Effects of exercise training on microangiogenesis of rat ischemic lower limbs

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Abstract

BACKGROUND: Previous studies demonstrated that ischemia can be compensated by establishing collateral circulation and microangiogenesis, and exercise training can ameliorate blood supply of ischemic lower limb. However, whether exercise training accelerate establishment of collateral circulation remains poorly understood.
OBJECTIVE: To explore the mechanism of exercise training on accelerating microangiogenesis of rat ischemic lower limb.
METHODS: Sprague-Dawley rats were randomly divided into the exercise training, model and sham-surgery groups. All animals were established left lower limb ischemia models except those in the sham-surgery group. Rats in the exercise training group were forced running 30 minutes per day at 1 week after model preparation and those in other groups were performed daily activities. The adductor of ischemic lower limb was obtained for the examination of microvessel density and the expressions of vascular endothelial growth factor and basic fibroblast growth factor. Simultaneously, bone marrow-derived endothelial progenitor cells were harvested for detection of microangiogenesis.
RESULTS AND CONCLUSION: Number of endothelial progenitor cells, expressions of vascular endothelial growth factor and basic fibroblast growth factor, as well as microvessel density of the exercise training group was obviously greater than those in the model and sham-surgery groups ($P < 0.01$). Compared with the model and sham-surgery groups, *in vitro* vasculogenesis of bone marrow-derived endothelial progenitor cells in the exercise training group was increased ($P < 0.05$). These findings demonstrated that lower limb ischemia increases microangiogenesis, and exercise training enhances this effect.

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INTRODUCTION

The incidence of chronic critical limb ischemia is increasing yearly, the treatment, especially for multi-segment arteriosclerotic obliteration or extensive arterial occlusion, are difficult[1]. Exercise training can ameliorate blood supply of ischemic lower limb, increase pain-free walking distance and maximum walking distance, accordingly, improves life quality. Currently, vascular growth factor and gene therapy have applied for accelerating microangiogenesis of the ischemic tissues in order to treat ischemic disease. Studies have demonstrated that exercise training accelerates microangiogenesis, which provides a new approach for the treatment of ischemic disease. In this experiment, we aimed to explore the effects of exercise training on the expressions of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), as well as microangiogenesis in ischemic lower limb, to explore relative mechanisms, and to provide theoretical basis for the application of exercise training on treating chronic limb ischemia.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

The experiment was performed at the Animal Center of Luzhou Medical College from May to July 2008.

Materials

Experimental animal

Totally 30 male, clean grade, Sprague-Dawley rats, weighted 200−220 g, aged 12 weeks, were provided by the Animal Center of Luzhou Medical College. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, published by the Ministry of Science and Technology of the People's Republic of China[2].

Exercise training device

A self-made drum-type mesh training device was utilized, which comprises a round mesh device (1.0 m length, 50 cm diameter), a fixed frame at the base, and a hand crank at one end. The device can rotate with speed of 5 r/min. This device can be used for training grip, rotation and walking of rats.
Main reagents are as follows:

Reagent	Source
Rabbit anti-mouse VWF factor	Santa Cruz, USA
Rabbit anti-mouse VEGF, bFGF polyclonal antibody	Zhongshan Golden-Bridge Biotechnology Co., Ltd., China
cck-8 kit	Dojindo Laboratories, Japan
ELSIA kit	Dalian Fanbang Company, China
SABC kit	Wuhan Boster Biological Technology, Co., Ltd., China

Methods

Grouping and model preparation

All 30 rats were randomly divided into the exercise training, model and sham-surgery groups, with 10 animals in each group. Rats in the exercise training and model groups were anesthetized with intraperitoneal injection of 3% pentobarbital (25 mg/kg), fixed, and the surgical site were cleaned. A 2-cm length longitudinal incision was cut at middle point of right inguinal region

towards to knee. The stem of femoral artery was isolated cling to inguinal region, ligated at the original site, cut down, and removed femoral artery below knee, and the branch vessels of deep femoral artery were ligated. The right lower leg skin was opened and closed without other treatment in the sham-surgery group. Rats in the exercise training group were forced running in the drum-type mesh training device at 1 week after model preparation with speed of 10 r/min and 20 m/min until fatigued. Fatigue was defined as the inability to keep up with the treadmill speed[3], about 30 minutes. Rats in the model and sham-surgery groups were only performed daily activities.

