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RESEARCH ARTICLE

Buyanghuanwu decoction promotes angiogenesis after cerebral ischemia/reperfusion injury: mechanisms of brain tissue repair

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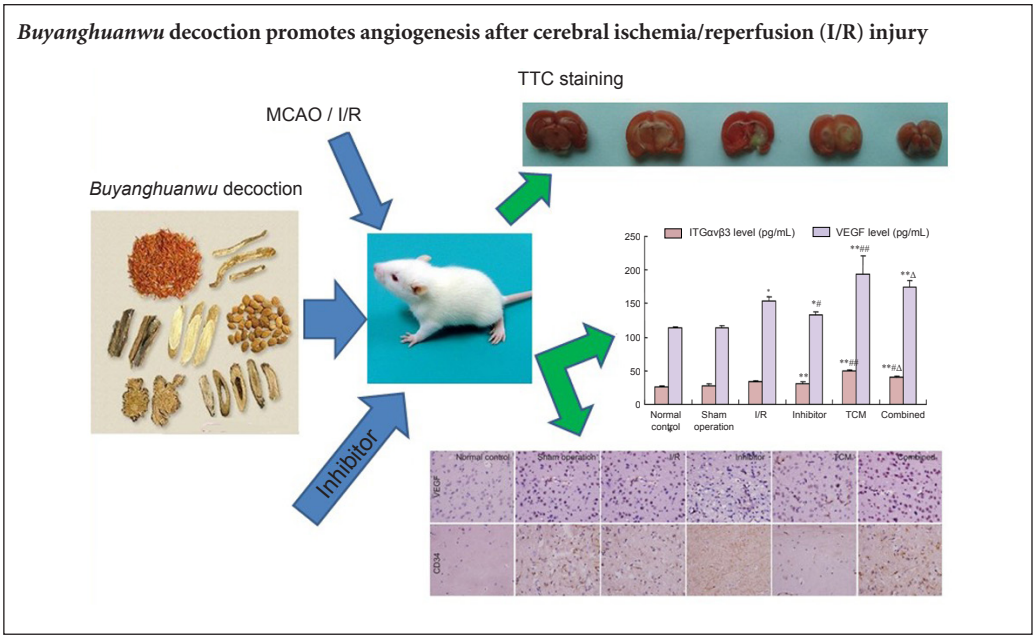
Graphical Abstract



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Abstract

Buyanghuanwu decoction has been shown to protect against cerebral ischemia/reperfusion injury, but the underlying mechanisms remain unclear. In this study, rats were intragastrically given *Buyanghuanwu* decoction, 15 mL/kg, for 3 days. A rat model of cerebral ischemia/reperfusion injury was established by middle cerebral artery occlusion. In rats administered *Buyanghuanwu* decoction, infarct volume was reduced, serum vascular endothelial growth factor and integrin αvβ3 levels were increased, and brain tissue vascular endothelial growth factor and CD34 expression levels were increased compared with untreated animals. These effects of *Buyanghuanwu* decoction were partially suppressed by an angiogenesis inhibitor (administered through the lateral ventricle for 7 consecutive days). These data suggest that *Buyanghuanwu* decoction promotes angiogenesis, improves cerebral circulation, and enhances brain tissue repair after cerebral ischemia/reperfusion injury.

Key Words: *nerve regeneration; Buyanghuanwu decoction; cerebral ischemia/reperfusion injury; ischemic cerebrovascular disease; integrin αvβ3; vascular endothelial growth factor; angiogenesis; CD34; neural regeneration*

Introduction

Buyanghuanwu decoction (BYHWD) consists of astragalus, angelica tail, radix paeoniae rubra, earthworm, rhizoma chuanxiong, safflower and peach kernel. BYHWD is often used to treat the sequelae of cerebrovascular events due to *qi* deficiency and blood stasis (such as stroke), including facial paralysis, aphasia, hemiplegia and paraplegia (Wen, 2009).

