

Atorvastatin treatment improves survival and effects of implanted mesenchymal stem cells in post-infarct swine hearts

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Aims

To investigate whether Atorvastatin (Ator) treatment improves the cardiac micro-environment that facilitates survival and differentiation of bone-marrow-derived mesenchymal stem cells (MSCs) implanted in the post-infarct myocardium.

Methods and results

Myocardial infarction was created by coronary ligation and immediately after reperfusion, autologous bone-marrow-derived MSCs were transplanted into the hearts of Chinese swine that were pretreated with or without Ator. Six weeks after transplantation, as evaluated by SPECT and MRI all the animals with Ator showed improved cardiac perfusion and contractility when compared with untreated. Increased survival and differentiation of implanted MSCs and decreased infarct area were observed in the Ator-treated, MSC-implanted animals. In the absence of Ator, MSC transplantation only achieved a modest improvement in perfusion and morphology. The combined treatment with Ator and MSCs significantly inhibited cardiac cell apoptosis, reduced oxidative stress, and suppressed expression of the inflammatory cytokines in the post-infarct myocardium.

Conclusion

Ator treatment may protect the myocardium undergoing acute infarction and reperfusion by creating a better environment for the survival and differentiation of implanted MSCs. The benefit of the Ator/stem cell combined therapy may result from the statin-mediated inhibition of apoptosis, oxidative stress, and inflammation in the infarcted myocardium.

Keywords

Mesenchymal stem cells • Acute myocardial infarction • Transplantation • Atorvastatin

Introduction

Recently, many experimental animal studies and clinical trials have shown the potential of stem cells for regenerating and repairing the myocardium and blood vessels with ischaemic injury, and for improving post-infarct perfusion and function of the damaged cardiac tissue.^{1–4} Several types of stem cells have been used for therapeutic cardiac regeneration, including the bone-marrow-

derived mesenchymal stem cells (MSCs), which have attracted a great attention from investigators because of their plasticity and availability.^{5,6} A major challenge to the success of MSC therapy is the low rates of survival and differentiation of implanted MSCs in the damaged tissue. It has been shown that majority of the donor cells may not survive the harsh micro-environment if the cells are implanted too early into the tissue after acute myocardial infarction (AMI).⁷ Therefore, for a successful stem cell therapy, it is

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important to protect implanted stem cells *in vivo* against the harmful environment generated by AMI.

Statins, an HMG-CoA reductase inhibitor, are one of the most prescribed drugs, with multiple biological activities independent of cholesterol-lowering action. Investigations have revealed statin's pleiotropic effects, such as the protection of endothelial function, increased nitric oxide bioavailability, antioxidant effects, anti-inflammatory reaction, and stabilization of atherosclerotic plaques.^{8–12} In this study, we hypothesized that pretreatment with Atorvastatin (Ator) improves the cardiac micro-environments created by AMI and reperfusion, thus facilitating the survival and differentiation of implanted MSCs *in vivo*.

Methods

Animals

Chinese mini-pigs (30 ± 5 kg) at about 10 months old were obtained from the Laboratorial Animal Center of Chinese University of Agriculture and housed in the animal facilities of Fu Wai Hospital. In compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA, all the experimental procedures were approved by the Care of Experimental Animals Committee of Chinese Academy of Medical Sciences and Peking Union Medical College, China. Twenty-eight Chinese mini-pigs were randomly allocated to treatment with or without Ator, and then to coronary ligation followed by implantation with or without MSCs. Each of the following groups contained seven pigs: Ator+no-MSCs (Ator only), Ator+MSCs, no-Ator+no-MSCs (control), and no-Ator+MSCs (MSCs only).

Outcome analysis

The primary outcome of this study to be determined was the left ventricular function, including the left ventricular ejection fraction (LVEF) detected by magnetic resonance imaging (MRI) and perfusion defect area by single-photon emission computed tomography (SPECT). The baseline data were collected at 1 week after transplantation and the endpoint data at 6 weeks after transplantation. The secondary outcome was the histological changes, including survival and differentiation potential of implanted MSCs *in vivo*, capillary density, apoptosis, oxidative stress, and inflammatory factor expression. All the data were collected and analysed by experienced investigators who were blind to the experimental assignment. The animal specimens from each experiment were coded with arabic number before and during the data analysis and evaluation.

Isolation and culture of bone-marrow-derived mesenchymal stem cells

Swine were sedated with ketamine (25 mg/kg intramuscular). About 50 mL of bone marrow aspirated from iliac crest were used for the preparation of mononuclear cells by centrifugation through 1.077g/mL Percoll (Sigma). Cells were then suspended at a density of $5 \times 10^5/cm^2$ in a low-glucose DMEM medium containing 10% fetal bovine serum (Gibco). The medium was changed every 3 days. At 80% confluence, cells were detached, labelled with 4',6-diamidino-2-phenylindole (DAPI; Sigma), and kept in warm DMEM before transplantation. The labelling efficiency was determined by fluorescent microscopy, which confirmed that all the cell nuclei were labelled.

Acute myocardial infarction model, cell transplantation, and Atorvastatin administration

A midline sternotomy was performed and the left anterior descending (LAD) coronary artery was dissected free just distal to the first diagonal branch and occluded by a vessel loop. At the end of 90 min, the snare loop was released and reperfusion was visually confirmed.

Thirty minutes after reperfusion, autologous bone-marrow-derived MSCs (3×10^7 cells per animal) were injected into the left ventricular wall of the infarcted hearts (300 μ L into 12 injected foci) and peri-infarct zones (200 μ L into eight injected foci) with a 28-gauge needle. The animals in the control group received intramyocardial injection of the same volume of cell-free DMEM. At the end of transplantation, the chest was closed, the animal was extubated appropriately, and allowed to recover. All the animals received post-operative antimicrobial therapy (Cephazoline 1.0 intramuscular twice daily for 3 days). As reported previously,¹¹ treatment with Ator (Lipitor, Pfizer Pharmaceutical Company, 0.25 mg/kg per day) was started 3 days prior to MSC transplantation and ended 4 days post-transplantation.

Cardiac perfusion and function

For SPECT ^{99m}Tc-sestamibi, about 296 MBq (8mCi) was administered to the pigs and the hearts were imaged 45–60 min later with a gamma camera. The dual head gamma camera (Varicam, GE) with a low energy and high resolution collimator at a 20% energy window was set to 140 KeV gamma peak. Thirty-two projection images per 40 s in a 64 \times 64 matrix were achieved by 180°-rotation arc from the 45°-right and anterior sector to the 45°-left and anterior sector. SPECT was reconstructed with Butterworth cut-off frequency of 0.45, with an order of 5 and the reconstructed data were created along three oblique axis (short axis, vertical long axis, and horizontal long axis) planes by setting the axes of the heart. Quantitative analysis was performed using Cedars quantitative perfusion SPECT (QPS). Perfusion defects were calculated using a scintigraphic bull's eye technique. In addition, all the animals were studied at both baseline and endpoint by cine MRI and contrast enhancement MRI (CE MRI). MRI was performed using a 1.5 T clinical MRI scanner (Siemens Avanto) with a phase array radiofrequency receiver coil. The cardiac function and geometry were detected by using MRI scanner according to the previous study.¹³

Histological analysis

At the end of the experiments, animals were sacrificed, cardiovascular tissues were collected, snap-frozen in OCT medium, and serial sections prepared at 5 μ m thickness. After fixation, tissue sections were incubated with antibodies against biomarkers for cardiovascular cells, including Von Willebrand factor (VWF, 1:200, DAKO), vascular smooth muscle actin (SM-actin, 1:50, DAKO), α -sarcomeric actin (1:50, DAKO), cardiac troponin T (cTn-T, 1:50, Sigma). After washing with PBS, sections were incubated with rhodamine-conjugated goat anti-mouse IgG or anti-rabbit conjugated FITC IgG. Immunofluorescence was detected and images were photographed under a laser scanning confocal microscope.

