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Original Article

Efficacy of Atorvastatin combined with adipose-derived mesenchymal stem cell transplantation on cardiac function in rats with acute myocardial infarction

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Mesenchymal stem cells (MSCs) have been extensively applied for the restoration of cardiomyocytes loss after acute myocardial infarction (AMI). However, the optimal therapeutic efficacy of MSCs in ischemic heart diseases has been hampered by their poor survival and low differentiated rates. Therefore, the improvement of MSC survival and differentiated rates is warranted and critical for the efficacy of MSCs in AMI. In this paper, MSCs isolated from rat inguinal fat tissues were termed as adipose-derived mesenchymal stem cells (ASCs), and the fourth passage of ASCs was pre-specified by co-culturing with cardiomyocytes in a transwell system termed as co-ASCs. Fourteen days later, GATA-4 (a transcription factor) and cardiac troponin-I were detected by cellular immunofluorescence. Atorvastatin (Ator group) or vehicle (control group) was administrated for the first 24 h after AMI production in rats. Fourteen days later, inflammatory parameters and cardiac function were evaluated. The other surviving rats were injected with a total of 1×10^6 co-ASCs/100 μl phosphate-buffered saline (PBS), 1×10^6 ASCs/100 μl PBS, or 100 μl PBS. Twenty-eight days after cell injection, survival and differentiated rates of transplanted cells and cardiac function were evaluated. The percentage of GATA-4 expression in co-ASCs was 28.5% ± 5.6% and of cardiac troponin-I was 22.8% ± 3.2%. Compared with the control group, the number of infiltrating inflammatory cells, myeloperoxidase activity, inflammatory cytokines (*VCAM-1*, *TNF-α*, *Hs-CRP*) mRNA expression, and Bax protein expression were significantly reduced in the three Ator groups, accompanied by a significant improvement of Bcl-2 protein expression and cardiac function ($P < 0.05$). Compared with the Ator2 + ASCs group and Con + co-ASCs group, the number of 4-6-diamidino-2-phenylindole-stained cells and cardiac troponin-I-positive transplanted cells, concomitant with cardiac function, were improved most prominently in the Ator3 + co-ASCs group ($P < 0.05$). Pre-amelioration of the cardiac milieu, in conjunction with pre-specification of ASCs, was beneficial for enhancing ASCs' therapeutic efficacy on cardiac function after AMI.

Keywords acute myocardial infarction; mesenchymal stem cell; Atorvastatin

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Introduction

Mesenchymal stem cells (MSCs) have been applied for the restoration of cardiomyocytes loss after acute myocardial infarction (AMI), and their capability for cardiogenesis and cardiac protection have been demonstrated [1–3]. However, the optimal therapeutic efficacy of MSCs on ischemic heart diseases has been hampered by their poor survival and low differentiated rates *in vivo* [4,5]. Therefore, many studies concerned with improving the survival or differentiated rates of MSCs have been conducted [3,6–10]. Previous studies have indicated that an amelioration of the cardiac milieu after AMI is crucial for the viability of transplanted MSCs and that pre-specifying MSCs can facilitate their *trans*-differentiation *in vivo* [10–12].

Therefore, our present study was undertaken to investigate the hypothesis of whether pre-specification of MSCs, in conjunction with pre-amelioration of the cardiac milieu before MSC transplantation, would be beneficial for enhancing the therapeutic efficacy of MSCs on cardiac function after AMI. Adipose-derived mesenchymal stem cells (ASCs) are one kind of MSCs. Because of the easy isolation, minimal invasion, and multiple differentiation potential of ASCs [13,14], our present study utilized ASCs as transplant cell candidates for AMI therapy.

Materials and Methods

ASCs' isolation and culture

ASCs' isolation and culture were performed as previously described [1]. Adipose tissues were isolated from the inguinal region of female Sprague–Dawley (SD) rats (100–120 g; obtained from the Laboratory Animal Center, Sun Yat-sen University, Guangzhou, China), washed with sterile D-Hanks' solution (Gibco, Paisley, UK), minced into small pieces, and digested with 0.1% type I collagenase solution (Sigma, St. Louis, USA) at 37°C for 1 h with agitation. Type I collagenase was inactivated with Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclon, Logan, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), and the infranatant was then centrifuged at 1000 *g* for 10 min. The cells were seeded at a density of 2×10^6 cells/ml in DMEM/F12, supplemented with 10% FBS, in T-75 tissue culture flasks (Corning, New York, USA) in humidified air with 5% CO_2 at 37°C. The medium was changed after the first 24 h. When the cells reached 80% confluence, adherent cells were digested with 0.125% trypsin and 0.01% EDTA and then replated at a 1:3 dilution. The fourth-passage ASCs were used in our study.

ASC immunophenotypes and differentiation

A total of 1×10^6 fourth-passage ASCs were washed three times with phosphate-buffered saline (PBS), suspended in 100 μl PBS containing 0.5% bovine serum albumin (BSA), and incubated with fluorescence-conjugated antibodies for 30 min on ice, while being shielded from light. The antibodies used were anti-rat CD29-fluorescein isothiocyanate (FITC), CD45-FITC, CD31-phycoerythrin (PE), and CD44-PE with a concentration of 0.5 mg/ml. After being washed three times with PBS, ASCs were suspended in PBS and analyzed by Elite flow cytometry using WinMDI2.9 software (Beckman Coulter, Fullerton, USA). Antibodies were obtained from BD Biosciences Pharmingen (Franklin Lakes, USA).