Recently, angiogenesis has become a hot spot in cerebrovascular disease studies, because enhancing angiogenesis in ischemic brain tissue appears to be an effective method for improving blood supply in the brain (Tian and Liu, 2010). Vascular endothelial growth factor (VEGF) plays a key role

in the formation of new blood vessels. Moreover, VEGF is involved in blood vessel production in the acute stage of brain ischemia (Infanger et al., 2008). VEGF, together with integrin αvβ3 (ITGαvβ3), promotes angiogenesis. ITGαvβ3 binds to matrix metalloproteinases and extracellular matrix components. ITGαvβ3 also enhances the degradation of extracellular matrix by matrix metalloproteinases, activates endothelial cells, and promotes endothelial cell proliferation. Endothelial cells also participate in the formation of new blood vessels (Zhang et al., 2000). In addition, $CD34^+$ cells promote neovascularization in the ischemic brain to facilitate neuronal reconstruction and regeneration (Taguchi et al., 2004). CD34 antibodies can be used to assess microvascular density in the newborn (Yang et al., 2005; Mahadevan et al., 2014; Tian et al., 2015).

Many studies have provided evidence that BYHWD promotes microvascular angiogenesis by inducing VEGF expression in the ischemic penumbra of the rat brain (Liu et al., 2007; Zhang et al., 2007; Lu et al., 2008; Li and Wang, 2011; Rao et al., 2014). However, the mechanisms underlying the reconstruction of microvascular networks by BYHWD are unknown. In the present study, we examined the effects of BYHWD on angiogenesis and the expression levels of VEGF, ITGαvβ3 and CD34 after ischemia/reperfusion (I/R) injury.

Materials and Methods

Experimental animals

A total of 90 clean healthy Sprague-Dawley rats, aged 12 weeks, male or female, weighing 280–300 g, were provided by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., Beijing, China (animal quality certificate No. 0261701). The rats were housed at room temperature under a 12-hour light/dark cycle. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Henan Animal Care and Use Committee, China.

Rats were equally and randomly divided into normal control group, sham operation group, I/R group (middle cerebral artery occlusion, MCAO), inhibitor group (MCAO + inhibitor), traditional Chinese medicine (TCM) group (MCAO + BYHWD) and combined group (BYHWD + inhibitor).

Establishment of I/R model

In accordance with a previous method (Nagasawa and Kogure, 1989) and that of Longa (Longa et al., 1989), rats (except those in the normal control group) were anesthetized with 10% chloral hydrate (0.3 mL/kg; Wuhan Hechang Chemical Co., Ltd., Wuhan, Hubei Province, China) intraperitoneally, and fixed in the dorsal position. After disinfection, a 2-cm incision was made on the neck. The right common carotid artery, the internal carotid artery, extracranial branches of the pterygopalatine artery, and the external carotid artery were bluntly dissected. The pterygopalatine artery was blocked, and the distal end of the external carotid artery was ligated. The distal ends of the common carotid and internal carotid arteries were clamped transiently with vascular clamps. An oblique incision was made at the distal end of the external carotid artery, 3–5 mm from the bifurcation of the external carotid and internal carotid arteries. A nylon fishing line, 0.235 mm in diameter, was inserted, and the vascular clamp on the internal carotid artery was removed. The nylon line was inserted a distance of 18 mm from the bifurcation of the external carotid and internal carotid arteries. The vascular clamp on the common carotid artery was removed to restore blood flow. The incision was sutured and disinfected. The end of the nylon line was maintained outside the body. Two hours later, the nylon line was gently removed to allow reperfusion of the middle cerebral artery. The normal control group did not undergo surgery. In the sham operation group, the incision was sutured after exposure of the internal carotid and external carotid arteries. A 0.1 mL volume of penicillin, 80,000 units, was given to the rats starting 3 days before surgery by intramuscular injection, every morning and evening. The rats were preoperatively fasted for 12 hours.

Behavioral scoring was performed when the rats awoke from anesthesia. The 5-point Longa scoring system is as follows: 0, no obvious symptoms of nerve injury; 1, cannot fully extend the left forepaw; 2, circling to the left; 3, falling towards the left when walking; 4, cannot spontaneously walk, loss of consciousness. Rats scoring 0 or 4 were not used further.

