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Intravenous administration of bone marrow-derived multipotent mesenchymal stromal cells has a neutral effect on obesity-induced diabetic cardiomyopathy

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ABSTRACT

Obesity is a major global health issue. Obese patients develop metabolic syndrome, which is a cluster of clinical features characterized by insulin resistance and dyslipidemia. Its cardiac manifestation, diabetic cardiomyopathy, leads to heart failure.
Bone marrow-derived multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSC) are envisioned as a therapeutic tool not only for cardiovascular diseases but also for other degenerative conditions.
Our aim was to evaluate whether the intravenous administration of MSC modifies cardiac dysfunction in obese mice. To this end, C57BL/6 mice were fed a regular (normal) or high-fat diet (obese). Obese animals received the vehicle (obese), a single dose (obese + 1x MSC) or three doses (obese + 3x MSC) of $0.5x10^6$ syngeneic MSC. Two to three months following MSC administration, cardiac function was assessed by cardiac catheterization, at basal condition and after a pharmacological stress.
Compared to normal mice, obese mice presented hyperglycemia, hyperinsulinemia, hypercholesterolemia and cardiac dysfunction after stress condition. Exogenous MSC neither improved nor impaired this cardiac dysfunction.
Thus, intravenous administration of MSC has neutral effect on obesity-induced diabetic cardiomyopathy.

Key terms: Obesity, metabolic syndrome, diabetic cardiomyopathy, multipotent mesenchymal stromal cells, cardiac function, dobutamine.

INTRODUCTION

Obesity is a major global health issue (Haidar and Cosman, 2011). Changes in lifestyle, predominantly hypercaloric diet ingestion and sedentary habits, produce a dramatic increase in its prevalence. Most obese patients develop metabolic syndrome, a cluster of clinical features characterized by insulin resistance and dyslipidemia (Bonora *et al.*, 2003). This pre-diabetic condition has been recognized as an independent risk factor for cardiovascular diseases, particularly hypertension, atherosclerosis and diabetic cardiomyopathy (Obunai *et al.*, 2007).

Diabetic cardiomyopathy was described in 1972 as a heart failure without signs of hypertension, coronary artery disease or valvular or congenital heart disease (Rubler *et al.*, 1972). During the last decade it has gained relevance because it leads to heart failure (Asghar *et al.*, 2009); its pathophysiology is still not well understood. However, it has been reported that lipid accumulation of cardiomyocytes changes their energy metabolism, increasing oxidative stress, impairing calcium handling and mitochondrial dysfunction, which promote cardiomyocyte death and interstitial fibrosis (Boudina and Abel, 2010). At present, clinical treatments for diabetic cardiomyopathy are aimed at delaying its progression, mainly by improving metabolic alterations using hypoglycemic agents, and cardiac performance using β-blockers and angiotensin-converting enzyme inhibitors (Miki *et al.*, 2013). Therefore, new therapies intended to reverse heart failure in obese individuals would have a significant impact on the health system (Bernardi *et al.*, 2012).

In both pre-clinical and clinical studies promising results were obtained when cell-based therapies were tested for the management of cardiac diseases (Jones *et al.*, 2012). Bone marrow-derived multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSC), appear as an appropriate tool for treating diabetic cardiomyopathy, since they manage oxidative stress, downregulate inflammation, secrete anti-apoptotic and mitogenic factors and might differentiate into cardiomyocytes (Ankrum and Karp, 2010). Furthermore, MSC may be transplanted without histocompatibility restraint directly into the injured heart, are anti-fibrotic and promote neovascularization. Due to the match between the pathophysiology of obesity-induced diabetic cardiomyopathy and the regenerative potential of MSC, we decided to evaluate whether the intravenous administration of MSC modifies cardiac dysfunction of obese mice. To this end, C57BL/6 mice were fed with regular (normal) or high-fat diet (obese). Obese animals received the vehicle (obese), a single dose (obese + 1x MSC) or three doses (obese + 3x MSC) of $0.5x10^6$ syngeneic bone marrow-derived MSC (Figure 1). Two to three months following MSC administration cardiac function was assessed by cardiac catheterization, at basal condition and after pharmacological stress.

