



doi:10.3969/j.issn.1673-5374.2013.03.001 [http://www.nrronline.org; http://www.sjzsyj.org]

Jin YL, Dong LY, Wu CQ, Qin J, Li S, Wang CY, Shao X, Huang DK. Buyang Huanwu Decoction fraction protects against cerebral ischemia/reperfusion injury by attenuating the inflammatory response and cellular apoptosis. *Neural Regen Res.* 2013;8(3):197-207.

Buyang Huanwu Decoction fraction protects against cerebral ischemia/reperfusion injury by attenuating the inflammatory response and cellular apoptosis^{*}

Yulian Jin^{1,2}, Liuyi Dong¹, Changqing Wu¹, Jiang Qin¹, Sheng Li¹, Chunyan Wang³, Xu Shao⁴,
Dake Huang⁵

1 Department of Pharmacology, Key Laboratory of Anti-inflammatory and Immunopharmacology, Ministry of Education; Key Laboratory of Chinese Medicine Research and Development, State Administration of Traditional Chinese Medicine, Anhui Medical University, Hefei 230032, Anhui Province, China

2 Anhui Provincial Children's Hospital, Hefei 230051, Anhui Province, China

3 Anhui Institute of Materia Medica, Hefei 230022, Anhui Province, China

4 Hefei Qi-xing Medicine and Technology Co., Ltd., Hefei 230088, Anhui Province, China

5 Synthetic Laboratory of Basic Medicine College, Anhui Medical University, Hefei 230032, Anhui Province, China

Abstract

Buyang Huanwu Decoction fraction extracted from *Buyang Huanwu* Decoction contains saponins of Astragalus, total paeony glycoside and safflower flavones. The aim of this study was to demonstrate the neuroprotective effect and mechanism of *Buyang Huanwu* Decoction fraction on ischemic injury both *in vivo* and *in vitro*. *In vivo* experiments showed that 50–200 mg/kg *Buyang Huanwu* Decoction fraction reduced infarct volume and pathological injury in ischemia/reperfusion rats, markedly inhibited expression of nuclear factor- κ B and tumor necrosis factor- α and promoted nestin protein expression in brain tissue. *Buyang Huanwu* Decoction fraction (200 mg/kg) exhibited significant effects, which were similar to those of 100 mg/kg Ginkgo biloba extract. *In vitro* experimental results demonstrated that 10–100 mg/L *Buyang Huanwu* Decoction fraction significantly improved cell viability, decreased the release of lactate dehydrogenase and malondialdehyde levels, and inhibited the rate of apoptosis in HT22 cells following oxygen-glucose deprivation. *Buyang Huanwu* Decoction fraction (100 mg/L) exhibited significant effects, which were similar to those of 100 mg/L Ginkgo biloba extract. These findings suggest that *Buyang Huanwu* Decoction fraction may represent a novel, protective strategy against cerebral ischemia/reperfusion injury in rats and oxygen-glucose deprivation-induced damage in HT22 cells *in vitro* by attenuating the inflammatory response and cellular apoptosis.

Key Words

neural regeneration; traditional Chinese medicine; *Buyang Huanwu* Decoction fraction; ischemia/reperfusion injury; brain injury; hippocampus; neurons; apoptosis; inflammatory reaction; oxidation; traditional Chinese herbal medicines; neuroprotection; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

- (1) *Buyang Huanwu* Decoction fraction extracted from *Buyang Huanwu* Decoction contains saponins of Astragalus (51%), total paeony glycoside (12%) and safflower flavones (8%).
- (2) *In vivo* and *in vitro* experiments verified that *Buyang Huanwu* Decoction fraction reduced brain injury in ischemia/reperfusion rats and relieved hippocampal neuron apoptosis following oxygen-glucose deprivation.

Yulian Jin[★], Master, Chief physician, Master's supervisor.

Yulian Jin and Liuyi Dong contributed equally to this work.

Corresponding author: Liuyi Dong, Ph.D., M.D., Associate professor, Department of Pharmacology, Key Laboratory of Anti-inflammatory and Immunopharmacology, Ministry of Education; Key Laboratory of Chinese Medicine Research and Development, State Administration of Traditional Chinese Medicine, Anhui Medical University, Hefei 230032, Anhui Province, China, dongly061@yahoo.com.cn.

Received: 2012-09-10
Accepted: 2012-11-20
(N20120530003/WLM)

(3) The neuroprotective effect of *Buyang Huanwu* Decoction fraction was associated with suppression of inflammatory factor secretion and expression, reduction of the inflammatory reaction of brain tissues, inhibition of apoptosis and the anti-oxidative properties of the fraction.

Abbreviations

BHDF, *Buyang Huanwu* Decoction fraction; NF- κ B, nuclear factor- κ B; TNF- α , tumor necrosis factor- α

INTRODUCTION

Stroke is the third most common cause of death worldwide and the leading cause of disability in adults^[1]. Therefore, the prevention and effective treatment of stroke is of utmost importance. A number of medications that help prevent stroke in high-risk patients are under investigation^[2]. However, there is currently no treatment that has been conclusively proven by clinical trials to be beneficial, except for thrombolytic treatment with recombinant tissue plasminogen activator for highly selective patients^[3]. Nonetheless, ischemia/reperfusion after thrombolysis therapy is a growing problem, which has restricted clinical applications of thrombolytic drugs^[4]. Currently, there is no valid therapeutic drug for the treatment of ischemia/reperfusion injury, especially for the therapy of neural damage. The occurrence of cerebral injury after ischemia/reperfusion is accompanied by significant cellular necrosis and apoptosis. Apoptosis is mainly located in the ischemic penumbral zone, and the ischemic core is characterized by cell necrosis^[5]. In addition, others have demonstrated that preclinical models of ischemic stroke in rats develop ischemic penumbral areas that are ultimately destined to become infarcted tissue without drug intervention^[6-7].

Over the last few years, intense interest has focused on the application of traditional Chinese herbal medicines in the treatment of ischemic stroke because many Chinese herbal medicines have been reported to possess cardioprotective or neuroprotective effects^[8]. Previous studies have demonstrated that *Buyang Huanwu* Decoction can antagonize free radical injury in cerebral ischemia/reperfusion models^[9-11]. *Buyang Huanwu* Decoction fraction (BHDF) is extracted from *Buyang Huanwu* Decoction and has been used to treat stroke for centuries^[12]. However, the mechanism of these beneficial effects has not been explained clearly. Strategies to identify and harness the cellular and molecular mechanisms of this protective effect hold tremendous potential and therapeutic benefit. Studies have shown that inflammation and apoptosis can aggravate ischemic brain

injury, which are important pathological mechanisms of stroke^[13-14]. The inflammatory response and apoptosis after cerebral ischemia/reperfusion is closely related with secondary brain damage. This study evaluated the neuroprotective effect of BHDF against cerebral injury and examined the most effective dose *in vivo* and *in vitro*, as well as its possible underlying mechanism of action.

