Buyang Huanwu Decoction Alleviates Cerebral Ischemia-Reperfusion Injury via Sirtuin 1/Autophagy Pathway

Han Li  
Guangzhou University of Chinese Medicine

Xin-Yi Lu  
Guangzhou University of Chinese Medicine

Dong Peng  
Guangzhou University of Traditional Chinese Medicine First Affiliated Hospital

Yang Zhang  
Guangzhou University of Chinese Medicine

Hao Lin  
Guangzhou University of Chinese Medicine

Shi-Jie Zhang  
shijiezhang@gzucm.edu.cn  
Guangzhou University of Chinese Medicine

Li Guan  
Guangzhou University of Chinese Medicine

Research

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Abstract

Background

Stroke accounts for a large proportion of deaths from disease around the world. Buyang Huanwu Decoction (BHD) is used to protect against stroke and stroke-induced disability for many years in China. However, the mechanism of BHD to protect against stroke is still confused.

Methods

The middle cerebral artery occlusion and reperfusion (MCAO-R) model was used to investigate in this study. The animals were administrated with BHD (5, 10 and 20 g/kg) or rapamycin respectively. Infarct size and modified neurological severity score (mNSS) on day 5 were calculated. Cellular changes around ischemic penumbra were showed by HE staining and Nissl staining. Protein expressions of nestin, brain-derived neurotrophic factor (BDNF), doublecortin on the X-chromosome (DCX) and autophagy-related protein in the cerebral peri-ischemic area were detected.

Results

In results, the post-treatment with BHD relieved brain infarct size and improved neurological deficits in MCAO-R rats. BHD protected against MCAO-R-induced neuronal necrosis, obviously enhanced autophagy (increased Beclin 1 and LC3II, decreased P62) and increased the protein expressions of nestin, BDNF and DCX. Meanwhile, BHD promoted the expression of Sirtuin1=SIRT1, an important regulator of autophagy.

Conclusions

In conclusion, our data suggested that post-treatment with BHD could protect rat brain from ischemia-reperfusion injury via SIRT1/autophagy pathway.

Background

Recently years, stroke has become one of the most common causes of death around the world. It has been the fifth leading cause of death from disease in western country but the second leading cause in China during the past 10 years(Benjamin et al. 2018, Zhou et al. 2016). Stroke occurred when the brain blood supply interrupted or reduced, which prevents brain tissue from getting oxygen and nutrients (Feigin et al. 2018). About 85% of stroke is caused by ischemia (Dai et al. 2013). Stroke can impair the neuron circuit and function (Han et al. 2018, Zhou et al. 2016). It disrupts not only the infarct area, but also the surrounding peri-ischemic areas (Hodges 1995). Stroke is a medical emergency, therefore, preventive and early action or other complications to reduce brain damage is crucial. However, limited drugs could protect the progression of stroke. Thus, finding new alternative therapeutic agents is required.
Buyang Huanwu Decoction (BHD) has been used for stroke in China for many years (Guo et al. 2015). Clinical trials have indicated that BHD could ameliorate the outcomes of stroke patients (Han et al. 2018, Hao et al. 2012, Zheng et al. 2018). BHD can protect against cerebral ischemia-reperfusion injury by promoting growth and differentiation of neuron (Jayakumar et al. 2015, Liu et al. 2013), inhibiting neural apoptosis and inflammation (Li et al. 2014), promoting angiogenesis and improving cerebral circulation (Zhang et al. 2016). Autophagy, a main degradation pathway, is essential for maintaining cellular homeostasis (Duan et al. 2018). Autophagy primarily exists in peri-ischemic areas when stroke happened (Liang et al. 2015). In addition, enhancement of autophagy had neuroprotective effect on cerebral ischemia (Sun et al. 2018). The proteins of sirtuin family can mediate autophagy (Chen et al. 2018). Among them, SIRT1 knockout mice indicated that SIRT1 is a critical regulator of autophagy (Tang et al. 2018). SIRT1 can regulate autophagic pathway under different conditions (Carloni et al. 2014, Wang et al. 2012b). In addition, SIRT1 may play an important role during stroke process. Whether SIRT1/autophagy pathway involved in the protective effect of BHD against stroke is still unknown.

In this study, we verified the neuroprotective effect of BHD against stroke in MCAO-R rats. In addition, we found BHD might produce neuroprotection effect on MCAO-R by regulating SIRT1/autophagy pathway in the peri-ischemic brain.

