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Time course effect of hypoxia on bone marrow-derived endothelial progenitor cells and their effects on left ventricular function after transplanted into acute myocardial ischemia rat

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Abstract. – OBJECTIVE: Ischemic heart disease is the most common cause of cardiovascular morbidity and mortality in the industrialized world, and the incidence has been increasing in developing countries. Stem cell transplantation has emerged as a potent new therapeutic strategy for acute/chronic ischemic heart disease and has been explored extensively. The present study aimed to investigate whether hypoxic preconditioning of endothelial progenitor cells (EPCs) before transplantation could ameliorate their survival and engraftment in ischemic tissue and the potential mechanisms.

MATERIALS AND METHODS: EPCs extracted were subjected to increasing hypoxia for 24-72 h, survival and function of the preconditioned EPCs were assayed in both *in vitro* and *in vivo*.

RESULTS: Hypoxia for 24 h caused significant enhancements in formation of tube like structure and motility of BM-EPCs ($p < 0.05$), as well as mRNA expressions of CXCR4, PI3K, AKT, and NF-κB, while these effects were reversed by prolonged hypoxia (48 and 72 h, $p < 0.05$). Hypoxia of BM-EPCs for 24 h did not result in increased apoptosis resistance, and cell apoptosis was even enhanced by prolonged hypoxia. *In vivo* transplantation experiments demonstrated the beneficial effect of hypoxic EPCs on left ventricular (LV) functions after acute myocardial ischemia (AMI).

CONCLUSIONS: Shorter-term hypoxia showed better survival, differentiation and function of BM-EPCs *in vivo*, further study was still needed to optimize the hypoxic pattern of BM-EPCs so as to better protect heart from myocardial ischemic injury. The present study showed evidence suggested that hypoxic preconditioning did exert further beneficial effects of BM-EPCs on preservation of LV function after AMI. Short-term exposure to hypoxia for about 24 h provided better condition for survival and function of BM-EPCs.

Key Words:
Acute myocardial ischemia (AMI), Bone marrow-derived endothelial progenitor cells (BM-EPCs), CXC chemokine receptors 4 (CXCR4), Hypoxia, Left ventricular (LV) function, Nuclear factor κB (NF-κB), Phosphatidylinositol 3-kinase (PI3K), Protein kinase B (AKT), Stromal cell-derived factor (SDF)-1α.

Introduction

Ischemic heart disease is the most common cause of cardiovascular morbidity and mortality in the industrialized world, about 1 million myocardial infarctions occur annually in the USA alone, and the incidence has been increasing in developing countries[1]. To protect the heart from ischemia reperfusion injury, various treatments have been attempted up to now[2]. Stem cells transplantation has emerged as a potent new therapeutic strategy for acute/chronic ischemic heart disease and has been explored extensively. Increasing evidence has established stem/progenitor cells-based therapies as a promising option for myocardial ischemia[3,4].

Endothelial progenitor cells (EPCs) or angioblast are a specific subtype of hematopoietic stem cells with the angiogenic ability, the most distinguishable characteristic over other stem cells. The number and migratory activity of circulating EPCs were demonstrated to be inversely correlated with risk factors for coronary artery disease[5], and were generally considered as a biomarker of cardiovascular risk. Previous studies indicated that EPCs were associated with endothelial repair after myocardial ischemia[6]. EPCs seemed to be mobilized in response to tissue ischemia[7], and were recruited to ischemic regions

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for neovascularization[8]. Shintani et al[9] found that EPCs number was significantly increased in patients with acute myocardial infarction. Meanwhile, study by Heeschen et al[10] discovered that numbers of EPCs were similar between patients with chronic ischemic cardiomyopathy and healthy control, whereas colony-forming capacity of EPCs was significantly impaired in the former patients. EPCs transplantation may be, therefore, as a potential therapy for ischemic disease.

Background

Transplantation of EPCs was demonstrated to be functionally beneficial to myocardial function in both acute myocardial infarction animal model and human[11,12]. However, despite of these benefits, the poor retention of transplanted stem/progenitor cells may pose a major barrier to successful stem cell therapies for myocardial ischemia[13], and the poor survival, homing, and proliferation performance of transplanted cells severely limit the clinical potential of stem cell therapy[14]. The survival, migration and proliferation of transplanted progenitor cells would require cell to adapt to the harsh, oxygen deprived environment in ischemic heart tissue[15]. The successful migration of the transplanted cells toward the site of injury is a critical step in cell engagement for tissue repair[16]. Multiple strategies have been adapted to enhance the survival, migration as well as proliferation of the transplanted stem/progenitor cells[17,18]. Previous reports have demonstrated that hypoxia preconditioning have a favorable effect on stem/progenitor cells transplantation in ischemic myocardium, and the survival and migration of transplanted stem/progenitor cells were greatly improved by hypoxic preconditioning in the ischemic heart[16,19].

Accumulating evidence has indicated that short-term exposure of stem cells to hypoxia can significantly enhance their viability, and thus improve their tissue repair capabilities after transplantation into the ischemic tissue[20,21]. However, to our knowledge, the effects of hypoxic preconditioning on functional benefit of EPCs in ischemic heart tissue have not been well reported, and the potential mechanism is still unclear. Moreover, the duration of hypoxia varied a lot, the optimal pattern of hypoxic preconditioning of EPCs is poorly characterized.

Aim

In the present study, we first demonstrated that the bone marrow-derive (BM)-EPCs extracted from rat, and BM-EPCs were subjected to the different time course of hypoxic preconditioning. We then determined the effects of time course hypoxia on differentiation, apoptosis, and migration activity of BM-EPCs. We further explored potential mechanisms of homing migration and survival of BM-EPCs involving mRNA expressions of CXC chemokine receptors 4 (CXCR4), as well as pro-survival factors PI_3K, AKT, and NF-κB, and functions of transplanted BM-EPCs that may be modulated by hypoxic conditioning.

