Aloperine protects against cerebral ischemia/reperfusion injury via activating the PI3K/AKT signaling pathway in rats

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Abstract. Cerebral ischemia is among the leading causes of death and long-term disability worldwide. The aim of the present study was to investigate the effects of aloperine (ALO) on cerebral ischemia/reperfusion (I/R) injury in rats and elucidate the possible underlying mechanisms. Therefore, a rat model of reversible middle cerebral artery occlusion (MCAO) was established to induce cerebral I/R injury. Following pretreatment with different doses of ALO, the histopathological changes in the brain tissue were evaluated by hematoxylin and eosin staining. The degree of cerebral infarction was determined using by 2,3,5-triphenyltetrazolium chloride staining. Additionally, the levels of oxidative stress- and inflammation-related factors were measured using commercially available kits. Cell apoptosis was assessed by TUNEL staining, while the expression levels of apoptosis- and PI3K/AKT signaling pathway-related proteins were determined by western blot analysis. The results demonstrated that ALO alleviated histopathological injury in the brain tissue and the area of cerebral infarction in a dose-dependent manner. Furthermore, significantly reduced levels of reactive oxygen species and malondialdehyde were observed in the ALO-treated rats post-MCAO/reperfusion, accompanied by increased levels of superoxide dismutase, catalase and glutathione. Consistently, treatment with ALO notably decreased the concentration of inflammatory factors, including TNF-α, IL-1β and IL-6, in a dose-dependent manner. In addition, ALO attenuated neuronal cell apoptosis, downregulated the expression of Bax and upregulated that of Bcl-2. I/R markedly reduced the expression levels of phosphorylated (p-)PI3K and p-AKT, which were dose-dependently restored by ALO intervention. Collectively, the aforementioned findings indicated that ALO could improve cerebral I/R injury and alleviate oxidative stress, inflammation and cell apoptosis via activating the PI3K/AKT signaling pathway, thus supporting the therapeutic potential of ALO against cerebral I/R injury in ischemic stroke.

Introduction

Stroke is a heterogeneous disease and among the leading causes of mortality worldwide, while cerebral ischemia is considered as the most common cause of stroke (1). Cerebral ischemia/reperfusion (I/R) injury may irreversibly impair brain function via partly interrupting blood supply to the brain, thus resulting in thrombosis, embolism or hypoperfusion, eventually leading to death or long-term disability (2). To date, there is no effective treatment available for cerebral ischemic stroke. Therefore, the prevention and treatment of cerebral I/R injury is becoming increasingly important in the treatment of ischemic cerebrovascular diseases.

A growing body of literature has suggested that energy metabolism disorders, oxidative stress, inflammation and apoptosis are involved in the pathogenesis of cerebral I/R injury (3,4). Neuroinflammation and oxidative stress are two well-known factors promoting neuronal injury and apoptosis during ischemic stroke (5). Traditional Chinese medicine (TCM) has been widely used to treat cerebral ischemia-related diseases, and has demonstrated notable therapeutic efficacy in clinical practice. TCM comprises several natural active substances (6). Aloperine (ALO) is a novel quinolizidine alkaloid derived from the leaves and seeds of the Sophora alopecuroides L. (Leguminosae) (Fig. 1A) (7). Matrine, one of the major alkaloids of the Sophora plant, has been demonstrated to be effective in ameliorating the histological consequences of middle cerebral artery occlusion (MCAO) in mice through its antioxidant activity and by inhibiting apoptosis (8). As a novel quinolizidine alkaloid derived from Sophora plant, ALO has been recognized as an effective treatment for several neurological diseases. For example, it was previously demonstrated that ALO could attenuate the production of reactive oxygen species (ROS) and cell apoptosis in a cell model of Alzheimer’s disease (9). A previous study reported that ALO could protect mice against I/R-induced renal injury via attenuating inflammatory infiltration and