MATERIALS AND METHODS

Animals

C57BL/6 male mice were housed at constant temperature (22 ± 2 °C) and humidity (60%), with a 12:12 hour light:dark cycle and unrestricted access to food and water. When required,

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animals were lightly anesthetized with sevofluorane (Abbott Laboratories, Illinois, USA) or 60 mg/Kg ketamine plus 4 mg/Kg xylazine. When sacrificed, animals were deeply anesthetized and received an overdose of ketamine/xylazine (60/4 mg/Kg). Animal protocols were approved by the Ethics Committee of the School of Medicine, Clínica Alemana, Universidad del Desarrollo.

Obesity induction

All mice were fed a regular diet up to one month of age. Then they were kept on a regular diet (normal) or switched to a high-fat diet (obese) until the end of the study (16 months of tested diet). Regular diet consisted of to 10 cal% fat, 20 cal% proteins and 70 cal% carbohydrates (Champion SA, Santiago, Chile). High-fat diet consisted of to 60 cal% fat, 20 cal% proteins and 20 cal% carbohydrates (D12492, Research Diets Inc., NJ, USA) ((Calligaris *et al*., 2013a; Ezquer *et al*., 2011).

Blood glucose, insulin, triglyceride and cholesterol quantification

After four hours of fasting, blood samples were collected from the tail vein of alert mice. Plasma glucose levels were determined with the glucometer system Accu-Chek Performa (Roche Diagnostic, Germany). Plasma insulin levels were assayed using an ultrasensitive mouse insulin ELISA kit (Mercodia, Uppsala, Sweden). Plasma triglyceride and cholesterol levels were determined using TG Color GPO/PAP and Colestat kits (Wiener Lab, Rosario, Argentina), respectively (Ezquer et al, 2011).

Cardiovascular parameter assessment at basal and stress conditions

Mice were deeply anesthetized and placed in supine position on a thermo-regulated plate. Body temperature was monitored using a rectal thermometer and gaseous oxygen was supplied. Hemodynamic parameters were measured by cardiac catheterization (Calligaris *et al*., 2013b; Lorenz and Robbins, 1997). The catheter used was a Mikro-Tip SPR-671 pressure sensor (Millar, Houston, USA) coupled to a PCU-2000 pressure/volt transducer (Millar) and connected to a PowerLab 4/30 data acquisition system (AdInstruments, Bella Vista, Australia). For cardiac function assessment under stress condition, a PE-10 plastic tube (Warner Instruments Co, CT, USA) was introduced into the jugular vein, connected to a KDS-KDS210P pump (Kdscientific Inc., MA, USA) and dobutamine was infused continuously at 12ng/g/min for two min. Dobutamine is a β-adrenergic agonist with a high affinity for β_1-receptors expressed in the heart. When systemically administered, it increases cardiac demand, producing cardiac stress. Data obtained were analyzed with LabChart 7Pro software (AdInstruments, Bella Vista, Australia).

MSC isolation and ex vivo expansion

Six to eight week-old female C57BL/6 mice were sacrificed by cervical dislocation. Bone marrow cells were obtained

