Transplantation of Adipose Tissue-Derived Stem Cells Overexpressing Heme Oxygenase-1 Improves Functions and Remodeling of Infarcted Myocardium in Rabbits

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Adipose tissue-derived stem cells (ADSCs) are a promising source of autologous stem cells that are used for regeneration and repair of infarcted heart. However, the efficiency of their transplantation is under debate. One of the possible reasons for marginal improvement in ADSCs transplantation is the significant cell death rate of implanted cells after being grafted into injured heart. Therefore, overcoming the poor survival rate of implanted cells may improve stem cell therapy. Due to limited improvement concerning direct stem cell therapy, gene-transfer methods are used to enhance cellular cardiomyoplasty efficacy. Heme oxygenase-1 (HO-1) can provide various types of cells with protection against oxidative injury and apoptosis. However, exact effects of autologous ADSCs combined with HO-1 on cardiac performance remains unknown. In this study, rabbits were treated with ADSCs transduced with HO-1 (HO-1-ADSCs), treated with non-transduced ADSCs, or injected with phosphate buffered saline 14 days after experimental myocardial infarction was induced, when autologous ADSCs were obtained simultaneously. Four weeks after injection, echocardiography showed significant improvements for cardiac functions and left ventricular dimensions in HO-1-ADSCs-treated animals. Structural consequences of transplantation were determined by detailed histological analysis, which showed differentiation of HO-1-ADSCs to cardiomyocyte-like tissues and lumen-like structure organizations. Apart from improvement in angiogenesis and scar areas, more connexin 43-positive gap junction and greater tyrosine hydroxylase-positive cardiac sympathetic nerves sprouting were observed in the HO-1-ADSCs-treated group compared with ADSCs group. These data suggest that the transplantation of autologous ADSCs combined with HO-1 transduction is a feasible and efficacious method for improving infarcted myocardium.

Keywords: adipose tissue-derived stem cells; gene transfer; heme oxygenase-1; myocardial infarction; rabbit


Myocardial infarction (MI), which is characterized by reduced blood supply to the heart and loss of functioning cardiomyocytes, has become the leading cause of morbidity and mortality in modern society. The limited capacity of the adult heart to self-regenerate and persistent deterioration of heart function yield poor prognosis for MI. Repairing a damaged heart would likely require regenerated myocardium, new blood vessels, nerve innervation, and electrical-mechanical coupling of the restored tissue (Beeres et al. 2008). Recent experimental studies have shown that adipose tissue-derived stem cells (ADSCs), a source of mesenchymal stem cells (MSCs), are self-renewing and capable of regenerating infarcted myocardium by inducing myogenesis and angiogenesis (Hwangbo et al. 2010; Lin et al. 2010; Yu et al. 2010). ADSCs and MSCs exhibit a highly similar gene expression profile (Noel et al. 2008), and the stem cells isolated from adipose tissues share the same morphology, phenotype and in vitro differentiation ability with bone marrow. Moreover, cultured populations of ADSCs can be expanded rapidly, and long-term cultured cells retain their mesenchymal pluripotency ( Ranera et al. 2011).

Despite the mentioned features of ADSCs above, the efficiency of their transplantation is under debate. One of the possible reasons for marginal improvement in ADSCs transplantation is the significant cell death rate of implanted cells after being grafted into injured heart. Therefore, overcoming the poor survival rate of implanted cells is clearly
needed to improve stem cell therapy. In line with this, heme oxygenase-1 (HO-1), the rate-limiting enzyme in the degradation of heme, is now considered to function as a cytoprotective agent due to its multiple catalytic byproducts (Shibahara 2003); in fact, HO-1 has been shown to protect against apoptosis after oxidative stress injury in multiple cell types (Takahashi et al. 1999; Abdel-Aziz et al. 2003; Zeng et al. 2010).

In this study, autologous ADSCs were isolated simultaneously when acute MI was induced in a rabbit model. After sufficient culture followed by HO-1 modification, stem cell transplantation was performed in sub-acute MI phase. The objective of this study was to evaluate the feasibility and effects of autologous ADSCs transplantation with HO-1 overexpression after induction of experimental MI in rabbits, particularly on heart function, neovascularization, nerve sprouting and gap-junction formation.

**Methods**

All animals were studied under guidelines published in Recommendations from the Declaration of Helsinki, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese PLA general hospital, Beijing, China.

**Study design**

The study design is presented in Fig. 1. Rabbits were treated with ADSCs transduced with HO-1 (HO-1-ADSCs group, n = 9), treated with non-transduced ADSCs (ADSCs group, n = 9), or injected with phosphate buffered saline (PBS group, n = 9). In HO-1-ADSCs group and ADSCs group, the fat tissue was excised for cell culture after the first echocardiography on Day 0; MI was induced on the same day. Cells were transduced with Ad.HO-1 on Day 12 for HO-1-ADSCs group. The second echocardiography was taken on Day 13, and the labeled cells were injected on Day 14. The third echocardiography, as well as hemodynamic evaluation, was performed on Day 42, and the rabbits were subsequently sacrificed. In PBS group, MI was induced on day 0 after the first echocardiography.

