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RESEARCH ARTICLE

# Role of a paracrine action of mesenchymal stem cells in the improvement of left ventricular function after coronary artery occlusion in rats

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**Purpose:** We aimed to determine whether soluble factors released by cultured mesenchymal stem cells (MSCs) improved cardiac function in an experimental model of myocardial infarction. **Methods:** MSCs were cultured in fresh medium. The conditioned medium, which contained factors secreted by MSCs, was collected after 4 days of culture. Fischer rats with 1-week-old myocardial infarction were divided into four groups that received: saline (n = 12); fresh medium (n = 10); conditioned medium (n = 8); or 2 million MSCs in fresh medium (n = 10) by direct intramyocardial injection. A total of 4 weeks later, left ventricular (LV) function was assessed by LV angiogram and by LV catheterization. Hearts were processed for histology. **Results:** Before treatment, LV angiogram assessment demonstrated that the baseline LV function was comparable among the four groups. At 4 weeks after treatment, LV angiogram and LV catheterization showed that LV ejection fraction was better in the fresh medium (49.5 ± 1.0%), conditioned medium (48.5 ± 2.1%) and MSCs groups (49.9 ± 4.2%) than in the saline group (43.7 ± 1.2%; $p < 0.05$). There were no significant differences in heart rate, blood pressure, postmortem LV volume, infarct size or septum thickness among the groups. The scar thickness was similar in the saline (395 ± 31 µm), fresh medium (404 ± 30 µm) and conditioned medium (397 ± 34 µm) groups, but significantly thicker in the MSCs group (560 ± 51 µm; $p < 0.05$). **Conclusion:** Fresh medium, conditioned medium and MSC injection all improved LV function at 4 weeks after treatment compared with saline treatment in a rat myocardial infarct model; only MSCs increased wall thickness. Since the culture medium contains nutrients and bovine serum, the roles of the soluble factors released by MSCs might be masked. The effect of these nutrients needs further investigation.

A paracrine effect of transplanted mesenchymal stem cells (MSCs) has been suggested to play a crucial role in the improvement of cardiac function in an experimental myocardial infarction model. We assessed the effects of saline, fresh culture medium, MSC-derived conditioned medium and MSCs on the left ventricular (LV) function in a rat myocardial infarction model. The results showed that fresh medium, conditioned medium and MSCs all improved LV function at 4 weeks after treatment compared with saline treatment. The mechanisms of beneficial effects of culture medium remain unclear and need further investigation.

Bone marrow-derived MSCs are multipotential cells, which have high self-renewal capability and are able to differentiate into a wide variety of lineages, including endothelial and myogenic cells [1]. Thus, MSCs are considered to be one of the potential cell sources for cell transplantation therapies of cardiac regeneration [2]. Although animal experiments and clinical trials have demonstrated that MSC transplantation therapies improve cardiac function after myocardial infarction, the underlying mechanisms of these therapies remain unclear. Grafted MSCs have been reported to form clusters of cell grafts and express cardiac muscle proteins on the border of the infarct in a rat myocardial infarction model [3] and induce neovascularization [4]. Our recently published data showed that MSC transplantation transiently improved LV function at 4 weeks after transplantation in a rat myocardial infarction model, when the grafted MSCs did not show complete myogenic differentiation and did not result in visible replacement of scar with sheets of muscle cells. The beneficial effect on LV function was lost at 6 months, although the grafted MSCs survived and expressed muscle markers and incorporated into blood vessels [5]. These results suggested that the improvement of cardiac function might

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be due to a paracrine effect of the transplanted MSCs. To address our hypothesis, in this study, we assessed the effects of conditioned medium from cultured MSCs on the LV function in a rat myocardial infarction model.

## Materials & methods

All animal studies were approved by the Institutional Animal Care and Use Committee of Good Samaritan Hospital and comply with the *Guide for the Care and Use of Laboratory Animals* [6]. The Association for Assessment and Accreditation of Laboratory Animal Care International accredits Good Samaritan Hospital.

