CLINICAL RESEARCH

Combined transplantation of endothelial progenitor cells and mesenchymal stem cells into a rat model of isoproterenol-induced myocardial injury

La transplantation combinée des cellules progénitrices endothéliales et mésenchymateuses améliore la fonction cardiaque dans un modèle d’atteinte myocardique induite par isoproteréol chez le rat

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KEYWORDS
Isoproterenol; Stem cells; Transplantation; Endothelial; Mesenchymal; Cardiac function

Summary
Background. — Endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) have different biological properties, but their potential for synergy in the treatment of injured myocardium has not been studied extensively.

Aim. — To determine if outcome could be improved by simultaneously transplanting MSCs and EPCs into a rat model of isoproterenol (ISO)-induced injured myocardium.

Methods. — Four weeks after ISO injection, 50 rats were separated randomly into five groups (n=10 per group) and allocated to receive a saline injection (control group), 200 μL medium alone, 200 μL medium plus 2 × 10^6 EPCs, 200 μL medium plus 2 × 10^6 MSCs, or 200 μL medium plus a combination of 1 × 10^6 EPCs and 1 × 10^6 MSCs.

Abbreviations: Ang-2, Angiopoietin-2; b-FGF, Basic fibroblast growth factor; BMNC, Bone marrow mononuclear cell; Dil-ac-LDL, Dil-labelled acetylated low-density lipoprotein; DMSO, Dimethyl sulfoxide; EGM-2, Endothelial cell growth medium-2; ELISA, Enzyme-linked immunosorbent assay; EPC, Endothelial progenitor cell; FISH, Fluorescence in situ hybridization; FITC-UEA-1, Fluorescein isothiocyanate-labelled Ulrex europaeus agglutinin-1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ISO, Isoproterenol; LVEDD, Left ventricular end-diastolic diameter; LVEDP, Left ventricular end-diastolic pressure; MSC, Mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, Reverse transcriptase polymerase chain reaction; VEGF, Vascular endothelial growth factor; vWF, von Willebrand factor.

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Background

Cardiovascular disease is the leading cause of morbidity and mortality, not only in the Western world, but also in developing countries, according to a report from the World Health Organization [1]. With the exception of heart transplantation, current therapeutic strategies, which aim to enable the heart to survive and work at a fraction of its original capacity, have limitations. The regeneration of functioning cardiac tissue is a desirable goal in the treatment of injured myocardium, and the biological properties of stem cells mean that they may have an important role to play in this regard [2].

Mesenchymal stem cells (MSCs) have been shown to induce myocardial regeneration and improve cardiac function when injected directly into infarcted myocardium [3]. However, although global left ventricular function had improved at four weeks after transplantation, this benefit was no longer present at six months after transplantation [4], indicating that the cells were dysfunctional — probably due to ischaemic conditions. Endothelial progenitor cells (EPCs) have been shown to release angiogenic factors and hence augment the cardiac performance in an animal model of acute myocardial infarction [2], probably by improving regional circulation [2,5]. However, the bone marrow EPCs are unable to differentiate into cardiomyocytes [6]. Based on the evidence that MSCs and EPCs have different biological properties, we hypothesized that the combined transplantation of MSCs and EPCs into injured myocardium might overcome these inherent problems and enhance outcome.

Most stem-cell therapy researches have been done in a model of myocardial infarction induced by coronary ligation. Isoproterenol (ISO)-induced myocardial injury is a standard model for the investigation of pharmacological protective effects against ischaemic reperfusion injury [7], but is seldom used for stem-cell therapy research; given
its well-maintained coronary vasculature, we thought that this model might provide transplanted stem cells with a better homing environment than the traditional model of myocardial infarction induced by coronary ligation. Therefore, using a rat model of ISO-induced myocardial injury, we aimed to test whether simultaneous transplantation of MSCs and EPCs could enhance angiogenic signals in patchy areas of myocardial infarction, improve the survival rate of transplanted cells and hence augment the cardiac performance.

**Methods**

**Induction of myocardial injury**

Seventy female rats (150–220 g) received ISO 250 mg/kg per day by inguinal subcutaneous injection on two consecutive days. The second injection was delivered at the opposite inguinal area to avoid local necrosis by ISO [8]. The rats were housed in cages under close monitoring for four weeks, then impaired cardiac function was verified by echocardiographic examination. Only rats with a notable decrease (>20% compared with baseline) in ejection fraction were enrolled for further study.

