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Physical training prior to myocardial infarction potentializes stem cell therapy, SDF-1/CXCR4 axis activation and inhibits the vasoconstrictor response in hypertensive rats

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ABSTRACT

Stem cell therapy is a promising strategy for recovering of injured cardiac tissue after acute myocardial infarction. The effects promoted by preventive physical training, beneficial for regeneration, are not yet understood on stem cell homing. In the present study, we evaluated the effect of preventive physical training on cell homing activation and associated mechanisms after acute myocardial infarction and therapy with adipose-derived stem cells in spontaneously hypertensive rats (SHR). Forty female SHR were allocated in sedentary (S), sedentary SHAM (S-SHAM), sedentary AMI (S-AMI), sedentary with cell therapy (S-ICT), aerobically trained (T), trained SHAM (T-SHAM), trained AMI (T-AMI) and trained with cell therapy (S-ICT) groups. Cell therapy was performed through the infusion of 2×10^5 ADSC/0.05 mL at the moment of AMI. Molecular markers of cell homing (SDF-1/CXCR4), inflammatory response (myeloperoxidase and cardiac expression of iNOS, gp91phox and NFkB), vasoconstrictor agents (Ang II and ET-1) and an angiogenesis inducer (VEGF) were measured. Functional capacity and echocardiographic parameters were also evaluated. Preventive physical training associated with cell therapy was able to reduce left ventricle ejection fraction losses in infarcted animals. Results demonstrated activation of the SDF-1/CXCR4 axis by physical training, besides a reduction in vasoconstrictor and systemic inflammatory responses. Physical training prior to AMI was able to induce a cardioprotective effect and optimize the reparative mechanism of cell therapy in an animal model of hypertension.

1. Introduction

Cardiovascular and chronic pulmonary diseases, as well as cancer and diabetes, are currently among the main causes of morbidity and mortality worldwide. The risk factors for cardiovascular diseases (CVD) include obesity, smoking, sedentary lifestyle, and systemic hypertension [1]. Of these, hypertension is considered to be the most important risk factor for CVD. Approximately 50% of deaths caused by stroke and 40% of deaths caused by cardiac ischemic disease result from hypertension, and myocardial infarction is among the main outcomes of CVD [2].

Cardiac damage caused by acute myocardial infarction (AMI) and other ischemic processes leads to inflammatory responses characterized by the expression of molecules, such as nuclear factor kappa B (NF-κB), gp91phox (catalytic subunit of the NADPH oxidase), and inducible nitric oxide synthase (iNOS); the release of the enzyme myeloperoxidase (MPO); and increased production of reactive oxygen species (ROS) at the injury site [3,4]. This typically progressive scenario is associated with molecular physiological responses, pathological ventricular remodeling, and the development of heart failure. These processes result in intense mobilization of immune cells and progenitor stem cells, which migrate to the site of tissue injury, where they proliferate and

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differentiate, a process known as cell homing. Stromal cell-derived factor 1 (SDF-1) plays a key role in this process, by stimulating the transmigration and adhesion of different progenitor cell lineages [4] by interacting with a specific receptor, CXC chemokine receptor type 4 (CXCR4) [5]. Stem cells infused for cardiac repair express CXCR4 in the presence of SDF-1 and are attracted to the injury site [6]. The most responsive stem cells that participate in this process are mesenchymal stem cells, which can be found in the bone marrow and several other tissues, such as the adipose and cardiac tissue. However, advanced atherosclerosis and heart failure create an inhospitable cellular environment associated with ischemia and infarction. Such environment induces intense cell mobilization and signaling molecule release, but does not favor tissue repair. Other cardiovascular risk factors, such as hypertension, obesity, smoking, and a sedentary lifestyle, can exacerbate this scenario.

Considering the effects exerted by the risk factors and signaling pathways associated with cardiac repair, strategies promoting enhanced antioxidant and angiogenic responses that favor cell homing may be able to improve disease prognosis and improve the success rates of cellular therapy, as previously reviewed by our group [7]. Also, in a study by our group, we demonstrated that aerobic training performed before AMI, may play a protective role in cardiac tissue, mainly mediated by an increase in antioxidant responses and in the redox state [8]. Aerobic physical training may promote endothelial progenitor cell (EPC) mobilization and vascular endothelial growth factor (VEGF) expression [9]. It also attenuates ROS production and oxidative damage, and increases antioxidant responses in myocardial cells after AMI [8].

Many therapeutic strategies, and non-pharmacological and surgical interventions have been evaluated in an effort to mitigate the adverse effects of AMI. Stem cell therapy (CT) is a promising cardiac repair therapy; it is associated with improvements in the contractile function, reductions in the infarction area [10], and secretion of cytokines and growth factors [11]. Therefore, the aim of the current study was to evaluate the effects of preventive physical training and sedentarism on the homing of mesenchymal stem cells derived from the adipose tissue (ADSC), and administered after AMI in female spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Animals

The study followed the Ethical Principles for Animal Experimentation [12]. Animal handling was performed according to the regulations of the Sociedade Brasileira para Ciência de Animais de Laboratório SBCAL/COBEA, and followed the Guidelines and Norms Regulating Research Involving Animals. The study was approved by the Comitê de Ética para Uso de Animais (Porto Alegre, Rio Grande do Sul) under protocol UP4582/10.

Forty 12-week-old female SHR (blood pressure = 188 ± 6 mm Hg) were used in the study. SHR were maintained under conventional animal facility conditions, with controlled temperature, and light and dark cycle (12 h). Rats were placed in animal boxes (four animals per box) receiving ration (Nuvilab CR1) and water *ad libitum*. Six days after acclimation, the animals were allocated into groups (5 per group) that were trained or maintained under sedentary conditions for 10 weeks, as follows: sedentary (S); sedentary, sham-operated (S-SHAM); sedentary, with induced AMI (S-AMI); sedentary, with CT (S-ICT); aerobically trained (T); trained, sham-operated (T-SHAM); trained, with induced AMI (T-AMI); and trained, with CT (T-ICT). The blood pressure was determined by plethysmography before the beginning of the training protocol. Animal body weight was determined before and after the experimental period.

2.2. Maximal exercise test (ET)

Functional capacity was measured by ET, as described elsewhere [13]. All SHR were first individually adapted to the treadmill (Inbramed), at a speed of 0.3 km/h, for 15 min for three consecutive days. To obtain the ET data, the physical training was prescribed for the period of 10 weeks, with a reassessment in week five to adjust the training velocity (as described below).