The second echocardiography was taken on Day 13, and PBS injection was performed on Day 14. The third echocardiography and hemodynamic evaluation were obtained on Day 42 and the rabbits were then sacrificed.

**Surgical preparation for MI**

Male New Zealand White rabbits were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) by intramuscular administration. After that, the rabbits were placed in the supine position and monitored by limb lead electrocardiograms. Under sterile conditions, the rabbits were subjected to a median sternotomy. This procedure was performed carefully so that injury to the parietal pleura was avoided. The sternum was gently retracted to maintain surgical exposure. The pericardium was incised to expose the anterior surface of the heart and the left coronary artery was ligated at 50% level from the apex along the course of the posterolateral division in the bifurcation pattern or 75% level from the apex along the course of the lateral division in the trifurcation pattern with a 6-0 Prolene suture, as described by Lee et al. (2002). Reliable ligation was confirmed by ST segment elevation on the electrocardiograms and regional cyanosis of the myocardial surface. After hemostasis was achieved, the sternum was closed with 3-0 nylon sutures. The muscle layer and skin incision were then closed with 4-0 nylon sutures. The rabbits remained upon the warming pad and were allowed to recover. Postoperatively, 0.03 mg/kg buprenorphine and 15 mg/kg penicillin were used for 3 days.

**Fat tissue preparation and cell separation**

After extracted from inguinal region during experimental MI procedure, adipose tissue was minced and incubated for 90 min at 37°C on a shaker with 0.05% trypsin and 0.1% collagenase I (Gibco, USA) at a concentration of 4 ml per gram of fat tissue in L-Dulbecco’s modified Eagle’s medium (L-DMEM; Gibco, USA). The digested tissue was sequentially filtered through 75 μm filters and centrifuged at 600 g for 10 min. The supernatant containing adipocytes and debris was discarded. Pelleted cells were suspended with PBS and layered over an equal volume of 1.073 g/ml Percoll solution (Sigma, USA). After centrifugation at 900 g for 30 min, mononuclear

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Fig. 1. Outline of experimental protocols. MI, myocardial infarction.
cells were recovered from the gradient interface; were suspended in growth medium containing L-DMEM, 20% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and then grown at 37°C in a humidified atmosphere containing 5% CO₂. Culture media were changed every 2 days to remove unattached cells. Plastic-adherent cells were referred to as ADSCs. After the ADSCs reached 90% confluence and were subsequently passaged. The ADSCs were harvested on Day 12 and were labeled for 20 to 30 minutes on ice using a manufacturer-recommended concentration of the mouse anti-human monoclonal fluorescent antibody for CD29, CD31, CD34, CD45, or CD90 (Santa Cruz, USA) as well as a control antibody. Flow cytometric analysis was performed as previously described (Liu et al. 2010).

Adenoviral transduction

Ad.HO-1 contains expression cassettes for human HO-1. It was constructed by subcloning the HO-1 cDNA into pAdTrackCMV and co-transforming with pAdEasy1 in E. coli BJ 5183 cells (a generous gift from Dr. Zhuo-zhuang Lu, Institute of Virology, Chinese Academy of Preventive Medicine). For construction of adenovirus containing green fluorescent protein (GFP), a shuttle vector containing human phosphoglycerate kinase gene promoter was used. The control virus lacking the HO-1 gene (Ad.β-gal) was separately prepared. Recombinant adenovirus was generated by homologous recombination and propagated in 293 cells, originally derived from human embryonic kidney cells. 293 cells have been used to produce therapeutic proteins and viruses for gene therapy. At stipulated time, the supernatant from 293 cells was collected and purified on cesium chloride (CsCl) gradient centrifugation and stored in 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L MgCl₂, and 10% (vol/vol) glycerol at 70°C until used for experiments.

After two passages, homogenous ADSCs that were devoid of hematopoietic cells were transfected with Ad.HO-1 at multiplicity of infection (MOI) of 100. After incubation at 37.8°C for 4 h, an equal volume of L-DMEM containing 20% FBS was added to the medium. The cells were subsequently cultured for additional 48 h.

Measurement of intracellular reactive oxygen species (ROS) and cell death induced by H₂O₂

Different groups, including control ADSCs, β-gal-ADSCs and HO-1-ADSCs, were in vitro set up for determining the mechanism of enhanced effectiveness in HO-1-ADSCs transplanted heart.

When ADSCs were treated with 0.5 mM H₂O₂ for 5 h, nuclear morphology for assessing apoptotic cell death apoptosis was performed by DAPI (fluorescent dye 4,6-diamidine-2-phenylindole, Roche, Switzerland) staining.

For measuring intracellular ROS, ADSCs were preincubated in serum free DMEM for 24 h, treated with 1 mM of H₂O₂ for 30 min, and preloaded with 10 μM DCFH-DA (Sigma, USA) for 30 min at 37°C. Fluorescence intensity was analyzed by Olympus FX1000S microscopy using 485 nm excitation and 538 nm emission filter.