### Materials And Methods

#### 1. Animals

Male SD rats, 270–280 g, were supplied by the Experimental Animal Centre of Guangzhou University of Chinese Medicine. All rats were raised in the center of laboratory animal of Guangzhou University of Chinese Medicine, with a 12-hour light/dark schedule, and give free access to food and water. All experimental procedures were carried out according to the Guangzhou University of Chinese Medicine Administrative Panel on Laboratory Animal Care. After one week of adaptive housing and feeding, animals were divided into 6 groups (n = 10 each group) randomly, which received sham operation as control, middle cerebral artery occlusion and reperfusion operation as model, rapamycin, BHD (5, 10 and 20 g/kg), respectively.

#### 2. Rat middle cerebral artery occlusion and reperfusion model

Transient focal cerebral ischemia model was induced by MCAO-R model, which as described previously (Su et al. 2014, Luo et al. 2017). Briefly, rats were anesthetized with 4% isoflurane and maintained with 1.5% isoflurane via Vaporizer for Isoflurane (RWD Life Science, Shenzhen, China), a midline neck incision was to expose the right common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA). A 4-0 silicone rubber-coated nylon monofilament was inserted into ECA, then gently advanced into the ICA about 17–19 mm from the carotid bifurcation to occlude the origin of middle cerebral artery (MCA). After 2 h of occlusion, the monofilament was gently removed to restore blood flow.
During the whole surgery body temperature of the rats were maintained at about 37 °C. Rats were anaesthetized by sodium pentobarbital and sacrificed at the 5th day after surgery for relevant detection.

3. Drug administration

BHD was prepared according to previous studies (Liao et al. 2018, Shen et al. 2016). All ingredients were purchased from the Guangzhou Zhixin Chinese Herbal Medicine Co. Ltd. (Guangzhou, China). All groups rats were treated with intraperitoneal injection of Hydroxychloroquine (HCQ, Selleck, S4430, 20 mg/kg) at 30 min after surgery (Huang et al. 2016). BHD powder and rapamycin (Rapa, Selleck, S1039) were dissolved with 0.9% saline. At the onset of the reperfusion (Chong et al. 2010), the treatment groups, including MCAO-R + BHD and MCAO-R + Rapa were administrated with BHD (5, 10 and 20 g/kg) by gavage and Rapa (10 mg/kg) via intraperitoneal injection respectively.

4. Neurological scores

A modified Neurological Severity Score (mNSS) test was used to calculated the neurological deficit on day 5 after surgery (Xia, Zhang and Zhao 2018, Yang et al. 2018). Higher score indicates more severe behavioral deficits.

5. Measurement of cerebral infarct size

Rat brains were rapidly sliced with a rat brain matrix (RWD Life Science, Shenzhen, China). The sections were stained with 2, 3, 5-triphenyltetrazolium hydrochloride (TTC; Sigma, T8877) (Song et al. 2013). Then photographic images were taken.

6. HE and Nissl stainings

Brain paraffin sections were prepared. Then, the sections were washed in PBS and stained with HE or Nissl reaction mixture. The sections were then washed with PBS. Images were analyzed by using a light microscope (Leica Microsystems, Wetzlar, Germany).

7. Western blot analysis

The brain peri-ischemic area and the corresponding area were homogenized in RIPA buffer. Protein samples were separated by SDS-polyacrylamide gels, and then transferred to PVDF membranes. The membranes were incubated with the primary antibody (SIRT1, LC3, Beclin 1, P62, DCX, ACTB) overnight, and then incubated with HRP-conjugated second antibody for 1 h. Blots Digital images were visualized with an Image Lab (Bio-Rad).

8. Immunofluorescence

Rat brains sections were blocked with BSA. Then they were incubated overnight at 4 °C with primary antibody (Nestin, LC3, BDNF, DCX). The slices were incubated with fluorescence-coupled secondary antibody. After rinsing, sections were incubated with DAPI. We detected fluorescence with laser scanning confocal microscope (ZEISS 8.0, Germany).

9. Statistical analysis
The data analyses were performed with SPSS 17. Data are presented as mean ± SD. One-way analysis of variance (ANOVA) and an unpaired t-test were used. Differences were considered as significant at $p < 0.05$.

**Results**

**BHD Ameliorates Infarction and Reduces Neurological Scores after MCAO-R**

The plan of this study was showed in Fig. 1. We firstly observed the infarct volumes of the brain. TTC staining showed that infarct volumes of in MCAO-R + BHD group were markedly decreased than those in MCAO-R group in a dose-dependent manner (Fig. 2A and B). Two BHD groups improved neurological outcomes after MCAO-R (Fig. 2C). The dosage of BHD was determined as 20 g/kg in further study. These data demonstrated that BHD could effectively ameliorate infarction and reduces neurological score after MCAO-R.

