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Original Article

Buyang Huanwu Decoction (补阳还五汤) Attenuates Glial Scar by Downregulating Expression of Leukemia Inhibitory Factor in Intracerebral Hemorrhagic Rats*

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ABSTRACT **Objective:** To evaluate the effect of Buyang Huanwu Decoction (补阳还五汤, BYHWD) on glial scar after intracerebral hemorrhage (ICH) and investigate the underlying mechanism. **Methods:** Collagenase type Ⅶ (0.5 U) was injected stereotaxically into right globus pallidus to induce ICH model. One hundred and twenty Sprague-Dawley rats were randomly divided into 3 groups according to a random number table, including normal group (n=40), ICH model group (n=40) and BYHWD group (n=40), respectively. After ICH, the rats in the BYHWD group were intragastrically administered with BYHWD (4.36 g/kg) once a day for 21 days, while the rats in ICH group were administered with equal volume of distilled water for 21 days, respectively. Double immunolabeling was performed for proliferating cell nuclear antigen (PCNA)$^+$/glial fibrillary acidic protein (GFAP)$^+$ nuclei. The expression of GFAP and leukemia inhibitory factor (LIF) was evaluated by immunohistochemistry and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). **Results:** The astrocytes with hypertrophied morphology around the hematoma was observed on day 3 after ICH. The number of GFAP positive cells and GFAP mRNA levels increased notably on day 3 and reached the peak on day 14 post-ICH (P<0.01). PCNA$^+$/GFAP$^+$ nuclei were observed around the hematoma and reached the peak on day 14 post-ICH (P<0.01). In addition, LIF-positive astrocytes and LIF mRNA level in the hemorrhagic region increased significantly till day 14 post-ICH (P<0.01). However, BYHWD not only reduced the number of PCNA$^+$/GFAP$^+$ nuclei, but also decreased GFAP and LIF levels (P<0.05). **Conclusion:** BYHWD could attenuate ICH-induced glial scar by downregulating the expression of LIF in the rats.

KEYWORDS Buyang Huanwu Decoction, intracerebral hemorrhage, glial scar, leukemia inhibitory factor, Chinese medicine

Intracerebral hemorrhage (ICH) indicates primary parenchymal hemorrhage resulting from cerebrovascular rupture, and is the second most common subtype of stroke. According to the latest epidemiological survey, ICH accounts for over 20% of all strokes in China.[1] Despite the advances in the treatment of ICH, the prognosis of ICH survivors is still poor and effective therapies remain limited.[2] Astrocytes are the most abundant cells in the brain and play an important role in maintaining neuronal homeostasis and the integrity of blood-brain barrier.[3] After ICH, astrocytes undergo important morphological changes such as hyperplasia and hypertrophy, and migrate to the lesion sites to form dense glial scar.[4] Although glial scar is considered to limit the spread of inflammation and toxic elements, it is detrimental for neuronal survival and regeneration.[5]

Leukemia inhibitory factor (LIF) has a broad range of biological activities including the regulation

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of neuronal differentiation.[6] LIF could promote astrocyte proliferation and activation *in vitro* and *in vivo*.[7-9] Recently, the upregulation of LIF induced by ICH was reported to promote glial scar.[10] Buyang Huanwu Decoction (补阳还五汤, BYHWD) is an ancient traditional Chinese formula that has been shown to ameliorate neurological damages of ischemic stroke.[11-13] Recently, increasing evidences have shown that BYHWD exerted neuroprotective effects against hemorrhagic injury.[14] Our previous study demonstrated that BYHWD was involved in angiogenesis in the ICH rats.[15] However, few studies have evaluated the efficacy of BYHWD on glial scar and explored the underlying mechanisms. Therefore, the present study aimed to investigate the effects of BYHWD on glial scar and the expression of LIF after ICH using the rat as the model.