To determine the survival and differentiating potential of implanted MSCs *in vivo*, left ventricle was cut into eight fragments from apex to base, and frozen sections at 5 μ m thickness were randomly chosen from every fragment for anti-cTn-T immunostaining. Cells were counted from randomized fields of every frozen section under a fluorescent microscope. Those DAPI-labelled cells positive for cTn-T were considered to differentiate into cardiomyocyte-like cells.

The capillary density was determined in the infarcted myocardium and in the peri-infarct zone, as described previously.¹⁴ Sections were stained using polyclonal anti-VWF antibody (1:200, DAKO). For quantification of positively stained vessels, sections were examined blindly by investigators with respect to the treatment. Capillaries were counted in five randomly chosen high-power fields (HPFs) in every chosen section. The results were expressed as capillaries per HPF.

Detection of apoptosis by TUNEL assay

For the assessment of apoptosis at the single-cell level, *in situ* detection of DNA fragmentation was performed using the TUNEL assay (Roche, Germany). Myocardial tissue sections were obtained from peri-infarct regions of all animals at endpoint. Briefly, paraffin tissue sections were deparaffinized, digested with trypsin, and incubated with TdT and fluorescein-labelled dUTP in a humid atmosphere for 60 min at 37°C. After incubation for 30 min with an antibody specific for fluorescein-conjugated alkaline phosphatase, the TUNEL stain was visualized with a substrate system in which nuclei with DNA fragmentation stained blue. To detect myocytes with or without apoptotic nuclei, sections were double-stained with a monoclonal antibody specific for desmin (1:100, DAKO) after TUNEL. Tissue sections were examined microscopically at $\times 400$ magnification and at least 400 cells were counted in a minimum of eight HPFs. The percentage of apoptotic cells was termed the apoptotic index.

Antioxidant enzyme activities and lipid peroxidation

The infarcted and control myocardial tissues were collected at endpoint and analysed for determination of the oxidative stress levels. The enzymatic activities of superoxide dismutase (SOD) were measured through xanthine-oxidation method according to the instructions (Jiancheng Institute, Nanjing, China) as described previously.¹⁵ The myocardial content of malondialdehyde (MDA), a secondary product of lipid peroxidation, was determined as thiobarbituric acid (TBA)-reactive substance as previously described¹⁶ (Jiancheng Institute, Nanjing, China).

Western blot analysis

For western blot analysis of the expression of Bax, Bcl-2, IL-1 β , IL-6, and TNF- α in the hearts of animals, equal amounts of proteins (60 μ g protein/lane) were electrophoresed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis in a Tris/HCl buffer system, and sequentially electrophoretically transferred to a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). Then blotting was conducted according to standard procedures with polyclonal rabbit antibodies against Bax (1:500) and Bcl-2 (1:500, both from Santa Cruz) or I goat anti-IL-1 β (1:1000), IL-6 (1:1,000), and TNF- α (1:1000, all from R&D system) antibodies. After incubation, membranes were washed in PBS, and probed with secondary antibodies conjugated with horseradish peroxidase (1:4000, Jackson Immuno Research Laboratories, PA). Specific bands of target proteins were visualized by chemiluminescence. Membranes were then stripped and re-blotted with a monoclonal mouse anti- β -actin antibody (1:10 000, Advanced ImmunoChemical). Target signals were normalized to the actin signal and analysed semiquantitatively with an NIH Image system.

Statistics

The animal experiments were designed using a 2×2 factorial model. Data were collected and analysed using the SPSS Software (version 13.0). Continuous variables were presented as mean \pm standard deviation. To reveal the benefit of the factorial design in terms of efficiency,

the primary estimate of effectiveness of an intervention should be based on a comparison of all animals allocated to receive it vs. those allocated not to receive it. For example, Ator administration is assessed by comparing the two groups with this intervention (Ator only plus Ator+MSCs) against the other two groups (MSCs only plus control).

Such comparisons are performed in a multivariable regression model, including the two interventions, the corresponding interaction term, as well as the value of the outcome variable at baseline [if the interaction term had not significant contributions ($P > 0.05$), then it would be omitted from the regression model]. Since factorial trials may have low power to detect interactions between the interventions, the magnitude in the effect of MSCs transplantation across the levels of Ator treatment was also calculated to adequately present the potential interaction.

Differences in the survival and differentiation efficiency of implanted MSCs between the Ator+MSCs group and MSCs only group were analysed by Student's *t* test and χ^2 test, respectively. Significant difference was established when *P*-values were < 0.05 .

Previous studies have demonstrated that the benefit of LVEF ($1-3.5 \pm 3\%$) derived from MSCs implantation immediately after AMI was not significantly different from the control animals. In addition, MSCs implantation with stable survival *in vivo* might increase LVEF by $6-14 \pm 3\%$. We hypothesized that the MSC implantation facilitated with Ator administration might improve LVEF by $6 \pm 3\%$. To achieve 80% power to detect a standardized difference of 0.03 at the two-sided 5% significance level, a sample size of 24 would be required based upon the power analysis. Respecting the possible death rate of 10–15% in the experiment, therefore, another four animals were needed. In total, the appropriate sample size in our study was determined as 28.

Results

All the animals survived the experimental procedures until sacrificing except for one animal which died within 24 h after AMI in the control, Ator-only, and MSC-only groups, respectively, and the dead animals were excluded from statistical analysis.

Administration of Ator improves post-infarct perfusion and promotes functional recovery in the heart with mesenchymal stem cell transplantation

Compared with the untreated animals, the Ator-treated ones showed a significant reduction in the area of myocardium with marked perfusion defect by 18.3% ($P = 0.001$), as defined by the quantitative SPECT analysis. Transplantation with MSCs modestly restored the post-infarct perfusion and the average perfusion defect area was reduced by 6.2% (95% CI -0.5 to 12.9%) in the MSC-transplanted animals compared with that without MSCs, which is not statistically significant ($P = 0.069$). Although there was no obvious synergy in restoring perfusion between Ator and MSCs ($P = 0.137$), the perfusion defect area in the myocardium with a combination of treatments with Ator and MSCs was much smaller than that in the myocardium treated with Ator alone, which only lead to a slight reduction in perfusion defect by 6.6% (95% CI -2.0 to 15.3%, $P = 0.118$). Furthermore, among all the animals treated without Ator, MSCs transplantation alone was not observed with significant improvement in perfusion defect compared with controls (by -0.9% , 95% CI -6.1 to 4.4%, $P = 0.721$) (Table 1 and Figure 1).

