Original Research

Preservation of the cardiac function in infarcted rat hearts by the transplantation of adipose-derived stem cells with injectable fibrin scaffolds

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Abstract

Cell-based therapy can improve cardiac function but is limited by the low cell retention and survival within ischemic tissues. Injectable cardiac tissue engineering aims to support cell-based therapies and enhance their efficacy for cardiac diseases. So far, no research has been devoted to studying the usefulness of the combination of fibrin glue (as scaffold) and adipose-derived stem cells (ADSCs) to treat myocardial infarction. In our study, the rat ADSCs were isolated from subcutaneous adipose tissues. The surface phenotype of these cells was analyzed by flow cytometry. The fibrin glue was then co-injected with ADSCs into the left ventricular wall of rat infarction models. The structure and functional consequences of transplantation were determined by detailed histological analysis and echocardiography. Most cultured ADSCs expressed CD105 and CD90, and were negative for CD34 and CD45. After injection, both the 24-h cell retention and four-week graft size were significantly higher and larger in the Fibrin + ADSCs group than those of the ADSCs group alone ($P < 0.01$). The heart function improved significantly in the Fibrin + ADSCs group compared with that of the ADSCs group four weeks after transplantation ($P < 0.01$). In addition, the arteriole densities within the infarcted area improved significantly in the Fibrin + ADSCs group compared with those in the ADSCs group four weeks after transplantation ($P < 0.01$). In conclusion, the ADSCs with the fibrin glue has the therapeutic potential to improve the function of infarcted hearts. The method of *in situ* injectable tissue engineering combining fibrin glue with ADSCs is promising clinically.

Keywords: adipose-derived stem cells, fibrin, tissue engineering, myocardial infarction

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Introduction

Myocardial infarction (MI) is a major cause of death worldwide. It results in cardiomyocyte loss, myocardial remodeling and the damage of cardiac function. These changes are associated with cardiac dysfunction and the progression to heart failure.[1] However, current therapies can only stall the progression of the disease. The shortage of heart donors is also a factor for innovating new therapies. Thus, new therapies are needed to regenerate damaged hearts to overcome the poor prognosis of patients with heart failure.

For nearly a decade, researchers have investigated the possibility of cell transplantation for cardiac repairs.[2] Both embryonic and adult stem cells have been regarded as candidate cells for cell therapy.[3,4] Unfortunately, ethical issues and potential problems with cell regulation have limited the use of embryonic stem cells. Bone marrow-derived mesenchymal stem cells (BM-MSCs) seem to have received most attention for cell therapy.[5,6] However, their number and maximal life span decline with increasing age.[2,7] The acquisition of BM-MSCs is a highly invasive procedure limiting the widespread use of BM-MSCs in cell therapy. Adipose tissue-derived stem cells (ADSCs) have a high proliferating potential, too.[8] In contrast, these cells can be obtained by minimally invasive procedures from subcutaneous fat. They can differentiate into multiple cell phenotypes, including cardiac myocyte, endothelial, bone, cartilage and neural progenitors.[9–11] It has been demonstrated that the transplantation of adipose-derived stem cells can induce angiogenesis, reverse wall thinning and improve cardiac function for animal models of MI.[12–14]

However, their poor viability,[15,16] limited cell retention and the failure to regulate the microenvironment[17] of the transplanted areas have obstructed the therapeutic potential of ADSCs. Since biomaterials can improve cell retention, survival and differentiation, they are widely applied in cardiac tissue engineering to support cell-based therapies and enhance their efficacy for cardiac diseases. In the last decade, cardiac tissue engineering has made considerable progress. Out of a variety of approaches in cardiac tissue engineering, the one of injectable cardiac tissue engineering is more minimally invasive than other approaches, such as *in vitro*-engineered tissue or epicardial patch implantation. It is, therefore, more clinically appealing.

Many biocompatible biomaterials have been used in injectable cardiac tissue engineering, including fibrin, chitosan, alginate and dextran.[18–20] Among them, fibrin is an ideal candidate. Fibrin, a critical blood component responsible for hemostasis, has been used extensively as a biopolymer scaffold in tissue engineering. It has many forms with different applications in tissue engineering, e.g. fibrin glue, fibrin hydrogels and fibrin microbeads.[21] The fibrin glue (approved by the United States Food and Drug Administration for certain human clinical applications) consists primarily of two components: enriched fibrinogen and thrombin. It is commonly served as a delivery vehicle in injectable cardiac tissue engineering.[22] Christman and Lee[22] discovered improved cell survival upon the transplantation of cells delivered by fibrin glue compared to the injection by cell alone. The fibrin glue was also shown to induce neovascularization within the ischemic myocardium and reduce infarct expansion.[23] More recently, Martens *et al.*[24] evaluated the utility of commercially available percutaneous catheters for the delivery of viscous cell (human bone marrow-derived mesenchymal stem cells)/fibrin glue suspensions. The results demonstrated the *in vivo* effectiveness of this preparedness and its ability to increase cell retention and survival in a nude rat model of MI. In addition to cell delivery, fibrin glue could also be used as a matrix to deliver therapeutic angiogenic agents into ischemic myocardium.[25] Nevertheless, no topic has addressed the usefulness of the injectable fibrin scaffold as a vehicle to deliver ADSCs for the treatment of the infarcted heart.

In this study, the injectable fibrin scaffold was used for ADSCs to test whether the combination of fibrin glue and ADSCs cells could improve cell retention, cell survival, angiogenesis, ventricular remodeling within the ischemic myocardium and cardiac function for a rat model of MI.

Materials and methods

Isolation and culture of ADSCs

Subcutaneous adipose tissue in the inguinal groove was harvested from female Sprague-Dawley (SD) rats (4-week-old). All the rats were purchased from the Experimental Animal Center, Academy of Military Medical Science (Beijing, China). All experiments in this study were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Military Medical Science, Beijing, China. Efforts were made to limit the number of animals used and minimize their suffering. Animals were individually housed in cages (accessible to water and food) with a room temperature of $24 \pm 2^{\circ}C$ (a normal day/night cycle). After anesthetization with sodium pentobarbital at 35 mg/kg, and incision in the skin, the fat in the groin was removed using a scalpel. The ADSCs were isolated from the adipose tissue as described by Zuk *et al.*[8] Briefly, the adipose tissue was washed three times with 0.1 mol/L phosphate-buffered saline (PBS, pH = 7.4), and then minced finely and digested with 0.1% collagnase type I (m/v, Sigma, St Louis, MO, USA) at 37°C for 45 min. After filtration with mesh filter and centrifugation at 800 *g* for eight minutes, we suspended isolated cells in a-MEM (α-MEM, GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and antibiotics, plated them onto a 100-mm dish and incubated them at 37°C with 5% CO_2. A small number of spindle-shaped cells were apparent in visible symmetric colonies by days 5–7.