RESULTS

In vivo studies

Quantitative analysis of experimental animals

A total of 96 rats were equally and randomly divided into the following six groups: sham group (sham operation, no ischemia/reperfusion), ischemia/reperfusion group (ischemia/reperfusion alone, with normal saline treatment), Ginkgo biloba extract group (ischemia/reperfusion with 100 mg/kg Ginkgo biloba extract treatment), and BHDF groups (ischemia/reperfusion with 50, 100, 200 mg/kg BHDF treatment). All 96 rats were involved in the final analysis.

Effect of BHDF on cerebral infarct volume

An ischemic zone, distinguished as a distinct pale stained area, was consistently identified in the cortex and striatum of the left cerebral hemisphere in rats subjected to cerebral ischemia reperfusion by 2,3,5-triphenyltetrazolium chloride. A statistically significant difference between the sham group and ischemia/reperfusion group in cerebral infarct volume was observed ($P < 0.05$). 50–200 mg/kg BHDF significantly attenuated the cerebral infarct volume ($P < 0.05$). Ginkgo biloba extract (100 mg/kg) had a similar effect on cerebral infarct volume as BHDF (Figure 1).

Effect of BHDF on pathological changes to brain tissues

Hematoxylin-eosin staining of the ischemic core of the middle cerebral artery occlusion lesion revealed that ischemic damage was absent in the sham group. BHDF (50–200 mg/kg) and Ginkgo biloba extract (100 mg/kg) treatment significantly decreased histological structure

breakdown and resulted in a reduced number of fibrinogen-fibrin complexes than that of the ischemia/reperfusion group. All slides were blinded and submitted to a pathologist for evaluation (Figure 2).

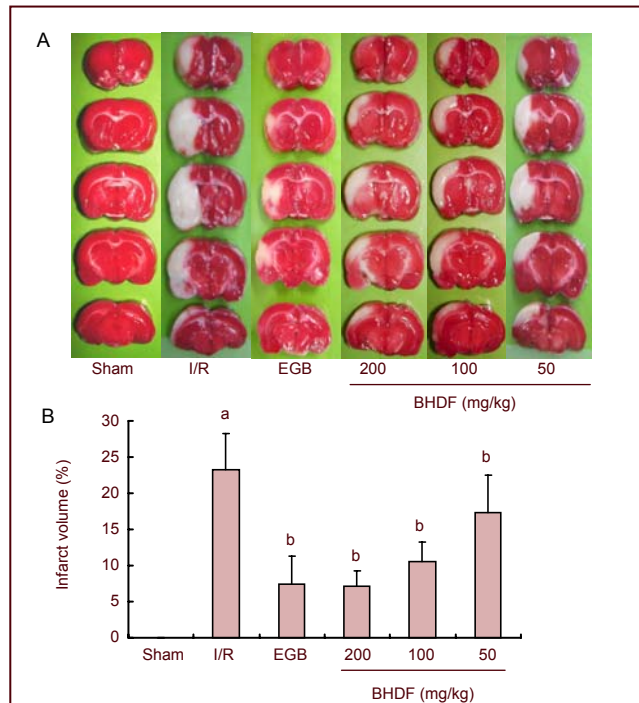


Figure 1 Effect of *Buyang Huanwu* Decoction fraction (BHDF) on cerebral infarct volume after cerebral ischemia/reperfusion (I/R) injury in rats.

(A) Representative photos of cerebral infarct volume (pale stained area).

(B) Histogram of the percentage of cerebral infarct volume. Values are expressed as mean \pm SD, $n = 8$. ^a $P < 0.05$, vs. sham group. ^b $P < 0.05$, vs. I/R group (one-way analysis of variance, Student's t -test). Infarct volume ratio = $(\sum \text{infarct area} \times \text{thickness}) / (\sum \text{brain slices area} \times \text{thickness}) \times 100\%$. EGB: Ginkgo biloba extract.

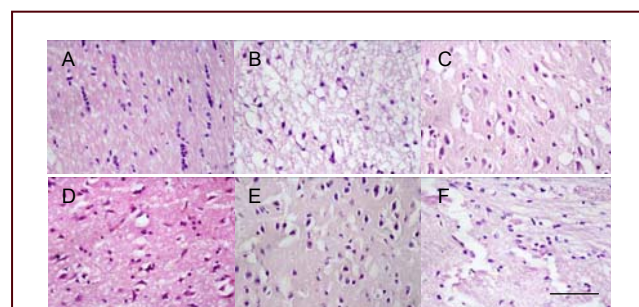


Figure 2 Effect of *Buyang Huanwu* Decoction fraction (BHDF) on pathological changes in the hippocampus after cerebral ischemia/reperfusion (I/R) injury in rats (hematoxylin-eosin staining, $\times 400$; scale bar: $50 \mu\text{m}$).

50–200 mg/kg BHDF and 100 mg/kg Ginkgo biloba extract treatment reduced histological structure breakdown and fibrinogen-fibrin complexes after I/R.

(A) Sham group; (B) I/R group; (C) Ginkgo biloba extract 100 mg/kg group; (D) BHDF 200 mg/kg group; (E) BHDF 100 mg/kg group; (F) BHDF 50 mg/kg group.

Effects of BHDF on the expression of nuclear factor- κB (NF- κB) p65 and tumor necrosis factor- α (TNF- α) in cerebral tissue

Neuronal damage and degeneration were quantified by counting the number of immunoreactive cells stained with antibodies against NF- κB , TNF- α and nestin in the middle of a defined CA1 area in the hippocampus per high magnification field (magnification $400 \times$) using a Biological Microscope Camera Analysis System. NF- κB p65 and TNF- α , which were positive as described by yellow or brown granules, were patchy and had a diffuse distribution, located in dendrites of hippocampal neurons. The expression of NF- κB p65 and TNF- α protein in the ischemia/reperfusion group was significantly greater compared with the sham group ($P < 0.05$). Compared with the ischemia/reperfusion group, BHDF (200, 100, 50 mg/kg) and Ginkgo biloba extract significantly reduced the expression of NF- κB p65 and TNF- α ($P < 0.05$) (Figures 3, 4).

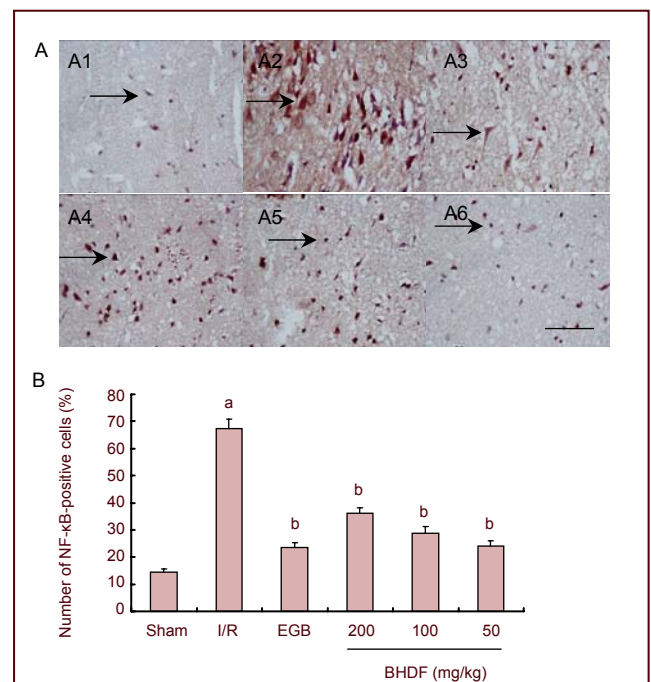


Figure 3 Effect of *Buyang Huanwu* Decoction fraction (BHDF) on the protein expression of nuclear factor- κB (NF- κB) p65 in the hippocampus after cerebral ischemia/reperfusion (I/R) injury in rats.

