Original Article
Endothelial progenitor cell (EPC) transplantation improves myocardial infarction via up-regulation of vascular endothelial growth factor and gap junction protein connexin 43 in rats

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Abstract: Background: Myocardial infarction (MI) has become one of the most deadly diseases worldwide. Therapeutic angiogenesis promotes the growth of capillary and collateral circulation, and therefore is considered a promising therapeutic method for MI by recovering the myocardial blood flow in the infarcted area. This study aimed to investigate the angiogenic effect and potential mechanism of endothelial progenitor cell (EPC) transplantation on in a rat model of MI. Methods: SD rats were randomly divided into healthy control, sham-operated, model and EPC group (n = 10 each). Model group was subjected to ligation of the anterior descending coronary artery. EPC group was treated by injection of immunofluorescence-confirmed EPC in the infarct following the construction of MI model. Sham-operated and model group was injected with same volume of saline. At 4 weeks after operation, ECG test and histological examination was performed. Myocardial apoptosis was detected by TUNEL assay. The expression of vascular endothelial growth factor (VEGF) and gap junction protein connexin 43 (Cx43) protein and mRNA was determined by immunohistochemical and qRT-PCR analyses, respectively. Results: When compared with model group, the EPC group exhibited lower VP, VT, VF and aggregate score (P = 0.006) at 4 week postoperatively. The myofibroblasts and cellular structure in EPC group was markedly recovered, and the vascular density in the infarct was significantly increased (P = 0.005). The myocardial apoptosis rate was markedly decreased (P = 0.002). The Cx43 and VEGF protein and mRNA expression was significantly enhanced (all P < 0.01). Conclusion: We confirm the therapeutic effects of EPC transplantation on MI, which might be achieved through stimulation of angiogenesis. Our study provides a theoretic basis for the clinical application of EPC transplantation in the treatment of MI.

Keywords: Myocardial infarction, endothelial progenitor cell, cardiac function, vascular endothelial growth factor, angiogenesis, ventricular remodeling

Introduction

Myocardial infarction (MI), commonly known as a heart attack, refers to the condition in which the blood flow to part of the heart is stopped leading to myocardial damages. MI has become one of the most deadly diseases worldwide [1]. The major goals of treatment for MI are the restoration of normal blood flow and myocardial function [2]. Currently, there are three common types of reperfusion therapy including interventional therapy, surgical bypass, and thrombolysis. Although these therapies can salvage the conditions in MI patients, they can not repair the damaged heat tissues, and stenosis or occlusion of blood vessel may recur. Typically, the self-repair of myocardium after MI is a slow process involving angiogenesis, which is crucial for the recovery of normal blood flow in the heart [3]. Therapeutic angiogenesis aims to promote the growth of capillary and collateral circulation, and therefore is considered as the best method to recover the myocardial blood flow and oxygen supply in the infarcted area, and to improve the cardiac function and ventricular remodeling.

Endothelial progenitor cells (EPCs), the precursor cells of vascular endothelial cells, possess multi-differentiation and self-propagation

potentials, and play an important roles in the repair of tissue and vascular damages [4, 5]. EPCs are derived from bone marrow, and migrate to the site of vascular injury along the peripheral circulation when stimulated by physiological or pathological factors [6, 7]. Nevertheless, in most circumstances, the stress-activated EPCs are not sufficient to compensate the loss of endothelial cells, which may require transplantation of EPCs. Studies have suggested that EPC transplantation can upregulate the expression of vascular endothelial growth factor (VEGF), promote the formation of new blood vessel, and thereby improve the blood supply at the site of injury [8]. In this study, we investigated the effect of EPC transplantation on angiogenesis in a rat model of MI in aim to provide an experimental basis for the application of EPC transplantation in the treatment of MI.

Materials and methods

Experimental animals

Six-week-old healthy male SD rats weighting 300-350 g were purchased from Slac Experimental Animals (certificate #: SCXK (Shanghai) 2012-0002, Shanghai, China), and housed under normal laboratory conditions with free access to food and water. This study was approved by the Animal Ethics Committee at the Jiangxi Provincial People's Hospital.

Main reagents and instruments

Peripheral blood lymphocyte isolation kit was purchased from Solar Biotech. (Beijing, China). Rat vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (bFGF), and human epidermal growth factor (hEGF) was purchased from Peprotech (Rocky Hill, NJ, USA). Rabbit anti-rat VEGF (vascular endothelial growth factor) and Cx43 (gap junction protein connexin 43) monoclonal antibody and biotinylated goat anti-rabbit IgG were purcahed from Abcam (Cambridge, MA, USA). TUNEL cell apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). TRIzol reagent and reverse transcription kit, and SYBR Green qPCR SuperMix were purchased from Invitrogen (Carlsbad, CA, USA). Primers were synthesized by Invitrogen. All other reagents were purchased from Sigma (Shanghai, China) unless stated otherwise.

