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Influences of urinary kallidinogenase on neuronal apoptosis in cerebral infarction rats through Nrf2/ARE oxidative stress pathway

Q.-Y. FAN[1], Z. QIU[2], X.-D. ZHANG[2]

[1]Department of Neurology, The First People's Hospital of Jiande, Jiande, China
[2]Department of Cerebral Surgery, The First People's Hospital of Jiande, Jiande, China

Qianya Fan and Zheng Qiu contributed equally to this work

Abstract. – OBJECTIVE: To investigate the influences of urinary kallidinogenase on neuronal apoptosis in rats with cerebral infarction through the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) oxidative stress pathway.

MATERIALS AND METHODS: A total of 30 male rats were divided into group A (model control group), group B (rat model of cerebral infarction) and group C (rat model of cerebral infarction + medical treatment with urinary kallidinogenase). The percentage of cerebral infarct volume and the apoptosis of brain cells in the three groups of rats were detected via 2,3,5-Triphenyltetrazolium chloride (TTC) staining, the pathological morphology of brain tissues in the three groups of rats was observed via hematoxylin and eosin (HE) staining, and the protein levels of Nrf2 and superoxide dismutase 1 (SOD1) in the brain tissues in the three groups of rats were measured using the Western blotting assay.

RESULTS: The degree of neurological deficit in group B was remarkably higher than that in group A ($p<0.05$), and it was markedly decreased in group C compared to that in group B, displaying statistically significant differences ($p<0.05$). Compared to that in group A, the cell apoptosis was significantly aggravated in group B, while a remarkably alleviated cell apoptosis was observed in group C compared to that of group B, and the differences were statistically significant ($p<0.05$). The cerebral infarct volume accounted for 34.87% of the whole brain volume in group B, and a mild cerebral infarction was detected in group C, with a percentage of cerebral infarct volume of 21.14%. Group B showed a more evident increase in the cerebral infarct volume than in group C ($p<0.05$). Compared to those of group A, pyknotic nuclei and neuron staining of brain tissue cells were evidently increased, and the neuronal cell injury was aggravated in group B. Moreover, prominently decreased pyknotic nuclei and neuron staining ($p<0.05$) as well as mild neuronal cell injury ($p<0.05$) were detected in group C compared to those in group B. The levels of Nrf2 and SOD1 protein in the brain tissues in group B were remarkably lower than those of group C ($p<0.05$).

CONCLUSIONS: Urinary kallidinogenase can inhibit the neuronal apoptosis in rats and protect the rats from cerebral infarction, whose mechanism is associated with the activation of the Nrf2/ARE oxidative stress pathway.

Key Words:
Urinary kallidinogenase, Nrf2/ARE oxidative stress pathway, Cerebral infarction, Neuronal apoptosis.

Introduction

Cerebral infarction is a common sudden brain disease that is prone to resulting in disability and death[1]. Ischemia and hypoxia of brain tissues induce local cerebral necrosis or malacia, thus affecting a variety of functions and activities of patients[2]. With regard to the complicated pathogenesis of cerebral infarction, Naess et al[3] studied and found that diabetes mellitus, shock, and other risk factors are the causes of cerebral infarction. It is also reported that 71.8% of patients with ischemic cerebrovascular disease are accompanied by symptoms such as carotid atherosclerotic plaque and increased intima-media thickness at the same time[4]. Improving blood circulation in the brain tissues, controlling hyperlipemia and diabetes mellitus and accelerating neurological function recovery are conventional therapies for cerebral infarction at present. In spite of the significant improvement of the degree of ischemia and relief of neuronal cell injury, these therapeutic methods have little efficacy in reducing the disability rate of patients with cerebral infarction[5,6]. Urinary kallidinogenase belongs to the recently-marketed Class I new drugs in China, which is widely

Corresponding Author: Xiaodong Zhang, BM; e-mail: 312964053@qq.com

applied in the treatment of cerebral infarction at present, but the treatment effect varies from case to case[7]. Experiments performed on rabbits with a microvascular injury have manifested that after the injection of urinary kallidinogenase, the cerebral vessels are dilated significantly, hemoglobin in the cerebral blood is increased prominently, the cerebral infarction area expands slowly, and the spontaneous abnormality of electrocorticogram is ameliorated effectively[8]. The nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway is an important antioxidant stress pathway, of which Nrf2 is the most active in the CNC family of transcription factors. In recent years, Kubo et al[9] revealed that urinary kallidinogenase exerts a protective effect on cerebral infarction. In this paper, a rat model of cerebral infarction was established, and the impacts of urinary kallidinogenase on the cerebral infarction and neuronal apoptosis in rats through the Nrf2/ARE signaling pathway were analyzed, providing a new direction for the treatment of the disease.

