Neuroprotective Effect of Buyang Huanwu Decoction on Rat Ischemic/Reperfusion Brain Damage by Promoting Migration of Neural Precursor Cells

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Abstract

Buyang Huanwu Decoction (BYHWD) is a classic formula widely used for treating stroke-induced disability, the highest morbidity of neurological disorders in China. However, the mechanism of its neuroprotection has not been fully clarified. Previous reports indicated that BYHWD may promote growth and differentiation of neural precursor cells (NPCs). The present study focused on the effects of BYHWD on migration of NPCs in rats with middle cerebral artery occlusion (MCAO). Rats were treated with different doses of BYHWD (12 and 24 grams/kg) from day 1 to day 21 after model building. BYHWD could increase the survival rate and decrease neurological scores and infarct volume as compared with the vehicle-treated MCAO rats. Moreover, BYHWD treatment significantly increased 5-bromo-2-deoxyuridine (BrdU)-positive cells in the subventricular zone (SVZ), subgranular zone (SGZ), and corpus striatum (CS) of the infarct brain. Interestingly, BYHWD could markedly enhance BrdU⁺/doublecortin⁺ cells not only in the SVZ and SGZ but also in CS, by up-regulating the protein expression of migration activators, including stromal cell derived factor-1, CXC chemokine receptor 4, vascular endothelial growth factor, Reelin, and brain-derived neurotrophic factor in the ipsilateral infarct area after MCAO. In addition, BYHWD treatment was able to promote the neuronal differentiation, which was closely related to the migratory process of NPCs in MCAO rats. These findings offer evidence for the first time that BYHWD may exert its neuroprotective effects partially by promotion of NPCs migration to ischemic brain areas.

Introduction

STEM CELLS HAVE BEEN PROPOSED as a new form of cell-based therapy in a variety of disorder and injuries of the central nervous system (CNS). Neural precursor cells (NPCs) have been identified in the CNS, where they reside mostly in the subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampus and are capable of sustained self-renewal and proliferation with multipotency toward CNS lineages.^{1,2} These NPCs proliferate in response to ischemia and migrate to the injury site, where some of them differentiate into neurons.³ Therefore, endogenous NPCs (eNPCs) may be used to replace dead cells in adults following brain injury, providing a novel mode of therapy for stroke. However, spontaneous brain regeneration is relatively limited and is not sufficient to induce significant behavioral improvement after brain injury. Nevertheless, eNPC responses can be increased considerably by tweaking the pathways governing cell pro-

liferation, migration, and differentiation. Considerable evidence suggests that such perturbations may lead to better functional outcome after brain injury.⁴⁻⁸

Because neurogenesis only persists in two distinct niches in the adult brain, the SGZ and SVZ, migration of NPCs plays a pivotal role in a variety of physiological and pathological events in adults.⁹⁻¹¹ Therefore, modulating migration of eNPCs for brain repair is a critical issue facing stem cell therapy. Increasing numbers of studies have demonstrated that NPCs, either endogenous or transplanted, are highly motile and display a unique tropism for areas of pathology in the adult brain,^{12,13} which reveals a therapeutic potential of promoting migration of NPCs for neural repair after injury.

Buyang Huanwu Decoction (BYHWD), a well-known traditional Chinese medicine formula, is composed of seven kinds of Chinese medicine—Huangqi (Radix Astragali seu Hedysari), Danggui (Radix Angelicae Sinensis), Chishao (Radix Paeoniae Rubra), Chuanxiong (Rhizoma Ligustici Chuanxiong),

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Taoren (Semen Persicae), Honghua (Flos Carthami), and Dilong (Pheretima)—all of which are recorded in the Chinese Pharmacopoeia. BYHWD has been used extensively for centuries to treat a variety of disorders, including stroke-induced disability and paralysis in China.^{14,15} A large number of studies have found its mechanisms on the treatment of stroke, including anti-inflammation,¹⁶ anti-oxidation,^{16–18} improvement of hemorheological disorders,¹⁹ inhibition of neural apoptosis,^{17,20} and others. Experimental reports show that BYHWD may promote growth and differentiation of NPCs^{21,22}; however, the role of BYHWD in migration of NPCs is still unclear.

The present study was designed to observe the effects of BYHWD on the migration of eNPCs in the middle cerebral artery occlusion (MCAO) animal model and to elucidate its role in the underlying mechanism of neuroprotective effects in treating stroke diseases.

Materials and Methods

Preparation of BYHWD

BYHWD is composed of Radix Astragali seu Hedysari (120 grams), Radix Angelicae Sinensis (6 grams), Radix Paeoniae Rubra (4.5 grams), Rhizoma Ligustici Chuanxiong (3 grams), Semen Persicae (3 grams), Flos Carthami (3 grams), and Pheretima (3 grams). These components were mixed in order with the ratio of 120:6:4.5:3:3:3 (dry weight). To keep the consistency of the herbal chemical ingredients, all of the herbal components were originally obtained from the standard naïve sources with good agricultural practice (GAP) grade, and the drugs were extracted with standard methods according to the Chinese Pharmacopoeia.²³ The herbs were decocted by boiling in distilled water at 100°C for 30 min. The solution was then freeze-dried under vacuum and made into a powder stored in aliquots at –20°C. The powder was dissolved in distilled water when administrated to rats.

Ethics statement

This study was approved by the Research Ethics Committee of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China. All animals were treated in accordance with the guidelines and regulations for the use and care of animals of the Center for Laboratory Animal Care, China Academy of Chinese Medical Sciences.

