Basic Investigations

Preliminary Study on the Mechanism of Acupoint Injection of Bone Marrow Mesenchymal Stem Cells in Improving Blood Flow in the Rat of Hind Limb Ischemia

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Objective: To explore the mechanism of acupoint injection of bone marrow mesenchymal stem cells (BM-MSCs) in improving blood flow in the rat with hind limb ischemia.

Methods: Twenty-four SD rats were randomly divided into 4 groups: normal control group (n=6), model group (n=6), BM-MSCs acupoint injection group (AI group, n=6) and BM-MSC intramuscular injection group (MI group, n=6). Sanyinjiao (SP 6), Housanli (ST 36), Zhaohai (KI 6), Huantiao (GB 30) and Yanglingquan (GB 34) were selected for the AI group, and five non-acupoints were selected on gastrocnemius and adductor of ischemic hind limbs in the MI group. BM-MSCs were injected to the latter two groups. The rat hind limb ischemia model was established with the method of blocking the femoral artery and its branches. Three weeks after injection of BM-MSCs, in each group, hindlimb adductor and gastrocnemius were taken from the ischemic side. Expressions of vascular endothelial growth factor (VEGF) and transfer growth factor-β_1 (TGF-β_1) in the skeletal muscle were determined with immunohistochemical method, and the small arteries in the skeletal muscle were labeled with α-SMA immunohistochemical staining method, the density of small arteries (number of arterioles / number of muscle fibers) and the number of the blood vessel with VEGF positive expression were calculated. The serum levels of VEGF and nitric oxide (NO) were detected.

Results: Compared with the model group, the expression of VEGF and TGF-β_1, and the density of small arteries and the number of VEGF-positive blood vessels in the AI group and the MI group significantly increased (both P<0.01). Compared with the MI group, the density of small arteries and the number of VEGF-positive blood vessels in the AI group significantly increased (both P<0.01); Compared with the model group and the normal control group, the serum expression quantity of NO and VEGF in the AI group and the MI group were significantly increased (P<0.01).

Conclusions: Acuppoint injection of BM-MSCs secrets more VEGF, TGF-β_1 and NO to increase angiogenesis and arteriogenesis, so as to improve blood flow of the rats of hind limb ischemic.

Keywords: *Acupoint injection; Bone marrow mesenchymal stem cells (BM-MSCs); Ischemia; VEGF; TGF-β_1; NO*

After arterial occlusion, sprouting new capillaries sprout (i.e. angiogenesis) and growing and remodeling the preexisting arterioles grow and remodel into physiologically relevant arteries (i.e. arteriogenesis).[1] In the process of angiogenesis and arteriogenesis, vascular endothelial growth factor (VEGF) and transfer growth factor-β_1 (TGF-β_1) play an important role.[2] In addition, a large number of evidences indicate that an effective angiogenesis requires participation of endogenous nitric oxide (NO),[3] and it has been confirmed that endothelial nitric oxide synthase(eNOS) and NO are necessary for the restoration of blood flow and formation of new blood vessels after ischemia.[4]

Previous studies showed that acupoint injection of bone marrow mesenchymal stem cells (BM-MSCs) could significantly improve perfusion in the rats of hind limb ischemic.[5] In this paper, expressions of VEGF and TGF-β_1 in the skeletal muscle, and serum levels of VEGF and NO were determined, and the density of small arteries (number of arterioles/ number of muscle fibers) and number of the blood vessel with VEGF positive expression were calculated. The serum levels of VEGF and NO were detected after acupoint injection of BM-MSCs in the hind limb ischemia model rat, so as to explore the mechanism of acupoint injection of BM-MSCs in improvement of blood flow.[1]

MATERIALS AND METHODS

Rat Bone Marrow Mesenchymal Stem Cells

SD rat BM-MSCs and the complete culture fluid BM-MSCs were both purchased from Cyagen

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Laboratory Animals
Twenty-four SD rats (SPF), body weight (200±20) g, either sex, were purchased from Beijing Weitong Lihua Company (Animal Certificate No.: SCXX (Jing) 2007-0001).

Main Experimental Instruments and Main Reagents
Cell incubator: MC0175CO cell incubator (SANYO Corporation), OLYMPUSix-71 inverted microscope (OLYMPUS), Nikon C-SHG1 image analysis system (Nikon), Automatic enzyme immunoassay instrument (U.S. awareness technology products), radioimmunoassay instrument (China).

