Promethazine inhibits neuronal apoptosis via PI3K/Akt signaling pathway in rats with cerebral infarction

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Abstract. – OBJECTIVE: To study the effect of promethazine on neuronal apoptosis in rats with cerebral infarction (CI) through the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signaling pathway.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly divided into the sham group (n=12), model group (n=12), and promethazine group (n=12). The external carotid artery was only exposed in the model group, and the ischemia-reperfusion model after CI was established using the suture method in the other two groups. Normal saline was intraperitoneally injected in the sham group and model group, while promethazine was intraperitoneally injected in the promethazine group. The rats were sampled after 1 week of intervention. The neurological deficits of rats were evaluated using the Zea-Longa score, and the cognitive function, the spatial learning and memory of rats were detected via the water maze test. Moreover, the expressions of B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) in brain tissues were detected via immunohistochemistry, and the relative protein expressions of PI3K p85, PI3K p110, and p-Akt were detected via Western blotting. The mRNA expressions of Bax and Bcl-2 were detected via quantitative Polymerase Chain Reaction (qPCR), and the apoptosis was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

RESULTS: The Zea-Longa score was significantly increased in the model group and promethazine group compared with that in the sham group (p<0.05), while it significantly declined in the promethazine group compared with that in the model group (p<0.05). The escape latency was significantly prolonged and the times of crossing platform were significantly reduced in the model group and promethazine group compared with those in the sham group (p<0.05), while the escape latency was significantly shortened and the times of crossing platform were significantly increased in the promethazine group compared with those in the model group (p<0.05). Compared with those in the sham group, the positive expression of Bax was significantly increased, while the positive expression of Bcl-2 was remarkably decreased in the model group and promethazine group (p<0.05). Compared with those in the model group, the positive expression of Bax was significantly decreased, while the positive expression of Bcl-2 was remarkably increased in the promethazine group (p<0.05). Besides, the model group and promethazine group had evidently higher relative protein expressions of PI3K p85, PI3K p110, and p-Akt than the sham group (p<0.05), while the promethazine group also had evidently higher relative protein expressions of PI3K p85, PI3K p110, and p-Akt than the model group (p<0.05). Compared with those in the sham group, model group, and promethazine group had remarkably increased relative mRNA expression of Bax, and remarkably decreased relative mRNA expression of Bcl-2 (p<0.05). Compared with those in the model group, the relative mRNA expression of Bax was remarkably decreased, while the relative mRNA expression of Bcl-2 was remarkably increased in the promethazine group (p<0.05). Finally, the apoptosis rate was significantly higher in
the model group and promethazine group than that in the sham group ($p<0.05$), while it was significantly lower in the promethazine group than that in the model group ($p<0.05$).

**CONCLUSIONS:** Promethazine inhibits neuronal apoptosis in CI rats by upregulating the PI3K/Akt signaling pathway, thereby exerting a protective effect.

**Key Words:** Promethazine, Cerebral infarction, PI3K/Akt signaling pathway, Apoptosis.

**Introduction**

Cerebral infarction (CI), as a clinically common acute cerebrovascular disease, has become one of the diseases seriously threatening human life and health. It is currently believed that the damage to the neurological function caused by CI severely affects the limb motor function of patients in mild cases and leads to the death of patients in severe cases, so the disability and mortality rates of CI are extremely high$^{1,2}$. According to epidemiological statistics, the morbidity rate of CI is increasing with the aging of the population in the world and changes in people’s lifestyles, and approximately 75% of patients suffer from sequelae caused by neurological deficits$^{1,3}$.

In other words, CI is characterized by high morbidity, disability, and mortality rates and high incidence of sequelae, making it a disease that seriously affects the lives of medical workers and other people. Therefore, how to effectively prevent CI, reduce the sequelae and death caused by neurological deficits after CI is of great significance$^{3,4}$.

Currently, it is believed that the pathological responses after CI are very complicated, including a series of cascade reactions, such as inflammation, lipid peroxidation, and release of free radicals, all of which can lead to neuronal apoptosis, necrosis, and other pathological results, thereby affecting neurological repair and leading to neurological deficits$^{5,6}$. In particular, the degree of neuronal apoptosis, as one of the most important pathological responses after CI, can determine the degree and scope of CI injury and the degree of neurological deficits in patients. Therefore, early effective anti-neuronal apoptosis is considered as one of the key steps in the treatment of CI.

The phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signaling pathway is an important cell signal transduction pathway, which plays an important regulatory role in such processes as cell proliferation, apoptosis, and necrosis, and has an important influence on the repair and reconstruction of neurological injury$^{7,8}$. It is now thought that the PI3K/Akt signaling pathway, as a classical anti-apoptotic pathway, can effectively reduce the incidence of apoptosis after injury. In particular, the PI3K/Akt signaling pathway in brain neurons is activated after CI, which can reduce the degree of neuronal apoptosis to play a protective role$^{9}$. As a commonly used sedative drug in clinics, promethazine functions as a H1 receptor blocker, exerting a certain protective effect on neuronal apoptosis, but its mechanism of action remains unclear. In this experiment, therefore, the protective mechanism of promethazine on neuronal apoptosis in CI rats through the PI3K/Akt signaling pathway was explored.

**Materials and Methods**

**Laboratory Animals**

A total of 36 specific pathogen-free Sprague-Dawley (SD) rats aged 1 month old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license No. SCXK (Shanghai, China) 2014-0003], and they were fed with normal feed and sterile filtered water every day in the Laboratory Animal Center under 12/12 h light-dark cycle, room temperature, and regular humidity. This research was approved by the Animal Ethics Committee of Shandong University Animal Center.

**Laboratory Reagents and Instruments**

The main reagents and instruments used were: promethazine hydrochloride injection (KingYork, Tianjin, China), anti-PI3K p110 antibody (Abcam, Cambridge, MA, USA), anti-PI3K p85 antibody (Abcam, Cambridge, MA, USA), anti-p-Akt antibody (Abcam, Cambridge, MA, USA), anti-B-cell lymphoma-2 (Bcl-2) antibody (Abcam, Cambridge, MA, USA) and anti-Bcl-2 associated X protein (Bax) antibody (Abcam, Cambridge, MA, USA), secondary antibodies (Abcam, Cambridge, MA, USA), immunohistochemistry kit and AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix Kit (Vazyme, Nanjing, China), HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China), optical microscope.
Animal Grouping and Treatment

The above 36 SD rats were randomly divided into the sham group (n=12), model group (n=12), and promethazine group (n=12) using a random number table. The rats were adaptively fed in the Laboratory Animal Center for 7 d before experiments.

The common carotid artery, external carotid artery, and internal carotid artery were exposed only in the sham group, and the CI model was established in the model group and promethazine group. After the operation, the promethazine hydrochloride injection was intraperitoneally injected (7.5 mg/kg) every day in the promethazine group, while an equal amount of normal saline was intraperitoneally injected every day in the sham group and model group. The rats were sampled after 7 d of intervention in each group.

Establishment of CI Model

After successful anesthesia via intraperitoneal injection of 10% chloral hydrate (3 mL/kg), the rats were fixed in a supine position, and the hair on the neck was shaved off, followed by disinfection and draping. An about 2 cm-long longitudinal incision was made in the median line of the neck, and the common carotid artery, external carotid artery, and internal carotid artery were carefully separated and exposed. The common carotid artery and external carotid artery were ligated with a silk thread, and the internal carotid artery was clamped with vascular forceps. The suture was inserted from the ligation site of the common carotid artery, the vascular forceps at the internal carotid artery were released, and the suture was slowly pushed into the branch of the middle cerebral artery. The internal carotid artery was ligated again, the suture was fixed, and the incision was washed with normal saline and sutured. Finally, the suture was slowly withdrawn after the vascular obstruction for 90 min.

Sampling

After successful anesthesia, the brain tissues were directly taken from 6 rats in each group, washed with normal saline and stored in the Eppendorf (EP; Eppendorf, Hamburg, Germany) tube at -80°C for later use. The samples were taken via perfusion fixation from the remaining 6 rats in each group: the skin was cut to expose the brain, and 400 mL of 4% paraformaldehyde was perfused. Then, the brain tissues were taken and fixed in 4% paraformaldehyde.

Zea-Longa Score and Morris Water Maze Test

After intervention for 2 weeks, neurological deficits were evaluated using the Zea-Longa score according to the symptoms and behaviors of the rats (Table I).

After the last intervention, the rats were placed in a water maze and allowed to walk freely. The escape latency and the times of crossing platform in the water maze were recorded. Then, the platform in the water maze was removed, and the rats were placed in the water maze at any point and allowed to walk freely. The times of crossing the original platform within 70 s and the residence time in the original quadrant were recorded.

