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ORIGINAL PAPER

In situ delivery of bone marrow cells and mesenchymal stem cells improves cardiovascular function in hypertensive rats submitted to myocardial infarction

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Abstract This work aimed to evaluate cardiac morphology/function and histological changes induced by bone marrow cells (BMCs) and cultured mesenchymal stem cells (MSCs) injected at the myocardium of spontaneously hypertensive rats (SHR) submitted to surgical coronary occlusion. Female syngeneic adult SHR, submitted (MI) or not (C) to coronary occlusion, were treated 24 h later with in situ injections of normal medium (NM), or with MSCs (MSC) or BMCs (BM) from male rats. The animals were evaluated after 1 and 30 days by echocardiography, histology of heart sections and PCR for the Y chromosome. Improved ejection fraction and reduced left ventricle infarcted area were observed in MSC rats as compared to the other experimental groups. Treated groups had significantly reduced lesion tissue score, increased capillary density and normal (not-atrophied) myocytes, as compared to NM and C groups. The survival rate was higher in C, NM and MSC groups as compared to MI and BM groups. In situ injection of both MSCs and BMCs resulted in improved cardiac morphology, in a more physiological model of myocardial infarction represented by surgical coronary occlusion of spontaneously hypertensive rats. Only treatment with MSCs, however, ameliorated left ventricle dysfunction, suggesting a positive role of these cells in heart remodeling in infarcted hypertensive subjects.

Keywords Bone marrow cells · Mesenchymal stem cells · Spontaneously hypertensive rats · Hypertension · In situ delivery

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Ischemic heart disease is the leading cause of death worldwide and also an important cause of heart failure. Atherosclerosis, the major determinant of ischemic cardiomyopathy, involves several complex processes, which can be initiated and accelerated by risk factors including systemic arterial hypertension, dyslipidemia, cigarette smoking, diabetes mellitus, age, male sex and family history of premature coronary heart disease [1].

Animal models have allowed the study of cardiovascular diseases in the early stages, as well as the pathogenic mechanisms and the effects of therapeutic interventions. Although there is no model that can entirely mimic human ischemic cardiomyopathy, the most frequently used animal model involves the induction of complete coronary occlusion [2]. Its performance in spontaneous hypertensive rats (SHR) accelerates left ventricular dilatation and haemodynamic alterations caused by the myocardial infarction [3].

The many available treatments for heart failure do not target the progressive loss of cardiomyocytes. Experimental studies have suggested that stem cells can exert beneficial effects on the failing heart by transdifferentiating into cardiac cell types and/or by providing a source of cardioprotective paracrine factors [4]. Several adult stem

cell types [5–9], animal models [10, 11] and delivery pathways [12–14] have been tested in the last years, in the search for ideal conditions which were not yet found. Bone marrow cells (BMCs) [15] and cultured mesenchymal stem cells (MSCs) [3, 16], administered mainly by in situ injection [10], are the most frequently used types of cells.

The aim of the present study was to evaluate the effects of two different kinds of adult stem cell populations, administered in situ, on cardiac morphology, function and histology of SHR submitted to surgical coronary occlusion, in an attempt to investigate this therapeutic approach in a more physiological animal model of myocardial infarction.

Materials and methods

Cell culture

Bone marrow cells (BMCs) were obtained from 3-week-old male SHR by flushing the cavity of femurs with normal culture medium (NM), consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St Louis, MO) with 10% fetal bovine serum (Cultilab, Sao Paulo, Brazil) and supplemented with 3.7 g/l sodium bicarbonate and 2.5 g/l HEPES (Sigma). No antibiotics were added. BMCs were centrifuged at 800 g for 10 min, and viability was assessed by Trypan blue staining.

Isolation, primary culture and expansion of MSCs were performed as we previously described for murine cells [17]. Briefly, bone marrow cells were collected, washed and cultured in concentration of 2.15×10^6 cells/ml in 25 cm^3 bottles (7 ml/bottle). The cells were kept at 37°C in NM, and non-adherent cells were removed after 72 h. MSCs were maintained by passage every 3 or 4 days, and were used for cell therapy between the 8th and 12th passages. For transplantation, MSC cultures were washed twice with $Ca^{2+}Mg^{2+}$-free Hank's balanced salt solution (Sigma), trypsinized, resuspended in 5 ml NM and washed. The cells were counted and resuspended in NM at 10^7 cells/ml.

Osteogenic and adipogenic differentiation of MSCs were induced as previously described [18]. Briefly, osteogenic differentiation was induced by culturing MSCs for up to 4 weeks in NM supplemented with 10^{-8} mol/l dexamethasone (Sigma), 5 μg/ml ascorbic acid 2-phosphate (Sigma) and 10 mmol/l β-glycerophosphate (Sigma). To observe calcium deposition, cultures were washed once with phosphate-buffered saline (PBS), and stained for 5 min at room temperature with Alizarin Red S stain (Sigma), pH 4.2. For adipogenic differentiation, MSCs were cultured for up to 4 weeks in NM supplemented with 10.8 mol/l dexamethasone and 5 μg/ml insulin (Sigma). Adipocytes were easily distinguished from undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and stained with Oil Red O (Sigma).

Immunophenotyping

Cells were trypsinized and incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies against rat CD29, CD44H and CD31 (Becton Dickinson, San Jose, CA), CD11 and CD45 (Caltag, Burlingame, CA). The cells were analyzed in a cytometer equipped with a 488 nm argon laser (FACScalibur, Becton Dickinson) using the CellQuest software. At least 10,000 events were collected.