NO kit was purchased from Nanjing Jiancheng Bio-Engineering Institute; VEGF and TGF-β1 kits were purchased from Beijing Bo Ao Sen (博奥森) Biotechnology Co., Ltd. α-SMA primary antibody was purchased by Abcam Company, USA.

Experimental Animals and Grouping
Twenty-four healthy SD rats were adaptively fed for 3 days and then were randomly divided into four groups, 6 in each group: normal control group, model group, BM-MSCs acupoint injection group (AI group), and BM-MSCs muscular injection group (MI group). They freely access to food and water and were raised under the same conditions.

Preparation of Animal Model
Referring to the method in the literature,[6] under aseptic condition, the rats were fixed at dorsal position on a rat plate after anesthesia (intraperitoneal injection of 10% chloral hydrate at the dose of 3.5 mL/kg). After vertically cutting from the left inguinal ligament to the knee joint, the femoral artery and its branches were isolated and ligated at both place near to the left common iliac artery and the popliteal artery using 5–0 suture silk, and then they were cut off at the middle of the both ligatures, thus establishing the ischemic hind limb model. Then the subcutaneous tissue and skin were sutured, without anti-infection conducted after surgery.

Treatment Methods
The 5 acupoints, Sanyinjiao (SP 6), Housanli (ST 36), Zhaohai (KI 6), Huantiao (GB 30), and Yanglingquan (GB 34) in the affected limb (the left side) were selected for the AI group, with the acupoints located according to the rat acupoint atlas of Hua, et al.[7] While 5 non-acupoints on the medial part of the thigh of the left ischemic hind limbs were selected in the model group. After surgery, BM-MSCs were injected immediately into 0.5–1.0 cm in depth. 5×10^6 BM-MSCs were injected to the rats in both the AI and the MI groups only once. Equal volume of phosphate buffer solution was injected into the same points in the model group as those in the MI group. No treatment was given to the right hind limbs of the rats in the AI, MI, and model groups, and they were taken as the normal control. No treatment was given to the rats in the normal control group.

Determination of Serum NO and VEGF
The rats were killed (spine dislocation) 3 weeks after the acupoint injection, and the blood was taken from the abdominal aorta. After standing for 30 min, the blood was centrifuged (1500 r/min, 15 min) and the supernatant was collected. According to the manual of NO and VEGF kits, serum contents of NO and VEGF were determined.

Detection of VEGF, TGF-β1 and TNF-α Expression
After blood flow measurement, 4 pieces of ischemic tissue from the adductor and gastrocnemius muscles were taken and put into 4% paraformaldehyde stationary liquid. After paraffin imbedding, they were cut into paraffin sections with 5 μm in thickness. The protein expression of TGF-β1 and VEGF were analyzed with immunohistochemical method according to the manual of kids. Under the microscope, brown particles in the cytoplasm were considered as positive expression. Nikon image analysis system used to analysis the results of immunohistochemical expression. Five high power fields (40×) of each section were randomly selected for analysis of VEGF and TGF-β1 expression. The positive expression area multiplied the average optical density (MOD) were calculated.

Measurements of the Density of α-SMA Labeled Small Arteries and VEGF-positive Capillaries
Twenty-one days after acupoint injection of BM-MSCs, the rats were anaesthetized and the adductor and gastrocnemius muscle of the hind limb were taken and fixed with formaldehyde, and then the transverse section of the muscle with 5 μm in thickness were made. When anti-smooth muscle actin immunohistochemical staining is used to label the smooth muscle of vascular wall, the part of α-SMA positive expression is considered to be location of the small arteries. Nikon C-SHG1 image analysis system was used, and 10 field of vision were selected under the high-power microscope (10×40), the density of small arteries (number of small arteries in the microscope / muscle bundles) was calculated .

The sections were stained with the manual of VEGF immunohistochemical kit, the cells with brown-yellow granules were regarded as positive cells, namely VEGF positive expression capillaries. Ten fields of vision were randomly selected under the high-power microscope (10×40) and the number of the VEGF positive expression capillary was counted, and the average number was calculated in each group.

Statistical Analysis
All the data were expressed as $\bar{x} \pm s$ and processed with SPSS11.0 software. Comparisons of parameters among the four groups were made by one way ANOVA followed by Scheffe's multiple comparison test. A

probability value <0.05 was considered statistically significant.