Small animal ventilator was purchased from Beijing Medical Equipment Factory (Beijing, China). ECG-6951D machine was purchased from Olympus. Real-time PCR detection system CFX96 was purchased from Bio-Rad (Hercules, CA, USA).

Isolation, culture and identification of EPCs

Rat EPCs were isolated as previously described [6]. Briefly, mononuclear cells were isolated from rat peripheral blood using peripheral blood lymphocyte isolate kit, and inoculated (1-4 × 10^6 cells/flask) in a cell culture flask containing 2% fetal bovine serum and induction agent (20 ng/mL rat VEGF, 2 ng/mL human bFGF2 and 20 ng/mL human EGF). After 2 days, the medium was replaced with new induction medium. The unattached cells was collected by centrifugation, inoculated in the cell culture flask, and incubated for an additional 48 hours. Further, EPCs were identified by immunofluorescence detection of cell surface antigen. Briefly, cell monolayer was fixed with paraformaldehyde for 15 min, and treated with 3% hydrogen peroxide to block endogenous peroxidase. Cells were perforated with 0.5% Triton X-100 in PBS at RT, washed 3 times with PBS, and blocked with 5% BSA for 30 min. Cells were incubated with anti-CD133 and anti-Flk-1 antibody overnight at 4°C. Cells were rinsed, incubated with secondary biotinated antibody, and stained with SABC-Cy3 and DAPI, respectively. The cell monolayer was treated with fluorescence quenching agent, and observed under a BioRad 788BR03915 fluorescence microscope.

Construction of rat MI model and grouping

Rats were given an intraperitoneal injection of 5% pentobarbital (0.8 mL/100 g). Following tracheal intubation, rats were maintained using a small animal ventilator (tidal volume: 7 mL, respiration ratio: 1:1, respiratory rate: 100/min) and connected to the electrocardiogram (ECG) electrodes. The anterior descending coronary artery was located between the left atrial appendage and the pulmonary artery cone, and ligated at approximately 3 mm from the aorta root. The AMI model (n = 10) was confirmed by ECG showing elevated ST segment and reduced heart rate. The sham surgery group (n = 10) was only stringed without the ligation of the atery. Ten healthy rats were used as normal
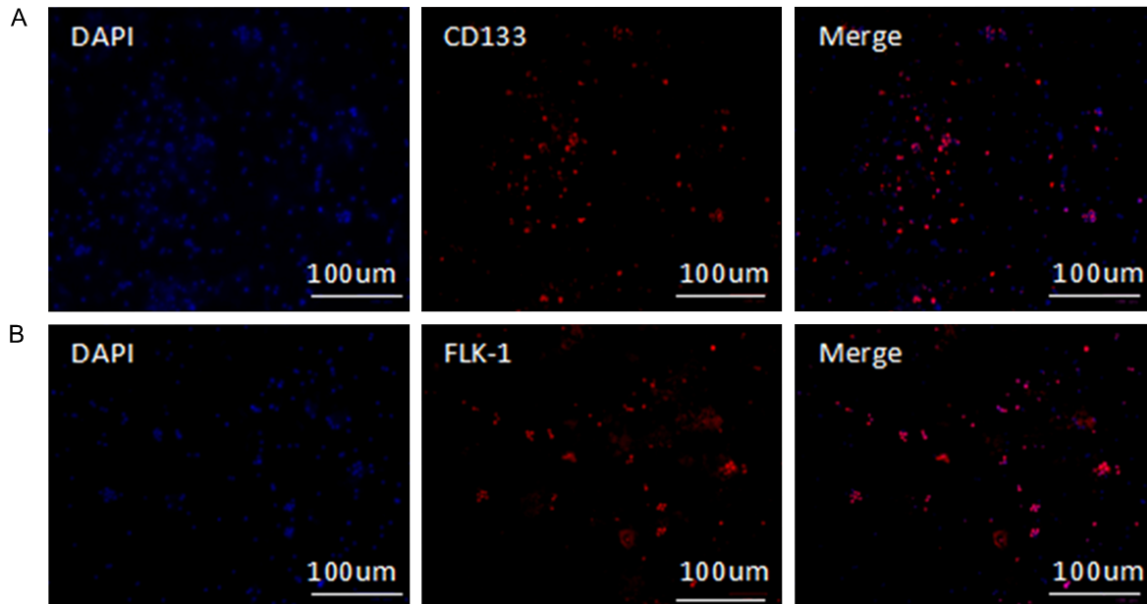
Figure 1. Identification of EPCs by immunofluorescence detection of cell surface antigen CD133 (A) and FLK-1 (B).

controls. At 2-3 min after surgery completion, rats in EPCs group (n = 10) was injected with 200 μL of 5 × 10^5/ml EPCs at 5 sites at the edge of the infarct. Rats in all other groups were injected with equal volume of PBS. Rats were given an intramuscular injection of penicillin for 3 days (400,000 U/day).

