

ORIGINAL REPORT

ENHANCEMENT OF HOMING CAPABILITY OF ENDOTHELIAL PROGENITOR CELLS TO ISCHAEMIC MYOCARDIUM THROUGH PHYSIOLOGICAL ISCHAEMIA TRAINING

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Objective: To investigate the effects of physiological ischaemia training on the quantity and activity of endothelial progenitor cells in rabbits with intermittent myocardial ischaemia.

Methods: A reversible coronary artery water balloon occluder (5.0 mm inner diameter) was placed around the proximal left ventricular branch and intermittent myocardial ischaemia was induced by deflation and inflation of the occluder. A wire electrode was inserted longitudinally into the epineurium of the sciatic nerve to generate physiological ischaemia training through isometric contraction induced by electrical stimulation. Rabbits were randomly divided into 3 groups: a sham operated group (SO), an intermittent myocardial ischaemia-only group (MI), and an MI plus physiological ischaemia training group (PT). Intermittent myocardial ischaemia was induced with 2-min ischaemia followed by 1-h reperfusion. Physiological ischaemia training was induced by electrical stimulation (40% maximum current strength, 1 ms, 40 Hz), 4-min per session, twice a day, 5 days per week for 4 weeks. At the end-points, endothelial progenitor cells were isolated and cultured for analysis of their migration ability. Endothelial progenitor cells were identified by dual-staining with Dil-labelled acetylated low-density lipoprotein and fluorescein isothiocyanate-labelled Ulex europaeus agglutinin-1. Circulating endothelial progenitor cells (CD34⁺/Flk-1⁺) were counted by fluorescence-activated cell sorter, and capillary density was evaluated by immunohistochemistry examination. **Results:** Group PT showed the highest migration capacity of endothelial progenitor cells (151 cells, standard deviation 16/high power field) and the highest increase in circulating endothelial progenitor cells ($p < 0.01$) after 4 weeks' physiological ischaemia training. Capillary density in the myocardium was also significantly enhanced in group PT ($p < 0.05$). Pearson's analysis demonstrated a positive correlation between the number circulating endothelial progenitor cells and capillary density in the myocardium after 4 weeks' physiological ischaemia training ($p < 0.05$).

Conclusion: Physiological ischaemia training may enhance the quantity and activity of endothelial progenitor cells in the blood, resulting in an increase in angiogenesis in the ischaemic heart region.

Key words: endothelial progenitor cells; angiogenesis; physiological ischaemia training; myocardial ischaemia; migration.

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INTRODUCTION

Endothelial progenitor cells (EPCs) play a vital role in angiogenesis during postnatal neovascularization in both pathological and physiological conditions (1, 2). Bone marrow-derived EPCs can migrate in the circulation to foci of neovascularization and differentiate into endothelial cells, contributing functionally to vasculogenesis during wound healing (3), limb ischaemia (4) and post-myocardial infarction (5, 6). EPCs are regarded as a biomarker of cardiovascular risk (7, 8). Research in cardiovascular rehabilitation has shown that aerobic exercise can lead to a significant increase in EPCs, augment capillary density in the ischaemic heart region, increase the left ventricular ejection fraction and protect the heart (9–11). Physiological ischaemia training (PIT), an isometric contraction training induced by electrical stimulus of normal skeletal muscles, which is a kind of static exercise, has been shown to improve angiogenesis in remote ischaemic areas (12, 13) with little influence on blood pressure and heart beat. However, it is not clear whether EPCs are involved in the process of angiogenesis in PIT. The aim of this study was to investigate the effects of PIT on the quantity and activity of EPCs, and its beneficial effects on angiogenesis in the ischaemia heart region.

MATERIAL AND METHODS

A total of 18 adult male New Zealand white rabbits (2.5–3.0 kg, age 3 months) were purchased from the Laboratory Animal Center of Nanjing Medical University. The rabbits were kept under conventional conditions in our animal facility with a 12-h light/dark cycle and free access to food and water. The procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes for Health (NIH Publication No. 85–23, revised 1996) and approved by the ethics committee of Nanjing Medical University and Jiangsu Province Hospital.