For adipogenic and osteogenic differentiation, the ASCs were plated at a density of 6×10^4 and 2×10^4 cells/cm^2, respectively. The conditioned media used were as follows: for adipogenesis, DMEM/F12 supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM isobutyl-methylxanthine, 10 μM insulin, and 100 μM indomethacin for 14 days; and for osteogenesis, DMEM/F12 supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 50 μM ascorbate-2-phosphate for 28 days. Adipogenesis and osteogenesis were evaluated using oil red O and alizarin red S staining, respectively. All chemical reagents were obtained from Sigma.

Cardiomyocytes isolation and ASCs pre-specification

Cardiomyocytes were isolated from neonatal SD rat hearts (3 days old). The ventricular myocardium was minced, digested with 0.05% type I collagenase for 3 h at 37°C with agitation, inactivated with DMEM/F12 supplemented with 10% FBS, and then centrifuged at 1000 *g* for 8 min. According to the difference of adhesive time consumption between cardiomyocytes and fibrocytes, cardiomyocytes were enriched using a differential adhesion technique. Bromodeoxyuridine (0.1 mM; Sigma) was added to the medium to inhibit proliferation of non-cardiomyocytes, as previously reported [15], and the produced cultures with 90%–95% of cardiomyocytes were assessed by microscopic observation.

Cardiomyocytes were co-cultured with ASCs at a ratio of 5:1 in transwell plates (pore size 0.4 μm; Corning) for 14 days. Cardiomyocytes were placed in the upper chamber and ASCs in the lower chamber of transwell plates. ASCs co-cultured with ASCs served as a control. The medium was changed every 48 h. The expression levels of cardiac troponin-I and transcription factor GATA-4 of co-cultured ASCs (co-ASCs) were detected by cellular immunofluorescence.

Cellular immunofluorescence was performed with rabbit anti-rat troponin-I (1:100) and mouse anti-rat GATA-4 primary antibodies (1:100) (Santa Cruz Biotechnology, Santa Cruz, USA). The secondary antibodies were FITC-conjugated goat anti-rabbit (1:300) and Cy3-conjugated goat anti-mouse IgG (1:300; Sigma). The cell nuclei were stained with 4-6-diamidino-2-phenylindole (DAPI; Sigma). The coverslips were examined using an Olympus BX51 microscope (Tokyo, Japan). Ten fields of each slide were selected to evaluate the percentage of ASCs expressing cardiac troponin-I and GATA-4. FITC- or Cy3-positive stained ASCs were counted, and the amount of cardiac-committed cells was expressed as a percentage relative to DAPI-positive ASCs.

AMI production

AMI production was performed as previously described [16]. Seventy male SD rats weighing 200–220 g were obtained from the Laboratory Animal Center, Sun Yat-sen University. All experimental procedures conformed to the Guidelines of Animal Experiments of the Chinese Committee of Medical Ethics. Permanent ligation of the left anterior descending coronary artery was performed under chemical ventilation (tidal volume, 6.0 ml; respiratory rate, 70 cycles/min). A similar procedure was performed in another 10 sham-operated rats without coronary artery ligation.

Pre-amelioration of the cardiac milieu

During the first 24 h after AMI, the surviving 63 operative rats were randomly assigned to three Atorvastatin (Ator) groups (Ator1, Ator2, and Ator3; $n = 15$ each group), in which rats were gavaged with 10 mg/kg/day of Atorvastatin (Lipitor; Pfizer, New York, USA), and a control group

Table 1 Real-time PCR primers and reaction conditions

Primer	Sequence	Number of cycles	Annealing temperature (°C)	Production size (bp)
β-Actin	Sense: 5′-GCATGGGTCAGAAGGATTCCT-3′ anti-sense: 5′-TCGTCCCAGTTGGTGACGAT-3′	40	55	106
VCAM-1	Sense: 5′-AAGGGAGGAAATTGGCACAA-3′ anti-sense: 5′-GGGATGAAGGTCATTTCCAAAC-3′	38	55	124
TNF-α	Sense: 5′-ACAAGGCTGCCCCGACTAC-3′ anti-sense: 5′-TCCTGGTATGAAATGGCAAACC-3′	38	55	120
Hs-CRP	Sense: 5′-TCTGTGCCACCTGGGAGTCT-3′ anti-sense: 5′-TGTCCCCACAATGTAGCCCT-3′	38	55	126

(Con, $n = 18$), in which rats were gavaged with an equal volume of vehicle for total of 14 days. Ten sham-operated rats were gavaged with an equal volume of vehicle agent. All the rats were maintained on standard rat chow.

