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# 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 [illegible] B (PI3K/Akt) signaling pathway.

**MATERIALS AND METHODS:** A total [illegible]36 Sprague-Dawley rats were randomly divide[illegible] to the sham group (n=12), model group (n=[illegible] and promethazine group (n=12). The extern[illegible] carotid artery was only expo[illegible]e mode[illegible] group, and the ischemia-re[illegible]asio[illegible]odel after CI was established usi[illegible]e sut[illegible]method in the other two groups. [illegible]model[illegible] mal saline was intrap[illegible]rite[illegible], i[illegible]te[illegible] sham group and m[illegible] gro[illegible]e promethazine was intrape[illegible]eally inj[illegible]in the promethazine grou[illegible] rats were [illegible]ed after 1 week of int[illegible]. The neurol[illegible]al deficits of rats w[illegible] eva[illegible]l using the Zea-Longa score, a[illegible]d the cogni[illegible]action, the spatial learning [illegible] memory of ra[illegible]e detected *via* the wa[illegible]aze test. Moreove[illegible]e expressions of B-[illegible]ympho[illegible]2 (Bcl-2) and Bcl-2 associated [illegible]otein ([illegible]) in brain tissues were detected w[illegible]m[illegible]istoch[illegible]stry, and the relative protein[illegible]ssions [illegible]3K p85, PI3K p110, and [illegible] wer[illegible]ect[illegible]*ia* Western blotting. The [illegible] expre[illegible]of Bax and Bcl-2 were detect[illegible]*via* quan[illegible]tive Polymerase Chain Reaction[illegible]CR), and the apoptosis was detected *via* t[illegible]nucleotidyl transferase-mediated P nick end labeling (TUNEL) assay.

**RESULTS:** The Zea-Longa score was signifi[illegible] increased in the model group and pro[illegible]e group compared with that in the sham group ($p<0.05$), while it significantly declined in the promethazine group compared w[illegible]hat in th[illegible]odel group ($p<0.05$). The es[illegible]late[illegible]was significantly prolonged and t[illegible]f crossing platform were significantly reduced in the model group and [illegible]ethazine group compared with those in [illegible] group ($p<0.05$), while the escape [illegible]te[illegible] 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 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



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**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 epidem- ical statistics, the morbidity rate of CI h increasing with the aging of the population he world and changes in people's lifestyles, an proximately 75% of patients suffer from seque caused by neurological deficits a CI. In oth words, CI is characterized l orbidity, disability, and mortality rat d hig cidence of sequelae, making it a p diseas medical workers and rese to effectively preven at C duce the sequelae and death d by neu al deficits after CI is of gr ificance[3,4].

Currently, d that the patholog- ical respon es after C very complicated, including eries of casca actions, such as inflam on, lipid peroxidati , and release of free cals, a which can lead to neuronal ap s, n s, and other pathological results, affecti eurological repair and lting urolo deficits[5,6]. In particular, gree onal apoptosis, as one of the st impor pathological responses after CI mines the degree and scope of CI injury a of neurological deficits in patients. erefore, early effective anti-neuronal apoptosis nsidered as one of the key steps in the treatm 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, a and necrosis, and has an importa on the repair and reconstruction urological injury[7,8]. It is now thought th PI3K/Akt signaling pathway, as a classica poptotic pathway, can effectively r ce the e of apoptosis after injury. In cular, the signaling pathway in h neuro is acti after CI, which can r e the d e of neuronal apoptosis to play a p tiv ie[9]. As commonly used seda drug tic, pr azine functions as t 1 recepto ake xerting a certain prot effect on n apoptosis, but its m ism action rema s unclear. In this exper ment, th e, the protective mechanism promethazi euronal apoptosis in CI ough the PI3K t signaling pathway explored.

## Materials and Methods

### Laboratory Animals

A total of 36 specific pathogen-free Dawley (SD) rats aged 1 month old ere p chased 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 SμperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China), optical microscope

(Leica DMI 4000B/DFC425C, Wetzlar, Germany), and fluorescence qPCR instrument (ABI 7500, Foster City, CA, USA).

