

## Original Research Article

# Probucol reduces the cerebral edema area and infarction volume in rat cerebral infarction model via PI3K/Akt pathway

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### Abstract

**Purpose:** To study the effects of probucol on rats with cerebral infarction through the phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (Akt) pathway.

**Methods:** Sprague-Dawley (SD) rats were divided into sham group (SO group, n = 7), model group (MO group, n = 7) and probucol group (PR group, n = 7). Infarction volume, messenger ribonucleic acid (mRNA), protein expressions of PI3K/Akt, neurological score, brain water content, degree of brain tissue lesions and neurological function score were determined.

**Results:** Neurological score was 0,  $2.54 \pm 0.67$  and  $1.34 \pm 0.21$  points, in SO, O and PR groups, respectively. In turning angle test, neurological function score gradually rose at 24 h after cerebral infarction in PR and MO groups, compared with that in the SO group ( $p < 0.05$ ), but significantly declined at 48 h in PR group compared with that in MO group ( $p < 0.05$ ). Brain water content was lowest in the SO group but highest in MO group; it was significantly lower in PR group than that in MO group ( $p < 0.05$ ). The mRNA and protein expressions of PI3K/Akt were highest in SO group and lowest in MO group; the expressions were higher in PR group than those in the MO group ( $p < 0.05$ ).

**Conclusion:** Probucol reduces the cerebral edema area and infarction volume by activating PI3K/Akt pathway, thereby exerting a significant therapeutic effect on rat model with cerebral infarction. Thus, this agent has the potential for use in the management of cerebral infarction.

**Keywords:** Probucol, Signaling pathway, Cerebral infarction, Molecular mechanism

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## INTRODUCTION

Cerebral infarction is a brain disease in clinics. In terms of its pathogenesis, the cerebral artery plaques continuously induce hypoxemia in brain tissues. Moreover, the emboli enters the cerebral vessels via circulation in some patients, so the

cerebral blood supply becomes disordered and the blood flow declines, thus leading to insufficient oxygen and blood supply to brain tissues [1]. With advances in medical science and an increase in the aging population, the number of patients with cerebral infarction has constantly been on the increase. According to

reports, about 2.3 million patients suffer from cerebral infarction-induced stroke in China every year, with a mortality rate of 50 %. So life expectancy of such patients cannot be guaranteed [2].

Currently, cerebral infarction is treated based on the specific disease conditions. Although the disease is alleviated to a certain extent, if the treatment opportunity of patients is delayed, the risk of exacerbation increases [3,4]. Probuocol is a kind of commonly-used drug for patients with cerebral infarction, which can enter the body and directly act on pL20-catenin/ $\alpha$ N-catenin, so as to avoid damage to neurogenesis, repair damaged nerves, accelerate the treatment of cerebral infarction, and minimize the impact of the disease [5].

Following the in-depth research on neurological function recovery in cerebral infarction, phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (Akt) signaling pathway has been newly discovered, and it is closely related to the neuronal development, reproduction and apoptosis in brain tissues. However, there is little literature about it [6]. In this paper, therefore, the percentage of infarction volume, messenger ribonucleic acid (mRNA) and protein expressions of PI3K/Akt, degree of brain tissue lesions, neurological score, brain water content, and neurological function score were detected in sham-operation group (SO group), model group (MO group) and probuocol group (PR group), so as to explore the effects of probuocol on cerebral infarction rats through the PI3K/Akt pathway.

## EXPERIMENTAL

### Animals

A total of 21 male Sprague-Dawley (SD) rats (250 - 300 g, 8 - 10 weeks old) were purchased from Guangdong Medical Laboratory Animal Center, and they were adaptively fed for 1 week before the experiment. This study was approved by the Animal Ethics Committee of School of Medicine, University of Electronic Science and Technology of China Animal Center (approval no. AEC#17-No.0325). All procedures were conducted in accordance with 'Animal Research: Reporting in Vivo Experiments guidelines 2.0' [7].