Materials and Methods

Animals

This study was reviewed and approved by the Animal Ethics Committee of Tianjin Cardiovascular Institute. Adult wistar rats, 8 weeks of age and weighting 250-280 g, were purchased from experimental animal center of Military Academy of Medical Sciences, China. All surgical procedures and care administered to the animals were approved by the Animal Care Committee and performed according to institutional guidelines.

Isolation and culturing of EPCs

Wistar rats were anesthetized with ketamine (80 mg/kg) and sacrificed by decapitation. The EPCs were isolated from rat bone marrow. Briefly, mononuclear cells were separated from the humerus, tibia, fibula and femur of Wistar rats by density gradient centrifugation using Histopaque 1083, according to the users' manual (Sigma-Aldrich, St Louis, MO, USA). Isolated cells were suspended in 2 mL of EBM-2 basal medium (1×10^9 cell/L, Lonza, Walkersville, MD, USA), then inoculated on rat plasma vitronectin (Sigma)-coated culture dishes, and maintained at 37°C in 5% CO_2. After 4 days in culture, non-adherent cells were removed and fresh medium was added. Cells were randomly distributed into the normoxic-preconditioning group (NBM-EPCs) and hypoxic-preconditioning group (HBM-EPCs), and were cultured under normoxic (5% CO_2, 95% humidified air) and hypoxic (1% O_2, 5% CO_2, 94% N_2) conditions for 3 days, respectively. The anoxic gas mixture was replaced every 24 h. Following the 7 days of culture, the preconditioned cells were harvested for analysis or transplantation.

Endothelial marker analysis of BM-EPCs

The expression of endothelial markers on BM-EPCs was analyzed by the IN Cell Analyzer 2000 System (GE Healthcare, Amersham, UK).

The adherent cells were detached into a single-cell suspension with 0.25% trypsin/EDTA, washed twice with PBS, counted and adjusted to 1×10^6 cells per mL. Aliquots containing 1×10^5 cells were re-seeded on a 96-well plate and incubated with Phycoerythrin (PE)-conjugated goat anti-mouse CD34 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and FITC-conjugated rabbit anti-mouse CD133 antibody (Bioss USA, Woburn, MA, USA) for 1 h at 37°C, and washed twice with PBS. The samples were subjected to image acquisition using the In Cell Analyzer 2000, and image analysis was performed with Image J software.

Endocytosis Analysis

The BM-EPCs were also characterized by endocytosis of 1,1-dioctadecyl-3,3,3,3- tetramethylindo-carbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL) and fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin-1 (FITC-UEA-1). The suspended cells were incubated sequentially with Dil-ac-LDL (10 μg/Ml, Molecular Probes Inc., Eugene, OR, USA), FITC-UEA-1 (10 μg/Ml, Sigma-Aldrich, St Louis, MO, USA), and 4,6-diamino-2- phenyl indole (10 μg/Ml, DAPI, Roche Molecular Biochemicals, Mannheim, Germany). Slides were examined using inverted fluorescence microscopy (Olympus IX71, Olympus Optical Co. Ltd, Tokyo, Japan) for double-positive cells.

Matrigel Tubule Assay

BM-EPCs harvested 3 days after normoxic culture and 1, 2, and 3 days after hypoxic culture were plated on matrigel (50Ml, BD Biosciences, Mountain View, CA, USA)-coated 96-well plates at 2×10^5 cells per plate, and cultured at 37°C for 6 to 8 h. Pictures were taken for quantification of network length and loops using WimTube software (Wimais GmbH, Munich, Germany).

Apoptosis Assay

Apoptosis was measured with the Annexin V/PI apoptosis detection kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions. BM-EPCs were collected after normoxic culture for 3 days and hypoxic culture for 1, 2, and 3 days. Each cell culture was plated on the tubes (1×10^5 cells), and incubated with PBS, FITC-Annexin V, PI, and FITC-Annexin V+ PI, respectively, then analyzed on a flow cytometer (FACSCanto, BD Biosciences, San Jose, CA, USA).

Cell Migration Assay

A Boyden chamber assay was used to assess BM-EPCs migration after normoxic or hypoxic culture for 1, 2 and 3 days. Briefly, resuspended cells were counted under a hemocytometer, 5×10^4 cells were plated in the upper of two chambers (Neuro Probe Inc., Cabin John, MD,USA) divided by a membrane with 8 mm pores, and 100 ng/mL stromal-derived factor-1α (SDF-1α, R&D Systems Inc., Minneapolis, MN, USA) was added to the lower chamber. After incubation at 37°C in 5% CO_2 atmosphere for 3.5 h, the membranes were detached and mounted on glass slides with a DAPI-containing mounting medium. The number of cells migrating through the filter was counted using a DF5000B Leica fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

Real Time (RT)-PCR Analysis

To further evaluate the effect of hypoxic preconditioning on BM-EPCs, mRNA expressions of CXCR4, protein kinase B (AKT), phosphatidylinositol 3-kinase (PI_3K), and nuclear factor κB (NF-κB) were assessed in BM-EPCs cultured under normoxic or hypoxic condition for 24, 48, and 72 h. Total cellular RNA was isolated from BM-EPCs under normoxic or hypoxic conditions with the UNIQ-10 column RNA extraction kit (Shanghai Biological Engineering Company Limited, Shanghai, China), according to the manufacturer's instructions and quantified using a UV spectrophotometer. Reverse transcription was performed using an oligo(dT)18 primer and M-MLV reverse transcriptase (Dalian Baosheng Biotechnology Co., Ltd., Dalian, China). Quantitative RT-PCR was performed with the 2× SYBR Premix Ex Taq (TaKaRa, Dalian, China) on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The specific primers used were summarized in Table I, and GADPH was used as internal control and amplified in parallel. The relative quantitative analysis was performed by comparison of the $2^{-\Delta\Delta Ct}$ values.

