MOTHERWORT PLAYS A PROTECTIVE ROLE IN CEREBRAL ISCHEMIA MODEL MICE BY ACTIVATING NRF-2/HO-1 SIGNALING PATHWAY

Shuai Wang[1], Junye Shen[2,*], Yingsong Zhou[3]
[1]Department of Rehabilitation Medicine, Hwa Mei Hospital, University of Chinese Academy of Sciences, Ningbo, PR China - [2]Department of Traditional Chinese Medicine, Hwa Mei Hospital, University of Chinese Academy of Sciences, Ningbo, PR China - [3]Faculty of Physical Education, Ningbo University, Ningbo, PR China

ABSTRACT

Objective*: To study the protective effect of leonurine has on cerebral-ischemia-modelling mice through activating the Nrf-2/HO-1 signalling pathway.*

Methods: *Sixty healthy and clean adult male Sprague Dawley mice were randomly divided into four groups: false-operation group, control group, low-dosage group and high-dosage group. A right middle cerebral artery occlusion was established in the control group, low-dosage group and high-dosage group according to the thread thrombus method of Zea-Longe. After the success of the modelling was determined by scored behavioural observations, the mice in the low-dosage group and the high-dosage group began to being intravenously injected with 5mg/kg and 10mg/kg of leonurine solution, beginning two hours after the operation and continuing once per day for seven days. The control group was injected with saline of the same volume once a day for seven days, beginning two hours after the operation. A modified neurological deficit score (Longa score) was used to evaluate the behaviour of all of the mice. The water content of brain tissue was measured using a dry-and-wet-weight method: the brain tissue of mice was collected following killing mice, the volume of cerebral infarction was calculated after TTC staining, and the levels of nuclear respiratory factor 2 (Nrf-2) and heme oxygenase-1 (HO-1) in the ischemic-side tissue of mice in each group were measured using a real-time quantitative fluorescence method.*

Results: *The Longa scores and brain-tissue water content of the control group were significantly higher than for the false-operation group. The Longa scores and brain-tissue water content in the dosed groups were significantly lower than for the control group and decreased with the increase of dose ($P<0.05$). The blood flow perfusion and the total power of the cerebral cortex for the control group were significantly lower than for the false-operation group. The blood perfusion and the total power of the cerebral cortex in the dosed groups were significantly higher than for the control group; this increased with the increase of dose, the differences being statistically significant ($P<0.05$). In the control group, the number of Nissl corpuscles in the hippocampal CA1 areas and CA3 areas decreased sharply, the volume decreased and the shape was very irregular; the number of Nissl corpuscles in the low-dosage group was higher than in the control group, the neurons were orderly and the nerve cells were less damaged; the number of Nissl corpuscles in the high-dosage group was higher than in the low-dosage group, and nerve injuries were alleviated. The levels of Nrf-2 and HO-1 in the ischemic-side tissue of the mice in the low-dosage group and the high-dosage group were significantly higher than the levels in the false-operation group. The levels of Nrf-2 and HO-1 on the ischemic-side tissue of the mice treated with leonurine were significantly higher than the levels for the control group; the difference was statistically significant with the increase of dose ($P<0.05$).*

Conclusion: *Leonurine has certain protective effects on the brain tissue of cerebral-ischemia-modelling mice; this protective effect may be enacted by activating Nrf-2/HO-1 signalling pathway.*

Keywords: *Leonurine, activation, Nrf-2/HO-1 signalling pathway, cerebral ischemia model, mouse, brain protection.*

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Introduction

Cerebral ischemic brain injury is a kind of brain dysfunction caused by the blockage of cerebral blood vessels and the sudden decrease of cerebral blood flow[(1)]. Clinical manifestations are sudden dizziness, tinnitus, eye flowers, walking instability and vertigo; severe symptoms include blurred consciousness, inexplicable fall, blindness or amblyopia, unilateral or bilateral limb weakness, abnormal feelings, and loss of speech fluency[(2)]. Characteristics of ischemic strokes are high incidence, high disability rates and high mortality; these factors represent economic burden and bereavement for countless families[(3)]. Because the pathogenesis of cerebral ischemic brain injury is not clear and the curative effect of drugs in

the treatment of cerebral ischemic brain injury is not satisfactory, the focus of medical scholars is to find effective drugs for the treatment of cerebral ischemic brain injuries[4]. Leonurus chinensis is the most commonly used drug for the treatment of dysmenorrhea, menstrual disorders and other gynaecological diseases[5]. In recent years, Leonurus chinensis has been proven effective in the treatment of ischemic cardiovascular and cerebrovascular diseases[6].

