

CHINESE MEDICAL SCIENCES JOURNAL

ORIGINAL ARTICLE

Effect of Intracoronary Infusion of Bone Marrow Mononuclear Cells or Peripheral Endothelial Progenitor Cells on Myocardial Ischemia-reperfusion Injury in Mini-swine

Chong-jian Li¹, Run-lin Gao^{1*}, Yue-jin Yang¹, Feng-huan Hu¹,
Wei-xian Yang¹, Shi-jie You¹, Lai-feng Song², Ying-mao Ruan²,
Shu-bin Qiao¹, Ji-lin Chen¹, and Jian-jun Li¹

¹Center of Coronary Heart Disease, ²Department of Pathology, Fuwai Hospital,
Chinese Academy of Medical Sciences & Peking Union Medical College,
Beijing 100037, China

Key words: bone marrow mononuclear cells; endothelial progenitor cells; myocardial ischemia-reperfusion injury

Objective To simulate and assess the clinical effect of intracoronary infusion of bone marrow mononuclear cells or peripheral endothelial progenitor cells on myocardial reperfusion injury in mini-swine model.

Methods Twenty-three mini-swine with myocardial reperfusion injury were used as designed in the study protocol. About $(3.54 \pm 0.90) \times 10^8$ bone marrow mononuclear cells (MNC group, $n=9$) or $(1.16 \pm 1.07) \times 10^7$ endothelial progenitor cells (EPC group, $n=7$) was infused into the affected coronary segment of the swine. The other mini-swine were infused with phosphate buffered saline as control ($n=7$). Echocardiography and hemodynamic studies were performed before and 4 weeks after cell infusion. Myocardium infarction size was calculated. Stem cell differentiation was analyzed under a transmission electromicroscope.

Results Left ventricular ejection fraction dropped by 0% in EPC group, 2% in MNC group, and 10% in the control group 4 weeks after cell infusion, respectively ($P<0.05$). The systolic parameters increased in MNC and EPC groups but decreased in the control group. However, the diastolic parameters demonstrated no significant change in the three groups ($P>0.05$). EPC decreased total infarction size more than MNC did ($1.60 \pm 0.26 \text{ cm}^2$ vs. $3.71 \pm 1.38 \text{ cm}^2$, $P<0.05$). Undermature endothelial cells and myocytes were found under transmission electromicroscope.

Conclusions Transplantation of either MNC or EPC may be beneficial to cardiac systolic function, but might not have obvious effect on diastolic function. Intracoronary infusion of EPC might be better than MNC in controlling infarction size. Both MNC and EPC may stimulate angiogenesis, inhibit fibrogenesis, and differentiate into myocardial cells.

Chin Med Sci J 2010; 25(3):176-181.

TRANSPLANTATION of stem cells of various types and origins has been shown to facilitate neovascularization and improve cardiac function after myocardial infarction.^{1,2} However, it is still unclear which type of stem cells is more effective in treating acute myocardial infarction.^{3,4} Most published clinical trials applied whole population of bone marrow mononuclear cells (MNCs) or peripheral endothelial progenitor cells (EPCs), yet reaching inconsistent results for various reasons. Therefore, we compared the effect of MNCs and EPCs used in intracoronary infusion for myocardial ischemia-reperfusion injury in the model of mini-swine so as to provide experimental evidence to selection of the optimal cell type in future clinical trials.

MATERIALS AND METHODS

Animal models

Chinese mini-swine (30±5 kg) about 10 months old were obtained from the Laboratorial Animal Center of Chinese Agricultural University. All the experimental procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996). Twenty-three mini-swine were divided into MNC group ($n=9$), EPC group ($n=7$), and control group ($n=7$).

