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Original Article

Effects of Peiyuan tongnao capsules and Dihuang yinzi decoction on mitochondrion-dependent apoptotic pathway in rats with MCAO model

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Abstract: Objective: This study aimed to explore the effects of the Peiyuan tongnao (PYTN) capsule and Dihuang yinzi (DHYZ) decoction on neurological impairment after cerebral ischemic injury and its underlying mechanisms. Methods: A total of 100 healthy male Sprague-Dawley rats were randomly divided into five groups: sham group, model group, nimodipine group, DHYZ group, and PYTN group. After 24 and 72 h of the MCAO model construction surgery, the rats were sacrificed, and the serum and brain tissue samples were collected. Luminex multiplex technology was used to analyze the levels of cytokines in the serum of rats. The morphological changes in the brain tissue sections were detected using hematoxylin-eosin staining, Nissl staining, and TUNEL assay. The expression levels of cyt-c, caspase-3, Bcl-2, and Bax were measured using Western blot analysis. Results: The mNSS scores in the DHYZ, PYTN, and nimodipine groups decreased significantly after 24 and 72 h, compared with the model group ($P < 0.01$). Moreover, the levels of IL-2, IL-4, IL-5, MIP-3α, and IL-18 decreased in the PYTN and DHYZ groups and the level of IL-4 decreased in the nimodipine group compared with the model group ($P < 0.05$). The levels of cyt-c, caspase-3, and Bax proteins reduced in the nimodipine, PYTN, and DHYZ groups, while the level of Bcl-2 increased after 24 and 72 h, compared with the model group ($P < 0.05$). Conclusions: PYTN capsules and DHYZ decoction alleviated neurological impairment, inhibited the expression of apoptotic genes and cytokines, and slowed reduced apoptosis and necrosis after ischemia in rats with MCAO.

Keywords: Apoptosis, Bcl-2, Bax, cyt-c, caspase-3, Chinese medicine, herbal, ischemia, middle cerebral artery occlusion, neuroprotective agents

Introduction

Stroke is associated with high morbidity, disability, and mortality. The global burden of the disease showed that cerebrovascular diseases caused 6.17 million deaths each year [1]. Stroke is the leading cause of mortality (149/10 per 100,000) with years of life lost (2633 years of life lost per 100,000) [2], leading to heavy social and economic burden. Hence, reducing mortality and disability is an urgent public problem.

Stroke is divided into ischemic and hemorrhagic types, with the former being the most common type. The key of acute ischemic stroke is the recovery of cerebral blood supply. The effective strategies include intravenous thrombolytic therapy and intravascular interventional therapy. Of all patients, 25% received intravenous thrombolytic therapy and 10%-12% received intravascular interventional therapy [3]. However, only a small number of patients are treated timely and effectively in clinical practice due to medical limitations and contradictions. For patients fail to receive thrombolytic and intravascular therapy, the remedy of reversible ischemic penumbra is the major sticking point. Attempts have been made to include thousands of potential neuroprotective compounds undergoing trials in clinical practice.

In cerebral ischemia due to thrombi, embolism, and hypoperfusion, oxygen and energy supply is not sufficient for normal cell metabolism,

leading to cell death, tissue necrosis, and irreversible damage to the neurological function. Cell apoptosis in the ischemic penumbra is the major mechanism of cerebral ischemic injury, and the remedy of apoptotic cells is an important neuroprotective approach. The neuroprotective effects of traditional Chinese medicine (TCM) have been extensively investigated. The classical prescription for stroke, Buyang huanwu decoction, was reported to promote angiogenesis by increasing the expression levels of angiopoietin-1 and stimulating the PI3K/Akt pathway through upregulation of the phosphorylated vascular endothelial growth factor receptor [4, 5]. The Chinese medicine Nao-Shuan-Tong could inhibit apoptosis by reducing the expression levels of caspase-3 and caspase-8 and the Bax/Bcl-2 ratio in a rat model of stroke [6]. The Dihuang yinzi decoction plays neuroprotective and anti-dementia roles by reducing the levels of synaptophysin and extracellular signal-regulated kinase [7].

The present study investigated the effect of Peiyuan tongnao capsule and Dihuang yinzi decoction on the expression levels of cyt-c, caspase-3, Bcl-2, and Bax to explore the mechanism underlying the neuroprotection provided by kidney-nourishing TCM drugs in acute stroke from the point of strengthening body resistance.

Material and methods

Animals

A total of 100 healthy male SPF SD rats, weighing 280 ± 20 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. [No. SCXK (jing) 2006-0009]. The experiment was conducted in Dongzhimen Hospital, Beijing University of Chinese Medicine. The rats had free access to water and food at a constant temperature of 25°C in 12-h/12-h light/dark cycle. The study was approved by the Institutional Animal Care and Use Committee and Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine (No. 17-15). All experiments were conducted in accordance with the regulations and guidelines of the Ministry of Science and Technology of the People's Republic of China.

Experimental drugs

Nimodipine tablets, 30 mg/tablet, were purchased from Bayer (Leverkusen, Germany) Logo Pharmaceuticals (Batch No. 113275, SFDA approval No.: H20003010). Dihuang yinzi decoction granules (DHYZ) were made by Tcmages Pharmaceutical Co., Ltd. Peiyuan Tongnao capsules (PYTN) were procured from Henan Lingrui Pharmaceutical Co., Ltd. (SFDA approval No.: Z20000022). The dosage was calculated using the flowing formula 1:

$$\text{Drugs/Weight}\,(\text{mg/kg})\,d_B = d_A \cdot \frac{R_B}{R_A} * \left(\frac{W_A}{W_B}\right)^{\frac{1}{3}} \quad (1)$$

where d_B is the dosage per weight (kg) of unknown animals B; d_A is the dosage per weight (kg) of known animals A; W_A and W_B are the weight of known animals; and R_A and R_B are the shape coefficients.