Evaluation of cardiac milieu parameters

After evaluation of cardiac function, five rats from each group were humanely killed at 14 days of Ator or vehicle administration. Tissues were harvested from the left ventricle peri-infarct areas for the following parameters evaluation. First, the number of infiltrated inflammatory cells was counted in 10 randomly selected areas with hematoxylin and eosin (HE) staining. Second, myeloperoxidase activity was analyzed with a commercial myeloperoxidase kit (A044, NJBI, Nanjing, China) according to the manufacturer's instructions. One unit of myeloperoxidase was defined as the quantity of an enzyme hydrolyzing 1 mmol of peroxide per min at 25°C. Third, *VCAM-1*, *TNF-α*, and *Hs-CRP* mRNA expression levels were evaluated by real-time-PCR, and normalized to those of *β-actin*. The real-time-PCR primers and reactive conditions are shown in **Table 1**. Fourth, Bcl-2 and Bax protein expression levels were detected by western blotting analysis. Protein was extracted using sodium dodecyl sulfate (SDS) sample buffer loaded in a 12% SDS polyacrylamide gel and transferred to a 4.5-μm polyvinylidene fluoride membrane. After blocking with 5% BSA blocking solution for 1 h at room temperature, the membranes were incubated with rabbit anti-rat Bcl-2 primary antibody (1:200), mouse anti-rat Bax primary antibody (1:300), and mouse anti-rat β-actin primary antibody (1:300) at 4°C overnight. After washing three times in tris buffered saline with tween (TBST), the membranes were incubated with goat anti-rabbit (1:5000) and goat anti-mouse (1:6000) secondary antibodies for 1 h at room temperature. Specific bands of target proteins were visualized using a chemiluminescence reagent (Millipore, Billerica, USA). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology.

Cell labeling and transplantation

Cells were labeled with DAPI at a final concentration of 50 μg/ml for 30 min. Fourteen days after Ator or vehicle administration, a total of 1×10^6 co-ASCs/100 μl PBS, 1×10^6 ASCs/100 μl PBS, or 100 μl PBS was injected at five different sites in peri-infarct areas. Sham-operated rats were injected with 100 μl PBS. This protocol created five groups: Ator1 group with PBS (Ator1 + PBS; $n = 8$), Ator2 group with ASCs (Ator2 + ASCs; $n = 8$), Ator3 group with co-ASCs (Ator3 + co-ASCs; $n = 7$), control group with co-ASCs (Con + co-ASCs; $n = 7$), and sham-operated group given PBS (Sham + PBS; $n = 5$).

Evaluation of survival and differentiated rates of transplanted cells

Rats were sacrificed 28 days after cell transplantation. The left ventricles were transversely sectioned and placed in an embedding medium (Tissue-Tek, Sakura, Japan). All specimens were cut into 10 μm and allowed to air dry for 2 h at room temperature. The sections were fixed in 4% paraformaldehyde for 30 min, rinsed three times in PBS, and blocked with 5% normal goat serum for 30 min. The goat serum was gently tapped off, and the sections were then incubated with 1:200 rabbit anti-rat troponin-I primary antibody (Santa Cruz Biotechnology) at 4°C overnight. They were rinsed three times with PBS and incubated with 1:300 FITC-conjugated goat anti-rabbit secondary antibody (Sigma) for 1 h at room temperature, while being shielded from light. The slides were observed under an Olympus BX51 microscope (Tokyo, Japan) to determine the survival and differentiated rates of transplanted cells.

Analysis of cardiac function

Cardiac function was evaluated by echocardiography at 1 day after AMI, 14 days after Ator or vehicle administration, and 28 days after cell transplantation. The left ventricular ejection fraction (LVEF), fractional shortening (FS), left ventricular end-systolic and end-diastolic volumes (LVVs and LVVd, respectively), and heart rate (HR) were measured using an

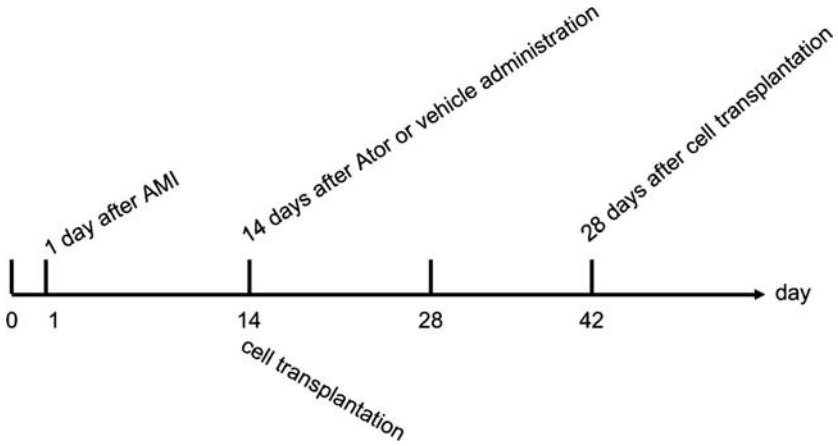

Figure 1 Schematic of the present study One day after AMI, Ator or vehicle was administrated for 14 days. At the 14 days after administration, cells were transplanted. Twenty-eight days after cell transplantation (at the time point of 42 days), transplanted cell survival and differentiated rates, and cardiac function were evaluated.

echocardiography system (ATL-HDI500; Philips Medical Systems, Bothell, USA) equipped with a 10-MHz image transducer. Cardiac output (CO) was calculated with the following formulation: CO = LVVd − LVVs. All procedures were performed by an experienced investigator.

Statistical analysis

All values were expressed as the mean ± SD, and analysis was performed using SPSS software (version 15.0; SPSS Science, Chicago, USA). Statistical significance among groups was evaluated using ANOVA *post hoc* test, and $P < 0.05$ was considered to be statistically significant.

Results

Experimental animal information and procedure

A total of seven rats, including two rats in the Ator1 group, two rats in the Ator2 group and three rats in the Ator3 group, died in the three Ator groups, and eight rats in the control group died during Ator or vehicle administration. After cell transplantation, three rats in the Ator1 + PBS group, one rat in the Ator2 + ASCs group, and two rats in the Con + co-ASCs group died; but no rats died in the Ator3 + co-ASCs or Sham + PBS group. **Fig. 1** is a schematic description of the present study.

