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Transplantation of SNAP-treated adipose tissue-derived stem cells improves cardiac function and induces neovascularization after myocardium infarct in rats

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ABSTRACT

Stem cell therapy has been considered a promise for damaged myocardial tissue. We have previously shown that S-nitroso-N-acetyl-D,L-penicillamine (SNAP) increases the expression of several muscular markers and VEGF in mesenchymal stem cells, indicating that transplantation of SNAP-treated cells could provide better functional outcomes. Here, we transplanted SNAP-treated adipose tissue-derived stem cells (ADSCs) in rat infarcted myocardium. After 30 days, we observed a significant improvement of the ejection fraction in rats that received SNAP-treated ADSCs, compared with those that received untreated cells ($p = 0.008$). Immunohistochemical reactions showed an increased expression of troponin T–C and von Willebrand factor, and organized vascular units in the infarcted area of tissue transplanted with treated ADSCs. SNAP exposure induced intracellular S-nitrosation, a decreased GSH/GSSG ratio, but did not increase cGMP levels. Collectively, these results indicate that SNAP alters the redox environment of ADSCs, possibly associated with a pre-differentiation state, which may improve cardiac function after transplantation.

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Introduction

Heart failure is the main cause of hospitalization in the United States. Despite the decline of mortality its prevalence continues to increase, mainly due to effectiveness of clinical treatment and aging of the population (Schocken et al., 2008).

In recent years several studies have demonstrated the potential of stem cells in tissue regeneration with the possibility of cardiac function improvement after transplantation (Al-Radi et al., 2003; Anversa et al., 2003; Orlic et al., 2002; Pittenger et al., 1999; Pittenger and Martin, 2004). The mechanisms by which these cells promote repair of myocardial damage are not well known. However, the idea that stem cells could promote myocardium regeneration by means of the transdifferentiation seems insufficient. For this reason complimentary mechanisms should be considered such as paracrine events and neovascularization (Korbling and Estrov, 2003).

Conceptually, a variety of stem cells can be used for myocardium regeneration (Boyle et al., 2006). Mesenchymal stem cells (MSCs) were first described by Friedenstein et al. (1970). Their main function is maintenance and renewal of adult mesenchymal tissue. These cells can be harvested from diverse sources (Fukuda, 2005; Korbling and Estrov, 2003; Wollert and Drexler, 2005) and although the similarities described, differences in the expression of some genes and in differentiation potential (Dominici et al., 2006; Rebelatto et al., 2008) should be considered to choose the best source for each determined transplant (Dai et al., 2005).

Adipose-derived stem cells (ADSCs) show similar characteristics to bone marrow mesenchymal stem cells (BM-MSCs). Furthermore, they are easily obtained, are present at a high frequency (0.5%), and are able to differentiate into various lineages (Zuk et al., 2002). Planat-Benard et al. (2004) observed that these cells can spontaneously differentiate and express transcription factors of cardiac cells. The ADSCs also express cardiac tissue proteins and have the ability to repair myocardial infarction (Strem et al., 2005), emphasizing the possibility of their use in cellular therapy (Jiang et al., 2002).

The *in vitro* differentiation of ADSCs in cardiomyocytes requires the addition of an inductive agent of differentiation. Nitric oxide (NO) induces the differentiation of embryonic stem cells in cardiomyocytes (Kanno

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et al., 2004). We have recently demonstrated that NO donors, such as DEA/NO (2-(N,N-diethylamino)-diazenolate-2-oxide) and SNAP (S-nitroso-N-acetyl-D,L-penicillamine), increase the expression of muscular and cardiac markers, VEGF and CD34 in BM-MSCs and ADSCs (Rebelatto et al., 2009), which might improve their effects in cellular therapy.

Here, we show that transplantation of SNAP-treated ADSCs in a rat model of myocardial infarction improves the cardiac ejection fraction when compared to transplantation of untreated ADSCs. The expression of troponins and von Willebrand factor, as well as the number of blood vessels in the infarcted area, are also increased in SNAP-treated cells transplantation. *In vitro* experiments indicate that SNAP triggers an early intracellular protein S-nitrosation and an oxidative redox state in ADSCs, which may be involved in observed beneficial effects.

Material and methods

Experimental animals and study design

All procedures were performed after approval by the Human and Animal Ethics Committees of *Pontifícia Universidade Católica of Paraná* (approval numbers 597 and 202, respectively). Myocardial infarction was induced in 60 male Wistar rats (200–230 g). Echocardiography was performed 7 days later for determination of ventricular function. On the eighth day, 33 rats presented ventricular ejection fraction below 40%. Eight rats died after randomization, and the remainder myocardial-infarcted animals underwent cell transplantation ($n=21$) or injection of medium (control, $n=4$). Five additional rats died after transplantation, and the rest were kept alive for 4 weeks, without immunosuppression. Some animals were transplanted with ADSCs previously transduced with *GFP* gene.