Model of intermittent myocardial ischaemia

A rabbit model of intermittent myocardial ischaemia (IMI) was applied. After induction of anaesthesia with 3% (v/v) sodium pentobarbital (1

ml/kg, intravenous), a left lateral thoracotomy was performed through the fourth intercostal space. The pericardium was opened and a reversible coronary artery water balloon occluder (5.0 mm inner diameter) placed around the proximal left ventricular branch (LVB), approximately 1 cm inferior to the left anterior coronary artery. The success of the implantation was confirmed by ST-segment fluctuation of more than 0.1 mV induced by balloon inflation and deflation, as shown in Fig. 1. After successful implantation of the occluder, the thoracotomy was closed and the rabbit was placed in a cage for recovery. Sham surgery involved the same cardiac exposure, but without implanting the coronary occluder. Antibiotics were administered for 5 days after surgery (4×10^5 U penicillin per day, intramuscular).

Model of physiological ischaemia training

In this study, physiological ischaemia training was induced by electrical stimulation of the sciatic nerve, as described in reference (12). Electrodes were implanted to generate electrical stimulation. With the animal in the prone position, a wire electrode was inserted longitudinally into the epineurium of the sciatic nerve and sutured with the epineurium. Meanwhile, a reference electrode was implanted into the ipsilateral gluteus maximus. Wires from these electrodes were taken under the skin to the nuchal region and connected to an external stimulator.

The intensity of electrical stimulation was determined by 40% maximum current strength, 1 ms, 40 Hz, to evoke contraction of the target muscle without joint motion.

Experimental design

Rabbits were randomly divided into 3 groups of 6: group SO (a sham operated group), group MI (which underwent intermittent myocardial ischaemia) and group PT (which underwent intermittent myocardial ischaemia followed by PIT). Intermittent myocardial ischaemia was applied twice a day, by 2-min ischaemia induced by balloon inflation and a subsequent 1-h reperfusion. PIT was also applied twice a day, with 4-min ischaemic isometric contraction induced by electric stimulus. The experiments were conducted for each group, 5 days a week, for 4 weeks.

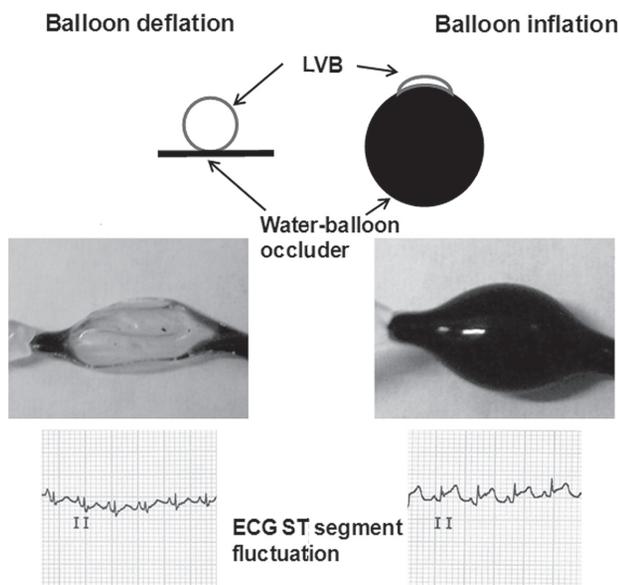


Fig. 1. Reversible myocardial ischaemia induced by water balloon deflation and inflation. When deflated, the blood flow in the left ventricle branch and the ST segment in the electrocardiogram (ECG) were normal. When inflated, the left ventricle branch was occluded, and the blood flow slowed down, as sequence ST segment was elevated.

At the end of the experiments, blood was collected and the rabbits were sacrificed. EPCs were isolated from peripheral blood samples, a portion of which were cultured for cell viability tests and the other part used for EPC quantification by fluorescence-activated cell sorter (FACS). The hearts were harvested and angiogenesis was analysed through domains of morphology (capillary density in the ischaemic area of the heart.)