Sample harvest and fixation

At 4 weeks after exercise training (30 minutes per day)[4], all rats were killed by overdose anesthesia, and a 1.0 cm × 0.5 cm × 0.5 cm size adductor of ischemic lower limb was obtained, fixed, embed, and cut into slices along the direction of muscular fiber by a constant temperature slicer.

Under aseptic condition, the bone marrow was removed from femur and tibia, diluted with phosphate buffered solution (1: 1), followed by density gradient centrifugation with lymphocyte separating medium, 2 000 r/min for 30 minutes. The middle white fog layer was sucked and placed in other tube, centrifuged with M199 (1: 5), 1 300 r/min, for 5 minutes, followed by twice washing. The supernatant was discarded, and the pellet was resuspended in M199. The cells were then seeded into M199-precoated fibronectin petri dish at a density of 1×10^6/L, and cultured in 5% CO_2 at 37 ℃. The culture medium was replaced after 3 days, and the attached cells were reserved for further experiment.

Hematoxylin-eosin staining

The slices were dewaxed, hydrated, and stained by hematoxylin, differentiated by hydrochloric acid, and counterstained by eosin, followed by dehydrated in ascending series of ethanol, cleared in xylene and mounted in neutral resin.

Cell identification

The morphological and quantity variations of cells were daily observed under an inverted phase contrast microscope, and the 1, 4, 7 and 20 days cells were seeded coverslips for examination of CD133 and Ⅷ factor expressions.

Detection of endothelial progenitor cell (EPC) microangiogenesis

In vitro microangiogenesis assay kit was used to determine the EPC microangiogenesis. Totally 5×10^7/L EPC were incubated into ECMatrix gel with density of 5 000−10 000 per well, cultured for 24 hours at the 37℃, and then the microangiogenesis was observed under an inverted microscope (× 200). Microangiogenesis was defined as the cells were lengthened and presented with a tube shape, and the length was 4-fold of width. The experimental procedures were performed by two operators using the double-blind method. Five layers of each well were randomly selected for the observation of tubular cells numbers.

Immunohistochemical detection of microvessel density (MVD), VEGF, and bFGF

The avidin-biotin-pcroxidase complex (SABC) method was applied for immunohistochemistry. Rabbit anti-mouse VWF factor (concentrated type), rabbit anti-mouse VEGF, and rabbit anti-mouse bFGF polyclonal antibodies served as first antibody, the SABC kit served as secondly antibody. The procedure was performed under guidance of instructions. The kytoplasm stained brown-yellow was considered as positive expression of VWF, which used for quantify MVD numbers. The highest density of colored region was observed under a low power lens, and totally 5 fields were randomly observed under a light microscope (× 200), the mean value served as MVD. The brown-yellow-stained kytoplasm was considered as positive expression of VEGF and bFGF, and 5 fields were randomly observed by two investigators under a light microscope (× 200) using double-blind method. The VEGF and bFGF scores were calculated[5].

Main outcome measures

The expressions of endothelial cell VWG, VEGF and bFGF.

Design, enforcement and evaluation

All authors, who received well-trained, designed, performed and evaluated the experiment using double-blind method.

Statistical analysis

Data were expressed as Mean±SD. Statistical analysis was performed using SPSS 13.0. SNK-*q* test was used for comparison between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Quantitative analysis of animals

All 30 rats were included in the final analysis.

Behavioral changes of rats following left lower leg ischemia

All experimental rats were survived after model preparation, and the left lower leg presented with claudication due to ischemia. One week later, the color Doppler showed that the artery flow was interrupted, which confirmed that the model was successfully established.