Infarct induction

All rats that underwent myocardial infarction were given general anesthesia of intramuscular ketamine (50 mg/kg, Ketamine™, Laboratórios König SA, Brazil) and xylazine (10 mg/kg, Sintec, Rhobiofarma, Brazil). These animals were endotracheally intubated with a flexible catheter (Abocath), and mechanically ventilated by using a small animal ventilator (HARVARD®, Inc., model 683, Massachusetts, USA). A left lateral thoracotomy was performed via the fourth intercostal space to expose the heart. The anterior descending coronary artery was localized, and ligated with a 7.0 non-absorbable Prolene suture (Ethicon, Brazil) just proximal to the bifurcation of the left coronary. The infarction was verified by the observed myocardial blanching distal to the ligation. The thorax was then sutured and the animals were monitored until they fully recovered from the anesthesia.

Echocardiography

Echocardiography was performed on the seventh day post-infarction and on the thirtieth day after cell transplantation. After anesthesia, by intramuscular injection, a two-dimensional transthoracic echocardiography was performed with an echocardiography system equipped with a 12-MHz phased-array transducer HP Sonos 5500 (Hewlett Packard, USA) with the use of a specific transducer for small animals (S 12 5–12 mHz linear 15 L6 7–15 mHz) in the anterolateral portion of the left lateral chest wall. The images were observed in two dimensions and ventricular chambers observed in two sections, longitudinal and transverse. Ejection fraction, end systolic and diastolic volume of the left ventricle, and the end systolic and diastolic area of left ventricle were analyzed by the Simpson's method. The study included animals that presented an ejection fraction of below 40% on the echocardiography on the seventh day after infarction. All measurements were performed on the same equipment and repeated three times by the same observer. The results are presented as the mean of three independent measurements.

Cell culture

ADSCs were isolated using the technique described by Puissant et al. (2005) with modifications. Primary human ADSCs were isolated and characterized as MSC as described elsewhere (Rebelatto et al., 2008) from subjects undergoing elective bariatric surgery and dermolipectomy procedures at the *Instituto de Cirurgia e Medicina do Paraná.* Patient consent was acquired prior to surgery. A cohort of 3 samples was used for cell culture and transplantation.

Transduction of ADSCs by lentivirus and flow cytometry

Four days prior to transplantation, ADSCs were transduced by a third-generation lentivirus containing the green fluorescent protein (*GFP*) gene. The lentiviral particles were kindly provided by the Institute of Molecular Biology of Parana (IBMP, Curitiba-PR, Brazil). Transductions were performed by a single exposure of lentivirus in the presence of 5 μg/ml of polybrene and a multiplicity of infection (MOI) of 5. Twenty-four hours after transduction the medium with the exceeding viruses was removed and the cells were maintained in standard culture media until transplanted.

The percentage of positive cells for the *GFP* gene was assessed by flow cytometry. Cells were trypsinized, washed twice with PBS solution and fixed in 300 μL of 1% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) in PBS. The evaluation was immediately performed in a FACS Calibur equipment (Becton Dickinson, San Jose, CA, USA) and data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

ADSCs treatment with nitric oxide donor SNAP

This work employed two types of experiments with ADSCs. 1) For the transplantation, following the establishment in culture of a homogeneous population of ADSCs (fourth passage), 0.4 mM SNAP (Calbiochem, CA, USA) was added in the first and third days and maintained until the fourth day. After this period, the cells remained for a further 16 days with medium without addition of SNAP. The medium was changed every two days until the twentieth day, when cells were prepared for the transplantation. 2) To analyze the effects of SNAP exposure to the ADSCs, cells were seeded at a density $1\times10^5/cm^2$. After adhesion, they were grown in medium containing 1% fetal calf serum for 24 h and then treated with 0.2 mM SNAP in medium containing 5% fetal calf serum (Gibco, Invitrogen Corporation, NY, USA) during 40 min.

Cell transplantation

Cell transplantation was performed 24 h after the determination of ventricular function by echocardiography. Rats underwent general anesthesia, a median thoracotomy was performed, and 1×10^6 ADSCs were resuspended in 300 μL of DMEM-F12 medium and injected using a tuberculin syringe with a needle into one point of the myocardium (left ventricle), specifically in the center of the scar covering the whole infarcted and peri-infarcted area. The animals were divided into three experimental groups: (A) 4 animals transplanted with medium (control group); (B) 11 rats transplanted with ADSCs treated with SNAP, and (C) 10 rats transplanted with ADSCs.

Animal euthanasia, perfusion-fixation of the heart

After 30 days of the transplant, the animals were euthanized under sedation, as described above. Briefly, the left ventricle was cannulated with a catheter connected to an infusion pump, and approximately 100 mL of a saline solution, followed by 400 mL of 2% PFA were infused. At the end of the infusion, the heart was removed and

sectioned into four pieces from the apex to the base. The segment with the infarcted region was fixed with 4% PFA for 24 h. Subsequently, the sections were immersed in three solutions of sucrose (Biotec, Paraná, Brazil) 10%, 20% and 30% in phosphate buffer (Gibco, Invitrogen Corporation, NY, USA) 0.1 M, pH 7.4, embedded and frozen in a block of cryopreservation medium of optimal cutting temperature (OCT) (Sakura Finetek, Zoeterwoude, The Netherlands) in liquid nitrogen, and stored at −80 °C.

