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Original Article

Huatuo Zaizao pill improves neurological function by enhancing angiogenesis in stroke rats

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Abstract: Huatuo Zaizao pill (HT), a traditional Chinese medicine, is widely used in China for the treatment of stroke patients. This study investigated the effect of HT on neurological function, ischemic area and angiogenesis in a rat model of cerebral ischemia-reperfusion (I/R) injury. Male rats were underwent a middle cerebral artery occlusion followed by reperfusion. Then the rats received intragastrically vehicle (for the sham group and model group) or HT (0.5, 1.0, or 2.0 g/kg), respectively. Neurological function was assessed by the Longa's method after 7-day treatment. Ischemic area was evaluated via triphenyl tetrazolium chloride staining. Double immunofluorescence staining for 4',6-diamidino-2-phenylindole and von Willebrand factor (vWF) was performed. Protein expressions of hypoxia inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang1), vascular endothelial receptor tyrosine kinase Tie2, stromal cell derived factor-1 (SDF-1), and CXC chemokine receptor 4 (CXCR4) in the peri-infarct regions of cortex were analyzed by Western blotting. Compared with the model group, treatment with HT improved the neurological function and reduced the brain ischemic area in cerebral I/R-injured rats. The number of vWF positive vessels was significantly increased in the HT groups as compared with the model group. Levels of HIF-1α, VEGF, Ang-1/p-Tie2 and SDF-1/CXCR4 were also enhanced after the 7-day HT treatment. These findings indicate that HT treatment could reduce the ischemic area and improve neurological function after stroke by enhancing angiogenesis.

Keywords: Huatuo Zaizao pill, cerebral ischemia-reperfusion, neurological function, angiogenesis

Introduction

Ischemic stroke is the leading cause of serious long-term disability. Therapeutic options for clinical management in stroke are limited. Accumulating data suggest that the ischemic brain exhibiting significant plasticity and angiogenesis can provide new opportunities for stroke recovery [1]. The process of angiogenesis begins with the proliferation and migration of endothelial cells to the zone of ischemic brain tissue, initiating the sprouting of new capillaries from pre-existing blood vessels. The formation of new blood vessels from angiogenesis will improve the impairment of microvasculature. Then, the reestablishment of functional microvasculature enhances neurogenesis and results in functional recovery after stroke [2].

Vascular endothelial growth factor (VEGF) is generally considered to be one of the major factors involved in the process of angiogenesis in the central nervous system (CNS) [3]. Angiopoietin-1 (Ang1) is an endogenous ligand for the vascular endothelial receptor tyrosine kinase Tie2. Signaling by Ang1 promotes vascular endothelial cell survival and the sprouting of blood vessels [4]. Hypoxia induces the activation of hypoxia inducible factor 1 (HIF-1) which in turn promotes expressions of a number of target genes including CXC chemokine receptor 4 (CXCR4) [5]. Stromal cell derived factor-1 (SDF-1) is a member of the alpha chemokine family. SDF-1 plays a crucial role in the mobilization and homing of hematopoietic stem cells to the bone marrow with its receptor CXCR4 [6].

Alternative and complementary medicine has gained global attention because of their widespread use in the field of medicine for treating central nervous system diseases. Huatuo Zaizao pill (HT), a typical traditional Chinese

medicine, consists of 5 Chinese herbs: *Ligusticum chuanxiong hort*, Borneol, *Evodia rutaecarpa (Juss.) Benth.*, *Carthamus tinctorius L.*, and *Angelica sinensis*. The pharmacopoeia of the people's republic of China Part I (Pharmacopoeia Commission of the People's Republic of China, 2015) demonstrates that HT can be used to promote the rehabilitation after stroke. Although HT has been used to promote the rehabilitation in stroke patients, the mechanism of action of HT remains poorly understood. In this study, whether HT could improve neurological function after stroke by enhancing angiogenesis in a rat model of cerebral I/R injury was investigated.

Materials and methods

Drug

Huatuo Zaizao pill was provided by Guangzhou Baiyunshan Qixing Pharmaceutical Co., LTD (Guangzhou China; batch number: 13193). The information of quality control about HT can be found in the pharmacopoeia of the people's republic of China Part I (Pharmacopoeia Commission of the People's Republic of China, 2015). The suspension of Huatuo Zaizao pill was prepared with 0.5% sodium carboxymethyl cellulose (CMC-Na).

