Original Article
The effects of sodium ferulate on inflammatory injuries induced by cerebral ischemia-reperfusion and the expressions of NF-κB and claudin 5 in rats

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Abstract: Objective: This study aimed to observe the effects of sodium ferulate on rats with focal cerebral ischemia-reperfusion injuries. Methods: 68 male SD rats were randomly divided into 6 groups: a mock surgical group, a model group, a sodium ferulate low dose group (25 mg/kg), a sodium ferulate medium dose group (50 mg/kg), a sodium ferulate high dose group (100 mg/kg), and a nimodipine (4 mg/kg) positive control group. The rat model of focal cerebral ischemia-reperfusion injury was established using middle-west embolization. The neurological deficit scores, the cerebral infarction volume, the pathomorphology, the ultraviolet-visible spectrophotometer, the expressions of brain inflammatory cytokines, the NF-κB, and the claudin 5 levels in the ischemic cerebral cortex were compared. Results: Compared with the model group, the neurological function scores, the brain water content, the infarct volume, and the levels of ROS, MDA, iNOS, NO, IL-6, IL-1β, TNF-α, and NF-κB in the brain tissue of the middle-and high-dose groups of sodium ferulate in brain tissue were significantly decreased, and the brain tissue morphology was improved (P<0.05). The expression of claudin 5 was higher than it was in the model group (P<0.05). Conclusion: Sodium ferulate has neuroprotective effects which can improve neurological symptoms in rats with focal cerebral ischemia-reperfusion injuries, reduce the volume of cerebral infarction, and reduce brain edema. Its protective effect may be related to the down-regulation of the expression of NF-κB, the up-regulation of the expression of claudin-5, and the reduction of oxidative stress damage in brain tissue.

Keywords: Sodium ferulate, cerebral ischemia, reperfusion injury, nuclear transcription factor-κB, transmembrane tight junction protein-5

Introduction

Ischemic stroke refers to localized ischemic necrosis in the brain caused by the cerebral ischemia and hypoxia when the blood supply is insufficient for the brain. It accounts for 60%-80% of all cerebrovascular diseases and has become a primary cause of disability and death in China [1]. Recombinant tissue plasminogen activator (rt-PA) for intravenous thrombolysis and interventional surgery are recognized as important means to rapidly improve ischemic stroke. They can effectively clear blood vessels, establish effective reperfusion, and restore microcirculation at the infarct site [2]. However, complications from ischemia-reperfusion injury may occur after blood flow reconstruction, thus

limiting its application. Brain tissue damage caused by cerebral ischemia-reperfusion is a complex pathological effect accompanied by changes in the structure and function of the blood-brain barrier (BBB). A study has shown that oxidative stress, the inflammatory response, calcium overload, mitochondrial dysfunction, and the like may be related to changes in BBB permeability during cerebral ischemia-reperfusion injury, resulting in brain neuron damage [3]. Another study indicated that oxidative stress and the inflammatory reaction after reperfusion injury, which damage the BBB, are important causes of the irreversible damage to neurons [4]. Nuclear transcription factor-κB (NF-κB) is an important member of the transcription factor protein family. It has been

shown to be involved in the transcriptional regulation of various inflammatory genes, mediating the expressions of inflammatory mediators and aggravating the inflammatory response to brain injury [5]. According to another study, the transmembrane tight junction protein-5 (claudin-5) was shown to play a key role in maintaining the integrity of the BBB and the stability of the environmental balance in the central nervous system [6]. Therefore, to provide protection for neurons, reduce brain edema, and repair the damage to the BBB, it has become a research hotspot for the development of new brain protection drugs that can alleviate oxidative stress damage in brain tissue, inhibit NF-κB activity, block the inflammatory response after cerebral ischemia-reperfusion, and up-regulate claudin-5 expression.

Sodium ferulate is an active ingredient of various medicinal plants such as Angelica, white peony, and Chuanxiong. It has anti-free radical and lipid peroxidation, anti-platelet aggregation, anti-inflammatory and immunoenhancer functions. Sodium ferulate is more and more widely used in adjuvant therapy for atherosclerosis, coronary heart disease, cerebrovascular disease, and the like [7]. However, little research has been done on the protective effects and mechanism of sodium ferulate in the acute phase of ischemic stroke. In this study, based on the preparation of a rat model of focal cerebral ischemia-reperfusion injury, the effects of sodium ferulate on the neurobehavior, cerebral infarction volume, pathological morphology, and BBB permeability in rats with cerebral ischemia-reperfusion injury were observed, and its neuroprotection mechanism was explored.

