Front. Agr. Sci. Eng. 2019, **6**(2): 197–205
https://doi.org/10.15302/J-FASE-2018245 Available online at http://journal.hep.com.cn/fase

RESEARCH ARTICLE

Leonurine protects ischemia-induced brain injury via modulating SOD, MDA and GABA levels

Shilei ZHENG[*], Jingru ZHU[*], Jiao LI, Shuang ZHANG, Yunfei MA (✉)

College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

Abstract The present study was designed to investigate the protective effects of leonurine, a compound purified from *Herba Leonuri* that is active on ischemic rat behavior and cortical neurons, and explore the underlying mechanism. The general rat activity, cortical neuron morphology, superoxide dismutase (SOD), malondialdehyde (MDA), γ-aminobutyric acid (GABA) and glutamate decarboxylase 67 (GAD67) levels were measured. We found leonurine significantly improve the general activity of rats in an open-field test, which was associated with attenuated neuronal damage induced by ischemia. Moreover, serum SOD activity was significantly greater, MDA level lower in the leonurine group as compared with ischemia group. In addition, GABA content in the cerebral cortex was significantly greater in high-dose leonurine group. Correspondingly, GAD67 protein level coincided with the GABA level. Taken together, our results demonstrated that leonurine attenuated brain injury during ischemia via antioxidative and anti-excitotoxicity effects by targeting GABA and leonurine might become a useful adjuvant neuroprotective agent.

Keywords cerebral ischemia, GABA, neuroprotection, leonurine, SOD

1 Introduction

Cerebral ischemia is one of the major causes of death and a salient risk factor for cerebrovascular disorders[1]. Cerebral ischemia causes numerous hemodynamic, biochemical and neurophysiological alterations that result in neuronal death. The mechanisms underlying cell death after cerebral ischemia are complex and major factors include glutamate-mediated excitotoxicity, calcium overload, oxidative stress and inflammation[2,3]. Glutamate is the major excitatory amino acid transmitter in the central nervous system (CNS)[4]. One study suggested that the accumulation of extracellular glutamate is a crucial determinant of injury in the setting of reversible middle cerebral artery occlusion[5]. γ-Aminobutyric acid (GABA), which is produced by glutamate decarboxylase (GAD), is the main inhibitory neurotransmitter of the CNS. GAD exists as two isoforms, GAD65 and GAD67. GAD67 accounted for most GABA synthesis in the anesthetized rat cortices[6]. During both ischemia and hypoxia high levels of glutamate were present and GABA levels increased in the CNS[7].

Leonurine ($C_{14}H_{21}N_3O_5$) is an active alkaloid present in *Herba Leonuri*, a traditional Chinese medicine, also called Chinese motherwort, widely used in China to treat dysmenorrheal, menoxenia and gynecological disorders. Recent studies suggested leonurine could improve antioxidant capacity of the myocardium, promote angiogenesis in ischemic myocardium and ameliorate endothelial dysfunction caused by hyperlipidemia[8,9]. Moreover, leonurine has recently been confirmed to be beneficial against ischemic stroke[10]. The salubrious effects of leonurine may be due various biological activities, such as anti-inflammatory, antiapoptotic and antioxidative activities[8–10]. However, few studies have investigated the effects of leonurine on GABA expression and the protective mechanism of leonurine on ischemia-induced brain injury remains unclear. Therefore, the aim of this study was to evaluate whether leonurine treatment could provide neuroprotection *in vivo* and to explore the underlying mechanisms of action.

In this study, we established a cerebral ischemia model through common carotid artery occlusion. To develop the model, we examined the general activity of rats, morphological changes of cortical neurons and density of NeuN-positive neurons. We further measured serum superoxide dismutase (SOD) and malondialdehyde (MDA) levels and investigated the effect of leonurine treatment on rat behavior and cortical neuron damage, oxidative stress and GABA expression.

Received June 10, 2018; accepted September 28, 2018

Correspondence: yunfeima@cau.edu.cn

[*]These authors contribute equally to the work

© The Author(s) 2018. Published by Higher Education Press. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0)

2 Materials and methods

To evaluate the protective effects of leonurine pretreatment on rat cerebral ischemia, an intraperitoneal injection of leonurine was administered each day for 3 d before conducting transient two-vessel occlusion. Subsequently, the general activity of rats, morphological changes of cortical neurons and density of NeuN-positive neurons were evaluated. Additionally, serum SOD and MDA levels were measured. The effect of leonurine on GABA and GAD67 expression was examined through enzyme-linked immunosorbent assay (ELISA) and western blotting analysis.

2.1 Animals and groups

All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, China Agricultural University. Sixty male Sprague Dawley rats (8 weeks old), weighing 230–250 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed individually in cages with a controlled temperature of 20±3°C and a relative humidity of 60%±5% for 3 d then randomly divided into five groups with 12 in each group: sham-operated (Sham), cerebral ischemia model (Ischemia), and low-(Leo-L), middle-(Leo-M), and high-(Leo-H)-dose leonurine groups.