ECG examination

At 4 weeks after the surgery, ECG examination was performed. Briefly, rats were anesthetized with 0.8 mL/100 g 5% pentobarbital. Two pacing electrodes (0.3-mm needle electrode coated with polytetrafluoroethylene) were inserted into the area of the apex beat with a distance of 0.5 cm between each other. Starting from IV intensity, intermittent stimulation (100 HZ, 1 second/each stimulus) was delivered by incrementally increasing the intensity using a XD-2A instrument. ECG examination was performed and indicators were recorded within 2 h including premature ventricular contraction (VPC), ventricular tachycardia (VT), and ventricular fibrillation (VF). VT was defined as the condition in which 4 or more VPCs occurred in a row. Arrhythmia score was defined as follows: 0 point, VPC = 0; 1 point, 0 < VPC ≤ 10; 2 point, 10 < VPC ≤ 50; 3 point, 50 < VPC; 4 point, VT = 1; 5 point, 2 ≤ VT ≤ 5; 6 point, 5 < VT; 7 point, VF = 1; 8 point, 2 ≤ VF ≤ 5; and 9 point, 5 < VF.

Hematoxylin & eosin (HE) staining

Rats were sacrificed at 4 week after surgery. Samples were obtained from the edge of myocardial infarct, treated with 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections. Routine histological HE staining was performed. Briefly, sections were deparaffinized in xylene and rehydrated in serial dilution of alcohol (100%, 90%, 80% and 70%). The sections were then stained with hematoxylin for 10 min, differentiated with 1% acid alcohol, and counterstained with 0.5% eosin for 3 min. The sections were further dehydrated with 80%, 90%, 100% alcohols, cleared with xylene, mounted in neutral gum, and observed under an inverted microscope. Vascular density was determined as previously described using Image pro-plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) [9]. The number of microvessels in 5 randomly selected visual fields at 100X magnification on each section was counted. The vascular density was calculated as the number of microvessels per unit of assessed area.

TUNEL apoptosis detection

After routine dewaxing and rehydration, myocardial sections were treated with proteinase K at 37°C for 30 min, washed with PBS, and incu-
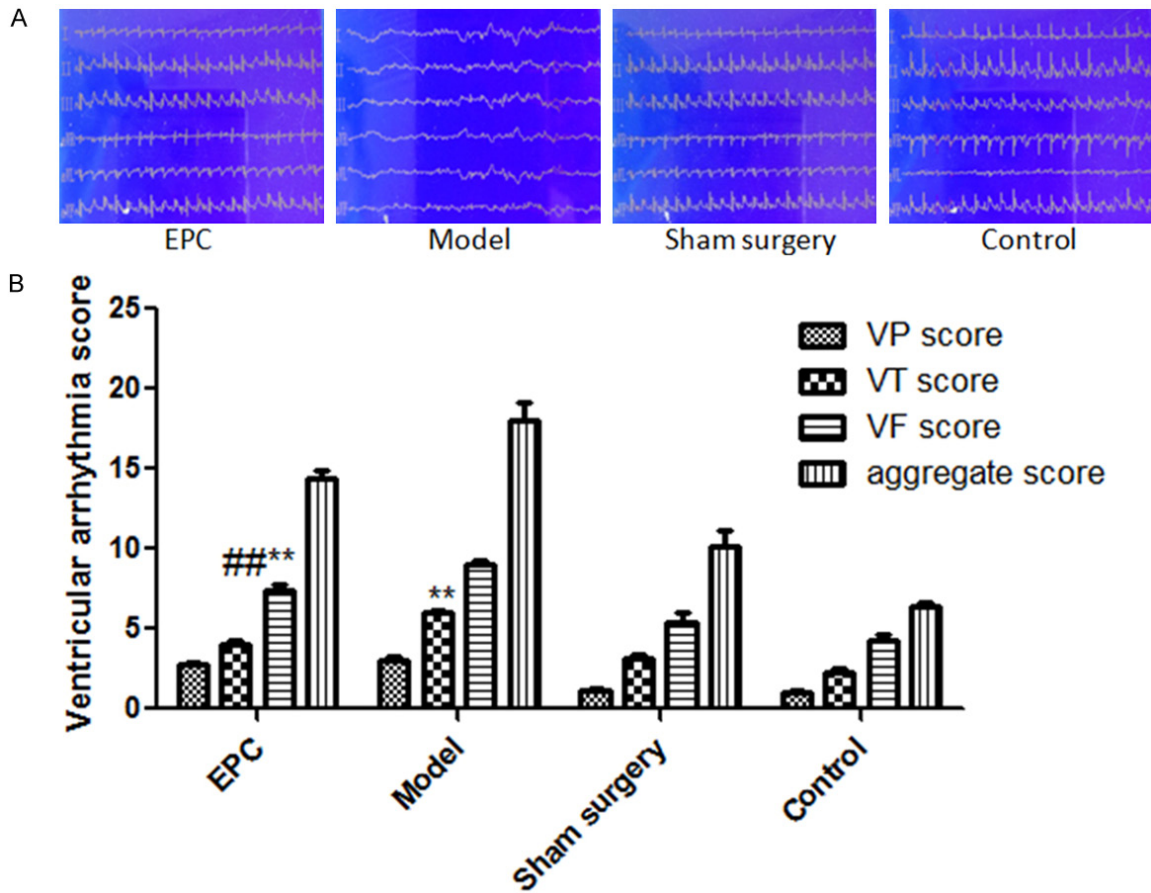
Figure 2. EPC transplantation improved arrhythmia at 4 weeks after operation. A. Comparison of ECG ECG signals in all groups. B. Comparison of ventricular arrhythmia score (n = 10 for each group). **, $P < 0.01$ compared with control group. ##, $P < 0.01$ compared with model group.