ASCs morphology, immunophenotypes and multiple differentiation

As shown in **Fig. 2**, most of the fourth-passage ASCs were positive for CD29 and CD44, which are specific markers of MSCs. In contrast, a minority of ASCs was positive for CD31 and CD45, which are indicated as endothelial and hematopoietic cell phenotypes, respectively. The ASCs showed a fibroblast-like morphology under a light microscope, and the ASCs were capable of multiple differentiation, as judged by their ability to differentiate into adipocytes, osteoblasts and cardiomyocytes. The percentage of GATA-4-positive co-ASCs was 28.5% ± 5.6% and of cardiac troponin-I-positive co-ASCs was 22.8% ± 3.2%. No cardiac protein was detected in the ASCs co-cultured with the ASCs group.

Cardiac milieu parameters evaluation

As shown in **Fig. 3**, at 14 days of Ator or vehicle administration, compared with the control group, there were fewer inflammatory cells in peri-infarct areas and lower myeloperoxidase activity, which quantitatively reflected that the neutrophil volume was significantly reduced in the three Ator groups ($P < 0.05$). For further evaluation of inflammatory reactions, the expression levels *VCAM-1*, *TNF-α*, and *Hs-CRP* mRNA were detected using real-time PCR, which showed that, compared with the control group, the mRNA expression was significantly downregulated in these three Ator groups ($P < 0.05$), suggesting that 14 days of Ator administration could effectively ameliorate the microenvironment. In order to evaluate anti-apoptotic and pro-apoptotic cytokines' expression, Bcl-2 and Bax were detected by western blotting analysis, respectively. The results revealed that, compared with the sham group, the anti-apoptotic protein Bcl-2 was downregulated, while the pro-apoptotic protein Bax was upregulated in the three Ator groups and control group ($P < 0.05$). However, compared with the control group, the downregulation of Bcl-2, concomitant with upregulation of Bax, was low in the three Ator groups ($P < 0.05$). Collectively, the aforementioned parameters indicated that, compared with vehicle treatment, 14 days of Ator administration could ameliorate the cardiac milieu after AMI.

Comparison of transplanted cell survival and differentiated rates

Twenty-eight days after cell transplantation, cardiac tissue immunofluorescence was conducted. **Fig. 4** showed that

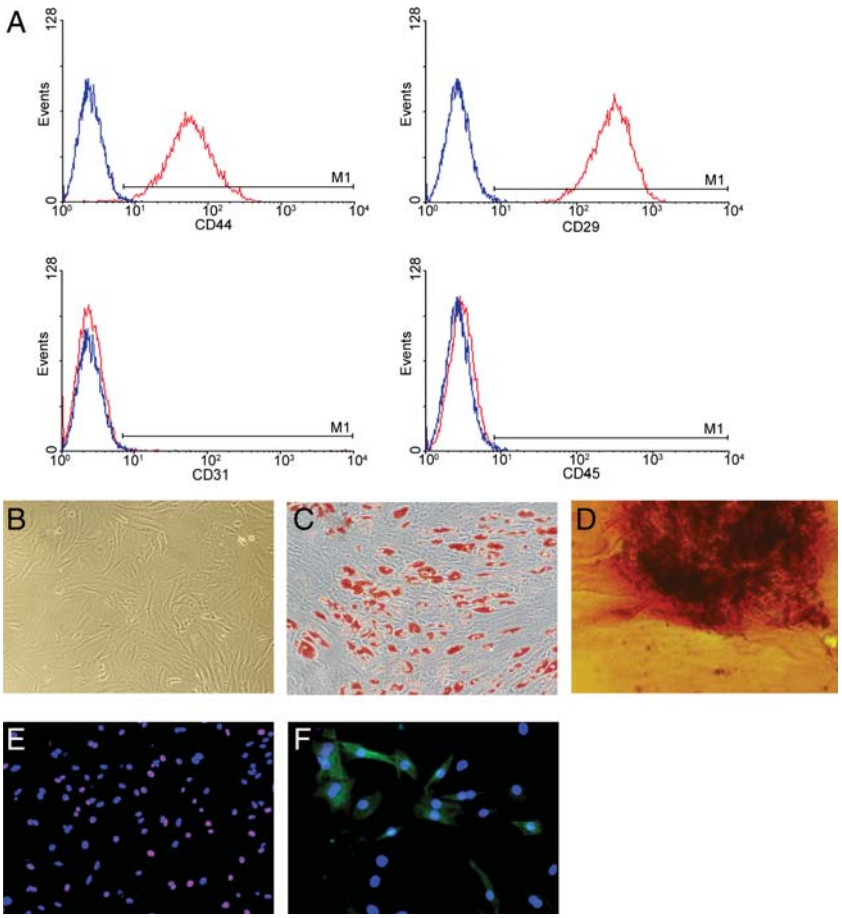

Figure 2 ASC immunophenotypes and multiple differentiation (A) The immunophenotypes of fourth-passage ASCs analyzed by flow cytometry. The red lines represent the specific fluorescently labeled antibodies, and the blue lines represent isotype controls. CD44 and CD29 were expressed by 98.5% ± 0.5% and 99.0 % ± 0.5% of ASCs, respectively, while 0.2% ± 0.1% and 0.3% ± 0.1% of ASCs expressed CD31 and CD45, respectively. (B) The morphology of fourth-passage ASCs. (C,D) ASCs of adipogenic and osteogenic differentiation as stained with oil red O and alizarin red, respectively. (E,F) The percentage of nuclei co-stained with blue (DAPI) and red (GATA-4), which was 28.5% ± 5.6% in co-ASCs, and the percentage of cytoplasma stained with green, which indicated cardiac troponin-I-positive co-ASCs and was 22.8% ± 3.2%. (B, C, E, F) Magnification, ×100; (D) magnification, ×200.

