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ORIGINAL ARTICLE

Intravenous transplantation of mesenchymal stem cells improves cardiac performance after acute myocardial ischemia in female rats

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Keywords
cardiac function, differentiation, mesenchymal stem cell, myocardial ischemia, ultrastructure.

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Summary

Mesenchymal stem cells (MSCs) are potential sources of cells for tissue repairing. However, little information is available regarding the therapeutic potency of intravenously transplanted MSCs for myocardial ischemia (MI). In the present study, MSCs were isolated from bone marrow of male rats and expanded *in vitro*. Three hours after ligation of left anterior descending artery, the transplanted group received an infusion of MSCs through the tail vein. At the same time, a coronary-ligated control group was injected with culture medium. Homing of MSCs to the heart was assessed by expression of the Y chromosome *sry* gene using fluorescent *in situ* hybridization (FISH). At 1 week or 8 weeks after transplantation, *sry* positive cells were present in cardiac tissue in the transplanted group, but not in the hearts of control group. Cardiomyocytes, smooth muscle cells, and endothelial cells that bore *sry* gene were identified in transplanted group at 8 weeks after transplantation. Ultrastructural observation revealed that a large number of capillary and some immature myocytes were found to survive in the ischemia region. MSCs transplantation also decreased LVEDP pressure and $-dP/dt$, but increased LVSP and $+dP/dt$. The cardiac infarct size was significantly smaller in transplanted group than in control group. Our data suggest that intravenously transplanted MSCs improve cardiac performance and promote the regeneration of blood vessels and cardiomyocytes.

Introduction

Bone marrow is a rich reservoir of stem cells and progenitor cells. Among these, a population of cells known as mesenchymal stem cells (MSCs) has been shown, *in vivo* and *in vitro*, to proliferate extensively and to differentiate along multiple lineages giving rise to muscle, brain, liver, cartilage, bone, fat and the vessel *in vitro* or *in vivo*, contributing to tissue regeneration [1–3]. Earlier studies showed that MSCs directly injected into the infarcted heart induce myocardial regeneration, and stem cells from bone marrow mobilized into the peripheral blood respond to tissue ischemia and promote tissue regeneration [4–7]. These results raise the possibility that intravenously transplanted MSCs participate in repair of the ischemic myocardium. However, little information is available regarding the therapeutic potential of allogeneic MSCs intravenously transplanted for MI. In the present study, we aimed to observe whether (i) allogeneic MSCs are able to homing to the ischemic myocardium and differentiate into cardiomyocytes, smooth muscle cells and endothelial cells, (ii) transplanted MSCs induce ultrastructural changes of ischemia myocardium and (iii) intravenous transplantation of MSCs decreases infarct size and improves cardiac function.

Materials and methods

Animals

Sprague–Dawley (SD) rats were obtained from experimental animal Center of Xi'an Jiaotong University (Shaanxi, China). All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). Female SD rats weighted 200–250 g were randomly divided into two groups: transplanted group given MSCs infusion and control group given culture medium infusion. The experimental protocols had been approved by the Care of Experimental Animals Committee of 1st Hospital, Xi'an Jiaotong University, Shaanxi, China.

Isolation and expansion of MSCs

Femurs and tibiae of 1-month-old male Sprague–Dawley (SD) rats were excised under sterile conditions. Bone marrow plugs were extracted by flushing the bone marrow cavity with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEDM-LG; GIBICO, Carlsbab, CA, USA). Mononucleated cells were isolated from the perfusates by density gradient fractionation (1.073 g/ml, Pharmacia, Jacksonville, FL, USA), washed and resuspended in DMEM-LG supplemented with 10% fetal bovine serum (Si-Ji-Qing, Hangzhou, Zhejiang, China), and cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The mesenchymal population was isolated on the basis of its ability to adhere to the culture plate and removed during subsequent medium changes. When the cultures reached 90% of confluence, cells were recovered by 0.25% trypsin (Amresco, Parkway Solon, OH, USA) and passages followed. Proliferation and growth in primary and passage culture were observed with phase contrast microscope (Olympus, Tokyo, Japan).

Identification of MSCs

Cultured MSCs were trypsinized and washed twice with PBS. The supernatant were discarded and the cells, stained with the monoclonal antibodies against phycoerythrin (PE)-conjugated CD34 (Santa Cruze, CA, USA), CD71 and FITC-conjugated CD90 (BD Pharmingen, San Diego, CA, USA), were suspended in 100 μl PBS. All incubations were performed at room temperature for 20 min. Control tubes were incubated with FITC- and PE- conjugated antibodies against mouse IgG1. After incubation, the cells were washed with PBS containing 0.1% BSA. Quantitative analyses were performed by using a flow cytometer (BD, Rockville, MD, USA).

Establishment of myocardial ischemic model

Female SD rats underwent ligation of the left coronary artery to produce MI. Briefly, the rats were anesthetized by inhalation of ether. The chest was opened, a 5–0 silk suture was passed with a tapered needle under the left anterior descending coronary artery 1–2 mm from the tip of the left atrium, and the ends of the suture were tied to induce MI.

Cell transplantation of MSCs

The transplantation was performed at 3 h after induction of MI. The male MSCs suspension (500 μl contains 1×10^7 cells) was slowly infused into the female MI rats via the tail vein. Meantime, the MI rats in the control group received an equal volume of culture medium through the tail vein.

Assessment of hemodynamics and infarct size

Hemodynamic studies were performed 8 weeks after coronary ligation. A P50 catheter connecting a pressure–electricity transducer (Power Lab 4.12 system; AD instrument, Sydney, Australia) was inserted in the right carotid artery and then advanced into the left atria and left ventricle for measurement of LV pressure and $\pm dP/dt$. After completion of hemodymamics measurements, the hearts were cut into transverse slices. Computerized planimetry by Image-Pro Plus 5.1(Wetzler, Leica, Germany)was used to measure and calculate (i) epicardial and endocardial circumference and circumference occupied by infracted wall (infarct size was determined as a percentage of total LV circumference); (ii) scar thickness (average of four equidistant measurements); (iii) septum thickness (average of four equidistant measurements).