Experimental transient focal cerebral ischemia–reperfusion model

Adult male Sprague–Dawley rats, weighing 250–280 grams (specific pathogen-free, certificate no. SCXK [JUN] 2007-004), were used in the study. The focal cerebral ischemic rat model was induced by MCAO, as previously reported.²⁴ Briefly, the rats were anesthetized with 350 mg/kg chloral hydrate, and rectal temperature was maintained at 37.0° by placing the animals on a heating bed. The right carotid bifurcation was exposed, and the external carotid artery was coagulated distal to the bifurcation. A nylon wire with a globose head was then inserted through the stump of the external cerebral artery and gently advanced (about 18 mm) to occlude the middle cerebral artery (MCA). After

90 min of occlusion, the nylon wire was gently withdrawn, and the incision was closed. The rats in the sham-operated group underwent the same procedure, but no arteries were occluded.

Experimental groups and mortality

In total, 120 rats were used; 20 animals were sham controls, and 100 rats underwent MCAO. Within 90 min of occlusion to induce ischemia–reperfusion, 22 animals died. There was a peri-infarct mortality rate of 22.0%. The remaining 78 rats were randomized into the following three groups—model and two BYHWD (29 rats in each group). The route of BYHWD delivery was oral administration intra-gastrically using syringe feeding. Treatment was given daily for a period of 21 days from day 1 to day 21 of MCAO. The dosage range of BYHWD treatment in MCAO rats (12 and 24 grams/kg) was based on the results of our previous study (Kong et al., unpublished observations) and according to previous studies.^{16,25}

Determination of neurological defect score and analysis of survival rates

The neurological behavior scores were carefully evaluated at 24 hr after surgery according to Bederson's method.²⁶ In brief, the neurological status was classified as: 0, no motor deficits (normal); 1, forelimb weakness and torso turning to the ipsilateral side when held by tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, unable to bear weight on the affected side at rest (severe); 4, no spontaneous locomotor activity or barrel rolling (critical). The neurological behavior scores were assessed twice a week. Each rat was scored by three examiners who were blinded to the identity of the rat or treatment protocol. Each group was given a survival rate every day until 7 days after MCAO.

Evaluation of infarct volume by 2,3,5-triphenyltetrazolium chloride

At the 7th day after MCAO, the rats were sacrificed by rapid decapitation under deep anesthesia. The whole brain was rapidly removed, sliced into 2-mm-thick coronal sections, and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) for 30 min at 37°C in the dark, followed by fixation with 4% paraformaldehyde (PFA) overnight. Brain slice areas lacking red staining defined the infarct area. The slices were photographed with a digital camera and analyzed by an image-processing system (Image-Pro Plus, Media Cybernetics). To correct for swelling due to cerebral edema, the infarct volume was normalized and expressed as a percentage of the ipsilateral hemispheric volume (%).

5-Bromo-2-deoxyuridine incorporation

To examine cell proliferation, we used the thymidine analog 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich), which is incorporated into DNA during the S phase of the cell cycle and remains in the DNA even when the cell has exited the active phases of the cell cycle. The rats were injected with 50 mg/kg of BrdU on the initial 3 consecutive days and 5–7 and 19–21 days after ischemia according to a previously reported protocol.²⁷

Immunohistochemistry staining

For histological analysis, animals were anesthetized as above and perfused transcardially with 4% PFA in phosphate buffer (PB). Brains were post-fixed with 4% PFA for 2 days, washed in PB, and cryoprotected in a 30% sucrose solution. The brains were embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA) and cut into 16- μ m-thick coronal sections mounted on the glass slides. The sections were stained according to the standard immunohistochemistry/immunofluorescence procedure in 0.1 M PB for 48 hr.^{28,29} The following antigens were stained: Stromal cell-derived factor (SDF)-1 (1:50; Santa Cruz Biotechnology Inc., Dallas, TX), CXC chemokine receptor (CXCR) 4 (1:100; Santa Cruz Biotechnology Inc.), vascular endothelial growth factor (VEGF) (1:100; Santa Cruz Biotechnology Inc.), Reelin (1:50; Santa Cruz Biotechnology Inc.), glial fibrillary acidic protein (GFAP) (1:100; Santa Cruz Biotechnology Inc.), microtubule-associated protein 2 (MAP-2) (1:150; Cell Signaling Technology, Beverly, MA) doublecortin (DCX) (1:100; Santa Cruz Biotechnology Inc.), and BrdU (1:100; Abcam, Cambridge, MA). Sections were incubated with primary antibodies overnight at 4°C in phosphate-buffered saline (PBS) with 5% donkey or goat serum and 0.5% Triton X-100. For immunohistochemical staining, the sections were carried out with commercial EnVision™ detection kits (DAKO, Denmark) according to the manufacturer's instructions. For immunofluorescence, the sections were exposed for 2 hr at room temperature to secondary species-specific antibodies conjugated with Alexa Fluor 488 (1:400; Molecular Probes Inc., USA) or Alexa Fluor 568 (1:400; Molecular Probes Inc., USA). For the negative control staining, PBS was used instead of the primary antibody.

For immunohistochemistry, the number of positive cells or staining intensity in the peri-ischemic zone was measured at a magnification of 200 $\times$. In all of the slices, five fields per sample and three to six tissues were quantified in each group. For SDF-1, CXCR4, and reelin, expression was analyzed using integral optical density (IOD) with Image-Pro Plus software. For BrdU and VEGF, positive cells were counted in five different fields per rat by an observer blind to the experimental treatment, and the quantitation was performed by calculating the positive cells per mm².

Confocal microscopy

Immunofluorescence was visualized using a Leica SP2 scanning confocal microscope (Leica Microsystems) and a 636 objective. Each fluorochrome was recorded individually in sequential scan mode to avoid channel mixing. For BrdU/DCX, BrdU/GFAP, and BrdU/MAP-2 quantifications, cells were selected at definite zones of the brain and analyzed for double immunoreactivity. Double labeling was assumed when cells exhibited direct co-localization of nucleus and cytosol from the same cell were individually labeled. Coronal sections were used for all quantifications. For double labeling, the average numbers of BrdU/DCX, BrdU/GFAP, and BrdU/MAP-2 positive cells were normalized by per mm².