bated with TUNEL reaction mixture (2 µL of TdT enzyme and 48 µL of labeling solution) at 37°C in dark for 60 min. Sections were washed with PBS and further stained with DAPI. The number of apoptotic cells and total cells in five randomly selected visual fields was counted and the mean apoptotic index was calculated.

Immunohistochemical detection

The expression level of CX43 and VEGF in myocardium was detected by immunochemical staining. Briefly, myocardial sections were routinely dewaxed and rehydrated. The sections were incubated in 3% hydrogen peroxide for 15 min to block the endogenous peroxidases. The sections were blocked with goat serum, and incubated with Rabbit anti-rat VEGF and CX43 monoclonal antibody (1:1000) at room temperature for 3 h. The sections were then incubated with biotinylated goat anti-rabbit secondary antibody at room temperature for 2 h and sub-

jected to DAB color development for 6 min. The sections were further counterstained with hematoxylin, dehydrated through serial alcohols, cleared with xylene, sealed with neutral gum. Cytoplasmic or membrane staining in yellow or brown was identified as positive staining. The positive rate of five randomly selected visual fields was calculated using the Image-Pro Plus 5.0 software.

Quantitative reverse transcription PCR (RT-PCR)

Myocardial tissue was homogenized. Total RNA was extracted using TRIzol reagent and reverse transcribed into cDNA using reverse transcription kit. The myocardial level of Cx43 and VEGF mRNA was measured by RT-PCR. The reaction condition: 95°C 15 s, followed by 45 cycles of 95°C 5 s and 60°C 30 s. The primer sequences were as follows: VEGF-F: 5'-CTGCTCTCTTGGGT-CCACTGG-3', VEGF-R: 5'-CACCGCCTTGGCTTGT-
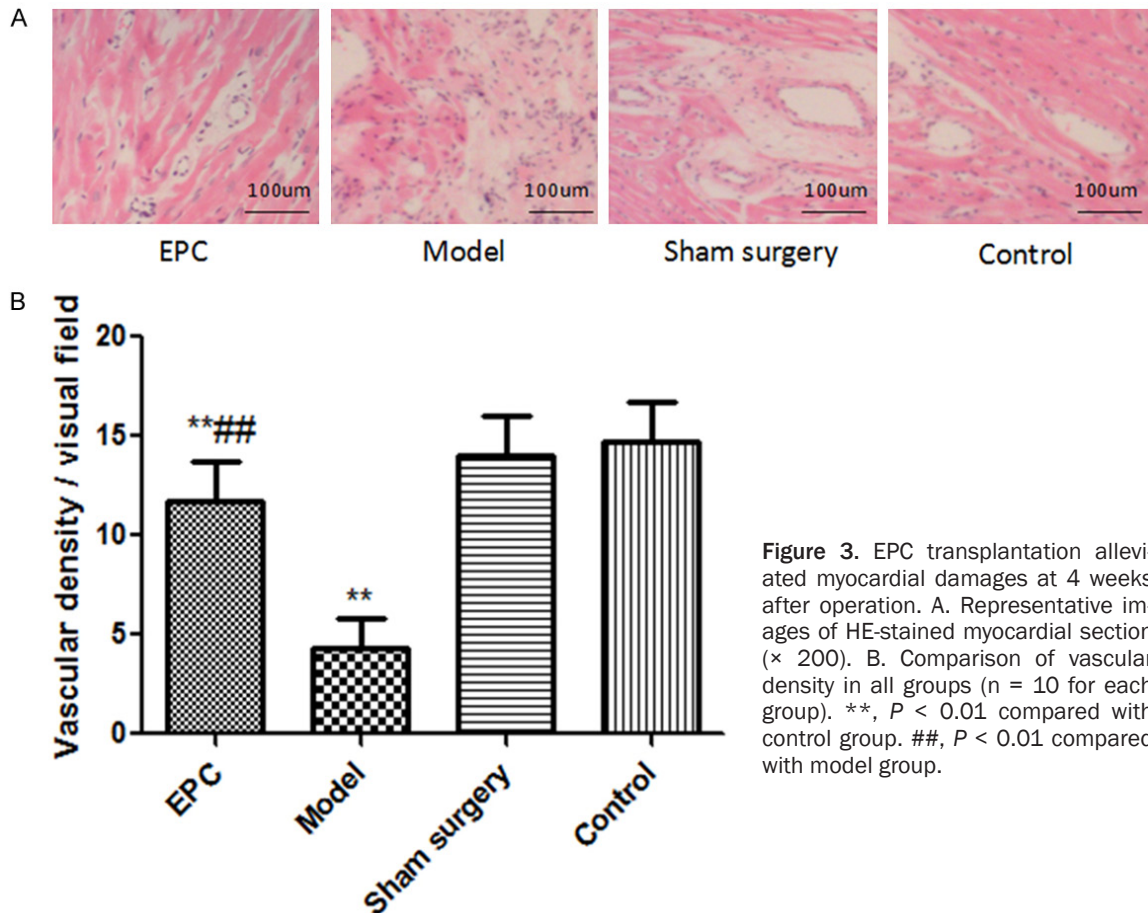
