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Buyang Huanwu decoction enhances cell membrane fluidity in rats with cerebral ischemia/ reperfusion☆

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Abstract

After bilateral carotid artery occlusion for 30 minutes and reperfusion for 2 hours, distinct pathological changes presented in the cerebral cortex and cerebellum of rats. Compared with normal rats, nerve cell membrane fluidity significantly decreased in ischemia/reperfusion rats as detected by spin-labeling electron spin resonance, consistent with order parameter S and rotational correlation time τc measurements. Brain nerve cells from rats with ischemia/reperfusion injury were cultured with 1–100 mg/mL *Buyang Huanwu* decoction. Results showed that *Buyang Huanwu* decoction gradually increased membrane fluidity dose-dependently to normal levels, and eliminated hydroxide ($OH^{\cdot}$) and superoxide ($O_2^{\cdot}$) free radicals dose-dependently. These findings suggest that *Buyang Huanwu* decoction can protect against cell membrane fluidity changes in rats with ischemia/ reperfusion injury by scavenging free radicals.

Key Words

Buyang Huanwu decoction; ischemia/reperfusion; electron spin resonance; cell membrane fluidity; free radical; brain; neural regeneration

Research Highlights

Buyang Huanwu decoction can increase injured nerve cell membrane fluidity, eliminate free radicals, and ameliorate injured nerve cell function in rats with cerebral ischemia/reperfusion.

Abbreviations

ESR, electron spin-labeling resonance

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INTRODUCTION

Buyang Huanwu decoction, a traditional Chinese medicine, has been shown to be effective against stroke and other cerebrovascular diseases[1]. In previous reports, *Buyang Huanwu* decoction could invigorate the body, promote blood circulation and activate meridians[2-3], exert a protective effect against neuronal injury and also promote regeneration of peripheral nerves *in vivo*[4-8]. The curative effect of *Buyang Huanwu* decoction comes from the comprehensive effect of the various ingredients in *Buyang Huanwu* decoction, which help repair injury to neuronal membrane fluidity, promote blood flow, remove blood stasis, clean and dilate reticular vessels and ameliorate brain function[2-8]. Using electron spin-labeling resonance (ESR), our previous report demonstrated that some effective constituents of *Buyang Huanwu* decoction could increase injured neuron membrane fluidity and be protective against ischemia/reperfusion injury[2]. Extracts of *Buyang Huanwu* decoction contain many effective constituents such as astragaloside IV, ferulic acid, chuanxiongzine, rutin, quercetin, calycosin, calycosin-7-o- glusoside, and paeonal[2, 9-13]. The present study investigated the mechanism involved in the protective effect of *Buyang Huanwu*

decoction (original recipe) against ischemic injury-induced cell membrane fluidity.

RESULTS

Quantitative analysis of experimental animals

Twenty Wistar rats were equally assigned to model and sham surgery groups. In the model group, the ischemia/reperfusion model was established. In the sham surgery group, bilateral common carotids were not ligated. All rats were included in the final analysis.

Morphology of rat brain tissues after ischemia/reperfusion

Results from hematoxylin-eosin staining showed no evidence of brain injury in the sham surgery group. Brain tissue slices were harvested from the cortex of the frontal lobe of the cerebrum as well as the cerebellum[14]. The structures of neurons were clear, and nucleoli were clearly visible. In the model group, tissue slices presented pathological changes similar to that in humans following a stroke, such as local hemorrhaging and neuronal deep dyeing in the cortex of the frontal lobe. Swelling, denaturation, necrosis or loss of neurons could be observed near the hemorrhage. In the cerebellum, metamorphosis and necrosis of Purkinje neurons was observed, and nuclei were not visible. Some Purkinje's neurons were even lost (Figure 1).

Nerve cell membrane fluidity decreased in rats following brain ischemia/reperfusion

Results from spin-labeling ESR showed corresponding changes to the above morphological changes, and cell membrane fluidity in stroke rats was different from control animals. The S and τc values were obtained by spin labeling 5-doxyl-stearlic acid methylester (5DS) and 16-doxyl-stearlic acid methylester (16DS), respectively, which reflected the molecular movement state of the outer cell membrane (by 5DS) and inside cell membrane (by 16DS)[15]. The order parameter S in stroke-model rats increased compared with sham surgery rats (0.738 ± 0.002 *vs*. 0.684 ± 0.008; $P < 0.01$). The rotational correlation time τc from the model group also augmented compared with the sham surgery group (8.472 ± 0.027 *vs*. 7.945 ± 0.082; $P < 0.01$). Both parameters revealed that injured cell membrane fluidity decreased because membrane fluidity was inversely proportional to S and τc[15].

