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Curcumin protects against ischemic spinal cord injury

*The pathway effect**

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Research Highlights

(1) This study established a rat model of spinal cord ischemia by ligating the $L_{3–7}$ lumbar artery below the left renal artery. This study only ligated the lumbar artery, retained the artery below the dominal aorta, and avoided complications such as urine and stool.
(2) Previous studies focused on the protective effect of curcumin on spinal cord injury, mainly regarding spinal cord contusion injury, but did not focus on the protective effects of curcumin on ischemic cells of the spinal cord. The present study investigated the protective effect of curcumin on ischemic cells of the spinal cord, and explored the possible mechanisms of this effect.
(3) Our results provide a theoretical basis and methodology for the prevention and treatment of spinal cord ischemia in the clinic.

Abstract

Inducible nitric oxide synthase and N-methyl-D-aspartate receptors have been shown to participate in nerve cell injury during spinal cord ischemia. This study observed a protective effect of curcumin on ischemic spinal cord injury. Models of spinal cord ischemia were established by ligating the lumbar artery from the left renal artery to the bifurcation of the abdominal aorta. At 24 hours after model establishment, the rats were intraperitoneally injected with curcumin. Reverse transcription-polymerase chain reaction and immunohistochemical results demonstrated that after spinal cord ischemia, inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA and protein expression significantly increased. However, curcumin significantly decreased inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA and protein expression in the ischemic spinal cord. Tarlov scale results showed that curcumin significantly improved motor function of the rat hind limb after spinal cord ischemia. The results demonstrate that curcumin exerts a neuroprotective fect against ischemic spinal cord injury by decreasing inducible nitric oxide synthase and N-methyl-D-aspartate receptor expression.

Key Words

neural regeneration; traditional Chinese medicine; curcumin; spinal cord injury; ischemic injury; N-methyl-D-aspartate receptor; inducible nitric oxide synthase; neuroprotection; grants-supported paper; neuroregeneration

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INTRODUCTION

Spinal cord ischemia commonly occurs in patients with spine injury, vascular surgery or thrombotic disease, or from complications of systemic diseases, such as malignant tumors, amniotic fluid embolisms or abnormal clotting mechanisms. Ischemic spinal cord injury is often combined with limb paralysis, sensory disturbance and difficult defecation, which severely impacts on patient quality of life. Thus, it is essential to prevent and treat ischemic spinal cord injury. At present, the drugs for preventing ischemic spinal cord injury mainly contain methylprednisolone and minocycline[1-2]. However, these drugs had adverse reactions, so they have been rarely used in the clinic. Therefore, it is critical to find a novel drug with few adverse reactions for preventing ischemic spinal cord injury.

Curcumin is an active substance isolated from the root of curcuma longa, and commonly used as an additive for pigment or food. Curcumin has extensive biological properties and plays an anti-inflammatory, anti-oxidant, free radical scavenging, and anti-tumor role. In addition, curcumin has been shown to inhibit ischemia/reperfusion injury and stabilize the cell membrane[3-14].

Curcumin molecular formula: $C_{21}H_{20}O_6$, molecular weight 368.37. Curcumin has protective effects against cerebral ischemia and myocardial ischemia[15-22]. It remains unclear whether curcumin has the same protective effect on spinal cord ischemia-induced cell injury.