The viability of ADSCs was determined by MTT (C,N-diphenyl-N-4, 5-dimethyl thiazol-2-yl tetrazolium bromide) assay (Sigma, USA). ADSCs were cultured in a 96-well microplate. After incubation with indicated concentrations of H₂O₂ for 1 h, the media were replaced with MTT solution (5 mg/mL in PBS). Incubation was further continued for 4 h, and then the supernatant was removed by aspiration. Dimethyl sulfoxide (DMSO, Sigma, USA) was added and absorbance was read at 570 nm on microplate reader, and the percent-age of cell viability was obtained.

Cells labeling and implantation

Before in vivo injection, cells were labeled with chloromethyl-1,1-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (CM-Dil, Invitrogen, USA) according to the manufacturer’s protocol. CM-Dil-labeled cells were also subcultured to confirm their vitality.

Two weeks after LCA ligation, all rabbits, whose left ventricular (LV) ejection fraction was confirmed to be reduced by the second echocardiography on Day 13, were anesthetized, intubated and respirated by using mechanical ventilatory support. Median sternotomy was repeated, and adherent tissue around the myocardium was carefully removed. After the heart was exposed, four 50 μL suspensions, each containing 2.5 × 10⁶ cells or PBS, were injected into 4 different locations within the infarcted border zone by using a needle with an outer diameter of 30 μm. The sternum, muscle and skin were closed in layers. Negative pressure drainage was performed. The animals were allowed to recover for 4 weeks.

Cardiac function and hemodynamic evaluation

Transthoracic echocardiography was performed at specified times described in the study design. Animals were lightly anesthetized and placed in the left lateral decubitus position. Standard two-dimensional and M-mode transthoracic images were recorded at the level of the papillary muscles in a blinded fashion using a 5.0 MHz probe connected to a Sequoia 512 system (Acuson, Mountain View, UK). The LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) were measured from M-mode recording. LV ejection fraction (EF) was calculated as follows: EF,% = [LVEDD¹ – LVESD¹]/LVEDD¹ × 100. LV fractional shortening (FS) was calculated as follows: FS, % = [LVEDD – LVESD]/LVEDD × 100. All measurements were performed according to the American Society for Echocardiology leading-edge technique and averaged on 3 consecutive cardiac cycles.

Cardiac hemodynamic studies were performed after the last echocardiographic examination. The carotid arterial catheter was inserted into the isolated right carotid artery and advanced into the left ventricle. The measurements were obtained by using a pressure transducer connected to a polygraph. For subsequent off-line analysis of left ventricular function, 3- to 5-second samples of pressure and dp/dt curves were recorded on-line to a microcomputer equipped with a physiological experiment system (Rm6240C, China). LV end-diastolic pressure (LVEDP) was measured during end stage of systole. Maximum rate of rise in left ventricular pressure (peak +dp/dt) and maximum rate of decrease in left ventricular pressure (peak –dp/dt) were recorded during systole and diastole, respectively. All variables were obtained in a blind fashion and averaged on 3 consecutive cardiac cycles.

Histopathological and immunohistochemical analysis

After the hemodynamic measurements, the rabbits were sacrificed and the segments of interest were embedded in paraffin blocks. Serial paraffin sections were deparaffinized, rehydrated, and antigen retrieved. Paraffin heart sections were stained with Hematoxylin and Eosin (HE staining). Masson’s trichrome staining was also performed to delineate scar tissue from viable myocardium. Five fields per section were calculated by image analysis system (Image-Pro Plus 4.0; Silver Spring, USA). The values were expressed as the ratio of Masson’s Trichrome-stained area to infarcted area. Then, we per-
formed connexin43 (Cx43, Abcam, UK) immunostaining for gap junctions, tyrosine hydroxylase (TH, Abcam, UK) staining to detect sympathetic hyper-innervation and factor VIII (fVIII, Abcam, UK) immunostaining for endothelial cells, respectively. Sections were incubated with peroxidase-conjugated streptavidin and stained with diaminobenzidine. Negative controls were prepared by omitting primary antibodies.

The number of microvessels (diameter < 20 μm) in the infarcted area was counted in paraffin-embedded sections with factor VIII immunostaining-positive staining under light microscopy (original area was counted in paraffin-embedded sections with factor VIII antibodies. Six high-power fields were chosen at random, and microvessels were counted in each field. The number of microvessels in each group was averaged, and vascular density was expressed as the number of microvessels per unit area (0.2 mm²).

The Cx43-positive gap junctions were identified by Cx43 immunostaining-positive linear structures located on the cell membranes of each myocardial cell, after which digital pictures of the tissue slides at a ×400 power magnification were obtained and the percent area of Cx43-positive gap junctions in infarcted border zone of each slide were quantified. Microscopic fields with the highest nerve density in the infarcted border zone were selected to quantify the TH-positive cardiac sympathetic nerves, and images were taken at a ×400 objective. The cardiac nerves were identified as tyrosine hydroxylase immunostaining-positive fibrillar structures between myocardial cells that were longer than 10 μm and stained brown.