Table 1 Cardiac perfusion defect (%) by SPECT

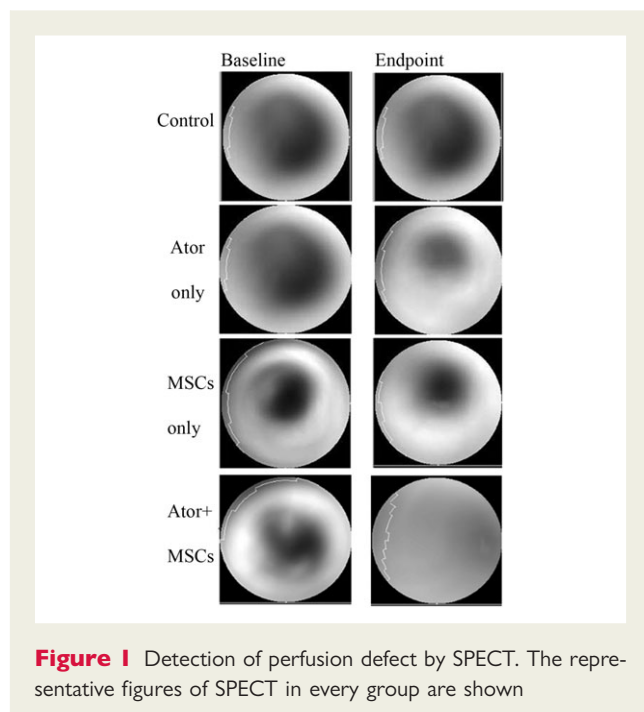
	Total Ator (n = 13)	Total no-Ator (n = 12)	Total MSCs (n = 13)	Total no-MSCs (n = 12)
Baseline	51.1 ± 15.0	51.4 ± 14.7	50.4 ± 15.5	52.2 ± 14.1
Endpoint	33.6 ± 11.6	48.8 ± 11.9	38.8 ± 15.2	43.4 ± 2.5
Adjusted difference ^a (95% CI)	18.3 (11.6 to 25.0)		6.2 (-0.5 to 12.9)	
P-value	0.000		0.069	
	Total Ator (n = 13)	Total no-Ator (n = 12)	Total MSCs (n = 13)	Total no-MSCs (n = 12)
	Ator only (n = 6)	Ator + MSCs (n = 7)	Control (n = 6)	MSCs only (n = 6)
Baseline	53.7 ± 14.9	51.2 ± 14.6	50.7 ± 14.5	52.2 ± 16.3
Endpoint	38.7 ± 13.1	29.3 ± 9.0	47.8 ± 11.1	49.8 ± 13.6
Decrease from Baseline	15.0 ± 7.0	19.6 ± 10.9	2.8 ± 6.1	2.3 ± 4.1
Adjusted difference ^b (95% CI)	6.6 (-2.0 to 15.3)		-0.9 (-6.1 to 4.4)	
P-value	0.118		0.721	

Ator, atorvastatin; MSCs, mesenchymal stem cells; SPECT, single-photon emission computed tomography.

The differences were calculated between endpoints of the intervention and its respective control.

^aAdjusted for values at baseline and the other intervention.

^bAdjusted for values at baseline.



Follow-up examination of cardiac images and function by MRI indicated that the end-diastolic volume (EDV, decreased by 0.3 mL, 95% CI -1.9 to 2.5 mL; $P = 0.8$) remained unchanged in all the Ator-treated animals compared with those untreated. However, other parameters were significantly improved: LVEF increased by 6.2% (95% CI 2.4 to 10.0%, $P = 0.003$), wall thickening increased by 46.2% (95% CI 34.7 to 57.6%, $P = 0.000$), end-systolic volume (ESV) decreased by 3.9 mL (95% CI 1.1 to 6.6 mL, $P = 0.008$), MI size decreased by 3.5 cm² (95% CI 2.4 to 4.5 cm², $P = 0.000$), dyskinetic segments decreased by 3.4 (95% CI 2.1 to 4.6, $P = 0.000$) and LV mass index decreased by 8.3 g/m² (95% CI 4.5 to 12.1 g/m², $P = 0.000$). In contrast, MSC transplantation did not have significant impacts on these parameters since MSC-receiving animals showed similar LV parameters as did untreated ones. However, a synergism appeared to occur between Ator and MSCs, as the combined treatment with Ator and MSCs significantly reduced the non-viable, infarct area ($P = 0.003$) and dyskinetic segments ($P = 0.023$) and improved wall thickening and motion ($P = 0.000$). All the cardiac contractile parameters tested in this study appeared to have better values in the animals dually treated with Ator and MSCs than those with Ator or MSCs alone except for EDV and ESV (Table 2).

Enhancement of cardiovascular morphogenesis and vascularization in the hearts with Ator and mesenchymal stem cell treatment

Histological examination by H&E and Masson's Trichrome staining showed that severe fibrosis and inflammatory cell infiltration occurred in the infarcted heart 6 weeks after transplantation. Ator treatment reduced both fibrosis and inflammation in the infarcted hearts with or without MSC transplantation. There was

more healthy muscle tissue present in infarcted regions in the Ator-treated animals when compared with the control animals or treated with MSCs alone (Figure 2). Fluorescent microscopy showed that the numbers of DAPI-labelled cell nuclei within cardiac tissues of animals with Ator/MSC dual treatment were significantly higher than that in the hearts receiving MSCs only (373.9 ± 90.3 vs. 70.5 ± 22.3 , $P < 0.0001$), suggesting that Ator treatment might increase the survival potential of implanted MSCs in post-infarct hearts (Figure 3A and B).

Immunofluorescent analysis of cardiac- and microvessel-specific proteins in both MSC and Ator+MSC treated groups indicated that several fractions of implanted MSCs contained an increased number of differentiated cardiomyocytes and microvessels (Figure 4A–C). The hearts with Ator+MSC treatment contained an increased number of cells bearing the differentiation markers for cardiomyocytes, especially in the population of DAPI-labelled cells compared with that in the animals treated with MSCs alone (44.4 ± 12.5 vs. $9.6 \pm 3.5\%$, $P < 0.001$) (Figure 4D).

The capillary densities in both infarcted (adjusted difference 2.2, 95% CI 1.3 to 3.0, $P = 0.000$) and peri-infarct regions (adjusted difference 3.4, 95% CI 1.9 to 4.9, $P = 0.000$) of Ator-treated animals were significantly higher than that in those untreated. Similarly, capillary densities in both infarcted (adjusted difference 2.1, 95% CI 1.2 to 2.9, $P = 0.000$) and peri-infarct region (adjusted difference 3.2, 95% CI 1.7 to 4.7, $P = 0.000$) of MSC-treated animals were significantly higher than that in no-MSCs ones. Interactions between Ator and MSCs were significant in capillary density of infarcted ($P = 0.000$) and peri-infarct region ($P = 0.000$). Further analysis showed that Ator-treated animals with transplantation of MSCs had a significantly increased capillary density in both infarcted (4.1 ± 0.7 vs. 1.9 ± 0.6 , adjusted difference 2.1, 95% CI 1.1 to 3.0; $P = 0.000$) and peri-infarct region (8.6 ± 1.2 vs. 5.4 ± 1.0 , adjusted difference 3.2, 95% CI 1.8 to 4.6; $P = 0.000$) compared with Ator only. In animals untreated with Ator, transplantation with MSCs only modestly enhanced capillary density in infarcted (1.8 ± 0.8 vs. 1.7 ± 0.6 , adjusted difference -0.1 , 95% CI -0.8 to 1.0; $P = 0.812$) and peri-infarct region (5.2 ± 1.4 vs. 5.3 ± 1.5 , adjusted difference 0.1, 95% CI -1.7 to 2.0; $P = 0.877$) compared with the control group (Figure 5).

