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Effect of Buyang Huanwu Decoction on Cerebral Ischemia-Reperfusion Injury of Rats by Regulating Endoplasmic Reticulum Stress

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Abstract:

Objective

To study the effect of Buyang huanwu decoction (BYHWD) on cerebral ischemia-reperfusion (I/R) injury in rats by regulating endoplasmic reticulum stress.

Methods

Sixty male SD rats were randomly divided into the operated group and the sham operated group. The rats in the operated group were established the middle cerebral artery occlusion (MCAO) model to simulate cerebral I/R injury. The successful model rats were randomly divided into model group, BYHWD group and Nimodipine group. The rats in the drug group were orally given BYHWD twice a day 14.3g·kg^{-1} after recovery from surgery. Rats in Nimodipine groups were given by intraperitoneal injection (10mg·kg^{-1}) once after waking up. Rats of treatment groups and model group were decapitated after reperfusion for 24 hours. Observation of neurological deficits in rats with Zea Longa score. TTC staining and toluidine blue staining method were used to determine the cerebral infarction volume and neurons damage of rats. Western blotting detects the GRP78 protein expression of rats' right cerebral cortex.

Results

The rats in the sham operated group did not have neurological deficits, no cerebral infarction, with abundant and regular neurons and Nissl bodies, and no obvious expression of GRP78. Compared with the sham operated group, the neurological function of the model group decreased significantly, infarct volume was significant ($P<0.05$), neurons and Nissls decreased, and GRP78 protein expression increased ($P<0.05$). Compared with the model group, the rats in the BYHWD group had reduced neurological deficits and cerebral infarction volume, with abundant nerve cells and Nissls ($P<0.05$), and significantly increased GRP78 expression. There was no significant difference between BYHWD group and Nimodipine group.

Conclusion

BYHWD can reduce cerebral I/R injury in rats by regulating endoplasmic reticulum stress.

Keywords: *Buyang huanwu decoction; cerebral ischemia-reperfusion injury; endoplasmic reticulum stress; GRP78; ischemic stroke;*

1. INTRODUCTION

Ischemic stroke is a kind of cerebral vascular disease that causes ischemia, hypoxia and infarction of the brain for cerebral vascular stenosis or occlusion. The patients with ischemic stroke were often manifested as dysfunction of the contralateral limb with weak numbness and accompanied by the loss of consciousness in clinic, which is globally recognized as a critical and severe disease [1,2]. The cerebral ischemia-reperfusion (I/R) injury is often occurred when the blood flow is re-perfused to the ischemic

area [3,4].

This cerebral I/R injury is affected by the multiple factors including reperfusion time and intensity, and is also related to the multiple mechanisms such as oxidative stress, inflammatory response, autophagy and apoptosis, which determines the final infarct volume of the brain [5,6,7]. In recent years, studies have found that endoplasmic reticulum stress（ERS）plays an important role in the occurrence and development of cerebral I/R injury, and has an important impact in the apoptosis and neuron survival [8,9]. Glucose regulated protein 78 kD (GRP78) is a marker protein for ERS [10]. Then, GRP78 level in the brain can reflect the state of neuron ERS.

Speaking in general terms, the ischemic stroke belongs to the category of "wind stroke" in Chinese medicine. Qi vacuity and inability to promote the blood flow leads to blood stasis, which ultimately results in blood stasis. Then, qi vacuity with blood stasis was considered as the main pathogenesis for ischemic stroke. Buyang huanwu decoction (BYHWD) is a classic prescription of Chinese medicine for treating the wind stroke of pattern qi vacuity with blood stasis, and has achieved the positive clinical effects. Previous studies in our laboratory have showed that BYHWD can alleviate cerebral I/R injury in rats, but whether the ERS involved in BYHWD's anti-cerebral I/R injury has not been reported. In the present study, the model of cerebral I/R injury for rats were established by occlusion of middle cerebral artery and the effects of BYHWD on the regulation of ERS were investigated by observing the behavioral score, neuronal morphological changes and the expression of ERS marker GRP78 protein for I/R injured rats.

