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Neuroprotective effect of pretreatment with ganoderma lucidum in cerebral ischemia/reperfusion injury in rat hippocampus

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Abstract

Ganoderma lucidum is a traditional Chinese medicine, which has been shown to have both anti-oxidative and anti-inflammatory effects, and noticeably decreases both the infarct area and neuronal apoptosis of the ischemic cortex. This study aimed to investigate the protective effects and mechanisms of pretreatment with ganoderma lucidum (by intragastric administration) in cerebral ischemia/reperfusion injury in rats. Our results showed that pretreatment with ganoderma lucidum for 3 and 7 days reduced neuronal loss in the hippocampus, diminished the content of malondialdehyde in the hippocampus and serum, decreased the levels of tumor necrosis factor-α and interleukin-8 in the hippocampus, and increased the activity of superoxide dismutase in the hippocampus and serum. These results suggest that pretreatment with ganoderma lucidum was protective against cerebral ischemia/reperfusion injury through its anti-oxidative and anti-inflammatory actions.

Key Words: *nerve regeneration; cerebral ischemia/reperfusion; ganoderma lucidum; anti-oxidative; anti-inflammatory; superoxide dismutase; malondialdehyde; interleukin-8; tumor necrosis factor-α; apoptosis; hippocampus; neural regeneration*

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Introduction

Cerebral ischemic disease is among the leading causes of senile dementia and death worldwide (Brouns and De Deyn, 2009). During ischemia, reduced glucose and oxygen transport to the brain causes cellular bioenergetic failure, which may lead to oxidative stress, inflammation, blood-brain barrier dysfunction, and eventually neuronal cell death, particularly in the hippocampus (Atlas et al., 2013). Evidence suggests that post-ischemic oxidative stress and inflammation are major events in the pathophysiology of ischemic damage (Chan, 1996; Lakhan et al., 2009). Excessive generation of free radicals and reactive oxygen species in the human brain results in lipid peroxidation of the cell membrane, protein denaturation, DNA damage, and oxidative injury to tissues (Ikeda and Long, 1990). The production of proinflammatory cytokines, such as tumor necrosis factor-α, interleukin-8 and interleukin-6, participates in tissue remodeling after injury and contributes to inflammation of the central nervous system (Wang et al., 2007, 2014; Terao et al., 2008; He et al., 2013).

Ganoderma lucidum is a white rot fungus used as a traditional remedy in the treatment of human diseases, such as hepatitis, liver disorders, hypercholesterolemia, arthritis, bronchitis, and tumorigenic diseases (Yuan et al., 2007; Zhou et al., 2012; Pan et al., 2014). The major active ingredients of ganoderma lucidum are polysaccharides, ergosterol, unsaturated fatty acids, and triterpenoids (Zhou et al., 2012; Pan et al., 2013a, 2014). Previous studies have shown that ganoderma lucidum-polysaccharides are anti-oxidative, hypoglycemic, anti-inflammatory, and have anti-tumor and immunomodulatory activities (Lin and Zhang, 2004; Li et al., 2011; Zhao et al., 2012). Oral administration of ganoderma lucidum has been shown to significantly reduce both cerebral infarct area and neuronal apoptosis in the ischemic cortex (Zhao et al., 2012). Recent pharmacological studies suggest that ganoderma lucidum stimulates the production of cytokines and exerts immunomodulatory effects (Ma et al., 2008). Administration of ganoderma lucidum to db/db mice also increases both serum and liver activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Pan et al., 2013a). Therefore, we hypothesized that ganoderma lucidum protects hippocampal neurons against cerebral ischemia/reperfusion injury because of both its antioxidant and anti-inflammatory activ-

ities. To test this hypothesis, we investigated the effect of the pretreatment with ganoderma lucidum on cerebral ischemia/reperfusion injury.