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Following anesthetization with intraperitoneal injection of 40 mg/kg pentobarbital sodium, the rats were fixed in a supine position. The left common carotid artery, the external carotid artery and the internal carotid artery were carefully exposed and dissected away from the adjacent nerves. A V-shaped oblique incision was made at the bifurcation of the external and internal carotid arteries with vascular scissors. Then, the artery clamp was reopened, while a paraffin bolt was inserted through the external carotid artery stump into the internal carotid artery until a slight resistance was felt. The time was set as the beginning of the embolism. The upper end of the common carotid artery was then ligated, and the paraffin bolt was gently pulled back through the incision of the external carotid artery 90 min after the embolism to allow reperfusion. After 24 h, I/R injury was evaluated using H&E and 2,3,5-triphenyltetrazolium chloride (TTC) staining. Rats in the model group exhibited notable histopathological changes and increased infarct volume, suggesting the success of I/R model. In rats subjected to sham surgery, the arteries were isolated but not ligated or occluded. During the surgical procedure, the body temperature of the rats was maintained at 37±5°C using a heating pad.

**Materials and methods**

**Animals.** A total of 60 specific pathogen-free grade male Sprague-Dawley rats (weight, 200-250 g) were provided by the Shanghai Laboratory Animal Center (Shanghai, China). All rats were housed under standard conditions with a 12-h light/dark cycle, and were given ad libitum access to food and water. The temperature and relative humidity were 22±2°C and 55±10%, respectively. All animal experimental procedures were approved by the Affiliated Hospital of North Sichuan Medical College (approval no. NSMC201901).

**Grouping and drug treatment.** Animals were randomly assigned into the following six groups (n=10 per group): Sham, I/R, I/R + 2 mg/kg nimodipine (I/R + NIM; positive control group), I/R + 2 mg/kg ALO, I/R + 25 mg/kg ALO and I/R + 50 mg/kg ALO. ALO (Santa Cruz Biotechnology, Inc.) was dissolved in saline supplemented with 10% acetic acid (2.5 mg/ml), as previously described (10). Rats in the ALO groups were treated with the corresponding doses of ALO by intraperitoneal injection for 7 consecutive days (4,8,13). Animals in the positive control group received intraperitoneal administration of 2 mg/kg NIM (Shandong Fangming Pharmaceutical Group Co., Ltd.) instead of ALO, while those in the sham group received the same volume of saline (containing 10% acetic acid). The doses of ALO were determined based on previous studies (14,15). NIM is a dihydropyridine calcium antagonist, which can reduce nerve cell apoptosis through calcium influx, increase the oxygen supply and blood supply to brain tissue by improving the deformability of red blood cells, reducing the permeability of the blood-brain barrier and improving blood rheology, so as to protect cerebrovascular and nerve cells (16,17). NIM can reduce brain inflammation and promote neuronal recovery by inhibiting the expression of inflammatory factors, such as TNF-α, IL-1β and IL-6, to relieve the inflammatory response of the body (18,19). NIM has been widely used as the positive control in the cerebral I/R injury (20,21). The MCAO surgery was performed at 2 h following the last drug administration.

**Induction of cerebral I/R injury.** Reversible MCAO surgery was performed using an improved Longa-Zea method (22). Following anesthetization with intraperitoneal injection of 40 mg/kg pentobarbital sodium, the rats were fixed in a supine position. The left common carotid artery, the external carotid artery and the internal carotid artery were carefully exposed and dissected away from the adjacent nerves. A V-shaped oblique incision was made at the bifurcation of the external and internal carotid arteries with vascular scissors. Then, the artery clamp was reopened, while a paraffin bolt was inserted through the external carotid artery stump into the internal carotid artery until a slight resistance was felt. The time was set as the beginning of the embolism. The upper end of the common carotid artery was then ligated, and the paraffin bolt was gently pulled back through the incision of the external carotid artery 90 min after the embolism to allow reperfusion. After 24 h, I/R injury was evaluated using H&E and 2,3,5-triphenyltetrazolium chloride (TTC) staining. Rats in the model group exhibited notable histopathological changes and increased infarct volume, suggesting the success of I/R model. In rats subjected to sham surgery, the arteries were isolated but not ligated or occluded. During the surgical procedure, the body temperature of the rats was maintained at 37±5°C using a heating pad.