### 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* intraperi[tone]al injection of 10% chloral hydrate (3 mL/[kg]) rats were fixed in a supine position, and th[e] hair on the neck was shaved off, followed by disi[nfec]tion and draping. An about 2 cm-long longitu[dinal] incision was made in the m[idd]le line of th[e] neck, and the common caro[tid] artery, external carotid artery, and interna[l] carotid a[rte]ry were carefully separated and e[xpo]sed. Th[e] common carotid artery and exter[nal] carotid artery were ligated with a silk th[read], and the internal carotid artery was clamp[ed] with vascular forceps. The suture was inse[rted] from the ligatio[n] of the common caro[tid] artery, the vascular forceps at the internal carotid arter[y] were released, and the suture w[as] slowly pushed [to] 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, 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 an 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 skull was cut open to expose the brain, and 400 mL 4% paraformaldehyde was perfused. Then, the brain tissues were taken and fixed in 4% paraformaldehyde.

### Zea-Longa Scoring 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 rats (Table I).

The last intervention, the rats were placed in the 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 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** Zea-Longa score.

| | Symptom |
|---|---|
| | No neurological deficits |
| | Mild: The right fore paw cannot be fully stretched in tail suspension |
| | Moderate: The body turns to the right while walking |
| 3 | Severe: The body leans to the right while walking |
| 4 | Unable to walk spontaneously, with loss of consciousness |

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. [illegible] the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with the sealing b[illegible] for 1.5 h, and incubated with the anti-PI3K p[illegible] (1:1000), anti-PI3K p85(1:1000) [illegible] anti-p-A[illegible] (1:1000) primary antibodies a[illegible]ry antibodies (1:1000). After the m[illegible]rane w[illegible]washed, the image was fully deve[illegible] using [illegible] minescent reagent for 1 m[illegible]

### QPCR

The total R[illegible]s extracted [illegible] brain tissues using [illegible]R[illegible]xtraction reagent, reversely tra[illegible]bed into [illegible]plementary deoxyribose n[illegible]c acid (cDN[illegible]ing the reverse transcr[illegible]n kit. The qPCR s[illegible]em (20 μL) was desi[illegible], and [illegible]reaction conditions were as fol[illegible] reac[illegible]at 53°C for 5 min, pre-denaturation [illegible]or 10 [illegible], denaturation at 95°C [illegible]10 s, [illegible]ling [illegible]2°C for 30 s, for a total [illegible]ycles. [illegible] value was calculated first and [illegible]n, the d[illegible]ence in the expression of the tar[illegible]e was calculated. The primer sequenc[illegible] Table II.

### [illegible]NEL Assay

[illegible]apoptosis in brain tissues was detected acc[illegible]ing to the instructions of the TUNEL apoptosis kit.

**Table II.** Primer sequences.

| Gene | Primer sequence |
|---|---|
| Bax | F: 5'-GTGGCGGGACATAGTCA[illegible]<br>R: 5'-CCCATTGGGACAGCT[illegible]A-3' |
| Bcl-2 | F: 5'-AACCTTCCTTCCTC[illegible]AG-3'<br>R: 5'-TGCTGTTCCTCTTCC[illegible]' |
| GAPDH | F: 5'-ACGGCAAGTT[illegible]ACGG[illegible]'<br>R: 5'-GAAGACGC[illegible]AGACTC[illegible]-3' |

### Statistical Analysis

The Statistical [illegible]odu[illegible]Servic[illegible]utions (SPSS) 20.0 s[illegible]are (IBM [illegible]Armonk, NY, USA) was [illegible] statistical a[illegible]s. The enumeration [illegible]w[illegible]pressed as m[illegible]an ± standard deviation. [illegible]he *t*-tes[illegible]used for the data in line with [illegible]al distribu[illegible]nd homogeneity of va[illegible], while the corre[illegible] *t*-test was used for t[illegible]ata in line with normal distribution and heterogeneity of v[illegible]nce, and the non-parametric [illegible] for the data [illegible]in line with normal distribution and homog[illegible]ty of variance. The rank-sum test [illegible]or the ranked data, and the Chi-square te[illegible]as 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