### Animal modeling and grouping

The rats were divided into three groups. In SO group (n = 7), the common carotid artery, internal carotid artery and external carotid artery were separated but not ligated, and the rats were fed

normally. In MO group (n = 7), the rat model of cerebral infarction was established. In PR group (n = 7), probuocol was given for treatment after the establishment of rat model of cerebral infarction. After all rats were anesthetized with 1% pentobarbital sodium. The skin of each rat was peeled off in a prone position, a median incision was made on the head, and the common carotid artery, internal carotid artery and external carotid artery were separated. The external carotid artery was ligated at the distal end, and the common carotid artery and internal carotid artery were clamped using the bulldog clamp. Then the occlusion line was inserted, the bulldog clamp was released, and the occlusion line was pushed 16 - 18 mm forward until there was resistance. After 2 h, the rats were anesthetized and the occlusion line was removed. According to the Zea-Longa score system [6], 1-3 points indicate the successful modeling. At the same time, the rats in PR group were intraperitoneally injected with probuocol (3 mg/kg) twice a day for 1 week. In SO group and MO group, the rats were injected with an equal amount of normal saline.

### Equipment and reagents

Enzyme-linked immunosorbent assay (ELISA) kits were purchased from MLBio (Shanghai, China). A refrigerated high-speed centrifuge was purchased from Beckman Coulter. An Olympus IX 71 microscope was provided by Olympus (Tokyo, Japan), and hematoxylin was provided by Micxy (Chengdu, China).

### Determination of brain water content

After the rats were sacrificed through cervical dislocation, the brain was quickly taken, and the frontal pole was excised. Then, the brain was serially sectioned at a thickness of 2 mm, and the brain water content was determined using wet/dry weight method. Brain water content was computed as in Eq 1.

$$\text{BWC (\%)} = \{(Ww - Dw)/Ww\}100 \dots\dots\dots (1)$$

where Ww and Dw are the wet and dry weights of brain tissue, respectively.

### Neurological score

The Zea-Longa neurological score format was given in each group (Table 1).

### Assessment of neurological function score

At 24 h before operation and at 24, 48 and 72 h after operation, the degree of neurological deficit was evaluated using the turning angle test. The

rats were placed in a space at an angle of 30°, and the direction of turning angle of rats was monitored. The test was repeated every 30 s for a total of 10 min. Neurological function score (NF) was calculated as in Eq 2.

$$NF (\%) = (NRT/1^0)100 \dots\dots\dots (2)$$

where NRT is the number of right turns.

**Table 1:** Neurological score

Score	Performance
0	Act normally
1	Unable to fully stretch the contralateral forepaw
2	Paralysis of right paws
3	The body leans rightwards when crawling and cannot stand firm
4	Loss of consciousness or inability to walk spontaneously

#### Evaluation of infarction volume via 2,3,5-triphenyltetrazolium chloride (TTC) staining

The rats were sacrificed by cervical dislocation, and the whole brains were excised quickly and serially sectioned at a thickness of about 2 mm. The sections were inserted in a water bath in 0.5 % TTC solution at 37 °C for 30 min, fixed with 4 % paraformaldehyde for 24 h, and photographed using a digital camera. The infarction area in each section was measured using Image J software, and the sections frozen at -20 °C for 10 min. Infarction volume was taken as the infarct area multiplied by infarct thickness (mm).

#### Determination of degree of brain tissue lesions using hematoxylin-eosin (H & E) staining

The left upper lobe of brain was fixed with formaldehyde solution, embedded in paraffin, sliced into 4 µm-thick sections, sealed and counterstained with H & E dye for 6 - 8 min and 10 s, respectively. Finally, the sections were observed and analyzed under a microscope.

#### Assessment of mRNA expressions of PI3K/Akt in brain tissues

The brain tissues were added to chloroform, shaken and centrifuged at 1,200 rpm and 4 °C until the solution became milky white. The tissues were then added to isopropanol, centrifuged, added to 75 % ethanol, centrifuged again, dried and kept at -80 °C. Then the RNA extracted using TRIzol® reagent was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using A3500 RT kits, and the expressions of target genes were determined using Biotium GelRed and Gelgreen and CFX-96 qRT-PCR instrument. The primers used were designed using NCBI/Primer (Table 2), and the reaction conditions are as follows: 60 °C for 10 min, 95 °C for 30 s, 72 °C for 30 s, 95 °C for 5 min, a total of 40 cycles for at least 3 times. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the PI3K/Akt expressions were determined using the  $2^{-\Delta\Delta CT}$  method.