2.3. Physical training

The physical training consisted of a 10-week moderate-intensity aerobic training program, five times a week. During the first 5 weeks of training, the adopted intensity was 60% of the maximum speed (km/h) determined by ET, with a progressive increase in duration up to 60 min per session. In the following 5 weeks, the intensity was incremented until the limit of 70% of the ET reading, in sessions of 60 min each, as previously described by our group [14]. Then, 48 h after the end of training, ET was performed again for the final assessment of the aerobic capacity of SHR. The sedentary group animals remained confined to the boxes. They did not undergo training but underwent the same assessment at the end of the experimental period as the trained animals.

2.4. Surgical intervention and CT

Surgical procedures for AMI and sham surgery were performed as described by Selye et al. [15], later adapted by our group [16], and with anesthesia, analgesia, and CT. Briefly, 22-week-old SHR underwent AMI (with or without CT) or sham surgery. The rats were anesthetized by an intraperitoneal injection of 1 mL/kg of body weight of ketamine (100 mg/mL, Dopalen) and 0.5 mL/kg of body weight of xylazine (2 g/100 mL, Anasedan). After intubation, the animals were placed on mechanical ventilation with ambient air, at a volume of 2.5 mL and ventilatory rate of 65 breaths per min, using a pressure fan for rodents (Harvard Apparatus, model 683). The anesthesia was maintained by using a vaporizer (Surgivet) with isoflurane (1 mL/mL, Isoforine, Cristália). For the induction of AMI, the left anterior descending coronary artery was permanently occluded with a single suture performed with a nylon wire 6.0 (Prolene, Johnson & Johnson). The CT was performed by infusion of ADSC subsequently to AMI. The animals from CT groups were infused with 2×10^5 cells in a volume of 100 μL, split into five injections in the anterior wall of the left ventricle, in the region close to the ischemic area. After the procedure, the animals received tramadol (20 mg/kg, every 6 h for 48 h, Teuto), and were maintained under medical oxygen and at 25–27 °C for 6 h.

2.5. Adipose tissue Collection, and ADSC acquisition and characterization

To acquire ADSC, a male SHR was anesthetized by intraperitoneal injection of ketamine (480 mg/kg) and xylazine (30 mg/kg). The epididymal fat was collected and washed in phosphate-buffered saline (PBS). ADSC isolation, expansion, and characterization were performed as described by Meirelles et al. [17].

To determine the identity and potential applicability of ADSC, the morphology, proliferative capacity, and adherence of the cultured cells were evaluated [18]. Cell immunophenotype was determined by using anti-mouse monoclonal antibodies CD90-PE (specific to ADSC) and CD45 FITC (negative for the strain), and the respective control isotype (BD Biosciences) [18] by flow cytometry (FACSCanto II flow cytometer; Becton Dickinson), using FACSDiva v6.1.3 software (Supplementary Data 1).

2.6. Monitoring the survival of transplanted cells

After CT, to monitor the retention and survival of ADSC infused into the cardiac tissue of female rats, the *Sry* gene located on the Y

chromosome was amplified by quantitative real-time polymerase chain reaction (qPCR). Briefly, 96 h after ADSC infusion, heart tissue fragments from the S-ICT and T-ICT groups were collected, and genomic DNA was isolated using Genomic DNA MiniKit (Invitrogen). TaqMan® assay (Applied Biosystems) was used to identify the target gene in the implanted cells. The concentration and purity of DNA were determined based on the optical density (260 nm) analysis using a spectrophotometer (SpectraMax M2e, Molecular Devices). For qPCR analysis, amplification was performed according to the manufacturer's recommendations, using a Stratagene Mx3005p thermocycler (Agilent Technologies). The Mann-Whitney *U* test was used to compare positive amplifications with the fragments used for DNA isolation ($\alpha = 0.05\%$). The reactions were positive in 80% of samples in the S-ICT group, and in 40% of samples in the T-ICT group; overall 60% of samples tested positive. There was no correlation between the amount of tissue used for analysis and the positivity of reaction (Supplementary Data 2).

2.7. Heart function analysis

Echocardiographic analyses were performed 48 h after the surgical procedures or 96 h after ET in the groups that did not undergo any surgery. The left ventricle ejection (LVEF) and shortening (LVSF) fractions, fractional area change (FAC), and the myocardial infarction size (MIS) after AMI were analyzed. The analysis was performed using EnVisor (Philips) echocardiograph with a 12-MHz transducer. The measurements and calculations of the heart function parameters followed those proposed by Peron et al. [19], under anesthesia with a mixture of 1–2% of isoflurane (100%, 1 mL/mL, Isoforine, Cristália) in 100% oxygen. The infarction area was delimited taking into account the movement of LV walls, by the observation of longitudinal, apical and transversal views of the LV. Regions with systolic thickness under normal, as well as portions with paradoxal movement, were considered as infarcted. The infarcted area (%) was thus determined by the ratio of these regions by total area of LV walls [20].

2.8. Euthanasia and collection of biological material

Blood samples for systemic analyses were collected 48 h after the surgical procedures and at the time of euthanasia in groups S-SHAM, S-AMI, S-ICT, T-SHAM, T-AMI, and T-ICT. In S- and T-groups, plasma was collected only at the time of sacrifice (these animals represent the "baseline" conditions). Approximately 0.5 mL of blood was collected from the lateral tail vein. For the final collection of blood and tissue for molecular analyses, SHR were anaesthetized using ketamine (100 mg/mL, Dopalen) and xylazine (2 g/100 mL, Anasedan). Next, the hearts were removed (euthanasia), weighed, and stored in liquid nitrogen. The aspiration tubes and syringes were treated with 1.8 mg/mL of EDTA (Invitrogen). Plasma and tissue samples were identified and stored at −80 °C.

2.9. Molecular analysis of systemic factors and tissue proteins

Plasma levels of SDF-1, VEGF, endothelin-1 (ET-1), angiotensin type II (Ang II), and MPO were determined in all groups using capture ELISA assays (Ebiosciences and Cusabio Biotech). The assays were performed according to the manufacturers' instructions. Sample absorbance at xx was measured using a spectrophotometer (Spectramax M2e, Molecular Devices) at 25 °C, with reduced background. Quantification was performed by 4-parameter linear regression (Excel, Microsoft). Data are expressed as pg protein per mL plasma.

Total protein extracts were prepared from cardiac tissue samples macerated in a mechanical homogenizer (Polytron, Marconi) in 5 mL of sucrose buffer on ice. The homogenized samples were centrifuged at 1700*g* for 10 min at 4 °C. The supernatants (approximately 3 mL) were collected, and the pellets were suspended in 1 mL of buffer, resuspended, centrifuged as described above, and the supernatant was collected. Protein concentration in the extracts was determined spectrophotometrically using Coomassie blue reagent (BioRad). The extract was stored at −20 °C until use.

