Basic Investigations

Effects of Buyang Huanwu Tang Combined with Bone Marrow Mesenchymal Stem Cell Transplantation on the Expression of VEGF and Ki-67 in the Brain Tissue of the Cerebral Ischemia-Reperfusion Model Rat

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Objective: To explore the mechanism of Buyang Huanwu Tang (补阳还五汤 Decoction Invigorating Yang for Recuperation) combined with bone marrow mesenchymal stem cells (MSCs) transplantation in protecting nerves of cerebral ischemic injury.

Methods: Local cerebral ischemia-reperfusion rat model was established with modified Zea-Longa thread-occlusion method, and MSCs were injected into the caudal vein, and Buyang Huanwu Tang (补阳还五汤) was administrated. Vascular endothelial growth factor (VEGF) and Ki-67 expression in the ischemic side of the brain in the cerebral ischemic-reperfusion rat were detected with immuno-histochemical staining method.

Results: VEGF and Ki-67 expressions were significantly up-regulated in the MSCs group and the combination group, with significant differences as compared with the model group and the sham operation group (P<0.05), and with the most strongest effect in the combination group.

Conclusion: Buyang Huanwu Tang (补阳还五汤) combined with MSCs transplantation repairs the injured blood vessels and lesion tissues possibly by up-regulation of VEGF and Ki-67 expression.

Keywords: Buyang Huanwu Tang; marrow mesenchymal stem cell; cerebral ischemia-reperfusion; immunohistochemical method; vascular endothelial growth factor (VEGF) and Ki-67

Recovery of blood flow supply after cerebral ischemia is undoubtedly a necessary treatment measure, but cerebral ischemia-reperfusion exacerbates the dysfunction and structure injury of the cerebral tissue, which is called cerebral ischemia reperfusion injury (CIRI). The previous study proved that Buyang Huanwu Tang (补阳还五汤) combined with bone marrow mesenchymal stem cells (MSCs) transplantation functions anti-cerebral ischemia-reperfusion injury by inducing differentiation of neuron-like cells, inhibiting nervous cell apoptosis and reducing the infarction area, etc.2-4

The study is aimed at investigating effects of Buyang Huanwu Tang (补阳还五汤) combined with MSCs transplantation on expression of vascular endothelium growth factor (VEGF) and Ki-67 related with synthetic metabolism of cells, so as to explore the mechanism from influencing regulative factors of angiogenesis.

MATERIAL AND METHODS

Experimental Animal and Grouping
Forty-eight SD rats, sanitary degree, aging 3 months, weighing 280–320 g, were purchased from the Henan Province Lab Animals Center, and randomly divided into 4 groups, sham operation group, model group, MSCs transplantation (MSCs) group, combined Buyang Huanwu Tang and MSCs transplantation group (combination group), 12 rats in each group.

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Experimental Instruments and Main Reagents
BHW-IV incubator (Beijing City Medical Equipment Factory), BH-2 light microscope (Olympus, Japan), electric heating thermostatic water-bath box (Beijing Changyuan Experimental Equipment Factory), Leica RM2135S paraffin microtome (Germany), image analysis software (Image-Pro Plus 5.0: Media Cybernetics, USA), DMEM culture medium (Gibco BRL Company), lymphocyte separating medium (Shanghai Hengxin Company), trypsin (Huamei Company), fetal calf serum (Hyclone Company, Batch number: sh30070.03), Sp-9000/9001/9002 immunohistochemical staining kits (Beijing Zhongshan Jingqiao Biological Technique Co. Ltd), rabbit anti-Ki-67 antibody (Beijing Boashen Biological Technique Co. Ltd, batch number: bs-0722R), rabbit anti-VEGF antibody (Wuhan Boster Biological Technique Co. Ltd, Batch number: BA0407).

Preparation of Experimental Drugs
Buyang Huanwu Tang (补阳还五汤) was prepared by the Section of Pharmaceutics, the First Affiliated Hospital, Henan College of Traditional Chinese Medicine. Danggui (当归 Radix Angelicae Sinensis) 60g, Taoren (桃仁 Semen Persicae) 30g, Chuanxiong (川芎 Rhizoma Chuanxiong) 30g were added with 720 ml water to extract volatile oil for use. The dregs of Rhizoma Chuanxiong 30g were added with 8640 ml water and decocted for another one hour and filtered. The two decoctions were mixed and concentrated to 600 ml, which was added with the volatile oil and 3 ml Tween-80, mixed again, and poured into bottles and sterilized at high temperature and high pressure, each milliliter containing 2.4 g the crude drugs.