2.2 Experimental ischemia

Before the operation, the rats were fasted for 12 h. We anesthetized rats in the sham-operated group, exposed both their common carotid arteries and separated them from the associated vagus and sympathetic nerves, and sutured the wound. Rats in the cerebral ischemia model group were subjected to transient two-vessel occlusion to induce cerebral ischemia after separating their common carotid arteries from the associated vagus and sympathetic nerves. We occluded both their arteries for 30 min using a bulldog clamp, then loosened the bulldog clamp and sutured the wound. Rats in the Leo-L, Leo-M, and Leo-H groups were administered an intraperitoneal injection of leonurine at a dose of 4, 8 and 16 $mg \cdot kg^{-1}$ bodyweight, respectively, each day for 3 d; subsequently, all the rats were subjected to transient two-vessel occlusion.

2.3 Open-field test

An open-field test (OFT) was conducted 24 h after the operation. The rats were transferred to the testing apparatus, which was an illuminated, soundproofed box (100 cm × 100 cm × 50 cm) with black inner walls. The bottom surface of the box was divided into 25 squares (15 cm × 15 cm). The 16 squares adjacent to the walls were defined as peripheral squares, whereas the other nine squares were defined as center squares. The apparatus was cleaned with 70% ethanol before the test of each animal. Each rat was placed onto a corner square of the arena and allowed to freely explore the open field for 5 min per trial. A camera was installed at the top right of the box to record the activity of the rats. The number of squares crossed and the number of rearings (defined as standing on the hind limbs without touching the wall) were recorded and the sum of the two numbers as a total score was used to evaluate the general activity of the rats.

2.4 Sample collection

After behavioral tests, six rats from each group were anesthetized by chloral hydrate (70 mg per 100 g bodyweight), exsanguinated by jugular vein and then euthanized. Blood samples were collected and centrifuged at 2000 × *g* for 10 min at 4°C. Serum samples were stored at –20°C for the determination of SOD activity and MDA content. Following rapid decapitation, tissues of the cerebral cortex were removed, frozen in the liquid nitrogen and stored at –80°C for the following experiments for ELISA and western blotting. The other six rats in each group were also deeply anesthetized with chloral hydrate and perfused transcardially with 200 mL of 5 $mmol \cdot L^{-1}$ sodium phosphate (pH 7.4)-buffered 0.9% (w/v) saline (PBS), followed by 300 mL of 4% (w/v) formaldehyde in 0.1 $mol \cdot L^{-1}$ sodium phosphate buffer (pH 7.4). The brains were removed to cut into several blocks and then postfixed with the same fixative for one day at 4°C. After cryoprotection with 30% (w/v) sucrose in PBS, the blocks were cut into 25-µm-thick sections on a freezing microtome for subsequent Nissl staining and immunohistochemistry.

2.5 SOD and MDA measurements

Serum SOD levels were determined by the xanthine oxidase method, using SOD detection kit (A001-1, NJJCBio Inc., Nanjing, China). Serum MDA content was measured by the thiobarbituric acid method, using MDA detection kit (A003-1, NJJCBio Inc.). All assays were conducted in accordance with the manufacturers' instructions.

2.6 Nissl staining and immunohistochemistry

For Nissl staining, frozen sections were mounted on gelatin coated slides, air-dried and then stored in 1:1 alcohol: chloroform overnight and subsequently rehydrated by placing them into 100% and 95% alcohol then distilled water. The slides were subsequently stained using 0.1% cresyl violet solution for 30 min. The sections were differentiated in 95% ethyl alcohol for 2–3 min, dehy-

drated again in 100% alcohol and finally cleared in xylene. The sections were observed using a microscope.

For immunohistochemistry, frozen sections were rinsed in PBS and then incubated in 2% hydrogen peroxide solution for 15 min to remove the endogenous peroxidase reactivity. All the incubations were performed at room temperature and rinsed with PBS containing 0.3% Triton X-100 (PBS-X). After rinsing with PBS, the sections were incubated overnight in a humidified chamber with a mouse monoclonal antibody to NeuN (1:500, MAB377, MilliporeSigma, Burlington, Billerica, MA, USA) in PBS-X containing 0.12% λ-carrageenan, 0.02% sodium azide and 1% donkey serum (PBS-XCD) and then incubated for 2 h with biotinylated donkey anti-mouse IgG (1:100, 715-065-151, Jackson Laboratories, West Grove, PA, USA). The sections were subsequently rinsed and incubated in ABC peroxidase solution (PK-6102, Vector Laboratories, Burlingame, CA, USA) for 30 min and the bound peroxidase brown color was finally developed by reaction for 20 min with 3,3′-diaminobenzidine-4HCl (Sigma-Aldrich, St. Louis, MO, USA) and H_2O_2. All the stained sections were mounted onto gelatinized glass slides, dried, dehydrated in an ethanol series, cleared in xylene and coverslip applied.

2.7 ELISA for GABA

To detect the contents of GABA in the cerebral cortex, the samples were weighed and added to nine times volumes of saline. After homogenization on the ice, the solution was centrifuged at 1000 × *g* for 10 min. The supernatants were collected and the concentrations of GABA detected by ELISA in accordance with the manufacture's instruction (the Rat GABA ELISA Assay Kit, H618, NJJCBio Inc., Nanjing, China).