For western blotting analysis, samples containing 150 µg of total protein extract were solubilized in NuPage buffer (Invitrogen) and separated on denaturing polyacrylamide gel (Invitrogen) by electrophoresis. These proteins were transferred onto a Hybond ECL nitrocellulose membrane (GE Healthcare) using a semi-dry system (Amersham Biosciences). After the transfer, the membranes were stained using Ponceau red, photographed, and washed in PBS to remove the dye. To block non-specific protein binding, the membranes were incubated for 1 h in 5% casein solution (skim powdered milk) in 1 PBS, pH 7.4 (blocking solution). The membrane was then incubated with anti-CXCR4, anti-gp91phox, anti-iNOS, or anti-NF-κB antibodies (100 mg antibody/20 mL blocking solution; Santa Cruz Biotech) for 16 h at 4 °C. The secondary antibody (anti-IgG, Millipore) was used at a dilution of 1: 5000 in xx. Membranes were incubated for 3 min in a solution containing enzyme substrate (ECL kit, GE Healthcare) and then placed on X-ray film (GE Healthcare) in the dark. The films were scanned and, together with images of Ponceau red staining, protein bands were quantified by densitometry using the publically available software Scion Image. All blots were normalized by densitometric analysis of the stained lane with Ponceau red and expressed by (arbitrary units, UA).

2.10. Statistical analysis

The results were analyzed by descriptive statistics and compared by the analysis of variance (ANOVA) followed by post-hoc Bonferroni's test. Correlations were determined using the Spearman's test. Longitudinal SDF-1 and MPO data were analyzed using generalized estimating equation with multiple comparisons with Bonferroni adjustment. The significance threshold was set at 5% for all tests. All calculations were performed using Statistical Package for the Social Sciences (SPSS, version 22.0).

3. Results

3.1. Preventive physical training improves functional capacity and in combination with CT preserves left ventricular function after AMI

The functional capacities of SHR were evaluated by ET at the beginning and end of the training period in sedentary and trained groups (Fig. 1). ET parameters of the trained groups showed significant improvement after 10 weeks of treadmill training ($p < 0.0001$ for all comparisons), while the parameters remained unchanged in groups that remained sedentary during the same period ($p = 1.000$ for all comparisons).

Echocardiographic parameters were evaluated in all groups at the end of the experimental protocol. As shown in Fig. 2(a), LVEF in AMI groups was significantly lower than in the other groups ($p < 0.0001$ for all comparisons). Among the non-infarcted groups, the T- and T-SHAM animals exhibited higher LVEF values than the S- and S-SHAM animals ($p = 0.001$ and $p < 0.0001$, respectively). Among the infarcted groups, the combination of physical training and CT resulted in a less severe decrease in LVEF in the T-ICT group than that in the S-AMI and T-AMI groups ($p = 0.01$ and $p = 0.006$, respectively), which was not found in comparison with S-ICT ($p > 0.05$ for both comparisons). As shown in Fig. 2(b), the AMI groups exhibited worse LVSF than the non-infarcted groups ($p < 0.0001$ for all comparisons). Among the non-infarcted groups, the T- and T-SHAM animals exhibited better LVSF than the S- and S-SHAM animals ($p < 0.0001$ for all comparisons). The trends in the FAC data (Fig. 2(c) were similar to those observed for the LVEF and LVSF data. The AMI groups differed from the non-infarcted groups ($p < 0.0001$ for all comparisons). The values obtained for the T-SHAM and T-groups were higher than those for the S and S-SHAM

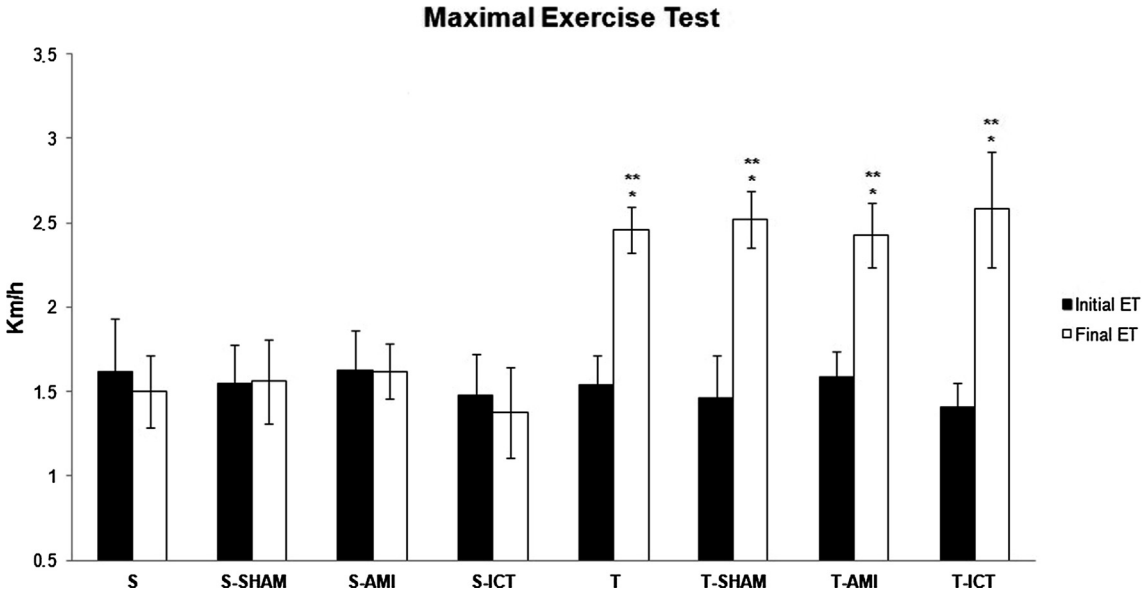

Fig. 1. Functional capacity analysis (ET) of SHR after 10 weeks of physical training or after an equivalent sedentarism period. Velocity (km/h) reached during a maximal exercise test, performed 48 h before the beginning of the experimental protocol (dark bars) and 48 h after the training period (white bars) is shown. Values are presented as the mean and standard deviation; the values were considered to be significantly different at $p < 0.05$: (*), for comparisons between initial and final ET; (**), for comparisons between the trained groups and their respective sedentary controls.

groups ($p < 0.0001$ for all comparisons). Fig. 2(d) shows the efficiency of AMI induction procedure. There was no difference in MIS values between groups subjected to AMI ($p > 0.05$ for all comparisons).