**Isolation and culture of stem cells**

Fifty male rats (250—300 g) were used as bone marrow mononuclear cell (BMNC) donors. Bone marrow was collected from the femur and tibia and placed in phosphate-buffered saline. Samples were separated using Ficoll-Paque (1.077 g/mL, Huajing, China) density gradient centrifugation. EPCs were cultured in dishes coated with fibronectin (10 ug/mL, Sigma, USA). Culture medium (endothelial cell growth medium-2 [EGM-2], Clonetics, USA) was replaced twice weekly. The MSC expansion method has been described previously [9]. Briefly, BMNCs were cultured in Dulbecco’s modified Eagle’s medium (Gibco, UAS), supplemented with 10% fetal bovine serum (Gibco, UAS), L-glutamine (2 mmol/L) and penicillin (100 U/mL). Non-adherent cells were removed with each medium change.

**Phenotyping of cultured stem cells**

By the ninth day, EPCs had replicated rapidly, displaying a typical ‘cobblestone-like’ appearance (Fig. 1A). Third passage MSCs had a spindle-shaped morphology (Fig. 1B).

At this stage, cultured EPCs were double stained for Dil-labelled acetylated low-density lipoprotein (ac-LDL, 10 μg/mL, Biomedical Technologies) and fluorescein isothiocyanate (FITC)-labelled Ulex europeaeus agglutinin-1 (UEA-1, 1 mg/mL, Sigma), at 37 °C for 4 and 1 h, respectively. The cells took up Dil-ac-LDL (shown in red at 570 nm, Fig. 1C) and were simultaneously stained positive for FITC-UEA-1 (shown in green at 490 nm, Fig. 1D). Using Photoshop software (version 7.0) a merged image was created from Fig. 1C and D, with double-positive staining shown in yellow (Fig. 1E), demonstrating typical EPC properties. The numbers of yellow and red cells were counted using 10 high-power fields of a fluorescent microscope (OLYMPUS IX71-A12FL, Japan). The purity of the EPCs was calculated as follows: (number of yellow cells/number of red cells) × 100%.

The phenotype of the EPC culture was further demonstrated by immunofluorescence staining, targeting von Willebrand factor (vWF, Biodesign International, USA) (Fig. 1F). More than 83.7% of cultured cells were double positive and expressed vWF.

Cultured EPCs and MSCs were analysed by fluorescence-activated cell sorting (FACScan flow cytometer, Becton Dickinson), with isotype-identical antibodies serving as controls. Flow-cytometric analysis showed that on the ninth day of culture, EPCs expressed CD34 (16.5%, Fig. 1G), CD133 (13.9%, Fig. 1H) and vascular endothelial growth factor receptor (VEGF; KDR; 57.8%, Fig. 1I), while third passage MSCs were strongly positive for CD90 (90.8%, Fig. 1J), but negative for CD45 (4.6%, Fig. 1K) and CD34 (0.8%, Fig. 1L).

**Optimizing stem-cell ratio**

To achieve better proliferative activity, the first passage EPCs obtained on the ninth day of culture and the third passage MSCs were cultured in serum-free medium in 96-well culture plates (200 μL/well) at ratios of 1:2 (1 × 10⁶ EPCs with 2 × 10⁶ MSCs), 1:1 (1.5 × 10⁶ EPCs with 1.5 × 10⁶ MSCs) and 2:1 (2 × 10⁶ EPCs with 1 × 10⁶ MSCs), respectively. After 72 h, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 g/L) were added to each well and the plates were incubated for another 4 h. The cell preparation was then mixed with 200 μL DMSO and shaken for 10 min, and optical density was measured at 570 nm. MTT staining showed that most proliferative activity was achieved when EPCs and MSCs were cultured at the 1:1 ratio (optical density = 0.65 ± 0.03 compared with 0.51 ± 0.05 for the 1:2 ratio and 0.43 ± 0.05 for the 2:1 ratio; P < 0.05, respectively). Therefore, on the day of cell transplantation, first passage EPCs obtained on the ninth day were mixed with third passage MSCs at a ratio of 1:1 (1 × 10⁶ EPCs and 1 × 10⁶ MSCs) for delivery.

