Therapy with mesenchymal stromal cells or conditioned medium reverse cardiac alterations in a high-fat diet–induced obesity model

P.S. DALTRO1,2, B.C. BARRETO1,3, P.G. SILVA4, P. CHENAUD NETO1, P.H.F. SOUSA FILHO4, D. SANTANA NETA4, G.B. CARVALHO1, D.N. SILVA1, B.D. PAREDES1, A.C. DE ALCANTARA1, L.A.R. FREITAS4,5, R.D. COUTO6, R.R. SANTOS1,7, B.S.F. SOUZA1,5,7, M.B.P. SOARES1,5,7, & S.G. MACAMBIRA1,5,7,8

1Center for Biotechnology and Cell Therapy, Salvador, BA, Brazil, 2Multicentric Program in Biochemistry and Molecular Biology, Federal University of Bahia, Salvador, BA, Brazil, 3Faculty of Biology, Federal University of Bahia, Salvador, BA, Brazil, 4Faculty of Medicine, Federal University of Bahia, Salvador, BA, Brazil, 5Gonçalo Moniz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Salvador, BA, Brazil, 6Faculty of Pharmacy, Federal University of Bahia, Salvador, BA, Brazil, 7National Institute of Science and Technology for Regenerative Medicine, Rio de Janeiro, RJ, Brazil, and 8Department of Biochemistry and Biophysics, Institute of Health Sciences, Federal University of Bahia, Salvador, BA, Brazil

Abstract

Background. Obesity is associated with numerous cardiac complications, including arrhythmias, cardiac fibrosis, remodeling and heart failure. Here we evaluated the therapeutic potential of mesenchymal stromal cells (MSCs) and their conditioned medium (CM) to treat cardiac complications in a mouse model of high-fat diet (HFD)–induced obesity.

Methods. After obesity induction and HFD withdrawal, obese mice were treated with MSCs, CM or vehicle. Cardiac function was assessed using electrocardiography, echocardiography and treadmill test. Body weight and biochemical parameters were evaluated. Cardiac tissue was used for real time (RT)–polymerase chain reaction (PCR) and histopathologic analysis.

Results/Discussion. Characterization of CM by protein array showed the presence of different cytokines and growth factors, including chemokines, osteopontin, cystatin C, Serpin E1 and Gas 6. HFD-fed mice presented cardiac arrhythmias, altered cardiac gene expression and fibrosis reflected in physical exercise incapacity associated with obesity and diabetes. Administration of MSCs or CM improved arrhythmias and exercise capacity. This functional improvement correlated with normalization of GATA4 gene expression in the hearts of MSC- or CM-treated mice. The gene expression of connexin 43, troponin I, adiponectin, transforming growth factor (TGF) β, peroxisome proliferator activated receptor gamma (PPARγ), insulin-like growth factor 1 (IGF-1), matrix metalloproteinase-9 (MMP9) and tissue inhibitor of metalloproteinases 1 (TIMP1) were significantly reduced in MSCs, but not in CM-treated mice. Moreover, MSC or CM administration reduced the intensity of cardiac fibrosis.

Conclusion. Our results suggest that MSCs and CM have a recovery effect on cardiac disturbances due to obesity and corroborate to the paracrine action of MSCs in heart disease models.

Key Words: cardiac dysfunction, cell therapy, mesenchymal stromal cells, obesity

Introduction

Obesity and excessive consumption of dietary fats have deleterious consequences to the metabolism and heart function, being strongly linked to the progression of type 2 diabetes mellitus (DM2) and heart disease [1,2]. Obesity has been associated with structural and functional changes in the heart, including arrhythmias, cardiac fibrosis and subclinical impairment of left ventricle systolic and diastolic functions. Some cardiac alterations associated with structural and electrical remodeling are irreversible and can lead to heart failure, increasing the risk for sudden death through mechanisms that are not completely elucidated [3].

