PROTECTIVE EFFECTS OF EDARAVONE ON NEURONAL APOPTOSIS IN VASCULAR DEMENTIA RATS BY PI3K/AKT SIGNALING PATHWAY

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ABSTRACT

Objective: To investigate the effect and mechanism of edaravone on vascular dementia (VD) in rats, and to provide a reference for the prevention and treatment of VD in clinic.

Methods: In accordance with the random number table method, 60 male Sprague-Dawley rats were randomly divided into three groups: Sham group (n=20), VD group (n=20) and VD+Edaravone group (n=20). The VD model was established by ligating the common carotid arteries of both sides of the rats twice in three days. Rats in the VD+Edaravone group were given 2.5 mg/kg/d of edaravone by gavage. After 28 days, the rats’ learning and memory ability was tested using the platform test and Y-maze test. The apoptosis-related genes Bax and bcl-2 were detected by RT-PCR and Western blotting. The brains of each group were evaluated by H&E staining. For tissue neuronal injury status: TUNEL staining was used to detect the neuronal apoptosis in the brain tissue of each group; Nissl staining was used to detect the number of Nissl bodies in hippocampal neurons of each group; and we also detected using immunohistochemical staining. The protein expression levels of Bax and bcl-2 in brain tissue of three groups of rats were used. Finally, we used immunoblotting to detect the effect of edaravone on PI3K/AKT signalling pathway in brain tissue of VD rats.

Results: Edaravone could significantly improve the pathological damage of rat brain tissue induced by VD, increase the number of Nissl bodies in hippocampus and improve the memory and learning ability of rats (p<0.05). In addition, edaravone can significantly inhibit the neuronal apoptosis induced by VD in hippocampus, promote the expression of Bcl-2 mRNA and protein and inhibit the expression of Bax mRNA and protein (p<0.05). Western blot results revealed that edaravone can significantly activate PI3K expression and phosphorylation of AKT.

Conclusion: Edaravone can significantly improve the neurological function of VD rats and inhibit the apoptosis of neuronal cells in the lesion area. The mechanism may be related to the activation of the PI3K/AKT signalling pathway by edaravone.

Keywords: Edaravone, vascular dementia, neurons, apoptosis, PI3K/AKT signalling pathway.

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Introduction

Vascular dementia (VD) refers to the loss of cognitive function caused by pathological changes of cerebrovascular diseases and cardiovascular diseases, which are mainly manifested as learning and memory disorders(1). VD is an age-related neurodegenerative disease and the second most common cause of dementia in the elderly after Alzheimer’s disease, characterized by significant cognitive impairment due to an insufficient supply of cerebral blood flow(2-3). In addition to age and gender, the development of VD is also associated with a variety of diseases such as hypertension and metabolic syndrome(4); smoking and drinking are also confirmed risk factors for VD(5). Current research has revealed that oxidative stress, inflammation, dysfunction of the neurotransmitter system, mitochondrial dysfunction, lipid metabolism disorders, abnormal growth factor levels and other factors can all lead to neuronal loss, accelerate neuronal apoptosis and ultimately aggravate the occurrence of VD(6-8). Therefore, designing drugs targeting these links can not only prevent VD from occurring early, but also suppress the increase of VD.

Edaravone is an oxygen free radical scavenger that prevents oxidative damage to cell membranes and inhibits cerebral edema and cerebral infarction...
following cerebral ischemia\(^9-10\). In recent years, more and more studies have gradually revealed that edaravone can be used for treatment not only in the acute phase of stroke, but also in a variety of reactive oxygen species (ROS)-related diseases\(^{11}\). For example, edaravone can alleviate neurological dysfunction following spinal cord ischemia-reperfusion injury caused by abdominal aortic obstruction in rabbits, pathological damage, and DNA metabolism disorders\(^{12-13}\). In the rat spinal cord contusion model, edaravone can improve neurological function and greatly preserve the white matter region of the spinal cord\(^{14-15}\). However, the role of edaravone in VD has yet to be reported.