Inhibition of apoptosis in the infarct heart treated with Ator and mesenchymal stem cells

In order to determine whether Ator and MSC treatment improve survival of MSCs and MSC-derived cells in the infarcted hearts, TUNEL analysis was conducted. The apoptotic index in the Ator animals was significantly lower than that in untreated ($3.4 \pm 2.1\%$ vs. $9.6 \pm 1.6\%$, adjusted difference 6.1%, 95% CI 4.8 to 7.4%, $P = 0.000$). Similarly, apoptosis of cardiac cells in MSC-implanted animals was lower than that in animals without MSCs ($5.3 \pm 3.9\%$ vs. $7.6 \pm 3.1\%$, adjusted difference 2.1%, 95% CI 0.8 to 3.4% $P = 0.003$). The interaction between Ator and MSCs was marginally significant ($P = 0.059$). Further analysis indicated that Ator-treated animals with transplantation of MSCs had a significantly decreased apoptotic index compared with those with Ator only (1.9 ± 0.3 vs. 5.2 ± 1.9 , adjusted difference 3.3, 95%

Table 2 Left ventricular function and geometry by MRI

Group		Total Ator (n = 13)		Total no-Ator (n = 12)	
		Ator only (n = 6)	Ator+MSCs (n = 7)	Control (n = 6)	MSCs only (n = 6)
LVEF (%)	Baseline	43.2 ± 7.8	42.4 ± 7.8	43.9 ± 6.6	43.5 ± 10.0
	Endpoint	44.1 ± 8.0	49.7 ± 10.4	42.0 ± 7.1	41.3 ± 8.8
	Increase from baseline	0.9 ± 2.8	7.2 ± 3.8	-2.0 ± 5.0	-2.2 ± 4.9
	Adjusted difference ^a (95% CI)	6.4 (2.2 to 10.6)		-0.3 (-6.7 to 6.0)	
	P-value	0.007		0.908	
EDV (mL)	Baseline	58.8 ± 5.6	55.1 ± 8.2	57.8 ± 5.8	57.2 ± 6.7
	Endpoint	67.3 ± 5.7	64. ± 7.5	67.2 ± 6.6	65.2 ± 5.8
	Increase from baseline	8.5 ± 2.0	9.4 ± 3.5	9.3 ± 2.7	8.0 ± 2.1
	Adjusted difference ^a (95% CI)	-0.4 (-4.2 to 3.3)		1.4 (-1.8 to 4.6)	
	P-value	0.805		0.356	
ESV (mL)	Baseline	8.7 ± 2.2	8.1 ± 2.1	8.3 ± 2.9	7.8 ± 3.4
	Endpoint	37.3 ± 8.1	33.1 ± 10.3	39.2 ± 7.3	38.5 ± 8.6
	Increase from baseline	3.7 ± 3.7	0.9 ± 2.8	6.5 ± 4.1	5.7 ± 2.2
	Adjusted difference ^a (95% CI)	-2.8 (-7.0 to 1.5)		-0.8 (-5.1 to 3.5)	
	P-value	0.176		0.680	
MI size (cm ²)	Baseline	7.3 ± 2.1	6.8 ± 2.0	6.7 ± 1.9	6.8 ± 2.1
	Endpoint	6.0 ± 2.1	3.5 ± 1.6	6.6 ± 2.1	7.1 ± 2.1
	Increase from baseline	-1.3 ± 0.7	-3.2 ± 1.5	-0.8 ± 0.3	0.2 ± 0.3
	Adjusted difference ^a (95% CI)	-2.1 (-0.7 to -3.5)		-0.3 (-0.7 to 0.1)	
	P-value	0.007		0.105	
Dyskinetic segments	Baseline	8.7 ± 2.2	8.1 ± 2.1	8.3 ± 2.9	7.8 ± 3.4
	Endpoint	7.2 ± 2.9	4.6 ± 1.1	8.2 ± 2.2	7.7 ± 3.4
	Increase from baseline	-1.5 ± 1.4	-3.6 ± 1.3	-0.2 ± 0.8	-0.2 ± 0.8
	Adjusted difference ^a (95% CI)	-2.2 (-3.8 to -0.5)		-0.1 (-0.9 to 1.0)	
	P-value	0.014		0.907	
Wall thickening (%)	Baseline	-26.0 ± 8.3	-21.3 ± 11.9	-26.5 ± 16.3	-24.0 ± 14.2
	Endpoint	-25.3 ± 14.3	26 ± 14.6	-27.2 ± 16.0	-21.8 ± 12.6
	Increase from baseline	-0.7 ± 9.2	47.9 ± 15.2	-0.7 ± 6.4	2.2 ± 9.9
	Adjusted difference ^a (95% CI)	8.6 (31.9 to 65.2)		3.1 (-3.3 to 9.6)	
	P-value	0.000		0.304	
LV mass index (g/m ²)	Baseline	68.3 ± 8.3	61.4 ± 9.1	64.2 ± 7.0	60.2 ± 7.9
	Endpoint	71.2 ± 7.9	65.4 ± 8.6	77.3 ± 8.1	75.3 ± 4.8
	Increase from baseline	7.8 ± 2.3	4.0 ± 2.0	13.2 ± 8.1	15.2 ± 4.2
	Adjusted difference ^a (95% CI)	4.0 (1.3 to 6.6)		0.1 (-7.6 to 7.8)	
	P-value	0.007		0.980	

LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; Ator, atorvastatin; MSCs, mesenchymal stem cells; ESV, end-systolic volume; EDV, end-diastolic volume; MI, myocardial infarction.

The differences were calculated between endpoints of the intervention and its respective control.

^aAdjusted for values at baseline.

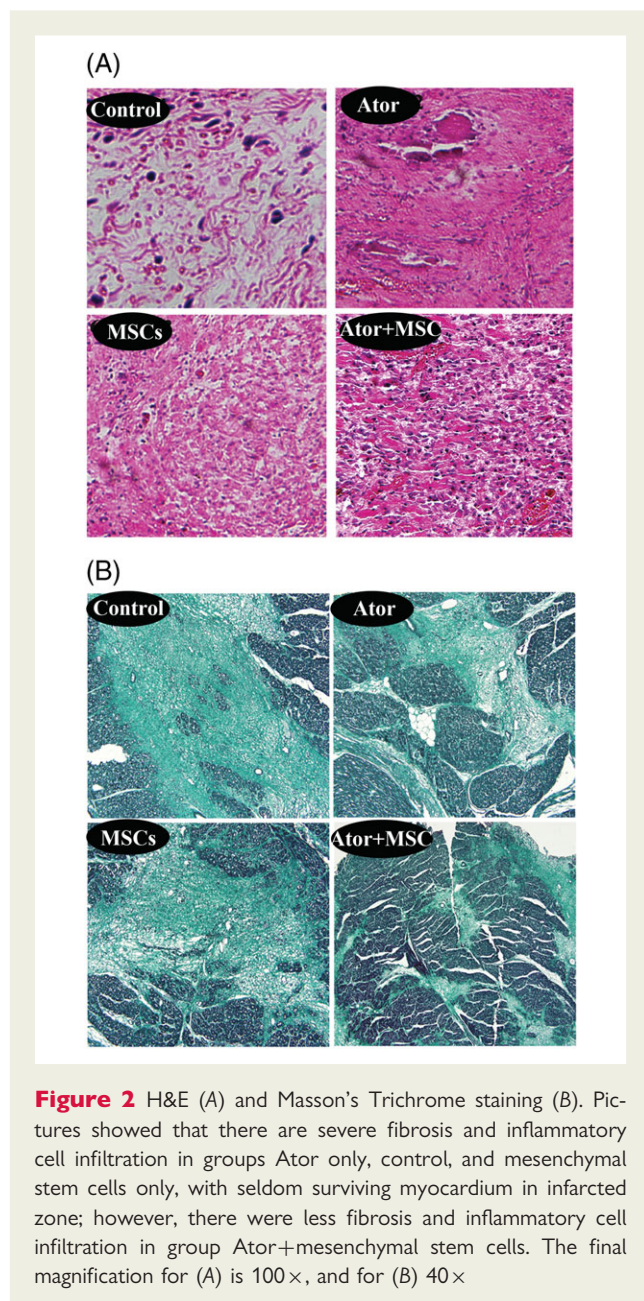
CI 1.7 to 4.9; $P = 0.001$). However, in Ator untreated animals, transplantation of MSCs slightly but not significantly decreased apoptosis with control animals (9.2 ± 1.4 vs. 10.1 ± 1.8 , adjusted difference 0.9, 95% CI -1.2 to 3.0; $P = 0.362$) (Figure 6A).