Material and methods

Materials

Animals: 120 clean grade, Sprague-Dawley (SD) male rats, weighing 250-280 g, were purchased from the Experimental Animal Center of Chongqing Medical University. The temperature of the feeding environment was 21-23°C, and the relative humidity was 40%-60%. The rats were free to eat and drink.

Instruments: A UV-1206 ultraviolet spectrophotometer was purchased from Shimadzu Corporation of Japan. A high-speed, low-tem-perature centrifuge was purchased from Jinan Hewei Biotechnology Co., Ltd. An electrophoresis instrument was purchased from Shanghai Jiapeng Technology Co., Ltd. An HM325 paraffin slicer was purchased from Beijing Hengsanjiang Instruments Sales Co., Ltd. A BIO-RAD 380 microplate reader was purchased from BIO-RAD, USA. An inverted optical microscope was purchased from Sunny Optical Technology (Group) Co., Ltd.

Drugs and reagents: Sodium ferulate was purchased from the Shanghai Hyundai Hasen (Shangqiu) Pharmaceutical Co., Ltd. 2,3,7-triphenyltetrazolium chloride (TTC) was purchased from Shanghai Hualan Chemical Technology Co., Ltd. Oxygen free radicals (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), inducible nitric oxide synthase (iNOS) and a nitric oxide (NO) kit were purchased from Beijing Enjihe Biotechnology Co., Ltd. Interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) kits were purchased from the Beijing Runnuos Medical Technology Co., Ltd. NF-Kb and claudin-5 polyclonal antibodies were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.

Methods

Establishment of a middle cerebral artery occlusion (MCAO) model: After 7 days for adaptive feeding, the rat brain MCAO model was prepared using the modified Longa 5 score method. After anesthesia, the rats were fixed in a supine position, and a midline incision was made to separate the common carotid artery on the right side and the branches of the internal and external carotid arteries. The common carotid artery and external carotid artery were ligated, and the internal carotid artery was inserted into the anterior cerebral artery and sutured layer by layer. After 90 minutes of ischemia, the suture was opened and the wire was pulled out to restore the blood perfusion. The outer end of the suture was cut, and then the outer skin of the neck was sutured layer by layer. The mock surgical group was operated on using the same methods except that the suture was not inserted.

Grouping and administration of the experimental animals: 168 rats were randomly divided into 6 groups (n=28) according to the random

number table method: the mock surgical group, the model group, the sodium ferulate low dose group (25 mg/kg), the sodium ferulate medium dose group (50 mg/kg), the sodium ferulate high dose group (100 mg/kg), and the nimodipine (4 mg/kg) positive control group. The rat models of focal cerebral ischemia-reperfusion were prepared in each of the groups in addition to the mock surgical group. The rats in each group were administered the drugs on the 2nd day after the modeling. The three sodium ferulate dose groups and the nimodipine group were administered the drugs though a tail vein injection after the models of focal cerebral ischemia-reperfusion. The rats in the mock surgical group and the model group were injected with the same volume of normal saline. All the rats were continuously administered the drugs for 7 days, once a day.

Rat neurobehavioral score: According to the Zea-Longa scoring method five-point scale, the neurological deficit was assessed using blinded scoring at 24 h after the reperfusion. Grade 0, no deficit; grade 1, failure to fully extend the right forepaw; grade 2, circling to the left; grade 3, falling to the left; and grade 4, no spontaneous walking and a depressed level of consciousness or death. The mice scoring from grades 1-4 with no bleeding in the middle cerebral artery after the decapitation were considered a successful model.