Animals

All animal care and experimental procedures were carried out in accordance with the recommendations in the Guide for the National Institutes of Health Guide (publication 86-23, revised in 1986) and were approved by the Committee of Yantai University for the Care and Use of Laboratory Animals (Yantai, China). Male Sprague-Dawley rats (Body weight: 280 ± 20 g) were housed in cages. The animal housing room was acclimatized to 24 ± 2°C, with 50 ± 10% humidity and a 12/12 h light-dark cycle and a free access to food and water. Rats were purchased from Beijing HFK Bioscience Co., Ltd (Beijing, China) 1 week prior to the experiment (Experimental animal certificate number: SCXK [Beijing] 2009-0015).

Cerebral I/R injury model

A middle cerebral artery occlusion model was established as previously described [7]. The middle cerebral artery was occluded by threading a monofilament nylon suture with a heat-rounded tip through the internal carotid artery, which was advanced further until it blocked the origin of the middle cerebral artery. Ninety minutes after ischemia, reperfusion was initiated by withdrawal of the monofilament. Sham-operated rats underwent an identical surgery except that the middle cerebral artery was not occluded. All animals were continuously monitored from surgery to recovery. The rats were allowed to recover from anesthesia and were then returned to their cages with free access to food and water.

Experimental design

The neurological function was evaluated according to the Longa's method [7] at 24 h after cerebral I/R injury by two professionally trained persons blinded to the experimental design. The criteria were as follows: 0, no deficit; 1, failure to extend the left forepaw fully; 2, circling to the left; 3, falling to the left; 4, no spontaneous walking with a depressed level of consciousness. Rats with neurological scores of 1 to 3 were considered successful models and used in this study. According to Longa's score, rats were randomly assigned to five experimental groups: sham-operated group, model group, and HT at doses of 0.5 g/kg, 1.0 g/kg, or 2.0 g/kg groups. Rats in HT groups were orally administered with HT once daily for 7 continuous days. The rats in the sham and model groups were given 0.5% sodium carboxymethyl cellulose (CMC-Na).

Neurological assessment

The neurological function was reevaluated at 7 d after treatment using Longa's method by the same two experimenters.

TTC staining

For quantitative ischemic area, six rats in each group were anesthetized with intraperitoneal injection of overdosed chloral hydrate. The brains were rapidly removed and cooled in cold saline for 5 min and were then coronally sectioned into five 2-mm-thick sections. The slices were stained in 2% 2,3,5-triphenylte trazolium chloride (TTC, Sigma, St. Louis, MO, USA) at 37°C in an incubator for 30 min and then fixed by 4% buffered formalin solution. The ischemic area was measured by an Image-Pro Plus 6.0

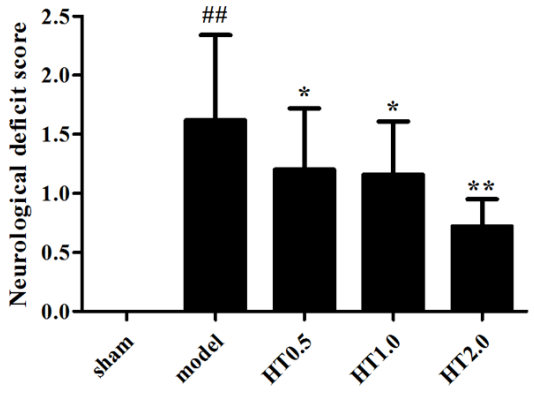

Figure 1. Effect of HT on neurological deficit score after cerebral I/R in rats. ##P < 0.01 vs. sham group; *P < 0.05, **P < 0.01 vs. model group.

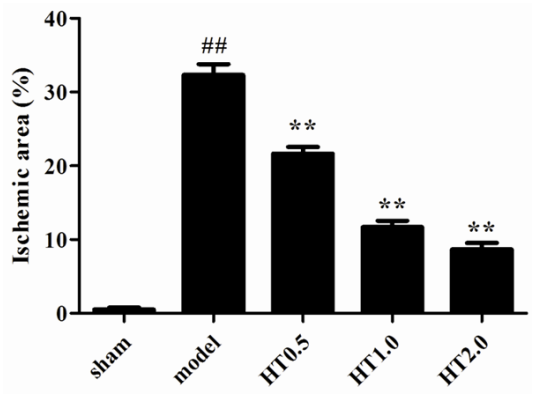

Figure 2. Effect of HT on ischemic area after cerebral I/R in rats. ##P < 0.01 vs. sham group; **P < 0.01 vs. model group.

software. The percentage of ischemic area to the total brain was calculated.

