Keap1-Nrf2/ARE pathway-based investigation into the mechanism of edaravone dexborneol in cerebral infarction model neuroprotection

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ABSTRACT

The neuroprotection of acute cerebral infarction (ACI) model by edaravone dexborneol (ED)-mediated Keap1-Nrf2/ARE signal pathway was investigated. Sham operation was set as a control to prepare the ACI model with cerebral artery occlusion. The abdominal cavity was injected with edaravone (ACI+Eda group) and ED (ACI+ED group). Then, neurological deficit scores, cerebral infarct volume, oxidative stress ability, inflammatory reaction level, and the status of the Keap1-Nrf2/ARE signal pathway of rats in all groups were explored. It was demonstrated that the neurological deficit score and cerebral infarct volume of rats in the ACI group apparently increased versus those in the Sham group (P<0.05), suggesting that the ACI model was successfully prepared. Versus those in the ACI group, the neurological deficit score and cerebral infarct volume of rats in the ACI+Eda and ACI+ED groups decreased. In contrast, the activity of cerebral oxidative stress superoxide dismutase (SOD) and glutathione-peroxidase (GSH-Px) increased. Malondialdehyde (MDA) and the expressions of cerebral inflammation indicators (interleukin (IL)-1β, IL-6, and tumor necrosis factor-α messenger ribonucleic acid (TNF-α mRNA)) and cerebral Keap1 reduced. The expressions of Nrf2 and ARE increased (P<0.05). Versus those in the ACI+Eda group, all indicators of rats in the ACI+ED group were improved more apparently and were more similar to those in the Sham group (P<0.05). The above findings suggested that both edaravone and ED could mediate Keap1-Nrf2/ARE signal pathway to play a neuroprotective role in ACI. Versus edaravone, ED improved ACI oxidative stress and inflammatory reaction level and played a neuroprotective role more apparently.

Introduction

The incidence of the ischemic cerebrovascular disease shows a growing trend year by year. It has become one of the main diseases that endanger people’s life and health, especially the elderly. Acute cerebral infarction (ACI) is the main cause of the increase in mortality and disability (1). ACI causes multiple different types of injuries and it results from the temporary or permanent reduction in cerebral blood flow (CBF). The occurrence of ACI is closely related to the cerebral artery. After cerebral infarction, a series of complex pathological cascade reactions emerge, such as inflammatory reaction, endothelial cell dysfunction, mitochondrial damage, and the generation of oxygenated free radicals (2). It is verified that the abnormal expressions of multiple cytokines occur after cerebral ischemia (3). Keap1-Nrf2/ARE is an oxidative stress signal pathway. It plays a significant role in maintaining the balance of oxidation-reduction reactions in cells after cerebral infarction. Besides, Keap1-Nrf2/ARE signal pathway can regulate downstream superoxide dismutase (SOD) to resist cell apoptosis, inflammatory reaction, and oxidative stress reaction (4). Hence, the activation of the Keap1-Nrf2/ARE signal pathway is the key to the clinical drug treatment for neuroprotection after cerebral infarction.

Edaravone is a kind of free-radical scavenger widely applied in the clinical treatment of ACI and excellent therapeutic results are achieved (5). During oxidative stress, superoxide anion, hydroxyl radical, hydrogen peroxide, and reactive oxygen species cause oxidative damage to lipids, proteins, or nucleic acid. Edaravone can reverse the cytotoxic effects mediated by free radicals to protect neuronal cells (6). In addition, edaravone can inhibit the further oxidation of lipid and endothelial cell injury, reduce the volumes of cerebral edema and cerebral infarction, and eventually reduce neurological deficit levels (7). Wu et al. (2021) (8) confirmed that edaravone combined with thrombolysis therapy could expand the treatment time window for ACI patients to reduce the risk of bleeding transformation. Edaravone can protect the brain and improve cerebral metabolism, while dexborneol has an anti-inflammatory effect. Franke et al. (2021) (9) showed that the occurrence of cerebral infarction was associated with inflammatory reaction to some extent. Therefore, edaravone combined with dexborneol can act on vascular endothelial cells to prevent cell injury, scavenge free radicals, resist oxidative stress, and fight against inflammatory reactions. At present, the mechanism of the treatment of cerebral infarction with edaravone combined with dexborneol is still unclear.