**Stem-cell administration**

Four weeks after injection of ISO, rats with apparent impaired cardiac performance (>20% decrease in ejection fraction) validated by echocardiography examination were randomized into five groups, and allocated to receive saline injection (control group), 200 μL medium alone (EGM-2 group), 200 μL medium plus 2 × 10⁶ EPCs (EPC group), 200 μL medium plus 2 × 10⁶ MSCs (MSC group), or 200 μL medium plus a combination of 1 × 10⁶ EPCs and 1 × 10⁶ MSCs (EPC/MSC group).

The rats were intubated (with appropriate anaesthesia), and ventilated with room air (60 cycles/min) with a tidal volume of 1 mL/kg (DW-2000, Shanghai). The heart was exposed by a left thoracotomy. Cultured stem cells were injected evenly into the left ventricle wall, and the chest was closed in layers. The rats were monitored closely for further 12 weeks.

**Echocardiographic and haemodynamic evaluation**

At baseline before ISO injection, four weeks after ISO injection and 12 weeks after cell therapy, two-dimensional echocardiography and M-mode imaging were...
Figure 1. Phenotype of cultured stem cells. On the ninth day, EPCs had a typical ‘cobblestone-like’ appearance (A, ×100). Third passage MSCs had a spindle-shaped morphology (B, ×200). Dil-ac-LDL (C; in red, ×400) and FITC-UEA-1 (D; in green, ×400) immunostaining was also performed for cultured EPCs at this stage, and a merged image was created (E; in yellow, ×400). EPCs were further phenotyped by vWF staining (F, ×100). Flow cytometric analysis showed that some EPCs were positive for CD34 (G) and CD133 (H), and that most expressed KDR (I). Flow cytometric analysis showed a high ratio of CD90+ cells (J), but low ratios of CD45+ cells (K) and CD34+ cells (L).

carried out at papillary muscle level [10] using an echocardiographic system with a 14 MHz transducer (Acu-son Sequoia 521, USA). Left ventricular end-diastolic diameter (LVEDD), ejection fraction and fractional shortening ratio were analysed by an independent investigator.

After 12 weeks of observation, all rats underwent invasive catheterization. Briefly, a 1.5 F fluid-filled catheter was fully heparinized, introduced through a right carotid artery cut-down and inserted into the left ventricle, with positioning guided by echocardiography. Left ventricular pressure tracings were digitized using an analog-to-digital converter and stored on computer for analysis. Left ventricular end-diastolic pressure (LVEDP) and maximum rate of left ventricular pressure rise/fall (±dP/dt max) were analysed blind, using dedicated software (MPC Systems, 2000M, China).

Measurement of regional myocardial blood flow

Coloured microspheres were used to evaluate average regional myocardial blood flow (RMBF). While the fluid-filled catheter was still in the left ventricle, it was connected to a peristaltic pump, and 0.3 mL orange coloured microspheres (diameter 15 μm; Dye-Trak, USA) were injected directly into the left ventricle before tissue harvesting. Aspiration of a blood sample via the catheter started several beats after coloured-microsphere injection, and continued for 1 min at a rate of 0.5 mL/min. Coloured microspheres were recovered and analysed according to the manufacturer's instructions. Average RMBF was calculated as follows: (number of coloured microspheres in myocardium × 0.5)/(number of coloured microspheres in blood × weight of myocardium).
Tissue harvesting

At the end of the 12-week observation period, the rats were sacrificed with an overdose of anaesthetic (ketamine 300 mg/kg) and the hearts were harvested. The left ventricle was sliced, perpendicular to the septum, into four sections, each approximately 4–5 mm thick. Because of the patchiness of the areas of myocardial infarction, and to avoid sample selection bias, sections were selected randomly for further analysis, which included immunostaining, RMBF, enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Tissue slices for RMBF and immunostaining were kept in 4% formalin, and those for ELISA and real-time RT-PCR were snap frozen and stored at −80 °C.

Assessment of engraftment and cell differentiation

Transplanted male cells in female rat hearts were detected using fluorescence in situ hybridization (FISH) on paraffin-embedded tissue slices selected at random. A probe specific for the rat Y chromosome Sry gene was synthesized (HaoYang Co, China), with the sequence 5′-ATATG GTGTA GGTTG TTGTG CCATT GCAGC-3′. Sections were deparaffinized and denatured at 95 °C for 10 min, chilled on ice for 5 min, then incubated with the probe at 37 °C for 12 h. Nuclei stained with 5′-FAM-NHS (flavo-green) were detected at 490 nm.