the number of DAPI-labeled transplanted cells was higher in both Ator groups than those in the control group ($P < 0.05$), which indicated the pro-survival effect of Ator administration. The percentage of cardiac troponin-I-positive transplanted cells was higher in the Ator3 + co-ASCs group (35% ± 3%) than that in the Ator2 + ASCs or Con + co-ASCs groups ($P < 0.05$). Although the percentage of cardiac troponin-I-positive transplanted cells in the Ator2 + ASCs group (24% ± 3%) was higher than that in the Con + co-ASCs group (19% ± 4%), there was no significant difference in the variation ($P = 0.125$).

Analysis of cardiac function parameters

The function parameters are listed in **Table 2**. One day after AMI production, LVEF, FS, and CO decreased, while HR increased in the three Ator groups and control group, indicating acute cardiac function impairment, and there was no difference in parameters among the AMI groups. However, 14 days after Ator administration, LVEF and CO were enhanced, concomitantly with LVVs and HR reduction, in each of the three Ator groups compared with those measured in the same groups one day after AMI ($P < 0.05$). However, there was no improvement in the control group compared with 1 day after AMI, and cardiac function in the control group was inferior to that of the three Ator groups at 14 days after Ator or vehicle administration ($P < 0.05$). Twenty-eight days after cell injection, compared with 14 days after Ator or vehicle administration, the improvement of LVEF and CO was more pronounced in the Ator3 + co-ASCs group than that in the Ator2 + ASCs and Con + co-ASCs groups, concomitant with significant LVVs and HR reduction ($P < 0.05$). Although LVEF and CO were higher than at 14 days after vehicle administration in the Con + co-ASCs group, the difference was not statistically significant ($P = 0.326$). Nevertheless, cardiac function deteriorated as CO decreased, accompanied by LVVs and HR increasing in the Ator + PBS group, compared with those measured at 14 days after Ator administration ($P < 0.05$).

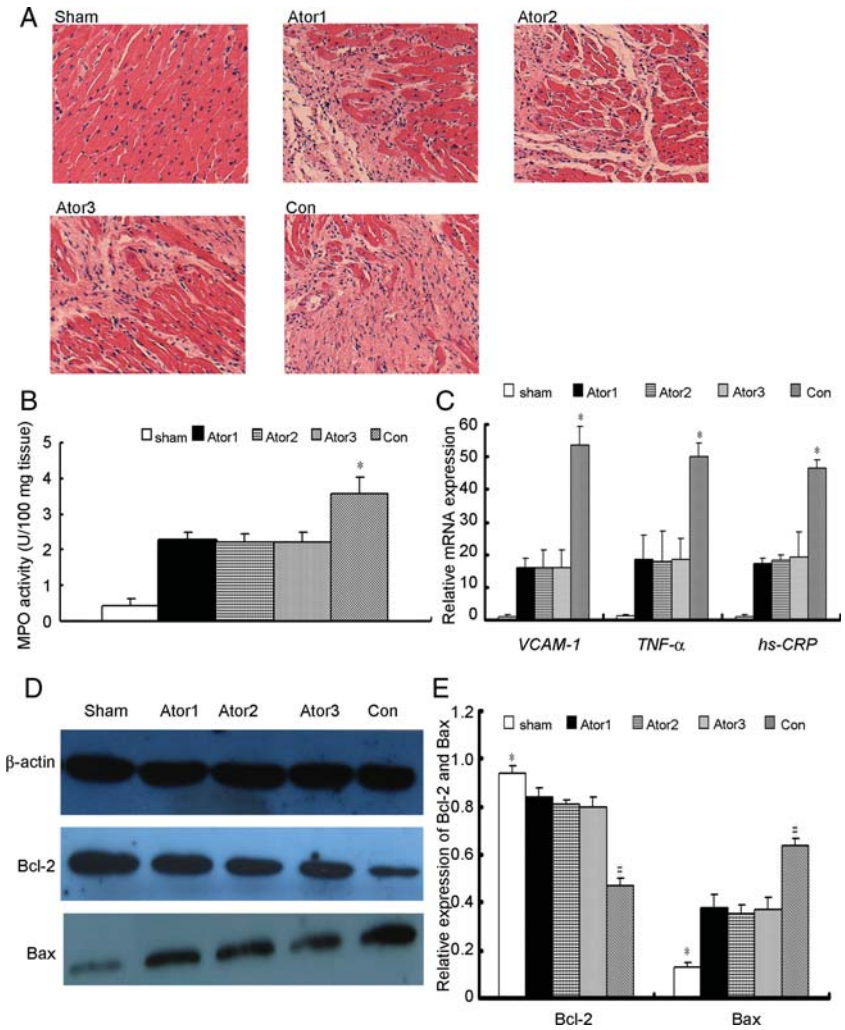

Figure 3 Evaluation of cardiac microenvironment parameters (A) A large number of inflammatory cells infiltrating in peri-infarct areas, and the number of inflammatory cells was less in the three Ator groups than in the control group. Magnification, ×100. (B) MPO (myeloperoxidase) activity comparison was used to evaluate quantitatively the neutrophil infiltrating volume among these groups, and the results indicated that MPO activity in the control group was significantly higher than in the three Ator groups (*$P < 0.05$). (C) Real-time PCR showed that *VCAM-1*, *TNF-α*, and *Hs-CRP* mRNA expression levels were higher in the AMI production groups than in the Sham group, whereas the mRNA expression of inflammatory cytokines in the three Ator groups was significantly reduced after 14 days of Ator administration compared with the control group (*$P < 0.05$). (D,E) The expression levels of both the anti-apoptotic cytokine Bcl-2 and the pro-apoptotic cytokine Bax were changed after AMI. Both downregulation of Bcl-2 and upregulation of Bax were significantly less in the three Ator groups than in the control group (*$P < 0.05$).