Materials and Methods

Laboratory Animals and Grouping

The rats used in this experiment were purchased from the Zhejiang Provincial Center for Disease Control and Prevention. 30 healthy male rats aged about 8 weeks old and weighing 0.18-0.22 kg were selected and fed (5 rats per cage) at 24°C, with a humidity of 45%, 12 h continuous lighting and free access to food and water. After adaptation to the new environment for a week, all the rats were applied to establish the model of cerebral infarction and divided into group A (model control group), group B (rat model of cerebral infarction) and group C (rat model of cerebral infarction + medical treatment with urinary kallidinogenase) with equal weight in each group. This investigation was approved by the Animal Ethics Committee of The First People's Hospital of Jiande Animal Center.

Main Reagents and Instruments

PVDF membrane (Millipore, Billerica, MA, USA), hematoxylin and eosin (HE) staining kit (Selleck, Houston, TX, USA), paraformaldehyde (Jingke Chemical Science-Tech Co., Ltd., Shanghai, China), rabbit anti-rat Nrf2 polyclonal antibody (Wanleibio), mouse anti-β-actin primary antibody (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit (Beyotime Biotechnology, Shanghai, China), Canon digital camera (Canon Inc., Tokyo, Japan), microsurgical instruments (Shanghai Jianzhong Medical Apparatus Co., Ltd., Shanghai, China), 3K30 ultracentrifuge (Sigma-Aldrich, St. Louis, MO, USA) and thermostat (Shanghai Yuejin Medical Optical Instruments Factory, Shanghai, China).

Animal Model Establishment

After fasting for 12 h before the operation, the three groups of rats were fixed in the supine position and anesthetized by intraperitoneal injection of 1% pentobarbital sodium (0.1 g/kg). Then the skin and fascia in the middle of the neck were cut to find the common carotid artery, and a small incision on the right common carotid artery, which was closer to the heart, was made using a pair of scissors. After that, an occluding suture was threaded into the common carotid artery from the incision until it encountered an apparent resistance at a depth of 17 mm. Subsequently, the artery was ligated, the end of the occluding suture was cut, and the incision was disinfected and sutured.

Dose and Route of Administration

At 3 h after successful modeling, the rats in each group were administered with the corresponding drugs. The rats in group A (control group) did not receive any treatment. The rats in group C were intraperitoneally injected with urinary kallidinogenase, while the same dose of normal saline (NS) was injected into the rats in group A and B. Subsequent experiments were performed after the injection of urinary kallidinogenase and NS for two consecutive weeks.

Neurological Function Score

After injection for 14 consecutive days, the limb status of the three groups of rats was observed, and the tail suspension test was conducted. The rats without spasm and disturbance of consciousness were enrolled for the present neurological scoring. The Zea Longa scoring standards were adopted for the evaluation of the neurological function (Table I). 0 point represents for normal neurological function without disturbance, 1-2 points for mild neurological dysfunction, 3-4 points for severe neurological dysfunction, and 5 points for death.

Table I. Neurological scoring standards.

Score	Scoring standard
0 point	Rats without neurological deficits
1 point	Rats unable to fully extend the left forelimb
2 points	Rats with left hemiplegia and circling to the left when crawling
3 points	Rats tumbling to the left when crawling
4 points	Rats unable to walk by itself and without consciousness
5 points	Dead rats

Observation of Percentage of Cerebral Infarct Volume in Rats via 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

The rats in group A, B and C were sacrificed after the intraperitoneal injection for 14 consecutive days, and the brain tissues were taken out under sterile conditions and preserved at -80°C for 5 min. The 2 mm-thick coronal plane sections starting from the frontal pole were stored in 1% of TTC solution, followed by incubation at 37°C in the dark. When stained successfully, the normal brain tissues were red, and the tissues in the infarction region were white. Next, images were processed and analyzed using professional image analysis software, and the percentage of cerebral infarct volume was calculated.

