Combinatorial effects of Naomai Yihao Capsules (脑脉一号胶囊) and vascular endothelial growth factor gene-transfected bone marrow mesenchymal stem cells on angiogenesis in cerebral ischemic tissues in rats

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Abstract

OBJECTIVE: To investigate the combinatorial effects of Naomai Yihao (NMYH) Capsules (脑脉一号胶囊) and vascular endothelial growth factor (VEGF) gene-transfected bone marrow mesenchymal stem cells (BMSCs) on angiogenesis in cerebral ischemic tissues in rats and the mechanism.

METHOD: BMSCs were isolated and cultured from bone marrow by an adherence method. Then, BMSCs were transfected with the eukaryotic expression plasmid pEGFP-VEGF165 by positive ionic liposome transfection. A rat model of middle cerebral artery occlusion (MCAO) was established. Rats were allocated to six groups: model, BMSC, VEGF gene-transfected BMSC transplantation (BMSC/VEGF), NMYH, combined NMYH and BMSC/VEGF (combined treatment group) and sham operation groups. The behavioral rating score (BRS) of rats and the expression of CD34 and VEGF in brain tissue were measured by immunohistochemistry on days 7, 14 and 21 after reperfusion. Angiogenesis was observed and evaluated with laser scanning confocal microscopy.

RESULTS: The BRS of rats in NMYH, BMSC transplantation and combined treatment groups was significantly lower than that of the model group (P<0.001), with no significant difference between NMYH and transplantation groups (P=0.619). The expression of CD34 and VEGF in NMYH, transplantation and combined treatment groups increased (P<0.001), with a significant difference between NMYH and transplantation groups (P<0.001). The blood vessel area in NMYH, transplantation and combined treatment groups was significantly increased (P<0.05), without a significant difference between NMYH and transplantation groups (P=0.873).

CONCLUSIONS: VEGF gene-transfected BMSCs improve angiogenesis in the cerebral ischemic area. NMYH Capsules promote angiogenesis in MCAO rats treated with BMSC transplantation, which show an improved BRS. The mechanism of angiogenesis may be related to up-regulation of VEGF expression.

Key words: Bone marrow mesenchymal stem cells; naomai yihao capsule; Vascular endothelial growth factor; Eukaryotic expression plasmid; Transplantation
INTRODUCTION
It is believed that an ideal treatment has not been developed for ischemic stroke, hence researchers attempt to seek a solution by combining the latest biomedical technologies with complementary/alternative methods. One potential solution is bone marrow mesenchymal stem cells (BMSCs) that can differentiate into neurons and glial cells to replace necrotic neural cells due to ischemia and hypoxia to promote the recovery of neurological function. BMSCs can also differentiate into vascular endothelial cells that produce various nutritional factors in autocrine and paracrine manners to promote angiogenesis in the ischemic area. Vascular endothelial growth factor (VEGF) is currently the strongest growth factor for vascular growth, hence it is expected that transplantation of VEGF gene-transfected BMSCs may have a robust effect on angiogenesis. However, survival and differentiation rates of newly transplanted cells are low because of local intensive inflammation, an oxidative stress reaction and proapoptotic and chemical factors caused by ischemia, which lead to the restriction of therapeutic effects. Fortunately, current studies show that there are various approaches by Chinese herbal medicine which promote the proliferation and differentiation of BMSCs and help to form a microenvironment suitable for the survival of transplanted cells. The Naomai Yihao (NMYH) Capsule is a product of Chinese herbs based on the Chinese medical theory to regulate the sea of blood in the brain. The capsule has been proven effective for the treatment of ischemic stroke in our previous clinical studies, and it improves the patients’ daily function impaired by cerebral ischemia and enhances the quality of life for stroke patients. Animal experiments also show that NMYH improves angiogenesis in focal cerebral ischemic rats treated with BMSC transplantation to enhance the behavioral rating score (BRS), in which stronger effects are expected with a longer treatment period.

In this study, we explored the combinatorial effect of NMYH Capsules and VEGF gene-transfected BMSCs on angiogenesis after cerebral ischemia, which may introduce a new approach for stem cell therapy assisted by Chinese herbal medicine to treat brain infarction.