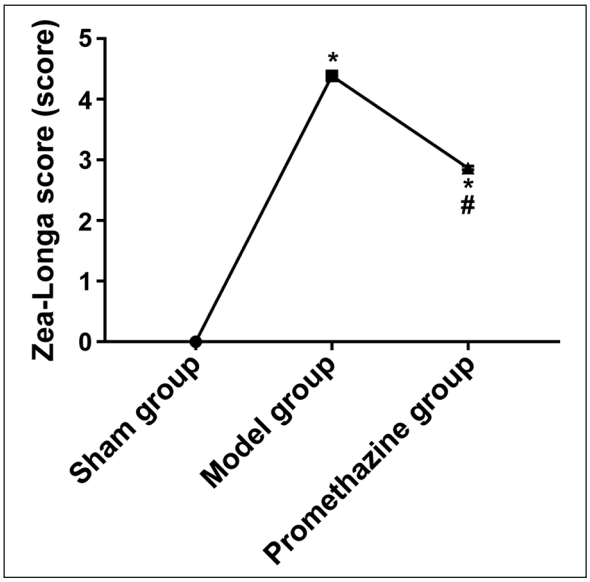


**Figure 1.** Zea-Longa score in each group. Note: *$p$<0.05 *vs.* sham group, $^{\#}p$<0.05 *vs.* model group.

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 in[illegible] ed a positive expression. The positive exp[illegible] of Bax was lower, and the positive expres[illegible] of Bcl-2 was higher in the sham group, but we detected the opposite situation in the model group. According to the statistical results (Fi[illegible] compared with those in the sham [illegible]p, mean optical density of Bax wa[illegible]gnificantly increased, while that of Bcl-2 [illegible]icantly declined in the model group and [illegible]ethazine group, and the differences [illegible]e stati[illegible]y significant ($p$<0.05). Beside[illegible]mpared w[illegible] in the model group, th[illegible]an opt[illegible]al densi[illegible] Bax significantly de[illegible]ed, wh[illegible]hat of Bcl-2 was significantly inc[illegible]d i[illegible]e prom[illegible]zine group, showing [illegible]tisti[illegible]gnific[illegible] differences ($p$<0.05).

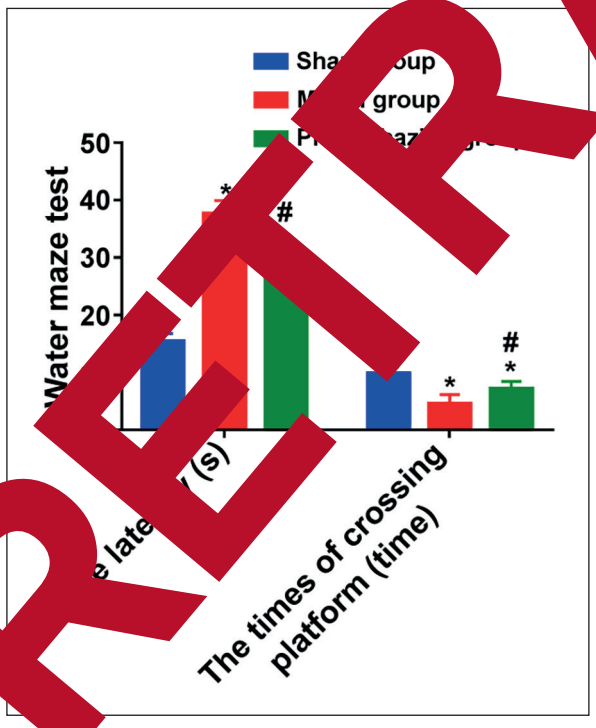


**Figure 2.** Water maze test in each group. Note: *$p$<0.05 *vs.* sham group, #$p$<0.05 *vs.* model group.

### Relative [illegible]te[illegible] Expressions [illegible]etected Via Wes[illegible]rn Bl[illegible]

The [illegible]tein expres[illegible]of p-Akt, PI3K p85, an[illegible]p110 were low[illegible]n the sham group, [illegible]e they were higher in the promethazine group [illegible]ure 5). Acc[illegible]ing to the statistical results [illegible]ure 6), the [illegible]el group and promethazine g[illegible] had sig[illegible]antly higher relative protein exp[illegible]3K p85, PI3K p110, and p-Akt than the [illegible] group and the differences were statistically significant ($p$<0.05), while the promethazine group also had significantly higher relative [illegible]ote[illegible]xpressions of PI3K p85, PI3K p110, and p-Akt than the model 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).