In this study, we constructed a VD rat model and injected a specific dose of edaravone by intraperitoneal injection to observe the effects of edaravone on neurological function and neuronal apoptosis in VD rats and to explore related mechanisms aimed to provide a reference for the prevention and treatment of clinical VD in the future.

**Materials and methods**

**Animal grouping and model building**

Sixty male SD rats weighing 281.78±8.62 g at 12-14 weeks were randomly divided into three groups, including the sham operation (Sham group, n=20), cerebral vascular dementia group (VD group, n=20) and the drug intervention group (VD + Edaravone group, n=20). There were no significant differences in the basic information such as age and body weight among the three groups of rats.

The VD rat model was made as follows:

- The rat was anesthetized by hydrated chloral hydrate (400 mg/kg) and fixed;
- Fixed on the plate after anaesthesia, a 50 mm incision was made on the left side of the neck midline, the skin muscles opened, the left neck total artery separated, and double ligatured with silk;
- Sutured and disinfected;
- Three days later, the symmetrical position of the left incision on the right side with the midline as the axis of symmetry was found and the skin muscles opened. Then, the right common carotid artery was fully exposed. Similarly, it was double-ligated with silk thread to predict the common carotid artery;
- Sutured and disinfected. The VD+Edaravone group took 2.5 mg/kg/d edaravone from the first day of surgery; in 28 days it was executed and taken after testing the learning memory function.

**Step-down Test**

Each group of rats was placed in a jumping apparatus for 3 minutes and connected to 36V AC after the rats adapted to their surrounding environment. The incubation period was recorded (seconds) for each group of rats, which was the first time they jumped off the platform. At the same time, the number of errors was recorded; that is, the number of times that the rat jumped off the platform after being clicked within 5 minutes. The test was repeated, and the number of errors was recorded as a rat memory score one day later.

**Y-maze Experiment**

Three groups of rats were placed in the maze and stimulated with 50 V voltage for a delay of 3 min after 3 min of adaptation. Subsequently, the safety zone and the electric shock zone were randomly replaced. If the rat encountered an electric shock and did not reach the safe zone, it was considered as an error reaction, and vice versa. The score was the total number of shocks before the correct response nine times out of 10 consecutive times. One day later, the correct response in 10 consecutive shocks was measured as a memory score.

**Nissl Staining**

The hippocampus tissues of each group of rats were deposited on a sucrose solution (30%) and then frozen section (30 μm). The cut brain tissue was attached to a glass slide (pretreated gelatin) and dried. Subsequently, each group of sections was stained in tar purple at 37 °C (0.5 h), then subjected to colour separation and dehydration by gradient concentration of ethanol and sealed after xylene treatment. Finally, sections were observed, and pictures taken under the light microscope.

**RT-PCR Detection of Apoptosis-related Gene Expression**

- The total RNA was extracted from brain tissue by the Trizol method and the concentration and purity of the extracted RNA were detected by ultraviolet spectrophotometer. A260/A280 = 1.8-2.0 can be used;
- mRNA was synthesized into cDNA by reverse transcription and stored in the refrigerator for -80 °C;
- RTPCR system: 10 × buffer 2.5 μl; cDNA 2 μl; forward primer (20 μmol/L) 0.25 μl; reverse primer (20 μmol/L) 0.25 μl; dNTPs (10 mmol/L) 0.5 μl; and Taq enzyme (2×106 U/L) 0.5 μl; ddH2O 19 μl. The amplification systems for RT-PCR were identical.
Protective effects of edaravone on neuronal apoptosis in vascular dementia rats by PI3K/AKT signaling pathway

**TUNEL Staining**

The cut brain tissue sections were baked in an oven at 60 °C for 30 min, followed by dewaxing (5 min X three times) with xylene and dehydrating three times with 100% ethanol, 95% ethanol and 70% ethanol. Subsequently, it was incubated with protein kinase K for half an hour, then terminal deoxyribonucleotidyl transferase TdT and lucif-erase-labelled dUTP were added after PBS rinse. After reacting at 37 °C for 1 h, a specific antibody labelled with horseradish peroxidase was added and incubated for an additional 1 h (37 °C) in an incubator. Subsequently, DAB was used as a sub-strate and reacted at room temperature for 10 min. After hematoxylin was stained with nuclei, photos were taken under a light microscope and counted.