MATERIALS AND METHODS

Experimental animals
Ninety-five specific pathogen free (SPF) Sprague-Dawley rats were used in this study. Rats were 3–4 months of age, weighed 250–330 g, and provided by the Experimental Animal Center of Southern Medical University, certificate number: 0051720.

Reagents and instruments
Naomai Yihao (NMYH) Capsules were composed of Zhi Tianma (Rhizoma Gastroae), Shuizhi (Hirudo), Huangqì (Radix Astragali), Zhi Nanxing (Arisaema cum Bile), Banxia (Rhizoma Pinelliae), Chuanqiong (Rihzama Chuanxiong) and Yimucao (Herba Leonuri), and prepared and provided by the Pharmacy Department of the 2nd Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China). The production lot was 090702. Each capsule contained the equivalent of 1 g crude drug. The providers of reagents, materials and instruments are as follows: pEGFP-VEGF165 plasmid, the Stem Cell Research Center of Sun Yat-sen University (Guangzhou, China); Lipo-fectamine 2000, Invitrogen Co Ltd. (Grand Island, NY, USA); plasmid extraction kits, Tiangen Biotech (Beijing) Co Ltd. (Beijing, China); rabbit anti-rat VEGF antibody (Lot No. BS-0279R) and rabbit anti-rat CD34 antibody (Lot No. BS-0646R), Beijing Biosynthesis Biotechnology Co Ltd. (Beijing, China); SABC (Lot No. SP-9001) and DAB (Lot No. ZLI-9032) kits, Zhongshan Goldenbridge Biotechnology Co Ltd. (Beijing, China); fluorescein isothiocyanate-labeled dextran (FITC-Dextran; molecular weight: 2 x 10^5), Sigma-Aldrich Co., (St. Louis, MO, USA); laser scanning confocal microscope (LSCM), LEICA Microsystems Ltd., (Wetzlar, Germany); and Image-Pro Plus 6.0 image analysis software (Model No. 41M60032-00032), Media Cybernetics Inc., (Bethesda, MD, USA).

Isolation, culture and VEGF gene transfection of BMSCs
Bilateral femoral bone marrow of SD rats aged 6–8 months was harvested and resuspended as single cells, then cultured in 0.1% fetal bovine serum (FBS) in low glucose Dulbecco’s modified Eagle medium (L-DMEM) in an incubator at 37°C with 5% CO₂. Differential attachment culture was used for cell purification, and cells were subcultured at 90% confluence. Third passage BMSCs were collected for transfection after optimizing the conditions as follows: 80%–90% confluence, 6 μg/mL DNA concentration and a 3 μg:4 μL ratio for DNA: Lipo-fectamine 2000. The 0.1% FBS L-DMEM was exchanged after 6 h, and incubation continued. Cells were collected after 48 h of transfection.

Establishment of the MCAO rat model
The MCAO rat model was established by the Zea Longa method with some modification. The middle cere-
Biopsy specimens were dewaxed and hydrated, then the antigens were recovered by microwave heating in citrate buffer (pH=6.0). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Primary antibodies (1:50; rabbit anti-rat VEGF and CD34) were added to the specimens after blocking with goat serum for 15 min. Then, specimens were stored overnight at 4°C, washed twice in cold PBS and incubated with a biotinylated goat anti-rabbit IgG at room temperature for 60 min followed by a horseradish peroxidase-labeled avidin working solution. Specimens were colored by a DAB chromogen, stained with hematoxylin, dehydrated in an alcohol gradient, transparentized with xylene and mounted with neutral gum. The stratum area of each processed specimen was observed with LSCM (400 x magnification) for positive cell counting in five random fields of vision.

**Group allocation and treatments**

Rats were allocated to operation groups, and the sham operation group in which the middle cerebral artery was not occluded. Operation groups included model, BMSC model, VEGF gene-transfected BMSC transplantation (BMSC/VEGF), NMYH, and combined NMYH and BMSC/VEGF (NMYH + BMSC/VEGF) groups. Each operation group was randomly divided into three subgroups based on the length of time for sampling (day 7, 14 and 21 groups). At each time point, five, five and eight specimens were respectively collected from each operation group, and another five specimens from the sham operation group that was not subdivided for the various time points.