Fluorescence *in situ* hybridization

One or 8 weeks after transplantation, the rat hearts were removed, embedded in optimal cutting temperature (OCT), snap-frozen in liquid nitrogen, and stored at −80 °C. Serial sections (5 μm) of the OCT blocks were collected on slides and fixed with 4% paraformaldehyde at 4 °C for 5 min. Fluorescence *in situ* hybridization (FISH) was performed to detect male MSCs in the female rats using a synthetic probe specific for the rat Y chromosome *sry* gene (Tianjin Hao-Yang Company, Tianjin, China). The probe sequence was 5′-ATAGT GTGTA GGTTG TTGTC CCATT GCAGC-3′. Sections were prepared by heat denaturalization at 95 °C for 10 min and chilled on ice for 5 min, then incubated with the probe at 37 °C for 12 h. Finally, the nuclei were stained with PI (red) and observed with a laser confocal microscope (Leica).

Immunofluorescence staining

The slides with *sry* positive cells were stained immediately. Cardiomyocytes were recognized with a mouse monoclonal anti-Troponin and desmin (NeoMarkers, Fremont, CA, USA), and vessel with a mouse monoclonal antismooth-muscle α-actin (NeoMarkers) and CD31 (BD). TRITC-conjugated rabbit antimouse IgG (Oxford, UK) was used as secondary antibody. After rinsing three times with PBS, the slides were permeabilized for 30 min with 0.2% Triton X-100 and blocked at room temperature for 30 min in PBS containing 5% horse serum, then incubated overnight at 4 °C with the primary antibodies and rinsed three times. Finally, they were incubated with the secondary antibodies at room temperature for 1 h and observed by laser confocal microscope. The number of blood vessels positive for CD31 was assessed in the peri-infarct area at 8 weeks after MSCs transplantation. The numbers in five high-power fields were averaged and expressed as the number of capillary vessels.

Ultrastructure investigation

Heart tissue was fixed with 3.0% glutaldehyde and 1.5% paraldehyde, washed with PBS, postfixed in osmium tetroxide, dehydrated in an ethanol series, embedded in epoxy resin and then observed through a transmission electron microscope (JEM-2000EX; JEOL Ltd., Tokyo, Japan).

Statistical analysis

All variables are expressed as mean ± standard deviation (SD). Comparisons of parameters between two groups were made by unpaired Student's *t*-test with SPSS software 10.1 (Statistical Product and Service Solutions, Chicago, IL, USA). All tests were 2-tailed, and significance was accepted at $P < 0.05$.

Results

Characteristics of cultured MSCs

Four days after MSCs seeding in culture plates, phase contrast microscope revealed small colonies of adherent cells with typical fibroblast-shaped morphology. These primary cells reached monolayer confluence after plating for 7–10 days, when they were passaged for the first time. The cells were noted to have great potential for proliferation after subculture. The fibroblast-like morphology was also maintained after passages and throughout the culture period (Fig. 1).

Identification of MSCs

Rats MSCs were identified by surface markers: $CD34^+$ 0.9%, $CD90^+$ 88.9%, $CD71^+$ 0.6%, $CD34^-CD90^+$ 82.1% and $CD71^+$ $CD90^+$ 0.6%. Flow cytometric analysis showed that they were strongly positive for CD90 but negative for CD71 and CD34 (Fig. 2).

Feature of myocardial ischemia model

One week after MI, the wall of left ventricle became thinner (Fig. 3a). The Masson's trichrome-stained sections showed serious hisopathological changes. Compared with normal myocardium (Fig. 3b), ischemic myocardium demonstrated denaturation atrophy and lysis of cardiac myocytes with extensive interstitial fibrosis (Fig. 3c).

Reduction of mortality and myocardial infarct size after MSCs transplantation

At 3 h after coronary ligation, 24 rats survived (80% survival rate). 24 were randomized to receive a tail vein injection of MSCs (transplanted group, $n = 12$) or culture medium

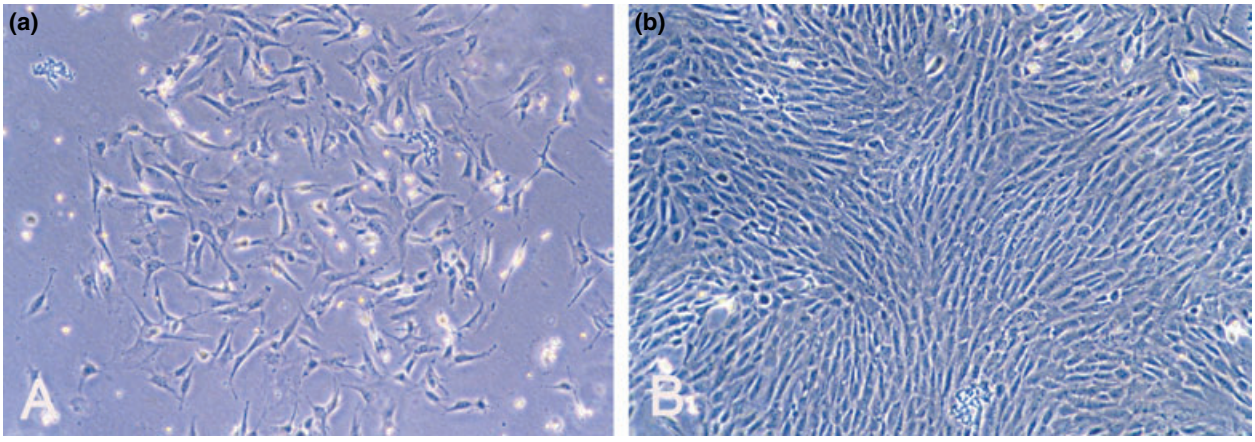


Figure 1 Cultured bone marrow MSCs *in vitro*. (a) Primary cultured MSCs (×4); (b) subcultured MSCs (×4).