Engrafted Sry-positive cells were phenotyped using immunofluorescence staining with an antibody against either cardiac troponin T ( monoclonal mouse anti-rat, Serotec, British) or vWF (rabbit polyclonal, Dako, Denmark), further characterizing the transplanted stem cells on the adjacent slice of FISH tissue section. Phycoerythrin-conjugated IgG antibody (BD, USA) was used as a secondary antibody.

Evaluation of myocardial fibrosis

Cardiac muscle fibrosis was detected using Masson’s trichrome staining of tissue slices selected at random. For each slice, 10 randomly selected fields were captured (magnification ×100) and images were digitized and analysed with a digital image analyser (MIQAS, Qiuwei Co, China).

Quantification of myocardial capillary density

Capillaries in the myocardium were semiquantified using immunohistochemical staining of endothelial cells with a polyclonal rabbit anti-vWF (Dako, Denmark), in tissue slices selected at random. A capillary vessel was defined as having a diameter <20 μm. The number of capillaries was counted under a light microscope (magnification ×250, OLYMPUS BH2, Japan) for 10 random fields in each transverse slice and presented as the mean number of blood vessels per unit area (preset at mm²).

Detection and quantification of apoptotic cells

Apoptotic cells were detected using tunnel staining (Roche, Germany), performed according to the manufacturer’s instructions, on tissue slices selected at random. Tissue sections were examined microscopically at ×400 magnification and greater than or equal to 100 cells were counted in 10 fields (OLYMPUS BH2, Japan). The apoptotic index was calculated as follows: (number of apoptotic cells/total number of cells) × 100%.

Quantification of angiogenic growth factor protein-expression levels

To assess the level of growth factor secretion, the randomly-selected heart tissue slices were crushed in ethanoic acid and centrifuged, and the supernatant was collected. VEGF (RD, USA), basic fibroblast growth factor (b-FGF, RD, USA) and angiopoietin-2 (Ang-2, ADL, USA) were measured by ELISA assay, according to the manufacturer’s instructions.

Quantification of Sry gene and angiogenic growth factor mRNA expression levels

Two-step real time RT-PCR, using SYBR® Green (Molecular Probes) as a dye, was carried out to analyse levels of VEGF, b-FGF, Ang-2 and Sry gene mRNA expression. Trizon (Gibco BRL, CA) was used to isolate mRNA, which was treated with DNase I and reverse-transcribed with random hexamers (Invitrogen), using Moloney murine leukaemia virus. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from the same tissue lysate was used as an internal control. The primer pairs for PCR amplification, targeting each specific gene, were designed using Primer Express 1.5 software (Applied Biosystems); the sequences are shown in Table 1. Real time RT-PCR was performed using the ABI 7000 system (Applied Biosystems, USA) with the following cycle conditions: 45 cycles of 30 s at 95 °C for denaturation, 45 s at 59 °C for annealing and 50 s at 72 °C for extension. Using ABI Prism 7300 SDS Software, ΔΔCt methods were used to quantify the mRNA expression level of each target gene.

Statistical analysis

Numerical values are presented as the mean ± standard deviation (S.D.). Comparisons between the five groups were made using a one-way analysis of variance, followed by the Scheffe-multiple-comparison test. Analyses were performed using SPSS version 10.0 statistical software (SPSS).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer pairs for angiogenic growth factors and the Sry gene.</th>
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</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Primers</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F 5′GGCATCTCTGACCCTGAACTGATA 3′ R 5′GGGTGTTGGAAGTCTCACA 3′</td>
</tr>
<tr>
<td>rat Sry</td>
<td>F 5′GGCTTCAAAGTATATATTGGG 3′ R 5′ATGCAATTGUGCCTTGA 3′</td>
</tr>
<tr>
<td>b-FGF</td>
<td>F 5′CGACCACACACGTAAACTA 3′ R 5′AGCAGGGCTCATCTCTTCT 3′</td>
</tr>
<tr>
<td>VEGF</td>
<td>F 5′GTTCGAGAAGGGGGAAGTTGG 3′ R 5′GGAGATCTGTGTTTTTGCAGGA 3′</td>
</tr>
<tr>
<td>Ang-2</td>
<td>F 5′GATGGCCAGCCTTATTTC 3′ R 5′ACATGCACTAAACCACCTGAC 3′</td>
</tr>
</tbody>
</table>

