RESEARCH ARTICLE

Houshiheisan and its components promote axon regeneration after ischemic brain injury

Yue Lu[1, 2, #], Flora Hsiang[1, 2, #], Jia-Hui Chang[1, 2], Xiao-Quan Yao[1, 2], Hui Zhao[1, 2], Hai-Yan Zou[1, 2], Lei Wang[1, 2], Qiu-Xia Zhang[1, 2, *]
1 School of Traditional Chinese Medicine, Capital Medical University, Beijing, China
2 Beijing Key Lab of TCM Collateral Disease Theory Research, Beijing, China

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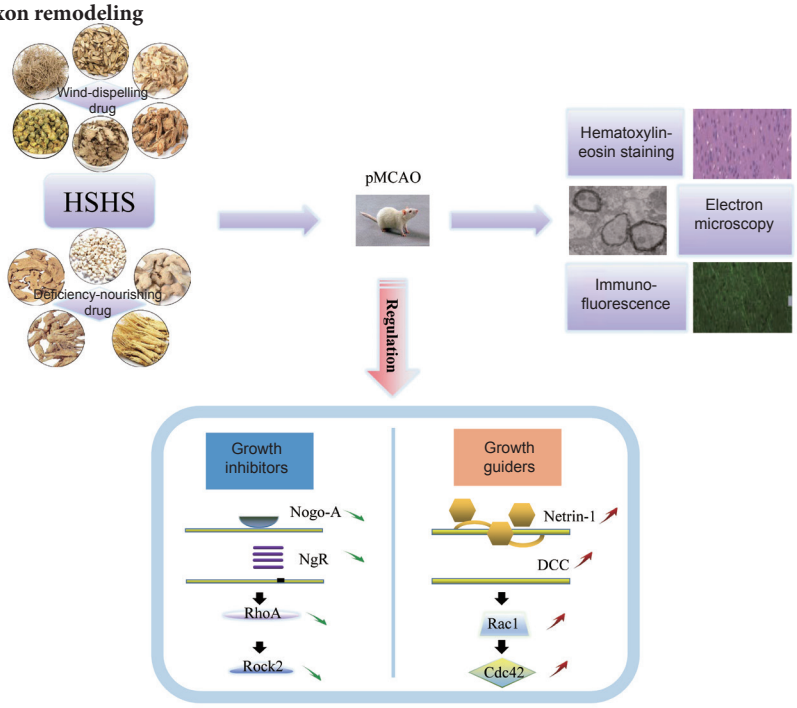
Graphical Abstract

Houshiheisan **(HSHS) and its components promote neural regeneration after cerebral ischemia possibly through axon remodeling**



***Correspondence to:**
Qiu-Xia Zhang, M.D., zqx26@163.com.

#These authors contributed equally to this study.

orcid:
0000-0001-6751-5699 (Qiu-Xia Zhang)

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Abstract

Houshiheisan, a classic prescription in traditional Chinese medicine, contains *Flos Chrysanthemi*, *Radix Saposhnikoviae*, *Ramulus Cinnamomi*, *Rhizoma Chuanxiong*, *Radix et Rhizoma Asari*, *Radix Platycodonis*, *Rhizoma Atractylodis macrocephalae*, *Poria*, *Rhizoma Zingiberis*, *Radix Angelicae sinensis*, *Radix et Rhizoma Ginseng*, *Radix Scutellariae* and *Concha Ostreae*. According to traditional Chinese medicine theory, *Flos Chrysanthemi*, *Radix Saposhnikoviae*, *Ramulus Cinnamomi*, *Rhizoma Chuanxiong*, *Radix et Rhizoma Asari* and *Radix Platycodonis* are wind-dispelling drugs; *Rhizoma Atractylodis macrocephalae*, *Poria*, *Rhizoma Zingiberis*, *Radix Angelicae sinensis* and *Radix et Rhizoma Ginseng* are deficiency-nourishing drugs. A large number of randomized controlled trials have shown that *Houshiheisan* is effective in treating stroke, but its mechanism of action is unknown. Axonal remodeling is an important mechanism in neural protection and regeneration. Therefore, this study explored the effect and mechanism of action of *Houshiheisan* on the repair of axons after cerebral ischemia. Rat models of focal cerebral ischemia were established by ligating the right middle cerebral artery. At 6 hours after model establishment, rats were intragastrically administered 10.5 g/kg *Houshiheisan* or 7.7 g/kg wind-dispelling drug or 2.59 g/kg deficiency-nourishing drug. These medicines were intragastrically administered as above every 24 hours for 7 consecutive days. *Houshiheisan*, and its wind-dispelling and deficiency-nourishing components reduced the neurological deficit score and ameliorated axon and neuron lesions after cerebral ischemia. Furthermore, *Houshiheisan*, and its wind-dispelling and deficiency-nourishing components decreased the expression of proteins that inhibit axonal remodeling: amyloid precursor protein, neurite outgrowth inhibitor protein A (Nogo-A), Rho family small GTPase A (RhoA) and Rho-associated kinase 2 (Rock2), and increased the expression of growth associated protein-43, microtubule-associated protein-2, netrin-1, Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42). The effect of *Houshiheisan* was stronger than wind-dispelling drugs or deficiency-nourishing drugs alone. In conclusion, *Houshiheisan*, and wind-dispelling and deficiency-nourishing drugs promote the repair of axons and nerve regeneration after cerebral ischemia through Nogo-A/RhoA/Rock2 and Netrin-1/Rac1/Cdc42 signaling pathways. These effects are strongest with *Houshiheisan*.