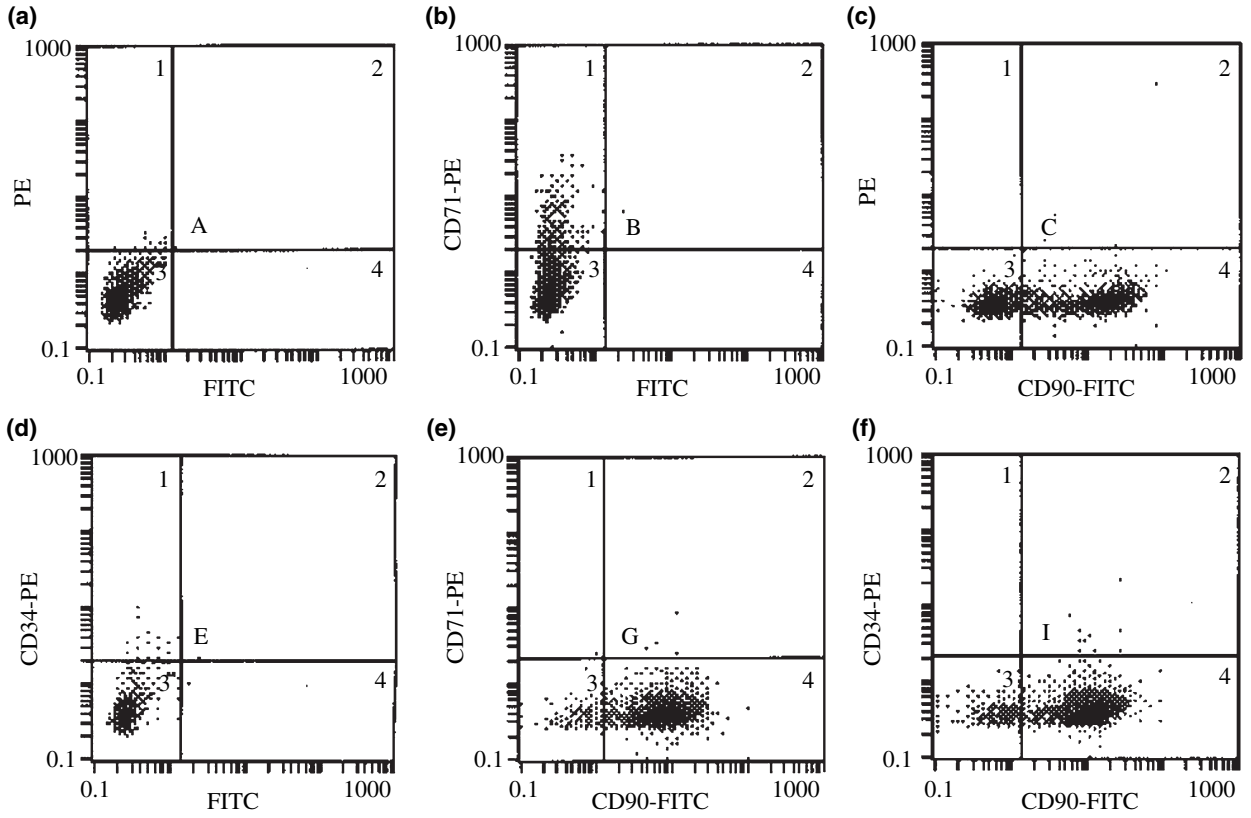


Figure 2 Flow cytometric analyses in subcultrued MSCs.

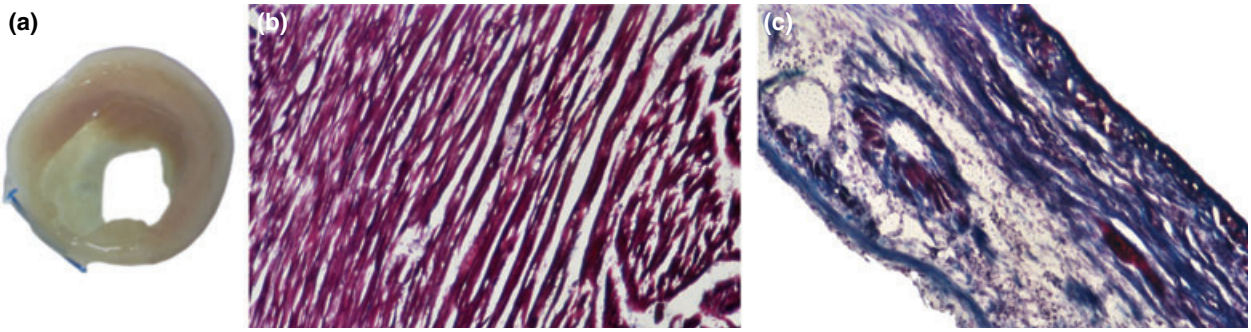


Figure 3 Morphological observation 1 week after myocardial ischemia. (a) Transection of ischemic myocardium, arrowheads indicate suture; (b) massion trichrome-stain of normal myocardium (×100); (c) massion trichrome-stain of infarcted myocardium (×100).

(control group, $n = 12$). The subsequent mortality for 8 weeks was 8.3% in the transplanted group and 25.0% in the control group. The mortality was significantly lower in the transplanted group than in the control group ($P < 0.05$). MSCs transplantation decreased the infarct size after myocardial infarction. Quantitative analysis also demonstrated that cardiac infarct size was significantly smaller in the transplanted group than in the control group: 26 ± 2.58 vs. 35 ± 2.79% ($P < 0.05$). In addition, transplanted group had a significant smaller septum thickness ($P < 0.05$) and a significant greater scar thickness ($P < 0.05$) compared with those in control rats (Fig. 4).

