# Autologous transplantation of bone marrow mononuclear cells improved heart function after myocardial infarction[1]

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**KEY WORDS** autologous transplantation; bone marrow cells; stem cell; left ventricular function; angiogenesis; nerve regeneration; myocardial infarction

## ABSTRACT

**AIM:** To investigate whether autologous transplantation of adult stem cells could improve post-infarcted heart function. **METHODS:** Bone marrow mononuclear cells (MNCs) were isolated from adult rabbits' tibias after coronary ligation. These cells were exposed to 5-azacytidine 10 μmol/L for 24 h on the third day of culture. After being labeled with bromodeoxyuridine (BrdU), the cells were auto-transplanted into bordering zone of the infarcted area at 2 weeks after injury. The animals were killed at 3 days, 2 weeks, 1 month, and 2 months after transplantation, respectively. The left ventricular functions, capillary density, and cardiac nerve density were measured and the differentiation of the engrafted cells was determined by immunostaining. **RESULTS:** BrdU-labeled MNCs were well aligned with the host cardiomyocytes. Parts of them were incorporated into capillary and arteriolar vessel walls. In addition to inducing angiogenic ligands (basic fibroblast growth factor, vascular endothelial growth factor) and imflammation cytokines (interleukin 1-β) during the early period of MNCs implantation, MNCs induced 2.0-fold increase in capillary density as well. Moreover, GAP43-positive and TH-positive nerve density were markedly higher in the MNCs-treated groups than that in the non-treated hearts. Left ventricular ejection fraction, LV+d$p$/d$t_{max}$, and LV-d$p$/d$t_{max}$ were 47 %, 67 %, and 55 % in MNCs-treated heart respectively, which was higher than that of the control heart, whereas left ventricular end-diastolic volume, left ventricular end-diastolic diameter, and left ventricular end-diastolic pressure were 45 %, 22 %, and 50 % respectively in MNCs-treated heart, which was lower than that of the control heart at 2 months after cell transplantation. **CONCLUSION:** Autologous transplantation of MNCs induced angiogenesis and nerve sprouting and improved left ventricular diastolic function.

## INTRODUCTION

Advances in the past five decades, including risk-factor modification, use of β blockers and angiotensin-converting-enzyme inhibitors, and effective percutaneous intervention ,have reduced the mortality and morbidity of ischemic heart disease. However, ischemic heart disease accounts for 50 % of all cardiovascular deaths and nearly 40 % of the heart failure incidents in the world. The progressive nature of ischemic heart disease, restenosis after percutaneous intervention, and some difficulty in gene therapy have exhausted patients and their doctors. Many researchers have investigated cell transplantation and considered it as an alternative treatment for heart disease. A variety of cell types have been proposed as useful candidates, such as skeletal

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muscle satellite cells, fetal cardiomyocytes, embryonic stem cells, and so on.

It is now accepted that an adherent population of cells isolated from bone marrow is multipotential progenitor cells, which can differentiate into muscle, cartilage, bone, fat, and tendon[1-6]. Orlic *et al* reported an experimental application of bone marrow stem cells for the regeneration of myocytes, endothelial cells, and smooth muscle cells in the infarcted heart[7]. Tomita *et al* demonstrated that autologous transplantation of bone marrow cells improved damaged heart function[8]. The repair of one damaged heart would likely require regenerated myocardium, new blood vessels, nerve innervation, and electrical and mechanical coupling of the restored tissue. The present study was designed to test therapeutic effect of marrow mononuclear cells (MNCs) on heart diastolic function, neovascularization, and nerve sprouting.

## MATERIALS AND METHODS

**Myocardial infarction** Sixty Japanese white rabbits (Grade II, Experimental Animal Center of Wuhan University) at 5 months of age were anesthetized with intraperitoneal injection of 3 % sodium pentobarbital (40 mg/kg). The hearts were then exposed through a 0.5-cm left thoracotomy. After pericardiotomy, the left anterior descending (LAD) ramification of the coronary artery was ligated with 5/0 silk sutures. The muscle layer and skin incision were closed with 3/0 silk sutures. Sham-operated (SO) rabbits did not have their coronary artery ligated. They only underwent thoracotomy and pericardiotomy.

**Isolation of bone marrow mononuclear cell** On the second day of myocardial infarction, about 3 mL of bone marrow aspirate was collected from the rabbits' tibias and pushed into a syringe containing 3000 U of heparin. The marrow sample was diluted with 3 mL of phosphate-buffered saline (PBS). Then the marrow cells were transferred to a 15-mL conical tube containing 3 mL of Percoll (density: 1073 g/L; Pharmacia Biotech), and the tube was centrifuged at 1500×*g* for 25 min. The mononuclear cells were collected from the interface, washed with the two volumes of PBS, and re-collected by centrifugation at 1500×*g* for 5 min. The cells were resuspended, counted, and plated at $1.6\times10^6$ cells/cm$^2$ in the 5 mL culture medium [DMEM/$F_{12}$ with 20 % fetal bovine serum (FBS, Gibco), benzylpenicilline (100 kU/L) and streptomycin (100 mg/L)].

**Cell culture and cell labeling** Cells were cultured in DMEM/$F_{12}$ supplemented with 20 % FBS and antibiotics at 37 ºC in humid air with 5 % $CO_2$. On the third day, cells were treated with 10 μmol/L of 5-azacytidine (Sigma) for 24 h. On the following day, the medium was removed and the adherent cells were washed with PBS in order to ensure complete removal of 5-azacytidine. The culture medium was changed twice a week. With the medium changes, almost all the hematopoietic stem cells were washed away.

To identify the transplanted cells in bordering area of the infarcted myodium, we labeled the cells with bromodeoxyuridine (BrdU, Sigma). Briefly, 10 μL of BrdU solution (BrdU, 50 mg; dimethyl sulfoxide 0.8 mL; water 1.2 mL) was added into each culture dish before the night of cell transplantation and was incubated with the cells for 16 h[8]. Monoclonal antibodies against BrdU (NeoMarkers) were used to determine labeling efficiency, and it was approximately 80 %.