MVD changes of rats following left lower leg ischemia

The circular blood capillary could be seen at intercellular space of skeletal muscle cells in the exercise training and model groups, and few blood capillary could be seen in the sham-surgery group. Immunohistochemical results showed that there were plenty of newly-born vessels in the exercise training and model groups, which mainly distributed in intercellular space of skeletal muscle cells, covered by one or more endothelial cells, and positive expressed VWF factor. The MVD in the exercise training group was greater than that of the model group ($P < 0.01$). Compared with the sham-surgery group, the MVD in the exercise training and model groups were greater ($P < 0.01$).

Expression of VEGF and bFGF following left lower leg ischemia

VEGF and bFGF were well expressed in the exercise training and model groups, showed brown-yellow deposits scattered

in kytoplasm, which revealed that there were positive expression of VEGF and bFGF. Compared with the model group, the expression of VEGF and bFGF were greater in the exercise training group ($P < 0.01$). The expression of VEGF and bFGF was negative or weak positive in the sham-surgery group, which was dramatically smaller than that of the model group ($P < 0.01$, Table 1).

Table 1 Expression of MVD, VEGF, and bFGF in muscle tissues ($x \pm s$, n=10)

Group	MVD (Number)	VEGF (Score)	bFGF (Score)
Exercise training	39.94±4.01[ab]	3.30±0.67[ab]	3.10±0.73[ab]
Model	27.23±3.64[a]	2.29±0.67[a]	2.39±0.69[a]
Sham-surgery	3.32±2.26	0.59±0.49	0.87±0.53

MVD: microvessel density; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; [a]$P < 0.01$, *vs*. sham-surgery group; [b]$P < 0.01$, *vs*. model group

Cell morphological identification

At 48 hours in primary culture, attached cell exhibited colony-like growth, presented with round, triangular, spindle-shaped, or irregular-shaped, with obviously mitosis figures. Thereafter, the cells displayed monolayer growth around colonies or multilayer growth in the center of colonies. At 7 days after culture, the cell colonies contacted each other. The cells showed a cobblestone like morphology at 9−11 days after culture.

The well grew cells were selected at 1, 4, 7 and 20 days and seeded coverslips for examination of CD133 and Ⅷ factor expressions by immunohistochemistry. The results showed that the number of positive CD133 cells were increased at 1, 4 and 7 days after culture, but dramatically decreased at 20 days. The positive Ⅷ factor cells were gradually increased from the 4 days.

EPC microangiogenesis

At 3 days after grouping culture, the EPC microangiogenesis was determined by *in vitro* microangiogenesis assay kit. Under an inverted phase contrast microscope, EPC grew into gel matrix and contacted each other like a net at 4 weeks after culture. In the exercise training group, the EPCs grew faster, even a few of three-dimensional network structure could be found. However, the tubular structure was tiny, and the net structure was sparse and incomplete in the sham-surgery group. Few tubular structures could be seen in the model group. Five fields were randomly observed by two investigators using double-blind method.

The findings demonstrated that tubular cell number of the exercise group was (2.65±1.46, 14.38±2.21, and 9.32±1.98) at 6, 12 and 18 hours after culture, which was greater than those of the sham-surgery (1.21±0.34, 9.45±1.72, 3.75±1.23) and model (0, 1.16±0.98, 0) groups ($P < 0.05$).

DISCUSSION

Ischemic limbs have natural compensation, the vascular system can self-remodeling by increasing oxygen supply for muscles, presented with collateral circulation formation, angiogenic growth factors and their receptors increasing at ischemic regions. The blood remodeling occurred when vascular system can not meet the need of metabolism, tissue activity, or peripheral arterial occlusion, which displays as collateral vessel expansion and blood capillary development. Recently studies demonstrated that all pathological changes caused by ischemia can be compensated *via* construction of collateral circulation and newly born of vessels.

Exercise can increase shear stress of vessel wall, accelerate VEGF-R expression, induce endothelial cell proliferation and differentiation, ultimately, promotes collateral circulation formation and microangiogenesis. In the present study, the MVD, VEGF and bFGF expressions in the ischemic tissues were obviously increased after exercise training, which demonstrated that exercise training can improve blood supply. In addition, exercise training can induce vessel adaptive change, such as increasing MVD, enhancing shear stress of blood current, and stimulating endothelial cell release various growth factors.