Drug intervention

The BYHWD was prepared as follows: 60 g of angelica and 30 g of rhizoma chuanxiong were boiled six times in 540 mL of water, and 200 mL of distillate was collected and filtered for further use. 1,200 g of astragalus, 60 g of radix paeoniae rubra, 30 g of earthworm, 30 g of safflower and 30 g of peach kernel were mixed together, and 10,800 mL of water was added. The mixture was decocted twice, each for 1 hour. After filtering, the decocted liquids were mixed. A rotary evaporator was used for vacuum concentration to approximately 400 mL. This preparation was added to 200 mL of the distillate, 3 mL of Tween-80 and distilled water to a final volume of 600 mL. The resulting liquid was dark brown, and 1 mL was equivalent to 2.4 g of raw material. Following high temperature and high pressure steam sterilization, samples were placed in sterile 250-mL sealed infusion bottles at 4°C. Drug decoction was conducted in the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, China.

Rats in the TCM and combined groups were given 15 mL/kg BYHWD. Rats in the normal control, sham operation, I/R and inhibitor groups were given an equal volume of normal saline. The treatment was given by gavage starting 3 days before surgery until 14 days after surgery, once a day.

Rats in the inhibitor and combined groups were injected with angiogenesis inhibitor (Endostar, recombinant human endostatin injection, liquid, 5 mg/mL, 5 μL/rat; approval No. S20050088; Shandong Simcere-Medgenn Bio-pharmaceutical Co., Ltd., Shandong Province, China) into the lateral ventricle using a microinjector 2 days before surgery (inhibitors were injected starting 1 day after BYHWD administration for 7

Table 1 Effect of *Buyanghuanwu* decoction on behavioral score in a rat model of cerebral ischemia

Group	Behavioral score
Normal control	0
Sham operation	0
I/R	1.00±0.35
Inhibitor	1.34±0.58$^{\#}$
TCM	0.30±0.22$^{\#\#}$
Combined	0. 61±0.29$^{\#\Delta}$

Scoring was performed according to the Longa 5-point standard scoring system as follows: 0, no obvious sign of nerve injury; 1, cannot fully extend the left forepaw; 2, circling to the left; 3, falling towards the left when walking; 4, cannot spontaneously walk, loss of consciousness. Animals with scores of 0 or 4 were not used. Data are presented as the mean ± SD, with 10 rats in each group. #$P < 0.05$, ##$P < 0.01$, *vs*. I/R group; Δ$P < 0.05$, *vs*. TCM group (analysis of variance followed by the least significant difference test). I/R: Ischemia/reperfusion; TCM: traditional Chinese medicine.

days). Under the stereotaxic instrument (ZH-blue star-A brain stereotaxic instrument, Huaibei Zhenghua Biological Instrument Equipment Co., Ltd., Huaibei, Anhui Province, China) and using a stereotaxic atlas, the lateral ventricle was localized for injection as follows: 1 mm posterior to the bregma, 1.5 mm lateral, and at a depth of 3.5 mm. The microinjector containing 5 μL Endostar was fixed on the positioning system. After adjusting the angle, the needle was slowly and vertically inserted into the skull with a pinhole of approximately 3.5 mm. The drug was slowly injected into the lateral ventricle over a period of 1–2 minutes. The skull surface was dried. The needle was maintained in place for a few minutes to ensure the drug completely entered the lateral ventricle. Subsequently, the microinjector was slowly retracted. After disinfecting with hydrogen peroxide, the incision in the scalp was sutured. The rats were maintained in separate cages after the operation and were injected with 80,000 units of penicillin by intramuscular injection for 3 days.

Enzyme linked immunosorbent assay (ELISA)

Fourteen days after MCAO surgery, five rats from each group were intraperitoneally anesthetized with 10% chloral hydrate (0.3 mL/100 g). A 5 mL sample of blood was collected from the abdominal aorta, incubated without stirring for 30 minutes, and then centrifuged at 1,700 × *g* for 15 minutes. The supernatant was collected and stored at −20°C. ELISA was performed in accordance with instructions in the ITGαvβ3 and VEGF ELISA kits (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China).