In addition to the electrical remodeling and severe arrhythmias, long-standing obesity is also associated with structural remodeling, characterized by eccentric hypertrophy and followed by diastolic dysfunction and fibrosis, despite coronary disease or hypertension [4–6]. When isolated from other cardiac
impairments, this disease state is referred to as diabetic cardiomyopathy [7]. Cell-based therapies are currently under investigation and hold promise to treat cardiac diseases. Mesenchymal stromal cells (MSCs) are among the most-studied cell types in the cardiovascular field. MSCs are multipotent cells easily isolated from different tissues in the adult, being expandable in vitro as plastic-adherent cells with fibroblast-like morphology, with potential for differentiation into tissues of mesodermal origin and displaying immunomodulatory properties [8,9]. Pre-clinical and clinical studies have shown beneficial effects of MSCs in injured hearts, leading to reduction of fibrosis [10], stimulation of angiogenesis [11] and restoration of contractile function [12]. In cardia diseases, the beneficial effects of MSCs are primarily explained as the result of release of paracrine factors [11,13–15], which regulate several biological processes. This hypothesis is further supported by the observation that MSC-conditioned medium (CM) also enhances cardiac tissue repair [16,17], being used for treatment of cardiac ischemia lesions, as previously shown [17,18].

Despite the growing body of evidence, there are no reports showing the effects of cell therapy and cardiac remodeling in the presence of arrhythmias and physical disability due to obesity. In the present study, we investigated the therapeutic effects of bone marrow–derived MSCs and its CM in an experimental model of cardiac alterations by obesity induction in C57Bl/6 mice fed with a diet enriched with lard saturated fat.

### Materials and methods

#### Animals and obesity induction

Six-week–old male C57Bl/6 mice were housed at the animal facility of the Center for Biotechnology and Cell Therapy, São Rafael Hospital (Salvador, Brazil), in a room with constant temperature (20 ± 2°C), controlled humidity (50%), free access to food and water ad libitum and exposure to a constant light–dark cycle of 12 h and 12 h. All animal protocols were approved by the Ethical Committee for Animal Research of Gonçalo Moniz Institute, Oswaldo Cruz Foundation. All mice were fed a standard mouse chow for up to 6 weeks of age. Mice were then divided into two groups: standard diet (Nuvital; n = 10) and high-fat diet (HFD; Pragsoluções Biociências; n = 28) for 36 weeks, for obesity induction. The composition of the diets is shown in Table I. The time of exposure to HFD was based on our previous study [19] in which we standardized the model of obesity and DM2 in C57BL/6 mice.

#### Treatment with MSCs and CM

After 36 weeks of obesity induction, HFD was withdrawn and replaced by regular chow. Obese mice were randomly divided into three subgroups depending on treatment administered: (i) MSCs (n = 10), in which intravenous (i.v.) administrations of MSCs (5 × 10⁵ cells/mouse), suspended in 100 μL, were performed through the tail vein, on 2 consecutive days; (ii) MSC CM (n = 10) or (iii) Dulbecco’s Modified Eagle’s

### Table I. Composition of standard diet and HFD.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>HFD 60% integral</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g %</td>
<td>kcal %</td>
</tr>
<tr>
<td>Protein</td>
<td>23.4</td>
<td>17.5</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>33.2</td>
<td>24.69</td>
</tr>
<tr>
<td>Fat</td>
<td>34.6</td>
<td>57.9</td>
</tr>
<tr>
<td>Total</td>
<td>3.87</td>
<td>100</td>
</tr>
<tr>
<td>Total Kcal/g</td>
<td>3.87</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g</th>
<th>kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>95.7</td>
<td>0</td>
</tr>
<tr>
<td>Gross fiber</td>
<td>4.2</td>
<td>0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>—</td>
<td>928</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
<td>400</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>8.6</td>
<td>774</td>
</tr>
<tr>
<td>Lard</td>
<td>24.7</td>
<td>2223</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>Sodium</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.60</td>
<td>0</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.76</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>159.26</td>
<td>4325</td>
</tr>
</tbody>
</table>