	Rat	Human
R (shape coefficient)	0.09	0.1
W (standard weight)	0.2	70

The three agents were prepared as 1 mL/100 g solution.

Experimental design

The 100 rats were divided into 5 groups according to the random digit table. Sham group (n = 20): controls undergoing same procedures as treated rats till the isolation of CCA; model group (n = 20): MCAO models constructed but given no treatments; Nimodipine group (n = 20): MCAO rats given Nimodipine tablets; DHYZ group (n = 20): MCAO rats given DHYZ decoction granules; and PYTN group (n = 20): MCAO rats given a standard dosage of PYTN capsules.

The rats in the sham and the model groups were intragastrically administered 0.9% sodium chloride solution every day, and the rats in the other 3 agent groups were given appropriate amounts of drugs. The samples were taken 24 and 72 h after the surgery, with 10 samples at every time point, to observe the effects of drugs at different time points.

Construction of MCAO rat models

The rats were fed and processed in the animal laboratory with a barrier system. All animals were given adaptive feed for 3 days, followed by 12-h fasting before the surgery. They were weighed, fixed on the pad, and anesthetized by intraperitoneal (i.p.) injection of 2% pentobarbital sodium (45 mg/kg). Then, the skin was pre-

pared, and the surgical site was disinfected with alcohol. The procedures were as follows.

A 2-cm incision was made on the skin slightly right to the cervical midline and fascia, and the muscles were bluntly separated. The right common carotid artery (CCA) and the external carotid artery (ECA) were isolated and ligated in order. The distal ECA was blown out with an electrotome. The internal carotid artery (ICA) was occluded. An incision was made in the ECA. An embolization thread was introduced through the incision and fixed with a slipknot close to the crotch of ECA. The artery clamp was removed, and the thread was slowly pushed upward from ECA to ICA, until the thread from the cross of ECA and ICA achieved 22 mm and the force of resistance was felt. After 90-min ischemia, the thread was removed, the ECA was ligated near the cross, and the slipknot was removed. The blood supply was recovered. Hence, the MCAO model was successfully constructed. Subsequently, the incision was sutured and sterilized with iodophor. Around 1 ml of penicillin was intraperitoneally injected immediately and once a day in 3 days after the surgery.

After the rats woke up, the neurological functions were scored using the Longa method on a 5-point scale [8]: 0, no neurologic deficit; 1, failure to extend the left forepaw fully; 2, circling to the hemiplegic side; 3, falling to the hemiplegic side; and 4, inability to walk spontaneously and having a depressed level of consciousness. The rats with 0 or 4 scores were illegible and excluded.

Neurological functions

The neurological functions were evaluated 24 and 72 h after the surgery using the mNSS scales.

Luminex multiplex technology for cytokine

Luminex multiplex technology was used to conduct the study on the levels of IL-2, M-CSF, IL-4, MIP-3α, IL-5, and IL-18 in the serum of rats. The Bio-Plex Pro Rat Cytokine 23-Plex Assay was purchased from Bio-Rad (CA, USA). The Luminex 200 instrumentation was supplied by Luminex Corporation (TX, USA). In brief, 25 μL of serum sample, 50 μL of antibodies, and 50 μL of diluted PE-conjugated streptavidin were added to each well. Using 125 μL assay buffer, the beads were resuspended and incubated for 2 min. The plates were incubated on a plate shaker at 800 rpm and analyzed using the Luminex system.

Hematoxylin-eosin stain

The brain tissues were fixed with 4% paraformaldehyde. The sections were subjected to conventional dewaxing in water, hematoxylin staining for 5 min, and eosin staining for 2 min. Finally, the sections were observed using a microscope (Axio Scope A1, ZEISS, Germany).

Nissl staining

Brain sections were stained with Nissl dye for 5 min using the standard procedure. The cell morphologies of the cerebral cortex were observed under a microscope (Axio Scope A1) to assess brain damage. Necrotic neurons were identified by the disappearance of Nissl bodies in the cytoplasm, shrunken intercellular spaces, and deep staining.

TUNEL assay

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche, Germany) in accordance with the manufacturer's protocol. TUNEL observations were conducted by a blinded investigator. The apoptotic index (AI) was defined as the number of apoptotic nuclei in 100 nuclei and calculated as follows: AI = (number of TUNEL-positive cells/total cells) × 100%.

Western blot analysis

The samples were mixed with an appropriate amount of RIPA lysate containing proteinase inhibitor and phosphatase inhibitor and resuspended on ice for 30 min. The proteins were extracted for acrylamide gel electrophoresis and transferred on to PVDF membranes with primary antibodies against cyt-c (ab13575), caspase-3 (ab13847), Bcl-2 (sc-492), Bax (ab-32503), and β-actin (ab6276). Subsequently, the PVDF membranes were soaked in the ECL colored solution for 1 min, exposed, developed, and fixed in the darkroom. The images were obtained with the Genegenome gel imaging system (Syngene International Limited). The gray value was calculated using the NIH ImageJ software. The ratio of objective proteins to internal controls indicated the relative expression of objective proteins.