Combined transplantation of endothelial progenitor cells and mesenchymal stem cells

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FISH was performed on tissue slices selected at random from each group. No Sry-positive cells were present in the control group (A, ×400) or the EGM-2 group (B, ×400). Sry-positive cells were detected in cardiac tissue in the EPC/MSC group (C, ×400), the MSC group (D, ×200) and the EPC group (E, ×400). Some Sry-positive cells (D1, ×400) in the MSC group were also positive for cardiac troponin T (D2 ×400). Some Sry-positive cells (E1, ×400) in the EPC group were also positive for vWF (E2, ×400).

Inc. Chicago, USA). A P-value less than 0.05 was considered statistically significant.

**Results**

**Rat model of isoproterenol-induced myocardial injury**

Fifty-five of 70 female rats survived ISO injection. Five rats were excluded from the study because of insufficient decrease in cardiac performance. Increased LVEDD with decreased ejection fraction and fractional shortening was demonstrated by echocardiography in 50 female rats, four weeks after ISO injection (LVEDD, pre 0.45 ± 0.05 cm versus post 0.67 ± 0.04 cm; ejection fraction, pre 77.47 ± 1.97% versus post 56.16 ± 4.23%; fractional shortening, pre 73.20 ± 6.30% versus post 36.69 ± 2.23%; P < 0.05, respectively). These 50 rats were divided randomly into the five treatment groups (10 per group). After stem-cell transplantation, the final numbers in each treatment group were as follows: control, 6; EGM-2, 7; MSC, 7; EPC, 8; and EPC/MSC, 7.

**Engraftment and differentiation of transplanted cells**

Twelve weeks after stem-cell transplantation, FISH revealed that Sry-positive cells were not present in heart tissue in the control group (Fig. 2A) or the EGM-2 group (Fig. 2B), but were present in heart tissue in the EPC/MSC group (Fig. 2C) and the MSC group (Fig. 2D). In the EPC group, Sry-positive cells were found in blood vessels (Fig. 2E). Sry-positive cells (Fig. 2D1) from the MSC group were shown to express cardiac troponin T (Fig. 2D2). Similarly, Sry-positive cells (Fig. 2E1) from the EPC group were shown to express vWF (Fig. 2E2).

Real time RT-PCR analysis showed that expression of the Sry gene was higher in the EPC/MSC group (ΔΔCt, 0.32 ± 0.04%) than in the EPC group (0.21 ± 0.07%; P < 0.05) or the MSC group (0.19 ± 0.06%; P < 0.05). There was no difference in Sry gene expression between the EPC and MSC groups (P > 0.05). Sry gene expression was not detectable in the EGM-2 and control groups.

**Cardiac function and haemodynamics**

As summarized in Table 2, the EGM-2 and control groups did not show a significant improvement in cardiac performance, 12 weeks after cell transplantation. However, similar degrees of improvement were seen in the MSC and EPC groups, with regard to fractional shortening, ejection fraction and LVEDD (P < 0.05, respectively). The greatest improvement in cardiac function was seen in the EPC/MSC group (P < 0.05) although the differences between the EPC/MSC group and the MSC and EPC groups were not statistically different in terms of LVEDD (P > 0.05). Intracardiac pressure recordings showed that the maximum rise and fall in left ventricular pressure (±dP/dt max) and LVEDP improved significantly in the MSC and EPC groups compared with the control and EGM-2 groups (P < 0.05, respectively), and that the difference in improvement was even more pronounced in the EPC/MSC group (P < 0.05, respectively); these three variables were not significantly different in the EPC and MSC groups (P > 0.05, respectively).
Capillary density and regional myocardial blood flow

Representative images of myocardial capillary density, identified by vWF-targeted immunostaining, are shown in Fig. 3A–E. The increase in total capillary density was significantly greater in the MSC group (33.67 ± 4.09 per millimetre square) than in the EGM-2 (26.00 ± 4.00 per millimetre square; P < 0.05) or control groups (24.33 ± 6.77 per millimetre square; P < 0.05), but was even more notable in the EPC group (38.33 ± 6.11 per millimetre square; P < 0.05 versus the MSC group). When MSCs and EPCs were combined, the increase in total capillary density (42.37 ± 9.26 per millimetre square) was not significantly greater than that seen in the EPC group (P > 0.05).