Further analysis of apoptosis-regulating proteins by western blotting revealed that expression of the pro-apoptotic protein Bax was significantly downregulated ($P = 0.000$) and antiapoptotic protein Bcl-2 was upregulated ($P = 0.000$) in Ator-treated animals compared with untreated. However, Bcl-2 expression remained unchanged ($P = 0.491$) in MSC-implanted animals when compared with animals without MSC transplantation. Ator-treated animals with transplantation of MSCs showed significantly decreased Bax

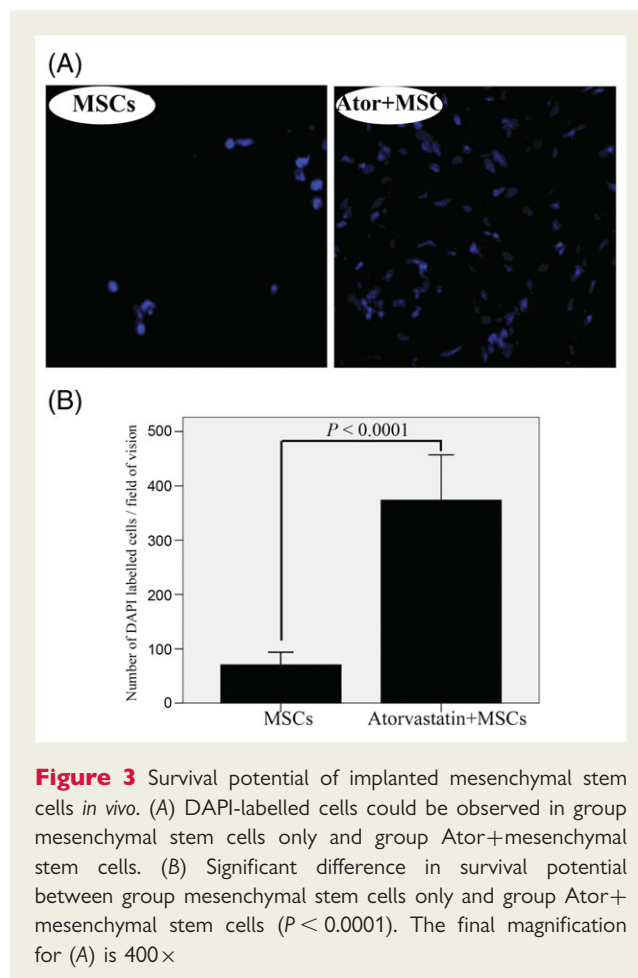
levels ($P = 0.001$), but their Bcl-2 expression was unchanged ($P = 0.736$) compared with that in Ator treatment alone. In Ator-untreated animals, MSC transplantation did not significantly alter expression of Bax ($P = 0.530$) and Bcl-2 ($P = 0.582$) in comparison with control animals (Figure 6B).

Reduced oxidative stress in the heart with Ator and mesenchymal stem cell treatment

Oxidative stress is known to be associated with reperfusion injury which triggers release of oxidative free radicals. Therefore, we



analysed SOD activities in the myocardium with infarction. We observed that SOD activities ($P = 0.001$) were increased while MDA contents ($P = 0.000$) were decreased significantly in Ator-treated animals compared with untreated. However, there were not significant differences between animals transplanted with or without MSCs. There was no significant synergy in regulation of SOD activities ($P = 0.524$) and MDA contents ($P = 0.653$) between Ator and MSC treatments. However, animals with a combined treatment of Ator and MSCs showed a tendency of increasing SOD activities with a lower level of MDA contents compared with those treated with Ator alone, although the values did not achieve statistical significance. Moreover, in Ator untreated animals, MSC transplantation did not significantly lower the oxidative stress levels (Table 3).



Inhibition of inflammatory cytokine expression in the heart with Ator and mesenchymal stem cell treatment

The three inflammatory cytokines IL-1 β , IL-6, and TNF- α were examined in the hearts with infarction. In the infarcted myocardium, Ator treatment inhibited expression of all the three cytokines. The levels of these cytokines were much lower than that in untreated controls ($P < 0.05$). There was no major difference in the cytokine expression between MSC-transplanted and untransplanted animals ($P > 0.05$). The synergy between Ator and MSCs were marginally significant in all three inflammatory factors ($P > 0.05$). Further analysis indicated that animals with treatment of Ator+MSCs reduced TNF- α expression ($P = 0.000$), but had no effect on IL-1 β ($P = 0.725$) and IL-6 ($P = 0.296$) compared with Ator alone. In addition, transplantation of MSCs alone decreased TNF- α expression, but did not alter IL-1 β and IL-6 expressions (Figure 7A and B).

Discussion

In the present study, we have demonstrated that treatment with Ator can exert protective effects on the myocardium undergoing infarction and reperfusion injury, especially in conjunction with