Key Words: *nerve regeneration; Houshiheisan; wind-dispelling drug; deficiency-nourishing drug; cerebral ischemia; Nogo-A/RhoA/Rock2 signaling pathway; axonal recovery; Netrin-1/Rac1/Cdc42 signaling pathway; neuroprotection; neural regeneration*

Introduction

Cerebral ischemia is a common neurological condition and leads to high rates of disability and mortality. At present, the only approved therapy is human tissue-type plasminogen activator (Lees et al., 2010). However, only 5% of patients can receive this treatment because it has to be administered within 4.5 hours of an ischemic event (Fonarow et al., 2011). Moreover, even with effective thrombolysis, most patients will have varying degrees of neurological deficits (Yamamoto et al., 2009). Stroke elicits disruption of myelin and impairs axonal conductivity, which exacerbates functional outcome (Benowitz and Carmichael, 2010). Axonal remodeling plays a crucial role in endogenous brain repair (Zhang et al., 2010). Some inhibitory factors of neurological regeneration, such as neurite outgrowth inhibitor protein A (Nogo-A), play an important role in restraining axon regeneration and lateral shoot formation (Schwab, 2004). Downregulation of RhoA/Rho-associated kinase, a downstream switch of Nogo-A, can promote axonal regeneration (Yang et al., 2013). Also, low expression levels of axonal growth factors may be another difficulty for neural regeneration. Netrin-1 is an important member of the netrin axonal guidance family, and is expressed in the mature central nervous system but not during development (Izzi and Charron, 2011). The Netrin family proteins can promote the recovery of neuronal function after cerebral ischemia (Bayat et al., 2012), while Netrin-1 can promote axon growth by activating Rho GTPases, including Rac1 and Cdc42, which are crucial for transmitting extracellular signals into axonal remodeling within the growth cone (Antoine-Bertrand et al., 2011). Furthermore, both promotive and inhibitory factors of axonal growth exert their functions by binding with their receptors and activating downstream molecular switches (Sakumura et al., 2005). Based on these findings, exploring alternative therapies that boost axonal repair is a matter of urgency for stroke treatment.

Houshiheisan (HSHS) was the first prescription for stroke created by Zhang Zhongjing and has been clinically used in China to promote safe and effective repair of neurological function for over 2000 years. According to basic theory of traditional Chinese medicine, HSHS is composed of a wind-dispelling drug (WDD) and a deficiency-nourishing drug (DND). *Flos* Chrysanthemi and *Radix et Rhizoma Ginseng* are considered as representative WDD and DND drugs, respectively. Our previous analysis of HSHS by high performance liquid chromatography showed that the main components are chlorogenic acid, apigenin-7-O-glucoside, and luteolin-7-O-glucoside (Chang et al., 2016). Importantly, chlorogenic acid ameliorates brain damage and edema by inhibiting matrix metalloproteinase-2 and 9 (Lee et al., 2012), while luteolin-7-O-glucoside inhibits inflammation after focal cerebral ischemia *in vivo via* conversion to luteolin (Zhang et al., 2012). Furthermore, HSHS can inhibit amyloid precursor protein (APP) deposition, reduce the release of inflammatory response factors, and up-regulate the expression of brain-derived neurotrophic factor, thereby improving neurological function (Bayat et al., 2012; Zhang et al., 2012; Wang et al., 2014). Our previous findings demonstrated that HSHS has a potent effect on neural regeneration. However, the mechanism of this effect has not been fully investigated. This study focused on the axonal signaling pathways of Nogo-A/RhoA/Rock2 and Netrin-1/DCC/Rac1, and explored the mechanisms by which HSHS promotes neural regeneration after cerebral ischemia *via* axon recovery.

Materials and Methods

Establishment of focal cerebral ischemia models

Seventy-two healthy, specific-pathogen-free, 3-month-old male Sprague-Dawley rats weighing 300–350 g, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd., China [Animal license No. SCXK (Jing) 2012-0001]. The ethical license number from the Institution Animal Care and Use Committee was AEEI-2016-054. Rats were housed in the laboratory animal center of the Capital Medical University, China [Animal license No. SYXK (Jing) 2010-0020]. Protocols conform to the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China. The rats were randomly divided into sham group (n = 12), permanent middle cerebral artery occlusion (pMCAO) group (n = 15), HSHS group (n = 15), WDD group (n = 15) and DND group (n = 15). The pMCAO model was based on our previously published method (Bayat et al., 2012). Rats were anesthetized *via* face mask inhalation of 1.5% to 2.5% isoflurane in a 2:1 N_2O:O_2 atmosphere and fixed in the supine position. The origins of the right common carotid artery, the internal carotid artery and the external carotid artery were exposed and isolated by blunt separation. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co., Ltd., Beijing, China) was inserted into the lumen of the internal carotid artery to block the right middle cerebral artery. Rats in the sham group were subjected to the same operation without insertion of the nylon suture. Five rats died within 7 days of pMCAO (two in the pMCAO group, two in the WDD group and one in the HSHS group).

HSHS preparation

HSHS contains 13 Chinese herbs, including *Flos Chrysanthemi, Radix Saposhnikoviae*, *Ramulus Cinnamomi*, *Rhizoma Chuanxiong*, *Radix et Rhizoma Asari*, *Radix Platycodonis*, *Rhizoma Atractylodis macrocephalae*, *Poria, Rhizoma Zingiberis*, *Radix Angelicae sinensis*, *Radix et Rhizoma Ginseng*, *Radix Scutellariae* and *Concha Ostreae*. According to the theory of traditional Chinese medicine, HSHS can be divided into two main components, WDD and DND. WDD consists of six Chinese herbs, including *Flos Chrysanthemi*, *Radix Saposhnikoviae*, *Ramulus Cinnamomi*, *Rhizoma Chuanxiong*, *Radix et Rhizoma Asari* and *Radix Platycodonis.* DND is composed of five Chinese herbs: *Rhizoma Atractylodis macrocephalae*, *Poria*, *Rhizoma Zingiberis*, *Radix Angelicae sinensis* and *Radix et Rhizoma Ginseng*. All the above Chinese herbs were purchased from Tong-ren-tang Chinese Medicine Co., Ltd. (Beijing, China) and authenticated by Associate Professor Jia Li (Capital Medical University, Beijing, China) according to the Chinese Pharmacopoeia (2015 edition). Their voucher specimens were deposited at the Beijing

Key Laboratory of Traditional Chinese Medicine Collateral Disease Theory Research (Beijing, China). Quality control of these Chinese herbs was previously performed by high performance liquid chromatography as published in our previous study (Bayat et al., 2012).