Enzyme-linked immunosorbent assay

At 7th and 21st day after MCAO, the rats were sacrificed by rapid decapitation under deep anesthesia. The whole

brain was removed rapidly, frozen in liquid nitrogen, and placed at -80°C. Total protein of the ischemic side of brain tissues was extracted following the instructions of the Bradford Kit. The expression levels of SDF-1, CXCR4, VEGF, and BDNF, were detected by an enzyme-linked immunosorbent assay (ELISA; R&D Systems, USA) according to the manufacturer's protocol, and absorbance was measured at 450 nm.

Western blot

The expression of SDF-1 and CXCR4 in ischemic tissues was determined by western blotting. Protein concentration was quantified using a bicinchoninic acid (BCA) protein quantity assay kit before 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under 4°C. The protein was transferred onto microporous polyvinylidene fluoride (PVDF) membranes in running buffer containing 20% methanol. After non-specific sites were blocked with 5% milk/Tris-buffered saline/Tween 20 (TBST), the membranes were incubated with anti-SDF-1 antibody (1:100; Santa Cruz Biotechnology Inc.), anti-CXCR4 antibody (1:100; Santa Cruz Biotechnology Inc.), and anti-GAPDH antibody (1:200; Santa Cruz Biotechnology Inc.) overnight, respectively. Then, a horseradish peroxidase (HRP)-linked antibody was employed as a secondary antibody after the membranes were washed in TBST. Densities of the bands were measured using an IBAS ReL2.0 image analyzer (Option Company, Belgium). The relative intensities of bands of SDF-1 and CXCR4 were quantified and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Each experiment was repeated at least three times, and there were six animals per group in each independent experiment. Data were represented in form of means $\pm$ standard deviation (SD). All of the data were analyzed using the SPSS statistical package (v. 13.0). Neurological defect scores were analyzed by a non-parametric Kruskal-Wallis test. Survival analyses were made by using the Kaplan-Meier method; the difference of survival rate between two groups was analyzed by the log-rank test. Mean values were compared through one-way analysis of variance (ANOVA), ANOVA, and multi-comparison. Data were tested through a homogeneity test for variance. If the variances were homogeneous, mean values were compared through ANOVA. The differences between two groups were analyzed, based on the test of least significance difference (LSD). If the variances were not homogeneous, mean values were compared using the Welch test. The differences between two groups were analyzed by the Games-Howell test. A value of $p < 0.05$ was considered as statistically significant.

Results

BYHWD increases survival rate and decreases neurological deficit score and infarction volume in MCAO rats

All animals in this study showed similar physiological values (*i.e.*, rectal temperature and mean arterial blood pressure) before, during, and after MCAO among the groups (data not shown). Most of the rats (60%) died within 7 days

after MCAO induction when treated with vehicle (distilled water, model group). However, those rats treated with BYHWD (12 and 24 grams/kg) showed an enhanced survival rate as compared to model group, with BYHWD (24 grams/kg) being the most effective ($p < 0.05$; Fig. 1A).

Prior to MCAO, scores of focal neurological function and general neurological function of rats were zero, similar to sham-treated rats. Cerebral ischemia for 90 min, followed by reperfusion, resulted in a significant neurological deficit and extensive cerebral infarct volume (Fig. 1B–D). At days 1 and 3, there was no significant difference in the neurological deficit scores among the MCAO group and the two BYHWD-treated groups (data not shown); however, rats in BYHWD-treated groups had much lower neurological scores at days 7–21 ($p < 0.05$ or $p < 0.01$, respectively; Fig. 1B) and reduced infarct volume than MCAO group ($p < 0.05$ or $p < 0.01$, respectively; Fig. 1D).

BYHWD enhances NPCs proliferation in MCAO rats

Because proliferation is the promise for NPCs in brain repair, we first investigated the effect of BYHWD on the

proliferation of NPCs by detecting the BrdU stained cells after transient focal cerebral ischemia. The numbers of BrdU⁺ cells in the SGZ and SVZ of the model group were significantly increased compared with that of sham group ($p < 0.05$ and $p < 0.01$; Fig. 2), suggesting that ischemia itself could stimulate the proliferation of NPCs. However, numbers of BrdU⁺ cells in the SGZ and SVZ were slightly increased at a dose of 12 grams/kg BYHWD and remarkably increased at a dose of 24 grams/kg BYHWD group ($p < 0.05$; Fig. 2). Moreover we tested the effects of BYHWD on NPCs proliferation in the corpus striatum (CS), where neurogenesis is relatively quiescent in adult animals and humans. The numbers of BrdU⁺ cells were evidently increased in MCAO rats compared with sham-treated rats ($p < 0.01$; Fig. 2). As for the two BYHWD groups, numbers of BrdU⁺ cells in the CS were extremely increased compared with model group, reaching nearly 20-fold that of the sham group ($p < 0.01$; Fig. 2). Although gradually declining over time in all groups, the numbers of BrdU⁺ cells were always higher in BYHWD-treated rats than in the sham or model groups. No BrdU⁺ cells were found in the contralateral striatum (data not shown).

