Abstract. OBJECTIVE: The objective of this study was to investigate the effects of edaravone combined with oxiracetam on neuronal apoptosis in rats with cerebral infarction (CI) and to explore the potential molecular mechanism.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly divided into sham-operation group (n=12), model group (n=12) and treatment group (n=12). Only the external carotid artery was exposed in sham-operation group, while the models of CI were established using suture method in the other two groups. After modeling, the rats in sham-operation group and model group were intraperitoneally injected with normal saline, and those in treatment group were administered with edaravone and oxiracetam solutions via intraperitoneal injection. Then, the specimens were obtained at 2 weeks after intervention. The cognitive function of the rats was evaluated using a water maze, Nissl staining was applied to observe the neuronal morphology, and the relative protein expressions of silent information regulator 1 (SIRT1) and NF-κB were measured by means of Western blotting. Furthermore, quantitative polymerase chain reaction (qPCR) was performed to determine the messenger ribonucleic acid (mRNA) expressions of interleukin-1 beta (IL-1β) and IL-6, the content of IL-1β and IL-6 was detected by enzyme-linked immunosorbent assay (ELISA), and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was conducted to examine the cell apoptosis.

RESULTS: Model group displayed a significantly longer escape latency and significantly fewer times of crossing the original platform than sham-operation group (p<0.05), whereas treatment group had a significantly shorter escape latency but significantly more times of crossing the original platform than sham-operation group (p<0.05). The relative protein expression level of SIRT1 was lowered significantly, while that of NF-κB was elevated significantly in model group in comparison with those in sham-operation group (p<0.05), and the opposite results were observed between model group and treatment group (p<0.05). Besides, the content of IL-1β and IL-6 in brain tissues was increased significantly in model group compared with that in sham-operation group (p<0.05), but it was decreased significantly in treatment group in comparison with that in model group (p<0.05). The relative mRNA expression levels of IL-1β and IL-6 were significantly higher in model group than those in sham-operation group (p<0.05). Moreover, model group exhibited more positive apoptotic cells and a significantly higher apoptosis rate than sham-operation group (p<0.05). No apparent abnormalities of neuronal morphology and structure were detected in sham-operation group, with many Nissl bodies. The neurons were damaged, with abnormal morphology and structure, and there were a small number of Nissl bodies in model group. The neurons were damaged in treatment group, but their morphology and structure were improved evidently compared with those in model group.

CONCLUSIONS: Edaravone combined with oxiracetam can inhibit the neuronal apoptosis in CI rats by regulating the SIRT1/NF-κB signaling pathway, thereby exerting a neuroprotective effect.

Key Words: Edaravone, Oxiracetam, Cerebral infarction, SIRT1/NF-κB signaling pathway, Inflammation, Apoptosis.

Introduction

Cerebral infarction (CI) refers to the ischemic and hypoxic changes in brain tissues caused by inadequate blood supply, with ischemia, hypoxia and reperfusion injury of brain tissues as major pathological processes, which usually leads to a series of complex pathological cascades such as inflammation and oxidation-reduction reaction1-2.
The morbidity rate of CI is rising with the aggravation of population aging, and CI has become a leading cause of dementia, cognitive decline, limb motor dysfunction and even death of elderly people.3,4

As one of the most common cerebrovascular diseases in clinic, CI induces great damage to cerebral nerve cells, and a train of complicated pathological cascades mediates the apoptosis and then necrosis of massive cerebral nerve cells. Therefore, effectively regulating the inflammation level after CI is essential for maintaining the apoptosis level and promoting repair after injury. The silent information regulator 1 (SIRT1)/nuclear factor-kappa B (NF-κB) signaling pathway is an important inflammatory signaling pathway in organisms, whose normal state is crucial for maintaining the normal inflammation level and neuronal apoptosis level.5,6

Edaravone and oxiracetam are clinically commonly used therapeutic drugs for cerebrovascular diseases; they have good anti-inflammatory and anti-apoptotic effects.7 It has been reported that edaravone could alleviate the degeneration of both motor neurons and muscles related to oxidative stress in amyotrophic lateral sclerosis (ALS) patients.8 Hence, the influence of edaravone combined with oxiracetam on the neuronal apoptosis in rats with CI via the SIRT1/NF-κB inflammatory pathway was investigated in this experiment.