Cell preparation

MNCs were isolated from 35 mL bone marrow aspirated from the iliac crest, by density gradient centrifugation (400 $\times$ g for 30 minutes) on a Ficoll-Paque PLUS 1.077 (Amersham Pharmacia, Freiburg, Germany). For cell tracking, the isolated MNCs were labeled with red fluorescent PKH26 (Sigma-Aldrich, Saint Louis, MO, USA) and re-suspended in 10 mL 4°C phosphate buffered saline (PBS). EPCs were separated out of 200 mL whole blood according to the previous report.⁵ In brief, total peripheral MNCs were isolated from 200 mL mini-swine peripheral blood by Ficoll density gradient centrifugation. The cells, suspended in specified endothelial medium supplemented with vascular endothelial growth factor (VEGF), were then plated on dishes coated with fibronectin. Attaching cells were detached and labeled with a series of endothelial specific antibodies and were analyzed by immunohistochemistry and immunofluorescence. On the day of infusion, the EPCs were labeled with chloromethylbenzamido dioctadecyl-tetramethylindo-carbocyanine (DiI, Molecular Probes, Eugene, OR, USA) and re-suspended in 10 mL 4°C PBS.

Ischemia-reperfusion injury and cell infusion

The model of ischemia-reperfusion injury in MNC group

were induced immediately after bone marrow aspiration, while the model in EPC group and the control group were induced on the day of intracoronary infusion. The left anterior descending (LAD) coronary artery was dissected free just distal to the first diagonal branch and occluded by a vessel loop. After 90 minutes, the loop was removed and reperfusion was visually confirmed. About $(3.54 \pm 0.90) \times 10^8$ MNCs or $(1.16 \pm 1.07) \times 10^7$ EPCs in 10 mL PBS were infused into LAD of mini-swine model in MNC group and EPC group, respectively. In the control group, only 10 mL PBS was injected. The infusion was performed *via* an on-the-wire balloon catheter, which, when inflated, could thoroughly prevent the backflow of cells. After cell infusion, coronary angiography was repeated to ascertain vessel patency and unimpeded flow of contrast material. Four weeks after cell infusion, the animals were euthanized by anesthetization shortly after echocardiography and hemodynamic studies.

Echocardiography and hemodynamic studies

Echocardiography and hemodynamic studies were performed twice: just before cell infusion and 4 weeks after it. Some indexes of left ventricle, including end systolic volume (LVESV), end diastolic volume (LVEDV), and ejection fraction (LVEF), were calculated based on two-dimensional echocardiography with a Hewlett-Packard Sonos 5500 (Agilent, Santa Clara, CA, USA). Left ventricular end systolic diameter (LVESD), end diastolic diameter (LVEDD), and interventricular septal thickness (IVS) were measured at the long axis of the mitral valve tendon by M-mode echocardiography. Left ventricular lateral wall thickness and anterior-septal wall thickness were mensurated at the short axis of the mid-papillary muscle. A fluid-filled pigtail catheter was inserted into the femoral artery for analysis of left ventricular end systolic pressure (LVESP), end diastolic pressure (LVEDP), $+dp/dt_{max}$, and $-dp/dt_{max}$ in accordance with electrocardiogram. Meanwhile, cardiac output was measured by inserting a Swan-Gans floating catheter into the pulmonary artery through heat dilution.

Immunohistochemical assessment and analysis of vessel amount

For immunohistochemistry the cardiac tissues were snap frozen in liquid nitrogen and 5 μ m thick sections were mounted on silane-coated glass slides. Masson's trichrome and HE staining were used to visualize vessels and cellular type. Serial paraffin-embedded sections were treated with rabbit anti-human von-Willebrand factor (vWF) antibody (Sigma-Aldrich). To analyze the vessel density, 5 fields (under high power) were sequentially chosen from the

ischemia-reperfusion area. Researchers not knowing the group classification of slides evaluated the density of arteries and capillaries by counting vessels in each field with an optical microscope.

To detect the differentiation of transplanted cells *in vivo*, cardiac tissue sections were incubated with antibodies against biomarkers for cardiovascular cells including vWF (1 : 200, Dakocytomation, Denmark), α -sarcomeric actin (1 : 50, Dakocytomation), and fibronectin (1 : 50, Sigma). After washing with PBS, the sections were incubated with rhodamine-conjugated goat anti-mouse IgG or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG according to the instructions of the reagents. Immunofluorescence was detected and the images were photographed with a laser scanning confocal microscope. The suspected myoblasts detected by monoclonal anti- α -sarcomeric actin were observed under a transmission electromicroscope.