Buyang Huanwu decoction dose-dependently increased membrane fluidity of nerve cells in rats with brain ischemia/reperfusion injury

The effects of *Buyang Huanwu* decoction on membrane fluidity in the two groups were measured by spin-labeling ESR (Table 1).

Prior to *Buyang Huanwu* decoction administration, the order parameter S in stroke-model rats increased compared with the order parameter S in sham surgery rats ($P < 0.01$).

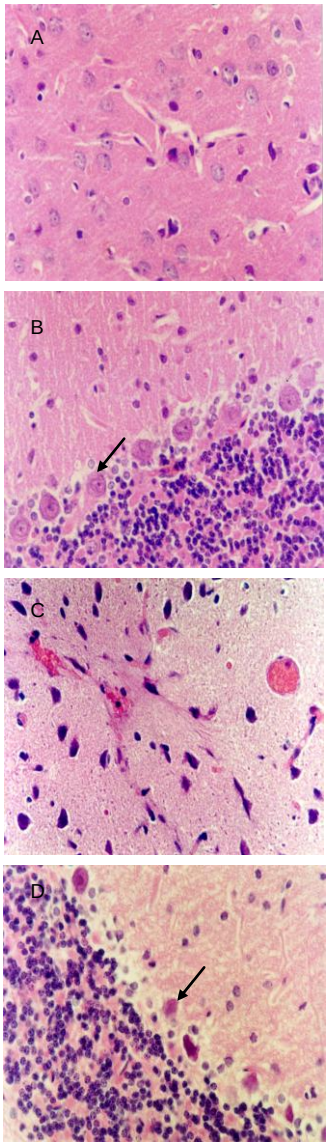

Figure 1 Morphology of brain tissue in rats (hematoxylin-eosin staining, × 400).

In the cerebrum (A) and cerebellum (B) in sham surgery group, the configuration of neurons was clear, and there was no evidence of injury. The arrow shows a normal Purkinje's neuron.

(C, D) In the cortex of model group, the configuration of neurons was not clear, and local hemorrhaging was observed.

In the cerebellum (D), the metamorphosis of Purkinje neurons was observed (as shown by the arrow). Some Purkinje neurons were lost.

Table 1 Effects of *Buyang Huanwu* decoction (BYHWD) on the order parameter S in the model and sham surgery groups (n=5)

Concentration of BYHWD (mg/mL)	Sham surgery group	Model group

[a]$P < 0.01$, *vs*. 0 mg/mL BYHWD; [b]$P < 0.01$, *vs.* sham surgery group with 0 mg/mL BYHWD. Data were expressed as mean ± SD. Statistical analysis was performed by independent sample *t*-test. The experiment was repeated five times.

This indicated that cell membrane fluidity declined and cell function was affected in stroke model rats. After increasing doses of *Buyang Huanwu* decoction administration, the order parameter S gradually decreased ($P < 0.01$). That is, cell membrane fluidity was restored to normal levels in a dose-dependent manner. The repair of membrane fluidity reflects the curative effect of *Buyang Huanwu* decoction. In the model group, the correlation coefficient between *Buyang Huanwu* decoction concentrations from 1 to 80 mg/mL and the order parameter S was 0.82 ($P < 0.05$; Figure 2), whereas in the sham surgery group, *Buyang Huanwu* decoction did not influence normal neuron function within the dose range.

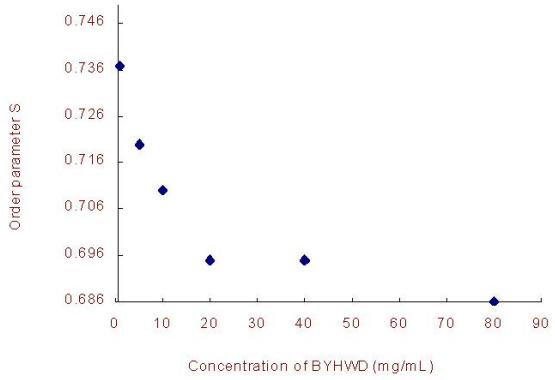

Figure 2 Correlation between *Buyang Huanwu* decoction (BYHWD) and the order parameter S in the model group ($r = 0.82$).

Statistical analysis was performed by Pearson correlation analysis.