Materials and Methods

Animals

Thirty-two healthy, aged, and specific-pathogen-free Wistar rats (male and female), 220 ± 10 g, were purchased from the Jining Lukang Co., Ltd. (Jining, Shandong Province, China) (license No. SCXK (Lu) 2008-0015) and housed in cages (4 rats per cage). All rats were allowed free access to food and water, and were maintained in the animal facility with filtered air under a 12-hour light/dark cycle at 23 ± 2°C and at a humidity of 45–55%. All procedures were approved by the Ethics Committee on Animal Experiments of Taishan Medical University and carried out in agreement with the Chinese Community guidelines for the Care and Use of Laboratory Animals. Rats were equally and randomly divided into four groups as follows: sham surgery, model, 3 or 7 days of pretreatment.

Preparation and administration of ganoderma lucidum

The ganoderma lucidum fungus mixture (water-soluble) was provided by Shandong Si Wei Co., Ltd. (Heze, Shandong Province, China) (license No. Z200220083). The preparation of ganoderma lucidum fungus mixture involved the inoculation of a pure culture of ganoderma lucidum mycelia into a solid culture medium (composed of bagasse and defatted rice bran) and cultured until just before the formation of the fruit body (for 3–4 months). The air-dried ganoderma lucidum fruit bodies were extracted with hot water and sterilized by filtration, as described previously (Gao et al., 2002; Kubo et al., 2005; Zhou et al., 2010). Ganoderma lucidum was administrated to rats at 20 mL/kg per day *via* gastric gavage (the polysaccharides is 2 mg/mL) (Hu et al., 2003). Rats of the 3- and 7-day pretreatment groups were administrated for their respective treatment exposure before the modeling. Rats in both the model and sham groups were administrated water at 20 mL/kg for 7 days.

Focal cerebral ischemia/reperfusion rat model

Animals in both the model and pretreatment groups were deprived of food for 12 hours before the surgical procedure. The transient focal cerebral ischemia model was induced by middle cerebral artery occlusion, as described previously (Longa et al., 1989). Briefly, rats were anesthetized intraperitoneally with chloral hydrate at 400 mg/kg. The right common carotid artery was exposed, carefully isolated from the vagus nerve, and ligated on the proximal side through a right paramedian incision. The external carotid artery, the occipital artery, and the pterygopalatine artery were ligated similarly. Ischemia was induced by advancing a nylon monofilament (0.26 mm) with its tip rounded into the interior carotid artery *via* the external carotid artery. After placement, the intraluminal suture was secured with a 4-0 silk suture tied around the external carotid artery. Reperfusion was produced when the intraluminal suture was withdrawn 1.5 hours after middle cerebral artery occlusion. Physiological parameters were monitored at baseline, during middle cerebral artery occlusion, and at reperfusion. Rectal temperature was maintained at 37°C with a heating lamp. Animals in the sham group were subjected to all the surgical procedures for ischemia/reperfusion except the occlusion.

The step-down test

The step-down test is widely used to measure passive avoidance for learning and memory (Longa et al., 1989). The procedure consisted of a training session and a test session 24 hours after training. Memory was measured 24 hours after ischemia. The apparatus (YLS-IA recorder for Multi-function autonomic activities in mice, Shandong academy of medical science, Jinan, Shandong Province, China) was a 40 cm × 40 cm plastic box with a 4.0 cm high and 10.0 cm wide platform in the left corner of the training box apparatus. The base of the apparatus was made of 0.1 cm caliber stainless steel bars spaced (in parallel) 1.0 cm apart. In the training session, animals were gently placed on the platform to habituate for 3 minutes. If the animals stepped down from the platform, they would receive a continuous scrambled foot shock (0.4 mA, 2 seconds), which made them immediately step up to the platform (*i.e.*, passive avoidance). The training procedure was carried out 30 minutes daily for 3 consecutive days. In test sessions, foot shock was not delivered after the animal step-down from the platform. The step-down latency and number of errors made in 10 minutes were recorded.