The immunostained percent areas of Cx43-positive gap junctions and cardiac sympathetic nerves were quantified using Image-Pro Plus 4.0 software. These densities were derived from gap junction or nerve areas divided by the total area examined and percent area, respectively.

Immunofluorescent analysis
Cryosections were fixed with acetone, and endogenous peroxide activity was quenched with 3% H₂O₂. After blocking with 2% normal goat serum, primary antibodies against cardiac Troponin T (cTnT; Abcam, UK), α-smooth muscle actin (α-SMA; Abcam, UK), and von-Willebrand factor (vWF; Abcam, UK) were used at 4°C overnight. Either fluorescein isothiocyanate or Cy3-conjugated immunoglobulin (CD31, α-SMA; Abcam, UK), and von-Willebrand factor (vWF; Abcam, UK) were used at 4°C overnight. Either fluorescein isothiocyanate or Cy3-conjugated immunoglobulin G (1:100; Sigma, USA) was then incubated for 1 hour at room temperature before being observed under an Olympus FV1000S confocal laser microscopy.

Western blot analysis
Cells were washed with ice-cold PBS, resuspended in lysis buffer and sonicated briefly. After centrifugation, the supernatant was prepared as protein extract, and protein concentrations were measured with a Bicin Chonic Acid (BCA) protein assay reagent. Whole cell extracts were fractionated by electrophoresis on 4-12% gradient gel (Invitrogen, USA) and transferred onto a Polyvinylidene fluoride (PVDF; Millipore, USA). For blocking nonspecific binding, the PVDF membrane was soaked in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk. Primary antibodies against HO-1 (1:1,000, Cell Signaling, USA) and β-actin (1:1,000, Sigma, USA) were used. The labeled proteins were detected using enhanced chemiluminescence reagents and exposed to film.

Statistical analysis
SPSS for Windows (version 15.0) was used for Statistical analysis. Data were described as mean ± standard deviation. Student’s t-test and one-way ANOVA with least significant difference post hoc analysis were performed. P < 0.05 was considered statistically significant.

Results
Characterization of ADSCs and Transfection
The ADSCs were isolated from the subcutaneous adipose tissue of rabbits based on the adherent properties of these cells. Cells expanded easily in vitro and exhibited a fibroblast-like morphology (Fig. 2A). After 12 days of culture, ADSCs expressed ADSC-related antigens, such as CD29 and CD90 (Fig. 2B), but they expressed neither hematopoietic lineage markers, such as CD34 and CD45, nor endothelial markers such as CD31. ADSCs were transfected with adenovirus at MOI of 100. Twenty-four hours later, the viral transduction efficacy was observed under fluorescent microscope by green fluorescence excitation (Fig. 2C), and the western blot assay confirmed the over-expression of HO-1 in the transduced ADSCs (Fig. 2D).

In vitro assay in H₂O₂-treated HO-1-ADSCs
We next analyzed the effects of H₂O₂, an apoptotic stimulus, on ADSCs expressing HO-1 (HO-1-ADSCs) and control ADSCs to elucidate the consequence of HO-1 expression. The fragmented or condensed chromatin was reduced in HO-1-ADSCs compared with the control ADSCs or β-gal-ADSCs after stimulation with 0.5 mM H₂O₂ for 4 h (Fig. 3A). The intracellular level of ROS was elevated after treating with H₂O₂ in ADSCs (145 ± 8% vs. non-treated ADSCs, P < 0.05) and in β-gal-ADSCs (137 ± 9% vs. non-treated ADSCs, P < 0.05), whereas the level was significantly reduced in HO-1-ADSCs (121 ± 7% vs. H₂O₂-treated β-gal-ADSCs, P < 0.05, Fig. 3B). Moreover, HO-1-ADSCs were found to be more resistant to H₂O₂ at different concentrations compared to control ADSCs or β-gal-ADSCs (P < 0.05) (Fig. 3C).

Clinical evaluation
The rabbits transplanted with HO-1-ADSCs showed more dynamic locomotor activity than PBS group after 10-14 days of cell injection. They were wandering in the cage and were more responsive, while the PBS-injected rabbits usually stayed motionless. The motionless trend of the rabbits treated with ADSCs was indistinguishable from that of PBS group. The pulse and the ventilation rates of the three groups were similar after MI induction on Day 0, but they were significantly different between HO-1-ADSCs group and PBS group on Day 42 (pulse rate: 168 ± 20 vs. 199 ± 19, P < 0.01, respectively and ventilation rate: 40 ± 6 vs. 49 ± 7, P < 0.01). Moreover, the HO-1-ADSCs group showed lower pulse rate than that in ADSCs group (168 ± 20 vs. 188 ± 20, P < 0.05). In contrast, no statistically significant difference was noted between ADSCs group and PBS group. Life-threatening arrhythmia was not detected in the monitored period during echocardiographic evalua-
Fig. 2. Characteristics of ADSCs. (A) Isolated ADSCs displayed fibroblast-shape. (B) ADSCs from rabbit inguinal region were analyzed by flow cytometry. The ADSCs were positive for CD29 and CD90, but negative for CD31, CD34, and CD45. (C) ADSCs were transduced to express HO-1 with GFP fluorescence. (D) Western blot analysis revealed that over-expression of HO-1 was detected in transduced ADSCs.

