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Protective effect of oxysophoridine on cerebral ischemia/reperfusion injury in mice★

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Abstract

Oxysophoridine, a new alkaloid extracted from *Sophora alopecuroides* L., has been shown to have a protective effect against ischemic brain damage. In this study, a focal cerebral ischemia/reperfusion injury model was established using middle cerebral artery occlusion in mice. Both 62.5, 125, and 250 mg/kg oxysophoridine, *via* intraperitoneal injection, and 6 mg/kg nimodipine, *via* intragastric administration, were administered daily for 7 days before modeling. After 24 hours of reperfusion, mice were tested for neurological deficit, cerebral infarct size was assessed and brain tissue was collected. Results showed that oxysophoridine at 125, 250 mg/kg and 6 mg/kg nimodipine could reduce neurological deficit scores, cerebral infarct size and brain water content in mice. These results provided evidence that oxysophoridine plays a protective role in cerebral ischemia/reperfusion injury. In addition, oxysophoridine at 62.5, 125, and 250 mg/kg and 6 mg/kg nimodipine increased adenosine-triphosphate content, and decreased malondialdehyde and nitric oxide content. These compounds enhanced the activities of glutathione-peroxidase, superoxide dismutase, catalase, and lactate dehydrogenase, and decreased the activity of nitric oxide synthase. Protein and mRNA expression levels of N-methyl-D-aspartate receptor subunit NR1 were markedly inhibited in the presence of 250 mg/kg oxysophoridine and 6 mg/kg nimodipine. Our experimental findings indicated that oxysophoridine has a neuroprotective effect against cerebral ischemia/reperfusion injury in mice, and that the effect may be due to its ability to inhibit oxidative stress and expression of the N-methyl-D-aspartate receptor subunit NR1.

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Key Words

neural regeneration; traditional Chinese medicine; brain injury; oxysophoridine; ischemia/reperfusion injury; oxidative stress; N-methyl-D-aspartate receptor; neuroprotection; grants-supported paper; neuroregeneration

Research Highlights

- (1) Oxysophoridine has a protective effect against ischemic brain damage. However, few studies have examined its role against cerebral ischemia/reperfusion injury.
- (2) This study is the first to demonstrate the neuroprotective mechanism underlying oxysophoridine against cerebral ischemia/reperfusion injury.

(3) Similar to nimodipine, oxysophoridine has a neuroprotective effect against cerebral ischemia/reperfusion injury, and its mechanism of action is related to its antioxidant properties and inhibition of the glutamic acid receptor pathway.

INTRODUCTION

Ischemic stroke is the third most common cause of death in humans^[1]. Therefore, further studies are needed to identify neuroprotective drugs that will prevent or ameliorate brain injury. Ischemic brain injury often causes irreversible damage^[2]. Focal cerebral ischemia leads to neuronal injury and death involving energy depletion, oxidative stress, excitotoxicity, and inflammation^[3-4]. The postsynaptic activation of excitatory N-methyl-D-aspartate glutamatergic receptors is known to be a critical event in neuronal death and brain damage induced by acute ischemic stroke^[5]. Glutamate excitotoxicity is a major pathway triggering brain damage following ischemic stroke^[6]. Because of the high Ca^{2+} permeability, N-methyl-D-aspartate receptors play a pivotal role in glutamate excitotoxicity following cerebral ischemia^[7]. Inagaki and colleagues^[8] reported that N-methyl-D- aspartate receptor activation can stimulate nitric oxide release from the aortic ring. Therefore, we speculate that ischemia/reperfusion injury may lead to N-methyl-D- aspartate receptor activation and subsequent brain damage.

Reperfusion at ischemic areas exacerbates ischemic brain damage^[9]. It is generally accepted that disturbances in the oxidant-antioxidant balance play a significant role in the vulnerability of the brain to ischemia/reperfusion injury^[10]. Reactive oxygen species are largely generated from mitochondrial energy metabolism *via* oxidative phosphorylation in the respiratory chain of eukaryotes. Because of the existence of antioxidant enzymes, such as glutathione, superoxide dismutase, and catalase, the redox balance is well maintained. After ischemia, there is an increase in superoxide anions, or other reactive oxygen species, from various sources, resulting in a disturbance to this delicate balance^[11]. The increase of reactive oxygen species consumes endogenous antioxidant compounds, such as glutathione and superoxide dismutase. When the injury is pronounced or persistent, compensatory responses become inadequate to correct the unbalanced redox state, giving rise to oxidative stress, with activation of subsequent signaling events leading to inflammatory responses and tissue damage^[12-15]. Mitochondrial dysfunction contributes to the

progression of cerebral ischemic injury and a number of cytoprotective agents, and can mediate their action through mechanisms that converge at the level of the mitochondrion^[16]. Nitric oxide is a well-characterized modulator of mitochondrial function^[17]. As the overall process of ischemia/reperfusion injury is extremely complex, the protective effects of Chinese medicinal herbs are receiving more attention in an effort to find agents for the treatment of ischemic cerebral vascular diseases.

Sophora alopecuroides L., a type of traditional Chinese medicine, has a wide variety of effects on ailments^[18]; for example, dispelling rheumatism and detoxification of the entire body. The extracts contain several different pigments such as matrine, oxymatrine, sophocarpine, Oxysophocarpine, sophramine, sophoridine, lehmannine, aloperine and oxysophoridine^[19]. Oxysophoridine is a new alkaloid that was recently purified from *Sophora alopecuroides* L.^[20].