Animal studies

Syngeneic spontaneously hypertensive rats (SHR) used in the experiments were housed and maintained under standard conditions, and were treated in accordance with the *Guidelines for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health. All procedures were approved by a local Research Ethics Committee. Myocardial infarction was induced in 45 females anesthetized by intraperitoneal (i.p.) injection of 50 mg/kg body wt ketamine (Parke-Davis, Ann Arbor, MI) and 10 mg/kg xylazine (Bayer, Newhaven, CT). After intubation, animals were positive-pressure ventilated with room air at 2.5 ml, 65 strokes/min with a pressure cycled rodent ventilator (HARVARD Apparatus, Model 683, Holliston, MA). For induction of myocardial infarction, a 2-cm left lateral thoracotomy was performed in the third intercostal space and the left anterior descending coronary artery was occluded with a single nylon (6.0) suture at approximately 1 mm from its origin below the tip of the left atrium as previously described [19, 20]. The chest was closed with silk suture. The animals were maintained in the ventilator until recovery. All rats received antibiotics (penicillin, 20,000 U) and Tramadol (20 mg/kg, every 6 h). The animals were matched for body weight (160–180 g) and treated 24 h after surgery with BMCs (BM group, $n = 5$), MSCs (MSC group, $n = 10$) or NM (NM group, $n = 7$). Two other groups served as control: one group was submitted to myocardial infarction and did not receive any treatment (MI group, $n = 6$), and another one was not operated or cell-treated (C group, $n = 8$).

Cell treatment

Twenty-four hours after coronary occlusion, the animals were re-anesthetized and an echocardiography was

performed to measure the infarction area (IA) and the ejection fraction (EF). Only animals with EF $\leq$ 50% and IA $\geq$ 30% were maintained in the experiment (37% were excluded). A total of 1×10^6 MSCs or BMCs were suspended in 100 µl NM and drawn into a 300 µl syringe with a 30-gauge needle. The animals were intubated, a re-thoracotomy was performed in the fourth intercostal space, and the cells or normal medium were injected in five points of the left ventricle anterior wall, in the viable myocardial bordering the infarcted area. Proper administration of the injected material was evaluated by the immediate appearance of a thin film of white liquid inflated underneath the visceral pericardium.

Systolic arterial pressure

Systolic arterial pressure (SAP) was measured using tail-cuff plethysmography before the MI surgery, to ensure similar levels of hypertension among the groups.

Echocardiography

Echocardiographic indices were obtained before treatment and one and 30 days after induction of myocardial infarction under the guidelines of the American Society of Echocardiography, by one observer blind to the treatments and with the use of a 10–13 MHz multi-frequency linear transducer in a SEQUOIA 512 (ACUSON Corporation, Mountain View, CA). Images were obtained with the transducer on the shaved chest (lateral recumbence). Rats were anesthetized as described above and scanned from below, at 2-cm depth with focus optimized at 1 cm. All measurements were based on the average of three consecutive cardiac cycles. Wall thickness and left ventricle (LV) dimensions were obtained from a short-axis view at the level of the papillary muscles. The LV mass was calculated by the use of the following formula, assuming a spherical LV geometry and validated in rats: LV mass = $1.047 \times [(\mathrm{LVd} + \mathrm{PWd} + \mathrm{IVSd})3 - \mathrm{LVd}3]$, where 1.047 is the specific gravity of muscle, LVd is LV end-diastolic diameter, PWd is end-diastolic posterior wall thickness and IVSd is end-diastolic interventricular septum thickness. Two-dimensionally guided pulsed-wave Doppler recordings of LV inflow were obtained from the apical four-chamber view. Maximal early diastolic peak velocity (*E*) and late peak velocity (*A*) were derived from mitral inflow. Peak *E* desacceleration time was also measured as an index of diastolic function. Isovolumic relaxation time (IVRT) was taken from aortic valve closure to the onset of mitral flow. Ejection fraction (EF%) was obtained by a modification of Simpson's method, which is more trustworthy in infarcted hearts. Its formula is

$$\mathrm{V} = \frac{\pi}{4} * \sum_{i=1}^{20} a_i * b_i * \frac{L}{20},$$

where L = LV length divided into 20 discs (i = 1 to 1 = 20) from basis to apex, with the diameter of each disc determined in 2 apical views (A and B). After these measurements in diastole and systole, the respective volumes were obtained, allowing EF evaluation (final diastolic volume—final systolic volume/final diastolic volume) $\times$ 100%.

The infarction area was delimited taking into account the movement of LV walls, by the observation of longitudinal, apical and transversal views of the LV. Regions with systolic thickness under normal, as well as portions with paradoxal movement, were considered as infarcted. The infarcted area (%) was thus determined by the ratio of these regions by total area of LV walls [21].

Tissue histology and morphometry

Thirty days after the treatments, the rats were anesthetized with sodium pentobarbital (80 mg/kg body weight), a transverse incision below the diaphragm and bilateral thoracotomy incisions were made and the LV was cannulated retrogradely with a needle to perfuse. The hearts were arrested in diastole by perfusion with a NaCl 0.9% plus 14 mM KCl solution, followed by buffered formalin (4%) for tissue fixation.

Excised hearts were immersed in 4% formalin for 24 h and then transected 5 mm below coronary ligation. Transversal slices were processed and embedded in paraplast. Sections of 5 µm were stained with hematoxylin-eosin (HE) for qualitative assessment, with picrosirius-hematoxylin (red stain) for fibrosis evaluation, and with periodic acid Schiff (PAS) for quantification of capillary density. Histo-morphometric analyses were performed blinded regarding the identity of experimental groups.

Fibrosis evaluation

Fibrosis in the LV was evaluated by dividing it in 8 quadrants, and determining a fibrosis score (0–4) for each quadrant. The aspect of collagen fibers was evaluated under polarized light, allowing evaluation of the molecular disposition of collagen and aspects related to myocardial function. Picrosirius-polarization staining was performed as previously described [12].