**Immmunohistochemical Staining**

The cut brain tissue sections were baked in an oven at 60 °C for 30 min, followed by dewaxing (5 min X three times) with xylene and dehydrating three times with 100% ethanol, 95% ethanol and 70% ethanol. Endogenous peroxidase activity was inhibited in methanol with a concentration of 3% hydrogen peroxide and the tissue was blocked with sheep serum for 1 h. Antibodies against Bax and bcl-2 were diluted 1:200 (PBS) and incubated overnight at 4 °C, then washed four times with PBS on a shaker. After the addition of the secondary antibody, colour staining was carried out with diaminobenzi-dine. After the colour staining was completed, from each group six samples were randomly selected, and each sample randomly selected five fields of view and took pictures under a 400X light microscope.

**Western Blotting Detection of Protein Expression**

The brain tissue of each group was thorough-ly ground in a lysis buffer followed by ultrasonic lysis. The supernatant of lystate was aspirated after centrifuged and dispensed into EP tube successively. After measuring the protein concentration by the BCA method and UV spectrometry, the volume was adjusted to the same concentration. The tissue was dispensed and put in the refrigerator at -80 °C. After total protein extraction, SDS-PAGE electrophoresis was performed. After the electrophoresis was completed, the protein in the gel was transferred to a cellulose acetate (PVDF) membrane and the primary antibody was incubated at 4 °C overnight, then incubated for 1 h in the goat anti-rabbit sec-ondary antibody without light. The protein bands were scanned and quantified using an Odyssey membrane sweeper, the level of the tested protein to be corrected by GAPDH.

**Data Analysis**

All data were analysed using SPSS 22.0 soft-ware. Measurement data were expressed as mean±standard deviation, and data comparison between the two groups was performed by t test. P<0.05 rep-reSENTs a statistically significant difference.

**Results**

**Step-down Test**

Compared with Sham, VD rats showed obvious phenotypes of learning and memory impairments such as shortened latency and increased number of errors, while the learning and memory impairments of VD+Edaravone rats were significantly improved showing an increase in latency and a decrease in the number of errors (p<0.05). See Table 2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Learning</th>
<th>Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Latency(s)</td>
<td>Number of errors (times)</td>
</tr>
<tr>
<td>Sham</td>
<td>20</td>
<td>41.34±2.46</td>
<td>2.13±1.21</td>
</tr>
<tr>
<td>VD</td>
<td>20</td>
<td>21.11±4.10'</td>
<td>5.45±1.34'</td>
</tr>
<tr>
<td>VD+Edaravone</td>
<td>20</td>
<td>36.59±4.28'</td>
<td>3.12±1.22'</td>
</tr>
</tbody>
</table>

Table 2: Results of the step-down results in each group.

Note: Sham: sham operation; VD: vascular dementia; VD+E- daravone: vascular dementia+ Edaravone intervention, *compared with Sham, †compared with VD, p<0.05, there is a statistical difference.