FIG. 1. Effect of Buyang Huanwu Decoction (BYHWD) on survival rate, neurological deficit score, and infarction volume in middle cerebral artery occlusion (MCAO) rats. The survival rate of MCAO rats, BYHWD-treated rats after stroke were calculated over 7 days ($n = 18$ for each group) (A). BYHWD administered at different doses (12–24 grams/kg) significantly decreased neurological deficit scores, whereas the scores of neurological function were zero in sham-treated rats. High scores represent severe injury (B). Six brain coronal sections, 2 mm thick, were selected for 2,3,5-triphenyltetrazolium chloride (TTC) staining (C). The infarct volume was quantified as a percentage of total volume, with large infarcts representing more severe injury. The percent of infarct volume was significantly less in BYHWD-treated groups compared with model group (D). (*) $p < 0.05$, (**) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n = 6$). Color images available online at www.liebertpub.com/rej

FIG. 2. Buyang Huanwu Decoction (BYHWD) promotes neural precursor cell (NPC) proliferation in the ischemia ipsilateral hemisphere after middle cerebral artery occlusion (MCAO). Cell proliferation was assessed by 5-bromo-2-deoxyuridine (BrdU) labeling. Representative microphotographs showed BrdU⁺ cells in the subventricular zone (SVZ), subgranular zone (SGZ), and corpus striatum (CS) of sham, model, and BYHWD-treated groups at day 7 (A). Quantitation of BrdU-positive cells showed that BYHWD significantly increased the number of BrdU⁺ cells in the SVZ, SGZ, and CS at days 7 and 21 (B–D). ([#]) $p < 0.05$, (^{##}) $p < 0.01$, versus the sham group. (^{*}) $p < 0.05$, (^{**}) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n = 6$). Color images available online at www.liebertpub.com/rej

BYHWD promotes NPCs migration in MCAO rats

To determine the migration of NPCs, immunohistochemical double labeling of BrdU and DCX proteins was used. The numbers of BrdU⁺/DCX⁺ cells were increased slightly in the model group compared with the sham group from day 7 until day 21 after MCAO, especially in SVZ, where the quiescent NPCs originate then migrate into the olfactory bulb via the rostral migratory stream (RMS) under physiological conditions in adults (Fig. 3A, B). More interestingly, compared with the model group, the numbers of BrdU⁺/DCX⁺ cells were significantly increased in BYHWD-treated groups with a dose-dependent tendency in all three observed brain zones ($p < 0.05$ or $p < 0.01$ in BYHWD 12 grams/kg; all $p < 0.01$ in BYHWD 24 grams/kg) (Fig. 3). The increase of BrdU⁺/DCX⁺ cells at day 7 after ischemia was more obvious than at day 21 after ischemia in the above three brain zones. On the contrary, there were relatively few

BrdU⁺/DCX⁺ cells in the contralateral SVZ and SGZ, and no BrdU⁺/DCX⁺ cells were seen in the contralateral CS (data not shown).

BYHWD up-regulates the pro-migration factors in MCAO rats

To determine what was responsible for the promotional effect of BYHWD on NPCs, we investigated the expression of SDF-1 and CXCR4, the most important pro-migration factors, in the penumbral areas of the focal cerebral ischemic rats. As shown in Figure 4, A and D, there were few staining of SDF-1 and its receptor CXCR4-positive signals in the brain sections of sham-treated rats. The signals were slightly increased in the vehicle-treated MCAO rats, although without statistic significance. However, SDF-1- and CXCR4-positive signals were remarkably increased in the CS of the 24 grams/kg BYHWD-treated group ($p < 0.05$;

FIG. 3. Buyang Huanwu Decoction (BYHWD) promotes neural precursor cells (NPCs) migration in the ipsilateral infarct area after middle cerebral artery occlusion (MCAO). Cell migration was assessed by representative confocal microscopic images showed doublecortin (DCX; red) and 5-bromo-2-deoxyuridine (BrdU; green) double-labeled. Representative microphotographs showed BrdU⁺/DCX⁺ cells in the subventricular zone (SVZ), subgranular zone (SGZ), and corpus striatum (CS) of sham, model, and BYHWD-treated groups at day 7 (A). Quantitation of BrdU⁺/DCX⁺ cells in the SVZ, SGZ, and CS at days 7 and 21 (B–D). ([#]) $p < 0.05$, (^{##}) $p < 0.01$, versus the sham group. (^{*}) $p < 0.05$, (^{**}) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n = 6$). Color images available online at www.liebertpub.com/rej

Fig. 4B, E). Moreover, we detected SDF-1 and CXCR4 levels by the ELISA method, and the results confirmed the same trend with immunohistochemical staining (Fig. 4C, F). Furthermore, western blot analysis was used to determine the amounts of SDF-1 and CXCR4 in the homogenate of the ischemic hemisphere, and the results were consistent with above two methods (Fig. 4G, H).

In addition, we investigated the expression of VEGF and Reelin, the two factors closely related to migration of NPCs, in the penumbral areas of the focal cerebral ischemic rats by immunohistochemical staining. Figure 5, A and D, shows the representative immunochemical staining of VEGF-positive cells and Reelin-positive cells in the brain sections. Compared to the sham group, positive staining signals of VEGF and Reelin in the MCAO rats were increased without statistical difference. BYHWD strongly raised the expression of VEGF and Reelin in CS of MCAO rats in a dose-dependent manner ($p < 0.05$ or $p < 0.01$ in BYHWD 12 grams/kg, all $p < 0.01$ in BYHWD 24 grams/kg, Fig. 5B, E). The ELISA results for VEGF showed the same trend in the lysate of the ischemic brain (Fig. 5C). In addition, the BDNF level in a homogenate of the ischemic hemisphere tested by ELISA also increased significantly in the BYHWD-treated group (Fig. 5F).

BYHWD stimulates NPCs neuronal differentiation in MCAO rats

Enhanced NPCs migrating to the ischemic zone of the brain may differentiate into the niche functional cells to facilitate repair, so we next observed the effect of BYHWD on NPCs differentiation. Differentiation analysis of proliferating cells was determined using double immunohistochemical labeling of BrdU and neuronal (MAP-2) or astroglial (GFAP) proteins (Figs. 6 and 7). The staining was performed on days 7 and 21 post-stroke, and the results showed that some of BrdU⁺/MAP-2⁺ and BrdU⁺/GFAP⁺ cells were present in the SVZ and SGZ of all groups and in the ischemic CS of those MCAO rats, with the vast majority of double-positive cells located in the CS.

