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Expression of cardiac function genes in adult stem cells is increased by treatment with nitric oxide agents

Carmen K. Rebelatto [a], Alessandra M. Aguiar [b,c], Alexandra C. Senegaglia [a], Carlos M. Aita [d], Paula Hansen [a], Fabiane Barchiki [a], Crisciele Kuligovski [b,c], Márcia Olandoski [a], José A. Moutinho [a], Bruno Dallagiovanna [b,c], Samuel Goldenberg [b,c], Paulo S. Brofman [a], Lia S. Nakao [d], Alejandro Correa [b,c,*]

[a] Núcleo de Cardiomioplastia Celular, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil
[b] Instituto Carlos Chagas, FIOCRUZ, Rua Algacyr Munhoz Mader 3775, Curitiba 81350-010, Brazil
[c] Instituto de Biologia Molecular do Paraná, Rua Algacyr Munhoz Mader 3775, Curitiba 81350-010, Brazil
[d] Núcleo de Investigação Molecular Avançada, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, Curitiba 80215-901, Brazil

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ABSTRACT

Mesenchymal stem cells (MSCs) have received special attention for cardiomyoplasty because several studies have shown that they differentiate into cardiomyocytes both *in vitro* and *in vivo*. Nitric oxide (NO) is a free radical signaling molecule that regulates several differentiation processes including cardiomyogenesis. Here, we report an investigation of the effects of two NO agents (SNAP and DEA/NO), able to activate both cGMP-dependent and -independent pathways, on the cardiomyogenic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived stem cells (ADSCs). The cells were isolated, cultured and treated with NO agents. Cardiac- and muscle-specific gene expression was analyzed by indirect immunofluorescence, flow cytometry, RT-PCR and real-time PCR. We found that untreated (control) ADSCs and BM-MSCs expressed some muscle markers and NO-derived intermediates induce an increased expression of some cardiac function genes in BM-MSCs and ADSCs. Moreover, NO agents considerably increased the pro-angiogenic potential mostly of BM-MSCs as determined by VEGF mRNA levels.

Stem cell therapy shows great promise for regeneration of damaged myocardial tissue [1]. The functional outcomes in injured heart is improved by this treatment [2], possibly due to muscle tissue regeneration and induction of neovascularization [3].

Of the various types of adult stem cells, mesenchymal stem cells (MSCs) have received most attention for cardiomyoplasty. Indeed, several studies report that MSCs differentiate into cardiomyocyte *in vivo* [4–6]. However, MSCs are heterogeneous populations so their differentiation capacity raises concerns about uncontrolled differentiation in patients. *In vitro* MSC differentiation to cardiomyocyte-like phenotypes has been described following 5-azacytidine treatment [6,7], coculture with cardiomyocytes [8,9], growth in a cardiomyogenic medium containing insulin, dexamethasone and ascorbic acid [10], and in a conditioned medium of cardiomyocytes after hypoxia/reoxygenation [11].

Nitric oxide (NO) is a free radical signaling molecule [for a review, see 12]. NO and/or its metabolites induce several differentiation processes [13–15], including cardiomyogenesis [16–18]. Treatment of embryonic stem (ES) cells with *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) or 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO), or the transduction with the iNOS gene increases both the number of spontaneously contracting cell clusters and the expression of cardiac myosin light chain (MLC) protein, an effect abolished by NOS inhibitors [16].

In view of (i) MSCs being multipotent cells able to differentiate into cardiomyocytes, (ii) NO being important in heart development and (iii) embryonic stem cells differentiating into cardiomyocytes after exposure to NO agents or endogenously produced NO, we investigated the effects of NO agents on cardiomyogenic differentiation of MSCs.