2. Materials and Methods

2.1. Animals

Sixty healthy adult SD rats (male, 300 ± 20g) were bought from Beijing Vitonlihua Laboratory Animal Co., LTD. (License Number: SCXK (Beijing) 2016-0011). These rats were fed in the experimental animal center with free access to food and water, under the laboratory conditions for the temperature of 25 ± 1℃ and 12-hours light-dark cycle. It is for a week that the rats were allowed to acclimatize to the laboratory environment before modeling. All of the experimental rats were approved by the Animal Ethics Committee of Hebei University of Chinese Medicine.

2.2. Reagents and Instruments

BYHWD medicines (per dose contains 120g of Astragalus, 5g of Red peony, 6g of Tangkuei head, 3g of Carthamus, 3g of Earthworm, 3g of Peach kernel, and 3g of Chuanxiong) were purchased from Beijing Tongrentang Pharmacy. Nimodipine (20mg, purity ≥98%, 20220109) was bought from Solarbio (Beijing, China). TTC dye and toluidine blue aqueous solution were obtained from Seville (Wuhan, Hubei, China). Protein Marker was supplied by ThermoFisher Scientific. Antibody to GAPDH was purchased from Beyotime (Shanghai, China). Antibody to GRP78 (ab108613) and goat anti-rabbit IgG (ab205718) were purchased from Abcam (USA). The tissue embedding machine, automatic rotary slicer and microscope photo system were supplied by Leica (Germany). Multifunctional microorifice plate reader was purchased from ThermoFisher Scientific (USA) and the multifunctional imaging system was obtained from Vilber (France).

2.3. Methods

2.3.1. Establishment of Model Screening

A total of 60 rats were randomly divided into the operated group by 50 and the sham group by 10. All of the operated group rats underwent middle cerebral artery occlusion (MCAO)/reperfusion, and the specific steps were as follows:

Firstly, SD rats were anesthetized with 4% sodium pentobarbital, and placed on the operating table in a supine position. Secondly, Cutting along the midline of the neck to separate the right muscle bluntly after the rats were disinfected, and expose the common carotid artery (CCA), internal carotid artery

(ICA) and external carotid artery (ECA). Thirdly, a thread was inserted through the ECA and the ICA to reach the middle cerebral artery (MCA) and occlude it after having ligated and severed the ECA. Finally, pulling out the thread gently to achieve reperfusion after 2 hours of ischemia, reperfusion lasted for 24 hours. Rats in the sham group just exposed the CCA, ICA and ECA without embolization. Zea Longa method was used to screen the rats when they were awake. The scoring criteria are as follows:

The rats had no behavioral problems, 0 score. The left paw was not fully extended when the tail was lifted, 1. The rats walked in circles to the left, unable to walk in a straight line, 2. The rats tumbled to the left or were unable to walk, 3. The rats were unable to stand or lose consciousness, 4. Of those, the rats with scores of 1 to 3 were assigned to follow-up experiments, while 0 score and 4 scores' are removed.

2.3.2. Grouping and Administration

The successful model rats were randomly divided into model group, BYHWD group and Nimodipine group. The rats in the drug group were orally given BYHWD twice a day 14.3g·kg^{-1} after recovery from surgery with once after resuscitation, and once again after 12 hours. Rats in Nimodipine group were given intraperitoneal injection (10mg·kg^{-1}) once after waked up. No drug intervention was performed in the model group. Rats in each treatment group and model group were killed by decapitation 24 hours after reperfusion.

2.3.3. Evaluation of Neurological Score with Zea Longa Method

24 hours after reperfusion, the rats in the treatment groups and model group were again evaluated for neurological deficit before decapitation, aiming to compare the improvement of neurological function defect between the treatment group and the model group. The scoring criteria are the same as above.

2.3.4. Detection of Cerebral Infarct Volume by TTC Staining

After 24 hours of reperfusion, rats in each group were deeply anesthetized with 4% pentobarbital sodium. The rat brains of each group were quickly collected on the ice and placed in the mold, then refrigerated at -20℃ for 18 minutes. Taking out the brain mold, each brain was cut into 2 mm coronal slices for 5. Put the brain slices into a glass dish, added TTC dye, and incubated in 37 ℃ incubator for 20 minutes. 4% paraformaldehyde fixed brain tissue slices for 24 hours and took pictures. Cerebral infarct volume (infarct volume = total infarct volume/whole brain *100%) was calculated by image-Pro Plus 6.0 Image analysis system.