The ADSCs were harvested for the third passage by flow cytometry analysis. The cells were washed in flow cytometry buffer (FBS, Biolegend, San Diego, CA, USA) and incubated for 30 min in FBS containing the following antibodies: fluorescein isothiocyanate (FITC)-conjugated antiCD105 (Biolegend), FITC-conjugated antiCD90 (Biolegend), phycoerythrin-conjugated antiCD34 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), FITC-conjugated anti-CD45 (Biolegend). For unlabelled primary antibodies, FITC-conjugated secondary antibodies were added. All analyses were performed using a BD flow cytometer (BD Bioscience, San Jose, CA, USA).

The ADSCs were labeled with nuclear dye 4′6-diamidino-2-phenylindole (DAPI, Sigma) before injection. A total of 1×10^7 cells were incubated with DAPI (a final concentration was 50 μg/mL) for 30 min at room temperature.

Fibrin glue

The fibrin glue was prepared according to previous reports.[26] Briefly, the fibrin glue used in this study was the commercially available fibrin sealant (Baxter Healthcare Corp., Los Angeles, CA, USA). Two component systems remained liquid for a couple of seconds before solidifying into a semi-rigid gel matrix. The first component includes concentrated fibrinogen and aprotinin, a fibrinolysis inhibitor. The second contains a mixture of thrombin and $CaCl_2$. It is mixed and delivered simultaneously by applying the supplied Duploject applicator. The ratio of fibrinogen to thrombin components was 1:1.

Cell viability in fibrin glue

A LIVE/DEAD stain was performed as described by Christman *et al.*[26] 100 μL of the thrombin solution containing 1×10^5 adipose-derived stem cells was combined with 100 μL of the fibrinogen solution and plated onto four-well culture plates. Culture media were added to the plates and changed every three days. At 24 and 72 h in culture, a LIVE/DEAD stain (Molecular Probes, Eugene, OR, USA) was used to label live and dead cells. Each culture time point was

performed in duplicate. Five microliters of 2 mmol/L ethidium homodimer-1 solution and 1.25 μL of 4 mmol/L calcein were added to 5 mL of PBS. Each plate was incubated with 200 μL of this solution for 30 min. To distinguish live from dead cells, the ADSC–fibrin glue cultures were then rinsed with PBS and examined using a LEICA-DMIRB fluorescent microscope (Germany). Images were then analyzed in Matlab using codes written to allow automated unbiased counting of red (dead) and green (live) ADSCs at 24 and 72 h.

Myocardial infarction model

An ischemia model for this study was described by Miyahara *et al*.[27] Briefly, female SD rats (225–250 g) were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally), and limb-lead electrocardiography was performed. The animals were then incubated and ventilated by a volume-regulated respirator during surgery. The surgical approach involved a left lateral thoracotomy as well as pericardectomy and identification of the left anterior descending coronary artery for permanent ligation. The left coronary artery was ligated with a 6-0 prolene suture in a place 2–3 mm far from its origin between the pulmonary artery conus and the left atrium. Subsequently, the infarcted area of the left ventricle (LV) paled immediately and beat weakly. The electrocardiography showed MI waves (typical ST-segment changes). Finally, the chest was closed. The 25–35% mortality was observed within the first 24 h following occlusion. The surviving rats were maintained on standard rat chow and water *ad libitum*. This laboratory has extensive experience with this model, and histological observation shows that the technique results in an acute infarction size of approximately 40–60% of the LV.

Injection surgeries

One week after MI, rats were randomized into four groups with the inclusion criterion of fractional shortening (FS) $<40\%$ by echocardiography one week after MI. Injections were performed along the border zone in two divided injections of 50 μL each through a 28-gauge needle. A volume of 100 μL of PBS was injected into the ischemic LV in the first group (group PBS, control group, $n = 10$), and 50 μL of fibrinogen and 50 μL of thrombin were simultaneously injected into ischemic myocardium in the second group

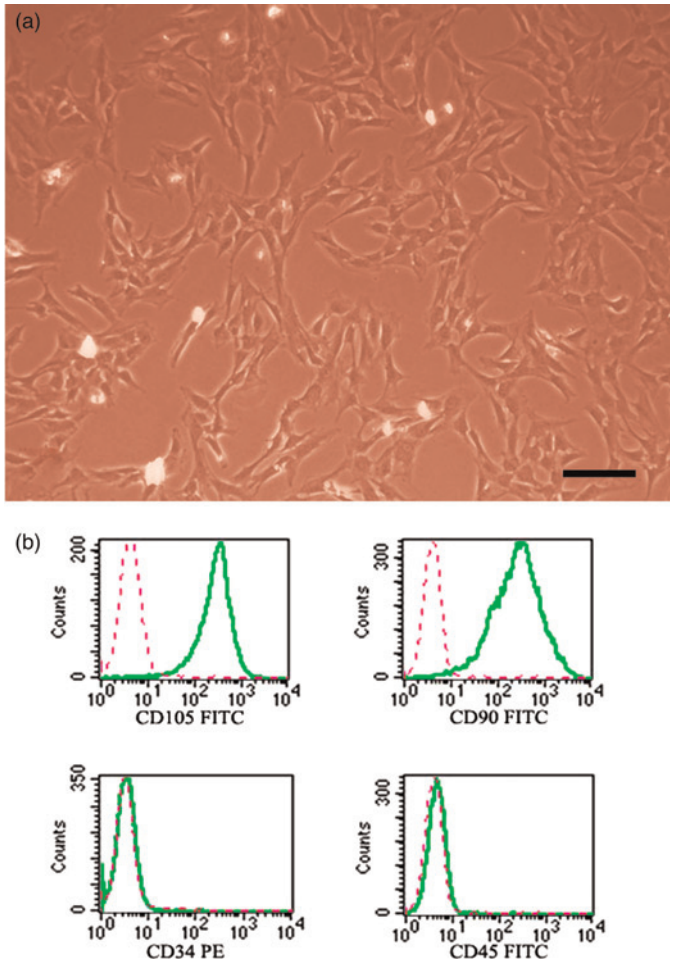

Figure 1 Characterization of adipose-derived stem cells. (a) The morphology of ADSCs. ADSCs expanded easily *in vitro* and exhibited a fibroblast-like morphology. Bars = 50 μm. (b) The flow cytometry analysis indicated that most of the cells expressed CD105 and CD90; the majority of cells were negative for CD34 and CD45. ADSCs, adipose-derived stem cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin (A color version of this figure is available in the online journal)