Buyang Huanwu decoction scavenges superoxide ($O_2^{\cdot}$) and hydroxide ($OH^{\cdot}$) free radicals

Using ESR spin trapping, the capacity of *Buyang Huanwu* decoction to scavenge active oxygen free radicals, including $O_2^{\cdot}$ and $OH^{\cdot}$ free radicals, was measured. Results showed that increasing concentrations of *Buyang Huanwu* decoction increased the percentage inhibition (I) of *Buyang Huanwu* decoction to scavenge $O_2^{\cdot}$ and $OH^{\cdot}$ free radicals. In 100 mg/mL of *Buyang Huanwu* decoction, the maximum inhibition percentage (I) to $OH^{\cdot}$ was 99.6% compared with 63.6% to $O_2^{\cdot}$, which indicated that *Buyang Huanwu* decoction was a more effective scavenger of $OH^{\cdot}$ free radical than $O_2^{\cdot}$ (Table 2).

Table 2 Inhibition percentage (I) (%) of *Buyang Huanwu* decoction to superoxide ($O_2^{\cdot}$) and hydroxide ($OH^{\cdot}$) free radicals (n=3)

Concentration of *Buyang Huanwu* decoction (mg/mL)	I to $O_2^{\cdot}$	I to $OH^{\cdot}$
1	9.4±3.5	17.4±3.9
5	28.5±3.1	78.3±4.6
20	32.5±2.8	92.9±3.2
40	52.2±4.4	94.8±3.7
80	61.5±5.1	96.6±1.4
100	63.6±4.7	99.6±0.3

Data were expressed as mean ± SD. Experiments were performed in triplicate.

DISCUSSION

Our previous studies have shown that some pure constituents of *Buyang Huanwu* decoction can increase injured neuron membrane fluidity and protect against ischemia/reperfusion injury[2]. The membrane fluidity is a basic property of cells. It is also an indicator of cell function, which is vulnerable to structural change. Using spin labeling ESR in the present study, cell membrane fluidity in model rats exhibited a dose-dependent increase in the presence of 1 mg/mL to 120 mg/mL *Buyang Huanwu* decoction. The increase in membrane fluidity with increasing concentrations of *Buyang Huanwu* decoction had a protective effect against injury in stroke rats.

The present study explored the ability of *Buyang Huanwu* decoction to scavenge active oxygen free radicals. We found that *Buyang Huanwu* decoction can directly scavenge $O_2^{\cdot}$ and $OH^{\cdot}$ free radicals. Free radicals are known to attack and injure cell membranes and result in a decrease in membrane fluidity and an increase in membrane rigidity[2, 15-17]. *Buyang Huanwu* decoction protected cell membrane fluidity by scavenging free radicals[2, 7-9, 16-27], which is one of the pharmacologic mechanisms by which *Buyang Huanwu* decoction protects against stroke injury.

In summary, the final effect of *Buyang Huanwu* decoction is a result of the compatibility and interactions of the various constituents in *Buyang Huanwu* decoction. Our results revealed that *Buyang Huanwu* decoction could protect injured neuronal membranes and increase

membrane fluidity by scavenging free radicals[19-28]. This is one of the effective mechanisms by which *Buyang Huanwu* decoction protects the brain against stroke injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment and *in vitro* cell experiment.

Time and setting

The experiments were performed in the State Key Laboratory of Natural and Biomimetic Drugs, Peking University, China, from June to October, 2006.

Materials

Animals

Twenty adult male Wistar rats, of clean grade, weighing 170–190 g, were purchased from the Animal Center of Health Science Base of Peking University (license No. SYXK (Jing) 2006-0025).

Drugs

Buyang Huanwu decoction was composed of the following dried Chinese medical herbs: 120 g Astragalus membranceus var. monghoticum roots, 6 g Angelica sinesis roots, 4.5 g Paeonia lactiflora, 3 g ligusticum chuanxiong, 3 g carthamus tinctorius, 3 g Prunus persica seeds and 3 g Pheretima aspergillum. They were all purchased from Herb Companies in China and identified by Peking University School of Pharmaceutical Sciences. According to the recipe from *Yi Lin Gai Cuo*, written by Wang Qingren[1], the above dried crude herbs were decocted all together for 1 hour. This process was repeated three times using 10, 8 and 6 times the amount of water. The decoction was collected and lyophilized to a powder for further use. The extraction rate of drug active ingredients (frozen dry) was 30% (w/w).

