SIRT1/NF-κB pathway on neuronal apoptosis in rats with ischemic stroke

Hongli Gao\(^1\), Lingling Yang\(^2\), YiShun Shao\(^3\)*

\(^1\) Department of Medical, Hubei Minzu University, Enshi 445000, China

\(^2\) Department of Neurology, The Eighth People's Hospital, Hengshui City 283800, China

\(^3\) Department of Internal Medicine, Affiliated Hospital of Huazhong University of Science and Technology, Wuhan 430074, PR China.

ABSTRACT

Recent studies have shown that neuronal apoptosis is one of the important mechanisms leading to cerebral ischemic injury. SIRT1/NF-κB is a carrier of protein units in the body, participates in the synthesis of DNA, RNA and important neurotransmitters in the brain, and is an important nutrient necessary to maintain the normal growth and development of the nervous system and the normal function of cells. This article aims to study the effect of the SIRT1/NF-κB pathway on neuronal apoptosis in rats with ischemic stroke. Rats were divided into sham operation group, model group, SIRT1/NF-κB group, each group was given intragastric administration at different doses 3 days before the operation and after the operation, and brain tissue was taken at 24, 48, 72, 96 h after operation HE staining, TTC staining and dynamic changes of SIRT1/NF-κB expression were measured. The results of the experiment showed that the result of Nao Xintong was low, and the SIRT1/NF-κB group could reduce the volume of cerebral infarction in rats (P <0.05), and reduce the expression of SIRT1/NF-κB positive cells. The experimental results show that Naoxintong SIRT1/NF-κB has a protective effect on neuronal apoptosis after focal cerebral ischemia-reperfusion in rats.

Keywords:
Neuronal Apoptosis, Cerebral Ischemic Injury, SIRT1/NF-κB, Protective Effect

Introduction

Since the 1990s, it has been found that in addition to necrosis, neuronal apoptosis also exists in nerve injury after cerebral ischemia. Apoptosis is a process of active cell death regulated by a variety of related genes and proteins. Among them, SIRT1/NF-κB is the family of apoptosis-related proteins that is currently the most valued (1). The SIRT1/NF-κB protein can suppress both necrosis and apoptosis. It is generally believed that the Bcl-2 gene family is an important endogenous anti-apoptotic factor that can maintain certain nerves after cerebral ischemia and reperfusion. Cell survival plays an important role (2). SIRT1/NF-κB is a commonly used Chinese medicine for the treatment of ischemic cerebrovascular disease. Pharmacological studies have confirmed that SIRT1/NF-κB can reduce blood viscosity, dilate blood vessels, improve cerebral blood flow, reduce blood lipids, and improve microcirculation. There are few studies on the effect of SIRT1/NF-κB on neuronal apoptosis after cerebral ischemic injury (3).

The main component of SIRT1/NF-κB is human urinary kininogenase, which can decompose to produce vasokinin and vasodilatin. Experimental studies have shown that the application of SIRT1/NF-κB can reduce the area of cerebral infarction and reduce the damage to nerve function. It may be related to promoting angiogenesis and nerve cell proliferation, migration, differentiation, and reducing apoptosis (4-7). Pharmacodynamic studies have shown that SIRT1/NF-κB can selectively dilate small arteries at the ischemic site, improve blood supply in the infarct, and promote neovascularization at the injury site, thereby playing a therapeutic role in acute cerebral infarction (8). The pathogenesis of cerebral ischemia is complex, and apoptosis is still an important part of the research on the mechanism of cerebral ischemia. Apoptosis refers to a process in which the body is stimulated under physiological conditions and undergoes a variety of signal transductions to cause programmed cell death (9). Domestic authors have rarely reported on the mechanism of SIRT1/NF-κB's protective effect on the brain from the perspective of apoptosis. This study explores the protective effect of SIRT1/NF-κB from the perspective of apoptosis and provides a basis for

*Corresponding author. E-mail: wzz1602@126.com

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the clinical application of SIRT1/NF-κB (5-7).