#### Determination of protein expressions of PI3K/Akt in brain tissues

Protein expressions of PI3K/Akt in brain tissues were determined using Western blotting. After the electrophoresis tank and other components were washed and dried, 12 % separation gel was prepared, and 1 cm-thick water layer was made. The coagel was placed in the electrophoresis apparatus for 1 h to prepare the spacer gel. Then the protein samples were loaded with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant pressure (80 V) for 2.5 h, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) via semi-dry transfer, immersed in Tris buffered saline-tween (TBST) containing 5 % skim milk powder, and slowly shaken for 1 h, and then sealed. Then the protein samples were incubated with rabbit anti-rat GAPDH primary antibody (diluted at 1: 1,000 with 5 % skim milk powder, Shanghai Beinuo Biology Co. Ltd, Shanghai, China), washed 3 times, incubated again with the corresponding secondary antibody at room temperature for 1.5 h, and shaken gently.

**Table 2:** Primer sequences used

Gene		Primer sequence
PI3K	F	5'-GGGCTTTCTGTCTCCTCTAAAC-3'
	R	5'-ATGTCTGGGTTCTCCCAATTC-3'
Akt	F	5'-CTACAACCAGGACCATGAGAAG-3'
	R	5'-TCTTGAGCAGCCCCCTGAAAG-3'
GAPDH	F	5'-TGAACGGGAAGCTCACTGG-3'
	R	5'-TCCACCACCCTGTTGCTGTA-3'

Finally, the protein samples were added with a mixture of electrochemiluminescence (ECL) A and B solution for about 1 min and washed with TBST 3 times, followed by automatic chemiluminescence imaging analysis (Thermo Fisher Scientific, Waltham, MA, USA).

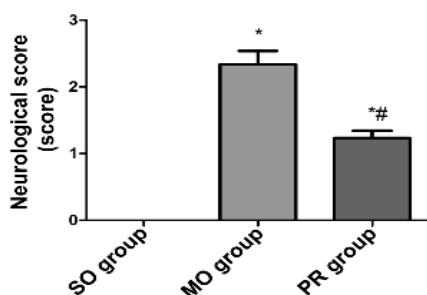
### Statistical analysis

SPSS statistical analysis software (version 26.0) was used for statistical analyses. Data were expressed as mean  $\pm$  standard deviation (mean  $\pm$  SD). Comparison between multiple groups was performed using One-way ANOVA test with Least Significant Difference;  $p < 0.05$  indicates statistically significant.

## RESULTS

### Neurological score

The neurological score (NS) was  $0, 2.54 \pm 0.67$  and  $1.34 \pm 0.21$  points for SO, MO and PR groups, respectively. NS was good in SO group, but severely impaired in MO group. The neurological score significantly declined in the PR group compared with that of MO group ( $p < 0.05$ , Figure 1).



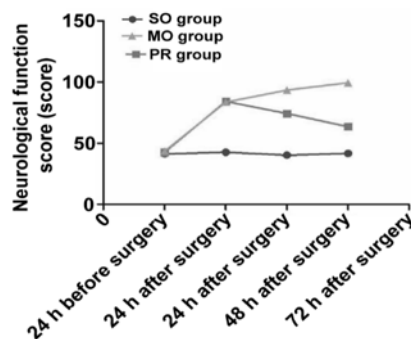
**Figure 1:** Neurological score for the three groups. \* $p < 0.05$  vs. SO group, # $p < 0.05$  vs. MO group

### Neurological function scores

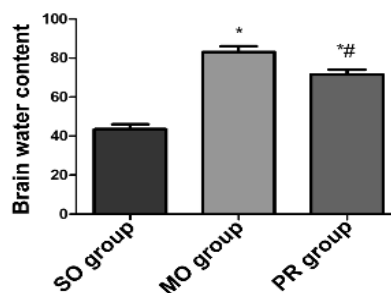
In the turning angle test, the neurological function score gradually rose at 24 h after cerebral infarction in PR group and MO group when compared with that in SO group ( $p < 0.05$ ), while it markedly declined at 48 h in PR group when compared with that in MO group ( $p < 0.05$ ) (Figure 2).

### Brain water content

The brain water content was lowest in SO group and the highest in MO group, and it was significantly lower in PR group than that in MO group ( $p < 0.05$ ) (Figure 3).