Oxysophoridine may reduce streptozotocin-induced diabetic rat aorta lesions, and have a protective effect on the ultrastructure of the aorta^[21]. Zhao *et al*^[22] reported that oxysophoridine has a significant protective effect on hippocampal neurons subjected to anoxic injury, and the protective mechanism was related to its reduction of calcium overload and its antioxidant properties. Previous studies addressing cerebral ischemia found that pretreatment with oxysophoridine has a significant protective effect against acute cerebral ischemic injury in mice and on hippocampal neurons subjected to anoxic injury, and that the effective mechanism of oxysophoridine may be attributed to its antioxidant behavior^[23-24]. However, to date, there has been no systematic evaluation on the treatment of ischemic stroke. During stroke, the majority of thrombi can be treated with thrombolytic drugs or self-dissolve. Therefore, it is of great significance to understand the role of oxysophoridine during ischemia/reperfusion injury.

The aim of this study was to observe the protective effects of oxysophoridine on cerebral ischemia/reperfusion injury in mice, in a broader attempt to explore the correlation between oxysophoridine and oxidative stress, as well as N-methyl-D-aspartate receptors.

RESULTS

Quantitative analysis of experimental animals

A total of 204 ICR mice were included in this study and randomly divided into six groups: sham group, model group (cerebral ischemia/ reperfusion model + distilled water), nimodipine group (cerebral ischemia/reperfusion model + 6 mg/kg nimodipine), low dosage (cerebral ischemia/reperfusion model + oxysophoridine 62.5 mg/kg), middle dosage (cerebral ischemia/reperfusion model + oxysophoridine 125 mg/kg), and high dosage (cerebral ischemia/ reperfusion model + oxysophoridine 250 mg/kg) oxysophoridine groups. Each group had 34 mice, including eight for the determination of cerebral

infarct volume, 10 for the determination of brain water content, eight for the measurement of antioxidant enzyme activity and the malondialdehyde content in the ischemic area, and the other eight for the detection of N-methyl-D-aspartate NR1 mRNA and protein expression using fluorescent quantitative PCR and western blot analysis. Twelve mice died during the modeling process; three in the model group, two in the nimodipine group, three in the low dosage group, two in the middle dosage group, and two in the high dosage group. All were randomly complemented. Therefore, 204 mice were involved in the final results analysis.

Protective effect of oxysophoridine on focal cerebral ischemia/reperfusion injury in mice (Figure 1)