Table 1. mNSS scores 24 and 72 h after the surgery ($\bar{x} \pm s$)

Groups	Scores of neurological function	
	24 h	72 h
Sham-operation group	0	0
Model group	12.4 ± 0.55##	9.8 ± 0.84##
Nimodipine group	11.0 ± 0.71**	8.2 ± 0.84*
DHYZ group	9.6 ± 0.55**	7.0 ± 1.22**
PYTN group	9.8 ± 0.84**	7.8 ± 0.45**

##$P < 0.01$, compared with the sham group. *$P < 0.05$, compared with the model group. **$P < 0.01$, compared with the model group.

Statistical analysis

Data were expressed as the mean ± standard deviation (mean ± SD). SPSS 22.0 was used for statistical analysis. The results were assessed for normal distribution and homogeneity of variance. One-way analysis of variance was used. *P* value less than 0.05 indicated a statistically significant difference.

Results

PYTN capsules and DHYZ decoction accelerated the recovery of neurological function

The neurological function score of rats increased noticeably in the model group compared with the sham group ($P < 0.01$; **Table 1**). The results of 24 and 72 h after the surgery showed that PYTN capsules, DHYZ decoction, and nimodipine significantly reduced the scores of neurological functions and accelerated the recovery of neurological functions in animals, compared with the model group ($P < 0.01$ or $P < 0.05$; **Table 1**).

PYTN and DHYZ reduced inflammatory response in rats with MCAO

After 24 h, the levels of MIP-3α, IL-4, and IL-5 increased in the model group compared with the sham group, while the level of M-CSF reduced ($P < 0.05$; **Figure 1**). Compared with the model group, PYTN and DHYZ increased the expression level of M-CSF and promoted angiogenesis and tissue repair ($P < 0.05$; **Figure 1A** and **Table 2**). After 72 h, the levels of IL-2, IL-18, MIP-3α, IL-4, and IL-5 increased noticeably in the model group compared with the sham group ($P < 0.01$ or $P < 0.05$; **Figure 1**). Compared with the model group, PYTN decreased the levels of IL-2, IL-18, and MIP-3α ($P < 0.05$; **Figure 1B-D**; **Tables 3**-**5**) and significantly reduced the levels of IL-4 and IL-5 ($P < 0.01$; **Figure 1E** and **1F** and **Tables 6** and **7**.), while DHYZ decreased the levels of IL-4, IL-5, and MIP-3α ($P < 0.05$; **Figure 1D-F** and **Tables 5**-**7**) and significantly reduced the levels of IL-2 ($P < 0.01$; **Figure 1B** and **Table 3**). Moreover, the level of IL-4 decreased in the nimodipine group compared with the model group ($P < 0.05$; **Figure 1E** and **Table 6**).

PYTN and DHYZ reduced the MCAO-induced histological damage in the cortex after ischemic injury in rats

Hematoxylin-eosin (HE) and Nissl staining showed the morphological changes in the brain tissue of rats after ischemic injury. In HE staining, voids were seen around neurons and small blood vessels in the model group compared with sham group. Neurons were degenerated and necrotic. Nuclei were found to shrink, fragment, and dissolve (**Figure 2A** and **2B**). In Nissl staining, the cerebral cortex cells in sham groups were complete in structure and a large number of Nissl bodies were absent in the neurons. In the model groups, a decrease in the number of neurons, atrophy of neuronal bodies, nuclear shrinkage, and a significant reduction in the number of Nissl bodies in the cytoplasm were observed (**Figure 2C** and **2D**). The PYTN, DHYZ, and nimodipine groups showed an improvement in neuron cells survived after ischemic injury ($P < 0.05$; **Figure 2E**).

Effects of PYTN and DHYZ on apoptotic neuronal tissue

Compared with the sham group, the apoptosis index in the model group significantly increased. Compared with the model group, the neuronal apoptosis index of the brain tissue in the PYTN and DHYZ groups significantly reduced ($P < 0.05$; **Figure 3**).

PYTN and DHYZ decreased the expression levels of cyt-c, caspase-3, and Bax, and increased the expression levels of Bcl-2 proteins in the cortex of rats with cerebral ischemia reperfusion

After 24 h of surgery, the expression levels of cyt-c, caspase-3, and Bax significantly increased in the model group compared with