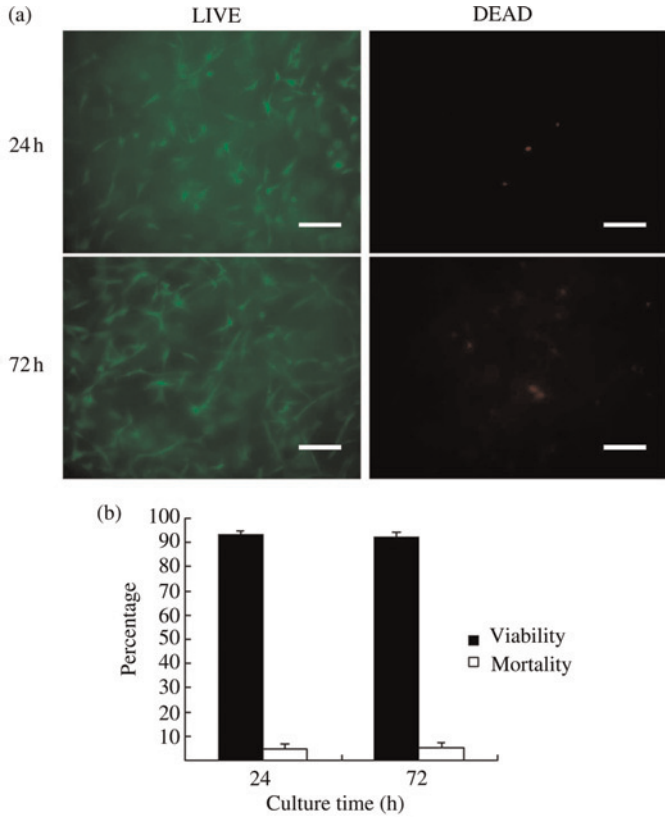

Figure 2 Cell viability in fibrin glue. (a) LIVE/DEAD stain of ADSCs at 24 and 72 h after culture in a fibrin glue (live cells stain green, dead cells stain red, bars = 100 μm). (b) ADSCs cultured in fibrin glue maintained $>90\%$ viability at 24 and 72 h. Cell death was rare in fibrin glue. ADSCs, adipose-derived stem cells (A color version of this figure is available in the online journal)

(group Fibrin, $n = 10$). A volume of 5×10^6 ADSCs in 100 μL of PBS was injected into the infarct in the third group (group ADSCs, $n = 18$, 8 for 24-h cell retention observation). A volume of 5×10^6 ADSCs in 100 μL of fibrin glue was injected into the infarct in the fourth group (group Fibrin + ADSCs, $n = 18$, 8 for 24-h cell retention observation). In the Fibrin + ADSCs group, ADSCs were suspended in 50 μL of the thrombin component, the thrombin-cell mixture was simultaneously injected into the myocardium with 50 μL of the fibrinogen component through supplied Duploject applicator. Injections were performed at an angle to reduce the chance of the injection into the lumen of the LV and verified by a slight lightening in the color of the myocardium as the solutions entered the infarcted wall.

Echocardiography

Four weeks after transplantation, animals were anesthetized for function detection with echocardiography (14.0 MHz, Sequoia 512; Acuson, München, Germany). Parasternal short axis views with M-mode were acquired at the ventricular base immediately distal to the mitral valve. LV end-diastolic diameter (EDD) and LV end-systolic diameter (ESD) were measured. LV FS and LV ejection fraction (EF) were calculated as follows: FS (%) = [(EDD − ESD)/EDD] × 100; EF (%) = [(EDD^3 − ESD^3)/EDD^3] × 100.[28] All procedures and analyses were performed by an experienced researcher to whom they were blinded. The animals were euthanized after functional measurements with an overdose of sodium pentobarbital in compliance with the above guidelines.

Histopathology

The hearts were excised rapidly and frozen in optimal cutting temperature freezing medium freshly. The sections (5 μm) were stained with masson trichrome (Sigma).

Five sections (5 μm), equally distributed through the infarcted area (from base to apex in an interval of 1 mm), were taken from each heart as representative samples. Each of them was measured for the presence of DAPI-labeled transplanted cells in 24 h and four-week postinjection. To measure cell retention within the myocardium in 24 h and graft size after four weeks of implantation, the area covered by the DAPI-labeled ADSCs was traced using a fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan). The ratio of area covered by DAPI-labeled cells on the infarcted

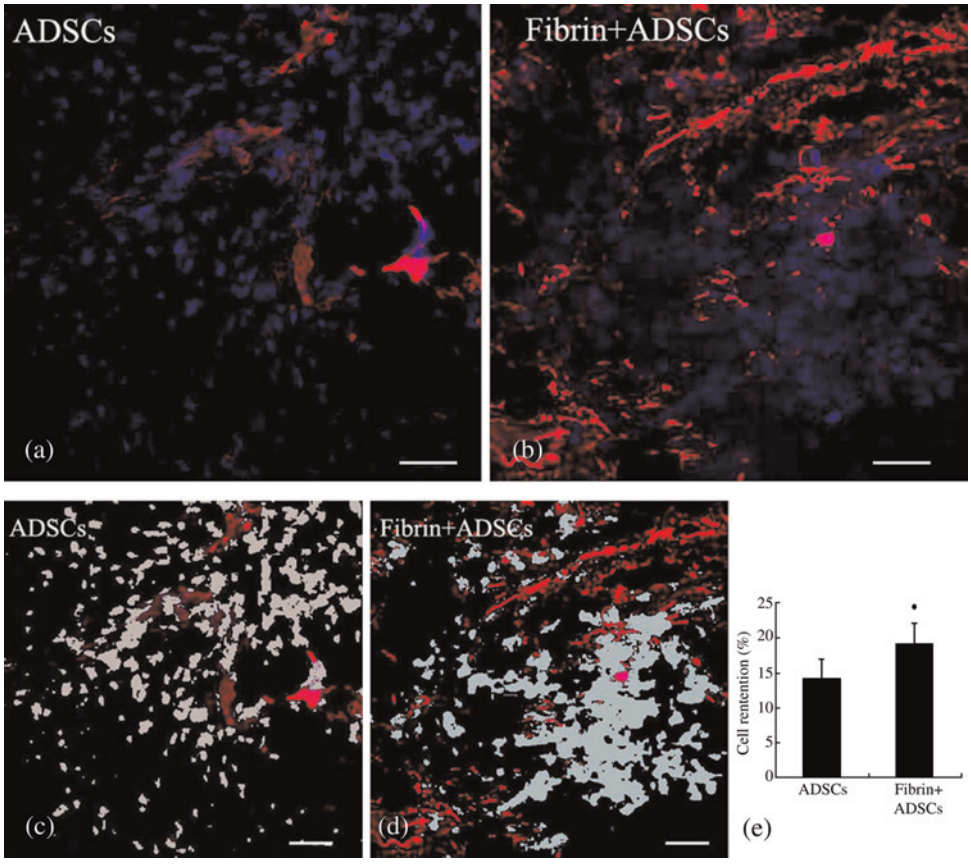

Figure 3 DAPI-labeled ADSCs in infarct area after 24 h of injection. (a) ADSCs group, DAPI-labeled ADSCs were scattered in the infarcted area. (b) Fibrin + ADSCs group, DAPI-labeled ADSCs were accumulated in infarcted area. Blue fluorescence illustrated DAPI-labeled ADSCs, red fluorescence illustrated actin-positive myocardium. Gray areas, marked by the RS image Pro, in (c and d) show the distributions of transplanted cells of (a) and (b) separately. (e) Diagram showing the statistical results. After 24 h of injection, ADSCs injected in PBS covered 14.16 ± 2.73% of the infarct, whereas ADSCs injected in fibrin glue covered 19.10 ± 3.13%. A significant difference exists between two groups ($P < 0.01$). $^*P < 0.01$ compared with ADSCs group. Bars = 50 μm. ADSCs, adipose-derived stem cells; DAPI, 4′6-diamidino-2-phenylindole; PBS, phosphate-buffered saline (A color version of this figure is available in the online journal)

area in section was measured by using RS Image Pro (version 4.5; Media Cybernetics, Inc., Trenton, NJ, USA). Cell retention and graft size were reported as percentage of DAPI labeling area in infarcted area.