Discussion

Our present study indicated the following: (i) pre-treatment with Ator could enhance transplanted cell survival rates via amelioration of the cardiac inflammatory reaction and regulation of anti-apoptotic and pro-apoptotic proteins expression after AMI; (ii) cardiac troponin-I-positive rate of transplanted cells *in vivo* could be enhanced by pre-specification via co-culturing with cardiomyocytes *in vitro*; and (iii) pre-specification of transplanted cells, in conjunction with pre-amelioration of the cardiac milieu after AMI, had better outcomes for cardiac function improvement after AMI.

Previously, many studies indicated that only a minority of transplanted cells could be retained in injured hearts after AMI, and these mechanisms were ascribed to the hostile cardiac milieu, which involved intensive inflammatory reactions, an oxidative stress burden, and an imbalance of pro- and anti-apoptotic cytokine expressions [17,18]. Therefore, to provide support for the ability of transplanted cells to withstand the hostile microenvironment is an essential prerequisite for cardiac function improvement with MSCs transplantation. Yang *et al*. [11,19] showed that Ator and simvastatin could improve MSCs' cardiac protection efficacy through amelioration of the cardiac milieu in an ischemic-reperfusion injury model. Ator and simvastatin are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, with pleiotropic effects including anti-inflammatory activities, anti-oxidant properties, regulation of pro-and anti-apoptotic cytokine expression, protection of the endothelium, and regulation of endothelial progenitor cell migration and

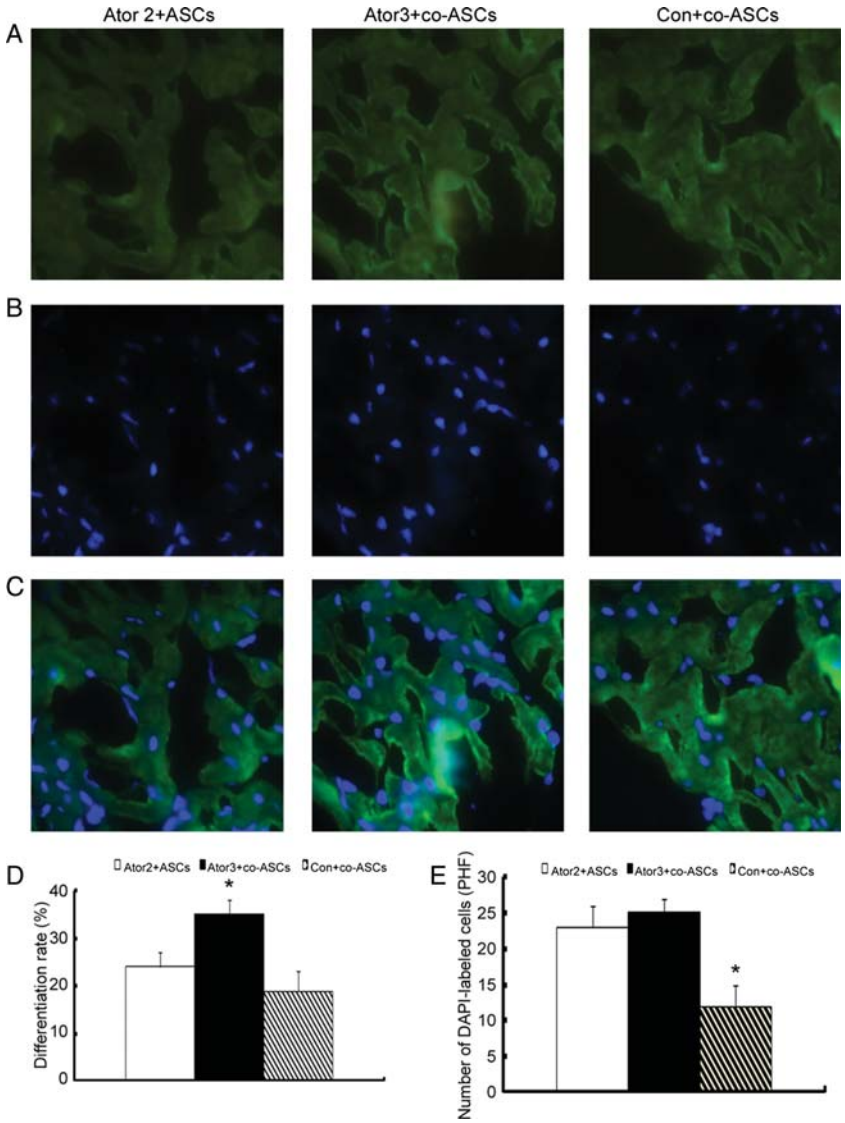

Figure 4 Comparison of transplanted cell survival and differentiated rates (A,E) The numbers of DAPI-labeled ASCs in both Ator pre-amelioration groups (Ator2 + ASCs, 23 ± 3/PHF; Ator3 + co-ASCs, 25 ± 2/PHF). They were higher than those in the Con + co-ASCs group (12 ± 3/PHF) (*$P <$ 0.05). (B) FITC-conjugated fluorescence images of cardiac troponin-I expression. (C) The picture is merged from (A) and (B). Co-localization of DAPI and FITC can be observed in some transplanted cells. (D) The percentage of transplanted cells' co-expression of DAPI and FITC was 35% ± 3% in the Ator3 + co-ASCs group, 24% ± 3% in the Ator2 + ASCs group, and 19% ± 4% in the Con + co-ASCs group. PHF, per high field. Magnification, ×100.