The theory of Ca^{2+} overloading-induced cell injury is a well-known mechanism underlying nerve cell injury following spinal cord injury[23]. During ischemia and hypoxia, because of a lack of energy, neurons release glutamic acid and aspartate, but reuptake is reduced. Simultaneously, abundant leakage of excitatory amino acids from dead cells increases the concentration of excitatory amino acids in the nerve gap. These excessive amounts of excitatory amino acids act on N-methyl-D-aspartate receptors on the cell membrane, resulting in overstimulation. Abundant Ca^{2+} influx *via* N-methyl-D-aspartate receptors, voltage controlled Ca^{2+} channels, and Na^{+}-Ca^{2+} exchange, results in intracellular Ca^{2+} overload. Subsequently, Ca^{2+} activates a series of cytotoxicity-related enzymes, such as nitric oxide synthase, protein kinase C, phospholipase, and proteases. Previous studies confirmed that inducible nitric oxide synthase could mediate apoptosis of ischemic/hypoxic nerve cells through p38MAPK and caspase-3[23-24]. Thus, Ca^{2+} overload is a main reason for nerve cell disintegration and necrosis in the late stage of nerve ischemia. If N-methyl-D-aspartate receptor activity could be inhibited, Ca^{2+} influx would be diminished, resulting in effective prevention of N-methyl-D-aspartate receptor-mediated ischemic injury. Pannu and Singh[24] showed that inducible nitric oxide synthase and N-methyl-D-aspartate receptor play an important role in ischemic nerve cell injury. Our previous studies verified that inducible nitric oxide synthase and N-methyl-D-aspartate receptors are involved in ischemic nerve cell injury during spinal cord ischemia, which aggravated ischemic nerve injury in the central nervous system[25-28].

This study established a rat model of spinal cord ischemia by ligating the lumbar artery. The protective effects of curcumin were investigated by assessing neurological function scores, and N-methyl-D-aspartate receptor and inducible nitric oxide synthase gene and protein expression in the ischemic spinal cord.

RESULTS

Quantitative analysis of experimental animals

A total of 30 Sprague-Dawley rats were equally and randomly divided into sham surgery group (isolation of lumbar artery + intraperitoneal injection of saline), ischemia group (spinal cord ischemia + intraperitoneal injection of saline), and curcumin group (spinal cord ischemia + intraperitoneal injection of curcumin). A total of 30 rats were

included in the final analysis.

Curcumin improved the motor function of the hind limb in rats with spinal cord ischemia

At 7 days after administration, Tarlov scale results demonstrated that the motor function score of the rat hind limb was significantly lower in rats with spinal cord ischemia (average 1.3 points) than that in the sham surgery group (5 points) (P < 0.01). The motor function score was significantly higher in the curcumin group (average 4.7 points) than that in the ischemia group (P < 0.01) (Table 1).

Table 1 Effect of curcumin on the motor function of the rat hind limb after spinal cord ischemia

Group	Motor function score					
	0	1	2	3	4	5
Sham surgery	0	0	0	0	0	10
Ischemia	3	2	4	1	0	0
Curcumin	0	0	0	0	3	7

Each group contained 10 rats. A high score represented good motor function. The data in Table 1 were number of rats in each group.

Curcumin downregulated inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA expression in the ischemic spinal cord of rats

RT-PCR results demonstrated that inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA expression was low in the sham surgery group. Inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA expression was significantly higher in the ischemic spinal cord than that in the sham surgery group (P < 0.01). Compared with the ischemia group, inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA expression was significantly downregulated in the ischemic spinal cord in the curcumin group (P < 0.05; Figure 1, Table 2).

Curcumin reduced inducible nitric oxide synthase and N-methyl-D-aspartate receptor protein expression in the ischemic spinal cord of rats

Immunohistochemical staining revealed that inducible nitric oxide synthase was expressed in the cytoplasm of neurons, glial cells and vascular endothelial cells. After ischemia, inducible nitric oxide synthase expression significantly increased (P < 0.01). Inducible nitric oxide synthase expression was significantly lower in the curcumin group than that in the ischemia group (P < 0.05). Inducible nitric oxide synthase expression was low in the sham surgery group (Figure 2, Table 3).

N-methyl-D-aspartate receptor expression in the cell membrane and cytoplasm was observed in neurons, glial cells and vascular endothelial cells. N-methyl-D-aspartate receptor expression significantly increased in the ischemia group (P < 0.01). N-methyl-D-aspartate receptor expression was lower in the curcumin group than that in the ischemia group (P < 0.05; Figure 3, Table 3). These results suggested that curcumin significantly reduced inducible nitric oxide synthase and N-methyl-D- aspartate receptor protein expression in rats with spinal cord ischemia.