**Materials and Methods**

**Laboratory Animals and Grouping**

A total of 36 SPF laboratory Sprague-Dawley (SD) rats aged 1 month old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd [license No.: SCXK (Shanghai, China) 2014-0003]. They were fed in the Laboratory Animal Center with normal diet and sterile filtered water every day under a 12/12 h light/dark cycle, and conventional room temperature and humidity. After 7 days of adaptive feeding, the SD rats were divided into sham-operation group (n=12), model group (n=12) and treatment group (n=12) using a random number table. In sham-operation group, only the common carotid artery, external carotid artery and internal carotid artery were exposed, without blocking the blood vessels, and an equal volume of normal saline was injected intraperitoneally every day after operation. The rats in model group and treatment group were utilized to prepare the models of CI, and then intraperitoneally injected with normal saline and edaravone injection (5.42 g/kg) and oxiracetam injection (0.216 g/kg), respectively, every day after operation. Specimens were obtained from each group after 14 consecutive days of intervention. This study was approved by the Animal Ethics Committee of Fujian Medical University Animal Center.

**Experimental Reagents**

Edaravone injection and oxiracetam injection were purchased from Simcere Dongyuan Pharmaceutical Co., Ltd. (Nanjing, China). Primary antibodies against SIRT1 and NF-κB and corresponding secondary antibodies were provided by Abcam (Cambridge, MA, USA). Nissl staining kit and enzyme-linked immunosorbent assay (ELISA) kit were bought from Beyotime (Shanghai, China). Moreover, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit and quantitative polymerase chain reaction (qPCR)-related kits were purchased from Vazyme (Nanjing, China).

**Establishment of CI Model**

The CI model was established as follows: 3% pentobarbital sodium solution (5 mL/kg) was intraperitoneally injected for anesthesia. After that, the rats were fixed in the supine position, and the neck was depilated, disinfected and then covered with a sterile towel. Next, a longitudinal incision (about 2 cm) was made at the anteromedian line of the neck, and the common carotid artery, external carotid artery and internal carotid artery were separated and exposed carefully. Subsequently, the common carotid artery and external carotid artery were ligated by silk sutures, and the internal carotid artery was clipped using vascular forceps. The thread was inserted into the ligated site of the common carotid artery, the vascular forceps on the internal carotid artery were released, and the thread was slowly pushed into the branch of middle cerebral artery. Later, the internal carotid artery was ligated again and fixed by the thread, and the incision was sutured after flushing with normal saline. Then, timing was stared, and the thread was withdrawn slowly at 90 min after vascular occlusion. The details of the CI Model establishment protocol were according to previous studies.9,10

**Cognitive function evaluation via Water Maze Test**

The rats were put in a water maze, where they were allowed to move freely, after the last intervention. The escape latency and the times of...
crossing the platform in the water maze of the rats were recorded. After that, the platform in the water maze was removed, and the rats were put into the water maze from any place. As the rats moved freely, the times of crossing the original platform and the residence time in the original quadrant were recorded within 70 s.

**Specimen Acquisition**

The rats were anesthetized by 3% pentobarbital sodium solution (5 mL/kg) via intraperitoneal injection. After successful anesthesia, specimens were directly taken from 6 rats in each group. Specifically, the brain tissues were taken out directly, washed with normal saline and placed into EP tubes, followed by preservation at -80°C for later use. As for the rest 6 rats in each group, the specimens were collected via perfusion-fixation. Specifically, the thoracic cavity was cut open to expose the heart, and 400 mL of 4% paraformaldehyde was perfused from the left atrial appendage. Finally, the brain tissues were taken out and immersed in 4% paraformaldehyde solution for fixation.

**Nissl Staining**

The tissues embedded in paraffin in advance were sliced into 5 μm-thick sections, followed by spreading in warm water at 42°C, collection and baking to prepare paraffin-embedded sections. Then, the sections obtained were soaked in xylene solution and gradient alcohol for routine deparaffinization until rehydration. Next, the sections were stained with hematoxylin dye for 5 min using the Nissl staining kit and then soaked in pure water for 10 min, followed by color separation in 95% ethanol for 5 s, transparentization with xylene for 10 s and mounting in neutral balsam.