To investigate whether edaravone dexborneol (ED) could mediate Keap1-Nrf2/ARE signal pathway to play a neuroprotective role in treating cerebral infarction, an ACI...
rat model was prepared in the research. Edaravone and ED were injected into rats’ abdomens to explore the effects of edaravone and ED on cerebral infarct volume, inflammatory reaction, oxidative stress reaction, and the status of the Keap1-Nrf2/ARE signal pathway in brain tissues. The research was conducted to provide the theoretical basis for improving the clinical cure rate and prognosis among patients with cerebral infarction.

Materials and Methods

Experimental materials

40 clean male Sprague-Dawley (SD) rats aged between 7 and 8 weeks were selected as the research objects. Their weight ranged from 230g to 270g and they were all purchased. Triphenyl tetrazolium chloride (TTC) stain was purchased from Yuxiu Biotechnology (Shanghai) Co., Ltd. Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione-peroxidase (GSH-Px) detection kits were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. PrimeScript™ RT Master Mix (Perfect Real Time) reverse transcription kit and TB Green® Premix Ex Taq™ (Tli RNase H Plus) quantitative detection kit was purchased from Takara Biomedical Technology Co., Ltd. (Beijing). Biquinolinic acid (BCA) protein concentration quantitative detection kit and electrophoregram chemiluminescence (ECL) detection kit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai). Keap1, Nrf2, ARE, and GAPDH first antibodies and IgG second antibody were purchased from Abcam plc. (UK).

Preparation of ACI model

The cerebral artery occlusion method was adopted to prepare the ACI rat model. 30mg/kg pentobarbital sodium with a density of 3mg/mL was injected to anesthetize rats. Rats fixed the operating table in the supine position. Rectal temperature was measured and maintained at about 36.9±0.4°C. An incision was made along the median cervical line to expose the right common carotid artery. The arterial end close to the heart was ligated. After that, an incision was made at the lateral carotid artery stump. Then, a bolt tread was inserted into the internal carotid artery of rats and advanced along the artery towards the incision was sutured layer by layer. After disinfection, the rats were kept in separate cages and performed anti-sepsis to remove the infection.

The infarct area. The infarct was treated for 1.5 hours and then the incision was removed slowly. After the surgery, the incision was sutured layer by layer. After disinfection, the rats were kept in separate cages and performed anti-sepsis.

Experimental grouping

5 rats were placed in the high plus maze experimental device 30cm above the ground (consisting of 2 50cm×10cm open arms, 2 50cm×10cm closed arms, and 10cm×10cm central open equipment). The activity of 5 rats in the maze was monitored in real-time and central area exercise time was recorded.

Forelimb foot fault placing test

The rats were placed on a 10cm×100cm grid plate 60cm above the ground (the area was 3cm×3cm). The rats were touched and stimulated to cross the grid within 1 minute. After that, the number of the placement of the forelimb into the grid hole was recorded. 0 points represented no placing error. 1 point suggested 1 or a few placing errors. 2 points indicated 1 to 2 placing errors. 3 points showed 2 to 3 placing errors. 4 points demonstrated 3 to 4 placing errors. 5 points revealed that rats were unable to move on the grid plate.

Parallel bar test

The rats were placed on 2 parallel wooden bars 1cm in diameter, 100cm in length, and 2.5cm in spacing. Then, they were stimulated to crawl and crawl time, distance, and the number of the placement of posterior limbs on the same wooden bar, sliding, and falling off was recorded. 0 points indicated no crawl error. 1 point represented 1 or a few crawl errors. 2 points demonstrated crawl errors. 3 points suggested 2 to 3 crawl errors. 4 points suggested 3 to 4 crawl errors. 5 points represented more than 5 crawl errors.

Vital sign scores for neurological deficit

Longa 5-point method was adopted to score the neurological deficit of rats in all groups 7 days after drug administration. Point 0 suggested no neurological deficit among rats. 1 point demonstrated that rats were unable to fully extend their contralateral front paws. 2 points indicated the occurrence of circle-drawing in rats’ contralateral front paws. 3 points showed that the dumping of rats’ contralateral front paws occurred. 4 points suggested that rats were unable to walk consciously and lost normal consciousness. A higher score for neurological deficit indicated severer behavioral disorders among rats.