METHODS

Preparation of BYHWD

BYHWD was prepared as described previously.[15] *Radix Astragali*, *Radix Angelicae Sinensis*, *Radix Paeonia Rubra*, *Rhizoma Chuanxiong*, *Semen Persicae*, *Flos Carthami* and *Lumbricus* were mixed at a ratio of 60:6:4.5:3:3:3:3 (dry weight), and extracted according to the methods described in Pharmacopoeia of the People's Republic of China (2015 ed). Briefly, all herbs were chopped, immersed in distilled water for 1 h and boiled for 30 min at 100 ℃. Then drug solution was vacuum-cooled and dried to form a brown powder (yield: 14.3%), which was dissolved in distilled water at a final concentration of 0.132 g/mL.

Animal Model of ICH

A total of 120 Adult male Sprague Dawley rats (specific pathogen-free grade, weight 250–300 g) were purchased from the Animals Laboratory Centre, China Three Gorges University [license No. SCXK (E) 2014-0011], and housed under temperature- and light-controlled laboratory conditions with free access to standard food and water. All procedures were approved by Ethic Committee of Medical Department, China Three Gorges University and conducted in accordance with the National Institutes of Health (NIH Publication No. 85–23, revised 1996). All rats were randomly divided into three groups using a random number table, including normal control group (n=40), ICH model group (n=40) and BYHWD group (n=40), respectively.

ICH model was induced by collagenase injection method as previously described.[16] After anesthesia by intraperitoneal injection of 10% chloral hydrate at a dose of 3.5 mL/kg body weight, rats were positioned in a stereotactic head frame (Stoelting, type 51600, USA), and a cranial burr hole was drilled on the right coronal suture 3.2 mm lateral to the midline. Then, collagenase Ⅶ (0.5 U in 2.5 μL 0.9% sterile saline; Sigma, MO, USA) was injected at a rate of 0.2 μL/min into the right globus pallidus using 5-μL Hamilton syringe according to the following coordinates relative to bregma: 1.4 mm posterior, 5.6 mm ventral, and 3.2 mm lateral. After the injection, the needle was left in the place for additional 10 min to prevent backflow. After the removal of the needle, the burr hole was sealed with bone wax. The normal control group received only a needle insertion. Two hours after surgery, the rats were intragastrically administered with BYHWD (4.36 g/kg) once a day.[15] The other rats were administered with equal volume of distilled water. The drug administration was lasting for 21 days.

Specimen Preparation

Rats were anesthetized with 10% chloral hydrate and perfused transcardially with 0.9% sterile saline by 250 mL ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer solution (PBS, pH 7.4). Brains were harvested and post-fixed in 4% paraformaldehyde for 24 h, cryoprotected with 30% sucrose at 4 ℃ until sinking to the bottoms, and then sectioned coronally (30 μm) at –20 ℃ with a cryostat (CM1900, Leica, Germany). For real-time reverse transcription-polymerase chain reaction (real-time RT-PCR), after rats were perfused with 0.9% sterile saline, the brains were immediately removed and the tissues in the striatum near the hematoma were harvested and preserved in liquid nitrogen.

Immunohistochemistry

Immunohistochemical staining was performed as described previously,[17] with the following primary antibodies: mouse anti-glial fibrillary acidic protein (GFAP, 1:500, Santa Cruz Biotech, CA, USA), rabbit anti-LIF (1:100, Santa Cruz Biotech, CA, USA), mouse anti-microtubule-associated protein (MAP, a marker for axonal growth, monoclonal, 1:200, Santa Cruz Biotech, CA, USA), and the following secondary antibodies were used: biotinylated goat anti-mouse (1:100, Santa Cruz Biotechn, CA, USA) for $GFAP^+$ detection, biotinylated goat anti-rabbit (1:100, Santa Cruz Biotechn, CA,

USA) for LIF detection, and Cy3-conjugated goat anti-mouse antibody (1:100, Jackson Immuno Research Laboratories, USA) for MAP2 detection.

Double Immunolabeling Staining

Double immunolabeling staining was performed using previously established protocols.[17] In brief, after blocking in 5% bovine serum albumin (BSA, Sigma, USA), the sections were incubated overnight at 4 ℃ with mouse anti-GFAP (1:100) and rabbit anti-proliferating cell nuclear antigen (PCNA, 1:200, Santa Cruz Biotech, CA, USA), or mouse anti-GFAP (1:100) and rabbit anti-LIF (1:50). The secondary antibodies were AF488-conjugated goat anti-mouse antibody (1:100, Jackson Immuno Research Laboratories, USA) and Cy3-conjugated goat anti-rabbit antibody (1:100, Jackson Immuno Research Laboratories, USA). The double immunolabeling images were acquired using a laser scanning confocal microscope (LSM-510, Zeiss).