The infarcted fraction of the LV was calculated according to Pfeffer *et al.*[29] The fraction of the LV infarcted was calculated from photographs of gross heart slices by measuring the length of the infarcted endocardial circumference and the entire endocardial circumference. Infarct size was reported as percentage of length of the infarcted endocardial circumference in the length of the entire endocardial circumference. The measurements from each slice were averaged together in every heart and including a zero measurement when the scar did not reach the most basal sections.

Immunofluorescent and immunohistochemical staining

Immunofluorescent staining was performed to identify myocardium using sarcomeric actin antibody. The differentiation of ADSCs in the infarcted heart was identified using cardiac troponin T (cTnT), vWAg antibody and smooth muscle α-actin (SMA). Blood vessel formation was detected by vWAg antibody.

Cryosections were fixed with acetone and endogenous peroxide activity was quenched with 3% H_2O_2. After blocking with 2% normal goat serum, sections were incubated with the primary antibodies (sarcomeric actin, Sigma, 1:200; cTnT, Sigma, 1:200; vWAg, Zymed, South San Francisco, CA, USA, 1:200; SMA, Sigma, 1:200) at 4°C overnight. Then, FITC or Cy3-conjugated IgG (Sigma, 1:100) were incubated for one hour at room temperature before observing under an Olympus FV1000S confocal laser microscopy. For immunohistochemical staining, sections were incubated with peroxidase-conjugated streptavidin and stained with diaminbenzidine. Negative controls were performed by omitting primary antibodies.

The number of arterioles was determined in the sections immunohistochemically stained with vWAg antibody. Five sections (5 μm), equally distributed through the infarcted area (from base to apex in an interval of 1 mm), were taken from each heart in the four groups: PBS group, Fibrin group, ADSCs group and Fibrin + ADSCs group after four weeks of injection. Five high magnification fields within the infarcted region of each section were chosen randomly. Arteriole densities were calculated accordingly. Arterioles in each section were quantified using the following criteria: (1) positive for vessel

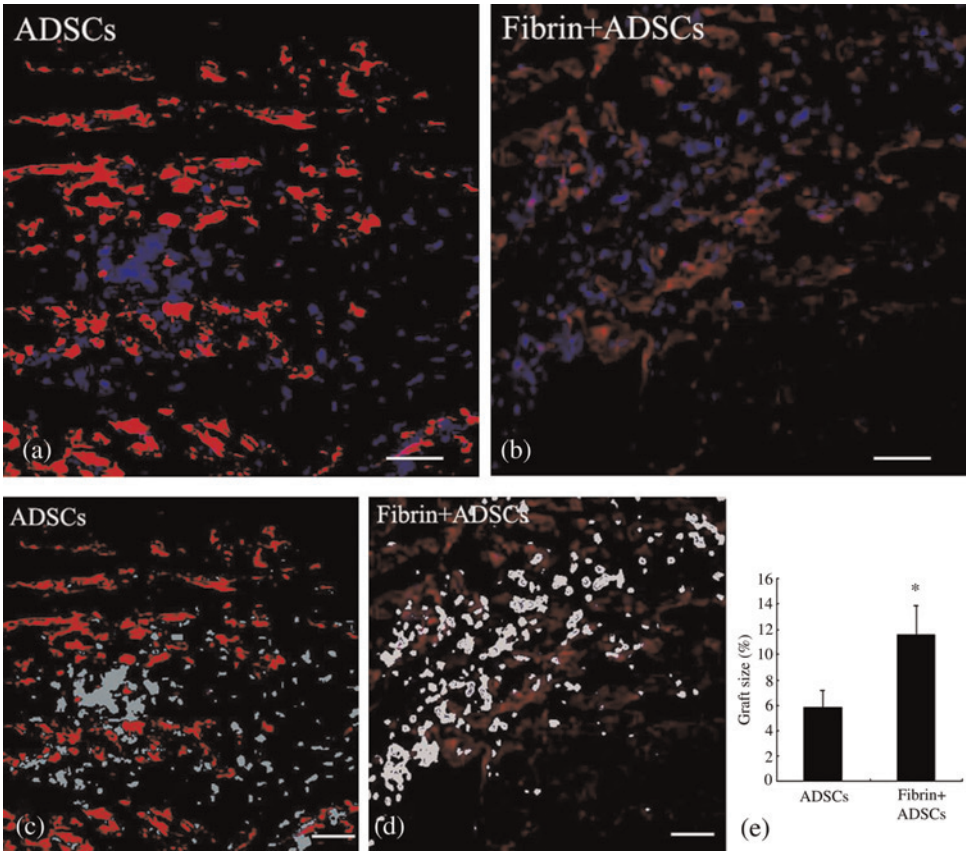

Figure 4 DAPI-labeled ADSCs after four weeks of injection. (a) ADSCs group, DAPI-labeled ADSCs were scattered in the infarcted area. (b) Fibrin + ADSCs group, DAPI-labeled ADSCs were accumulated in the infarcted area. Blue fluorescence illustrated DAPI-labeled ADSCs, red fluorescence illustrated actin-positive myocardium. Gray areas, marked by the RS image Pro, in (c and d) showed the graft size of (a) and (b) separately. (e) Diagram showing the statistical results. After four weeks, the ADSC population upon the injection in the fibrin glue significantly outnumbered that in the infarcted area upon the injection in PBS. The cells injected in the fibrin glue covered 11.52 ± 2.34% of the infarcted areas. In contrast, only 5.85 ± 1.35% was covered by the cells injected with PBS. $^*P < 0.01$ compared with ADSCs group. Bars = 50 μm. ADSCs, adipose-derived stem cells; DAPI, 4′6-diamidino-2-phenylindole; PBS, phosphate-buffered saline (A color version of this figure is available in the online journal)

endothelium labeling; (2) within the infarct scar; (3) a visible lumen; and (4) a diameter between 20 and 100 μm. The measurements from each slice were averaged together in every heart and including a zero measurement when the scar did not reach the most basal sections.