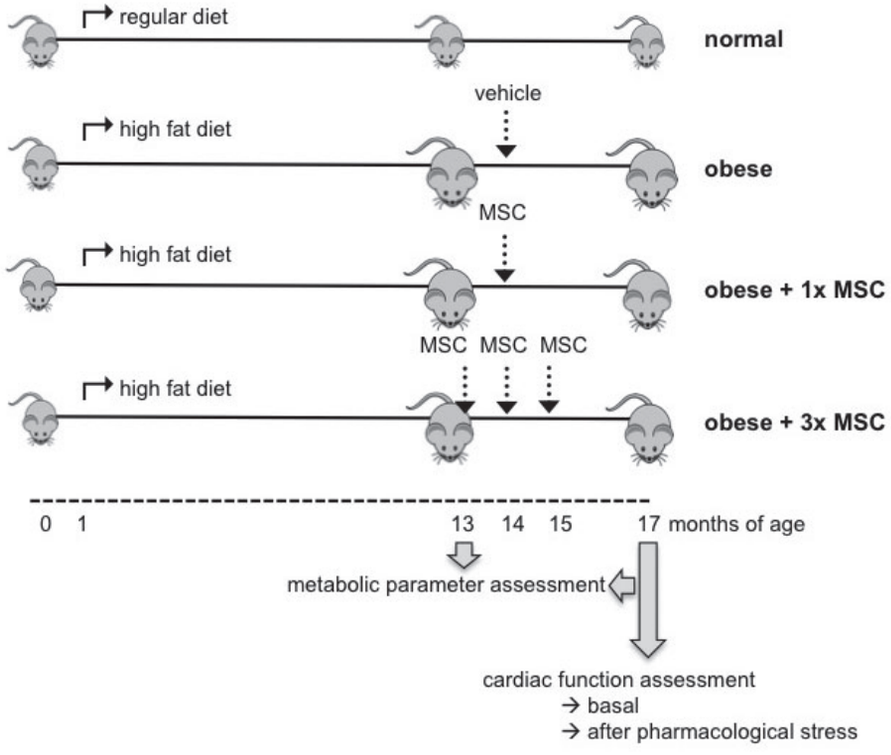

Figure 1. Study design
Male C57BL/6 mice were fed a regular diet up to one month of age. Then they were switched (obese) or not (normal) to a high-fat diet. Obese mice received a single dose of 0.5 x 10^6 syngeneic bone marrow-derived MSC at 13 months of age (obese + 1x MSC), or three doses of 0.5 x 10^6 syngeneic bone marrow-derived MSC at 13, 14 and 15 months of age (obese + 3x MSC).

by flushing femurs and tibias with sterile PBS. After centrifugation, cells were resuspended in alpha-MEM (Gibco, NZ) supplemented with 10% selected fetal bovine serum (Gibco) and 80 ug/mL gentamicin (Sanderson Laboratory, Chile) and plated at a density of $1x10^6$ nucleated cells/cm^2. Non-adherent cells were removed after 72 hours by media change. When foci reached confluence, adherent cells were detached with 0.25% trypsin, 2.65 mM EDTA (Gibco), centrifuged and subcultured at 7,000 cells/cm^2. After two subcultures, adherent cells were characterized and transplanted (Ezquer *et al*., 2008).

Phenotype of administrated MSC

Since there are currently no consensus markers for murine MSC as there are for human MSC (Conget and Minguell, 1999; Dominici *et al*., 2006), immunophenotyping was performed by flow cytometry analysis after immunostaining with monoclonal antibodies against CD45.2 (FITC-conjugated) from BD Pharmingen, USA, CD11b (PE-conjugated), Sca-1 (APC-conjugated) and CD90.2 (PE-conjugated), all from eBioscience, CA, USA.

MSC differentiation potential was assessed after cell exposure to standard adipogenic or osteogenic differentiation media for 14 and 21 days, respectively (Conget and Minguell, 1999). Images were acquired with a Nikon T1-SM microscope.

MSC intravenous administration

A total of $0.5x10^6$ MSC were resuspended in 0.2 mL of 5% mouse plasma and administered via the tail vein to lightly anesthetized mice. Control animals received 0.2 mL of vehicle.

Statistical analysis

Data are presented as mean ± S.E.M. To determine the statistical significance of intergroup differences, Student's t-test was used to compare mean values between normal and obese mice and one-way ANOVA was used to compare mean values among all groups. A value of $p<0.05$ was considered as statistically significant.

RESULTS

Metabolic parameters of obese mice

Compared to normal mice, obese mice presented overweight, hyperglycemia, hyperinsulinemia and hypercholesterolemia, up until the end of the study period (Table 1).

Phenotype of administered MSC

Administrated MSC were $CD45^-$, $CD11b^-$, $Sca\text{-}1^+$ and $CD90.2^+$ (Figure 2A); they differentiated into adipocytes (neutral lipid droplet-containing cells) and osteoblasts (hydroxyl-apatite-secreting cells,) meeting the criteria of mouse MSC definition (Figure 2B) (Sung *et al*., 2008).

Cardiac function of obese mice after MSC administration

Under basal conditions, no significant changes were observed in contractile (dP/dt_{max}) or relaxation (dP/dt_{min}) heart capability between normal and obese mice (Figure 3A).

When cardiac function was evaluated after a pharmacological stress (dobutamine stimulation), obese mice showed a statistically significant reduction in both hemodynamic parameters compared to normal mice (Figure 3B).

Exogenous MSCs did not significantly modify the cardiac performance of obese mice under basal condition or after dobutamine stimulation (Figure 3B). When dP/dt_{min} and dP/dt_{max} values obtained at basal condition were compared to data obtained under stress condition, it was observed that obese animals that received three doses of MSC experienced a slight but not significant increase in $\Delta dP/dtmin_{(dobutamine\text{-}basal)}$, suggestive of a function improvement (normal: 3,231 ± 1,000; obese: 1,609 ± 758; obese + 1x MSC: 1,883 ± 632; obese + 3x MSC: 2,423 ± 886).