Cell Counting

For each animal, GFAP, MAP2 and LIF positive cells and $PCNA^+/GFAP^+$ nuclei were quantified in 10 consecutive sections from the hemorrhagic core at 0.24 mm from the bregma. The number of positive cells or nuclei was counted in an area of four 250 μm × 250 μm in non-overlapping fields immediately adjacent to the hematoma using a magnification of × 40 as previously described.[10] The total number of positive cells or nuclei was expressed as the number of nuclei per mm^2 (No./mm^2). Analysis was performed by two investigators who were blinded to the experimental cohort.

Quantitative RT-PCR

Total RNA was extracted from 100 mg tissue near the hematoma using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then, 2 μg of total RNA was reversely transcribed into cDNA using a quantitative RT-PCR kit (Fermentas, Vilnius, Lithuania) and cDNA was amplified by PCR using SYBR Premix ExTaq™ PCR kit according to the manufacturer's protocol. The primers for GFAP, LIF and β-actin are shown in Table 1. The mRNA level of each target gene was normalized to that of β-actin. $2^{-\triangle\triangle CT}$ method was used to calculate relative mRNA expression levels.[18]

Statistical Analysis

Values were expressed as mean ± standard deviations ($\bar{x} \pm s$). Differences between groups were evaluated statistically by using Student's *t* test, and differences in the same group at different time points were evaluated statistically by using one-way analysis of variance (ANOVA). Statistical significance was defined at $P<0.05$.

Table 1. Primers for RT-PCR

cDNA	Primer sequence (5'-3')	Product (bp)
GFAP	Sense: 5'- GGATCTGGAGAGGAAGGTTGAG -3'	207
	Antisense: 5'- GTTTCTTGCATGTTACTGGTGG -3'	
LIF	Sense: 5'-ACGGCAACCTCATGAACCA-3'	135
	Antisense: 5'-GGAAACGGCTCCCCTRGA-3'	
β-actin	Sense: 5'-CGTTGACATCCGTAAAGAC-3'	201
	Antisense: 5'-TGGAAGGTGGACAGTGAG-3'	

RESULTS

BYHWD Attenuated ICH-Induced Glial Scar

After ICH induction, several astrocytes with hypertrophied morphology were detected around the hematoma on day 3, and much more astrocytes with hypertrophied morphology were detected on day 14. Quantitative analysis showed that both GFAP- positive cells and GFAP mRNA levels increased on day 3 and reached the peak on day 14 post-ICH. However, BYHWD significantly reduced GFAP levels (Figure 1A, $P<0.05$). Next, double immunolabeling staining demonstrated that $PCNA^+/GFAP^+$ nuclei could be detected in peri-hematoma region. Quantitative analysis demonstrated that the increase of $PCNA^+/GFAP^+$ nuclei after ICH was markedly attenuated by BYHWD (Figrue 1B, $P<0.01$). Finally, The results showed that the number of MAP2 positive cells in peri-hematoma region increased significantly after BYHWD treatment (Figrue 1C, $P<0.05$).

BYHWD Inhibited Expression of LIF

After ICH, LIF positive astrocytes were detected on day 3, and their number peaked on day 14 after ICH (Figures 2A and 2B, $P<0.01$). Double immunolabeling demonstrated that LIF immunoreactivity was co-localized with GFAP immunoreactivity in astrocytes (Figure 2C). Real-time RT-PCR showed striking upregulation of LIF mRNA on day 3 and persisted till day 14 after ICH. However, after BYHWD treatment both LIF positive cells and LIF mRNA levels decreased significantly (Figures 2A and 2B, $P<0.05$).