In particular, we investigated the effects of two NO agents (the nitrosothiol SNAP and the NO donor DEA/NO) on the cardiomyogenic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived stem cells (ADSCs). We report that exposure to these compounds induced an increased expression of some muscle genes and vascular endothelial growth factor (VEGF). The low expression of muscle markers and the considerable induction of VEGF suggest that, rather than a direct differentiation to the cardiac phenotype, MSCs treated with NO

* Corresponding author. Address: Instituto Carlos Chagas, FIOCRUZ, Rua Algacyr Munhoz Mader 3775, Curitiba 81350-010, Brazil. Fax: +55 41 3316 3267.
E-mail addresses: alejandro@tecpar.br, alejandrocorreadominguez@gmail.com (A. Correa).

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agents may improve cardiac function acting pro-angiogenically, possibly through paracrine signaling.

Materials and methods

Isolation, culture and characterization of adherent cells from BM and AT. Human bone marrow (BM) was obtained from the iliac crest of ten patients, aged between 40 and 74 years (60.4 ± 9.9) and suffering from dilated myocardiopathy. Subcutaneous abdominal adipose tissue (AT) was obtained from ten patients, aged between 19 and 60 years (38.0 ± 12.6), undergoing elective bariatric surgery and dermolipectomy procedures.

Adherent cells were obtained from BM and AT, cultured and characterized as described by Rebelatto and colleagues [19]. All samples were collected after informed consent following guidelines on research involving human subjects, as approved by the Ethics Committee of Pontifícia Universidade Católica of Paraná (approval number 597).

Cell treatment with NO agents. To explore whether NO promoted the differentiation of BM-MSCs and ADSCs into cardiomyocytes or cardiomyocyte-like cells, BM-MSCs and ADSCs biological replicates were exposed to NO by exogenous supplementation of *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (Calbiochem, CA, USA) and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO) (Calbiochem, CA, USA). Confluent cultures of BM-MSCs and ADSCs maintained in 5% fetal calf serum (FCS) were exposed to NO by exogenous supplementation with 0.4 mM SNAP and 0.1 μM DEA/NO. NO agents were added to cell cultures on day 0, replaced on day 2 and maintained until day 4. Thereafter, cultures were continued in medium without NO agents. Medium was replenished every 2 days until day 20 [41, modified]. After 20 days, BM-MSCs and ADSCs were analyzed for cardiac and muscular-specific gene expression by indirect immunofluorescence, flow cytometry, RT-PCR and real-time PCR. Non-induced cell cultures were used as negative controls. Treatments were performed in biological triplicate.

Flow cytometry. BM-MSCs and ADSCs were basically treated as previously described [19, modified]. Briefly, cells were stained with the following anti-human monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD44; phycoerythrin (PE)-conjugated anti-CD34; allophycocyanin (APC)-conjugated anti-CD29; and peridinin chlorophyll protein (PerCP)-conjugated anti-CD45. All antibodies were from BD Biosciences (San Jose, CA, USA). For intracellular staining, cells were first permeabilized with the Fix&Perm Cell permeabilization kit (Caltag Laboratories, CA, USA) and incubated separately with the following primary anti-human antibodies: anti-cardiac troponin T (1/100), anti-ventricular myosin heavy chain (VMHC) (1/10) (both from Chemicon International, CA, USA), anti-myosin (skeletal, slow) (1/2000) (Sigma–Aldrich, MO, USA), anti-cardiac (fetal) actin (1/10) (Fitzgerald, MA, USA) and anti-connexin-43 (1/20) (BD Biosciences Pharmingen, CA, USA). The cells were washed then stained with a secondary goat anti-mouse IgG (BD Biosciences Pharmingen, CA, USA). Controls for the flow cytometry setup procedure included cells incubated with FITC-, PE-, PerCP- and APC-conjugated isotype antibodies (all from BD Biosciences Pharmingen). Cytometric evaluation was performed in FACSCalibur equipment (Becton Dickinson, San Jose, CA, USA). Data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA). Results are expressed as the ratio between immunopositive treated and immunopositive untreated cells. Values are the mean of two independent experiments.