**Cell transplantation** At two weeks after myocardial infarction, the cells were harvested with 0.25 % trypsin-0.02 % edetic acid, washed with PBS, resuspended in serum-free culture medium, and counted. The cells were kept on ice until they were implanted, usually within 30 min.

The heart was secondly exposed through the previous incision after intraperitoneal administration of 3 % sodium pentobarbital (40 mg/kg). Cell suspension 25 μL containing $1\times10^6$ cells were injected using a 100 microliters volume of syringe into the LAD risk area of the left ventricular free wall. Altogether 4 injections were made, in other words, 100 μL of cell suspension were slowly injected. In the control animals, one hundred microliters of DMEM/$F_{12}$ were injected into the LAD risk area. The dynamic electrocardiograph was monitored daily for 3 consecutive days after surgery. Three rabbits had premature ventricular beats at the first day after transplantation. None of them had supraventricular arrhythmia, ventricular tachycardia, or ventricular fibrillation. And all animals survived the procedure.

**Left ventricular function measurements** The 60 animals analyzed for cell engraftment in the study were killed with a highdose of kalium chloride at 3 d, 2 weeks, 1 month, 2 months after cell transplantation. Before the animals were killed, echocardiography was performed in conscious rabbits by using a Sequoia 512 (Acuson) equipped with a 6-MHz cardiac transducer (7V3). The anterior chest area was shaved, and M-mode tracings were recorded from the parasternal

short axis view at the level of papillary muscles. From M-mode tracings, anatomical parameters in diastole and systole were obtained. $LVEDV=[7.0/(2.4+D_d)]D_d^3$; $LVESV=[7.0/(2.4+D_s)]D_s^3$; EF=(LVEDV-LVESV)/LVEDV.

Subsequently, the right carotid artery of each animal was cannulated with a microtip pressure transducer for the measurements of left ventricular pressures, LV+d$p$/d$t_{max}$ (max rate of pressure rise) and LV-d$p$/d$t_{max}$ (max rate of pressure decay) in the closed-chest preparation.

**Measurement of cytokines** After hemodynamic measurements, blood samples were drawn from the right carotid artery before death. Plasma concentrations of interleukin 1-β (IL-1β), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) were measured by specific enzyme linked immunosorbent assay (ELISA) kit (R&D Systems,USA). Each assay was carried out in duplicate for each animal. Absorbance was measured at 450 nm by means of a microplate reader.

**Cardiac anatomy and infarct size** At specific times, the hearts were arrested in diastole with the administration of 10 % kalium chloride through the right carotid artery. Then the myocardium was perfused with 10 % formaline. The LV chamber was filled with fixative at a pressure equal to the *in vivo* measured end-diastolic pressure. The LV intracavitary axis was measured. The hearts were cut into three sections from the base to the apex of the left ventricle. The mid-section was used to measure LV thickness. The lengths of the endocardial and epicardial surfaces delimiting the infarcted region, and the endocardium and epicardium of the entire LV, were measured in each section. Subsequently, their quotients were computed to yield the average infarct size in each case. Tissue samples at the transplantation sites were collected, embedded in paraffin, and cut into slices of 4 μm thick. Parts of them were stained with hematoxylin and eosin, and the adjacent sections were processed for immunohistochemistry.

**Identification of transplanted MNCs in the treated hearts** Monoclonal antibodies against BrdU (NeoMarkers) were used to localize the transplanted bone marrow mononuclear cells. Briefly, the sample was treated with 1 % Triton X-100 for 10 min at room temperature and 50 % formamide for 10 min at 65 ºC to denature DNA. After being rinsed with PBS three times, the sample was incubated with antibodies against BrdU in a moist chamber at 4 ºC for 18 h, followed by FITC-conjugated goat-anti mouse IgG at 37 ºC for 1 h. After thorough washing, the second primary antibody was applied for an additional 18 h. These included either a mouse monoclonal anti-cardiac specific troponin T (NeoMarkers), or a mouse monoclonal antiphospholamban (Santa CruZ), a mouse monoclonal anti-factor VIII (NeoMarkers), or a mouse monoclonal anti-smooth muscle α-actin (NeoMarkers). This was followed by TRITC-conjugated goat-anti mouse IgG at 37 ºC for 1 h. Fluorescence imaging was performed with a Leica TCS-ST2 confocal microscope.

**Measurement of capillary density** To analyze the vessels, we randomly chose 5 fields (5 $mm^2$) from the risk area in the direction of LAD. After treatment with mouse monoclonal anti-factor VIII, the density of capillaries in each field was evaluated by counting vessels in the 5 randomly chosen unit areas (500 $mm^2$) under ocular micrometers (Olympus).

**Measurement of cardiac nerve density** Briefly, we used anti-growth-associated protein 43 (GAP43) antibody and anti–tyrosine hydroxylase (TH) antibody for immunocytochemical staining. We determined nerve density by a computer-assisted image analysis system (Image-Pro Plus 4.0). The computer automatically detected the stained nerves in these fields by their brown color and then calculated the area occupied by the nerves in the field. The nerve density was the nerve area divided by the total area examined ($\mu m^2/mm^2$). The mean density of nerves in these 3 selected fields was used to represent the nerve density of that slide.

**Statistical analysis** Data were expressed as Mean±SD. Statistical Analysis System software (SAS) was used for all analysis. Comparisons of continuous variables between >2 groups were performed by a one-way ANOVA. If the $F$ distribution was significant, a $t$-test was used to specify differences between groups. $P<0.05$ were considered significant.