It has been found that Leonurus chinensis extract can, to a certain extent, improve coronary blood flow and protect cerebral ischemia. Leonurine is one of the main components of Leonurus chinensis and plays an important role in protecting the myocardium. In recent years, it has been found that Leonurine can prevent cerebral obstruction and delay its progress[7]. Studies have shown that oxidative stress plays a certain role in ischemic brain injury and is the key factor in brain injury and nerve function damage[8]. The NRF-2/HO-1 signalling pathway is one of the intrinsic in-vivo antioxidant systems and can maintain the oxidation balance of the body[9]. The purpose of this study was to evaluate the protective effect of leonurine on the brains of cerebral-ischemia-modelling mice and to explore the relationship between leonurine and the Nrf-2/HO-1 signalling pathway.

Materials and methods

General information

Sixty adult male Sprague Dawley mice of healthy and clean grade were randomly selected for purchase from the Experimental Animal Centre of Guilin Medical College.

The biological licence number of the experimental animals was (245.50±112.35) weeks old, the average body weight was (245.50±112.35) g, the room temperature was (23.00±1.00) degrees Celsius, the relative humidity was (50.00±10.00) %, the noise level was ≤45dB, and drinking water was provided freely. There was no significant difference in age and body mass between mice.

Methods

The mice were randomly divided into four groups: control group, false-operation group, low-dosage group and high-dosage group.

The mice in the control group, low-dosage group and high-dosage group were randomly divided into four groups: control group, low dose group and high dose group. A right middle cerebral artery occlusion model was established using the thread thrombus method of Zea-Longe. The mice were anaesthetised using 3.5% chloral hydrate, fixed in a supine position on the operating table, and then their neck hair was removed and sterilized. The skin was cut off along the middle of the neck, the right common carotid artery was separated, the external carotid artery and internal carotid artery were separated along the carotid artery, and then all of the branches of the external carotid artery were collected. After ligating and hanging the sterilised nylon line at the top, the external carotid artery was cut and lifted from the internal carotid artery, a small incision was cut into the end of the external carotid artery, and the sterilised nylon line was slowly inserted into the internal carotid artery. After inserting it 8-9 mm into the artery, the large living knot was ligated and ligated tightly, and the suture was sterilised. The mice in the false-operation group underwent a false operation, that is, the cervical artery was freed but the nylon line was not inserted; the others were the same.

After the model's success was determined by behavioural score, the mice in the low-dosage group and the high-dosage groups began being intravenously injected with 5mg/kg and 10mg/kg of leonurine solution. This began two hours after the operation and continued once a day for seven days. The control group began being injected with the same volume of saline once a day for seven days, beginning two hours after the operation.

Observation indicators

The behavioural scores for all the mice were evaluated according to a modified neurological deficit score (Longa score), where the highest score was 5 points. A score of 0 described mice being able to move freely and no obvious defects being found;

- Described tail lifting and inability to extend the forelimb;
- Described contralateral forelimb flexion;
- Described mice moving in mild contralateral circles;
- Described the mice moving in severe contralateral circles;
- Described the mice falling contralaterally.

Blood flow perfusion in the cerebral cortex of mice was measured using a laser speckle flow dynamic imager. The mice were anesthetised with 3.5% chloral hydrate. Then, the mice were fixed in a prone position on the operating table. After disinfection, the skin was cut off, the periosteum was exposed, the laser scanner was placed in the upper nine cm of the

mouse's brain, a range of 15 mm2 was measured, and the changes in cerebral cortical blood flow were recorded for each group.

A brain stereotactic apparatus was used to record EEGs of mice in each group: the mice were anesthetized with 3.5% chloral hydrate, fixed in a prone position on the operating table, the skin was cut open after disinfection, the periosteum was exposed, the mice were placed in the brain stereotactic instrument, EEGs were recorded over 30 minutes for each group, and the total power was analysed.

The water content of brain tissue was measured using the dry-and-wet-weight method. After killing the mice, the brain was quickly removed, and 3-mm-thick brain tissue was removed to detect the water content of the brain. The brain tissue was divided into the lesion side and the opposite side of the lesion. After weighing the fresh brain tissue at 100 °C for 24 hours, the brain tissue was weighed at room temperature, and the water content of the brain tissue on both sides was calculated, according to the following equation: (wet weight - dry weight)/wet weight × 100%. Paraffin sections were established to observe the structural changes to Nissl corpuscles in brain tissue: anesthetised mice were anesthetised with 3.5% chloral hydrate, fixed in the supine position on the operating table, the heart was perfused and fixed, the paraffin sections of tissue were made, and the sections of brain tissue were stained with Nissl staining to observe the structural changes to Nissl corpuscles in the brain tissue of mice in each group. Inspection index detection: the results showed that the mice were anesthetized with 3.5% chloral hydrate, the cervical vertebrae was dislocated and the mice were killed, and the brain tissue of ischemic area was washed with DEPC water. The total RNA of brain tissue was extracted to detect the concentration of RNA, and a cDNA library was constructed. The levels of nuclear respiratory factor 2 (Nrf-2) and heme oxygenase 1 (HO-1) in the ischemic side of mice in each group were detected using real-time quantitative fluorescence analysis.