Numbers of studies have reported that focal ischemia also induces a marked proliferation of astrocytes, reflecting massive gliosis and inflammatory reaction. Therefore, most of the BrdU⁺/GFAP⁺ cells in our experiment indicated gliosis, but only a small part indicated astroglial differentiation from NPCs. Meanwhile, we found that the number of both BrdU⁺/MAP-2⁺ and BrdU⁺/GFAP⁺ cells gradually increased over time in all experimental groups from day 7 to day 21 post-stroke, showing a significantly higher number of

FIG. 4. Buyang Huanwu Decoction (BYHWD) up-regulates expression of SDF-1 and CXCR4 in the ischemic hemisphere after middle cerebral artery occlusion (MCAO). Representative microphotographs showed expression of SDF-1 (A) and CXCR4 (D) from the corpus striatum (CS) of ischemic hemisphere in sham, model, and BYHWD-treated MCAO rats by immunohistochemical staining. Quantitation of SDF-1 (B) and CXCR4 (E) results showed that BYHWD significantly increased the expression of the factors in the CS at days 7 and 21 after MCAO. SDF-1 and CXCR4 levels in the lysate of the ischemic hemisphere were also detected by enzyme-linked and immunosorbent assay (ELISA) (C and F) and western blot analysis (G and H). (*) $p < 0.05$, (**) $p < 0.01$, versus the sham group. (#) $p < 0.05$, (##) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n=6$). Color images available online at www.liebertpub.com/rej

double-positive cells in BYHWD-treated rats in a dose-dependent manner ($p < 0.05$ or $p < 0.01$ in BYHWD 12 grams/kg, all $p < 0.01$ in BYHWD 24 grams/kg; Figs. 6 and 7). Distribution analysis of BrdU⁺/MAP-2⁺ and BrdU⁺/GFAP⁺ cells revealed that most of the cells located in the

ischemic CS reached about five to 10 times those in the SVZ and SGZ of the BYHWD-treated groups, which is different from the distribution of BrdU⁺/DCX⁺ cells. No co-localization of BrdU⁺ with MAP-2⁺ cells was found in SVZ at any time point analyzed (data not shown).

FIG. 5. Buyang Huanwu Decoction (BYHWD) increases expression of vascular endothelial growth factor (VEGF), Reelin, and brain-derived neurotrophic factor (BDNF) in the ischemic hemisphere after middle cerebral artery occlusion (MCAO). Representative microphotographs showed expression of VEGF (**A**) and Reelin (**D**) from the corpus striatum (CS) of the ischemic hemisphere in sham, model, and BYHWD-treated MCAO rats by immunohistochemical staining. Quantitation of VEGF (**B**) and Reelin (**E**) results showed that BYHWD significantly increased the expression of the factors in the CS at days 7 and 21 after MCAO. VEGF and BDNF levels in the lysate of the ischemic hemisphere were also detected by enzyme-linked immunosorbent assay (ELISA) (**C** and **F**). ([#]) $p < 0.05$, (^{##}) $p < 0.01$, versus the sham group. (^{*}) $p < 0.05$, (^{**}) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n = 6$). Color images available online at www.liebertpub.com/rej

Discussion

BYHWD, as a well-known traditional Chinese medicine formula, made up of seven types of Chinese medicines, has been widely used for therapy of ischemic cerebral disease in China. But the mechanism of its neuroprotection is not clear. Previous reports indicated that the neuroprotective effects of BYHWD may be associated with stimulation of neurogenesis,^{21,22,30–32} inhibition of apoptosis,^{17,20} and activation of blood circulation.^{19,33} Cai and Tong have reported that BYHWD could promote proliferation of the NPCs at the hippocampus and SVZ in the ischemic brains.^{22,31} Other research performed *in vitro* has suggested that BYHWD may promote

growth and differentiation of cultured NPCs.^{21,34} Most of the reports focus mainly on the proliferation and differentiation of NPCs. To further clarify the mechanisms of BYHWD action on stroke, we have demonstrated herein the role of BYHWD on migration of NPCs in MCAO rats. The main findings of our study are: (1) Besides promotion of NPCs proliferation and differentiation, BYHWD may enhance migration of NPCs to damaged areas of the brain to facilitate repair. (2) The promotional effect of BYHWD on NPCs migration may be achieved by up-regulating the pro-migration factors, such as SDF-1–CXCR4 signaling, Reelin, and VEGF in MCAO rats. These findings suggest that BYHWD may exert neuroprotective activity in MCAO rats,

FIG. 6. Buyang Huanwu Decoction (BYHWD) promotes neuronal precursor cells (NPCs) neuronal differentiation in the ipsilateral infarct area after middle cerebral artery occlusion (MCAO). Representative microphotographs showed colocalization of BrdU and MAP-2 cells in the subgranular zone (SGZ) and corpus striatum (CS) of ischemic hemisphere in sham, model, and BYHWD-treated MCAO rats by immunohistochemical staining. Quantitation results showed that BYHWD significantly increased BrdU⁺/MAP-2⁺ cells in the SGZ and CS at days 7 and 21 after MCAO. (#) $p < 0.05$, (##) $p < 0.01$, versus the sham group. (*) $p < 0.05$, (**) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n = 6$). Color images available online at www.liebertpub.com/rej

and its action may be partially caused by promoting migration of NPCs.