(A) Representative photographs of NF- κB p65 expression in the hippocampus (immunohistochemistry, $\times 400$; scale bar: $50 \mu\text{m}$). Arrows indicate NF- κB p65-positive cells.

(A1) Sham group; (A2) I/R group; (A3) Ginkgo biloba extract (EGB) 100 mg/kg group; (A4) BHDF 50 mg/kg group; (A5) BHDF 100 mg/kg group; (A6) BHDF 200 mg/kg group.

(B) Histogram of the number of NF- κB p65-positive cells. Values are expressed as mean \pm SD, $n = 8$. ^a $P < 0.05$, vs. sham group; ^b $P < 0.05$, vs. I/R group (one-way analysis of variance, Student's t -test).

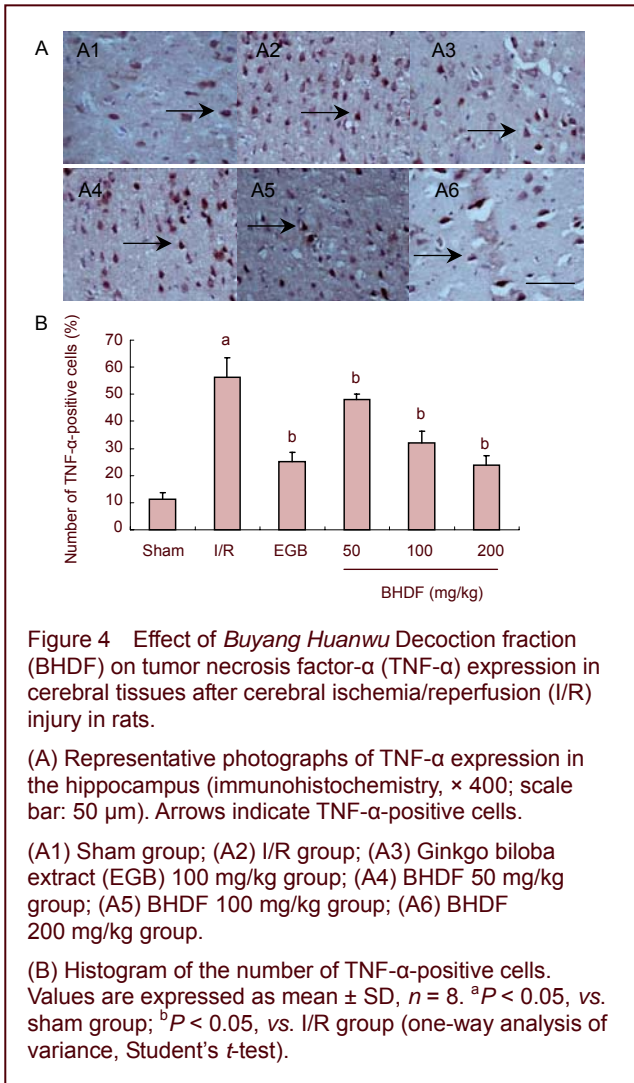


Figure 4 Effect of *Buyang Huanwu* Decoction fraction (BHDF) on tumor necrosis factor- α (TNF- α) expression in cerebral tissues after cerebral ischemia/reperfusion (I/R) injury in rats.

(A) Representative photographs of TNF- α expression in the hippocampus (immunohistochemistry, $\times 400$; scale bar: 50 μ m). Arrows indicate TNF- α -positive cells.

(A1) Sham group; (A2) I/R group; (A3) Ginkgo biloba extract (EGB) 100 mg/kg group; (A4) BHDF 50 mg/kg group; (A5) BHDF 100 mg/kg group; (A6) BHDF 200 mg/kg group.

(B) Histogram of the number of TNF- α -positive cells. Values are expressed as mean \pm SD, $n = 8$. ^a $P < 0.05$, vs. sham group; ^b $P < 0.05$, vs. I/R group (one-way analysis of variance, Student's t -test).

Effect of BHDF on nestin expression in cerebral tissues

Nestin, which was positive as indicated by yellow or brown granules, was mainly located in the cytoplasm. The sham group had no positive expression of nestin. Nestin expression in the ischemic region of the ischemia/reperfusion group had weak expression. Compared with the ischemia/reperfusion group, BHDF treatment (200, 100, 50 mg/kg) and Ginkgo biloba extract treatment significantly enhanced nestin protein expression ($P < 0.05$; Figure 5).

In vitro experiments

Effect of BHDF on HT22 hippocampal neuron cell survival rate, lactate dehydrogenase activity and malondialdehyde content

As shown in Table 1, cell viability in the model group was significantly lower than in the control group ($P < 0.01$). BHDF at 10, 30 and 100 mg/L also significantly enhanced cell viability ($P < 0.01$) compared with the model group. A significant increase in lactate

dehydrogenase release and malondialdehyde levels in HT22 cells was detected in the model group ($P < 0.01$). However, BHDF treatment at 10, 30 and 100 mg/L, and Ginkgo biloba extract (100 mg/L) treatment markedly reduced the release of lactate dehydrogenase and malondialdehyde levels ($P < 0.05$ or $P < 0.01$).

