Effect of brain functional recovery decoction on expression of vascular endothelial growth factor and Ang-1 protein in a rat cerebral ischemia reperfusion model

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RESULTS: VEGF mRNA expression was greater in the model group compared with the sham operation group ($P < 0.05$); Ang-1 protein expression was more obvious in the treatment group than the model group ($P < 0.05$).

CONCLUSION: BFRD promoted VEGF mRNA and Ang-1 protein expression in the brains of rats with cerebral ischemia, suggesting increased angiogenesis.

Key words: Vascular endothelial growth factors; Brain ischemia; Angiopoietin-1; Stroke; Brain functional recovery decoction

INTRODUCTION

Stroke is a disease that causes serious harm to human health, and with today’s aging population, the incidence rate of stroke increases every year. Stroke is categorized into hemorrhagic and ischemic stroke, with ischemic stroke accounting for 60%-80% of stroke cases. Blood supply should be restored to the ischemic area as soon as possible to preserve neurons, vascular endothelial cells, and glial cells. VEGF plays a role in the prevention and treatment of ischemic stroke. Angiopoietin-1 (Ang-1) is an angiogenic growth factor and plays an important role in microvascular remodeling; it improves tissue perfusion around ischemic regions, induces angiogenesis, and promotes brain ischemia area functional recovery. Molecular biological methods have been used to show the effects of focal cerebral ischemia reperfusion and Traditional Chinese Medicine on VEGF and Ang-1 expression in the rat brain.
MATERIALS AND METHODS

Animal grouping
The rats were randomly divided into a focal cerebral ischemia reperfusion model group (model group, 30 rats), model group of focal cerebral ischemia reperfusion treated with Chinese medicine (Traditional Chinese Medicine group, 30 rats), and sham operation group (sham group, 30 rats). Animals were replaced in groups where there was accidental death. The groups were further subdivided into focal cerebral ischemia 1 h reperfusion groups after 2 h, 24 h, 3, 7, and 14 d, with a total of 5 groups and 6 rats in each group. The sham operation group did not undergo additional treatment.

Model establishment
A total of 90 healthy adult rats, weighing 200-300 g, were provided by the Animal Experimental Center of Xi’an Jiaotong University, Department of Medicine. All experimental rats were housed at room temperature (25 ± 1) °C and (50% ± 1%) humidity. The rats were allowed free access to food and water, and the feeding experiment was performed after a week of maintenance. For the Traditional Chinese Medicine group and model group, middle cerebral artery occlusion was established according to previously described methods. 

"Briefly, the rats were fixed in a supine position on the operating table and were abdominally injected with 10% chloral hydrate anesthesia (0.3 mL/100 g), and a neck incision was made to reveal the carotid artery, external carotid artery, and internal carotid artery extra-cranial end. A ring groove was made in the external carotid artery stump, and a 0.22-mm-diameter nylon rope was inserted around the stump with polyurethane treatment, which was inserted 11-20 mm along the incision to the intracranial internal carotid artery. When resistance of the thread tip at the origin of the middle cerebral artery was encountered, ischemia was induced for 1 h, after which the suture was pulled back to the external carotid artery end to achieve perfusion. Intramuscular gentamicin was administered to prevent infection. For the sham operation group, the suture was only inserted to 5 mm, which did not induce middle cerebral artery occlusion, and the remaining steps were the same as the other groups. Successful model establishment was characterized; a 5-grade score was applied after the rats awoke from surgery according to the Longa standards."

Effect of brain ischemia reperfusion on the neurological behavior scores of rats
The sham operation group did not exhibit symptoms of ischemic injury. The model group and the drug group, however, exhibited symptoms of neurological dysfunction.

Treatment
The sham operation group and model group were treated with distilled water (2 mL) two times daily in the First Affiliated Hospital of Xi’an Jiao Tong University (see basic components of drug production in the Chinese medicine group discussion section). The Chinese medicine treatment group was administered 2 mL BFRD two times each day (three times the equivalent adult dosage).

VEGF mRNA in situ hybridization
In situ hybridization was performed using the VEGF mRNA in situ hybridization kit from Wuhan Boshide Company (Wuhan, China). Cells were cultured on polylysine slides, frozen, and then fixed with a 4% paraformaldehyde solution. Citric acid was used to expose the mRNA nucleic acid fragments, after which the cells were incubated in 3% fresh pepsin. Pre-hybridization was performed by incubating overnight in pre-hybridization solution at 38 °C. A protective film was placed over the glass to cover the slides. The glass slides were biotinylated mouse anti-digoxin antibody was added to the slide and incubated overnight in hybridization solution at 38 °C, and then washed and followed by the addition of Strept Avidin-Biotin Complex (SABC), and then the color reaction was initiated with 3, 3’-diaminobenzidine (DAB). The slides were then dehydrated through an alcohol gradient, cleared in xylene, and coverslipped.