1177
Medium (DMEM; n = 8; Gibco), consisting of 100 µL/mouse of CM or DMEM, respectively, administered by i.v. route, following a protocol of three courses of three injections with a 1-day interval between each injection, and 9 days between each course. Mice were euthanized 2 months after the end of the therapy, under anesthesia with 5% ketamine (Vetanarcol; Konig) and 2% xylazine (Sedomin; Konig). The experimental design is shown in Figure 1.

**Culture of MSCs and preparation of CM**

MSCs were obtained from the marrow of femurs and tibiae of C57Bl/6 mice, after culture and expansion in DMEM supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific), 100 IU/mL penicillin (Pen) and 100 mg/mL streptomycin (Strep; ThermoFisher Scientific). Culture medium was replaced every 3 days, and, upon reaching 90% confluence, adherent cells were passaged with trypsin-ethylenediaminetetraacetic acid (EDTA) 0.05% (ThermoFisher Scientific) and transferred to tissue culture flasks containing medium supplemented with 10% FBS and 1% Pen/Strep. CM was prepared using MSCs in passage 4, with 80–90% confluence. Cells were washed twice with phosphate-buffered saline (PBS) and transferred to a serum-free DMEM without phenol red, supplemented with Pen/Strep, during 48 h. The CM was then collected and concentrated 25× by centrifugation at 3000g for 50 min at 20°C using a Centriprep 3 kd filtration system (Millipore) and was stored at −80°C until use. Equivalent medium kept under the same conditions in flasks without cells was prepared and used as control.

**Phenotypic and multipotency characterization of MSCs**

MSCs were trypsinized, resuspended in 0.9% saline solution and used for immunophenotyping. MSCs (5 × 10⁵ cells) were incubated for 5 min with anti-CD16/CD32 (BD Biosciences), followed by incubation at 4°C for 30 min with the following antibodies (diluted at 1:100): Phycoerythrin-Cyanine5.5 (Sca1-PE-Cy5.5) (Caltag Medsystems); allophycocyanin (CD90.2-APC), Phycoerythrin (CD117-PE, CD45-APC), CD34-Alexa Fluor 647 and CD44-PE (BD Biosciences) and CD29-APC and CD105-PE (BioLegend). Isotype-identical antibodies were used as controls. After incubation and two washes with PBS, data were acquired and analyzed on an LSR Fortessa flow cytometer (BD Biosciences). At least 50 000 events were collected and analyzed.

To evaluate the differentiation potential of MSCs, we performed the trilineage differentiation assay (adipogenic, osteogenic and chondrogenic) using specific kits, following manufacturer’s instructions (ThermoFisher Scientific). Cultures were stained with oil red O, alcian blue and alizarin red S stainings.
(Sigma-Aldrich) to assess adipogenic, chondrogenic and osteogenic differentiation, respectively.

Characterization of CM

The analysis of protein contents of CM was done using the Proteome Profile Array Mouse XL Cytokine Array Kit (R&D Systems), according to the manufacturer’s instructions. Detection was done by chemiluminescence reaction and quantified by densitometry using ImageJ software (National Institutes of Health) and Gilles Carpentier’s Protein Array Analyzer macro [20].

Assessment of body weight, fasting glucose, glucose tolerance test and total cholesterol

Induction of obesity was monitored by monthly measurements of body weight and fasting glucose levels, in addition to a quarterly evaluation of glucose tolerance, with samples collected from tail vein blood. The individual weight measurements were monitored with the aid of a semi-analytical balance (Tepron—Equipment). The fasting glucose test was performed 7 h following the last food intake, whereas the glucose tolerance test was performed after a 4-h fast. Tests were performed using an AccuChek Active glucometer system (Roche Diagnostics). For assessment of total cholesterol, plasma samples were collected at 0, 36 and 51 weeks in the experimental model. Blood samples were collected and placed in microtubes and centrifuged at 1000 rpm at 4°C for 10 min to obtain the plasma, which was stored at −80°C for further analysis in automated equipment, LabMax 560 (LABtest Diagnostic SA), through the enzymatic colorimetric method.