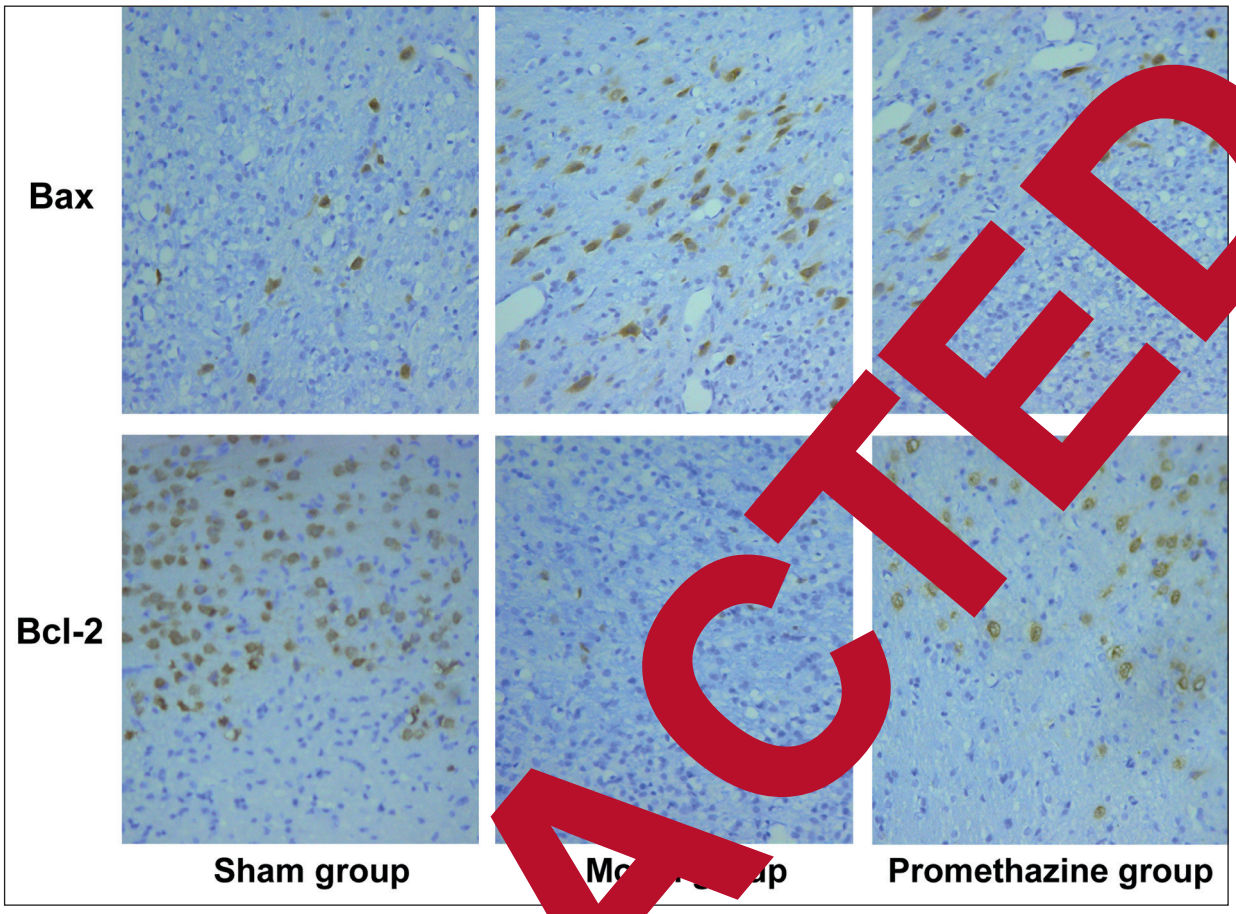


**Figure 3.** Immunohistochemical detection (×200).

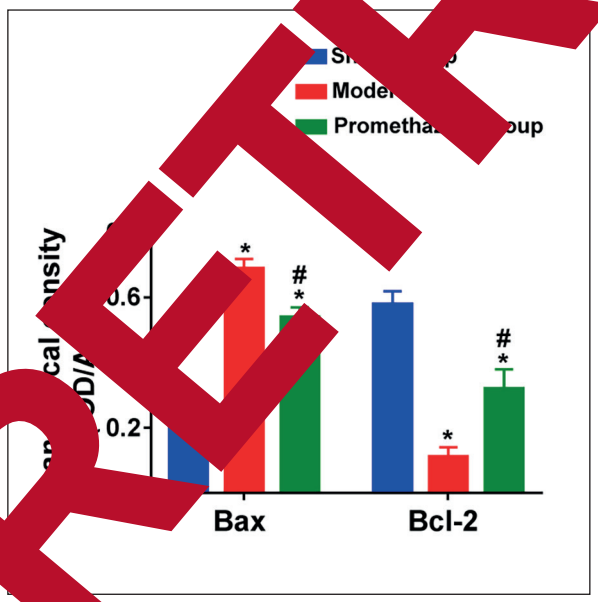


**Figure 4.** Mean optical density of positive expression in each group Note: *$p$<0.05 *vs.* sham group, #$p$<0.05 *vs.* model group.