After 24 h of reperfusion, rats received an injection of 10 μL cell suspension into the right striatum (AP=0 mm, MR=2.0 mm, DV=4.5 mm) using a stereotactic apparatus. The injection for BMSC/VEGF and NMYH + BMSC/VEGF groups contained 5 × 10⁵ VEGF gene-transfected BMSCs. The injection for the BMSC model group contained 5 × 10⁶ BMSCs. The injection for NMYH and sham operation groups contained L-DMEM without serum. After transplantation, rats in NMYH and NMYH + BMSC/VEGF groups were administered the NMYH herbal compound by gavage at 1.5 g/kg. Sham operation, BMSC and BMSC/VEGF groups were simultaneously administered normal saline at an equivalent volume. The drug delivery frequency was once per day. The drug dosage and liquid volume were adjusted weekly according to the body mass of the rats until specimens were collected.

**Behavioral rating scale (BRS) for the nerve function of rats**

The nerve function of rats after reperfusion was assessed by the mNSS scale that includes muscle strength and tone, consciousness, masonic movement and reflex on days 7, 14 and 21 after treatment. The highest BRS score is 18. Higher scores indicate a more serious nerve injury.

**Detection of the expression of VEGF and CD34 by immunohistochemistry**

Biopsy specimens were dewaxed and hydrated, then the antigens were recovered by microwave heating in citrate buffer (pH=6.0). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Primary antibodies (1:50; rabbit anti-rat VEGF and CD34) were added to the specimens after blocking with goat serum for 15 min. Then, specimens were stored overnight at 4°C, washed twice in cold PBS and incubated with a biotinylated goat anti-rabbit IgG at room temperature for 60 min followed by a horseradish peroxidase-labeled avidin working solution. Specimens were colored by a DAB chromogen, stained with hematoxylin, dehydrated in an alcohol gradient, transparentized with xylene and mounted with neutral gum. The stratum area of each processed specimen was observed with LSCM (400 x magnification) for positive cell counting in five random fields of vision.

**Observation of angiogenesis with LSCM**

After 2 days of treatment, rats were anaesthetized and injected with FITC-dextran (50 mg/mL) in the tail vein (1 mL for each rat). FITC-dextran is maintained in a free state in plasma during vascular circulation. One minute after injection, rats were sacrificed by decapitation. Brain hemispheres were fixed in paraformaldehyde for 48 h at 4°C and then stored in a 20% sucrose solution. Serial coronal sections of specimens were prepared with a frozen sectioning machine after OTC embedding, in which the section region was between 5.2 and 5.8 mm from the anterior fontanel. Five 100 μm sections of each specimen were cut for every 2 mm of tissue. LSCM (200 x magnification) was applied to scan both the ischemic and contralateral sides (five regions each were selected for each side) under the following settings: 488 nm excitation wavelength, 522 nm emission wavelength, 512 x 512 pixels X-Y plane, 4 x average frame scanning, 1 μm Z-axis as the step sequence with scanning for 30 layers. The vessel diameter and area were measured with the image analysis software Image-Pro Plus 6.0.

**Statistical analysis**

Statistical analysis was performed by SPSS version 11.0. After testing for data normality and variance homogeneity, multiple factor analysis of variance was used to analyze the relationship between transplantation, NMYH capsules, observed time points and their interactive effects. Measurement data were presented as the means ± standard deviation (SD). The significance level was α=0.05.

**RESULTS**

**BRS scores of nerve function in rats**

The BRS score of the sham operation group was 0 because there were no neurological deficits found. All sub-


CD34 expression
There was no CD34 expression in the sham operation group. CD34 was expressed in all operation groups on day 7 after the operation, which peaked on day 14 and began to decrease on day 21. The pattern of CD34 expression in all operation groups was the same as that of the model group (See Table 1). Transplantation, NMYH capsules and observation time were influential factors for CD34 expression (P<0.001). In addition, the NMYH+BMSCs/VEGF group showed the highest CD34 expression among operation groups with interacting effects (P<0.001). High CD34 expression is a marker of angiogenesis, thus these results implied that NMYH promotes vascular regeneration in the ischemic area after BMSC/VEGF transplantation. See Figure 1.