Statistical methods

The data of this study were analysed using the SPSS20.0 software package. All of the measurement data were expressed by ($\bar{x}\pm s$), a t test was used to compare the data between groups, the percentage of data counted was expressed using a percentage test, and the comparison between groups was tested. A Ridit test was used to compare the grade data. A p value of <0.05 was considered statistically significant.

Results

Comparisons of Longa scores and brain-tissue water content

The Longa score and brain-tissue water content of mice in the control group were significantly higher than those in the false-operation group, while the Longa score and brain-tissue water content of mice in the leonurine group were significantly lower than those in the control group. The differences were statistically significant with the increase of dose (P<0.05). These results are shown in Table 1.

Group	n	Longa Score	Brain water content (%)
False-operation group	15	0.00±0.00	78.15±3.45
Control group	15	3.52±0.35[a]	88.50±5.05[a]
Low-dosage group	15	2.85±0.41[ab]	83.16±4.15[ab]
High-dosage group	15	1.72±0.45[abc]	81.12±3.76[abc]

Table 1: Comparison of Longa scores and brain water content ($\bar{x}\pm s$).

Note: a indicates comparison with the false-operation group, [a]P<0.05; b represents [b]P<0.05 compared with the control group; c indicates that [c]P<0.05 compared with the low-dosage group.

Comparisons of blood flow perfusion and total power of the cerebral cortex

The blood flow perfusion volume and the total power of the cerebral cortex were significantly lower in the control group than in the false-operation group, while blood flow perfusion volume and the total power of cerebral cortex were significantly higher in the leonurine group than in the control group; differences increased with the increase in dose and the difference was statistically significant (P<0.05). These results are shown in Table 2.

Group	n	Blood flow perfusion volume	Total power of cerebral cortex
False-operation group	15	335.26±3.45	2.07±0.02
Control group	15	135.56±11.25[a]	0.58±0.01[a]
Low-dosage group	15	146.26±12.52[ab]	0.81±0.05[ab]
High-dosage group	15	205.13±11.45[abc]	1.21±0.08[abc]

Table 2: Comparisons of blood flow perfusion and total power of cerebral cortex ($\bar{x}\pm s$).

Note: a indicates that compared with the false-operation group, [a]P<0.05; b represents [b]P<0.05 compared with the control group; c indicates [c]P<0.05 compared with the low-dosage group.

Comparison of the structures of the focal cerebral nerve cells

In the false-operation group, the number of Neshusundefineds bodies in the hippocampi of the mice was clear and clear, and no significant

damage was found in the structure; the number of Neshusundefineds bodies in the hippocampal CA1 region and the CA3 region of the control group decreased, the volume was reduced, and the shape was highly irregular. In the low-dosage group, the number of Nissl bodies was lower than that of the control group, the neurons were more orderly, and damage to nerve cells was light. These results are shown in Fig. 1.

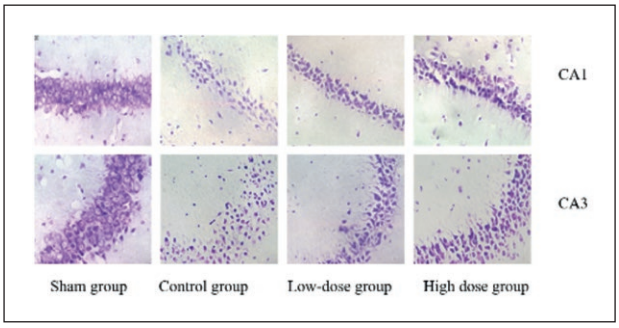

Figure 1: Comparison of the structure of focal brain Nissl corpuscles for each group of mice following Nissl staining.

Comparisons of Nrf-2 and HO-1 levels in the ischemic lateral tissues

The levels of Nrf-2 and HO-1 in the ischemic side tissues of mice in the control group, the low-dosage group and the high-dosage group were significantly higher than those in the false-operation group. The levels of Nrf-2 and HO-1 in the ischemic side tissues of mice in the leonurine group were significantly higher than in the control group and increased with the increase in dose. The differences were statistically significant (P<0.05). These results can be seen in Table 3.

