Effect of Qingre Huayu Decoction on Autophagy and Apoptosis of Neurons in Rats with Ischemic Brain Injury by Regulating Ca^{2+}/CaMKKβ-AMPK-mTOR Pathway

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Abstract [**Objectives**] To explore the neuroprotective mechanism of Qingre Huayu Decoction on rats with acute cerebral ischemia injury. [**Methods**] SD rats were divided into sham operation group, ischemia model group, low, medium and high dose groups of Qingre Huayu Decoction, with 10 rats in each group. Referring to the MCAO operation model, both the sham operation group and the model group were given normal saline by gavage, and the Qingre Huayu Decoction group was given different doses of Qingre Huayu Decoction by gavage. After the operation, the rats were scored for neurological deficit, neurons were stained with HE, apoptotic cells were detected with TUNEL, and the levels of autophagy and apoptotic proteins in the Ca^{2+}/CaMKKβ-AMPK-mTOR pathway in brain tissue were detected with Western-blot. [**Results**] Compared with the model group, the neurological function score of Qingre Huayu Decoction Group decreased significantly ($P<0.05$), the pathological damage of neurons in Qingre Huayu Decoction Group decreased, the proportion of apoptosis-positive cells detected by TUNEL decreased ($P<0.05$), and the expression of CaMKKβ and AMPK increased, expression of mTOR decreased, expression of Beclin-1 and LC3 increased, and expression of Caspase-3 decreased in Qingre Huayu Decoction Group ($P<0.05$). [**Conclusions**] Qingre Huayu Decoction may play a neuroprotective role by activating Ca^{2+}/CaMKKβ-AMPK-mTOR pathway and regulating the level of apoptosis and autophagy.

Key words Qingre Huayu Decoction, Ischemic brain injury, Apoptosis, Autophagy

1 Introduction

Ischemic cerebrovascular disease (ICD) is a kind of neurological dysfunction disease caused by the interruption of local blood circulation in the brain, and is more common in middle-aged and elderly people. With the aging of China's population, cerebrovascular diseases have become one of the main diseases for death and disability in China, among which ischemic stroke is the most prominent, accounting for about 70% of cerebrovascular diseases[1]. ICD has the characteristics of high morbidity, high mortality and high disability rate. At present, ICD lacks effective prevention and treatment methods, which brings a heavy medical burden to the patient families.

Traditional Chinese medicine has accumulated rich experience in the prevention and treatment of stroke, and "toxic damage to brain collaterals" is an important pathological mechanism for the understanding, research, diagnosis and treatment of stroke in traditional Chinese medicine encephalology. Qingre Huayu Decoction is based on the theory of "toxic damage to brain collaterals" by Academician Wang Yongyan and Ren Jixue, a master of traditional Chinese medicine. It has the effect of "removing blood stasis and clearing heat, cleaning phlegm and detoxifying", and gets to the pathogenesis and treatment point of stroke. With the progress of modernization research of traditional Chinese medicine, the advantages of traditional Chinese medicine with multiple levels, multiple targets and multiple pathways have been further explored. It is found[2-3] that most traditional Chinese medicines for promoting blood circulation and removing blood stasis can exert brain protection effect by regulating autophagy and apoptosis pathways, and regulating autophagy and apoptosis is a potential therapeutic strategy for cerebral ischemic injury.

In this experiment, the effect of Qingre Huayu Decoction on autophagy and apoptosis of Ca^{2+}/CaMKKβ-AMPK-mTOR pathway in rats with acute cerebral ischemia injury was observed, and its protective mechanism was explored.

2 Materials and methods

2.1 Experimental materials 50 SPF-grade SD rats weighing (250 ± 50) g were selected and purchased from Hunan SJA Laboratory Animal Co., Ltd., and the license number of the experimental unit was SYXK 2012-0001. After one week of adaptive feeding, they were transferred to experimental modeling. The medicine was purchased from the Chinese herbal medicine room of Affiliated Hospital of Jinggangshan University, and Qingre Huayu Decotion consists of several drugs (*Cornu Bubali* 30 g, leech 12 g, *Radix Paeoniae Rubra* 15 g, *Salvia miltiorrhiza* 15 g, *Pheretima* 10 g, *Ligusticum wallichii* 12 g, *Achyranthes bidentata* Blume 10 g, *Concretio Silicea Bambusae* 10 g, wine-treated rhubarb 6 g, and *Arisaema cum* bile 6 g). Decoction method of tradi-

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tional Chinese medicine is as follows: Traditional Chinese medicine was added with water to 500 mL; when the medicine was decocted until about 150 mL was left, it was poured out and stored; then 300 mL of water was added to the decocted traditional Chinese medicine to obtain 50 mL of medicine liquid; finally, the medicine liquid obtained by two decoctions was mixed for preservation and later use.