Infarction size calculation

The infarcted area was visualized by tetrazolium red staining. Left ventricular wall and infarcted area were manually traced in cross sections. The total cardiac area and infarcted area were calculated using the image processing system BHEC-100 version 1.0 according to the previous study.⁶ The infarction percentage was presented as the ratio of total infarcted area to total myocardial area of left ventricle.

Statistical analysis

Continuous variables are presented as means $\pm$ SD. Inter-group comparison was analyzed by the unpaired Student's *t* test. Comparison of echocardiography and hemodynamic parameters before and after cell infusion was performed using one-way analysis of variance (ANOVA). Bonferroni post hoc test was used to specify pairwise differences in the case of a significant *F* ratio. All the statistical tests applied in this study were two-tailed, and $P < 0.05$ was considered statistically significant. All statistical analyses were conducted with SPSS 11.0.

RESULTS

Cell identification *in vitro*

Immunohistochemistry study showed that the cultured EPC expressed typical endothelial markers including vWF, Flk-1, and CD31. The cell surface adhesion molecule CD34 was expressed in 26.0% $\pm$ 2.8% of cells as detected by fluorescence-activated cell sorting after 10 days culture. Trypan blue exclusion staining *in vitro* showed that 99.9%

of MNCs or EPCs were viable before infusion. The ratio of fluorescent labeling for MNC and EPC *ex vivo* was both 100%.

Cardiac function

No significant difference was found in baseline parameters of echocardiography and hemodynamics among MNC group, EPC group, and control group. The volume and thickness of left ventricle were well maintained after cell infusion in both MNC group and EPC group ($P > 0.05$). On the contrary, cardiac output and $+dp/dt_{\max}$ decreased, and interventricular septal, lateral, and anterior septal wall thickness decreased significantly at 4 weeks after infusion in control group ($P < 0.05$). Four weeks after infusion, LVEF decreased by 2% (68% $\pm$ 10% vs. 66% $\pm$ 7%, $P > 0.05$) in MNC group, about 0% (69% $\pm$ 7% vs. 69% $\pm$ 8%, $P > 0.05$) in EPC group, and 10% (70% $\pm$ 9% vs. 59% $\pm$ 7%, $P < 0.05$) in control group, respectively. The difference of LVEF change among the groups was significant ($P < 0.05$). The systolic parameters, such as cardiac output, LVESP, and $+dp/dt_{\max}$, increased in MNC group and EPC group, while decreased in control group 4 weeks after cell infusion. However, the diastolic parameters (LVEDP and $-dp/dt_{\max}$) did not change significantly in either group ($P > 0.05$) (Table 1).

Engraftment and differentiation

Some implanted MNCs expressed vWF and the pre-labeled MNCs corresponded largely to vWF positive cells, indicating their participation in angiogenesis (Fig. 1). The number of small vessels in either MNC group or EPC group was significantly higher than that in the control group [13.39 $\pm$ 6.96/high power field (HPF) vs. 3.50 $\pm$ 1.90/HPF, 12.39 $\pm$ 4.72/HPF vs. 3.50 $\pm$ 1.90/HPF, both $P < 0.05$]. But there was not significant difference between MNC group and EPC group ($P > 0.05$). The responsive intensity of immunohistochemical stain for fibronectin was significantly lower in MNC group and EPC group than that in control group (both $P < 0.05$), while that for α -sarcomeric actin was similar among all the three groups (Table 2).

The suspected myoblasts detected by monoclonal anti- α -sarcomeric-actin in EPC group displayed a typical striation pattern in confocal microscopy, but aligned in multiple directions in MNC group. In EPC group, thick and thin myofilaments were seen in some cells in the infarcted area. In spite of the typical hexagonal lattice formed sporadically, the majority of myofilaments showed an uneven distribution and an irregular orientation with disproportional thin filaments, and intercellular fusion was partially formed leading to an intercalated disc structure (Fig. 2). In MNC group, the nuclei of vascular endothelial

cells presented small number of loose heterochromatin without apparent distinction from euchromatin. And the small and dense mitochondria as well as more ribosome and glycogen granules were seen in the cytoplasm (Fig. 3). In control group, myocardial cells were found mostly with an isotropic distribution of myofibril, except for a few sarcomeres which had few myofilaments. The majority of these myocardial cells displayed an intact structure without visible tissue degeneration (Fig. 4).