Evaluation of capillary density

Capillary density was quantified by microscopic examination of the sections using a 10 $\times$ 10 grid optically

superimposed on each of 20 non-overlapping fields at 400× magnification, randomly distributed on the antero-septal side of the left ventricle. Capillary density was evaluated adjacent to the cardiac scars as the total number of capillaries in the fields, expressed as number of capillaries per field (units/mm^2) [5].

Molecular evaluation

To analyze the presence of the injected cells in the heart tissue, the infarcted heart was divided in four pieces. Genomic DNA was extracted from each piece using the proteinase K method [22] and submitted to PCR. The quality of the DNA samples was analyzed with GAPDH primers (Table 1). The PCR reaction mixture contained approximately 100 ng genomic DNA, 1U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 10 pmol of each oligonucleotide primers, 1 μl of 5 mM dNTP, 2.5 μl of 10× PCR buffer, and 1 μl of 50 mM $MgCl_2$, in a final volume of 25 μl. The PCR was carried out in a Techne thermocycler (Barloworld Scientific, Staffordshire, UK) for 40 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 1 min) with final extension of 7 min (72°C). Presence of the Y chromosome was analyzed by nested PCR. The reaction mixture and amplification cycles were the same as for GAPDH PCR, except that it contained 10 pmol of rat Sry-specific oligonucleotide primers (Table 1). In the first round, SRY external primers were used to amplify a fragment of 272 bp. The second round (nested PCR) included two SRY internal primers added to the 2 μl of the first round amplification product. Both reactions were conducted in the same conditions. The PCR products were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Statistical analyses

Data are reported as mean ± SEM. According to the parameter, one or two way ANOVA was used to compare groups, followed by the Student-Newman-Keuls test. The Kaplan Meyer method was used to determine the survival curve. The significance level was established at $P < 0.05$.

Results

Establishment and characterization of rat MSCs

The cell population isolated consisted of adherent cells with fibroblastoid morphology which, when properly stimulated, gave origin to adipocytes and osteocytes in culture (Fig. 1a). The cells were positive for CD29 and CD44, and negative for CD45, CD11b and CD31 (Fig. 1b).

Body weight

Body weight was similar in all groups before coronary occlusion (173.8 ± 4, 166.6 ± 4; 169.7 ± 1; 168 ± 5 and 172 ± 5 g) but lower 30 days after the procedure (166.7 ± 3; 157.2 ± 1; 160.7 ± 7; 158.2 ± 5 and 162.8 ± 7 g) in C, MI, NM, BM and MSC groups, respectively ($P > 0.05$).

Cardiovascular evaluation

As expected, all animals were hypertensive at the beginning of the protocol (mean systolic arterial pressure: 167 ± 3 mmHg), with no differences among the groups. Figure 2a shows the LV infarcted area 24 h and 30 days after coronary occlusion. The result was similar between groups one day after coronary occlusion (~40% of the left ventricle wall). Treatment with mesenchymal stem cells determined a significant reduction in the LV infarcted area (MSC: 26 ± 4%) 30 days after coronary occlusion as compared to groups that did not receive cells (MI: 52 ± 2% and NM: 41 ± 5%), and also to the group that received BMCs (BM: 45 ± 4%).

Left ventricle ejection fraction, evaluated at 24 h and 30 days after coronary occlusion, is shown in Fig. 2b. It was reduced by ~40% one day after coronary occlusion in

Table 1 Sequences of forward and reverse oligonucleotide primers

Primer	Sequence	
GAPDH	*GAPDH F: 5′ACCACAGTCCATGCCATCAC 3′*	
	GAPDH R: 5′TCCACCACCCTGTTGCTGTA 3′	
SRY	External	SRY R ext: 5′GTAGGTTGTTGTCCCATTGC 3′
		SRY F ext: 5′GAGAGAGGCACAAGTTGGC 3′
	Internal	SRY F int: 5′AAGCAGCTGGGATATCAGTGG 3′
		SRY R int: 5′TTTTGTTGAGGCAACTTCACG 3′