2.8 Western blotting analysis for GAD67

Western blotting was performed as described previously[11]. Briefly, proteins were extracted using a Whole Cell Lysis Assay Kit (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China). Cells were lysed in a lysis buffer supplemented with a 1 × protease inhibitor cocktail. A BCA Protein Assay Kit (CWBIO, Beijing, China) was used for protein quantification. Equivalent amounts of proteins were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8%–12%), transferred onto a polyvinylidene difluoride membrane and immunoblotted using primary antibodies against GAD67 (1:500, MAB6406, Millipore, USA) and β-actin (1:5000, 50201, Kemei Borui Science And Technology Co., Ltd., Beijing, China). The β-actin antibody was used to confirm equal loading conditions. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, respectively and protein bands were finally visualized using a Tanon 5200 chemiluminescence system (5200, Tanon Science & Technology Co., Ltd., Shanghai, China).

2.9 Statistical analysis

A total of 18 representative images of cortex were randomly taken from immunocytochemical staining of each group under a 40 × power microscope. The images were analyzed with image analysis software (Image Pro Plus, version 4.5). The total number of NeuN-positive cells were counted, and the cell densities (number of cells per mm^2) were calculated. Statistical analyses were performed using SPSS 17.0 (SPSS, IBM Co., Chicago, IL, USA). All data are presented as the mean±SD. Differences among the groups were analyzed by one-way analysis of variance and multiple comparisons. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of leonurine on the general activity of rats

The total score of the OFT (Fig. 1) was significantly lower in Ischemia than in Sham ($P < 0.05$). Compared with the Ischemia, the Leo-H exhibited significant improvements in general activity ($P < 0.05$). However, no significant differences in OFT scores were observed between the Leo-L and Leo-M groups and the Ischemia group ($P > 0.05$).

The histogram shows that the total score of the OFT was significantly lower in Ischemia than in Sham. Compared with Ischemia, Leo-H exhibited significant improvements in general activity.

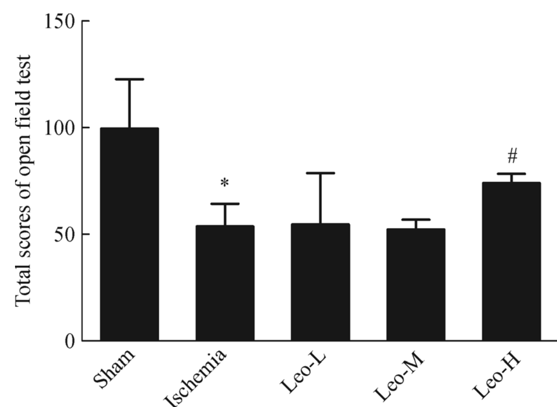

Fig. 1 Effect of leonurine on the neurobehavior of rats according to open-field test. Sham, Ischemia, Leo-L, Leo-M and Leo-H stand for the sham-operated, cerebral ischemia model, low-dose leonurine, middle-dose leonurine and high-dose leonurine groups, respectively; * indicates the comparison between the ischemia model and sham-operated groups ($P < 0.05$); # indicates the comparison between the leonurine treatment and ischemia model group ($P < 0.05$).

3.2 Effect of leonurine on SOD and MDA levels

Serum SOD levels were significantly lower and MDA levels were significantly higher in Ischemia than in Sham ($P < 0.05$). Compared with the Ischemia group, the leonurine treatment groups showed a significant concentration-dependent increase in the serum SOD level (Fig. 2(a)) and a significant concentration-dependent decrease in the MDA level (Fig. 2(b)).

The histogram shows that serum SOD levels were significantly lower and MDA levels were significantly higher in Ischemia than in Sham. Compared with Ischemia, Leo-L, Leo-M and Leo-H showed a significant concentration-dependent increase in the serum SOD level and a significant concentration-dependent decrease in the MDA level.

3.3 Effect of leonurine on the morphology and density of neurons

The cerebral cortex can be divided horizontally into six layers: the molecular, external granular, external pyramidal, internal granular, internal pyramidal and multiform layers. Significant morphological changes were observed in the cortical neurons in the internal pyramidal layer. Nissl staining results revealed extensive neuronal vacuoles changes. The neurons of the rats in Ischemia showed cytoplasmic vacuoles, the disruption of Nissl bodies and the fragmentation of the nucleus. Leonurine treatment attenuated the neuronal damage induced by cerebral ischemia, which was characterized by the appearance of fewer cytoplasmic vacuoles and less nuclear fragmentation (Fig. 3).

Nissl staining results show the normal neurons in Sham, the abnormal neurons with the appearance of cytoplasmic vacuoles, the disruption of Nissl bodies and the fragmentation of the nucleus in Ischemia. Treatment with Leo-L, Leo-M, and Leo-H attenuated the neuronal damage induced by cerebral ischemia, which was characterized by the appearance of fewer cytoplasmic vacuoles and less nuclear fragmentation. Arrowheads indicate representative neurons in each group.