DISCUSSION

In the past decade, the neurovascular unit, which consists of neurons, endothelial cells, and astrocytes,

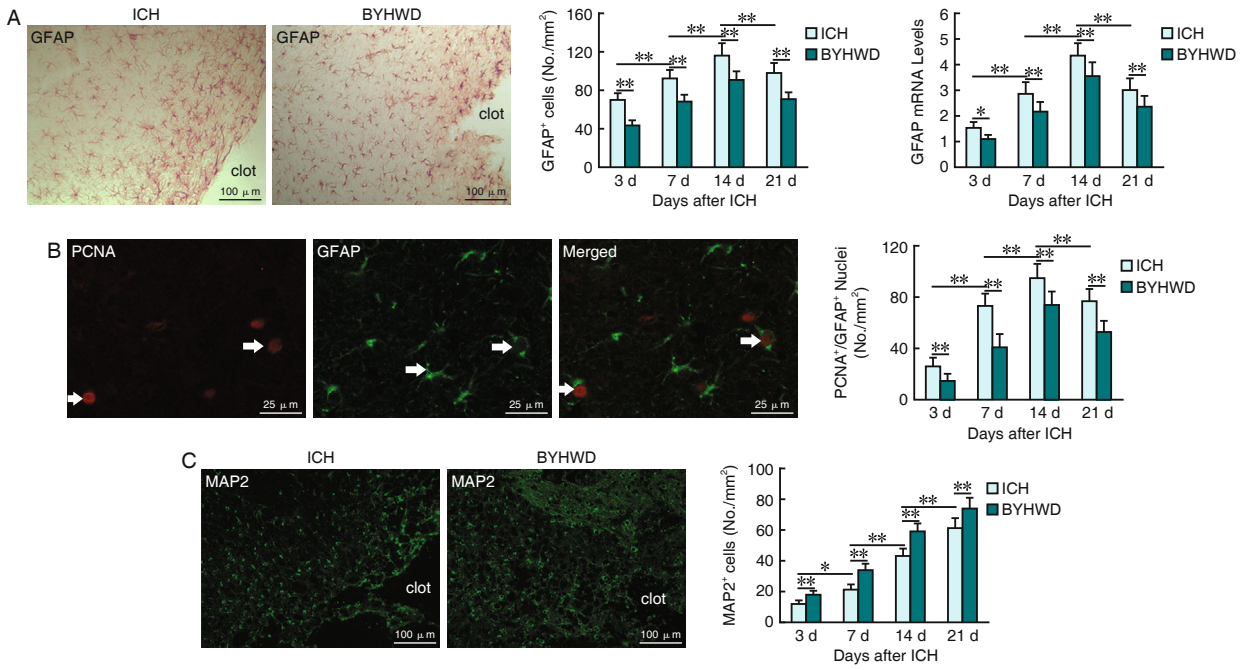

Figure 1. BYHWD Attenuated ICH-Induced Glial Scar ($\bar{x} \pm s$)

Notes: (A) BYHWD downregulated the expression of GFAP after ICH. (B) BYHWD decreased the number of PCNA$^+$/GFAP$^+$ nuclei after ICH. (C) BYHWD increased the number of MAP2 positive cells after ICH. $^*P$<0.05, $^{**}P$<0.01. n=5 in each group.

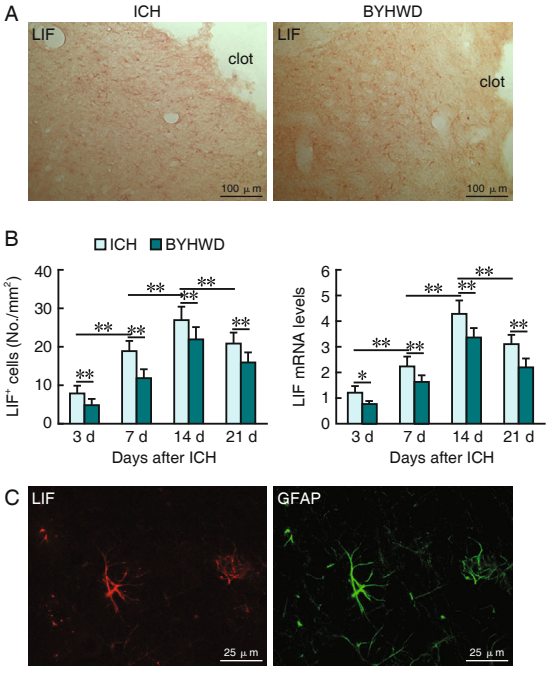

Figure 2. BYHWD Downregulated Expression of LIF after ICH ($\bar{x} \pm s$)