Acute Myocardial Infarction Model

The rat acute myocardial infarction model was developed in 34 adult wistar rats by ligating the left anterior descending coronary artery as described previously[22]. Briefly, animals were anesthetized with ketamine hydrochloride (80 mg/kg) in combination with Xylazine Hydrochloride (10 mg/kg). An indwelling needle (Gauge No. 16)

Table I. Sequences of primer used for RT-PCR.

Gene	Primer (5′→3′)		Length (bp)
CXCR4	Forward	CGCTACCTTGCCATTGTCC	158
	Reverse	CAGATGTACCTGCCGTCCC	
AKT1	Forward	GTGTGGCAAGATGTGTATGAGAA	192
	Reverse	TGAGTAGGAGAACTGGGGAAAG	
PI3K	Forward	AAACGGAAAGGGAAGCTGTGA	240
	Reverse	TCTCAATCACGTGGACCTGC	
NF-κB	Forward	TTACGGGAGATGTGAAGATGC	103
	Reverse	TGAAGGTGGATGATGGCTAAG	
GAPDH	Forward	CCCATCACCATCTTCCAGGAGCA	411
	Reverse	GGCAGGGATGATGTTCTGGAGAGCC	

was inserted into trachea, and connected with a ventilator (Hallowell EMC, Hallowell Engineering and Manufacturing Corp, Pittsfield, MA, USA), with the respiratory rate and tidal volume adjusted according to body weight[23]. A left thoracotomy was performed through the fourth intercostal space, and the heart was exposed. The pericardium was opened and LAD was ligated with a 7-0 polypropylene suture 2 mm from its origin (the edge of the left atrium). The color of the LV endocardial surface was monitored to ensure an infarct size of about 30% of the area at risk[22].

BM-EPCs Transplantation

After the establishment of acute myocardial infarction model, 34 Wistar rats were randomly divided into the three groups: control group (n=12), normoxic group (n=10), and hypoxic group (n=12). Animals in three groups were respectively given intracardiac injections of 200 μL PBS, 2×10^6 NBM-EPCs and HBM-EPCs suspended in 200 μL PBS, correspondingly, at the five different regions of the margin of infarcted myocardium with a 30-Gauge needle. After cellular transplantation, the thoracotomy site was closed in layers, and animals were weaned from the ventilator and allowed to recover. Each rat was injected with buprenorphine (0.5 mg/kg) subcutaneously for postoperative analgesia, and cultured another four weeks for the following experiments.

Hemodynamic Measurement

Four weeks after cells transplantation, animals were anesthetized and mechanically ventilated as mentioned above. For measurement of cardiac function, a pressure-volume conductance catheter (SPR-869; Millar Instruments Inc. Houston, TX, USA) was introduced through the right carotid artery into the left ventricle (LV), as described previously[23]. Data were analyzed using LabChart, version 6 software (AD Instruments, Colorado Springs, CO, USA) and PVAN Cardiac Pressure-Volume Analysis software (Millar Instruments, Houston, TX, USA).

Echocardiographic Measurement

Changes in LV morphology and function were assessed by transthoracic echocardiography (Philips Sonos 5500, Koninklijke Philips Electronics N.V., Groenewoudseweg, Eindhoven, Netherlands) with a S12 probe four weeks after cell transplantation. LV end-systolic and end-diastolic internal diameters were measured using an M-mode echocardiogram, and LV ejection fraction was calculated.

Statistical Analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing. Data were expressed as the mean value ± standard deviation (SD). Comparisons between groups were analyzed by paired samples *t*-test, and comparisons among groups were performed by one-way analysis of variance (ANOVA). Multiple comparisons between groups were determined by least significant difference test (LSD-t). The *p* values of less than 0.05 were considered significantly difference.

Results

Morphology and Proliferation of BM-EPCs

The initially seeded cells were round and after cultured for 2 days attached cells appeared. 4 days after cultivation, cell number increased sig-

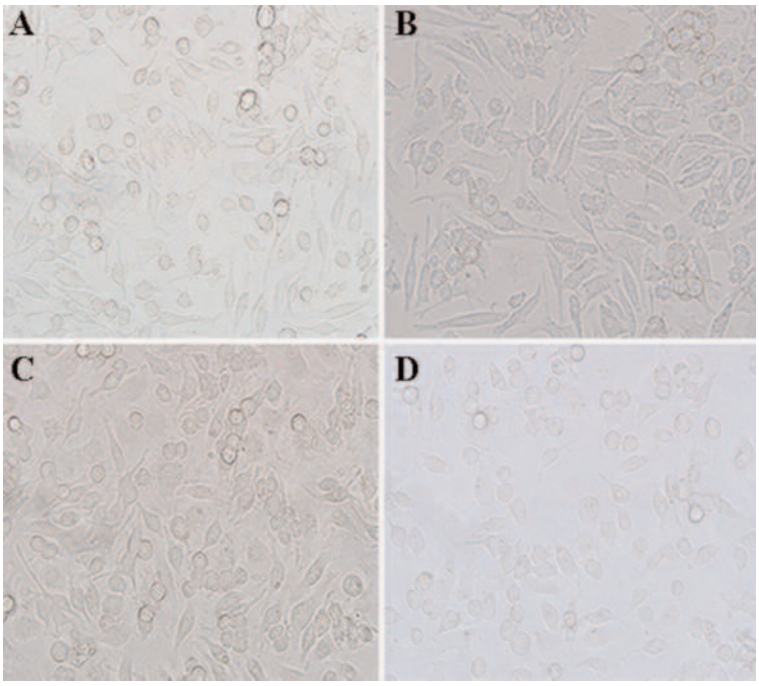

Figure 1. The cell morphology of in vitro expanded BM-EPCs. ***A,*** BM-EPCs grew under normoxic condition for 7 days. ***B,*** BM-EPCs after hypoxic preconditioning for 1 day. ***C,*** BM-EPCs after hypoxic preconditioning for 2 days. ***D,*** BM-EPCs after hypoxic preconditioning for 3 days (Magnification ×100).

