Arch Iranian Med 2007; 10 (4): 467 – 473

Original Article

Autologous *In Vitro* Expanded Mesenchymal Stem Cell Therapy for Human Old Myocardial Infarction

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Background: **Stem cell transplantation after myocardial infarction has been claimed to restore cardiac function. Mesenchymal stem cells attract a lot of attention because of the feasibility of *in vivo* and *ex vivo* differentiation to cardiomyocytes and endothelial cells as well as their trophic effect on tissue repair. In this study, we investigated the efficacy of autologous bone marrow derived mesenchymal stem cells in improving heart function in patients with old myocardial infarction.**

Methods: **Eight patients with old myocardial infarction and proper inclusion criteria were injected with mesenchymal stem cells at the time of coronary artery bypass grafting or percutaneous coronary intervention (test group) and compared with eight matched patients who received the same treatment without mesenchymal stem cell injection (control group). Evaluation of heart function was done by echocardiography plus single-photon emission computed tomography before and six months after the procedure. Serial clinical examination was performed every month through New York Heart Association class.**

Results: **The mean New York Heart Association class and single-photon emission computed tomography scan results decreased significantly in the test group (*P*=0.000 and 0.002, respectively) and in the control group (*P*=0.049 and 0.007, respectively) after the procedure at six months follow-up. Left ventricular ejection fraction increased significantly in the test group (*P*= 0.005) but not in the control group. In comparison between the test and control groups the results of New York Heart Association class assessment and single-photon emission computed tomography demonstrated significant improvement in the test group (*P*=0.005 and 0.013, respectively). There were no significant differences between the baseline variables in the two groups.**

Conclusion: **Transplantation of *ex vivo* expanded bone marrow derived mesenchymal stem cell in patients with old myocardial infarction is a safe and feasible procedure. These cells improve the cardiac function without serious adverse effects.**

Archives of Iranian Medicine, Volume 10, Number 4, 2007: 467 – 473.

Keywords: Infracted myocardium • mesenchymal stem cell

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Accepted for publication: 7 February 2007

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Introduction

Acute myocardial infarction represents the greatest cause of morbidity and mortality in the developed countries.[1] Despite the recent advances in clinical and interventional treatments for myocardial infarction

(MI), there is no effective treatment to replace infarcted myocardium. Human cardiomyocytes are postmitotic cells and can not proliferate after birth.[2] While putative cardiac progenitor cell populations may exist in adult myocardium,[3] the endogenous regenerative capacity of these cells is inadequate to counterweight the loss of cardiomyocytes that occur with cardiac injury. Therefore replacing damaged cardiomyocytes with stem cells possessing the potential to differentiate into cardiomyocyte is an enticing possibility.

These transplanted cells may replace infarcted myocardium and increase the number of functional cardiomyocytes, limit the scar expansion, and reduce post-infarction heart failures.[4–6] Transplanted cells may also be developed into a novel revascularization strategy and enhance myocardial angiogenesis.[7]

Mesenchymal stem cells (MSCs) were initially believed to solely maintain the marrow stroma necessary for hematopoietic stem cell (HSC) survival and function.[8] Subsequent studies have convincingly demonstrated their differentiation, under appropriate conditions, into adipocytes, osteocytes, chondrocytes, tenocytes, skeletal myocytes, and neurons.[9 – 11]

Recently, emerging evidence has suggested that MSCs are also capable of differentiation into cardiomyocytes.[12, 13] Chen et al demonstrated that autologous bone marrow (BM)-MSC transplantation improves the cardiac function in patients with acute myocardial infarction.[14] In addition to their documented cardiomyogenic potential, MSCs have the potential to down regulate and inhibit immune response in both recognition and elimination phases through antigen presenting cells (APC), T cells, B cells, and natural killer cells.[15–18] In the presence of human MSC, immature or partially immature APC are produced.[19] Immature APC silence T cells or induce them to enter an anergic state either by eliminating T cells or modulating them toward a regulatory (T $CD4^{+}CD25^{+}$) phenotype.[20]

MSCs exhibit a cell surface phenotype of low immunogenicity,[21] and this hypoimmunogenicity stems from absence of the surface HLA class II antigen(s),[22] and costimulatory molecules such as CD80, CD86, and CD40.[23] Because of the immunosuppressive/immunomodulatory potential, MSCs down-regulate activated immune cell reactivity and thereby reduce tissue damage. MSCs play the role of stimulators and "cell factories" in injured and inflamed tissues when they are in contact with the local microenvironment. They promote tissue repair by differentiating into the injured cell types, thus compensating for their loss as well as secretion of trophic factors.[24]

Animal and human studies to date have shown stem cell injection at the site of MI is safe.[14, 25–26] However, we need further assurance in patients with old myocardial infarction as well. To examine our theory, we decided to perform a pilot study on patients with old myocardial infarction, to demonstrate the safety and therapeutic functionality of MSCs at the damaged site.