Acute ischemic stroke is caused by cerebral artery occlusion through the loss or the reduction of cerebral blood flow, leading to an infarction of brain tissue. Although clinical therapies targeting the molecular elements leading to cell death have been identified, current therapeutics are inefficient. Therefore, other methods, such as stem cell therapy, for stroke injury are being pursued avidly by researchers. Recent experimental findings raise the possibility that functional improvement after stroke may be achieved through neural replacement by eNPCs residing in the adult brain. In this study, our results showed the increased number of BrdU-positive NPCs in SVZ, SGZ, and CS during the first week after focal ischemic injury; however, the number declined within the period we observed (3 weeks), which is in line with the data of previous studies.^{35–38} Interestingly, we found that newly generated NPCs moved to the damaged area, such as the hippocampus, striatum, neocortex, and other damaged regions of the CNS, where some of these cells were double positive for a proliferation marker (BrdU) and migrating markers (DCX) 1–3 weeks after the injury. However, the number of eNPCs was extremely low and the survival of these new neurons generated from NPCs in the lesioned area was minimal in our study, which is similar to previous reports of about 0.2% of the lost striatal cells.^{39,40} Therefore, modulating eNPCs for brain repair is a critical issue facing stem cell therapy. The premise of the endogenous repair strategy is to take advantage of the inherent properties of NPCs to proliferate, migrate to the site of the

injury, and differentiate into mature cells in the injured brain. However, most research has focused primarily on the proliferation and differentiation of the NPCs. For endogenous NPCs to contribute to recovery, it is necessary for NPCs to migrate from the SVZ through the brain parenchyma into the peri-infarct region and further, and this must occur in sufficient numbers to generate sufficient numbers of cells. The data presented in our experiments showed that there were increased BrdU⁺/DCX⁺ cells in the ischemic sites of BYHWD-treated rats, suggesting the promotional effect on NPCs migration.

NPCs migration is an essential process for neurogenesis that occurs in the adult CNS,⁴¹ especially in ischemic injury of the brain. Following injury in the CNS, NPCs preferentially migrate to damaged areas of the brain to facilitate repair, indicating that agents present in the damaged tissue guide the migration of precursor cells.^{42–44} Chemokines, such as SDF-1 α , are important mediators of this process through the SDF-1–CXCR4 signaling pathway.^{45,46} Endothelial cells and perivascular astrocytes within damaged tissue have been proposed as the sources of SDF-1. A growing body of evidence has shown that endogenous NPCs express CXCR4 and depend on niche-generated SDF-1. More importantly, these CXCR4-expressing NPCs can be recruited from the stem cell niche to damaged tissue through long-distance migration. SDF-1 also serves to maintain embryonic and adult NPCs.^{47,48} It seems that the first two steps in NPC-based tissue repair (activation and migration) are under partial control of the SDF-1–CXCR4 signaling pathway. Because previous research has reported that

FIG. 7. Buyang Huanwu Decoction (BYHWD) increases the number of BrdU⁺/GFAP⁺ cells in the ipsilateral infarct area after middle cerebral artery occlusion (MCAO). Representative microphotographs showed co-localization of 5-bromo-2-deoxyuridine (BrdU) and glial fibrillary acidic protein (GFAP) cells in the subventricular zone (SVZ), subgranular zone (SGZ), and corpus striatum (CS) of ischemic hemisphere in sham, model, and BYHWD-treated MCAO rats by immunohistochemical staining. Quantitation results showed that BYHWD significantly increased BrdU⁺/GFAP⁺ cells in the SVZ, SGZ, and CS at days 7 and 21 after MCAO. ([#]) $p < 0.05$, (^{##}) $p < 0.01$, versus the sham group. (^{*}) $p < 0.05$, (^{**}) $p < 0.01$, versus the model group. Data represent means $\pm$ standard deviation (SD) ($n = 6$). Color images available online at www.liebertpub.com/rej

BYHWD may potentially promote neurogenesis in brain ischemic model, we hypothesized that BYHWD could play a role in the SDF-1–CXCR4 system. Consistent with this hypothesis, we found that BYHWD markedly enhanced NPCs migration to the infarct areas and significantly increased the expression of SDF-1 and CXCR4 in the ischemic hemisphere with a dose-dependent tendency, which is confirmed by ELISA, immunohistochemistry, and western blot analysis.

Additionally, our data showed that BYHWD could increase the levels of VEGF, Reelin, and BDNF near the injury areas in MCAO rats. VEGF and BDNF secreted from NPCs, microglia, astrocytes, and vascular endothelial cells have emerged as multifunctional molecules that are involved in regulation of proliferation, migration, differentiation, survival, and synaptic plasticity, and can influence complex processes in the adult brain.^{49–52} Reelin controls the migration and laminar arrangement of neurons in various structures, including the neocortex, hippocampus, and cerebellum.^{53,54} The above results have suggested the promotional effect on NPCs migration by BYHWD partly coming from the elevated levels of those factors. The latest reports⁵⁵ have demonstrated that injury-induced migration of neuroblasts

shares similarities with the constitutive migration of neuronal precursors in the RMS with regard to vasculature association and involvement of BDNF in the initiation of the migratory phase; however, the precise effect by BYHWD on the surrounding environment and cells needs further study.

In conclusion, our results reveal for the first time that the neuroprotective effect of BYHWD in MCAO rats may depend on modulation of NPCs migration by up-regulating pro-migration factors. These findings provide a novel insight into the role of BYHWD, a famous traditional Chinese medicine formula, in stroke pathogenesis and suggest that it might be an attractive and suitable therapeutic agent for treating this disease.