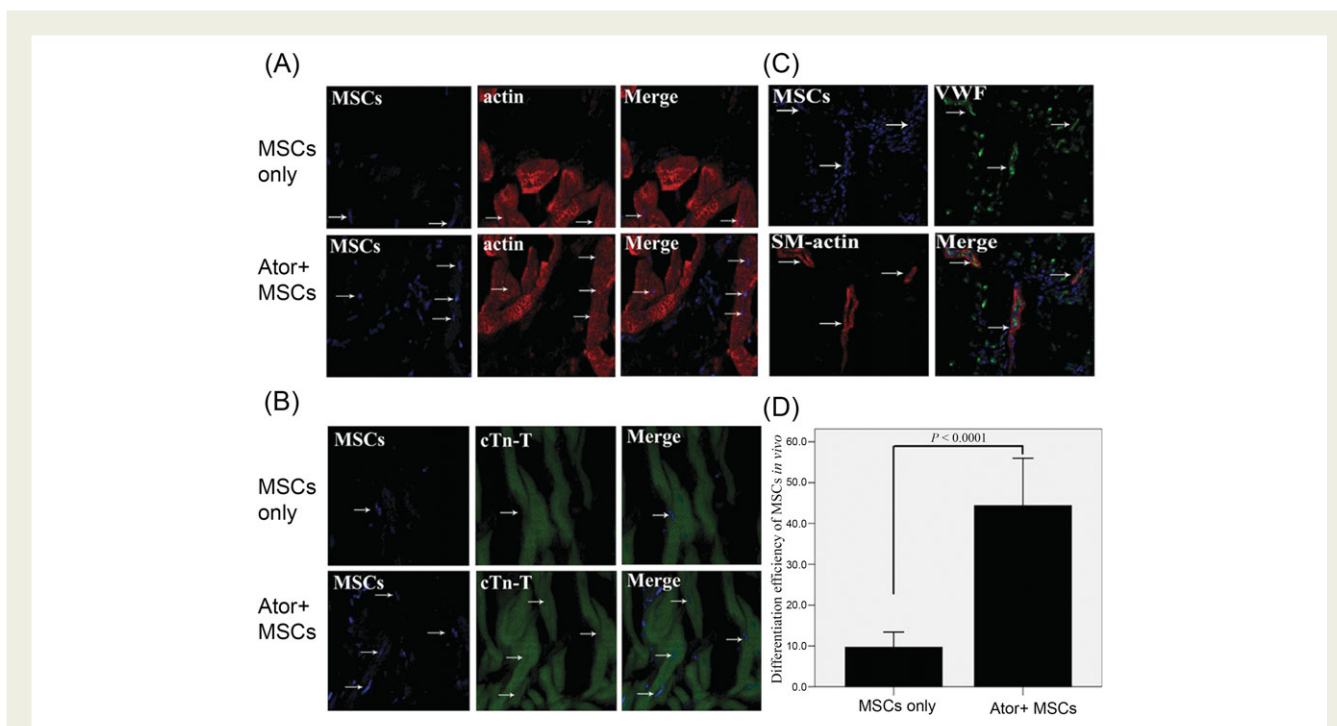


Figure 4 Cardiomyogenesis and angiogenesis of implanted mesenchymal stem cells *in vivo*. (A and B) Several fractions of DAPI-labelled cells are positive for α -sarcomeric actin (actin), cardiac troponin T (cTn-T). (C) Several fractions of DAPI-labelled cells are positive for vascular smooth muscle-actin (SM-actin) and von Willebrand factor (VWF), indicating that they took part in angiogenesis. (D) Significant difference in differentiation efficiency of mesenchymal stem cells *in vivo* between group mesenchymal stem cells only and group Ator+mesenchymal stem cells ($P < 0.0001$). The final magnification for (A and C) is 400 \times

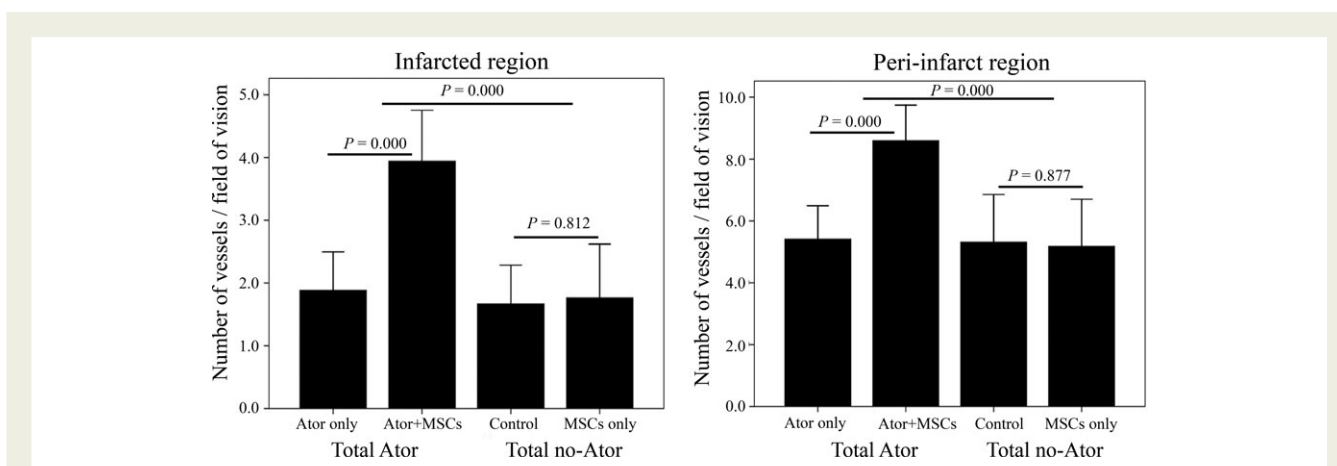


Figure 5 Capillary densities in infarcted and peri-infarct zone. Capillary densities in both infarcted and peri-infarct region of Ator-treated animals were significantly higher than that in Ator untreated. Similarly, capillary densities in animals with mesenchymal stem cell transplantation were significantly higher than that in no-mesenchymal stem cells ones. Further analysis indicated that Ator+mesenchymal stem cells remarkably increased capillary density in both infarcted and peri-infarct region compared with Ator only. However, within Ator-untreated animals, mesenchymal stem cell transplantation alone did not significantly enhance capillary density compared with the control group

MSC transplantation. Our data clearly showed that immediate MSC transplantation after infarction–reperfusion did not significantly improve cardiac perfusion and function. This may be due to a poor survival rate of implanted cells in the post-infarct environment that may release cytotoxic factors. However, as

shown in this study, a low dose and a short-term administration of Ator around the time of cell transplantation may improve the efficacy of stem-cell therapy. We found that the statin treatment may have multiple benefits on the micro-environment of the myocardium, including increased perfusion rate, and the inhibitory