Immunofluorescence assay

Three rats in each group were deeply anesthetized with chloral hydrate and disposed by cardiovascular perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Serial coronal sections from bregma -1.80 to -4.30 were selected for immunofluorescent staining. Sections were incubated with the primary polyclonal goat against von Willebrand factor (vWF) antibody (1: 200, Dako, Carpinteria, CA, USA) for 30 min at 37°C. After having been washed with PBS for 3 times, the slides were then incubated with Alexa Fluor 594-labeledsecondantibody (Cell Signaling Technology, MA, USA) for 1 h at 37°C. Washed with 3% bovine serum albumin (BSA) and mounted with a ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology, MA, USA). Brain sections were investigated using a fluorescent microscope (Olympus IX83, Tokyo, Japan). Images were acquired with an Image-Pro Plus 6.0 software. The number of vWF positive vessels in the peri-infract cortex were counted using a microscope (200×). In all of the slices, five fields of peri-infarct regions of cortex per brain samples were quantified by an observer blinded to the experimental treatment.

Western blotting

The peri-infarct regions of cortex were collected and stored at -80°C until further use. Brain samples were lysed in RIPA (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) buffer and centrifuged at 12,400 g for 20 min at 4°C. Proteins were then loaded (80 μg per lane) and subjected to 6% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 30 min at 80 V, then 120 V till the end. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corporation, MA, USA). After blocking with 5% nonfat milk for 2 h, the membranes were incubated overnight at 4°C with the primary antibodies: VEGF (1:1000, Cell Signaling Technology, MA, USA), HIF-1α (1:1000, Abcam, Cambridge, MA, USA), Ang-1 (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA), p-Tie2 (1:1000, Bioworld Technology, Louis Park, USA), SDF-1 (1:1000, Cell Signaling Technology, MA, USA), CXCR4 (1:1000, Abcam, Cambridge, MA, USA). The membranes were then washed with TBS-0.05% Tween 20 and processed with the anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (all at 1:2000, Beyotime Institute of Biotechnology). Bands were visualized using the enhanced chemiluminescent substrate kit (Beyotime Institute of Biotechnology), and bands were quantified using Image Quant LAS 4000 (GE Healthcare Bio-Sciences AB, Tokyo, Japan). The housekeeping protein of β-actin was used as a loading control.

Statistical analysis

All results were presented as the mean ± standard deviation. Least significant difference (LSD) test was applied for comparisons of the difference between 2 groups. Values of $P < 0.05$ were considered statistically significant. The SPSS 17.0 Statistical Software was used for the analyses.

