Original Article

# Comparison Between Transepicardial Cell Transplantations: Autologous Undifferentiated Versus Differentiated Marrow Mesenchymal Stem Cells

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## Abstract

***Background:*** *Marrow-derived mesenchymal stem cells (MSCs) have been heralded as a source of great promise for the regeneration of the infarcted heart. There are no clear data as to whether or not in vitro differentiation of MSCs into major myocardial cells can increase the beneficial effects of MSCs. The aim of this study was to address this issue.*

***Methods:*** *To induce MSCs to transdifferentiate into cardiomyocytes and endothelial cells, 5-Azacytidine and vascular endothelial growth factor (VEGF) were used, respectively. Myocardial infarction in rabbits was generated by ligating the left anterior descending coronary artery. The animals were divided into three experimental groups: I) control group, II) undifferentiated mesenchymal stem cell transplantation group, and III) differentiated mesenchymal stem cell transplantation group. The three groups received peri-infarct injections of culture media, autologous undifferentiated MSCs, and autologous differentiated MSCs, respectively. Echocardiography and pathology were performed in order to search for improvement in the cardiac function and reduction in the infarct size.*

***Results:*** *Improvements in the left ventricular function and reductions in the infarcted area were observed in both cell transplanted groups (Groups II and III) to the same degree.*

***Conclusion:*** *There is no need for prior differentiation induction of marrow-derived MSCs before transplantation, and peri-infarct implantation of MSCs can effectively reduce the size of the infarct and improve the cardiac function.*

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***Keywords:*** *Myocardial infarction • Stem cell • Bone morrow • Differentiation*

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## Introduction

The remodeling process following myocardial infarction (MI) will eventually lead to the impairment of the left ventricular function.[1,2]

The use of bone marrow-derived mesenchymal stem cells (MSCs) is an auspicious method which prevents deleterious remodeling and improves the left ventricular (LV) function.[3,4]

5-Azacytidine, which is a DNA demethylating agent, and vascular endothelial growth factor (VEGF) can induce MSCs to transdifferentiate into cardiomyocytes and endothelial cells, respectively.[5,6] Some authors have used a mesenchymal stem cell pretreatment with 5-Azacytidine to increase the probability that these cells will be directed toward a cardiomyocyte-differentiation pathway,[7-9] but the significance of this approach is not yet clear.[10] On the other hand, it has been suggested that an immature, more plastic cell may be more effective than an ex vivo pre-differentiated, committed cell.[11]

On account of the fact that there are no published data from a systematic study which might indicate whether or not in vitro differentiation induction of MSCs has more beneficial effects for the regeneration of the infarcted myocardium and improvement of LV function rather than undifferentiated MSCs, we sought to address this issue by choosing a rabbit model, suggested as a good experimental model for infarction-related researches.[12]

## Methods

This study was performed in accordance with the guidelines published in Guide for the Care and Use of Laboratory Animals (NIH publication 8523, revised 1996).

### Cell isolation and expansion

Male New Zealand white rabbits were anesthetized with ketamin (50 mg/kg I.M.) and xylazine (5 mg/kg I.M.). Then 5-8 ml of marrow was aspirated from the iliac crest with an 18-gauge needle connected to a syringe containing 3000 units of heparin. Bone marrow-derived mononuclear cells (BM-MNCs) were isolated by centrifugation on a Ficoll gradient. Next, BM-MNCs were washed twice with phosphate-buffered saline (PBS), pelleted by centrifugation, and resuspended in culture medium DMEM (Sigma) supplemented with 20% fetal bovine serum (FBS). The cells were subsequently introduced into 25 $cm^2$ flasks and incubated with 95% air and 5% $CO_2$ at 37 °C. The medium was changed subsequently twice per week. Non-adherent mononuclear and red blood cells were removed during the first few medium changes. The attached cells grew and developed colonies in approximately 5-7 days. Mesenchymal stem cells (MSCs) were passaged prior to confluency by detachment using 0.25% trypsin/EDTA (Gibco).