Figure 3. EPC transplantation alleviated myocardial damages at 4 weeks after operation. A. Representative images of HE-stained myocardial section (× 200). B. Comparison of vascular density in all groups (n = 10 for each group). **, $P < 0.01$ compared with control group. ##, $P < 0.01$ compared with model group.

CACAT-3'; Cx43-F: 5'-TGAAAGAGAGGTGCCCAG-AC-3', Cx43-R: 5'-GCAGCCAGGTTGTTGAGTG-3'; GAPDH-F: 5'-TTCAACGGCACAGTCAAGG-3', and GAPDH-R: 5'-CTCAGCACCAGCATCACC-3'. The relative expression of mRNA was expressed as its intensity to that of β-actin. The experiment was performed in triplicates.

Statistical analysis

All data were expressed as mean ± standard deviation and analyzed by SPSS 17.0 (IBM SPSS, Chicago, IL, USA). Difference between groups was analyzed by one-way ANOVA followed by SNK in case of significant difference. P values smaller than 0.05 were considered statistically significant.

Results

EPC transplantation improved arrhythmia

As shown in **Figure 1**, the isolated cells were positive for surface antigens CD133 and Flk-1, indicating the identification of EPCs. The MI rats were treated with EPC transplantation,

and subjected to ECG examination after 4 week. The spectrum of the ECG signals in all groups was shown in **Figure 2**. The sham surgery and control group exhibited similar ECG signals. When compared with sham surgery group, wider R waves and elevated ST segment and J point were observed in model group, suggesting MI-induced arrhythmia. As shown in **Figure 2**, the model group had higher VP, VT, VF and aggregate score compared with control group ($P = 0.001$ for VT). At 4 week after EPC transplantation, rats displayed normal ECG signals, indicating the therapeutic effects of EPC transplantation on MI. The EPC group exhibited lower VP, VT, VF and aggregate score compared with model group ($P = 0.006$).