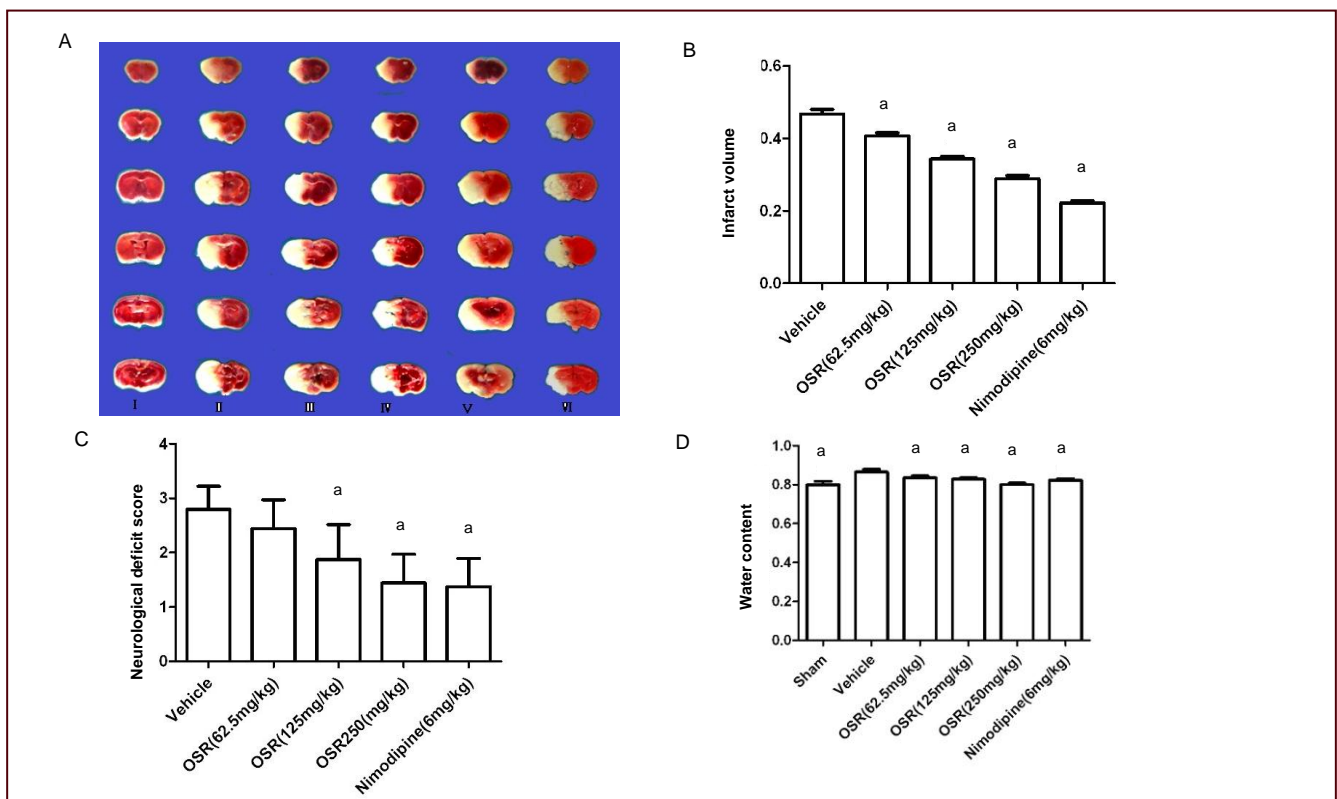


Figure 1 Effects of oxysophoridine (OSR) on cerebral ischemia/reperfusion injury in mice at 24 hours after middle cerebral artery occlusion.

Animals were treated intraperitoneally with distilled water or oxysophoridine (62.5, 125, 250 mg/kg), or intragastrically with nimodipine (6 mg/kg) 7 days before middle cerebral artery occlusion for 2 hours.

(A) Cerebral infarct size (white area) by tetrazolium chloride staining. (I) Sham group; (II) model group; (III) OSR (62.5 mg/kg) group; (IV) OSR (125 mg/kg) group; (V) OSR (250 mg/kg) group; (VI) nimodipine (6 mg/kg) group. Normal brain tissue appeared uniformly red while the infarcted region appeared white.

(B) OSR (62.5, 125, 250 mg/kg) or nimodipine reduced cerebral infarct volume. Infarct volume was expressed as a percentage of total brain volume.

(C) OSR (125, 250 mg/kg) or nimodipine significantly decreased neurological deficit scores. The higher scores indicate more serious brain damage^[25].

(D) OSR (62.5, 125, 250 mg/kg) or nimodipine significantly reduced brain water content. Brain water content = (wet weight – dry weight)/wet weight.

Data were expressed as mean \pm SD. There were eight mice in (B), 34 mice in (C) and 10 mice in (D) per group. ^a $P < 0.01$, vs. model group (one-way analysis of variance followed by least significant difference- t test). Vehicle: Model group.

Administration of oxysophoridine at 62.5, 125, 250 mg/kg and nimodipine could lead to the reduction in cerebral infarct volume (white area in Figure 1A, B), neurological deficit scores (Figure 1C) and brain water content (Figure 1D) when compared with the model group ($P < 0.01$).

In addition, oxysophoridine showed a dose-dependent effect in neuroprotection, with 250 mg/kg oxysophoridine having the greatest effect (Figure 1).

Oxysophoridine regulated oxidative products and antioxidant enzymes in ischemic brain tissue of mice with cerebral ischemia/reperfusion injury

To assess the effect of oxysophoridine on energy metabolism and oxidative stress after reperfusion, levels of adenosine-triphosphate, malondialdehyde, glutathione-peroxidase, superoxide dismutase and catalase were measured.

In the oxysophoridine and nimodipine groups, adenosine-triphosphate content, and the activities of glutathione-peroxidase, superoxide dismutase and catalase in the ischemic brain tissue, were increased when compared with the model group ($P < 0.01$; Figures 2A–D); while levels of malondialdehyde in ischemic brain tissue were reduced ($P < 0.05$ or $P < 0.01$; Figure 2E). Oxysophoridine-induced neuroprotection occurred in a dose-dependent manner, with 250 mg/kg oxysophoridine inducing the greatest effect (Figure 2).

Oxysophoridine regulated nitric oxide content, and nitric oxide synthase and lactate dehydrogenase activities in ischemic brain tissue of mice with cerebral ischemia/reperfusion injury

To assess the effect of oxysophoridine on oxidative stress after reperfusion, nitric oxide level, nitric oxide synthase and lactate dehydrogenase activities were measured.