HE Staining

After injection for 14 consecutive days, the three groups of rats were subjected to the following treatment: the tissues of cerebral infarction were taken out under sterile conditions and fixed with 4% of formaldehyde for 1 d. Then, they were prepared into 4 μm-thick paraffin-embedded coronal sections and stained with HE to observe the tissue morphology in the cerebral infarction region.

Brain Cell Apoptosis in Each Group of Rats Detected via TUNEL Assay

The prepared paraffin-embedded sections underwent TUNEL staining to detect the brain cell apoptosis in each group. 5 non-overlapping fields of vision in three sections were selected from three sections for observation under an optical high power microscope, in which the yellowish-brown cells were apoptotic cells. Brain cell apoptosis rate = number of apoptotic cells/total number of cells × 100%.

Protein Levels of Nrf2 and Superoxide Dismutase 1 (SOD1) Detected via Western Blotting

The brain tissues (0.1 g) were taken from each group of rats to extract the proteins, and the protein concentration in the brain was determined according to the instructions of bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Next, the proteins were transferred onto a membrane after separation *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), added with skim milk powder and sealed at 22-25°C for 1 h. Before the addition of primary antibodies and after the addition of secondary antibodies, the membrane was washed with Tris-Buffered Saline and Tween 20 (TBST) once and incubated at about 4°C for 24 h and at about 22°C for 1 h, respectively. After that, the membrane was washed, and the color was developed using enhanced chemiluminescence (ECL), followed by photography and preservation.

Statistical Analysis

In this paper, the Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the statistical analysis of data. The *t*-test was performed for comparisons of neurological function score, cell apoptosis, cerebral infarct volume as well as Nrf2 and SOD1 proteins among group A, B, and C. Univariate analysis was applied to analyze data in different groups, and calculation data were presented as mean ± standard deviation ($\bar{\chi}\pm s$). $p<0.05$ suggested that the difference was significant.

Results

Changes in Neurological Function in Each Group of Rats

The rats in group A could move normally, those in group B had an apparent limb movement disorder, and those in group C had a mild limb movement disorder. The neurological deficit in group A was more prominent than that in group B ($p<0.05$), and it was markedly alleviated in group C compared to that in group B ($p<0.05$) (Figure 1).

Comparison of Cerebral Infarction Status Among the Three Groups of Rats

The rats in both group B and C had white infarction foci in the brain tissues. There was a serious cerebral infarction in group B, with a cerebral infarct volume of 34.87%, while there was a mild

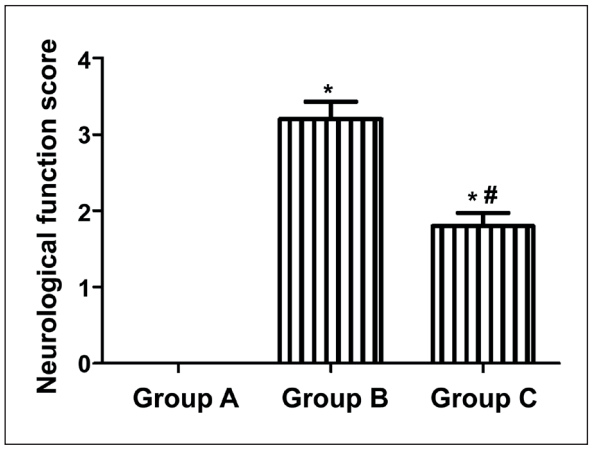

Figure 1. Neurological function score in group A, B, and C. *p<0.05 *vs.* group A, #p<0.05 *vs.* group B.

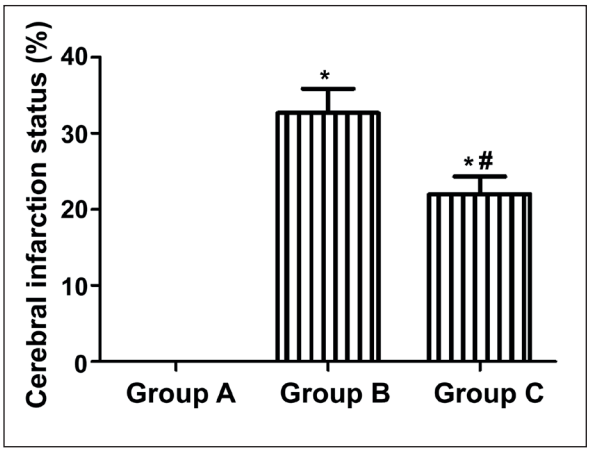

Figure 2. Cerebral infarction status in group A, B, and C. *p<0.05 *vs.* group A, #p<0.05 *vs.* group B.

cerebral infarction in group C, with a cerebral infarct volume of 21.14%. The increase in the cerebral infarct volume in group B was more remarkable than that in group C (p<0.05) (Figure 2).