## RESULTS

***In vitro* differentiation of bone marrow mononuclear cells** Two major types of cells, mesenchymal stem cells (MSCs) and hematopoietic stem cells were obtained by centrifugation media for mononuclear separation. Because the latter were round and did not attach to the culture dish, they were washed away with the culture medium changes. The MSCs were spindle-shaped, with big nucleus and small cytoplasm, and

attached to the dish tightly as fibroblastic cells. After 5-azacytidine treatment, the size of MSCs increased gradually. Part of MSCs lengthened themselves in one direction and confluenced into multinuclear myotubes at 2 to 3 weeks after initial plating. And striations were discernible by microscopy (Fig 1).

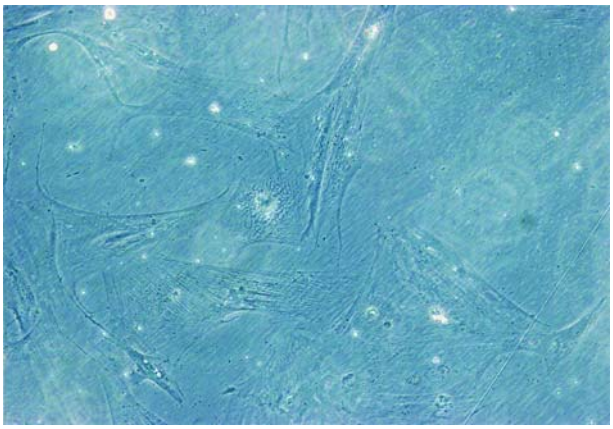

**Fig 1. After 5-azacytidine treatment, striations in the cytoplasm were discernible by microscopy at the 21th of cell culture. (Phase-contrast photograph ×200)**

***In vivo* engraftment of bone marrow mononuclear cells** Rabbits receiving intracardiac injection of BrdU-labeled MNCs were killed after 3 d to 2 months. At d 3, BrdU-stained cells were first observed in the transplanted area. With time *in situ*, the quantity of BrdU-positive cells increased greatly. The newly formed myocytes were in active growing phase and had a wide size distribution. The density of regenerative cardiomyocytes was high in the LAD risk area, lower in the papillary muscle, and lowest in the infarcted area. The majority of $BrdU^+$ cells were well aligned with the surrounding cardiomyocytes and the minority of brown nuclei confluenced with the host nuclei (Fig 2).

We also determined the expression of proteins specific for cardiomyocytes. Engrafted MNCs were first found to express cTnT at 2 weeks, whereas all identified MNCs were positive for this protein expression at 2 months. Similarly, MNCs were positive for phospholamban, a phosphoprotein that played a key role in modulating the cardiac sarcoplasmic reticulum $Ca^{2+}$ ATPase (Fig 3).

To characterize further the properties of these myocytes, we determined the expression of Connexin 43. It was clearly detectable in the interface between regenerated cardiomyocytes and host cells at 14 d after transplantation and it acquired a more mature pattern of

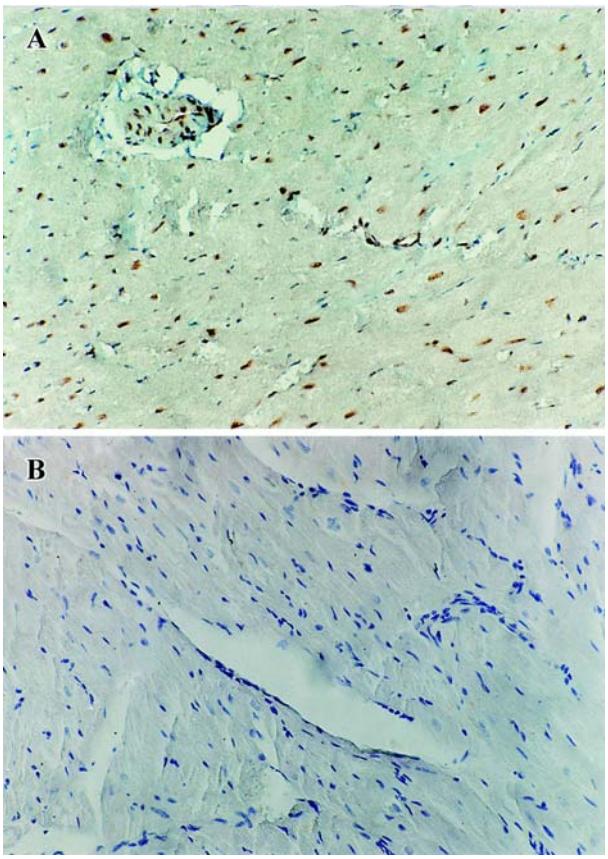


**Fig 2. Immunohistochemical staining of tissue section for BrdU (×200). A: In the MNCs-treated heart, the majority of BrdU-positive cells were well aligned with the surrounding cardiomyocytes and the minority of brown nuclei confluenced with the host nuclei. B: There were BrdU-negative cells in the non-treated heart.**

distribution at 28 d (Fig 4). Fig 5 showed that some vessels walls were composed of BrdU-positive smooth muscle cells and endothelial cells, which were located in bordering area of the infarcted myocardium. Collagen accumulation and lymphocyte infiltration were not evident.

**MNCs secret cytokines and induce angiogenesis** We measured the plasma concentrations of some inflammatory cytokines and angiogenic ligands by ELISA technique at 3, 14, and 28 d after autologous transplantations. The results showed that the levels of IL-1β and VEGF were significantly higher (4.5 fold, 3.4 fold, respectively) in the transplanted hearts than those of the control after 3 d, whereas they reverted to the baseline level after 14 d. And bFGF was upregulated at 14 d after MNCs injection, whereas it reverted to the baseline level after 1 month (Tab 1).