In this study, MSCs were separated through BM aspiration, expanded in culture medium (*in vitro*) and then were injected into myocardium or coronary arteries of the patients. After injection, we assessed the safety of the procedure and the patients' improvement from a clinical point of view in comparison with the control group.

Materials and Methods

Eight patients with old MI (1 female, 7 males) with a mean age of 49 year (range: 36 – 66 years) participated in this study with their consent, and approval of the Ethical Committee of Digestive Diseases Research Center, Tehran University of Medical Sciences (FWA00001331). These patients had been living with heart failure for an average of 72 months (range: 3 to 286 months). Five patients received MSCs by intramuscular injection at the time of coronary artery bypass grafting (CABG) and three patients received the cells by intracoronary route during percutaneous coronary intervention (PCI) procedure.

Inclusion criteria were: patients with a history of MI, age under 70 years, absence of viable myocardium on scintigraphic study of infarcted region, akinesia or dyskinesia in echocardiography and or ventriculography.

Exclusion criteria were: age over 70, accompanying advanced disease, and cardiac shock.

Control group consisted of eight matched patients with proper inclusion criteria who did not undergo the trial MSC therapy.

Sample collection and MSC expansion

Forty mL of BM was obtained from the patients 2 – 3 months prior to injection. The BM mononuclear cells were separated by the Ficoll density gradient method.[27] Vented flasks (75 cm^2) with 21 mL MSC medium, consisting of

Dulbecco's modified eagles medium (DMEM) with 10% fetal bovine serum (FBS), and 10% penicillin/streptomycin (all from Gibco, TX,USA), were seeded with 1×10^6 mononuclear cells/mL for primary culture. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and were fed by complete medium replacement every four days, until the fibroblast like cells at the base of the flask reached confluence (Figure 1).

On reaching confluence, the adherent cells were resuspended using 0.025% trypsin and reseeded at 1×10^4 cells/mL (first passage). These were incubated again until confluence, and were once again trypsinizes and reseeded 1×10^4 cells/mL. The number of passage of cells depending on the required amount of cells could be repeated.

At the end of the last passage, when the cells reached confluence, they were washed with tyrode salt and incubated with M199 medium for an hour. cells were detached with trypsinization and washed with normal saline supplemented with 1% human serum albumin and heparin and resuspended at 1 – 2×10^6 /mL density. This washing process eliminates trace amounts of FBS as well.

Immunophenotyping

At the end of the last passage, surface expression of CD166, CD105, CD44, and CD13, which are MSC surface markers and CD34 and CD45, which are HSC and CD31 endothelial surface markers respectively—were determined on culture-expanded MSCs. The monoclonal antibodies used were anti-CD44, CD45, and CD34 fluorescein isothiocyanate (FITC), anti-CD13 and CD31 phycoerythrin (PE) (all from Dako, Glostrup,Denmark), and anti-CD166 FITC and anti-CD105 R-phycoerythrin (RPE) (from Serotec, Dusseldorf, Germany). Relevant isotope control antibodies were also used. Flowcytometry was performed on a FACScan (Becton Dickinson, CA, USA) and data were analyzed with CellQuest software (version 3.1).

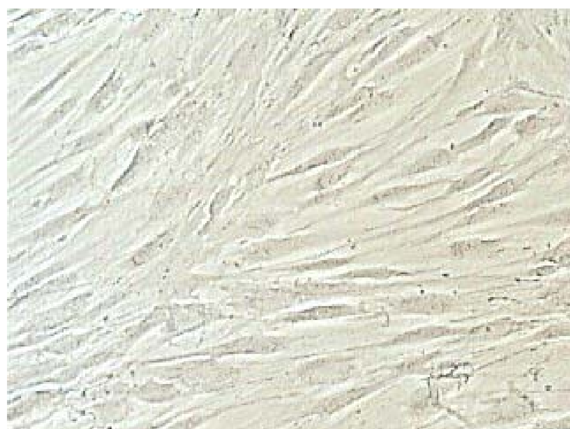

Figure 1. *Ex vivo* expanded mesenchymal stem cells culture.