Indirect immunofluorescence. For indirect immunofluorescence analysis, BM-MSCs and ADSCs were treated as previously described [20]. In brief, treated and untreated cells were washed with PBS, fixed with 2% paraformaldehyde for 2 h and permeabilized with 0.2% Triton X-100. After blocking with 1% BSA, the cells were incubated for 3 h at room temperature with primary mouse anti-human cardiac troponin T (1/100) (Chemicon International, CA, USA), VMHC (1/10) (Chemicon), cardiac (fetal) actin (1/10) (Fitzgerald, MA, USA), α-actinin (sarcomeric) (1/250) (Sigma–Aldrich, MI, USA), myosin (skeletal, slow) (1/2000) (Sigma–Aldrich), connexin-43 (1/20) (BD Biosciences Pharmingen, CA, USA); goat anti-human troponin I (1/50) (Santa Cruz Biotechnology, CA, USA) and rabbit anti-human desmin (1/10) (Sigma–Aldrich). The cells were incubated with secondary antibodies: rabbit anti-goat IgG Texas Red (Santa Cruz Biotechnology, CA, USA) at dilution 1:100, goat anti-rabbit IgG at dilution 1:100 and goat anti-mouse IgG Texas Red at dilution 1:1500 both from Molecular Probes (Oregon, USA). Nuclei were counterstained by 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). Slides were examined on a Nikon E-600 microscope. Reaction controls involved incubating the cells with the secondary antibody only, and all do not showed nonspecific staining. Digital images were captured using CoolSNAP-PRO*cf* (Media Cybernetics) camera controlled by an Image Pro-Plus software system from Diagnostic Instruments. Indirect immunofluorescence was done at least in three independent experiments and technical duplicate.

Total RNA extraction and reverse transcription-polymerase chain reaction. Total RNA extraction, RT-PCR and qPCR (real-time quantitative PCR) were all performed as described by Rebelatto and colleagues [19]. Primer sets used for RT-PCR and qPCR analyses are shown in Table 1. Three independent experiments were performed

Table 1
Primer sets used for RT-PCR and qPCR analyses. Abbreviations: MLC-A, myosin light chain atrium; MLC-V, myosin light chain ventricle; VEGF, vascular endothelial growth factor.

Target	Reference	NCBI seq	Amplicon (bp)	TM (°C)	Primer sequence
GAPDH	[39]	2597	150	55	F: 5′-GGCGATGCTGGCGCTGAGTAC-3′ R: 5′-TGGTTCACACCCATGACGA-3′
Alpha cardiac actin	[40]	NM_005159	226	55	F: 5′-GCAAGGACCTGTATGCCAACAATG-3′ R: 5′-GCCTCATCGTACTCTTGCTTGCTA-3′
Conexina-43	[41]	M65188	154	60	F: 5′-CCTTCTTGCTGATCCAGTGGTAC-3′ R: 5′-ACCAAGGACACCACCAGCAT-3′
MLC-A	[42]	BC027915	239	62	F: 5′-GCTCTTTGGGGAGAAGCTCA-3′ R: 5′-CGTCTCCATGGGTGATGATG-3′
MLC-V	[42]	BC031006	200	62	F: 5′-GGCGCGTGAACGTGAAAAAT-3′ R: 5′-CAGCATTTCCCGAACGTAAT-3′
Cardiac troponin	[42]	BC002653	152	62	F: 5′-GGCAGCGGAAGAGGATGCTGAA-3′ R: 5′-GAGGCACCAAGTTGGGCATGAACGA-3′
VEGF	[42]	M27281	101	60	F: 5′-CTACCTCCACCATGCCAAGTG-3′ R: 5′-TGCGCTGATAGACATCCATGA-3′

for each stem cell source. GAPDH was used as an internal housekeeping control for expression.