DISCUSSION

Diabetic cardiomyopathy is revealed by cardiac remodeling (concentric hypertrophy), fibrosis, progressive diastolic and systolic dysfunction and impaired contractile reserve in stress test performance (Abel *et al*., 2008; Daniels *et al*., 2010). These cardiac alterations have been related to increased oxidative stress, altered calcium homeostasis, progressive mitochondrial dysfunction starting with a reduction of ATP production, to activation of apoptotic signals (release of cytochrome c) and lipotoxicity (apoptosis-induced by ceramide) (Boudina and Abel, 2010).

It has been reported that VEGF, HGF, FGF and matrix metalloproteinases produced by MSC promote myocardium regeneration and improve functionality in animal models of acute myocardial infarction (Samper *et al*., 2012). It has also been suggested that MSC might contribute to the management of diseases where tissue damage is linked to oxidative stress directly (Valle-Prieto and Conget, 2010; Van Linthout *et al*., 2011) or through the secretion of IGF-1, a factor that inhibits oxidative stress production in cardiomyocytes (Kajstura *et al*., 2001). In the present study, MSC administration reversed neither the contractile nor the diastolic dysfunction of obese mice. Moreover, exogenous MSC did not impair either metabolic or cardiac complications of obese mice. To discount that the observed lack of effects is explained by the inability of

TABLE 1

Metabolic parameters of normal and obese mice.
**: *P*<0.01 student T-test obese *vs.* normal mice at the same age. n=8

	Normal		Obese	
Age (months)	13	17	13	17
Body weight (g)	34.6 ± 1.2	34.6 ± 1.4	56 ± 1.9**	58.9 ± 1.8**
Glucose (mg/dl)	115 ± 4	120 ± 5	159 ± 8**	160 ± 6**
Insulin (µg/l)	0.5 ± 0.3	1 ± 0.4	2.8 ± 0.6**	5.6 ± 1.1**
Tryglicerides (mg/dl)	111 ± 5	96 ± 5	120 ± 6	100 ± 6
Cholesterol (mg/dl)	105 ± 6	141 ± 5	230 ± 7**	289 ± 8**

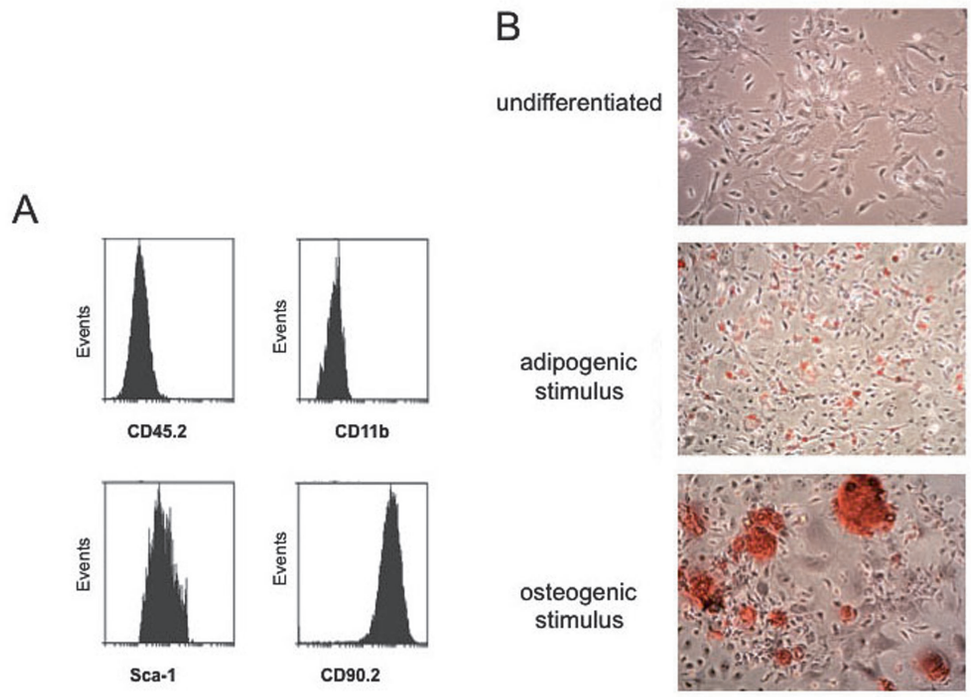

Figure 2. Phenotype of administrated MSC
MSC isolated from 6-8 week-old female C57BL/6 mice were incubated with anti-CD45.2, anti-CD11b, anti-Sca-1 and anti-CD90.2 antibodies, and analyzed by flow cytometry (A). They were also exposed to adipogenic or osteogenic differentiaton media, stained for neutral lipids or hydroxyl-apatite minerals, and observed under light microscope (B). Data are representative of 5 MSC cultures. Bar = 50 µm.