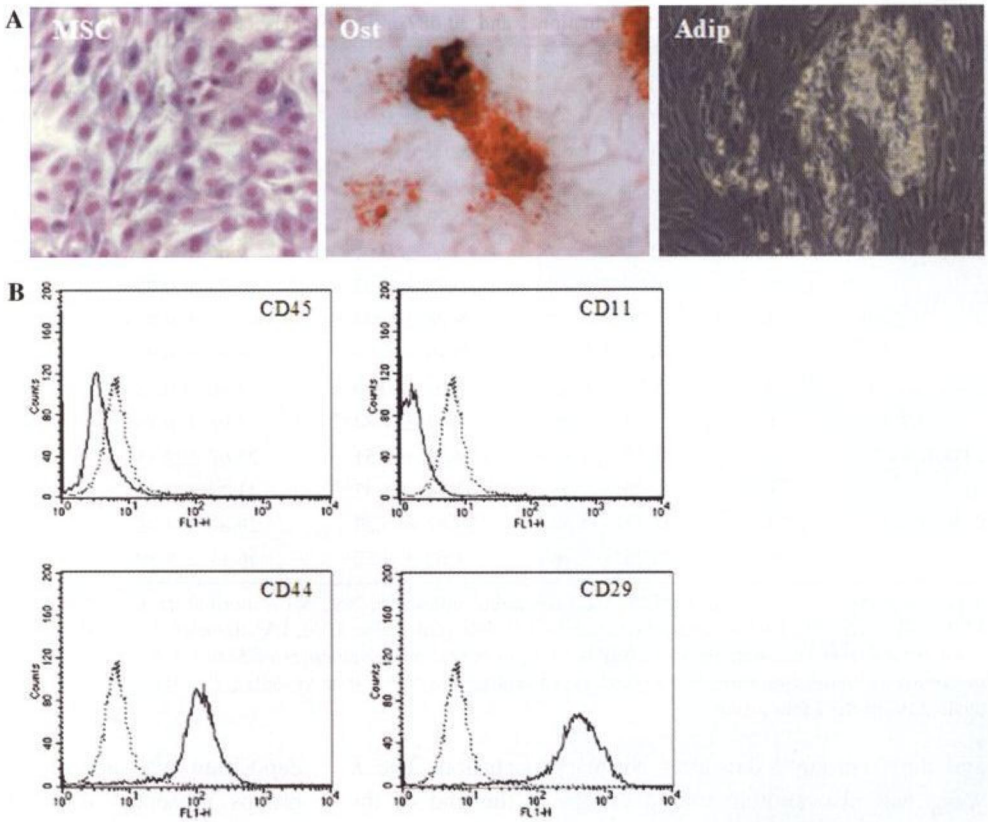

Fig. 1 (**a**) Cultured rat mesenchymal stem cell (MSCs) assumes a flat morphology (400×). The cells differentiate in osteoblasts (ost, 1,000×) and adipocytes (adip, 400×) when cultured with specific induction media. Bar, 20 μm. (**b**) Immunophenotyping of cultivated MSCs. The cells are negative for CD31 (not shown), CD45 and CD11b, and positive for CD29 and CD44. ...., control reactions; ___ , incubation with antibodies

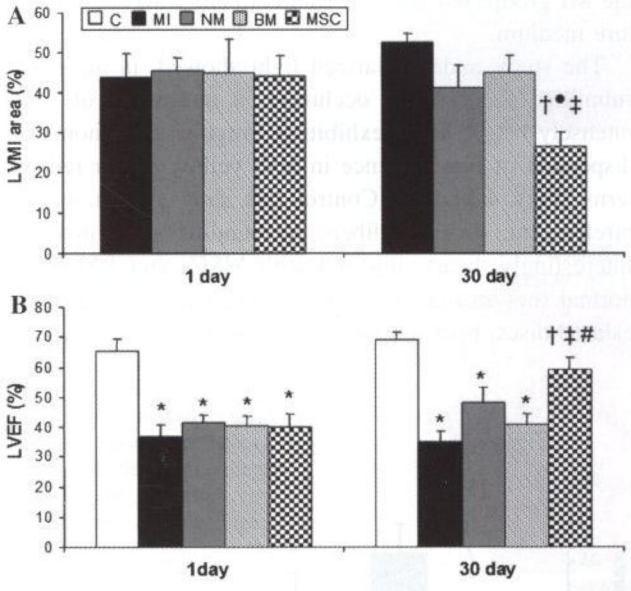

Fig. 2 Left ventricle (LV) wall myocardium infarction (MI) area (**a**) and ejection fraction (EF) (**b**) in C, MI, NM, BM and MSC groups in the initial and final echocardiographic evaluations. $*P < 0.05$ vs. control group; $\dagger P < 0.05$ vs. MI group; $\ddagger P < 0.05$ vs. BM group; $\bullet P < 0.05$ vs. NM; $\#P < 0.05$ vs. initial evaluation in the same group

the animals submitted to myocardial infarction (36.6 ± 4%; 41.5 ± 2%; 40.2 ± 3%; 39.9 ± 4% in MI, NM, BM and MSC, respectively), as compared to control animals (65 ± 4%). The left ventricle ejection fraction was lower at the end of the protocol in the MI (34 ± 3%), NM (48 ± 5%) and BM (41 ± 4%) groups, as compared to the C group (69 ± 3%). Thirty days after coronary occlusion, however, the rats injected with cultivated mesenchymal stem cells (MSC group) presented an improvement in the left ventricle ejection fraction (59 ± 4%) in comparison to MI, NM and BM rats, whereas no difference was observed between MSC and C animals.

Other variables analyzed by echocardiography (LV mass, LVd, *E* wave, *A* wave, *E*/*A* ratio, EDT and IVRT) are shown in Table 2. Left ventricle mass and LVd were unchanged 1 day after coronary occlusion in all groups, as compared to the C group. The LV mass was increased in the BM group in relation to the other groups at the end of the protocol (30 days after coronary occlusion). At the same time, the LV area was increased in all infarcted groups in comparison to the C group and to the initial evaluation in infarcted groups (1 day after coronary occlusion). Left ventricle diameter during diastole (LVd) was also higher in MI, MSC and BM rats in relation to C rats. The BM group presented increased LVd as compared to MI, NM and MSC groups. However, LVd was similar in the infarcted rats injected with cultivated mesenchymal stem cells (MSC group) as compared to C animals.

Diastolic function, evaluated by *A* wave, *E* wave, *E*/*A* ratio, EDT and IVRT, was similar in the infarcted groups

Table 2 Echodoppler cardiovascular evaluation 1 and 30 days after coronary occlusion

Parameter	Day	C	MI	NM	BM	MSC
LV mass (g)	1	0.92 ± 0.03	0.95 ± 0.03	0.95 ± 0.005	1.06 ± 0.02	0.95 ± 0.02
	30	0.93 ± 0.03	1.00 ± 0.02	1.03 ± 0.04	1.24 ± 0.01*†•#	0.99 ± 0.07‡
LVDIA (cm)	1	0.599 ± 0.02	0.60 ± 0.02	0.62 ± 0.02	0.66 ± 0.01	0.62 ± 0.02
	30	0.60 ± 0.02	0.76 ± 0.02*#	0.73 ± 0.04*	0.85 ± 0.02*†•#	0.69 ± 0.03‡
E-wave (m/s)	1	0.60 ± 0.03	0.64 ± 0.05	0.66 ± 0.036	0.66 ± 0.01	0.59 ± 0.02
	30	0.59 ± 0.01	0.75 ± 0.03	0.71 ± 0.05	0.75 ± 0.04	0.64 ± 0.04
A-wave (m/s)	1	0.34 ± 0.02	0.24 ± 0.03	0.291 ± 0.035	0.276 ± 0.02	0.241 ± 0.02
	30	0.37 ± 0.02	0.16 ± 0.04*	0.31 ± 0.04†	0.12 ± 0.04*•	0.31 ± 0.04†‡
E/A ratio	1	1.75 ± 0.09	3.02 ± 0.63	2.36 ± 0.183	2.77 ± 0.28	2.53 ± 0.20
	30	1.63 ± 0.08	6.47 ± 1.34*#	3.03 ± 0.77†	5.67 ± 0.62*•#	2.11 ± 0.48†‡
EDT (ms)	1	31.13 ± 1.89	28.00 ± 2.51	25.67 ± 2.25	32.00 ± 1.15	26.33 ± 1.93
	30	30.38 ± 2.33	43.14 ± 3.37*#	41.22 ± 3.43*#	45.33 ± 1.86*#	43.43 ± 1.81*#
IVRT (ms)	1	34.13 ± 1.54	33.67 ± 3.20	29.80 ± 1.02	35.80 ± 1.71	36.60 ± 1.58
	30	37.75 ± 2.76	31.00 ± 3.52	36.11 ± 1.89	30.33 ± 1.36	35.18 ± 2