Results

Human BM-MSCs and ADSCs were previously isolated and characterized by our group [19] and shown that these cells are similar in morphology, immunophenotype and differentiation capacity. Here, we investigated by flow cytometry, cellular immunofluorescence, RT-PCR and real-time PCR analysis if exposure of these cells to the NO agents SNAP and DEA/NO induces the expression of cardiac function genes.

In order to define an efficient, but non-lethal concentration of the NO agents, cells were treated with 4, 0.4 and 0.04 mM SNAP and 1, 0.1 and 0.01 μM DEA/NO. Cells were exposed to the NO agents for 4 days and left in culture in the absence of the compounds for up to 20 days. The time points analyzed were 2 (i.e., during the NO agents treatment), 7 and 20 days. Expression of various cardiac markers was assayed by RT-PCR (Table 1) in two biological samples of BM-MSCs and ADSCs. The highest concentration of SNAP was cytotoxic. Overall, cells cultured for 20 days, treated with 0.4 mM of SNAP and 0.1 μM of DEA/NO expressed α-cardiac actin, cardiac troponin T and connexin-43 in both BM-MSCs and ADSC more consistently than the other tested conditions (data not shown). Thus, these concentrations and time of treatment were chosen for further analyses.

Indirect immunofluorescence analysis showed that both NO agents induced the expression of some cardiac and muscle markers by day 20; some markers were observed in a few isolated cells and others in most of the cells (Fig. 1). Interestingly, connexin-43, cardiac (fetal) actin and α-actinin (sarcomeric) were detected even in non-treated cells, suggesting that MSCs spontaneously express these markers after 20 days in culture. The findings for BM-MSCs and ADSCs exposed to SNAP and DEA/NO were similar.

The percentage of immunopositive cells for muscle markers was determined in duplicate by flow cytometry. The number of cells expressing muscle markers, putatively differentiated cells or in the process of differentiation, never exceeds 20% of the total number of treated cells. Because some markers were spontaneously expressed, results are reported as the ratio of immunopositive treated to immunopositive non-treated cells on day 20 (Table 2). The percentage of CD34-positive cells increased in BM-MSCs treated with both SNAP and DEA/NO and in ADSCs treated with SNAP. CD44 increased slightly in ADSCs treated with both NO agents. Overall, expression of muscle markers showed a tendency to increase, and mainly in BM-MSCs, following exposure to NO agents (Table 2).

The expression of some muscle marker genes and of VEGF was also assessed by RT-PCR and qPCR (Table 1). RT-PCR results were highly dispersed. MLC-V expression was detected in only one ADSC sample treated with SNAP whereas expression of the genes for connexin-43, VEGF and troponin T was detected in most of the samples analyzed (Fig. 2). When qPCR was performed, it was