Cardiac functional analysis

The cardiac function evaluation included the following: echography, electrocardiography (EKG) and treadmill test performed every quarter. For echography and EKG recordings, mice were anesthetized with inhaled isoflurane (0.5%). Transthoracic echocardiography was performed using the Vevo 770 Echosystem (Visual Sonics) equipped with a 30 MHz transducer (Model 707B RMV; Visual Sonics) to acquire images in different acoustic windows, on supine-positioned mice maintained on a thermo-regulated plate (37°C). EKG acquisition was performed using a bipolar I lead, obtained from the Bio Amp PowerLab System (PowerLab 2/20; ADInstruments), allowing for the recording of biological signals in animals with complete electrical isolation. All acquired data was analyzed on Windows Chart 5 (PowerLab). Recordings were bandpass-filtered (1–100 Hz) to minimize environmental signal disturbances at a sampling rate of 1 kHz. A single-animal motor-driven treadmill chamber (LE 8700; Panlab), in which the speed and shock intensity (mA) were controlled by a potentiometer (LE 8700-treadmill control; Panlab), was used to evaluate the animal physical capacity to exercise. Mean room temperature was maintained at 21 ± 1°C. After an adaptation period of 30 min in the treadmill chamber, mice were exercised at five different velocities (7.2, 14.4, 28.8, 43.2 and 57.6 m/min), with initial speed increases of 5 min and from the third speed 10 min of exercise at each speed. Velocity was increased until the animal could no longer sustain a given speed and remained more than 10 s on an electrified stainless-steel grid, which provided an electrical stimulus to maintain the mice in motion.

Histopathologic analysis

Hearts from 38 animals (eight from DMEM-treated mice and 10 from each remaining group) were divided equally such that half of the atria and ventricles was used for histology whereas the other half was used for RNA extraction. Hearts were removed and fixed in buffered 10% formalin. Heart sections were analyzed using optical microscopy after paraffin embedding, followed by Sirius red staining to evaluate the fibrosis. Images were digitized using a color digital video camera (CoolSnap) adapted to a BX41 microscope (Olympus). The images were analyzed using Image Pro 7.0 (Media Cybernetics).

Blinded analysis was performed on 10 fields captured per heart, identifying areas of fibrosis and avoiding blood vessels (200× magnification). The percentage of fibrosis was estimated in each field using Image-Pro Plus software, where the percentage of the red area was compared with the remaining areas of the field.

Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Hearts were removed and used for RNA extraction, as described in the previous section. RNA was extracted from heart tissue with TRIzol reagent (Invitrogen) and the concentration was determined using photometric measurement. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize complementary DNA (cDNA) from 1 µg of RNA, according to the manufacturer’s recommendations. Synthesis of cDNA and RNA expression analysis was performed by Real-Time polymerase chain reaction (PCR) using TaqMan Gene Expression Assay for Adipoq (Mm00456425_m1), Tnf (Mm00443258_m1), Gata4 (Mm00484689_m1), Tgfβ1 (Mm00441724_m1), Pparg (Mm01184322_m1), Mmp9 (Mm00442991_m1), Timp1 (Mm00441818_m1), Cx43 (Mm00439105_m1), Tnni, Timp1, Cx43, Tgfβ1, Pparg, Mmp9, Timp1,
Hgf (Mm01135193_m1), Socs3 (Mm 00545913_s1), Col1a1 (Mm 00801666_g1), Igf1 (Mm00439560_m1) and Vgfl (Mm 00437304_m1). All reactions were run in duplicate on an ABI 7500 Real Time PCR System (Applied Biosystems) under standard thermal cycling conditions. A non-template control (NTC) and non-reverse transcription controls (No-RT) were also included. The samples were normalized with Hprt (endogenous control). The threshold cycle (2^ΔΔCt) method of comparative PCR was used to analyze the results [21]. Data was analyzed using GraphPad software version 6.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) for the number of animals in each group. Student t test was used to compare quantitative variables from the two groups at one point. When two or more groups were compared, analysis of variance (ANOVA) was applied, followed by Bonferroni post-test. Significant difference was considered when \( P < 0.05 \). Statistical analysis was performed with GraphPad Prism 5.0 software.