differentiation [20,21]. Our present results revealed that, compared with vehicle administration, 14 days of 10 mg/kg/day Ator administration could not only decrease inflammatory cells infiltration, myeloperoxidase activity, the expression of inflammatory cytokine mRNA, and regulation of anti- and pro-apoptotic cytokines expression, but also improve cardiac function, as reflected by LVEF and CO elevation, and LVVs and HR reduction in the three Ator groups. Compared with the studies by Yang *et al*. [11,19], there were some discrepancies needed to be addressed. First, the experimental model used by Yang was created by an ischemia–reperfusion procedure, while ours was permanent ligation of coronary artery. Second, cells were transplanted immediately after ischemia–reperfusion procedure in Yang's study, while in our study cell transplantation was 14 days after AMI. Finally, the MSCs used in Yang's study were not pre-specification, while the ASCs in our study were pre-specification. Accordingly, we speculated that cardiac function improvement might be attributed to cardiac milieu amelioration, which had also been found in previous studies [22,23].

Twenty-eight days after cell transplantation, the survival rates of transplanted cells were significantly higher in both Ator pre-treatment groups than in control group, as indicated by the number of DAPI-labeled cells in the cardiac tissue. Due to differences in animal models, prescribed drugs, and the duration of observational time, our present study cannot compare with any other previous study regarding the improvement of transplanted cell survival rates. And since DAPI has a relative short half-life time

Table 2 Comparison of cardiac function parameters

Group	Sham	Ator1	Ator2	Ator3	Control
One day after AMI					
LVEF (%)	68.25 ± 1.97	44.63 ± 3.22	45.05 ± 2.40	45.24 ± 2.73	46.02 ± 2.62
FS (%)	36.11 ± 2.66	25.58 ± 2.29	26.35 ± 2.89	26.95 ± 2.73	25.27 ± 2.99
LVVs (ml)	1.81 ± 0.15	3.53 ± 0.24	3.58 ± 0.26	3.56 ± 0.24	3.45 ± 0. 26
LVVd (ml)	5.81 ± 0.12	6.52 ± 0.27	6.51 ± 0.22	6.51 ± 0.24	6.43 ± 0.29
CO (ml)	4.00 ± 0.09	2.99 ± 0.13	2.93 ± 0.12	2.95 ± 0.13	2.98 ± 0.14
HR (bpm)	433 ± 52	533 ± 47	537 ± 55	532 ± 45	533 ± 50
Fourteen days after Ator or vehicle administration					
LVEF (%)	69.53 ± 1.84	50.15 ± 3.09§	50.70 ± 3.29§	50.64 ± 3.46§	44.65 ± 2.95$^{\#}$
FS (%)	36.22 ± 3.20	26.03 ± 3.08	27.06 ± 3.25	27.85 ± 3.34	24.01 ± 3.36$^{\#}$
LVVs (ml)	1.76 ± 0.11	3.04 ± 0.17§	3.07 ± 0.16§	3.09 ± 0.15§	3.48 ± 0.23$^{\#}$
LVVd (ml)	5.78 ± 0.17	6.27 ± 0.21	6.23 ± 0.20	6.26 ± 0.16	6.22 ± 0.27
CO (ml)	4.02 ± 0.10	3.23 ± 0.12§	3.16 ± 0.10§	3.17 ± 0.13§	2.74 ± 0.10$^{\#}$
HR (bpm)	430 ± 44	512 ± 40§	505 ± 48§	508 ± 45§	548 ± 50$^{\#}$
Twenty-eight days after cell transplantation					
Group	Sham + PBS	Ator1 + PBS	Ator2 + ASCs	Ator3 + co-ASCs	Con + co-ASCs
LVEF (%)	68.62 ± 1.33	48.53 ± 2.53	54.02 ± 3.13*	56.78 ± 3.66*$^{\&}$	46.17 ± 2.03
FS (%)	37.08 ± 1.01	24.64 ± 1.67	28.58 ± 1.97	29.02 ± 2.20	24.66 ± 1.36
LVVs (ml)	1.80 ± 0.12	3.27 ± 0.22*	2.90 ± 0.23	2.73 ± 0.22*	3.30 ± 0.26
LVVd (ml)	5.79 ± 0.11	6.29 ± 0.22	6.22 ± 0.21	6.28 ± 0.13	6.17 ± 0.28
CO (ml)	3.99 ± 0.12	3.02 ± 0.12*	3.32 ± 0.11	3.55 ± 0.10*$^{\&}$	2.87 ± 0.12
HR (bpm)	445 ± 49	530 ± 37*	493 ± 46*	471 ± 40*$^{\&}$	538 ± 47

bpm, beat per minute.