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Author Disclosure Statement

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References

- Gage FH. Mammalian neural stem cells. *Science* 2000;287:1433–1438.
- Ma DK, Bonaguidi MA, Ming GL, Song H. Adult neural stem cells in the mammalian central nervous system. *Cell Res* 2009;19:672–682.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 2002;110:429–441.
- Ara J, De Montpellier S. Hypoxic-preconditioning enhances the regenerative capacity of neural stem/progenitors in subventricular zone of newborn piglet brain. *Stem Cell Res* 2013;11:669–686.
- Zhuang P, Zhang Y, Cui G, Bian Y, Zhang M, Zhang J, Liu Y, Yang X, Isaiah AO, Lin Y, Jiang Y. Direct stimulation of adult neural stem/progenitor cells in vitro and neurogenesis in vivo by salvianolic acid B. *PLoS One* 2012;7:e35636.
- Sejstred Y, Hildrestrand GA, Kunke D, Rolseth V, Kroeide SZ, Neurauder CG, Suganthan R, Atneosen-Asegg M, Fleming AM, Saugstad OD, Burrows CJ, Luna L, Bjoras M. Endonuclease VIII-like 3 (Neil3) DNA glycosylase promotes neurogenesis induced by hypoxia-ischemia. *Proc Natl Acad Sci USA* 2011;108:18802–18807.
- Osman AM, Porritt MJ, Nilsson M, Kuhn HG. Long-term stimulation of neural progenitor cell migration after cortical ischemia in mice. *Stroke* 2011;42:3559–3565.
- Nakano-Doi A, Nakagomi T, Fujikawa M, Nakagomi N, Kubo S, Lu S, Yoshikawa H, Soma T, Taguchi A, Matsuyama T. Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. *Stem Cells* 2010;28:1292–1302.
- Kim JH, Lee JE, Kim SU, Cho KG. Stereological analysis on migration of human neural stem cells in the brain of rats bearing glioma. *Neurosurgery* 2010;66:333–342; discussion 342.
- Okano H, Sawamoto K. Neural stem cells: Involvement in adult neurogenesis and CNS repair. *Philos Trans R Soc Lond B Biol Sci* 2008;363:2111–2122.
- Watson DJ, Walton RM, Magnitsky SG, Bulte JW, Poptani H, Wolfe JH. Structure-specific patterns of neural stem cell engraftment after transplantation in the adult mouse brain. *Hum Gene Ther* 2006;17:693–704.
- Schmidt NO, Koeder D, Messing M, Mueller FJ, Aboody KS, Kim SU, Black PM, Carroll RS, Westphal M, Lamszus K. Vascular endothelial growth factor-stimulated cerebral microvascular endothelial cells mediate the recruitment of neural stem cells to the neurovascular niche. *Brain Res* 2009;1268:24–37.
- Yip S, Shah K. Stem-cell based therapies for brain tumors. *Curr Opin Mol Ther* 2008;10:334–342.
- Hao CZ, Wu F, Shen J, Lu L, Fu DL, Liao WJ, Zheng GQ. Clinical efficacy and safety of buyang huanwu decoction for acute ischemic stroke: A systematic review and meta-analysis of 19 randomized controlled trials. *Evid Based Complement Alternat Med* 2012;2012:630124.
- Wang L, Jiang DM. Neuroprotective effect of Buyang Huanwu Decoction on spinal ischemia/reperfusion injury in rats. *J Ethnopharmacol* 2009;124:219–223.
- Liu Y, Lin R, Shi X, Fang Z, Wang W, Lin Q, Zhang J, Zhang H, Ji Q. The roles of buyang huanwu decoction in anti-inflammation, antioxidation and regulation of lipid metabolism in rats with myocardial ischemia. *Evid Based Complement Alternat Med* 2011;2011:561396.
- Fan L, Wang K, Cheng B. Effects of buyang huanwu decoction on apoptosis of nervous cells and expressions of Bcl-2 and bax in the spinal cord of ischemia-reperfusion injury in rabbits. *J Tradit Chin Med* 2006;26:153–156.
- Yang S, Gao Q, Xing S, Feng X, Peng L, Dong H, Bao L, Zhang J, Hu Y, Li G, Song T, Li Z, Sun J. Neuroprotective effects of Buyang Huanwu decoction against hydrogen peroxide induced oxidative injury in Schwann cells. *J Ethnopharmacol* 2011;137:1095–1101.
- Wang WR, Lin R, Zhang H, Lin QQ, Yang LN, Zhang KF, Ren F. The effects of Buyang Huanwu Decoction on hemorheological disorders and energy metabolism in rats with coronary heart disease. *J Ethnopharmacol* 2011;137:214–220.
- Li XM, Bai XC, Qin LN, Huang H, Xiao ZJ, Gao TM. Neuroprotective effects of Buyang Huanwu Decoction on neuronal injury in hippocampus after transient forebrain ischemia in rats. *Neurosci Lett* 2003;346:29–32.
- Sun J, Bi Y, Guo L, Qi X, Zhang J, Li G, Tian G, Ren F, Li Z. Buyang Huanwu Decoction promotes growth and differentiation of neural progenitor cells: Using a serum pharmacological method. *J Ethnopharmacol* 2007;113:199–203.
- Cai G, Liu B, Liu W, Tan X, Rong J, Chen X, Tong L, Shen J. Buyang Huanwu Decoction can improve recovery of neurological function, reduce infarction volume, stimulate neural proliferation and modulate VEGF and Flk1 expressions in transient focal cerebral ischaemic rat brains. *J Ethnopharmacol* 2007;113:292–299.
- Pharmacopoeia Commission of the Ministry of Health of the People's Republic of China. *Pharmacopoeia of the People's Republic of China*. 2010;1.
- Yu X, Chen D, Zhang Y, Wu X, Huang Z, Zhou H, Zhang Z. Overexpression of CXCR4 in mesenchymal stem cells promotes migration, neuroprotection and angiogenesis in a rat model of stroke. *J Neurol Sci* 2012;316:141–149.
- Yang G, Fang Z, Liu Y, Zhang H, Shi X, Ji Q, Lin Q, Lin R. Protective effects of chinese traditional medicine buyang huanwu decoction on myocardial injury. *Evid Based Complement Alternat Med* 2011;2011:930324.
- Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurologic examination. *Stroke* 1986;17:472–476.
- Liu J, Solway K, Messing RO, Sharp FR. Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J Neurosci* 1998;18:7768–7778.
- Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, Mori T, Gotz M. Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. *Proc Natl Acad Sci USA* 2008;105:3581–3586.
- Boda E, Vigano F, Rosa P, Fumagalli M, Labat-Gest V, Tempia F, Abbracchio MP, Dimou L, Buffo A. The GPR17 receptor in NG2 expressing cells: Focus on in vivo cell maturation and participation in acute trauma and chronic damage. *Glia* 2011;59:1958–1973.
- Wang HW, Liou KT, Wang YH, Lu CK, Lin YL, Lee IJ, Huang ST, Tsai YH, Cheng YC, Lin HJ, Shen YC. Deciphering the neuroprotective mechanisms of Bu-yang Huan-wu decoction by an integrative neurofunctional and genomic approach in ischemic stroke mice. *J Ethnopharmacol* 2011;138:22–33.