RESULTS

Comparison of VEGF and TGF-β_1 Expressions in the 4 Groups

Compared with the normal control and model groups, the protein expression of VEGF and TGF-β1 in both the AI and the MI groups were increased significantly (P<0.01). Compared with the MI group, the protein expression of VEGF and TGF-β1 in the AI group were increased significantly (P<0.01 or P<0.05, Table 1).

Table 1. Comparison TGF-β1 and VEGF expressions in the 4 groups（$\bar{x} \pm s$）

Group	n	TGF-β1	VEGF
normal control	6	11.67±1.98	6.29±0.80
Model	6	11.99±4.89	12.89±2.88**
MI	6	20.80±5.03**▲▲	17.94±2.09**▲▲
AI	6	25.63±3.23**▲▲△	26.57±4.59**▲▲△△

Notes: **P<0.01 vs the normal control group; ▲▲ P<0.01 vs the model group; △P<0.05, △△ P<0.01 vs the MI group.

Comparison of Serum Contents of NO and VEGF among the 4 Groups

Compared with the normal control and model groups, the serum contents of NO and VEGF in the MI and AI groups were increased significantly (P<0.01). Compared with the MI group, the contents of NO and VEGF in the AI group did not have significant difference (P>0.05, Table 2).

Table 2. Comparison of serum VEGF and NO contents among the four groups（$\bar{x} \pm s$, n=6）

Group	NO (μmol/L)	VEGF (ng/mL)
normal control	25.64±3.93	47.91±6.86
Model	27.69±3.77	55.21±11.05
MI	35.48±5.56**△△	83.09±23.34**▽
AI	38.41±5.48**△△	85.67±29.67**▽

Notes: **P<0.01 vs the normal group ; △△P<0.01 , △P< 0.05 vs the model group.

Comparison of VEGF-positive Capillaries and Density of Small Arteries among the 4 Groups

Compared with the normal control and model group, the VEGF-positive capillaries and the density of small arteries in AI and MI groups are increased significantly (P<0.01). Compared with the MI group, VEGF-positive capillaries and the density of small arteries in AI group was increased significantly (P<0.01, Table 3, Figure 1, and Figure 2).

Table 3. VEGF-positive capillaries and the density of small arteries among the 4 group（$\bar{x} \pm s$, n=6）

Group	Density of small arteries	VEGF-positive capillaries
normal control	0.85±0.06	9.38±1.19
Model	0.90±0.09	10.50±1.20
MI	1.40±0.11**▲▲	17.00±2.62**▲▲
AI	1.80±0.07 **▲▲△△	19.875±2.5**▲▲△△

Notes: **P<0.01 vs the normal control group; ▲▲ P<0.01 vs the model group; △△ P<0.01 vs the MI group.

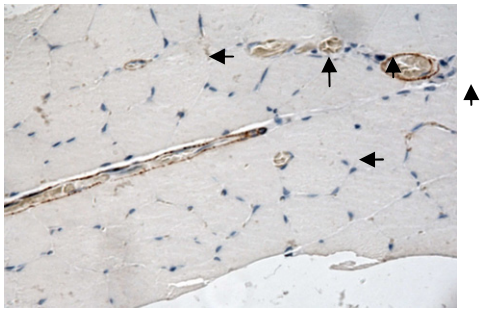

Figure 1. Anti-α-SMA-labeled small arteries (×400)
The arrow indicates capillaries

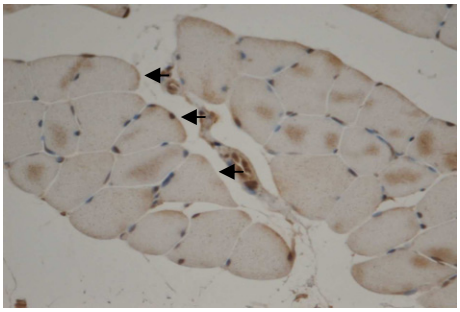

Figure 2. VEGF-positive capillaries (×400)
The arrow indicates small arteries

Notes: Histologic sections of muscle tissues in which bone marrow mesenchymal stem cells (BM-MSCs) were implanted. Sections were stained with immunohistochemical staining method. Figure1.Section was stained with anti α-SMA antibodies.(Antibody was diluted to 1:200). Figure 2.Section was stained with anti VEGF antibodies.(Antibody was diluted to 1:200).