Improvement of cardiac function after MSCs transplantation

8 weeks after transplantation, hemodynamic studies were performed in the control ($n = 6$), and transplanted ($n = 6$)

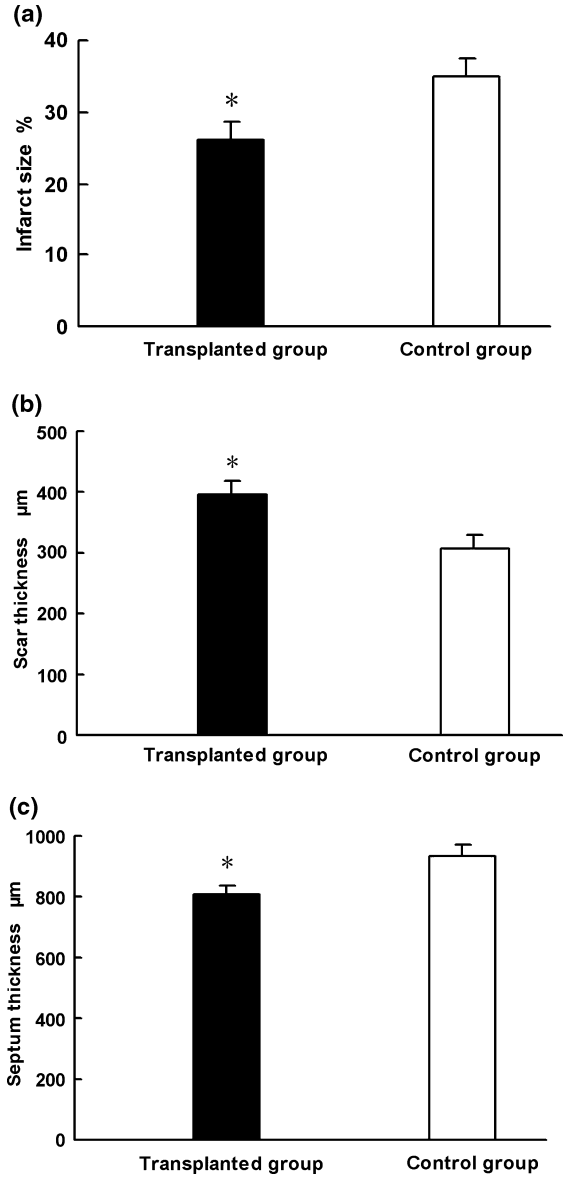


Figure 4 The effect of MSCs transplantation on infarct size 8 weeks after myocardial ischemia. (a) Infarct size (%); (b) scar thickness (μm); (c) septum thickness (μm). Values are mean ± SD. $*P < 0.05$ vs. control group.

groups (Table 1). Heart rate did not significantly differ between the two groups. However, transplanted group had a significant higher LV systolic pressure (LVSP) and a significant lower LV end-diastolic pressure (LVEDP) compared with those in control rats. LV maximum dP/d*t* was significantly higher and LV minimum dP/d*t* tended to be lower in the transplanted group as well. Transplantation of MSCs significantly improved ventricular contractility reflected by the increase in +dP/d*t* and LV systolic pressure (Fig. 5).

Table 1. Effects of MSCs transplantation on hemodynamics.

	Transplanted group	Control group
HR (bpm)	364 ± 18	376 ± 15
LVSP (mmHg)	105.67 ± 21.48*	89.45 ± 18.69
LVEDP (mmHg)	10.21 ± 3.62*	16.18 ± 2.48
+dP/d*t* (mmHg/s)	4879.56 ± 318.53*	3968.38 ± 389.62
−dP/d*t* (mmHg/s)	−4716.63 ± 582.39*	−3815.66 ± 528.35

Values are mean ± SD. Transplanted group, myocardial ischemia rats given mesenchymal stem cells; control group, myocardial ischemia rats given culture medium; HR, heart rate; LVS P, left ventricle systolic pressure; LVEDP, left ventricle end diastolic pressure; +dP/d*t*, LV maximum dP/d*t*; −dP/d*t*, LV minimum dP/d*t*. $*P < 0.05$ vs. control group.

Homing of MSCs to ischemic myocardium

One or 8 weeks after transplantation, *sry* positive cells were observed in the cardiac tissue of transplanted group (Fig. 6), but no *sry* positive cell was presented in the heart of control group. FISH examination identified transplanted cells either in the infarcted or border zone but not in remote viable myocardium.

Differentiation of MSCs after transplantation

To demonstrate the contribution from *sry* positive cells to the repair of cardiac tissue, we tested for the expression of markers of fully differentiated cardiomyocytes, smooth muscle cells and endothelial cells (Fig. 7). One week after transplantation, the *sry* positive cells in transplanted group were indicated in absence of Troponin, Desmin, smooth muscle α-actin and CD31. However, 8 weeks after transplantation, some Y chromosome- containing cells stained positively for Troponin and Desmin and formed part of the array of cardiac muscle fibers. This indicated that some engrafted MSCs had differentiated into cardiac-like cells. On the other hand, *sry* positive cells stained with smooth muscle α-actin or CD31 were also identified in transplanted group. This confirmed that some MSCs had differentiated into smooth muscle cells, endothelial cells and formed vascular structures. Quantitative analysis demonstrated that capillary density was significantly higher in the transplanted group than in the control group (Fig. 8).

