its chemical activity was measured using a caspase-3 activity assay kit (Roche, Basel, Switerland). The expression Bax and Bcl-2 was detected by Western blot.

Statistical Analysis

The data were exhibited as mean ± standard deviation (SD). The GraphPad Prism 8.0 (La Jolla, CA, USA) statistical software was used for the standard statistical analysis including Student's *t*-test, one-way ANOVA. A value of $p < 0.05$ or $p < 0.01$ considered to indicate a statistically significant difference.

Results

Effects of VIS on Spatial Learning and Memory

The chemical structure of VIS is shown in Figure 1. The Morris water maze test was used to study spatial learning and memory. In our study, a model of I/R injury in rats was established, which exhibited persistent changes in neurological function. The results showed that, compared with sham group, the time and distance traveled to reach the target quadrant were reduced at 2-, 3-, 4- and 5-days after surgery respectively, (Figure 2A, B). In addition, treatment with VIS (30 and 60 mg/kg) of the rats in the I/R injured model group exhibited significantly enhanced spatial learning and memory, compared with the untreated I/R injured model group. However, as shown in Figure 2A-C, time and distance traveled to reach the target quadrant of LOW group were also improved compared to model group, although not statistically significant ($p > 0.05$). According to the results, in the memory test, the time spent in the target quadrant in the I/R injured model group (28.9s ± 1.92s) was significantly shorter than that of the sham group (67.32s ± 1.08s, $p < 0.01$), while MOD (30 mg/kg) and HIG (60 mg/kg) group reversed the outcome (Figure 2C). These suggest that VIS can rescue the memory and learning ability of rats after I/R injury, and the 60 mg/kg dose of VIS is most evident.

Antioxidant Activity Effects of VIS on I/R Injured Rats

At 24 hours after the reperfusion, it was observed that the expressions of MDA in the I/R injured model group were significantly higher than those in the sham group, while CAT, SOD and GSH-Px activities in the brain tissues of rats were markedly lower in model group than those in the sham group ($p < 0.01$). The alterations in MDA

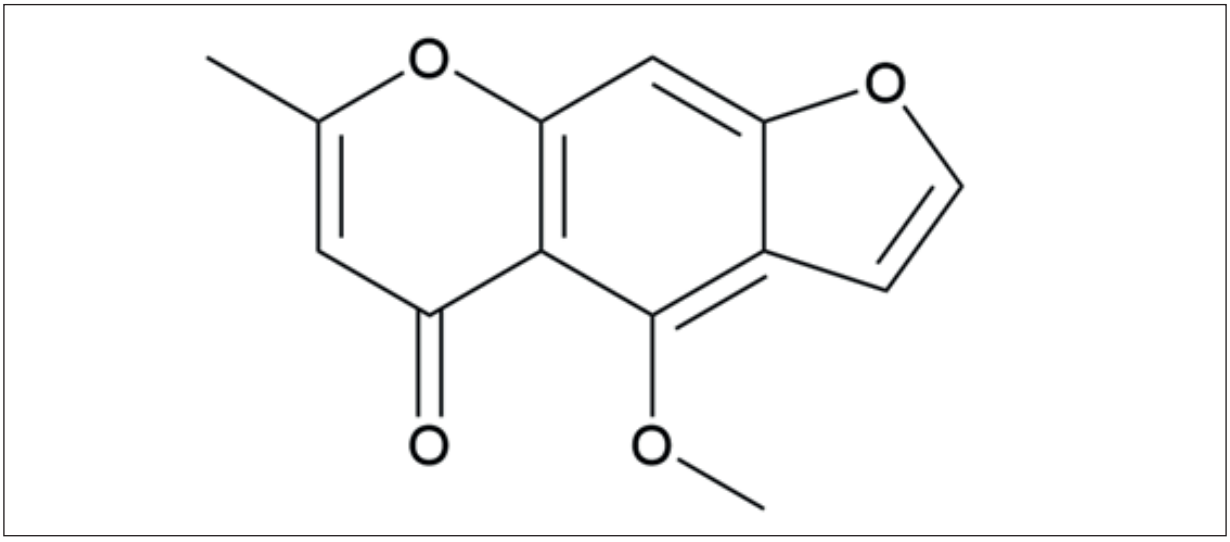

Figure 1. Chemical structure of visnagin.

content, CAT, SOD and GSHPx activities were apparently reversed after the administration of VIS (10, 30 and 60 mg/kg) ($p < 0.05$ or $p < 0.01$), which presented a dose-dependent relationship to some extent (Figure 3A-D).