Isolation, cultivation and identification of endothelial progenitor cells

A 20 ml blood sample was taken from the central artery of a rabbit's ear, and mononuclear cells were isolated by density gradient centrifugation, as described by Chen et al. (14). Two million mononuclear cells were plated on culture dishes coated with human fibronectin (Sigma, St Louis, USA), in endothelial basal medium (EBM-2) supplemented with EGM-2MV SingleQuots (Lonza, Cologne, Germany). After 4 days in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), and adherent cells were cultured continuously with fresh media that was replaced every 3 days.

After 7 days in culture, adherent cells were confirmed as EPCs by double-staining with Dil-labelled acetylated low-density lipoprotein (Dil-Ac-LDL, Molecular Probes, Carlsbad, CA, USA) and fluorescein isothiocyanate (FITC)-labelled Ulex europaeus agglutinin (UEA)-1 (Sigma). Cells were first incubated with ac-LDL at 37°C for 4 hours and then fixed with 1% paraformaldehyde for 10 min. After being washed, the cells were reacted with UEA-1 (10 µg/ml) for 1 hour. The samples were stained, then examined with a laser confocal microscope (LSM510, Zesis, Jena, Germany). Double-stained cells were confirmed to be EPCs.

Migration assay

Isolated EPCs were harvested by centrifugation, resuspended in 500 µl endothelial basal medium (EBM) and counted; 2×10^4 EPCs were then placed in the upper chamber of a modified Boyden chamber assay. The chamber was placed in a 24-well culture dish containing EBM with supplements and vascular endothelial growth factor (VEGF) (50 ng/ml). After incubation for 24 h at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells that migrated into the lower chamber and attached to the lower side of the filter were counted manually in 3 random microscopic fields by 3 independent blinded investigators.

Count of circulating endothelial progenitor cells

To quantify peripheral circulating EPCs by FACS, 5 ml blood was drawn. Mononuclear cells were isolated by density gradient centrifugation and incubated for 30 minutes on ice with PE-conjugated anti-mouse CD34 (BD Biosciences, San Jose, CA, USA) and FITC-conjugated anti-mouse Flk-1 (R&D Systems, Minneapolis, MN, USA). After incubation, the residual erythrocytes were lysed (BD FACS Lysing Solution, BD Biosciences, San Jose, CA, USA), and the remaining cells were washed with PBS. Data were analysed using CellQuest software (Becton Dickinson, San Jose, CA, USA) and all staining used isotype-matched control antibodies purchased from BD Pharmingen (San Diego, CA, USA) as reference. At least 1,000,000 cells were acquired during every event, and CD34⁺/Flk-1⁺ cells were evaluated in the lymphocyte/monocyte population and expressed as a percentage of gated cells.

Assessment of capillary density

The rabbits were sacrificed by intravenous injection of potassium chloride after 4 weeks' intervention. The hearts were harvested, embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN, USA) and cut transversely into slices approximately 5 µm thick. The segments between the anterior and posterior papillary muscle