Brain tissue water content and infarct volume measurement: After completion of "1.2.3", 6 rats were randomly selected from each group. After the rats were anesthetized, the brains were removed, and the cerebellums and the lower brainstems were removed. The brain tissue was weighed before (W) and after (D) baking at 110°C for 48 h. The brain water content (%) = [(W-D) /W] × 100% was used to calculate the water content of the brain tissue. In each group, the other 6 rats were randomly selected. After the rats were anesthetized, the brains were removed and cut into 5 pieces (1.50 mm each). They were immersed in 2% TTC solution and incubated for 30 min at 37°C in a water bath. Image-Pro Plus 5.1 software was used to analyze the percentage of brain tissue infarction.

Pathomorphological examination using HE staining: After 24 hours of reperfusion in the rats, 6 rats were randomly selected from each

group. After the rats were anesthetized, the brains were removed, and the lower brain stem, cerebellum and olfactory bulb were removed. The brains were fixed in about 200 ml of a 4% paraformaldehyde solution for 3 days and embedded in paraffin. After continuous coronal sectioning, dewaxing and hydration, the brains were subjected to conventional HE staining. The morphological changes of the ischemic cortical neurons in the rat brain tissue were observed under an inverted lighted microscope.

The quantification of oxidative stress related indicators in the brain tissue: The brains of 10 rats in each group were removed under anesthesia with the hippocampus tissue stripped. After grinding and centrifuging at 3000 r/min for 15 min, the supernatant was taken. The activities of SOD, CAT, GSH-Px, iNOS and the contents of ROS, MDA and NO were determined using a UV-visible spectrophotometer after the processing according to the kit's instructions.

Brain tissue inflammatory cytokine measurement: The brain tissue homogenate prepared by "1.2.6" was used to determine the contents of IL-6, IL-1β, and TNF-α using an enzyme-linked immunosorbent assay according to the kit's instructions.

The detection of the NF-κB and claudin 5 protein expressions in the ischemic cerebral cortex using western blotting (WB): The brain tissue homogenate prepared by "1.2.6" was centrifuged at 12000 r/min for 20 min, and the precipitate was taken for protein denaturation and quantification. After the SDS-PAGE electrophoresis, the proteins were electrotransferred to PVDF membranes. At room temperature, 5% skim milk powder was sealed for 2 hours. The antibodies were incubated at 4°C overnight (diluted 1:200), and a horseradish peroxidase-labeled secondary antibody (1:3000) was incubated for 1 h. An ECL kit was used to color the proteins. The expressions of the NF-κB and claudin 5 proteins were semi-quantitatively analyzed based on the gray value of the bands using Image J.

Statistical analysis

The statistical analysis was performed using SPSS 19.0 statistical software. The measure-
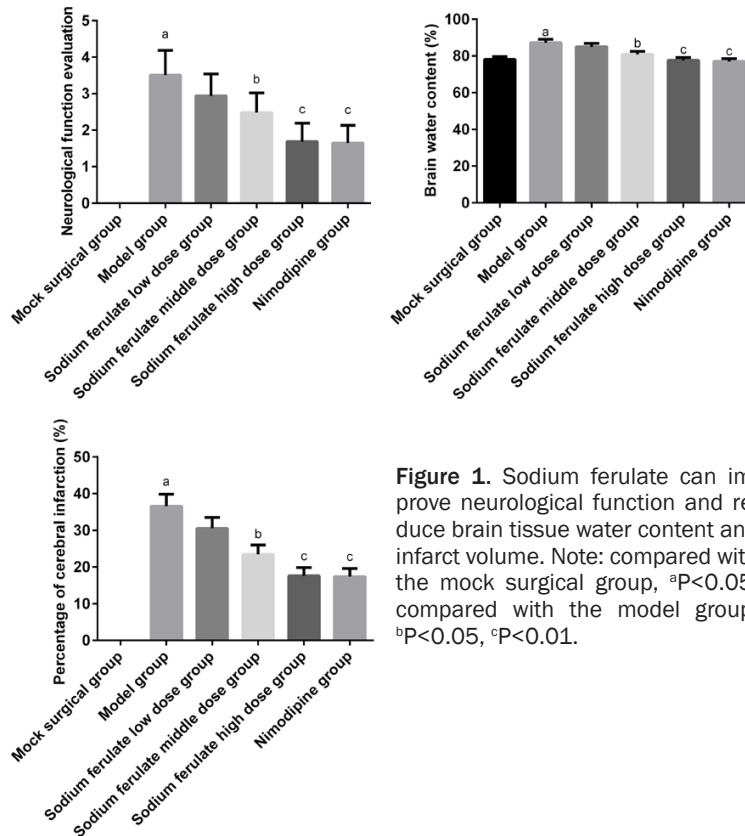
Figure 1. Sodium ferulate can improve neurological function and reduce brain tissue water content and infarct volume. Note: compared with the mock surgical group, [a]P<0.05; compared with the model group, [b]P<0.05, [c]P<0.01.