The immunohistochemistry results (Fig. 4) showed changes in the cell density of NueN-positive neurons in the cerebral cortices of rats. Compared with Sham, the density of NeuN-positive neurons significantly decreased by 33.5% in Ischemia ($P = 0.006$). The density of NeuN-positive neurons was higher in the Leo-L and Leo-M groups than in the Ischemia group; however, the difference was nonsignificant ($P > 0.05$). Compared with the Ischemia group, the density of NeuN-positive neurons significantly increased by 25.4% in the Leo-H group ($P < 0.05$).

The density of NeuN-positive neurons significantly decreased in Ischemia compared with Sham. Compared with Ischemia, the density of NeuN-positive neurons significantly increased in Leo-H.

3.4 Effect of leonurine on GABA expression levels in the cerebral cortex

The GABA expression level in the cerebral cortex (Fig. 5) was evaluated through ELISA. GABA expression was markedly higher in Ischemia than in Sham ($P < 0.05$). Moreover, GABA expression was significantly greater in the Leo-H group compared with the Ischemia group ($P < 0.05$), although no significant differences were observed between the Leo-L or Leo-M and Ischemia groups.

GABA expression was markedly higher in Ischemia than in Sham. Moreover, GABA expression was significantly greater in Leo-H compared with Ischemia.

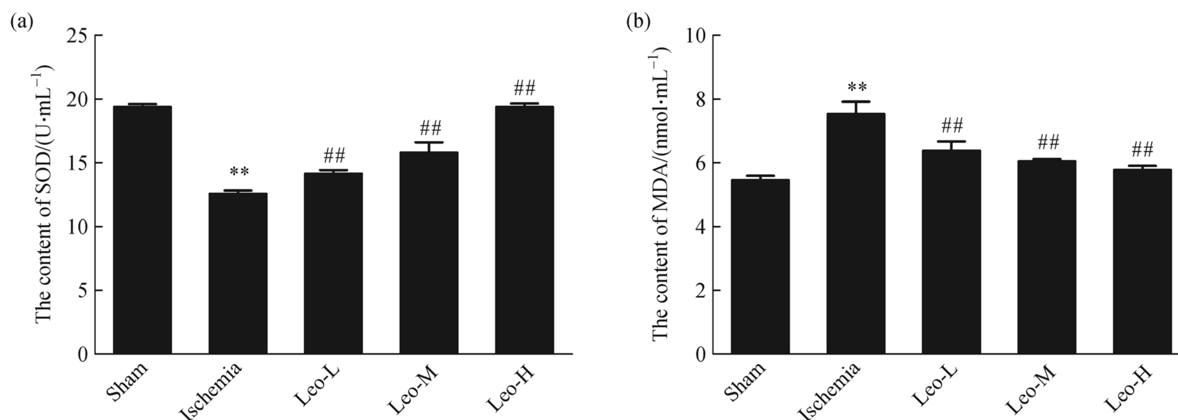

Fig. 2 Effect of leonurine on serum MDA and SOD levels in rats. Sham, Ischemia, Leo-L, Leo-M and Leo-H stand for the sham-operated, cerebral ischemia model, low-dose leonurine, middle-dose leonurine and high-dose leonurine groups, respectively; ** indicates the comparison between the ischemia model and sham-operated groups ($P < 0.01$); ## indicates the comparison between the leonurine treatment and ischemia model groups ($P < 0.01$).