Nissl staining for the histopathological assessment of the hippocampus

Brain sections from sacrificed animals were exposed to the Nissl stain for the assessment of neuronal cell loss at the dorsal CA1 subfield of the hippocampus, as previously described (Atlas et al., 2013). Animals were deeply anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and then transcardially perfused with cold saline followed by 4% paraformaldehyde in PBS (0.1 mol/L; pH 7.4). After post-fixation *in situ* overnight, brains were removed, washed in PBS, cryoprotected with 30% sucrose in PBS, and frozen in powdered dry ice. Coronal sections (20 μm) were cut at the level of the dorsal hippocampus (3.3–4.0 mm posterior from the bregma) (Paxinos and Watson, 2005) with a cryostat. Every fourth section was collected and stained with cresyl violet. For Nissl staining, the sections were mounted on slides (Superfrost-plus, Fisher Scientific, Pittsburgh, PA, USA), dehydrated and rehydrated in graded ethanols and xylenes, respectively and then incubated in 1% cresyl violet for 30 seconds. Sections were then decolorized in acetic acid, dehydrated, and coverslipped with Permount. Sections were observed with a binocular microscope (Olympus, Tokyo, Japan).

Detection of oxidative stress in the hippocampus and serum

The level of malondialdehyde is used to measure the amount of lipid peroxidation, and this compound was determined spectrophotometrically, as previously described (Ohkawa et

al., 1979). Briefly, 10 mg hippocampal tissues were homogenized with 0.1 mL sodium phosphate buffer (0.2 mol/L, pH 7.4). Acetic acid (1.5 mL, 20%, pH 3.5), thiobarbituric acid (1.5 mL, 0.8%), and sodium dodecyl sulfate (0.2 mL, 8.1%) were added to 0.1 mL of processed tissue sample and serum. The mixture was then heated at 100°C for 60 minutes, cooled with tap water and 5 mL of n-butanol plus pyridine (15:1, v/v) in 1 mL of distilled water, and then shaken vigorously. After centrifugation at 1,500 × *g* for 10 minutes, the organic layer was removed and its absorbance was measured at 532 nm using a spectrophotometer (Third Instrument Factory, Shanghai, China). Superoxide dismutase activity in hippocampal homogenates and serum was measured by the inhibition of nitroblue tetrazolium (Assay kit from Beyotime Institute of Biotechnology, China) reduction caused by the xanthine-XO system as the superoxide generator (Zhou and Prognon, 2006). Briefly, superoxide dismutase activity was assessed during the ethanol phase of the lysate after 1.0 mL ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and then centrifuged. One unit of superoxide dismutase was defined as the amount of enzyme that caused 50% inhibition of the nitroblue tetrazolium reduction rate. A calibration curve was derived with purified superoxide dismutase as the standard to calculate the activity of superoxide dismutase present in the samples.

Immunohistochemistry for tumor necrosis factor-α and interleukin-8 in the CA1 region of the hippocampus

Animals were transcardially perfused with a saline solution containing heparin (10 U/mL) followed by 4% paraformaldehyde dissolved in 0.1 mol/L phosphate buffer. The hippocampus was removed from the cranium, paraffin-embedded, and sectioned at a thickness of 4 μm for histology. Immunohistochemistry was performed using the Histostainplus kit (Beijing Zhongshan Biotechnology, Beijing, China). Briefly, brain sections were incubated in a peroxidase quenching solution (3% hydrogen peroxide in absolute methanol), rinsed twice with PBS and then incubated with serum blocking solution for 20 minutes. Sections were then incubated with the monoclonal antibodies, mouse anti-rat tumor necrosis factor-α (1:100; Boster, Wuhan, Hubei Province, China) or interleukin-8 (1:100; Boster), overnight at 4°C. After primary antibody incubation, the samples were rinsed with 0.3% skim milk in PBS containing 0.05% Triton X-100, then incubated with biotinylated goat anti-mouse IgG diluted in PBS containing 0.3% skim milk, followed by the enzyme conjugate diluted in PBS containing 0.3% skim milk. The bound antiserum was visualized by incubating the slides with 3,3′-diaminobenzidine. Finally, the sections were dehydrated and covered by a coverslip, and were then viewed, photographed, and analyzed by Image analysis software Image-proplus (Media Cybernetics, Bethesda, MD, USA). Photomicrographs were taken, and the absorbance was calculated. Omission of the primary or secondary antibody served as the negative control (Griffiths et al., 1991).

Statistical analysis

All data were expressed as mean ± SD and were analyzed by one-way analysis of variance followed by Dunnett's *post hoc* test. All analyses were performed with SPSS 17.0 (SPSS Chicago, IL, USA). Significance was reached at values of $P < 0.05$.