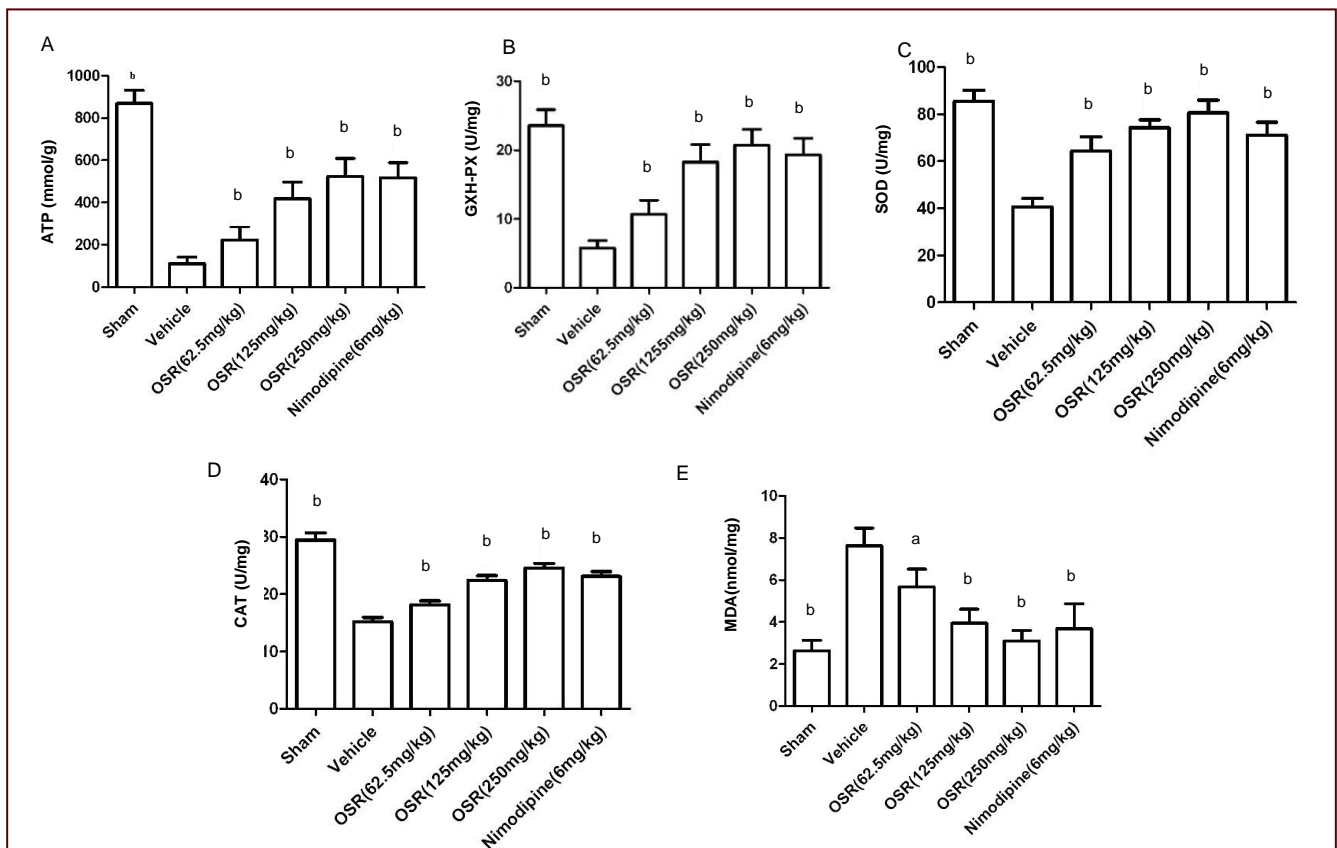


Figure 2 Effects of oxysophoridine (OSR) on adenosine-triphosphate (ATP), malondialdehyde (MDA), glutathione-peroxidase (GSH-PX), superoxide dismutase (SOD) and catalase (CAT) in the mouse ischemic brain at 24 hours after middle cerebral artery occlusion.

Animals were treated intraperitoneally with distilled water or oxysophoridine (62.5, 125, 250 mg/kg), or intragastrically with nimodipine (6 mg/kg) 7 days before middle cerebral artery occlusion for 2 hours.