Preparation of Local Cerebral Ischemia Rat Model
Referring to the modified Zea-Longa method, the cerebral ischemia-reperfusion injury rat model was prepared. After the rat was anaesthetized with intraperitoneal injection of 10% chloral hydrate (35 mg/100 g) and routinely disinfected, an incision was made along the cervical median, then the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were separated. The proximal end of CCA was clipped with a bulldog clamp and ECA was ligated. A nylon thread plug was inserted into CCA and delivered to the intracranial part via ICA until a little resistance was felt. The plug was inserted into (19±0.5) mm away from the bifurcation of ICA and ECA. After ischemia of 2 hours, the nylon thread was slightly withdrawn, and when a resistance was felt, indicating that one end of the thread came to the trunk of ICA. Then the clamp was loosened, thus, the reperfusion of the middle cerebral artery was carried out. For the rat in the sham operation group, the blood vessels were separated only, without thread inserted. After the operation, the rats were free access to food and water.

Preparation of MSCs
Marrow mononuclear cells (MNCs) were separated with the density gradient centrifugation method. The marrow of the rat was slowly added into the upper layer of lymphocyte separating medium with a specific gravity of 1.077 g/cm³ in an equal volume (1:1). After horizontal centrifugation at 2000 rpm for 25 min, the mononuclear cells layer was carefully sucked and added with phosphate buffer solution (PBS) of 3–5-fold volume, centrifuged at 1500 rpm for 15 min, washed twice, the supernatant was removed. Finally, the MNCs were stained with trypan-blue, and the living cells were counted under the light microscope and regulated as 1.0x10⁶/ml and inoculated in a T-75 culture flask with DMEM culture solution containing 10% fetal serum, which was placed in a CO₂ incubator with saturated humidity and 5% CO₂, at 37°C. After 5 days, the culture medium was replaced and the non-adherent cells were discarded, and afterwards, the medium was completely replaced once each 3 days, and then cellular morphological changes were observed and recorded under an inverted microscope. After complete confluence, the adherent cells were digested with 0.25% (mass/volume) trypsin and passed, with the medium completely replaced once each 3 days. After continuous passage, MSCs were amplified and purified.

Transplantation of MSCs and Administration Methods
After local cerebral ischemia of 2 hours and reperfusion for one day, 1x10⁶ MSCs suspension was injected into the caudal vein for each rat in both the MSCs and combination groups; and 1 ml saline solution for the sham operation and model groups. Saline was administrated for the rats of the sham operation, model group and MSCs groups in a dose of 15 ml/kg by intra-gastric perfusion, and corresponding drugs for the
combination group in 15 ml/kg. The administration started 3 days before modeling, once each day, for 10 consecutive days.

**Sampling and Preparation of Tissue Specimens**
After cerebral ischemia of 2 hours and reperfusion for 7 days, the rat was killed by decapitation and the brain was taken and fixed by perfusion of 4% formaldehyde, then the brain tissue were cut into sections of 2 mm in thickness and routinely embedded with paraffin, which were made coronal sections of 3 µm in thickness. For each rat, three sets of the sections were taken for staining. The first set of the section was stained with HE and used for location of cellular layers and cell nuclei groups, the second and third sets were respectively used for VEGF and Ki-67 immunohistochemical staining.

**HE Staining and Immunohistachamical Reaction**
The sections were stained with Hematoxylin-eosin (HE) and observed under the light microscope. Immunohistochemical staining was conducted according to directions of the kids. Five different high power fields (400x) for each section was randomly selected and positive cells number in each visual field were counted and the means were calculated. The titer of the first antibody of VEGF was 1:50 and the positive granules in the cytoplasm showed pale brown under the optic microscope, and the titer of the first antibody of Ki-67 was 1:100 and the positive granules in the nuclear showed pale brown. For the positive control, except cerebral tumor tissue was used, all other procedures were completely as same as the above routine methods with same reactions and staining methods. All the sections after staining, alcohol dehydration, xylene clearing and neutral gum mounting were carried out.

**Statistical Analysis**
The data were expressed as $\bar{x} \pm s$, and processed with SPSS13.0 statistical software, and variance analysis and comparison between groups were made.

**RESULTS**

**Pathomorphological Changes**
HE staining showed that in the sham operation group, the structure and form of the nerve cell were normal, with round or oval nuclei, obvious nuclei and nucleoli, the cytoplasm with no red-staining, and with no edema of cells and intercellular substance; In the model group, degeneration and necrosis of a great number of nerve cells, with cell body shrinking, pyknosis, breaking and dissolution of the nucleus, concentrated cytoplasm and red-staining, obvious edema of intercellular substance, and infiltration of inflammatory cells were seen under the light microscope; In both the MSCs group and the combination group, the number of degenerative and necrotic nerve cells were reduced with the degree obviously alleviated. VEGF and Ki-67 immune positive granules all were pale brown and dark brown, which could be seen in the neurons and ganglia cells of the cerebral cortex, hippocampus region, striatum and other areas, and in the cytoplasm of a part of vascular endotheliocytes (Fig.1, and 2).