**Western Blotting Assay**

The cryopreserved brain tissues were added with lysis buffer for 1 h of ice bath and centrifuged at 14,000 g for 10 min in a centrifuge, and the proteins were quantified using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Next, the absorbance and standard curve of the proteins were obtained through a microplate reader, based on which the protein concentration in tissues was calculated. Subsequently, the proteins in tissue specimens were denatured and separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The position of the Marker proteins was observed, and the electrophoresis was stopped when the Marker proteins reached the bottom of glass plate in a straight line. Later, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and reacted with blocking buffer for 1.5 h. After that, anti-SIRT1 primary antibody (1:1000), anti-NF-κB primary antibody (1:1000) and secondary antibodies (1:1000) were added in sequence. Finally, the image was fully developed with chemiluminescent reagent in the dark for 1 min after rinsing.

**QPCR Assay**

The fresh brain tissues were added with TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer’s protocol to extract the total RNA. Next, the total RNA obtained was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) and a designed reaction system (20 μL) under the following reaction conditions: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s and annealing at 62°C for 30 s for 35 cycles. Real-time PCR was performed with a FastStart Universal SYBR Green Master kit (Roche, Basel, Switzerland). With GAPDH as endogenous control, the relative expression of the genes was calculated using the 2-ΔΔCt method. The detailed primer sequences were shown in Table I.

**Detection of Content of Inflammatory Factors in Brain Tissues Via ELISA**

The freshly obtained brain tissues were mashed. Based on the instructions of the ELISA kit, samples were loaded, and standard substance, biotinylated antibody working solution and enzyme conjugate working solution were added. Finally, the plate was washed, and the tissues were detected using the microplate reader at 450 nm.

**TUNEL Assay**

The tissues embedded in paraffin in advance were prepared into paraffin-embedded tissue sections (5 μm thick) through spreading in warm

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Table I. Primer sequences.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
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<tr>
<td>IL-1β</td>
<td>Forward: 5′ATGGCAGAAGTGACCTAACGCTC3′ Reverse: 5′ATGGCAGAAGTGACCTAACGCTC3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5′ATGGCAGAAGTGACCTAACGCTC3′ Reverse: 5′ATGGCAGAAGTGACCTAACGCTC3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5′ACGGCAATTCACGGACAG3′ Reverse: 5′GAAGAGGCCATAGTACCCAGC3′</td>
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water at 42°C, collection and baking. Then, the paraffin-embedded sections were routinely deparaaffinized in xylene solution and gradient alcohol until rehydration. Finally, the cell apoptosis in brain tissues was detected according to the instructions of the TUNEL assay kit.

**Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis in this study, and the measurement data were expressed as mean ± standard deviation. The $t$-test was performed for the data meeting normal distribution and homogeneity of variance, corrected $t$-test was adopted for the data in line with normal distribution and heterogeneity of variance, and non-parametric test was used for the data not meeting normal distribution and homogeneity of variance. The ranked data were subjected to the rank sum test, and the enumeration data were examined by chi-square test.

**Results**

**Water Maze Test Results**

Model group displayed a significantly longer escape latency and significantly fewer times of crossing the original platform than sham-operation group, showing statistically significant differences ($p<0.05$). However, the escape latency was significantly shortened, but the times of crossing the original platform were increased significantly in treatment group in contrast with those in model group, and there were statistically significant differences ($p<0.05$) (Figure 1).

**Protein Expressions Detected Via Western Blotting Assay**

The protein expression of SIRT1 was high, while that of NF-κB was low in sham-operation group. In contrast, model group had a lower protein expression of SIRT1 but a higher protein expression of NF-κB (Figure 2A). The relative protein expression level of SIRT1 was lowered significantly, while that of NF-κB was elevated significantly in model group in comparison with those in sham-operation group, with statistically significant differences ($p<0.05$). Moreover, the opposite results were observed between model group and treatment group, and the differences were statistically significant ($p<0.05$) (Figure 2B).

**Content of Inflammatory Factors in Brain Tissues Determined Through ELISA**

The content of IL-1β and IL-6 in brain tissues was increased significantly in model group compared with that in sham-operation group, showing a statistically significant difference ($p<0.05$), but it was decreased significantly in treatment group in comparison with that in model group, with a statistically significant difference ($p<0.05$) (Figure 3).

**Messenger RNA (mRNA) Expressions Measured by qPCR Assay**

The relative mRNA expression levels of IL-1β and IL-6 were significantly higher in model group than those in sham-operation group and treatment group, and all the differences were statistically significant ($p<0.05$) (Figure 4).