3.2. Preventive physical training and CT improve activation of the SDF-1/CXCR4 axis

Plasma levels of SDF-1 were evaluated in the S-SHAM, S-AMI, S-ICT, T-SHAM, T-AMI, and T-ICT groups, 48 h and 96 h after surgery; and at the end of the experimental period only in the non-surgery groups (S

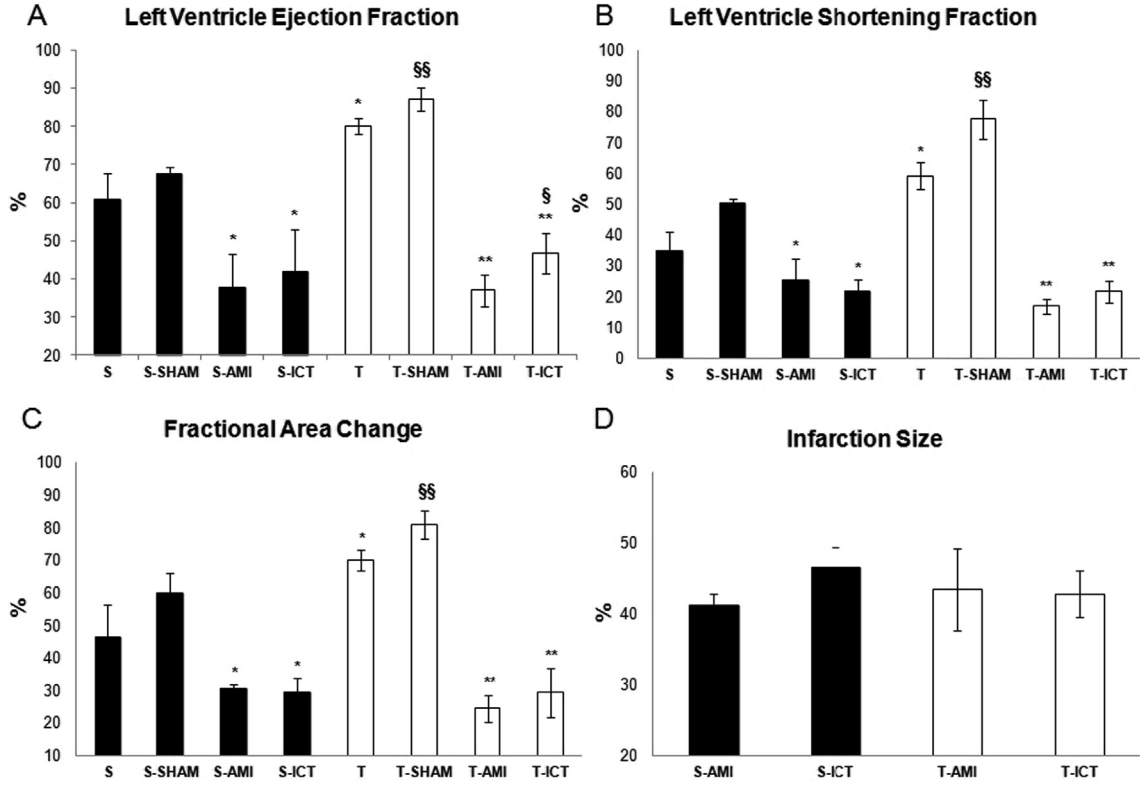

Fig. 2. Evaluation of echocardiographic parameters in SHR after 10 weeks of physical training or equivalent sedentarism period, AMI, and ADSC therapy. Evaluations were performed 48 h after ET, at the end of physical training (T) or equivalent sedentarism period (S), and 48 h after surgical interventions in other groups. LVEF (%) (a), LVSF (%) (b), FAC (%) (c), and MIS (%) (d) are shown. Values are presented as the mean and standard deviation; the values were considered to be statistically different at $p < 0.05$; (*) vs. S; (**) vs. T; (§) vs. S-AMI and T-AMI; (§§) vs. S-SHAM.

