

Transplantation of bone marrow stromal mesenchymal cells in the treatment of acute myocardial infarction

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Abstract

Introduction: Aim of the study to investigate the efficacy of bone marrow stromal mesenchymal cells (BMSCs) in the early stages of acute myocardial infarction (AMI) in a rat model.

Material and methods: Bone marrow was collected from lower limbs of male Wistar rats. Cells were prepared and cultured. Myocardial infarction was induced in female Wistar rats by coronary occlusion. After 30 min, rats received an intramyocardial injection of BMSCs ($n = 9$) or culture medium (control, $n = 11$). Echocardiography was performed at baseline and 30 days later, before heart excision. RT-PCR was performed and presence of chromosome Y, NKX2.5, troponin I, GATA4, VEGF- α , VEGF-R1, and KDR was investigated in lesion areas and in the posterior wall.

Results: At day 30, a significant decrease in ejection fraction (LVEF) ($46.6 \pm 16.2\%$ vs. $35.6 \pm 16.0\%$, $p = 0.035$) and a marked increase in end-diastolic (LVEDd) (6.74 ± 0.75 mm vs. 8.92 ± 1.59 mm, $p = 0.012$) and end-systolic (LVESd) diameters (5.32 ± 0.99 mm vs. 7.47 ± 1.68 mm, $p = 0.012$) were evident in the control group. Bone marrow stromal mesenchymal cells-treated rats showed no difference in LVEF ($36.8 \pm 12.1\%$ vs. $44.8 \pm 12.3\%$, $p = 0.262$), LVEDd (7.92 ± 0.89 mm vs. 7.76 ± 1.34 mm, $p = 0.671$), or LVESd (6.65 ± 1.15 mm vs. 6.21 ± 1.42 mm, $p = 0.624$). Chromosome Y was detected only in lesion areas of BMSC-treated hearts. Expression of NKX2.5, troponin I, GATA4, VEGF- α , VEGF-R1, and KDR was significantly higher in lesion areas of treated hearts.

Conclusions: Cell therapy with BMSCs seems effective in the early stages of AMI, preventing LVEF worsening and remodelling. Injected cells give origin to muscular and endothelial cells. It is necessary to clarify whether new cells directly derive from injected cells, or originate from resident or circulating cells by stimulation or chemotaxis.

Key words: stromal mesenchymal cells, myocardial infarction, cell transplantation.

Introduction

Left ventricular failure is a problem affecting a large number of people. Five hundred thousand new cases are reported each year in the USA alone [1]. During the last ten years, cell therapy has emerged as one of the most promising pathways to follow.

To date, several cell types have been investigated, from fetal and adult cardiomyocytes [2-4], through skeletal myoblasts and adult stem cells [5-10], to the latest studies on embryonic stem cells [11-13] and cardiac resident stem cells [14, 15].

Despite the spreading, even if poorly controlled, use in humans, many questions are still to be solved.

First of all, whether the injected cells are able to transdifferentiate into muscular cardiac cells, integrate in the native tissue and give origin to new myocardium, or they are simply the starting point of a cascade of events able to stimulate resources already present in the recipient.

Several experimental studies have shown an improvement in left ventricular performance after cell transplantation, even if only a small quantity of the injected cells could be found in place some weeks after implantation.

Human trials [16, 17], after an initial promising functional improvement, have shown comparable medium-term results, supporting the hypothesis that many steps are still to be made before clinical use.

In our study we tested the efficacy of transplantation of bone marrow stromal mesenchymal cells (BMSCs) in the early stages of acute myocardial infarction, using a rat model.

Material and methods

Cell preparation

Adult male rats (Wistar) were sacrificed by overload of anaesthetic and their tibias and femurs were dissected and cleaned of all soft tissue in a sterile hood. The epiphysis of each bone was clipped, and the bone marrow was flushed out of the tibia and femur using sterile Dulbecco's Modified Eagle's Medium (DMEM) without serum, containing 100 IU/ml penicillin (Sigma) and 100 µg/ml streptomycin (Celbio). Cells were then isolated and prepared as previously described [18, 19]. Bone marrow was seeded on plastic flasks (25 cm², Iwaki) in a final volume of 10 ml, supplemented with fetal bovine serum (final concentration 10%) and incubated in a humidified incubator at an atmosphere of 5% CO₂ at 37°C. After 4 days the supernatant was removed and replaced with Dulbecco's Modified Eagle's complete Medium (complete DMEM; Celbio), supplemented with 100 IU/ml penicillin (Sigma), 100 mg/ml streptomycin (Sigma) and 10% fetal calf serum (FCS; Celbio); the medium was then replaced every three days. Cell cultures were carried out in complete DMEM for 3 weeks. About 3 weeks after culture initiation, several clones of fibroblastic cells emerged. These cells were trypsinized and pooled together before being distributed in other flasks. These multiclonal cultures were kept in an incubator for 7 days until 70-80% confluence was attained. Then, semi-confluent cells were trypsinized and