Results

Characterization of MSCs and CM

Bone marrow-derived MSCs successfully underwent adipogenic, osteogenic and chondrogenic differentiations (Figure 2A–2C) and were characterized using flow cytometry, which showed the expression of MSC cell markers CD29, CD44, CD105 and Sca-1 in the majority of the cells, and low frequency of cells expressing hematopoietic cell markers CD11b and CD45 (Figure 2D). The CM of MSCs was prepared and characterized using a protein array kit for analysis of 111 different cytokines (Supplementary Tables S1-S3). The protein array analysis was able to detect the presence of 19 cytokines, including chemokines, growth factors, adhesion molecules and other proteins involved in apoptosis, inflammation and angiogenesis (Figure 3).

Figure 2. Characterization of bone marrow MSCs from C57Bl/6 mice. MSCs were differentiated into oil red O–positive adipocytes (a), alizarin red S–positive osteocytes (b) and alcian blue–positive chondrocytes (c). (d) Flow cytometric analyses showed that MSCs (passage 8) were positive for CD105, Sca-1, CD29 and CD44 and negative for CD11b and CD45.
HFD removal in obese mice reduces body weight, fasting glucose, glucose tolerance test and total cholesterol

The introduction of HFD for 36 weeks caused a significant increase in body weight, fasting glucose, glucose tolerance test (GTT) and total cholesterol, as compared with standard diet–fed mice. Statistically significant differences in body weight and fasting glucose were found after 28 weeks of induction (Figure 4A and 4B). After 36 weeks, HFD was withdrawn and all animals were fed with a standard diet, while receiving MSCs, CM or DMEM administrations. Eight weeks after the end of the treatment (51 weeks) all mice improved their blood levels of glucose and cholesterol, while decreasing body weight, with no significant differences between the different treatment groups (Figure 4).

HFD induced glucose intolerance, when compared with mice fed with standard diet, as shown by an impairment in reducing plasma glucose levels to the initial glucose concentration values after 120 min of intraperitoneal injection of glucose (Figure 5A and 5B). Glucose intolerance was also normalized at the 51st week, independently of treatment (Figure 5C).

MSCs regulate the expression levels of inflammatory cytokines and transcription factors in hearts of obese mice

Next, we performed an analysis by quantitative reverse transcription PCR (RT-qPCR) of cardiac tissues at 51 weeks. Adiponectin gene expression was reduced in DMEM-treated HFD mice compared with standard diet controls (Figure 7A). Treatment with MSCs or CM restored the levels of adiponectin gene expression to levels similar to nonobese controls.
The gene expression of tumor necrosis factor (TNF) α, vascular endothelial growth factor (VEGFα), transforming growth factor (TGF) β1 and insulin growth factor 1 (IGF-1) was increased in the hearts of obese mice treated with DMEM, compared with nonobese controls (Figure 7B–7E). Following the completion of the HFD-induced obesity (36 weeks), mice returned to standard diet and received MSCs or CM or DMEM treatments until 51 weeks. Values are expressed as mean ± SEM of 8 to 10 mice per group. HFD mice versus standard diet mice; ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05. Standard diet mice versus DMEM-treated; †P ≤ 0.05.