Figure 2. HE staining was used to determine the pathological changes of the brain tissue in each group (HE staining × 400 times). The cerebral tissue of rats with cerebral ischemia-reperfusion was cytoplasmic and fusiform. Cavitation degeneration, cytoplasmic red staining, a decreased number of neurons, and inflammatory cell infiltration of the brain tissue and other brain damage manifestations were observed, while sodium ferulate significantly improved brain damage in rats. (A-F) are the mock surgical group, the model group, the low dose sodium ferulate group, the medium dose sodium ferulate group, the high dose sodium ferulate group, and the nimodipine group.

ment data were expressed as $\overline{x}\pm s$, and a one-way analysis of variance was used for the comparison between multiple groups, and a LSD-t test was used for the comparisons between two groups. The count data were expressed as [n (%)], and an χ^2 test was used. P<0.05 was considered statistically significant.

Results

Sodium ferulate improved neurological functions and reduced brain tissue water content and infarct volume

Compared with the mock surgical group, the neurological function score, the brain water content, and the infarct volume of the model group were increased (*P*<0.05). Compared with the model group, the neurological function scores, the brain water content, and the infarct volume of the sodium ferulate medium and high dose groups were all decreased ($P_{medium\ dose\ group}$<0.05; $P_{high\ dose\ group}$<0.01), and the high dose group was not significantly different from the nimodipine group (*P*>0.05) (**Figure 1**).