2.2 Methods

2.2.1 Animal grouping and drug administration. The rats were divided into four groups by random number table method: sham operation group, ischemia model group and low, medium and high dose group of Qingre Huayu Decoction, with 10 rats in each group. The middle cerebral artery obstruction (MCAO) model was constructed in rats using the documented operation method[4], and about 8 mm fishing line was inserted during the operation in the sham operation group, which was not enough to block the middle cerebral artery blood flow. Each dose group of Qingre Huayu Decoction was given equal volume of Qingre Huayu Decoction by gavage 2 h after operation, and the dosage was based on the equivalent dose coefficient conversion method for human and animal body surface area[5]. The low, medium and high dose groups of Qingre Huayu Decoction were given Qingre Huayu Decoction at 0.7, 1.4, 2.8 mL/100 g, respectively. Both the sham operation group and the model were given equal volume of normal saline by gavage twice a day at regular intervals, and samples were sacrificed after two consecutive weeks.

2.2.2 Neurological function scoring criteria. After the rats were modeled and awake, Longa's 5-level neurological deficit scoring method[6] was used to score the neurological deficit of the rats, and those with a score of 1 to 3 points were included in the experimental study.

2.2.3 Section preparation and staining. After deep anesthesia, the rats were fixed in supine position. The completely removed brain tissue was fixed with 4% PFA at 4 ℃ for 48 h. After transferring it into 30% sucrose solution until the brain tissue sank to the bottom, the tissue was cut into 20 μm of sections using a cryoslicer, and then adsorbed on anti-detachment slides and stored at −20 ℃. After conventional HE staining, the pathological changes of nerve cells were observed in the field of vision of 10 × 20.

2.2.4 Cell apoptosis detection by TUNEL. After the prepared paraffin sections were debenzenized, they were sequentially soaked in absolute ethanol of different concentrations, incubated in proteinase K. After washing the sections, TUNEL reaction mixture was added, the sections were sealed with PBS solution, and the sections were observed under a fluorescent microscope to calculate the proportion of apoptotic cells.

2.2.5 Detection of autophagy and apoptotic proteins in brain tissue by Western-blot. The rat brain tissue samples were completely taken out and homogenized, and then the total protein of the samples was extracted. Then, after the protein content was measured, SDS-PAGE electrophoresis, membrane transfer, immune reaction, chemiluminescence, development and fixation were carried out, and the gray value of the target band was analyzed and calculated using a gel image processing system.

2.2.6 Statistical methods. The data were processed by SPSS 22.0 software, and the measurement data were expressed by mean ± standard deviation ($\bar{x} \pm s$). t test was used for comparison between two groups. When comparing multiple groups, a test for homogeneity of variance was performed first. One-way ANOVA test was used when the variances were uniform, and non-parametric rank-sum test was used when the variances were uneven. $P < 0.05$ indicated that the difference was statistically significant.

3 Results and analysis

3.1 Results of neurological deficit scoring of rats in each group The results showed that the neurological function score of the sham operation group was 0; compared with the sham operation group, both the ischemia model group and the Qingre Huayu formula group showed obvious nerve function damage ($P < 0.01$); compared with the model group, the neurological function score of the Qingre Huayu Decoction group decreased significantly ($P < 0.05$), and the high-dose group decreased significantly, as shown in Table 1.