Immunohistochemistry

For immunohistochemistry, cryosections of 5–7 μm were obtained. Tissue sections were washed three times with a solution of 10 mM TBS (Invitrogen, CA) containing 0.1% triton for 5 min. The specimens were blocked with 10 mM TBS containing 0.1% triton (Nuclear, São Paulo) and 5% goat serum (Gibco, Invitrogen Corporation, NY, USA) for 1 h. Subsequently, sections were incubated for 2 h with primary antibodies mouse anti-GFP conjugated with FITC (1/50), goat anti-human troponin I (1/50) and goat anti-troponin T–C (1/50), all from Santa Cruz Biotechnology (CA, USA), rabbit anti-human desmin (1/10) (Sigma-Aldrich, Missouri, USA) and rabbit anti-human von Willebrand factor (1/200) (Sigma-Aldrich, Missouri, USA). The sections were washed with TBS +0.1% triton, blocked with 10 mM TBS containing 0.1% triton and 5% goat serum for 40 min and incubated with secondary antibodies rabbit anti-goat IgG Texas Red (Santa Cruz Biotechnology, CA, USA) (1/100), goat anti-rabbit IgG (Molecular Probes, Oregon, USA) (1/400) for 1 h and 30 min at room temperature. Nuclei were stained with 4′,6-diamino-2 phenylindole (DAPI) and the slides were mounted using fluorescence mounting medium (Dako, CA, USA). The anti-GFP was used in the sections of animals that were transplanted with cells transduced with the *GFP* gene. The controls of the immunohistochemical reaction were made using only the secondary antibody. Slides were analyzed in the E-600 Nikon microscope and images captured using the camera-*CoolSNAP PRO cf* (Media Cybernetics) and Image Pro-Plus software (Diagnostic Instruments).

Indirect immunofluorescence

After the short SNAP exposure, cells were fixed with 4% PFA and then incubated with anti-nitrosocysteine (1/400) (Sigma-Aldrich, Missouri, USA), overnight at 4 °C, in a humidified chamber. The secondary antibody used was anti-rabbit IgG-Alexa 488 (1/600) (Molecular Probes, Oregon, USA), in PBS containing 0.01% saponin (Sigma-Aldrich, Missouri, USA), during 40 min at 4 °C. Actin was labeled with phalloidin conjugated with Texas red (Sigma-Aldrich, Missouri, USA). In some experiments, 2 mM $HgCl_2$ was added to the cells for 30 min, before the fixation step, to analyze the specificity of the anti-nitrosocysteine to the nitrosocysteine epitope. Images were captured by a confocal microscopy (Nikkon, Eclipse 800 equipped with a Biorad camera, Radianna 2100).

GSH and GSSG determination

Cells (treated in T75 flasks) were pelleted and stocked at −80 °C until analysis. For the GSH and GSSG determination, the light-protected pellets were thawed in ice, and suspended in 80 μL of a buffer consisting of 50 mM phosphate pH 2.7, 50 μM sodium octanosulfonate (Sigma-Aldrich, Missouri, USA). Cells were lysed by 3 freeze-thaw cycles in liquid nitrogen/water bath. Lysates were centrifuged (10,000 rpm at 4 °C for 5 min) and the supernatants were 0.22 μm-filtered. Thirty microliters of the supernatants were injected in an HPLC Shimadzu system consisting of a quaternary pump (LC20AD) controlled by the LC Solution software, a manual injector (Rheodyne 7125), and a coulometric detector (ESA Chelmsford), operating at 470 and 910 mV, for GSH and GSSG detection, respectively.

Intracellular levels of cGMP

Cells were treated as described in 24 wells plates (TPP, Trasadingen, Switzerland). Cyclic GMP levels were assessed by a commercial kit (GE Healthcare), according to the manufacturer.

Statistical analysis

This work has a primary outcome based on analysis of immunohistochemistry for *in vivo* evaluation of expression of neovascularization and muscle markers and secondary in the evaluation of ventricular function with the data obtained from an ejection fraction of the left ventricle. Statistical analysis was performed by a non-parametric Kruskal Wallis test for the assessment of ejection fraction of the three study groups and by non-parametric Wilcoxon test to compare pre and post ejection fraction within each group. The data were analyzed with the software Statistica 8.0. The GSH/GSSG ratios were analyzed by one way ANOVA, using GraphPad Prism software.

Results

The myocardial-infarcted animals were divided into three groups: (A) control; animals that were transplanted with medium ($n=4$) (B) animals that received ADSCs treated with SNAP ($n=9$) and (C) animals that received untreated ADSCs ($n=7$). Some ADSCs samples were transduced with the *GFP* gene. A total of eight rats from groups B and C were transplanted with *GFP*-transduced cells. The efficiency of transduction with GFP gene was 28 and 31% for SNAP-treated and untreated ADSCs, respectively (data not shown).