Results

Pretreatment with ganoderma lucidum improved learning and memory in rats with cerebral ischemia/reperfusion injury

Results in the step-down test showed that compared with the sham surgery group, the mean latency was significantly lower and the number of errors was significantly higher in rats of the model group ($P < 0.05$; **Figure 1**). However, the 3- and 7-day pretreatment with ganoderma lucidum significantly prolonged the mean latency and decreased the number of errors in the step-down test compared with the model group ($P < 0.05$; **Figure 1**). Furthermore, both pretreatments did not affect rat behavior (**Figure 1**).

Pretreatment with ganoderma lucidum reduced ischemia-induced neuronal loss in the hippocampus

Nissl staining showed that in the sham surgery group, CA1 pyramidal neurons exhibited a typical shape and regular surface structure, and were clearly visible and orderly arranged (**Figure 2**). In the model rats, pyramidal neurons were disarranged and exhibited shrinkage, a dark staining appearance with small cytoplasm, or neuronal loss (**Figure 2**). Cell junctions became loose and the intercellular spaces were widened. Pretreatment with ganoderma lucidum, particularly for 7 days, greatly reduced ischemia-induced neuronal loss in the hippocampus (**Figure 2**).

Pretreatment with ganoderma lucidum decreased malondialdehyde contents and increased superoxide dismutase levels in the hippocampus and serum in rats with cerebral ischemia/reperfusion injury

Compared with the sham surgery group, malondialdehyde and superoxide dismutase levels were significantly increased and decreased, respectively in the hippocampus and serum of the model group ($P < 0.05$; **Figure 3**). Pretreatment with ganoderma lucidum for 3 or 7 days significantly decreased and increased the levels of malondialdehyde and superoxide dismutase, respectively in the hippocampus and serum compared with the model group ($P < 0.05$; **Figure 3**). The malondialdehyde content and superoxide dismutase level in the hippocampus tissue and serum in rats with cerebral ischemia/reperfusion injury was similar at each pretreatment timepoint (**Figure 3**).

Pretreatment with ganoderma lucidum suppressed the expression of tumor necrosis factor-α and interleukin-8 in the hippocampus of rats with cerebral ischemia/reperfusion injury

Immunohistochemistry revealed that tumor necrosis factor-α and interleukin-8 were expressed at very low levels in the sham surgery group (**Figure 4**). The immunoreactivity

of these two cytokines was significantly higher in the hippocampal CA1 region of the model group compared with the sham surgery group ($P < 0.05$; **Figure 4**). Pretreatment with ganoderma lucidum significantly reduced the immunoreactivity of both cytokines in the hippocampus ($P < 0.05$; **Figure 4**). The immunoreactivity of both cytokines in the hippocampus of rats with cerebral ischemia/reperfusion injury was similar at each pretreatment time point (**Figure 4**).

Discussion

Despite numerous therapeutic trials, stroke is still the leading cause of death in the world. The present treatment for stroke is to perfuse with recombinant tissue plasminogen activator (Lakhan et al., 2009). However, a narrow therapeutic time window and risk of hemorrhage has hindered the success of this treatment (Hickenbottom and Barsan, 2000). Therefore, a useful and safe-to-use protective agent is particularly important in treating and alleviating the unfavorable outcomes of stroke. The present study demonstrated that pretreatment with ganoderma lucidum was protective against cerebral ischemia/reperfusion injury through its anti-oxidative and anti-inflammatory actions.

To determine the aspects of neurobehavioral protection, the animals were subjected to the step-down test, which is widely used for evaluating passive avoidance memory in rats. In this study, pretreatment with ganoderma lucidum for 3 and 7 days increased the latency time and decreased the error number compared with the control group. Therefore, these results suggested that ganoderma lucidum could improve memory retention. Ganoderma lucidum has been shown to improve learning and memory in senescence-accelerated mice prone 8, and thus neuroactive components that may exist in ganoderma lucidum extracts may cross the blood-brain barrier to promote neuronal function (Wang et al., 2004; Zhou et al., 2012). The hippocampus plays a critical role in several fundamental memory operations (Eichenbaum, 2001). Oral administration of ganoderma lucidum-polysaccharides significantly reduces the cerebral infarct area, neurological functional deficits, and neuronal apoptosis in ischemic cortex (Zhou et al., 2010). To confirm the protective potential of ganoderma lucidum, neuronal injury was analyzed by Nissl staining. The present study showed that in addition to marked improvements in memory, rats pretreated with ganoderma lucidum also exhibited less neural death in the hippocampal CA1 region compared with model rats. This result further confirmed the protective effect of this compound against ischemia.