### *MSC isolation & expansion*

Allogeneic MSCs were isolated, characterized and cultured according to the previous established methods [7,8]. Briefly, the MSCs were isolated from the femoral and tibial bones of donor ACI rats (a cross between the August and Copenhagen–Irish strains), as described previously [7,8]. MSCs were expanded in fresh medium composed of 44.5% Ham's F12 medium (F12), 44.5% minimum essential medium (MEM), 10% fetal bovine serum and 0.1% antibiotic/antimycotic. The conditioned medium was collected at 4 days after MSCs culture. All cells and medium were kept frozen until use. MSC viability ranged from 90 to 95%, as assessed by trypan blue.

### *Surgical preparation*

Female Fischer CDF rats were anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (5 mg/kg), intubated and mechanically ventilated with room air (rate 60 cycles/min, tidal volume 1 ml per 100 g body weight, Harvard Apparatus Rodent Ventilator, Model 683, MA, USA). The heart was exposed by a left thoracotomy through the fourth intercostal space. The free wall of the left ventricle was exposed after the pericardium was excised. The left anterior descending coronary artery was encircled with a silk suture and ligated. The rats recovered under care, and buprenex (0.001 mg/100 g body weight, daily) was administered for 2 days as analgesic.

The rats with 1-week-old myocardial infarction were re-anesthetized and hearts re-exposed (as described above). After locating the infarcted area, MSCs ($2 \times 10^6$ cells in ~70 µl fresh medium; n = 10), conditioned medium (~70 µl; n = 8), fresh medium alone (~70 µl; n = 10) or saline (~70 µl; n = 12) were injected directly into the scar area with a 28-gauge needle attached to an insulin syringe. After successful injection, typified by the formation of a bleb covering the infarct zone, the chest was closed and animals recovered under care.

### *Assessment of LV function by angiography*

Left ventricular contrast angiography was performed with a XiScan 1000 C-arm x-ray system (XiTec, Inc; 3-inch field of view) before treatment at 1 week after induction of myocardial infarction at baseline and at 4 weeks after treatment. After rats were anesthetized, a catheter was inserted into the left jugular vein, followed by the injection of 1 ml nonionic contrast. Under view of anterior–posterior and lateral projections, video images were acquired on half-inch super-VHS videotape at 30 frames/second under constant fluoroscopy. LV volumes in systole and diastole were calculated from the video images in a blinded manner. All parameters were averaged over three consecutive cycles in both projections. Ejection fraction (%) was calculated as:

$$100 \times \frac{\text{vol. in diastole} - \text{vol. in systole}}{\text{vol. in diastole}}$$

and averaged over both projections.

### *Hemodynamic measurements*

After angiography, a 2F high-fidelity, catheter-tipped micromanometer (model SPR-869, Millar, Inc) was inserted into the right carotid artery to record arterial blood pressure and heart rate, then advanced into the ascending aorta and into the LV to record peak maximum and minimum LV pressure change over time (dP/dt).

### *Postmortem LV volume & histological measurements*

After the completion of the hemodynamic measurement, hearts were arrested in diastole by an intravenous injection of 2 mEq KCl administered under deep anesthesia. The excised hearts were pressure-fixed with formalin (pressure equal to 13 cm water column). After fixation, postmortem LV volumes were measured by filling the cavity with water and weighing, repeated three times.

Following LV volume measurement, the hearts were cut into three transverse slices and embedded in paraffin and processed for histology. Hematoxylin and eosin, picrosirius red staining and immunohistochemical staining with primary antibody against sarcomeric actin (Dako M0874, 1:75) were performed on sections (5 µm thickness) of

the paraffin-embedded tissue. Computerized planimetry of the histological images of the stained sections was used to measure and calculate:

- Scar thickness (average of five equidistant measurements) and septum thickness (average of three equidistant measurements);
- Epicardial circumference and endocardial circumference, and circumference occupied by the infarcted wall. The infarct size was expressed as percentage of total LV circumference;
- Expansion index was calculated as defined by Hochman and Choo [9], which is expressed as:

$$\frac{\text{LV cavity area}}{\text{total LV area}} \times \frac{\text{Septum thickness}}{\text{Scar thickness}}$$

### Statistics

All data are presented as mean ± standard error of the mean (SEM). Comparisons of results among groups were made by one way ANOVA. If an f-value of less than 0.05 was obtained, differences among the means were determined by Tukey's Studentized Range test.

### Results

Two rats in the saline group and one in the fresh medium group died after anesthesia administration at 4 weeks after treatment. One in the saline group, two in the fresh medium group and one in the MSC group died after angiography. One in the saline group and one in the conditioned medium group were excluded for histology because of sample damage during processing.