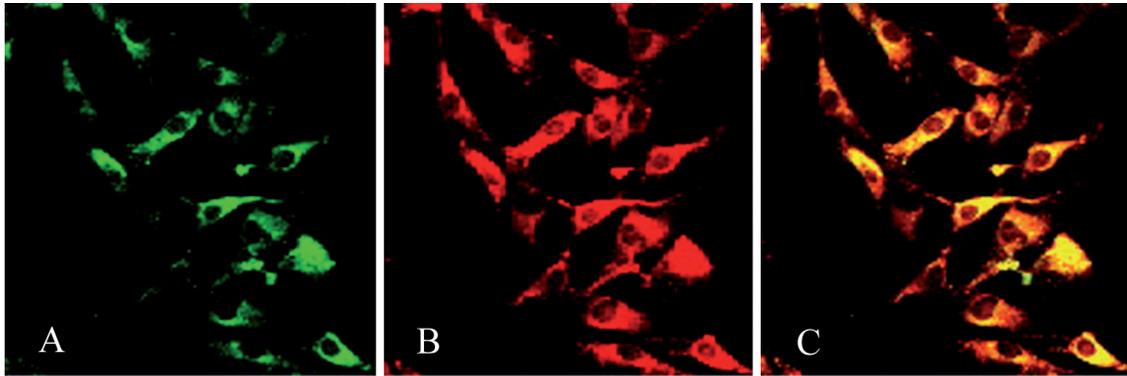


Fig. 2. (A) Fluorescence microscopy ($\times 200$) illustrates that adherent cells were positive for fluorescein isothiocyanate-labelled Ulex europaeus agglutinin-1 binding. (B) The adherent cells were positive for uptake of Dil-labelled acetylated low-density lipoprotein (Dil-ac-LDL). (C) Merged image of (A) and (B). The cells that were dual positive for UEA-1 and Dil-ac-LDL were characterized as pure endothelial progenitor cells.

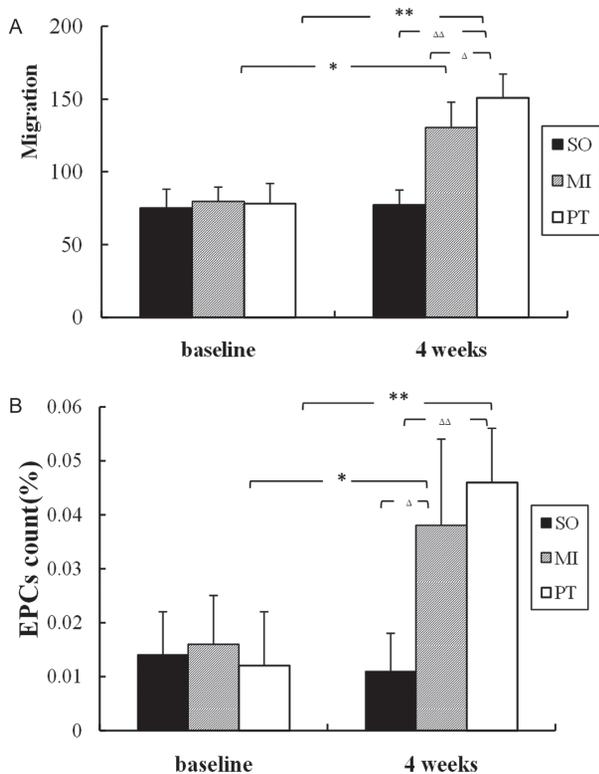


Fig. 3. (A) Quantities of migration endothelial progenitor cells (EPCs) before and after 4 weeks' training. Values are means and standard deviations (SD). Compared with the baseline, quantities increased significantly for groups MI ($*p < 0.05$) and PT ($**p < 0.001$), but showed little difference for group SO ($p > 0.05$). Among all groups, the increase in quantity of EPCs in group PT is the largest, much more than those of the other 2 groups ($\Delta p < 0.05$ vs group MI, $\Delta\Delta p < 0.001$ vs group SO) at the end-points. (B) Quantities of circulating EPCs before and after 4 weeks' training. Similar to the case of migration EPCs, groups PT and MI also showed significant increases in circulating EPC quantities while group SO showed little change (at the end-point, $\Delta\Delta p < 0.001$ group PT vs group SO, $\Delta p < 0.01$ group MI vs group SO). Among all groups, the quantity of circulating EPCs in group PT increased the most ($**p < 0.001$ in group PT, $*p < 0.01$ in group MI, end-point vs baseline). MI: intermittent myocardial ischaemia-only group; PT: MI plus physiological ischaemia training group; SO: sham operated group.

were selected. Some sections were fixed overnight in 10% formalin and embedded in paraffin for immunohistochemistry analysis. Some sections were frozen for immunofluorescence analysis.

Capillary density was evaluated by immunohistochemical staining to detect CD31 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) expression by endothelial cells. Here, a capillary was defined as a round structure with a diameter of less than 10 μm . Five fields from each sample were randomly selected for counting by 3 independent blinded pathologists. The number of positive cells in each high-power field was converted to cells per square mm.