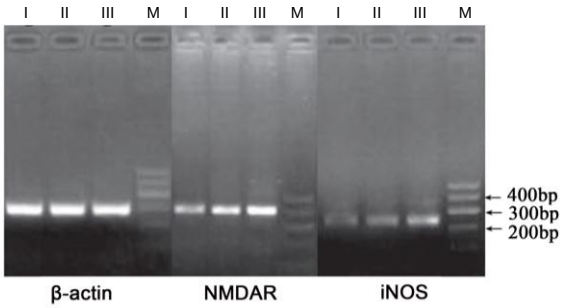

Figure 1 Electrophoretogram of inducible nitric oxide synthase (iNOS) and N-methyl-D-aspartate receptor (NMDAR) mRNA expression in the ischemic spinal cord of rats at 7 days after treatment with curcumin.

M: Marker; I: sham surgery group; II: curcumin group; III: ischemia group. iNOS and NMDAR mRNA expression was less in the sham surgery group; iNOS and NMDAR mRNA expression significantly increased in the ischemia group; iNOS and NMDAR mRNA expression in the curcumin group was between the sham surgery group and ischemia group.

Table 2 Effects of curcumin on inducible nitric oxide synthase (iNOS) and N-methyl-D-aspartate receptor (NMDAR) mRNA expression (absorbance ratio of NMDAR or iNOS to β-actin) in each group

Group	NMDAR	iNOS
Sham surgery	0.26±0.08	0.15±0.06
Ischemia	0.76±0.16[b]	0.69±0.16[b]
Curcumin	0.42±0.13[ac]	0.28±0.10[ac]

Results were expressed as mean ± SD. Each group contained 10 rats. [a]P < 0.05, [b]P < 0.01, *vs.* sham surgery group; [c]P < 0.05, *vs.* ischemia group. One-way analysis of variance was used. Paired comparisons were performed using least significant difference test.

DISCUSSION

Ischemic spinal cord injury is commonly induced by vascular surgery, and presents as acute paraplegia or delayed paraplegia. The rate of paraplegia induced by aortic aneurysm surgery is 0.9–40.0%[29], which is a severe complication, and this greatly impacts on patient condition.

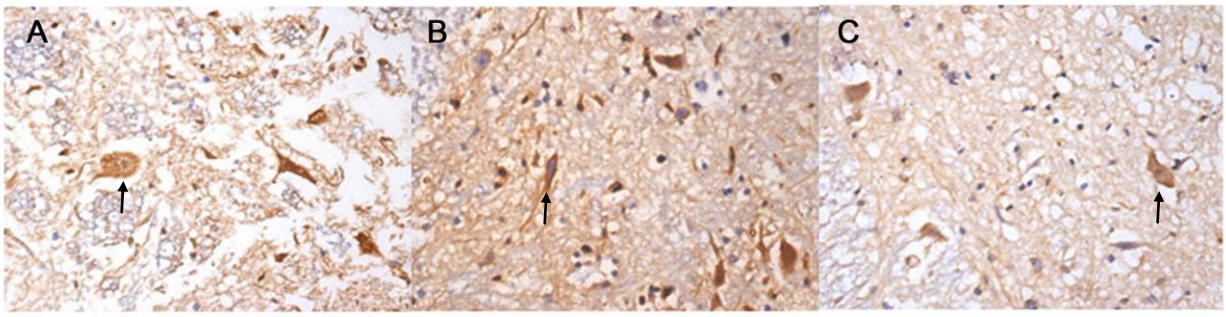

Figure 2 Inducible nitric oxide synthase (iNOS) expression decreased in ischemic spinal cord of rats after treatment with curcumin (immunohistochemistry, × 400).

Arrows show iNOS-positive brown products, which mainly express in the cytoplasm of neurons, glial cells and vascular endothelial cells. (A) Sham surgery group: a few positive expression; (B) ischemia group: significantly increased iNOS expression; (C) curcumin group: iNOS expression was between sham surgery group and ischemia group.