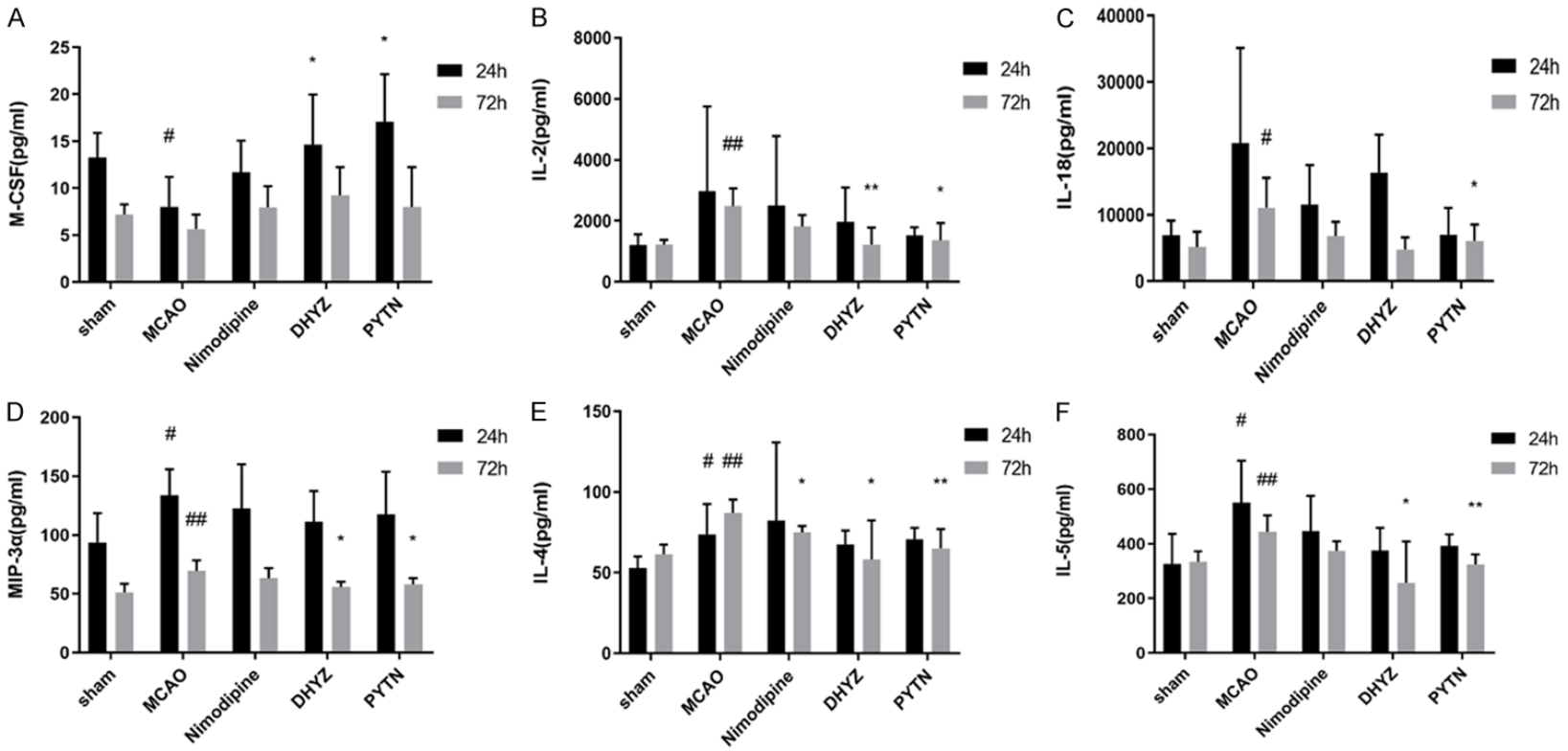

Figure 1. PYTN capsule and DHYZ decoction reduced inflammatory response. The serum levels of (A) M-CSF, (B) IL-2, (C) IL-18, (D) MIP-3α, (E) IL-4, and (F) IL-5. $^{\#}P < 0.05$, compared with the sham group; $^{\#\#}P < 0.01$, compared with the sham group; $^{*}P < 0.05$, compared with the model group, and $^{**}P < 0.01$ compared with the model group.

Table 2. Levels of the analyzed rat M-CSF ($\bar{x}$ ± s)

Groups	Analytes, M-CSF (pg/mL)	
	24 h	72 h
Sham-operation group	13.29 ± 2.59	7.22 ± 1.08
The model group	8.02 ± 3.17#	5.67 ± 1.53
Nimodipine group	11.69 ± 3.38	7.95 ± 2.27
DHYZ group	14.63 ± 5.37*	9.22 ± 3.03
PYTN group	17.08 ± 5.06*	8.01 ± 4.24

#*P* < 0.05, compared with the sham group; **P* < 0.05, compared with the model group.

Table 3. Levels of the analyzed rat IL-2 ($\bar{x}$ ± s)

Groups	Analytes, IL-2 (pg/ml)	
	24 h	72 h
Sham-operation group	1204.15 ± 357.36	1227.20 ± 146.86
The model group	2971.00 ± 2775.79	2485.40 ± 578.57##
Nimodipine group	2495.130 ± 2281.93	1815.60 ± 383.77
DHYZ group	1972.080 ± 1117.84	1221.83 ± 558.64**
PYTN group	1530.40 ± 267.53	1363.44 ± 564.29*

##*P* < 0.01, compared with the sham group; **P* < 0.05, compared with the model group; ***P* < 0.01 compared with the model group.

Table 4. Levels of the analyzed rat IL-18 ($\bar{x}$ ± s)

Groups	Analytes, IL-18 (pg/ml)	
	24 h	72 h
Sham-operation group	6918.80 ± 2192.72	5152.20 ± 2290.46
The model group	20824.60 ± 14276.33	11091.80 ± 4449.58#
Nimodipine group	11509.40 ± 6002.83	6776.80 ± 2172.76
DHYZ group	16312.40 ± 5772.51	4793.00 ± 1812.20
PYTN group	7025.20 ± 4027.15	6040.80 ± 2524.35*

#*P* < 0.05, compared with the sham group; **P* < 0.05, compared with the model group.

Table 5. Levels of the analyzed rat MIP-3α ($\bar{x}$ ± s)

Groups	Analytes, MIP-3α (pg/ml)	
	24 h	72 h
Sham-operation group	93.71 ± 24.82	51.18 ± 7.36
The model group	133.56 ± 22.29#	69.36 ± 9.07##
Nimodipine group	122.64 ± 37.67	63.12 ± 8.79
DHYZ group	111.24 ± 26.10	55.71 ± 4.46*
PYTN group	117.74 ± 35.95	57.94 ± 5.39*

#*P* <0.05, compared with the sham group; ##*P* < 0.01, compared with the sham group; **P* < 0.05, compared with the model group.