### LV stroke volume & ejection fraction by angiography

The baseline LV stroke volumes and ejection fractions were comparable among the four groups before treatment at 1 week after myocardial infarction (Table 1). At 4 weeks after treatment, the LV ejection fraction was similar in groups receiving fresh medium (49.5 ± 1.0%), conditioned medium (48.5 ± 2.1%) and MSCs (49.9 ± 4.2%), and was significantly greater than the group receiving saline (43.7 ± 1.2%; $p < 0.05$) (Table 1).

### Hemodynamics

At 4 weeks after treatment, heart rate, blood pressure and -dP/d*t* (LV peak negative change in pressure over time) did not significantly differ between the four groups (Table 1). +dP/d*t* (LV peak positive change in pressure over time) was similar in groups receiving conditioned medium and MSCs, but it was significantly greater in the fresh medium group compared with the group receiving saline ($p < 0.05$) (Table 1).

**Table 1. Parameters of left ventricular function and postmortem morphometery.**

| | Saline group | Fresh medium | Conditioned medium | MSCs |
|---|---|---|---|---|
| ***Angiographic analysis (n)*** | ***10*** | ***9*** | ***8*** | ***10*** |
| Stroke volume (μl) at baseline | 120 ± 4 | 128 ± 4 | 132 ± 3 | 143 ± 7 |
| Stroke volume (μl) after treatment† | 129 ± 6 | 163 ± 6 | 170 ± 6 | 164 ± 8 |
| LVEF (%) at baseline | 54.7 ± 1.2 | 54.4 ± 0.5 | 53.4 ± 1.6 | 53.7 ± 1.5 |
| LVEF (%) after treatment | 43.7 ± 1.2 | 49.5 ± 1.0* | 48.5 ± 2.1* | 49.9 ± 4.2* |
| ***Hemodynamics (n)*** | ***9*** | ***7*** | ***8*** | ***9*** |
| Heart rate (beats/minute) | 231 ± 10 | 227 ± 10 | 245 ± 8 | 219 ± 7 |
| Systolic blood pressure (mmHg) | 145 ± 7 | 161 ± 9 | 158 ± 6 | 135 ± 7 |
| Diastolic blood pressure (mmHg) | 109 ± 3 | 113 ± 4 | 117 ± 2 | 104 ± 3 |
| +dP/d*t* (mmHg/second) | 2746 ± 700 | 5350 ± 627* | 4895 ± 296 | 4769 ± 625 |
| -dP/d*t* (mmHg/second) | 1970 ± 601 | 3450 ± 328 | 2635 ± 274 | 3105 ± 320 |
| ***Postmortem analysis (n)*** | ***12*** | ***10*** | ***8*** | ***10*** |
| Postmortem LV volume (μl) | 351 ± 15 | 379 ± 14 | 374 ± 12 | 367 ± 22 |
| ***Histology (n)*** | ***11*** | ***10*** | ***7*** | ***10*** |
| Infarct size, % of LV circumference | 37.8 ± 3.8 | 39.7 ± 3.1 | 36.9 ± 4.8 | 33.6 ± 4.3 |
| Scar thickness (μm) | 395 ± 31 | 404 ± 30 | 397 ± 34 | 560 ± 51‡ |
| Septum thickness (μm) | 993 ± 43 | 1047 ± 55 | 1086 ± 66 | 1053 ± 27 |

**Statistically significant vs saline, $p < 0.05$.*

*‡Statistically significant vs saline, fresh medium and conditioned medium, $p < 0.05$.*

*†After treatment is 4 weeks after treatment.*

*dP/dt: LV peak change in pressure over time; LV: Left ventricular; LVEF: Left ventricular ejection fraction; MSC: Mesenchymal stem cell.*

**Figure 1. Representative picrosirius red-stained sections of hearts show that cardiac muscle stains yellow, and collagen stains red.**


The blue staining shows the blood vessels. **(B**, **D**, **F & H)** These are the higher magnification (×40) of the boxed area in **A** (saline group), **C** (fresh medium group), **E** (conditioned medium group) and **G** (MSC group), respectively. The dotted lines show the border of myocardial infarction with thin-walled, transmural collagenous scars. Note that some areas of the MSC-treated scar area are thicker than in the other groups and have a higher cell density.