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In the low-dose Naoxintong group and the high-dose Naoxintong group, the central area of ischemia became smaller, the neuron degeneration and necrosis decreased in the marginal area, and most of the surviving cells were relatively normal. Therefore, in this paper, the rats were divided into sham operation group, model group, SIRT1/NF-κB group, each group was given intragastric administration at different doses 3 days before and after the operation, 24, 48, 72, 96 h after operation The brain tissue was taken for HE staining, TTC staining, and the dynamic changes of SIRT1/NF-κB expression were measured. The results showed that both high and low doses of Naoxintong can significantly alleviate neuronal cell injury and neuronal apoptosis in the cerebral ischemic area after cerebral ischemia-reperfusion in rats, and reduce neuronal apoptosis and expression of SIRT1/NF-κB There was a positive correlation, suggesting that Naoxintong reduced the apoptosis of neurons in the ischemic penumbra of rats and decreased the expression of SIRT1/NF-κB.

**Materials and methods**

**Main Instruments and Reagents**

The main instruments and reagents used were SIRT1/NF-κB immunohistochemistry (Beijing Zhongshan Biotechnology Co., Ltd.); TUNE L kit (Roche company); DAB kit (Beijing Zhongshan reagent company); PBS phosphate buffer salt (Beijing Zhongshan biological reagent company); citrus rafter Salt repair solution (Beijing Zhongshan Biological Reagent Company); Anti-ERK rabbit-derived polyclonal antibody (Cell Signaling Technology Company); HRP-conjugated goat a ti-rabbit IgG secondary antibody (Chemicon).

**Brain Tissue Perfusion Fixation**

At each time point, 10% chloral hydrate was used, the dose reference (0.35 ml / 100g), intraperitoneal injection of anesthesia, and after the anesthesia became effective, the rats were fixed in the supine position on the rat board. Open the abdominal cavity, expose the abdominal organs, use ophthalmic forceps to quickly free the subcutaneous muscular layer and fascia, expose the abdominal aorta, close the abdominal aorta with arterial clips, hold the xiphoid process with vascular forceps and pull up, expose the diaphragm, use Tissue scissors cut a small mouth in the middle of the diaphragm to form an artificial pneumothorax. Gradually cut the diaphragm and the ribs on both sides along the small mouth to fully expose the heart. Hold the perfusion needle and insert it into the left ventricular apex at a 45-degree angle under direct vision Cut off the right atrial appendage, and at the same time inject approximately 100-150ml of room temperature saline (NS) through the perfusion needle. When the rat's forelimbs and lungs gradually become white and the outflow liquid is basically clear, stop pushing into the NS, and then push (4 ℃) After about 100ml of 4% paraformaldehyde solution, decapitate the rat and take the right side.

**Establishment of Rat Traumatic Brain Injury Model**

70 healthy male rats, weighing 300-350 g, were provided to the Experimental Animal Center. Rats were randomly divided into a brain trauma group and a control group. The trauma group fixed the abdominal supine position of the rat to the sponge mattress (10 cm × 10 cm × 20 cm) after anesthesia according to the Marmarou method, along the midline sagittal suture and herringbone suture. A stainless-steel pad (8 mm in diameter and 2 mm in thickness) was fixed between the coronal suture and the herringbone in the rat. A copper rod weighing 450 g and 18 mm in diameter was freely dropped from a height of 1.5 m. The control group was given only scalp Cut open without injury. Each group was
divided into 7 time-phase groups at 10.30 min, 3, 6, 24, 48, and 72h after injury, including 6 rats in each phase of the brain trauma group and 4 rats in the control group.

Immunochemistry
Rats were perfused with 4% paraformaldehyde through the left ventricle after anesthesia. After the whole body was stiff, the brain tissue was decapitated and fixed for 24 h. Staining was performed in strict accordance with the SP method. The DAB solution was developed (17). The intensity of the immunoreactivity of positive cells was analyzed by Motic Med 6.0 digital medical image analysis system and its integrated optical density value was expressed.

In situ cell DNA break detection (TUNEL method)
Specimen preparation steps are the same as immunohistochemistry. TUNEL labeling reaction mixture (negative control only add an equal amount of ribonucleic acid reaction solution, the rest are the same), incubate at 37Y for 1 h, add AP transformation solution dropwise, incubate at 37T for 30 min, DAB chromogenic solution is protected from light, under the microscope Observe that the cells with brown nuclei are positive. The number of positive cells in the random count in the field of view of the eyepiece grid micrometer at 200 times the light microscope is used as the analysis object for comparison.