Ultrastructural observation after transplantation

One week after transplantation, ultrastructural observation of some cells in the peri-infarct area of the transplanted group revealed large numbers of surface villi, cytoplasm enriched in rough endoplasmic reticulum and mitochondria, and large irregular nuclei. In addition, cell connection was formed gradually as well (Fig. 9). Eight

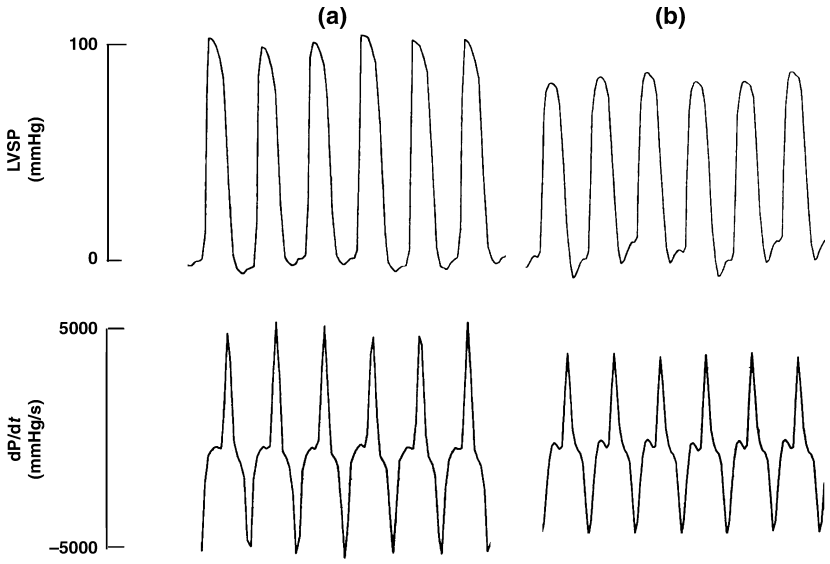


Figure 5 MSCs transplantation improved left ventricular (LV) function. Continuous chart strip recordings of hemodynamic measurement in anesthetized animals. (a) Transplanted rats; (b) control rats. Measurement was conducted in rats 8 weeks after transplantation.

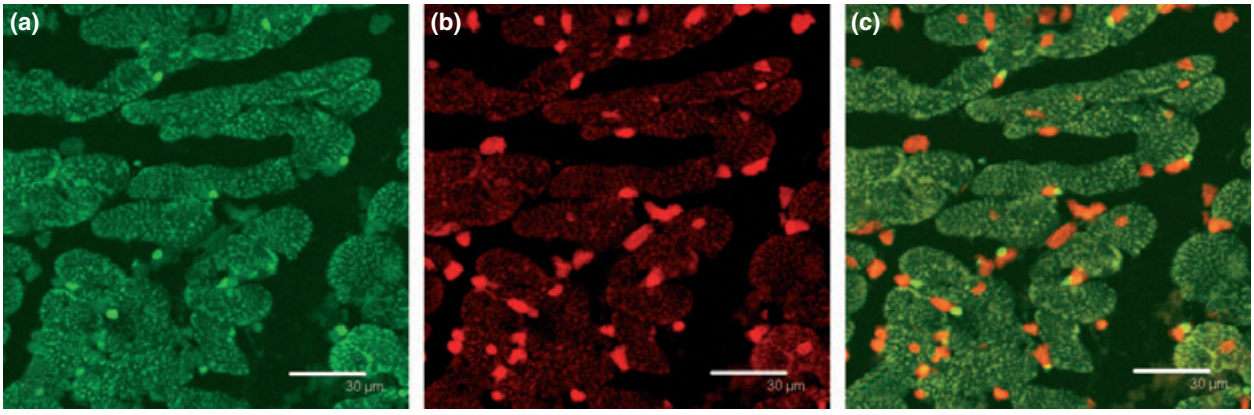


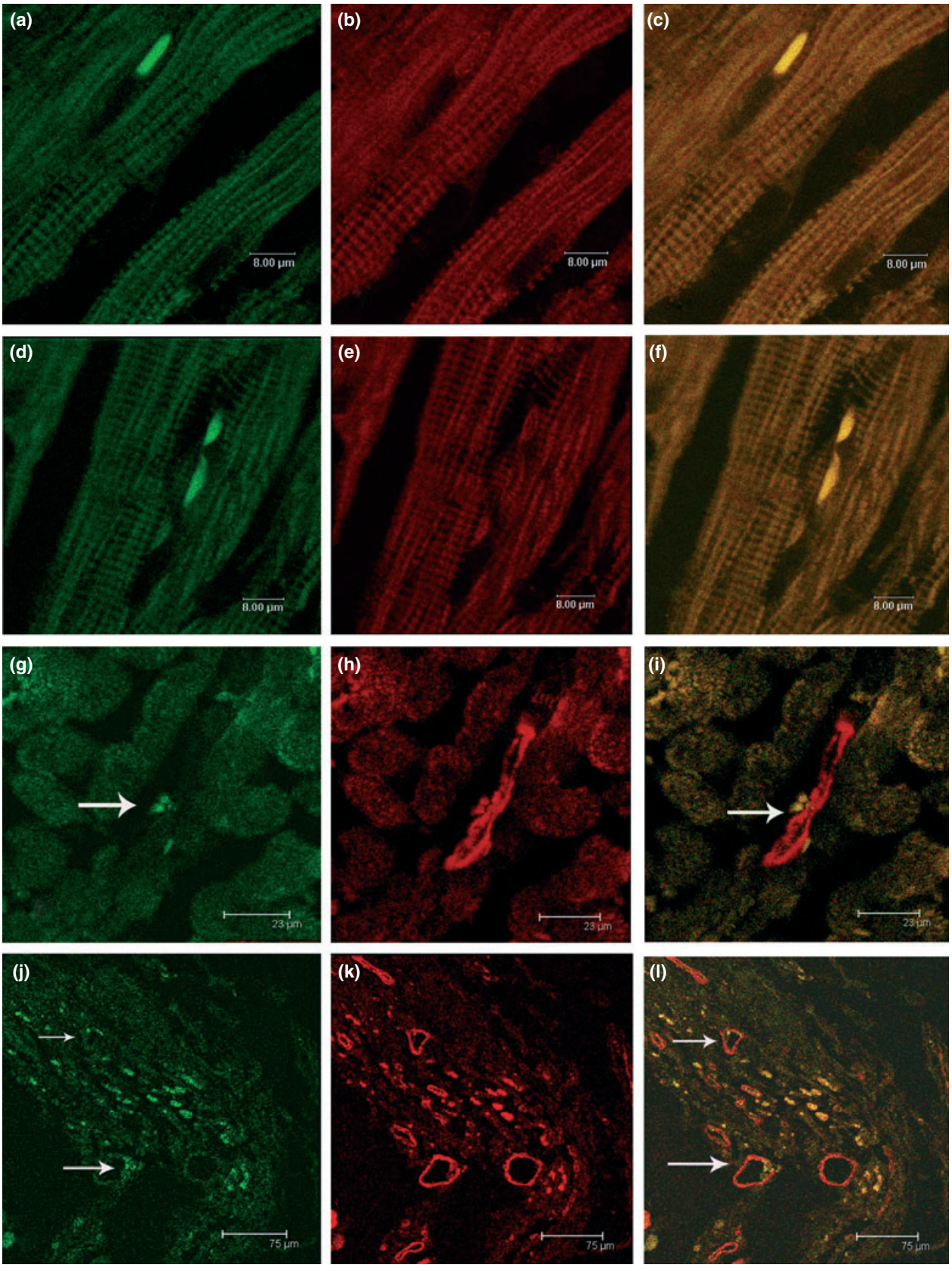
Figure 6 Fluorescence *in situ* hybridization (FISH) analysis of the *sry* positive cells in ischemic myocardium of female rats. Hybridization signals (green) of *sry* gene were detected in the heart (a); Nuclei (red) were stained with PI (b); image (c) is overlay of (a) and (b). Scale bar = 30 μm.