Behavioral detection

The changes in depression- and anxiety-like behaviors among rats in all groups 7 days after drug administration were evaluated through open field test and high plus maze test. Besides, the exercise capability of rats in all groups 7 days after drug administration was evaluated through a forelimb foot fault placing test and parallel bar test.

Open field test

Five rats were selected and placed into a square open cardboard box with a size of 100cm×100cm×60cm. The activity of 5 rats in the maze was monitored in real-time and central area exercise time was recorded.

High plus maze test

Five rats were randomly enrolled into the sham operation group (Sham) (10 rats), ACI group (10 rats), ACI+Eda group (10 rats), and ACI+ED group (10 rats). According to the procedures mentioned in Section 2.2, ACI models of ACI, ACI+Eda, and ACI+ED groups were prepared and the right common carotid arteries of rats in the Sham group were isolated without inserting bolt tread. After that, the abdominal cavities of the rats in the Sham and ACI groups were injected with an appropriate amount of physiological saline. After modeling, the abdominal cavities of the rats in the ACI+Eda group were injected with 8mg/kg/d edaravone and the abdominal cavities of the rats in the ACI+ED group were injected with 8mg/kg/d edaravone and 2mg/kg/d dexborneol.

Determination of cerebral infarct volume

Cerebral infarct volumes of rats in all groups were stai...
nked with the TTC method 7 days after drug administration. Besides, the neuroprotective effect of the drug was evaluated. 30mg/kg pentobarbital sodium with a density of 3mg/mL was used to anesthetize rats. After quick decapitation, brain tissues were extracted and frozen for 20 minutes. Next, 5 brain tissue sections with a thickness of about 2mm were cut consecutively along the coronal plate on the floe. After that, the frozen brain tissues were placed into 4% TTC staining solution and then stained away from light at 37°C for 10 minutes. The stained tissues were soaked in a formaldehyde solution for 1 day. The infarct areas were stained light yellow or white, while non-infarct areas turned red or dark red. The stained brain tissues were photographed with a digital camera. Besides, the cerebral infarct volume of rats was detected with Image J image analysis software according to Equation 1.

\[
\text{Total infarct volume} = \frac{(CIV - INIV)}{CIV} \times 100\% \quad [1]
\]

In Equation 1, CIV and INIV referred to contralateral infarct volume and ipsilateral noninfarct volume, respectively.

**Determination of oxidative stress levels**

MDA, SOD, and GSH-Px detection kits were employed to detect the oxidative stress levels in brain tissues of rats in all groups 7 days after drug administration. 30mg/kg pentobarbital sodium with a density of 3mg/mL was used to anesthetize rats. After quick decapitation, brain tissues were extracted and hippocampus tissues were isolated. Next, the content of MDA and the activity of SOD and GSH-Px were detected according to the instructions of MDA, SOD, and GSH-Px kits.

**Real-time fluorescent quantitative polymerase chain reaction (PCR)**

The expressions of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, Keap1, Nrf2, and GAPDH in brain tissues of rats in all groups were detected 7 days after drug administration. 30mg/kg pentobarbital sodium with a density of 3mg/mL was used to anesthetize rats. After quick decapitation, the peripheral cortex of brain tissues was extracted and added with RIPA lysis buffer for the extraction of total protein from brain tissues. In addition, the BCA method was adopted to detect the expression of total protein. An appropriate amount of extracted protein was taken to prepare spacer gel with a reasonable concentration and isolate gel loading electrophoresis. After that, target protein bands were transferred to polyvinylidene fluoride (PVDF) membrane and then sealed with the confining liquid containing 10% skimmed milk powder for 3 hours. Besides, the diluted Keap1 (1:1000), Nrf2 (1:1000), ARE (1:1000), and GAPDH (1:5000) first antibodies were added and then incubated at 4°C for 8 hours. After the PVDF membrane was washed with tris buffered saline tween (TBST), the diluted horse-radish peroxidase (HRP)-labeled IgG second antibody (1:2000) was added and then incubated at room temperature for 1 hour. After the PVDF membrane was rinsed with TBST, an ECL detection kit was used for luminescence of the target bands, which were photographed with a gel imager. Finally, the gray-scale values of target gene bands were determined by Image J.