**Cell Apoptosis Examined Using TUNEL Assay**

Model group had more positive apoptotic cells and a significantly higher apoptosis rate than sham-operation group, displaying statistically significant differences ($p<0.05$). Furthermore, there were fewer positive apoptotic cells and a significantly lower apoptosis rate in treatment group than those in model group, with statistically significant differences ($p<0.05$) (Figure 5).
Discussion

CI is a fairly common cerebrovascular disease caused by blockage of supply vessels to the brain in clinic, which results in ischemic and hypoxic changes, as well as ischemia/reperfusion injury of brain tissues, thus having important effects on brain tissues, nerve cells and the structure and function of synapses and myelin sheaths in these patients. CI generally leads to limb motor dysfunction, cognitive impairment, vascular dementia and even death of patients\(^{12-14}\), so it is one of the vital killers of the life and health of elderly people, seriously jeopardizing the mobility, quality of life, and life health of the elderly. Its incidence rate becomes higher and higher.
Edaravone combined with oxiracetam in cerebral infarction rats

Along with the aggravation of population aging, increase in pace of human life and change in lifestyles. Therefore, CI has been a hotspot and difficulty of research around the world. Additionally, efficiently preventing and treating CI, further clarifying the pathological mechanism and responses of CI and promoting neuroprotection and neural repair after CI are hot spots in studies. The pathological responses following CI are a series of complex cascades, in which the inflammation plays a pivotal role and exerts crucial regulatory effects in other pathological responses and neuronal apoptosis. After its occurrence, large quantities of inflammatory factors and cytokines are released into the injury site under the hypoxic and ischemic conditions and the action of ischemia/reperfusion injury, thereby triggering inflammatory infiltration of nerve tissues at the injury site. Under such circumstances, inflammatory factors can further activate the inflammation-associated signaling pathways to stimulate the release of inflammatory factors and activate the signaling pathways related to neuronal apoptosis at the same time, finally causing massive neuronal apoptosis. SIRT1 plays important regulatory roles in such processes as the regeneration, repair, metabolism and senescence of nerve cells under physiological conditions. It is highly expressed in brain tissues, so as to maintain the normal metabolism and physiological function of the nervous system. Meanwhile, SIRT1 possesses favorable anti-inflammatory effects, whose high expression can preferably inhibit the expression of inflammatory factors after brain injury. More importantly, SIRT1 has a good inhibitory effect on NF-κB under physiological conditions and forms the SIRT1/NF-κB signaling pathway with NF-κB, thereby regulating the inflammation in a favorable manner. The highly expressed SIRT1 can effectively repress the transcription of NF-κB and restrain its entrance into the nucleus to function as a vital transcription factor, thus inhibiting the expression of inflammatory factors downstream of the signaling pathway. In this study, a large number of inflammatory factors were highly expressed, and there was massive neuronal apoptosis in the brain tissues of rats with CI. Besides, the protein expression of SIRT1 declined markedly, while that of NF-κB rose notably, suggesting that the SIRT1/NF-κB signaling pathway is repressed, which may be one of the important causes of a large amount of neuronal apoptosis due to excessive inflammation in the brain tissues, as well as severe cognitive impairment of rats with CI. Both edaravone and oxiracetam are commonly applied drugs for cerebrovascular diseases in clinical practices. They have preferable neuroprotective effects, which regulate the inflammation, cell apoptosis and release of excess oxygen free radicals in a good manner. It was found in the present study that the intervention of CI with edaravone and oxiracetam could clearly ameliorate the morphology of nerve cells and relieve cognitive impairment of rats with CI simultaneously, which agrees with previous researches.

The novelty of this study was that we revealed the effects of edaravone combined with oxiracetam on CI and investigated the potential mechanism which might be involved in this process for the first time. It was discovered that edaravone combined with oxiracetam was able to effectively increase the protein expression of SIRT1 and reduce that of NF-κB, thus having a promoting effect on the SIRT1/NF-κB signaling pathway, which may be a potential mechanism of edaravone combined with oxiracetam in repressing the inflammation and neuronal apoptosis after CI.

Conclusions

Therefore, briefly, edaravone combined with oxiracetam inhibits the neuronal apoptosis in CI rats by regulating the SIRT1/NF-κB signaling pathway, thereby exerting a neuroprotective effect.

Figure 5. Cell apoptosis examined via TUNEL assay showed the apoptosis rate in each group. Note: *p<0.05 vs. sham-operation group, **p<0.05 vs. model group.
Conflict of Interests
The authors declare that they have no conflict of interest.

References