Fig. 3. Improvement of oxidative stress injury in HO-1-ADSCs in vitro. (A) Representative DAPI staining images in cells treated with 0.5 mM H$_2$O$_2$ for 5 h. The arrowheads indicated positive regions. β-gal-ADSCs refers to as ADSCs transduced with the control null virus (Ad.β-gal). (B) Quantitative analysis of intracellular ROS level detected using DCF fluorescent dye after treatment with 1 mM H$_2$O$_2$. *P < 0.05 for comparison to untreated ADSCs (n = 6). †P < 0.05 for comparison to H$_2$O$_2$-treated β-gal-ADSCs (n = 6). (C) Quantitative analysis of cell viability using MTT assay in control ADSCs, β-gal-ADSCs and HO-1-ADSCs after 1-hour treatment with H$_2$O$_2$ at different concentrations. *P < 0.05 for comparison to ADSCs (n = 6). †P < 0.05 for comparison to β-gal-ADSCs (n = 6).

HO-1 Helps Autologous ADSCs Better Improve Infarcted Heart

In the follow-up period after the injection, no animals in HO-1-ADSCs group and ADSCs group were lost, but one animal in PBS group died.

**Improvement of left ventricle function in rabbits with myocardial infarction**

As shown in Table 1, echocardiographic parameters obtained on Day 0 and Day 13 did not reveal significant differences among the three groups. However, compared
with Day 0, the parameters on Day 13 illustrated a statistically significant decrease in cardiac functions (known as EF and FS) in all groups, indicating the validity of the MI model. In-group comparison of Day 0 and Day 42 values and that of Day 13 and Day 42 values showed significant deterioration in LV dimensions (known as LVEDD and LVESD) and relative maintenance in cardiac functions in PBS group, suggesting the presence of LV remodeling. Similar deteriorations in both EF and LVEDD values were found in ADSCs group, whereas improved EF values and marginal changes in LV dimensions were detected in HO-1-ADSCs group. Comparisons of the three groups on Day 42 revealed that LV dimensions were omnidirectionally improved as well as cardiac functions in HO-1-ADSCs group. For ADSCs group, there was partial improvement in FS and LVESD values, as compared with PBS group. Furthermore, ADSCs group was better than ADSCs group in EF values (P < 0.05).

Consistent with the results determined by echocardiography, cardiac function assessed by cardiac catheterization 4 weeks after cell transplantation also showed an improvement in HO-1-ADSCs group and ADSCs group (Table 2). The peak +dp/dt values were significantly higher and LVEDP was statistically lower in ADSCs group than in PBS group (P < 0.05). In HO-1-ADSCs group, not only values of peak +dp/dt and LVEDP, but also peak −dp/dt and heart rates, were significantly different from those in PBS group (P < 0.05). Moreover, LVEDP and peak −dp/dt were significantly higher in HO-1-ADSCs group compared with ADSCs group 4 weeks after cell transplantation (P < 0.05).

**Immunohistochemical staining for structural improvement**

Fig. 4A showed distributions of indicated pathological properties in each group. HE staining revealed that the myocardial structure in peri-infarcted area was more intact in both cell-treated groups, whereas the myocardial structure was chaotic in the control PBS-treated heart. No infil-

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### Table 1. Echocardiographic parameters of the groups during the protocol.