Statistical analysis

Cell retention and graft size were compared using the Student's *t*-test. Analysis of variance and Turkey's multiple comparison tests were used to determine possible significant differences in the cardiac function, wall thickness, infarct size and vessel density between groups. Statistical analyses were performed with SPSS 13.0. The significance was accepted at $P < 0.05$. The results are reported as mean $\pm$ standard deviation.

Results

Characterization of adipose-derived stem cells

The ADSCs were isolated from the subcutaneous adipose tissue of rats on the basis of adherent properties of these cells. Cells expanded easily *in vitro* and exhibited a fibroblast-like morphology (Figure 1a). After approximately three to four passages, most adherent cells expressed CD105 and CD90. In contrast, the majority of adherent cells were negative for CD34 and CD45 (Figure 1b). These results were similar to those from BM-MSCs.[30] By adopting methods reported previously,[31] we have confirmed that these adipose-derived adherent cells were multipotent, as evidenced by their ability to differentiate into adipocytes and osteoblasts (data not shown). Thus, we confirmed that the majority of adherent cells isolated from adipose tissue were ADSCs.

Cell viability in fibrin glue

LIVE/DEAD stain demonstrated that ADSCs survived and proliferated well in fibrin glue (Figure 2a). It showed normal morphology with ADSCs maintaining >90% viability at 24 and 72 h (Figure 2b). Cell death was rare in fibrin glue.

Cell retention and graft size

After 24 h of injection, ADSCs injected in PBS covered 14.16 $\pm$ 2.73% of the infarct (Figure 3a), whereas ADSCs

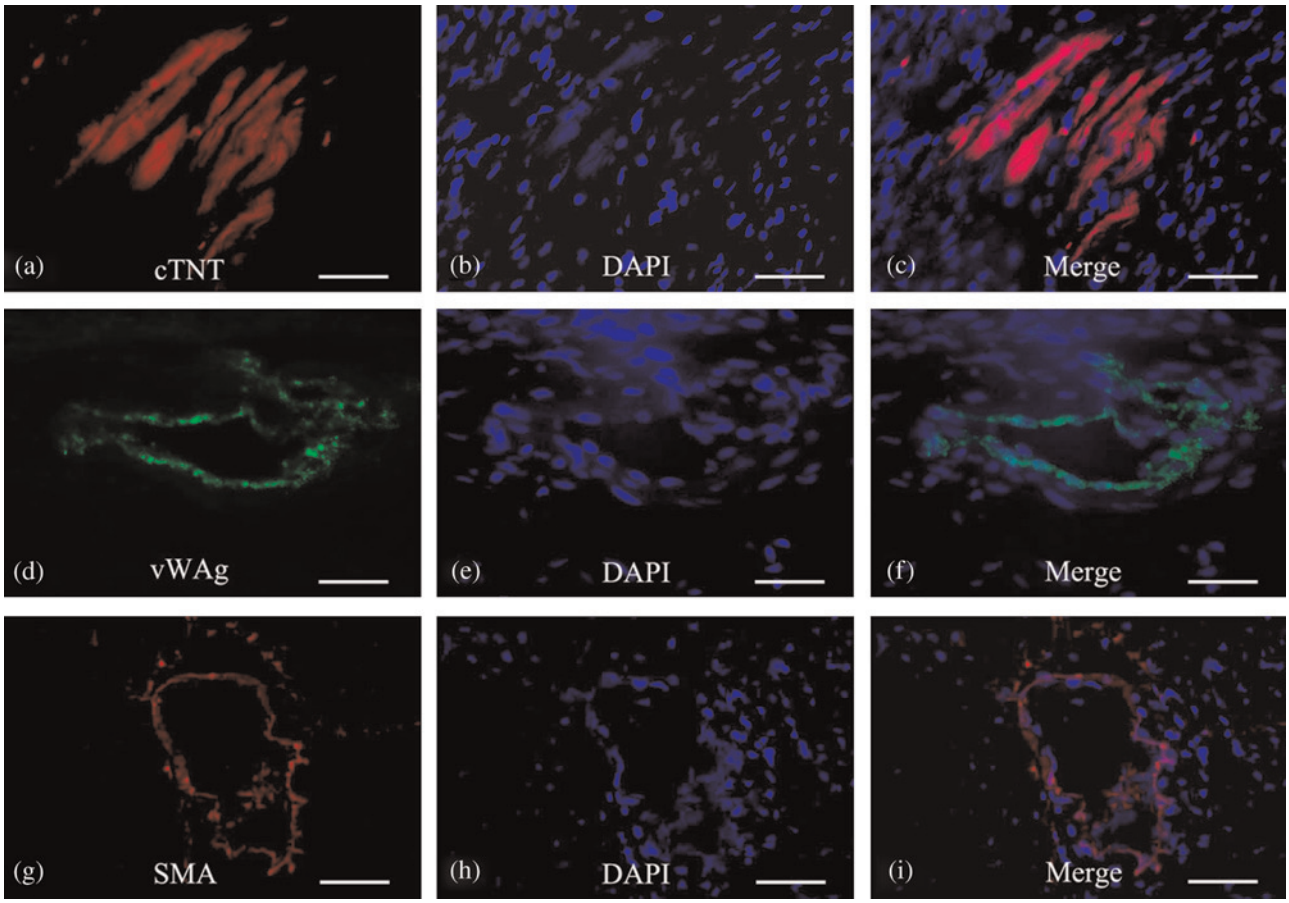

Figure 5 Differentiation of ADSCs in infarcted heart. (a–c) The cTnT immunofluorescence staining of DAPI-labeled ADSCs at four weeks of transplantation occasionally appeared to show co-localization of both DAPI-positive and cTnT-positive cells in the infarcted areas. In addition, occasional DAPI-positive cells were found to be vWAg- (d–f) or SMA- (g–i) positive, suggesting that they formed small blood vessels within the infarcted zone. The findings suggested the potential ability of transplanted ADSCs to differentiate into cardiomyocyte-like, endothelial and vascular smooth muscle cells. More efforts are needed to confirm this finding. Bars = 20 μm. ADSCs, adipose-derived stem cells; DAPI, 4′6-diamidino-2-phenylindole; cTnT, cardiac troponin T; SMA, smooth muscle α-actin (A color version of this figure is available in the online journal)

injected in fibrin glue, 19.10 ± 3.13% (Figure 3b). A significant difference exists between the two groups ($P < 0.01$) (Figure 3e). After four weeks, the ADSC population in the infarcted area, upon injection in the fibrin glue, significantly outnumbered that in the infarcted area upon injection in PBS (Figure 4). The cells injected in fibrin glue covered 11.52 ± 2.34% (Figure 4a) of the infarcted area. In contrast, only 5.85 ± 1.35% (Figure 4b) was covered by the cells injected with PBS.