**Y-maze Experiment**

Compared with the VD group, the Sham group showed a significant phenotype of learning and memory impairments such as a decrease in the number of correct responses and an increase in the cumulative number of shocks. After edaravone intervention, the rats’ learning and memory

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**Target gene**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Index sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GACATGCCGCTGGGAGAAC-3', 5'-AACCAGGGATGCCCTTTAGT-3'</td>
</tr>
<tr>
<td>Bax</td>
<td>5'-TGCTGCCCTTTCTGCTCCT-3', 5'-AAGTGCTGGGTAGGGAAGT-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5'-GCCACCGAACCCTAGGTC-3', 5'-ACACTTCTCCGAAAGTTCCA-3'</td>
</tr>
</tbody>
</table>

Table 1: Each index primer sequence in RT-PCR.
improvement was significantly improved, showing the number of times increased for correct response and the cumulative number of clicks decreased (p<0.05), as shown in Table 3.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Numbers</th>
<th>Learning (cumulative shocks (times))</th>
<th>Memory (correct responses (times))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>20</td>
<td>60.23±12.34</td>
<td>8.99±0.45</td>
</tr>
<tr>
<td>VD</td>
<td>20</td>
<td>145.34±23.11*</td>
<td>5.12±0.44*</td>
</tr>
<tr>
<td>VD + Edaravone</td>
<td>20</td>
<td>80.38±12.48*</td>
<td>7.59±0.81*</td>
</tr>
</tbody>
</table>

Table 3: Results of the Y-maze experiment results in each group.

Note: Sham: sham operation; VD: vascular dementia; VD+Edaravone: vascular dementia + Edaravone intervention, *compared with Sham, #compared with VD, p<0.05, there is a statistical difference.

**H&E Staining Results of Brain Tissue in Each Group**

As shown in Fig.1, the pyramidal neurons in the hippocampal CA1 area of the Sham group were arranged neatly with a complete nuclear nucleus and clear nucleoli. In the VD group, a variety of histological changes occurred in the hippocampus of CA1 such as atrophy or even disappearance of neurons, nuclear pyknosis and neurons arranged in a disordered and irregular pyramidal layer. The neuronal pathological damage in the brain tissue of the VD+Edaravone group was significantly reduced compared with the VD group.

**Results of Nissl Staining of Hippocampal Neurons in Each Group**

As shown in Figure 2, the results of Nissl staining showed that glial cell proliferation and Nissl body loss were observed in hippocampus of VD rats. But the number of glial cells in the rats’ hippocampus was significantly reduced, and the number of Nissl bodies was also significantly increased (p<0.05) after intervention with edaravone.

**TUNEL Staining on Rat Brain Tissue in Each Group**

In addition, we performed TUNEL staining on rat brain tissue. The results showed (Figure 3) that the apoptosis of rat brain tissue cells increased significantly after VD (p<0.05), and the number of apoptosis in rat brain tissue decreased to one-third of the VD group after edaravone intervention (p<0.05).
Expression Levels of Apoptosis-related Apoptosis Proteins in Rat Brain Tissue in Three Groups

In addition, we used immunoblotting to detect protein expression levels of apoptosis-related genes Bax and bcl-2 in the brain tissue of three groups of rats, and the results were consistent with RT-PCR. Edaravone can significantly inhibit the up-regulation of Bax and the down-regulation of bcl-2 by VD (p <0.05) (Figure 5).

Immunohistochemical staining of Bax and bcl-2 in hippocampus of rats in three groups

To visually observe the expression of Bax and bcl-2 in the rat hippocampus, we used the immunohistochemical technique to stain the brain tissue of three groups of rats. The results showed (Figure 6) that the Bax positive staining in the hippocampus of the VD group was deeper than the Sham group and the positive staining of bcl-2 was shallower than the Sham group, while the VD+Edaravone group was exactly the opposite. This result further confirms the results in Figures 5 and 6.

Effect of edaravone on PI3K/AKT signalling pathway in rat brain tissue

In order to further explore the mechanism of edaravone exerting neurological effects, we used immunoblotting to detect the expression of PI3K/AKT in the brain tissue of each group. The results showed (Figure 7) that the expression levels of PI3K and phosphorylated AKT (p-AKT) in the brains of the VD group were significantly down-regulated (p<0.05) while PI3K and p-AKT expression was inhibited after edaravone intervention (p<0.05).