The brain is particularly vulnerable to oxidative stress injury because of its high consumption of oxygen, abundant polyunsaturated fatty acids, and low levels of endogenous antioxidants (Madamanchi et al., 2005; Schreibelt et al., 2007). Free radicals may attack protein and polyunsaturated phospholipids in membranes, including plasma membranes and cellular organelles, leading to the disruption of these organelles. Therefore, inducing anti-oxidative effects is considered to be a promising treatment for ischemic stroke (Hall and Murdoch, 1990; Powers and Jackson, 2008). Superoxide dismutase is the primary protective enzyme against tissue damage caused by reactive oxygen species. This enzyme catalyzes the dismutation of superoxide anion to hydrogen peroxide and prevents the formation of the hydroxyl radical (Huang et al., 2012). Superoxide dismutase activity in serum has been shown to be reduced in stroke patients, and increased antioxidant activity may be beneficial in the acute treatment of cerebral ischemia (Spranger et al., 1997). Our study showed that the reduction in superoxide dismutase activity after cerebral ischemia/reperfusion injury was prevented by administration of ganoderma lucidum. Brain malondialdehyde is one of the most sensitive indicators of lipid peroxidation (Cini et al., 1994). In the present study, malondialdehyde was significantly elevated in the model group, suggesting the involvement of lipid oxidation in cerebral injury. However, ganoderma lucidum significantly reduced the level of malondialdehyde. Overall, these results indicate an antioxidant effect of ganoderma lucidum. Therefore, this compound may induce a protective mechanism by increasing the endogenous defensive capacity of the brain to combat oxidative stress induced by ischemia/reperfusion.

Inflammation is an important pathological process in ischemia, particularly during the acute phase (Candelario-Jalil, 2009; Lakhan et al., 2009). Focal cerebral ischemia elicits a strong inflammatory response involving tumor necrosis factor-α, which induces the synthesis of subsequent proinflammatory cytokines, such as interleukin-6 and interleukin-8 (Cieślak et al., 2013; Zhang et al., 2013). These proinflammatory molecules induce multiple inflammatory cascades and contribute to the progression of brain damage following ischemic insult. Ganoderma lucidum has been shown to suppress lipopolysaccharide-mediated expression of tumor necrosis factor-α in murine RAW 264.7 cells (Dudhgaonkar et al., 2009). Ganoderma lucidum-polysaccharides significantly reduces the levels of both serum interleukin-6 and tumor necrosis factor-α and increases the levels of serum interleukin-2, interleukin-4, and interleukin-10 in rats (Pan et al., 2013b). Other studies found that ganoderma lucidum suppresses oxidative stress-induced secretion of interleukin-8 from breast cancer cells. In the present study, immunoreactivity of tumor necrosis factor-α and interleukin-8 was significantly reduced in the hippocampal CA1 region by the pretreatment of ganoderma lucidum compared with the model group. These results suggest that ganoderma lucidum protects neuronal cells from inflammation-induced injury after ischemia.

In conclusion, results of the present study indicate that ganoderma lucidum produces a distinct protective effect against cerebral ischemia/reperfusion injury in rats. This protective effect may be due to both its anti-oxidative and anti-inflammatory properties. Overall, ganoderma lucidum may be a potentially safe traditional Chinese medicine treatment for stroke patients.