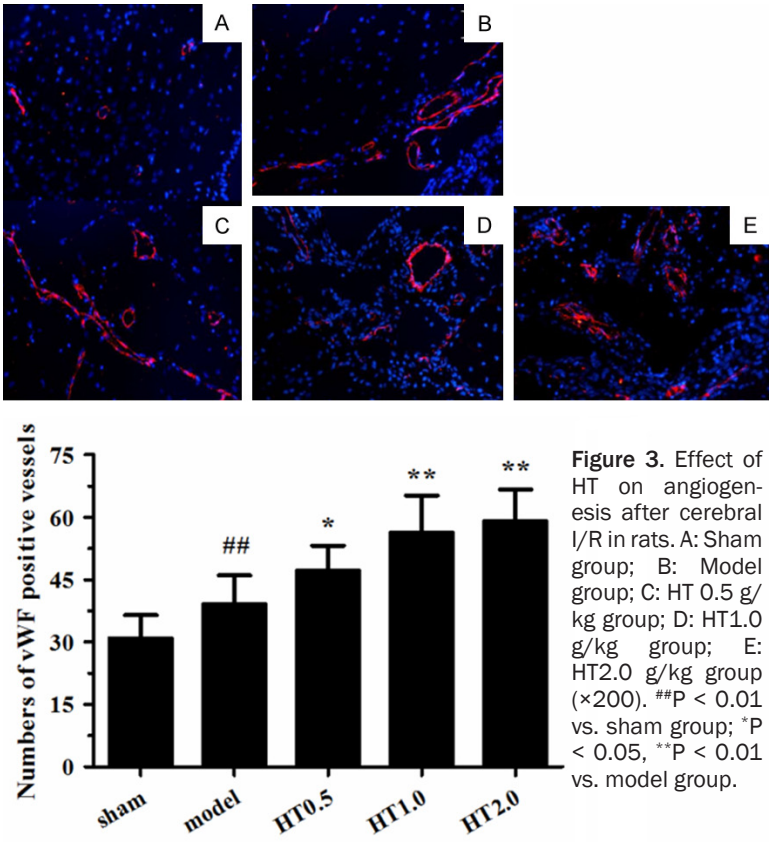

Figure 3. Effect of HT on angiogenesis after cerebral I/R in rats. A: Sham group; B: Model group; C: HT 0.5 g/kg group; D: HT1.0 g/kg group; E: HT2.0 g/kg group (×200). $^{\#\#}P < 0.01$ vs. sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

Results

Effect of HT on neurological deficit score after cerebral I/R in rats

Neurological function following cerebral I/R assessed by Longa's method was performed at 7 d after treatment with HT. The animals in model group showed a significantly high neurological deficit score. Neurological deficit scores in the HT-treated animals were reduced when compared with that of model group ($P < 0.05$ or $P < 0.01$, **Figure 1**).

Effect of HT on ischemic area after cerebral I/R in rats

There were obvious ischemic areas observed in the rats of model group. At 7 d after HT treatment, a significant reduction of ischemic area was found when compared with model group ($P < 0.01$). The results were shown in **Figure 2**.

Effect of HT on angiogenesis after cerebral I/R in rats

The number of vWF positive vessels in the peri-infarct regions of cortex was evaluated using the immunofluorescence method. There were few vWF positive vessels observed in the sham group. Compared with the sham group, a significantly higher number of vWF positive vessels were found in the model group ($P < 0.01$). HT treatment markedly increased the number of vWF positive vessels in the peri-infarct regions of cortex when compared with the model group ($P < 0.05$ or $P < 0.01$, **Figure 3A**, **3B**).

Effect of HT on expressions of HIF-1α, VEGF, Ang-1/p-Tie2, SDF-1/CXCR4 after cerebral I/R in rats

The protein expressions of HIF-1α, VEGF, Ang-1/p-Tie2, SDF-1/CXCR4 were assayed via Western blotting after 7-day HT treatment. Western blotting analysis showed that cerebral I/R markedly increased the levels of HIF-1α, VEGF, Ang-1/p-Tie2, SDF-1/CXCR4 in the peri-infarct regions of cortex as compared with the sham group ($P < 0.05$ or $P < 0.01$). Compared with the model group, HT treatment significantly enhanced expressions of HIF-1α, VEGF, Ang-1/p-Tie2, SDF-1/CXCR4 ($P < 0.05$ or $P < 0.01$, **Figures 4**-**6**).

Discussion

HT is a traditional Chinese medicine that has been used to treat and prevent cerebrovascular diseases in China. The present results showed that HT could improve neurological function and reduce the ischemic area after stroke by enhancing angiogenesis. Furthermore, the findings also demonstrated that the mechanism of action of HT was related to the upregulation of HIF-1α, VEGF, Ang-1/p-Tie2 and SDF-1/CXCR4 in the peri-infarct regions of cortex. To our knowledge, this was the first demonstration of the effectiveness of HT on angiogenesis and the expressions of HIF-1α, VEGF, Ang-1/p-Tie2 and SDF-1/CXCR4 following cerebral I/R injury. These results provided evidence that HT might benefit the post ischemic tissue repair by promoting angiogenesis in ischemic stroke patients.