AntiInflammatory Effects of VIS on I/R Injured Rats

At 24 hours after the reperfusion, the anti-inflammatory effects of VIS on I/R injured rats were analyzed. The results of this study showed that IL-6, NF-κB p65 unit, IL-1β and TNF-α levels in sham group were significantly lower than those in the model group ($p < 0.01$). Furthermore, these inflammatory factors exhibited marked downregulation in VIS-treated (30 and 60 mg/kg) groups, compared with those in I/R injured model group (Figure 4A-D), suggesting that the protective effect of VIS on cerebral I/R injury appears to be associated with the inhibition of the production of inflammatory factors. However, no significant differences in IL-1β and IL-6 expression were detected between model group and LOW group on the I/R injured rats ($p > 0.05$), which showed a slightly dose related relationship.

Anti-Apoptosis Effects of VIS on I/R Injured Rats

At 24 hours after the reperfusion, the anti-apoptosis effects of VIS on I/R injury were measured. The expression of apoptosisregulated proteins, including Bax and Bcl2 was detected by Western blot analysis (Figure 5A). Following oneway ANOVA analysis, the present study showed that expression of Bax from model group was upregulated compared with sham group, while Bcl2 expression was significantly decreased ($p < 0.01$). VIS treatment to the I/R injured rats decreased the expression of Bax and increased Bcl-2 at the protein level in a dosedependent manner (Figure 5B and 5C). In addition, the activity of caspase3, an executive molecule in the apoptotic cascade, was found to be increased in model group ($p < 0.01$), and the treatment with VIS (30 and 60 mg/kg group) significantly inhibited this cascade, as shown in Figure 5D.

Discussion

Cerebral ischemia-reperfusion (I/R) injury includes stroke, is one of the major causes of mortality and neurofunction deficits worldwide. In the current study, visnagin significantly alleviates learning and memory deficits in cerebral I/R injured rats. Its neuroprotection effect may be associated with suppressing oxidative stress and the inhibition of inflammation or apoptosis.

Early-stage vessel recanalization is the main therapy for acute brain ischemia. However, when blood supply returns to brain tissue after a period of lack of oxygen, the ischemic injury is not alleviated as expected. The underline mechanisms of cerebral I/R injury have been associated with a series of chain reactions[13]. Oxidative stress is one of those mechanisms, which is involved in the regulation of life activities, including cell proliferation and apoptosis. Under physiological conditions, reactive