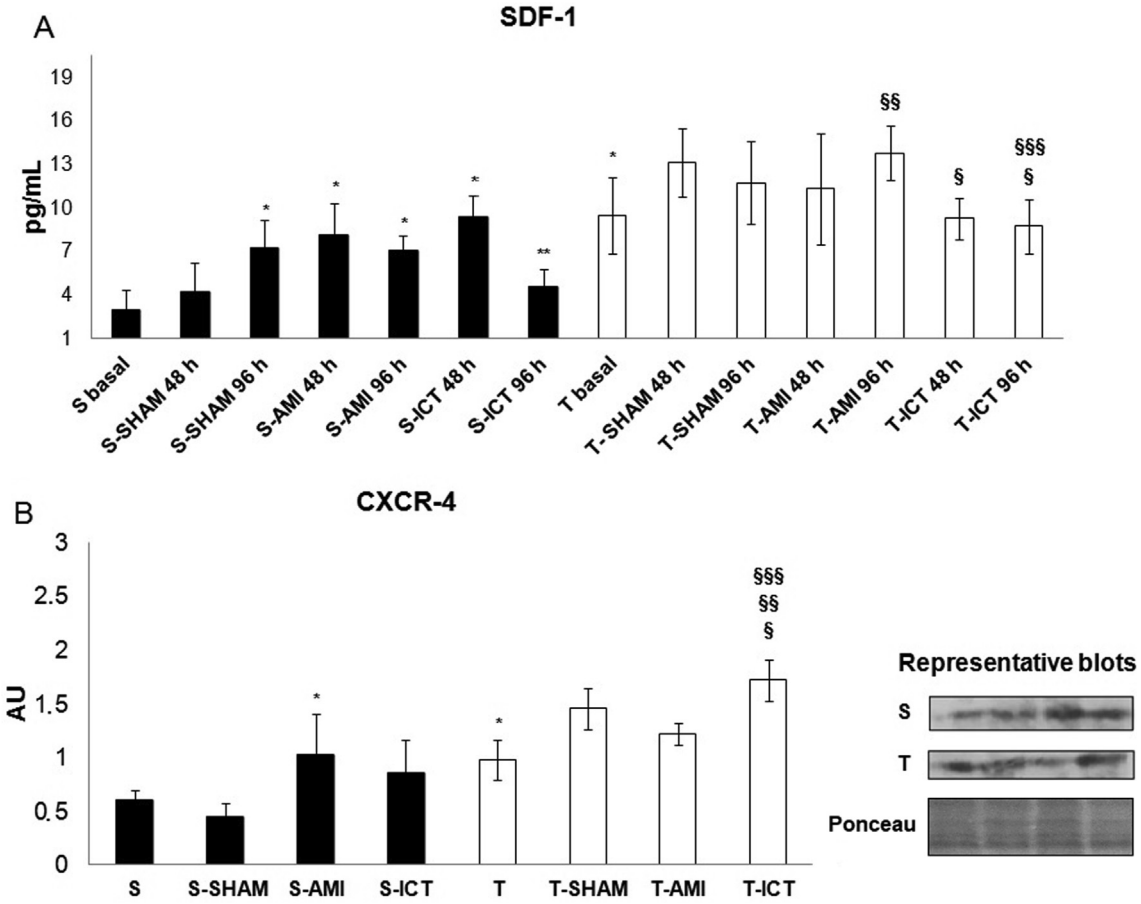

Fig. 3. Analysis of the SDF-1/CXCR4 axis activation in SHR after 10 weeks of physical training or equivalent sedentarism period, AMI, and ADSC therapy. (a) The systemic release of SDF-1 (pg/mL) was evaluated by ELISA immunoassays in plasma collected 48 h and 96 h after sham and AMI surgeries (S-SHAM, S-AMI, S-ICT, T-SHAM, T-AMI, and T-ICT groups), and at the end of experimental protocol in non-surgery groups (S and T basal). (b) CXCR4 expression (arbitrary units, UA) was determined by western blotting and normalized by densitometric analysis of the lane stained with Ponceau red, in cardiac tissue collected at the end of the experimental protocol in all evaluated groups. Representative blots are presented. Values are presented as the mean and standard deviation; the values were considered to be significantly different at $p < 0.05$. (a) (*) vs. S basal; (**) vs. S-ICT 48 h; (§) vs. T-AMI 96 h; (§§) vs. T basal; (§§§) vs. S-ICT 96 h; (b) (*) vs. S basal; (§) vs. T basal; (§§) vs. T-AMI; (§§§) vs. S-ICT.

and T, basal). As shown in Fig. 3(a), plasma SDF-1 levels were impacted by physical training (sedentary vs. trained; $p < 0.0001$). The effect of the time post-surgical procedure on SDF-1 levels was apparent in the sedentary groups. In the S-SHAM 96 h, S-AMI 48 h, S-AMI 96 h, and S-ICT 48 h samples, SDF-1 levels were higher than those in the S group (basal) ($p < 0.0001$ for all comparisons). However, there was no difference between the S-ICT 96 h samples after AMI induction and the S group ($p = 0.149$). Among the trained animals, SDF-1 levels in the T-AMI group 96 h after AMI were higher than in the T group (basal) ($p < 0.0001$). In T-ICT 48 h and 96 h samples, the SDF-1 levels were reduced to levels similar to those in the T group ($p = 1.000$ and $p = 0.560$, respectively).

Cardiac expression of CXCR4 in all studied groups is show in Fig. 3(b). The effects of ET on the expression of this receptor were indeed apparent. In the sedentary groups, CXCR4 expression was higher in the S-AMI group than in the S group ($p = 0.007$). However, no difference was apparent when the S-ICT group was compared with the S group ($p = 0.308$). In the trained groups, CXCR4 expression in the T-ICT group was higher than in the T ($p < 0.0001$) and T-AMI ($p = 0.001$) groups. A difference in CXCR4 expression ($p = 0.004$) was observed between the S and T groups. The same relationship was observed between the groups that received CT (S-ICT and T-ICT; $p < 0.0001$), demonstrating the effect of exercise training on CXCR4 protein level. Further, there was a significant correlation ($r = 0.483$; $p = 0.002$) between CXCR4 expression and the concentration of SDF-1.

3.3. Preventive physical training and CT reduce vasoconstrictor responses in SHR

To analyze the effects of physical training and surgery on angiogenic and vasoconstrictor responses, plasma levels of VEGF, ET-1, and Ang II were evaluated. In the sedentary groups, VEGF levels (Fig. 4(a)) decreased in response to surgery. VEGF levels in the S-SHAM, S-AMI, and S-ICT groups were reduced in comparison with the S group ($p < 0.0001$ for all comparisons). The same trends were observed in the trained groups, with the T-SHAM, T-AMI, and T-ICT groups exhibiting lower values than the T group ($p < 0.0001$ vs. T-SHAM and T-ICT; $p = 0.003$ vs. T-AMI). Additionally, VEGF levels in the T-AMI group were higher than those in the S-AMI group ($p = 0.001$).

For the vasoconstrictor response, as shown in Fig. 4(b), Ang II levels were higher in the S group than in the S-SHAM, S-AMI, and S-ICT groups ($p < 0.0001$ for all comparisons), as well as the T group ($p < 0.0001$). The latter comparison represented the effect of physical training alone on the serum levels of the hormone.

The analysis of ET-1 levels is shown in Fig. 4(c). In the sedentary groups, there were no effects of the different treatments on the levels of the peptide (S, S-SHAM, S-AMI, and S-ICT; $p > 0.05$ for all comparisons). In the trained groups, systemic ET-1 levels in the T-SHAM and T-AMI groups were higher than in the T group ($p = 0.003$ and $p < 0.0001$, respectively), but the levels in T group were not different from those of the T-ICT group ($p = 1.000$). Physical training reduced