Detection Index and Method
Neuronal apoptosis was detected by intraperitoneal injection of 10% chloral hydrate (0.4mL / 100g body mass) after the third day of behavioral evaluation. After perfusion of the heart, the brain was decapitated and the occipital brain lobe was removed at low temperature. Brain tissue, fixed in 4% paraformaldehyde solution, embedded in paraffin, sliced, dewaxed, digested with digestive enzyme K for 15 min, washed with water, washed with PBS for 3x5 min, and added TUNEL reaction mixture dropwise Solution, mix according to 1: 9. Incubate with normal serum for 20 min to prevent non-specific binding, drop POD conversion agent after the drop, and DAB develops color. Hematoxylin counterstains the cell nucleus for the 30s, and is dehydrated, transparent, and mounted. Count the number of positive cells stained brownish-yellow under a 400x optical microscope, select 5 non-repetitive fields and take the average.

Statistical Method
The statistical software SPSS 12.0 for windows was used to analyze the experimental data. The measurement data were expressed as the mean soil standard deviation, and the analysis of variance was used. The LSD-t test was used for the pairwise comparison. P <0.05 was considered statistically significant.

Specimen Preparation and Histological Evaluation
The experimental animals were decapitated and their brains were removed on time. After brain tissue was fixed with 4% neutral paraformaldehyde for 2 hours, they were dehydrated and embedded in paraffin. Coronal slices were cut back at the intersection of the optic nerve. Animal brain tissue sections were stained using the TUNEL method (reagent kit purchased from Roche Corporation, Cat. No. 1684817), and nerve cell nuclei stained yellow or brown-yellow were positive. Apoptosis index refers to the percentage of apoptotic cells in at least 500 cells in each brain tissue section. Anti-Caspase-3 polyclonal antibody (kit purchased from Neomarkers, CPP32, Ab4) was used for immunohistochemical staining, and a score quantification table was used to evaluate the expression level of SIRT1/NF-κB in each group. The scoring criteria are 0 (no expression), 1 (positive), 2 (strong positive).

Results and discussion
Expression of SIRT1/NF-κB Protein in Traumatic Brain Injury of Rats
Compared with the neurological deficit score, the rats were awake within 1 h after surgery. The animals in the sham-operated group were normally active without neurological deficit. The neurological deficit score was 0. The neurological deficit score was the most severe in the ischemic group; and Compared with the simple ischemia group, the Eureklin group could improve the neurobehavioral score of focal cerebral ischemia rats (P <0.05 or <0.01), and showed a dose-effect relationship with increasing dose. One rat in the ischemic group and the medium-dose group
of urekine died and were eliminated. See picture 1. The SIRT1/NF-κB protein was expressed in the brain trauma group at 10 minutes after injury, which was significantly higher than the control group at 3 hours after injury (P <0.05), and reached a peak at 6 hours (F <0.01), and then gradually decreased after injury. The expression level at 24 h was still significantly higher than that of the control group (P <0.05). A large number of nuclei in the trauma group were stained brown-yellow under the microscope at 6 h after the injury. (Table 1 and Figure 1).

**Table 1.** The graph showing the nuclear data of the control group

<table>
<thead>
<tr>
<th>Group</th>
<th>Score</th>
<th>Average rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Model group</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>SIRT1/NF-κB group</td>
<td>7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Figure 1.** The graph showing the nuclear data of the control group

**Trauma Control**

After 20 days of the administration, the serum NO content and ET-1 content of the model group were significantly lower than that of the sham operation group (P <0.05), while the NO / ET-1 of the model group was significantly lower than that of the sham operation group (F <0.05). Compared with the model group, the middle and high doses of the experimental group and the control group had significantly higher serum NO levels. NO / ET-1 increased significantly, while plasma ET-1 content decreased significantly (all P <0.05); the above indicators, the high-dose group was significantly higher than the low-dose group and control group (P <0.05). No positive cells were detected in the control group. The expression of SIRT1/NF-κ in the trauma group increased significantly at 3 hours after injury, reached a peak at 6 hours (P <0.01), and then gradually decreased, but was still significantly higher at 72 hours than the normal group (P <0.05). Observe the trauma group under the microscope SIRT1/NF-κ-positive cells nucleate into brownish yellow, and the control data of the trauma group are shown in Figure 2 and Table 2.