Author contributions: *Zhang WX, Zhang QL and Du YF conceived and designed the experiments. Zhang WX, Deng W, Li YL, Xing GQ and Shi XJ performed the experiments. Zhang*

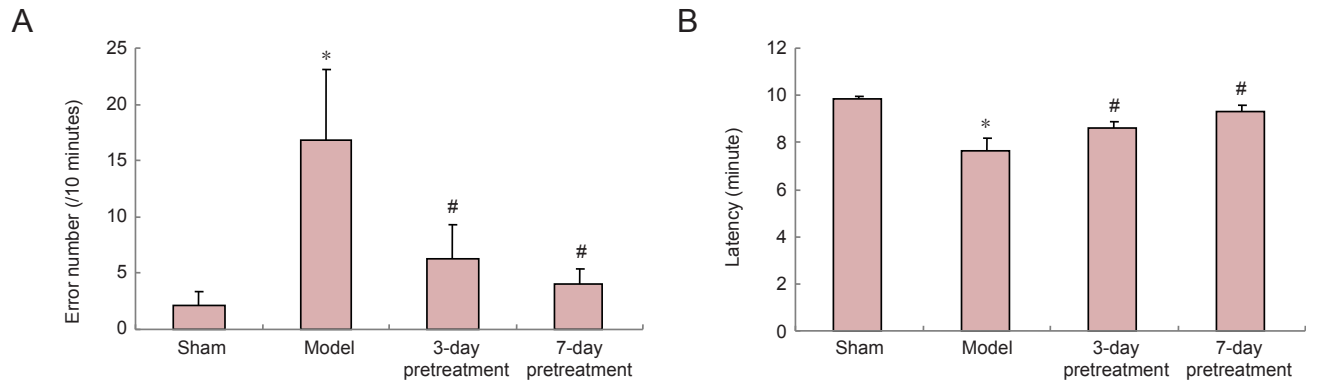

Figure 1 Pretreatment with ganoderma lucidum improves learning and memory after cerebral ischemia/reperfusion injury.
(A) The number of errors of the step-down test. (B) Mean latency of the step-down test. All data were expressed as mean ± SD (n = 8 rats per group) and were analyzed by one-way analysis of variance followed by Dunnett's *post hoc* test. *P < 0.05, *vs.* sham surgery group (sham); #P < 0.05, *vs.* model group.

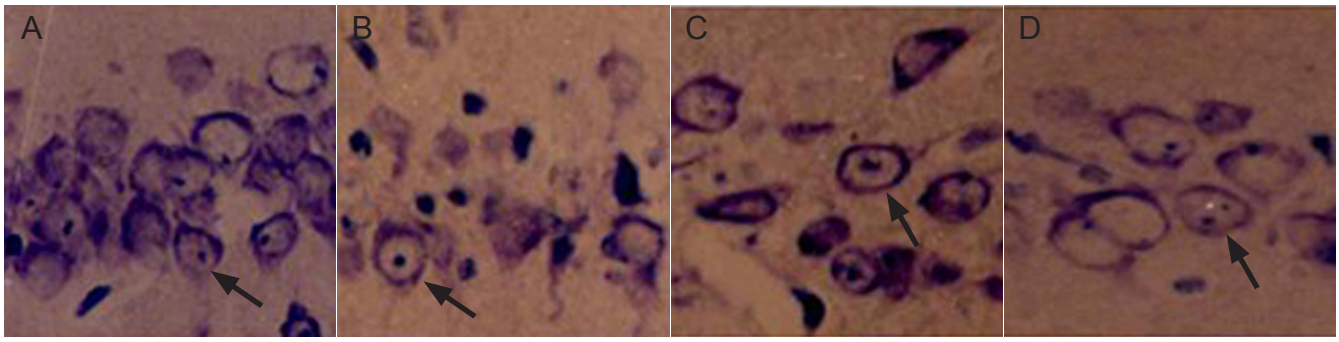

Figure 2 Pretreatment with ganoderma lucidum greatly reduces ischemia-induced neuronal loss in the hippocampus (× 400).
Nissl staining of (A) sham surgery group, (B) model group, and (C) 3-day or (D) 7-day pretreatment with ganoderma lucidum. Arrows indicate neurons.