### Cardiomyocyte and endothelial cell differentiation of MSCs

The differentiation of cardiomyocytes from MSCs was performed as described previously.[13,14] The second passage of MSCs was seeded into flasks at a concentration of 20000 cells/$cm^2$. On the second day, the medium was changed and the cells were treated for 24 h with a medium containing 6 µM/L 5-Azacytidine. Thereafter, the cells were washed and incubated with DMEM containing 10% FBS. The medium was changed every 3 days. Differentiation was confirmed by immunostaining. For fluorescent immunostaining, the cells were washed with PBS and fixed by incubating in 4% paraformaldehyde for 20 min before being washed three times with PBS, permeabilized with 0.1% triton X-100 for 15 min, and rinsed three times in PBS. Proteins were blocked by incubating the cells in 1% bovine serum albumin (BSA). After that, the cells were incubated overnight at 4 °C with primary antibodies including anti-sarcomeric α-actinin (1:200; Sigma) and anti-sarcomeric myosin, MF20 (1:20; Developmental studies, Hybridoma Bank). Afterwards, the cells were washed three times with PBS and incubated with the 1:100 diluted fluorescence isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma) for 3 h at room temperature. After having been rinsed in PBS, the cells were analyzed using a fluorescence microscope (Olympus). Non-induced MSCs were stained as negative control.

To induce MSCs to differentiate into endothelial cells, the second passage of MSCs was treated for 2 weeks with a medium containing 20 ng/ml VEGF. The medium was changed every 3 days. To confirm the differentiation, fluorescent immunostaining was carried out for VEGF receptor-2 (KDR) with the following primary antibody: anti-VEGF receptor-2 (KDR) (1:100; Sigma). Phycoerythrin (PE)-conjugated anti-mouse antibody (1:100; Sigma) was used as a secondary antibody. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). As negative control, non-induced MSCs were stained through a similar method.

### Myocardial infarction (MI) generation and experimental groups

After anesthesia, the rabbits were mechanically ventilated and their hearts were exposed through left thoracotomy. The left anterior descending coronary artery was ligated with a 6-0 polypropylene, just below the tip of the left auricle. The regional pallor of the anterior cardiac surface confirmed myocardial ischemia.

The animals were divided into the following three experimental groups: I) control group (n = 6), II)

undifferentiated mesenchymal stem cell (UMSC) transplantation group (n = 7), and III) differentiated mesenchymal stem cell (DMSC) transplantation group (n = 7). They were respectively injected with 200 µL of culture media, $10^6$ undifferentiated MSCs, and $5\times10^5$ cardiomyocyte plus $5\times10^5$ endothelial differentiation of MSCs at four sites bordering the infarcted area 14 days post-MI.

### *Cardiac function assessment*

Transthoracic echocardiography was performed using an echocardiographic system (Toshiba SSA-380A) provided with a 7.5 MHz linear transducer before MI, 14 days post-MI before injection and 28 days post-injection. LV anterior wall thickness (AWT, mm), posterior wall thickness (PWT, mm), body weight-corrected LV end-diastolic dimension (EDD/BW), LV ejection fraction (EF, %), and

fractional shortening (FS, %) were measured by two blind examiners.

### *Pathology*

Twenty-eight days after injection, the animals were heparinized (500 U/kg) and sacrificed by an overdose of pentobarbital, after which, body weight and LV weight were measured. LV was then fixed in 10% buffered formalin and cut into 6 transverse slices from base to apex. The slices were embedded in paraffin, and two 7 µm tick serial sections were cut from each slice. These two sections were stained with hematoxylin-eosin and Masson`s trichrome and scanned. For each transversely sliced preparation with infarction, the LV wall area as well as the infarcted area was calculated as described previously.[15]

### *Statistical analysis*

All the values are expressed as mean±SD. The differences in echocardiographic data between the three groups were determined by two-way repeated-measures analysis of variance (ANOVA), followed with a post hoc Tukey-Kramer`s test. After the evaluation of the homogeneity of variance and normal distribution of post-sacrifice data, a statistical comparison between the post-sacrifice data was performed using the one-way ANOVA, followed by Tukey-Kramer's multiple comparison test. Values of $p<0.05$ were considered statistically significant.

## *Results*

### *In vitro differentiation of MSCs into cardiomyocytes and endothelial cells*

Within 2 weeks after 5-Azacytidine treatment, fluorescent immunostaining of the differentiated cells revealed that cardiomyocyte markers, including sarcomeric myosin and α-actinin, were expressed in the cells (Figure 1a-d).