the sham group, while the level of Bcl-2 decreased (*P* < 0.01; **Figure 4**). The expression levels of cyt-c, caspase-3, and Bax reduced, while the level of Bcl-2 increased in the PYTN and DHYZ groups compared with the model group (*P* < 0.05; **Figure 4A-D**). After 72 h, the expression levels of cyt-c, caspase-3, and Bax significantly increased in the model group compared with the sham group, while the level of Bcl-2 decreased (*P* < 0.01; **Figure 4**). The expression levels of caspase-3 and Bax reduced and the level of Bcl-2 was upregulated in the PYTN group compared with the model group (*P* < 0.05; **Figure 4B-D**). The expression levels of cyt-c and Bax declined and the level of Bcl-2 protein increased in the DHYZ group compared with the model group (*P* < 0.05; **Figure 4A**, **4C** and **4D**). The levels of cyt-c and Bax reduced in the nimodipine group compared with the model group (*P* < 0.05; **Figure 4A** and **4C**). The level of caspase-3 notably decreased, while that of Bcl-2 significantly increased in the nimodipine group compared with the model group (*P* < 0.01; **Figure 4B** and **4D**).

Discussion

The PYTN capsule is a common Chinese patent medicine for treating ischemic stroke. In TCM, the brain is considered the sea of marrow and the kidney is the organ that generates marrow and dominates bone. Tonifying kidney and benefiting marrow can protect the brain. PYTN capsules reinforce the kidney and activate blood, which is a symptomatic treatment. Dihuang yinzi decoction is a classical prescription invigorating the kidney. Many studies found pharmacological effects of some important ingredients of PYTN. One of the major ingredients of PYTN was catalpol, which not only decreased the expression level of Bax and increased the expression level of Bcl-2 in ischemic cells but also inhibited caspase-3 activa-

Table 6. Levels of the analyzed rat IL-4 ($\bar{x} \pm s$)

Groups	Analytes, IL-4 (pg/ml)	
	24 h	72 h
Sham-operation group	52.85 ± 7.24	61.39 ± 6.08
The model group	73.80 ± 18.75#	86.94 ± 8.29##
Nimodipine group	82.19 ± 48.54	74.95 ± 3.97*
DHYZ group	67.55 ± 8.52	58.05 ± 24.28*
PYTN group	70.83 ± 6.90	65.10 ± 11.78**

##$P < 0.01$, compared with the sham group; #$P < 0.05$, compared with the sham group; *$P < 0.05$, compared with the model group; **$P < 0.01$ compared with the model group.

Table 7. Levels of the analyzed rat IL-5 ($\bar{x} \pm s$)

Groups	Analytes, IL-5 (pg/ml)	
	24 h	72 h
Sham-operation group	326.70 ± 109.52	334.15 ± 38.36
The model group	550.27 ± 154.17#	443.90 ± 60.51##
Nimodipine group	446.08 ± 129.81	374.22 ± 34.90
DHYZ group	375.96 ± 82.47	257.29 ± 151.22*
PYTN group	393.33 ± 40.79	325.07 ± 36.38**

##$P < 0.01$, compared with the sham group; #$P < 0.05$, compared with the sham group; *$P < 0.05$, compared with the model group; **$P < 0.01$ compared with the model group.

tion and cyt-c release to protect cells from apoptosis [9]. The other crucial ingredient echinacoside significantly inhibited apoptosis-related proteins caspase-3 and Bax; increased the activity of antioxidases SOD, GSH-PX, and CAT; and decreased the MDA level, and therefore, protecting neurons via the antioxidative effect and inhibiting cell apoptosis [10]. The extracts of *Cinnamomi Cortex* significantly enhanced the activities of SOD and GPx and decreased the levels of LDH and MDA in ischemic cardiac muscle cells, protecting cardiac muscles from ischemia-reperfusion injury [11]. In addition, the extracts of *Crataegi Fructus* lessened oxidative stress-related injury [12]. *Paeoniae Radix Rubra* has two active ingredients terpene glycoside and paeoniflorin (PF). The former can decrease the levels of caspase-3 and Bax/Bcl-2 to inhibit cell apoptosis by activating the PI3K/Akt/mTOR signaling pathway [13] while the latter can regulate the Ca^{2+}/CaMKII/CREB signaling pathway [14], which may be the mechanism of affecting the downstream apoptosis-related proteins. Licorice root was also reported to have some brain-protective effect [15].

The MCAO model is a classical animal model used for simulating ischemic stroke, which was proposed by Koizumi [16] and improved by Longa [8]. The mNSS scale can comprehensively assess multiple defects, including motion, sensation, reflex, and balance. Therefore, it can more completely reflect the range of neurological impairment after cerebral ischemia in rats [17, 18]. The scores of neurological functions showed a downward trend. The functions of the injured cerebrum are disrupted after ischemia and recover gradually via collateral circulation and compensation of cerebral functions after the recovery of blood supply. This is consistent with the clinical phenomenon that the symptoms are severe in the acute phase and gradually disappear in the recovery phase. However, the neurological functions recovered faster in the treatment groups compared with the model group, which might decrease the high disability in stroke, which was verified in further clinical trials.

The inflammatory response was an important part of the physiological mechanism of the brain after ischemic injury [19]. The inflammatory cascade eventually leads to apoptosis and even cell necrosis [20]. By reducing the inflammatory response, the severity of post-ischemic injury and the degree of neurological damage could be reduced. In this study, the 72 h groups could obviously reduce the degree of the inflammatory response, while the effect of the 24 h group drug was not obvious, which might be related to the progressive exacerbation of the inflammatory response in the acute phase. Statistical significance was obtained within 72 h, which showed that TCM could reduce the inflammatory response, and was more beneficial in the early and continuous use.

The present study explored the role of TCM in relieving apoptosis after cerebral ischemia, with the Bcl-2 families and apoptotic-related protein caspase-3 and cyt-c as the observational indices. Apoptosis is the key to cerebral ischemic injury, and the alleviation of apoptosis is an approach to prevent irreversible tissue necrosis.