There was a significant difference between groups with respect to absolute difference in the pre and post transplant ejection fraction. A significant difference between B and C ($p=0.008$) was found, while groups A and C ($p=0.249$) presented similar behavior (Fig. 1). This data suggests that SNAP exposure to the ADSCs had a role in the final functional outcome. The ventricular remodeling occurred in all groups measured by the increment of ventricular size. On average, this variation was 18.8% in group A, 16.4% in group B and 17.1% in group C ($p=0.907$). Although group B presented the lowest increment of ventricular size, no significant difference was observed between groups. In addition, there was no difference between groups regarding

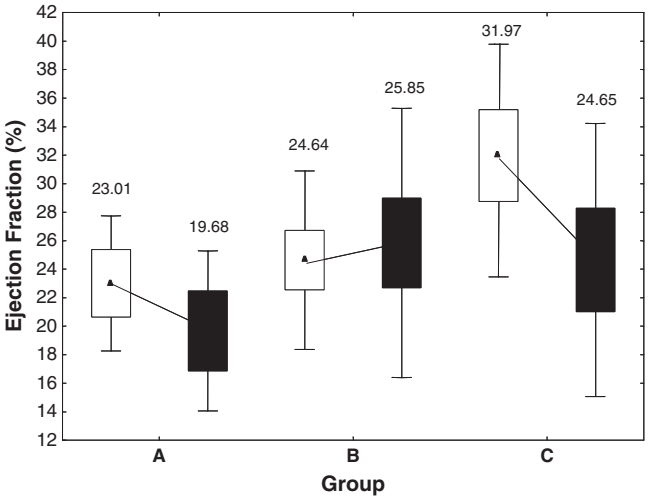

Fig. 1. Mean pre- and post-transplant ejection fraction. Myocardial infarcted rats were divided in groups A (control), B (animals transplanted with ADSCs treated with SNAP) and group C (animals transplanted with untreated ADSCs). Their mean cardiac ejection fractions (annotated above the bars) were determined by echocardiography one day before (white bars) and thirty days after (black bars) the cell transplant. Ejection fractions are presented as mean ± SE in the box area and mean ± SD in Whisker plot.

the absolute difference in the end diastolic volume of the left ventricle ($p = 0.999$; data not shown).

The ejection fractions of animals in groups B and C that were transplanted with *GFP*-transduced cells were compared to determine if *GFP* transduction could have negatively influenced the ejection fraction by providing an early cell death of transplanted ADSCs. Although some *GFP* expression could be detected in the infarcted region of all transplanted rats (Fig. 2), no difference in ejection fraction was found between these two transplanted groups ($p = 0.518$), indicating that transplanted cells were integrated to the injured tissue and that cardiac function was independent on *GFP* expression.

To investigate if SNAP-treated cells were more prone to differentiate into cardiac cells in the infarcted area, we analyzed the expression of desmin, troponin T–C and troponin I by immunohistochemistry. Positive cells to desmin were observed in both groups, B and C in regions with and without myocardial infarct, i.e., with and without transplanted cells (data not shown). These results indicate that desmin is a nonspecific marker and could not be considered appropriate for analysis of expression of cardiac proteins in transplanted cells. The presence of troponin I was observed in the infarcted area of both B and C groups (Fig. 3a, b). A specific marker to cardiac cells, troponin T–C, was predominantly present in the infarcted region of the animals in group B (Fig. 3c, d). The anti-von Willebrand, specific for endothelial cells, was observed in cells around blood vessels in infarcted and non-infarcted (data not shown) regions in both groups B and C, but more prominently in SNAP-treated cells (Fig. 3e, f). It was not observed unspecific staining by secondary antibodies. A semi-quantitative analysis was carried out to evaluate the number of vessels in the infarcted region of animals from groups B and C. Sixty-eight random fields were counted in both groups, and a greater number of vessels ($n = 81$) was observed in group B when compared with group C ($n = 63$) which might reflect the more extensive von Willebrand factor staining in group B.

So far, the results demonstrate that transplantation of SNAP-treated ADSCs, compared with transplantation of untreated ADSCs, induces an improvement in cardiac ejection fraction, an increased cardiac expression of troponin T–C and von Willebrand factor, and an increased neovascularization. Next, we aimed to analyze the early events triggered by SNAP in ADSCs, which could be associated with the above effects. Since NO activates guanylate cyclase (GC), leading to the production of cGMP, we measured its intracellular level 40 min after exposure to SNAP. No significant difference between control (untreated) and SNAP-treated ADSCs was found (676 *versus* 710 fmol/well, respectively, $n = 2$). We employed 1 μM DEA/NO, a compound that spontaneously releases NO, as a positive control. With this NO donor, cGMP levels increased to 976 fmol/well. This result demonstrates that SNAP does not induce cGMP production in ADSCs under our experimental conditions. Thus, an alternative pathway to NO signaling is a cGMP-independent mechanism, relying on protein S-nitrosation. Indeed, the images obtained with anti-nitrosocysteine antibody show an intense immunostaining, with a granular pattern dispersed in the cytoplasm, as determined by the extension of actin labeling with phalloidin (Fig. 4). Control cells, i.e., not exposed to SNAP, showed no anti-nitrosocysteine immunostaining (Fig. 4). To investigate whether the observed reaction was due to nitrosocysteine residues recognition, we incubated the fixed treated cells with $HgCl_2$, to disrupt the nitrosocysteine bond. Indeed, no staining was observed after this incubation (data not shown), confirming the antibody specificity to nitrosocysteine epitopes.