Methods

Establishment of ischemia/reperfusion model

Rats were anaesthetized with urethane (1 200 mg/kg, intraperitoneal). Bilateral common carotids were exposed and isolated from nearby tissues, then occluded for 30 minutes, followed by reperfusion for 2 hours[2, 28]. In the sham surgery group, protocols were the same as the model group but bilateral common carotids were not occluded.

Morphology of brain tissue

After model establishment, the entire brain tissue was quickly harvested, washed with D-Hanks medium, then incised sagittally into two parts at 0–4°C. One part was fixed in 10% (v/v) formalin and sagittal slices (10 μm thick) were prepared for morphological observation by microscopy (Leica, Heidelberg, Germany) after hematoxylin-eosin staining. The remaining brain tissue was used to detect membrane fluidity.

Cell preparation

The cerebral meninges of the second part of the brain tissue was stripped off and filtrated through a filter (Pingan Filter Making Company, Hebei, China) to prepare single cells. These single cells were collected after centrifugation at 500 r/min at low temperature for 3 minutes, and re-suspended with D-Hanks medium. The concentration of cells was $5–8 \times 10^7$/mL. Cell viability was over 90% as determined by trypan blue staining[2].

Spin-labeling ESR to determine the influence of Buyang Huanwu decoction on membrane fluidity of nerve cells

The reagents 5DS (Sigma, St. Louis, MO, USA) and 16DS (Sigma) were used as spin labels to mark brain cells acquired by the aforementioned procedure[2, 15]. First, nerve cells from the sham surgery and model groups were mixed with spin labeling reagent 5DS (4×10^{-5} M) and incubated at 37°C for 0.5 hour to label cell membranes. Cells were divided into eight subgroups and numbered from 1 to 8 (each 600 μL). Normal saline was added to the sham surgery and model subgroup 1 and *Buyang Huanwu* decoction at concentrations of 1, 5, 10, 20, 40, 80 and 120 mg/mL were added to the model and sham surgery subgroups 2 to 8, respectively (each 240 μL). All cells in the subgroups were incubated again at 25°C for 120 minutes to observe the dose-effect relationship between *Buyang Huanwu* decoction and cell membrane fluidity. Finally, each subgroup was centrifuged at 3 500 r/min for 3 minutes and the sediment was placed in a quartz capillary for ESR measurements. The experimental course for 16DS was the same for 5DS. Conventional ESR spectra were detected using a Bruker 300 X-band electron paramagnetic resonance spectrometer (Bruker Corporation, Billerica, MA, USA). ESR spectra signals were obtained with 10 mW microwave power and a 100 kHz modulation frequency, modulated amplitude at 0.2 mT. The sweep width was 10 mT at the central field 349 mT. ESR spectra were recorded, stored and manipulated on a computer. The order parameter “S” was calculated according to the ESR spectrum acquired by the 5DS label, and rotational correlation time “τc” acquired by the 16 DS label. Cell membrane fluidity was inversely correlated with S and τc[2, 15].

Spin trapping ESR to determine the capacity of Buyang Huanwu decoction to scavenge $OH^{\cdot}$ and $O_2^{\cdot}$

free radicals

$O_2^{\cdot}$ free radicals were produced by a reaction system[16-17]. Distilled water was added into the system as a control. Peak height of the spectrum, marked as H_0, was detected by spin trapping-ESR. *Buyang Huanwu* decoction was dissolved in distilled water at various concentrations: 1, 5, 20, 40, 80 and 100 mg/mL. The peak height was marked as H_x. If *Buyang Huanwu* decoction could decrease H_x compared with H_0, free radicals were scavenged. Scavenging potency or inhibition percentage (I) was calculated as follows:

$$I = [(H_0 - H_X) / H_0] \times 100\%$$

$OH^{\cdot}$ free radicals could be produced by the fenton reaction as previously described[16-17]: Inhibition percentage (I) to $OH^{\cdot}$ free radicals was also calculated with the same above-mentioned method.

Statistical analysis

Order parameter S data were expressed as the mean ± SD and processed by SPSS 11.0 software (SPSS, Chicago, IL, USA). Statistical analysis was performed by independent sample *t*-test and Pearson correlation analysis. A value of $P < 0.05$ represented statistical significance.

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Author contributions: Chenxu Li designed, conducted and evaluated the experiments based on previous studies.

Conflicts of interest: None declared.

Ethical approval: The experimental procedures were approved by the Animal Use and Care Advisory Committee of Health Science Center of Peking University, China.

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