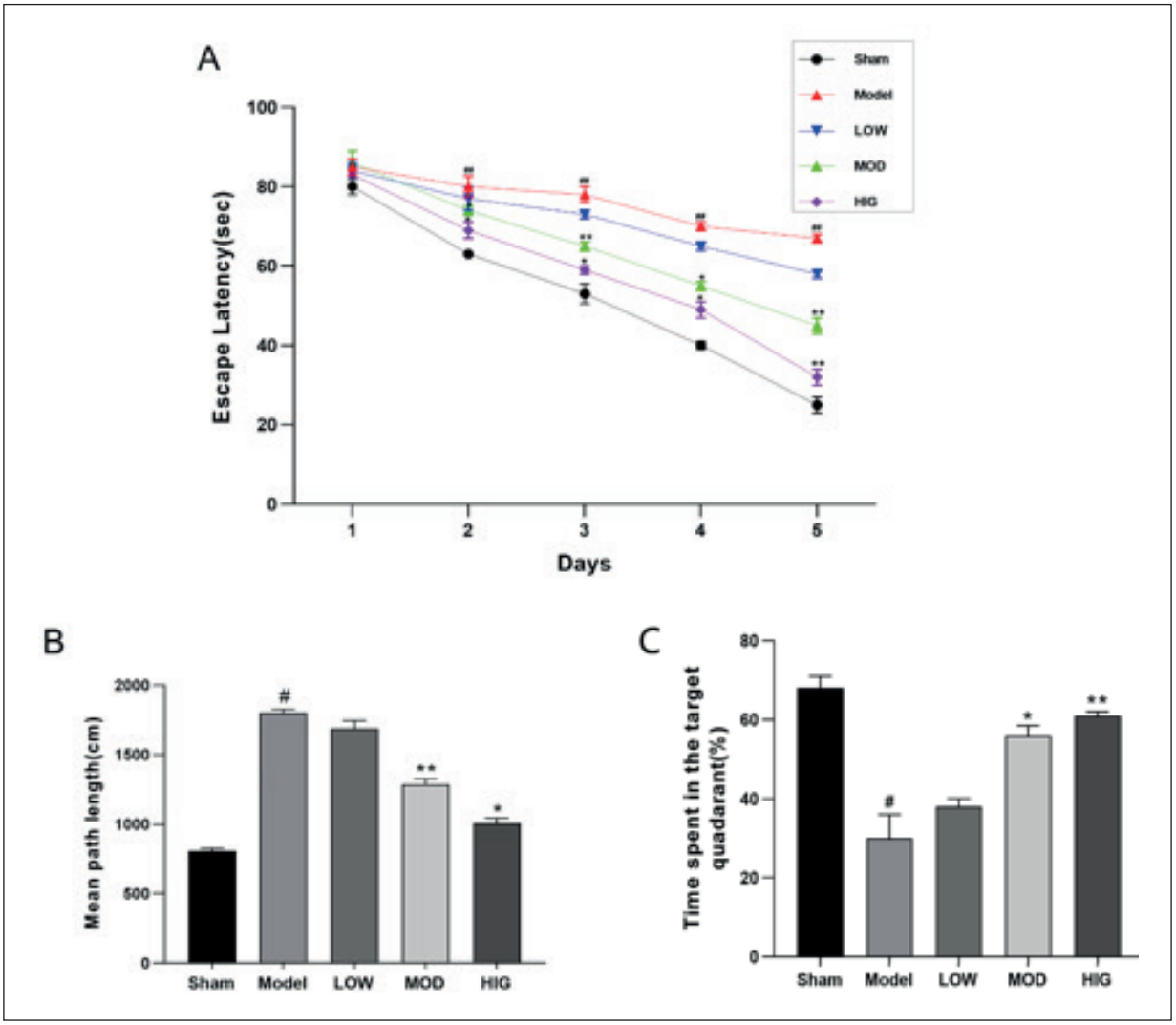

Figure 2. VIS ameliorated I/R induced cognitive dysfunction in rats (n = 5, mean ± standard deviation). (***A***) Escape latency, (**B**) Mean path length, (**C**) Time that spent in the target; Sham, sham group; Model, cerebral I/R injured model group; LOW, visnagin (10mg/kg) treated-group; MOD, visnagin (30 mg/kg) treated-group; HIG, visnagin (60 mg/kg) treated-group. ## $p < 0.01$, *vs.* sham group; ** $p < 0.01$, * $p < 0.05$, *vs.* model group.

oxygen species (ROS) maintain a dynamic balance with other compounds. The reperfusion injury may promote the production of ROS and initiate excessive oxidative stress. Nrf2 is a transcription factor regulating the expression of antioxidant proteins that protect against oxidative stress injury. Under oxidative stress, Nrf2 is transported to the nucleus and binds to the antioxidant response element (ARE), and activates the transcription of antioxidant genes, such as HO-1, SOD, CAT, and GSH-Px[14]. SOD plays a pivotal role in the oxidative stress defense system. It will scavenge superoxide anion free radicals through reduction reactions, and block the chain reactions of free radicals, thus protect cells from free radical injury. CAT and GSH-Px are another two antioxidant molecules, they interact with SOD, maintaining the intracellular redox balance of cells, and preventing further damage from oxidative stress[15,16]. MDA is an endogenous product from lipid peroxidation of unsaturated fatty acids in phospholipids and is used as a marker of oxidative stress. In the present study, cerebral I/R injured rats model exhibited a significant increase in MDA expressions associated with the reduction of GSH and other antioxidant enzymes, which consequently aggravate the cognitive dysfunction and demonstrated that oxidative stress plays a negative role in neuropathologic lesions.