MSCs treated with VEGF expressed KDR (Figure 1e and 1f). Non-induced undifferentiated MSCs were negative for these antigens.

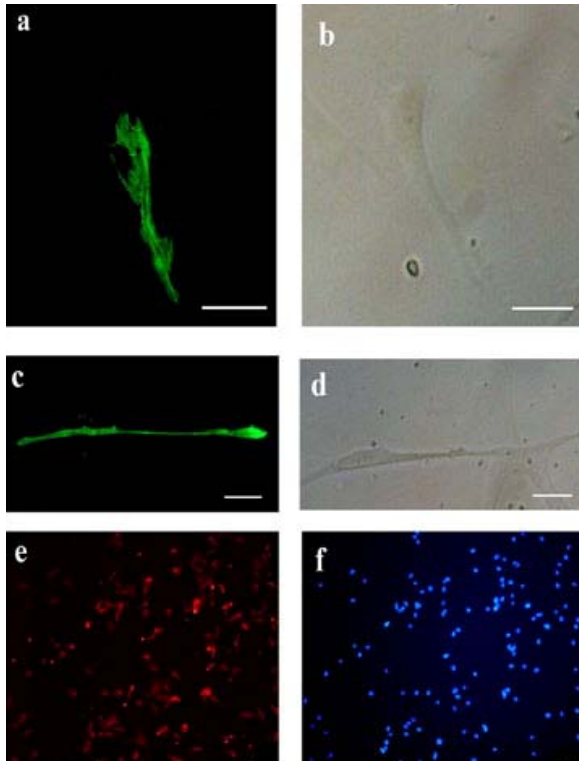


Figure 1. Fluorescent immunostaining of differentiated MSCs toward cardiomyocytes and endothelial cells. (a and c) FITC-positive cells stained for sarcomeric α-actinin (a) and sarcomeric myosin (MF20) (c) 2 weeks after induction with 5-Azacytidine. (b and d) Phase contrast photographs related to (a) and (c), respectively. (e) PE-positive cells stained for KDR, 2 weeks after VEGF treatment. (f) DAPI-stained nuclei related to panel (e). Non-induced undifferentiated MSCs were negative for these antigens. Scale bars: 50 µm in panels (a), (b), (c) and (d); 100 µm in panels (e) and (f)
MSCs, Mesenchymal stem cells; FITC, Fluorescence isothiocyanate; MF20, A myosin heavy chain marker; PE, Phycoerythrin; KDR, Kinase insert domain-containing receptor; VEGF, Vascular endothelial growth factor; DAPI, 4′,6-diamidino-2-phenylindole

### *Echocardiography*

Before MI and 2 weeks post-MI, echocardiographic parameters between Groups I, II, and III were not statistically different. Twenty-eight days post-injection, a significant increase in LV anterior wall thickness, EF, and FS, as well as a significant decrease in EDD/BW was observed in the cell-transplanted groups compared with those of the control group (Figure 2a, 2c-e). However, there was no significant difference in these parameters between Groups II and III. Posterior wall thickness was similar between Groups I, II, and III at all times (Figure 2b).