Apoptosis is a type of necrosis characterized by pyknosis and karyorrhexis. The intrinsic apoptotic pathway is strongly related to the Bcl-2

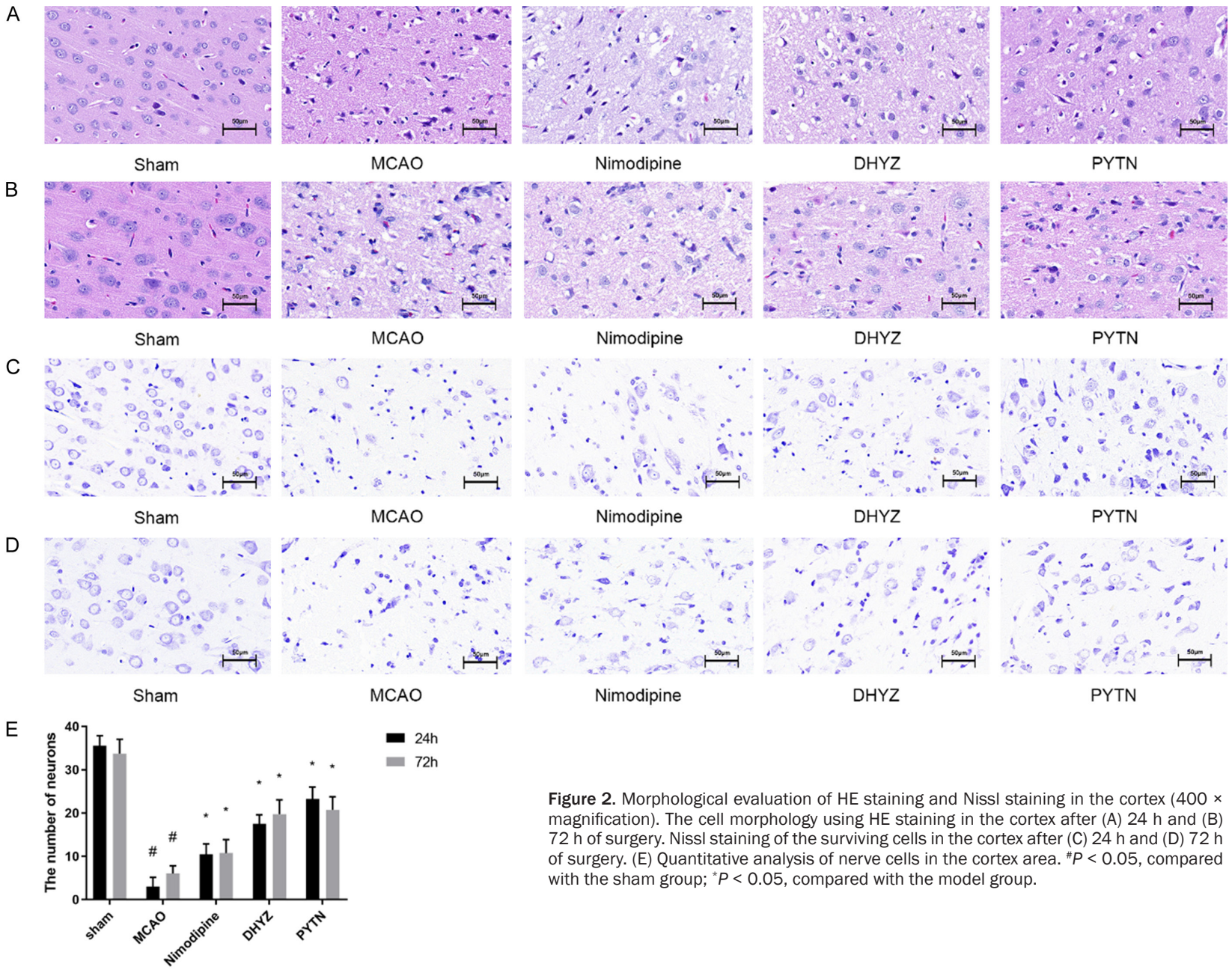

Figure 2. Morphological evaluation of HE staining and Nissl staining in the cortex (400 × magnification). The cell morphology using HE staining in the cortex after (A) 24 h and (B) 72 h of surgery. Nissl staining of the surviving cells in the cortex after (C) 24 h and (D) 72 h of surgery. (E) Quantitative analysis of nerve cells in the cortex area. $^{\#}P < 0.05$, compared with the sham group; $^{*}P < 0.05$, compared with the model group.