In the clinic, physicians have tried to use various measures[30-34] for the prevention and treatment of ischemic spinal cord injury[35-37], including *in vitro* bypass or shunts, intercostal artery replacement, cerebrospinal fluid drainage, hypothermia and drugs (such as antioxidants, free radical scavengers, excitatory amino acid receptor antagonists, corticosteroids, and calcium channel blockers). However, outcomes remain unsatisfactory.

Table 3 Curcumin effects on inducible nitric oxide synthase (iNOS) and N-methyl-D-aspartate receptor (NMDAR) protein expression (H-score) in ischemic spinal cord of rats

Group	iNOS	NMDAR
Sham surgery	0.16±0.09	0.48±0.14
Ischemia	0.95±0.27[a]	2.24±0.27[a]
Curcumin	0.47±0.18[b]	0.83±0.18[b]

Results are expressed as mean ± SD. Each group contained 10 rats. [a]$P < 0.01$, *vs*. sham surgery group; [b]$P < 0.05$, *vs*. ischemia group. One-way analysis of variance was used. Paired comparisons were performed using least significant difference test. A high score shows high expression of immunopositive products.

This study was performed, with some modifications, in accordance with Zivin and DeGirolami[23], which established models of lumbar spinal cord ischemia induced by abdominal aorta occlusion. Models of spinal cord ischemia were established by ligating the lumbar artery from the left renal artery to the abdominal aorta bifurcation. The lumbar artery from the abdominal aorta is an important source of blood supply for the spinal cord. After ligation, limbs experienced movement disorder, and the degree of injury was even. Simultaneously, the lumbar artery was cut off to prevent recanalization of the lumbar artery. This study only ligated the lumbar artery, and retained the blood supply of the artery below the abdominal aorta bifurcation. Thus, this did not injure other organs dominated by the abdominal aorta, avoided complications such as urine and stool disorders, and reduced the impact on detected indexes. Tarlov scale results showed that neurological function scores significantly reduced in rats from the ischemia group, which indicated that the model closely mimicked the clinical state, was stable and had few interference factors. Therefore, this model was ideal for studying ischemic spinal cord injury.

Results from this study demonstrated that inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA and protein expression were significantly higher in the ischemia group than those in the sham surgery group. Inducible nitric oxide synthase and N-methyl-D-aspartate receptor was mainly expressed in the cell membrane and cytoplasm, and less in nuclei, suggesting that inducible nitric oxide synthase and N-methyl-D-aspartate receptor are definitely expressed in neurons, as shown using the neuron specific marker NeuN, and play a crucial role in ischemic spinal cord injury, which was consistent with previous results[4-7, 38-39].

Ischemic and hypoxic spinal cord disease is a kind of vascular disease, and is often combined with complications in the nervous system to different degrees. The decrease in blood supply in the spinal cord led to neuronal ischemia, hypoxia, and exhausted adenosine triphosphate storage, excessive release of glutamate, which overstimulated N-methyl-D-aspartate receptors, produced excitatory postsynaptic potential, intracellular Ca^{2+} influx, and subsequent calcium overload. As a result, a series of biochemical cascades occurs, including activation of protein kinases, phospholipase A2, phospholipase C and nitric oxide synthase.