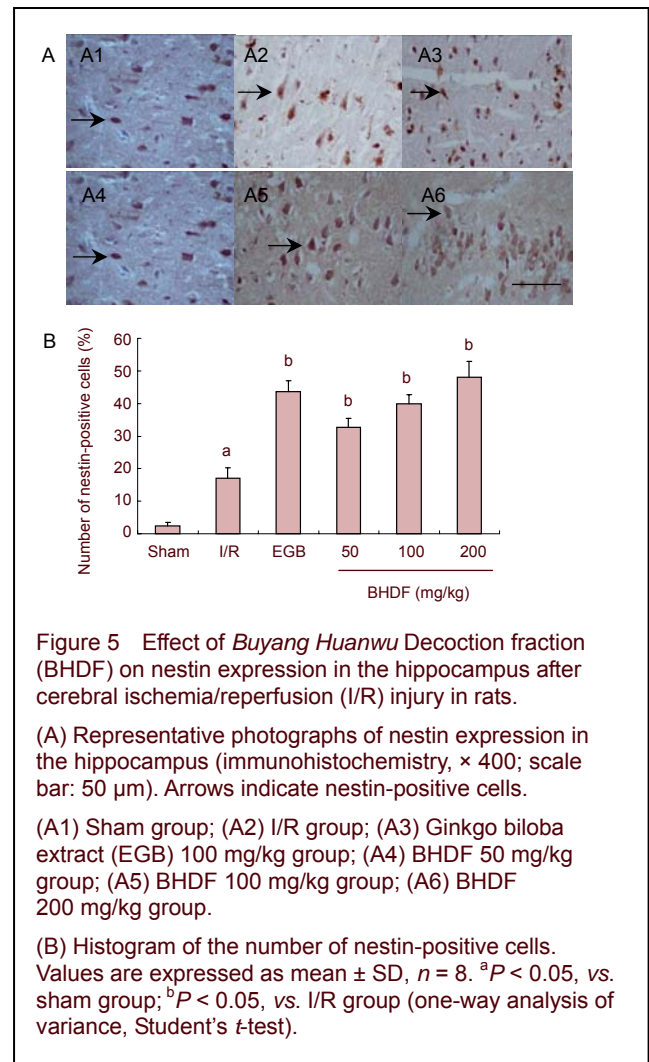


Figure 5 Effect of *Buyang Huanwu* Decoction fraction (BHDF) on nestin expression in the hippocampus after cerebral ischemia/reperfusion (I/R) injury in rats.

(A) Representative photographs of nestin expression in the hippocampus (immunohistochemistry, $\times 400$; scale bar: 50 μ m). Arrows indicate nestin-positive cells.

(A1) Sham group; (A2) I/R group; (A3) Ginkgo biloba extract (EGB) 100 mg/kg group; (A4) BHDF 50 mg/kg group; (A5) BHDF 100 mg/kg group; (A6) BHDF 200 mg/kg group.

(B) Histogram of the number of nestin-positive cells. Values are expressed as mean \pm SD, $n = 8$. ^a $P < 0.05$, vs. sham group; ^b $P < 0.05$, vs. I/R group (one-way analysis of variance, Student's t -test).

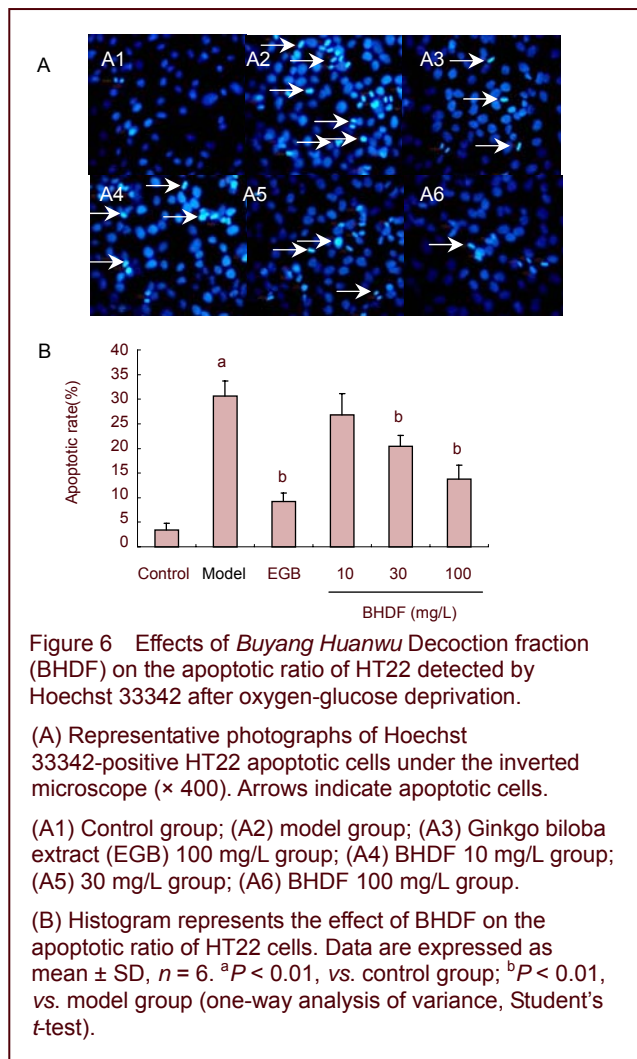
Table 1 Effect of *Buyang Huanwu* Decoction fraction (BHDF) on HT22 hippocampal neuron survival rate, lactate dehydrogenase activity and malondialdehyde content induced by oxygen-glucose deprivation

Group	Dose (mg/L)	Survival rate (%)	Lactate dehydrogenase activity (U/mL)	Malondialdehyde content (μ M)
Control	--	100.0 \pm 0.6	333.4 \pm 101.9	4.3 \pm 0.7
Model	--	62.5 \pm 0.4 ^a	490.3 \pm 138.8 ^a	8.0 \pm 0.7 ^a
Ginkgo biloba extract	100	84.3 \pm 1.1 ^c	385.7 \pm 141.0 ^c	5.4 \pm 1.2 ^c
BHDF	10	71.2 \pm 0.8 ^c	427.4 \pm 87.2	6.9 \pm 0.8
	30	74.9 \pm 1.1 ^c	409.3 \pm 59.9 ^b	6.3 \pm 1.5
	100	80.3 \pm 1.0 ^c	367.3 \pm 121.8 ^c	6.1 \pm 1.6 ^b

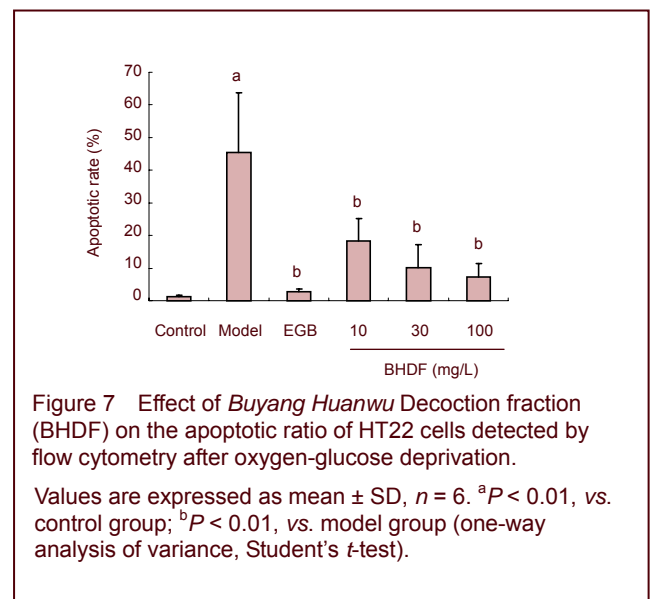
Data are expressed as mean \pm SD, $n = 6$. ^a $P < 0.01$, vs. control group; ^b $P < 0.05$, ^c $P < 0.01$, vs. model group (one-way analysis of variance, Student's t -test).