Sodium ferulate improved brain tissue damage in rats with cerebral ischemia

There were no abnormalities in the brain tissue morphology or cell structures in the mock surgical group. The cell bodies were round and had no red staining. The model group's brain tissue showed neuronal cytoplasmic looseness, a fusiform shape, vacuolar degeneration, and a large amount of red in the cytoplasms. A decrease of neurons and inflammatory cell infiltration were observed. The pathological changes of the brain tissue in each dose group of sodium ferulate showed different degrees of improvement. The improvement in the high dose group was the most apparent, and the difference between the high dose group and the nimodipine group was not significant (*P*>0.05) (**Figure 2**).
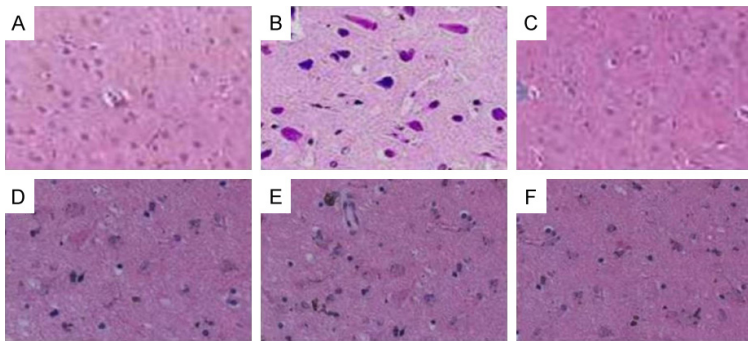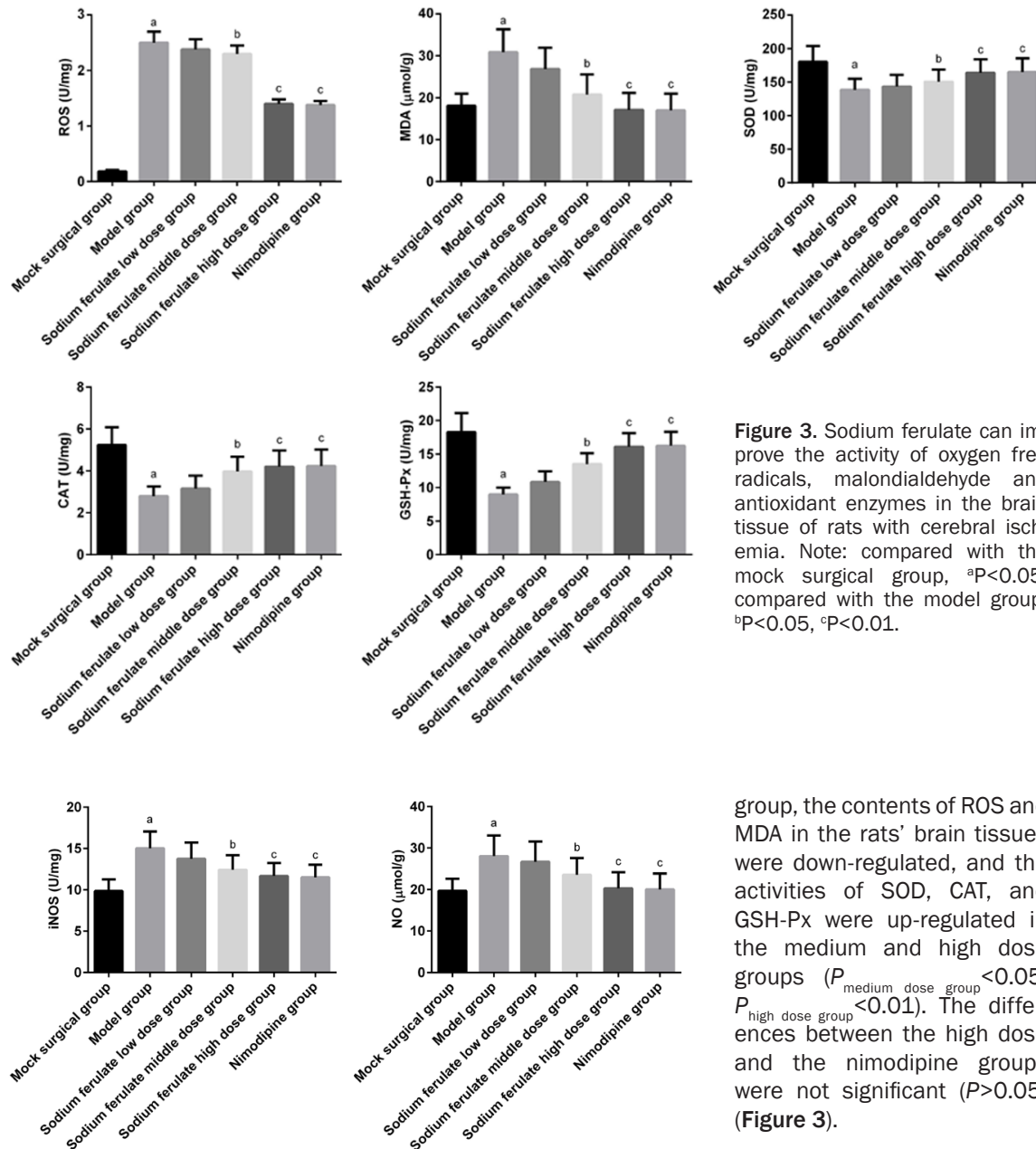
Figure 3. Sodium ferulate can improve the activity of oxygen free radicals, malondialdehyde and antioxidant enzymes in the brain tissue of rats with cerebral ischemia. Note: compared with the mock surgical group, [a]P<0.05; compared with the model group, [b]P<0.05, [c]P<0.01.

Figure 4. Sodium ferulate can improve the levels of iNOS and NO in the brain tissues of rats with cerebral ischemia. Note: compared with the mock surgical group, [a]P<0.05; compared with the model group, [b]P<0.05, [c]P<0.01.

group, the contents of ROS and MDA in the rats' brain tissues were down-regulated, and the activities of SOD, CAT, and GSH-Px were up-regulated in the medium and high dose groups ($P_{\text{medium dose group}}$<0.05; $P_{\text{high dose group}}$<0.01). The differences between the high dose and the nimodipine groups were not significant (P>0.05) (**Figure 3**).