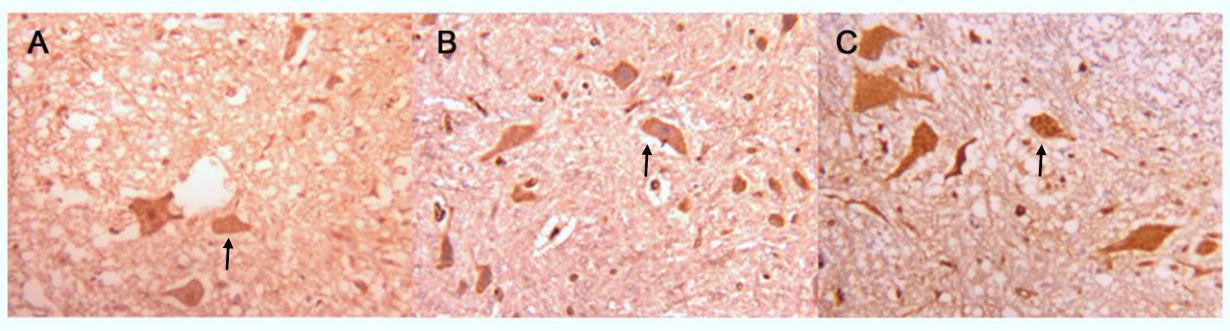

Figure 3 N-methyl-D-aspartate receptor (NMDAR) expression decreased in the ischemic spinal cord of rats after treatment with curcumin for 7 days (immunohistochemistry, ×400).

Arrows show NMDAR-positive products, which are mainly expressed in the cytoplasm and cell membrane of spinal cord neurons, glial cells and vascular endothelial cells. There was little NMDAR-positive expression in the sham surgery group (A), and increased expression of NMDAR in the ischemia group (B). However, NMDAR expression in the curcumin group (C) was between the sham surgery group and ischemia group.

These reactions contribute to the large release of excitatory amino acids in the presynaptic membrane, which activate postsynaptic N-methyl-D-aspartate receptors, increase opening of receptor gated ion channels, and result in more Ca^{2+} influx into cells.

In addition, activation of key enzymes required for nerve cell injury and the production of abundant oxygen free radicals causes nerve cell injury[40-44]. Simultaneously, Ca^{2+} influx induces endonuclease activation, which can initiate cell apoptosis, and further aggravate tissue injury[45-46]. N-methyl-D-aspartate receptors are extensively distributed in the central nervous system, and are essential receptors for noxious stimulation by the spinal cord. Inducible nitric oxide synthase promotes the increase in nitric oxide production, produces toxic substances *via* a series of reactions, and causes injury to protein, nucleic acids and lipid membranes[47], resulting in cell apoptosis[48-49]. Therefore, drugs used to inhibit inducible nitric oxide synthase and N-methyl-D-aspartate receptors can protect nerve cells.

The pathophysiological mechanism of ischemic spinal cord injury is very complicated. Recently, studies have focused on lipid peroxidation[50-52], inflammation[53-54] and cell apoptosis[55-57] in addition to the toxic effect of excitatory amino acids.

Curcumin is a food additive[58] approved by the World Health Organization, US Food and Drug Administration, and many other countries, which has been shown to prevent various diseases[59]. The anti-inflammatory, antioxidant and inhibitory effects on cell apoptosis of curcumin have garnered much interest: (1) curcumin has been considered as a natural antioxidant. Antioxidation was strongly associated with the phenolic hydroxyls, which can capture or scavenge free radicals. Curcumin has been demonstrated to remove superoxide anions, which are involved in peroxidation, H_2O_2 and nitric oxide, to maintain the activation of superoxide dismutase, catalase and glutathione peroxidase, which suppress lipid peroxidation and protect cell membrane structure against damage[60-62].

(2) For decades, curcumin has been shown to play an important role in the treatment of inflammation-mediated diseases, such as tumor, atherosclerosis, diabetes, and rheumatoid arthritis. Curcumin exerts anti-inflammatory effects possibly by inhibiting mediators of inflammation such as cyclooxygenase (COX-1 and COX-2), lipoxygenase, inducible nitric oxide synthase, cytokine products (interferon-C and tumor necrosis factor) and other transcription factors (nuclear factor-κB)[63-65]. The main structural components of curcumin that exert the anti-inflammatory effect are the 4-hydroxy benzene ring, 3,5-electron donor group, and unsaturated ketones[66-69].