Intergroup comparison of infarction size

The total myocardial area was similar among the three groups ($P>0.05$). The total infarction size and the infarction percentage in control group were significantly larger than those in MNC group and EPC group (all $P<0.05$). In addition, the total infarcted area in EPC group was much smaller than that in MNC group after cell infusion ($P<0.05$) (Table 3).

Table 1. Comparison of left ventricular echocardiography and hemodynamic parameters before and 4 weeks after cell infusion[§]

Item	Control group (n=7)		MNC group (n=9)		EPC group (n=7)	
	Before infusion	4 weeks after infusion	Before infusion	4 weeks after infusion	Before infusion	4 weeks after infusion
Ejection fraction	0.70±0.09	0.59± 0.07*	0.68± 0.10	0.66± 0.07	0.69± 0.07	0.69± 0.08
End diastolic diameter (cm)	3.04±0.59	3.25± 0.90	2.94± 0.19	3.18± 0.40	2.96± 0.28	3.01± 0.50
End systolic diameter (cm)	1.92±0.66	2.06± 0.80	1.90± 0.16	2.03± 0.44	1.81± 0.24	1.84± 0.68
Interventricular septal thickness (cm)	0.66±0.06	0.56± 0.05*	0.69± 0.08	0.69± 0.06	0.71± 0.04	0.68± 0.04
Lateral wall thickness (cm)	0.62±0.05	0.52± 0.09*	0.66± 0.08	0.67± 0.04	0.71± 0.14	0.70± 0.13
Anterior septal wall thickness (cm)	0.70±0.14	0.51± 0.12*	0.67± 0.06	0.61± 0.11	0.71± 0.12	0.65± 0.12
Cardiac output (L/min)	1.27±0.03	1.10± 0.08*	1.10± 0.10	1.16± 0.12	1.21± 0.08	1.24± 0.06
End diastolic pressure (mm Hg)	7.48±3.04	10.61± 4.89	7.78± 2.21	6.27± 2.21	11.08± 5.18	10.72± 2.88
End systolic pressure (mm Hg)	94.29±9.78	87.06±11.81	81.13±15.58	89.09±15.44	79.18±18.27	89.71±24.25
+dp/dt _{max} (kmm Hg/s)	1.76±0.31	1.41± 0.22*	1.72± 0.53	1.76± 0.49	1.22± 0.65	1.34± 0.52
- dp/dt _{max} (kmm Hg/s)	1.25±0.26	1.10± 0.10	1.38± 0.47	1.37± 0.57	0.92± 0.47	1.01± 0.56

§: Plus-minus values are means±SD.

MNC: mononuclear cell; EPC: endothelial progenitor cell.

* $P<0.05$ compared with the result before cell infusion.

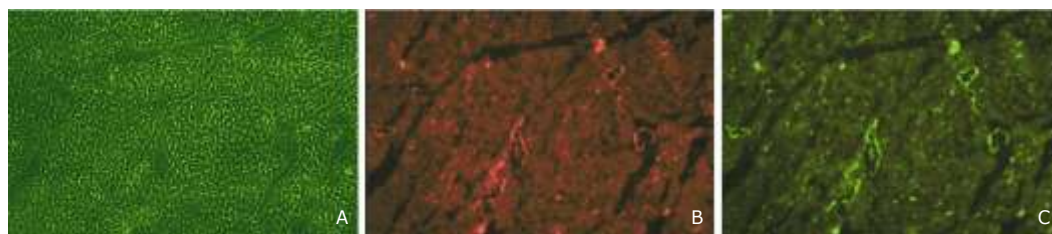


Figure 1. Vessel density of myocardial infarction boundary area in bone marrow MNC group. Masson's trichrome and HE staining $\times 100$
A. Vessel density in optical microscope;
B. MNCs pre-labeled with red fluorescence distribute along the capillary wall;
C. Compared with figure B, most implanted bone marrow MNCs correspond with vWF-positive cells.
vWF: von-Willebrand factor.

