Direct intramyocardial injection of mesenchymal stem cell sheet fragments improves cardiac functions after infarction

Chung-Chi Wang1†, Chun-Hung Chen2†, Wei-Wen Lin3, Shiaw-Min Hwang4, Patrick C.H. Hsieh5, Po-Hong Lai2, Yi-Chun Yeh2, Yen Chang1*, and Hsing-Wen Sung2*

1Division of Cardiovascular Surgery, Veterans General Hospital-Taichung, and College of Medicine, National Yang-Ming University, Taipei, Taiwan, Republic of China; 2Department of Chemical Engineering/Bioengineering Program, National Tsing Hua University, Hsinchu, Taiwan, Republic of China; 3Division of Cardiology, Veterans General Hospital-Taichung, and Department of Life Science, Tunghai University, Taichung, Taiwan, Republic of China; 4Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China; and 5Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan, Republic of China

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1. Introduction

Myocardial infarction leads to loss of tissue and impairment of cardiac performance.1 Recently, the identification of stem cells capable of contributing to tissue regeneration has raised the possibility that cell therapy could be employed for repair of damaged myocardium.2–4 It was shown that cell transplantation via local intramuscular injection is a promising therapy for patients with myocardial infarction.1–3 Several studies performed on rodents and pigs suggested that bone marrow-derived mesenchymal stem cells (MSCs) own the potency to differentiate into a variety of lineages, including endothelial cells, vascular smooth muscle cells, and cardiomyocytes in the heart.5,6

Prior to cell transplantation, a large scale of the desired cell types must be expanded in vitro on tissue culture poly(styrene (TCP5) dishes. Upon confluence, detachment of the cultured cells from TCP5 dishes usually requires using a proteolytic enzyme such as trypsin or dispase.7 However, the use of enzymes commonly dissociates the cultured cells

KEYWORDS
Myocardial infarction; Cell transplantation; Cell sheet; Tissue regeneration; Angiogenesis

Aims Cell transplantation is a promising approach for patients with myocardial infarction. However, following injection, retention of the transplanted cells in the injected area remains a central issue, which can be deleterious to cell transplantation therapy. We hypothesized that the use of cell sheet fragments, with the preservation of extracellular matrix (ECM), may significantly increase cell retention and thus improve cell therapy.

Methods and results Mesenchymal stem cell (MSC) sheet fragments with ECM were fabricated. Experimental myocardial infarction was created in male syngeneic Lewis rats. Thirty minutes after myocardial infarction, an intramyocardial injection was conducted with a needle directly into the peri-infarct areas. There were four treatment groups (n = 10): sham; phosphate buffered saline; dissociated MSCs; and MSC sheet fragments. Echocardiography and pressure measurements were assessed post-operatively. At retrieval, the hearts were fixed for histological evaluation. After injection, the MSC sheet fragments remained intact, while the complete cell sheets were torn into pieces. The results obtained in the echocardiography and pressure measurements revealed a superior heart function in the MSC sheet fragment group compared with the dissociated MSC group (P < 0.05). The MSC sheet fragments were able to conform and align their inherent ECM along the interstices of the muscular tissues at the injection sites, while only a few cells were identified in the dissociated MSC group at 12 weeks post-operatively. Additionally, transplantation of the MSC sheet fragments stimulated a significant increase in vascular density (P < 0.05) and enhanced the graft/host cell connection.

Conclusion The MSC sheet fragments may serve as a cell delivery vehicle by providing a favourable ECM environment to retain the transplanted cells and improve the efficacy of therapeutic cell transplantation.

* Corresponding author. Tel: +86 886 3 574 2504; fax: +86 886 3 572 6832. E-mail address: hwsung@che.nthu.edu.tw (H.-W.S.) or ychang@vghtc.gov.tw (Y.C.)
† The first two authors contributed equally to this work.

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and disrupts their micro-intercellular junctions, extracellular matrix (ECM), and integrative adhesive agents. Additionally, following injection of the dissociated cells, retention of the transplanted cells in the injected area remains a central issue. These facts can be deleterious to cell-transplantation therapy.

In the study, we hypothesized that the use of continuous cell sheets, with the preservation of endogenous ECM, intercellular junctions, and integrative adhesive agents, may be used to overcome the above-mentioned problems and is therefore beneficial for cell transplantation. Cell sheet engineering has been developed as an alternative approach for tissue engineering by Okano’s group. They demonstrated that monolayered MSCs attached onto the anterior wall of the scarred myocardium reversed wall thinning in the scar area and improved cardiac functions in rats with myocardial infarction.