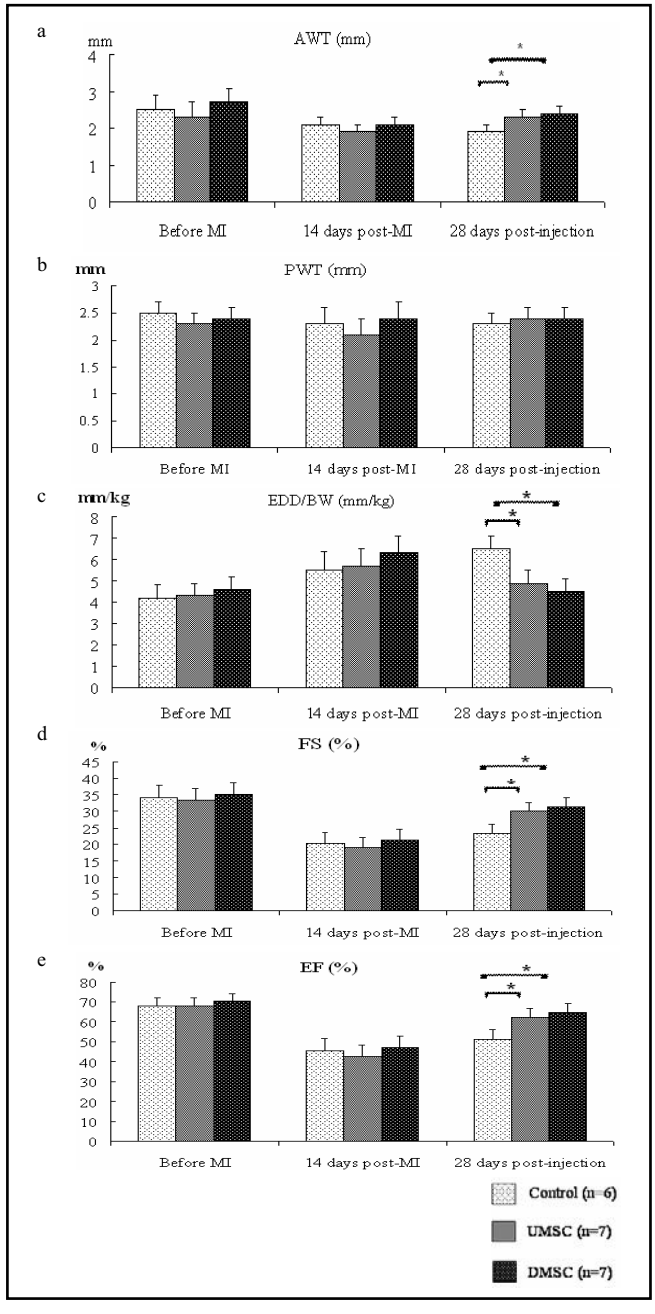


Figure 2. Improvement in cardiac function after cell transplantation.
AWT, anterior wall thickness; PWT, posterior wall thickness; EF, ejection fraction; FS, fractional shortening; BW, body weight; Control, culture media-injected group; DMSC, differentiated mesenchymal stem cell-transplanted group; UMSC, undifferentiated mesenchymal stem cell-transplanted group
*p value < 0.05

## *Pathology*

Twenty-eight days post-injection, we observed scar tissue consisting of collagen and fatty tissue in the infarcted zone of all the groups. The infarct sizes in Groups I, II, and III were 16.8±3.0 %, 5.9±2.0 %, and 5.5±1.7 %, respectively. A significant reduction in the infarcted area occurred in the cell-transplanted groups versus the control group (p<0.0001) (Figure 3d). Nonetheless, a statistical analysis of the infarcted area of the two transplanted groups (II and III) did not show a significant difference (p=0.951). Olysia, Soft Imaging System, Germany. (Figure 3d)

No significant differences were observed in LV weight and LV wall area between the three groups (Figure 3b and c). Respective transverse diameters of the cardiomyocytes in the left ventricular anterior wall and left ventricular posterior wall of the three groups were similar, but the anterior wall cardiomyocyte diameter was significantly greater than that of the posterior wall cardiomyocytes (Figure 3a).