Table 1 Neurological deficit score and neurological apoptosis ratio of rats in each group ($\bar{x} \pm s$, $n = 10$)

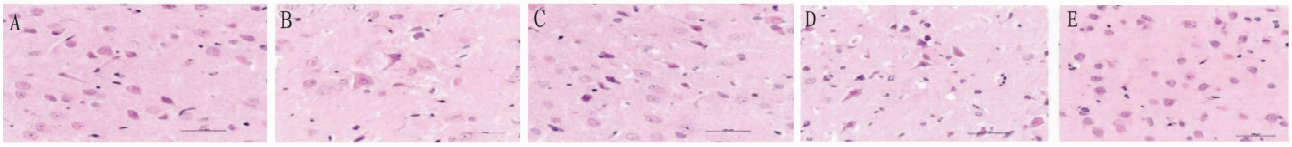
Group	Neurological function score // points	Apoptosis ratio // %
Sham operation group	0	3.25 ± 0.26
Ischemia model group	2.85 ± 0.15△	48.60 ± 5.35△
Qingre Huayu Decoction-low	2.61 ± 0.14△▲	42.67 ± 5.30△▲
Qingre Huayu Decoction-medium	2.23 ± 0.16△▲	38.03 ± 4.27△▲
Qingre Huayu Decoction-high	1.96 ± 0.13△▲	36.76 ± 4.18△▲

NOTE Compared with the sham operation group, △$P < 0.01$; compared with the ischemia model group, ▲$P < 0.05$.

3.2 Results of neuronal cell apoptosis in each group of rats A small number of stained TUNEL-positive cells were seen in the sham operation group, and the TUNEL-positive cells in the ischemia model group and Qingre Huayu Decoction group were significantly more than those in the sham operation group ($P < 0.01$); compared with the ischemia model group, the proportion of TUNEL-positive cells in each dose group of Qingre Huayu Decoction decreased ($P < 0.05$), as shown in Table 1.

3.3 HE staining results of each group In the sham operation group, the neuron morphology of rats was complete, the cell membrane was round or oval, the nucleolus size was different, and the nucleus staining was clear. In the ischemic model group, the neurons were disordered, the intercellular space was widened, the cell body was shrunk, the nucleus was pyknotic, the nucleus was heteromorphic, the chromatin was deeply stained, and the nucleolus disappeared. A small number of neuronal nucleus abnormali-

ties, deep chromatin staining, shortened intercellular space were seen in each group of Qingre Huayu Decoction, and the overall pathological damage decreased compared with the ischemia model group. The improvement in Qingre Huayu Decoction-high dose group was more obvious, as shown in Fig. 1.



NOTE A. sham operation group; B. ischemia model group; C. Qingre Huayu Decoction-low; D. Qingre Huayu Decoction-medium; E. Qingre Huayu Decoction-high. The same in Fig. 2.

Fig. 1 HE staining results of each group

3.4 Autophagy and apoptotic protein levels in Ca^{2+}/CaMKKβ-AMPK-mTOR pathway in rat brain tissue Compared with the sham operation group, the protein levels of CaMKKβ and AMPK in the model group were significantly reduced, the level of mTOR increased, the protein expression of Beclin-1 and LC3 decreased, and the protein expression of Caspase-3 increased ($P<0.01$). After treatment with Qingre Huayu Decoction, CaMKKβ and AMPK increased, mTOR level decreased, Beclin-1 and LC3 protein expression increased, and Caspase-3 protein expression decreased ($P<0.05$), as shown in Fig. 2 and Table 2 – 3.