Differentiation of ADSCs in infarcted heart

The cTnT immunofluorescence staining of DAPI-labeled ADSCs at four weeks of transplantation occasionally appeared to show co-localization of both DAPI-positive and cTnT-positive cells in the infarcted areas (Figure 5c). In addition, occasional DAPI-positive cells were found to be vWAg- or SMA-positive, suggesting that they formed small blood vessels within the infarcted zone (Figures 5f and i). The aforesaid findings suggested the potential ability of transplanted ADSCs to differentiate into cardiomyocyte-like, endothelial and vascular smooth muscle cells. More efforts are needed to confirm this finding.

Cardiac function

The improved heart function could be observed in Fibrin, ADSCs and Fibrin + ADSCs group. It is compared with the PBS control group by echocardiography. EDD (Figure 6a), ESD (Figure 6b), FS (Figure 6c) and EF (Figure 6d) were analyzed, respectively. LV EDD in hearts of the Fibrin + ADSCs group was 5.91 ± 0.24 mm versus 6.60 ± 0.38 mm in hearts of the ADSCs group, 6.70 ± 0.41 mm in hearts of the Fibrin group and 7.66 ± 0.30 mm in hearts of the PBS group. LV ESD in hearts of the Fibrin + ADSCs group was 4.24 ± 0.26 mm versus 5.04 ± 0.22 mm in hearts of the ADSC group, 5.24 ± 0.35 mm in hearts of the Fibrin group and 6.50 ± 0.20 mm in hearts of the PBS group. FS in the heart of the Fibrin + ADSCs group was 28.16 ± 2.23% versus 24.11 ± 1.97% in the heart of the ADSCs group, 23.24 ± 2.44% in the heart of the Fibrin group and 15.90 ± 1.10% in the heart of the PBS group. EF in the heart of the Fibrin + ADSCs group was 61.77 ± 4.14% versus 55.75 ± 3.41% in the heart of the ADSCs group, 53.14 ± 3.59% in the heart of the Fibrin group, 38.80 ± 2.91% in hearts of the PBS group. The rats with transplanted ADSCs in fibrin glue exhibited the best improved heart function compared with others ($P < 0.01$). The group treated with fibrin glue exhibited similar heart function to the one receiving ADSCs ($P > 0.05$).

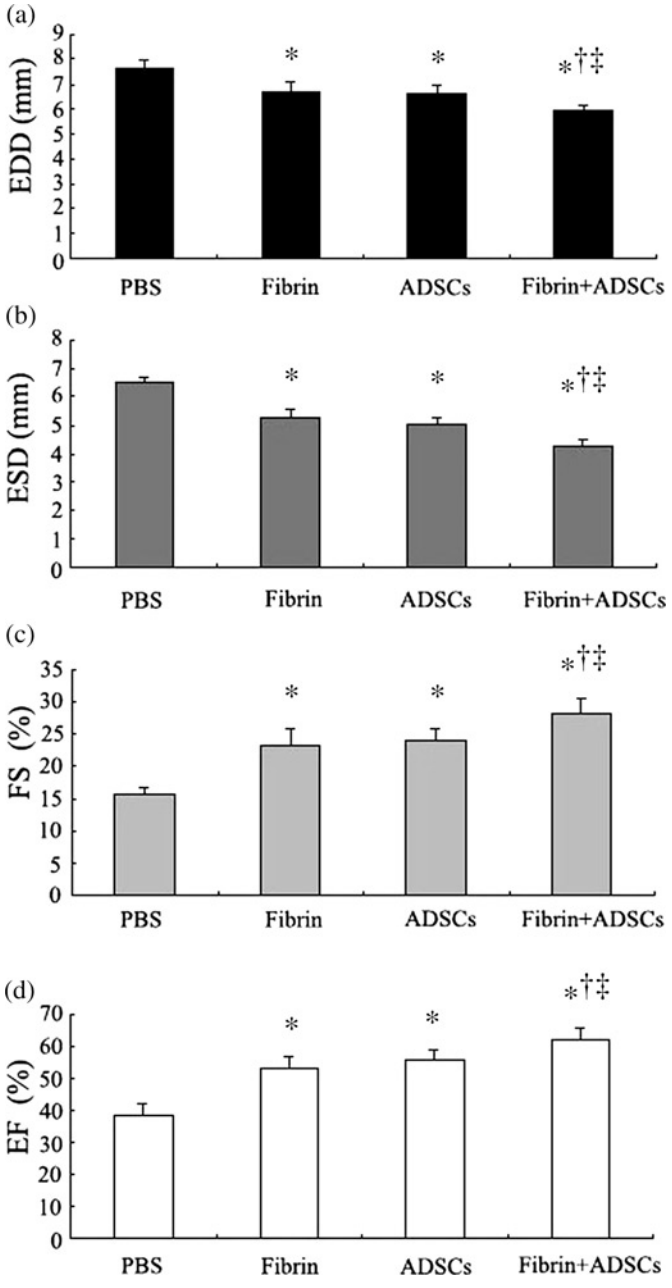

Figure 6 Echocardiography. (a) EDD, (b) ESD, (c) FS and (d) EF. Heart function was better in the rats that were treated with Fibrin, ADSCs and Fibrin + ADSCs compared with that using the control (PBS). The Fibrin + ADSCs group showed a significantly better heart function compared with the Fibrin group and ADSCs group. The group treated with fibrin glue exhibited similar heart function to the one receiving ADSCs (*$P < 0.01$ compared with PBS, †$P < 0.01$ compared with Fibrin, ‡$P < 0.01$ compared with ADSCs). EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; EF, ejection fraction

Histopathology

Infarct size and wall thickness

The infarct size in the PBS group was 45.13 ± 2.49% (Figure 7a). Injection with fibrin glue could reduce the infarct size to 35.96 ± 2.11% (Figure 7b). Similarly, the infarct size could be reduced to 33.33 ± 2.30% when ADSCs were injected in PBS (Figure 7c). The co-transplantation of ADSCs with fibrin glue could reduce the infarct size to 28.50 ± 3.60% (Figure 7d). All treatments have significantly reduced the infarct size compared with the PBS group ($P < 0.01$, respectively). The co-transplantation of ADSCs with fibrin glue significantly reduced the infarct size compared with either Fibrin or ADSCs group alone ($P < 0.01$). No statistical difference was observed between Fibrin and ADSCs group ($P > 0.05$) (Figure 7f).