2.3.5. Observation of Nissl and Neuronal Morphological Changes by Toluidine Blue Method

The rats were anesthetized after 24 hours of reperfusion. Brains of the rats in each group were collected on ice and fixed in 4% paraformaldehyde solution for one week. After that, the brain tissue was embedded in paraffin and the coronal section was performed with a thickness of about 4 μm. The paraffin sections were deparaffinized to water, washed with distilled water, immersed in toluidine blue solution for 5 minutes, washed with tap water, differentiated with 95% alcohol, transparent with xylene, and mounted with neutral gum. Observe the morphological changes of Nissl bodies and neurons under a 400-fold upright microscope, and select 6 different positions to take pictures and count for analysis.

2.3.6. Detection of GRP78 Protein Expression with Western Blotting

The cortical tissue of each group of rats was added to the RIPA lysis buffer, homogenized, centrifuged, and the supernatant was taken to obtain total protein. BCA method determined the protein concentration of the sample. Then, the protein was separated by SDS-PAGE electrophoresis and transferred to the PVDF membrane by wet transfer. The PVDF membrane was sealed in 5% skimmed milk powder at room temperature for 3 hours, and then incubated with the primary antibody of GRP78 (1:5000) for 2 hours. The membrane was washed 3 times with TBST for 7 minutes each time, and then incubated with secondary antibody (1:20000) for 1 hours. After that, the PVDF was exposed by ECL and the protein bands were analyzed by VisionCapt software.

2.4. Statistical Method

All data were analyzed by GraphPad Prism 8.0.2 software and presented as the mean ± SD. The differences between groups of all experimental data were used by One-way analysis of variance (ANOVA) and Tamhane's T2 was used for post-hoc tests. *P<0.05* was considered statistically significant.

3. RESULTS

3.1. Model Success Rate

There were 50 rats to perform I/R and the neuronal score was 1-3 for 41 rats. Thus, the successful model rate of I/R injury is 82%.

3.2. Effect of BYHWD on Neurological Deficits in Rats with Cerebral I/R Injury

Table 1 shows the neurological deficits of the rats in each group. The score of rats in the sham operated group was 0, and no neurological deficits. Compared with the sham operated group, the scores of rats in the model group were significantly increased (*P<0.05*). Compared with the model group, the neurological deficits score of BYHWD group decreased (*P<0.05*), and the score of Nimodipine group was also lower than that of model group (*P<0.05*). These results indicate that BYHWD can effectively improve the behavior for cerebral I/R injury.

Table1. *Effect of BYHWD on neurological deficits of rats with cerebral I/R injury (Mean ± SD, n=10)*

Groups	Scores
Sham	0
Model	2.72±0.43$^{\#}$
BYHWD	1.65±0.62*
Nimodipine	1.71±0.34*

$^{\#}$*P<0.05 vs sham group.* **P<0.05 vs model group*

3.3. Effect of BYHWD on Cerebral Infarction Volume in Rats with Cerebral I/R Injury

Figure 1 shows the effect of BYHWD on cerebral infarction volume in rats with cerebral I/R injury. Normal brain tissue was red and the infracted area occurred the white. No cerebral infarction lesions were observed in the sham group, while the significant infarcts were observed in the model group. Compared to the model group, the volume of cerebral infarction in BYHWD group decreased *(P<0.05)*, and the infarct volume in Nimodipine group was also small *(P<0.05)*. The results show that BYHWD can effectively reduce the volume of infarction in the ischemic area.