VEGF expression
There was no VEGF expression in the sham operation group. VEGF expression peaked in all operation groups on day 7 after the operation and decreased from day 14 to 21. The pattern of VEGF expression in operation groups was the same as that of the model group (See Table 1). Transplantation, NMYH capsules and observation time were influential factors for VEGF expression (P<0.001). In addition, the NMYH+BMSCs/VEGF group showed significantly higher VEGF expression among operation groups (P<0.001), which implied that NMYH promoted angiogenesis in the ischemic area after transplantation of VEGF gene-transfected BMSCs. There were interacting effects between transplantation and observation time (P<0.05), which indicates that improved therapeutic effects can be expected as time progresses. See Figure 2.

Angiogenesis
Microvessel diameters in cerebral tissue increased after ischemia, compared with those of the non-ischemic side. Neither BMSC transplantation nor administration of NMYH capsules showed a significant effect on changes in microvessel diameter, and showed no interacting effects (P=0.198). However, both BMSC transplantation and NMYH capsules significantly affected vessel area (P<0.05). The combination of BMSC/VEGF transplantation and NMYH resulted in the largest vessel area, compared with those of other groups, but both showed no interacting effects (P=0.873). (See Table 2 and Figure 3).

DISCUSSION
Therapeutic angiogenesis is a current research focus of therapies for ischemic disease. BMSCs can directly differentiate into vascular endothelial cells, and secrete autocrine and paracrine growth factors to promote angiogenesis in the ischemic area[12]. VEGF is the strongest angiogenesis factor that efficiently and directly binds two receptors on vascular endothelial cells, namely Flt and Flk/KDR that accelerate endothelial cell migration and angiogenesis by stimulating the proliferation of endothelial cells and producing plasminogen activator and collagenase[13]. In addition, VEGF also directly enhances neurogenesis and protects neurons[14] by stimulating axonal growth and reducing neural cell death induced by hypoxia or excitatory amino acids. VEGF gene expression is regulated by hypoxia-inducible fac-

Table 1 BRS scores and positive VEGF and CD34 expression among operation groups ( \( \bar{x} \pm S, n=5 \) )

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (Day)</th>
<th>BRS</th>
<th>VEGF</th>
<th>CD34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>7</td>
<td>9.13±1.25</td>
<td>32.40±3.91</td>
<td>15.20±2.77</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.75±0.71</td>
<td>20.20±2.59</td>
<td>19.20±3.11</td>
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<tr>
<td></td>
<td>21</td>
<td>5.36±1.03</td>
<td>15.80±1.92</td>
<td>14.20±2.17</td>
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<tr>
<td></td>
<td>7</td>
<td>8.75±0.89</td>
<td>48.80±3.96</td>
<td>23.40±2.07</td>
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<tr>
<td>BMSCs</td>
<td>14</td>
<td>6.43±0.74</td>
<td>29.40±3.36</td>
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<td>4.53±0.62</td>
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<td>23.80±2.49</td>
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<td></td>
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<td>8.63±0.92</td>
<td>55.60±6.02</td>
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<tr>
<td>BMSCs/VEGF</td>
<td>14</td>
<td>6.50±1.07</td>
<td>32.20±3.96</td>
<td>29.20±3.33</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>4.27±1.01</td>
<td>25.20±2.39</td>
<td>25.40±3.05</td>
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<tr>
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<td>7.75±0.89</td>
<td>50.60±4.16</td>
<td>24.80±2.39</td>
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<tr>
<td>NMYH</td>
<td>14</td>
<td>6.38±1.30</td>
<td>28.20±3.35</td>
<td>27.80±3.96</td>
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<tr>
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<td>24.20±2.77</td>
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<tr>
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<td>7</td>
<td>7.50±0.93</td>
<td>58.20±6.22</td>
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<td>NMYH+BMSCs/VEGF</td>
<td>14</td>
<td>5.38±0.92</td>
<td>33.20±3.27</td>
<td>34.20±4.60</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>3.36±1.02</td>
<td>29.20±3.35</td>
<td>30.40±4.04</td>
</tr>
</tbody>
</table>

Table 2 Outcome of angiogenesis among groups ( \( \bar{x} \pm S, n=3 \) )