frozen at 500 000 cell/ml in fetal calf serum containing 10% dimethylsulfoxide (DMSO, Sigma), using a specific frozen gradient (Misterfrosty, Sigma) and stored in liquid nitrogen until use.

For cell transplantation we used BMSCs at 80% confluency. After trypsinization cells were recovered with gentle centrifugation (600 rpm for 5 min) and resuspended in DMEM without serum at 1 × 10⁶ cell/100 µl immediately before injection in the rat hearts.

The animal model

The experiments complied with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, and published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Female Wistar rats were anaesthetised with ketamine (50 mg/kg, *i.p.*) and xylazine (10 mg/kg, *i.p.*) and ventilated with an endotracheal tube. The age of the animals was about 4 weeks, and their weight was between 200 and 250 g. An acute myocardial infarction (AMI) was then created by ligation of the left coronary artery with a 7-0 polypropylene snare (Ethicon Somerville, NJ, USA) through a left lateral thoracotomy, as previously described [20, 21].

Within 30 min after AMI induction, 1 000 000 BMSCs suspended in 100 µl of culture medium were injected with an insulin syringe into ten different sites in infarcted hearts, both at the margins of the ischaemic area, and in the middle of the injured area. Control rats with AMI received the same volume of cell-free medium in the same areas.

Nine rats received BMSC-conditioned culture medium, while eleven rats received culture medium alone. The rats were randomly assigned to one of the two groups.

The animals were then observed during a 30-day period. After functional assessment performed at day 30, the hearts were explanted, cut into three segments and immediately frozen in liquid nitrogen-cooled isopentane for biochemical analysis.

Functional assessment

Immediately after transplantation and 30 days after the procedure all the rats were studied by 2D echocardiography, using a commercially available 13 MHz linear-array probe, connected to a numeric ultrasound device (*Sonoline G50, Siemens Medical Solutions USA, Inc.*). The ketamine-anaesthetized animals were placed in supine or lateral position on a warming pad. Parasternal 2D and M-mode views were used to calculate left ventricular ejection

fractions (LVEF) by Simpson method, left ventricular end-systolic (LVESd) and left ventricular end-diastolic (LVEDd) diameters.

All measurements were averaged on 3 or more consecutive cardiac cycles and analyzed by an observer who was blinded to the treatment status of the animals.

DNA and RNA extraction

Two specimens of about 20-30 mg (wet weight) were cut off from the ischaemic region and oxygenated region from each control and injected hearts (the ischaemic regions of the left anterior ventricle were clearly visible as white tissue whereas the oxygenated regions of the left posterior ventricle were recognized as red tissue). From these samples total RNA and genomic DNA was extracted simultaneously using the NucleoSpin kit with a specific DNA extraction buffer (Macherey Nagel). The extracted RNA was treated with DNase I (DNase Free, Ambion) to remove any traces of contaminating DNA. RNA and DNA were quantified by UV spectrophotometer and their integrity were verified by agarose gel electrophoresis [22].

Quantitative RT-PCR and genomic PCR

4 µg of total RNA was retrotranscribed using a High Capacity cDNA synthesis kit (Applied Bio-

systems) for 2 h at 37°C. Quantitative real-time RT-PCR was performed on an Abi Prism 7000 instrument (Applied Biosystems) using Taqman Universal PCR Master Mix with Cyber Green as fluorescent dye (Applied Biosystems) following the manufacturer's instructions. Primers were designed using Primer Express software (Applied Biosystems) using gene sequences deposited in the public databases (Table I). For quantitative RT-PCR a final concentration of 100 nM for each primer was used. At this concentration only the specific PCR product was amplified and quantified as measured by the dissociation curves. Primers were designed to have a similar amplicon size and similar amplification efficiency as verified by serial dilution of cDNA samples. The quantification was relative to the β-actin expression.

To verify the presence of male cells into female hearts, PCR on genomic DNA was done to detect the presence of SRY locus using the primers shown in Table I in RedTaq Mastermix (Sigma) as previously reported [23].