**Statistical processing**

SPSS 19.0 software was employed for the statistical analysis of experimental data. All experimental data were denoted by mean±standard deviation (x̄ ± s). The differences among groups were compared by one-factor analysis of variance (ANOVA). P<0.05 indicated that the differences were statistically significant.

**Results**

**Influences of ED on neurological deficit among ACI rats**

![Figure 1. Comparison of neurological deficit scores for ACI rats in different groups. Note: The comparison with the sham group suggested *P<0.05. The comparison with the ACI group indicated #P<0.05. The comparison with the ACI+Eda group showed ΔP<0.05.](Image 508x350 to 531x362)
Longa 5-point scoring method was adopted to score the neurological deficit of rats in all groups (Figure 1). It was demonstrated that neurological deficit scores for rats in ACI, ACI+Eda, and ACI+ED groups apparently increased versus that in the sham group (P<0.05), which suggested that the ACI rat model was successfully constructed. In contrast to that in the ACI group, neurological deficit scores for rats in ACI+Eda and ACI+ED groups were apparently reduced (P<0.05). Versus that in the ACI+Eda group, the neurological deficit score for rats in the ACI+ED group notably declined (P<0.05).

**Influences of ED on infarct volume among ACI rats**

The difference in cerebral infarct volumes of rats among different groups was detected by TTC stain (Figure 2). Cerebral infarct volumes of rats in ACI, ACI+Eda, and ACI+ED groups increased versus that in the sham group (P<0.05), which demonstrated that the ACI rat model was successfully constructed. In contrast to that in the ACI group, cerebral infarct volumes of rats in ACI+Eda and ACI+ED groups apparently decreased (P<0.05). Versus that in the ACI+Eda group, cerebral infarct volumes of rats in the ACI+ED group notably reduced (P<0.05).

**Influences of ED on anxiety- and depression-like behaviors and exercise capability among ACI rats**

The changes in depression- and anxiety-like behaviors among rats in all groups were evaluated through open field test and high plus maze test (Figure 3). It was demonstrated that central area exercise time, the number of entering open arms, and the dwell time of open arms among rats in ACI, ACI+Eda, and ACI+ED groups all declined versus those in the sham group (P<0.05). In contrast to those in the ACI group, central area exercise time, the number of entering open arms, and the dwell time of rats in ACI+Eda and ACI+ED groups all notably increased (P<0.05). Versus those in the ACI+Eda group, central area exercise time, the number of entering open arms, and the dwell time of rats in the ACI+ED group all significantly enhanced (P<0.05).

The difference in exercise capability of rats in different groups was assessed through the forelimb foot fault placing test and parallel bar test (Figure 4). It was demonstrated that forelimb placing error scores and crawl error scores for rats in ACI, ACI+Eda, and ACI+ED groups all notably increased versus those in the sham group (P<0.05). In contrast to those in the ACI group, forelimb placing error scores and crawl error scores for rats in ACI+Eda and ACI+ED groups all dramatically declined (P<0.05). Versus those in the ACI+Eda group, forelimb placing error scores and crawl error scores for rats in the ACI+ED group both significantly decreased (P<0.05).

**Influences of ED on oxidative stress reactions among ACI rats**

The differences in the expressions of SOD, MDA, and GSH-Px in brain tissues of ACI rats in different groups were detected (Figure 5). It was demonstrated that the activity of SOD, B. Content of MDA. C. Activity of GSH-Px. The comparison with the sham group indicated *P<0.05. The comparison with the ACI group indicated #P<0.05. The comparison with the ACI+Eda group showed ΔP<0.05.
and ACI+Eda groups both apparently reduced, while the content of MDA obviously improved versus those in the sham group ($P<0.05$). There were no remarkable differences in SOD, MDA, and GSH-Px in the brain tissues of rats between sham and ACI+ED groups ($P>0.05$). In contrast to those in the ACI group, the activity of SOD and GSH-Px among rats in ACI+Eda and ACI+ED groups both apparently increased, while the content of MDA notably decreased ($P<0.05$). In contrast to those in the ACI+Eda group, the activity of SOD and GSH-Px in the ACI+ED group both obviously improved, while the content of MDA apparently reduced ($P<0.05$).