Effect of BHDF on the apoptotic ratio of HT22 hippocampal neurons

Hoechst 33342 staining was performed to determine whether the death of HT22 cells was caused by apoptosis. Results indicated that there was a significant increase in apoptotic cell death in the model group ($P < 0.01$) compared with the control group. BHDF treatment at 30 and 100 mg/L, and Ginkgo biloba extract decreased the number of apoptotic cells ($P < 0.01$; Figure 6). In the DNA histogram, the amplitude of the sub-G₁ DNA peak represents the number of apoptotic cells. In the model group, HT22 cells displayed a typical sub-diploid peak by flow cytometry, and the percentage of apoptosis reached $45.35 \pm 18.44\%$ ($P < 0.01$). Compared with the model group, BHDF treatment (10, 30, 100 mg/L) and Ginkgo biloba extract significantly attenuated the number of apoptotic cells ($P < 0.01$; Figure 7).



ischemia is thought to closely mimic the events of an embolic stroke. This method allows rapid occlusion and gradual reperfusion^[15]. At the acute phase of stroke, neurons are both necrotic or apoptotic, and differentiated neural stem cells are damaged, leading to neural function defects. Although the hippocampal CA1 region is a very important structure for learning and memory, it is among the most vulnerable brain regions after focal cerebral ischemia/reperfusion. The rapid decrease of oxygen and glucose in the ischemic region can trigger delayed neuronal death^[16], and reperfusion may further exacerbate the injury.



Chinese herbs, such as *Buyang Huanwu* Decoction, are commonly used clinically to treat nervous system diseases such as central infarction and cerebral hemorrhage. Centuries of clinical practice and certification have indicated that *Buyang Huanwu* Decoction has various effects, such as an anti-cerebral ischemia/reperfusion injury effect, anti-apoptotic and anti-free radical effect, and contributes to repair following nervous system injury^[12, 17]. Accordingly, we hypothesized that BHDF would offer a protective effect on neuronal cells following cerebral ischemia/reperfusion injury. Our data demonstrated that BHDF at a final dose of 50, 100 and 200 mg/kg significantly reduced ischemia reperfusion-induced cerebral infarct volume in rats.

Stroke triggers an inflammatory reaction that progresses for hours after onset, and this inflammation plays a central role in the pathogenesis of neuronal injury in ischemic stroke^[18]. There is ample evidence indicating that NF- κ B is activated in cerebral ischemia and reperfusion, specifically in neurons^[19-20].

DISCUSSION

The middle cerebral artery occlusion model of cerebral

Therefore, inhibition of NF- κ B may represent a treatment strategy in ischemic stroke. Post-ischemic neurodegeneration may be accelerated by a cytokine-receptor mediated apoptotic pathway, as shown in the brains of transgenic rats overexpressing TNF- α ^[21]. Nestin, a marker of neural stem cells, has previously been shown to provide neuroprotection when transplanted into the adult brain in rodent models of stroke^[22]. Interestingly, our immunohistochemistry results indicated that BHDF therapy was associated with decreased numbers of inflammatory cells in the penumbra and decreased expression of NF- κ B and TNF- α , which may be a consequence of the reduced infarction volume and increased expression of nestin. The immunohistochemistry results indicate that BHDF can also induce the differentiation of multipotent stem cells. Multipotent stem cells have a high self-renewal capacity and differentiation potential. If differentiation potential is towards neurons, this may provide a new source of stem cells for repair after neural system injury.

In the context of hypoxia, specific genes that contribute to cellular events and injury in neurons are known to respond, such as genes associated with apoptotic cell death, inflammation, necrosis, and neurogenesis^[23]. Previous investigators have demonstrated that BHDF can reduce cell death and apoptosis in cultured embryonic cortical cells exposed to compounds that mimic many of the events in cerebral ischemia, including glutamate excitotoxicity, sodium dithionite, hypoxia, and oxygen-glucose deprivation.

Apoptosis, also called programmed cell death, is controlled by genes and mediated by specific RNA and proteins. Previous investigators have demonstrated that there are two pathways to execute apoptosis, the mitochondrial pathway and the death receptor pathway^[24]. Lactate dehydrogenase release, a marker of apoptosis and necrosis, is an indicator of plasma membrane damage and is commonly used for the determination of neurotoxicity^[23, 25]. Malondialdehyde is regarded as a hallmark of oxidative damage. Reactive oxygen species play important roles in the pathogenesis of central nervous system injury^[26]. BHDF improves neuronal survival in hypoxic conditions in a dose-dependent manner, and inhibits lactate dehydrogenase release from HT22 hippocampal neurons exposed to sodium dithionite toxicity.

Viable cells displayed normal nuclear size and uniform fluorescence, whereas apoptotic cells showed

condensed nuclei or nuclear condensation. In addition, chromosomal condensation and morphological changes in the nucleus of HT22 cells were observed using the chromatin dye Hoechst 33342^[27]. This protocol describes a rapid and simple method for the identification of apoptotic cells. Owing to changes in cell membrane permeability, compared with viable cells, apoptotic cells show an increased uptake of the vital DNA dye Hoechst 33342. The DNA dye can distinguish between necrotic cells that have lost membrane integrity from apoptotic cells that still have intact membranes as assayed by dye exclusion. Flow cytometry is a generally accepted tool to analyze apoptosis, and it provides results that are reliable for apoptotic measurements^[28]. The mechanisms underlying neuronal cell death in the brain after oxygen-glucose deprivation are complex as they appear in a variety of forms and depend on multiple factors. Hoechst 33342 staining and flow cytometric detection of the sub-G₁ peak in cells revealed that the control group showed a similar mean percentage of apoptotic cells using both methods (3.5% by morphology *versus* 1.2% by flow cytometry) after 24 hours of culture. In the model group, the mean percentage of apoptotic cells quantified by morphology was lower than by flow cytometry (34.29% by morphology *versus* 45.35% by flow cytometry after oxygen-glucose deprivation). However, in the treatment groups, the mean percentage of apoptotic cells quantified by morphology was higher than that by flow cytometry in each group treated with same dose. In our system, flow cytometric sub-G₁ peak analysis appeared to underestimate apoptosis. The mechanism of the anti-apoptotic effect of BHDF may be related to the regulation of the transcription of pro- and anti-apoptotic molecules, and an increase in the expression of anti-apoptotic proteins.

In vivo and *in vitro* studies have demonstrated neuroprotective effects of BHDF against a variety of insults. Our current results, indicating the cerebroprotective properties of BHDF during ischemic stroke, validate the role of anti-inflammatory compounds in ischemic stroke. Apoptosis is characterized by decreased cell viability, condensation of nuclear chromatin and increased phosphatidylserine translocation^[29]. Stroke is a leading cause of death and disability in many industrial nations, yet treatments are few and often restricted to a small number of patients^[30]. Such studies are expected to accelerate development of therapeutic strategies to ameliorate neuronal death and cognitive deficits not only following focal cerebral

ischemia, but also in other neurodegenerative disorders.

In summary, BHDF exerts a significant neuroprotective effect against cerebral ischemia/reperfusion injury in rats and HT22 cells damaged by oxygen-glucose deprivation *in vitro* by attenuating the inflammatory response and cellular apoptosis.

MATERIALS AND METHODS

Design

A randomized, observational, controlled animal experiment.

Time and setting

This experiment was performed at the Department of Pharmacology, Anhui Medical University, China between May 2010 and July 2011.