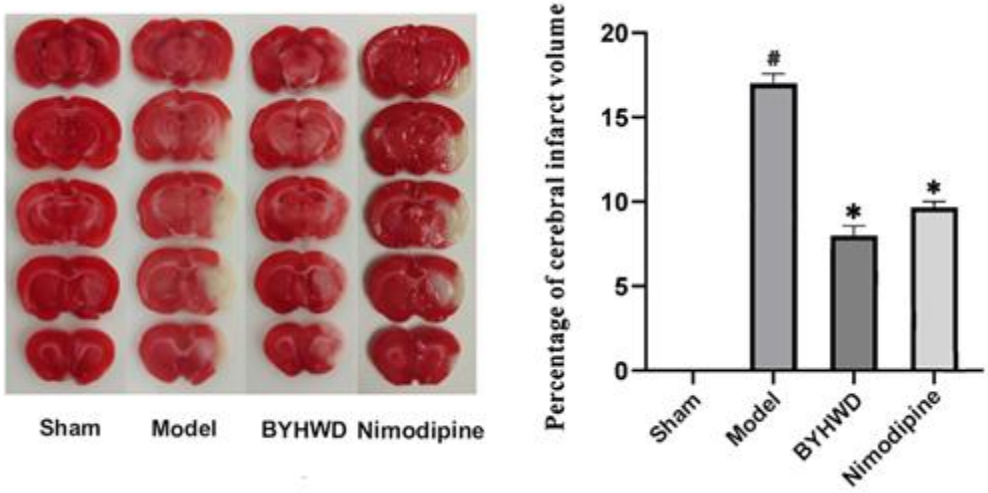

Figure1. *Effect of BYHWD on cerebral infarction volume in rats with cerebral I/R injury. Left: TTC staining images of brain sections of rats in each group. Right: Percentage of cerebral infarction volume in each group (Mean ± SD, n=3).* $^{\#}$*P<0.05 vs sham group.* **P<0.05 vs model group.*

3.4. Effect of BYHWD on Neurons and Nissl in Rats with Cerebral I/R Injury

Figure 2 shows the effect of BYHWD on neurons and Nissl in rats with cerebral I/R injury. The nucleus is blue, Nissl is dark blue and the stained background is light blue. Neurons in the sham operated group are regular, with abundant Nissl bodies. Compared with the sham operated group, the neurons in the model group were deformed and necrotic, and the number of neurons and Nissls were significantly reduced *(P<0.05)*. Compared with the model group, the cell condition of BYHWD group was improved, the number of neurons was abundant (*P<0.05*), and Nissl bodies were more. These dates show that BYHWD can effectively reduce the damage of neurons and Nissl in MCAO model.

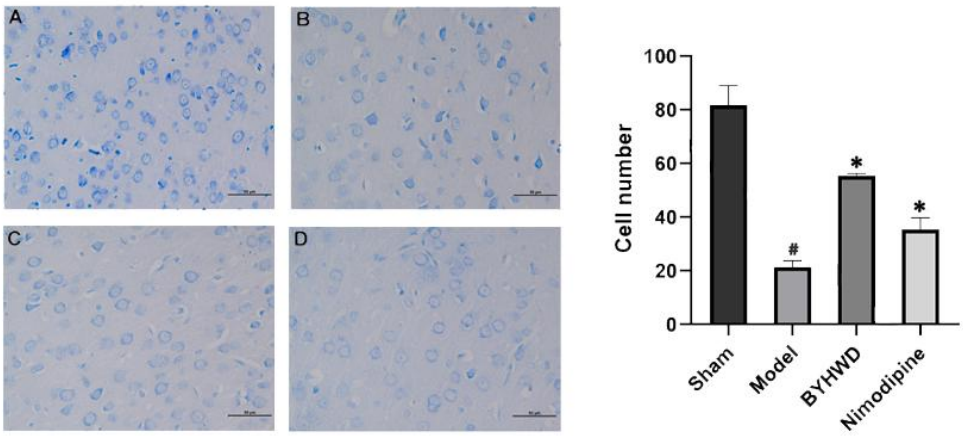

Figure2. *The effect of BYHWD on Neurons and Nissl in rats with cerebral I/R injury. A: Sham. B: Model. C: BYHWD. D: Nimodipine. Left: Toluidine blue staining in cortical areas of ischemic cerebral tissue of rats in each group (X400). Right: Analysis of the number of neurons in cortical areas of ischemic cerebral tissue in each group (Mean ± SD, n=6). #P<0.05 vs sham group. *P<0.05 vs model group.*

3.5. Effect of BYHWD on GRP78 Protein Expression

Figure 3 shows the Western blotting results for rats' cortical GRP78 protein expression. Compared with the sham operated group, the GRP78 protein expression of model group was significantly increased (*P<0.05*). Compared with the model group, the expression of BYHWD group showed significantly increased (*P<0.05*), and Nimodipine group as to improve the expression of GRP78 protein. These results indicate that BYHWD may increase the expression level of GRP78 protein and reduce cerebral I/R injury.