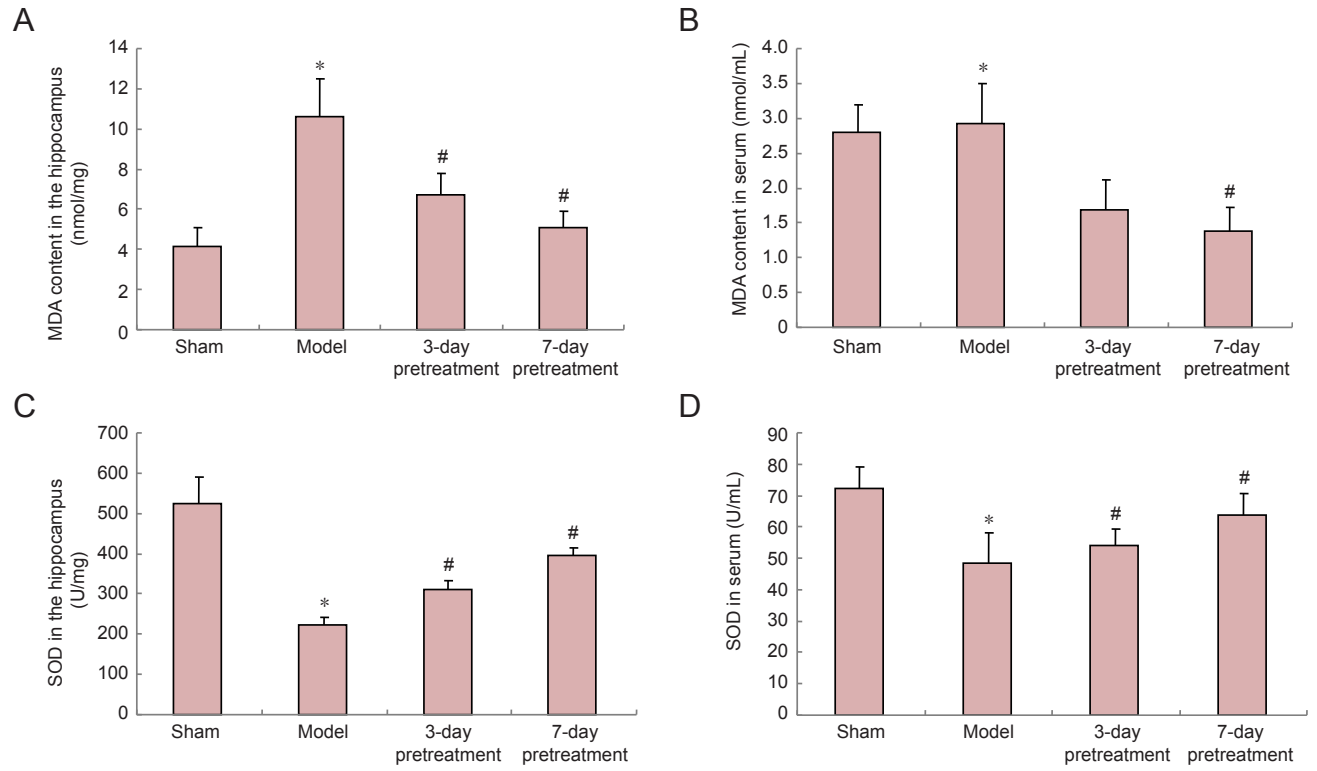

Figure 3 Pretreatment with ganoderma lucidum decreases malondialdehyde (MDA) content and increases superoxide dismutase (SOD) level in the hippocampus and serum of rats with cerebral ischemia/reperfusion injury.
(A, C) Levels of MDA and SOD in the hippocampus. (B, D) Levels of MDA and SOD in the serum. All data were expressed as mean ± SD (n = 8 rats per group) and were analyzed by one-way analysis of variance followed by Dunnett's *post hoc* test. *P < 0.05, *vs.* sham surgery group (sham); #P < 0.05, *vs.* model group.