Microangiogenesis is a complex process, involving endothelial cell division, basilar membrane and extracellular matrix degradation and endothelial cell migration. Microangiogenesis is mainly characterized by the protrusion and outgrowth of capillary buds and sprouts from pre-existing blood vessels, which depends on VEGF stimulation. VEGF is a highly specific mitogen for vascular endothelial cells, which not only promotes endothelial cell division, proliferation, migration, and chemotaxis, but also facilitates mononuclear macrophage migration, accordingly, secretes various vascular growth factors, thus, indirectly accelerates microangiogenesis[6]. bFGF directly induces microangiogenesis, which further promotes the formation and mature of vessel[7], thereby, encourages microangiogenesis at the ischemic region. VEGF mobilizes bone marrow-derived EPCs proliferation and localizes in acute ischemic events, *in situ* differentiates into mature endothelial cell, and forms new vessels[8]. Studies confirmed that exercise effectively mobilizes marrow-derived EPC, which secretes VEGF that can promote microangiogenesis[9]. In the present study, the number of EPC of the exercise training group was greater than that of other groups, and the capability of microangiogenesis was better, which demonstrated that exercise training contributes to EPC differentiation, which may result from stimulation of exercise training on intra-cellular signal pathway or inhibition of microangiogenesis at the ischemic limbs.

Exercise training can ameliorate blood supply of ischemic lower limb, increase pain-free walking distance and maximum walking distance, thus, improve life quality. Currently, it has aroused increasing interests for the treatment of ischemic lower limbs, especially for arterial occlusive disease below knee joint. Studies have found that blood flow increased after cerebral ischemia, induced extensive expression of VEGF, and exercise training can promote collateral circulation and enlarge blood flow after cerebral ischemia. For this reason, theoretically, it is possible to induce VEGF expression using exercise training[10-13]. Here, VEGF concentration was not reach a peak at 28 days in the exercise training group, but combined with the bFGF and MVD, all these three factors were greater than those of the model and sham-surgery groups, this finding demonstrated that lower limb ischemia accelerates

microangiogenesis. In addition, the differences between the exercise training and model groups showed that exercise training can enhance microangiogenesis. However, the experimental samples were small, and the observation only performed for 28 postoperative days, thus, the variation of VEGF required further investigation.

The experimental results demonstrated that, athletic sports, especially running, can promote microangiogenesis. This is benefit for explaining effects of sports on encourage microangiogenesis and enriching theoretical research of “remedial microangiogenesis” and rehabilitative treatment. Exercise training can amendment body microcirculation, mitigate inflammatory lesions, as well as enhance cardiorespiratory function, which can play its role in the treatment of chronic occlusive arterial disease.

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大鼠缺血下肢微血管生成与运动训练

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摘要

背景：研究表明缺血可通过侧支循环的建立和血管的新生得到代偿，运动训练可以改善患者缺血下肢血液供应，但运动是否能够促进侧支循环的建立至今少有报道。

目的：探讨运动训练促进大鼠缺血下肢微血管生成的机制。

方法：将 SD 大鼠随机分为运动训练组、模型组和假手术组。除假手术组外，其余组均建立大鼠左下肢缺血模型。建模 1 周后运动训练组大鼠跑步训练 30 min/d。模型组和假手术组均为日常活动。运动训练 4 周后取各组大鼠大腿内收肌组织块免疫组化检测微血管密度、血管内皮生长因子和碱性成纤维细胞生长因子的表达，同时取骨髓内皮祖细胞，检测其成血管生成能力。

结果与结论：运动训练组内皮祖细胞、大鼠肌组织血管内皮生长因子、碱性成纤维细胞生长因子的表达和微血管密度均高于模型组和假手术组（$P < 0.01$）。运动组骨髓内皮祖细胞的体外血管生成能力比假手术组及模型组增加（$P < 0.05$）。结果提示下肢缺血刺激可以促进微血管新生，而运动训练可以增强该效应。

关键词：缺血；运动训练；血管新生；微血管密度；血管内皮生长因子；碱性成纤维细胞生长因子；组织构建

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