Materials

Drugs and reagents

BHDF containing total astragalosides 51%, total paeony glycoside 12%, and carthamus tinctorius flavonoids 8% was provided by Hefei Qi-xing Medicine and Technology Co., Ltd. (Solid powder, Lot No. 20090504, Hefei, Anhui Province, China).

Ginkgo biloba extract (tablets; 2.4 mg/pill; approval No. GYZZ Z20028024; lot No. 080201) was provided by the Herb Production Factory of Ningbo City (Ningbo, Zhejiang Province, China).

Animals

Ninety-six healthy adult male Sprague-Dawley rats aged 6–8 weeks and weighing 250–320 g were used. The animals were obtained from the Experimental Animal Center of Anhui Medical University (License No. SCXK (Wan) 2005-001). The animals were housed in clear plexiglas cages with stainless steel wire lids and filter tops, in a temperature-controlled (20–24°C) room. Sprague-Dawley rats were maintained on a 12-hour light/dark cycle (lights on at 7:00 a.m.). Rats were tested during the light phase of the light/dark cycle. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[31].

Methods

In vivo experiments

Establishment of the rat middle cerebral artery

occlusion model: Rats were anesthetized with an intraperitoneal injection of chloral hydrate (10% (w/v), 0.3 mL/100 g), then fixed in a supine position. After removing the fur around the neck, a midline incision was performed at the cervical region. The middle cerebral artery occlusion model was performed as previously described in detail with slight modifications^[32-34]. With the tip rounded by heating near a flame, a length of 20.0–22.0 mm of 3-0 surgical monofilament nylon suture was inserted through the right common carotid arteries into the internal carotid artery until it blocked the origin of the middle cerebral artery. Rats were subjected to 2 hours of focal ischemia followed by 22 hours of reperfusion and were allowed to survive for 24 hours. The suture was not advanced to occlude the middle cerebral artery in the sham group. Rectal temperature was maintained at 37°C with air conditioning throughout the surgical procedure. Finally, animals were allowed to regain consciousness and were maintained at a room temperature of 25°C with free access to food and water. Neurological symptoms were examined and only rats demonstrating circling contralateral to the middle cerebral artery occlusion, detection of hemiplegia and abnormal posture^[35] were included in the study. All rats were euthanized 24 hours after ischemia.

Drug treatment: BHDF (50, 100, 200 mg/kg; reference to the pre-experiment) or Ginkgo biloba extract (100 mg/kg) were administered to the appropriate treatment group by gavage for 7 days (once a day). Drugs were administered 7 days prior to undergoing middle cerebral artery occlusion. Animals in the sham group and the ischemia/reperfusion group were administered the same volume (1 mL/100 g) of 0.9% (w/v) saline by gavage.

Measurement of infarct volume: The rats were given an overdose of chloral hydrate and decapitated. Brains were quickly removed, rinsed in cold PBS, placed at –80°C for 3 minutes and sliced into 2 mm thick coronal sections. Tissue sections were incubated for 30 minutes at 37°C in a solution of 2% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) in PBS in the dark. The borders of the infarct in each brain slice were outlined and the area quantified using NIH image J software. To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. Infarct volume was calculated by integrating the infarct areas for all slices of each brain^[36]. Infarct volume ratio was calculated according to the following

formula: Infarct volume ratio = $(\sum \text{infarct area} \times \text{thickness}) / (\sum \text{brain slices area} \times \text{thickness}) \times 100\%$.

Preparation of brain samples: middle cerebral artery occluded rats were anesthetized and perfused through the heart, first with PBS, and then with PBS containing 4% (w/v) paraformaldehyde. Rat brains were removed rapidly, and then fixed with 10% (v/v) formalin and embedded in paraffin blocks. Sections from infarcted area of each group were sliced into 4- μm -thick slices using a microtome (RM-2145; Leica Slicer, Solms, Germany) for hematoxylin-eosin^[37-38] and immunohistochemical staining.

Immunohistochemical staining: Immunohistochemical staining was used to determine the protein expression of NF- κB , TNF- α and nestin. A commercially available avidin-biotin-peroxidase complex staining kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Paraffin-embedded cerebral tissue blocks were sectioned at 4 μm . Sections were deparaffinized in xylene and dehydrated through graded ethanol, then washed thrice with PBS for 5 minutes. The sections were treated with 3% (v/v) H_2O_2 for 10 minutes at room temperature to quench endogenous peroxidase activity. After blocking with 5% (w/v) normal goat serum in PBS for 30 minutes, they were then incubated in primary antibodies for 1 hour at 37°C. The primary antibodies were rabbit polyclonal antibodies (1:200): anti-NF- κB /p65 (sc-8008), anti-TNF- α (sc-1350) and anti-nestin (sc-20978), all purchased from Beijing Zhongshan Biological Technology Co., Ltd. (Beijing, China). PBS was used to replace the antibody in the negative control group. After three washes in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (1:1 000; Beijing Zhongshan Biological Technology Co., Ltd.) for 30 minutes, followed by incubation with streptavidin-biotin complex for 60 minutes at 37°C. Ten non-overlapping random fields of view of each section from each group were captured using a microscope (Nikon 80i, Tokyo, Japan). Images were analyzed using the Jetta 801D morphological image analysis system (Version 6.0; Nanjing, Jiangsu Province, China). The parameters remained unchanged for each treatment group. The positive track point was set as the brown localization expressed in the cytoplasm or membrane. The total number of positive cells in each visual field was calculated as follows: number of positive cells/total cells \times 100%.

***In vitro* experiments**

HT22 hippocampal neuronal cell cultures: HT22 cells (provided by Nanfang Medical University, Guangzhou,

Guangdong Province, China), a murine hippocampal cell line, were plated at a density of 10^5 – 10^6 cells per well in plates. The cells were cultured in Dulbecco's modified Eagle's medium (ThermoScientific, Barrington, IL, USA) containing 10% (v/v) fetal calf serum to ensure normal neuronal development and survival, and medium was replaced every 2 days. Cells were incubated at 37°C in a 5% CO_2 and 95% air humidified atmosphere until the day of the experiment.

Oxygen-glucose deprivation model: HT22 hippocampal neurons in the logarithmic growth phase were used for all experiments. Sodium dithionite (1 mM; Wuxi Zhanwang Chemical Reagent Co., Ltd., Wuxi, Jiangsu Province, China) was used to induce oxygen-glucose deprivation in all experiments^[39-40]. HT22 hippocampal neuronal cells were randomly divided into six groups: (1) Control group (no oxygen-glucose deprivation): cells were cultured with Dulbecco's modified Eagle's medium, (2) Model group (oxygen-glucose deprivation alone, with no treatment): cells were cultured for 24 hours in fresh medium containing sodium dithionite (1 mM), without glutamate, (3) BHDF groups: cells were treated with BHDF (10, 30, 100 mg/L) containing Dulbecco's modified Eagle's medium, and then incubated for 24 hours. The supernatant was then removed and replaced with fresh medium containing sodium dithionite (1 mM) without glutamate for an additional 24 hours, (4) Ginkgo biloba extract group: cells were treated with 100 mg/L Ginkgo biloba extract, and the same method was performed as for the BHDF groups, except that BHDF was replaced with Ginkgo biloba extract.

