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ORIGINAL ARTICLE

Exercise Training Potentiates The Cardioprotective Effects of Stem Cells Post-infarction

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Background Preconditioning of cell recipients may exert a significant role in attenuating the hostility of the infarction milieu, thereby enhancing the efficacy of cell therapy. This study was conducted to examine whether exercise training potentiates the cardioprotective effects of adipose-derived stem cell (ADSC) transplantation following myocardial infarction (MI) in rats.

Methods Four groups of female Fisher-344 rats were studied: Sham; non-trained rats with MI (sMI); non-trained rats with MI submitted to ADSCs transplantation (sADSC); trained rats with MI submitted to ADSCs (tADSC). Rats were trained 9 weeks prior to MI and ADSCs transplantation. Echocardiography was applied to assess cardiac function. Myocardial performance was evaluated *in vitro*. Protein expression analyses were carried out by immunoblotting. Periodic acid-Schiff staining was used to analyse capillary density and apoptosis was evaluated with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

Results Echocardiography performed 4 weeks after the infarction revealed attenuated scar size in the both sADSC and tADSC groups compared to the sMI group. However, fractional shortening was improved only in the tADSC group. *In vitro* myocardial performance was similar between the tADSC and Sham groups. The expression of phosphoSer473Akt1 and VEGF were found to be higher in the hearts of the tADSC group compared to both the sADSC and sMI groups. Histologic analysis demonstrated that tADSC rats had higher capillary density in the remote and border zones of the infarcted sites compared to the sMI rats.

Conclusions Preconditioning with exercise induces a pro-angiogenic milieu that may potentiate the therapeutic effects of ADSCs on cardiac remodelling following MI.

Keywords Exercise training • Myocardial infarction • Preconditioning • Cell-Based therapy • Adipose-Derived stem cells

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Introduction

Myocardial infarction (MI) has a pivotal role in cardiovascular morbidity and mortality. Although several therapeutic approaches have been proven to ameliorate post-infarction cardiac remodelling, the prognosis remains poor, and left ventricular (LV) dysfunction often progresses to heart failure [1].

The cell therapy with stem cell (SC) has emerged as an attractive approach to ameliorate myocardial remodelling and improve cardiac function following MI [2]. There are several sources of SC including the umbilical cord [3], bone marrow [4], epithelium [5], dental pulp [6], and more recently, adipose tissue [7]. The abundance of adipose tissue, and its easy accessibility are the advantages in choosing adipose-derived stem cells (ADSC) [8]. Moreover, studies in animal models of MI have revealed the ability of ADSC to engraft and differentiate into various types of cells as well as to secrete a wide range of angiogenic and anti-apoptotic factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 [9–11].

Unfortunately, experimental and clinical studies have demonstrated modest or no beneficial effect of SC in recovering cardiac function. This limitation has stimulated the search for approaches that improve or increase the therapeutic potential of SCs, such as genetically modifying SC, or preconditioning the SC with factors that potentiate their survival [7]. Moreover, preconditioning the recipient heart before SC transplantation has been proposed as a strategy to attenuate the hostility of the infarction milieu, thereby increasing the survival and efficacy of transplanted SCs in attenuating cardiac remodelling [7]. The effectiveness of *in vivo* preconditioning in increasing SC survival was demonstrated in a study involving low-level laser therapy for bone marrow-derived SCs that were injected directly into the myocardium [12]. It has also been reported that statins alleviate inflammatory response by decreasing cytokines, thereby improving SC survival [13].

Recently, two studies analysed the impact of post-infarction exercise training on the effects of SC therapy. Cosmo et al. [14] demonstrated that the combination of exercise and SC induced a favourable condition for attenuating the ventricular dilation resulting from MI, in which exercise training was started 23 days after SC in infarcted myocardial. Chirico et al. [15] demonstrated that a single exercise session performed 7 days following MI increased cell retention in the transition zone between the infarcted myocardium and the remote tissue. However, this did not result in improvement in myocardial function. In another protocol of Chirico's study, infarcted rats were submitted to SC following daily sessions of exercise for a total of 4 weeks. The authors failed to identify any benefit of SCs and exercise on myocardial remodelling.