Group	n	HO-1 mRNA	Nrf-2 mRNA
False-operation group	15	1.48±0.08	1.06±0.12
Control group	15	2.41±0.17[a]	1.86±0.17[a]
Low-dosage group	15	3.16±0.15[ab]	2.16±0.08[ab]
High-dosage group	15	4.02±0.37[abc]	3.21±0.09[abc]

Table 3: Comparison of Nrf-2 and HO-1 levels in the ischemic lateral tissues ($\bar{x}\pm s$).
Note: a indicates that compared with the false-operation group, [a]P<0.05; b represents [b]P<0.05 compared with the control group; c indicated that [c]P<0.05 compared with the low-dosage group.

Discussion

Ischemic brain injuries are mainly due to types of brain damage, including brain-neuron injuries and nerve-function disorders, which are caused by blood entering the brain tissue leading to changes in the behaviour of the body and disordered brain activity[10]. Cerebrovascular disease is one of the three major diseases leading to the death of a patient. It is mainly used for the treatment of cerebral ischemic brain damage, but the curative effect is limited. Leonurine shows effects of promoting blood circulation, regulating menstruation, promoting urination, relieving swelling, and removing heat and toxic materials; it has mainly been used to treat menoxenia, dysmenorrhea, lochia, oedema, sores and swelling, and it is a common drug in gynaecological clinical use in the context of Chinese medicine[11]. Leonurine is the main effective component extracted from Leonurus chinensis and can expand the peripheral blood tube and increase blood flow; this has the effects of promoting blood circulation, removing blood stasis, promoting diuresis and detumescence, and assisting in treating ischemic brain injury[12].

The Longa score is one of the most common methods for evaluating the severity of cerebral ischemia, which can reflect the neurological and behavioural changes in mice. Brain oedema is one of the basic symptoms of ischemic brain damage, and is an important index for cerebral ischemia. Cerebral ischemic brain damage can occur in the brain, reducing blood flow perfusion, exciting the injured nerve and increasing neuron degeneration. In this study, the Longa scores and water content of the brain tissue of the control group were significantly higher than in the false-operation group, and the Longa scores and the water content of the brain tissue of the mice treated with leonurine were significantly lower than in the control group. The differences were statistically significant with the increase of the dose (P<0.05). The blood flow perfusion and the total power of the cerebral cortex in the control group were significantly lower than in the false-operation group, and the blood flow perfusion and the total power of the cerebral cortex in the mice dosed with leonurine were significantly higher than in the control group. The differences were statistically significant with the increase of the dose (P<0.05). This suggests that leonurine can reduce brain damage in mice with cerebral ischemia, reduce neurological function and abnormal behaviour, and improve brain activity[13]. The study found that the hippocampus is the most damaged area following cerebral ischemic brain injury. Nissl bodies are a kind of basophilic substance present in the cytoplasm. In normal conditions, Nissl bodies have uniform distribution and a complete structure. When the nerve is damaged, the Nissl bodies can decompose and dissolve. The results showed a significant reduction in the number

of Nissl bodies[14]. In this study, the number of Nissl bodies in the hippocampi of the mice in the false-operation group was clear and clear, and no significant damage was observed in their structure; the numbers of Nissl bodies in the CA1 and CA3 regions of the hippocampi of the control group were reduced, the volume was reduced, and the shape was very irregular. The number of the Nissl bodies in the low-dosage group was much higher than in the control group, the neurons were more orderly and the damage to nerve cells was light; the number of Nissl bodies the higher-dose group was lower than in the low-dosage group, and the damage to the nerve was relieved. This suggests that leonurine can repair damage to cerebral ischemia-induced neurons in mice.

Oxidative stress is one of the most substantial factors in cerebral-ischemic brain injury, and the Nrf-2/HO-1 signalling pathway is an important pathway for oxidative stress endogenous antioxidants[15]; HO-1 and Nrf-2 are important components of the NRF-2/HO-1 signalling pathway. The enzyme HO-1 is a speed-limiting enzyme in the heme-catabolism process, and Nrf-2 is a member of the leucine zipper transcription activator family; it is highly sensitive to oxidative stress. The levels of Nrf-2 and HO-1 in the cerebral ischemia-side tissues of the control group, the low-dosage group and the high-dosage group were significantly higher than those in the false-operation group. The difference was significant ($P<0.05$). This suggests that the Nrf-2/HO-1 signalling pathway can be used to protect the brain tissue and relieve brain damage caused by cerebral ischemia. That is to say that leonurine has been demonstrated to have a protective effect on the brain tissue of the cerebral-ischemia-modelling mice, and that protective effect can be enacted by activating the Nrf-2/HO-1 signalling pathway.

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Corresponding Author:
Junye Shen
Email: hi924k@163.com
(China)