**H&E staining.** The rats were euthanatized with intraperitoneal injection of 200 mg/kg pentobarbital sodium at 24 h post-I/R, and their whole brains were removed and washed with saline. Then, the whole brain cortical tissue sample from each animal (n=5 for each group) was soaked in 4% paraformaldehyde overnight at room temperature. Following dehydration through an ethanol gradient, the tissues were transparentized with xylene, and the brain blocks were embedded in paraffin. The paraffin-embedded tissue samples were then cut into 5-μm coronal sections using a microtome (Leica Microsystems GmbH), deparaffinized in xylene and rehydrated through a descending ethanol series. Following dehydration via a graded ethanol and xylene series, the histological structure of the brain tract was observed under a light microscope (magnification, x400; Olympus Corporation). Finally, three tissue sections from each animal were stained with H&E using standard techniques according to a previous study (23) and the stained slides were observed under a light microscope (magnification, x400; Olympus Corporation).

**Measurement of cerebral infarct volume.** To evaluate the cerebral infarct size, TTC staining was performed. Briefly, the rats were euthanatized with 200 mg/kg pentobarbital sodium at 24 h post-I/R and thoroughly perfused with saline. The brains (n=5 for each group) were removed quickly, and the olfactory bulb, cerebellum and brainstem were excised and cut into five 2-mm coronal sections. Subsequently, the brain sections were fixed in 4% paraformaldehyde for 20 min, followed by fixation in 4% paraformaldehyde for 6 h at 4°C. Images of the TTC-stained sections were captured under a light microscope (magnification, x50). TTC stains the non-infarcted region with a deep red pigment, while the infarcted brain area appears white. Infarct size was calculated using ImageJ software (1.52r; National Institutes of Health) and the result was expressed as a percentage of infarct area to total brain area. The infarct volume percentage in the ischemic cerebral hemisphere was calculated using the following equation: [(Contralateral hemisphere volume-volume of
non-ischemic ipsilateral hemisphere/contralateral hemisphere volume] x100, as previously reported (24).

**Evaluation of oxidative stress-related markers.** The brain tissues (n=5 for each group) were obtained at the end of reperfusion and the ischemic hemispheres were then cut into small pieces. These pieces were homogenized with RIPA lysis buffer (Beyotime Institute of Biotechnology), centrifuged at 12,000 x g for 10 min at 4°C, and the supernatant was collected. The levels of ROS (cat. no. E004-1-1), malondialdehyde (MDA; cat. no. A003-1-2) and reduced glutathione (GSH; cat. no. A006-1-1), as well as the activities of superoxide dismutase (SOD; cat. no. cat. no. A001-1-2) and catalase (CAT; cat. no. A007-2-1), were measured in tissue homogenate supernatant with corresponding commercially available kits (Nanjing Jiancheng Bioengineering Institute) using chemical colorimetry, according to the manufacturer's recommendations.

**Measurement of inflammatory factors.** To determine the levels of inflammation-related factors, including TNF-α (cat. no. F16960), IL-1β (cat. no. F15810) and IL-6 (cat. no. F15870), the brain tissue homogenate supernatant was collected as described above and ELISA was carried out using corresponding kits (Shanghai XiTang Biotechnology Co., Ltd.) according to the manufacturer's instructions. The optical density values were measured at 450 nm and were read using a plate reader (BioTek Instruments, Inc.).

**TUNEL staining.** The sections were fixed with 4% paraformaldehyde for 20 min at room temperature, deparaffinized, rehydrated and then permeated with protease K for 10 min at room temperature to increase cell membrane permeability. To evaluate cell apoptosis in the hippocampus post-I/R, TUNEL assay was performed using a commercially available kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The sections were washed by PBS and then incubated with TUNEL solution for 1 h at 37°C in a dark chamber, followed by the counterstaining with 4', 6-diamidino-2-phenylindole (DAPI) for the nuclei for rehydrated and then permeated with proteinase K for 10 min with corresponding commercially available kits (Shanghai XiTang Biotechnology Co., Ltd.) according to the manufacturer's instructions. The optical density values were measured at 450 nm and were read using a plate reader (BioTek Instruments, Inc.).