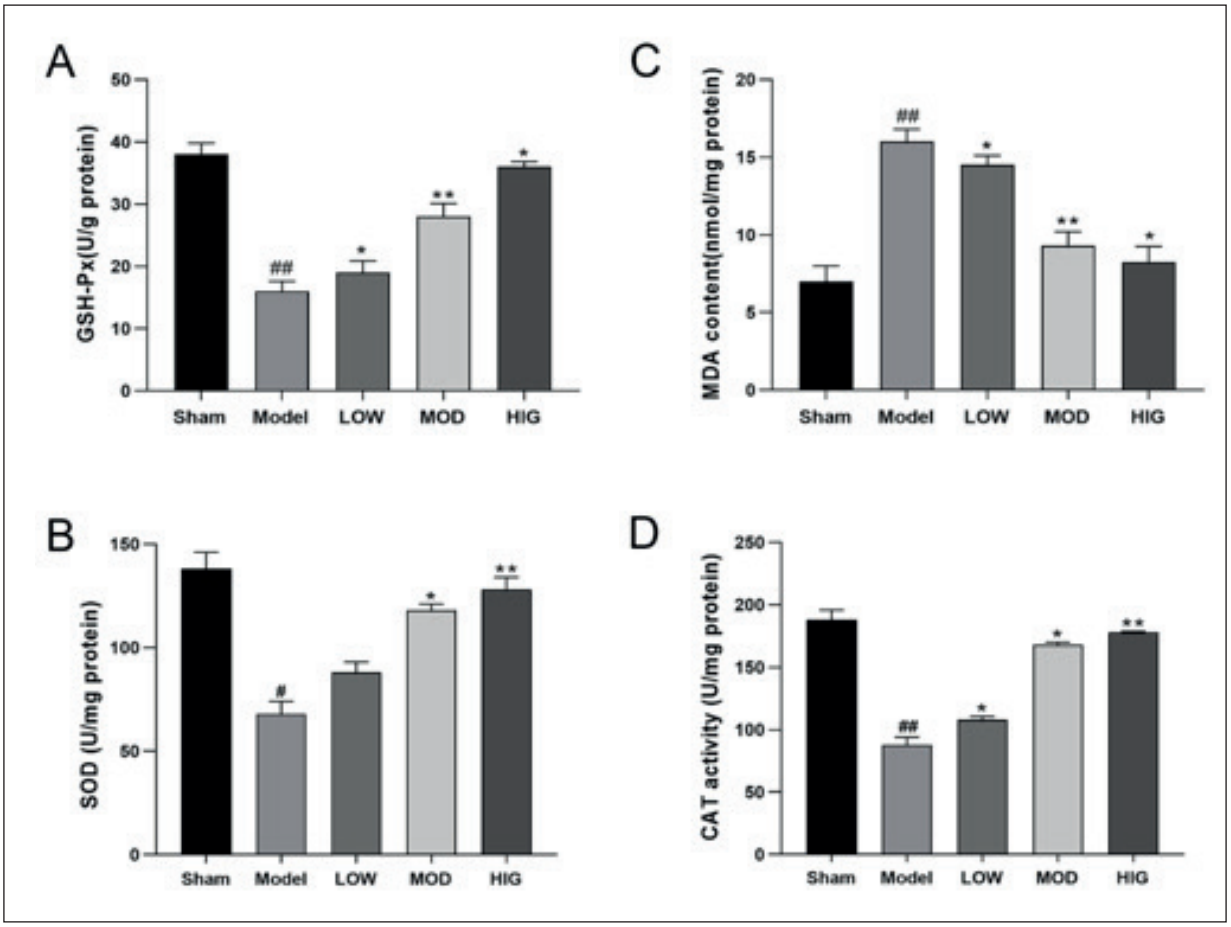

Figure 3. Protective effects of VIS against oxidative stress. The concentrations of GSH-Px (**A**), SOD (**B**), MDA (**C**) and CAT (**D**) in the I/R injured rats. Data are presented as the mean ± standard deviation. Sham, sham group; Model, cerebral I/R injured group; LOW, visnagin (10 mg/kg) treated-group; MOD, visnagin (30 mg/kg) treated-group; HIG, visnagin (60 mg/kg) treated-group. ## $p < 0.01$, *vs.* sham group; # $p < 0.05$, *vs.* sham group; ** $p < 0.01$, * $p < 0.05$, *vs.* model group.