EPC transplantation alleviated myocardial damages

The histopathology of heart tissues in different groups was compared by HE staining. As shown in **Figure 3A**, the myofibroblasts in sham surgery and control groups were orderly organized without any damages. The cardiomyocytes had
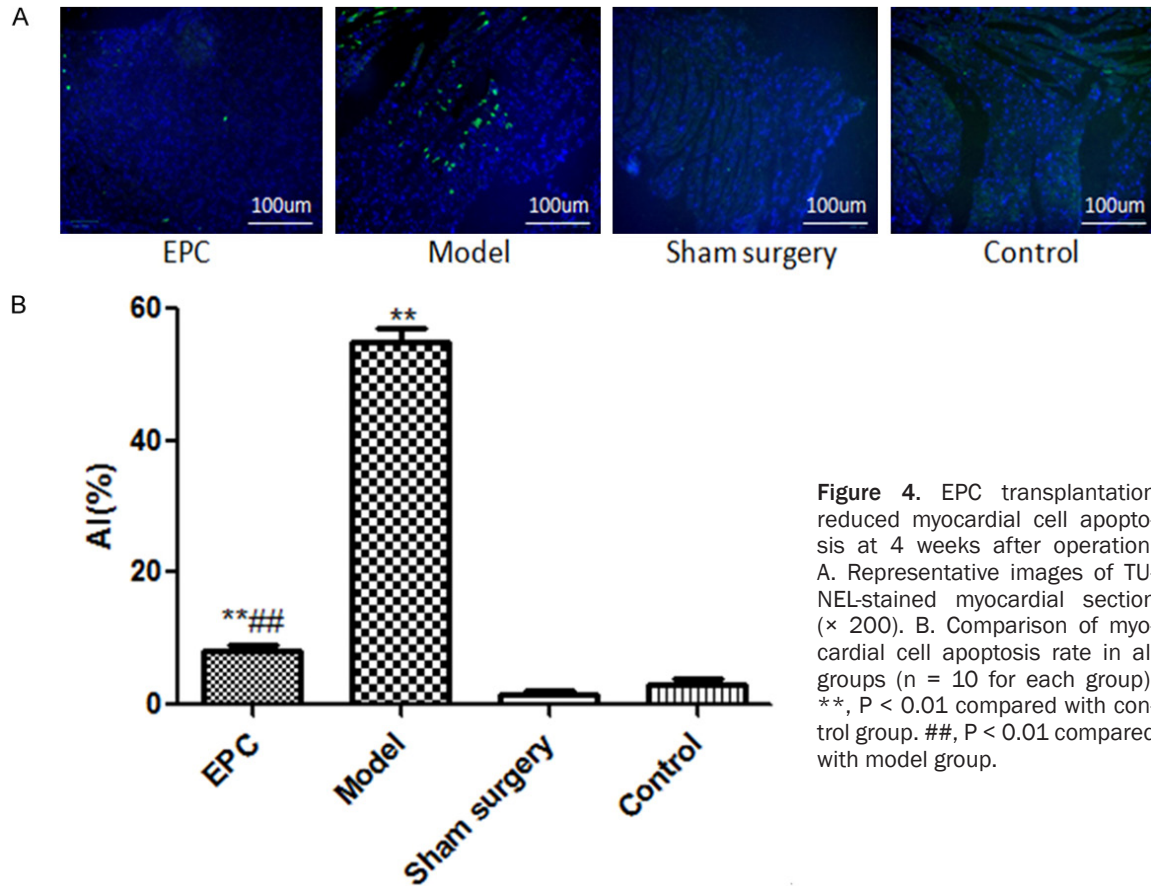
Figure 4. EPC transplantation reduced myocardial cell apoptosis at 4 weeks after operation. A. Representative images of TUNEL-stained myocardial section (× 200). B. Comparison of myocardial cell apoptosis rate in all groups (n = 10 for each group). **, P < 0.01 compared with control group. ##, P < 0.01 compared with model group.

rich cytoplasm and uniform intercellular space. In the model group, damaged myofibroblasts and inflammatory infiltration was observed. A large amount of cardiomyocytes were destroyed and cell nuclei had been disappeared. The vascular density in the infarct in model group (4.7±2.4) was significantly reduced compared with control group (13.8±3.12, P = 0.003). In contrast, the myofibroblasts and cellular structure in EPC group was markedly recovered, suggesting that EPC transplantation had effectively alleviated myocardial damages. As shown in **Figure 3**, the vascular density in the infarct in EPC group (12.3±2.7) was significantly increased compared with model group (4.7±2.4, P = 0.005), and was comparable to that in control group (13.8±3.12, P = 0.253), suggesting that EPC transplantation stimulated the post-infarction angiogenesis.