Since NO-derived species are able to alter the redox environment of the cell, we assessed whether SNAP exposure could alter the cellular redox state, determined by the GSH/GSSG ratio. Our results indicate that SNAP decreases the GSH/GSSG ratio in ADSCs by *ca.* 50% compared to the untreated cells (Fig. 5).

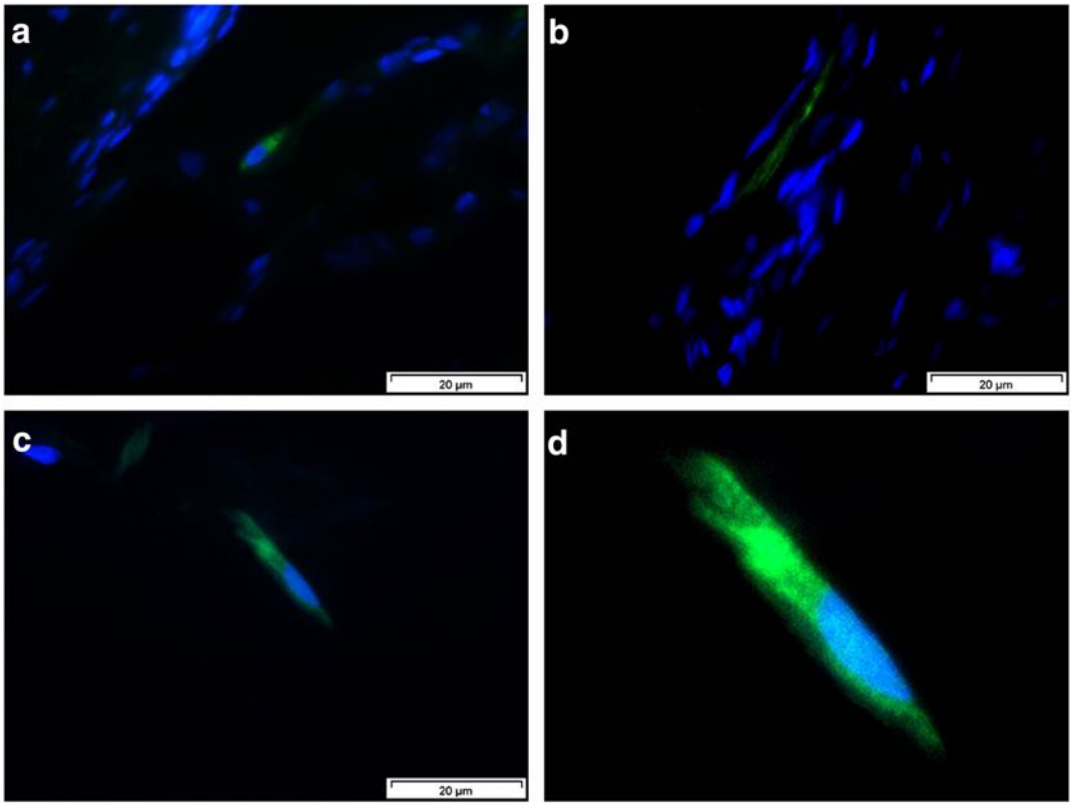

Fig. 2. GFP expression in ADSCs-transplanted infarcted myocardium. Indirect immunofluorescence indicates the presence of *GFP*-transduced ADSCs (green) in infarcted area of untreated (a) and SNAP-treated ADSCs (b, c). In subpanel (d) the image is magnified from subpanel (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