Statistical analysis

Data are expressed as mean and standard deviation (SD). Analysis of variance was applied to assess differences among the groups, and multiple comparisons were performed using the forward least significant difference (LSD) *post hoc* test. A paired Student's *t*-test was used for comparing the number of EPCs before and after training in each group. Pearson's correlation coefficient was used to test the relationship between the number of EPCs and capillary density. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, USA).

RESULTS

Identification of endothelial progenitor cells

EPCs were characterized as adherent cells, positive for both Dil-ac-LDL and UEA-1 staining under a laser scanning confocal microscope. Double-positive cells were recognized as differentiating EPCs, as shown in Fig. 2.

Effect of physiological ischaemia training on endothelial progenitor cells migration

Among the 3 groups, little statistical difference in quantity of migration cells was evident at baseline ($p > 0.05$) (Fig 3A). However, after 4 weeks' PIT, the quantity of migration cells in both PT and MI groups increased significantly, while in group SO little increase was found after the experiment. It should be noted that the migration cells in group PT increased the most (196%, SD 22, $p < 0.05$). The percentage increase was approximately 30% more than that in group MI (166%, SD 27 in MI, $p < 0.05$). As a result, group PT showed the largest migration cell quantity among all groups (group PT: 151, SD 16, group MI: 130, SD 17, group SO: 77 cells, SD 10/high-power field, $p < 0.05$). Thus, PIT

Table I. Peripheral blood-endothelial progenitor cells number (%) in the experiment. Compared with baseline, circulating endothelial progenitor cell quantity after the experiment increased significantly for groups PT and MI, while there was no increase for group SO (** $p < 0.001$ in group PT; * $p < 0.05$ in Group MI; $p > 0.05$ in group SO). Of all the groups, PT showed the largest increase in the quantity of EPC after the experiments ($\Delta\Delta p < 0.001$ group PT vs group SO, $\Delta p < 0.01$ group MI vs group SO)

Group	Baseline	4 weeks	p-value
	Mean (SD)	Mean (SD)	
PT	0.012 (0.006)	0.046 (0.007)** $\Delta\Delta$	0.000
MI	0.016 (0.009)	0.038 (0.016)* Δ	0.035
SO	0.014 (0.008)	0.011 (0.007)	NS

SD: standard deviation; MI: intermittent myocardial ischaemia-only group; PT: MI plus physiological ischaemia training group; SO: sham operated group.

further increased the migration cells based on MI, which proves the positive effect of PIT on the migration activity of EPCs.

Effects on peripheral blood-endothelial progenitor cells number

The changes in CD34⁺/Flk-1⁺ peripheral blood-endothelial progenitor cells (PB-EPCs) in group PT, compared with groups SO and MI, are shown in Table I and Fig. 3B. Before the experiments, the percentages of PB-EPCs showed no statistical difference among all groups ($p > 0.05$). An increase in EPCs was observed in groups PT and MI. EPC levels were enhanced in group SO: 0.014%, SD 0.008, group MI: 0.016%, SD 0.009 and group PT: 0.012%, SD 0.006 to 0.011%, SD 0.007%, 0.038%, SD 0.016 and 0.046%, SD 0.007, respectively (baseline vs end-point of the experiment, $p > 0.05$). It shows that among all groups, group PT increased the PB-EPCs the most ($p < 0.01$).

Effects on capillary density

As shown in Fig. 4, at the end of the fourth week, capillary density in the ischaemic heart region in group PT (824.0, SD106.5/mm²) was much higher ($p < 0.01$) than those in the other two groups (group MI, 508.2, SD 92.7/mm²; and group SO, 329.3, SD 74.9/mm²).