EPC transplantation reduced myocardial cell apoptosis

As shown in **Figure 4**, TUNEL apoptosis assay demonstrated that nearly no apoptotic cells were detected in both sham surgery and control groups. In contrast, the apoptosis rate in model group was significantly higher compared with control group (P = 0.000). Although the apoptosis rate in EPC group was also significantly higher than that in control group (P = 0.008), it was markedly decreased when compared with model group (P = 0.002), indicating that EPC transplantation greatly reduced MI-induced myocardial cell apoptosis.

EPC transplantation stimulated Cx43 and VEGF expression

Immunohistochemical results showed that sham surgery and control groups had similar myocardial level of Cx43 and VEGF (P > 0.05, **Figure 5A** and **5B**). The model group had significantly reduced Cx43 expression (P = 0.008), but notably enhanced VEGF level (P = 0.006) compared with control group. At 4 week after EPC transplantation, the Cx43 and VEGF expression was significantly higher compared with model group (P = 0.003 and 0.000, respectively, **Figure 5C**). When compared with control
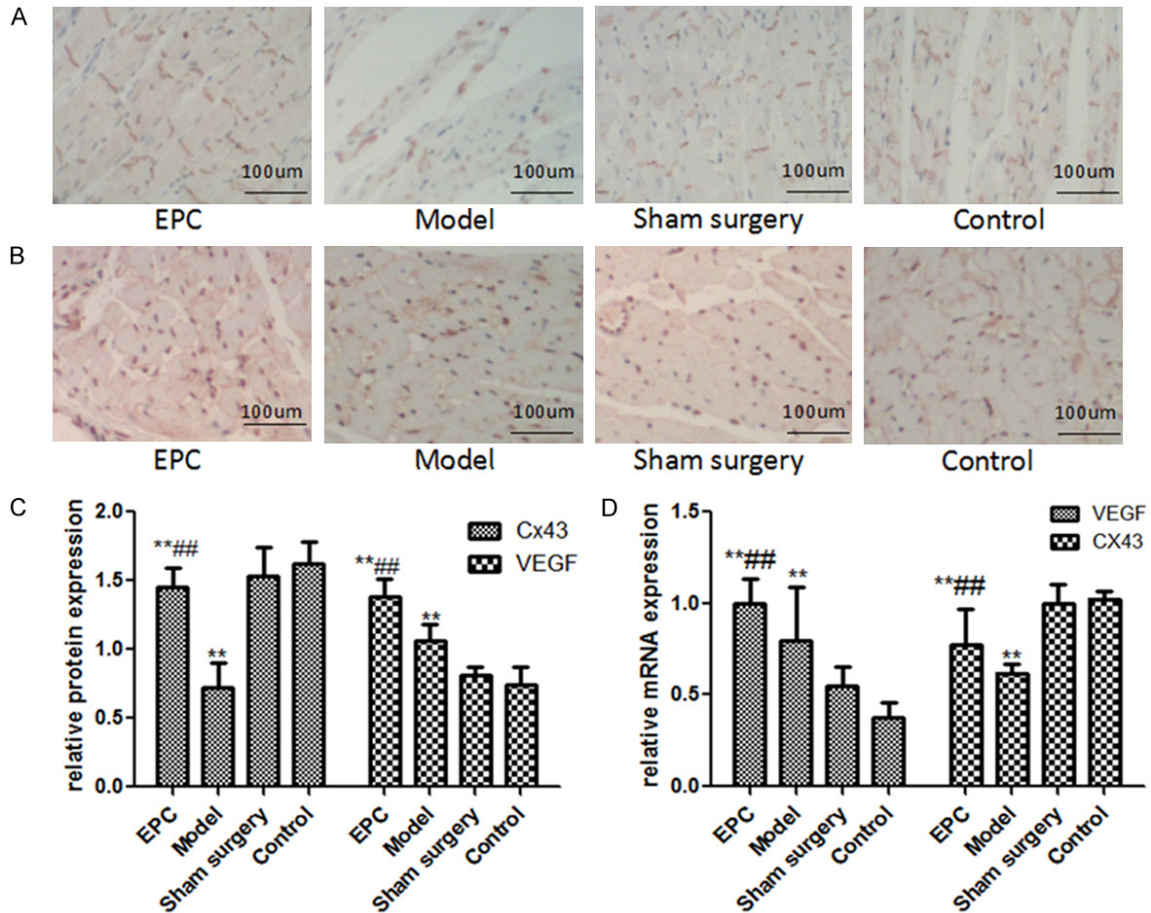
Figure 5. EPC transplantation promoted Cx43 and VEGF expression at 4 weeks after operation. Expression of Cx43 (A) and VEGF (B) in myocardial section was detected by immunochemical staining of myocardial section (× 200). (C) Comparison of Cx43 and VEGF protein expression in all groups (n = 10 for each group). (D) qRT-PCR analyses of Cx43 and VEGF mRNA expression in all groups (n = 10 for each group). **, $P < 0.01$ compared with control group. ##, $P < 0.01$ compared with model group.