Sodium ferulate improved the levels of inducible nitric oxide synthase and nitric oxide in the rat brains

Sodium ferulate improved the activity of oxygen free radicals, malondialdehyde and antioxidant enzymes in the rat brains

Compared with the mock surgical group, the ROS and MDA contents in the brain tissues of the model group were up-regulated, and the activities of SOD, CAT and GSH-Px were down-regulated (P<0.05). Compared with the model

Compared with the mock surgical group, the levels of iNOS and NO in the brain tissues of the model group were up-regulated (P<0.05). Compared with the model group, the contents of iNOS and NO in the brain tissues of the middle and high dose group were down-regulated ($P_{\text{medium dose group}}$<0.05; $P_{\text{high dose group}}$<0.01). The high dose group was not significantly different from the nimodipine group (P>0.05) (**Figure 4**).
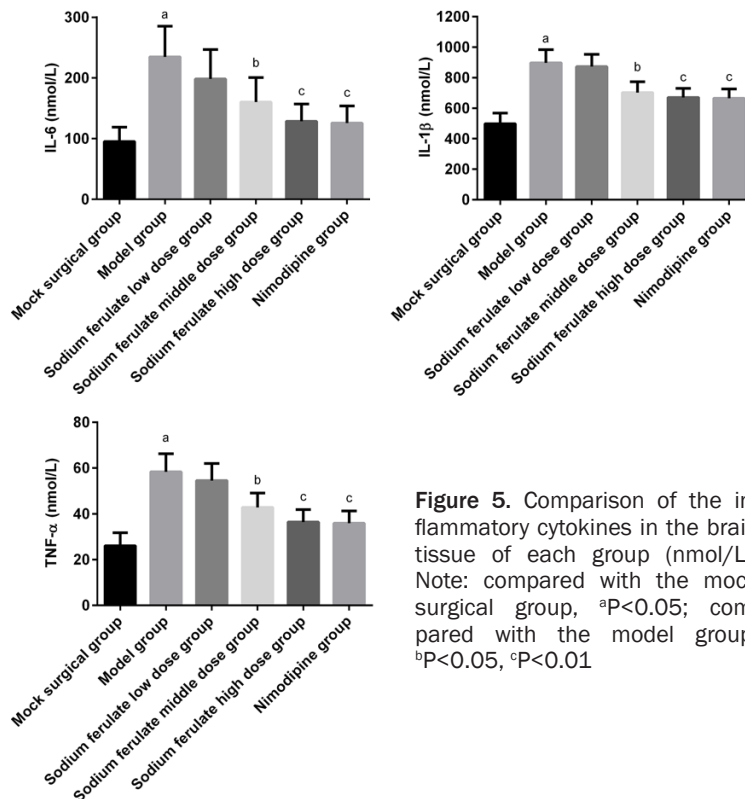
Figure 5. Comparison of the inflammatory cytokines in the brain tissue of each group (nmol/L). Note: compared with the mock surgical group, [a]$P<0.05$; compared with the model group, [b]$P<0.05$, [c]$P<0.01$

Sodium ferulate improved the level of inflammatory cytokines in the brain tissues of the rats with cerebral ischemia

Compared with the mock surgical group, the levels of IL-6, IL-1β, and TNF-α in the brains of the model group were up-regulated ($P<0.05$). Compared with the model group, the levels of IL-6, IL-1β, and TNF-α in the brain tissue of the middle and high dose groups were down-regulated ($P_{\text{medium dose group}}<0.05$; $P_{\text{high dose group}}<0.01$), but the difference between the high dose group and nimodipine group was not significant ($P>0.05$) (**Figure 5**).

Comparison of the NF-κB and claudin-5 protein expressions in the brain tissues of the rats in each group

Compared with the mock surgical group, the expressions of the NF-κB protein in the brain tissue of the model group were increased ($P<0.05$), and the expression of the claudin-5 protein was decreased ($P<0.05$). Compared with the model group, the expressions of the NF-κB protein in the brains of the rats in the middle and high dose groups were decreased ($P_{\text{medium dose group}}<0.05$; $P_{\text{high dose group}}<0.01$), and the expression of claudin-5 protein was increased ($P_{\text{medium dose group}}<0.05$; $P_{\text{high dose group}}<0.01$). No difference was observed between the high dose group and the nimodipine group. ($P>0.05$) (**Figure 6**).