### Influences of ED on inflammatory reactions among ACI rats

A real-time fluorescent quantitative PCR technique was utilized to detect the differences in the expressions of IL-1$\beta$, IL-6, and TNF-\(\alpha\) mRNA in the brain tissues of rats in all groups (Figure 6). It was demonstrated that the expressions of IL-1$\beta$, IL-6, and TNF-\(\alpha\) mRNA in brain tissues of rats in ACI and ACI+Eda groups notably improved versus those in the sham group ($P<0.05$). There were no remarkable differences in the expressions of IL-1$\beta$, IL-6, and TNF-\(\alpha\) mRNA in brain tissues of rats between sham and ACI+ED groups ($P>0.05$). Versus those in the ACI group, the expressions of IL-1$\beta$, IL-6, and TNF-\(\alpha\) mRNA in brain tissues of rats in ACI+Eda and ACI+ED groups dramatically decreased ($P<0.05$). Versus those in the ACI+Eda group, the expressions of IL-1$\beta$, IL-6, and TNF-\(\alpha\) mRNA in brain tissues of rats in the ACI+ED group notably reduced ($P<0.05$).

### Treatment of ACI with ED-mediated Keap1-Nrf2/ARE signal pathway

Real-time fluorescent quantitative PCR technique was adopted to detect the differences in the expressions of Keap1 and Nrf2 mRNA in the brain tissues of rats in all groups (Figure 7). It was demonstrated that the expression of Keap1 mRNA in brain tissues of rats in ACI and ACI+Eda groups apparently increased, while the expression of Nrf2 mRNA notably reduced versus those in the sham group ($P<0.05$). There were no remarkable differences in the expressions of Keap1 and Nrf2 mRNA in brain tissues of rats between sham and ACI+ED groups ($P>0.05$). In contrast to that in the ACI group, the expressions of Keap1 mRNA in brain tissues of rats dramatically declined, while the expression of Nrf2 mRNA obviously improved in ACI+Eda and ACI+ED groups ($P<0.05$). Versus those in the ACI+Eda group, the expression of Keap1 mRNA in brain tissues of rats dramatically declined, while the expression of Nrf2 mRNA obviously improved in the ACI+ED group ($P<0.05$).

Western blot technique was adopted to detect the differences in the expressions of proteins Keap1, Nrf2, and ARE in the brain tissues of rats in all groups (Figure 8). It was demonstrated that the expressions of Keap1 apparently improved, while the expressions of Nrf2 and ARE obviously reduced in the brain tissues of rats in ACI and ACI+Eda groups versus those in the sham group ($P<0.05$). There were no remarkable differences in the expressions of Keap1, Nrf2, and ARE in brain tissues of rats between sham and ACI+ED groups ($P>0.05$). Versus those in the ACI group, the expressions of Keap1 apparently decreased, while the expressions of Nrf2 and ARE obviously...
increased in brain tissues of rats in ACI+Eda and ACI+ED groups ($P<0.05$). In contrast to those in the ACI+Eda group, the expressions of Keap1 notably reduced, while the expressions of Nrf2 and ARE significantly increased in the brain tissues of rats in the ACI+ED group ($P<0.05$).

Discussion

The fatality and disability of ACI are high. Hence, effective treatment method for ACI is the focus of current research. At present, the main clinical treatment methods for ACI include intravenous thrombolytic therapy, intravenous infusion of neuroprotectant, and arterial endovascular treatment (10,11). Due to the limitations of the treatment time window, contraindications, and other factors, most ACI patients can’t receive intravenous thrombolytic treatment and arterial endovascular treatment. Therefore, intravenous infusion of neuroprotectants is of great concern (12). Neuroprotection therapy can inhibit neuronal apoptosis by reducing pathological cell injuries. Edaravone is the most widely used neuroprotectant in clinical practice. It can effectively alleviate the injuries of nerve cells and vascular endothelial cells, inhibit the apoptosis of nerve cells, and improve the neurological function of patients with ischemic cerebral diseases by scavenging free radicals and inhibiting the over-oxidation of lipids and the generation of hydroxyl radicals (13). Li et al. (2021) (14) verified that oxidative stress and inflammatory reaction often show high levels in patients’ bodies after ACI, which eventually affects their cognitive function and induces depression- and anxiety-like behaviors. ED has the anti-oxidant effect that edaravone possesses and an anti-inflammatory effect that dexbornedole possesses. However, its neuroprotective effect on ACI is still unclear.