Statistical analysis

All variables were tabulated using Microsoft Excel (Microsoft Corp). Statistical analysis was computed using SPSS, release 13.0 for Windows (SPSS Inc, Chicago, IL). A non-parametric Wilcoxon test was

Table I. Primers used in the study

| Gene | Primers (5'-3') | Accession number | Reference |
|------------|--|------------------|----------------|
| GATA4 | CATGTGTCCCATACCTGATCTGA CTAAACCCCCAGGAAGCA | NM_144730 | This work |
| NKX2.5 | CGGCGTCGCCACCAT CTGCTCCAGGTTCCAGGATGTC | NM_053651 | This work |
| CD34 | GGCCGACGCTGGTACTAGTG CACTCGGGCCTAACCTCAGA | XM_223083 | This work |
| TROPONIN I | CCAGGAATCTGCAATCCATT CCGCATCGCTGCTCTCA | NM_017144 | This work |
| VEGF-A | CAAAAACGAAAGCGCAAGAAA CGCTCTGAACAAGGCTCACA | NM_031836 | This work |
| VEGFR-1 | CTGCCAGAAGTCGTATGGTTA CACCGAATAGCGAGCAGATTT | NM_019306 | This work |
| KDR | GCTGCGGGTCTCACTACCA GGGTATGGTACTTCTTGAGGAT | NM_013062 | This work |
| β-ACTIN | TTCAACACCCAGCCATGT GTGGTACGACCAGAGGCATACA | NM_031144 | Cobb 2005 |
| β2M | CGTGCTTGCCATTCAGAAAA GAAGTTGGGCTTCCCATTCTC | NM_012512 | Thain 2005 |
| SRY* | GCCTCCTGGAAAAAGGGCC GAGAGAGGCACAAGTTGGC | | Muramatsu 2005 |
| GAPDH* | ATGCTGGTCTGAGTATGTCG GTGGTGCAGGATGCATTGCTGA | NM_017008 | Muramatsu 2005 |

*These primers were used on genomic DNA

employed to compare numerical echocardiographic data of LVEF, LVEDd and LVESd. Biochemical data were analyzed by the Kruskal-Wallis test. All data are expressed as mean \pm standard deviation (SD). A p value < 0.05 was considered significant.

Results

Functional results

Baseline ejection fraction did not differ significantly between the two groups ($46.6 \pm 16.2\%$ in the control group vs. 36.8 ± 12.1 in the BMSC-transplanted group, $p = ns$).

Four of the rats included in the study died before day 30. All of them had a baseline LVEF lower than 30%. Three of them belonged to the control group, while only one belonged to the BMSC-transplanted group. In any case the animals that died before day 30 were not further studied.

One month later, LVEF had significantly lowered in control rats, reaching a mean value of $35.6 \pm 16.0\%$ ($p = 0.035$). Moreover, LVEDd and LVESd in the control group were significantly augmented (LVEDd 6.74 ± 0.75 mm vs. 8.92 ± 1.59 mm, $p = 0.012$; LVESd 5.32 ± 0.99 mm vs. 7.47 ± 1.68 mm, $p = 0.012$), suggesting an important left ventricular dilation and loss of geometry (Figure 1).

The same significance was not observed in the BMSC-treated group: LVEF in this group showed even a slight tendency to improvement ($36.8 \pm 12.1\%$ vs. $44.8 \pm 12.3\%$, $p = 0.262$), while left ventricular size and shape remained essentially unchanged (LVEDd 7.92 ± 0.89 mm vs. 7.76 ± 1.34 mm, $p = 0.671$; LVESd 6.65 ± 1.15 mm vs. 6.21 ± 1.42 mm, $p = 0.624$) (Figure 2).

Biochemical results

Thirty days after coronary ligation, the animals were sacrificed and samples of both ischaemic and normal regions were collected from every heart. To verify the presence of male cells in BMSC-transplanted hearts, PCR on genomic DNA extracted from specimens prepared as described above was performed. The presence of chromosome Y was demonstrated only in the ischaemic region of the hearts treated with BMSCs, showing that the injected cells survived until day 30 (Figure 3).