**BHD Protects Against Neural Death after MCAO-R**

As shown in Fig. 3A and B (HE and Nissl staining), large areas of neuronal necrosis were induced by cerebral ischemia-reperfusion. MCAO-R group exhibited massive neurons died, with disappearance of the cytoplasmic bodies, swelling of cell bodies, nuclear condensation and sparse nissl bodies (Normal neurons showed clear and large cell nucleus and bodies). BHD reversed these changes. These data demonstrated that BHD could protect against neural death after MCAO-R.

**BHD Activates SIRT1 and Autophagy in Rat Cerebral Peri-ischemic Area after MCAO-R.**

In order to determine whether SIRT1/autophagy pathway was involved in the protective effect of BHD, SIRT1 and autophagic markers (LC3, P62 and Beclin-1) were detected by western blot (Fig. 4A-E). Results showed that the expression of SIRT1 in MCAO-R group and MCAO-R + BHD group was elevated. MCAO-R + BHD group showed a significantly enhanced elevation of SIRT1 than in MCAO-R group. BHD promotes SIRT1 expression in cerebral ischemia-reperfusion-induced injury. Meanwhile, the expressions of LC3-II and Beclin-1 were increased, while P62 was down-regulated in rat cerebral peri-ischemic area of MCAO-R group slightly. Then post-conditioned with BHD dramatically increased the status of autophagy, when compared with MCAO-R group. Rapa treatment showed a similar effect compared with BHD group. In addition, we elucidated the distribution pattern of Nestin and LC3 by tissue immunostaining. The expression pattern of LC3 is similar to western blot analysis (Fig. 5A and B). Nestin, a protein marker for neural stem cell, was also elevated by BHD. These data suggested that the protective effect of BHD might be related with SIRT1/autophagy pathway.

**BHD Improves BDNF and DCX Expressions in Rat Cerebral Peri-ischemic Area after MCAO-R.**
The expressions of BDNF and DCX (a microtubule-associated protein expressed by neuronal precursor cells) were further determined by immunostaining or western blot. MCAO-R induced the downregulations of BDNF and DCX. Post-conditioned with BHD caused a significant increase in BDNF and DCX expressions in MCAO-R rats (Figs. 6 and 7). Collectively, these results supported that neuroprotective effect of BHD in MCAO-R rats.

Discussion

In this study, we found that BHD could ameliorate infarction and reduce neurological scores at the 5th day after MCAO-R in rats. BHD treatment showed the similar trends on regulating autophagy with Rapa. Furthermore, our results found that SIRT1 was up-regulated in rat cerebral peri-ischemic area. In addition, BHD treatment improves Nestin, BDNF and DCX expressions in MCAO-R rats, which supported the neuroprotective effect of BHD. Thus, our results demonstrated that BHD activated SIRT1/autophagy pathway to produce neuroprotective effect against stroke.

The lack of blood flow during stroke making complicated pathophysiological which leads to neurons damaged, such mechanisms mainly includes excitotoxicity, mitochondrial dysfunction, Calcium overload, oxidative stress, protein misfolding, inflammatory changes and neuronal apoptosis(Hossmann 2006). Nowadays, clinical treatment of stroke mainly includes intravenous injection of tissue plasminogen activator during acute period, restore blood flow of the penumbral tissue, giving neurotrophic factor to protect neurons and symptomatic treatment. However, a large number of stroke patients are unable to receive the acute treatments owing to the narrow time windows(George and Steinberg 2015). Besides, blood flow recovery in a short time usually cause more damage to the neurons(Zhang et al. 2005). Therefore, discover more neurotrophic drugs plays a crucial role to treat stroke.

MCAO has been a valuable model of stroke since 1981,which is a model that can better simulate human stroke(Lopez and Vemuganti 2018). mNSS test is a classic test used to calculated the neurological deficit in rat after MCAO-R. Higher score indicates more severe behavioral deficits. TTC staining of the brain tissue is used to described the level of brain damage after MCAO-R in rat. BHD significantly ameliorated neurological deficit and the level of brain damage in rat after MCAO-R. After MCAO-R,neurons became cytoplasmic bodies disappeared, cell bodies swelling, nuclear condensed and nissl bodies sparse. But BHD ameliorate this condition. Thus, BHD can protect neurons against MCAO-R.