In line with capillary density data, the increase in average RMBF was significantly greater in the EPC group (6.66 ± 0.46 mL/min) than in the MSC group (3.61 ± 0.59 mL/min; P < 0.05), the EGM-2 group (2.35 ± 0.61 mL/min; P < 0.05) and the control group (2.47 ± 0.38 mL/min; P < 0.05). The combination of MSCs and EPCs induced a significantly greater increase in average RMBF (7.14 ± 0.89 mL/min) than MSCs alone (P < 0.05), but the increase was not significantly greater than that induced by EPCs alone (P > 0.05).

Myocardial fibrosis

Masson’s trichrome staining showed patchy areas of fibrosis in the heart tissue in all groups (Fig. 3F–J). After cell transplantation, there was less collagen deposition in heart tissue in the MSC group (Fig. 3H, 31.81 ± 4.54%) and the EPC group (Fig. 3I, 30.28 ± 4.34%) than in the EGM-2 group (Fig. 3G, 36.18 ± 3.85%; P < 0.05) and the control group (Fig. 3F, 34.52 ± 5.19%; P < 0.05). No significant difference was detected between the EGM-2 and control groups, or between the EPC and MSC groups (P > 0.05, respectively). There was significantly less fibrosis in the EPC/MSC group (Fig. 3J, 25.59 ± 6.86%; P < 0.05) than in the other four groups.

Apoptotic index

As shown in Fig. 3K–O, each of the three groups that received stem-cell therapy had a significantly lower apoptotic index (EPC/MSC, 11.35 ± 1.70%; MSC, 21.12 ± 2.65%; EPC, 20.80 ± 2.58%) than the EGM-2 group (31.24 ± 10.52%; P < 0.05) and the control group (33.06 ± 8.01%; P < 0.05). The apoptotic index in the EPC/MSC group was significantly lower than that in the MSC and EPC groups (P < 0.05, respectively), but there was no significant difference between the EPC and MSC groups, or between the EGM-2 and control groups (P > 0.05, respectively).

Angiogenic growth factor protein and mRNA expression

Data on levels of angiogenic growth factor protein and mRNA expression are summarized in Table 2. VEGF, b-FGF and Ang-2 protein levels were slightly increased in the MSC group compared with the EGM-2 and control groups (P < 0.05, respectively). Both the EPC/MSC and the EPC groups had a significantly greater increase in the level of protein expression than the MSC group (P < 0.05). The EPC group had a significantly higher level of b-FGF protein expression than the EPC/MSC group (P < 0.05).

Real-time RT-PCR analysis of the targeted genes for these proteins revealed a higher level of mRNA expression in the MSC group than in the EGM-2 and control groups (P < 0.05, respectively), and mRNA expression levels were even greater in the EPC/MSC and EPC groups. However, the level of b-FGF mRNA expression in the EPC group did not differ from that in the EPC/MSC group (P > 0.05, respectively).

Discussion

Using a rat model of injured myocardium induced by ISO injection, we have shown that the combined transplantation of EPCs and MSCs, compared with transplantation of EPCs or MSCs alone, results in a greater improvement in cardiac function in terms of increase in ejection fraction and fractional shortening, decrease in LVEDP and changes in maximum left ventricular pressure.

In investigating the underlying mechanisms, we observed: the presence of Sry-positive cells in heart tissue, indicating that the transplanted cells had either differentiated or integrated into heart tissue; upregulation of expression of angiogenic growth factors, including VEGF, b-FGF and Ang-2; an association between the increased expression of these angiogenic factors and an increase in capillary density, which in turn led to an improvement in average RMBF; a potential reversal process, as histological examination revealed less fibrosis and fewer apoptotic cells.