DISCUSSION

There are 2 mechanisms for vascular growth in adults after embryogenesis: 1) Angiogenesis, proliferation of local capillaries increases local blood perfusion; 2) arteriogenesis, dilation of preexisting high resistance collateral blood vessels decreases flow resistance to increase blood perfusion in the ischemic region.[8] BM-MSCs are effective in augmenting the vascular response to arterial occlusion, increasing capillary counts and collateral blood flow in the hindlimb. In addition, BM-MSCs have been shown to form capillary-like structures in an in vitro Matrigel assay. Furthermore, MSC transplantation has been shown to induce angiogenesis in a rat model of hindlimb ischemia.[9]

BM-MSCs in the bone marrow stroma differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells,

endothelial progenitor cells and vascular smooth muscle cells. There exist three mechanisms by which endothelial progenitor cells can enhance collateral development. 1) Support: Progenitor cells secrete multiple cytokines, growth factors, and chemokines that can facilitate arteriogenesis by a) influencing the matrix in a way conducive for collateral development, b) inhibiting endothelial and smooth muscle cell apoptosis and stimulating their migration and proliferation, and c) recruiting proarteriogenic inflammatory and progenitor cells. 2) Incorporation: Progenitor cells could directly incorporate into the developing collateral and thereby physically contribute to collateral formation; the biological importance of this mechanism is presently a source of controversy. 3) Fusion: Fusion of progenitor cells with tissue specific cells has been demonstrated, but no data indicate that this functionally contributes to collaterogenesis.[8]

The results show that compared with the MI group, VEGF-positive capillaries and the density of small arteries in the AI group were increased significantly (P<0.01). It is indicated that in the process of acupoint injection of bone marrow mesenchymal stem cells to improve blood flow in rat hind limb there exists not only angiogenesis but also arteriogenesis. Because the injected bone marrow mesenchymal stem cells were not labeled, so we can not determine it how to participate in angiogenesis and arteriogenesis.

This study demonstrated that acupoint injection of BM-MSCs could significantly increase serum contents of NO and VEGF, and the expression of VEGF and TGF-β1 in the ischemic skeletal muscle. In the MI group and the AI group the local injection method was adopted, so serum levels of VEGF and NO in the two groups did not have significant changes.

VEGF, an important factor for promotion of blood vessel formation, plays an important role in angiogenesis. In addition, they regulate angiogenesis and vascular maintenance during embryogenesis and in adults.[10] VEGF has been known as one of the key factors in angiogenesis and acts on several aspects of this process, including endothelial proliferation, migration, and tube formation. VEGF can enhance endothelial regeneration, restore endothelial function, and stimulate angiogenesis.

TGF-β is a polypeptide growth factor which plays an important role in tissue repair and angiogenesis. TGF-β1 is a major member of the TGF-β family, and it is a key cytokines in regulation of normal cell growth and other functions. TGF-β1 can inhibit apoptosis of the MSCs induced by serum deprivation and hypoxia,[11] promote the proliferation of BM-MSCs and maintain its biological activity in long time.[12] Therefore, TGF-β1 secreted by BM-MSCs not only reduce the apoptosis of BM-MSCs but also promote its cell growth, in turn, the high survival BM-MSCs secrets more TGF-β1, with a good mutual promoting role.

NO plays a critical role in ischemia-induced angiogenesis. Thus, impairment of this metabolic pathway is related with the development of peripheral arterial occlusive disease. Studies have shown that: 1) NO levels are increased in the ischemic limb; 2) Pharmacological inhibition or gene disruption of eNOS decreases NO levels and inhibits ischemia-induced angiogenesis; 3) Exogenous NO restores ischemia-induced angiogenesis; Moreover, agonist-dependent NO release restores ischemia-induced angiogenesis in these pathological situations[1].

Research has shown that VEGF, TGF-β1 stimulates the release of NO from cultured human umbilical venous endothelial cells and upregulates the expression of nitric oxide synthase.[13] Whether VEGF and TGF-β_1, paracrine by BM-MSCs, stimulate the release of NO is not known yet.

Thus, firstly, acupoint injection of BM-MSCs produces more VEGF, TGF-β1 and NO to promote angiogenesis and arteriogenesis; secondly, produces more TGF-β_1 which not only reduces the apoptosis of BM-MSCs but also promotes its cell growth; lastly, promotes synapse extension through secretion of VEGF and plays a role in neural protection and neural newborn, so as to reduce the peripheral nerve injury produced by hypoxic-ischemic,[14] thereby increasing blood flow of the ischemic hindlimb.

In short, injection of BM-MSCs into acupoint produces more VEGF, TGF-β1 and NO to play the role of angiogenesis and arteriogenesis.

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