2,3,5-Triphenyltetrazolium chloride (TTC) staining

Fourteen days after MCAO surgery, rats were rapidly decapitated after sampling the blood, and the whole brain was quickly and carefully removed and placed at –20°C for 20 minutes. The first cut was made at the midpoint between the cerebral anterior pole and the optic chiasm, the second cut was at the optic chiasm, the third cut was in the infundibular stalk, and the fourth cut was between the infundibular stalk and the posterior lobe caudal pole. A total of 5–6 slices were obtained, and each slice was 2-mm thick. The sections were placed in 2% TTC, covered with foil and incubated in a 37°C water bath for 10–30 minutes. The brain slices were intermittently turned over to ensure the sections fully contacted the dye solution. Sections were photographed, and the size of the infarcted region was evaluated. The non-ischemic area was pale red, while the infarcted tissue was white. All sections were fixed in 4% paraformaldehyde and photographed. The cerebral infarct volume ratio was calculated as infarct volume/whole brain volume (%) (five rats in each group).

Immunohistochemical staining

Fourteen days after MCAO surgery, five rats from each group were intraperitoneally anesthetized with 10% chloral hydrate (0.3 mL/100 g). The rats were fixed in the dorsal position and perfused with paraformaldehyde. The whole brain was carefully taken out after decapitation, and the cerebellum was removed and fixed in 4% paraformaldehyde. Paraffin sections were dewaxed, hydrated, and incubated with 0.01 M pH 6.0 citrate buffer for 20 minutes. Sections were washed with PBS and blocked in normal goat serum at room temperature for 10 minutes. Sections were then incubated with anti-rat polyclonal antibodies (VEGF, 1:300; CD34, 1:500; Wuhan Boster Biological Engineering Co., Ltd., Wuhan, Hubei Province, China) at 37°C for 1 hour, washed with PBS, and then incubated with anti-rabbit polyclonal antibody (1:500; Wuhan Boster Biological Engineering Co., Ltd.) at 37°C for 15 minutes. After washing with PBS, sections were incubated with 3,3′-diaminobenzidine and observed under the light microscope (Olympus, Tokyo, Japan). Brown or red staining indicated a positive reaction. The sections were then counterstained with hematoxylin, dehydrated, permeabilized, dried, and mounted with neutral resin. The numbers of VEGF- and CD34-positive cells per mm^2 were calculated.

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and expressed as the mean ± SD. Differences were analyzed using analysis of variance followed by the least significant difference test. $P < 0.05$ was considered statistically significant.

Results

BYHWD improved neurological function in rats with cerebral ischemia

Compared with the I/R group, the neurological function score was higher in the inhibitor group ($P < 0.05$), but lower in the TCM and combined groups ($P < 0.01$ or $P < 0.05$). Moreover, the neurological function score was lower in the TCM group than in the combined group ($P < 0.05$; **Table 1**).

BYHWD reduced infarct volume in rats with cerebral ischemia

TTC staining showed that compared with the I/R group, infarct volume was larger in the inhibitor group ($P < 0.05$).

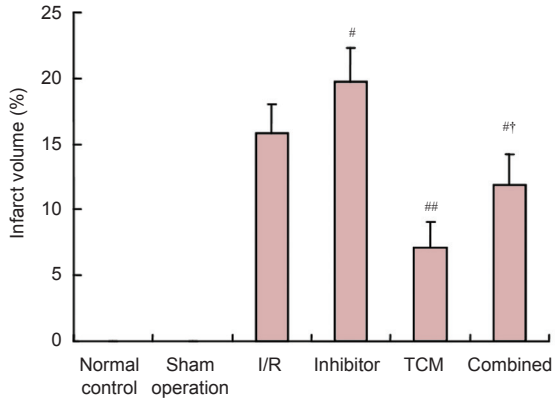

Figure 1 Effect of *Buyanghuanwu* decoction on infarct volume in a rat model of cerebral ischemia (2,3,5-triphenyltetrazolium chloride staining).
The cerebral infarction volume ratio = infarct volume/whole brain volume (%). Data are expressed as the mean ± SD, with five rats in each group. #$P < 0.05$, ##$P < 0.01$, *vs.* I/R group; †$P < 0.05$, *vs.* TCM group (analysis of variance followed by the least significant difference test). I/R: Ischemia/reperfusion; TCM: traditional Chinese medicine.