**Western blot analysis.** For immunoblotting, the brain tissues were lysed on ice and centrifuged at 4°C and at 12,000 x g for 10 min. The supernatant was extracted using RIPA buffer (Beyotime Institute of Biotechnology) containing protease inhibitor cocktail (Beyotime Institute of Biotechnology) and the protein concentration was examined by means of a bicinchoninic acid protein quantification kit (Beyotime Institute of Biotechnology). For each sample, equal amounts of protein (40 µg/lane) were separated by 10% SDS-PAGE and transferred to a nitrocellulose blotting membrane (Cytiva). Possible non-specific binding was blocked by 5% skimmed milk for 1.5 h at room temperature and the membranes were then incubated overnight at 4°C with specific primary antibodies. On the following day, horseradish peroxidase-conjugated secondary antibody (cat. no. 7074S; 1:3,000; Cell Signaling Technology, Inc.) were added to the membranes and incubated for 1 h at room temperature. Protein bands was scanned and visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.). The relative intensity of each band was quantified using ImageJ software (1.52r; National Institutes of Health). The protein expression was normalized to GAPDH levels. Anti-Bax (cat. no. 14796S; 1:1,000), anti-phosphorylated (p-)PI3K (cat. no. 17366S; 1:1,000), anti-PI3K (cat. no. 4249S; 1:1,000), anti-p-AKT (cat. no. 4060T; 1:1,000), anti-AKT (cat. no. 4691T; 1:1,000) and anti-GAPDH (cat. no. 5174T; 1:1,000) antibodies were obtained from Cell Signaling Technology, Inc. Anti-Bcl-2 (cat. no. sc-7382; 1:1,000) antibody was provided by Santa Cruz Biotechnology, Inc.

**Statistical analysis.** All experiments were repeated independently in triplicate. Data are presented as the mean ± standard deviation. One-way analysis of variance was used to assess multiple differences, followed by a Tukey's post hoc test with GraphPad Prism 6.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate statistically significant differences.

**Results**

**ALO treatment significantly alleviates brain injury and reduces the cerebral ischemic area in MCAO rats.** To investigate the potential neuroprotective effect of ALO in cerebral ischemic stroke, the histopathological changes in the brain tissues were evaluated using H&E staining following treatment of MACO rats with ALO. In the sham group, the outline of the cortex neurons was clear and their structure was compact, with abundant cytoplasm. In addition, I/R injury was associated with unequivocal signs of pyknotic and shrunken nuclei, nuclear loss and numerous vacuolated spaces, which were markedly restored following ALO preconditioning in a dose-dependent manner (Fig. 1B and C; P<0.001). Additionally, treatment with high doses of ALO had the same effects as NIM treatment. Consistently, as shown in Fig. 1D and E, I/R challenge notably increased the infarct volume compared with the sham group (P<0.001), while significantly decreased infarct volumes were observed in the ALO and NIM intervention groups compared with the I/R group (P<0.001). These findings suggested that ALO treatment significantly improved brain injury and the cerebral ischemic area in MCAO rats.

**ALO preconditioning ameliorates MCAO/reperfusion-induced oxidative stress and inflammation.** Subsequently, the levels of oxidative stress- and inflammation-related markers in rat brain tissue homogenates were assessed using commercially available kits. As shown in Fig. 2A-E, I/R injury markedly increased the contents of ROS and MDA (P<0.001), while it reduced SOD, CAT and GSH compared with the sham group (P<0.001). Furthermore, compared with the I/R group, pretreatment with ALO dose-dependently decreased ROS and MDA and increased SOD, CAT and GSH (P<0.01, P<0.001).
Simultaneously, the levels of inflammatory factors, including TNF-α, IL-1β and IL-6, were notably enhanced following I/R exposure compared with the sham group (P<0.001), whereas ALO or NIM preconditioning markedly reduced the levels of the aforementioned inflammatory factors (P<0.001). These findings indicated that ALO treatment markedly attenuated oxidative stress and neuroinflammation in rats post-MCAO/reperfusion.