HSHS, WDD and DND were prepared separately with our previously published methods (Bayat et al., 2012). Briefly, the air-dried herbs were mixed according to the prescription and then refluxed twice with 30% ethanol (1:10, w/v) for 2 hours. After filtration, the solution was evaporated under vacuum at 50°C to remove ethanol to give liquid extracts of HSHS, WDD and DND. One milliliter of HSHS, WDD and DND extract was produced from 1.7 g, 1.25 g and 0.42 g of crude herbs, respectively.

Drug administration

According to a previous study (Bayat et al., 2012), the rats in the HSHS, WDD and DND groups were given 10.5 g/kg HSHS, 7.7 g/kg WDD and 2.59 g/kg DND, respectively. The rats in the sham and pMCAO groups were given the same volume of normal saline (1 mL/100 g). The doses of HSHS, WDD and DND were calculated using the body surface area normalization method and normal clinical usage of crude drugs. Drugs were intragastrically administrated for the first time at 6 hours after the operation, and then every 24 hours for 7 consecutive days. Brain tissues were removed at 7 days after pMCAO for analysis (**Figure 1**).

Neurofunctional scoring

Neurological deficit was assessed using the improved Zea Longa five-grade scoring method (Longa et al., 1989): 0 = no apparent neurological symptoms; 1 = failure to fully stretch the left forelimb; 2 = rotating to the left; 3 = falling to the left side during walking; 4 = loss of consciousness or walking only by stimulation.

Beam walking test

The beam walking test was performed at 3 and 7 days after pMCAO (Roof et al., 2001). Rats were trained from 3 days before the operation to ensure they could successfully cross a bar 100 cm long, 3 cm wide and located 60 cm above the floor. The assessment was evaluated with the following grading criteria: 0 = unable to stay on the crossbar; 1 = able to stay on the crossbar without movement; 2 = trying to traverse the beam but falling off; 3 = traversing the beam with more than 50% left hindlimb slips; 4 = traversing the beam with less than 50% left hindlimb slips; 5 = walking across the beam with only one left hindlimb slip; 6 = crossing the beam successfully without any hindlimb slips.

Neuropathological assessments

Three rats were randomly selected from each of the five experimental groups at 7 days after modeling for hematoxylin-eosin staining. After deep anesthesia, rats were transcardially perfused with 500 mL 0.9% saline, followed by 500 mL of 4% paraformaldehyde in 0.1% M phosphate buffered solution (PBS, pH 7.4). Brains were then carefully dissected, fixed in 4% paraformaldehyde, embedded in paraffin, and 3 μm-thick sections prepared for hematoxylin-eosin staining. Stained sections were observed by optical microscopy (E100, Nikon, Tokyo, Japan) at 400× magnification.

The ultrastructure of ischemic peri-infarcts in the hippocampus was observed by transmission electron microscopy (JEM-1230, Jeol, Japan). One rat was randomly selected from each of the five groups, and perfused transcardially with 2% paraformaldehyde mixed with 2% glutaraldehyde for 1 hour, followed by 3% glutaraldehyde for 2 hours. Brains were then removed and ultrathin sections prepared. The ratio of axon diameter to total fiber diameter is called the g-ratio, and data were collected from 10 fields of view from each section and averaged using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) (Zheng et al., 2015).

Immunofluorescence for microtubule-associated protein-2 (MAP-2)

MAP-2 plays a critical role in axon growth (Wang et al., 2017). Sections for immunofluorescence staining of MAP-2 were heated with 0.01 M citrate buffer, and incubated with primary antibody (MAP-2, mouse monoclonal, 1:3000; Abcam, Cambridge, MA, USA) at 4°C for 40 hours and then at 37°C for 1 hour. Sections were then washed with PBS and incubated with 50 μL secondary antibody (goat anti-mouse IgG <H+L>-FITC, 1:200; SouthernBiotech, Birmingham, AL, USA) at 37°C for 2 hours. Sections were mounted using Dapi-Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) and preserved at 4°C. Sections were observed by fluorescence microscopy (Olympus, Tokyo, Japan), and images were collected by digital photomicroscopy (Leica, Wetzlar, Germany). Five fields of view were randomly selected from each section for quantitative analysis, and the average value of integral optical density calculated using the NIS-Elements Basic Research image acquisition system (Nikon).

Western blot assay

Total protein was extracted from ischemic hippocampi and quantified using a bicinchoninic acid protein assay kit. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene fluoride membranes (Neobioscience Biotech Co., Ltd., Beijing, China). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hour, and incubated with primary antibodies, including APP (1:100,000; rabbit monoclonal; Epitomics, Burlingame, CA, USA), growth associated protein-43 (GAP-43) (1:100,000; rabbit monoclonal; Epitomics), Nogo-A (1:5000; rabbit polyclonal; Abcam), NgR (1:5000; rabbit monoclonal; Abcam), Rock2 (1:10,000; rabbit polyclonal; Abcam), RhoA (1:15,000; rabbit monoclonal; Cell Signaling Technology, Danvers, MA, USA), Netrin-1 (1:2000; rabbit monoclonal; Abcam), deleted in colorectal cancer (DCC) (1:5000; rabbit polyclonal; Abcam), Rac1 (1:15,000; mouse monoclonal; Abcam), Cdc-42 (1:5000; rabbit polyclonal; Abcam), and GAPDH (1:10,000; mouse monoclonal; GeneTex, Irvine, CA, USA) at 4°C overnight. After several washes with