Discussion

VD is defined as a progressive neurodegenerative disease with cognitive decline, the onset of which is primarily due to cerebrovascular factors. Numerous studies have shown that chronic cerebral hypoperfusion is closely related to the occurrence and the development of VD and Alzheimer's disease. In recent years, suitable VD animal models have been established to simulate the pathogenesis of clinical VD in experimental studies, which has
largely helped us understand the causal relationship between cerebral hypoperfusion and cognitive impairment\(^{(18)}\). Currently, the most widely used VD experimental model is the permanent occlusion of the bilateral common carotid arteries in rats\(^{(19)}\). A large number of animal experiments have revealed that learning and memory disorders caused by ischemia, abnormal cerebral blood flow, energy metabolism disorders and pathological damage of brain neurons are the key links in the development of VD\(^{(20)}\). Recent studies have shown that low cerebral perfusion can lead to a series destruction of homeostasis and further lead to apoptosis and necrosis of neuronal cells, eventually leading to a series of neurological disorders and even defects and other symptoms\(^{(21)}\).

Apoptosis, known as programmed cell death, refers to programmed cell death controlled by genes in order to maintain homeostasis under physiological or pathological conditions\(^{(22)}\). Studies have shown that apoptosis is closely related to a variety of neurological diseases, including Alzheimer's disease, VD, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis and stroke\(^{(23-25)}\). There are a large number of neuronal cells that re-express the cell in the brain tissue of patients with VD. These abnormal cell cycle neurons may accelerate the loss of neurons and further increase the nerve fibre tangles\(^{(26)}\). In addition, studies have reported that the number of glial cells in the hippocampus of patients with VD can be abnormally increased; and abnormally increased glial cells can secrete a variety of inflammatory factors (including IL-10 and TNF-α). Increased toxicity of neuronal cells may eventually aggravate the apoptosis of neurons in the hippocampus of patients with VD by inducing a series of cascades of inflammation cascades\(^{(27)}\). Apoptosis is a tightly regulated process involving changes in the expression of a diverse set of genes. The two currently recognized major genes regulating apoptosis include Bax and bcl-2\(^{(28)}\). Bcl-2 is a key member of the anti-apoptotic bcl-2 family and plays a crucial role in the regulation of mitochondria-mediated cell death. Up-regulation of bcl-2 protects nerve cells from neurotoxic damage. In contrast, Bax is a member of the pro-apoptotic family, which promotes cytochrome c release in mitochondria through mitochondrial translocation into the mitochondrial membrane, thereby promoting apoptosis. The ratio of Bax to bcl-2 was significantly increased during apoptosis\(^{(29-30)}\).

In this study, we constructed a rat VD model by using bilateral common carotid artery clamping at different time points and administered a dose of edaravone to VD rats by gavage, aiming at observation and discussion of the effect of edaravone on neurological function and neuronal apoptosis in VD rats. Results showed that edaravone can significantly improve the neurological function of VD rats caused by cerebral hypoperfusion and improve the memory function and learning ability of rats. In addition, molecular biology experiments revealed that edaravone can also inhibit VD-induced neuronal apoptosis in the hippocampus mainly by up-regulating the mRNA and protein levels of bcl-2, while down-regulating the mRNA and protein levels of Bax. Further, our study found that the neuroprotective effect of edaravone may be related to the activation of the PI3K/AKT signalling pathway in the brain tissue of VD rats.

Even so, our research still has some limitations:
- To explore the direct role of edaravone;
- The initiation pathway of apoptosis is not the same as neuronal apoptosis in VD. Whether the initiation is dependent on external pathways (such as Fas or tumour necrosis factor pathway) or mitochondrial pathways, these issues need to be further explored.

In conclusion, our study demonstrates that edaravone can ameliorate neurological abnormalities and neuronal apoptosis induced by vascular dementia in rats by activating the PI3K/AKT signalling pathway.

References


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