nificantly, and BM-EPCs formed a number of island-like cell clusters, with two types of cells recognized, namely fusiform or spindle cells located at the margin of the colony and polygonal cells in the center. These clusters were gradually disappeared after normoxic culture for another 3-5 days, and BM-EPCs under normoxic condition grew bigger, displayed a typical fusiform morphology, and some were arranged in-line (Figure 1A). The BM-EPCs grew under hypoxic condition for 24 h were dominated by spindle cells and did not show marked change in morphology when compared with the normoxic cultured ones (Figure 1B). However, cell morphology changed a lot after hypoxic preconditioning for 48 and 72 h (Figure 1C and D). Hypoxic BM-EPCs grew bigger, shorter and thicker than the normoxic cultured ones at 72 h hypoxia (Figure 1D).

Analysis of Endocytosis and Endothelial Cell Markers of BM-EPCs

After expanded in vitro for 7 days, as presented in Figure 2, BM-EPCs showed the capability to incorporate DiI-acLDL and bind UEA-1, consistent with the proliferation characteristics of EPCs. We next examined the expression of endothelial lineage markers on BM-EPCs, and our results showed that EPCs expressed endothelial markers CD34 (red, Figure 3A) and CD133 (green, Figure 3B).

Time Course Hypoxia on Tube Formation of BM-EPCs

The characteristics of BM-EPCs were also assessed by matrigel Assay. As shown in Figure 4, cells incubated in matrigel produced tube-like structures, a property of mature endothelial cells, when cultured in either normoxic or hypoxic condition. Formation of tube like structures was more prominent on 24 h hypoxia (Figure 4). Quantitative analysis showed the total tubes formed and length of the tubes on 24 h hypoxic preconditioned BM-EPCs were significantly greater than that of the normoxic ones, while prolonged hypoxic preconditioning for 48 h and 72 h significantly reduced the formation of tube like structures when compared with the normoxic group (Figure 4 E), suggesting that short-term hypoxic preconditioning for 24 h could enhance the ability of EPCs to form tube-like structures *in vitro*.

Time Course Hypoxia on Apoptosis of BM-EPCs

When culture cells were exposed to hypoxia for 72 h, the apoptosis of BM-EPCs was showed to be enhanced with hypoxic preconditioning time (Figure 5). The ratio of apoptotic cells (Annexin V+) was not significantly increased after 24 h of hypoxia, while, after 48 and 72 h hypoxic preconditioning, data were found to be significantly higher than that of normoxic cells (Figure 5E).

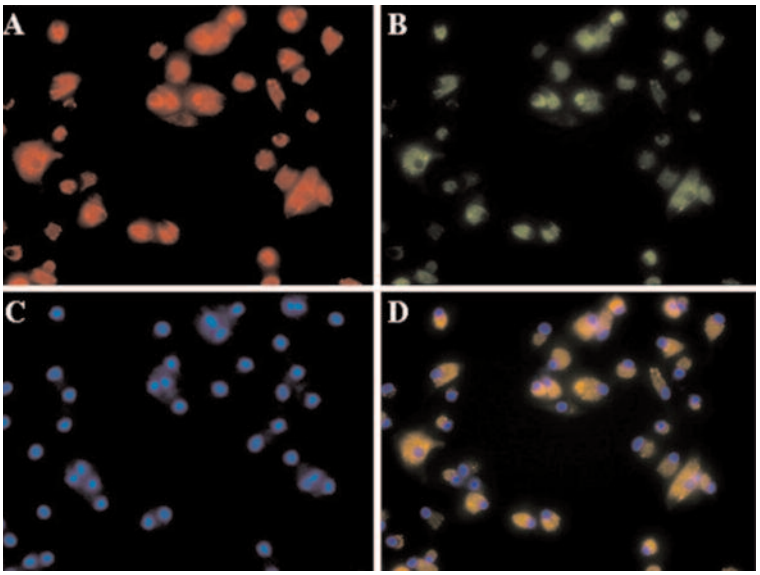

Figure 2. Identification of BM-EPCs by DiI-acLDL incorporation and FITC-UEA-1 bind affinity. BM-EPCs taken up DiI-acLDL were in red fluorescence ***(A)***, bound to FITC-UEA I were displayed in green fluorescence ***(B)***, Cultured BM-EPCs stained with DAPI (C, blue), and the double-stained cells for DiI-Ac–LDL and FITC-UEA-1 were showed in yellow fluorescence ***(D***, DAPI nuclear stain) (Magnification ×400).

Time Course Hypoxia on Cell Motility of BM-EPCs in Response to SDF-1α

The motility of BM-EPCs under either normoxic or hypoxic condition was examined by testing their ability to migrate through a porous filter. As shown in Figure 6, BM-EPCs under normoxic condition were able to migrate through the filter at a low rate of 123 ± 27 cells per field of view. Hypoxic preconditioning for 24 h showed a significant increase in BM-EPCs migrating through the filter (506 ± 66 cells per field of view, $t = 7.596$, $p < 0.05$), while migration of BM-EPCs was markedly attenuated by the prolonged hypoxia for 48 and 72 h, and the data were still higher than that of normocix control although did not reach statistical significance (Figure 6E).