The ventricular wall thickness at the center of MI after four weeks of implantation was reported as follows:

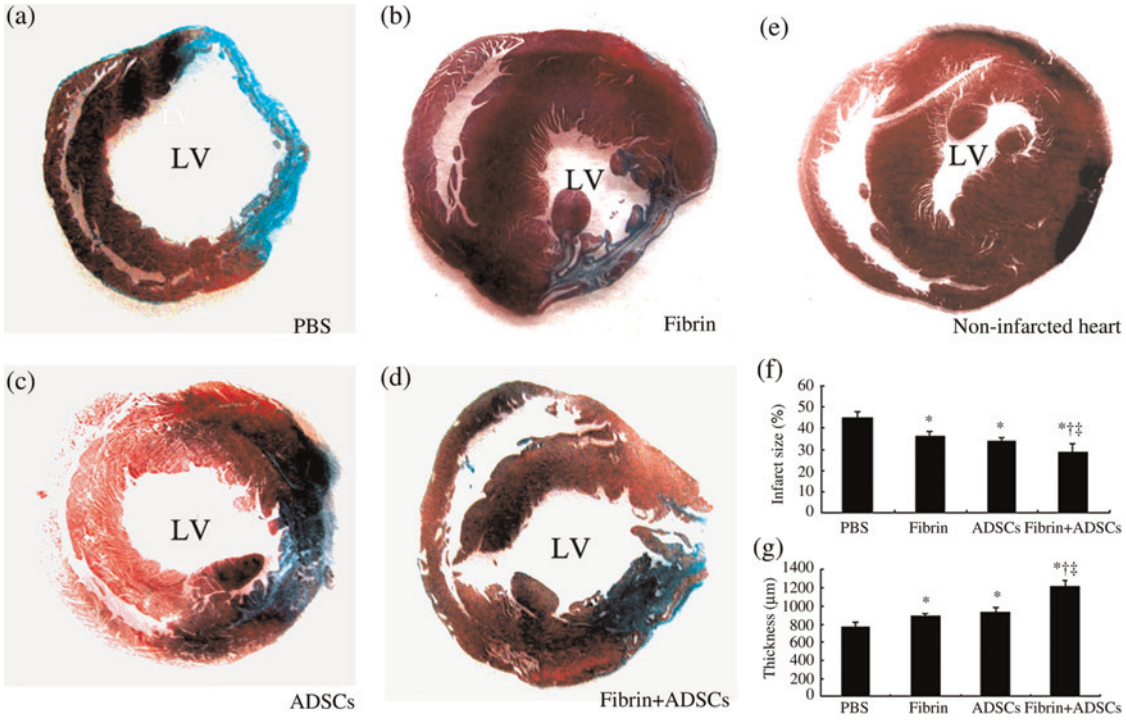

Figure 7 Infarct size and infarct wall thickness. (a–e) Masson trichrome staining of representative sections showed the infarcted wall of the four groups and the non-infarcted group. (a) PBS; (b) Fibrin; (c) ADSCs; (d) Fibrin + ADSCs; and (e) non-infarcted control. The diagram shows the statistical results. (f) All treatments have significantly reduced the infarct size compared with the PBS group. The co-transplantation of ADSCs with fibrin glue significantly reduced the infarct size compared with either Fibrin or ADSCs groups alone. No statistical difference was observed between Fibrin and ADSCs groups. (g) In contrast with the PBS group, the thickness in the three experimental groups was significantly higher. The co-transplantation of ADSCs with fibrin glue had the best effect to preserve the ventricular wall thickness compared with ADSCs or fibrin glue treatment. No significant difference was observed between Fibrin and ADSCs groups ($^{*}P < 0.01$ compared with PBS, $^{\dagger}P < 0.01$ compared with Fibrin, $^{\ddagger}P < 0.01$ compared with ADSCs). ADSCs, adipose-derived stem cells; PBS, phosphate-buffered saline; LV, left ventricle (A color version of this figure is available in the online journal)

771.3 ± 45.3 μm in the PBS group, 880.7 ± 26.1 μm in the Fibrin group, 930.5 ± 50.8 μm in the ADSCs group and 1212.0 ± 74.8 μm in the Fibrin + ADSCs group. In contrast with the PBS group, the thickness in the above three experimental groups was significantly higher ($P < 0.01$). The co-transplantation of ADSCs with fibrin glue had the best effect to preserve the ventricular wall thickness compared with ADSCs or fibrin glue treatment ($P < 0.01$). No significant difference was observed between Fibrin and ADSCs groups ($P > 0.05$) (Figure 7g).

Neovasculature formation

Neovasculature formation potential was assessed in the sections immunohistochemically stained with vWAg antibody. Since collateral arterioles often bordered the scar post-MI, we counted vessels within the infarcted area to assess the effect of neovasculature by different treatments. The arteriole densities in the central area of MI post four weeks of implantation were reported as follows: $30.60 \pm 4.86/mm^2$ in the PBS group, $55.30 \pm 5.58/mm^2$ in the Fibrin group, $61.30 \pm 6.13/mm^2$ in the ADSCs group, and $108.70 \pm 11.38/mm^2$ in the Fibrin + ADSCs group. The arteriole density in scar areas increased significantly in Fibrin (Figure 8b), ADSCs (Figure 8c), and Fibrin + ADSCs groups (Figure 8d) as compared with that in the PBS group (Figure 8a) ($P < 0.01$). This increase was even more pronounced in the Fibrin + ADSCs group ($P < 0.01$). No significant difference was observed between Fibrin and ADSCs groups ($P > 0.05$) (Figure 8e).

Discussion

After MI, both the cardiomyocytes and extracellular matrix are pathologically modified. In ischemic myocardium, transplanted stem cells can rarely stay and survive. This appears to be a main problem affecting the therapeutic effects of MI by stem cell transplantation.[32] One strategy against this in preclinical experiment adopts the approach of injectable cardiac tissue engineering (embedding stem cells in biodegradable materials to provide a temporarily favorable environment). It is more attractive from a practical standpoint than any other approaches in cardiac tissue engineering. The present study demonstrated that the transplantation of adipose-derived stem cells with fibrin glue enhanced cell retention and survival. It improved the infarcted heart function, reduced the infarct size, increased wall thickness and promoted angiogenesis for a rat model of MI. The finding suggests that the fibrin glue is a promising vehicle to deliver ADSCs to the infarcted heart wall. Thus, the method of injectable tissue engineering combining fibrin glue with ADSCs has clinical potential. So far, there has been no report on the application of ADSCs with injectable fibrin glue to cardiac repairs.