**Reversion of HFD-induced heart fibrosis in the heart after MSC and CM administration**

The presence and degree of fibrosis were evaluated in the heart sections of obese mice and nonobese controls (Figure 8A–8D). DMEM-treated obese mice had a significantly higher percentage of cardiac fibrosis at week 51 when compared with mice fed with standard diet (Figure 8E). Both MSC and CM administrations promoted a significant reduction in fibrosis in the hearts of obese mice when compared with DMEM-treated obese mice (Figure 8E). Analysis of gene expression in the heart tissue showed...
reduction of type I collagen by MSC and CM administration, reaching levels similar to those of mice fed with standard diet (Figure 8F). Finally, the gene expression of MMP9 and TIMP1, two genes associated with extracellular matrix remodeling, was normalized by treatment with MSCs, but not with CM (Figure 8G and 8H).

Table II. Presence of arrhythmias at 36 weeks of HFD induction (pretreatment) and 2 months after treatments (51 weeks).

<table>
<thead>
<tr>
<th>Normal sinus rhythm</th>
<th>First degree AV block</th>
<th>Second degree AV block</th>
<th>Junctional rhythm</th>
<th>Intraventricular conduction disturbance</th>
<th>Ventricular bigeminism</th>
<th>Polymorphic ventricular tachycardia</th>
<th>AV dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 wk</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>51 wk</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

*The period of 36 weeks comprises the pretreatment period during HFD administration, while 51 weeks consists of the post-treatment period, 8 weeks after the end of treatment.

Figure 6. Cardiac conduction disturbances in arbitrary units in C57Bl/6 mice before (pre-treatment) and 2 months after treatment (post-treatment). Degree of severity: 0, no cardiac conduction disturbances; 1, first-degree atrium-ventricular block; 2, intraventricular conduction disturbance; 3, junctional rhythm; 4, intraventricular conduction disturbance; 5, ventricular bigemism; 6, polymorphic ventricular tachycardia; and 7, AV dissociation. *P ≤ 0.05. Pretreatment versus bone marrow stromal cell (BMSC)-treated group vs CM-treated group. #P ≤ 0.05; ##P ≤ 0.01 DMEM-treated versus BMSCs-treated mice versus CM-treated mice.

Table III. Exercise capacity (run distance in m) of mice submitted to standard diet or HFD.

<table>
<thead>
<tr>
<th></th>
<th>Preinduction</th>
<th>36 wk</th>
<th>51 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard (n = 10)</td>
<td>615 ± 29.3</td>
<td>648 ± 25.6</td>
<td>627 ± 37.3</td>
</tr>
<tr>
<td>HFD (n = 28)</td>
<td>668 ± 20.0</td>
<td>317 ± 15.8c,d</td>
<td>—</td>
</tr>
<tr>
<td>HFD + DMEM (n = 08)</td>
<td>—</td>
<td>—</td>
<td>426 ± 21.3c,h</td>
</tr>
<tr>
<td>HFD + MSC (n = 10)</td>
<td>—</td>
<td>—</td>
<td>524 ± 30.8d</td>
</tr>
<tr>
<td>HFD + CM (n = 10)</td>
<td>—</td>
<td>—</td>
<td>471 ± 12.2b,e,g</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

1P ≤ 0.05.
2P ≤ 0.01.
3P ≤ 0.001; HFD mice versus standard diet-fed mice.
4P ≤ 0.001; HFD mice in 36 weeks versus HFD mice in preinduction period.
5P ≤ 0.05.
6P ≤ 0.01; HFD mice in 36 weeks versus MSCs-treated mice and CM-treated mice.
7P ≤ 0.05.
8P ≤ 0.001; HFD mice in preinduction period versus DMEM-treated mice and CM-treated mice.
Figure 7. RT-qPCR in heart tissue cytokines and transcription factors. Representative values of Adipoq (a), Tnf (b), Vegfa (c), Tgf-β1 (d), Socs3 (e), Gata4 (f), Pparg (g), Igf1 (h), Tnni3 (i) and Cx43 (j). Expression obtained 8 weeks after the end of treatments, which corresponded to 51 weeks of study. (Values are expressed as mean ± SEM of 7 to 9 mice/group.) Standard diet–fed mice versus treated mice group; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. DMEM-treated mice versus MSC-treated or CM-treated mice; #P ≤ 0.05; ##P ≤ 0.01; ###P ≤ 0.001. MSC-treated mice versus CM-treated mice; ‡P ≤ 0.05; ‡‡P ≤ 0.01.
Discussion