Table 2. Small blood vessels and responsive intensity of α -sarcomeric actin or fibronectin in the three groups[§]

Group	n	Small blood vessels (HPF)	α -sarcomeric actin (%)	Fibronectin (%)
Control group	7	3.50±1.90	10.83±1.46	11.95±6.04
MNC group	9	13.39±6.96*	10.86±2.45	8.52±2.96*
EPC group	7	12.39±4.72*	9.92±2.26	8.16±3.32*

§: Plus-minus values are means±SD.

HPF: high power field.

* $P<0.05$ compared with control group.

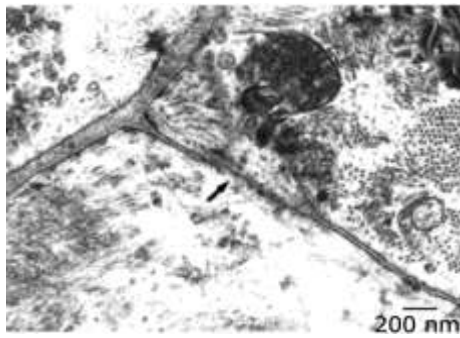


Figure 2. Transmission electromicroscope shows thick and thin myofilaments in some cells in the infarcted area in EPC group. Although typical hexagonal lattice is formed sporadically (shown by an arrow), the majority of myofilaments demonstrate an uneven distribution and irregular orientation with disproportional thin filaments, and intercellular fusion is partially formed leading to an intercalated disc structure.

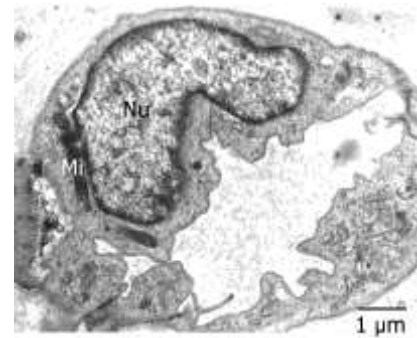


Figure 3. The nucleus of a vascular endothelial cell in MNC group presents small amount of loose heterochromatin without apparent distinction from euchromatin. The small and dense mitochondria as well as more ribosome and glycogen particles are detected in the cytoplasm of the endothelial cell. Nu: nucleus; Mi: mitochondrion.

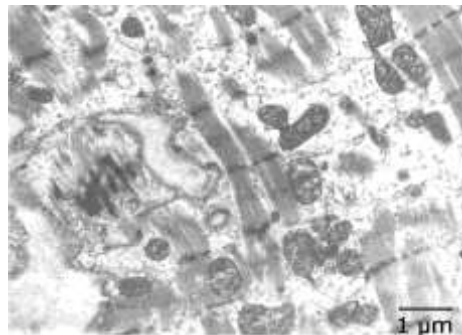


Figure 4. The myocardial cells in the control group mostly show an isotropic distribution of myofibrils, and display an intact structure without visible tissue degeneration except for a small amount of sarcomeres with fewer myofilaments.

Table 3. Comparison of infarction size in the three groups[§]

Group	<i>n</i>	Total cardiac area (cm ²)	Total infarction area (cm ²)	Total infarction percentage (%)
Control group	7	36.56± 4.52	4.14±1.30	11.38±3.24
MNC group	9	46.32±13.54	3.71±1.38*	8.36±3.80*
EPC group	7	39.18± 1.14	1.60±0.26*#	4.09±0.63*

§: Plus-minus values are means±SD.

**P*<0.05 compared with control group; #*P*<0.05 compared with MNC group.

DISCUSSION

The present study compared the effect of intracoronary infusion of MNCs with the infusion of EPCs in mini-swine model of myocardial ischemia-reperfusion. In the functional indexes after cell transplantation, researchers in most of the preclinical studies focused on LVEF or fractional shortening.⁷⁻¹⁰ Recently, Moelker et al¹¹ reported that no significant improvement in global or regional left ventricular function was observed 4 weeks after intracoronary infusion of bone marrow cells, even though a reduction in infarction size was noted. In the present study, both MNC and EPC infusion were found well preserve the global left

ventricular function and the wall thickness of infarcted area. However, the diastolic parameters did not show significant difference in either group, coinciding with the fact that no literature has reported the effect of cell therapy on diastolic function so far. Our findings suggest that both MNC and EPC therapies could increase capillary density and decrease infarction size, thereby reducing the impairment of systolic rather than diastolic function after ischemia-reperfusion injury. Based on the primary results of the present study, EPC infusion appears to decrease infarction size more significantly than MNC infusion, indicating that the former might be more efficient in limiting infarct origin.