Time Course Hypoxia on mRNA Expressions of Various Factors in BM-EPCs

As presented in Figure 7, RT-PCR analysis showed varying induction of transcriptions of CXCR4, PI3K, AKT, and NF-κB after 24 h hypoxic preconditioning of BM-EPCs. By contrast, after the prolonged hypoxic preconditioning for 48 and 72 h, mRNA expressions of CXCR4, PI_3K, AKT, and NF-κB were significantly attenuated (Figure 7 A, B, C, and D; $p < 0.05$).

Hypoxia preserved LV Function in Rat Acute Myocardial Ischemia Model

To ensure the survival and function of BM-ECPs, we chose our sub-lethal time of 24 h to further examine whether hypoxia-preconditioned BM-ECPs had a greater therapeutic potential on the LV function in acute myocardial ischemia (AMI) of rats. After 28 days of transplantation, as shown in Table II, LV end-diastolic and end-systolic diameters were found to be significantly reduced by administration of normoxic BM-EPCs ($p < 0.05$), while normoxic BM-EPCs transplantation resulted in a significant increase in maximum pressure, dP/dt_{max}, dV/dt_{max}, stroke work, cardiac output,

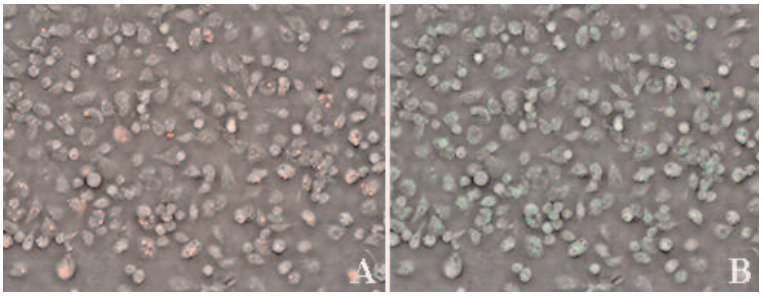

Figure 3. Identification of BM-EPCs by fluorescent antibody labeling of endothelial cell markers. BM-EPCs were positively stained for endothelial markers CD34 (red, ***A***) and CD133 (green, ***B***) (Magnification ×40).

and LV ejection fraction on day 28 compared to that of the control ($p < 0.05$). By contrast, hypoxic preconditioned BM-EPCs, when transplantation into the myocardial infarcted rats, resulted in significantly lower LV end-diastolic and end-systolic diameters, along with the significantly higher maximum pressure, dP/dt_{max}, dV/dt_{max}, stroke work, cardiac output, and LV ejection fraction, when compared with the control group rats. Moreover, LV end-systolic diameter was further reduced by transplantation of hypoxic preconditioned BM-EPCs, while maximum pressure, dP/dt_{max}, and LV ejection fraction were further significantly increased, as compared with normoxic BM-EPCs transplanted group (Table II).

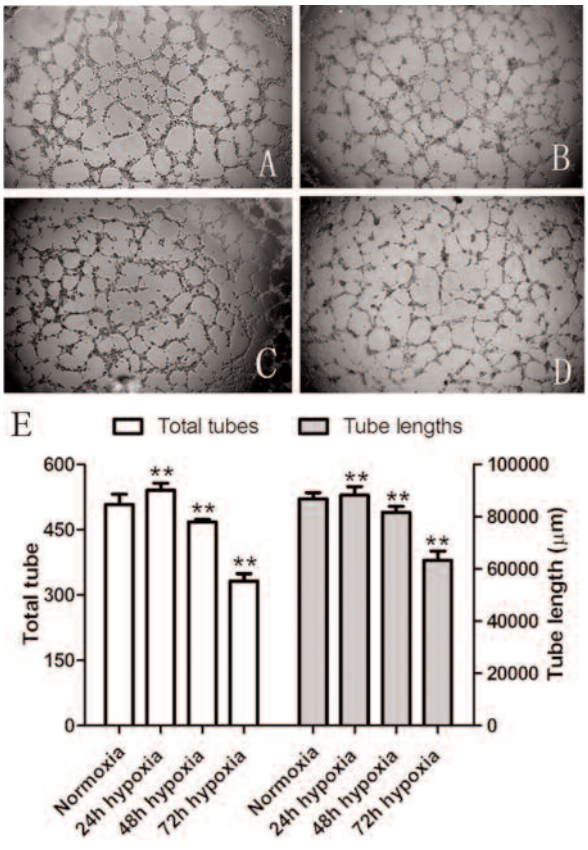

Figure 4. Tube formation abilities of the BM-EPCs under normoxic or hypoxic condition. ***A,*** BM-EPCs grew under normoxic condition for 7 days. B) BM-EPCs after 1 day hypoxic preconditioning. ***C,*** BM-EPCs after hypoxic preconditioning for 2 days. ***D,*** BM-EPCs after hypoxic preconditioning for 3 days (Magnification ×100). ***E,*** Quantitative analysis of the tube formation of BM-EPCs under either normoxic or hypoxic condition ($^{**}p < 0.01$ vs the normoxic group).