Safety assessment

To make sure the cells were not contaminated, bacteriological tests were performed on the samples for every passage and at the time of injection. Viability of the cells was assessed by methylene blue dye exclusion test just before injection.

Injection of MSCs

A mean volume of 5.4 mL (range: 2.1 – 6.5) containing mean of 5.55×10^6 (range: 2.1 to 9.1) prepared cells were injected intramyocardial or intracoronary as follows:

The cardiopulmonary bypass was done using blood cardioplegia. After the coronary bypass surgery, the infarcted site was exposed and MSCs were injected into the infarcted and peri-infarcted regions by needle gauge 22 (each 0.2 mL). Afterward, the heart was reperfused and the surgery was completed. In the patients who undergone PCI, we replaced the guiding catheter for crossing the lesion with a wire and balloon then we positioned the over the wire balloon at the occlusion site. At this time one milliliter of prepared MSCs were injected through the central lumen of the balloon into the infarcted zone. To prevent backflow of MSCs, the balloon remained inflated for 2 – 3 minutes. This cycle was repeated for five to six times. After finishing the cell injection, the procedure was completed with stenting the lesion with an appropriate size, bare metal stent. No immunosuppressive drug was administered to the patients. In the control group, the procedure was done as the same as the test group without MSC administration.

We followed up the patients at least a year for:

- Evaluation of diastolic dysfunction, regional wall motion, and left ventricular ejection fraction (LVEF) by echocardiography before and six months after procedure.

- Assessment of viable myocardium (scar size) through thallium scan by single-positron emission computed tomography (SPECT) before and six months after procedure.

-Serial clinical examination based on the New York Heart Association (NYHA) classification every month.

Statistical analysis

All data were presented as mean±standard deviation (SD). Intraindividual comparison between the before and after variables was done with a paired samples *t*-test. Comparison of parametric data between the two groups was performed with independent samples *t*-test. A value of $P<0.05$ was considered significant. This analysis was performed using SPSS software version 11.5.

Results

The demographic characteristics of the patients in the two groups and the root of injection of MSCs are shown in Table 1. The viability of the cells was over 95% and the results of bacteriological analysis were negative for all samples. The result of flowcytometry analysis of expanded cells, for CD13 (mean: 79%), CD44 (mean: 72%), CD105 (mean: 59%), and CD166 (mean: 52%) was positive and for the hematopoietic markers, CD34, CD45, and CD31 was negative (Figure 2).

We tried to select a matched control group so baseline clinical characteristics between the two groups did not differ significantly (Table 2). No deaths occurred during the 18-month follow-up in both groups. Table 3 shows the cardiac function parameters as mean±SD, and *P* values before and after cell therapy during the mean follow-up of 18 months (range: 12 – 25 months) in the test group. As shown in Table 3, the mean of NYHA and infarction size were reduced and global LVEF increased significantly ($P= 0.000$, $P= 0.002$, and $P= 0.005$, respectively) after cell therapy.

In the control group, the mean of NYHA was decreased near significant levels ($P= 0.049$) after procedure but LVEF was not changed significantly. The result of thallium scan (SPECT) showed significant improvement in viable myocardium [($P= 0.007$) (Table 4)].

To compare the test and control groups, the clinical improvement detected by NYHA assessment in MSC treated patients was significantly higher than the control group [($P= 0.005$) (Table 2)]. Infraction size was reduced significantly in test group at six months follow-up ($P= 0.013$). Left ventricular EF at six months after procedure did not show significant differences between the two groups.