<table>
<thead>
<tr>
<th>Group</th>
<th>Vessel diameter (( \mu m ))</th>
<th>Vessel area (( \mu m^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral</td>
<td>1.29±0.09</td>
<td>830.15±117.74</td>
</tr>
<tr>
<td>Model</td>
<td>1.31±0.10</td>
<td>698.75±117.28</td>
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<tr>
<td>BMSCs</td>
<td>1.46±0.11</td>
<td>893.91±110.93</td>
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<tr>
<td>BMSCs/VEGF</td>
<td>1.46±0.10</td>
<td>915.34±188.31</td>
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<tr>
<td>NMYH</td>
<td>1.47±0.11</td>
<td>917.58±169.34</td>
</tr>
<tr>
<td>NMYH+BMSCs/VEGF</td>
<td>1.49±0.12</td>
<td>1111.01±192.13</td>
</tr>
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</table>
Guo JW et al. Effects of Naomai Yihao & VEGF gene-transfected BMSCs on angiogenesis in brain ischemic tissues, in which angiogenesis occurs upon stimulation by ischemia or hypoxia with increased VEGF expression that gradually reduces within 2 – 4 days. As a result, this limited angiogenesis cannot restore endothelial cell integrity and build effective collateral circulation[15]. Therefore, sufficient and timely supply of VEGF is beneficial during the critical period of angiogenesis[11].

In our study, BMSCs transfected with the eukaryotic expression vector pEGFP-VEGF165 were transplanted into cerebral ischemic rats, and it was found that the gene expression of VEGF and CD34 in the transfected groups was higher than that in the non-transfected groups. This outcome implies that angiogenesis in the ischemic area is related to VEGF expression.

Chinese medicine has shown progress as a treatment to enhance the effectiveness of BMSC transplantation to treat ischemic diseases. Recent studies indicate that Chinese medical herbs promote blood circulation and remove blood stasis to facilitate angiogenesis by increasing the level of growth factors in the brain[16], which increases blood supply in microcirculation. Chinese medical herbs that eliminate heat and detoxify can reduce the expression of inflammatory factors and permeability of the blood-brain barrier[6], which are favorable to the survival of transplanted cells in the microenvironment. Bi et al[7] found that salvia miltiorrhiza monomer, namely Salvianolic acid B (Sal B), increases cell survival rates in BMSC transplantation which were 2 and 3 times higher than those of the non-transplanted group on days 7 and 28, respectively. It was hypothesized that the mechanism was Sal B protecting BMSCs from damage by tumor necrosis factor.

The NMYH capsule is a medicinal Chinese herbal compound based on the therapeutic principle of regulating blood flow[17], in which the clinical effectiveness has been assessed and proven by a prospective multi-center randomized controlled trial[8]. NMYH capsules include Huangqi (Radix Astragali), Chuanqiong (Rhizama Chuanxiong), Zhi Nanxing (Arisaema cum Bile) and Shuizhi (Hirudo), and their active components inhibit inflammatory responses[18], reduce apoptosis[19], eliminate oxygen free radicals and improve microcirculation[20]. Therefore, the combination of NMYH capsule administration and BMSC transplantation may promote angiogenesis and increase the survival of transplanted cells in ischemic cerebral tissue and reduce neuronal apoptosis.

Our study showed that the expression of VEGF and CD34 significantly increased in the NMYH group, compared with that of the model group, and the expression of VEGF and CD34 in the combined NMYH + BMSC/VEGF group increased significantly, compared with that of BMSC transplantation alone. These findings imply that NMYH may play a role in the treatment of cerebral ischemia. The mechanism may be enhanced blood supply to ischemic cerebral tissues by improving angiogenesis after transplantation of VEGF gene-transfected BMSCs, and an improvement
of impaired nerve function. The interacting effects between VEGF expression and treatment duration indicated that enhanced therapeutic effects may be observed after a longer time-period. The absence of an interacting effect between NMYH and BMSC/VEGF transplantation to improve BRS and the vessel area of rats is possibly related to the limitation of the observation period and NMYH dosage. Therefore, further study should explore the effects on angiogenesis after BMSC/VEGF transplantation at various dosages of NMYH with a longer observation period.

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