Immunohistochemical detection of Ang-1 protein
The paraffin sections were incubated in 3-Aminopropyl-Triethoxysilane (APES) at 58 °C-60 °C for 30-60 min to detect the antigen. The sections were dewaxed in 30% H2O2. Ten copies were mixed with distilled water, 3 times distilled water. The sections were immersed in 0.01 M citrate buffer (pH 6.0) and microwaved in 5-minute intervals, which was repeated twice. After cooling, the sections were washed twice in poly butylenes succinate (PBS) (pH 7.2-7.6), and then biotinylated goat anti-mouse IgG was diluted with PBS and the sections were incubated at 30 °C for 20 min and subsequently washed three times in PBS for two min each. A color reaction was obtained with DAB (1 mL; DAB Kit; AR1022). Then, the ABC reagent was mixed with distilled water and added to the section, incubated at room temperature for 10 min, and the amount of staining was monitored under a microscope.

Positive cell count
Positive cells (×400) with a brown-stained cytoplasm were randomly selected from non-consecutive sections from 3 rats, and each section was quantified with an average of 10 discontinuous fields of view.

Statistical analysis
Data were expressed as mean ± standard deviation (±), and analyzed using SPSS 12.0 (SPSS Inc. SPSS Statistics for Windows, Version 12.0. Chicago, IL, USA). P < 0.05 was the level of statistical significance. The differences between groups were tested with analy-
sis of variance and post hoc least significant difference tests.

RESULTS

Detection results
In the sham operated group, the neurons were regularly arranged, with no infarction foci, no abnormalities in neuronal structure and morphology, no edema, and occasional neural cell degeneration (Figure 1).

![Image](http://www.journaltcm.com)

Figure 1 Neuronal structure and morphology in the sham operation group
A, B: sham operation group. The sham operation group was treated with distilled water (2 mL) two times daily.

VEGF protein expression
In the model group and Chinese medicine group, VEGF mRNA expression significantly increased at 2 h after reperfusion ($P < 0.01$), reached a peak at 24 h ($P < 0.01$), and remained high up to 7 d ($P < 0.01$). In the cortical region around the ischemic necrotic core, scattered positive cells were observed. Positive cells were also observed in the lateral ventricle, as well as around the hippocampus (Figure 2).

Following reperfusion, VEGF mRNA expression at 24 h, 3, 7, and 14 d was higher than in the model group, respectively ($P < 0.05$, Table 1).

Ang-1 protein expression
Differences in Ang-1 protein expression among the model group, Chinese medicine group, and sham operation group at 2 h after reperfusion were not statistically significant ($P > 0.05$). However, Ang-1 protein expression increased at 24 h after reperfusion ($P < 0.01$), and peaked at 7 d after reperfusion ($P < 0.01$). A brown staining was visible in neurons and glial in the cortex and hippocampus (Figure 3).

![Image](http://www.journaltcm.com)

Figure 2 VEGF expression at 24 h in the groups ($\times 400$)
A: the model group; B: Traditional Chinese Medicine group. The model group was treated with distilled water (2 mL) two times daily; the Chinese medicine treatment group was administered 2 mL BFRD two times each day. VEGF: vascular endothelia growth factor.

![Image](http://www.journaltcm.com)

Figure 3 Expression of Ang-1 protein in groups ($\times 400$)
A: the model group; B: Traditional Chinese Medicine group. The model group was treated with distilled water (2 mL) two times daily; the Chinese medicine treatment group was administered 2 mL brain functional recovery decoction two times each day.

From 2 h to 7 d after reperfusion, Ang-1 protein expression significantly increased in the model group; expression increased at 2 h after reperfusion ($P < 0.05$), became even more significant at 24 h ($P < 0.01$), and remained high at 7 d compared with the sham operation group. At 3 d after reperfusion, expression increased and reached a peak, with levels remaining high at 7 d ($P < 0.05$, Table 2).

![Image](http://www.journaltcm.com)

Table 1 VEGF expression in rats ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>2 h</th>
<th>24 h</th>
<th>3 d</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>6</td>
<td>4.1±0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>7.2±1.3$^a$</td>
<td>25.2±4.2$^a$</td>
<td>20.3±3.5$^a$</td>
<td>11.2±2.6$^a$</td>
<td>7.5±1.7$^a$</td>
</tr>
<tr>
<td>Traditional Chinese Medicine</td>
<td>6</td>
<td>13.2±4.2$^a$</td>
<td>40.6±5.1$^a$</td>
<td>36.0±3.2$^a$</td>
<td>16.9±2.4$^a$</td>
<td>10.0±2.1$^a$</td>
</tr>
</tbody>
</table>

Notes: the model and sham group was treated with distilled water (2 mL) two times daily; the Chinese medicine treatment group was administered 2 mL BFRD two times each day. Compared with the sham operated group, $^aP < 0.01$; compared with the model group, $^bP < 0.01$, $^cP < 0.05$. VEGF: vascular endothelia growth factor; BFRD: brain functional recovery decoction.