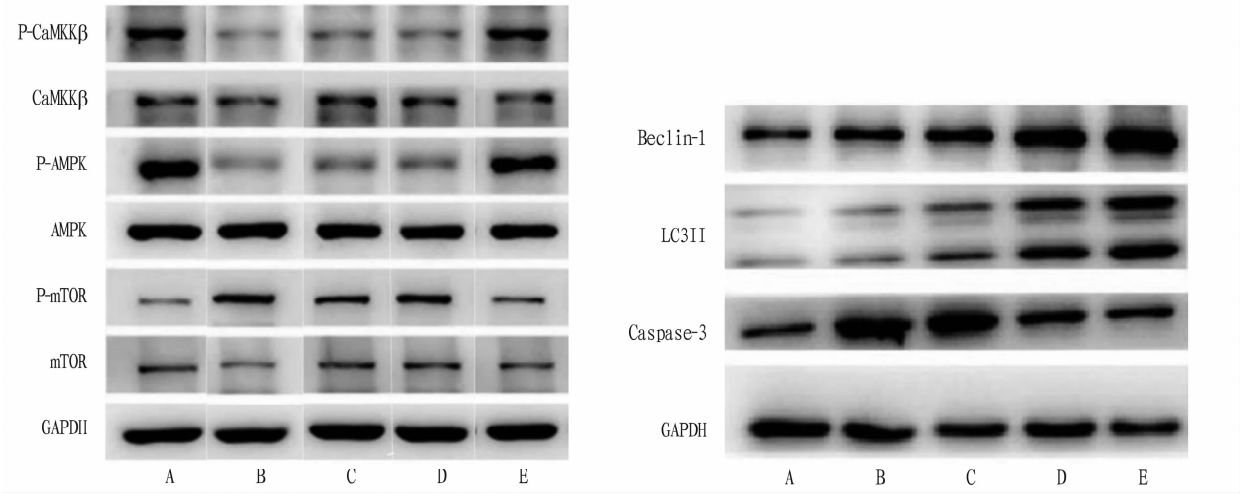


Fig. 2 Changes of autophagy and apoptotic protein levels in each group

Table 2 Ca^{2+}/CaMKKβ-AMPK-mTOR protein results for each group ($\bar{x}\pm s$)

Group	CaMKKβ	AMPK	mTOR
Sham operation	2.21 ± 0.02	4.15 ± 0.05	1.23 ± 0.02
Ischemia model	0.89 ± 0.01	1.02 ± 0.02	4.47 ± 0.07
Qingre Huayu Decoction-low	1.07 ± 0.01▲	2.22 ± 0.03▲	3.53 ± 0.04▲
Qingre Huayu Decoction-medium	1.26 ± 0.02▲	2.68 ± 0.04▲	2.65 ± 0.03▲
Qingre Huayu Decoction-high	1.74 ± 0.03▲	4.01 ± 0.06▲	1.68 ± 0.02▲

NOTE Compared with the ischemia model group, ▲$P<0.05$. The same below.

Table 3 Results of autophagy and apoptotic protein levels of rats in each group ($\bar{x}\pm s$)

Group	Beclin-1	LC3-1	LC3-11	Caspase-3
Sham operation	1.22 ± 0.01	1.05 ± 0.03	1.11 ± 0.04	1.23 ± 0.02
Ischemia model	4.26 ± 0.62	2.52 ± 0.27	2.47 ± 3.24	4.16 ± 0.54▲
Qingre Huayu Decoction-low	4.70 ± 0.39▲	2.62 ± 0.21▲	2.53 ± 0.24▲	3.84 ± 0.36▲
Qingre Huayu Decoction-medium	4.56 ± 0.34▲	2.68 ± 0.15▲	2.65 ± 0.28▲	3.01 ± 0.31▲
Qingre Huayu Decoction-high	5.44 ± 0.62▲	2.81 ± 0.23▲	2.80 ± 0.29▲	2.48 ± 0.22▲

4 Discussion

In recent years, many studies have reported that apoptosis and autophagy play an important role in the occurrence and development of ischemic stroke, and apoptosis is one of the main forms of neu-

ronal death after cerebral ischemia[7]. Apoptosis is an active death process under the regulation of specific genes, and it is one of the important mechanisms to maintain the stability of intracellular environment. Autophagy is a reversible form of cell death, which can regenerate cells. Unlike necrosis and apoptosis, which eventually lead to cell death, autophagy plays a protective role in the continuous renewal and evolution of various substances in cells. Under physiological conditions, basic autophagy plays an important role in maintaining the stability of cell genome, promoting the senescence of damaged cells, and recycling macromolecular substances in cells. However, when insufficient or excessive autophagy occurs in cells, it will lead to the imbalance of intracellular environment and eventually lead cells to death[8]. Therefore, regulating apoptosis and autophagy may become a potential therapeutic strategy for ischemic stroke.

Ca^{2+} is the most common and important second messenger. As an important regulatory ligand, CaM is its main intracellular receptor to dynamically regulate Ca^{2+}-dependent target proteins and their downstream pathways. Ca^{2+}/calmodulin-dependent protein kinase beta (CaMKKβ) is a member of the CaM kinase family that specifically phosphorylates and activates CaM kinases I and IV. AMP-activated protein kinase (AMPK) is an important energy receptor in cells, which plays an important role in regulating energy balance. AMPK and CaMKK are highly expressed in central nervous system tissues and are activated during stress. AMPK can be activated in the upstream kinase mode through CaMKKβ. Activated AMPK can inhibit mToR activity and ultimately induce autophagy. mTOR is a relatively conserved serine/threonine protein kinase in evolution, which can be affected by many factors such as growth factors, cytokines, nutrition and metabolic status. By phosphorylating its downstream target proteins, mTOR participates in gene transcription and protein expression, and then affects biological activities such as autophagy and apoptosis. Studies have shown that[9] when the concentration of Ca^{2+} in the cytoplasm of nerve damage increases, CaMKKβ can be activated, and the activation of CaMKKβ can positively act on AMPK to activate it, thus negatively regulating mTOR and then regulating autophagy.