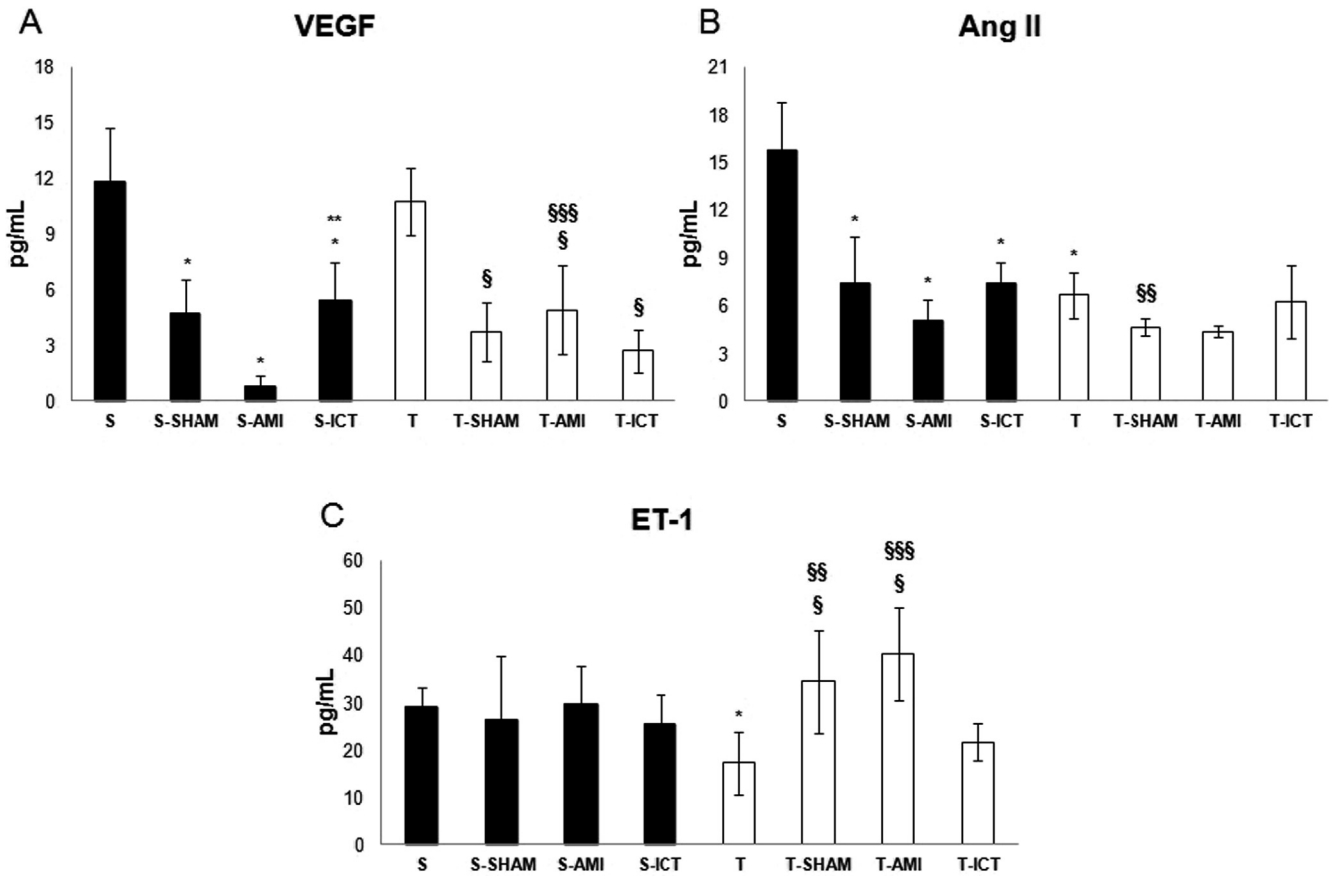

Fig. 4. Analyses of angiogenic and vasoconstrictor responses in SHR after 10 weeks of physical training or equivalent sedentarism period, AMI, and ADSC therapy. Systemic release of VEGF (a), Ang II (b), and ET-1 (c) was analyzed by ELISA immunoassays in plasma collected at the end of physical training or equivalent sedentarism period (S and T groups, respectively), and 96 h after surgical interventions (S-SHAM, S-AMI, S-ICT, T-SHAM, T-AMI, and T-ICT groups). Values are presented as the mean and standard deviation; the values were considered to be significantly different at $p < 0.05$; (*) vs. S; (**) vs. S-AMI; (§) vs. T; (§§) vs. S-SHAM; (§§§) vs. S-AMI.

ET-1 levels in the S group compared to the T group ($p = 0.016$). Among the trained groups, increased ET-1 levels were noted after AMI, but these values returned to the baseline in the group that received the CT.

3.4. Preventive physical training and CT maintain active inflammatory profile in the cardiac Tissue, but not systemically

To analyze the inflammatory responses caused by the above interventions, the expression of the following proteins was evaluated: MPO, iNOS, gp91phox, and NF-κB. Plasma levels of MPO were evaluated in the S-SHAM, S-AMI, S-ICT, T-SHAM, T-AMI, and T-ICT groups at 48 and 96 h after surgery, and at the end of the experimental period in the groups that had not undergone surgery (S and T basal).

As shown in Fig. 5(a), among the sedentary groups, MPO levels in the animals that received ADSC therapy (S-ICT after 48 h and 96 h) were significantly lower than those in the S group ($p < 0.0001$ for both comparisons). When the trained groups were compared with the sedentary groups, MPO levels in the T group were lower than those in the S group ($p < 0.0001$). Likewise, MPO levels in the S-SHAM and S-AMI groups were significantly higher than those in the T-SHAM and T-AMI groups 48 h and 96 h post-surgery ($p < 0.0001$ for all comparisons).

The expression levels of iNOS are shown in Fig. 5(b). Among the sedentary animals, iNOS expression in the S-AMI group was higher than that in the S ($p < 0.0001$) and S-ICT groups ($p = 0.021$). Further, iNOS expression in the S and S-ICT groups was not different ($p = 0.373$). Among the trained groups, iNOS levels in the T-SHAM and T-ICT groups were higher than those in the T group ($p < 0.0001$ for both comparisons). Additionally, iNOS expression was higher in the T-SHAM group than that in the S-SHAM group ($p < 0.0001$), and in the T-ICT group than that in the S-ICT group ($p < 0.0001$).

Tissue levels of gp91phox are shown in Fig. 5(c). Among the sedentary groups, gp91phox levels in the S-AMI group were enhanced compared to the S and S-ICT groups ($p < 0.0001$ and $p = 0.002$, respectively). Among the trained groups, gp91phox expression in the T-ICT group was higher than that in the T group ($p = 0.002$). A comparison between the sedentary and trained animals revealed that gp91phox expression in the T-ICT group was higher than that the S-ICT group ($p = 0.005$).

Cardiac expression levels of NF-κB are shown in Fig. 5(d). In the sedentary groups, surgery reduced the expression of NF-κB in the S-SHAM, S-AMI, and S-ICT groups when compared with the S group ($p < 0.0001$ compared with S-SHAM and S-AMI; $p = 0.016$ compared with S-ICT). In the trained groups, only T-SHAM NF-κB levels were lower than those in the T group ($p < 0.0001$). A comparison of the sedentary and trained groups indicated that the NF-κB levels were higher in the T, T-SHAM, T-AMI and T-ICT groups than in the corresponding sedentary groups ($p < 0.0001$ for all comparisons), demonstrating the positive effects of physical training on NF-κB expression. Further, NF-κB levels were positively correlated with those of the homing axis protein CXCR4 ($r = 0.528$; $p < 0.0001$).

4. Discussion

In the current study, we reported the effect of preventive exercise training and physical inactivity on the mobilization of molecular factors after the induction of AMI and infusion of ADSC in SHR. Preventive