$^{\S}P < 0.05$ compared with the same group at 1 day after AMI; $^{\#}P < 0.05$ compared with other groups at 14 days after Ator or vehicle administration; $^{*}P < 0.05$ compared with the same group at 14 days after Ator or vehicle administration; $^{\&}P < 0.05$ compared with other groups, except the Sham + PBS group, at 28 days after cell transplantation.

comparing with other innovative tracking methods considered somewhat to affect the comparison of the *in vivo* tracking. However, in line with Ator's being widely prescribed in clinics [24,25], we considered that it seemed more feasible and beneficial to conduct a clinical pilot study of the usage of Ator to pre-ameliorate the cardiac milieu before MSCs' implantation. Collectively, the improvement of transplanted cell survival rate by pre-amelioration of the cardiac milieu with Ator was feasible.

The *trans*-differentiation into cardiomyocytes of transplanted cells *in vivo* is another key point concerning MSCs' therapeutic efficacy on cardiac function, and the precise mechanisms affecting MSCs' *trans*-differentiation *in vivo* are complicated and still unclear. Therefore, to our knowledge, it might be achievable and feasible to pre-specify transplanted MSCs *in vitro* by using cardiomyocytes-oriented tactics, rather than direct injection of naive MSCs into a complicated cardiac milieu that might not be suitable for cardiomyocytes-oriented differentiation. Recent report of Behfar *et al*. [10] found that compared to injection of naïve-state MSCs, cardiopoietic MSCs with a pre-specified cocktail medium achieved superior functional and structural benefits in chronic myocardial infarction patients. Therefore, it seems to be more rational to pre-specify MSCs before implantation. MSCs can be induced into cardiomyocytes by culturing them with cocktails of a conditioned medium, gene engineering, and direct or indirect co-culturing with cardiomyocytes. Cardiomyocytes can secrete cytokines, hormones, or soluble factors that can regulate the signal pathway to induce cardiomyocytes-oriented differentiation of MSCs, and it might more closely resemble *in vivo* cardiac circumstances by co-culturing ASCs with cardiomyocytes, although the clear mechanism is still unclear due to the complicated *in vivo* nature. Our present study showed that, after 14 days of co-culturing, a minority of ASCs expressed GATA-4 and cardiac troponin-I. Twenty-eight days after cell injection, the cardiac troponin-I-positive rate of transplanted cells *in vivo* increased, as indicated that the percentage of cardiac troponin-I-positive cells in the Ator3 + co-ASCs group was 35% ± 3%. Comparing with the Ator3 + co-ASCs group, the cardiac troponin-I-positive rates in the Ator2 + ASCs group and Con + co-ASCs group were lower, and we speculated that the discrepancy was associated with either the lack of pre-specification or the lack of pre-amelioration. Since the *in vivo* circumstance

was complicated and there was also possible that transplanted cells could infuse with host cardiomyocytes. However, in our present study, the higher rate of cardiac troponin-I-positive transplanted cells in Ator3 + co-ASCs group was indicative of the favorable results with pre-specification. Collectively, the improvement of the differentiated rate of ASCs *in vivo* was feasible by pre-specification of ASCs through co-culturing with cardiomyocytes.

Finally, the most important primary point of our study was to evaluate the efficacy of cardiac function improvement with ASCs transplantation after pre-amelioration of the cardiac milieu and pre-specification of transplanted cells. Cardiac function parameters revealed that, 14 days after Ator administration, LVEF, LVVs, and CO, which indicated cardiac systolic function, were significantly enhanced compared with those measured at 1 day after AMI; however, these same parameters in the control group were deteriorative and inferior to those in the three Ator groups at the same time point. Previous studies have demonstrated the efficacy of statins on cardiac function protection after AMI via multiple mechanisms including amelioration of oxidative stress, downregulation of inflammatory reactions, regulation of pro- and anti-apoptotic protein expressions, protection of endothelial function, and increasing of blood perfusion [21,25,26]. Consistent with previous studies, the three Ator groups had better outcomes for cardiac function after 14 days of treatment, which we considered to be a result of cardiac milieu amelioration. Twenty-eight days after cell injection, improvement of LVEF and CO was most pronounced in the Ator3 + co-ASC group, concomitant with the highest survival and differentiated rates of ASCs *in vivo*. LVEF and CO were also enhanced in the Ator2 + ASC group, whereas the increment in the Ator2 + ASC group was less robust than that in the Ator3 + co-ASC group. This disparity might be attributed to the higher differentiated rate of ASCs in the Ator3 + co-ASC group, which we considered to be associated with the *in vitro* pre-specification of ASCs. Although LVEF and CO were higher at 28 days after cell injection, compared with 14 days of vehicle administration in the Con + co-ASCs group, the variation was not statistically significant ($P = 0.326$). We speculated that this result might be due to the insufficiency of the transplanted cells' viability and differentiation in the Con + co-ASCs group. However, it could also be associated with the insufficiency of the observational time. Conversely, compared with 14 days after Ator administration, CO decreased, accompanied by LVVs and HR being elevated, in the Ator1 + PBS group, which might be due to the loss of cardiomyocytes and the inability of the cardiomyocytes to regenerate. And according to Ator's pleiotropic effects, we speculated that discontinuation of Ator treatment in Ator1 + PBS group might also obviate the benefits, although our present study was lack of direct comparison of Ator1 + PBS with Con + PBS.

Collectively, our present study indicated that the protocol of pre-amelioration of the cardiac milieu, in conjunction with pre-specification of transplanted cells, was beneficial for enhancing ASCs' therapeutic efficacy on cardiac function after AMI.

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