**ALO intervention inhibits cell apoptosis in the brain tissues of rats following MCAO/reperfusion injury.** To evaluate the effect of ALO on cell apoptosis post-MCAO/reperfusion, TUNEL staining was performed on rat brain sections. A significant increase in the number of apoptotic cells was observed in the I/R group compared with the sham group (Fig. 3A and B; P<0.001). Of note, treatment with ALO dose-dependently reduced the number of apoptotic cells in the brain tissues of rats with MCAO/reperfusion injury (P<0.001). Concurrently, compared with the sham group, the expression levels of the pro-apoptotic protein Bax was notably upregulated in the I/R group, accompanied by decreased expression of the anti-apoptotic protein Bcl-2 (Fig. 3C; P<0.001). Importantly, the effect of MCAO/reperfusion on the expression of apoptosis-related proteins was restored by ALO preconditioning in a dose-dependent manner (P<0.001). These findings indicated that ALO could attenuate neuronal apoptosis in response to cerebral I/R injury in rats.

**ALO protects against cerebral I/R injury via activating the PI3K/AKT signaling pathway.** To uncover the potential mechanisms underlying the role of ALO in cerebral ischemic stroke, the expression levels of PI3K/AKT pathway-related proteins were determined by western blot analysis. As shown in Fig. 4, I/R injury significantly reduced the protein expression levels of p-PI3K and p-AKT compared with the sham group (P<0.001), whereas this inhibitory effect was reversed following treatment with ALO or NIM (P<0.05 and P<0.001, respectively). Overall, the aforementioned data suggested that ALO treatment could protect against cerebral I/R injury via activating the PI3K/AKT signaling pathway.

**Discussion**

Brain tissue is highly sensitive to injury and neuronal apoptosis due to I/R (25). TCM has become increasingly important in the treatment of cerebral I/R injury. It has been reported that an alkaloid-free ethyl acetate extract from the root of *Sophora flavescens* Ait. exerted neuroprotective effects on focal cerebral ischemia in rats (26). In addition, oxymatrine and matrine, as the main alkaloids extracted from the traditional Chinese herb, *Sophora flavescens* Ait., have been shown to be effective in improving hypoxic-ischemic brain injury in neonatal rats and MCAO mice by antioxidant and anti-apoptotic activities (8,27). As a novel quinolizidine alkaloid derived from *Sophora alopecuroides* L., ALO has important beneficial effects in the treatment of neurological diseases.
shown to alleviate oxygen-glucose deprivation and reperfusion-induced cultured rat hippocampal neuron injury and ameliorate early brain injury following subarachnoid hemorrhage (11,12). To the best of our knowledge, the present study was the first to investigate the effects of ALO on cerebral I/R injury in rats subjected to MCAO and its potential regulatory mechanisms. The results of the present study demonstrated the beneficial effect of ALO against neuronal injury in a rat model of MCAO/reperfusion, and further suggested that these protective effects may be mediated by the PI3K/AKT pathway.

The pathophysiological mechanisms underlying neuronal injury in cerebral I/R are complex and multifactorial, and a considerable body of evidence has demonstrated that oxidative stress plays a dominant role in the occurrence and development of this condition (28,29). It has been reported that increased production of ROS and lipid peroxidation can be detected at the early stages of cerebral I/R (13,30). MDA, the end product of lipid peroxidation, is a marker of oxidative stress. SOD and CAT are crucial enzymes of the antioxidant defense system produced under oxidative stress, which together protect neuronal cells against ROS-induced cell death (31,32). GSH, a tripeptide with a free sulfhydryl group, reduces oxidative stress and helps to maintain the normal reduced redox state in cells via directly interacting with ROS (33). Emerging evidence has suggested that the excessive accumulation of ROS accompanied by an exhausted antioxidant defense system are involved in the pathogenesis of cerebral I/R injury (34). ALO is a quinolizidine alkaloid derived from the leaves and seeds of the Sophora plant, which has several pharmacological functions in human diseases through its antioxidant activity (7,35). Compelling evidence has indicated that ALO exerts protective effects against hydrogen peroxide-induced oxidative stress and apoptosis in human retinal pigment epithelial cells (36). In addition, a study revealed that ALO could inhibit ROS formation and cell apoptosis in an Alzheimer's disease cellular model (9). Additionally, the significant neuroprotective effects of ALO on oxygen-glucose deprivation/reperfusion-injured neonatal rat primary cultured hippocampal neurons were attributed to its antioxidant activity (11). In the present study, pretreatment of rats post-MCAO/reperfusion with ALO markedly reduced the levels of oxidative stress, supporting the potential protective effects of ALO against cerebral I/R injury.