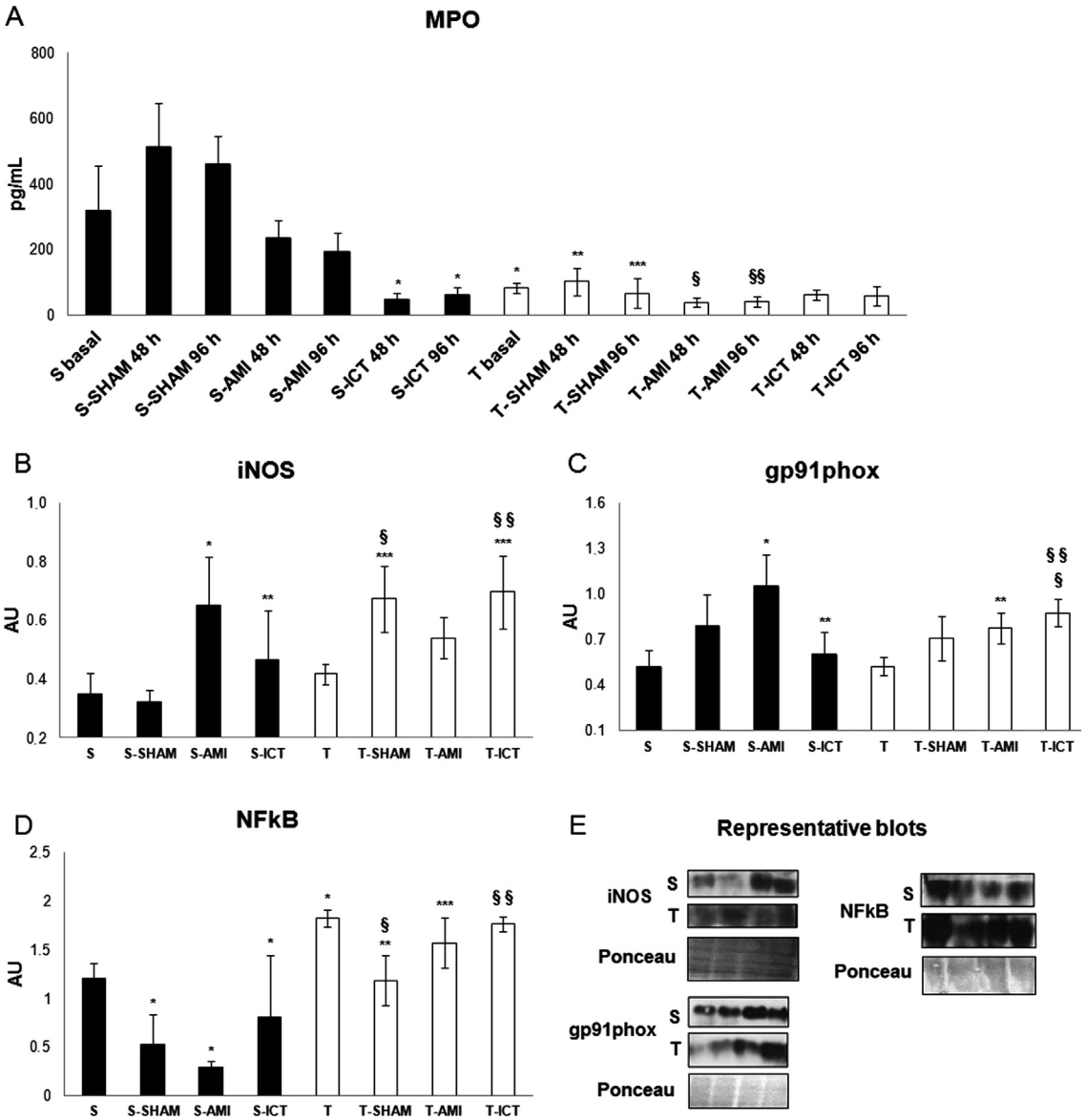

Fig. 5. Inflammatory responses assessment in SHR animals after 10 weeks of physical training or equivalent sedentarism period, AMI and ADSC therapy. (A) Myeloperoxidase enzyme level (MPO pg/mL) measurements were performed by ELISA immunoassay from plasma collected at 48 and 96 h after SHAM and AMI surgeries (S-SHAM, S-AMI, S-ICT, T-SHAM, T-AMI, T-ICT groups) and at the end of experimental protocol in non-surgery groups (S and T basal). The expression of iNOS (B), gp91phox isoform of NADPH oxidase (C) and NFkB (D) were measured by Western Blot and normalized by densitometric analysis of the lane stained with Ponceau red, from cardiac tissue collected at the end of experimental protocol in all evaluated groups. (E) Representative blots of analyzed proteins in all sedentary (S) and trained groups (T). Values are presented as mean and standard deviation, and they were considered different when $p < 0.05$. (A) (*) vs. S basal; (**) vs. S-SHAM 48 h; (***) vs. S-SHAM 96 h; (§) vs. S-AMI 48 h; (§§) vs. S-AMI 96 h; (B) (*) vs. S, (**) vs. S-AMI, (***) vs. T, (§) vs. S-SHAM and (§§) vs. S-ICT; (C) (*) vs. S, (**) vs. S-AMI, (§) vs. T and (§§) vs. S-ICT; (D) (*) vs. S, (**) vs. S-SHAM, (***) vs. S-AMI, (§) vs. T, (§§) vs. S-ICT.

physical training clearly exerted a cardioprotective effect, enhancing the repair processes associated with CT. These findings reflect the importance of clinical trials for CT regimens.

The physical training program promoted significant improvement in the functional capacity of trained animals, as previously demonstrated by our group in the SHR model in another study [8]. The improvement in functional capacity observed in the current study was accompanied by improvement of the cardiac function in the trained groups without AMI. LVEF, LVSF, and FAC values were significantly higher in trained animals than in sedentary animals, but training alone did not prevent a reduction in these parameters in infarcted animals that did not receive CT. These findings are in agreement with a study by Veiga et al. [21], who demonstrated that 8 weeks of exercise training (swimming) of Wistar Kyoto rats performed before myocardial infarction induction did not reduce the necrotic area, or improve FAC or LVEF. However, in the current study, trained animals that received ADSC exhibited higher LVEF than those subjected to AMI that did not receive CT. This difference was not observed in sedentary animals, suggesting that physical training exerts a potentiating effect on CT, partially attenuating the loss of heart function. Similarly, no improvements in the cardiac function are induced by ADSC infusions in C57BL/6 mice that had not been subjected to preventive physical training [22]. Thus, data presented in the current study suggest that physical training prior to AMI positively influences the process by which stem cells mediate cardiac function restoration, although the analysis period was brief. Based on these observations, physical training may affect molecular signaling in cell-homing mechanisms.

The mobilized factors that promote cardiac repair after ischemic events include cytokines, including chemokines and interleukins, secreted by the immune and progenitor cells. Majority of these

chemokines are associated with the activation of the cellular SDF-1 response. In the current study, physical training induced an increase in systemic levels of this protein. SDF-1 levels, as previously demonstrated [23], increase in response to AMI-induced ischemic damage of the cardiac tissue. Data in the current study supported this notion. As shown, SDF-1 levels in sedentary animals with AMI and CT were significantly higher than those in the non-infarcted group 48 h after the ischemic damage. It is important to note that in the sedentary group 96 h after ADSC infusion (S-ICT 96 h), SDF-1 levels returned to the baseline, supporting the idea that this factor is released upon tissue injury and then its levels decrease because of tissue response. In human, the mobilization of various types of stem cells, including $CXCR4^{+}$, has been shown to increase after the onset of ischemia [24,19]. However, after the acute phase of AMI, SDF-1 levels in the peripheral blood tend to decrease [25]. Further, persistent production of this protein by the myocardium is associated with heart function loss during the adaptive period after AMI [25], indicating that cardiac tissue injury affects the SDF-1/CXCR4 axis in the human heart.