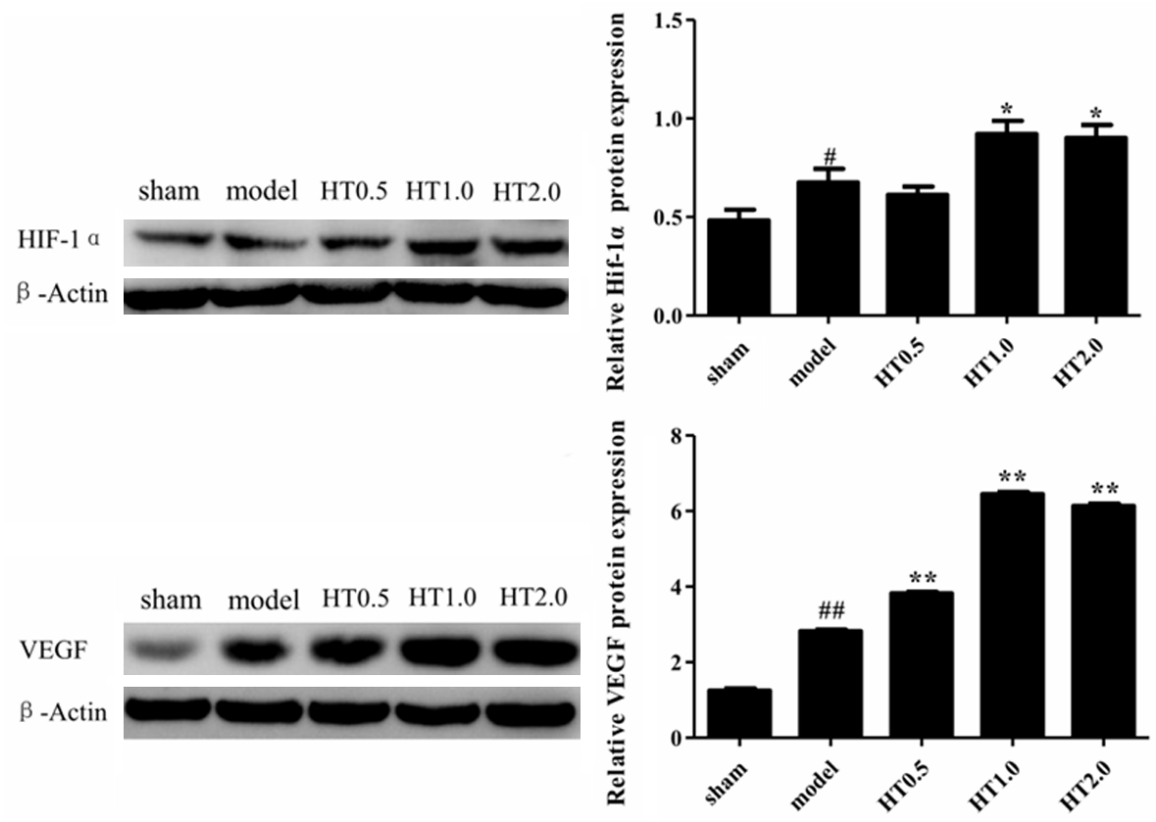

Figure 4. Effect of HT on expressions of HIF-1α and VEGF after cerebral I/R in rats. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

Middle cerebral artery occlusion in rats is the most common and preferred focal I/R injury model for imitating human ischemic stroke [8]. The 5-point scale used to evaluate the neurological function impairment was set up by the same authors who created transient middle cerebral artery occlusion with the suture [7]. In the present study, this neurologic grading scale was applied to assess the degree of neurological deficits following cerebral I/R in rats. We found that HT treatment reduced neurological deficit scores in rats with transient cerebral I/R, suggesting that HT can improve neurological function after ischemic stroke.

TTC staining is a rapid and reliable method which discriminates the intact and ischemic tissue after brain ischemia [9]. In this experiment, TTC staining showed the larger ischemic area in model group, demonstrating a successful preparation of the cerebral I/R model. Compared with the model group, the ischemic area was reduced in HT groups which indicated that HT had therapeutic effects on attenuating the cerebral I/R injury.

vWF is a protein produced by vascular endothelial cells and a marker of angiogenesis [10]. Krupinski and colleagues demonstrated that the number of new vessels within ischemia penumbral regions correlated with a longer survival in ischemic stroke patients [11]. Angiogenesis has been shown to reach a peak between 3 and 7 days post-cerebral ischemia [12]. The current study investigated the expression of vWF in the peri-infarct regions of cortex after 7-day HT treatment. The increase of the number of vWF-positive vessels in HT-treated groups demonstrated that HT enhanced the angiogenesis and thereby improved neurological function after stroke.

Seven days after administration, HT could improve the neurological function and enhance the angiogenesis in the peri-infarct regions of cortex of cerebral I/R rats. The mechanism of HT's pharmacological action might be associated with altering the microenvironment of the ischemic regions of brain. VEGF appears to be the main factor involved in the formation of new vessels during microvascular remodeling [13].