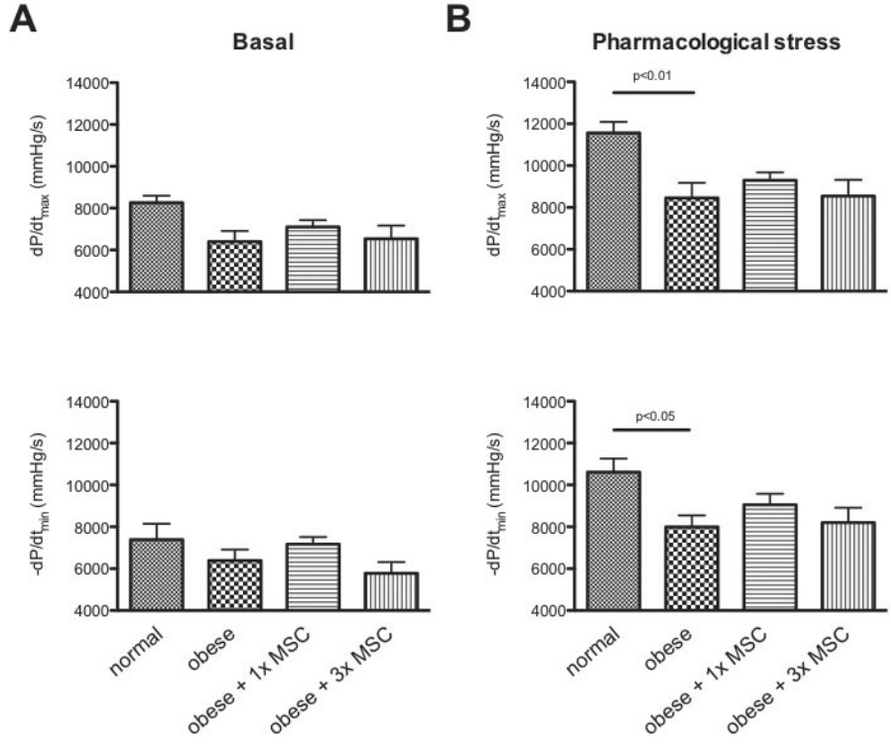

Figure 3. Cardiac function of obese mice after MSC administration
Two to three weeks following MSC administration (17 months of age), hemodynamic parameters were determined at basal condition (A) and after pharmacological stress stimulation (B). p values for one-way ANOVA test. (n=8).
dP/dt_{max}: maximum positive pressure development. dP/dt_{min}: maximum negative pressure development.

MSC to reach the heart of obese mice, we administered a single dose of MSCs that constitutively express GFP to 14 month-old obese mice. Three months later we were able to find rare donor cells in the myocardium but not in the blood of obese mice. Due to the fact that mice remained under the high-fat diet during the entire study period, it appears that MSC are unable to protect cardiomyocytes from metabolic impairment, modifying neither contraction-relaxation performance nor the progression of diabetic cardiomyopathy (Calligaris *et al.*, 2013a).

In order to assess whether the observed lack of effects depended on the dose used, we tripled the amount of MSC administered. Nevertheless, no further effect was observed. We did not test a higher dose because the number of cells already administered (8×10^6) was already in the upper limit of what is currently tested in clinical trials ($1\text{-}10 \times 10^6$) (Elnakish *et al.*, 2012).

An earlier study described a beneficial effect of MSC administration on streptozotocin-induced diabetic rats (Zhang *et al.*, 2008). While exogenous MSC promoted angiogenesis and attenuated cardiac remodeling, no functional results were reported. This brief report is the first study conducted since then to describe the effect of MSC on obesity-induced diabetic cardiomyopathy at the functional level. We show that at the conditions tested (route, time and doses), donor MSC have neutral effect on diabetic cardiomyopathy induced by obesity.

Further studies should be conducted in order to improve the putative effect of MSC on cardiac dysfunction of obese mice, such as MSC pre-conditioning in order to increase their homing and/or resistance to oxidative stress microenvironments (Samper *et al.*, 2012), and other administration routes such as intramyocardial or intracoronary (Elnakish *et al.*, 2012; Mathiasen *et al.*, 2012).

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