Assessment of cell supernatant: Supernatants were collected to measure lactate dehydrogenase release and malondialdehyde content. Lactate dehydrogenase release was used to measure neurotoxicity^[41]. Lactate dehydrogenase is a cytoplasmic enzyme released from cells with compromised cell membranes. Malondialdehyde can induce cell damage in various ways and levels reflect the content of free radicals produced by lipid peroxidation^[41]. Lactate dehydrogenase activity and malondialdehyde content were detected using a colorimetric reaction, which was read at an absorbance of 440 and 532 nm, respectively, using a microplate reader (Haimen Boyang, Haimen, Jiangsu Province, China). The assays were performed using commercially available kits (Nanjing Jiancheng Biological Company, Nanjing, Jiangsu Province, China). Both assays were conducted according to the manufactures' instructions.

The MTT assay: Cell survival was assayed using the microculture tetrazolium method^[42]. Approximately 10^5 to 10^6 cells/well were dispensed within 96-well culture plates in 10 μ L volumes. In each of the above cultured wells, 10 μ L of MTT working solution (Sigma) was added to every well, and then incubated continuously for 4 hours. All culture medium supernatant was removed from the wells and then each of the plates was replaced with 100 μ L of dimethyl sulfoxide (Sigma). The absorbance value of each well was measured using a microplate reader (Haimen Boyang) at 550 nm. Cell survival rate was calculated according to the following formula: Cell survival rate = absorbance value of observed group/absorbance value of control group \times 100%.

Nuclear staining for the assessment of apoptosis: For quantification of apoptosis-related DNA fragments, we used Hoechst 33342 (Shanghai Shize Biological Technology Co., Ltd., Shanghai, China) to determine and detect apoptotic cell death, but not necrotic cell death^[43]. At the end of oxygen-glucose deprivation treatment, the supernatants were removed. HT22 cells from each well were fixed with a 1:3 mixture of acetic acid and methanol at 4°C for 20 minutes. The supernatants were removed. After three rinses with PBS, cells were stained with 10 μ g/mL Hoechst 33342 (Sigma) for 20 minutes at room temperature. Cultures were then washed three times with PBS and mounted in an anti-fluorescein fading medium (Amresco, Solon, OH, USA). Slides were visualized under a fluorescent microscope (Nikon). Experiments were repeated at least three times using separate batches of cultures. Apoptotic cells in each group were quantified by counting the mean number of positive cells per six fields at 200 \times magnification using an inverted microscope. The apoptotic ratio was equal to the number of apoptotic cells/total cells \times 100%.

Flow cytometry analysis of apoptosis: After cells were treated and collected, HT22 cells were digested with trypsin (2.5 g/L; Difco, Franklin Lakes, NJ, USA) and centrifuged at 1 500 r/min for 10 minutes, and then the supernatant was discarded. Cells were rinsed with PBS three times and fixed with 70% (v/v) ice-cold ethanol in the dark at 4°C overnight. On the following day, neurons were centrifuged at 1 500 r/min for 10 minutes, washed twice with PBS and adjusted to a concentration of 1×10^6 cells/mL. After RNase (0.5 mL, 1 mg/mL in PBS; Sigma) was added, the specimens were stained with propidium iodide (50 mg/L; Sigma) in the dark at 4°C for 30 minutes, and then subjected

to flow cytometry analysis using the FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). Experiments were repeated at least three times using separate batches of cultures.

Statistical analysis

All data were expressed as mean \pm SD, and were analyzed using SPSS 15.0 for Windows software (SPSS, Chicago, IL, USA). One-way analysis of variance and Student's *t*-test were used for comparisons between groups. A level of $P < 0.05$ was considered to be statistically significant.

Acknowledgments: We gratefully acknowledge Danna Yao, Ph.D., from Nanfang Medical University for providing the HT22 cell line. We thank Dr. David J. Blake from Fort Lewis College for critical reading of this manuscript.

Funding: This project was supported by a grant from the Major Programs of Anhui Science and Technology Special Funds, No. 08010302099; and the Doctor Funds of Anhui Medical University, No. XJ200813.

Author contributions: Liuyi Dong and Yulian Jin participated in experimentation, prepared the animal model, analyzed experimental data, and wrote the manuscript. Changqing Wu, Jiang Qin, Sheng Li, Chunyan Wang, Xu Shao and Dake Huang participated in experimentation, analyzed experimental data and performed statistical analysis. Liuyi Dong was responsible for the experimental concept and design, validation and guidance of the study. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: All animal experiments were approved by the Animal Ethics Committee, Anhui Medical University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

REFERENCES

- [1] Wong KS, Chen C, Ng PW, et al. Low-molecular-weight heparin compared with aspirin for the treatment of acute ischemic stroke in Asian patients with large artery occlusive disease: a randomised study. *Lancet Neurology*. 2007;6(5):407-413.
- [2] Hohnloser SH, Duray GZ, Baber U, et al. Prevention of stroke in patients with atrial fibrillation: current strategies and future directions. *European Heart J (Supplements)*. 2008;10:4-10.