Immunohistochemistry

The paraffin-embedded tissues were sliced into 5 μm-thick sections, flattened, and fished up in warm water at 42°C, baked, and prepared into paraffin sections. Then, the sections were soaked and routinely deparaffinized in xylene solution and gradient alcohol, placed in the citric acid buffer, and repeatedly heated in a microwave for 3 times (3 min/time, braised for 5 min each time) for complete antigen retrieval. After the sections were washed, the endogenous peroxidase blocker was added dropwise for the reaction for 10 min. Then, the sections were washed again and sealed with goat serum for 20 min. After the goat serum was discarded, the anti-Bax (1:200) and anti-Bcl-2 (1:200) primary antibodies were added for incubation in a refrigerator at 4°C overnight.

Table I. Zea-Longa score.

<table>
<thead>
<tr>
<th>Score</th>
<th>Symptom</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No neurological deficits</td>
</tr>
<tr>
<td>1</td>
<td>Mild: The right fore paw cannot be fully stretched in tail suspension</td>
</tr>
<tr>
<td>2</td>
<td>Moderate: The body turns to the right while walking</td>
</tr>
<tr>
<td>3</td>
<td>Severe: The body leans to the right while walking</td>
</tr>
<tr>
<td>4</td>
<td>Unable to walk spontaneously, with loss of consciousness</td>
</tr>
</tbody>
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On the next day, the sections were washed, reacted with secondary antibodies for 10 min, fully washed, and reacted with the streptavidin-peroxidase solution for 10 min, followed by color development with diaminobenzidine (DAB; Solarbio, Beijing, China). Finally, the nuclei were counterstained with hematoxylin, and the sections were sealed and observed.

**Western Blotting**

The cryopreserved brain tissues were added with lysis buffer, subjected to ice bath for 1 h, and centrifuged in a centrifuge at 14,000 g for 10 min. The protein was quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The absorbance of protein was detected using a microplate reader, the standard curve was plotted, and the protein concentration was calculated basing on this curve. After protein denaturation, the protein was separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the position of the Marker protein was observed. The electrophoresis was terminated when the Marker protein reached the bottom of the glass plate in a straight line. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with the sealing buffer for 1.5 h, and incubated with the anti-PI3K p110 (1:1000), anti-PI3K p85 (1:1000), and anti-p-Akt (1:1000) primary antibodies and secondary antibodies (1:1000). After the membrane was washed, the image was fully developed using the chemiluminescent reagent for 1 min.

**QPCR**

The total RNA was extracted from brain tissues using an RNA extraction reagent, reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit. The qPCR system (20 μL) was designed, and the reaction conditions were as follows: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 s, denaturation at 95°C for 10 s, annealing at 62°C for 30 s, for a total of 35 cycles. The ΔCt value was calculated first and then the difference in the expression of the target gene was calculated. The primer sequences are shown in Table II.

**TUNEL Assay**

The apoptosis in brain tissues was detected according to the instructions of the TUNEL apoptosis kit.

**Statistical Analysis**

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

**Results**

**Zea-Longa Score**

As shown in Figure 1, the Zea-Longa score was the lowest in the sham group and the highest
in the model group. The Zea-Longa score was significantly increased in the model group and promethazine group compared with that in the sham group, and there were statistically significant differences \((p<0.05)\), while it significantly declined in the promethazine group compared with that in the model group, showing a statistically significant difference \((p<0.05)\).

**Water Maze Test**

As shown in Figure 2, the escape latency was significantly prolonged and the times of crossing platform were significantly reduced in the model group and promethazine group compared with those in the sham group, and there were statistically significant differences \((p<0.05)\), while the escape latency was significantly shortened and the times of crossing platform were significantly increased in the promethazine group compared with those in the model group, displaying statistically significant differences \((p<0.05)\).

**Immunohistochemical Detection**

As shown in Figure 3, the dark brown indicated a positive expression. The positive expression of Bax was lower, and the positive expression of Bcl-2 was higher in the sham group, but we detected the opposite situation in the model group. According to the statistical results (Figure 4), compared with those in the sham group, the mean optical density of Bax was significantly increased, while that of Bcl-2 significantly declined in the model group and promethazine group, and the differences were statistically significant \((p<0.05)\). Besides, compared with that in the model group, the mean optical density of Bax significantly declined, while that of Bcl-2 was significantly increased in the promethazine group, showing statistically significant differences \((p<0.05)\).