Due to the growing prevalence of obesity, it is crucial to develop new treatments to control cardiac repercussions and comorbidities. Here we show that treatment with MSCs efficiently reverses cardiac abnormalities induced by obesity in mice fed with a HFD. This was shown by recovery of physical exercise capacity, arrhythmias reversal, regulation of gene expression and reduction of fibrosis in the heart tissue.
Importantly, most of the therapeutic effects found in MSC-treated mice were also observed in mice treated with MSC CM.

In the present study, C57Bl/6 male mice fed with a diet enriched with lard saturated fat developed obesity and myocardial disturbances, which mimics the physiological effects associated with human adiposity through nutritional behavior [22–24]. Previous studies have shown that lifestyle changes, such as exercise and balanced diet with low levels of saturated fatty acids, trans fatty acid and cholesterol, are essential to control blood glucose and reduce cardiovascular risk [25,26]. Here we show that obese mice in which HFD was replaced by the standard diet had a reduction in body weight, glucose levels and total cholesterol levels, regardless of the treatment, in accordance with our previous study [19]. In spite of that, diet change did not improve the established cardiac alterations, such as conduction disturbances, decreased exercise capacity, cardiac fibrosis and transcriptional profile. In contrast, administration of MSCs was able to induce significant morphological and functional improvements in the hearts of HFD-fed mice.

After administration of MSCs or CM, we observed reduced collagen levels and decreased fibrosis. These effects probably are associated with the decreased severity of arrhythmias observed because increased deposition of ECM among cardiomyocyte layers may interrupt their electrical coupling, resulting in weakened cardiac contraction and increased risk of arrhythmia [27]. We investigated by RT-qPCR analysis the expression of TGF-β, MMP-9 and TIMP-1 genes, factors that could be associated with fibrosis development. It is known that TGF-β is a central mediator involved in the repair process, and its excessive secretion results in altered deposition of extracellular matrix (ECM) proteins and collagen deposition in the heart [28], by stimulation of fibroblasts to myofibroblasts differentiation, while activating Smad3 signaling pathway to promote ECM proteins synthesis and matrix preservation through increased expression of tissue inhibitors of metalloproteinases (TIMPs) [29]. We found that treatment with MSCs was able to restore the expression of TIMP-1 and MMP-9 to levels similar to control mice.

Similarly, PPARγ gene expression levels were also elevated in HFD mice, but were modulated in mice treated with MSCs. PPARγ, a nuclear receptor and transcription factor, was previously found to be upregulated in the hearts of patients with metabolic syndrome and cardiac hypertrophy, in response to injury [30,31].

In the present study, we analyzed the cardiac expression of genes involved in the process of cardiac hypertrophy and remodeling, including adiponectin, IGF-1 and GATA-4. Adiponectin has been previously demonstrated to be cardioprotective and to inhibit cardiac remodeling through anti-inflammatory and pro-survival mechanisms [32,33]. We found that adiponectin expression in the hearts of mice treated with MSCs or CM was similar to control mice, but was reduced in DMEM-treated HFD mice. In contrast, GATA4, a transcription factor that regulates the expression of cardiac-specific genes, was found to be increased by HFD in our study and by others, being associated with cardiac hypertrophy [6]. Importantly, treatments with MSCs or CM were able to restore GATA-4 messenger RNA (mRNA) levels to a pattern similar to control mice. Futhermore, GATA-4 is involved in the regulation of troponin I [34], which, when highly expressed, has stronger correlation with increased risk and severity of cardiac arrhythmias [35–37]. In the present study, only MSC-treated mice had the same troponin I expression levels as control mice after the treatment, suggesting its participation in cardiac recovery. IGF-1 has also been associated with cardiac repair during remodeling [38] by inducing expression of TGF-β, which directly stimulates collagen expression [39]. Thus, the reduction of IGF1 expression in bone marrow stromal cell (BMSC)-treated mice described in the present study could be one of the mechanisms involved in the improvement of cardiac function.