Values are expressed as mean ± SEM. MI, myocardial infarction; NM, MI + normal medium; BM, MI + treatment with bone marrow cells; MSC, MI + treatment with mesenchymal cells; LV, left ventricular; LVd, LV diameter during diastole; *E*-wave, maximal early diastolic peak velocity; *A*-wave, late peak velocity; *E/A* ratio, ratio between the velocities of *E* and *A* waves; EDT, peak *E* desacceleration time; IVRT, LV isovolumetric relaxation time. * $P < 0.05$ vs. control group; † $P < 0.05$ vs. MI; ‡ $P < 0.05$ vs. BM; • $P < 0.05$ vs. NM; # $P < 0.05$ vs. initial evaluation in the same group

and the C group 1 day after coronary occlusion. The *E* wave was also similar among groups at the end of the protocol. The *A* wave was reduced and the *E/A* ratio was increased in MI and BM groups as compared with C, NM and MSC groups 30 days after coronary occlusion. The *E/A* ratio was also higher in MI and BM rats comparing the final (30 days) vs. the initial (1 day) evaluation. Thirty days after coronary occlusion, EDT was increased in all infarcted groups in comparison to the C group and to their respective initial evaluation (1 day). IVRT remained unchanged during the whole protocol.

Survival rates were similar among the C (100.0%), NM (90.0%) and MSC (87.5%) groups, but these animals presented higher survival rates as compared to the MI (56.2%) and BM (66.7%) groups ($P < 0.05$).

Molecular analyses

PCR and nested PCR showed the presence of the Y chromosome in normal male heart tissue (not shown), but no amplification was obtained in heart samples collected from the female rats in any of the experimental groups. Positive results with GAPDH control primers (not shown) proved the adequate quality of the DNA samples prepared.

Ventricle fibrosis evaluation

Myocardial scars following ischemia-induced lesion were always extended to the anteroseptal LV wall. Fibrosis deposition was not observed in control rats, while MI groups presented different scar extension and fibrosis. Figure 3 depicts the fibrosis scores, which were higher in the MI group, but reduced by treatment with cells or culture medium.

The study under polarized light showed, in all groups submitted to coronary occlusion, a meshwork of thick, intensely bright fibers exhibiting irregular and anomalous dispersion of birefringence in both yellow and/or red patterns (Figs. 4 and 5). Control rats showed thin, weakly birefringent, greenish fibers surrounding each myocyte. Interestingly, hearts injected with MSCs and BMCs had normal (not-atrophied) myocytes, presenting visible intercalated discs, between fibrosis and scar.

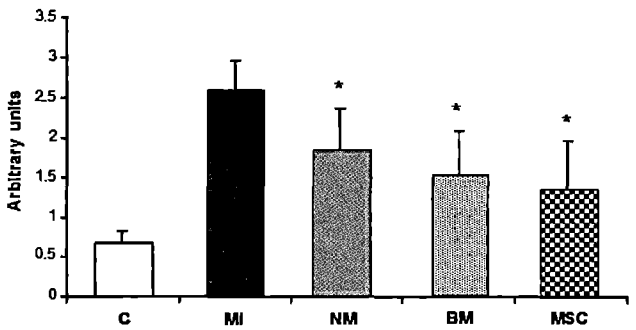

Fig. 3 Fibrosis score in the studied groups. It was higher in MI rats as compared to C rats, but similarly lower in the MSC, BMC and NM groups. *$P < 0.05$ vs. MI group