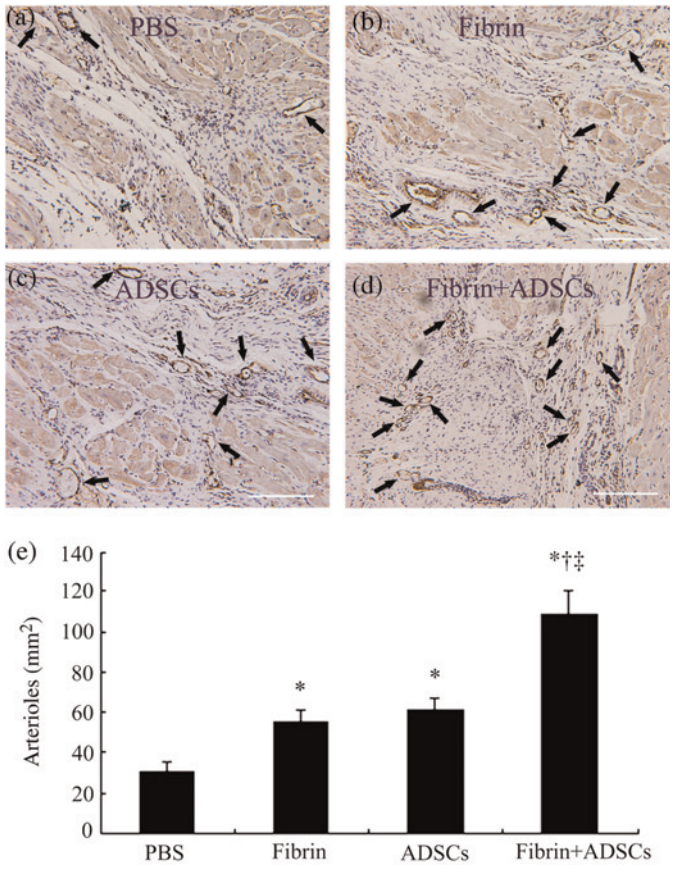

Figure 8 Arteriole density in the infarct area four weeks after injection. Arterials were identified in PBS (a), Fibrin (b), ADSCs (c) and Fibrin + ADSCs (d) groups in the infarct area. (e) The diagram shows the statistical results. The arteriole density increased significantly in Fibrin, ADSCs and Fibrin + ADSCs groups as compared with the PBS group. The arteriole density could be observed to increase most in the Fibrin + ADSCs group. There was no significant difference between Fibrin and ADSCs groups ($^{*}P < 0.01$ compared with PBS, $^{\dagger}P < 0.01$ compared with Fibrin, $^{\ddagger}P < 0.01$ compared with ADSCs). Bars = 100 μm. Black arrows show the arterials. ADSCs, adipose-derived stem cells; PBS, phosphate-buffered saline (A color version of this figure is available in the online journal)

This study demonstrated that 24-h cell retention and four-week graft size with fibrin glue were significantly higher and larger than those with PBS control. The 24-h cell retention by using fibrin glue group was 19.10 $\pm$ 3.13%, whereas that using the PBS group was 14.16 $\pm$ 2.73%. This result indicated that fibrin glue could significantly improve cell retention. Four weeks after the ADSCs were injected in fibrin glue, the area of the infarcted wall covered by transplanted ADSCs was significantly larger compared with that injected in PBS. It indicated the potential for fibrin glue to increase cell survival. As a temporary semi-rigid scaffold, fibrin glue provides a temporary extracellular matrix to enhance cell survival. The proliferation of ADSCs and neovasculature formation induced by fibrin glue helps to improve ADSCs survival. The findings were similar to the report of Christman *et al.*[26]

A reliable cell source to restore cardiac function after MI is crucial. However, current stem cell studies diverge on the cell type optimal for cardiac repairs and regeneration. In this study, ADSCs were used as seeding cells in the injectable cardiac tissue engineering. The advantages of ADSCs include their minimally invasive accessibility, relative abundance, rapid expansion and easy culturing for ischemic myocardium repairs and regeneration. After Zuk *et al.*[8] reported the type of multipotent msenchymal stem cells in fat tissue in 2001, these cells have been used in tissue engineering of myocardium, skin and bone.[33] In this study, the co-localization of ADSCs with cardiac muscle-specific troponin T protein occasionally appeared. However, more efforts are needed to confirm whether these differentiated cells were cardiomyocytes. In addition, occasional DAPI-positive cells were found to be vWAg- or SMA-positive, suggesting that they formed small blood vessels within the infarcted zone. This differentiation seemed to have contributed to the significantly higher microvessel density in the infarcted region. Heart function, wall thickness and microvessel density within the infarcted area four weeks after implantation in the ADSCs group alone was better than those in the PBS group. Four possible reasons might account for the results: (1) the paracrine mechanism;[34] (2) the increase in vessel density;[35] (3) the inhibition of apoptosis through induction of angiogenesis;[36] and (4) the improvement of the local myocardial elasticity.[35]

The injection of biomaterials alone (i.e. alginate, collagen and self-assembling peptide) into the infarcted areas could limit LV remodeling and improve LV function.[22,37,38] In the present study, the infarct size can be reduced by either the injection of fibrin glue or ADSCs in fibrin glue. Such a reduction could hamper infarct expansion later. Our study demonstrated that fibrin glue, ADSCs and ADSCs in fibrin glue preserved infarct wall thickness. It is noteworthy that fibrin glue alone was sufficient to retain the left ventricular thickness four weeks after transplantation and to prevent further deterioration of cardiac function. Among the three groups of treatment, the left ventricular thickness increased significantly to 1212.0 $\pm$ 74.8 μm in the group co-injecting fibrin glue with ADSCs. This indicates that ADSCs were not an exclusive factor to restore myocardial function. First, fibrin glue may have provided the internal structural and mechanical support of the damaged area. Second, it induced neovasculature formation that led to a smaller infarcted region. In summary, fibrin inhibited infarcted ventricle remodeling and improved cardiac function after MI in rats.

In the present study, ADSCs were labeled by fluorochrome DAPI. Although the strategy of gene transfection is complicated, its safety profile, host immune response and transfection efficiency remain controversial and need further study. Our previous study suggested that this method could be used as a qualitative tracing method for the *in vivo* study of transplanted cells in lieu of precise quantitative study. In addition, Leiker *et al.*[39] assessed the nuclear affinity labeling method to track implanted stem cells for myocardial implantation in a porcine model with three variances, i.e. adenovirus-mediated expression of enhanced green fluorescence protein (EGFP), beta-galactosidase (LacZ) and nuclear staining with DAPI. The result demonstrated that DAPI-labeled MSCs retained full viability, ceased proliferation and exhibited an increased differentiation potential. Therefore, although the fluorochrome labeling technique is to be desired, the method of labeling

transplanted cells with fluorochrome seems feasible in our study.

The main limitation to the present study is the application of a small animal model of MI. Further studies need a larger animal model to evaluate the prospect of this approach. Furthermore, the beneficial effects in terms of cardiac function were only evaluated in the four-week period following injection. A longer duration is necessary to evaluate heart performance and function.

In summary, our study demonstrated that the injectable cardiac tissues engineered by co-transplanting ADSCs with fibrin glue could improve the morphology and function of the heart in a rat model of MI. The fibrin could deliver cells directly into the infarcted heart walls without massive bleeding and fill the irregular geometry of necrosis tissues. Our results suggest that the method of *in situ* injectable tissue engineering combining fibrin glue with ADSCs is promising clinically to treat MI.

Author contributions: XZ: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; HW: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; XM: data analysis and interpretation, manuscript writing; AA: collection and/or assembly of data, data analysis and interpretation; BW: data analysis and interpretation; FL: data analysis and interpretation; BC: collection and/or assembly of data; CW: conception and design, data analysis and interpretation, manuscript writing, financial and administrative support, and final approval of manuscript; YM: conception and design, data analysis and interpretation, manuscript writing, financial and administrative support, and final approval of manuscript. XZ and HW contributed equally to this work.

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