The biochemical analyses of cardiac contractile cell markers were carried out by real-time RT-PCR. The data showed a significant difference between the infarcted area of BMSC-transplanted hearts and the other samples considered, for NKX2.5, troponin I and GATA4 (Figure 4). In fact, after

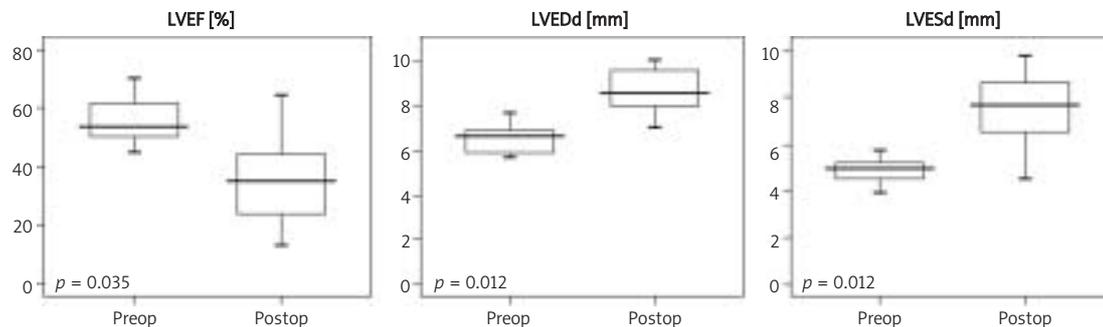


Figure 1. Baseline and postoperative day 30 echocardiographic measures in control group. Box-plots with relative statistical values

LVEF – left ventricular ejection fraction, LVEDd – left ventricular end diastolic diameter, LVESd – left ventricular end systolic diameter, Preop – preoperative, Postop – postoperative

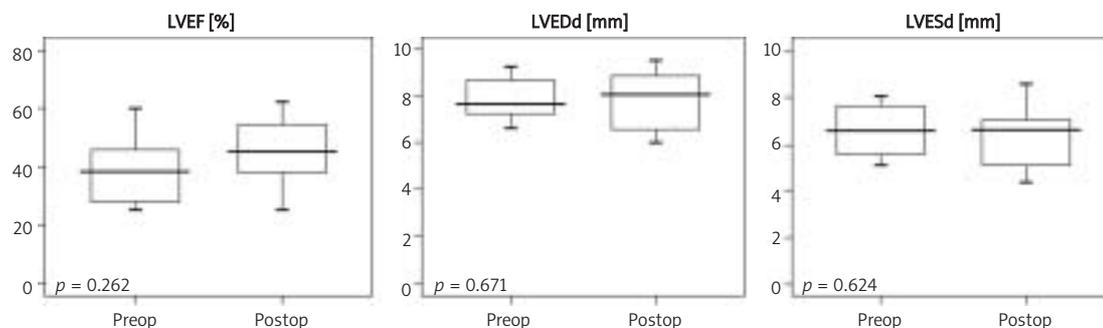


Figure 2. Baseline and postoperative day 30 echocardiographic measures in BMSC group. Box-plots with relative statistical values

LVEF – left ventricular ejection fraction, LVEDd – left ventricular end diastolic diameter, LVESd – left ventricular end systolic diameter, Preop – preoperative, Postop – postoperative

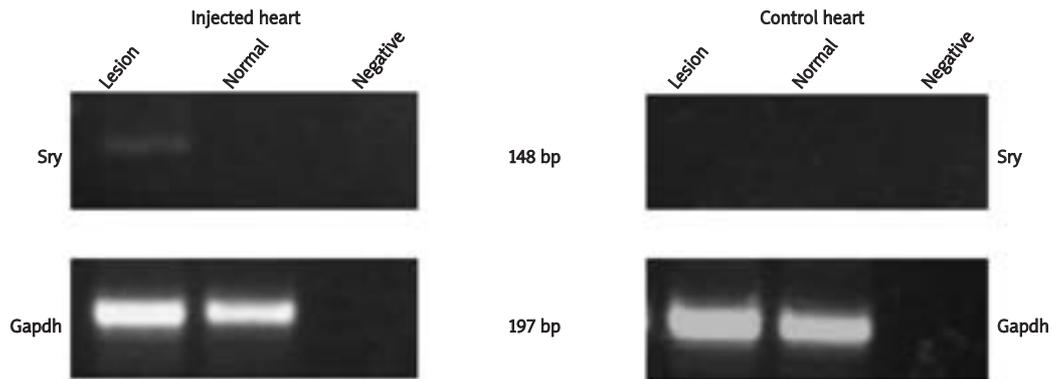


Figure 3. RT-PCR showing the presence of chromosome Y in the lesion area of injected hearts. GAPDH was used as control

transplant, gene expression for these typical myocardial cell markers was up-regulated. The same significant difference was observed for endothelial cell markers VEGF- α , VEGF-r1, and KDR, which are more specifically related to angiogenesis (Figure 4).