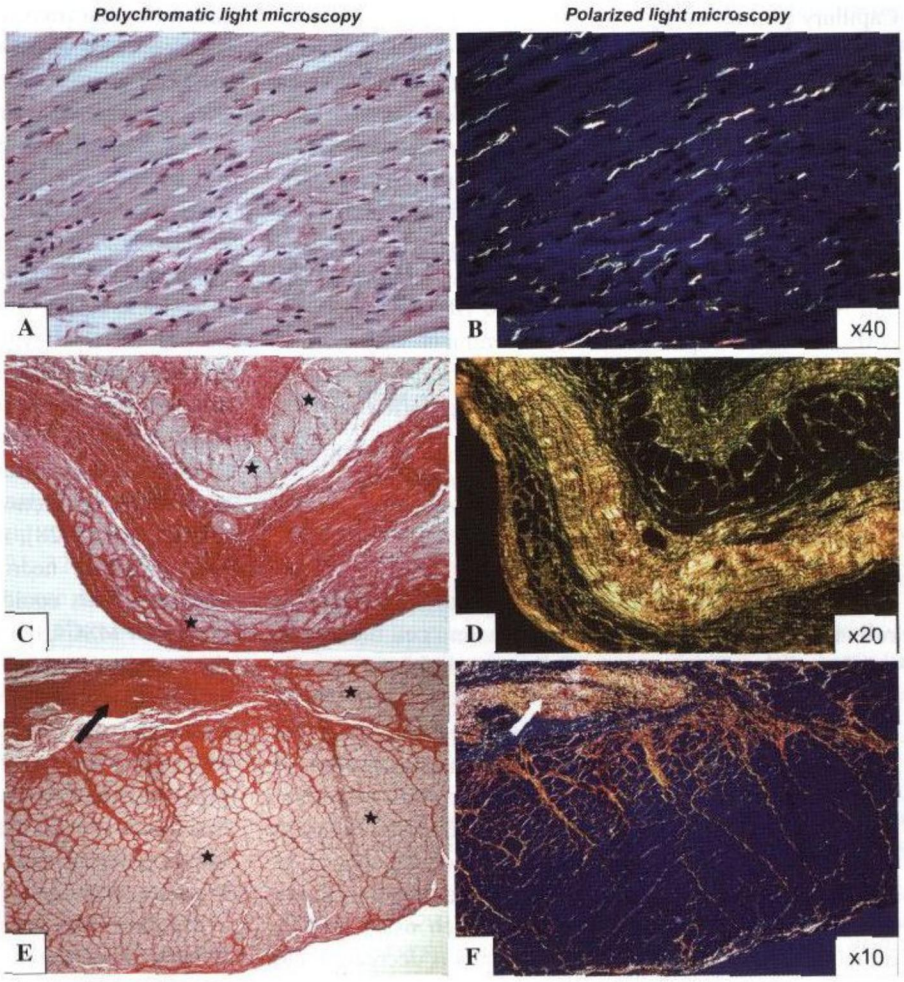

Fig. 4 Representative photomicrographies of different *myocardial configurations* observed in sections stained with picrossirius-hematoxylin *and observed under* polychromatic light microscopy or polarized light microscopy. (**a**) and (**b**) Control group. (**c**) and (**d**) Scar region, predominantly in the myocardium, where epicardic and subendocardic regions presents few viable myocytes (*). (**e**) and (**f**) Region of fibrosis (arrow), more frequent in the *MSC, BM and NM groups*, presenting larger amount of viable myocytes (*)

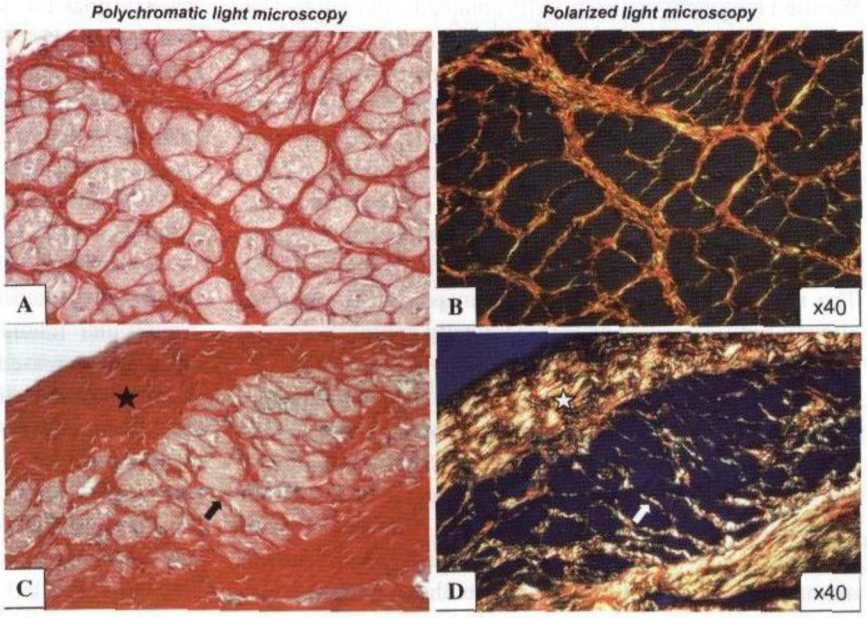

Fig. 5 Representative photomicrographies of *myocardial configurations* observed in studied groups. (**a**) and (**b**) Thickening of endomysium around viable myocytes. (**c**) and (**d**) Viable myocytes between collagen fibers. Note the great collagen deposition by strongly birefringent, yellow collagen fibers (*) in contrast with the weakly birefringent greenish *fibers (arrow) that involve each* myocyte

Capillary proliferation

The injection of MSCs and BMCs around the infarcted area in the LV resulted in important increase of capillary density. Capillary quantification was performed in viable myocardium (not in the scar), and MI rats did not show differences in capillary density as compared to control rats (273 ± 49 vs. 262 ± 28 capillaries/mm^2, respectively). Cell injections increased capillary density, compared to MI and NM groups (MSC: 487 ± 29, and BM: 453 ± 38 vs. NM: 366 ± 73 capillaries/mm^2). Treatment with normal medium increased capillary density in the NM group as compared to the MI group (366 ± 73 capillaries/mm^2 vs. 262 ± 28 capillaries/mm^2).

Discussion

Adequate definition of the type and route of delivery of cells is of fundamental importance for stem cell therapy of cardiovascular diseases to be fully successful. Stem cells can be delivered through coronary arteries, coronary veins, peripheral veins or intra myocardial injection. The main results of the present study showed that in situ injection of MSCs can successfully reduce the myocardial infarction area, increase the ejection fraction and reduce mortality in an animal model of myocardial infarction associated to systemic hypertension. The effects were not so clearly observed with the same delivery approach using BMCs, although in both cases the fibrosis area was decreased and capillary density increased.