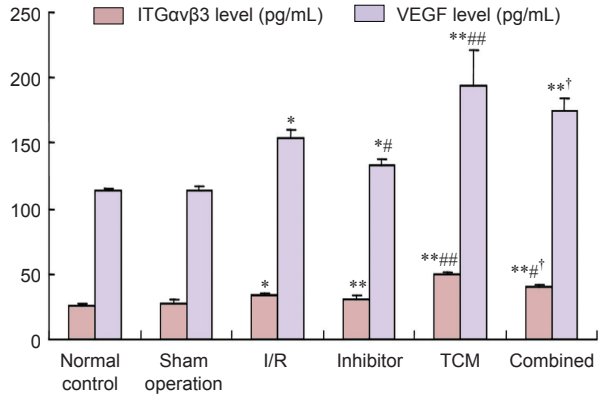

Figure 2 Effect of *Buyanghuanwu* decoction on serum ITGαvβ3 and VEGF levels in a rat model of cerebral ischemia (enzyme linked immunosorbent assay).
Data are expressed as the mean ± SD, with five rats in each group. *$P < 0.05$, **$P < 0.01$, *vs.* normal control and sham operation groups; #$P < 0.05$, ##$P < 0.01$, *vs.* I/R group; †$P < 0.05$, *vs.* TCM group (analysis of variance followed by the least significant difference test). ITGαvβ3: Integrin αvβ3; VEGF: vascular endothelial growth factor; I/R: ischemia/reperfusion; TCM: traditional Chinese medicine.