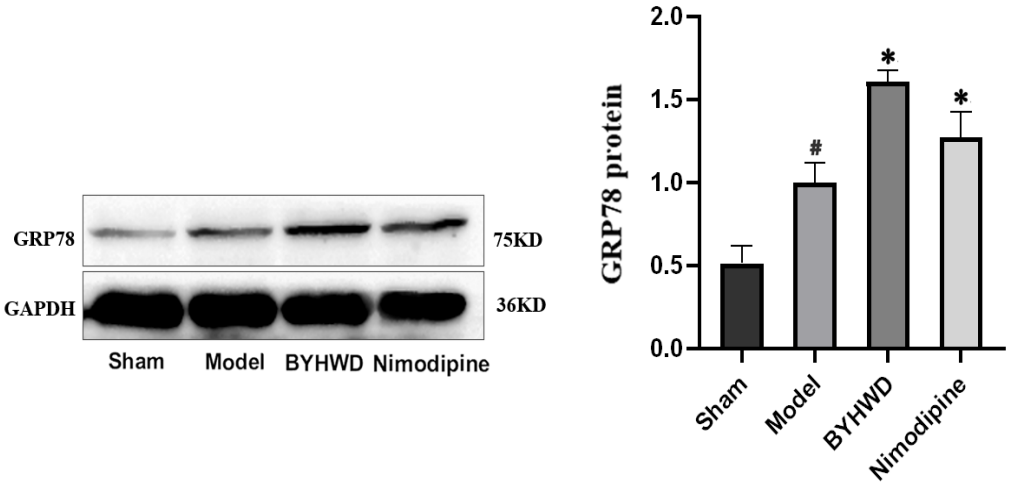

Figure3. *The effect of BYHWD on the expression of GRP78 protein in the cortex of rats with cerebral I/R injury. Left: Bands of the GRP78 protein expression in each group of rats. right: GRP78 protein expression of each group of rats (Mean ± SD, n=3). #P<0.05 vs sham group. *P<0.05 vs model group.*

4. DISCUSSION

Ischemic stroke is a common disease and frequently-occurring disease that seriously threatens human life and health. It has the characteristics of high incidence, high mortality and disability, and high recurrence rate. The blood flow restored to the ischemic area is currently the main treatment strategy for ischemic stroke. However, it often induces more serious damage, that is, cerebral I/R injury, when the blood flow is re-perfused to the ischemic area. Cerebral I/R injury is an important pathophysiological process affecting ischemic stroke, which can result neurological deficits, leading to contralateral limb dysfunction or loss of consciousness, and ultimately determine the infarct volume of the brain. In this experiment, MCAO model was performed to simulate cerebral I/R injury, and it was found that rats in the model group had severe neurological dysfunction with obvious infarction volume, while BYHWD could effectively improve the neurological deficits in the operated group. At the same time, the results of TTC staining showed that BYHWD could effectively reduce the volume of cerebral infarction in rats. All of these indicate that BYHWD can treat ischemic stroke and reduce cerebral I/R damage.

Endoplasmic reticulum (ER) is an organelle ubiquitous in eukaryotic organisms, including rough endoplasmic reticulum and slippery endoplasmic reticulum, which can secrete and fold proteins, synthesize lipids, store and release Ca^{2+} function, and plays a vital role in maintaining the homeostasis of the intracellular environment [11]. However, when the body undergoes pathological or physiological changes, such as ischemia and hypoxia, disorder of Ca^{2+} balance, drug toxins and other influencing factor, the homeostasis of endoplasmic reticulum will be destroyed. Then, a large number of unfolded proteins are accumulated and the ERS is occurred [12]. The Nissl body is mainly composed of rough endoplasmic reticulum, which is an important part of protein synthesis in neurons and can effectively reflect neurological functions. Under physiological conditions, there are abundant Nissl bodies in neurons, but if pathological changes occur, Nissl bodies will decrease and disintegrate, which will affect the function of neurons. In this experiment, the morphological changes of Nissl and neurons were observed by the toluidine blue method, and it was found that BYHWD can reduce the degradation of Nissl body and reduce the damage and death of neurons, indicating that BYHWD may play a protective role in reducing neurological function attenuation and death by maintaining the function of the rough endoplasmic reticulum.