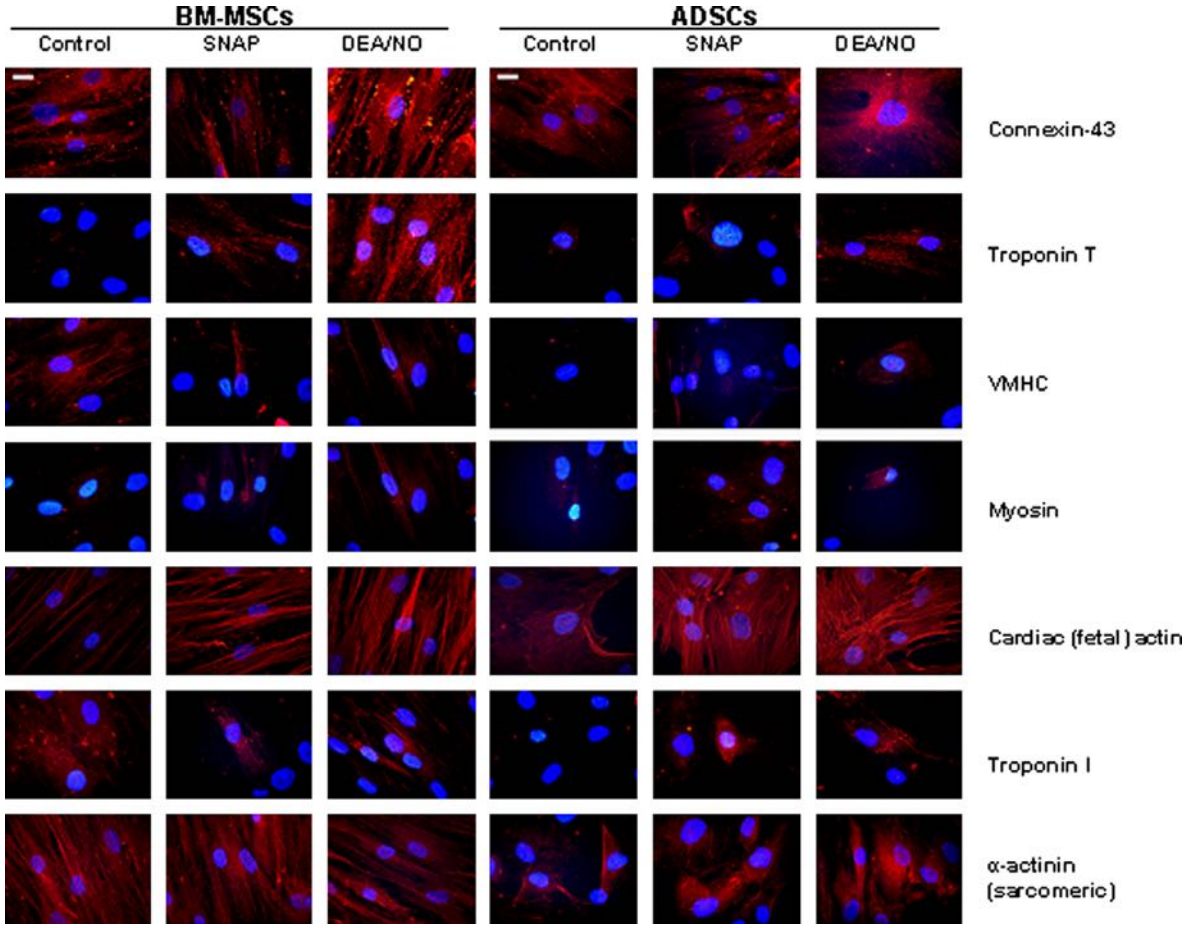

Fig. 1. Indirect immunofluorescence showing the expression of muscular and cardiac markers. BM-MSCs (A) and ADSCs (B) were treated with SNAP and DEA/NO. On day 20, indirect immunofluorescence analyses were performed for the markers indicated on the right (Texas Red). DAPI stain (blue) show the nuclei. Representative results of three independent experiments are shown. VMHC, ventricular myosin heavy chain. Scale bar = 20 μM.

Table 2
Quantification by flow cytometry of immunopositive cells. Results are expressed as the ratio between immunopositive treated and immunopositive untreated cells for the indicated markers. Values are mean of two independent experiments. Abbreviations: BM-MSC, bone marrow mesenchymal stem cell; ADSC, adipose derived stem cell; VMHC, ventricular myosin heavy chain.

	CD44	CD34	CD45	CD29	Connexin-43	Troponin T	VMHC	Myosin	Cardiac actin
Mean ratio ADSC									
SNAP/control	1.9	3.5	0.8	1.2	0.7	1	0.8	0.6	1.4
DEA/NO/control	2.1	0.8	0.8	1.2	1.2	1.2	0.8	0.9	1.4
Mean ratio BM-MSC									
SNAP/control	1	6.2	1.6	1	0.8	2	1.8	1.4	1.6
DEA/NO/control	1	4.3	1.3	1	1.2	1.6	1.3	1.6	2

possible to detect an increased of connexin-43 expression in BM-MSCs treated with NO agents. More interestingly, the mRNA levels of the potent angiogenic factor VEGF were considerably increased in all BM-MSCs samples following treatment with either SNAP or DEA/NO. Only one ADSC sample gave results in agreement with these data (Fig. 3).