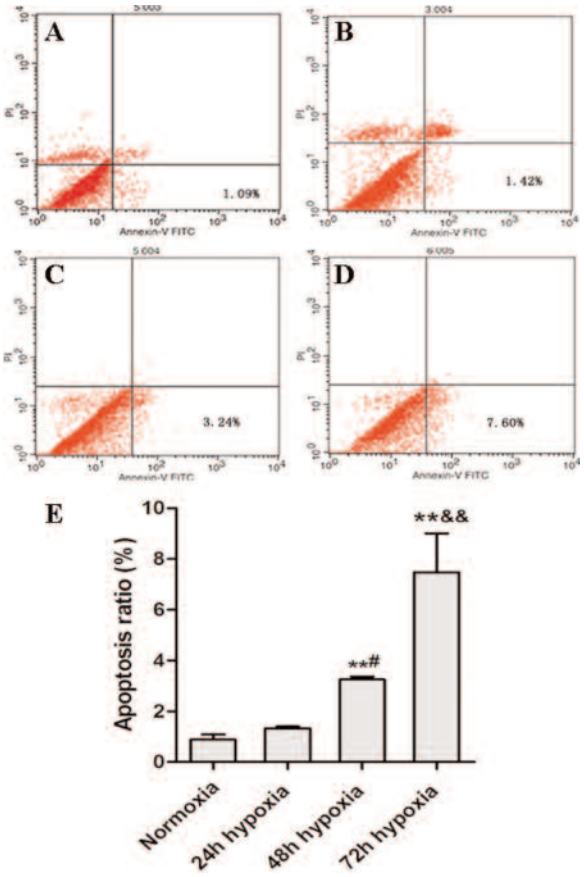

Figure 5. Time course effects of hypoxic preconditioning on apoptosis of BM-EPCs as analyzed by flow cytometry. ***A,*** BM-EPCs grew under normoxic condition for 7 days. ***B,*** BM-EPCs after hypoxic preconditioning for 1 day. ***C,*** BM-EPCs after hypoxic preconditioning for 2 days. ***D,*** BM-EPCs after hypoxic preconditioning for 3 days. ***E,*** The ratio of apoptotic BM-EPCs after normoxic or time course hypoxic preconditioning. $^{**}p < 0.01$ vs normoxic group, $^{\#}p < 0.05$ vs 24 h hypoxic group, and $^{\&\&}p < 0.01$ vs 48 h hypoxic group.

Discussion

Vasculogenesis and angiogenesis are two distinctive morphogenetic mechanisms of postnatal revascularization that playing a critical role in the pathophysiology of ischemic diseases, and are of major important for myocardial impairment after ischemia and infarction[24, 25]. Endothelial progenitor cells (EPCs) or angioblasts, a circulating peripheral blood- or bone marrow-derived subtype of progenitor cells, may exert both types of effect by releasing growth factors that promote angiogenesis and/or by becoming physical elements of newly formed vessels that contribute to vasculogenesis[26]. EPCs were showed to be rapidly re-

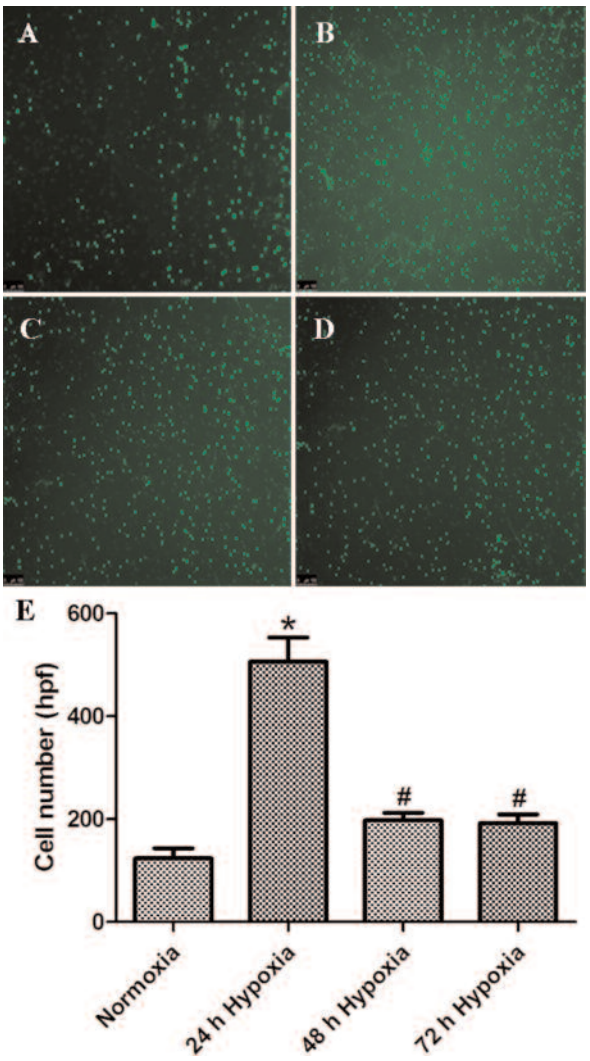

Figure 6. Transfilter assay of BM-EPCs toward SDF-1α. ***A,*** BM-EPCs grew under normoxic condition. ***B,*** BM-EPCs after hypoxic preconditioning for 24 h. ***C,*** BM-EPCs after hypoxic preconditioning for 48 h. ***D,*** BM-EPCs after hypoxic preconditioning for 72 h. ***E,*** Cell number of BM-EPCs migrating through the filter under the fluorescence microscopy (cells per field of view). $^{*}p < 0.05$ vs the normoxic group, and $^{\#}p < 0.05$ vs the 24 h hypoxic group.

cruited to ischemic myocardium, and there were a large accumulation of EPCs in active angiogenic foci that participate in neovascularization after ischemic insults[24,27]. Progenitor cells were expected to be of most benefit to cardiac regeneration or performance when used to treat jeopardized myocardium[28]. The incorporation of bone marrow-derived angioblasts showed a great physiological benefit by inducing vasculogenesis and angiogenesis, and thus protect ischemic myocardium from apoptosis and remodeling[26]. Clinically, intracoronary infusion of EPCs was demonstrated to be safe and feasible in acute myocardial infarction patients[29]. However, only few transplanted cells homed to the targeted ischemic tissues, and the local hypoxia, oxidative stress and inflammation further restricted the survival and proliferation of transplanted cells, which in turn resulted in inadequate functional recovery of the ischemic myocardium[3]. It is, therefore, of crucial important to enhance the migration and survival of the implanted EPCs to maximize the efficiency and efficacy of EPC-based therapy.