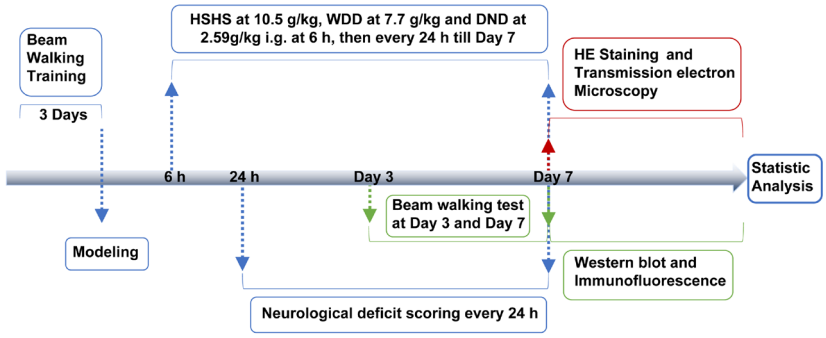


Figure 1 Schedule of experimental manipulations.
HSHS: *Houshiheisan*; WDD: wind-dispelling drug; DND: deficiency-nourishing drug; h: hours; i.g.: intragastrically; HE: hematoxylin-eosin.

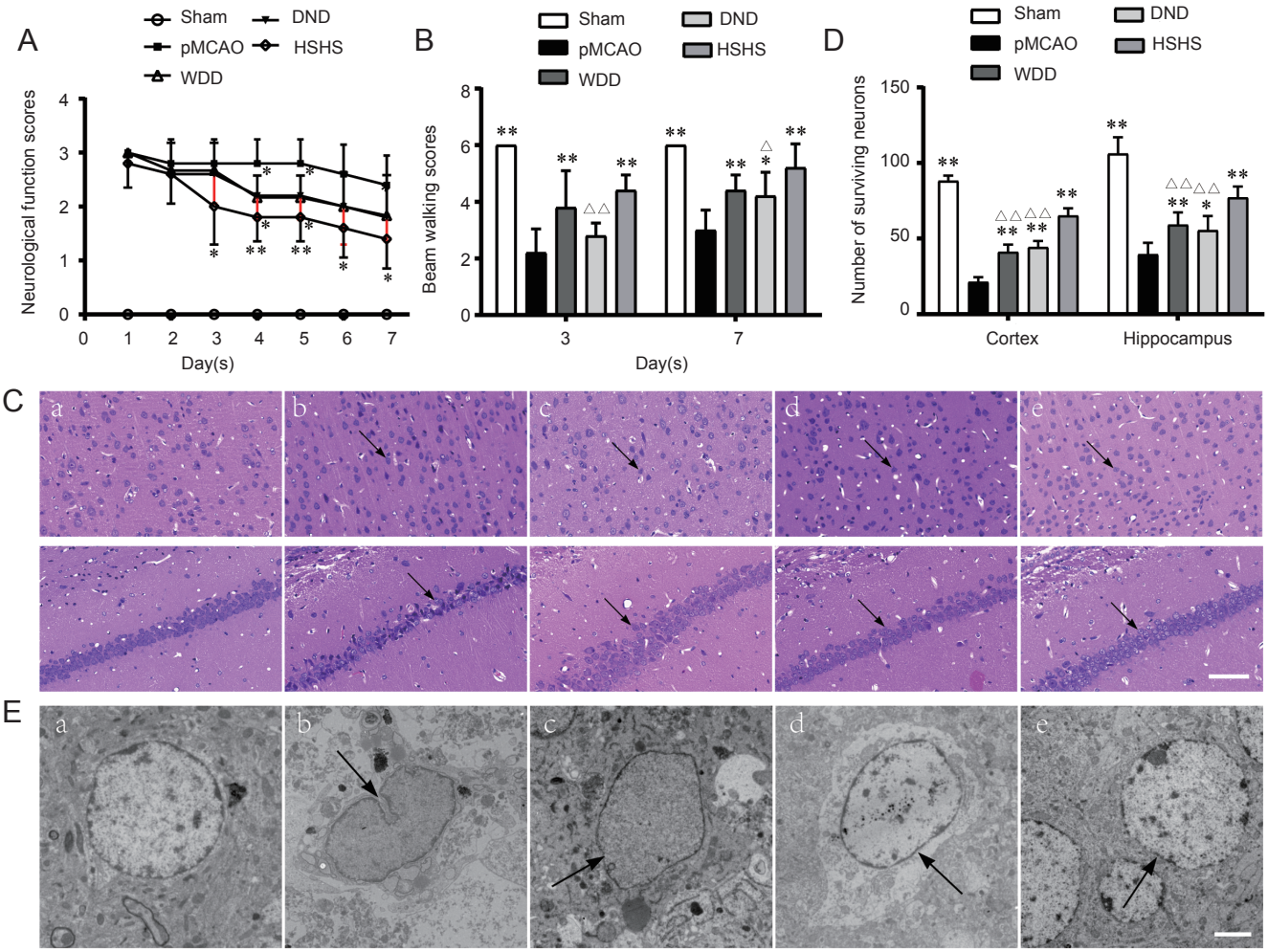
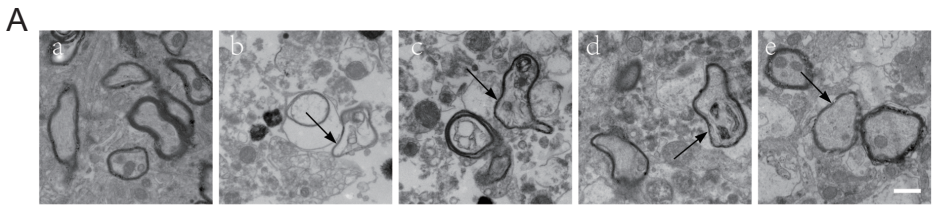


Figure 2 Neurological function and pathological changes in each group after ischemia.
(A) Neurological function scores from 1 to 7 days after pMCAO; (B) Beam walking scores at 3 and 7 days after pMCAO. Higher scores in (A) & (B) represent better neurological function, while low scores mean more serious neurological damage. (C) Hematoxylin-eosin staining of surviving neurons (arrows) in peri-infarct cortex (upper) and hippocampi (lower) at 7 days after cerebral ischemia (original magnification: 400×; Scale bars: 50 μm). (a–e) Sham, pMCAO, WDD, DND, and HSHS groups, respectively. (D) Quantification of the number of surviving neurons. (A, B, D) Data are expressed as the mean ± SD, and were analyzed by analysis of variance followed by the least significant difference *post hoc* test. $*P < 0.05$, $**P < 0.01$, *vs.* pMCAO group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, *vs.* HSHS group. (E) Transmission electron microscopy images showing neurons with an irregular nuclear membrane (arrow) in peri-infarct hippocampi at 7 days (original magnification: 1500×, scale bars: 2 μm). (a–e) Sham, pMCAO, WDD, DND, and HSHS group, respectively. pMCAO: Permanent middle cerebral artery occlusion; WDD: wind-dispelling drug; DND: deficiency-nourishing drug; HSHS: *Houshiheisan*.

Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated in horseradish peroxidase-linked goat-anti-rabbit antibody (IgG, 1:20,000; Neobioscience Biotech Co., Ltd.) for 1 hour at 37°C and washed. Blots were visualized using the ECL chemiluminescent system (Millipore, Burlington, MA, USA) and quantified using Image Quant TL software (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

Data are shown as the mean ± SD, and were analyzed with



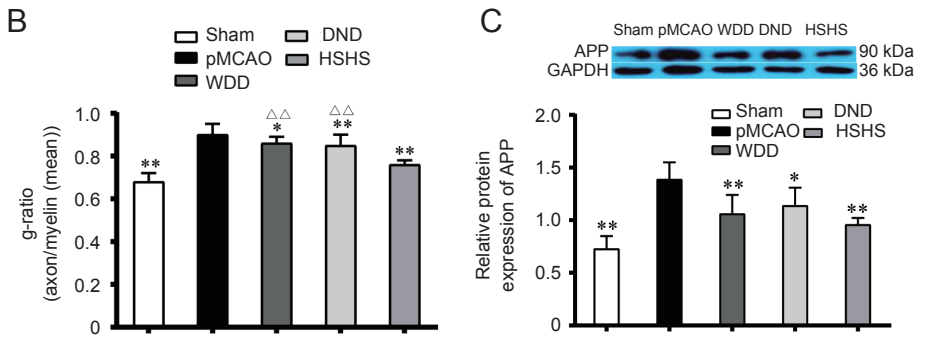


Figure 3 Myelin sheath and axonal changes 7 days after cerebral ischemia.
(A) Transmission electron microscopy images: Myelinated nerve fibers were disorganized; layers of the myelin sheath were wrinkled and axoplasms of axons were degenerated in the peri-infarct hippocampus (arrow) of the pMCAO group (original magnification: 4000×; scale bar: 1 μm); (a–e) Sham, pMCAO, WDD, DND, and HSHS groups, respectively. (B) g-ratio of rats in each group. (C) APP protein expression in peri-infarct hippocampi detected by western blot assays. Data are expressed as the mean ± SD, and were analyzed by analysis of variance followed by the least significant difference *post hoc* test. $*P < 0.05$, $**P < 0.01$, *vs.* pMCAO group; $\Delta\Delta P < 0.01$, *vs.* HSHS group. pMCAO: Permanent middle cerebral artery occlusion; WDD: wind-dispelling drug; DND: deficiency-nourishing drug; HSHS: *Houshiheisan*.

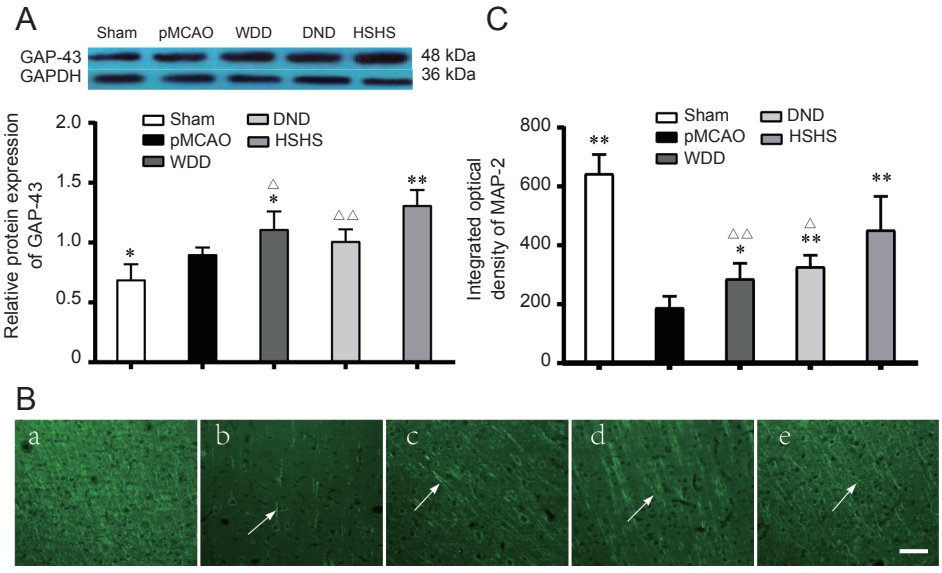


Figure 4 MAP-2 and GAP-43 expression in the peri-infarct hippocampus of rats in each group at 7 days after cerebral ischemia.
(A) GAP-43 protein expression in peri-infarct hippocampi detected by western blotting. (B) Immunofluorescence staining of peri-infarct hippocampi showing MAP-2 positive neurons (arrow). Scale bar: 50 μm; original magnification: 400×. (a–e) Sham, pMCAO, WDD, DND, and HSHS group, respectively. (C) Quantification of MAP-2 immunofluorescence staining. Data are expressed as the mean ± SD, and were analyzed by analysis of variance followed by the least significant difference *post hoc* test. $*P < 0.05$, $**P < 0.01$, *vs.* pMCAO group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, *vs.* HSHS group. pMCAO: Permanent middle cerebral artery occlusion; WDD: wind-dispelling drug; DND: deficiency-nourishing drug; HSHS: Houshiheisan; MAP-2: microtubule-associated protein-2; GAP-43: growth associated protein-43.