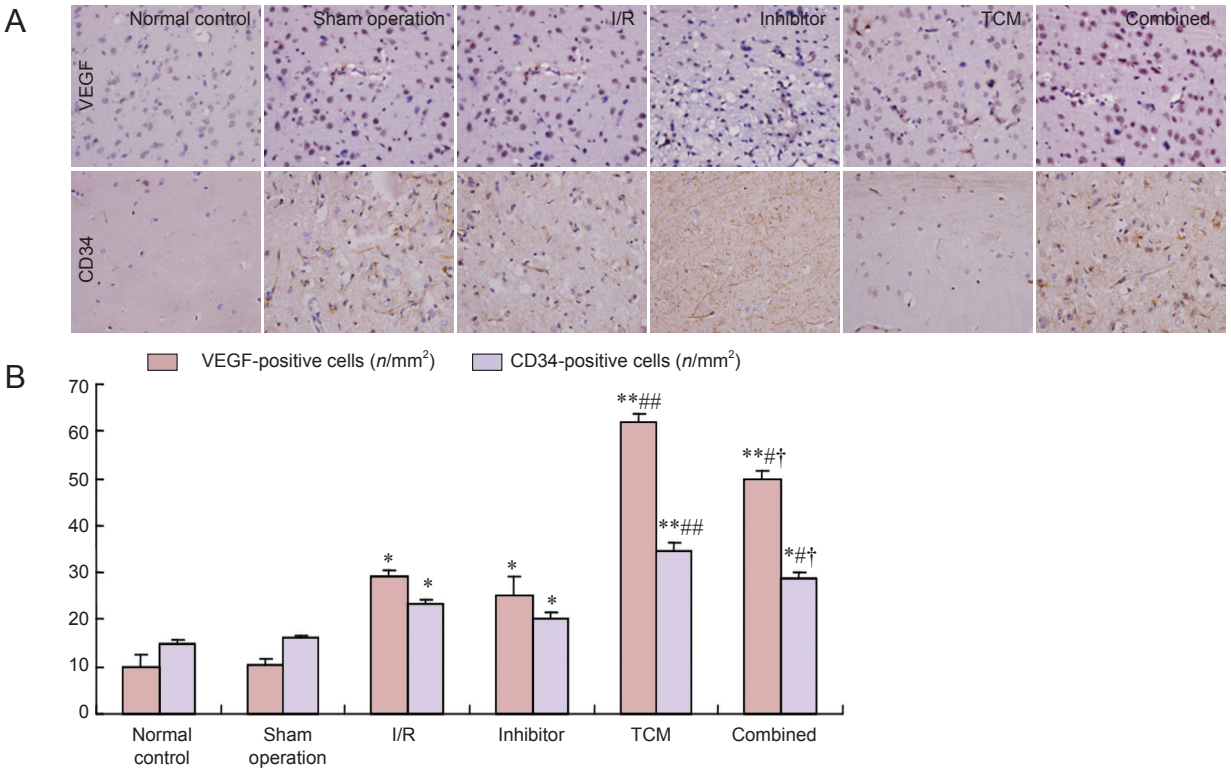

Figure 3 Effect of *Buyanghuanwu* decoction on the expression of VEGF and CD34 in the penumbra of brain tissue in a rat model of cerebral ischemia (immunohistochemical staining).
(A) Expression of VEGF and CD34 in brain tissue (× 400). (B) Number of VEGF- and CD34-positive cells. Data are expressed as the mean ± SD with five rats in each group. *$P < 0.05$, **$P < 0.01$, *vs.* normal control and sham operation groups; #$P < 0.05$, ##$P < 0.01$, *vs.* I/R group; †$P < 0.05$, *vs.* TCM group (analysis of variance followed by the least significant difference test). VEGF: Vascular endothelial growth factor; I/R: ischemia/reperfusion; TCM: traditional Chinese medicine.

Infarct volumes were lower in the TCM and combined groups compared with the I/R group ($P < 0.01$ or $P < 0.05$). The infarct volume was lower in the TCM group than in the combined group ($P < 0.05$; **Figure 1**).

BYHWD increased serum levels of VEGF and ITGαvβ3 in rats with cerebral ischemia

Compared with the normal control and sham operation groups, the levels of VEGF and ITGαvβ3 in serum were

higher in the I/R, inhibitor, TCM and combined groups ($P < 0.01$ or $P < 0.05$). Compared with the I/R group, the concentration of VEGF was lower in the inhibitor group ($P < 0.05$), but there was no significant difference in ITGαvβ3 levels between these two groups ($P > 0.05$). The levels of VEGF and ITGαvβ3 in serum were significantly increased in the TCM and combined groups ($P < 0.01$, $P < 0.05$). Compared with the TCM group, the levels of VEGF and ITGαvβ3 in serum were significantly lower in the combined group ($P < 0.01$, $P < 0.05$; **Figure 2**).

BYHWD increased expression of VEGF and CD34 in the brain of rats with cerebral ischemia

Immunohistochemistry revealed that compared with the normal control and sham operation groups, the expression levels of VEGF and CD34 in brain tissue were greater in the I/R, inhibitor, TCM and combined groups ($P < 0.01$ or $P < 0.05$). Compared with the I/R group, the expression levels of VEGF and CD34 in brain tissue were higher in the TCM and combined groups ($P < 0.01$ or $P < 0.05$), but there was no significant difference between the I/R and inhibitor groups ($P > 0.05$). Compared with the combined group, the expression levels of VEGF and CD34 in brain tissue were significantly higher in the TCM group ($P < 0.05$; **Figure 3**).

Discussion

Angiogenesis serves as a mechanism for wound healing and tissue repair. Studies have shown that the vascular density after cerebral ischemia in brain tissue is relatively high, and angiogenesis promotes the recovery of neural function (Zhang et al., 2000). In the present study, vascular density was higher in the I/R group than in the normal control and sham operation groups, suggesting that spontaneous angiogenesis after cerebral ischemia occurred. Vascular density was further increased in the TCM and combined groups. This suggests that BYHWD plays a protective role in I/R injury, and possibly promotes angiogenesis by protecting vascular endothelial cells, regulating vascular growth factors and promoting the formation of new blood vessels.