weeks after transplantation, large number of capillary among the myocardium of the peri-infarct area was observed in ischemic myocardium. Meanwhile, ultrastructural observation also revealed that some immature myocytes with large nuclei and loosen myofibrils were found to survive in the ischemic region (Fig. 10).

Discussion

Ischemic heart disease is a life-threatening event that may cause sudden cardiac death and heart failure. Although angioplasty and thrombolytic agents can relieve the symptom of the infarction, no medication or procedure used clinically has shown efficacy in replacing myocardial scar with functioning contractile tissue. There is the need for new therapeutics to regenerate cardiac tissue [8]. Earlier studies showed that MSCs directly injected into the myocardium or those injected into coronary arteries improve cardiac function after myocardial ischemia [9]. However, little information is available regarding the therapeutic potential of intravenously delivered MSCs for myocardial ischemia. In the present study, we aimed at observing therapeutic potency of intravenously transplanted mesenchymal stem cells for acute myocardial ischemia.

In this study, we found that bone marrow MSCs in culture appeared fibroblastic and homogeneous in size and morphology by the third passage. Flow cytometry analyses showed that the rat MSCs were strongly positive for CD90, but negative for CD34 and CD71. This indicated that mesenchymal lineages surface markers were present in the cultures but hematopoietic cell markers were not. Therefore, the cultured cells based on their



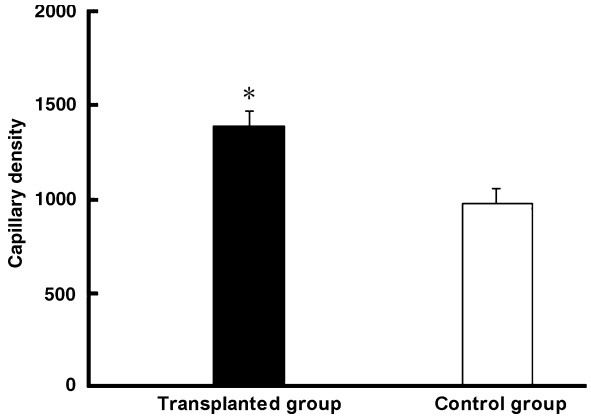


Figure 8 Quantitative analysis of capillary density in peri-infarct area. Values are mean ± SD. $*P < 0.05$ vs. control group.

morphology and surface markers might be MSCs [10]. It was reported that examination of the interaction of allogeneic MSCs with cells of the immune system indicates little rejection by T cells [11,12]. In our study, the purified male MSCs could survive 8 weeks in exogenous host hearts without addition of any immunosuppressant. Persistence of allogeneic MSCs *in vivo* suggests their potential therapeutic use for multiple recipients.

We found that *sry* positive cells were presented in the cardiac tissue of transplanted group, but no *sry* positive cell was seen in the cardiac tissues of the control group at 1 or 8 weeks after transplantation. It indicates that the tissue injury may play important roles in migration of MSCs to heart. This extraordinary ability of MSCs to home to sites of acute tissue injury has been demonstrated in the settings of bone fracture [13,14] and cerebral ischemia [15,16]. The present findings about donor cell entrapment in the heart are in accordance with previous reports as well [17,18]. Our findings suggest that MSCs are preferentially attracted to and retained in the ischemic myocardium, colonize there, and might contribute to