It is well known that tissue hypoxia could mobilize EPCs from bone marrow into the peripheral circulation[9]. Hypoxic preconditioning was reported to improve cellular engraftment and survival in low-oxygen atmospheres, and local hypoxic condition stimulated migration activity and differentiation of EPCs, as well as enhanced the angiogenic functions of EPCs in the ischemic hind limb of immunodeficient nude rats[31]. However, litter is known about the time course effect of hypoxic preconditioning on the differentiation, migration and apoptosis of the BM-EPCs, as well as LV function after transplantation of hypoxic preconditioned BM-EPCs into the ischemic myocardium and the potential mechanism. Accordingly, we investigated whether time course hypoxia itself would influence the differentiation, migration and apoptosis of BM-EPCs during *in vitro* expansion and whether hypoxic preconditioning of BM-EPCs exerted a functional benefit through SDF-1α and CXCR4 axis. The EPCs were isolated from rat bone marrow, and were identified by their positive stain for CD34 and CD133 surface markers, as well as dual affinity to acLDL and UEA-1. For hypoxic pretreatment, BM-EPCs were exposed to low oxygen (1% O_2) for 24 to 72 h. Our results showed a significant increase in cell motility of BM-EPCs after hypoxic preconditioning for 24 h, while migration of BM-EPCs was markedly attenuated by the prolonged hypoxia for 48 and 72 h. The shorter term hypoxia of BM-EPCs for 24 h also enhanced the tube formation of BM-EPCs in vitro, a property of mature endothelial cells, while BM-EPCs formed less tube like structures after hypoxic preconditioning for 48 and 72 h compared to the normoxic ones. Meanwhile, hypoxic preconditioning performed in our study resulted in the enhanced apoptosis of BM-EPCs, and the percent of apoptotic cells after hypoxic preconditioning for 48 and 72 h was found to be

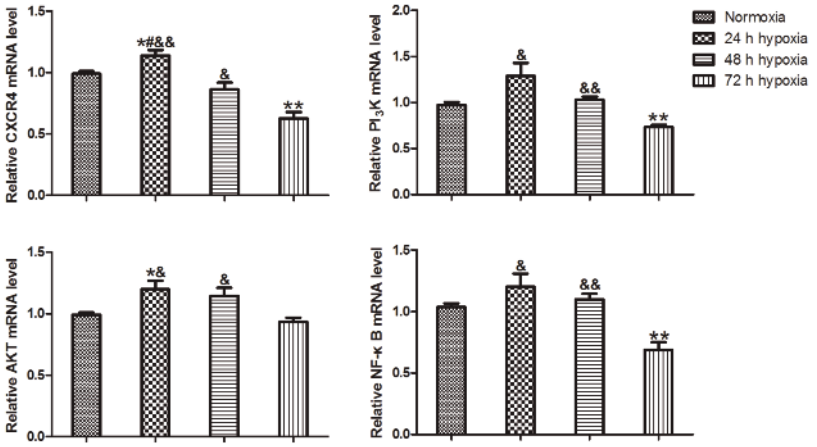

Figure 7. RT-PCR analysis of expressions of SDF-1α receptor CXCR4, as well as pro-survival factors PI3K, AKT, and NF-κB in BM-EPCs grown under normoxic or hypoxic condition. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the normoxic group, $^{\#}p < 0.05$ vs the 48 h hypoxic group, and $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs the 72 h hypoxic group.

significantly higher than normoxic control. Corroborating with these results, our RT-PCR results showed that mRNA expressions of CXCR4, AKT, PI_3K, and NF-κB were found to be induced by hypoxic preconditioning, while prolonged hypoxia down-regulated their transcription in a time-dependent manner. To our knowledge, the time course hypoxic preconditioning of EPCs has been less reported up to now. Unlike the results of our study, Akita et al[31] reported that hypoxic preconditioning of human EPCs under anoxic condition (95% N_2 and 5% CO_2) for 7 days increasingly promote growth and differentiation of EPCs. The previous study on embryonic stem cells (ESCs) found that the retinoic acid-induced ESCs remained fully viable when exposed to 1% O_2 hypoxia for at least 12 h, while increasing exposure to 1% O_2 for 24 to 48 h resulted in time-dependent cell death[32]. Preliminary experiments on mesenchymal stem cells (MSCs) performed by Liu and colleagues (2012) showed that 48 h duration of 3% O_2 exposure did not adversely affected the viability and growth of MSCs. Take all these together, the effects of hypoxic preconditioning on growth and differentiation of stem/progenitor cells may vary with the varying durations and concentrations of hypoxia, as well as the types of stem/progenitor cells used[33]. Our study showed that the 1% O_2 hypoxia duration of about 24 h may be more effective for BM-EPCs preconditioning, and further study was still needed to find the optimal pattern of hypoxic preconditioning for BM-EPCs.

Table II. Echocardiographic assessment and invasive hemodynamic measurements of LV morphology and function after BM-EPCs transplantation.