Notes: (A) LIF positive astrocytes were detected in the peri-hematomal tissue. (B) BYHWD reduced LIF positive cells and mRNA levels. (C) LIF immunoreactivity was co-localized with GFAP-immunoreactivity in the astrocytes. $^*P$<0.05, $^{**}P$<0.01. n=5 in each group.

has been gained more attention for understanding the pathology of stroke.[19,20] As mentioned in the introduction, glial scar, which originates from astrocytes proliferation and activation, play a detrimental role for neuronal survival and regeneration.[5] Furthermore, BYHWD could promote differentiate of neural stem cells into neurons rather than astrocytes, and facilitate neurorehabilitation after central nervous system injury.[21,22] In this context, this study aimed to investigate the effects of BYHWD on glial scar after ICH. The results showed that not only morphologically reactive astrocytes but also increased GFAP levels and PCNA$^+$/GFAP$^+$ nuclei could be detected around the clot, which could be attenuated by BYHWD. However, BYHWD could increase the number of MAP2 positive cells after ICH. Increasing evidences have demonstrated that inhibition of glial scar could ameliorate the outcomes after ICH[23] and spinal cord injury.[24] In addition, our previous study showed that enhancement of glial scar was associated with an aggravation of neurological deficit.[10] Accordingly, it is proposed that BYHWD might promote neurite outgrowth by inhibiting glial scar after ICH.

LIF, a member of the interleukin-6 cytokine family, is expressed in a variety of cell types such as astrocytes and neurons after cerebral ischemia.[17] After spinal cord injury, the upregulation of LIF led to severe hindlimb motor dysfunction.[25] LIF was reported to contribute to glial scar via activation of signal transducer and activator of transcription 3 signaling after ICH.[10] In line with these studies, the results of

this study showed that ICH led to the upregulation of LIF mRNA and protein levels, consistent with the induction of GFAP. Recently, activation of the Notch-1 signaling pathway was involved in ICH-induced glial scar in rats.[26] Whether Notch-1 signaling pathway participates in the process of LIF-mediated glial scar after ICH remain further investigated.

BYHWD is beneficial for ischemic[11-13] and hemorrhagic[14,15] stroke. Ding, et al[27] reported that BYHWD inhibited the expression of GFAP after cerebral ischemia. In this study, we showed that BYHWD could attenuate glial scar but promote axonal regeneration. It is well accepted that inflammation and reactive oxygen species (ROS) are main positive regulators of glial scar.[28,29] After ICH, both cell death and microglia activation lead to inflammation outbreak and ROS generation.[30] However, BYHWD could alleviate inflammatory response and decrease ROS generation.[31,32] Accordingly, BYHWD might negatively regulate ICH-induced glial scar by inhibiting inflammation and decreasing ROS level. *Radix Astragali* and *Radix Angelicae Sinensis*, two major component of BYHWD, have been demonstrated to attenuate inflammation.[33,34] Moreover, the hydroalcoholic extract of *Radix Astragali* significantly decreased activation of microglia and astrocytes.[35] Therefore, it is likely that they are the major components of BYHWD to inhibit glial scar after ICH.

In summary, our findings demonstrate that BYHWD attenuates glial scar by downregulating the expression of LIF after ICH, which may be the mechanism by which BYHWD exerts neuroprotective effect after ICH in rats.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Conception and design: Zhou HJ and Kang X. Acquisition of data: Kang X, Zhong JH and Cui HJ. Analysis and interpretation of data: Zhou HJ, Tang T. Drafting the article: Kang X. Critically revising the article: Yang J, Tang T, Zhou JH and Mei ZG. Statistical analysis: Kang X. Study supervision: Zhou HJ. All authors reviewed final version of the manuscript and approved it for submission.

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