**Table 2.** Trauma group control data

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>0 h reperfusion</th>
<th>6h reperfusion</th>
<th>Reperfusion for 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock surgical group</td>
<td>Saline 4 mL</td>
<td>1.15±0.55</td>
<td>1.17±0.75</td>
<td>2.54±1.13</td>
</tr>
<tr>
<td>Model group</td>
<td>Saline 4 mL</td>
<td>1.42±1.23</td>
<td>27.60±6.09</td>
<td>17.58±5.69</td>
</tr>
<tr>
<td>SIRT1/NF-κ group</td>
<td>100 mg/kg</td>
<td>2.14±0.96</td>
<td>11.32±4.63</td>
<td>12.33±5.32</td>
</tr>
</tbody>
</table>

**Figure 2.** Trauma group control data

**In situ DNA Breakage to Detect Apoptosis**

The scores of neurological deficits in rats are shown in Table 3. There were no obvious neurological impairments in rats in groups A and B. Groups C and D had the highest behavioral scores after 24 hours of reperfusion, and limb nerve defects were the most severe. The score of each group began to decrease. Compared with groups A and B, the behavioral scores of rats at each time point were significantly higher (P <0.01); compared with group C, the symptoms of nerve damage at each time point in group D were improved (P <0.05), reperfusion 3 The neurological function recovered best at h and 48 h (P <0.05). Under the microscope, TUNEL-positive cells showed brown nuclei with dark staining. They appeared in the CA1 area after 3 hours of injury, and increased at a later time point, reaching a peak at 48 hours (P <0.01). The data that appears in the area is shown in Figure 3.
It is generally believed that the overexpression of the SIRT1/NF-κB gene can inhibit Ca2+ overload, prevent the transmission of apoptosis gene signals or the induction of gene products, and inhibit neuronal apoptosis induced by oxygen free radicals, excitatory amino acids, etc (4). SIRT1/NF-κB protein is the product of SIRT1/NF-κB proto-oncogene and is an anti-apoptotic protein that can prevent the release of cytochrome C from mitochondria into the cytoplasm, thereby inhibiting cell apoptosis. According to the research in this article, it was found that some nerve cells in the peripheral area of cerebral ischemia undergo apoptosis. With the extension of the reperfusion time, apoptotic cells gradually increase within a certain time, reaching the peak at 24-72h, and the apoptosis inhibitory gene SIRT1/NF-κB. In the early period of reperfusion, the peak is reached, and neuronal apoptosis plays an important role in cerebral ischemia-reperfusion injury (11-13).

According to experimental studies, apoptosis of some nerve cells in the peripheral area of cerebral ischemia occurred. With the extension of reperfusion time, apoptotic cells gradually increased within a certain time, reaching a peak at 24-72h. The apoptosis inhibitory gene SIRT1/NF-κB was Reperfusion peaks early, and neuronal apoptosis plays an important role in cerebral ischemia-reperfusion injury. Cerebral ischemia-reperfusion belongs to the category of "stroke" in traditional Chinese medicine. Chinese medicine believes that Qi and blood are the material basis for brain growth and development and various functions. Qi and blood disorders are also the main pathogenesis of encephalopathy, and Qi and blood circulation are treatments. Chinese medicine believes that Qi and blood are the material basis for brain growth and development and produces various functions. Qi and blood disorders are also the main pathogenesis of encephalopathy, and benefiting Qi and activating blood circulation is the basic principle of treatment of stroke. It must not reach the blood vessel, the blood vessel has no gas, it must stay and stasis ", so put forward the theory of Qi deficiency and blood stasis, emphasizing based on promoting blood circulation and removing blood stasis medicine combined with Qi medicine to promote the operation of Qi and blood. Radix Angelicae is a qi-invigorating medicine, which is beneficial to qi-solidifying the surface, diminishing swelling, and supporting the effect of poisoning the muscles.

In summary, SIRT1/NF-κB protein has a significant inhibitory effect on neuronal apoptosis in cerebral ischemia, and its mechanism of action may be to enhance free radical scavenging enzyme activity, reduce free radical production, inhibit lipid peroxidation, or upregulate. The expression level of phosphorylated SIRT1/NF-κB protein in the ERK pathway is related. The discussion of the mechanism of folic acid on apoptosis of nerve cells in this experiment can lay a foundation for the further study of the protective effect of folic acid on cerebral ischemia.

Acknowledgments
Not applicable.

Interest conflict
The authors declare that they have no conflict of interest.

References
2. Song Y, Wu Z, Zhao P. The protective effects of activating Sirt1/NF-κB pathway