In our recent study, a novel method, using a thermo-responsive hydrogel [methylcellulose (MC) blended with distinct salts] coated on TCPS dishes, was developed for harvesting living cell sheets. Apparently, the physical size of a complete cell-sheet harvested from the coated TCPS dish is too bulky for intramuscular injection. Employing a similar cell-sheet culture system, construction of MSC sheet fragments was reported in the study. Cell morphology, endogenous ECM, intercellular junctions, and integrative adhesive agents of the MSC sheet fragments, before and after injection through a needle, were examined in vitro. Transplantation of the MSC sheet fragments to induce tissue regeneration and improve cardiac functions in a syngeneic rat model with infarcted myocardium was investigated. The dissociated MSCs obtained by traditional trypsinization were used as a control.

2. Methods

2.1 Preparation of the cell-sheet culture system

Aqueous MC solutions (12% by w/v) were prepared by dispensing the weighed MC powders (M7027, Sigma-Aldrich, St Louis, MO, USA) in heated water with the addition of phosphate buffered saline (PBS, 5.0 g/l, Sigma-Aldrich) at 50 °C. The prepared MC solutions were autoclaved and used to coat TCPS dishes (BD Falcon 353653, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and subsequently gelled at 37 °C. To improve cell attachments, a 100 μL aqueous collagen type I (0.5 mg/mL bovine dermis collagen, Sigma-Aldrich) was evenly spread over the gelled MC.

2.2 Characterization of Mesenchymal stem cell Sheets

Bone marrow MSCs were isolated from femora and tibia of Lewis rats. The isolated MSCs were spindle-shaped and attached to the culture dish tightly. The DNA-demethylating agent 5-azacytidine (5-Aza) was added on the third day and incubated with MSCs for 24 h. Subsequently, the induced MSCs were labelled for later identification by adding 100 μg/mL 5-bromo-2’-deoxyuridine (BrdU) containing media to 50% confluent cultures for 24 h. The labelled MSCs were then seeded evenly on the above-mentioned cell-sheet culture system at a density of 5 x 10⁶ cells/cm² for 7 days. The grown MSC sheets were fixed in 4% paraformaldehyde at 37 °C for 15 min and then stained with a monoclonal antibody against BrdU (Caltag Laboratories, Burlingame, CA, USA), collagen type I (clone I-BHS, MP Biomedical Inc., Solon, OH, USA), collagen type III (clone 3G4, Chemicon International Inc., Temecula, CA, USA), fibronectin (clone IST-9, Abcam Inc., Cambridge, UK), laminin (clone ZEB, Chemicon), Pan-CAM (clone CH-19, Abcam), N-CAM (clone 389, Zymed, Invitrogen, San Francisco, CA, USA), or connexin 43 (Cx43, clone 4E6.2, Chemicon). A Cy-5 conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was then used as the secondary antibody. The cell sheets were stained to visualize F-actins and nuclei by phalloidin (Oregon Green® 514 phalloidin, Molecular Probes, Invitrogen, Karlsruhe, Germany) and propidium iodide (PI, P4864, Sigma-Aldrich), respectively. The stained cell sheets were examined using an inverted confocal laser-scanning microscope (CLSM, TCS SL, Leica, Wetzlar, Germany) with excitations at 488, 543, and 633 nm, respectively. Superimposed images were performed with an LCS Lite software (version 2.0, Leica).

2.3 Construction and characterization of Mesenchymal stem cell sheet fragments

To obtain the MSC sheet fragments, a stainless screen (40 mesh, from the cell dissociation sieve-tissue grinder kit, CD1-1KT, Sigma-Aldrich) was used to compress and fragment the MSC sheet grown on the cell-sheet culture system at room temperature (Figure 1). The obtained MSC sheet fragments were washed with cold PBS several times to remove the remaining MC hydrogel and then collected via centrifugation.