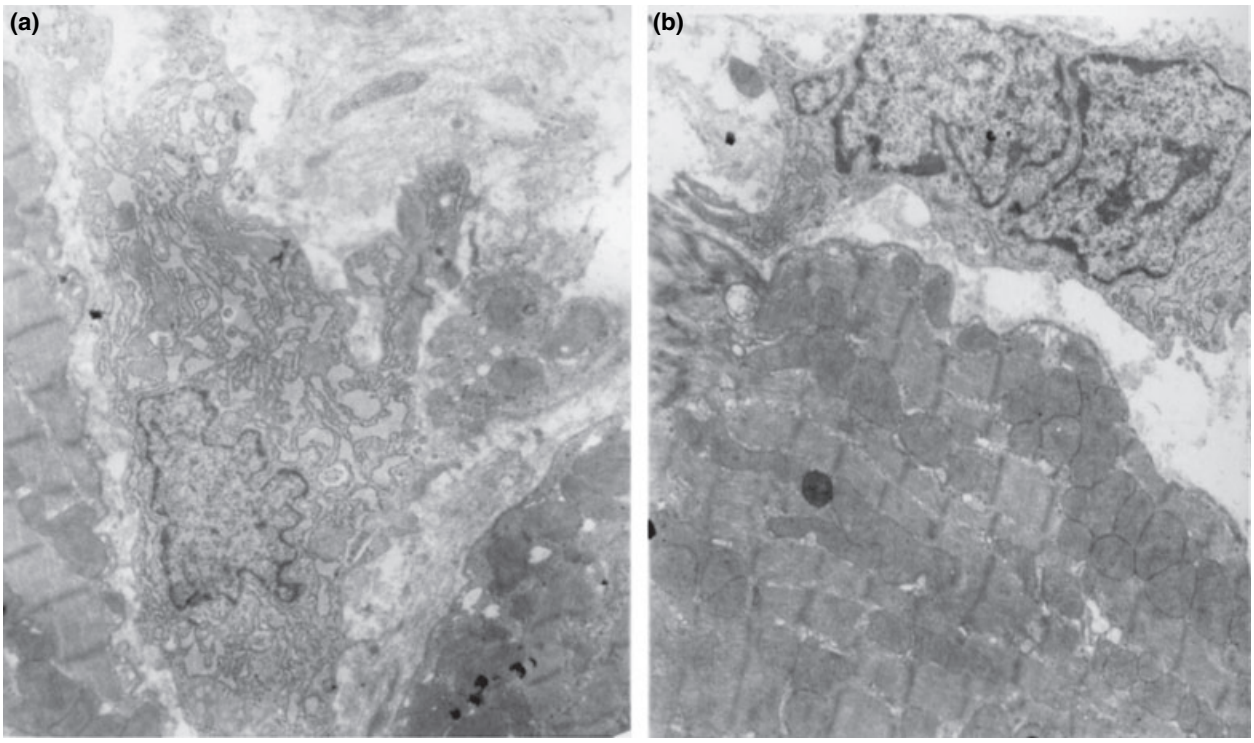


Figure 9 Ultrastructure of ischemia myocardium at 1 week after MSCs transplantation. (a) Large number of villus in surface, rough surfaced endoplasmic reticuli in endoplasm, and large nucleus with irregular shape were observed in few cells located in the peri-infarct area; (b) formation of cell connection (TEM × 5000).

Figure 7 Differentiation of transplanted MSCs in ischemic myocardium of female rats. Tissue sections were stained for hybridization signals (green) of *sry* gene (a, d, g, J). Sections were stained for cardiac troponinT (b), Desmin (e), smooth muscle α-actin (h) and CD31 (k). Images (c), (f), (i), and (l) are overlays of (a) and (b), (d) and (e), (g) and (h), (j) and (k) respectively. (a–f) Scale bar = 8 μm; (g–i) scale bar = 23 μm; (j–l) scale bar = 75 μm.