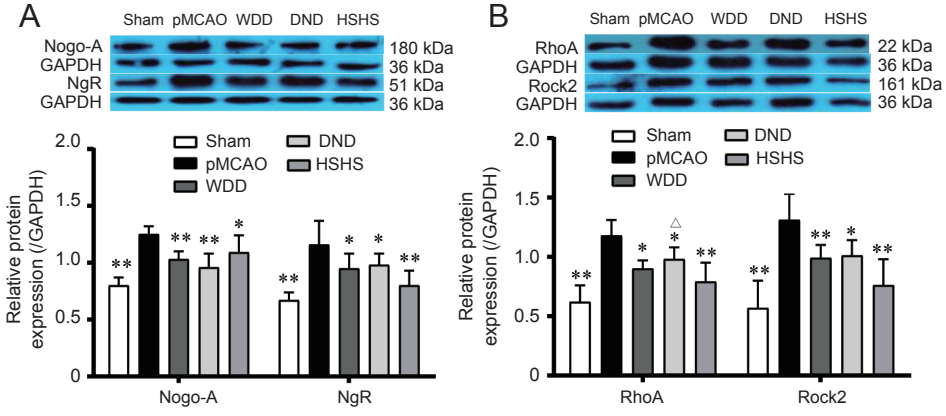


Figure 5 Nogo-A/RhoA/Rock2 expression in the peri-infarct hippocampus of rats in each group at 7 days after cerebral ischemia.
(A) Nogo-A and NgR protein expression in peri-infarct hippocampi detected by western blotting. (B) RhoA and Rock2 expression in peri-infarct hippocampi detected by western blotting. Data are expressed as the mean ± SD, and were analyzed by analysis of variance followed by the least significant difference *post hoc* test. $*P < 0.05$, $**P < 0.01$, *vs.* pMCAO group; $\Delta P < 0.05$, *vs.* HSHS group. pMCAO: Permanent middle cerebral artery occlusion; WDD: wind-dispelling drug; DND: deficiency-nourishing drug; HSHS: *Houshiheisan*.

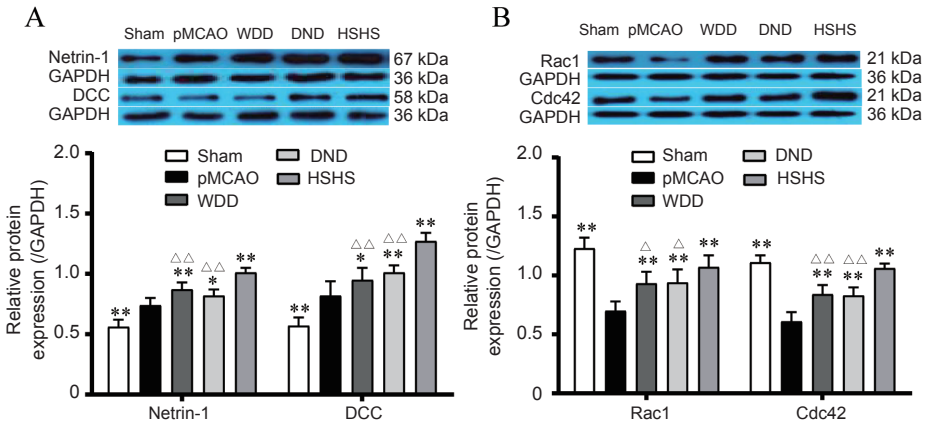


Figure 6 Netrin-1/DCC/Rac1/Cdc42 expression in the peri-infarct hippocampus of rats in each group at 7 days after cerebral ischemia.
(A) Netrin-1 and DCC protein expression in peri-infarct hippocampi detected by western blot assay. (B) Rac1 and Cdc42 expression in peri-infarct hippocampi detected by western blot assay. Data are expressed as the mean ± SD, and were analyzed by analysis of variance followed by the least significant difference *post hoc* test. $*P < 0.05$, $**P < 0.01$, *vs.* pMCAO group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, *vs.* HSHS group. pMCAO: Permanent middle cerebral artery occlusion; WDD: wind-dispelling drug; DND: deficiency-nourishing drug; HSHS: *Houshiheisan*.

SPSS 19.0 software (IBM, Armonk, NY, USA). One-way analysis of variance followed by the least significant difference *post hoc* test was utilized to compare mean difference among multiple groups. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of HSHS on improving neurological function and relieving neuronal damage in cerebral ischemia model rats

Neurological function was evaluated by the Zea Longa five-level neurological deficit score every 24 hours for 7 days after pMCAO. Compared with the pMCAO group, the neurological deficit score was significantly decreased in HSHS-treated rats from 3–7 days after pMCAO (neurological deficit score: Day 3, $F_{(3,16)} = 2.024$, $P < 0.01$; Day 4, $F_{(3,16)} = 4.515$, $P < 0.01$; Day 5, $F_{(3,16)} = 4.515$, $P < 0.01$; Day 6, $F_{(3,16)} = 2.260$, $P < 0.01$; Day 7, $F_{(3,16)} = 2.384$, $P < 0.01$) (**Figure 2A**), while WDD and DND treatment reduced the neurological deficit score from 4–5 days ($P < 0.05$) (**Figure 2A**).

The beam walking test was performed to observe fine motor control. Compared with the pMCAO group, the scores were higher in the HSHS and WDD groups at 3 and 7 days (Day 3, $F_{(3,16)} = 6.713$, $P < 0.01$; Day 7, $F_{(3,16)} = 7.515$, $P < 0.01$) (**Figure 2B**). Scores in the DND group showed a prominent improvement at 7 days ($P < 0.05$; **Figure 2B**). It was noteworthy that scores in DND group were markedly lower at 3 and 7 days compared with the HSHS group ($P < 0.05$; **Figure 2B**), which indicated that HSHS had a greater effect than DND.