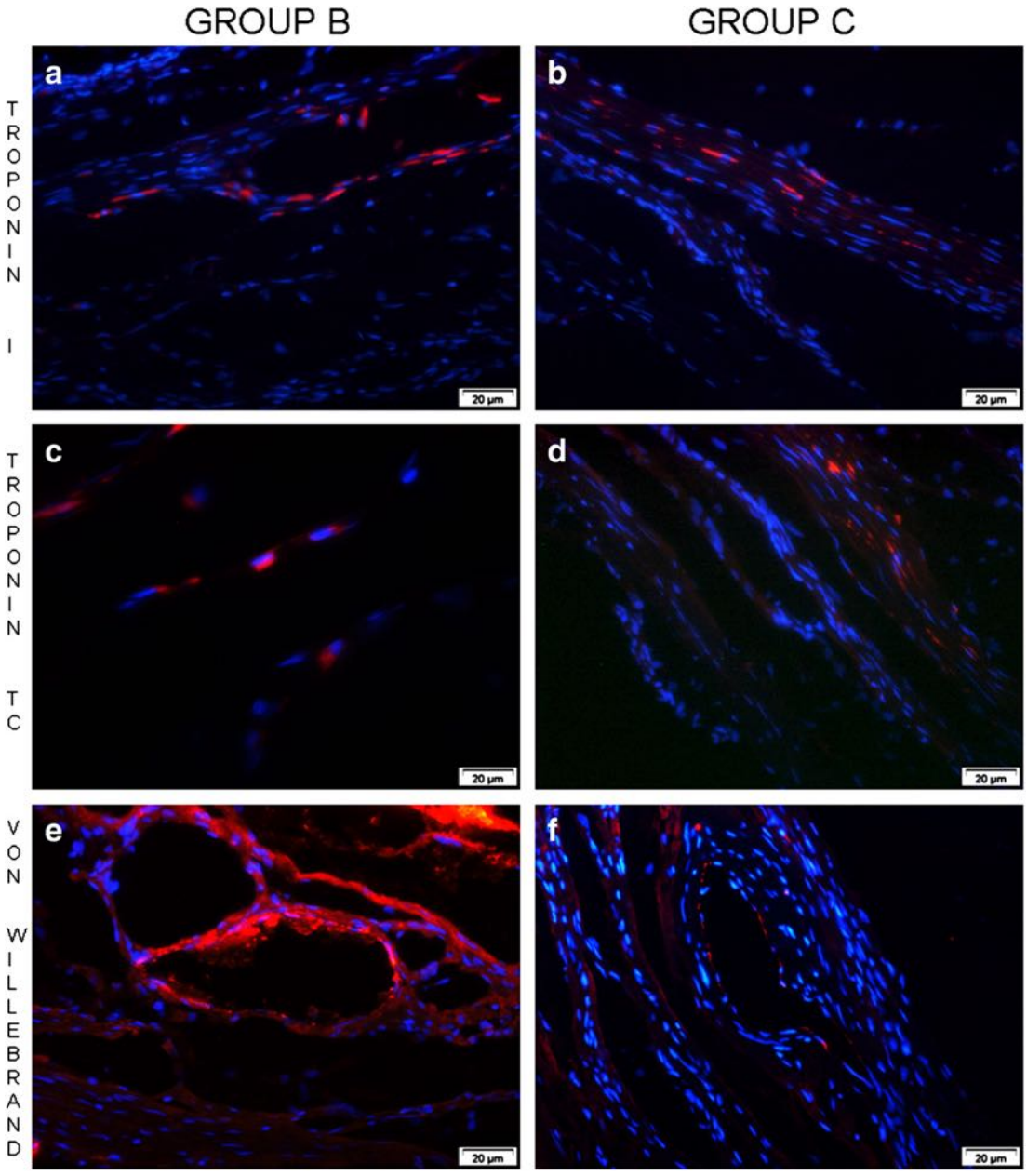

Fig. 3. Expression of troponin I, troponin T–C and von Willebrand factor in infarcted cardiac tissue after ADSCs transplantation. SNAP-treated (a, c, e) and untreated (b, d, f) ADSCs were transplanted in the infarcted myocardium and the expression of troponin I (a, b), troponin T–C (c, d) and von Willebrand factor (e, f) was analyzed by indirect immunofluorescence.

Discussion

Cellular therapy has emerged as an alternative to cardiac repair and formation of new blood vessels (Pittenger and Martin, 2004). Based on studies showing that NO utilization could facilitate cardiomyogenesis (Kanno et al., 2004) and that treatment of ADSCs with NO agents increases the expression of cardiac specific genes (Rebelatto et al., 2009), our focus was to study the effects of SNAP-treated ADSCs transplantation on cardiac repair after myocardial infarct in Wistar rats.

In this study, a significant increase in the ejection fraction was observed in the group that received ADSCs treated with SNAP compared with the group that received untreated ADSCs. We did not observe a significant difference in the ejection fraction variation between groups A (control) and C (transplanted with untreated ADSCs), in contrast to other studies which show that mesenchymal stem cell transplantation can improve cardiac function in different experimental models (Caplan, 2009; Dai et al., 2005; Nagaya et al., 2005; Strem et al., 2005; Valina et al., 2007). Differences between these publications and our results can be explained by methodological differences such as isolation and cultivation techniques (Giordano et al., 2007), methods of infarct induction, moment of transplant after infarction, route of infusion and number of transplanted cells.

Despite promising results in the recovery of ventricular function with mesenchymal stem cell transplantation, some studies have not shown positive results. Van der Bogt et al. (2009) performed cellular transplant with bone marrow mesenchymal stem cells and ADSCs in rats with myocardial infarction and did not observe benefits on ejection fraction, in line with our results. The low number of transplanted cells, 5×10^5, might have interfered in the result. We transplanted 1×10^6 cells by intramyocardial infusion. Possibly, a higher number of transplanted stem cells could result in a significant functional improvement. Indeed, Nagaya et al. (2005) observed that satisfactory increments of ejection fraction were obtained only after increasing the number of stem cells to 5×10^6.