![Image](http://www.journaltcm.com)

Table 2 Ang-1 protein expression in rats ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>2 h</th>
<th>24 h</th>
<th>3 d</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
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<td>5.2±1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>5.7±0.8$^b$</td>
<td>8.7±2.3$^b$</td>
<td>10.4±2.5$^b$</td>
<td>13.1±2.9$^b$</td>
<td>20.2±3.1$^b$</td>
</tr>
<tr>
<td>Traditional Chinese Medicine</td>
<td>6</td>
<td>7.2±1.2$^c$</td>
<td>11.4±2.3$^c$</td>
<td>18.7±3.2$^c$</td>
<td>17.1±2.4$^c$</td>
<td>28.2±4.5$^c$</td>
</tr>
</tbody>
</table>

Notes: the model and sham group was treated with distilled water (2 mL) two times daily; the Chinese medicine treatment group was administered 2 mL BFRD two times each day. Compared with the sham operated group, $^bP < 0.01$, $^cP < 0.05$; compared with model group, $^bP < 0.05$, $^cP < 0.01$. BFRD: brain functional recovery decoction.
DISCUSSION

BFRD is mainly composed of the Huangqi (Radix Astragali Mongolici), Danggui (Radix Angelicae Sinensis), Chuanxiong (Rhizoma Chuanxiong), Dilong (Phereetima Aspergillum), Chishao (Radix Paeoniae Rubra). Huangqi (Radix Astragali Mongolici) is the primary drug in this Chinese medicine prescription. “Qi” herbs most,” ”blood” gas called into the lung to enhance the atmosphere, gas blood is handsome; Wang Qingren “Qi and blood.” Research has shown that Huangqi (Radix Astragali Mongolici) has the following functions: (a) free radical scavenging, reducing quality of plasma endothelin content, improving brain function; (b) reduces content of excitatory amino acids, reduces ischemic brain damage; (c) increases Bcl-2 expression after cerebral ischemia, increases expression of p53 and caspase-3 antagonists, effectively inhibits brain ischemia-induced neuronal apoptosis. Chuanxiong (Rhizoma Chuanxiong) is considered a “blood gas medicine.

In recent years, studies have shown that Ligustrazine can expand blood vessels and improve cerebral circulation to protect brain cells. Dilong (Phereetima Aspergillum) are the active ingredient in Lumbrokinase-like substances and exhibit strong fibrinolytic activity in vitro and in vivo. It promotes expression of tRNA, and has a strong anti-coagulation and thrombolytic effect. Reduced fibrinolytic activity can, therefore, promote thrombus dissolving. Total paonxy saponin has been shown to reduce and prevent neural apoptosis, thereby effectively preventing cerebral ischemia and the expansion of a cerebral embolism.

VEGF is a protein composed of two xylose molecules. The peptide contains two disulfide bonds to constitute two dimers. There are two VEGF receptors, namely FLT-1 and FLT-1/KD, which are mainly expressed in neurons and glial cells, as well as vascular endothelial cells. VEGF directly binds to vascular endothelial cells and induces expression of tissue the base factor and collagenase, thereby stimulating release of vWF from endothelial nerves and altering the extracellular matrix. Conducive to the growth of vascular endothelial cells, so that endothelial cells from apoptosis. Ang-1 expression in perivascular cells, including pericytes, endothelial cells, and vascular smooth muscle cells, promotes activity of vascular pericytes and endothelial cells, thereby promoting stability, maturation, and remodeling of new blood vessels.

Results from the present study showed an increase in VEGF mRNA expression in the Chinese medicine group at 2 h after reperfusion compared with the model group, which peaked at 24 h, and remained high at 14 d. VEGF expression in the Traditional Chinese Medicine group was significantly greater than in the model group. In the Chinese medicine group at 2 h after reperfusion, Ang-1 protein content increased compared with the sham group; compared with the model group, Ang-1 expression increased at 2 h after reperfusion and reached the peak at 14 d. Ang-1 protein expression in the Chinese medicine group was significantly greater than in the model group. Results showed that BFRD improved VEGF mRNA expression and Ang-1 protein expression after cerebral ischemia. BFRD also improved the microvascular system in the reconstruction process, suggesting that BFRD could be used to promote vascular focal cerebral ischemia reperfusion injury. Further studies are needed, however, to determine the impact of other angiogenic factors.

REFERENCES