	Control group	Normoxic group	Hypoxic group	F value	*p*
n	12	10	12		
Maximum pressure (mmHg)	97.46 ± 5.87	88.54 ± 4.99*	108.77 ± 8.16$^{*\#}$	26.29	<0.01
dP/dtmax (mmHg/s)	3972.41 ± 332.46	4274.53 ± 316.62*	5485.88 ± 287.52$^{*\#}$	77.83	<0.01
dV/dtmax (μL/s)	1230.28 ± 179.91	1655.39 ± 331.03*	2145.13 ± 447.47*	20.58	<0.01
Stroke work (mmHg/mL)	3861.99 ± 752.07	4581.00 ± 647.81*	6344.75 ± 1008.56*	28.46	<0.01
Cardiac output (μL/min)	12847.61 ± 1540.77	14318.79 ± 2546.65*	18911.97 ± 2957.43*	20.40	<0.01
LV end diastolic diameter (cm)	0.983 ± 0.105	0.827 ± 0.101*	0.753 ± 0.081**	8.194	<0.01
LV end-systolic diameter (cm)	0.811 ± 0.105	0.635 ± 0.088*	0.502 ± 0.087$^{**\#}$	15.029	<0.01
LV ejection fraction (%)	0.388 ± 0.111	0.546 ± 0.077*	0.671 ± 0.085$^{**\#}$	12.974	<0.01

LV: Left ventricular; dP/dt_{max}: The maximum first derivative of developed LV pressure; dV/dt_{max}: The maximum first derivative of developed LV volume; $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control group, $^{\#}p < 0.05$ vs the normoxic group.

Cell migration to the injury region is an early essential step for stem cell therapy and was considered as an inherently inefficient process[14,16]. The chemokine SDF-1/CXCR4 axis has been well documented to play a key role in EPCs mobilization in response to hypoxia[34] and correlate with the proliferation and survival of EPCs[35]. Accumulating evidence has indicated that SDF-1α was constitutively expressed in the myocardium and was up-regulated immediately after acute myocardial infarction, and EPCs migrated to infarcted myocardium following SDF-1 gradients recognized by the cell surface receptor, CXCR4[36], suggesting that SDF-1α induced the migration of EPCs and the migration was CXCR4 dependent[34]. However, CXCR4 was found to be less expressed in *in vitro* expanded MSCs[37], which in turn resulted in inefficient response of transplanted MSCs to homing signals emanated from the ischemic tissue[38]. Besides, PI_3K/Akt pathway was also found to contribute to the migration, adhesion and survival of MSCs *in vitro*[31]. Zheng et al[34] have reported that SDF-1α/CXCR4 mediated the migration of EPCs via PI_3K/Akt/eNOS signal transduction pathway. The exposure of EPCs under hypoxic condition was shown to enhance the cell migratory activity *in vitro* and angiogenesis *in vivo*[31], and the PI_3K/AKT signaling pathway was also found to be activated by hypoxic preconditioning in MSCs[19]. Hypoxic preconditioning of mice by sub-lethal oxygen exposure for 24 h prior to focal permanent ischemia showed reduction in infarct volumes[39]. In accord with previous studies on stem/progenitor cells[33,40], our present study indicated that BM-EPCs could migrate toward SDF-1α, and hypoxic preconditioning for 24 h significantly enhanced migratory function of BM-EPCs in response to SDF-1α. The mRNA expressions of CXCR4, a key molecule in regulating EPCs homing and recruitment to ischemic tissue, as well as pro-survival factors PI_3K, AKT, and NF-κB were demonstrated to be up-regulated by shorter hypoxia for 24 h. Thus, hypoxia-induced CXCR4 secretion as well as PI_3K/Akt pathway activation may at least in part account for the enhanced motility of BM-EPCs under hypoxia.

To further clarify the effects of hypoxic preconditioned BM-EPCs after transplanted into the infarcted myocardium, BM-EPCs grew under normoxic and hypoxic conditions were transplanted into the rat acute myocardial infarction model established by ligation of the left anterior descending coronary artery, respectively. Our results showed that, after 28 days of transplantation, LV diastolic and systolic diameters were found to be significantly reduced in the normoxic BM-EPCs group, while maximum pressure, dP/dt_{max}, dV/dt_{max}, stroke work, cardiac output, and LV ejection fraction were significantly higher than that of the control group, partially consistent with the results reported by Kawamoto et al[10], which indicated that intravenous administration of ex vivo expanded EPCs had a favorable impact on the preservation of LV function in rats with myocardial ischemia. The previous meta-analysis results also showed the modest improvements in LV ejection fraction after intracoronary delivery of hypoxic preconditioned autologous bone marrow mononuclear[4]. The findings of our results indicated that hypoxic preconditioning further enhanced the beneficial effects of BM-EPCs on preservation of the LV function, as demonstrated by further reduced LV end-systolic diameter, as well as further increased maximum pressure, dP/dt_{max}, and LV ejection fraction, when compared with the normoxic preconditioning. The above results suggested that hypoxic preconditioning of BM-EPCs could provide further therapeutic benefits on left ventricular function after AMI.

Conclusions

Our study showed that time course hypoxia of BM-EPCs for 24 to 72 h caused morphologically and functionally changes. Tube formation ability and motility activity of BM-EPCs were significantly enhanced by 24 h hypoxia, while the prolonged hypoxia for 48 and 72 h significantly inhibited the formation of tube-like structures and motility of BM-EPCs. The early apoptosis of BM-EPCs was found to be significantly enhanced by increasing hypoxia for 48 and 72 h. Hypoxic preconditioning increased the transcriptions of the SDF-1α receptor, CXCR4, as well as pro-survival factors PI3K, AKT, and NF-κB. *In vivo* study of transplantation of hypoxic preconditioned BM-EPCs resulted in the significantly improved LV function in the rat acute myocardial infarction. All these results showed evidence suggested that hypoxic preconditioning did exert further beneficial effects of BM-EPCs on LV function after AMI. The short-term hypoxia for about 24 h provided better condition for BM-EPCs survival and differentiation, and further study is still

needed to find the optimal hypoxic pattern of BM-EPCs preconditioning so as to protect heart from ischemic injury.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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