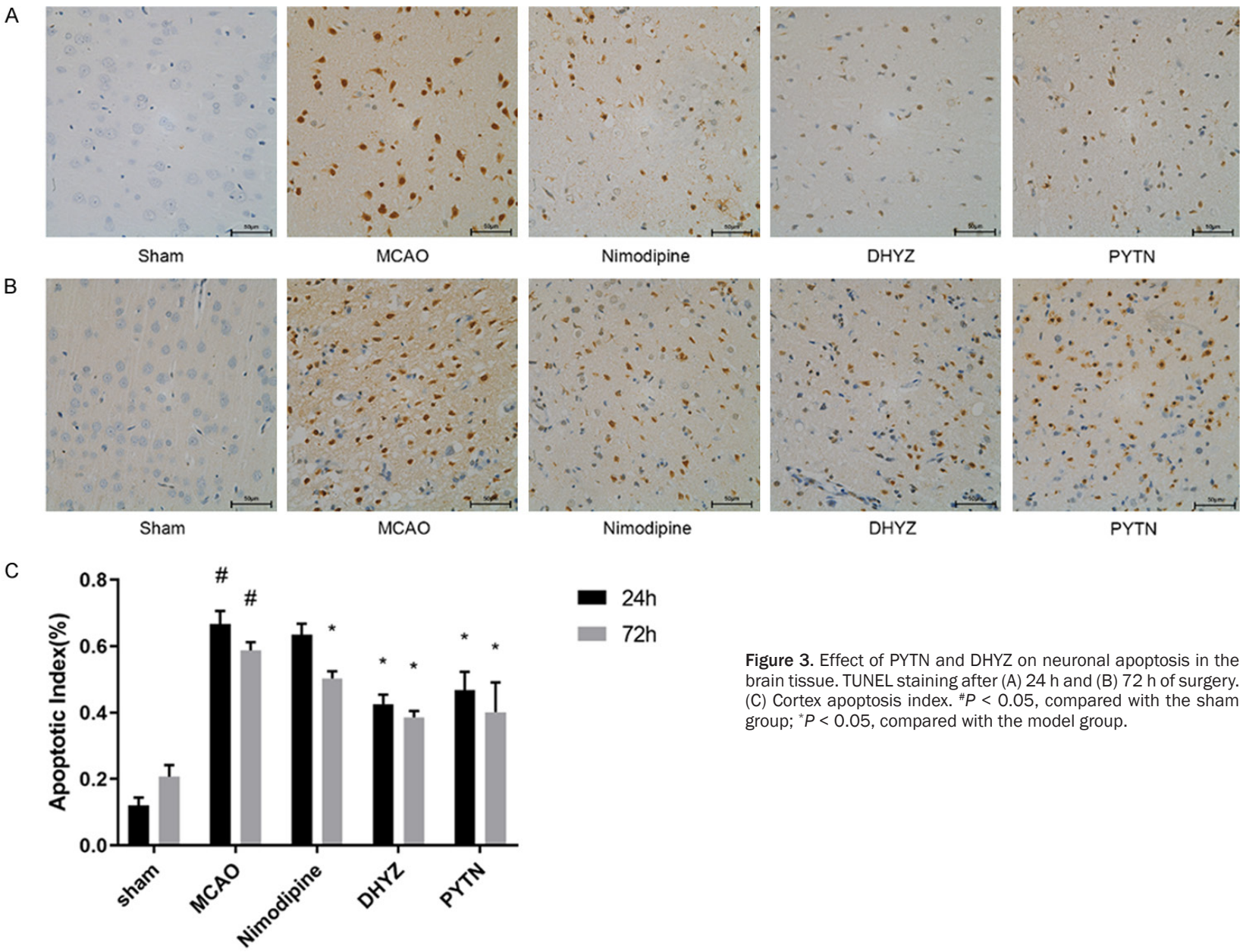

Figure 3. Effect of PYTN and DHYZ on neuronal apoptosis in the brain tissue. TUNEL staining after (A) 24 h and (B) 72 h of surgery. (C) Cortex apoptosis index. $^{\#}P < 0.05$, compared with the sham group; $^{*}P < 0.05$, compared with the model group.