Autophagy, a dynamic process, in which a cell degraded its own substance though surrounded by a lysosome and a bilayer membrane, fluctuates constantly(Lee et al. 2010). In central nervous system, moderate autophagy activation might be a manifestation of endogenous neuroprotective mechanisms (Hou et al. 2019). LC3-I converted to LC3-II, indicating the onset of autophagy (Corona Velazquez and Jackson 2018). SQSTM1/P62 binds directly to LC3 and is then degraded by the autophagy-lysosomal pathway (Li et al. 2018, Wang et al. 2018). Beclin-1 is a specific gene involved in autophagy and always be seem as a marker of autophagy(Ashkenazi et al. 2017). In this study, BHD and Rapa significantly
increased LC3-II and Beclin-1 compared with MCAO-R group, while down-regulated P62 expression in rat cerebral peri-ischemic area. Confocal microscopy showed that LC3 isoforms were located in living post-ischemic cells. BHD increased the enhancement of rat peri-ischemic brain autophagy at the 5th day after MCAO-R.

SIRT1, a NAD + dependent deacetylase, is well-known as a modulator of aging (Lapierre et al. 2015). SIRT1 has the function of anti-inflammatory, anti-apoptotic, antioxidation, DNA repair, maintain energy metabolism, adjust autophagy when stroke happened (Zhang, Zhang and Wu 2018). SIRT1 can interact with several essential components of the autophagy (Chen et al. 2018). Besides, research has shown that SIRT1 plays a role of brain protection might through activating autophagy pathways during stroke (Wang et al. 2012a). In this study, BHD were proven to elevate SIRT1 expression, which might be the upstream of autophagy.

Nestin is expressed from neural stem cells and neural progenitor cells (Bojnordi et al. 2017, Dey et al. 2017), which might participate in neurogenesis after stroke (Su et al. 2014). And Brain-derived neurotrophic factor (BDNF), identified as one of the critical growth factors promotes the neuronal survival and regulate the different neuronal functions such as differentiation, migration and synaptic function in CNS (Song et al. 2013). DCX is used as a marker for neurogenesis (Shahsavani et al. 2018). Results of Immunofluorescence showed that the expression of Nestin, BDNF and DCX decreased significantly after MCAO-R, whereas the stimulation of BHD could greatly enhance their expressions compared with MCAO-R group, which provided the evidence that BHD played a critical role of neuroprotection.

**Conclusion**

Our results have provided in vivo evidences that autophagy was activated state at the 5th day after cerebral ischemia-reperfusion and post-conditioning with BHD showed the similar trends on regulating autophagy with Rapa. Furthermore, our results found that SIRT1 was up-regulated when MCAO-R happened and BHD exacerbated this phenomenon. In addition, BHD treatment improves Nestin, BDNF and DCX expressions in MCAO-R rats, which supported the neuroprotective effect of BHD. Thus, our results demonstrated that BHD activated SIRT1/autophagy pathway to produce neuroprotective effect against stroke. However, the underlying mechanisms are still need to further explore.

**Abbreviations**

BHD Buyang Huanwu Decoction

MCAO-R middle cerebral artery occlusion and reperfusion

mNss modified neurological severity score

BDNF brain-derived neurotrophic factor
DCX doublecortin on the X-chromosome
SIRT1 Sirtuin1
Rapa rapamycin
HE staining hematoxylin-eosin staining
LC3 Microtubule-associated protein 1 Light chain 3
SQSTM1/P62 Sequestosome1
SD Rat Sprague-Dawley Rat
CCA common carotid artery
ECA external carotid artery
ICA internal carotid artery
MCA middle cerebral artery
TTC 2, 3, 5-triphenyltetrazolium hydrochloride
PBS Phosphate Buffered Saline
SDS Sodium Dodecyl Sulfate
PVDF Polyvinylidene Fluoride
HRP Horseradish Peroxidase
BSA Bovine Serum Albumin
DAPI 4,6-diamino-2-phenyl indole
TCM Tradition Chinese Medicine

**Declarations**

**Ethics approval and consent to participate**

The Animal Ethics Committee approved experimental protocols of Guangzhou University of Chinese Medicine, and experiments were performed in compliance with relative protocols.

**Consent to publish**
All of authors consent to publication of this work in Chinese Medicine.

**Availability of data and materials**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' Contributions**

Han Li and Yang Zhang carried to most of the experiments and wrote the manuscript. Shi-Jie Zhang designed the experiments. Dong Peng, Hao Lin, Shi-Jie Zhang and Li Guan modified manuscript.

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Not applicable.

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