Cell morphology, endogenous ECM, intercellular junctions, and integrative adhesive agents of the MSC sheet fragments, before and after injection through a needle, were examined. Briefly, the MSC sheet fragments (2 x 10⁶ cells in total) were resuspended in 500 μL of culture medium, loaded in a syringe, injected through a 27-gauge needle, and subsequently seeded onto a 24-well plate (Costar® 3524, Corning, Corning, NY, USA). Changes in morphology of the MSC sheet fragments on the plates with time were investigated and photographed every day for up to 5 days (n = 10). The dissociated MSCs (at the same cell density) obtained by traditional trypsinization were used as a control (n = 10).

2.4 Animal study

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Acute myocardial infarction was created in male syngeneic Lewis rats weighing 350–400 g. Thirty minutes after myocardial infarction, the rats were randomly divided into four treatment groups: sham (without LCA ligation); PBS (300 μL); dissociated MSCs (5 x 10⁶ cells) suspended in 300 μL of PBS; MSC sheet fragments (5 x 10⁶ cells in total) suspended in 300 μL of PBS. An intramyocardial injection of PBS or the dissociated MSCs directly into the border zones (or the peri-infarct areas) of the ischaemic left ventricular (LV) wall was performed with a 30-gauge needle, while that of the MSC sheet fragments was conducted with a 27-gauge needle. After treatment, the rats were allowed to recover under care. Animals were coded so that all measurements were made without knowledge of treatment groups. The study was continued until at least 10 rats survived at least 3 months in each of the 4 coded groups.

2.5 Left ventricular function assessment by echocardiography

Echocardiography was performed at 4, 8, and 12 weeks postoperatively for all studied groups. Rats were anaesthetized with sodium pentobarbital (30 mg/kg) and isoflurane (2.0%) was used as a supplement to maintain mild anaesthesia. Cardiac ultrasonography was performed with a commercially available echocardiographic system (SONOS 5500, Agilent Technologies, Andover, MA, USA) equipped with a 12 MHz broadband sector transducer. The two-dimensional (2D) images and M-mode tracings were recorded from the short-axis views at the level of mid-papillary muscles. From M-mode tracings, anatomical parameters in diastole and systole
were obtained. Dimension data were presented as the average of measurement of 10 consecutive beats. The fractional shortening (FS) of LV was calculated as follows:

$$LVFS(\%) = \frac{LVEDD - LVESD}{LVEDD} \times 100\%$$

where LVEDD and LVESD correspond to LV dimensions in end-diastole and in end-systole, respectively.

### 2.6 Left ventricular catheterization

Pressure measurements were performed at 12 weeks postoperatively. Rats were anaesthetized with isoflurane (4.0%) and intubated for continuous ventilation with room air supplemented with oxygen and isoflurane (3.0%). The apex of LV was cannulated with a physiological pressure transducer (MLT844, Millar Instruments Inc., Houston, TX, USA). The pressure waveforms were recorded with a data-acquisition system (Powerlab ML870, AD Instruments, Colorado Springs, CO, USA). The aforementioned measurements (echocardiography and catheterization) were conducted by investigators blinded to the experimental conditions.

### 2.7 Histological examinations

LV myocardium specimens of each studied group were retrieved at 12 weeks postoperatively. The specimens used for light microscopy were fixed in 10% phosphate buffered formalin and embedded in paraffin. The embedded specimens were then sectioned into a thickness of 5 μm at five different transversal levels at the site of tissue necrosis, encompassing the entire lesion, and stained with Masson’s trichrome. The stained sections were used to measure and calculate the thickness values of the peri-infarct and infarct areas in each studied group with a computer-based image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA) at a ×10 magnification and converted to millimetre. The infarct size was expressed as the percentage of total LV circumference. Additional sections were stained for factor VIII with an immunohistological technique with a monoclonal anti-factor VIII antibody (DAKO Corp., Carpinteria, CA, USA). The vascular density in the peri-infarct area of all animals was quantified with the same image analysis system at a ×100 magnification and converted to vessels/mm². We repeated these measurements at five different areas for each studied sample and calculated a mean.

For immunofluorescent staining, after rehydration and microwave antigen retrieval with 0.1 mol/L sodium citrate, the 5 μm sections were washed and incubated at 4°C for 12 h with the anti-BrdU antibody resuspended in the dilution buffer. The sections were then double-stained with antibodies against bovine collagen type I, Cx43, α-sarcromeric actin (clone SC5, Serotec, Kidlington, Oxford, UK), factor VIII, α-smooth muscle actin (α-SMA, clone 1A4, DAKO), fibronectin, cleaved caspase-3 (clone 5A1, Cell Signaling Technology, Beverly, MA, USA), macrophage (CD68, clone ED1, Serotec, Oxford, UK), and the early marker of myocyte development Nkx2.5 (clone N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the primary antibody incubation, the following secondary antibodies (Molecular Probes) were used: goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 546, goat anti-mouse Alexa Fluor 633, goat anti-rabbit Alexa Fluor 633 and rabbit anti-goat Alexa Fluor 633. An expanded immunolabelling protocol and the antibodies used can be found in Table 1.