As regards the transdifferentiation potential *in vivo* of adult stem cells, as indicated by some preliminary clinical

trials, EPCs or bone marrow multipotential stem cells tend to express myocardiocyte specific markers after intracoronary or intramyocardial transplantation.^{12,13} Although bone marrow stem cells demonstrate a strong proliferation capacity *in vitro*, whether they have potential of transdifferentiation *in vivo*, in the absence of specific marker and in the particular microenvironment of local tissues, remains a laboratory-based presumption.^{14,15} It is still uncertain if stem cells do develop into myocardial cells, mainly due to the inconsistent criteria in distinguishing the regenerated myocardial cells and myocardium-like cells. Presence of sarcomere structure in cytoplasm and nuclei at cell center is a morphological gold standard in identifying mature cardiomyocytes. Therefore, to find out whether MNCs or EPCs developed into myocardial cells or myocardium-like cells, the present study traced the morphological development of these cells by observing fluorescence-labeled areas in suspected tissues with transmission electromicroscope, and then observed the ultrastructure of differentiated cells to find characteristic sarcomeres. The results indicated that EPCs and MNCs infused in the infarcted area might differentiate to either vascular endothelial cells or myocardial cells. The mechanism of stem cell therapy for myocardial injury remains unclear.

REFERENCES

1. Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function *via* side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 2001; 104:1046-52.
2. Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation* 2001; 103:634-7.
3. Schachinger V, Assmus B, Britten MB, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction. Final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol* 2004; 44:1690-9.
4. Tossios P, Krausgrill B, Schmidt M, et al. Role of balloon occlusion for mononuclear bone marrow cell deposition after intracoronary infusion in pigs with reperfused myocardial infarction. *Circulation* 2008; 29:1911-21.
5. Li CJ, Gao RL, Liu YQ, et al. Differentiation and culture of endothelial progenitor cells from mini-swine peripheral blood *in vitro*. *Chin J Pathophysiol* 2005; 21:1230-3.
6. Li CJ, Gao RL, Yang YJ, et al. Implantation of autologous bone marrow mononuclear cells into ischemic myocardium enhances coronary capillaries and systolic function in miniswine. *Chin Med Sci J* 2008; 23:234-8.
7. Silva GV, Litovsky S, Assad J, et al. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation* 2005; 111:150-6.
8. Hirsch A, Nijveldt R, van der Vleuten PA, et al. Intracoronary infusion of autologous mononuclear bone marrow cells in patients with acute myocardial infarction treated with primary PCI: Pilot study of the multicenter HEBE trial. *Catheter Cardiovasc Interv* 2008; 71:273-81.
9. Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOW transfer to enhance ST-elevation infarct regeneration) trial. *Circulation* 2006; 113:1287-94.
10. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002; 106:1913-8.
11. Moelker AD, Baks T, van den Bos EJ, et al. Reduction in infarct size, but no functional improvement after bone marrow cell administration in a porcine model of reperfused myocardial infarction. *Eur Heart J* 2006; 27:3057-64.
12. Dimmeler S, Burchfield J, Zeiher AM. Cell-based therapy of myocardial infarction. *Arterioscler Thromb Vasc Biol* 2008; 28:208-16.
13. Assmus B, Schächinger V, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 2002; 106:3009-17.
14. Katusic ZS. Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. *Arterioscler Thromb Vasc Biol* 2004; 24:2021-7.
15. Rossig L, Urbich C, Dimmeler S. Endothelial progenitor cells at work: not mature yet, but already stress-resistant. *Arterioscler Thromb Vasc Biol* 2004; 24:1977-9.