(3) Curcumin has many targets during inhibition of cell apoptosis. Curcumin suppresses the growth of some tumor cell strains including drug-resistant cell strains and the expression of cyclin-dependent kinases (Cdk4 and Cdk6), induces tumor cell apoptosis by activating caspase-8, stimulates mitochondrial release of cytochrome C, activates caspase-9 and caspase-3, and activates poly-ADP ribose polymerase. Curcumin regulates tumor cell proliferation, invasion, metastasis and angiogenesis, decreases the expression of COX-2, matrix

metalloproteinase-9, tumor necrosis factor, cyclin D1 and adhesion molecule[70-72].

In this study, Tarlov scale results showed that neurological function scores were significantly higher in the curcumin group than in the ischemia group, suggesting that curcumin has a certain protective effect on ischemic spinal cord injury. Curcumin has been shown to diminish inducible nitric oxide synthase expression in tumor cells and tissues[73]. Results from the present study demonstrated that curcumin reduced inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA and protein expression in the ischemic spinal cord, and confirmed that curcumin protected ischemic cells in the spinal cord probably by affecting the N-methyl-D-aspartate receptor-Ca^{2+}-inducible nitric oxide synthase pathway. Numerous previous studies have shown that curcumin could inhibit inflammation, oxidation and suppress cell apoptosis[3-14]. Whether the protective effect of curcumin on ischemic spinal cord injury was associated with anti-inflammation, antioxidation, inhibition of cell apoptosis, or inhibition of the neurotoxic effects of excitatory amino acid remains unclear, and deserves further investigation.

In summary, our experimental results demonstrated that curcumin has a protective effect on spinal cord ischemia. The protective mechanism of curcumin most likely involves a reduction in inducible nitric oxide synthase and N-methyl-D-aspartate receptor expression, and suppression of the neurotoxic effect of excitatory amino acids, which protects cells in the spinal cord from ischemia.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were conducted in the Fujian Institute of Neurosurgery, China in November 2011.

Materials

A total of 30 healthy clean male adult Sprague-Dawley rats weighing 250 ± 30 g were purchased from Shanghai Silaike Experimental Animal Co., Ltd. (Shanghai, China; license No. SCXK (Hu) 2003-0003). All animals were allowed free access to food and water before experimentation and housed at 23 ± 2°C, a humidity of about 56% in a 12-hour light/dark cycle, without glare or loud sound. Ultraviolet disinfection and ventilation were conducted every day. The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[74].

Methods

Preparation of rat model of spinal cord ischemia

The rats were intraperitoneally anesthetized with 10% (v/v) chloral hydrate 0.3 mg/kg, and fixed on the operating table in a supine position. After shaving or sterilization, an abdominal midline incision was made, and then all lumbar arteries from the left renal artery to the abdominal aorta bifurcation were bluntly isolated under a microscope (Olympus, Tatsuno, Japan). The lumbar artery was ligated with a No. 0 silk suture to establish models of permanent spinal cord ischemia[23] (Figure 4). The abdominal cavity was closed after washing with penicillin. Twenty-four hours later, hind limb motor function was evaluated using the Tarlov scoring system[75]. The animals scoring < 2 points were considered successful models. In the sham surgery group, the lumbar artery was only isolated, but not ligated.

Drug intervention

Curcumin (powder, purity 99%, lot No. 293963; Sigma, St. Louis, MO, USA) was used in this study. At 24 hours after model induction, 80 mg curcumin was dissolved in 0.4 mL dimethyl sulfoxide (DMSO). The total volume was 40 mL by adding saline. Curcumin solution (2 mg/mL) was prepared and rats were intraperitoneally injected with curcumin solution 30 mg/kg at 9 a.m. every day for 7 consecutive days. The sham surgery group and ischemia group were administered an equal volume of saline.

Score of motor function of rat hind limb

At 1 week after administration, motor function of the rat hind limb was evaluated using the modified Tarlov method[75] by three people who did not participate in the experiments. Modified Tarlov scoring: 0, no activity, no weight loading; 1, with activity, no weight loading; 2, frequent or powerful activity, no weight loading; 3, hind limb can support body weight, can walk one or two steps; 4, can walk, with only slight handicap; 5, normal walking.