**MRNA Expression Detected Via QPCR**

Compared with those in the sham group, the relative mRNA expression of Bax was remarkably increased, while the relative mRNA expression of Bcl-2 was remarkably decreased in the model group and promethazine group, and there were statistically significant differences \((p<0.05)\). Compared with those in the model group, the relative mRNA expression of Bax was remarkably decreased, while the relative mRNA expression of Bcl-2 was remarkably increased in the promethazine group, and there were statistically significant differences \((p<0.05)\) (Figure 7).

**Apoptosis Detected Via TUNEL Assay**

The apoptosis rate was significantly higher in the model group and promethazine group than that in the sham group, and the difference was statistically significant \((p<0.05)\). The apoptosis rate was significantly lower in the promethazine group than that in the model group, and the difference was statistically significant \((p<0.05)\) (Figure 8).
Figure 3. Immunohistochemical detection (×200).

Figure 4. Mean optical density of positive expression in each group Note: *p<0.05 vs. sham group, †p<0.05 vs. model group.

Figure 5. Protein expressions detected via Western blotting.
Discussion

As a common cardio-cerebrovascular disease in the elderly, CI is characterized by high morbidity, disability, mortality, and recurrence rates, making it one of the major diseases threatening human life and health. The pathological responses after CI are very complicated. It is currently believed that apoptosis, namely programmed cell death, plays an important role in the pathological responses after CI. After CI, ischemia, and hypoxia of local brain tissues can rapidly activate the neuronal apoptosis pathway, leading to abnormal expressions of Bax and Bcl-2. Under the influence of the injury factors and ischemia-hypoxia environment, the expression of Bax (a pro-apoptotic gene) is abnormally high in brain tissues, while that of Bcl-2 (an anti-apoptotic gene) is abnormally low, both of which act on the downstream apoptotic effector molecule Caspase3, ultimately resulting in an increase in neuronal apoptosis. In particular, apoptosis is one of the most important pathological responses for nerve cells in the peripheral region of injury. In addition, the combined effects of many pathological responses after injury, such as inflammation, lipid peroxidation, and amino acid toxic reaction, further aggravate the neuronal apoptosis. Therefore, effectively reducing the degree of neuronal apoptosis after CI can effectively protect nerve cells, benefiting the repair of the nervous system after CI.

It has been confirmed in studies that the PI3K/Akt signaling pathway, an important cell signal transduction pathway, plays an important regulatory role in cell proliferation, differentiation, apoptosis, and necrosis and other pathophysiological processes. Phosphoinositol triphosphate as a second messenger can further
activate and phosphorylate Akt to be p-Akt, thus exerting an effect of signal transduction, which plays an important regulatory role in the gene transcription and protein translation of the downstream Bax and Bcl-2, so that the downstream apoptotic effector molecule Caspase-3 is controlled to exert an anti-apoptotic effect. The results of the present study further confirmed that after CI, the expressions of Bax and Bcl-2 in the injury area were significantly abnormal, and there was a high expression of Bax and low expression of Bcl-2, aggravating neuronal apoptosis. At the same time, the neuronal PI3K/Akt signaling pathway was activated under the injury stimulus, and there were more p-p85, p-p110, and p-Akt, indicating that the PI3K/Akt signaling pathway is activated in the body after CI, thereby resisting neuronal apoptosis.

According to further studies, in CI rats in the promethazine group, the Zeal-Longa score was reduced, the escape latency was significantly shortened, and the times of the crossing platform were significantly increased after promethazine intervention, suggesting that promethazine can well improve the neurological deficits and cognitive dysfunction after CI. At the same time, CI rats in the promethazine group had decreased expression of Bax, increased expression of Bcl-2, and lowered apoptosis rate after promethazine intervention. Moreover, the key molecules in the PI3K/Akt signaling pathway were detected, and it was found that the phosphorylation of p85, p-p110, and p-Akt could significantly upregulate the phosphorylation of p85, p-p110, and p-Akt, indicating that promethazine can significantly upregulate the PI3K/Akt signaling pathway. Therefore, it can be concluded that promethazine inhibits neuronal apoptosis in CI rats by upregulating the PI3K/Akt signaling pathway, thereby exerting a protective effect.

Conclusions

We showed that promethazine inhibits neuronal apoptosis in CI rats by upregulating the PI3K/Akt signaling pathway, thereby exerting a protective effect.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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