Table 2 Cardiac function and haemodynamic profiles.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ejection fraction (%)</th>
<th>Fractional shortening (%)</th>
<th>LVEDD (cm)</th>
<th>LVEDP (mmHg)</th>
<th>+dp/dt (mmHg/s)</th>
<th>−dp/dt (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55.68 ± 3.34</td>
<td>36.82 ± 1.89</td>
<td>0.69 ± 0.06</td>
<td>22.11 ± 5.70</td>
<td>4380.41 ± 634.96</td>
<td>5189.02 ± 504.59</td>
</tr>
<tr>
<td>EGM-2</td>
<td>55.04 ± 3.53</td>
<td>35.45 ± 1.44</td>
<td>0.69 ± 0.09</td>
<td>23.68 ± 4.37</td>
<td>4191.31 ± 568.30</td>
<td>5003.07 ± 585.37</td>
</tr>
<tr>
<td>MSC</td>
<td>62.85 ± 4.84</td>
<td>42.36 ± 2.91</td>
<td>0.62 ± 0.04</td>
<td>18.59 ± 2.16</td>
<td>4470.55 ± 484.53</td>
<td>4605.51 ± 473.30</td>
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<tr>
<td>EPC</td>
<td>63.43 ± 4.49</td>
<td>43.86 ± 2.54</td>
<td>0.61 ± 0.05</td>
<td>18.98 ± 2.38</td>
<td>4573.71 ± 443.45</td>
<td>4546.51 ± 360.38</td>
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<td>EPC/MSC</td>
<td>68.23 ± 2.69</td>
<td>47.09 ± 2.17</td>
<td>0.60 ± 0.05</td>
<td>14.76 ± 2.26</td>
<td>4996.74 ± 523.16</td>
<td>4233.74 ± 437.28</td>
</tr>
</tbody>
</table>

*P < 0.05 vs EGM-2 group; †P < 0.05 vs EGM-2 group, MSC group and EPC group.
Figure 3. Immunostaining against vWF to identify capillaries (A–E), Masson's trichrome staining to detect myocardial fibrosis (F–J; collagen stained in green, myocardium stained in red) and Tunnel staining to detect apoptotic cells (K–O) in the control, EGM-2, MSC, EPC and EPC/MSC groups, respectively.

The pathological process of ISO-induced myocardial damage is characterized by patchy areas of myocardial infarction with well-maintained coronary vasculature. This model has also been shown to exhibit dose-dependent progression of postmyocardial infarction remodelling [8]. Although the underlying mechanism is not fully understood, there are certain similarities with traditional myocardial infarction attributable to ischaemic coronary disease, as oxidative stress is the main trigger for cardiac injury [7]. Using this ISO-induced myocardial injury model for the first time, we were able to demonstrate that the combination of EPCs and MSCs may induce neovascularization and hence augment cardiac performance. Our reasons for undertaking combined transplantation of EPCs and MSCs were based on the different biological properties of these stem cells. Improved myocardial contractility has been observed in a clinical trial where both EPCs and MSCs were used for transcoronary transplantation in patients with myocardial infarction [11]. The mechanism by which combined transplantation of EPCs and MSCs improves cardiac function significantly is not fully understood, but we have acquired a number of pieces of evidence that support this effect.

Firstly, we found evidence of transplanted cells incorporated into cardiac tissue. Sry-positive cells may have differentiated into cardiac tissue cells, as they exhibited positive immunostaining for troponin T, although we cannot exclude the possibility that these MSCs had merged with pre-existing cardiac tissue cells. The same phenomenon was observed for EPCs implanted into cardiac tissue, as

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGF protein (pg/mg)</th>
<th>VEGF mRNA (%)</th>
<th>b-FGF protein (pg/mg)</th>
<th>b-FGF mRNA (%)</th>
<th>Ang-2 protein (ng/mg)</th>
<th>Ang-2 mRNA (%)</th>
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<tr>
<td>Control</td>
<td>110.03 ± 4.31</td>
<td>2.91 ± 1.53</td>
<td>426.87 ± 25.46</td>
<td>11.86 ± 3.37</td>
<td>1.18 ± 0.24</td>
<td>9.93 ± 6.79</td>
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<td>EGM-2</td>
<td>121.38 ± 2.93</td>
<td>3.21 ± 1.10</td>
<td>415.30 ± 37.66</td>
<td>12.46 ± 2.14</td>
<td>1.01 ± 0.10</td>
<td>9.81 ± 4.83</td>
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<td>MSC</td>
<td>134.48 ± 4.88</td>
<td>4.93 ± 2.10*</td>
<td>545.50 ± 65.76</td>
<td>17.81 ± 5.65*</td>
<td>1.31 ± 0.10</td>
<td>12.84 ± 4.10*</td>
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<tr>
<td>EPC</td>
<td>154.25 ± 32.91†</td>
<td>7.97 ± 2.11†</td>
<td>972.41 ± 204.21†</td>
<td>38.66 ± 14.50†</td>
<td>1.46 ± 0.05†</td>
<td>18.40 ± 6.97†</td>
</tr>
<tr>
<td>EPC/MSC</td>
<td>185.75 ± 13.20‡</td>
<td>27.05 ± 1.84‡</td>
<td>732.85 ± 51.25‡</td>
<td>36.43 ± 12.68‡</td>
<td>1.73 ± 0.15‡</td>
<td>32.01 ± 13.80‡</td>
</tr>
</tbody>
</table>