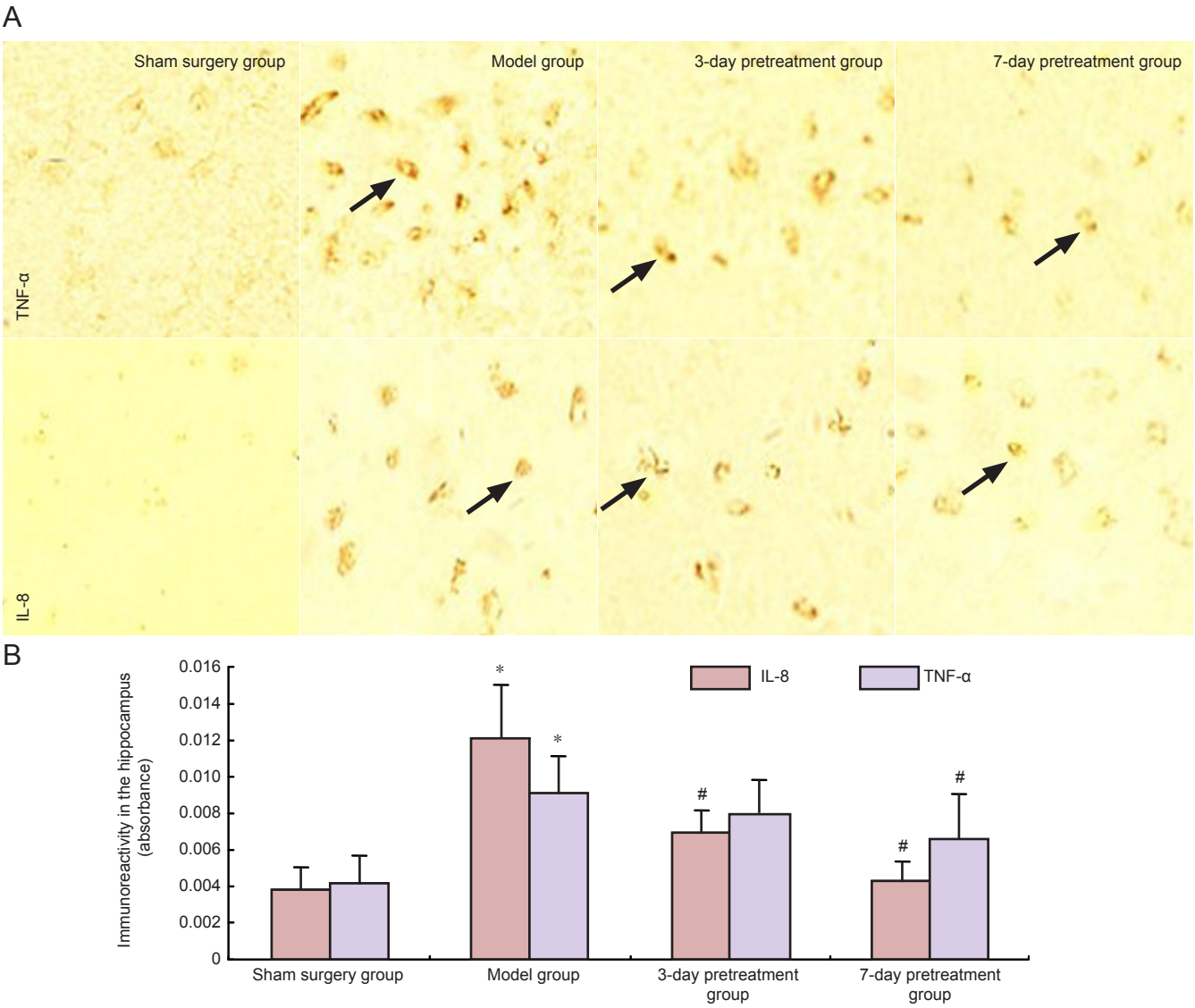

Figure 4 The effect of G. lucidum pretreatment on tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) immunoreactivity in the CA1 of the rat hippocampus.
(A) TNF-α and IL-8 immunoreactivity in the CA1 of the rat hippocampus (immunohistochemical staining, × 400). Arrows point to the positive expression. (B) The quantitative expression of TNF-α and IL-8 in the CA1 of the rat hippocampus. Data are expressed as mean ± SD, $n = 8$ rats in each group. Statistical evaluation of the data was performed using one-way analysis of variance followed by Dunnett's *post hot* test for comparisons among more than two groups. $*P < 0.05$, *vs.* sham surgery group; $\#P < 0.05$, *vs.* model group.

WX and Zhang QL provided reagents/materials/analysis tools. Zhang WX, Zhang QL and Du YF wrote the manuscript. All authors approved the final version of the paper.
Conflicts of interest: *None declared.*

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