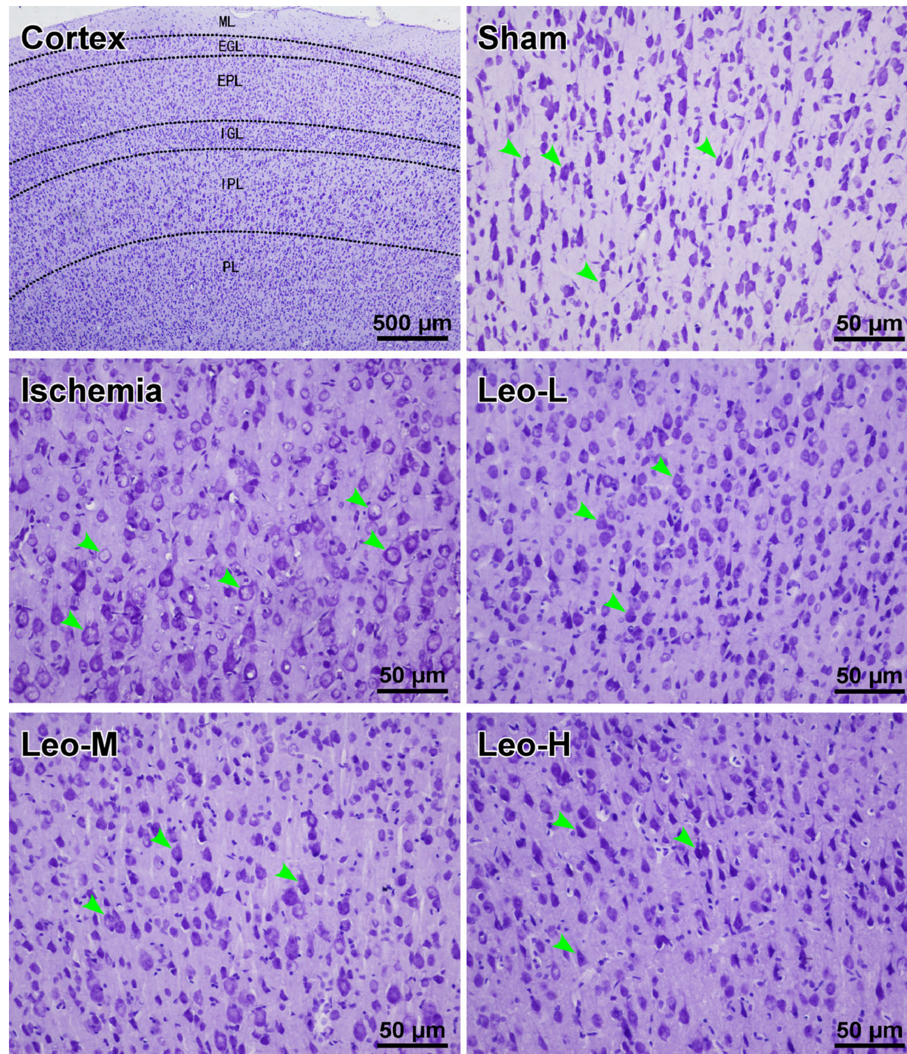

Fig. 3 Effect of leonurine on cortical morphological architecture according to Nissl staining. Sham, Ischemia, Leo-L, Leo-M and Leo-H stand for the sham-operated, cerebral ischemia model, low-dose leonurine, middle-dose leonurine and high-dose leonurine groups, respectively.

3.5 Effect of leonurine on GAD67 expression levels in the cerebral cortex

The expression of GAD67 in the cerebral cortex was analyzed by western blotting. GAD67 expression (Fig. 6) was significantly greater in Ischemia than in Sham ($P < 0.05$). Leonurine treatment increased GAD67 expression particularly and was significantly greater in Leo-H than in Ischemia ($P < 0.05$).

GAD67 content was significantly greater in Ischemia than in Sham. Leonurine treatment (Leo-L, Leo-M and Leo-H) increased GAD67, which was significantly greater in Leo-H than in Ischemia.

4 Discussion

Cerebral ischemia (stroke) is one of the major causes of death and long-term disability worldwide and impairs normal neurological functions; this impairment is triggered by a complex series of biochemical and molecular mechanisms that cause a reduction or complete blockade of blood flow, resulting in deficiency of glucose and oxygen supply to the affected region[12]. The relevant factors for neuronal cell damage caused by cerebral ischemia include calcium overload, energy failure, excitotoxicity, oxidative stress, neuroinflammation and apoptosis[12–14].