Tissue sections were also stained for anti-factor VIII antibody to detect endothelial cells. The density of

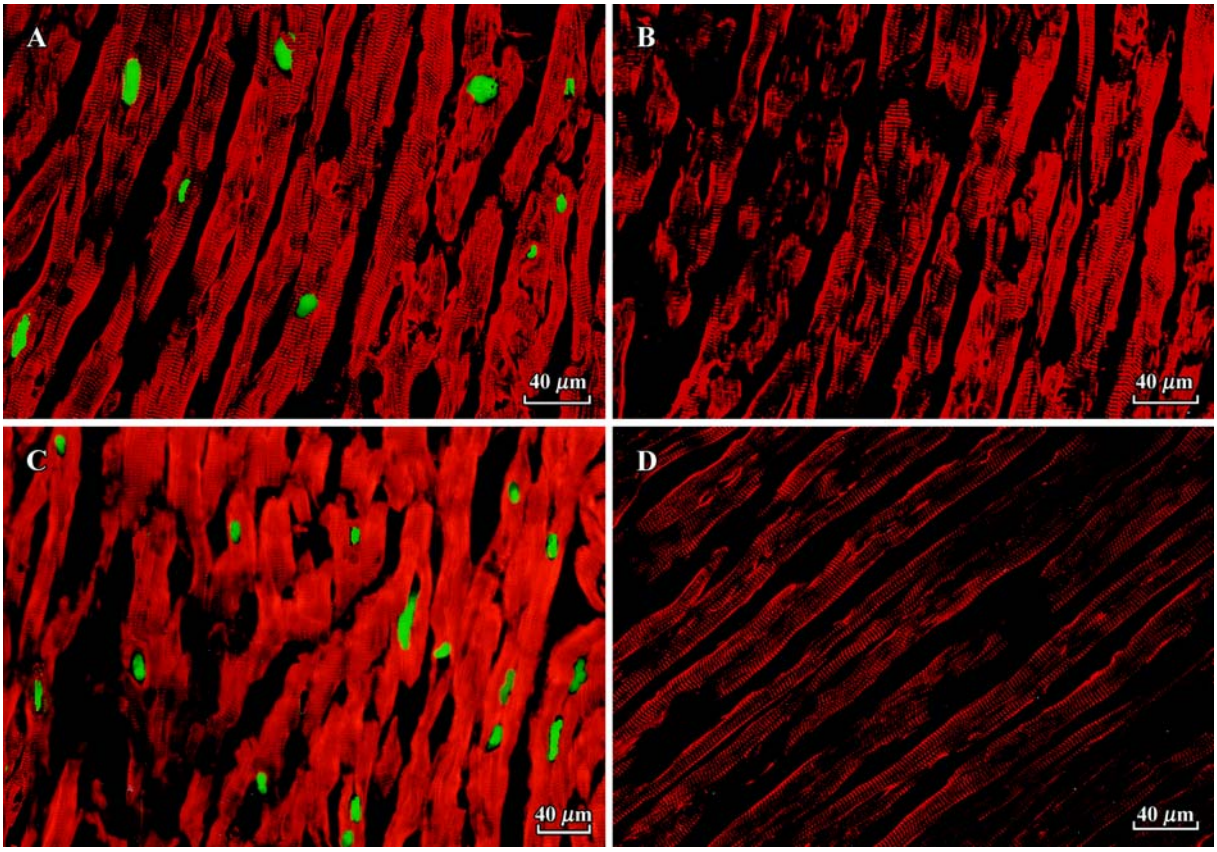


**Fig 3. Immunofluorescence images of differentiating cardiac cells (×400). A: Section injected with BM-MNCs was double-stained with antibodies to BrdU (FITC-labeled secondary antibody, green) and cardiac troponin T (TRITC-labeled secondary antibody, red). There were some cardiomyocytes with BrdU-labeled nuclei (green), indicating that engrafted MNCs expressed cTnT. B: Section injected with control medium. Section was negative-stained with antibodies to BrdU and positive-stained with cardiac troponin T (red). C: Section injected with BM-MNCs. Section was double-stained with antibodies to BrdU (FITC-labeled secondary antibody, green) and phospholamban (TRITC-labeled secondary antibody, red). There were some cardiomyocytes with BrdU-labeled nuclei (green), indicating that engrafted MNCs expressed phospholamban, one diastolic protein restricted to cardiac muscle. D: Section injected with control medium. Section was negative-stained with antibodies to BrdU and positive-stained with phospholamban (red).**

capillaries was greater (1.6 fold, 2.0 fold, respectively) in the MNCs-treated hearts than that of the control after 1 month and 2 months (Tab 2).

**MNCs induce cardiac nerve sprouting** GAP43-positive nerve density and TH-positive nerve density were significantly higher in the MNCs-treated groups than the non-treated groups at 2 months after injection (Fig 6, 7). Although nerve densities at peri-infarct myocardium were lower than those remote from infarction in both GAP43-positive nerves and TH-positive nerves, the difference did not reach statistical significance (Tab 3).

**Echocardiography and hemodynamics of the MNCs-treated hearts** Myocardial regeneration attenuated left ventricular cavitary dilation and mural thinning, improved anatomical remodeling and ventricular function in the infarcted heart. Anatomically, MNCs-induced myocardial repair decreased thickness of LV free wall by 39 % and 45 % and transmural infarcted area by 18 % and 27 % than non-treated groups after 1 month and 2 months, respectively (Tab 2).

Echocardiographically, LV end-diastolic diameter (LVEDD) and LV end-diastolic volume (LVEDV) were smaller in MNCs-treated group. At 1 month after transplantation, LVEDD of sham-operated, nontreated group, and MNCs-treated group was (1.38±0.25) cm, (2.05±0.30) cm, and (1.70±0.35) cm, respectively; at 2 months, LVEDD of groups was (1.38±0.25) cm, (2.00±0.35) cm, and (1.55±0.40) cm, respectively. LVEDV was 40 % lower in treated- than in nontreated-group at the end of first month, while LVEDV was 45 % lower in treated- than in nontreated-group after the second month (Tab 4, Fig 8).