Hematoxylin-eosin staining revealed severe necrosis, infiltration of inflammatory cells and vascular edema in the cortex and hippocampus in the non-treatment pMCAO group. There were different degrees of improvement in morphology after HSHS, WDD and DND treatment at 7 days after cerebral ischemia (**Figure 2C**). Quantitative light microscopy analysis showed that survival of neurons in the cortex and hippocampus of HSHS, WDD and DND groups was significantly increased compared with the pMCAO group (survival neurons: cortex, $F_{(4,20)} = 174.610$, $P < 0.001$; hippocampus, $F_{(4,20)} = 40.263$, $P < 0.001$; **Figure 2D**). Many more neurons survived in the HSHS group than in the WDD and DND groups ($P < 0.01$; **Figure 2D**).

Transmission electronic microscopy showed structural abnormalities of neurons following pMCAO. HSHS, WDD and DND treatments alleviated neuronal deformation compared with the pMCAO group (**Figure 2E**).

Effect of HSHS on reducing myelin sheath and axonal damage after cerebral ischemia

Pathological changes to myelin sheaths and axons were observed by transmission electron microscopy at 7 days after pMCAO (**Figure 3A**). Myelin sheath damage was severe in pMCAO rats but was was markedly relieved after administration of HSHS, WDD or DND (**Figure 3**).

The g-ratio was increased after focal cerebral ischemia and was decreased after HSHS, WDD or DND treatments compared with the pMCAO group (one-way analysis of variance: g-ratio, $F_{(4,45)} = 47.247$, $P < 0.01$) (**Figure 3**). More importantly, HSHS-treated rats had a lower g-ratio compared with WDD- and DND-treated rats (*post hoc*, $P < 0.01$; **Figure 3**). The degree of axon damage was detected by western blot assay for APP. APP levels were significantly increased after pMCAO, but HSHS, WDD and DND treatments reduced APP expression compared with the pMCAO group (one-way analysis of variance: APP, $F_{(4,20)} = 14.000$, $P < 0.01$; **Figure 3**).

HSHS promoted axon recovery in the ischemic hippocampus by up-regulating the expression of GAP-43 and MAP-2

GAP-43 is an indicator of axonal recovery. The expression of GAP-43 was up-regulated after pMCAO, and was further elevated after HSHS or WDD treatment (GAP-43, $F_{(4,20)} = 19.394$, $P < 0.001$; **Figure 4A**). Notably, the expression of GAP-43 was higher after HSHS treatment than after WDD or DND treatment ($P < 0.05$; **Figure 4A**).

MAP-2 is another useful marker for evaluation of axonal recovery (Roof et al., 2001). MAP-2 staining was significantly decreased in the pMCAO group compared with the sham group and was up-regulated to different degrees after HSHS,

WDD and DND treatments (one-way analysis of variance: APP, $F_{(4,20)} = 32.638$, $P < 0.01$; **Figure 4B, C**). Meanwhile, HSHS had a significant effect on the expression of MAP-2 compared with WDD or DND alone (*post-hoc*, $P < 0.05$; **Figure 4C**).

HSHS promoted axon recovery by down-regulating the Nogo-A/NgR/RhoA/Rock2 signaling pathway in the pMCAO rat hippocampus

Nogo-A is a potent axonal inhibitory factor (Schwab, 2004). The expression of Nogo-A and its receptor, NgR, were clearly increased in ischemic rats without treatment, but this expression decreased after administration of HSHS, WDD or DND (one-way analysis of variance: Nogo-A, $F_{(4,20)} = 12.763$, $P < 0.01$; NgR, $F_{(4,20)} = 9.476$, $P < 0.01$; **Figure 5A**).

The inhibitory effect of Nogo-A/NgR depends on the activity of downstream molecules, RhoA and Rock. The expression of RhoA and Rock2 [a subtype of Rock with cardio-cerebral vascular distribution (Iizuka et al., 2012)] was detected by western blot assay. Both RhoA and Rock2 levels were significantly increased in the pMCAO group at 7 days after cerebral ischemia, and HSHS, WDD and DND treatments down-regulated the expression of RhoA and Rock2 compared with the pMCAO group (one-way analysis of variance: RhoA, $F_{(4,20)} = 14.025$, $P < 0.01$; Rock2, $F_{(4,20)} = 10.834$, $P < 0.01$). Interestingly, the expression of RhoA after HSHS treatment was significantly lower than that after DND treatment (*post-hoc*, $P < 0.05$; **Figure 5B**).

HSHS promoted axon growth by up-regulating the Netrin-1/DCC/Rac1/Cdc42 signaling pathway in the pMCAO rat hippocampus

Netrin-1 promotes axonal remodeling by binding with its receptor, DCC (Blasiak et al., 2016). Netrin-1 and DCC levels were increased in the pMCAO group compared with the sham group, and HSHS, WDD, and DND treatment further elevated netrin-1 and DCC levels compared with the pMCAO group (Netrin-1, $F_{(4,20)} = 48.947$, $P < 0.01$; DCC, $F_{(4,20)} = 41.473$, $P < 0.01$; **Figure 6A**). Moreover, HSHS up-regulated the expression of Netrin-1 and DCC compared with WDD or DND alone ($P < 0.01$; **Figure 6A**).

Rac1 and Cdc42 are downstream molecular switches of Netrin-1 and DCC (Ji et al., 2016). Ischemic injury significantly reduced the expression of Rac1 and Cdc42, while HSHS, WDD and DND up-regulated Rac1 and Cdc42 expression to different degrees (Rac1, $F_{(4,20)} = 21.108$, $P < 0.01$; Cdc42, $F_{(4,20)} = 41.471$, $P < 0.01$). Further comparison revealed that HSHS had a greater effect on up-regulating the expression of Rac1 and Cdc42 compared with WDD or DND alone ($P < 0.01$) (**Figure 6B**).