*P < 0.05 vs EGM-2 group; †P < 0.05 vs EGM-2 group and MSC group; ‡P < 0.05 vs EGM-2 group, MSC group and EPC group.
Sry-positive cells detected by FISH were also positive for immunofluorescence staining against vWF in the EPC group.

In addition, the levels of angiogenic growth factor protein and mRNA expression were higher in the EPC and EPC/MSC groups than in MSC group. The factors in question (VEGF, b-FGF and Ang-2) have been shown to augment neovascularization and hence increase capillary density [12]. b-FGF is a powerful mitogen that stimulates the migration and proliferation of various vascular cell types, including smooth-muscle cells, endothelial cells and fibroblasts [13]. Ang-2 can stimulate the degradation of the base membrane by proteases secreted by activated endothelial cells, which aids migration, proliferation, and the formation of solid endothelial cell sprouts into the stromal space [14]. We were surprised to observe a higher level of b-FGF protein expression in the EPC group than in the EPC/MSC group, but a similar level of b-FGF mRNA expression in these two groups. This difference in post-translational regulation might be due to the interaction between EPCs and MSCs; the detailed underlying mechanism requires further investigation.

The beneficial effect of the increase in angiogenic factors was evidenced by the higher Sry gene expression levels detected by real time RT-PCR, indicating a synergetic effect of the combination therapy in enhancing either engraftment rate or possibly proliferative activity.

Finally, we observed the lowest level of fibrosis deposition in cardiac tissue in the EPC/MSC group, indicating a potential reversal of cardiomyopathy. We did not investigate the mechanism underlying this effect but propose that it may have been associated with the paracrine effects of the angiogenic factors secreted by transplanted cells and the lower numbers of apoptotic cells.

Recently, Suuronen et al. reported that the transplantation of EPCs resulted in better cardiac function, increased arteriole density and less myocardial fibrosis in a rat model of myocardial infarction than the transplantation of MSCs, or EPCs plus MSCs [15]. These results are strikingly different from our data, and can be attributed to a number of factors: a much shorter observation period post-transplantation; the use of far fewer EPCs and MSCs; a relatively superior baseline cardiac performance before cell transplantation (relatively smaller decrease in cardiac function compared with baseline before coronary ligation); and the use of a different animal model, involving different pathological processes.

Given the immunogenic properties of EPCs, it is advisable that the immunosuppressant cyclosporine A is administered. However, it is still uncertain whether cyclosporine A enhances the engraftment rate of cell therapy [16], and one report has shown that it can even suppress the differentiation process [17].

In conclusion, our study has shown for the first time that the intramyocardial infusion of culture-expanded EPCs together with MSCs may represent a novel and more efficient therapeutic strategy for the treatment of ISO-induced cardiomyopathy.

Limitations of the study

Our study was limited by the small sample size. In addition, we should have attempted to use BMNCs as control cells. Also, we were unable to label all the stem cells before transplantation, to enable us to identify the cells in vivo; this made the evaluation of engrafted stem cells more difficult. The ISO-induced cardiac injury model also has its own drawback, characterized by patchy areas of necrosis rather than a fixed risk area, which can make results difficult to interpret. Nevertheless, the data showed that the combined transplantation of EPCs and MSCs into a rat model of isoproterenol (ISO)-induced injured myocardium improved cardiac function after 12 weeks. We excluded all rats with a drop in myocardial performance of less or equal to 20% to minimize the variability inherent in this animal model. Further study is warranted to elucidate detailed underlying mechanisms, and an extended observation period is required to validate long-term outcome.

References