HE Staining for Brain Tissues in the Three Groups of Rats

According to the HE staining results, compared to those of group A, pyknotic nuclei and the neuron staining of brain tissue cells were evidently increased, and the neuronal cell injury was aggravated in group B. While compared to those in group B, pyknotic nuclei and neuron staining markedly decreased (p<0.05); an alleviated neuronal cell injury (p<0.05) was detected in group C (Figure 3).

Comparison of TUNEL Assay Results of Brain Cell Apoptosis in Rats

The TUNEL assay results manifested that group B had large quantities of yellowish-brown apoptotic cells compared to that of group A, and group C had significantly fewer yellowish-brown apoptotic cells in the brain tissues than group B (Figure 4). Compared to that of group A, the cell apoptosis was markedly aggravated in group B, while a remarkably alleviated cell apoptosis was observed in group C, in comparison to that in group B (p<0.05) (Figure 5).

Impacts of Urinary Kallidinogenase on Nrf2 and SOD1 Protein Levels in Rat Brain Tissues Detected via Western Blotting

The levels of Nrf2 and SOD1 proteins in rat brain tissues were detected *via* Western blotting. It was indicated that the levels of Nrf2 and SOD1 proteins in the brain tissues in group B were significantly lower than those in group A and group C (p<0.05). Also, group C had significantly higher levels of Nrf2 and SOD1 proteins than group A (p<0.05) (Figures 6 and 7).

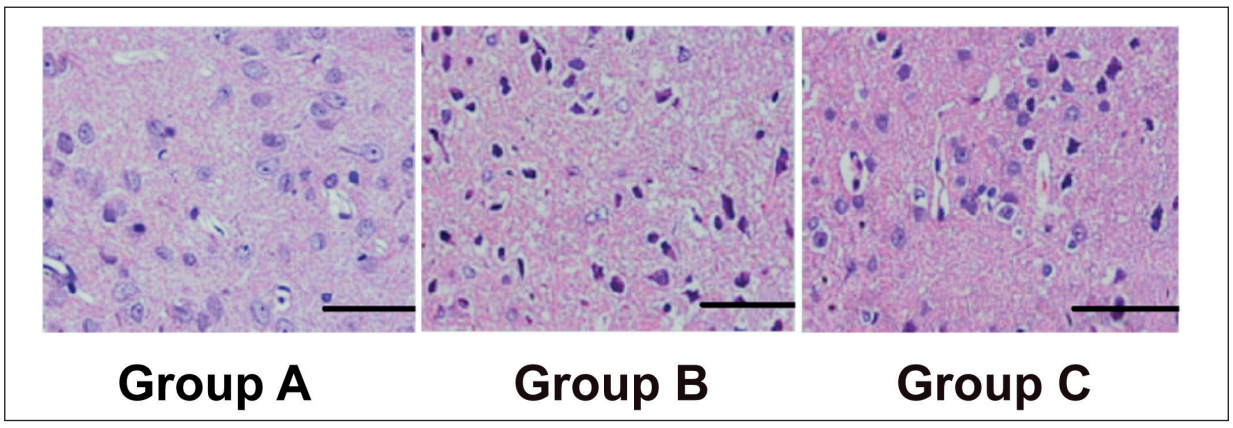

Figure 3. HE staining for brain tissues in group A, B, and C (×200).