**VEGF Expression in the Rat Brain Tissue of the Groups** (Table 1)
Table 1 shows that almost no VEGF expression was found in the brain tissue in the sham-operation group, but compared with the sham operation group, the positive cell number significantly increased in the model, MSCs and combination groups, particularly, the expression was the strongest in the combination group, with significant differences between the MSCs group and the model group ($P<0.05$), between the combination group and the model group ($P<0.01$), and between the combination group and the MSCs group ($P<0.05$).
Ki-67 Expression in the Rat Brain Tissue of the Groups (Table 1)
It can be seen from Table 1 that Ki-67 expression was almost not seen in the brain tissue in the sham operation group, but could be seen in the model, MSCs and combination groups, with significant differences between the MSCs group and the model group ($P<0.05$), between the combination group and the model group ($P<0.01$), and between the combination group and the MSCs group ($P<0.05$).

DISCUSSION

Pathophysiologival changes for ischemic cerebral vascular diseases are complicated and varied, with no very clear mechanism, but the recent study indicates that the “compensatory angiogenesis” after cerebral ischemia is beneficial to formation of collateral circulation, improving blood supply around foci, saving the dying neurocytes, etc., which is an self-protective mechanism after cerebral ischemia. Angiogenesis is referred to the process of growing new capillaries on the present blood vessels in a budding type, and it is a programmed cascade event. VEGF is one of key angiogenesis-promoting factors and it is a vascular endothelial growth factor with specific action on vascular endotheliocytes, and it can promote division, proliferation and transfer of vascular endotheliocytes. Exogenous VEGF can reduce the infarction area of ischemic cerebral injury, increase the nerve generation in striatum and the density of capillaries in brain tissue of the half-hazed zone, and improve nervous functions. Ki-67 antigen is a nucleoprotein expressed by cells with proliferation and division ability, and it is a mark for cell proliferation, and it has become an important index for judging normal and tumor stem cells in basic and clinical medicine.

In the present study, angiogenesis was researched by detection of VEGF and Ki-67 expression induced by Buyang Huanwu Tang combined with MSCs transplantation in the cerebral ischemia-reperfusion model rat. The results indicated that after cerebral ischemia, both VEGF and Ki-67 were expressed to a certain extent, suggesting that angiogenesis and cell proliferation are a self-protective mechanism after cerebral ischemia, MSCs transplantation promotes angiogenesis-related factors expression, Buyang Huanwu Tang combined with MSCs transplantation induced the most strong expression.

There are reports about the Chinese drugs for supplementing qi and activating blood circulation and MSCs promoting angiogenesis. For example, Cai XB, et al. report that Huangqi (黄芪 Radix Astragali) has a certain action of promoting regeneration of blood vessels; Meng H, et al. report that Danggui (当归 Radix Angelicae Sinensis), Danshen (丹参 Radix Salviae Miltiorrhizae), Chuanxiong (川芎 Rhizoma Chuanxiong) have functions of promoting proliferation of microvascular endothelial cells and capillaries. However, there are no many reports about Buyang Huanwu Tang (补阳还五汤) promoting angiogenesis. MSCs are a kind of stem cells with trans-blastodermic differentiating potentiality in the bone marrow. In resent years it has found that MSCs can survive in the central nervous system and differentiate to nerve-like cells, and have improving action on the defect of nervous functions of cerebral ischemia, brain trauma and other diseases. Because of rich source, convenient collecting, and easy to be separated and cultured, and transplantation by many ways including venous transplantation and intracerebral transplantation, it has become an ideal selection for cell alternation therapy. Chen, et al. adopt human MSCs to treat cerebral infarction in the rat, results indicates that transplanted MSCs can survive in the body of host, and successfully induce regeneration of blood vessels in the ischemic area, significantly increasing the number, length and superficial area of the blood vessels as compared with those of the ischemic contra-lateral or the control group; and the expression of VEGF and its receptor raises. Dynamic observation by MRI shows that the volume of blood flow, number and density of the blood vessels in the transplantation side of the brain are increased, and the recovery of
nervous functions is related with up-regulation and the prolonged high-expression time-course.

In Brief, Buyang Huanwu Tang (补阳还五汤) combined with MSCs transplantation promoting angiogenesis by strengthening the expression of endogenous vascular factors is one of important ways for protecting nerves. Harmony of qi with blood, free flow of blood provide good environment and condition for functions of MSCs, and the drugs for supplementing qi and activating blood circulation combined with stem cells have cooperative pharmacologic action.

Because of advantages of high self-renewing ability and multi-directional differentiating potency and convenient collection, and weaker bad reactions after transplantion, etc., MSCs have aroused general concern. In resent years, it has become a research hot point of cells, gene engineering and regeneration medicine. Buyang Huanwu Tang (补阳还五汤) combined with MSCs can up-regulate the angiogenesis cascade event-related factor VEGF, but the mechanisms of VEGFR-1, a receptor of VEGF, angiotensin Ang-1 or Ang-2, and integrin αvβ3, integrin ligand Eph-B4 remain to be researched further.

REFERENCES


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