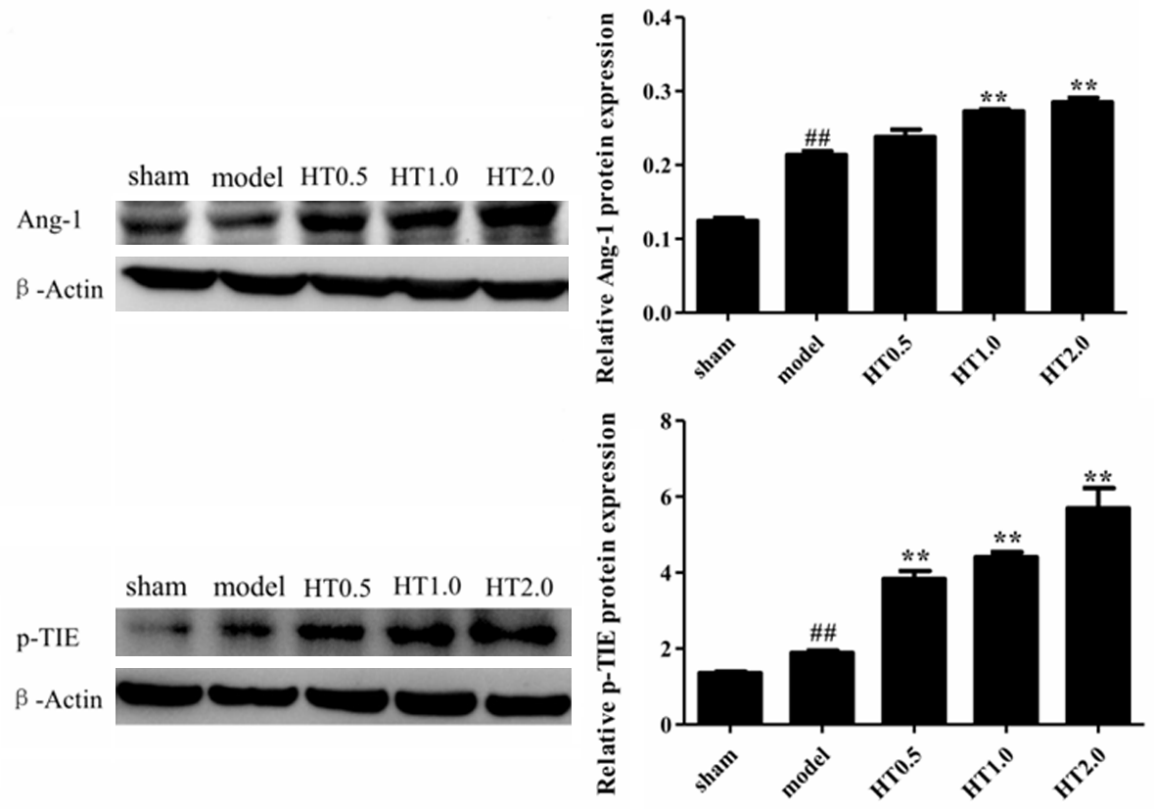

Figure 5. Effect of HT on expressions of Ang-1/p-Tie2 after cerebral I/R in rats. ##P < 0.01 vs. sham group; **P < 0.01 vs. model group.

VEGF binds to its receptor on endothelial cells to induce angiogenesis. Apart from VEGF and its receptor, the other identified endothelial cell specific growth factor/receptor system is the angiopoietin/tie system [14]. Ang-1 promotes angiogenesis and remodeling of blood vessels through its receptor tyrosine kinase Tie2 which is expressed on blood vessel endothelial cells [15]. It also reported that cerebral ischemia induces the expression of HIF-1α, which in turn triggers SDF-1 expression [16]. HIF-1α regulates the transcription of over 60 genes involved in the response to hypoxia [17]. SDF-1 is a ligand of the CXCR4. SDF-1 stimulating CXCR4 modulates the angiogenic activity and homing capacity of endothelial progenitor cells. Upregulation of SDF-1α expression represents a promising avenue for ischemic stroke therapy with a wider treatment window [18]. In our study, the expressions of HIF-1α, VEGF, Ang-1/p-Tie2 and SDF-1/CXCR4 in the peri-infarct regions of cortex were significantly increased in HT-treated rats. These findings demonstrated that the mechanism of action of HT was associated with up-regulating the expressions of HIF-1α, VEGF, Ang-1/p-Tie2 and SDF-1/CXCR4.