<table>
<thead>
<tr>
<th></th>
<th>Day 0 (before MI)</th>
<th>Day 13 (after MI)</th>
<th>Day 42 (after Impl)</th>
<th>P value</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HO-1-ADSCs group, n=9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF, %</td>
<td>61.6 ± 5.6</td>
<td>34.8 ± 3.1</td>
<td>43.5 ± 3.5*#</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FS, %</td>
<td>37.2 ± 5.8</td>
<td>29.1 ± 4.7</td>
<td>31.4 ± 3.6*</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>13.9 ± 1.1</td>
<td>15.0 ± 2.0</td>
<td>16.2 ± 2.5*</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>NS</td>
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<tr>
<td>LVESD, mm</td>
<td>8.9 ± 1.1</td>
<td>10.2 ± 1.3</td>
<td>11.2 ± 1.0*</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ADSCs group, n=9</strong></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>EF, %</td>
<td>61.0 ± 5.4</td>
<td>36.4 ± 3.7</td>
<td>40.5 ± 2.5</td>
<td>&lt; 0.01</td>
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<tr>
<td>FS, %</td>
<td>38.2 ± 5.1</td>
<td>31.9 ± 4.0</td>
<td>32.9 ± 4.3*</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>14.6 ± 1.5</td>
<td>15.1 ± 1.1</td>
<td>17.2 ± 2.3</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>&lt; 0.01</td>
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</tr>
<tr>
<td>LVESD, mm</td>
<td>9.2 ± 1.1</td>
<td>10.9 ± 2.1</td>
<td>12.3 ± 1.6*</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>&lt; 0.01</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PBS group, n=9</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>EF, %</td>
<td>59.3 ± 4.2</td>
<td>37.4 ± 3.2</td>
<td>37.9 ± 4.7</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>FS, %</td>
<td>36.8 ± 4.4</td>
<td>28.9 ± 5.8</td>
<td>26.5 ± 2.2</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>NS</td>
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<tr>
<td>LVEDD, mm</td>
<td>14.4 ± 1.0</td>
<td>15.0 ± 1.6</td>
<td>19.9 ± 4.0</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>&lt; 0.01</td>
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<tr>
<td>LVESD, mm</td>
<td>8.8 ± 1.1</td>
<td>10.1 ± 1.6</td>
<td>14.8 ± 2.5</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
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</table>

*P < 0.05 for comparison to PBS group on Day 42. #P < 0.05 for comparison to ADSCs group on Day 42. P1 refers to comparison of Day 13 and Day 0 values. P2 refers to comparison of Day 42 and Day 0 values. P3 refers to comparison of Day 42 and Day 13 values.

MI, myocardial infarction; EF, ejection fraction; FS, fractional shortening; Impl, implantation; LVEDD, left ventricle end-diastolic diameter; LVESD, left ventricle end-systolic diameter; NS, not significant.

### Table 2. Left ventricle hemodynamic parameters of the groups on Day 42.

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVEDP (mmHg)</th>
<th>Peak +dp/dt (mmHg/s)</th>
<th>Peak −dp/dt (mmHg/s)</th>
<th>Heart rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HO-1-ADSCs group</strong></td>
<td>4.52 ± 1.19*</td>
<td>2,476.73 ± 512.23*</td>
<td>2,096.35 ± 419.43*</td>
<td>171 ± 20*</td>
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<tr>
<td><strong>ADSCs group</strong></td>
<td>9.58 ± 2.37*</td>
<td>2,008.19 ± 534.74*</td>
<td>1,367.84 ± 365.07</td>
<td>195 ± 32</td>
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<tr>
<td><strong>PBS group</strong></td>
<td>13.17 ± 4.03</td>
<td>1,429.21 ± 455.75</td>
<td>1,102.87 ± 404.83</td>
<td>203 ± 21</td>
</tr>
</tbody>
</table>

*P < 0.05 for comparison to PBS group. #P < 0.05 for comparison to ADSCs group. LVEDP, left ventricular end-diastolic pressure; +dp/dt, maximum rate of rise in left ventricular pressure during systole; −dp/dt, maximum rate of decrease in left ventricular pressure during diastole.
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tration of lymphocytes and small macrophages were observed within cells in the injected region. Interestingly, mature adipose tissue was observed in the infarcted area in all three groups. In Fig. 4B, Masson’s trichrome staining of the gross specimens illuminated remarkable wall thinning in the infarction sites and LV dilation in PBS group (mean infarcted size in the heart was 37.7 ± 2.4% of the LV). Nevertheless, both cell-treated groups attenuated the development of wall thinning and the enlargement of left ventricular lumen. ADSCs transplantation demonstrated a modest protective effect, limiting the infarcted size to 34.6 ± 2.8% of the LV. Furthermore, transplantation of HO-1-ADSCs significantly limited infarcted size to 28.8 ± 3.1% of the LV (p < 0.01 compared with the two other groups).

Neovascularization in the infarcted myocardium after the treatment was examined as described Materials and Methods. Quantitative analyses revealed that microvessel density in the HO-1-ADSCs group was significantly higher than that of the values obtained for the heart in ADSCs group and PBS group (p < 0.01) and that the microvessel density of the heart in ADSCs group was significantly higher than that of the heart in PBS group (p < 0.01) (Fig. 4C).

The expression of Cx43 in peri-infarcted areas at 1 month post-injection was determined to characterize the properties of these myocytes further. In the quantification of Cx43-positive gap junction at 432 digital images on 27 slides immunostained with Cx43, the percent areas of Cx43-positive gap junctions did not significantly differ between ADSCs group (2.11 ± 0.74%) and PBS group (1.93 ± 0.97%). However, the density of Cx43-positive gap junction was higher in HO-1-ADSCs group (2.72 ± 1.29%) compared with other two groups. (Fig. 4D)

TH-positive nerve density was significantly higher in both cells-treated groups. By contrast, PBS group rarely exhibited TH-positive sympathetic nerve twigs. The calculated percent areas of cardiac sympathetic nerves in the tissue of HO-1-ADSCs group (0.71 ± 0.58%) was signifi-