The specific cell type used for treating cardiovascular diseases can result in different outcomes [10, 15, 23, 24]. We used mesenchymal stem cells obtained from 8th to 12th passage of cultured bone marrow cells, which showed plasticity and immunophenotype similar to those previously described [18, 23]. Bone marrow preparations, more frequently used in clinical trials of cell-based therapies, are a heterogeneous population of mature mononuclear cells, blasts and stem/progenitor cells. MSCs, on the other hand, represent the expansion of a specific subpopulation, whose therapeutic potential has been increasingly recognized [23]. The number of cells used for treatment (10^6/animal) has already been described as therapeutically appropriate in rat models of cardiovascular disease [25].

Since hypertension accelerates left ventricular dilatation and haemodynamic alterations following myocardial infarction in rats [3], we decided to use SHRs submitted to coronary occlusion as a more physiological model of the association of these pathologies. There is no report in the literature concerning the use of stem cell therapy in this animal model. We observed that in these animals, ventricular dilatation was not accelerated as in normotensive rats following myocardial infarction, probably due to pre-existing LV hypertrophy and increased hypertrophic response to myocardial infarction [26]. Absolute left ventricular dimensions in SHR have been shown to increase out of proportion to body growth, consistent with concentric hypertrophy [27], a finding we did not observe—LV mass was not different comparing the final vs. the initial evaluation. This difference is probably due to the younger age of animals and the short time of follow up (30 days) in the present study. The only group that presented higher LV mass at the final evaluation was the BM group. We ascribed this difference, even though it was not statistically different from the other groups, to their higher baseline LV mass.

Intracoronary infusions do not appear to infiltrate the myocardial infarction zone or reduce the myocardial infarction size [28], so the direct delivery of cells into the area of tissue necrosis seems to be the best delivery approach. This could be shown in the present study with the use of MSCs, but not BMCs, in hypertensive rats, since the former effectively reduced the LV infarction area. This structural benefit was also translated into functional advantage, as observed by the higher LV ejection fraction and similar LVd of the MSC group as compared to the C group. Using normotensive pigs, similar structural and functional benefits have been observed with percutaneous injection catheter delivery of MSCs, persisting until 8 weeks after the cell therapy [10].

Baseline diastolic parameters were similar among groups, as expected. However, diastolic dysfunction was observed 30 days after coronary occlusion in MI and BM groups, but not in C, NM and MSC groups, as indicated by decreased *A*-wave and increased *E/A* ratio. These data showed that LV remodeling, common in infarcted hearts, was modified by the in situ injection of NM and MSCs. The unexpected effect of NM may be due to growth factors present in the serum. The evaluation of diastolic function using echodopplercardiography has been reported by few investigators. The present work showed improved results as compared to similar experiments with in situ administration of MSCs in pigs [10].

Reduction of fibrosis score may suggest reestablishment of the correct arrange of cytoskeleton, supporting the better ventricular function. Interestingly, treatment with culture medium increased capillary density, and this may explain the reduction of fibrosis score in this group when compared to MI. The increase in capillary density is in accordance with a putative angiogenic effect of both MSCs and BMCs [10, 24].

Cultured mesenchymal stem cells and BMCs treatments did not induce arrhythmias, differently from results recently reported [29]. One possible explanation for this difference could be related to the fact that we injected

smaller volumes distributed in more myocardial points. Also, in that study arrhythmia was assessed by telemetry, a more sensitive method than ours, since we evaluated the cardiac rhythm by echocardiography twice during the whole protocol. Since mortality was not different in animals that presented arrhythmia vs. those that did not have this complication in the study above, and no higher mortality was seen in our groups injected with stem cells, the significance of those findings is uncertain.

The therapeutic role of MSCs in myocardial repair is under intense investigation, and may involve multiple factors such as direct differentiation into cardiac cells, cell fusion or the secretion of cytokines and growth factors with paracrine activities [30]. Our results favor this latter model, since transplanted male cells were not found in the heart of treated females when they were analyzed by PCR and nested PCR 30 days later. Although surprising, similar results have recently been reported [31, 32]. Prockopp and collaborators, for instance, using a mouse model of acute MI, observed that cardiac function and fibrosis were significantly improved, but there was no evident MSC engraftment in the heart despite of the use of three different and highly sensitive assays [31]. These results give support to the hypotheses that theses cells can exert beneficial effects after coronary artery occlusion through the secretion of cardioprotective and reparative factors.

In an attempt to explain these results, the gene expression profiles of cultured MSCs were analyzed with microarrays. The expression of genes coding for anti-apoptotic, angiogenic/arteriogenic, and matrix-mediating factors, particularly IL-6, leukemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) family members in the MSCs was observed [31]. More recently, Sze et al. [33] determined the secretion proteome of human embryo-derived MSCs. The study revealed the presence of 201 unique gene products, representing important signaling pathways in cardiovascular biology, bone development and hematopoiesis. The MSC secretory products identified may act as paracrine modulators of tissue repair and replacement in cardiovascular diseases. Since our MSC cultures are in all aspects (morphology, kinetics, immunophenotyping and plasticity) identical to the cell populations used in those studies, we may assume that they secret similar profiles of cytokines and growth/differentiation factors. The fact the bone marrow cells did not result in amelioration of left ventricle dysfunction is also an evidence of a mechanism exclusive of MSCs in heart remodeling.

Systemically administered MSCs determine different responses in comparison to their in situ administration. We, in SHR [34], and others, in normotensive rats [23], observed increased LVd after myocardial infarction and treatment with systemically injected MSCs. Immunodeficient mice submitted to the same procedure showed improved cardiac function and reduced myocardial fibrosis [31]. These results raise the possibility of different actions of the cells delivered by different routes, and show that in situ delivery of MSCs is more effective for regeneration of the damaged myocardium.