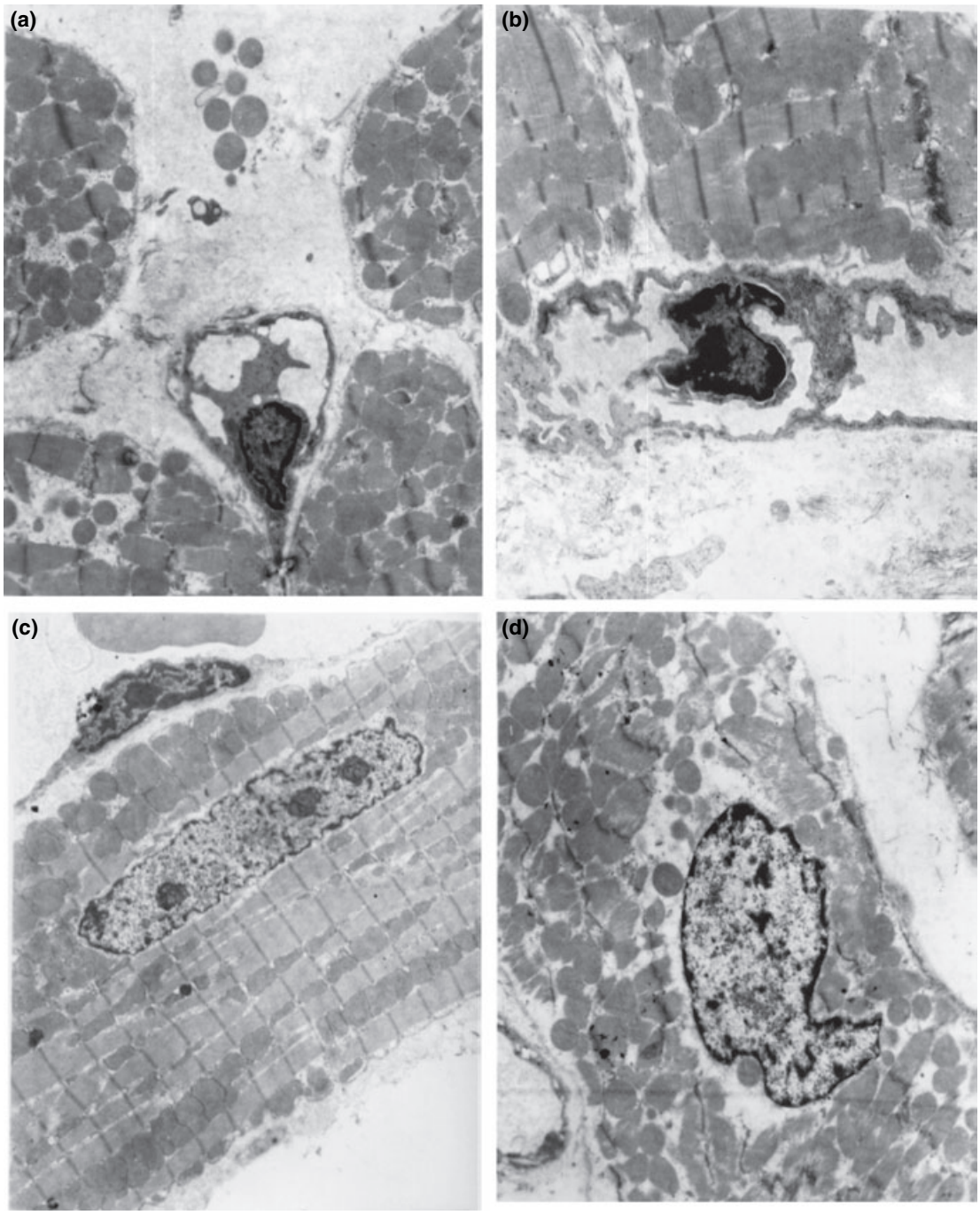


Figure 10 Ultrastructure of ischemia myocardium at 8 weeks after MSCs transplantation. (a, b) Neovascularization in ischemic myocardium; (c) mature myocardial cell; (d) immature myocardial cell (TEM × 5000).

healing of the heart. Although the factors responsible for MSC migration have not yet been defined clearly, the transient nature of the phenomenon is consistent with the involvement of an inflammatory mediator similar to those responsible for macrophage and neutrophil infiltration in injured tissue. It is possible that the intense inflammatory reaction after myocardial ischemia causes a local accumulation of mast cells and may initiate migration of MSCs to myocardium [19]. The ischemic tissue may express appropriate receptors or ligands to facilitate trafficking and adhesion of stem cells to the site of injury where initiation of a differentiation results in the

generation of cells of the appropriate lineage [20–22]. However, it is still unclear what environmental cues initiate homing of MSCs to ischemic heart.

To demonstrate a contribution from *sry* positive cells to the repair of cardiac tissue, we tested for the expression of markers of fully differentiated cardiomyocytes, smooth muscle cells and endothelial cells. With *sry* tracing after transplantation, we found that no *sry* positive cells were stained positively for Troponin Desmin, smooth muscle α-actin and CD31 at 1 week after transplantation. However, at 8 weeks after transplantation, *sry* positive cells stained with Troponin, Desmin, smooth muscle α-actin and CD31 were observed in transplanted group. It indicates that MSCs had differentiated into cardiac-like cells and blood vessel. This is consistent with previous studies, which demonstrated the capacity of MSCs to give rise to new myocytes, smooth muscle cells and endothelial cells in ischemic myocardium [23,24]. The molecular players involved in differentiation of stem cells are likely to differ depending on the tissue, degree of injury, and stem cells involved [25]. After an injury, various growth factors that stimulate cell replication and substitution in the injured tissue are released by the surrounding cells and facilitate differentiation of stem cells [2]. Therefore, the differentiation process is pronounced in injured tissue.

A number of studies have demonstrated that bone marrow stem cells can survive and differentiate into functional cells in the myocardium by immunohistochemical analysis [5,23,24,26]. However, little information is available regarding ultrastructural changes of ischemia myocardium after intravenous transplantation of MSCs. In the present study, we observed that few cells located in the peri-infarct area of ischemia myocardium had large number of villus in surface, rough surfaced endoplasmic reticuli and mitochondria in endoplasm, and large nucleus with irregular shape at 1 week after MSCs transplantation through TEM. In addition, cell connection was gradually formed between 'few cells' and myocardial cells as well. The ultrastructure of 'little cells' was similar to that of cultured MSCs, which demonstrated large number of villus in surface and rough surfaced endoplasmic reticuli and mitochondria in endoplasm as well [27]. It is inferred that the 'few cells' may be transplanted MSCs migrating to ischemia myocardium via the blood circulation. Tissue-directed differentiation within the injured organ is probably influenced both by cell-to-cell contact and by growth factors [28]. Therefore, we found that the majority of engraftment was adjacent to the region of infarct in which blood supplement and inflammation reaction existed. Ultrastructural observation also revealed that vessel formation and some immature myocytes with large nuclei and loosen myofibrils were found to survive in the peri-infarct area at 8 weeks after MSCs transplantation. It indicated that the intravenously transplanted MSCs might promote neovascularization and cardiomyocyte regeneration. Although the mechanisms by which MSCs develop into cardiomyocyte-like cells remain unclear, it is possible that the direct attachment with host cardiomyocytes in the ischemic myocardium contributes to the cardiogenic differentiation of transplanted MSCs.

The present study showed that MSCs transplantation significantly reduced mortality and infarct size. Furthermore, MSC transplantation improved cardiac function after acute myocardial ischemia, as indicated by a significant increase in LVSP and decrease in LVEDP, a tendency for an increase in $+dP/dt$ and a decrease in $-dP/dt$. Improvement of cardiac function may be attributable to myocardial regeneration. Meanwhile, newly formed vessels after MSCs transplantation improve tissue perfusion around the ischemic boundary zone, resulting in functional recovery after acute myocardial ischemia as well. Nevertheless, when MSCs were intravenously transplanted in an acute phase of myocardial ischemia, MSCs homing to ischemic myocardium, modestly but significantly, improved cardiac function. Thus intravenous delivery of MSCs may be beneficial for the treatment of acute myocardial ischemia.

In conclusion, allogeneic MSCs intravenously transplanted were preferentially attracted to the ischemic myocardium and might promote regeneration of vessel and cardiomyocytes. Finally, MSCs transplantation decreased the infarct size and improved cardiac performance after acute myocardial ischemia. Thus, MSCs transplantation may be a new therapeutic strategy for the treatment of myocardial infarction. It will be one of the future tasks to find the most practical and specific way of evolving and targeting the healing potency of stem cells for tissue regeneration [29].

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