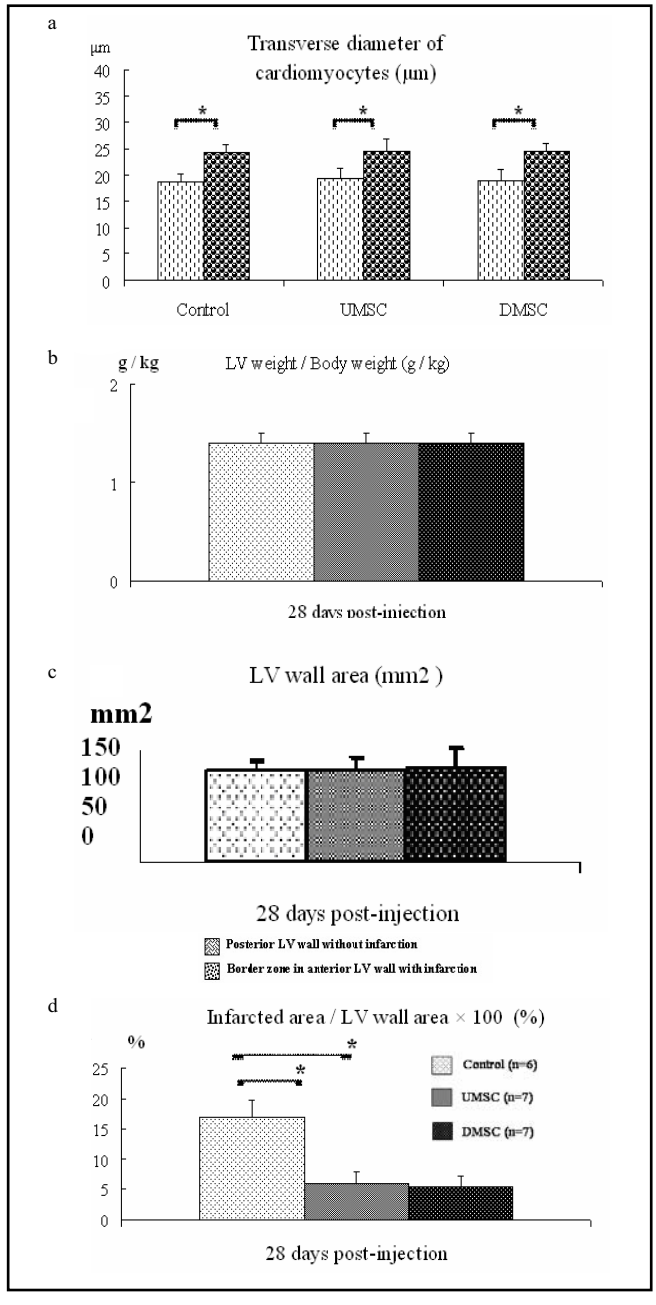


Figure 3. Changes in the infarct size in the cell-transplanted groups
Control, culture media-injected group; UMSC, undifferentiated mesenchymal stem cell-transplanted group; DMSC, differentiated mesenchymal stem cell-transplanted group; LV, left ventricular
*p value < 0.05

## Discussion

The significant decrease in the scar tissue in the cell-transplanted groups compared to that in the control group can be attributed to a reduction in collagen synthesis and/or formation of new cardiomyocytes. Since the total LV area and weight as well as the responsible for the reduction of the infarcted areas in the cell-transplanted hearts compared to the control hearts. Recently, it has been transverse size of cardiomyocytes were similar in all the experimental groups, the hypertrophy of the preexisting myocytes could not be demonstrated that in bone marrow-derived mononuclear cell-treated hearts as opposed to saline-treated hearts, the expression of repair However, the improvement in cardiac function may be ascribed to the self-renewal ability of MSCs and para-secretion of growth factors, in addition to the prevention of apoptosis in ischemic myocardium.[16-18] Paracrine factors secreted by MSCs and incorporation of MSCs into newly forming blood vessels by differentiation of MSCs in situ can related cytokines such as transforming growth factor (TGF)-β, a mediator stimulating h collagen synthesis, is down-regulated and this is likely to contribute to an improved LV function as well as a reduction in the scar tissue.[15]

enhance the neovascularization.18-20 An increase in the blood supply in the infarcted area, as a result of neovascularization, would prevent further necrosis or apoptosis of hypertrophied but otherwise viable myocardium, and increase the viability of implanted cells, thus improving ventricular function.[21]

Bittira et al. reported that the pretreatment of marrow stromal stem cells with 5-aza-2`deoxycytidine, which is an active metabolite of 5-Azacytidine, had a better phenotypic outcome compared with untreated stromal cells when they were injected directly into the scar created by cryoinjury.[22] They concluded that when MSCs were implanted directly within the scar, because of the nonmyogenic milieu of the scar, pretreating MSCs with 5-Aza could enhance their myogenic differentiation.[22] Nevertheless, they did not measure the infarcted area in their experimental groups. Moreover, the main difference between their study and the clinical setting is the cryoinfarction model.

In conclusion, directing MSCs toward cardiac cells before transplantation is not necessary. A peri-infarct injection of marrow-derived MSCs can effectively reduce the size of the infarct and improve the cardiac function.

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The study protocol was approved by the Institutional Review Board and Ethics Committee of Tehran Heart Center.

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