Specimen collection and tissue section preparation

After scoring, the rats were intraperitoneally anesthetized with 10% (v/v) chloral hydrate (0.3 mg/kg). The thoracic cavity was opened, and a puncture was made through the left ventricle into the aorta. Subsequently, 80–100 mL

saline was perfused. The vertebral canal was cut through the middle of the back over the appropriate vertebrae. $L_{2–4}$ spinal cord tissue was obtained and fixed in 4% (w/v) paraformaldehyde for 12 hours, followed by paraffin sectioning. After perfusion with saline through the heart, the specimens for RT-PCR were treated with liquid nitrogen, and stored at –80°C.

Inducible nitric oxide synthase and N-methyl-D-aspartate receptor mRNA expression in the ischemic spinal cord of rats as detected by RT-PCR

Using the Trizol method, total RNA was extracted on ice. RNA concentration and the absorbance ratio at 260 nm/280 nm were measured using a spectrophotometer (3100 Pro; GE, Bethesda, MD, USA). Total RNA (1 μg) was reverse-transcribed in accordance with the RT-PCR kit (Promega, Madison, WI, USA). Taking 2 μL of the transcript as a template, the corresponding primer (β-actin as an internal reference) was added for PCR amplification. The reaction volume was 25 μL. All primer sequences are shown in Table 4.

Amplification conditions were as follows: predenaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, total 25 cycles, followed by extension at 72°C for 7 minutes. PCR products were electrophoresed on a 1.5% (w/v) agarose gel and observed with a gel imaging system (Gel Doc XR; Bio-Rad, Hercules, CA, USA). β-Actin served as an internal reference. PCR products were semiquantitatively analyzed using Quantity one analysis software (Gel Doc XR; Bio-Rad).

Inducible nitric oxide synthase and N-methyl-D-aspartate immunoreactivity expression in the ischemic spinal cord of rats as detected by immunohistochemistry

After dewaxing and hydration, the sections were incubated with H_2O_2 at room temperature for 10 minutes to deactivate endogenous peroxidase, and blocked with normal goat serum (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature for 20 minutes. Subsequently, the sections were incubated with rabbit anti-inducible nitric oxide synthase polyclonal antibody or N-methyl-D-aspartate receptor polyclonal antibody (1:80; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 4°C overnight, with biotinylated goat anti-rabbit IgG (1:500; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 37°C for 20 minutes, with ABC mixture (1:99; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Sections were visualized with 3,3′-diaminobenzidine (DAB) (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.), counterstained with hematoxylin, and mounted. Sections incubated with only phosphate buffered saline (PBS) were used as the negative control. Immunohistochemical staining was scored as previously described[76]. Five high-power visual fields (× 400) were randomly selected under a microscope. H-score= $\sum$ Pi(i + 1). “i" represents staining (dark or light). Sections were scored from 0 to 3 as follows: 0, no coloration; 1, light brown; 2, brown; 3, dark brown. Pi represents cell percentage of each staining degree (0–100%). Four points indicated full marks. The sections were examined by three independent observers who were blinded to the treatment groups.

Table 4 PCR primer sequences of inducible nitric oxide synthase (iNOS), N-methyl-D-aspartate receptor (NMDAR) and β-actin

Primer	Sequence (5′–3′)	Product length (bp)
NMDAR	Forward: TGC AAG TGG GCA TCT ACA ATG G Reverse: TTG TTG CTG TTG TTT ACC CGC	400
iNOS	Forward: CAC GGA GAA CAG CAG AGT TGG Reverse: TGT GGT GAA GGG TGT CGT G	342
β-actin	Forward: GGT ATG GGT CAG AAG GAC TCC Reverse: TGA TCT TCA TGG TGC TAC GAG CC	250

Statistical analysis

The data were analyzed with SPSS 13.0 (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Intergroup comparison was conducted with one-way analysis of variance followed by least significant difference test. A value of $P < 0.05$ was considered statistically significant.

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