Discussion

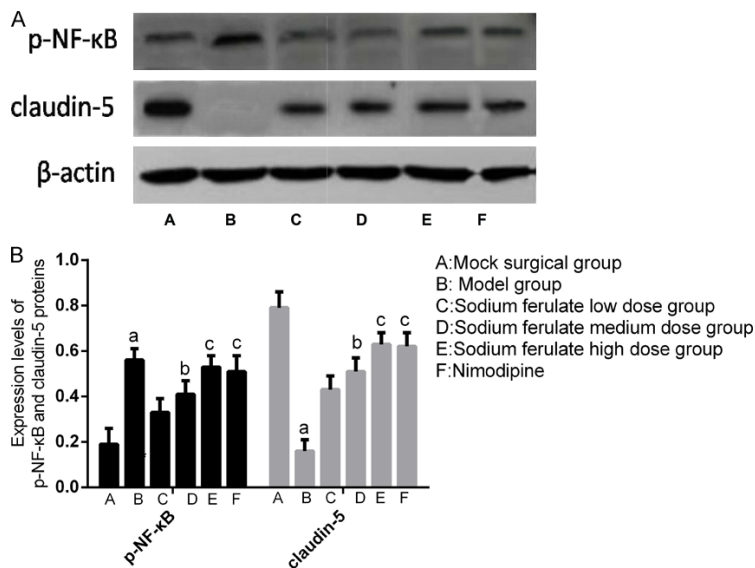Owing to the increasing elderly population and a change in

Figure 6. Western blot was used to determine the changes of the p-NF-κB and claudin-5 protein expressions in brain tissues of each group. Sodium ferulate can significantly reduce the p-NF-κB expression and increase the claudin-5 expression. A-F represent the mock surgical group, the model group, the sodium ferulate low dose group, the sodium ferulate medium dose group, the sodium ferulate sodium high dose group, and the nimodipine group.

lifestyles, ischemic stroke has become a major disease that causes disability and death among the Chinese, which seriously threatens human health and safety [8]. Therefore, it is of great significance to seek effective drugs to protect brain tissue. The results of this study showed that sodium ferulate significantly improves neurological symptoms, reduces the volume of cerebral infarction, and improves the brain edema of rats with focal cerebral ischemia-reperfusion injury. Its protective effect may be related to the down-regulation of NF-κB expression, the up-regulation of claudin-5 expression, and the reduction of oxidative stress in brain tissue.

Sodium ferulate, also known as Angelica, is the main active ingredient of the traditional Chinese medicine compound Angelica Kushen pills. Studies have shown that sodium ferulate has the function of regulating neuroimmunity, inhibiting atherosclerosis and the anti-lipid peroxidation metabolism, and it is anti-inflammatory, anti-oxidative stress, and anti-apoptosis [9, 10]. Xie Tao's research indicated that sodium ferulate can protect against cerebral ischemia-reperfusion injury through an anti-oxidative damage mechanism, thereby reducing the volume of cerebral infarction in rats with cerebral ischemia-reperfusion [11]. A study by Zhou Qin et al. indicated that sodium ferulate could improve the neurological function in rats after focal cerebral ischemia and reperfusion by promoting angiogenesis [12]. In this study, the neurological function of the model group rats after cerebral ischemia-reperfusion injury was missed. The permeability of BBB increased and caused cerebral edema. A large number of infarcts appeared and the neuronal structural damage was severe. After treatment with sodium ferulate, the neurological functions of the rats were restored, the water content and the infarct volume of the brain tissue was reduced, and the destruction of the structure and histomorphology of the neurons was also significantly improved in a dose-dependent manner. It was suggested that sodium ferulate played an important role in neuroprotection.