Discussion

Regeneration of neurons after cerebral ischemia, has been extensively investigated (He et al., 2016a; Yin et al., 2016). This study investigated the effect and molecular mechanisms of HSHS and its components WDD and DND in neural regeneration and axon remodeling in the pMCAO rat model.

Neuroprotection plays an important role in neural regeneration after ischemic stroke (Shi et al., 2016). HSHS and its components significantly alleviated ischemic injury in both neurons and axons. Importantly, HSHS and its components decreased the expression of APP, a neuronal transmembrane glycoprotein transported by rapid axonal transport, which rapidly accumulates after cerebral ischemia (Coleman, 2005). Decreased levels of APP indicate less axon damage. Our results indicate that HSHS and its components have protective effects on axons, and that HSHS was more potent than WDD or DND alone in protecting the myelin sheath.

HSHS promotes neuronal recovery after cerebral ischemia by up-regulating certain neurotrophic factors (Bayat et al., 2012). Axonal remodeling is the main manifestation of neuronal recovery after cerebral ischemia (Liu et al., 2017). GAP-43, a growth-related protein promoting neuronal growth, development, nerve regeneration and synaptic remodeling, is a marker of axonal recovery after cerebral ischemia (Li et al., 2017). MAP-2 is another important molecule in regulating axonal reorganization (Roof et al., 2001). Our results show that HSHS and WDD up-regulate the expression of GAP-43 and MAP-2, which means that HSHS and WDD had positive effects on axonal recovery. Furthermore, the effect of HSHS was significantly stronger than that of WDD. However, DND did not produce a significant effect. These results illustrate that HSHS promotes neural regeneration by promoting axonal recovery and that the effect may derive from the complementarity of WDD and DND.

Axon recovery is regulated by guidance and inhibitory molecules (Schmidt and Minnerup, 2016; Dun and Parkinson, 2017). However, axonal growth in adult neurons after cerebral ischemia is limited because of a lack of intrinsic capacity. More importantly, some inhibitory neurite outgrowth factors can induce axonal dysplasia in central neurons after ischemic lesion (Papadopoulos et al., 2002; Pernet and Schwab, 2012). Nogo-A, a myelin-derived transmembrane protein, is a strong inhibitor on axonal growth by inducing axonal dysplasia (Bandtlow, 2003; Schweigreiter et al., 2004). Under physiological conditions, Nogo-A is highly expressed in immature neurons, which reduces when the neurons become mature. However, ischemic stimulation can evoke an increase of Nogo-A in mature neurons (Buss et al., 2005; Rolando et al., 2012; Feng et al., 2016). Nogo-A can lead to collapse of the growth cone (GrandPré et al., 2000; Lindau et al., 2014). In an NgR-knockdown ischemic animal model, axon growth was more active and was accompanied by recovery of neurological function (Wang et al., 2010). The present study showed an obvious increase in Nogo-A/NgR levels at 7 days after pMCAO, while HSHS, WDD and DND treatments had an inhibitory effect on Nogo-A and NgR expression. RhoA/Rock2, the Rock subtype mainly expressed in brain tissue, is a downstream molecule in the Nogo-A/NgR pathway (Liu et al., 2011a). HSHS, WDD and DND had beneficial effects on down-regulating the expression of RhoA/Rock2, while HSHS had a greater effect than DND on the expression of RhoA. Overall, HSHS, WDD and DND contributed to axon growth by down-regulating the expression of the inhibitory Nogo-A/NgR/RhoA/Rock2 signaling pathway, and the effect of HSHS

was more powerful than that of DND in inhibiting RhoA.

Netrin-1, the vertebrate homolog of Unc6, is the strongest known chemoattractant for the promotion of axon extension and is a regulator of axonal guidance and angiogenesis (Lai Wing Sun et al., 2011). The combination of Netrin-1 and its receptor, DCC, can precisely regulate the growth direction of the axonal growth cone (Shoja-Taheri et al., 2015). In this study, the expression of Netrin-1 and DCC was spontaneously elevated after cerebral ischemia, which is consistent with previous results (Liu et al., 2011b; Bayat et al., 2012). HSHS, WDD and DND treatments upregulated the expression of Netrin-1/DCC to varying degrees to promote recovery after cerebral ischemia. HSHS promoted the release of axon guidance factors more effectively than WDD and DND after focal cerebral ischemia. Furthermore, the Netrin-1/DCC-mediated axonal recovery needed a downstream effector to exert its function. Rac1/Cdc42 is a molecular switch in axon regeneration (Sit and Manser, 2011). In our study, the expression of Rac1/Cdc42 markedly decreased after pMCAO, while HSHS, WDD and DND treatments up-regulated Rac1/Cdc42 to promote axonal recovery after cerebral ischemia. Importantly, HSHS had a greater effect on the molecular switches than WDD or DND.

Based on these results, we confirmed that HSHS, WDD and DND can reduce axon damage, promote axon recovery, and facilitate axonal regeneration by regulating the expression of Nogo-A/RhoA/Rock2 and Netrin-1/Rac1/Cdc42 after cerebral ischemia. HSHS had better therapeutic effects than WDD or DND alone after focal cerebral ischemia in the following ways: (1) protecting neurons, reducing axonal damage; (2) promoting axonal recovery; (3) up-regulating guidance pathways to encourage axon growth. The potent effect of HSHS on promoting neural regeneration was probably derived from the complementarity of WDD and DND, with WDD participating in all the therapeutic effects by promoting axonal recovery and performing better than DND. SHS has been used in China to treat stroke for approximately 2000 years, or since the Han Dynasty. Recently, doctors of traditional Chinese medicine have expanded the clinical application of HSHS to treat neurodegenerative diseases, such as cerebral ischemia (He et al., 2016b) and Parkinson's disease (Pang, 2015). Our findings indicate that WDD and DND combined can achieve better therapeutic effects on ischemic cerebral stroke than when used separately.

The limitation of our study was that we only explored the mechanism of HSHS on axonal recovery at 7 days after cerebral ischemia; more time points should be observed in future research.

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