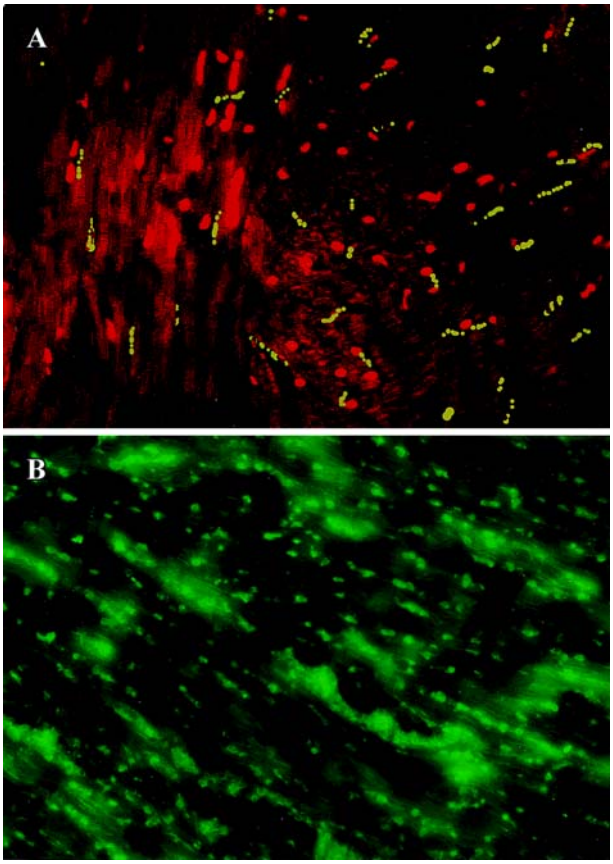


**Fig 4. Myocardial repair and connexin 43 (×400). Section was double-stained with antibodies to BrdU (TRITC-labeled secondary antibody, red) and connexin 43 (FITC-labeled secondary antibody, green). A: In the MNCs-treated heart, connexin 43 (yellow-green) was clearly detectable in the interface between regenerated cardiomyocytes and host cells. B: In the non-treated heart, connexin 43 (green) was present in the infarcted tissue.**

Measurements of hemodynamics before death showed the MNCs-induced repair improved ventricular performance. In rabbits treated with MNCs, contractile function and dilatational function developed with time in the left ventricle. At the end of first month, EF, LV+d$p$/d$t_{max}$, and LV-d$p$/d$t_{max}$ were 40 %, 55 %, and 54 % higher in treated- than in nontreated-group, respectively. After the second month, EF, LV+d$p$/d$t_{max}$, and LV-d$p$/d$t_{max}$ were 47 %, 67 %, and 55 % higher in treated- than in nontreated-group, respectively. Importantly, left ventricular end-diastolic pressure (LVEDP) were 44 % and 50 % lower in treated-animals after 1 month and 2 months respectively (Tab 4).

## DISCUSSION

**Main findings** Our study was designed to test the therapeutic effectiveness of autologous implantation of bone marrow mononuclear cells in adult rabbit with myocardial infarction. The main findings in our research included that (1) autologous implantation of BM-MNCs could express systolic and diastolic proteins specific for cardiomyocytes, as well as improved left ventricular ejection fraction and left ventricular dias-

**Tab 1. The plasma concentrations of IL-1β, VEGF, and bFGF at d 3, 14, and 28 after BM-MNCs transplantation. *n*=5. Mean±SD. $^bP<0.05$ *vs* non-treated group.**

| Groups | IL-1β/ng·L$^{-1}$ | VEGF/ng·L$^{-1}$ | bFGF/ng·L$^{-1}$ |
|---|---|---|---|
| Sham-Operated | | | |
| d 3 | 8.4±4.0 | 20.8±12.4 | 50.4±12.5 |
| d 14 | 5.3±3.7 | 22.6±9.9 | 49.8±10.7 |
| d 28 | 6.3±4.0 | 19.6±11.3 | 51.7±19.9 |
| Non-treated | | | |
| d 3 | 20.1±6.8 | 95.6±48.7 | 192.7±49.2 |
| d 14 | 9.4±4.9 | 35.1±8.2 | 72.3±23.6 |
| d 28 | 7.2±5.7 | 23.7±14.2 | 48.9±9.8 |
| MNCs-treated | | | |
| d 3 | 97.4±23.5$^b$ | 328.8±33.8$^b$ | 962.8±189.3$^b$ |
| d 14 | 16.8±5.3 | 47.5±12.3 | 303.7±56.3$^b$ |
| d 28 | 10.9±6.7 | 28.3±10.6 | 56.4±10.2 |

IL-1β= interleukin 1-β; VEGF= vascular endothelial growth factor; bFGF = basic fibroblast growth factor.

**Tab 2. Comparison of capillaries density, thickness of LVFW and transmural infarcted area between groups. *n*=5. Mean±SD. $^bP<0.05$ *vs* non-treated group.**

| Groups | Capillary density (vessels/ 500 mm$^2$) | Thickness of LVFW (cm) | Transmural infarcted area (mm$^2$) |
|---|---|---|---|
| Sham-operated | | | |
| Month-1 | 51±2 | 2.5±0.3 | - |
| Month-2 | 50±3 | 2.5±0.3 | - |
| Non-treated | | | |
| Month-1 | 58±2 | 1.38±0.4 | 60±3.0 |
| Month-2 | 62±3 | 1.45±0.4 | 58±3.2 |
| MNCs-treated | | | |
| Month-1 | 91±4$^b$ | 1.9±0.5$^b$ | 49±3.2$^b$ |
| Month-2 | 126±5$^b$ | 2.2±0.3$^b$ | 42±2.4$^b$ |