Oxidative stress is an important mechanism of cerebral ischemia-reperfusion injury, and excess ROS in the body is the root cause of oxidative stress damage in the body [13]. Under normal physiological conditions, ROS can be reduced to produce water and carbon dioxide under the action of antioxidant enzymes such as SOD, CAT, and GSH-Px. However, after cerebral ischemia-reperfusion, when the oxygen supply is restored, phospholipase A2 is activated and causes a large amount of ROS secretions. ROS can directly damage the brain tissue, and even promote brain tissue apoptosis in various ways. It can also attack cell membranes by destroying unsaturated fatty acids. which are the main component of the cell membranes through oxidation. Lipid peroxidation metabolite MDA is the product of this process, and it can be used to reflect the degree of damage to the body [14]. SOD, CAT, and GSH-Px activity can be used to reflect the body's antioxidant capacity [15, 16]. Studies have shown that under normal physiological conditions, the NO content is low. However, iNOS expressed under pathological conditions can catalyze the mass production of NO, while NO can react with ROS in vivo to form ONOO- and N2O3 at high concentrations. These products can induce and aggravate cellular oxidative damage [17]. In this study, sodium ferulate can reduce the content of ROS, MDA, and NO in the brain tissue of rats with ischemia-reperfusion, decrease the activity of iNOS, and increase the activity of SOD, CAT, and GSH-Px. It is suggested that sodium ferulate can effectively inhibit the oxidative stress response of the body, which is an important mechanism to alleviate brain damage after cerebral ischemia-reperfusion in rats.

The NF-κB activation pathway is a newly discovered inflammatory pathway. The inflammatory response mediated by NF-κB is currently a hot spot in the field of central nervous system research [18]. NF-κB is an important effector regulating the inflammatory response. It is mainly present in the cytoplasms in a non-activated state. When cerebral ischemia occurs, NF-κB is activated resulting in the activation of inflammatory cells, and the abundant expressions of a series of inflammatory cytokines such as IL-6, IL -1β, and TNF-α, and the up-regulation of adhesion molecules in brain tissue. The up-regulated adhesion molecules make leukocytes adhere to vascular endothelial cells and block microvessels. Aggregated leukocytes simultaneously release a large number of free radicals, which directly cause endothelial cells, BBB, neuron damage, and cerebral edema [19]. In this study, cerebral ischemia-reperfu-

sion injury caused an increase of inflammatory cells and pro-inflammatory factor levels, and an over-expression of NF-κB proteins in brain tissue. NF-κB protein expression was significantly down-regulated after treatment with sodium ferulate. The release of downstream inflammatory factors was also significantly reduced in a dose-dependent manner. It is suggested that sodium ferulate can inhibit neuroinflammation by down-regulating the expression of NF-κB, which is the core mechanism for alleviating brain damage after cerebral ischemia-reperfusion in rats.

BBB is the structural basis for maintaining an environmental balance in the central nervous system, while the claudin-5-based tight junction protein is an important component of BBB and an important substance for maintaining BBB structure and function [20]. Due to the release of a large number of inflammatory factors during ischemia-reperfusion, the expression and location of the tight junction protein claudin-5 is abnormal. As a result, the integrity of tight junctions was destroyed, BBB permeability was changed, and cerebral edema occurred [21]. After treatment with sodium ferulate, claudin-5 protein expression was significantly up-regulated in a dose-dependent manner. It is suggested that sodium ferulate can promote the expression of the claudin-5 protein and restore the integrity of the tight junction structure, thereby improving the permeability of BBB and exerting ideal neuroprotective effects.

Meanwhile, as this paper is a preliminary study, it still has the following shortcomings: (1) It was found that cerebral ischemia reperfusion injury is related to oxidative stress injury and the NF-κB signaling pathway, which were all detected. However, the relationship between them and the molecule pathway related to oxidative stress were not detected, which makes the paper seem to lack logic. A further analysis will be made in the next study. (2) The indicators of the experimental study, especially NF-κB, require rapid cryopreservation. Due to the lack of experimental experience, the brain specimens were not photographed in time, which makes the argument of the paper a little poor. In future experiments, we will pay closer attention to the details of the experiments, and the specimens will be well preserved to further

observe the gross morphological changes in brain tissue.

In summary, sodium ferulate has neuroprotective effects. It can improve neurological symptoms in rats with focal cerebral ischemia-reperfusion injuries, reduce the volume of cerebral infarction, and reduce brain edema. Its protective effects may be related to the down-regulation of NF-κB expression, the up-regulation of claudin-5 expression, and the reduction of oxidative stress damage in brain tissue.

Disclosure of conflict of interest

None.

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