Cerebral I/R injury is often accompanied with inflammation. An exacerbation of inflammatory response often leads to activation of toxic enzymes and activation of the apoptotic cascade. Previous studies[17] suggest that neuroinflammatory response was initiated by the production of several cytokines, chemokines, matrix metalloproteinases (MMPs), and ROS signaling. As one of transcription factor protein, NF-κB plays an important role in the inflammatory response. Furthermore, most inflammatory genes are regulated by NF-κB signaling pathway, including TNF-α, IL-6, IL-1β, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and inhibitor of kappa B (IκB) proteins. Indeed, the activity of NF-κB is mainly modulated by IκB. In inactive state, functional NF-κB dimers are located in cytoplasm and combining with IκB to form the inactive trimer. Upon stimulation, activated IκB kinase (IκK) phosphorylates its substrate IκB, leading to the exposure of nuclear localization site of NF-κB (p65). This allows free NF-κB to relocate from the cytoplasmic into the nucleus and bind to specific target genes and increasing the expression of pro-inflammatory factors[18]. Previous study[19] indicated that VIS decreased the expressions of inflammatory factors, for instance TNF-α, IL-1β, and IL-6 and their downstream receptors in doxorubicin-induced cardiomyopathy. Consistent with these reports, our study presented that the induction of focal cerebral I/R caused a remarkable elevation of protein levels of inflammatory factors, including NFκB p65, TNF-α, IL-1β and IL-6 in the brain. VIS reduced the serum activities of NF-κB p65 unit, TNF-α, IL -1β and IL-6 in the cerebral I/R injured model rats effectively, suggesting the persistent suppression of inflammatory factors.

Apoptosis is a key mechanism of cell death, which is characterized by the reduction of cellular volume, chromatin and nuclear condensation,