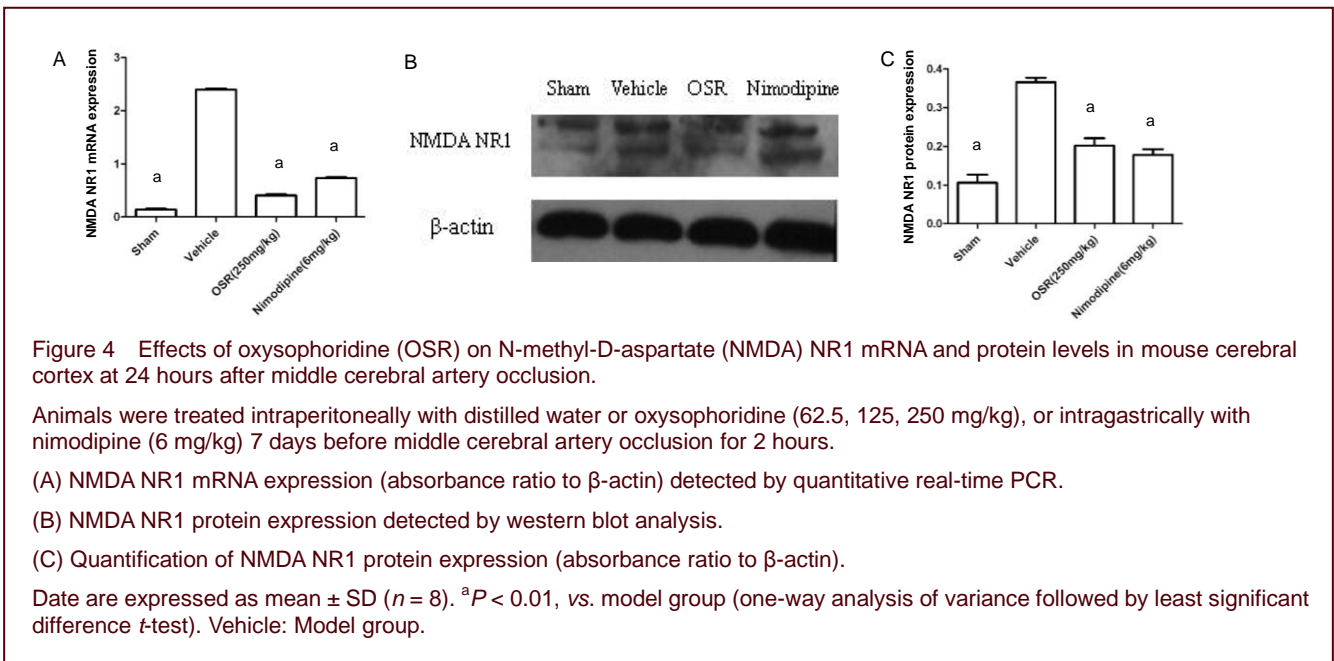
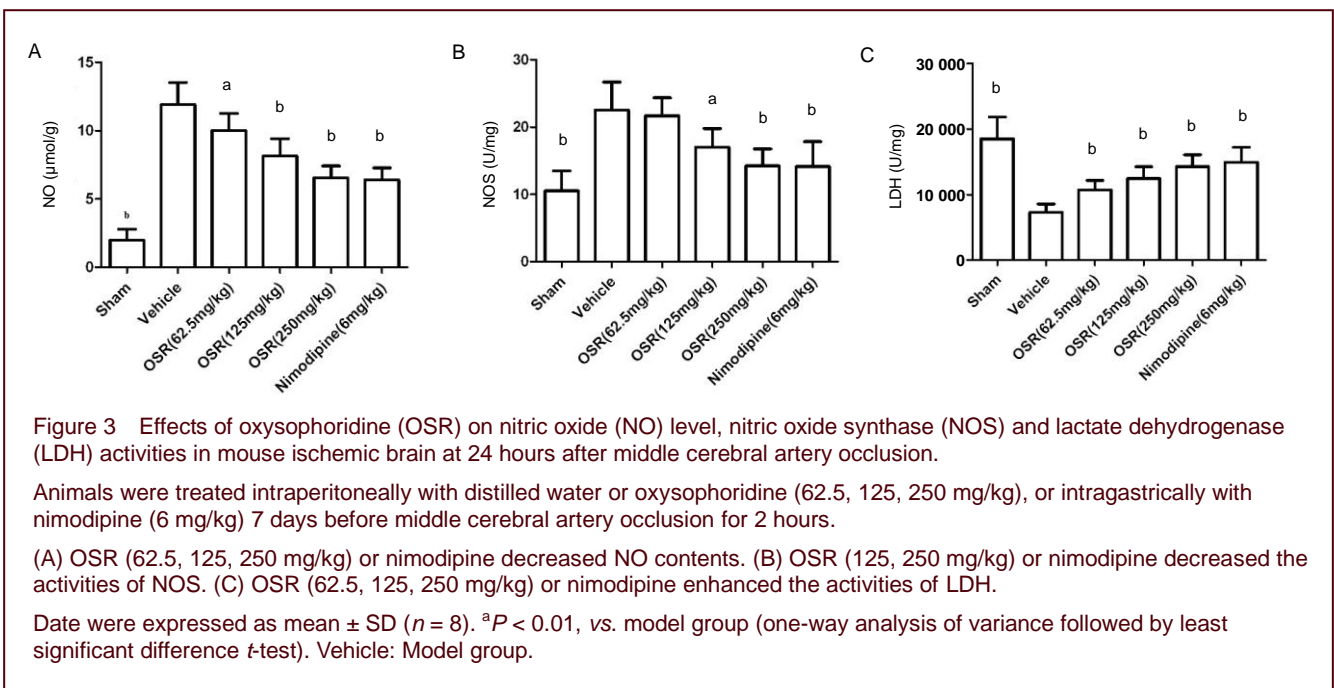
OSR (62.5, 125, 250 mg/kg) or nimodipine increased ATP content (A) and enhanced the activities of GSH-PX (B), SOD (C), and CAT (D), and inhibited the increase of MDA content (E).

Data were expressed as mean \pm SD ($n = 8$). ^a $P < 0.05$, ^b $P < 0.01$, vs. model group (one-way analysis of variance followed by least significant difference t -test). Vehicle: Model group.

In the oxysophoridine (125, 250 mg/kg) and nimodipine groups, nitric oxide level and nitric oxide synthase activity in ischemic brain tissue were reduced compared with the model group ($P < 0.01$; Figures 3A, B); while lactate dehydrogenase activity was higher than the model group ($P < 0.01$; Figure 3C). Oxysophoridine showed a dose-dependent neuroprotective effect, and 250 mg/kg oxysophoridine induced the most obvious effect (Figure 3).

Oxysophoridine inhibited N-methyl-D-aspartate NR1 mRNA and protein expression in the cerebral cortex of mice with cerebral ischemia/reperfusion injury

Figure 4A shows the expression of N-methyl-D-aspartate NR1 mRNA detected by quantitative real-time PCR. N-methyl-D-aspartate NR1 mRNA expression in the oxysophoridine (250 mg/kg) and nimodipine (6 mg/kg) groups were significantly reduced compared with the model group ($P < 0.01$). Figures 4B and C show the expression of N-methyl-D-aspartate NR1 protein detected by western blot analysis. Results indicated that N-methyl-D-aspartate NR1 mRNA and protein expression levels in the oxysophoridine (250 mg/kg) and nimodipine (6 mg/kg) groups were significantly reduced compared with the model group ($P < 0.01$).