This work confirms that cell therapy offers a promising approach for prevention and treatment of heart failure, through establishment of new blood vessels supplying surviving muscle cells and replacement of dead cells.

We chose to transplant BMSCs 30 min after coronary ligation, since, like otherwise described [24-27], stem cell therapy seems much more effective in preventing left ventricular remodelling if administration occurs in the early stages.

Cell therapy appears to be safe even in immunocompetent animals: in fact at 30 days no adverse reactions to therapy or macroscopic tumour development could be observed, after heterologous cell transplantation. This finding is not surprising considering that BMSCs are at a very early stage of differentiation and therefore they express fewer

histocompatibility proteins on their membrane [28, 29].

Moreover, this is further confirmation of the already described evidence that human, baboon, and murine MSC failed to elicit a proliferative response from allogeneic lymphocytes.

The presence of chromosome Y in the excised hearts demonstrated the survival of the injected cells at least until day 30, even if it was not possible to quantify the exact percentage of cell survival.

In fact the genomic PCR assessment of SRY is not quantitative but it is only able to indicate masculine cell survival in female host tissue. In any case, considering the PCR band intensity, we can assume that male cell survival after 30 days was very low. For this reason, we transplanted a large number of cells, assuming that the majority of them would die from membrane mechanical trauma during the injection procedure, or from oxygen loss due to poor blood supply in the ischaemic area.

Stem cells' spontaneous mobilization from bone marrow after an AMI and their contribution to new cardiac tissue formation have been previously described [30, 31]; however, the evidence of this study is that injected cells seem to give origin to both cardiac muscular cells and endothelial cells, as shown by the overexpression of the investigated genes. As a matter of fact, previous studies have focused on the capacity of engrafted BMSCs to differentiate into cardiac lineage cells [32-36]. Moreover, considering the relevant expression of NKX2.5 and GATA-4, as it occurs in the early stages of cardiac differentiation, we could assume activation of cardiac resident stem cells by paracrine action of the injected cells, as previously described by other authors [37-39].

In addition, it can be hypothesised that limitation of left ventricular remodelling and function loss could also be due to better oxygen supply to the ischaemic perilesional area. The formation of new capillary vessels in the jeopardized region could limit infarct size, thus giving more chances for

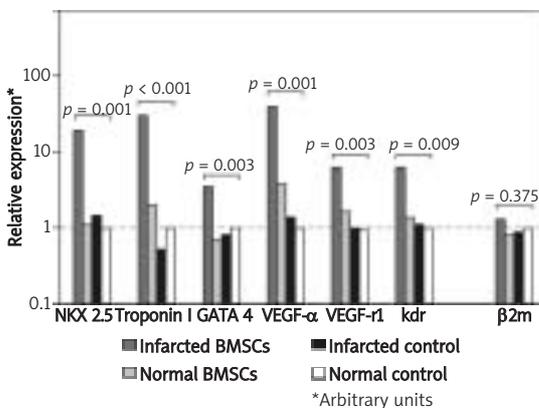


Figure 4. Histogram showing gene expression of cardiac and vascular biochemical markers (see text for details) in BMSC and control group. β 2m was used as control

function recovery [24, 25, 40]. A capillary count was not performed, but the onset of new vessels could be assumed by the relevant expression of VEGF and VEGF receptors in the BMSC-transplanted hearts.

The most important limitation of this study is the small sample size (sixteen rats), which results from the mortality that occurred after creation of AMI and throughout the thirty-day follow-up period. In fact we observed four early deaths among the animals included in the study. Most of the rats with a low baseline ejection fraction belonging to the control group failed to survive even 30 days, confirming the efficacy of BMSC transplantation.

In conclusion, early stage cell therapy with BMSCs seems to be effective in reducing left ventricular remodelling and function loss after AMI.

At the moment we cannot affirm that injected cells transformed into cardiomyocytes or fused with them, but it is only possible to assume that the presence of BMSCs in host tissue matrix can modify the repair processes. The injected cells seem to stimulate the proliferation of both endothelial cells and muscular cardiac cells with still unknown mechanisms.

Certainly, further studies are needed to clarify whether the BMSC tissue integration process occurs by transdifferentiation of the injected cells, or their role is to activate or enhance homing of circulating or resident stem cells.

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No commercial sources.

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