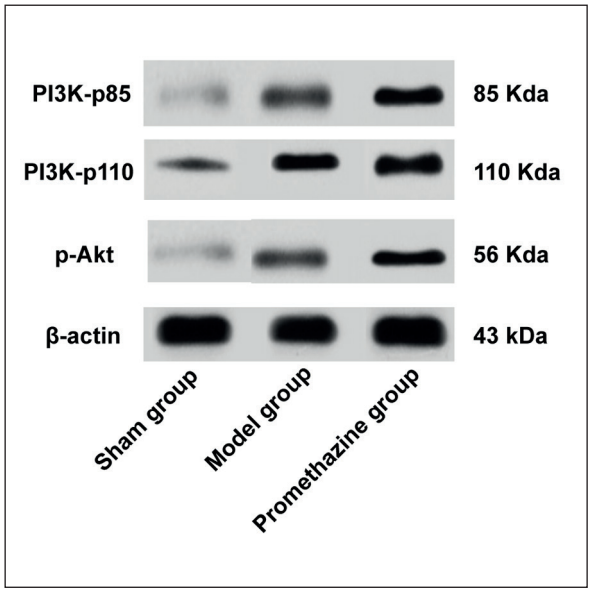


**Figure 5.** Protein expressions detected *via* Western blotting.

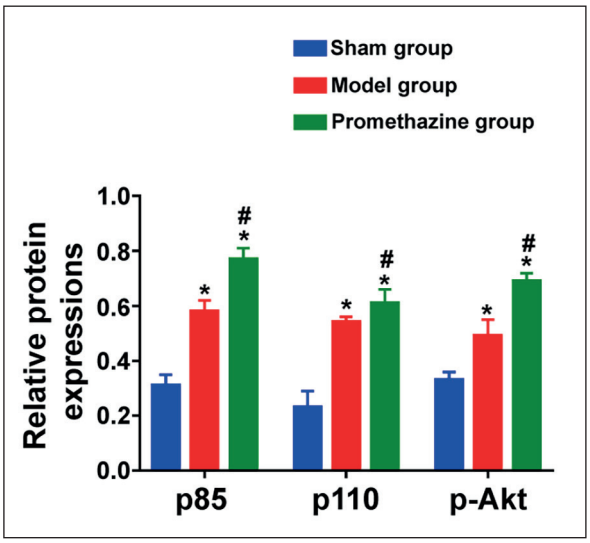


**Figure 6.** Relative protein expressions in each group. Note: **p*<0.05 *vs.* sham group, #*p*<0.05 *vs.* model group.

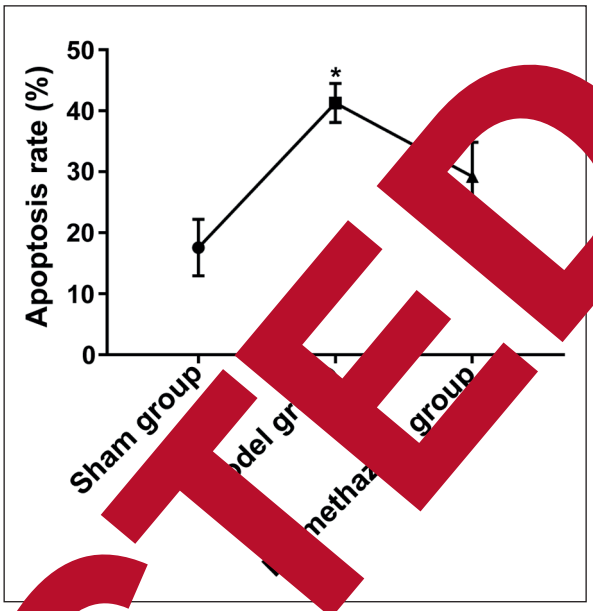


**Figure 8.** Apoptosis rate in each group. Note: **p*<0.05 *vs.* sham group, #*p*<0.05 *vs.* model group.

## 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 brain tissues can rapidly activate the neuronal apoptosis pathway, leading to abnormal expressions of Bax and Bcl-2[10,11]. 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[12,13]. 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, benefitting the repair of the nervous system after CI[14].

It has been confirmed in studies[15,16] 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

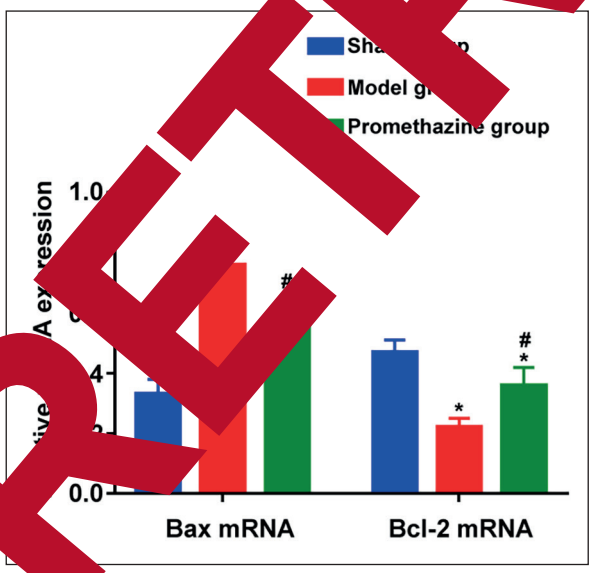


**Figure 7.** Relative mRNA expression in each group. Note: **p*<0.05 *vs.* sham group, #*p*<0.05 *vs.* model group.

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[17]. 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 ... rological deficits and cognitive dysf... after CI. At the same time, CI rats ... promethazine group had decreased exp... sion of Bax, increased expression of Bc... and lowered apoptosis rate aft... methazi... intervention. Moreover, the ... cules in... the PI3K/Akt signaling p... ay w... detected, and it was found ... the pr... could significantly u... th... lation of p85, p110 ... Ak... n indicates that promethazin... ses the a... ion of the PI3K/Akt signa... thway. The... it can be concluded ... t p... thazine inhibits neuronal apop... in CI r... upregulating the PI3K/Ak... naling pathw... hereby exerting a prote... e effect.

## Con... ions

... show... romethazine inhibits neuron... poptosis ... CI rats by upregulating the PI3... t signaling pathway, thereby exerting a n... t.


### Conflict of Interest

The Authors declare that they have no conflict of interests.

### Funding Acknowledgements

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## Referen...


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