Antibodies against BrdU and bovine collagen type I were used to localize the MSC sheet fragments injected in the area of the peri-infarct, while those against α-sarcromeric actin and Nkx2.5 were used to identify the MSC-derived cardiomyocyte-like cells. To characterize further the properties of these cardiomyocyte-like cells, we determined the expression of Cx43 and cleaved caspase-3. Moreover, endothelial and smooth muscle cells were identified by the expression of factor VIII and α-SMA, respectively. In addition to the labelling of smooth muscle cells in arterioles, α-SMA was used to identify myofibroblasts, which were characterized by elongated cell morphology without visible lumen organization. The stained sections were counterstained to visualize nuclei by Sytox blue (Invitrogen) or PI and examined with excitations at 458, 488, 543, and 633 nm, respectively, using an inverted confocal laser scanning microscope (Leica).
2.8 Statistical analysis

Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (SAS Institute Inc., Cary, NC, USA). All data are presented as a mean value with its standard deviation indicated (mean ± SD). Differences were considered to be statistically significant when the P-values were less than 0.05.

3. Results

3.1 Characteristics of Mesenchymal stem cells in cell sheets

BrdU incorporation was evident in approximately 80% (80.3 ± 1.7%, n = 10 batches, Figure 2A) of the cultured MSCs. ECM molecules [collagen type I (Figure 2B) and collagen type III (Figure 2C)] and integrative adhesive agents [fibronectin (Figure 2D) and laminin (Figure 2E)] were clearly identified. Additionally, Cx43 (Figure 2F), Pan-CAM, and N-CAM (data not shown) were clearly identified at the cell borders within the MSC sheets at approximately 21 days after 5-Aza treatment.

3.2 Characterization of Mesenchymal stem cell sheet fragments

Upon confluence, the grown MSC sheets were taken out from the incubator with media present. Subsequently, a sterilized stainless screen was used to compress and fragment the MSC sheets grown on the cell-sheet culture system at room temperature (Figure 1). Immediately after compressing the stainless screen, fragmented cell sheets (detaching from the surface of the culture system) were obtained. The dissociated MSCs, complete MSC sheets and MSC sheet fragments were separately suspended in culture medium and subsequently loaded into a syringe. After injection through a 27-gauge needle, the dissociated MSCs and MSC sheet fragments still remained intact, while the complete cell sheets were torn into pieces (data not shown).

After injection through the needle, the dissociated MSCs and MSC sheet fragments were individually seeded onto 24-well plates. Their photomicrographs together with immunofluorescent images taken at distinct culture periods are shown in Figure 3A and B, respectively. As shown, the MSC sheet fragments adhered to the surface of the culture plate instantly, while it took awhile for the dissociated MSCs to settle down and stick to the plate surface (Figure 3A). The MSC sheet fragments attached tightly onto the surface of the culture plate within 1 h (50 ± 2 min), which was significantly faster than the dissociated MSCs (within 4 h, 220 ± 10 min). Additionally, the time required for the seeded cells to reach confluence was shorter for the MSC sheet fragments (3 days) than for the dissociated MSCs (4–5 days).

Analyses of the immunofluorescent images indicated that there was no fibronectin deposited on the plate surface initially for the dissociated MSCs (Figure 3B). Six hours later, fibronectin was organized into short linear streaks and the cells started to attach to the plate surface. In contrast, a robust fibronectin meshwork inherent with the ECM was clearly observed within the MSC sheet fragments in the beginning. This fibronectin meshwork started to attach to the surface of the culture plate within 1 h and the cells on the sheet fragments continuously produced fibronectin and deposited it onto the plate surface.

3.3 Animal study

The overall surgical mortality rate, defined as animal death within 24 h after surgery, was 4.3% (2 of 47 rats), and the late mortality rate (death between 24 h and 12 weeks...
after surgery) was 6.6% [3 of 45 (PBS group, n = 1; dissociated-MSC group, n = 1; MSC-sheet-fragment group, n = 1)].