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References

1. Ridker PM, Stampfer MJ, Rifai N (2001) Novel risk factors for systemic atherosclerosis: a comparison of c-reactive protein, fibrinogen, homocysteine, lipoprotein(a), and standard cholesterol screening as predictors of peripheral arterial disease. JAMA 285:2481–2485
2. Doggrell SA, Brown L (1998) Rat models of hypertension, cardiac hypertrophy and failure. Cardiovasc Res 39:89–105
3. Nishikimi T, Yamagishi H, Takeuchi K, Takeda T (1995) An angiotensin II receptor antagonist attenuates left ventricular dilatation after myocardial infarction in the hypertensive rat. Cardiovasc Res 29:856–861
4. Wollert KC, Drexler H (2006) Cell-based therapy for heart failure. Curr Opin Cardiol 21:234–239
5. Becker C, Lacchini S, Muotri AR, da Silva GJ, Castelli JB, Vassallo PF, Menck CF, Krieger JE (2006) Skeletal muscle cells expressing VEGF induce capillary formation and reduce cardiac injury in rats. Int J Cardiol 113:348–354
6. Kucia M, Ratajczak J, Reca R, Janowska-Wieczorek A, Ratajczak MZ (2004) Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. Blood Cells Mol Dis 32:52–57
7. Lin F, Cordes K, Li L, Hood L, Couser WG, Shankland SJ, Igarashi P (2003) Hematopoietic stem cells contribute to the regeneration of renal tubules after renal ischemia-reperfusion injury in mice. J Am Soc Nephrol 14:1188–1199
8. Rehman J, Li J, Orschell CM, March KL (2003) Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 107:1164–1169
9. Zhang FB, Li L, Fang B, Zhu DL, Yang HT, Gao PJ (2005) Passage-restricted differentiation potential of mesenchymal stem cells into cardiomyocyte-like cells. Biochem Biophys Res Commun 336:784–792
10. Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, Lehrke S, Baumgartner WW, Martin BJ, Heldman AW, Hare JM (2005) Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci USA 102:11474–11479
11. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P (2001) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 98:10344–10349
12. Boomsma RA, Swaminathan PD, Geenen DL (2006) Intravenously injected mesenchymal stem cells home to viable myocardium after coronary occlusion and preserve systolic function without altering infarct size. Int J Cardiol [Epub ahead of print]

13. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109:1543–1549
14. Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura K (2004) Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 287:H2670–H2676
15. Davani S, Marandin A, Mersin N, Royer B, Kantelip B, Herve P, Etievent JP, Kantelip JP (2003) Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 108(Suppl 1):II253–II258
16. Shyu KG, Wang BW, Hung HF, Chang CC, Shih DT (2006) Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. J Biomed Sci 13:47–58
17. Meirelles Lda S, Nardi NB (2003) Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 123:702–711
18. Da Silva Meirelles L, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213
19. De Angelis K, Leirner AA, Irigoyen MC, Cestari IA (2001) Nonstimulated cardiomyoplasty improves hemodynamics in myocardial-infarcted rats. Artif Organs 25:939–943
20. Johns TN, Olson BJ (1954) Experimental myocardial infarction. I. A method of coronary occlusion in small animals. Ann Surg 140:675–682
21. Nozawa E, Kanashiro RM, Murad N, Carvalho AC, Cravo SL, Campos O, Tucci PJ, Moises VA (2006) Performance of two-dimensional Doppler echocardiography for the assessment of infarct size and left ventricular function in rats. Braz J Med Biol Res 39:687–695
22. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, New York
23. Beyer Nardi N, da Silva Meirelles L (2006) Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handbook Exp Pharmacol 174:249–282
24. Kudo M, Wang Y, Wani MA, Xu M, Ayub A, Ashraf M (2003) Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. J Mol Cell Cardiol 35:1113–1119
25. Lovell MJ, Mathur A (2004) The role of stem cells for treatment of cardiovascular disease. Cell Prolif 37:67–87
26. Zdrojewski T, Gaudron P, Whittaker P, Poelzl S, Schiemann J, Hu K, Ertl G (2002) Ventricular remodeling after myocardial infarction and effects of ACE inhibition on hemodynamics and scar formation in SHR. Cardiovasc Pathol 11:88–93
27. Pfeffer J, Pfeffer M, Fletcher P, Braunwald E (1979) Alterations of cardiac performance in rats with established spontaneous hypertension. Am J Cardiol 44:994–998
28. Wollert KC, Drexler H (2004) Cell therapy for acute myocardial infarction: where are we heading? Nat Clin Pract Cardiovasc Med 1:61
29. Fukushima S, Varela-Carver A, Coppen SR, Yamahara K, Felkin LE, Lee J, Barton PJ, Terracciano CM, Yacoub MH, Suzuki K (2007) Direct intramyocardial but not intracoronary injection of bone marrow cells induces ventricular arrhythmias in a rat chronic ischemic heart failure model. Circulation 115:2254–2261
30. Wang XJ, Li QP (2007) The roles of mesenchymal stem cells (MSCs) therapy in ischemic heart diseases. Biochem Biophys Res Commun 359:189–193
31. Iso Y, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, Sobel BE, Delafontaine P, Prockop DJ (2007) Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun 354:700–706
32. Noiseux N, Gnecchi M, Lopez-Ilasaca M, Zhang L, Solomon SD, Deb A, Dzau VJ, Pratt RE (2006) Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. Mol Ther 14:840–850
33. Sze SK, de Kleijn DP, Lai RC, Tan EK, Zhao H, Yeo KS, Low TY, Lian Q, Lee CN, Mitchell W, El Oakley RM, Lim SK (2007) Elucidating the secretion proteome of human ESC-derived mesenchymal stem cells. Mol Cell Proteomics [Epub ahead of print]
34. Braga LMGM, Rosa K, Rodrigues B, Malfitano C, Camassola M, Chagastelles P, Lacchini S, Fiorino P, De Angelis K, Schaan B, Irigoyen MC, Nardi NB (2007) Systemic delivery of adult stem cells improves cardiac function in spontaneously hypertensive rats. Clin Exp Pharmacol Physiol 35; [Epub ahead of print]