BYHWD has been shown to affect cell survival (Zhang, 2006). In our study, BYHWD inhibited apoptosis induced by cerebral I/R injury. BYHWD has been shown to promote neurological recovery and angiogenesis after intracerebral hemorrhage in mice by enhancing VEGFR2 phosphorylation though the PIK/AKT signaling pathway, and this effect can be blocked by SU5416, a potent selective inhibitor of VEGFR2 (Cui et al., 2015). In the present study, we focused on the effects of BYHWD on angiogenesis in the brain after I/R injury by evaluating VEGF expression. We found that compared with the normal control and sham operation groups, the levels of VEGF and CD34 in serum and brain tissue were higher in the I/R, inhibitor, TCM and combined groups, and especially in the TCM group. These results suggest that the changes in VEGF and CD34 expression were associated with angiogenesis after I/R injury. BYHWD appears to promote the expression of VEGF, which induces an increase in the number of CD34-positive cells in blood vessels. This shows that BYHWD promotes angiogenesis by regulating the expression of VEGF, and plays an important role in protecting brain tissue.

VEGF, as an angiogenic factor, plays an important role at all stages of angiogenesis. Recently, angiogenesis has become a hot spot in cerebrovascular disease research. Our results show that BYHWD induces VEGF expression after I/R injury. This suggests that VEGF signaling is involved in the pro-angiogenic effects of BYHWD. However, the underlying mechanisms are unclear and require further study.

Integrins are important cell adhesion molecules. ITGαvβ3 is a dimeric glycoprotein consisting of an αv (CD51, 150 kDa) subunit and a α3 (CD61, 105 kDa) subunit, and participates in numerous processes, including angiogenesis, inflammation, and blood clotting. ITGαvβ3 regulates angiogenesis by promoting the proliferation, activation, migration and survival of endothelial cells. ITGαvβ3 is expressed in vascular endothelial cells in the ischemic area, and ITGαvβ3 mRNA expression is increased after cerebral ischemia (Abumiya et al., 1999).

In the present study, we found that the serum levels of ITGαvβ3 and VEGF were increased in the I/R group compared with the normal control group, suggesting that ITGαvβ3 participates in angiogenesis after cerebral I/R injury. The concentration of VEGF was much higher than the concentration of ITGαvβ3 in the I/R group, indicating that the increase in ITGαvβ3 might be associated with increased expression of VEGF. In addition, compared with the I/R group, the serum levels of ITGαvβ3 and VEGF were increased in the TCM group, and the concentration of VEGF was much higher than that of ITGαvβ3. Endostatin decreased the expression of VEGF and ITGαvβ3 in rats administered BYHWD. These results show that BYHWD promotes angiogenesis by increasing levels of ITGαvβ3 and VEGF, thereby protecting brain tissue.

In our previous study, BYHWD and its individual components protected against cerebral I/R injury by modulating the levels of thromboxane B2 and 6-Keto-PGF1a, which are metabolites of arachidonic acid (Zhang and Zhang, 2004). BYHWD has been shown to modulate angiogenesis in the ischemic region by increasing the expression of VEGF, transforming growth factor-β and angiopoietin-1 (Zhang and Zhang, 2004). However, the signaling pathways mediating the pro-angiogenic effects of BYHWD are unclear and require further investigation.

In summary, BYHWD increased the expression of VEGF and ITGαvβ3 as well as microvessel density in the ischemic brain. The increased levels of VEGF and ITGαvβ3 enhanced angiogenesis and improved neurological recovery. This suggests that the administration of VEGF to promote angiogenesis after stroke may have therapeutic efficacy and may reduce cerebral infarct volume.

Author contributions: *YKZ designed the study and performed the analyses. ZQZ conceived and designed this study, and revised the paper. JYS was primarily responsible for writing the paper, collected and integrated data. YQJ conducted the*

experiments, collected data and was responsible for the animal model. All authors approved the final version of the paper.

Conflicts of interest: *None declared.*

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