It is well known that cerebral ischemia results in anxiety,

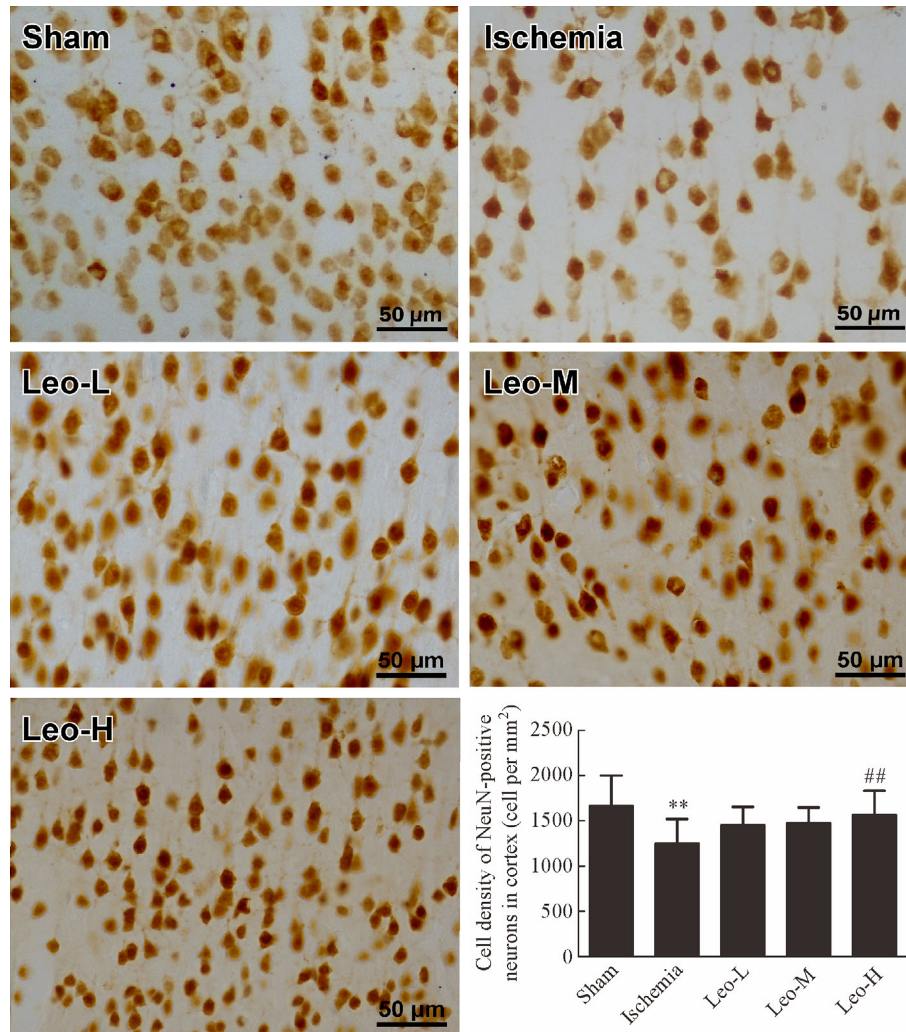

Fig. 4 Effect of leonurine on NeuN-positive cells in the cerebral cortex of rats. Sham, Ischemia, Leo-L, Leo-M and Leo-H stand for the sham-operated, cerebral ischemia model, low-dose leonurine, middle-dose leonurine and high-dose leonurine groups, respectively; ** indicates the comparison between the ischemia model and sham-operated groups ($P < 0.01$); ## indicates the comparison between the leonurine treatment and ischemia model groups ($P < 0.01$).

behavior disorders and neuronal damage[13,14], and the OFT is commonly used to examine psychological functions[15]. In this study, the OFT results showed that cerebral ischemia inhibited the mobility and exploratory activities in rats, indicating that anxiety-like behavior was induced after cerebral ischemia (Fig. 1). Moreover, the number of NeuN-positive neurons was obviously less, and the neurons in the cerebral cortex were significantly damaged and exhibited swelling, degeneration, pyknosis of the Nissl body, necrosis and apoptosis, after cerebral ischemia treatment (Fig. 3). Meanwhile, the significant changes in SOD activity and MDA level further indicated the oxidative stress caused by cerebral ischemia. Our results demonstrate that the rat model of cerebral ischemia used in this study was successfully established.

Leonurine has been reported to have cardioprotective effects against ischemia-induced myocardial injury and can reduce the infarction area of the cerebral cortex and repair neurological damage[10,16]. To investigate the neuroprotective effect of leonurine treatment on cerebral ischemia and the underlying mechanisms of action, we measured serum SOD activity and MDA levels, GABA and GAD67 contents in the cortex, and then analyzed the effect of leonurine treatment on rat behavior, cortical neuron, oxidative stress and GABA expression. The results (Fig. 1) showed that leonurine obviously improved the general activity of rats in the OFT. This observation corresponded with attenuated neuronal damage induced by ischemia. GABA content and GAD67 protein level in the cerebral cortex in the Leo-H group as well as Serum SOD was significantly greater and the MDA level was obviously lower in the leonurine group as compared with Ischemia group, which indicated the presence of oxidative stress and GABA signals in Ischemia.

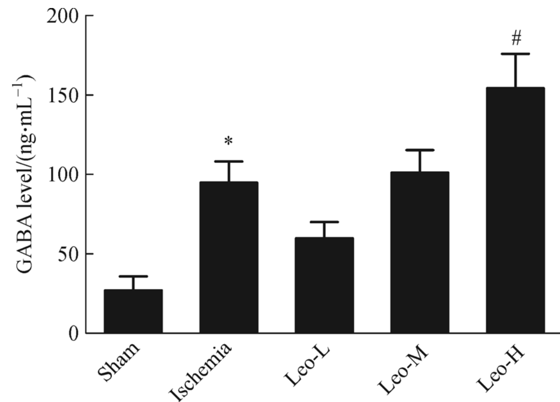

Fig. 5 Effect of leonurine on GABA content in the cerebral cortex of rats. Sham, Ischemia, Leo-L, Leo-M and Leo-H stand for the sham-operated, cerebral ischemia model, low-dose leonurine, middle-dose leonurine and high-dose leonurine groups, respectively; * indicates the comparison between the ischemia model and sham-operated groups ($P < 0.05$); # indicates the comparison between the leonurine treatment and ischemia model groups ($P < 0.05$).

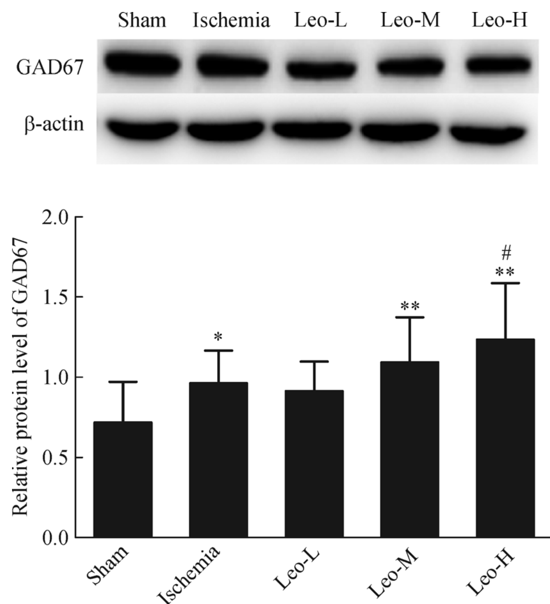

Fig. 6 Effect of leonurine on GAD67 content in the cerebral cortex of rats. Sham, Ischemia, Leo-L, Leo-M and Leo-H stand for the sham-operated, cerebral ischemia model, low-dose leonurine, middle-dose leonurine and high-dose leonurine groups, respectively; * indicates the comparison between the ischemia model and sham-operated groups ($P < 0.05$); ** indicates the comparison between the ischemia model and sham-operated groups ($P < 0.01$); # indicates the comparison between the leonurine treatment and ischemia model groups ($P < 0.05$).