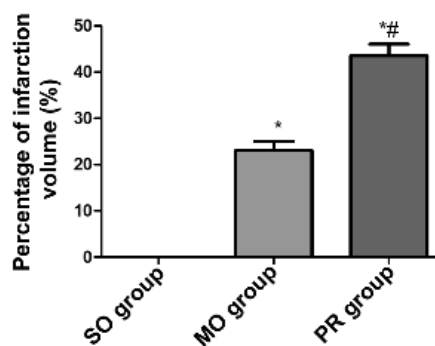
**Figure 2:** Comparison of neurological function scores among the three groups



**Figure 3:** Brain water contents in the three groups. \* $p < 0.05$  vs. SO group, # $p < 0.05$  vs. MO group

### Infarction volume

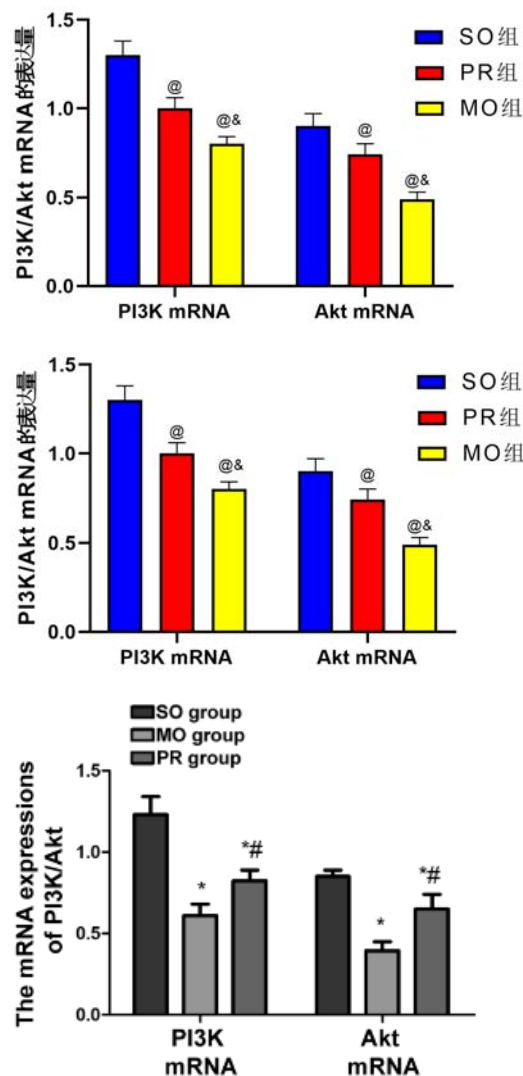
No infarction was found in brain tissues in the SO group, but there were obvious lesions in brain tissues in the PR group and MO group. Infarction volume was lower in the PR group than in the MO group ( $p < 0.05$ ) (Figure 4). The brain tissues without edema were arranged regularly in the SO group; brain tissues which were severely damaged and with more edema and karyopyknosis were arranged in the MO group, and the necrosis of brain tissues was relieved in the PR group when compared with that of the MO group ( $p < 0.05$ ).



**Figure 4:** Infarction volume in the three groups. \* $p < 0.05$  vs. SO group, # $p < 0.05$  vs. MO group

### PI3K/Akt mRNA expression levels in rat brain tissues

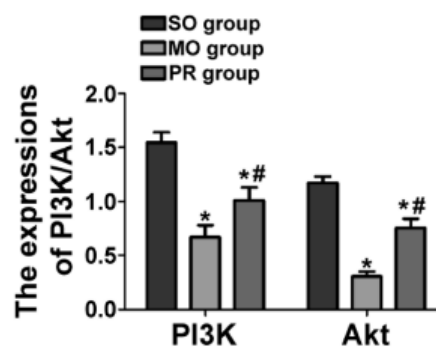
The mRNA expressions of PI3K/Akt were highest in SO group and lowest in MO group, and they were remarkably higher in PR group than those in MO group ( $p < 0.05$ ) (Figure 5).



**Figure 5:** PI3K/Akt mRNA expressions in rat brain tissues. \* $p < 0.05$  vs. SO group, # $p < 0.05$  vs. MO group

### PI3K/Akt protein expressions in rat brain tissues

The protein expressions of PI3K/Akt were highest in the SO group and lowest in the MO group, and they were remarkably higher in the PR group than in the MO group ( $p < 0.05$ ) (Figure 6).