3.4 Left ventricular function assessment

The results of echocardiography (Table 2) and catheterization (Figure 4A and B) revealed a superior heart function in the group treated with the MSC sheet fragments compared with that treated with the dissociated MSCs. The MSC-sheet-fragment group showed a significantly higher LVFS than the dissociated-MSC group at 8 and 12 weeks postoperatively, whereas LVFS deteriorated with time in the PBS group (Table 2). Additionally, LVESD and LVEDD were relatively smaller in the MSC-sheet-fragment group than in the dissociated-MSC and PBS groups. However, LVESD and LVEDD increased significantly over 12 weeks in the PBS group but not in the dissociated-MSC group. In the pressure measurements, the MSC-sheet-fragment group demonstrated significant improvements in the LV end-systolic pressure (LVESP, Figure 4A) and in the LV end-diastolic pressure (LVEDP, Figure 4B) when compared with the dissociated-MSC and PBS groups.

3.5 Morphological and histological findings

Masson’s trichrome staining demonstrated a severe (moderate) degree in myocardial fibrosis and LV dilation in the PBS (dissociated-MSC) group, while the group treated with the MSC sheet fragments attenuated the development of myocardial fibrosis and the enlargement of LV cavity (Figure 5A). Quantitative analyses demonstrated that the size of the infarct in the group treated with the MSC sheet fragments was significantly smaller than its counterparts treated with the dissociated MSCs or PBS (Figure 5B), while their thickness values [the infarct (Figure 5C) and the peri-infarct (Figure 5D)] and the vascular density [the peri-infarct (Figure 5E)] were significantly greater.

In the immunofluorescent staining, examination of control sections taken from the sham group revealed that most cardiomyocytes aligned parallel with the host myocardium and expressed Cx43 (Figure 6A). Only a few BrdU-positive cells were identified in the peri-infarct area in the group treated with the dissociated MSCs (Figure 6C). In contrast, the MSC sheet fragments [identified by a large amount of BrdU-positive cells adhered to fibronectin (Figure 6E) together with the implanted bovine collagen type I (Figure 6D)] were clearly observed within the injured heart muscle and adapted to the host organ structure without distorting the cardiac wall geometry. There was very little detectable cleaved caspase-3 (an evidence of apoptotic cell death, Figure 6F). The inflammatory cells infiltrated into the peri-infarct area were identified by immunostaining with a CD 68 antibody (Figure 6G). Quantification results demonstrated that the percentage of macrophages present at the site of intramuscular injection was <5% for the MSC-sheet-fragment group.

A portion of the identified BrdU-positive cells in the MSC sheet fragments were stained further positively for α-sarcomeric actin and Nkx2.5 (Figure 6H), indicating that some of the transplanted MSCs had been differentiated into cardiomyocyte-like cells and expressed cytoplasmic α-sarcomeric actin and the transcription factor Nkx2.5. Nkx2.5 has been used to stain developing myocytes; we confirmed this by performing immunofluorescence on isolated neonatal myocytes (data not shown). In addition, BrdU-positive MSCs that expressed factor VIII alone or with α-SMA were identified (Figure 6I), indicating a role for MSCs in angiogenesis. Moreover, a significant portion of the...
The ability of cell attachment and proliferation of the mesenchymal stem cell (MSC) sheet fragments was still preserved after injection through a needle. (A) Photomicrographs and (B) immunofluorescence images of the dissociated MSCs and the MSC sheet fragments after injection through a needle and then seeded on TCPS dishes taken at distinct time points. Scale bars, (A) 200 μm and (B) 40 μm.