Discussion

Cellular therapy for myocardial injury has improved ventricular function in both animal and clinical studies.[25 – 26] Several studies demonstrate HSCs have the capacity to differentiate into cardiomyocytes and have significant effect on heart function.[5, 28, 29] Whereas other results seemed to point at HSCs inability to transdifferentiate into cardiac myocytes in myocardial infarctions.[30–32] However, HSC differentiation into cardiomyocyte is a subject of debate.[33] We focused here on the therapeutic effects of MSCs on heart function. Many studies have proved that MSCs have the potential to differentiate into cardiomyocytes.[6,34] Even in some studies the MSC differentiation into cardiomyocyte has been quantified.[35] MSC has the highest potentiality for adhesion, because of the expression of different adhesion molecules,[36] to vessel walls and this may cause infiltration of these cells into the infarct zone. Besides, with the direct differentiation of MSC into cardiac cells they increase heart function by exerting a significant trophic effect on heart repair including: neo-vascularization,[37] neo-nerve sproting,[38] inhibition of scar formation, and inhibition of apoptosis.[30] Tang et al showed that MSCs implanted into ischemic myocardium stimulated an increased production of vascular endothelial growth factor (VEGF), increased vascular density and blood

Table 1. The demographic characteristics of the participants and injected MSCs.

T and C	Injection route		Age		Sex		Follow-up months		Cell volume	Cell No. $\times 10^6$
	T	C	T	C	T	C	T	C	T	T
1	IM	IM	48	52	M	M	25	20	4.50	2.30
2	IM	IM	45	60	M	F	19	25	6.50	9.10
3	IM	IM	48	45	M	F	17	18	6.00	7.20
4	IM	IM	36	42	F	M	17	18	4.00	4.00
5	IM	IM	66	60	M	M	12	10	6.00	2.10
6	IC	IC	53	55	M	M	21	20	5.50	6.50
7	IC	IC	42	58	M	M	19	20	4.50	6.70
8	IC	IC	58	54	M	M	18	15	6.50	6.50

T=test; C=control; IM=intramyocardial; IC=intracoronary.