### Postmortem LV volumes & histological measurements

At 4 weeks after treatment, there were no significant differences in the postmortem LV volume, infarct size as a percentage of the LV circumference or septum thickness among the 4 groups (Table 1). The scar wall thickness was comparable in the groups receiving saline (395 ± 31 µm), fresh medium (404 ± 30 µm) and conditioned medium (397 ± 34 µm), but was significantly thicker in the group receiving MSCs (560 ± 51 µm; $p < 0.05$) (Table 1).

Hematoxylin and eosin, and picrosirius red staining showed that the scars were transmural and thin and composed of collagenous tissues with a thin discontinuous layer of subendocardial cardiac myocytes in all of the four groups (Figure 1). MSC treatment increased scar thickness due to an increase in cell density, including cells that stained positive for α-sacromeric actin besides an increase of collagen deposition (Figure 2). The cell density in the infarct area was 4280 ± 291 cells/mm$^2$ in the MSC group. It was significantly higher than the cell density in the other three groups (2124 ± 116 cells/mm$^2$ in the saline group, 2112 ± 101 cells/mm$^2$ in the fresh medium group and 2267 ± 119 cells/mm$^2$ in the conditioned medium group; $p < 0.05$).

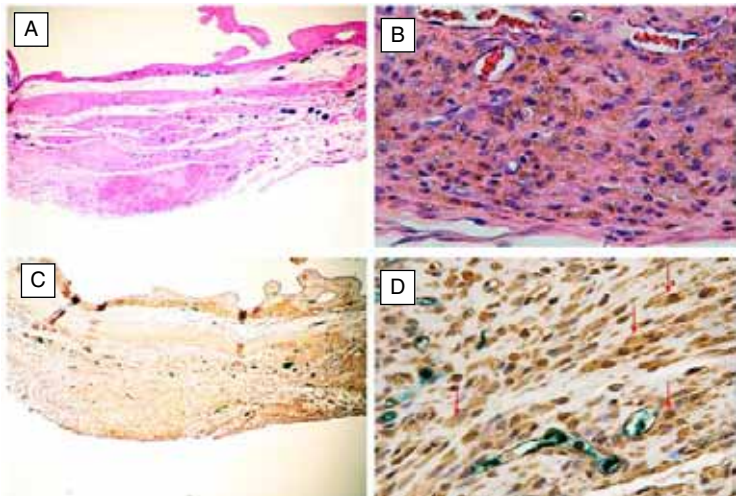
## Discussion

This study demonstrates that allogeneic MSCs, fresh medium and conditioned medium all improve global LV function at 4 weeks after treatment. Combined transplantation with MSCs and fresh medium significantly increased scar thickness. These results suggest that there are some substances in the fresh medium that may benefit the grafted MSCs or the heart, and improve the LV function.

Cellular cardiomyoplasty aims to regenerate damaged myocardium and induce revascularization of the injured region through cell transplantation. Bone marrow contains a useful cell source for cell transplantation therapies [10,11]. Bone marrow-derived MSCs are multipotent cells and represent less than 0.01% of all nucleated bone marrow cells. Although they can be readily expanded *in vitro*, there is little evidence of *in vivo* proliferation due to contact inhibition [1]. Thus, MSCs need to be expanded *ex vivo* for cell therapy. The isolation and *ex vivo* expansion of MSCs are time consuming, making using of autologous cells difficult. The type of MSC that we used was allogenic and has been suggested to be immune-privileged [1]. Thus, an 'off-the-shelf' approach of allogeneic MSCs has the potential for clinical application.

MSCs are able to differentiate into cardiomyocytes in defined culture media [12]. Toma and colleagues transplanted human MSCs into normal left ventricle of CB17 SCID/beige adult mice and observed that the engrafted human

**Figure 2. Representative pictures to show the high density of cells in the scar area in the MSC-treated myocardial infarction.**




**(A)** (×40) and **(B)** (×400) are H&E staining of the boxed area in **Figure 1G**. **(C)** (×40) and **(D)** (×400) are the immunohistochemical staining for sacromeric actin of the boxed area in **Figure 1G**. Note that there are numerous cells within the scar area, and that these cells are sacromeric actin-positive staining (red arrows).