31. Tong L, Tan XH, Shen JG. [Comparative study of Buyang Huanwu Decoction and the different combinations of its ingredients on neurogenesis following ischemic stroke in rats]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2007;27:519–522.
32. Cheng YS, Cheng WC, Yao CH, Hsieh CL, Lin JG, Lai TY, Lin CC, Tsai CC. Effects of buyang huanwu decoction on peripheral nerve regeneration using silicone rubber chambers. *Am J Chin Med* 2001;29:423–432.
33. Zhao LD, Wang JH, Jin GR, Zhao Y, Zhang HJ. Neuroprotective effect of Buyang Huanwu decoction against focal cerebral ischemia/reperfusion injury in rats—time window and mechanism. *J Ethnopharmacol* 2012;140:339–344.
34. Sun JH, Gao YM, Yang L, Wang X, Bao LH, Liu WJ, Yew D. Effects of Buyang Huanwu Decoction on neurite outgrowth and differentiation of neuroepithelial stem cells. *Chin J Physiol* 2007;50:151–156.
35. Kernie SG, Parent JM. Forebrain neurogenesis after focal Ischemic and traumatic brain injury. *Neurobiol Dis* 2010;37:267–274.
36. Kokaia Z, Lindvall O. Neurogenesis after ischaemic brain insults. *Curr Opin Neurobiol* 2003;13:127–132.
37. Kuge A, Takemura S, Kokubo Y, Sato S, Goto K, Kayama T. Temporal profile of neurogenesis in the subventricular zone, dentate gyrus and cerebral cortex following transient focal cerebral ischemia. *Neurol Res* 2009;31:969–976.
38. Zhang RL, Zhang ZG, Zhang L, Chopp M. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience* 2001;105:33–41.
39. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 2002;8:963–970.
40. Lichtenwalner RJ, Parent JM. Adult neurogenesis and the ischemic forebrain. *J Cereb Blood Flow Metab* 2006;26:1–20.
41. Gage FH. Neurogenesis in the adult brain. *J Neurosci* 2002;22:612–613.
42. Takeuchi H, Natsume A, Wakabayashi T, Aoshima C, Shimato S, Ito M, Ishii J, Maeda Y, Hara M, Kim SU, Yoshida J. Intravenously transplanted human neural stem cells migrate to the injured spinal cord in adult mice in an SDF-1- and HGF-dependent manner. *Neurosci Lett* 2007;426:69–74.
43. Ke Y, Chi L, Xu R, Luo C, Gozal D, Liu R. Early response of endogenous adult neural progenitor cells to acute spinal cord injury in mice. *Stem Cells* 2006;24:1011–1019.
44. Belmadani A, Tran PB, Ren D, Miller RJ. Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *J Neurosci* 2006;26:3182–3191.
45. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* 2004;10:858–864.
46. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, Snyder EY, Khoury SJ. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci USA* 2004;101:18117–18122.
47. Li M, Chang CJ, Lathia JD, Wang L, Pacenta HL, Cotleur A, Ransohoff RM. Chemokine receptor CXCR4 signaling modulates the growth factor-induced cell cycle of self-renewing and multipotent neural progenitor cells. *Glia* 2011;59:108–118.
48. Kokovay E, Goderie S, Wang Y, Lotz S, Lin G, Sun Y, Roysam B, Shen Q, Temple S. Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. *Cell Stem Cell* 2010;7:163–173.
49. Waterhouse EG, An JJ, Orefice LL, Baydyuk M, Liao GY, Zheng K, Lu B, Xu B. BDNF promotes differentiation and maturation of adult-born neurons through GABAergic transmission. *J Neurosci* 2012;32:14318–14330.
50. Petridis AK, El Maarouf A. Brain-derived neurotrophic factor levels influence the balance of migration and differentiation of subventricular zone cells, but not guidance to the olfactory bulb. *J Clin Neurosci* 2011;18:265–270.
51. Calvo CF, Fontaine RH, Soueid J, Tammela T, Mäkinen T, Alfaro-Cervello C, Bonnaud F, Miguez A, Benhaim L, Xu Y, Barallobre MJ, Moutkine I, Lyytikka J, Tatlisumak T, Pytowski B, Zalc B, Richardson W, Kessaris N, Garcia-Verdugo JM, Alitalo K, Eichmann A, Thomas JL. Vascular endothelial growth factor receptor 3 directly regulates murine neurogenesis. *Genes Dev* 2011;25:831–844.
52. Thau-Zuchman O, Shohami E, Alexandrovich AG, Leker RR. Vascular endothelial growth factor increases neurogenesis after traumatic brain injury. *J Cereb Blood Flow Metab* 2010;30:1008–1016.
53. Forster E, Zhao S, Frotscher M. Laminating the hippocampus. *Nat Rev Neurosci* 2006;7:259–267.
54. Forster E, Bock HH, Herz J, Chai X, Frotscher M, Zhao S. Emerging topics in Reelin function. *Eur J Neurosci* 2010;31:1511–1518.
55. Grade S, Weng YC, Snayyan M, Kriz J, Malva JO, Saghatelyan A. Brain-derived neurotrophic factor promotes vasculature-associated migration of neuronal precursors toward the ischemic striatum. *PLoS One* 2013;8:e55039.

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