Fig. 4. Structural improvement by histochemical evaluation.
(A) Immunostaining of ventricular tissue in the borderline region of myocardial infarction for HE, Cx43, TH and fVIII at indicated magnification power. Furthermore, calculated percent areas of infarct size (A), Cx43-positive gap junction (B), tyrosine hydroxylase-positive cardiac sympathetic nerves (C) and vascular density (D) was performed respectively among groups. The arrowheads indicated positive regions. Bar = 50 μm. Error bars represented s.d. (n = 6). HE, Hematoxylin and Eosin; Cx43, Connexin 43; TH, tyrosine hydroxylase; fVIII, factor VIII.
cantly higher than those found in the ADSCs group (0.54 ± 0.62%, \( p < 0.01 \)) and PBS group (0.23 ± 0.27%, \( p < 0.05 \)). (Fig. 4E)

**Immunofluorescence staining for In vivo differentiation by HO-1-ADSCs**

The cTnT immunofluorescence staining of CM-Dil-labeled ADSCs at 4 weeks post-transplantation occasionally appeared to show co-localization of CM-Dil-positive cells and cTnT-positive ones in the infarcted areas. In addition, engrafted CM-Dil-positive cells were found to be vWAg positive (D-F) or SMA positive (G-I). The arrowheads indicate positive regions. Bar = 50 \( \mu \)m. ADSCs, adipose-derived stem cells; CM-Dil, chloromethyl-1,1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; cTnT, cardiac troponin T; SMA, smooth muscle \( \alpha \)-actin.

![Fig. 5. Immunofluorescence staining for differentiation of HO-1-ADSCs in infarcted heart.](image)

(A-C) After four weeks of transplantation, it occasionally appeared to show co-localization of both CM-Dil-positive and cTnT-positive cells in the infarcted areas. In addition, engrafted CM-Dil-positive cells were found to be vWAg positive (D-F) or SMA positive (G-I). The arrowheads indicate positive regions. Bar = 50 \( \mu \)m. ADSCs, adipose-derived stem cells; CM-Dil, chloromethyl-1,1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; cTnT, cardiac troponin T; SMA, smooth muscle \( \alpha \)-actin.

**Discussion**

This study has demonstrated the favorable effects of autologous ADSC transplantation on cardiac function and ventricular remodeling in a rabbit MI model. Cellular cardiomyoplasty, which seek to replace or regenerate cardiomyocytes through cell transplantation, may be achieved in several ways, at least including transplanting stem cells that differentiate into cardiomyocytes or promote angiogenesis (Segers and Lee 2008), protecting cardiomyocytes as well as vascular endothelial cells via a paracrine mechanism (Takahashi et al. 2006) and mobilizing autologous resident stem cells to the site of injury by using cytokines (Zhao et al. 2009). Furthermore, electrophysiological stabilization and neural structure, referring to as gap junction and nerve sprouting respectively, are indispensable for evaluating effects and safety of cellular therapy in heart disease, especially in large animal and in humans (Cai et al. 2009).

For better imitating clinical situation, autologous adipose samples were obtained at the same moment MI was induced in this study. *In vitro* culture for isolated stromal vascular fraction could result in purity of mesenchymal property, accounting for characteristics of ADSCs, as shown in Fig. 1. Allogeneic stem cells, in spite of their paracrine protection abilities, reportedly have poor survival and subsequent differentiation after transplantation (Dai et al. 2005). Certain types of autologous stem cells had been
rendered as effectiveness for heart repair with ease in cell isolation and delivery (Lin et al. 2004; Tokac et al. 2010). The present study confirms that autologous ADSCs and their use in ethical procedures (Gimble et al. 2007) are effective and safe. Also, our experiments had identified time-window of the HO-1 expression as well as similar cell viabilities before transplantation among groups (data not shown). Furthermore, in vitro culture for 12 days after isolation can lead to sufficient numbers of cells for implantation, showing that clinical application of the experimental procedure is feasible.

Theoretically, the empty vector effects can be definitely determined by setting up a $\beta$-gal-ADSCs injection group. It is easy to perform that null-ADSCs group in small animals’ model like mice and rats. However, for large animal including rabbits and pigs, it is practically difficult and ethically inappropriate to carry out, which makes it worse when performing autologous stem cell transplantation. Moreover, in vitro study of our work had illustrated non-significant differences on anti-ROS ability between $\beta$-gal-ADSCs and ADSCs. Based on the consideration to facilitate autologous cell transplantation in a more practical state, the establishment of a separate set of $\beta$-gal-ADSCs cells was not involved in the study design.

Many studies used ligation of the left anterior descending artery (Tang et al. 2009), whereas others employed modified methods for different targets (Feng et al. 2009). From the anatomical viewpoint of the rabbit heart, the myocardial region supplied by anterior descending artery is relatively limited (Podesser et al. 1997), indicating that it might narrow the potential superiority of cellular cardiomyoplasty. Previous research had identified lateral artery as major vascular of left ventricle, such ligation in mid-level of this vessel can provided a reliable and safe model of rabbit myocardial infarction. The present study validated this model by echocardiography and pathological assessment. Such a remarkable infarct model was considered more appropriate. In addition, the timing of 1-2 weeks after myocardial infarction was generally viewed as optimum for cell transplantation (Bartunek et al. 2006).