Immediately after AMI, SDF-1 binds to its receptor. This results in the activation of various intracellular processes, MAP kinases, and specific transcription factors (such as NF-κB), leading to the activation of cell proliferation, and secretion of cytokines and growth factors [26]. In the current study, SDF-1 levels in previously trained animals were significantly higher than those in sedentary animals. The behavior of this cytokine was similar in the trained and untrained groups after intervention; however, in trained animals, the kinetic response was faster. In trained animals, SDF-1 levels returned to the baseline (T group) within only 48 h of cell infusion (T-ICT 48 h group), indicating that exercise training enhanced modulation of the acute response. Sarto et al. [27] reported that SDF-1 levels increase in patients with chronic heart failure who underwent aerobic training for 8 weeks. In this context, results of the current study indicate that training is able to modulate SDF-1 activation, contributing to myocardial repair mechanisms.

In response to increased SDF-1 levels in the bloodstream, resident and infused stem cells tend to express CXCR4, which activates cellular homing. We noted increased CXCR4 expression in animals from the trained groups compared to the sedentary ones. We also found that the receptor was more highly expressed in the cardiac tissues of trained animals that received ADSC infusions than in sedentary stem cell treated group (S-ICT), a finding suggestive of the potentiation of activation of the SDF-1/CXCR4 axis. Regarding the chronic effects of exercise, Xia et al. [28] noted increased regenerative potential of endothelial progenitor cells in older individuals via the activation of CXCR4, after 12 weeks of treadmill training. Although factor(s) that trigger the activation of the SDF-1/CXCR4 axis remain unclear, it is known that oxygen deprivation generated by hypoxia secondary to ischemia has a significant effect on this process [29].

Hypoxia and ischemia caused by arterial occlusion are natural biological stimuli of endogenous angiogenesis. Vascular endothelial growth factors, well-studied angiogenesis stimulators, belong to a family of glycoproteins. VEGF (mainly isoform 1) is frequently used in experimental and clinical trials concerning therapeutic angiogenesis [30]. In the current study, we sought to analyze mobilization of these growth factors in response to physical training and CT. However, the obtained data revealed that physical training did not influence VEGF levels at baseline (without surgery). After AMI, VEGF levels in the sedentary group were lower than those in the trained group. Nevertheless, CT caused no changes in VEGF expression in the time interval analyzed in the current study. It is possible that the increased VEGF levels represent a long-term effect in CT animals. VEGF levels are reportedly elevated in anti-inflammatory environments [31], which may explain the reduction in VEGF levels within 96 h after AMI observed in the current study.

Among the risk factors for CVD, hypertension, and elevated levels of Ang II and ET-1 are closely associated with the reduction in vasodilation in response to endothelial stimuli. The current study revealed that Ang II expression was increased in sedentary animals without any intervention (group S) but was reduced in response to physical training, as has been also noted by Silva et al. [32] in a study involving trained SHR. However, CT had no effect on the chemokine levels. Further, in trained animals without surgery, ET-1 levels were reduced when compared to those in sedentary animals, as was also noted by Lee et al. [33]. In addition, when subjected to AMI, the trained animals exhibited increased systemic release of ET-1. However, in the trained group that received ADSC (T-ICT), ET-1 levels were similar to those observed in trained animals without surgery. This effect was not apparent in the sedentary animals. This finding suggests that the combination of preventive physical training and ADSC therapy reduces the vasoconstrictor response after AMI.

Physical training was previously described as a regulator of proinflammatory interleukins in an SHR model [34]. In the current study, levels of MPO, a marker of inflammation in the coronary disease syndrome [35], were substantially reduced in animals subjected to physical training. Pro-inflammatory interleukins strongly induce iNOS activation, as observed in the animal groups that underwent surgery. However, preventive exercise training did not reduce the levels of activated iNOS in these animals compared with sedentary animals at baseline. Although MPO levels were significantly reduced after CT in trained animals, iNOS expression in the cardiac tissues did not decrease under these conditions, possibly because of the effect of a secreted factor capable of maintaining iNOS activation. Results presented in the current study indicated that AMI, with or without CT, in SHR subjected to physical training, results in elevated expression of gp91phox. In this regard, in the current study, except for MPO levels, aerobic preventive training maintained elevated the levels of all the factors and enzymes associated with inflammation in the cardiac tissue, especially after CT. This observation may indicate that inflammation is required for the activation of cellular homing mechanisms during the early stages of cardiac repair after infusion of stem cells. In fact, we also observed an increase in the expression of NF-κB upon preventive physical training, compared with sedentary animals. Although NF-κB is known for its role in the development of inflammation [36], this factor also seems to play an important role in cardioprotective responses to ischemic events and in adaptive responses to ROS production, as shown by Hikoso et al. [37]. Further, a cardioprotective effect of NF-κB was noted upon its activation by the SDF-1/CXCR4 axis, mainly because of its role in cell survival after stress [38]. In support of this hypothesis, the current study revealed an association between NF-κB expression and CXCR4 in the cardiac tissue.

Mesenchymal stem cells isolated from the adipose tissue are well studied and have been used in various pre-clinical and clinical protocols. However, the effects of physical training have seldom been explored in the setting of CT involving mesenchymal cells isolated from the bone marrow or other tissues. The current study is the first one to show cardioprotective effects of physical training undertaken prior to AMI induction in the context of modulatory factors that can influence the success of CT.

Further studies are clearly required to further elucidate the molecular basis of the effects of long-term physical exercise. The available clinical evidence pertaining to its beneficial effects with respect to primary disease prevention is significant. Considering the current status of cell-based therapies for the treatment of CVD, it is important to emphasize that we are still at the dawn of the era of regenerative medicine. Even though many pathophysiological mechanisms and practical issues remain to be resolved, it is important to remember that great progress has been made in the field in a relatively short time.

5. Conclusions

In the current study, we demonstrated that aerobic physical training performed before experimental AMI may potentiate stem-cell regenerative capacity by activating cell homing axis and by increasing the

expression of inflammatory factors in the cardiac tissue of SHR. Further, the adopted training program reduced the short time vasoconstrictor and inflammatory responses and, more importantly, it partially preserved heart function in infarcted animals. Future studies with long term analysis after myocardial infarction and more molecular parameters, as fibrosis development in cardiac tissue and others heart failure markers, should improve our understanding on the importance of physical training as a protective factor for heart function.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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