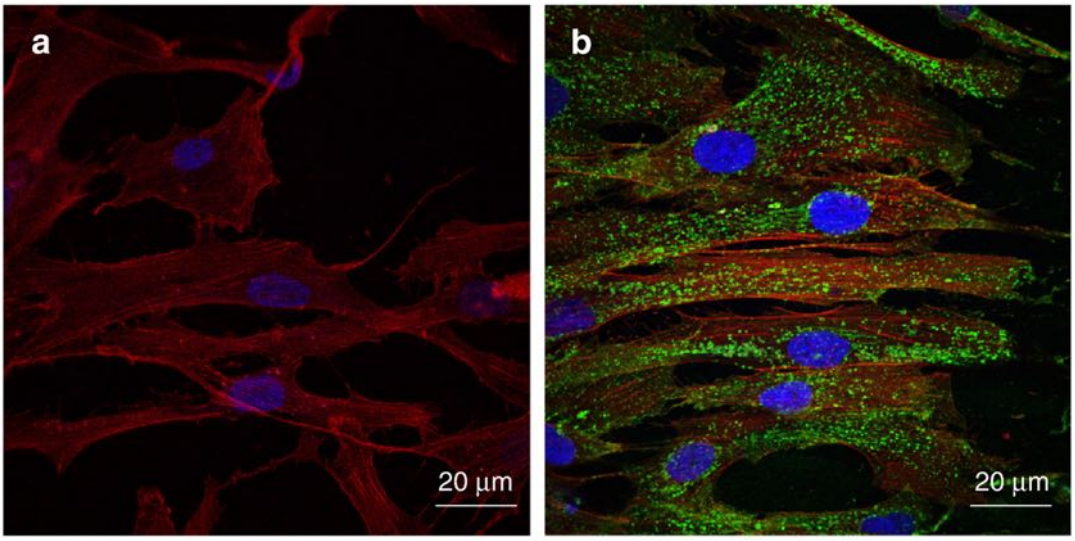

Fig. 4. Protein S-nitrosation by SNAP-treatment in ADSCs. Cells were untreated (a) or treated with 0.2 mM SNAP (b) during 40 min. After fixation and permeabilization, actin was labeled with phalloidin conjugated with Texas Red. The reaction was performed with anti-nitrosocysteine antibody and rabbit anti-IgG conjugated with Alexa 488. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We aimed to investigate the transplant of pre-committed ADSCs for the cardiac phenotype. Other studies showed that committed cells could lead to increased cytoprotection effects of cardiomyocytes present in the heart and to increased therapeutic efficiency of mesenchymal stem cells in myocardial regeneration (Chamberlain et al., 2007). Mazo et al. (2008) had demonstrated that undifferentiated ADSCs were more efficient than predifferentiated ADSCs to cardiac regeneration. These differences can be related to different methods of predifferentiation used in each study. We chose the nitrosothiol SNAP, since this agent had been previously shown to increase cardiomyogenesis in both embryonic and adult stem cells (Kanno et al., 2004; Rebelatto et al., 2009). We hypothesized that cardiomyogenesis might have been facilitated by the ADSCs pre-commitment. Here, we demonstrated a significant increase in the ejection fraction in the group that received ADSCs treated with SNAP compared with the group that received untreated ADSCs. This increase in contractile function indicates that SNAP-treated cells contribute to the improvement of the damaged area, since we have observed an increased expression of some cardiac muscle markers in the infarcted area. Troponin I was expressed in untreated and SNAP-treated ADSCs. Due to the small number of positive cells and the antibody specifity for the human protein, we suggest that cells expressing troponin I were the transplanted human ADSCs. However, it was not possible to visualize the co localization of *GFP* and troponin I-positive cells. The anti-troponin T–C utilized is specific for cardiac tissue and its expression was observed in ADSCs treated with SNAP and only in the infarcted area. Thus, we suggest that SNAP had acted on the pre-differentiation of ADSCs to muscular cells. The expression of cardiac muscular proteins by ADSCs has been described after differentiation induction by several methods. For example, Zuk et al. (2002) had demonstrated that ADSCs *in vitro* could express myogenic transcription factor 1 (myod1), desmin, myogenin, myogen regulator factor and myosin heavy chain (MHC) after addition of hydrocortisone. Also, we have demonstrated the expression of cardiac α-actin, troponin T, troponin I and connexin-43 on ADSCs and bone marrow mesenchymal stem cells after SNAP addition (Rebelatto et al., 2009) and the improvement of cardiac function of infarcted rats has been associated with expression of α-MHC, β-MHC, α-actin and myosin light chain (MLC2v) after 30 days of transplant (Nagaya et al., 2005).