Table 2 Serial echocardiography

<table>
<thead>
<tr>
<th>Time point and parameter</th>
<th>Sham</th>
<th>PBS</th>
<th>Dissociated MSCs</th>
<th>MSC sheet fragments</th>
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</thead>
<tbody>
<tr>
<td>4 weeks, n value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>52.9 ± 3.5</td>
<td>32.4 ± 3.1</td>
<td>33.0 ± 2.3</td>
<td>34.0 ± 2.6</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.4 ± 0.3</td>
<td>4.3 ± 0.8</td>
<td>3.9 ± 0.8</td>
<td>3.7 ± 1.0</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>5.2 ± 0.3</td>
<td>6.0 ± 0.8</td>
<td>5.8 ± 0.5</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>8 weeks, n value</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>55.3 ± 4.1</td>
<td>30.6 ± 3.9</td>
<td>35.1 ± 3.0</td>
<td>42.2 ± 3.4***</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.5 ± 0.3</td>
<td>4.5 ± 0.8</td>
<td>3.9 ± 0.5</td>
<td>3.6 ± 0.9**</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>5.5 ± 0.3</td>
<td>6.4 ± 0.9</td>
<td>6.0 ± 0.7</td>
<td>5.7 ± 0.9**</td>
</tr>
<tr>
<td>12 weeks, n value</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>57.5 ± 4.7</td>
<td>26.3 ± 4.7</td>
<td>36.5 ± 4.5</td>
<td>46.2 ± 4.2***</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.5 ± 0.4</td>
<td>5.5 ± 0.7</td>
<td>4.0 ± 0.7</td>
<td>3.2 ± 0.8**</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>5.9 ± 0.7</td>
<td>7.4 ± 0.9</td>
<td>6.3 ± 0.6</td>
<td>6.0 ± 0.6**</td>
</tr>
</tbody>
</table>

LVFS: left ventricular fractional shortening; LVESD: left ventricular end-systolic dimension; LVEDD: left ventricular end-diastolic dimension.

*P < 0.05 vs. the dissociated-MSC group.

**P < 0.05 vs. the PBS group.
Injectable cell sheets for cardiac repair

4. Discussion

Retention of cells after intramyocardial injection is crucial to the efficacy of therapeutic cell transplantation. We demonstrated that the use of continuous cell sheets may serve as a cell-delivery vehicle by providing a favourable ECM environment to retain the cells at the injected sites. In the preparation of cell sheets, we found that the obtained MSC sheet fragments preserved the endogenous ECM. The ECM contained adhesive glycoproteins that bind to both cells and other matrix macromolecules and thereby help cells attach to the ECM. Additionally, junction proteins such as Pan-CAM, N-CAM, and Cx43 were identified at the intercellular borders. It is known that ECM provides not only a physical support but also outside-in signals that regulate many cellular functions such as adhesion, migration, proliferation, and differentiation and must be maintained for optimal cellular benefits.22

The obtained MSC sheet fragments were in square with a length corresponding to the width of opening of the screen mesh used (380 μm). The number of cells grown on a complete cell sheet (with an area of 2.45 cm²) was approximately 2 × 10⁵ cells, after reaching confluence. Based on this information, the number of cells on each MSC sheet fragment was estimated to be 120 cells/sheet.

The MSC sheet fragments suspended in the syringe appeared to be pliable and deformable, while no aggregation or precipitation of cell sheets was observed. During injection through a 27-gauge needle (with an inside diameter of 400 μm), the MSC sheet fragments were able to conform and align their physical shapes instantly and squeeze through the channel of the needle, whereas the complete cell sheets often got stuck in the needle.

After injection through the needle, the ability of cell attachment and proliferation of the MSC sheet fragments was still preserved (Figure 3A). Additionally, the time required for the MSC sheet fragments to attach and proliferate on the surface of a culture plate was significantly shorter than the dissociated MSCs. For most types of cells, opportunities for their attachment and proliferation depend on the surrounding ECM and integrative adhesive agents (e.g. fibronectin), which are inherent with the seeded MSC sheet fragments (Figure 3B). These results demonstrated that after injection through the needle, the MSC sheet fragments retained their activity upon transferring to another growth surface.

The results obtained in the echocardiography (Table 2) and pressure measurements (Figure 4) revealed a superior heart function in the group treated with the MSC sheet fragments when compared with that treated with the dissociated MSCs. By 12 weeks after injection of the dissociated MSCs, the LV cavity was enlarged and a little contraction was observed in the infarcted wall (from M-mode tracings). In contrast, in the group treated with the MSC sheet fragments, the enlargement of LV cavity was attenuated and the contractile function in the infarcted region of the wall still remained. The improvement in LV function was further indicated by a significant increase in LVEFP and a decrease in LVEDP. The significant improvements in LV function in the group treated with the MSC sheet fragments may be attributed to a greater increase in angiogenesis (Figure 5) that increases local perfusion into the myocardium, thus attenuating expansion of the infarct. It was reported that neovascularization within the infarcted tissue appears to be an integral component of the remodelling process.4,6,23