The role of suppressor of cytokine signaling (SOCS) proteins in the pathogenic mechanisms of diabetes are one of the most investigated factors of the last few years [40]. Although the correlation between SOCS3 expression and insulin resistance has been demonstrated before by detection of high levels of SOCS3 in insulin-sensitive tissues from diabetic patients [41,42] and animals that developed insulin resistance [43,44], in the present study, cardiac expression level of SOCS3 was similar in control mice and HFD MSC-treated mice, suggesting an improvement in cardiac intracellular signaling to insulin.

Alterations in connexin 43 expression and function have been described in experimental models of diabetes [45], hypercholesterolemia [46] and HFD [47]. These include altered pattern of phosphorylation, leading to increased propensity to proteolytic degradation [45]. It has been demonstrated that altered connexin 43 expression favors arrhythmia [48]. The increased connexin 43 mRNA levels induced by HFD in our study may reflect a compensatory response to decreased levels of functional protein, as previously described [46]. Interestingly, connexin-43 mRNA levels were found to be decreased to levels similar to control mice, in the groups treated with MSCs or CM, which correlated with improvements in arrhythmias. Another compensatory mechanisms could be the elevated expression of VEGF detected in HFD mice because it is known that cardiac insult, fibrosis and arrhythmias
lead to VEGF production increase, which in turn regulate the connexin 43 expression [49,50].

The CM from MSCs contains several secreted factors and this suggests a probable mechanism of paracrine action. MSC CM was previously shown to promote cardiac repair in different experimental models [17,51]. The characterization of CM by protein array revealed the presence of cytokines, growth factors, chemokines, angiogenic factors, anti-apoptotic proteins and adhesion molecules, as described previously [52]. It has been shown that MSCs secrete a wide range of cytokines and growth factors with anti-inflammatory, anti-apoptotic, angiogenic and anti-fibrotic effects, which may contribute to the observed therapeutic effects of cell therapy in the heart [53,54]. Besides soluble mediators, MSCs also release extracellular vesicles (EVs) that contribute to tissue repair and regeneration through the transfer of a repertoire of mRNAs, micro RNAs, DNA, proteins and lipids [55–57]. This process may be also involved in the mechanisms of action of MSCs and CM in our model, which will require further studies for proper investigation.

In conclusion, our results suggest that MSCs and CM exert a therapeutic effect on cardiac alterations associated with obesity. The alterations found in our experimental mode, include cardiac arrhythmias, conduction disturbances and fibrosis, which were sustained even after diet change and weight loss, but reversed after cell therapy. The effects of MSC CM support the relevance of MSC’s secreted factors in the mechanisms of action involved.

Acknowledgments
We thank Dr. Carine Azevedo (FIOCRUZ) for technical assistance in morphometrical analysis. This work was supported by grant 465656/2014-5 from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), by Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) and Financiadora de Estudos e Projetos (FINEP).

Disclosure of interests: The authors indicate no potential conflicts of interest.

References


Chintalgattu V, Nair D, Katwa LC. Cardiac myofibrillarbs: a novel source of vascular endothelial growth factor (VEGF) and its receptors Flt-1 and KDR. J Mol Cell Cardiol 2003;3:277–86.


Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2017.07.002.