DISCUSSION

In the present study, the antioxidant and neuroprotective potential of a Chinese herbal medicine was studied in mice subjected to middle cerebral artery occlusion-induced oxidative stress. Compared with chemical drugs, Chinese herbal medicines show lower toxic side effects^[26]. This study demonstrates for the first time that oxysophoridine possesses neuroprotective effects against middle cerebral artery occlusion-induced cerebral infarction in mice. In this study, we used the method of middle cerebral artery occlusion-induced cerebral infarction in mice, which is a well-documented method that has been used in animals in an attempt to mimic the events of human cerebral ischemia.

Neurological scores are used to evaluate the success of model establishment, and previous studies have shown a positive correlation between neurological scores, infarct volume size and cerebral blood flow changes^[27]. In our experiments, the neurological scores of model animals were mainly 2 or 3, and the percentage of cerebral infarct volume to the entire brain was approximately 46.67%, confirming the consistency of our model. The cerebral infarct volume is an important indicator for the evaluation of ischemic brain damage; the greater the infarct volume, the more serious the brain damage. Ischemic brain damage may lead to cerebral edema, including cytotoxic edema and vasogenic edema^[28]. Cytotoxic edema is the result of sodium and water retention, while vasogenic edema is due to blood-brain barrier damage. Thus reduced brain edema is an important sign of ischemic brain damage. Our study demonstrates that oxysophoridine significantly reduced neurological deficits and cerebral infarct size, and prevented increases in brain water content. This finding suggests that oxysophoridine has a neuroprotective effect against cerebral ischemia/reperfusion injury in mice.

Cerebral ischemia results in a rapid depletion of energy stores that triggers a complex cascade of cellular events such as cellular depolarization and Ca^{2+} influx, resulting in excitotoxic cell injury and death^[29-30]. Cerebral ischemia leads to a lack of glucose and oxygen supply in brain tissue, and a reduction in adenosine-triphosphate production^[31-32]. Therefore, brain adenosine-triphosphate content can reflect the energy status of brain tissue. In our study, oxysophoridine played a neuroprotective effect through increasing adenosine-triphosphate content in the brain after cerebral ischemia/reperfusion injury.

Free radical-induced lipid peroxidation is considered to be an important mechanism of cerebral ischemia/reperfusion injury^[33]. When cerebral ischemia occurs, oxygen and glucose are deprived, and free radicals and lipid peroxides are generated. However, a large number of studies have revealed greater radical and lipid peroxide generation occurs during reperfusion^[34]. Both free radicals and lipid peroxidation of unsaturated fatty tissue can damage cells and the mitochondrial membrane, as well as destroy the blood-brain barrier. The damage to cell membranes leads to lactate dehydrogenase release from the neuronal cytoplasm to the cellular gap, thus reducing the activity of lactate dehydrogenase in the brain. Free radical increase and lipid peroxidation of the brain beyond compensatory ability can lead to antioxidant enzyme mass consumption, thus causing serious injury. The main antioxidant enzymes, including glutathione-peroxidase, superoxide dismutase and catalase, possess the ability to clear free radicals and lipid peroxides. Therefore, the malondialdehyde content, and activities of glutathione-peroxidase, superoxide dismutase, catalase and lactate dehydrogenase, indirectly reflect the content of oxygen free radicals in the brain and the severity of brain damage. The higher the malondialdehyde content and activities of glutathione-peroxidase, superoxide dismutase, catalase and lactate dehydrogenase, the higher the amount of oxygen free radicals in the brain and the more severe the brain damage. Our experimental findings showed that oxysophoridine treatment significantly reduced malondialdehyde content, and enhanced the activities of glutathione-peroxidase, superoxide dismutase, catalase, and lactate dehydrogenase when compared with the model group. The increase in lactate dehydrogenase activity was the evidence that oxysophoridine alleviated nerve cell membrane damage. These findings suggest that the neuroprotective mechanism of oxysophoridine in cerebral ischemia/reperfusion injury may be related to antioxidant actions.

Nitric oxide, a free radical neurotransmitter, has a two-phase role during the pathophysiological process of cerebral ischemia/reperfusion injury. On one hand, low concentrations of nitric oxide can dilate blood vessels to regulate cerebral blood flow and improve microcirculation, and it also has an antiplatelet role, all of which have a protective effect on brain tissue^[35]. In contrast, high concentrations of nitric oxide through inhibition of glycolysis and mitochondrial enzymes may reduce neuronal energy generation, which has a neurotoxic effect on brain tissue^[36]. Nitric oxide is synthesized by

nitric oxide synthase catalysis, and nitric oxide synthase expression is directly related to nitric oxide production. Therefore, we can detect the levels of nitric oxide synthase to observe the changes in nitric oxide. Nitric oxide synthase in the central nervous system has three forms: neuronal nitric oxide synthase, endothelial nitric oxide synthase and inducible nitric oxide synthase. Endothelial nitric oxide synthase produces a small amount of nitric oxide and has a protective effect on brain tissues, while inducible nitric oxide synthase produces large amounts of nitric oxide and thus has a neurotoxic effect on brain tissues. In this experiment, we could not clearly identify which subtype of nitric oxide synthesized the nitric oxide. However, our results are sufficient to confirm that oxysophoridine can reduce nitric oxide synthase activity, inhibit nitric oxide synthesis and prevent nitric oxide-induced brain damage. Nitric oxide serves as a free radical, and the neuroprotective mechanism underlying oxysophoridine against cerebral ischemia/reperfusion injury may be related to its antioxidant actions.