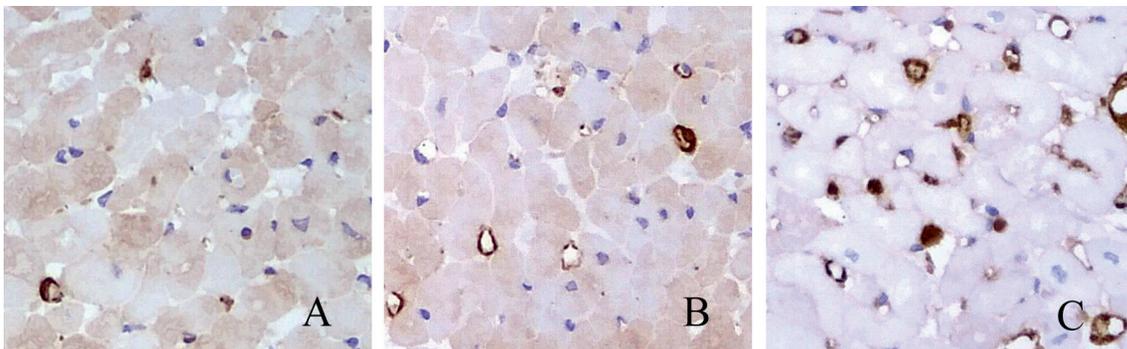


Fig. 4. The histology of capillary density in: (A) group SO, (B) group MI, and (C) group PT, indicated by brown stained lumens. MI: intermittent myocardial ischaemia-only group; PT: MI plus physiological ischaemia training group; SO: sham operated group.

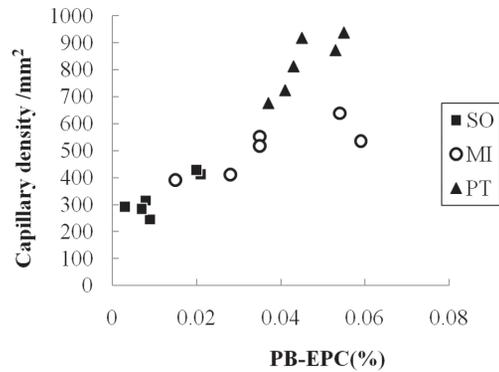


Fig. 5. Positive correlations of peripheral blood-endothelial progenitor cells (PB-EPCs) with capillary density were observed in the experiment in groups MI and PT. $r_{MI} = 0.812$, $p < 0.05$; $r_{PT} = 0.844$, $p < 0.05$. MI: intermittent myocardial ischaemia-only group; PT: MI plus physiological ischaemia training group; SO: sham operated group.

Association between endothelial progenitor cells and capillary density

The correlation coefficient between the quantity of EPCs and capillary density was 0.81 in group MI and 0.84 in group PT (Fig. 5). This indicates a high correlation between the quantity of EPCs and collateral formation.

DISCUSSION

PIT, the isometric contraction of normal skeletal muscles induced by electrical stimulation, was first proposed in our laboratory as a novel and safe technique for use in cardiac rehabilitation. Our preliminary experiments on rabbits show that PIT has little influence on heart rate and blood pressure. When intramuscular pressure is close to, or larger than, arterial pressure, a reversible local ischaemia can be induced. Evidence of the safety and efficacy of isometric exercise in patients with chronic heart failure is well documented in the literature. It has been demonstrated by Fisher et al. (15) that, even with electrically evoked ischaemic isometric calf plantar flexor exercise at 30% maximum voluntary contraction, blood pressure would not

be changed. Petrofsky et al. (16) studied elderly people (65.8 years old, SD 8.8) with severe heart failure (New York Heart Association IV group, ejection fraction = 19.7%, SD 6.4), and found that a short maximum voluntary isometric contraction of the lower extremities muscles with persistent physiological breathing did not have an abnormal effect on systemic and pulmonary haemodynamics. Tayer et al. (17) studied the effects of isometric training on older adults with hypertension and found a significant reduction in resting systolic pressure and mean arterial pressure after training. A stable blood pressure and heart beat is helpful in preventing cardiac accidents during training.