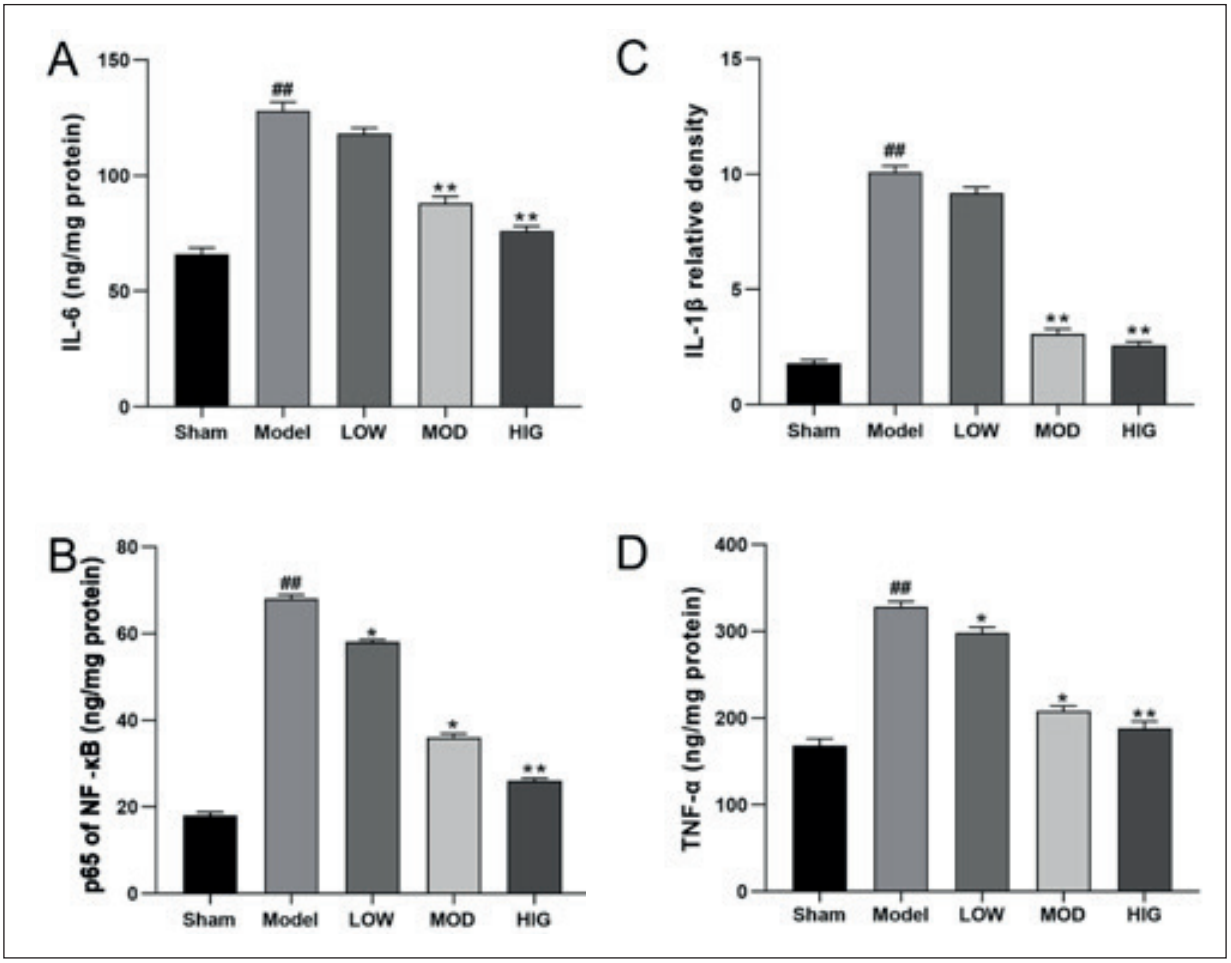

Figure 4. Protective effects of VIS against inflammatory. The activities of IL-6 (**A**), NFκB p65 (**B**), IL-1β (**C**) and TNF-α (**D**) on I/R injured rats. Data are presented as the mean ± standard deviation. Sham, sham group; Model, cerebral I/R injured model group; LOW, visnagin (10 mg/kg) treated-group; MOD, visnagin (30 mg/kg) treated-group; HIG, visnagin (60 mg/kg) treated-group. ## $p < 0.01$, *vs.* sham group; ## $p < 0.01$, *vs.* sham group; ** $p < 0.01$, * $p < 0.05$, *vs.* model group.

DNA cleavage, and cell division into apoptotic bodies. Mitochondrial pathway and death receptor pathway are the two well-known apoptotic pathways, which are achieved through activation of the caspase family. Caspase-3 is a crucial member of the family, increased activation of caspase-3 means irreversible apoptosis. Apoptotic signals induce the cleavage of caspase-3 and activated caspase-3 may initiate the caspase cascade reaction[20]. The proportion between anti-apoptotic gene Bcl-2 and proapoptotic gene Bax determined the destiny of the cells to apoptosis or not[21]. Under the condition of cerebral ischemia, Bax is translocated to the outer mitochondrial membrane and increases the permeability of mitochondrial outer membrane, activating the apoptotic pathway, triggering the caspase-3 cascade, ultimately leading to DNA degradation and brain injury[22,23]. Instead, Bcl-2 inhibits cell apoptosis by maintaining the integrity of the mitochondrial membrane and preventing the activation of downstream apoptotic signaling[24]. The consensus in academic community confirmed that the inhibition of cell apoptosis can alleviate cerebral I/R injury and thereby have a protective effect on brain tissues. Our findings are compatible with these results. The present study demonstrated that visnagin protected cerebral I/R injury by inhibiting the activation of caspase 3 thus preventing apoptosis in the I/R injured model rats. However, no statistically significant changes in antiapoptotic effect were observed between the model group and low dose (10 mg/kg) group. This could be due to the fact that the 10 mg/kg VIS dose might be insufficient to maintain an effective concentration in the brain. Therefore, further studies are needed to determine effective doses