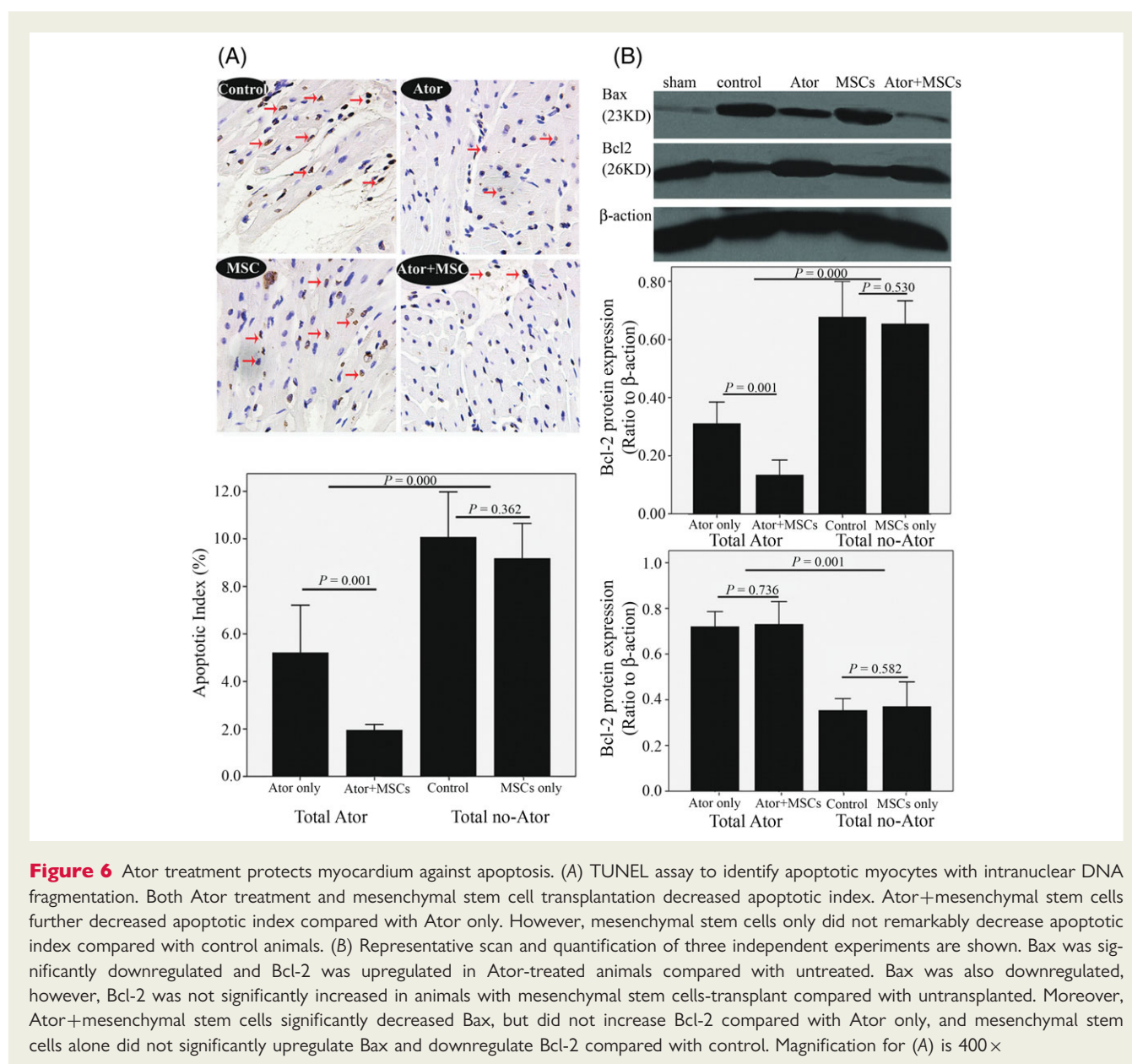


Figure 6 Ator treatment protects myocardium against apoptosis. (A) TUNEL assay to identify apoptotic myocytes with intranuclear DNA fragmentation. Both Ator treatment and mesenchymal stem cell transplantation decreased apoptotic index. Ator+mesenchymal stem cells further decreased apoptotic index compared with Ator only. However, mesenchymal stem cells only did not remarkably decrease apoptotic index compared with control animals. (B) Representative scan and quantification of three independent experiments are shown. Bax was significantly downregulated and Bcl-2 was upregulated in Ator-treated animals compared with untreated. Bax was also downregulated, however, Bcl-2 was not significantly increased in animals with mesenchymal stem cells-transplant compared with untransplanted. Moreover, Ator+mesenchymal stem cells significantly decreased Bax, but did not increase Bcl-2 compared with Ator only, and mesenchymal stem cells alone did not significantly upregulate Bax and downregulate Bcl-2 compared with control. Magnification for (A) is 400×

effects on apoptosis, oxidative stress, and inflammatory reactions. The beneficial effects may occur as a synergism between Ator and MSCs. Treatment with either Ator or MSCs alone could not achieve these effects. The Ator effects are supported by the observation that Ator administration enhances the survival and differentiation of implanted MSCs, decreases the infarcted area, promotes angiogenesis, and reverses negative ventricular remodelling.

The adult bone marrow-derived MSCs can proliferate *in vitro* while retain their differentiation potential^{17,18} or the ability to colonize the myocardium¹⁹ and differentiate into cardiomyocytes.²⁰ As a prerequisite for benefits derived from cell transplantation, the donor cells should engraft in the targeted region and stably survive, thus producing bioactivities. Our results showed that most of the implanted MSCs could not survive in the post-infarct myocardium, suggesting that the local micro-environment of the heart with fresh AMI and reperfusion may not be suitable

for stem cells to survive *in vivo*. Apoptosis, oxidative stress, and intense inflammatory reaction are known to be the adverse factors contributing to donor cell death. Li *et al.*⁷ have recently reported that the effects of cell transplantation were better when cells were injected 2 weeks after cryo-injury compared with immediate injection, which was presumably due to excessive cell death caused by the early post-infarction inflammation. Our results also showed that immediate transplantation of cells after AMI and reperfusion could not significantly improve cardiac function and morphology.

It is, however, unknown currently as to why MSC transplantation alone could not produce the benefits we were expecting. Several factors may contribute to this: (1) there is a difference in experimental subjects; (2) a difference in the transplantation time reported previously by Chen *et al.*²¹ (18.4 days+8.4 h after AMI) and Katritsis *et al.*²² (242.4 ± 464.0 days, ranged 8–1560 days).

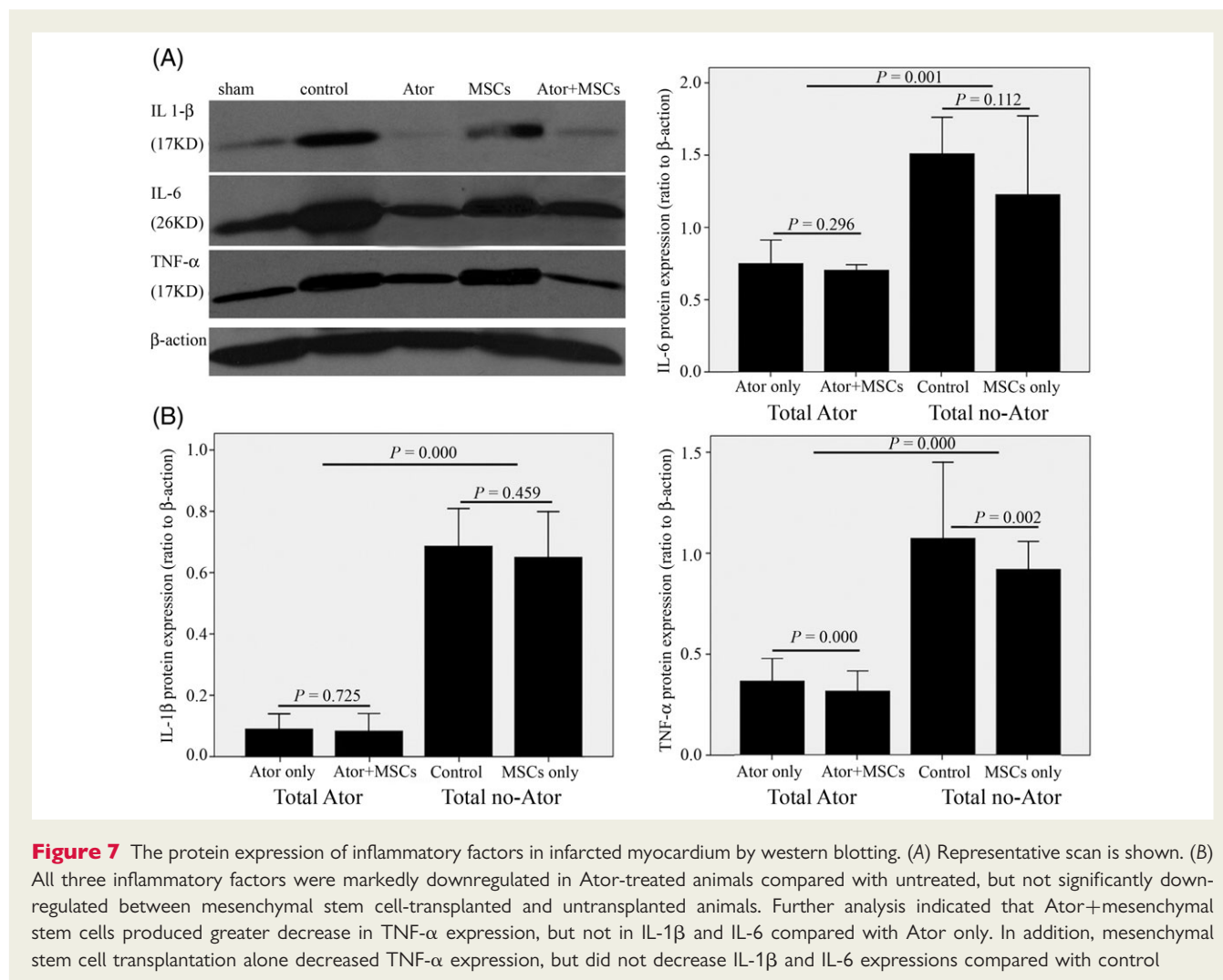
Table 3. Assessment of oxidative stress in infarcted myocardium