N-methyl-D-aspartate is a specific type of ionotropic glutamate receptor that plays key roles in neuronal plasticity, learning, and memory in the central nervous system. In addition, most N-methyl-D-aspartate receptors are associated with high permeability to Ca^{2+} ^[37]. N-methyl-D-aspartate receptors contain an NR1, NR2 and NR3 subunit. NR1 is a functional subunit of the N-methyl-D-aspartate receptor complex, and has all the pharmacological and electrophysiological properties of N-methyl-D-aspartate receptors. NR1 gene disorders directly affect the biological activity of N-methyl-D-aspartate receptors, and NR1 expression can be a direct indicator of N-methyl-D-aspartate receptor function and status.

Cerebral ischemia/reperfusion injury may cause the upregulation of NR1 receptors and excitatory amino acids on the cell membrane, and induce neuronal damage. The injury mechanism associated with NR1 receptors depends on the following two aspects^[37-38], 1) permeability damage and 2) Ca^{2+} overload-related injury. Yoo *et al*^[40] showed that Barberry extract down-regulated NR1 receptor expression and had a protective effect on cerebral ischemia/reperfusion injury. In this study, compared with the model group, oxysophoridine treatment could significantly reduce NR1 expression, suggesting that oxysophoridine plays a neuroprotective role during cerebral ischemia/reperfusion injury by down-regulating N-methyl-D-aspartate receptor NR1 expression.

In conclusion, the neuroprotective effect of oxysophoridine on cerebral ischemic damage in middle cerebral artery occlusion-reperfusion mice is most likely due to its antioxidant properties, followed by the inhibition of the N-methyl-D-aspartate NR1 receptor. Treatment with oxysophoridine is not limited to one factor but involves many mechanisms, most of which may be interrelated. These experimental findings provide a scientific basis to study the mechanism of oxysophoridine against middle cerebral artery occlusion-induced cerebral ischemia/reperfusion injury in mice.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

The experiment was performed at the Medical Science-Technology Research Center, Ningxia Medical University, China from August 2011 to January 2012.

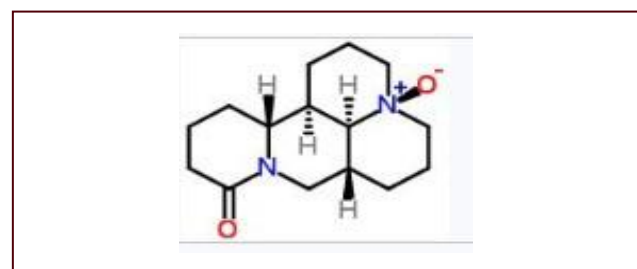
Materials

Animals

A total of 204 male ICR mice were provided by the Experimental Animal Center of Ningxia Medical University, China. All animals, aged between 8–12 weeks, weighing 25–30 g (license No. SCXK 2005-001), were housed in the Departmental Animal House and exposed to a 12-hour light/dark cycle. All experiments were performed in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[41].

Drugs

Oxysophoridine was supplied by the Institution of Chemistry and Chemical Engineering, Ningxia Agricultural College, China (purity 98%, lot No. 960368). Oxysophoridine was isolated as white crystals, with the molecular formula of $\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}_2$ and a molecular weight of 264.36. The molecular structural formula is shown below:



Methods

Establishment of middle cerebral artery occlusion model

Mice were subjected to left-sided middle cerebral artery occlusion as previously described^[42-44]. Briefly, mice were anesthetized with an intraperitoneal injection of 3.5% (w/v) chloral hydrate (Sigma, St. Louis, MO, USA) in 0.9% (w/v) NaCl (0.1 mL/10 g), and placed in dorsal recumbency. Under sterile conditions, a ventral neck incision was made, and the external carotid artery and internal carotid artery were exposed and carefully isolated. A nylon monofilament (15 mm in length and 0.15 mm in diameter) was inserted in the lumen of the left external carotid artery and internal carotid artery, to occlude the origin of the left middle carotid artery. After 2 hours of ischemia, the mice were re-anesthetized, the occlusive suture was removed, and reperfusion was allowed for 24 hours. Body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a heating lamp linked to a rectal thermometer during all phases of the surgery.

Drug administration

Oxysophoridine and nimodipine (Bayer Healthcare Company Ltd., Beijing, China) were dissolved in 0.9% (w/v) NaCl solution. In the oxysophoridine treatment groups, oxysophoridine (62.5, 125, 250 mg/kg) was administered intraperitoneally at a volume of 0.1 mL/10 g; in the nimodipine group, nimodipine (6 mg/kg) was given intragastrically at a volume of 0.1 mL/10 g^[21]. In the sham and model groups, equal volumes of distilled water were given. All drug solutions were freshly prepared before use. Both oxysophoridine and nimodipine were administered once a day for 7 days before middle cerebral artery occlusion.

Neurobehavioral testing

Mice were tested for neurological deficits at 24 hours after middle cerebral artery occlusion and reperfusion. These mice were scored as previously described with minor modifications^[45]. Score 0: no observable neurological deficits; score 1: failure to extend the right forepaw; score 2: circling to the contralateral side; score 3: inclining to the contralateral side; score 4: unable or difficult to ambulate. The higher scores indicated the worse neurobehavioral dysfunction, and *vice versa*^[46]. All experimental data were statistically analyzed using a single-blinded method.