Discussion

Our study shows that the expression of some muscle markers in MSC is increased by treatment with NO agents. However, unlike ES cells [16], MSCs exposed to the NO agents did not fully differentiate into cardiomyocyte-like cells, confirming that the potential of MSCs to differentiate is more restricted than that of ES cells. The results of flow cytometry and immunofluorescence were consistent concerning the relative increases in expression, i.e., the differences between NO-treated and untreated cells after 20 days in culture.

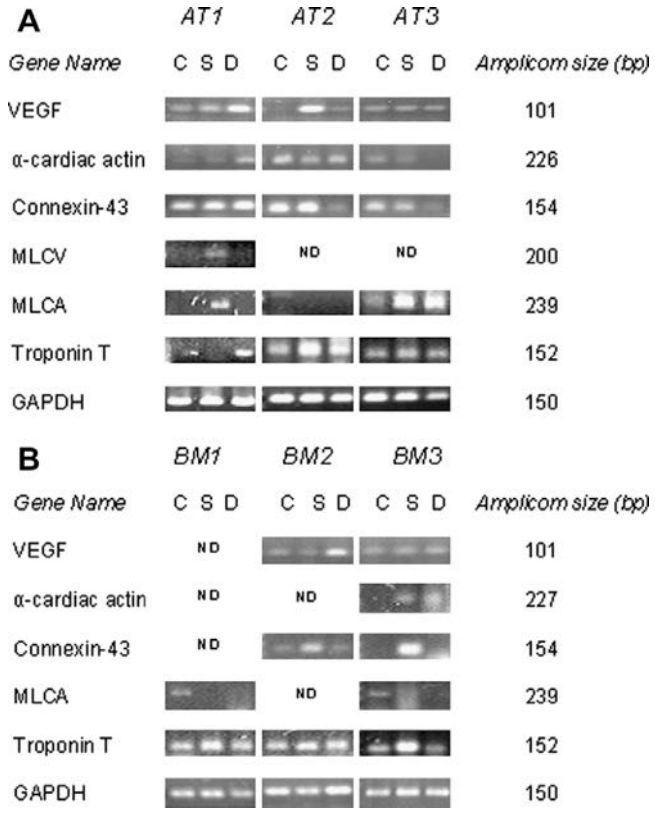

Fig. 2. Analysis of VEGF and cardiac markers mRNA levels by RT-PCR. Three independent experiments of AT (A) and BM (B) are shown. Non-induced cells were used as negative controls. GAPDH was used as an internal control. Abbreviations: BM, bone marrow; AT, adipose tissue; C, control; S, SNAP; D, DEA/NO; ND, not detected.

One interesting finding was that untreated (control) ADSCs and BM-MSCs expressed some muscle markers at mRNA and protein levels. Spontaneous expression of muscle markers by MSCs has also been observed at the molecular level by other groups [7,21–23]. Tondreau and colleagues [24] suggested that expression of certain proteins by MSCs depends on time in culture, rather than on specific induction factors. These studies, combined with the findings reported by Verfaille's group, support the idea that MSCs are, at the molecular level, "pluridifferentiated" cells [4]. Although the corresponding mRNAs are present in untreated ADSCs and BM-MSCs, they might be functionally inactive or the proteins they encode might have non-canonical functions [25].

BM-MSCs seem to be more promising than ADSCs for cardiomyoplasty, as assessed by qPCR analysis. This may be a consequence of the pre-commitment of these cells for the cardiomyogenic lineage, because all the BM donors were dilated cardiomyopathy patients. Local or systemic production of inflammatory mediators might influence not only MSC migration to injured tissues [26,27], but also MSC proliferation, differentiation [28] and engraftment [29].