There are limitations in this study. Whether the increase of angiogenesis induced by HT treatment can translate into an improvement of blood flow? Whether an improvement of blood flow can play a role in neurological function recovery after HT administration? The present findings cannot answer these questions and therefore it is the limitation of this study. Further experiments are required to be done to answer these questions.

In conclusion, this study suggests that administration of HT improves neurological function and reduce the ischemic area in cerebral I/R rats. The mechanism of action of HT is related to augmenting angiogenesis via up-regulating the expression of HIF-1α, VEGF, Ang-1/p-Tie2 and SDF-1/CXCR4. Therefore, these findings may provide evidence that HT might benefit the post ischemic tissue repair by promoting angiogenesis in stroke patients.

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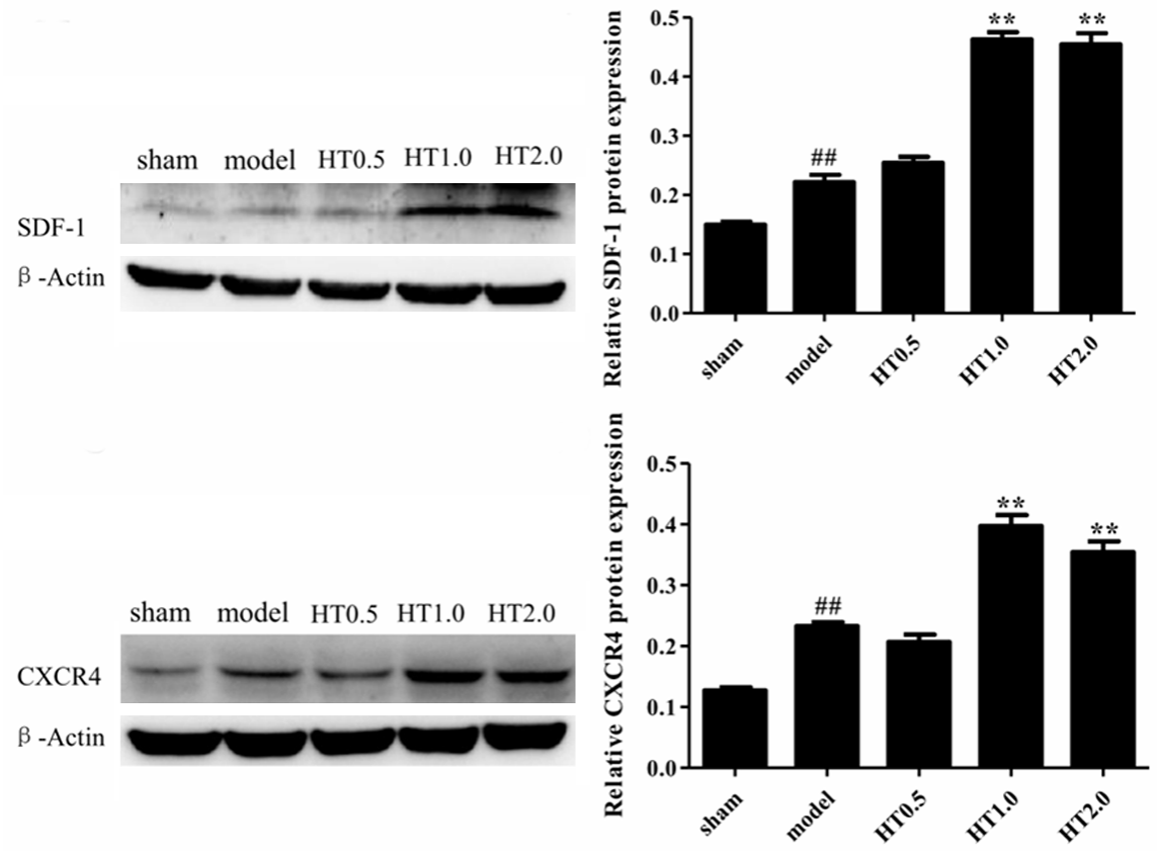

Figure 6. Effect of HT on expressions of SDF-1/CXCR4 after cerebral I/R in rats. ##P < 0.01 vs. sham group; **P < 0.01 vs. model group.

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Disclosure of conflict of interest

None.

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