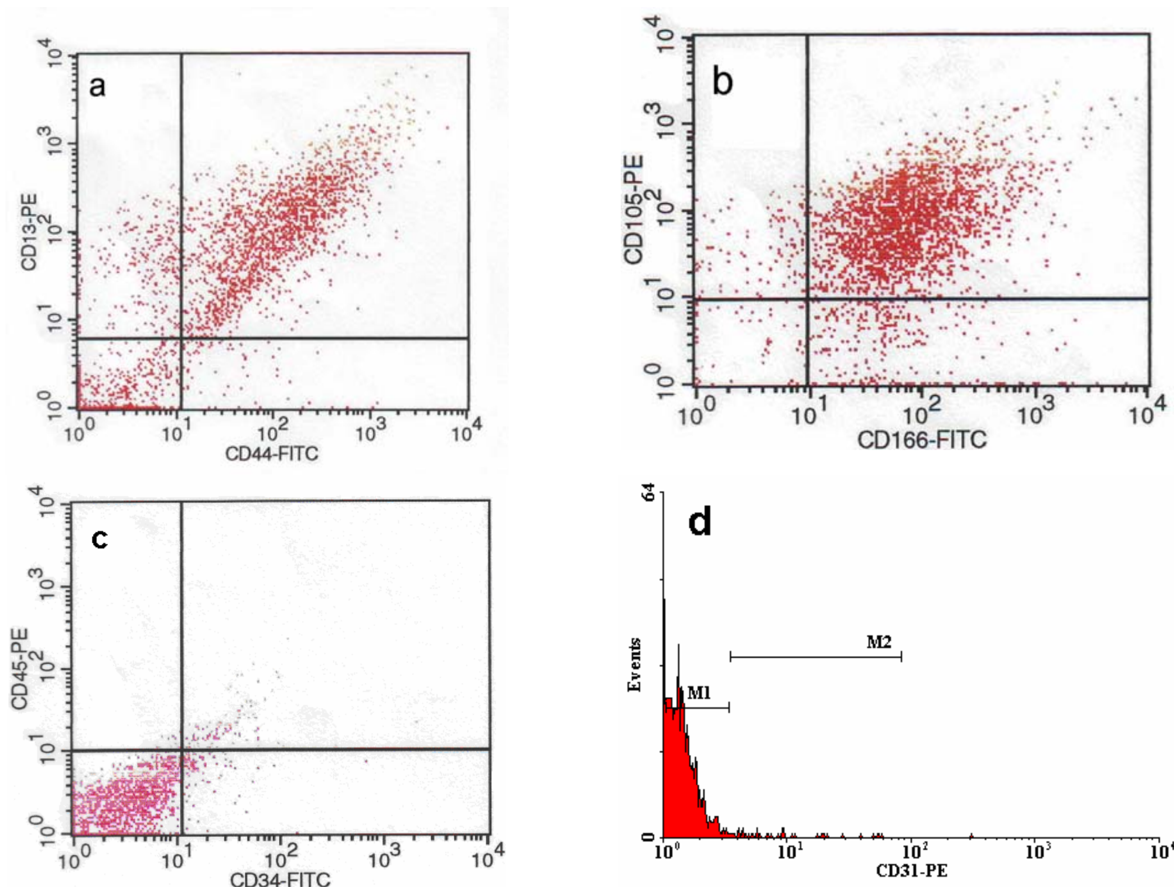

Figure 2. MSC cultures are: a) CD13/CD44, and b) CD105/CD166 positive. c) CD45/34 and d) CD31 negative.

flow, and decreased apoptosis.[37] They also argued that some MSCs differentiated directly into endothelial cells. MSCs also stimulate host progenitors to divide and differentiate into functional regenerative units.

It is difficult to assess what portion of the increased heart function can be ascribed to MSC derived cardiomyocytes and what contribution is attributable to those other factors. What is clear from these studies is that the administration of MSCs to the infarcted area results in increased heart function compared with controls.

MSCs' immunosuppressive quality induce a local inhibitory milieu through effect of soluble factors and of mechanisms mediated by cell-to-cell contact.[40]

MSCs are unable to generate lymphocyte proliferation in response to alloantigen stimulation.[41] This property of MSCs make them, at least potentially and theoretically, safe to use allogenically. Makkar et al injected the allogenic MSCs intramyocardially in the porcine model without immunosuppression.[26] They demonstrated that direct injection of allogenic MSCs resulted in successful engraftment and differentiation into cardiomyocytes and improved heart function after myocardial infarction.

Some studies used stem cell therapy in acute MI in both animal and clinical trails.[14, 42] Strauer et al determined that the best time for transplant should be between 7 to 14 days after the acute MI.[43] There are few reports about stem cell therapy in patients with old MI.[44, 45] However, their results demonstrated that stem cell therapy in old MI significantly improved the cardiac function as well.

Based on the results of the present study, we

Table 2. Comparison of cardiac function in the two groups.

Groups	NYHA (class)		LVEF (%)		Thallium scan (No. of segment)	
	Before	After	Before	After	Before	After
Test group	2.75	1.38	38.75	48.75	11	7.75
Control group	2.75	2.13	41.88	42.50	10.88	9.75
P value	NS	0.005	NS	NS	NS	0.013

NS=not significant; NYHA=New York Heart Association; LVEF=left ventricular ejection fraction.

Table 3. Cardiac function parameters in mean 18 months follow-up in test group.

Parameters	Base line±SD	After MSC±SD	*P* value
NYHA (class)	2.75±0.70	1.38±0.51	0.000
LVEF (%)	38.75±13	48.75±6.4	0.005
Thallium scan (segment No.)	11±2	7.75±1.1	0.002

Table 4. Cardiac function parameters in mean 18 months follow-up in control group.

Parameters	Base line±SD	After pro.±SD	*P* value
NYHA (class)	2.75±0.70	2.13±0.35	0.049
LVEF (%)	41.88±8.42	42.50±8.86	NS
Thallium scan (segment No.)	10.88±1.95	9.75±1.58	0.007

can confirm that the injection of expanded MSC intramuscular or intracoronary is a safe and feasible procedure. Our patients in comparison with matched control group showed significant improvement in cardiac function during the 18-month follow-up. It is worthy to note that when comparing LVEF between the two groups no significant differences are notable after six months of follow-up (Table 4). The reason is that our patients in the test group had lower baseline LVEF.

Although the level of LVEF in both groups showed some increase, this variation was greater in the test group.

MSC injection after myocardial infarction is a feasible procedure. They reduce the stiffness of the subsequent scar and attenuate postinfarction remodeling and preserve some cardiac function in patients with old MI. This pilot study is just the beginning of investigation. To arrive at concrete, useful results we must concentrate and modify the: cellular dose needed, number of injection(s) required, proper use of costimulators, defining the best subtypes of cells (among the stem cell population), and finding a noninvasive way of labeling and tracking these cells.

Acknowledgment

We would like to thank Mrs. Maryam Bashtar and Miss. Bita Ansaripour for their technical assistance .This work was supported by a grant (418/A/5184) of the Hematology-Oncology and BMT Research Center, School of Medicine/ Tehran University.

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