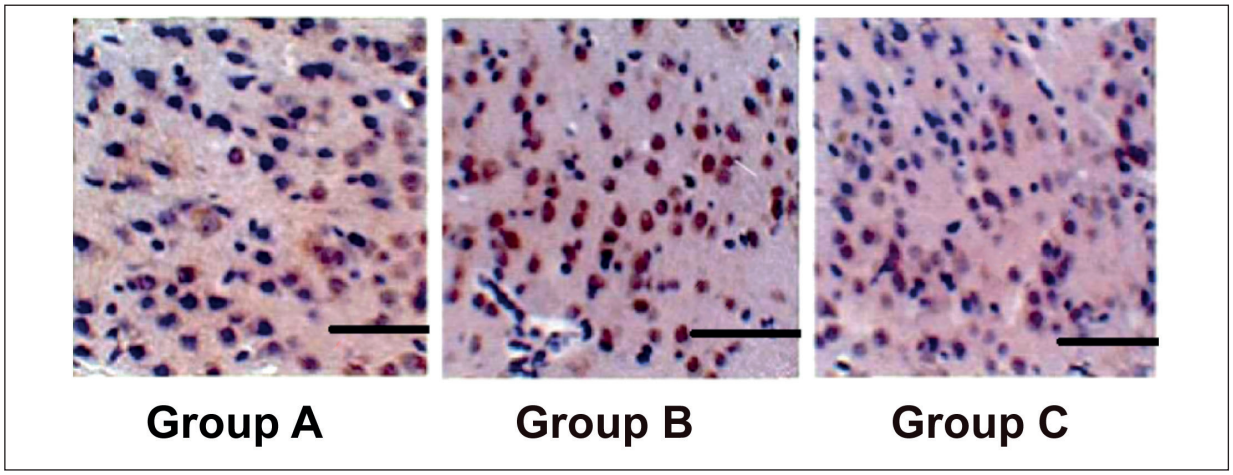

Figure 4. Comparison of brain cell apoptosis in rats (×200).

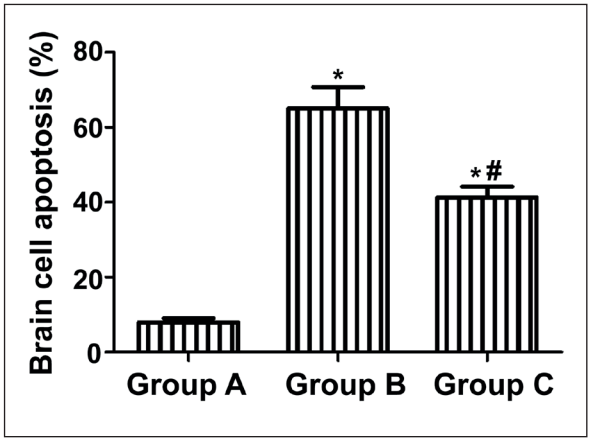

Figure 5. Comparison of brain cell apoptosis in rats. **p*<0.05 *vs.* group A, #**p*<0.05 *vs.* group B.

Discussion

As the most common sudden brain disease, cerebral infarction, also known as ischemic stroke, refers to the ischemic necrosis of brain cells caused by the insufficient blood supply to the brain tissues due to blood circulation disorders induced by embolic embolism in the cerebral vascular lesions or blood[10-12]. Cerebral infarction mostly occurs suddenly in a resting state or in sleep, with such clinical symptoms as speech disorder and paralysis or paraesthesia of the unilateral limb[13,14]. Currently, theories related to the pathogenesis of the disease include calcium overload in brain tissue cells, overexpression of inflammatory factors, the disequilibrium of energy metabolism *in vivo*, acidosis, brain tissue apoptosis and free radical injury *in vivo*. The study of Rancan et al[15] has shown that long-term cerebral ischemia will result in neuronal damage and apoptosis.

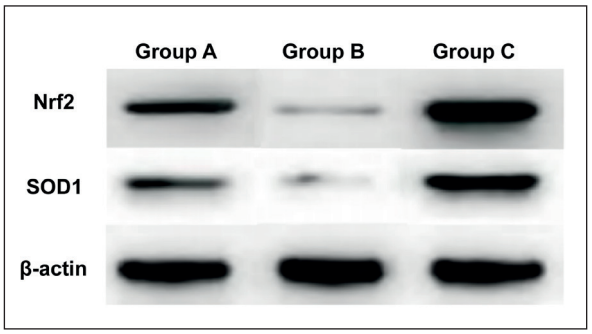

Figure 6. Expression of Nrf2 and SOD1 proteins in the brain tissues in group A, B, and C.