ERS is a protective stress response of the body, which restores the homeostasis of the endoplasmic reticulum through the unfolded protein response (UPR) [13]. GRP78 is a marker protein for ERS. Under normal conditions, GRP78 interacts with endoplasmic reticulum transmembrane receptor inositol-requiring enzyme-1α (IRE1α), activating transcription factor 6 (ATF6) and the endoplasmic reticulum-resident kinase (PERK), and they are all in an inactive state [14]. However, when the aforementioned pathological changes occur in the endoplasmic reticulum, GRP78 separates from the endoplasmic reticulum transmembrane receptors, and instead binds to the ever-increasing new unfolded proteins. At the same time, the isolated transmembrane receptors resume their activity and trigger URP, thereby achieve the role of maintaining the homeostasis of the endoplasmic reticulum [15,16]. In addition, studies have found that the expression of GRP78 in rats of MCAO model is highest at 24 hours after surgery, and then gradually declines [17]. In this experiment, the GRP78 protein expression of each group of rats was detected by Western Blot technology. The results showed that the GRP78 protein expression of the sham operated group was low, while the GRP78 protein expression of the model group was significantly increased compared to the sham operated, indicating that the cerebral I/R damage triggered ERS. While the expression of GRP78 in the BYHWD group was more significant than that in the model group, indicating that BYHWD promoted the expression of GRP78, and may promote the correct folding of the protein by up-regulating the expression of GRP78, and reduce the production of unfolded protein or misfolded protein, thus can help restore endoplasmic reticulum homeostasis and reduce cerebral I/R damage. This result is consistent with the data in promoting the expression of GRP78 to reduce the impact of cerebral I/R damage on the cerebral cortex and improve neurological function [18,19]. Meanwhile, our experimental results also show that Nimodipine can also enhance the expression of GRP78, reduce neurons damage, and plays a protective role in the brain.

There was a basic theory in traditional Chinese medicine that the heart governs the blood and vessels, which means that the heart-qi promotes and regulates the blood circulation in the vessels, flows into the body, and nourishes the various organs. If the heart-qi push is weak, the blood flow in the brain slows

down, then the "blood stasis" is occurred and result in the "stroke". BYHWD is a classic prescription of Chinese traditional medicine for the treatment of stroke with qi vacuity and blood stasis pattern, and has been achieved some clinical data. The prescription uses Astragalus, Tangkuei head, Red peony, Chuanxiong, Peach kernel, Carthamus, Earthworm and other herbs. Among of these herbs, Astragalus is used to supplement heart-qi, and is the monarch. Tangkuei head is used for promoting blood circulation and dredging collaterals and as minister. Red peony, Carthamus and Peach kernel are assistants of qi and blood circulation. The Earthworm is good for the whole body, playing the effect of freeing the network vessels. The prescription uses lots of Astragalus as the monarch medicine to supply heart-qi and improve the function of "heart governing the blood and vessels". "Blood flow if qi is sufficient", BYHWD promotes blood flow in the brain by supplementing the heart-qi and reduces the production of pathological substances such as "blood stasis", so as to reduce the cerebral infarction volume of rats, improve the neurological deficit, reduce neurons damage and help restore the brain microenvironment homeostasis.

In summary, BYHWD may reduce cerebral I/R injury by enhancing the protective effect of ERS, reduce neurons death, decrease cerebral infarction volume, and improve neurological function. From the perspective of Chinese medicine, it may be related to BYHWD to supplement heart-qi, improve the function of "heart governing the blood and vessels", and promote blood circulation in the brain.

AUTHORS CONTRIBUTION

Gao Weijuan conceived and designed the experiment. Hou Xiaochan, Zhang Ziwei, Shan Yudong, Jin Xiaofei, Yu Wentao and Zhou Xiaohong conducted the test and wrote the manuscript.

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