LVFW=left ventricular free wall

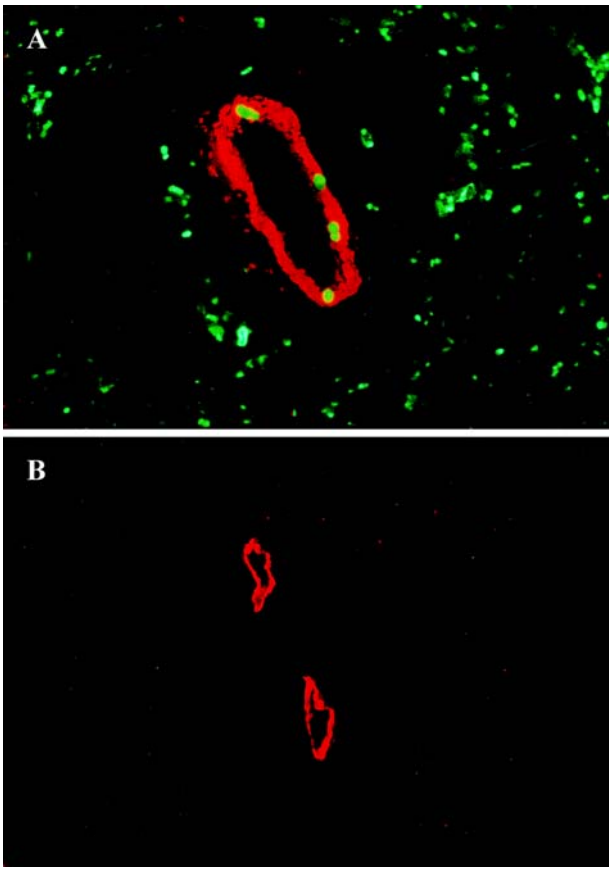


**Fig 5. Differentiation of BM-MNCs into vascular smooth muscle cells (×400). Section was double-stained with antibodies to BrdU (FITC-labeled secondary antibody, green) and smooth muscle α-actin (TRITC-labeled secondary antibody, red). A: In the MNCs-treated heart, some vessels' walls were composed of smooth muscle cells whose nuclei were labeled by BrdU (green). B: In the non-treated heart, vessels' walls were only positive for antibody to smooth muscle α-actin (red).**

tolic function; (2) implantation of BM-MNSc could induce myocardial regeneration, attenuate LV cavitary dilation and mural thinning, thus prevent myocardial remodeling after myocardial infarction; (3) BM-MNCs were incorporated into capillary and arteriolar vessel walls. In addition to supplying angiogenic ligands (bFGF, VEGF) and imflammation cytokines (IL-1β), MNCs induced angiogenesis as well; (4) MNCs induced cardiac nerve sprouting; and (5) fibrocollagenous accumulation and lymphocyte infiltration were negligible.

**Comparison with previous studies** In our experiment BM-MNCs can be detected *in vitro* and *in vivo* to express phospholamban — one diastolic protein restricted to cardiac muscle. Furthermore, the data received from echocardiography and hemodynamic studies showed a striking gain in heart function, especially

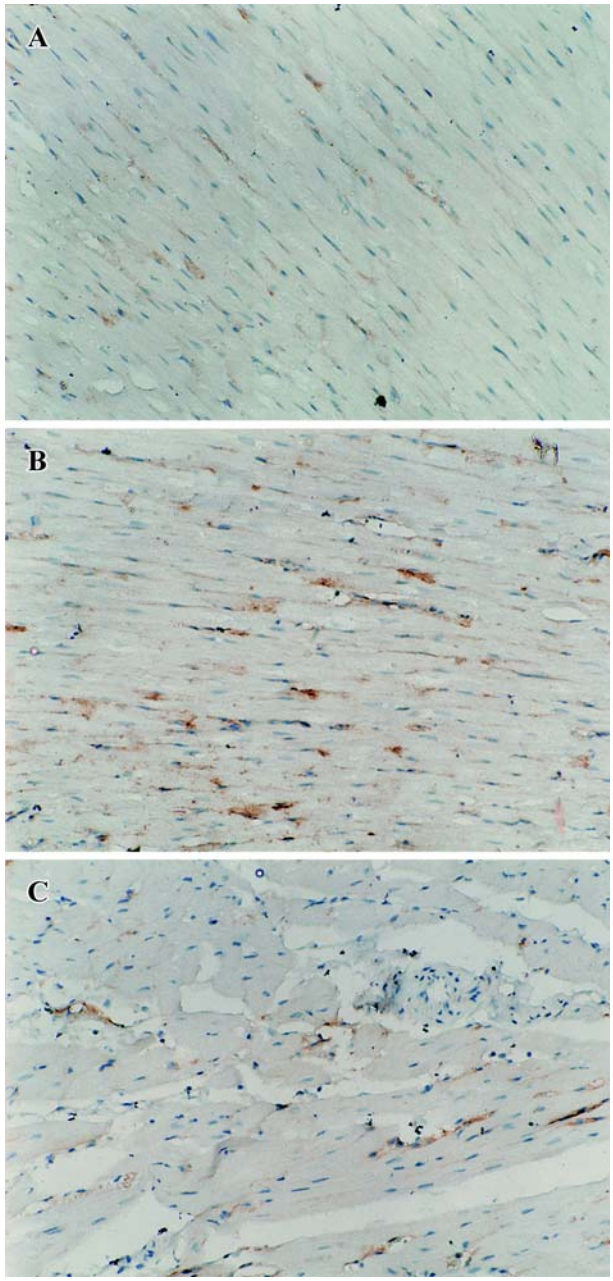


**Fig 6. Immunohistochemical staining of ventricular tissue with an antibody against GAP43 (×200). A: Tissue from the peri-infarct area in the MNCs-treated group. B: Tissue remote from infarct in the MNCs-treated group. C: Tissue from the peri-infarct area in the non-treated group.**

left ventricular diastolic function, including decrease in LVEDV, LVEDD, and LVEDP, and increase in LV-d$p$/d$t_{max}$ as well. Compared with the differentiation of skeletal muscle satellite cells in the injured myocardium[9-12], the expression of diastolic protein and the improvement of LV diastolic function observed in BM-MNCs implantation was distinct and encouraging. Although it was theoretically possible that BM-MNCs had potency to