**Figure 6:** PI3K/Akt protein expressions in brain tissues. Protein expressions of PI3K/Akt in brain tissues: comparison of PI3K/Akt protein expressions among the three groups. \* $p < 0.05$  vs. SO group, # $p < 0.05$  vs. MO group

## DISCUSSION

Cerebrovascular diseases refer to the loss of brain function induced by cerebrovascular lesions, which can be classified into ischemia and bleeding in the clinic. Due to the limitation of cerebral blood circulation, large-area hypoxia and ischemia occur in local brain tissues, leading to necrosis or softening of brain tissues, endangering physical health [8]. Cerebral infarction is a brain disease caused by long-term hypoxemia in brain tissues. Currently, cerebral infarction is more common in middle-aged and elderly people, and it often occurs with other senility-related diseases. With the rapid rise in social development as well as economic status, cerebral infarction has been observed to more frequently occur in older people. Its incidence however is now showing a younger trend [9].

Cerebral infarction has become the major cause of the increasing death rate among middle-aged and elderly people in China, so its diagnosis and treatment are extremely important. Moreover, it is necessary to repair the hypoxic-ischemic brain tissues as soon as possible for such patients, so as to improve the prognosis.

At present, it has been confirmed in a large amount of literature that the pathogenesis of cerebral infarction is related to the PI3K/Akt signaling pathway. It is involved in the regulation of neuronal proliferation, differentiation and apoptosis in the pathological process of cerebral infarction, and also plays an indispensable role in the occurrence and development of other brain diseases, such as glioma [10]. The key to the treatment of cerebral infarction is to prevent ischemia-reperfusion. At present, irreversible neuronal damage is reduced by drug preconditioning in the clinic, so as to protect

brain tissues. Studies have found that [11] drug therapy can reduce the area affected by ischemic lesions in cerebral ischemia. In this study therefore, the cerebral infarction model was established to observe the effects of probucol on rats with cerebral infarction *via* the PI3K/Akt pathway.

It was discovered that the neurological deficit was severe in rats with cerebral infarction. After injection with probucol, the neurological function score declined, and the limb function was improved. It was observed *via* HE staining that the brain tissues had severe edema and karyopyknosis, and there was massive neuronal apoptosis in the MO group. In the PR group, edema was improved and neuronal apoptosis was relieved [12]. Neurological deficit often occurs in a variety of brain diseases, and some studies have shown that neurological deficit is serious in the infarction region due to local ischemia in cerebral ischemia patients [13]. Probucol can selectively promote the stability of endothelial function, and improve the neurological deficit in the human body. It has been confirmed that after gavage with probucol in rats with cerebral infarction for 14 d, the limbs can move normally, and the rats can walk independently without disturbance or discomfort. It can be seen that probucol can delay the occurrence and development of the disease and improve the nervous system. Cerebral infarction is often accompanied by mild-severe atheromatous plaques, leading to obstruction of blood flow [14]. Besides, it is reported that a large number of dead neurons are found in brain tissues of rats with cerebral infarction, because a large number of free radicals are produced in the ischemic region in cerebral ischemia, accompanied by mitochondrial damage and inflammatory response, ultimately causing neuronal apoptosis [15].

Probucol is a commonly used drug for patients with cerebral infarction, which can inhibit the thrombosis and reduce the secretion of smooth muscle cells. Studies have demonstrated that after treatment with probucol, the inflammation and blood lipid levels greatly decline in patients with cerebral ischemia, and the quality of life is improved [16].

In recent years, the PI3K/Akt signaling pathway has become a research emphasis of many scholars. It is involved in cell proliferation and differentiation, and also has an inseparable relationship with many diseases [17].

In this study, the results showed that the protein expressions of PI3K/Akt were significantly

decreased in the MO model, but increased in the PR group. According to a large volume of literature, activating the PI3K/Akt signaling pathway can prevent cerebral ischemia, which reduces apoptosis, and can also reduce cardiomyocyte hypertrophy and renal fibrosis [18]. Researchers found that the PI3K/Akt signaling pathway is significantly inhibited in rats with cerebral infarction, and the cerebral infarction area is increased with massive neuronal apoptosis, indicating that the PI3K/Akt signaling pathway may suppress the occurrence of cerebral infarction [19]. In addition, experiments confirmed that probucol can delay the occurrence and development of cerebral ischemia, and alleviate the condition of the disease through the activation of the PI3K/Akt pathway [20].

## CONCLUSION

Probucol reduces the cerebral edema area and infarction volume in rats by activating PI3K/Akt pathway. Thus, it can potentially be developed for the management of cerebral infarction in humans.

## DECLARATIONS

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Guo Huang and Yamei Li contributed equally to this work.

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