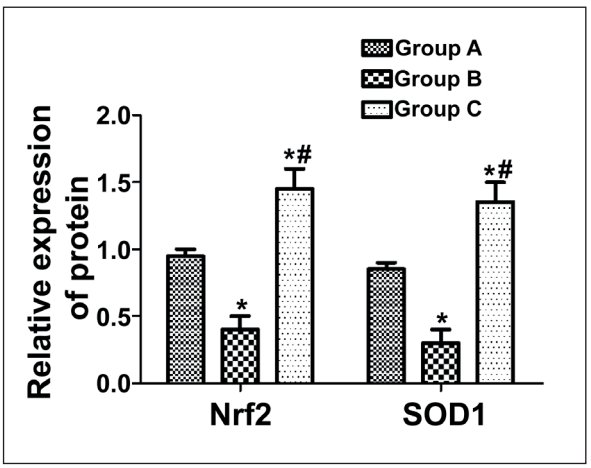

Figure 7. Differences in Nrf2 and SOD1 proteins in the brain tissues in group A, B, and C. **p*<0.05 *vs.* group A, #*p*<0.05 *vs.* group B.

In this work, the rat model of cerebral infarction was established, and it was revealed that the rats in group A could move normally, those in group B had an apparent limb movement disorder, and those in group C had a mild limb movement disorder. The neurological deficit in group A was more prominent than that in group B ($p<0.05$), and it was alleviated markedly in group C compared to that in group B ($p<0.05$). Studies have shown that urinary kallidinogenase possesses a very high application rate, which can improve local blood supply by selectively dilating blood vessels at the lesions and ameliorate the neurological deficits. In this role, it not only has basically no impacts on the normal parts, but it also can inhibit platelet aggregation and promote neovascularization.

White infarction foci occurred in the brain tissues in both group B and group C after TTC staining. There was a severe cerebral infarction in group B, with a cerebral infarct volume of 34.87%, while mild cerebral infarction was detected in group C, with a cerebral infarct volume of 21.14%. Compared to that of group C, the cerebral infarct volume was increased more significantly in group B ($p<0.05$), and there were statistically significant differences among the three groups ($p<0.05$). According to the HE staining results, compared to those in group A, pyknotic nuclei and the neuron staining of brain tissue cells were markedly increased evidently, and the neuronal cell injury was aggravated in group B. In comparison to those in group B, however, pyknotic nuclei and the neuron staining of cells were markedly decreased ($p<0.05$), and the neuronal cell injury was alleviated ($p<0.05$) in group C. The TUNEL assay results indicated that there were massive yellowish-brown apoptotic cells in group B compared to that in group A, and group C had significantly evidently fewer yellowish-brown apoptotic cells in the brain tissues than group B (Figure 4). Compared with that in group A, the cell apoptosis was aggravated notably in group B, while remarkably alleviated cell apoptosis was observed in group C in comparison with that in group B ($p<0.05$). Urinary kallidinogenase, a type of glycoprotein extracted from fresh urine of males, is capable of relieving vasodilation by regulating the blood pressure[16,17]. It is illustrated in animal experiments that urinary kallidinogenase can significantly reduce the cerebral infarction area and effectively save the cells in ischemic penumbra, thereby recovering the neurological function[18,19].

The detection of protein expressions in the brain tissues of rats *via* Western blotting showed that the levels of Nrf2 and SOD1 proteins in the brain tissues in group B were markedly lower than those in group A and group C ($p<0.05$). The group C had significantly higher levels than group A ($p<0.05$), suggesting that Nrf2 and SOD1 proteins in the brain tissues of rats injected with urinary kallidinogenase are markedly increased, and the oxidative stress generated during cerebral infarction can increase the Nrf2 and SOD1 proteins at the same time. The anti-oxidative stress capacity of the Nrf2/ARE pathway plays a vital role in organisms[20]. According to the study of Lutsky et al[21], the cerebral infarction is correlated with oxidative stress. Under normal conditions without injury, SOD1 protein is restrained by Nrf2 in the body. In the case of impairment, however, Nrf2 can interact with small proteins in the nucleus, promote its binding to ARE and increase the content of antioxidant enzyme SOD1 in the body, thereby enhancing the resistance of cells against oxidative stress. Such a process indicates that the Nrf2/ARE pathway is activated.

Conclusions

We found that urinary kallidinogenase can activate the Nrf2/ARE oxidative stress pathway, repress the neuronal apoptosis in rats with cerebral infarction and relieve the symptoms of cerebral infarction, thus protecting the brain neurons.

Conflict of Interests

The authors declared that they have no conflict of interests.

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