Lipid peroxidation injury of the tissue induced by oxygen free radicals is one of the mechanisms underlying cerebral ischemic injury[17]. The MDA level is an indicator of lipid peroxidation. SOD is an endogenous mitochondrial antioxidant enzyme that removes excess free radicals that cause cerebral ischemic injury. Cerebral ischemia induces the production of numerous free radicals, resulting in an increase in the MDA level, and consumes SOD to reduce its activity, leading to a decrease in the SOD level[17,18].

Excitatory amino acids, including glutamic acid, aspartic acid and 2-carboxymethyl-3-isopropyl, are crucial neurotransmitters in the mammalian brain; however, high extracellular concentrations of these acids are neurotoxic[4,19,20]. Significant protection against excitotoxic cell death was observed in the absence of exogenous GABA[6,7,21]. Our results showed that expression of GAD, a rate-limiting enzyme in the synthesis of GABA, increased in the brain after ischemia, which might contribute to the improvement of behavior and the consequent survival of neurons in rats, with the consequent hyperactivity of GABAergic transmission[22].

Although the use of thrombolysis is limited to patients with stroke, it is currently the only effective therapy for cerebral ischemia. Therefore, neuroprotective agents with an extended therapeutic window that can prevent multiple neurochemical cascades, which ultimately cause irreversible brain damage, are urgently required[23]. Chinese herbal medicines with antioxidant properties are believed to exert a therapeutic effect. Leonurine, an active alkaloid extracted from *Herba Leonuri*, has shown biological effects, such as antioxidant, anticoagulant, antiapoptotic and protective effects, against ischemic heart diseases[24,25]. In our study, pretreatment with leonurine reduced neurobehavioral scores, increased NeuN-positive neuron density, attenuated neuronal damage, enhanced SOD activities and reduced the MDA level in the ischemic rats, suggesting that the neuroprotective effects of leonurine on cerebral ischemia are exerted through antioxidant effects, especially in the Leo-H group (16 $mg \cdot kg^{-1} \cdot d^{-1}$) (Fig. 2).

Furthermore, our results revealed that GABA and GAD67 expression increased significantly in the Leo-H group although GABA was a little reduced in the Leo-L group. The synthesis and release of GABA in the rat brain after cerebral ischemic injury are influenced by multiple causes. Free radical production influences the expression of GABA and SOD may reduce GABA release[16]. However, the specific mechanisms of the GABA effect remain unclear. Recently, it has been reported that leonurine exerts neuroprotection by reducing the damage caused by excitatory toxicity[26]. The increased expression levels of GABA and GAD67 in cerebral ischemia after Leo-H pretreatment suggested that increased GABA expression is one of the mechanisms underlying the neuroprotection of leonurine.

In conclusion, the present study shows that pretreatment with leonurine can improve ischemia-induced anxiety-like behavior and neuron injury by modulating SOD, MDA and GABA levels. Our study provides further evidence that leonurine is an excellent potential therapeutic agent for

cerebral ischemia, and the neuroprotective effect of leonurine is likely exerted through antioxidant effects and excitotoxicity antagonism, which indicated a correlation between oxidative stress-GABA signal pathway activation and the neuroprotective effects of leonurine. Further studies are required to elucidate these mechanisms.

Acknowledgements This work was supported by the National Natural Science Foundation of China (31772686 and 31502025) and the Public Service Sectors Agriculture Research Projects of Ministry of Agriculture of China (201403051-07).

Compliance with ethics guidelines Shilei Zheng, Jingru Zhu, Jiao Li, Shuang Zhang, and Yunfei Ma declare that they have no conflicts of interest or financial conflicts to disclose.

All institutional and national guidelines for the care and use of laboratory animals were followed.