- [3] Alexandrov AV, Hall CE, Labiche LA, et al. Ischemic stunning of the brain: early recanalization without immediate clinical improvement in acute ischemic stroke. *Stroke*. 2004;35(2):449-452.
- [4] Warach S, Latour LL. Evidence of reperfusion injury, exacerbated by thrombolytic therapy, in human focal brain ischemia using a novel imaging marker of early blood-brain barrier disruption. *Stroke*. 2004;35(11 Suppl 1):2659-2661.
- [5] Wang GX, Li GR, Wang YD, et al. Characterization of neuronal cell death in normal and diabetic rats following experimental focal cerebral ischemia. *Life Sci*. 2001;69(23):2801-2810.
- [6] Bråtane BT, Walvick RP, Corot C, et al. Characterization of gadolinium-based dynamic susceptibility contrast perfusion measurements in permanent and transient MCAO models with volumetric based validation by CASL. *J Cereb Blood Flow Metab*. 2010;30(2):336-342.
- [7] Henninger N, Sicard K, Schmidt K, et al. Comparison of ischemic lesion evolution in embolic versus mechanical middle cerebral artery occlusion in Sprague Dawley rats using diffusion and perfusion imaging. *Stroke*. 2006;37(5):1283-1287.
- [8] Hua Q, Zhu XL, Li PT, et al. Refined Qing Kai Ling, traditional Chinese medicinal preparation, reduces ischemic stroke-induced infarct size and neurological deficits and increases expression of endothelial nitric oxide synthase. *Biol Pharm Bull*. 2008;31(4):633-637.
- [9] Chen J, Zeng YY, Zeng HL, et al. Inhibition on inflammatory cytokine expressions in heart and lung from brain death rats pretreated with Buyang Huanwu Decoction. *Zhong Cao Yao*. 2010;41(5):757-761.
- [10] Zhang SP, Liang Y, Deng CQ. Effect of Buyang Huanwu Decoction and its active fractions on caspase expression after cerebral ischemia-reperfusion in rats. *Zhong Cao Yao*. 2006;37(7):1041-1045.
- [11] Liu BY, Cai GX, Liu W, et al. Effect of Buyang Huanwu Decoction on vascular endothelial growth factor and its receptor Flk1 in rats after focal cerebral ischemia. *Zhong Cao Yao*. 2007;38(3):394-397.
- [12] Sun J, Bi Y, Guo L, et al. Buyang Huanwu Decoction promotes growth and differentiation of neural progenitor cells: using a serum pharmacological method. *J Ethnopharmacol*. 2007;113(2):199-203.
- [13] Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. *J Neuroimmunol*. 2007;184(1-2):53-68.
- [14] Pan W, Kastin AJ. Tumor necrosis factor and stroke: role of the blood-brain barrier. *Prog Neurobiol*. 2007;83(6):363-374.
- [15] Mecca AP, O'Connor TE, Katovich MJ, et al. Candesartan pretreatment is cerebroprotective in a rat model of endothelin-1-induced middle cerebral artery occlusion. *Exp Physiol*. 2009;94(8):937-946.
- [16] Dijkhuizen RM, Knollema S, Van der Worp HB, et al. Dynamics of cerebral tissue injury and perfusion after temporary hypoxia-ischemia in the rat: evidence for region-specific sensitivity and delayed damage. *Stroke*. 1998;29(3):695-704.
- [17] Zhao LD, Wang JH, Jin GR, et al. Neuroprotective effect of Buyang Huanwu decoction against focal cerebral ischemia/reperfusion injury in rats--time window and mechanism. *J Ethnopharmacol*. 2012;140(2):339-344.
- [18] Jiang W, Zhang S, Fu F, et al. Inhibition of nuclear factor- κ B by 6-O-acetyl shanzhiside methyl ester protects brain against injury in a rat model of ischemia and reperfusion. *J Neuroinflammation*. 2010;7:55.
- [19] Huang CY, Fujimura M, Noshita N, et al. SOD1 downregulates NF- κ B and c-Myc expression in mice after transient focal cerebral ischemia. *J Cereb Blood Flow Metab*. 2001;21(2):163-173.
- [20] Stephenson D, Yin T, Smalstig EB, et al. Transcription factor nuclear factor- κ B is activated in neurons after focal cerebral ischemia. *J Cereb Blood Flow Metab*. 2000;20(3):592-603.
- [21] Pandya JD, Sullivan PG, Pettigrew LC. Focal cerebral ischemia and mitochondrial dysfunction in the TNF α -transgenic rat. *Brain Res*. 2011;1384:151-160.
- [22] Jin K, Mao X, Xie L, et al. Transplantation of human neural precursor cells in Matrigel scaffolding improves outcome from focal cerebral ischemia after delayed postischemic treatment in rats. *J Cereb Blood Flow Metab*. 2010;30(3):534-544.
- [23] Yu Z, Liu J, Guo S, et al. Neuroglobin-overexpression alters hypoxic response gene expression in primary neuron culture following oxygen glucose deprivation. *Neuroscience*. 2009;162(2):396-403.
- [24] Hengartner MO. The biochemistry of apoptosis. *Nature*. 2000;407(6805):770-776.
- [25] Schreihöfer DA, Redmond L. Soy phytoestrogens are neuroprotective against stroke-like injury in vitro. *Neuroscience*. 2009;158(2):602-609.
- [26] Ano Y, Sakudo A, Kimata T, et al. Oxidative damage to neurons caused by the induction of microglial NADPH oxidase in encephalomyocarditis virus infection. *Neurosci Lett*. 2010;479(1):39-43.
- [27] Yin WL, He JQ, Hu B, et al. Hydrogen sulfide inhibits MPP⁺-induced apoptosis in PC12 cells. *Life Sci*. 2009;85(7-8):269-275.
- [28] Stemberger J, Witt V, Printz D, et al. Novel single-platform multiparameter FCM analysis of apoptosis: Significant differences between wash and no-wash procedure. *Cytometry A*. 2010;77(11):1075-1081.
- [29] Zhang X, Chen J, Davis B, et al. Hoechst 33342 induces apoptosis in HL-60 cells and inhibits topoisomerase I in vivo. *Arch Pathol Lab Med*. 1999;123(10):921-927.
- [30] Niizuma K, Endo H, Nito C, et al. Potential role of PUMA in delayed death of hippocampal CA1 neurons after transient global cerebral ischemia. *Stroke*. 2009;40(2):618-625.
- [31] Guidance Suggestions for the Care and Use of Laboratory Animals. The Ministry of Science and Technology of the People's Republic of China. 2006-09-30.
- [32] Longa EZ, Weinstein PR, Carlson S, et al. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989;20(1):84-91.

- [33] Zierath D, Hadwin J, Savos A, et al. Anamnestic recall of stroke-related deficits: an animal model. *Stroke*. 2010; 94(11):2653-2660.
- [34] Zhai FG, Zhang XP, Guan Y, et al. Expression profiles of microRNAs after focal cerebral ischemia/reperfusion injury in rats. *Neural Regen Res*. 2012;7(12):917-923.
- [35] Bederson JB, Pitts LH, Tsuji M, et al. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*. 1986; 17(3):472-476.
- [36] Swanson RA, Morton MT, Tsao WG, et al. A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Met*. 1990;10(2):290-293.
- [37] Liang H, Liu P, Wang Y, et al. Protective effects of alkaloid extract from *Leonurus heterophyllus* on cerebral ischemia reperfusion injury by middle cerebral ischemic injury (MCAO) in rats. *Phytomedicine*. 2011;18(10):811-818.
- [38] Isayama K, Pitts LH, Nishimura MC. Evaluation of 2,3,5-triphenyltetrazolium chloride staining to delineate rat brain infarcts. *Stroke*. 1991;22(11):1394-1398.
- [39] Li YL, Zhao L, Li L. Protective effect of diphenyl ethylene glycoside on hippocampal neuron cells ischemic injury model rats. *Zhongguo Yaofang*. 2006;17(1):12-14.
- [40] Zhang LL, Xue Y, Mark K. Minocycline provides neuroprotection against glutamate neurotoxicity in HT22 cells. *Int J Emerg and Crit Care Med*. 2005;2:978-981.
- [41] Li L, Lau BH. A simplified in vitro model of oxidant injury using vascular endothelial cells. *In Vitro Cell Dev Biol Anim*. 1993;29A(7):531-536.
- [42] Zhuang H, Pin S, Li X, et al. Regulation of heme oxygenase expression by cyclopentenone prostaglandins. *Exp Biol Med*. 2003;228(5):499-505.
- [43] Jin Y, Yan EZ, Fan Y, et al. Neuroprotection by sodium ferulate against glutamate-induced apoptosis is mediated by ERK and PI3 kinase pathways. *Acta Pharmacol Sin*. 2007;28(12):1881-1890.

(Edited by Tu QY, Bai H/Qiu Y/Wang L)