The present study showed that PIT could increase circulating EPCs. In the literature, it was demonstrated that physical training could increase systemic EPCs. For example, Steiner et al. (18) reported that a 12-week period of regular physical exercise resulted in a 2.9-fold increase in circulating EPCs, and therefore promoted cardiovascular health. Walther et al. (19) reported that after a school year of regular, high-intensity exercise training, the numbers of EPCs were significantly increased in high-school and grade 6 students. Rehman et al. (20) also reported that a single episode of exercise could acutely increase circulating EPCs by 4-fold from baseline. Meanwhile, Adams et al. (21) reported that, for patients with coronary artery disease, exercise-induced myocardial ischaemia could significantly increase EPCs, which reach a maximum after 24–48 h and then return to baseline within 72 h. Our study, however, showed that PIT could also increase circulating EPCs, which is in accordance with physical training in heart disease rehabilitation.

It was demonstrated that physical training could enhance the migration of EPCs. Hoetzer et al. (22) reported that 3 months' regular aerobic exercise resulted in a 50% increase in migratory capacity. A 3-month exercise programme offered to sedentary middle-aged to elderly subjects resulted in significant upregulation of colony-forming and migratory capacity EPCs (23). Van Craenenbroeck et al. (24) studied the effects of exercise on 35 sedentary men with chronic heart failure (CHF; left ventricular ejection fraction <or=45%), with or without Type D personality. It was found that exercise induced a highly significant enhancement of migratory capacity in all groups. In our research, the relationship between PIT and the function of EPCs was investigated. It was found that, similar to the circulating EPCs, PIT also could increase both the quantity of migration EPCs and the migratory activity of EPCs. This is in accordance with the situation in physical training.

Exercise training is currently regarded as an intervention for primary and secondary prevention of coronary artery disease (25, 26). Many researchers have demonstrated that exercise can enhance capillary density. In early studies, capillary density was found to be higher in wild rabbits or rats than in domesticated ones, suggesting that the higher level of physical activity of wild animals is associated with higher capillary density (27). Garciaarena et al. (28) reported that endurance training could increase myocardial capillary density by approximately 45% in spontaneously hypertensive rats. The present study, however, showed that PIT could also improve capillary density in the ischaemic heart region, which is in ac-

cordance with the circulating EPCs and EPC migration activity, and hence might be applied as a promising new approach in cardiac rehabilitation.

The study shows that IMI could increase circulating EPCs, while PIT together with IMI could mobilize more EPCs homing to ischaemic myocardium. Similarly, IMI could increase capillary density in the ischaemic heart region, and PIT with IMI could augment capillary density further. Our research also showed that PIT alone could also increase circulating EPCs. The statistics showed that circulating EPCs were positively correlated with capillary density in the ischaemic heart region. This may be due to the EPCs homing. When EPCs homed to the injured tissue, they could promote angiogenesis and then improve the local microenvironment (5, 6). Meanwhile, the improved microenvironment in turn could promote homing and functioning of EPCs. Many studies have demonstrated that homing of EPCs results in angiogenesis in the foci. Our study showed that the homing of EPCs was involved in the effect of PIT on angiogenesis in the remote ischaemic heart region, during which the quantity and activity of EPCs are shown to be the key factors.

Fig. 3 shows that, for EPC migration, IMI made the largest contribution to EPC mobilization, while further performing PIT lead to a modest additional improvement. However, the increase in capillary density was much more pronounced in the PT group compared with the group undergoing IMI alone. This may also be caused by the improvement in the local microenvironment due to the migration of EPCs. With the increase in capillary density, however, myocardial ischaemia would be significantly alleviated, which would reduce the need for EPCs. It should be noted that the results shown in Fig.3 were from the data at 4 weeks. At that time, a large number of capillaries had already been generated and the need for mobilizing EPC was reduced, thus made the increase in capillary density much greater than that in EPCs.

It should be noted that this paper focuses on research in rabbits. As a novel rehabilitation method, initial animal research is necessary and indispensable. The results will be extremely useful for subsequent study in humans. This research is currently being extended to patients with ischaemic heart disease. Corresponding testing and data collection is being carried out.

In conclusion, in rabbits with myocardial ischaemia, 4 weeks of PIT increased PB-EPCs, augmented migration of EPCs, and improved angiogenesis in the ischaemic heart region. This study focused on the effect of PIT on EPCs. The molecular mechanism of this effect is unknown, and will be the subject of future research.

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