Group		Total Ator (n = 13)	Total no-Ator (n = 12)	Total MSCs (n = 13)	Total no-MSCs (n = 12)
SOD (U/mg protein)	Endpoint	103.9 ± 14.0	85.4 ± 9.3	98.2 ± 16.2	91.1 ± 13.5
	Adjusted difference ^a (95% CI)	18.5 (8.6 to 28.3)		7.1 (-2.7 to 16.9)	
	P-value	0.001		0.149	
MDA (nmol/mg protein)	Endpoint	5.6 ± 1.2	8.7 ± 0.8	7.0 ± 1.8	7.3 ± 2.0
	Adjusted difference ^a (95% CI)	3.2 (2.3 to 4.0)		0.4 (-0.5 to 1.2)	
	P-value	0.000		0.395	
Group		Total Ator (n = 13)		Total no-Ator (n = 12)	
		Ator only (n = 6)	Ator + MSCs (n = 7)	Control (n = 6)	MSCs only (n = 6)
SOD (U/mg protein)	Endpoint	98.8 ± 13.4	109.0 ± 13.8	83.4 ± 8.8	87.4 ± 10.2
	Difference (95% CI)	10.2 (-7.3 to 27.7)		4.0 (-8.3 to 16.2)	
	P-value	0.224		0.485	
MDA (nmol/mg protein)	Endpoint	5.7 ± 1.3	5.5 ± 1.1	9.0 ± 0.8	8.5 ± 0.8
	Difference (95% CI)	0.2 (-1.4 to 1.7)		-0.6 (-1.6 to 0.5)	
	P-value	0.818		0.259	

Ator, atorvastatin; MSCs, mesenchymal stem cells; SOD, superoxide dismutase; MDA, malondialdehyde.

The differences were calculated between endpoints of the intervention and its respective control.

^aAdjusted for the other intervention



The immediate transplantation of stem cells after AMI and reperfusion may expose the cells to an harsh environment leading to a lower survival rate as discussed earlier; (3) a difference in the number of implanted MSCs. Chen *et al.* transplanted $4.8\text{--}6.0 \times 10^{10}$ bone marrow-MSCs into the infarcted hearts, which were 10^3 times more than that in our study; (4) a difference in the follow-up time. The studies of Chen *et al.* and Katritsis *et al.* had a follow-up time of 3–6 months and 4 months, respectively, which have passed the acute inflammatory phase.

The observation that Ator treatment around AMI and reperfusion could decrease the myocardial infarct sizes is very interesting. The statin may exert this action partly via its pleiotropic effects (e.g. anti-apoptosis, anti-oxidative stress, and anti-inflammation) consistently with previous reports.^{11,23,24} In addition, our results indicate that Ator administration enhanced capillary density of infarcted region and peri-infarct region, especially in those animals receiving Ator+MSCs, which is consistent with the results of Landmesser *et al.*²⁵ Furthermore, Landmesser *et al.* observed a marked enhancement of EPC mobilization after statin treatment, which might contribute angiogenesis in post-infarct myocardium. However, in our study Ator alone appear to slightly increase

capillary density which was significantly enhanced only by Ator+MSCs, indicating the angiogenesis was mostly due to MSC transplantation. Our current observation that the infarcted area was significantly decreased by Ator treatment is not consistent with the previous report by Landmesser *et al.*,²⁵ which only showed modest improvement. The difference may be attributed to the experimental model used in their study. The infarct model of their study was created by permanent ligation of LAD artery; while our model was reperfused 90 min after the ligation of LAD artery. It is likely that at a low-dosage Ator may exert protective effects on the hearts with reperfusion injury.²⁶

Our data also demonstrated that the low-dose and short-term administration of Ator could increase the potential of survival and differentiation of implanted cells in post-infarct myocardium *in vivo*, accompanied with significant benefits in cardiac function, indicating that stem-cell engraftment, survival, and differentiation are closely relied on the local milieu *in vivo* after AMI. It is certain that the local milieu play a critical role in cell engraftment and bioactivity, and therefore is responsible for the functional responses in cellular cardiomyoplasty. Although stem cells are studied clinically for cardiac repair, a high level of engrafted cell

death occurs after grafting into injured hearts which hampered the progress in cellular cardiomyoplasty.²⁷ Most cells were lost within 24 h of transplantation, only 15% survived for 12 weeks.²⁸ The quick loss after implantation is mainly due to cell leakage out of the myocardium, or wash-out through the vascular system. Furthermore, regenerated cells from donor cells do not survive repeated bouts of ischaemia.²⁹ The molecular mechanism for stem cell death in ischaemic heart is mainly due to ischaemia; moreover ischaemia/reperfusion, apoptosis, and inflammatory response also play important roles,³⁰ which is consistent with our results.

Therefore, protection of graft cells from acute death in ischaemic myocardium is important for clinical applications. Up to date there have been several methods to improve the fates of implanted cells, including Akt or bcl-2 gene transfection.^{31,32} These methods are established based on the levels of donor cells. However, they may not be suitable for clinical application because of the difficulty of gene delivery to the myocardium. Therefore, the interventions aimed in improving the quality of local micro-environments to facilitate survival and biological behaviour of implanted cells may be similarly effective and clinically practicable. Although at lower doses Ator administration may not have major detectable benefits in cardiac function, in this study, we have observed some protective effects of Ator, including the decrease in infarcted area, inhibition of apoptosis and oxidative stress, and amelioration of inflammatory response in post-infarct myocardium. The survival and differentiation of implanted MSCs were indeed enhanced in the animals treatment with Ator+MSCs as shown in this study.

In conclusion, for the first time, this study has documented experimental evidence showing that a combination of pharmacologic intervention with statin and stem-cell transplantation may have a synergism in regeneration and repair of normal myocardial function and morphology post-infarction. Ator treatment could effectively enhance survival of implanted MSCs in the infarcted tissue, accompanied by functional benefits resulting from cell transplantation. The data from the present study also provide a potentially new method for the application of stem-cell transplantation in AMI patients who are the candidates for emergent coronary intervention.

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