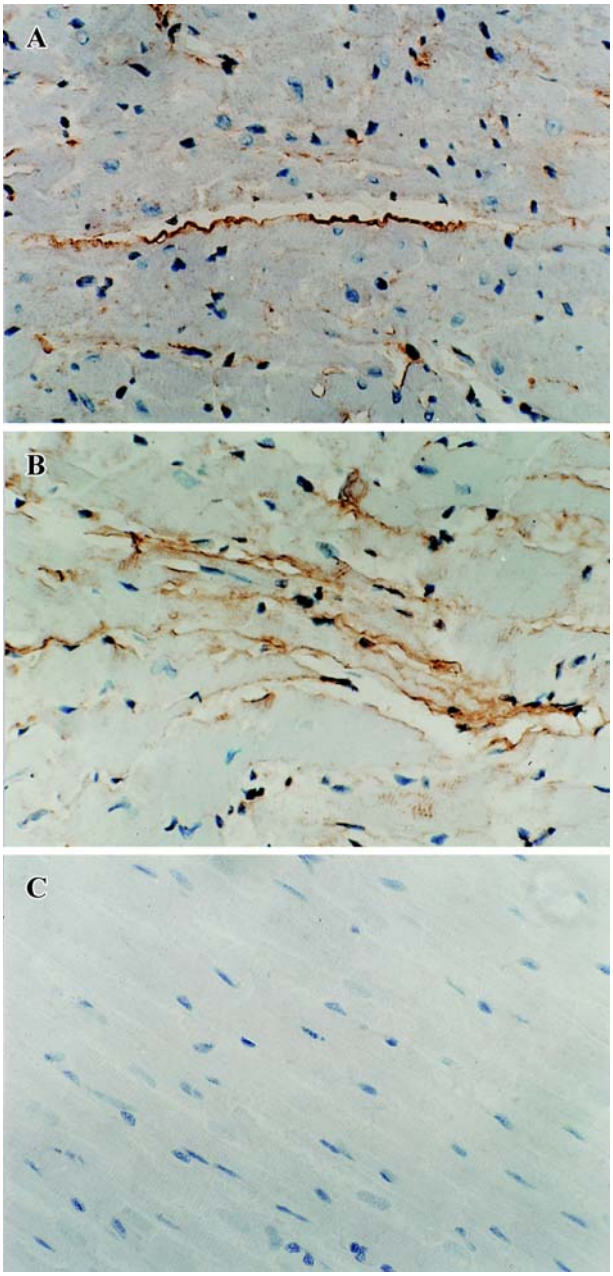


**Fig 7. Immunohistochemical staining of ventricular tissue with an antibody against TH (×400). A: Tissue from the peri-infarct area in the MNCs-treated group. B: Tissue remote from infarct in the MNCs-treated group. C: Tissue from the peri-infarct area in the Non-treated group.**

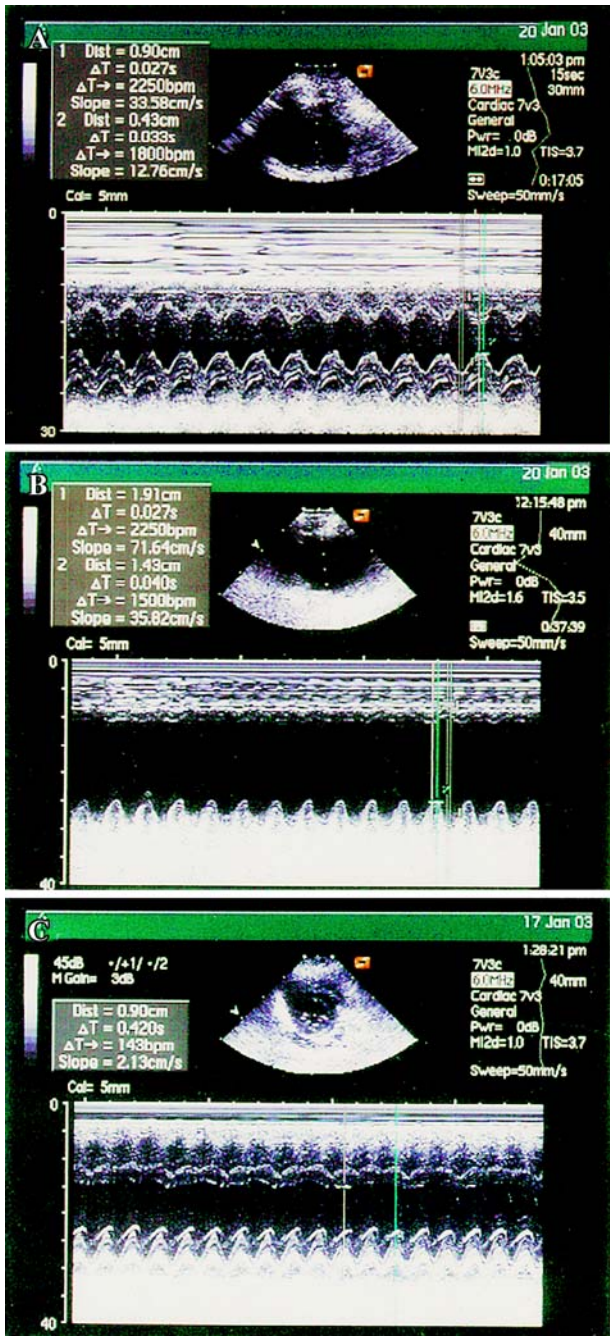


**Fig 8. M-mode echocardiogram. A: the Sham-operated rabbit. B: the non-treated rabbit. C: the MNCs-treated rabbit.**

differentiate to skeletal muscle cells and cardiomyocytes, "milieu-dependent differentiation" played a key role in the differentiation process of BM-MNCs. In other words, autocrine or/and paracrine growth signals in the ischemic myocardium in addition to the electrical and mechanical stimulation present in the adult heart would be involved in myocardial regeneration[7,13-15].