Assessment of cerebral infarct size

Mice were sacrificed by decapitation after 24 hours of reperfusion. The brains were cut into 6×1 mm coronal slices, 1 mm away from the frontal pole. The slices were

placed in 2% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma) in 0.9% (w/v) PBS and stained at 37°C for 30 minutes. Normal brain tissue appeared uniformly red while the infarcted region appeared white^[47-48]. Each stained brain slice was drawn using a computerized image analyzer (Image-Pro plus; Media Cybernetics, Inc., Bethesda, MD, USA). Infarct volume was expressed as a percentage of total brain volume.

Assessment of brain water content

For vasogenic edema, animals were sacrificed 24 hours after middle cerebral artery occlusion/reperfusion by chloral hydrate overdose. The brains were divided into the ipsilateral hemisphere (ischemic side) and contralateral hemisphere. The ipsilateral hemisphere was weighed to obtain the wet weight and then dried at 110°C for 24 hours. The brain water content in the ipsilateral hemisphere was calculated as follows: water content = (wet weight – dry weight)/wet weight^[49].

Determination of adenosine-triphosphate content

The ischemic hemisphere was weighed and homogenized (10% w/v) with cold 0.9% (w/v) NaCl. Adenosine-triphosphate concentration was measured according to the manufacturer's specifications (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; lot No. 20110818).

Determination of nitric oxide synthase activity and nitric oxide levels

The ischemic hemisphere was homogenized at a ratio of 1:9 (w/v) in ice-cold saline after blotting and weighing. The homogenate was centrifuged at $3\,500 \times g$ at 4°C for 15 minutes. The supernatant was used to determine the activity of nitric oxide synthase and the level of nitric oxide using a spectrophotometer (Nanjing Jiancheng Bioengineering Institute; lot No. 20110810, 20110803).

Determination of antioxidant enzyme activity and malondialdehyde content

The ischemic hemisphere was homogenized at a ratio of 1:9 (w/v) in ice-cold saline after blotting and weighing. The homogenate was centrifuged at $3\,500 \times g$ at 4°C for 15 minutes, and then the supernatant was used to determine the activity of glutathione-peroxidase, lactate dehydrogenase, catalase, superoxide dismutase and the level of malondialdehyde using the spectrophotometer (Nanjing Jiancheng Bioengineering Institute; lot No. 20110801, 20110712, 20110817, 20110801, 20110811).

Quantitative real-time PCR

Quantitative real-time PCR was used to assess gene

expression for the glutamate receptor subunits N-methyl-D-aspartate NR1 in ischemic tissue. To standardize gene expression across samples, we compared the expression levels of one well-known housekeeping gene (β -actin) within the samples. For reverse transcription, we used 5 μ g of total RNA and the Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Conditions for amplification were as follows: initial denaturation for 5 minutes at 94°C, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final extension stop of 72°C for 10 minutes. The mouse-specific (sense and antisense) primers for N-methyl-D-aspartate NR1 and β -actin were 5'-CCT ACA AGC GAC ACA AGG ATG-3' and 5'-GGC TCT GCT CTA CCA CTC TTT C-3' (N-methyl-D-aspartate NR1 109 bp); 5'-GAG ACC TTC AAC ACC CCA GC-3' and 5'-ATG TCA CGC ACG ATT TCC C-3' (β -actin 285 bp). The mRNA levels of N-methyl-D-aspartate NR1 were evaluated in the model group and oxysophoridine (250 mg/kg) group by real-time PCR using the SYBR-Green method^[50].

Western blot analysis

Protein was extracted from the cerebral cortex from the ischemic side and quantified using the KEYGEN Total Protein Extraction Kit (ChenGen Biotech, Nanjing, China; lot No. P0013B). Aliquots containing 80 mg of total protein were boiled for 8 minutes in loading buffer, and each aliquot of protein (80 μ g) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked at 4°C for 2 hours, followed by incubation at 4°C overnight with a rat anti-mouse polyclonal antibody against N-methyl-D-aspartate NR1 (1:200; Abcam, Cambridge, UK), β -actin (1:500; Abcam) and goat anti-rat IgG (1:5 000; Zhongshan Golden Bridge, Beijing, China). Blots were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) in the dark, and were imaged by EM-CCD in a dark box. The absorbance of protein bands were quantitatively analyzed using Bio-RAD image analysis software (NIS-Elements BR 3.1). The level of N-methyl-D-aspartate NR1 was normalized to the level of β -actin.

Statistical analysis

All data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were expressed as mean \pm SD. Multi-group comparisons were made by analysis of variance and the least significant difference *t*-test was used for further analysis of pairwise comparison. Statistical significance was set at $P < 0.05$.

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Author contributions: Hongbo Wang and Kuai Zhang participated in experimentation, prepared the animal models, and analyzed experimental data. Yuxiang Li wrote the manuscript. Tengfei Wang and Lin Ma analyzed experimental data and performed statistical analyses. Ning Jiang, Yanrong Wang, Tao Sun and Jianqiang Yu were responsible for the experimental concept and design, validation and guidance of the study. All authors participated in experimentation and approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Ningxia Medical College in China.

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