MSCs expressed myogenic markers at 1 week after injection [13]. Shake and colleagues injected autologous MSCs into myocardial infarction in a pig model [14]. The transplanted MSCs expressed muscle-specific proteins, assessed by immunohistochemistry at 4 weeks after injection. Moreover, the MSC therapy attenuated contractile dysfunction and pathological thinning after myocardial infarction in this model. These results suggested that MSCs may be used for cellular cardiomyoplasty to augment the function of diseased myocardium. However, the mechanism by which bone marrow-derived MSC therapies improve cardiac function remains controversial. Recent studies have highlighted the potential paracrine effects of grafted MSCs in the infarcted myocardium. Grafted bone marrow-derived cells may release paracrine factors to improve cardiac function. Kamihata and colleagues injected bone marrow-derived mononuclear cells into the infarcted myocardium in a swine myocardial infarction model [15]. Grafted cells significantly upregulated the expression of basic fibroblast growth factor, vascular endothelial growth factor and angiopoietin in the myocardium, and significantly improved regional blood flow, capillary density and cardiac function. Tang and colleagues injected autologous MSCs directly into the peri-infarct zone in a rat myocardial model [16]. At 2 weeks after injection, the expression of basic fibroblast growth factor, vascular endothelial growth factor and stem cell homing factor significantly increased, and the pro-apoptotic protein Bax decreased, assessed by western blot analysis in the ischemic myocardium. These findings were accompanied by an increase in capillary density and improvement in cardiac function. These results suggested that a paracrine action of the engrafted cells might be the major mechanism by which cardiac function is improved by MSC transplantation therapy. In order to provide evidence for the hypothesis that the secreted soluble paracrine cytoprotective factors of MSCs play a crucial role in the cardiac function improvement, Gnecchi and colleagues collected the conditioned medium from cultured bone marrow-derived MSCs overexpressing Akt (Akt-MSCs), and demonstrated that the Akt-MSC-conditioned medium markedly inhibits hypoxia-induced apoptosis and triggers vigorous spontaneous contraction of adult rat cardiomyocytes *in vitro* [17]. The Akt-MSC-conditioned medium significantly limited infarct size and improved ventricular function after intramyocardial injection into infarcted hearts. These data support the hypothesis of paracrine mechanisms of myocardial protection and functional improvement in MSC transplantation therapy.

In our present study, we also observed that conditioned medium and MSC treatment significantly improved cardiac function compared with the saline-treated group, which was consistent with the reported results in Gnecchi's study [17]. The interesting and somewhat surprising finding of this study is that treatment with serum-containing fresh medium also improved cardiac function. One of the explanations is that the fetal bovine serum is a complex mixture of a large number of unknown constituents, including a broad spectrum of macromolecules, carrier proteins for lipoid substances and trace elements, attachment and spreading factors, low molecular weight nutrients, hormones and growth factors [18,19]. The hormones and growth factors contained in the fresh medium might work as paracrine factors to improve cardiac function. Another possible explanation is that the injected fresh medium provides essential nutrients required by injured cells within the ischemic area. This was supported by the fact that the MSCs that were suspended in the fresh medium significantly increased the scar thickness compared with the saline group. By contrast, our previous study demonstrated that MSCs that were suspended in saline did not increase scar thickness in the same myocardial infarction model [5].

It is likely that the increase in cell number and wall thickness in the cell transplant group was due to an increased mass of the wall of the scar due to the cells themselves, coupled with an improved survival rate by implanting them with fresh medium (rather than in saline as performed in our previous study [5]).

In summary, this study demonstrates that serum-containing culture medium has a similar beneficial effect on cardiac function as conditioned medium or MSCs in rat myocardial infarction. The mechanism of the benefit is unclear. Since many commercially available serums are of a high uniform quality and contain ill-defined components, future studies are needed to define the factors within the culture medium that can improve the cardiac function of the infarcted heart.

**Executive summary**

- Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent cells and a potentially useful cell source for cell transplantation therapy of damaged myocardium.
- Paracrine action of the engrafted MSCs might be the major mechanism by which cardiac function is improved by MSCs transplantation therapy.
- This study demonstrates that serum-containing culture medium has a similar beneficial effect on cardiac function as conditioned medium or MSCs in rat myocardial infarction.
- The hormones and growth factors contained in the fresh medium might work as paracrine factors to improve cardiac function.

## Acknowledgements

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