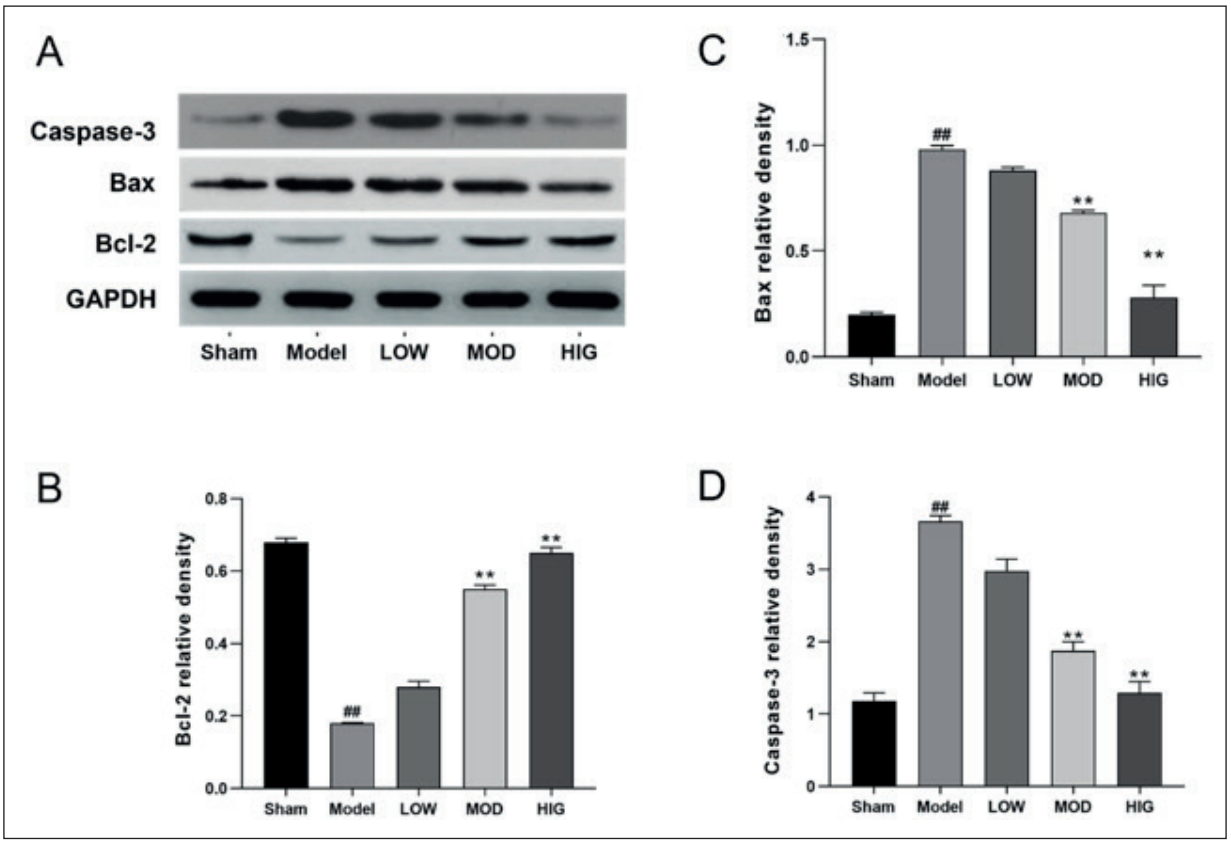

Figure 5. Anti-apoptotic effects of VIS. (**A**) Western blot images showed expressions of caspase-3, Bax and Bcl2 on I/R injured model rats from different groups. (**B**, **C** and **D**) Relative proteins density of Bcl2, Bax and caspase-3, respectively. The data were normalized to the internal reference protein (GAPDH). Data are presented as the mean ± standard deviation. Sham, sham group; Model, cerebral I/R injured group; LOW, visnagin (10 mg/kg) treated-group; MOD, visnagin (30 mg/kg) treated-group; HIG, visnagin (60 mg/kg) treated-group. ## $p < 0.01$, *vs.* sham group; ** $p < 0.01$, *vs.* model group.

of VIS, release the potential value of traditional Chinese medicines.

Conclusions

In summary, for the first time, we have demonstrated that VIS attenuates injuries of cerebral I/R in rats. The potential mechanisms of visnagin toward the neuroprotective may be the anti-oxidative, anti-inflammation and anti-apoptotic effects in I/R injured rats. Other underlying pathways are waiting to be unveiled.

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Conflicts of interest

The authors declare that they have no competing interests.

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Data Availability

Data available on request due to privacy.

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