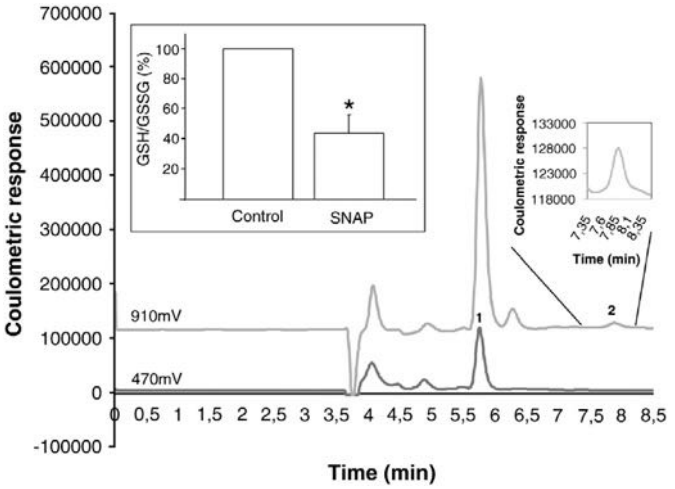

Fig. 5. Decreased intracellular GSH/GSSG ratio by SNAP treatment in ADSCs. Representative chromatograms of the simultaneous detection of GSH (peak 1, 470 mV trace) and GSSG (peak 2, 910 mV trace) in ADSCs cytosolic fraction are shown. Inset shows the GSH/GSSG ratio in control and SNAP-treated ADSCs. The values are representative of 5 independent experiments. *$P<0.05$.

In addition, our results show that SNAP-treated cells were either more prone to differentiate to endothelial cells or that they induced other tissue cells to differentiate into endothelial cells, by a paracrine signaling for instance. Our data showed more cells expressing von Willebrand factor and organized in vascular units in infarction area of animals that received treated ADSCs than in the group that received untreated cells. Recent studies suggest that mesenchymal stem cells can be differentiating toward endothelial cells (Fraser et al., 2006; Kinnaird et al., 2004; Tang et al., 2006). However, we were unable to co-localize *GFP* and von Willebrand factor in our experiments. This is not surprising considering that the efficiency of transduction was approximately 30%, and about 3% of these cells remain at the lesion site (Freyman et al., 2006). On the other hand, SNAP has been associated with VEGF increment (Maulik, 2006) and this marker is involved in function improvement, vessels number increment and remodeling limitation as demonstrated by Sadat et al. (2007). These authors had demonstrated that ADSCs secrete angiogenic factors that are responsible for vascular formation and cellular protection. Also, Tang et al. (2005) had demonstrated that ventricular function increment and vessels formation after mesenchymal cell transplant were associated with elevation of VEGF, fibroblast growth factor (FGF) and stem cell derived factor 1-α (SDF-1α) expression. We had

previously reported an increased genic expression of VEGF by ADSCs after SNAP exposure (Rebelatto et al., 2009). Indeed the vasculogenesis has been more often observed than the differentiation in muscle cells (Kamihata et al., 2002).

Here we showed that SNAP induces ADSCs S-nitrosation that may contribute to the functional effects observed. We did not identified the proteins modified by SNAP in ADSCs, but in endothelial cells, S-nitrosation by exogenous NO donors, like DEA/NO, takes place mainly in mitochondrial proteins, presenting a staining pattern similar to that observed by us (Yang and Loscalzo, 2005). Recently, SNAP has been shown to stabilize HIF1α, leading to VEGF expression, by a mechanism dependent on HIF1 S-nitrosation (Park et al., 2008).

SNAP is a nitrosothiol which has been described to release NO, leading to cardiovascular benefits, such as vasodilation (Moncada and Higgs, 1993; Nathan, 1992) and also protection against oxidative stress (Monastyrskaya et al., 2002). However, its chemical properties, regarding NO release, are quite complex, and are extremely dependent on the chemical environment (Janssens et al., 1999) such as the presence of thiols (Hu and Chou, 2006). SNAP itself cannot cross the cell membrane (Zhang and Hogg, 2005). Thus, it can be suggested that SNAP-derived NO enters the cell, possibly by transnitrosation mediated by membrane proteins (Zai et al., 1999). NO or NO-derived species then nitrosates intracellular thiols, such as glutathione. Nitrosoglutathione may then further react with other biomolecules, and becomes oxidized, decreasing the GSH/GSSG ratio. These transnitrosation reactions may account for the lack of GC activation observed in our experiments. Moreover, nitrosoproteins may transfer the NO moiety to other thiols, acting as NO carriers/storage. It has been demonstrated in endothelial cells that the half-life of nitrosoproteins is about 1 h (Yang and Loscalzo, 2005).

Finally, the SNAP-induced oxidative redox state in ADSCs is in agreement with the findings described by Jones (for a review, see Jones, 2006). It has been demonstrated that a slightly oxidative reduction potential, measured by the intracellular concentrations of glutathione and glutathione disulfide accompanies the differentiation phenotype, probably by altering the redox state of target protein modules involved in transcription. On the other hand, more reduced potentials are associated with cell proliferation. Thus, the decreased GSH/GSSG ratio found after SNAP treatment in ADSC may be an important factor to induce differentiation.

In conclusion, this study indicates that transplanted SNAP-treated ADSCs induce a functional improvement and neovascularization in rats that underwent severe myocardial infarction, possibly by events involving ADSCs protein S-nitrosation and decreased GSH/GSSG ratio. Specific proteins involved in this process remain to be identified.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

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