In the research, medium-sized cerebral artery occlusion was employed to prepare the ACI rat model. It was found that neurological deficit score and cerebral infarct volume were both remarkably superior to those of rats in the sham group. Medium-sized cerebral artery occlusion can affect the normal behaviors of rats and cause chemical and histological changes in the cranial nerve. It can be used for the simulation of human ACI symptoms (15). It was suggested that the ACI rat model was successfully prepared. Edaravone can improve the neurological function of ACI rats and reduce cerebral infarct area. According to the research results, neurological deficit score and cerebral infarct area apparently decreased after the treatment for ACI rats with edaravone and ED. Besides, it was indicated that the evaluation of depression- and anxiety-like behaviors and exercise capability were dramatically improved. ED had a more significant therapeutic effect on ACI rats. The above findings demonstrated that ED could restore the neurological function of ACI rats and reduce cerebral infarct volume more effectively than edaravone.

Zheng et al. (2020) (16) showed that the inflammatory reactions and injuries induced by oxygen free radicals, abnormal apoptosis of neurons, the toxicity of excitatory amino acid, and abnormal metabolism of brain tissues all got involved in the ACI process. Oxidative stress is the main cause of the aggravation of ACI injury and reactive oxygen species lead to ischemic tissue injuries. In the research, the activity of SOD and GSH-Px and the content of SOD in the hippocampus tissues of rats were detected. SOD is a free-radical scavenger that can inhibit brain cell injury caused by oxygen free radicals and the generation of peroxide during ischemia-reperfusion. Finally, the hypoxia tolerance of the cerebral cortex is enhanced (17). GSH-Px is a peroxidase that can reduce toxic peroxide to a non-toxic hydroxyl compound to protect the structure and function of the cell membrane (18). MDA is a metabolite during lipid peroxidation. It indirectly reflects free radical expression (19). It was found that the activity of SOD and GSH-Px was enhanced, while the content of MDA was reduced after the treatment for ACI rats with edaravone and ED. ED had a more significant effect. What’s more, it was demonstrated that the expressions of IL-1β, IL-6, and TNF-α in brain tissues of ACI rats significantly reduced after the treatment with edaravone and ED. The result indicated that both edaravone and ED could enhance the anti-oxidant and anti-inflammatory abilities of the hippocampus areas of ACI rats, especially ED.

Keap1-Nrf2/ARE signal pathway is an important transduction pathway of oxidative stress signals. It can activate the expressions of downstream factors to resist inflammations, oxidative stress, and cell apoptosis (20). Nrf2 is an essential endogenous protective factor that indirectly resists oxidation and maintains homeostasis (21). What’s more, Nrf2 can protect the tissues injured by inflammatory reactions and pathological inflammatory reactions (22-24). It has been validated that the activated Nrf2-ARE could mediate the generation of SOD, Nrf2-ARE, and other free radical scavenging enzymes (25). The research results demonstrated that the expression of Keap1 declined, while the expressions of Nrf2 and ARE increased in brain tissues after treating ACI rats with edaravone and ED. ED had a more significant regulatory effect. It was shown that both edaravone and ED could activate Keap1-Nrf2/ARE signal pathway to play a neuroprotective role in ACI. ED was relatively more effective.

ED could down-regulate the expression of Keap1 and activate the Nrf2/ARE signal pathway to reduce cerebral infarct volume and the incidence of depression- and anxiety-like behaviors and enhance exercise capability and anti-oxidative stress and anti-inflammatory abilities of ACI patients. Furthermore, it achieved neuroprotective effects on ACI patients. In the research, only the neuroprotective effects of edaravone and ED on ACI rats were compared without an in-depth investigation into the differences in the effects of different doses of ED. In follow-up research, more experiments should be carried out to search for the most appropriate dose of ED. In general, the research results provided a reference for understanding the pathogenesis of ACI and searching for an effective therapeutic drug.

References


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