There is recent evidence that mesenchymal progenitor cells might have beneficial effects on post-infarcted myocardium scarring through paracrine factor-stimulated angiogenesis, rather than by cardiomyogenic differentiation itself [20,30,31]. The increased expression of connexin-43 observed in cardiomyocytes was attributed to secretion of paracrine factors by transplanted mesenchymal progenitor cells from BM [32]. Through this indirect mechanism, MSC transplantation could lead to the recovery of cardiac performance and to induction of neovascularization [20], although some degree of differentiation into cardiomyocytes may also take place. Our qPCR results are consistent with this possibility. We show that untreated BM-MSCs and ADSCs produce the mRNAs for connexin-43 and VEGF and, more importantly, their expressions were greatly increased after exposure to NO agents, most substantially with DEA/NO. VEGF is a critical angiogenic factor which may both contribute to endothelial lineage cell survival through VEGF-mediated phosphorylation of protein kinase B and endothelial nitric oxide synthase [33–35] and also accelerate development of microvessels and enhance regional blood flow in ischemic tissue [36].

We determined the percentages of immunopositive cells by flow cytometry and found an increase of CD34 expression in BM-MSCs exposed to both NO agents and in ADSCs exposed to SNAP. CD34 has been considered to be a marker of activated stem cells [37], and $CD34^+$ cell levels are significantly correlated to VEGF levels in healthy controls [38].

In conclusion, this work indicates that NO and/or its derivatives increase the expression of various muscle markers and VEGF in BM-MSCs and ADSCs, and is consistent with the putative pro-angiogenic role of MSCs. Thus, NO agents treated-MSCs transplantation may have beneficial effects in cardiac patients.

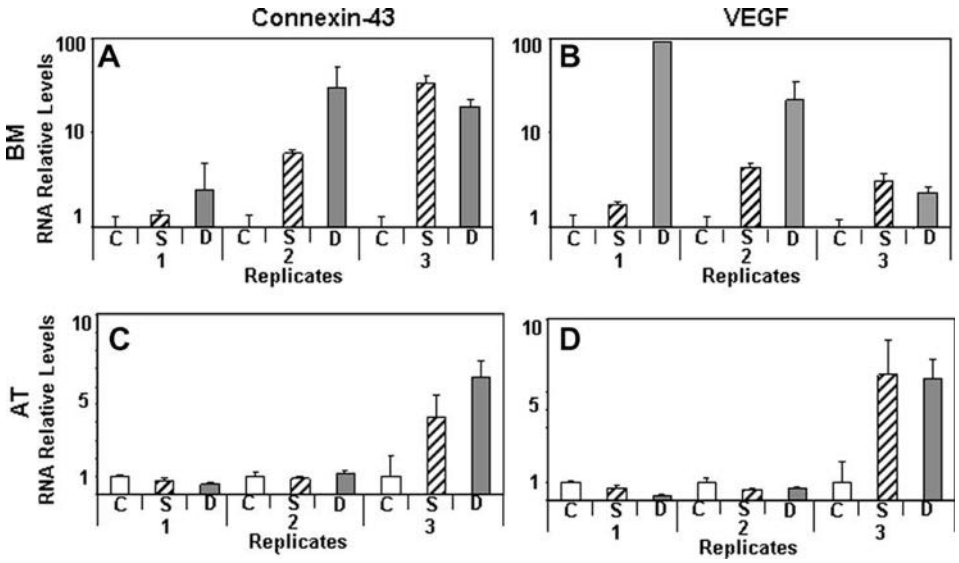

Fig. 3. qPCR for connexin-43 and VEGF mRNAs. Expression of connexin-43 (A, C) and VEGF (B, D) in BM (A, B) and AT (C, D). Three independent experiments are shown. qPCR values are expressed as means ± SD of technical triplicates. GAPDH was used as an internal housekeeping control for expression. Abbreviations: C, control; S, SNAP; D, DEA/NO.

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