Caspase-dependent apoptosis is one of the two main mechanisms of apoptosis, and the expression changes of Caspase are closely related to apoptosis[10]. Among Caspase family proteins, Caspase-3 is the final effector involved in the apoptosis process. Beclin-1 and LC3 are the main proteins involved in autophagy, among which Berlin-1, as the direct executor of autophagy, can not only promote the formation of autophagosome, increase the occurrence of autophagy, but also participate in neuronal apoptosis[11]. The protein molecules represented by the LC3 complex are involved in the formation of autophagosome membrane and further participate in the degradation stage of autophagy[12]. Beclin-1 and LC3, as representative indicators reflecting the occurrence and intensity of autophagy, play an important role in the progression of ischemic stroke. Studies have shown[13] that in the rat model of acute cerebral infarction, the up-regulation of LC3 and Beclin 1 is involved in the induction of autophagy, and the administration of autophagy inhibitor (3-MA) can inhibit the activation of autophagy and alleviate cerebral ischemia injury. This study showed that normal and few expressions of CaMKKβ, AMPK, mTOR, Beclin-1, LC3 and Caspase-3 proteins were found in the brain tissues of rats in each group; compared with the ischemia model group, CaMKKβ and AMPK increased, mTOR level decreased, Beclin-1 and LC3 protein expression increased, Caspase-3 protein expression decreased, TUNEL staining positive cells decreased, and HE staining pathological damage was reduced in each dose group of Qingre Huayu Decoction, indicating that Qingre Huayu Decoction may play a protective role in brain by regulating apoptosis and autophagy protein levels.

The etiology and pathogenesis of ischemic stroke are complex. Academician Wang Yongyan put forward the theory of "toxic damage to brain collaterals" and pointed out that toxic damage to brain collaterals is the key to the pathogenesis[14]. The disease is always caused by blood stasis, heat toxin, phlegm toxin, and fire toxin. The brain marrow is damaged, leading to "brain collaterals stasis", and "phlegm stasis and heat stasis" is an important link. However, "phlegm and blood stasis → heat accumulation → toxic change → brain injury" is an important pathological chain reaction of ischemic stroke, which is consistent with the abnormalities such as excitotoxic substances, oxygen free radicals and inflammatory immune factors in the brain proposed in clinical medical practice. Phlegm and blood stasis will accumulate heat for a long time, and heat accumulation leads to poison. These pathological factors affect each other and form a vicious circle of new pathogenic factors, which ultimately aggravates the disease.

Under the guidance of the pathogenesis theory of "toxic damage to the brain collaterals", Qingre Huayu Decoction is formulated according to the characteristics of the pathogenesis of ischemic stroke. In the decoction, *Cornu Bubali* can clear heat, remove blood stasis and remove the effects of poison; *Salvia miltiorrhiza*, also as a monarch drug, can activate blood circulation, dredge collaterals and clear the heart; *Radix Paeoniae Rubra*, *Ligusticum wallichii* and leech can strengthen the effect of *Salvia miltiorrhiza* in promoting blood circulation and dredging collaterals, *Pheretima* and wine-treated rhubarb can not only promote blood circulation, but also clear away heat and remove the effects of poison, they are used as ministerial drug; it is supplemented with *Arisaema cum* bile to resolve phlegm for resuscitation, *Concretio Silicea Bambusae* to clear away heat and resolve phlegm, and *Achyranthes bidentata* Blume to reduce blood heat. All medicines are used in combination to play the functions of clearing away heat and detoxifying, removing blood stasis and dredging collaterals. Qingre Huayu Decoction relieves patients' symptoms by interrupting the malignant pathological cycle of "blood stasis, phlegm and retained fluid, heat toxicity, and secondary stasis". Previous studies have found that it

has a good curative effect on ischemic stroke[15].

To sum up, this study found that Qingre Huayu Decoction may play a role in reducing cerebral ischemic injury by regulating apoptosis and autophagy, which provides a good experimental basis for the theory of "toxic damage to brain collaterals" to prevent and treat ischemic stroke.

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