group, EPC group had significantly higher VEGF expression ($P = 0.001$), but substantially lower Cx43 expression ($P = 0.025$). The relative expression of VEGF and CX43 mRNA in the myocardium was further determined by RT-PCR (**Figure 5D**). Consistent with immunohistochemical results, Cx43 mRNA level in model group was significantly decreased ($P = 0.002$), but VEGF level was increased ($P = 0.009$) when compared with control group. EPC transplantation had markedly upregulated the expression of Cx43 and VEGF mRNA compared with model group ($P = 0.006$ and 0.000, respectively).

Discussion

Myocardial infarction causes myocardial ischemia and hypoxia due to a sharp reduction or interruption of blood flow to part of the heart, which ultimately leads to extensive apoptosis of cardiomyocytes. In this study, a large amount of MI-induced apoptotic cardiomyocytes were observed in the model group. Effective treatment of MI focuses on how to improve myocardial ischemia and hypoxia, and restore myocardial blood supply [10, 11]. The angiogenesis in the infracted area is therefore essential for MI therapeutics [11]. As the precursor cells of vascular endothelial cells, EPCs are not only involved in blood vessel formation in early embryonic tissues, but also play an important role in the repair process after vascular injury. Studies have shown that EPCs can differentiate into endothelial cells and form a tubular upon *in vitro* induction, suggesting their excellent potential of proliferation and differentiation [12]. In this study, MI rats were treated by EPC transplantion in the infacted area. It was found

that these rats exhibited normal ECG signals, notably alleviated myocardial injury and inflammation, and markedly reduced apoptosis of cardiomyocytes.

The process of angiogenesis involves a variety of regulators. VEGF is a signal protein and potent angiogenic factor, and thereby plays a key role in ischemic diseases [13]. VEGF has been well known as an essential growth factor for vascular endothelial cells by promoting the mitosis of endothelial cells [14]. Myocardial ischemia can stimulate the expression of VEGF in myocardial tissues in rats, which promotes the production of other angiogenesis factors, improve the repair of vascular endothelium, and alleviate tissue ischemia and hypoxia [15, 16]. Studies have shown that the expression of VEGF in ischemic myocardium after MI is positively correlated with the density of capillaries and the number of endothelial cells [17], suggesting that VEGF level as an indicator for the extent of angiogenesis. In this study, VEGF expression in model group was significantly higher than that in control group, suggesting that cardiomyocytes had started the self-repair process via upregulation of VEGF expression in response to acute myocardial injury. EPC transplant significantly enhanced the expression of VEGF when compared to the model group. Conssitetnly, HE staining confirmed that the EPC treatment markedly increased the vascular density in infacted area. Previous studies have suggested that thymosin-β4 (Tβ4) can markedly improve cardiac function, stimulate the VEGF expression, and increase capillary density in a mouse MI model [18]. Tβ4 has been known as an essential paracrine factor secrected by EPCs [19]. Therefore, it is worthy further investigation to elucidate the potential role of Tβ4 in the therapeutic effect of EPCs on MI.

Cx43 is the most widely expressed gap junction protein. Under normal physiological conditions, Cx43 is abundantly expressed in the myocardium of right and left ventricle in healthy human heart [20-22]. Changes in Cx43 expression and distribution might be associated with cardiac malformations [23]. As a gap junction protein, Cx43 is involved in cell differentiation, proliferation and migration via regulation of intercellular signaling and exchange of functional substances [24]. In the current study, it was found that Cx43 expression in EPC group was significantly

higher when compared with all other groups, suggesting that EPC transplantation had stimulated Cx43 expression in infacted area. The increased Cx43 expression, in return, might promote the differentiation, proliferation and migration of EPCs.

Conclusion

All together, in this study, we evaluated the therapeutic effect of EPC transplantation in a rat MI model and found that rats in EPC group exhibited normal ECG signals, notably alleviated myocardial injury and inflammation, and markedly reduced apoptosis of cardiomyocytes. In an attempt to elucidate the relevant mechanism, we revealed that EPC transplantation significantly promoted Cx43 and VEGF expression in infarcted myocardium and increased the myocardial vascular density, suggesting that the therapeutic effect of EPCs might be achieved through stimulation of angiogenesis. Our study confirms the therapeutic effect and potential mechanism of EPC transplantation, and shall provide a theoretic basis for its clinical application in the treatment of MI.

Acknowledgements

Disclosure of conflict of interest

None.

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