Only a few BrdU-positive cells were identified at the sites of injection in the group treated with the dissociated MSCs (Figure 6C). It is known that when treated with the dissociated cells, the number of surviving cells identified at the injection sites appeared to be quite small, such that often in the histological sections surprising few labelled implanted cells were found in various models.24–27 It was reported that mechanical leakage and vascular washout may account for a major portion of cell loss after intramuscular injection with the dissociated cells.10 Also, the dissociated cells settle in the cavities surrounded by the fibrous scar without any intercellular communication and begin their basic but...
delayed establishment of microenvironment, which proves fatal for their primary survival.\textsuperscript{28}

In contrast, there were a large number of BrdU-positive cells retained at the sites of injection in the group treated with the MSC sheet fragments. This is because the MSC sheet fragments were able to conform and align their inherent ECM along with the interstices of the muscular tissues at the injection sites, thus providing a better incorporation of the transplanted cells to the host tissue. Also, as demonstrated in our \textit{in vitro} study, the MSC sheet

![Figure 5 Intramyocardial injection of mesenchymal stem cell sheet fragments reduced the infarct size and increased the vascular density. (A) Photomicrographs of each studied group (stained with Masson’s trichrome) and its microvessels (in the inset stained with Factor VIII, scale bars, 40 μm) retrieved at 12-week postoperatively. (B) The infarct size and (C) the thickness values in the infarct and (D) in the peri-infarct together with (E) the vascular density in the peri-infarct for each studied group retrieved at 12-week postoperatively. *Statistical significance at a level of $P < 0.05$.](image-url)
fragments had a better ability of cell attachment and proliferation than the dissociated MSCs upon transferring to another growth surface.

It was reported that ECM can be effective matrix materials for myocardial reconstruction and angiogenesis induction in the infarct zone. Additionally, MSCs have been shown to express angiogenic growth factors in a paracrine way to stimulate neovascularization at the sites of the cell graft. These facts may explain why there was a greater vascular density observed in the group treated with the MSC sheet fragments than that treated with the dissociated MSCs (Figure 5D). Such vascular density was thought to maintain the viability of the grafted cells and residual cardiomyocytes for a successful cellular cardiomyoplasty. The cardioprotective effects of MSCs are known to be mediated not only by their differentiation into cardiovascular cells, but also by their ability to supply large amounts of angiogenic, anti-apoptotic, and mitogenic factors.

In the study, the cell population of the MSC sheet fragments retained in the injected sites at retrieval was not calculated precisely, as a cell by cell count in such dense conglomerates was impossible. Additionally, we have to consider that the BrdU labelling intensity can be lost with cell division before terminal differentiation, which may cause a risk of false-negative results of the implanted cells.

5. Conclusions

The results obtained in the study indicated that there were significantly more cells retained in the peri-infarct area in the group treated with the MSC sheet fragments than that treated with the dissociated cells. Additionally, transplantation of the MSC sheet fragments induced a significant increase in vascular density and enhanced the graft/host cell connection. Taken together, these factors may contribute to attenuate expansion of the infarct, thus improving the LV functions.

Conflict of interest: none declared.

Funding

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Figure 6  There were a large number of BrdU-positive cells retained at the site of injection in the group treated with the mesenchymal stem cell (MSC) sheet fragments, while only a few BrdU-positive cells were identified in the dissociated-MSC group. Immunofluorescence images of the control groups (sham and PBS) and the groups treated with the dissociated MSCs or the MSC sheet fragments in the areas of the peri-infarct retrieved at 12-week postoperatively. Area defined by a square is shown at a higher magnification in the inset. (A) Cx43, α-sarcomeric actin and nuclei; (B) α-sarcomeric actin and nuclei; (C) BrdU and nuclei; (D) collagen type I, α-sarcomeric actin and nuclei; (E) BrdU, fibronectin and nuclei; (F) cleaved caspase-3, BrdU and nuclei; (G) BrdU, macrophage (anti-CD 68), and nuclei; (H) Nkx2.5, α-sarcomeric actin and nuclei; (I and J) factor VIII, α-SMA, BrdU, and nuclei; (K) Cx43, collagen type I and nuclei; (L) Cx43, α-sarcomeric actin, and nuclei. Scale bars, 80 μm (B and D), 20 μm (A, C, and E-L).
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