Moreover, we found that some vessels' walls actually incorporated BrdU labeled MNCs and the plasma concentration of IL-1β, bFGF, and that VEGF were upregulated during the early period of MNCs implantation. Kamibata *et al* also reported that some neocapillaries actually incorporated BM-MNCs and BM-MNCs synthesized bFGF, VEGF, or Ang-1[16,17]. On one hand, MNCs contain various kinds of cell lineages, such as hematopoietic cells, osteoblasts, and myogenic cells,

**Tab 3. Comparison of nerve density between groups at 2 months after injection. Mean±SD. [b]*P*<0.05 *vs* non-treated group.**

| Groups | GAP43 ($\mu m^2/mm^2$) | | TH ($\mu m^2/mm^2$) | |
|---|---|---|---|---|
| | Peri-infarction area (*n*=108) | Area remote from infarct (*n*=110) | Peri-infarction area (*n*=113) | Area remote from infarct (*n*=110) |
| Sham-operated | 2534±1267 | 2165±1443 | 593±352 | 650±329 |
| Non-treated | 1989±923 | 2359±1390 | 587±363 | 716±438 |
| MNCs-treated | 5557±2395[b] | 6781±3073[b] | 1838±879[b] | 2542±1240[b] |

Peri-infarction area=the LAD risk area of the left ventricular free wall;
Area remote from infarct=left ventricular myocardium apart from the peri-infarction area.

**Tab 4. Comparison of left ventricular function between groups. *n*=5. Mean±SD. [b]*P*<0.05 *vs* Non-treated group.**

| Groups | EF (%) | LV+d$p$/d$t_{max}$ ($mmHg \cdot s^{-1}$) | LV-d$p$/d$t_{max}$ ($mmHg \cdot s^{-1}$) | LVEDP (mmHg) | LVEDV (mL) | LVEDD (cm) |
|---|---|---|---|---|---|---|
| Sham-Operated | | | | | | |
| Month-1 | 72±10 | 3000±96 | 2800±92 | 5±2.3 | 4.98±0.9 | 1.38±0.3 |
| Month-2 | 72±10 | 3000±96 | 2800±92 | 5±2.3 | 4.98±0.9 | 1.38±0.3 |
| Non-treated | | | | | | |
| Month-1 | 35±7 | 1350±76 | 1300±70 | 18±5.0 | 12.75±1.5 | 2.05±0.3 |
| Month-2 | 40±12 | 1550±85 | 1500±80 | 16±4.5 | 11.55±2.0 | 2.00±0.4 |
| MNCs-treated | | | | | | |
| Month-1 | 49±5[b] | 2100±80[b] | 2000±90[b] | 10±3.0[b] | 7.50±1.5[b] | 1.70±0.4[b] |
| Month-2 | 59±5[b] | 2500±90[b] | 2300±95[b] | 8±3.5[b] | 6.55±1.0[b] | 1.55±0.4[b] |

EF=ejection fraction; LVEDP=left ventricular end-diastolic pressure; LVEDV=left ventricular end-diastolic volume; LVEDD=left ventricular end-diastolic diameter.

as well as 16 % of endothelial-lineage cells. It suggests that MNCs have the ability to form mature vascular endothelial cells. On the other hand, MNCs secrete a broad spectrum of angiogenic cytokines. IL-1β upregulated the expression of VEGF and its receptor KDR/flk-1 via activation of protein tyrosine kinases[18], and bFGF and VEGF were shown to have strong angiogenic activity. So implantation of MNCs is thought to be committed to induce angiogenesis, improve cardiac function of the infarcted myocardium by enhancing blood flow.

Furthermore, our present study showed that MNCs induced cardiac nerve sprouting. One possible explanation was that MNCs differentiated into nerve cells and migrate to remote sites[19]. A second possible explanation was that MNCs differentiated into cells that overexpressed nerve growth factor or other growth factors. GAP43-positive nerve twigs formed around these blood vessels in the myocardium. The colocalization of neovascularization with GAP43-positive nerve suggested that the same trophic factors might have stimulated both angiogenesis and nerve sprouting[20,21]. This finding suggested anther possible mechanism for improving left ventricular function after MNCs transplantation — cardiac nerve sprouting and sympathetic hyperinnervation. However, nerve sprouting, its heterogeneity and sympathetic hyperinnervation were known as one of the substrates for cardiac arrhythmia [22]. In our study, we only performed short-term electrocardiograph monitoring on the experimental animals; therefore, it was unclear that MNCs injection could perpetuate arrhythmia in patients or animals.

Connexin 43, which was responsible for intercellular connections and electrical coupling through the generation of plasma-membrane channels between myocytes, was apparent in the cell cytoplasm and at the surface of closely aligned differentiating cells[23,24]. The expression of connexin 43 implied the newly formed myocardium would be electrically coupled with the rest of the heart and contract together mechanically, which distinguished MSCs from skeletal myoblasts, because the latter did not express Connexin 43. This result was also consistent with the functional competence of the heart.

**Study limitations** Although our study showed that intracardiac injections of autologous MNCs appeared to be effective, the delivery method involved open-heart surgery, and the risk associated with open-heart surgery are great and unfit for clinical application. The optimal delivery method was still to be investigated. In the recent studies[25-27], percutaneous delivery (via the Biosense Electromechanical NOGA mapping catheter) of MNCs appeared to be feasible, relatively safe, and worthy of further research. Second, we did not perform long-term electrocardiograph monitoring on the experimental animals. So, we cannot establish a direct link between MNCs transplantation and arrhythmogenesis.

**Conclusion** Bone marrow mononuclear cells should be considered as an alternative cell source to repair the damaged myocardium. Autologous transplantation of MNCs into the bordering zone of infarcted myocardium could realize cardiomyocyte regeneration, induce angiogenesis and nerve sprouting, and improve left ventricular function.

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