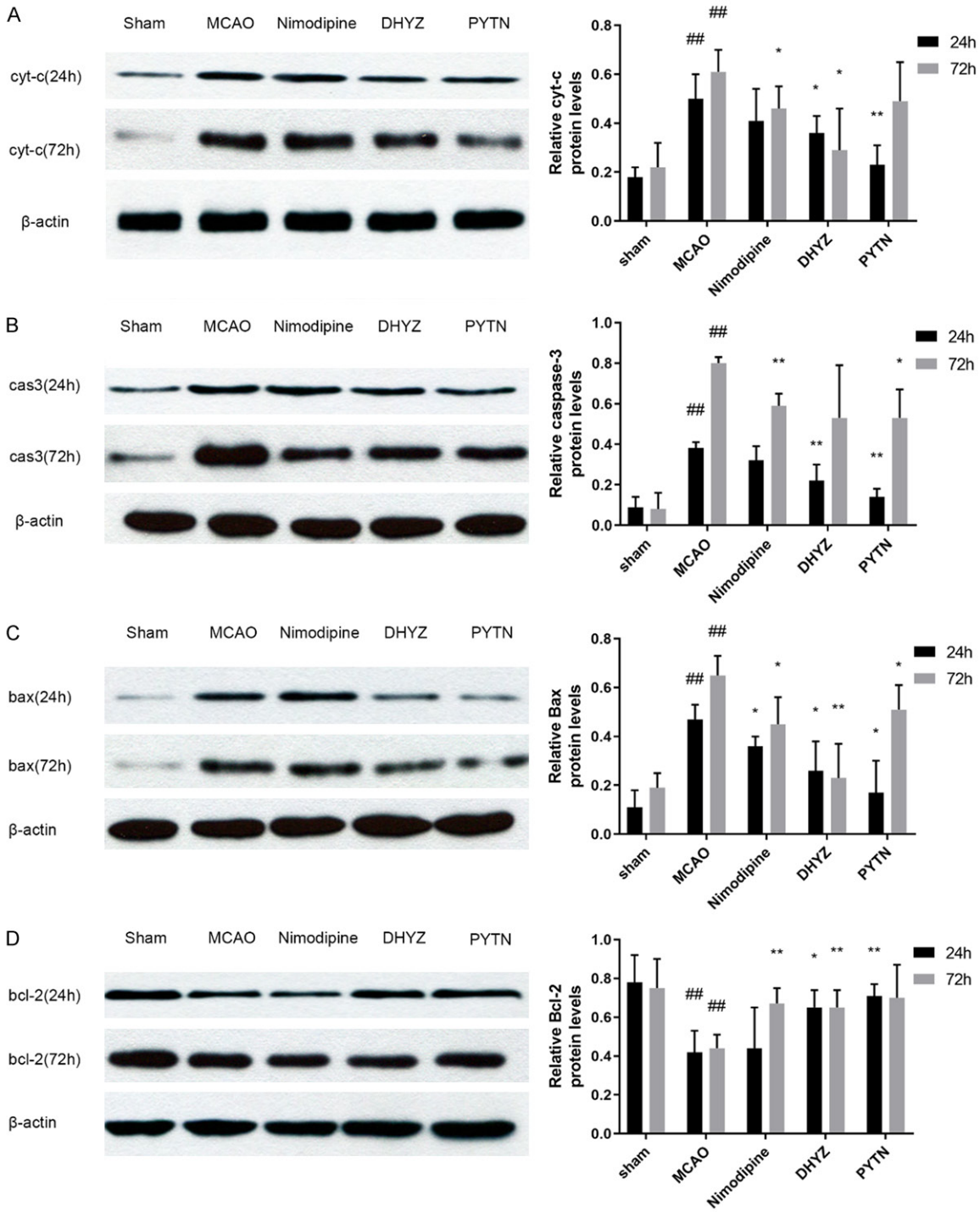

Figure 4. Protein expression levels and Western blot analysis of cyt-c, caspase-3, Bcl-2, and Bax levels in the cortex. Western blot and quantitative analyses of protein levels of (A) cyt-c, (B) caspase-3, (C) Bax, and (D) Bcl-2. $^{\#\#}P < 0.01$, compared with the sham group; $^{*}P < 0.05$, compared with the model group, $^{**}P < 0.01$, compared with the model group.

family proteins and primarily to the balance between pro-apoptotic members and anti-apoptotic members. Caspases belong to the protease system leading to cell apoptosis and are an important effector in the apoptotic pathway. A large number of apoptotic factors are

found in the mitochondria, which can activate caspase-dependent and caspase-independent apoptotic pathways.

Cyt-c is a pro-apoptotic factor in the upstream of apoptotic pathway, which activates caspase-9. The active caspase-9 further activates caspase-3, caspase-6, and caspase-7. The cascade activation finally induces cell shrinkage and DNA breakage and inhibits DNA repair. In addition, extracellular signals activate death receptors in the extrinsic pathway and subsequently activate caspase-3, caspase-6, and caspase-7, leading to cell apoptosis, similar to the intrinsic pathway [21-23].

The PYTN capsule and DHYZ groups had significantly lower cyt-c, caspase-3, and Bax expression levels and higher Bcl-2 levels 24 h and 72 h after the surgery, suggesting that the early use of TCM could slow down apoptosis. In addition, nimodipine also affected the expression of apoptosis-related factors 72 h after the surgery, and its blood vessel protection might be helpful for the recovery of ischemic injury. The protein levels were higher 72 h, compared with 24 h, after the surgery, suggesting that cell death in the ischemic penumbra aggravated 24-72 h after the surgery. However, drugs can delay and control the ischemic injury, and early intervention with TCM exerts an obvious effect.

Based on TCM, DHYZ invigorates mainly the kidney and PYTN capsule has effects on promoting the blood flow. Hemorrhagic agents *Radix Paeoniae Rubra* and *Ligusticum chuanxiong* can significantly decrease the levels of MMP-9 and PAI-1, upregulate the Bax level, and downregulate the Bcl-2 level, thus decreasing the apoptosis of ischemic brain tissues and protecting the blood-brain barrier [24]. Tanshinone protects the blood-brain barrier by inhibiting MMP-9 and claudin-5 in patients with acute ischemic stroke after intravenous thrombolysis [25].

This finding suggests that PYTN capsules and DHYZ decoction may involve the expression of cyt-c, caspase-3, Bax and Bcl-2, which is related to the classical pathway of apoptosis. However, in this study, research on pathway mechanism is not complete enough, and the mechanism of drugs regulating the upstream and downstream of the apoptosis pathway can be further explored, and verify the mechanism of association between pathways related to apoptosis.

Conclusions

The present study found that the early application of PYTN capsules and DHYZ in rats with MCAO slowed reduced cell apoptosis in the ischemic cortex and relieved neurological impairment. It suggested a neuroprotective mechanism of invigorating the kidney in the acute phase of stroke from the point of strengthening body resistance, thus providing experimental evidence for invigorating the kidney in the early stage of acute cerebral infarction. Further investigations are required to validate the findings.

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Disclosure of conflict of interest

None.

Abbreviations

MCAO, transient middle cerebral artery occlusion; mNSS, Modified neurological severity score; Bcl, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PYTN, Peiyuan tongnao capsule; DHYZ, Dihuang yinzi decoction; PI3K, Phosphoinositide 3-kinases; Akt, PKB, Protein kinase B; SPF, Specific Pathogen Free; SD, Sprague Dawley; SFDA, State Food and Drug Administration of China; IL, Interleukin; M-CSF, Macrophage colony-stimulating factor; MIP-3α, Macrophage inflammatory protein-3α; RIPA, Radioimmunoprecipitation assay; PVDF, polyvinylidene difluoride; ECL, electrogenerated chemiluminescence; NIH, National Institutes of Health; SOD, superoxidase dismutase; GSH-PX, Glutathione peroxidase; MDA, Malondialdehyde; CREB, cAMP response element-binding protein; MMPs, Matrix metallopeptidases.

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