Pathophysiologically, anatomic change helps preservation of heart function, but it can also finally cause functional deterioration. Once anatomic decompensation occurs, only minor structural deterioration may dramatically alter function. The present study did not find any statistically improvement for either EF or LVEDD in ADSCs group compared with PBS group, but we observed mild functional preservation of FS as well as LVESD, suggesting that direct ADSCs transplantation may just improved systolic function. As is known, cardiac remodeling after myocardial infarction was characterized as diastolic ventricular enlargement and function deterioration. It was also indicated that systolic function repair was insufficient to provide better improvement. Nevertheless, remarkable structural and functional modifications for both systolic and diastolic stage occurred in HO-1-ADSCs group, and these were further confirmed by hemodynamic evaluation. The data suggested that ADSCs combined with over-expressed HO-1 brought forward better amelioration in heart remodeling than ADSCs group. In addition, we demonstrated that the genetically modified ADSCs with HO-1 were more resistant to apoptosis induced by $\text{H}_2\text{O}_2$, characterizing another more preferable effect of ADSCs in the rabbit MI model.

Gene modification has been widely performed in pre-clinical studies related to cellular therapy, especially in bone-marrow MSCs. Via transduction with certain protective cytokines or variants, such as VEGF (Tang et al. 2010), IGF (Haider et al. 2008) and AKT (Gneechi et al. 2005), among others. As to the ADSCs, lentiviral transduction was used for labeling in tracking cell detain, as previously published (Bai et al. 2010). The present study found that a MOI of 100, which was established on a preliminary experiment (data not shown), was able to yield highly efficient transduction and amplified expression of HO-1 protein. A previous study related to HO-1 overexpression had shown that a hypoxia-regulated HO-1 vector improved grafted MSCs survival in ischemic heart and enhanced cardiac function (Tang et al. 2005). In addition, Zeng et al. (2008) found that HO-1 overexpression confers anti-inflammation and anti-fibrogenic properties on infarcted myocardium, and that the paracrine factors secreted from HO-1-modified MSCs dramatically limited infarct size and apoptosis in a rat MI model. Characterization of these released factors may have an important implication in elucidating the repair mechanism of stem cells. Cells modified by HO-1 have also been shown to secrete multiple angiogenic growth factors, such as vascular endothelial growth factor and hepatocyte growth factor, which might be suitable for regenerative cell therapy for ischemic diseases. In addition, one in vitro study has shown that HO-1 can resist injuries through scavenging intracellular ROS in certain cells (Mamiya et al. 2008). The in vitro results of the present study suggested that adenoviral transfer of HO-1 may contribute to the protection of ischemic myocardium, possibly by decreasing ROS. The long-term improvement of functional and histological changes cannot rule out the role of HO-1, but HO-1 expression time is limited. Long-term changes, including structure and function, should be more likely to result from stem cells protection, such as paracrine mechanisms and cell differentiation. In addition, in vitro $\text{H}_2\text{O}_2$ study just touched on one potential mechanism which requires further study.

It was perceived by some researchers that cardiac nerve sprouting potentially mediated electrophysiological turbulence resulting in malignant cardiac arrhythmia (Cao et al. 2000), whereas other scholars precluded higher incidences of arrhythmia via studies of nerve sprouting alone or in combination with a pacemaker (Kim et al. 2004). The gap junction was generally referred to as an essential marker for effective treatment of cellular cardiomyoplasty (Valiunas et al. 2004), rendering that differentiation from
ADSCs to cardiomyocytes might be insufficient for functional improvement. We did not detect any malignant cardiac arrhythmia during procedure as well as auxiliary tests. Specifically, changes in cardiac nerve innervations were not observed in ADSCs group, suggesting that death of acutely implanted ADSCs caused low retention of cells in infarcted regions and, consequently, insufficient cardiac innervations. When ADSCs survive better in acute stage of implantation, as designed in HO-1-ADSCs group, both nerve sprouting and gap junction increased, consistent with structural and functional improvement. Therefore, any method aimed at elevating cardiac innervations and gap junction will certainly benefit in suppression of cardiac remodeling and preservation of heart function (Hahn et al. 2008). A recent study reported reduced gap-junction expression and delayed local conduction at sites of human MSC transplantation (Kim et al. 2010). However, issues for the cell to cell coupling with the recipient heart in that study were not be resolved, suggesting that reduced gap-junction may result from allogenic suppression and low implanted cell retention.

In conclusion this study further supported the role of autologous ADSCs in cellular therapy after MI, and suggested that HO-1 modification to ADSCs could enhance the effectiveness of the therapy both in function and structure.

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Conflict of Interest

The authors have no conflict of interest.

References


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