References

1. Dirnagl U, Iadecola C, Moskowitz M A. Pathobiology of ischaemic stroke: an integrated view. *Trends in Neurosciences*, 1999, **22**(9): 391–397
2. Mehta S L, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. *Brains Research Reviews*, 2007, **54**(1): 34–66
3. Nakka V P, Gusain A, Mehta S L, Raghubir R. Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. *Molecular Neurobiology*, 2008, **37**(1): 7–38
4. Aarts M M, Tymianski M. Molecular mechanisms underlying specificity of excitotoxic signaling in neurons. *Current Molecular Medicine*, 2004, **4**(2): 137–147
5. Takagi K, Ginsberg M D, Globus M Y, Dietrich W D, Martinez E, Kraydieh S, Busto R. Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology. *Journal of Cerebral Blood Flow and Metabolism*, 1993, **13**(4): 575–585
6. Mason G F, Martin D L, Martin S B, Manor D, Sibson N R, Patel A, Rothman D L, Behar K L. Decrease in GABA synthesis rate in rat cortex following GABA-transaminase inhibition correlates with the decrease in GAD(67) protein. *Brain Research*, 2001, **914**(1–2): 81–91
7. Madl J E, Royer S M. Glutamate dependence of GABA levels in neurons of hypoxic and hypoglycemic rat hippocampal slices. *Neuroscience*, 2000, **96**(4): 657–664
8. Liu X, Pan L, Wang X, Gong Q, Zhu Y Z. Leonurine protects against tumor necrosis factor-α-mediated inflammation in human umbilical vein endothelial cells. *Atherosclerosis*, 2012, **222**(1): 34–42
9. Liu X H, Pan L L, Deng H Y, Xiong Q H, Wu D, Huang G Y, Gong Q H, Zhu Y Z. Leonurine (SCM-198) attenuates myocardial fibrotic response via inhibition of NADPH oxidase 4. *Free Radical Biology & Medicine*, 2013, **54**: 93–104
10. Loh K P, Qi J, Tan B K, Liu X H, Wei B G, Zhu Y Z. Leonurine protects middle cerebral artery occluded rats through antioxidant effect and regulation of mitochondrial function. *Stroke*, 2010, **41**(11): 2661–2668
11. Wang Q H, Zhang S, Qin L M, Zhang W J, Liu F H, Xu J Q, Ma Y F, Teng K D. Yimu San improves obstetric ability of pregnant mice by increasing serum oxytocin levels and connexin 43 expression in uterine smooth muscle. *Journal of Zhejiang University: Science B*, 2017, **18**(11): 986–993
12. Zemke D, Smith J L, Reeves M J, Majid A. Ischemia and ischemic tolerance in the brain: an overview. *Neurotoxicology*, 2004, **25**(6): 895–904
13. Zádori D, Klivényi P, Szalárdy L, Fülöp F, Toldi J, Vécsei L. Mitochondrial disturbances, excitotoxicity, neuroinflammation and kynurenines: novel therapeutic strategies for neurodegenerative disorders. *Journal of the Neurological Sciences*, 2012, **322**(1–2): 187–191
14. Wang Y, Wan C, Yu S, Yang L, Li B, Lu T, Bi Y, Jiang J, Cui G. Upregulated expression of NF-YC contributes to neuronal apoptosis via proapoptotic protein bim in rats' brain hippocampus following middle cerebral artery occlusion (MCAO). *Journal of Molecular Neuroscience*, 2014, **52**(4): 552–565
15. Lo Iacono L, Gross C. Alpha-Ca^{2+}/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. *Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 2008, **28**(24): 6250–6257
16. Liu H, Zhang X, Du Y, Ji H, Li S, Li L, Xing Y, Zhang X, Dong L, Wang C, Zhao K, Ji Y, Cao X. Leonurine protects brain injury by increased activities of UCP4, SOD, CAT and Bcl-2, decreased levels of MDA and Bax, and ameliorated ultrastructure of mitochondria in experimental stroke. *Brain Research*, 2012, **1474**: 73–81
17. Hua J S, Li L P, Zhu X M, Wang Y J. Effects of moxibustion pretreating on SOD and MDA in the rat of global brain ischemia. *Journal of Traditional Chinese Medicine*, 2008, **28**(4): 289–292
18. Akman T, Guven M, Aras A B, Ozkan A, Sen H M, Okuyucu A, Kalkan Y, Sehitoglu I, Silan C, Cosar M. The neuroprotective effect of glycyrrhizic acid on an experimental model of focal cerebral ischemia in rats. *Inflammation*, 2015, **38**(4): 1581–1588
19. Puyal J, Ginet V, Clarke P G. Multiple interacting cell death mechanisms in the mediation of excitotoxicity and ischemic brain damage: a challenge for neuroprotection. *Progress in Neurobiology*, 2013, **105**: 24–48
20. Ahmad A S, Shah Z A, Doré S. Protective role of arginase II in cerebral ischemia and excitotoxicity. *Journal of Neurology and Neuroscience*, 2016, **7**(2): 88–93
21. Erdö S, Michler A, Wolff J R. GABA accelerates excitotoxic cell death in cortical cultures: protection by blockers of GABA-gated chloride channels. *Brain Research*, 1991, **542**(2): 254–258
22. Monnerie H, Le Roux P D. Glutamate alteration of glutamic acid decarboxylase (GAD) in GABAergic neurons: the role of cysteine proteases. *Experimental Neurology*, 2008, **213**(1): 145–153
23. Loh K P, Huang S H, Tan B K H, Zhu Y Z. Cerebral protection of purified *Herba Leonuri* extract on middle cerebral artery occluded rats. *Journal of Ethnopharmacology*, 2009, **125**(2): 337–343
24. Wang Z, Zhang P L, Ju Y. Effect of leonurine on the activity of creatine kinase. *Journal of Asian Natural Products Research*, 2004, **6**(4): 281–287

25. Qi J, Hong Z Y, Xin H, Zhu Y Z. Neuroprotective effects of leonurine on ischemia/reperfusion-induced mitochondrial dysfunctions in rat cerebral cortex. *Biological & Pharmaceutical Bulletin*, 2010, **33**(12): 1958–1964
26. Liu C, Yin H, Gao J, Xu X, Zhang T, Yang Z. Leonurine ameliorates cognitive dysfunction via antagonizing excitotoxic glutamate insults and inhibiting autophagy. *Phytomedicine*, 2016, **23**(13): 1638–1646