Accumulating evidence has shown that the inflammatory response is triggered immediately after ischemia, thus resulting
in the deterioration of delayed cerebral injury and poor functional recovery of neurons (37). Oxidative stress increases the levels of inflammatory cytokines, including TNF-α, IL-6 and IL-1β, and is closely associated with the injury caused by the excessive secretion of these mediators (38). A cascade of physiological events during the inflammatory responses after cerebral I/R may result in neuronal cell death and neurological dysfunction, eventually leading to brain injury (39,40). Apoptosis is considered as an important type of neuronal cell death that occurs after cerebral I/R injury (41,42). A study revealed that ALO could attenuate human pulmonary vascular smooth muscle cell proliferation via suppressing inflammatory response (43). Mao et al (44) demonstrated that ALO could restrain cardiomyocyte apoptosis and alleviate coronary microembolization-induced rat myocardial injury. A previous study demonstrated that ALO could protect mice against I/R-induced renal injury via suppressing inflammatory infiltration and tubular cell apoptosis (10). Consistent with the aforementioned findings, the present study revealed that cerebral MCAO/reperfusion could markedly enhance the secretion of inflammatory cytokines (TNF-α, IL-6 and IL-1β) and promote cell apoptosis in brain tissues. These effects were effectively inhibited following treatment with ALO, indicating that the protective effect of ALO on cerebral I/R injury could be associated with the inhibition of inflammation and apoptosis.

The PI3K/AKT signaling pathway is a known regulator of a wide range of cellular functions, such as proliferation, oxidative stress, inflammation and apoptosis (45). Mounting evidence indicates that the activation of PI3K/AKT signaling plays an important role in cerebral I/R injury via promoting the repair and survival of ischemic nerve cells (46,47). In the
nervous system, PI3K is involved in the survival and differentiation of neuronal cells, and PI3K activation can modulate the expression of downstream target genes via activating AKT expression (48). A previous study demonstrated that AKT could exert a critical protective effect on cerebral I/R injury in rats, since its overexpression was found to reduce the volume of the infarcted brain tissue (49). Therefore, the development of effective PI3K/AKT activators may be a focus of future research on ischemic cerebrovascular diseases. ALO was found to attenuate coronary microembolization-induced myocardial injury in rats via activating the PI3K/AKT signaling pathway (44). Additionally, ALO was shown to regulate inflammatory responses in colitis via suppressing the PI3K/AKT pathway in a P2P2A-dependent manner (50). The present study revealed that ALO treatment markedly upregulated the expression of p-PI3K and p-Akt in rats post-MCAO/reperfusion, suggesting that ALO may protect against cerebral I/R injury via activating the PI3K/AKT signaling pathway.

Taken together, the findings of the present study demonstrated that ALO exerted anti-neuroinflammatory, antioxidant and anti-apoptotic effects during cerebral I/R injury via activating the PI3K/AKT signaling pathway. These data support the therapeutic potential of ALO in cerebral ischemic stroke. However, the lack of behavioral measures and neurological deficit scores constitute limitations of the present study, and will be addressed in future studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL, XC and LX searched the literature, designed the experiments and conducted the experiments. XC and RZ analyzed and interpreted the data. ZL wrote the manuscript. XC and RC confirmed the authenticity of all the raw data. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experimental procedures were approved by the Animal Care and Use Committee of the Affiliated Hospital of North Sichuan Medical College (approval no. NSMC201901).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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