Although initial studies have signalled a beneficial role of exercise post-MI in improving the action of SCs in the injured myocardium, it remains to be established whether prior-MI exercise training has any effect on SC therapy. Consequently, this study aimed to evaluate whether exercise training potentiates the cardioprotective effects of ADSC transplantation following myocardial infarction MI in rats. This hypothesis was based on studies that provided evidence that exercise before injury reduces pro-apoptotic genes and increases angiogenesis factors [16,17]. Indeed, animal studies have suggested that prior exercise can precondition the myocardium, thereby protecting the heart against damage and ventricular dysfunction induced by MI. Thus, one study in mice using permanent coronary artery ligation showed that infarct area was thicker, whereas fibrosis and apoptosis in the remote LV myocardium were blunted by prior voluntary wheel running [18]. Furthermore, rats submitted to regular swimming exercise training before induction of MI showed a lower scar area and inflammation, together with improvement of myocardial vascularisation and function [16,17,19].

Material and Methods

Ethics

All the experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). All *in vivo* experiments were performed under ketamine (50 mg/kg) and xylazin (10 mg/kg) anaesthesia, and all efforts were made to minimise the suffering of animals. Animals were anaesthetised with isoflurane (3%, at 2 L/min oxygen flow) for invasive procedures. Animal hearts were isolated following rapid decapitation. This experimental protocol (number: 2130010214) was approved by the Institutional Research Ethics Committee of the Federal University of São Paulo, Brazil.

Study Design

Figure 1 illustrates the experimental design of this study. Female Fisher-344 rats weighing 140–190 g were randomly assigned to an exercise training protocol or non-trained status. The following experimental groups were studied: Sham (n = 35); non-trained rats submitted to MI (sMI, n = 35); non-trained rats submitted to MI and SC therapy (sADSC, n = 35); and trained rats submitted to MI and SC therapy (tADSC, n = 35). Trained animals were submitted to a 9-week training protocol while the non-trained rats remained without any activity over the same period. After the end of training/no-training period, MI rats were submitted to permanent coronary occlusion. Sham rats too were operated upon similarly, although the coronary occlusion procedure was avoided. All rats remained without training following MI. The ADSC transplantation was conducted 48 hours following MI. For immunoblotting analyses, rats were killed 1 hour or 1 week post-ADSC transplantation. These timepoint analyses were designated based on our previous studies, in which we showed short-term biomolecular effects of SC transplantation in myocardium [20,21]. Another group of rats that were subjected to the same protocol were followed-up for 4 weeks post-ADSC transplantation for histological and functional analysis.

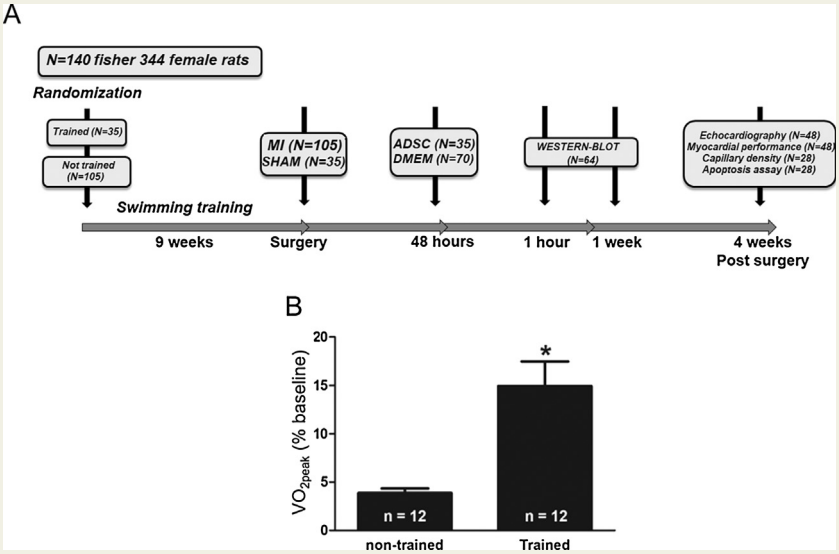

Figure 1 (*A*) Experimental design. (*B*) Maximum aerobic performance (mean ± standard error) on sedentary and trained animals. VO_2 peak: peak oxygen consumption. Data demonstrates the relative changes between the assessment before starting the exercise and following the completion of the observation period. *$p < 0.05$ *vs.* no-trained; unpaired Student's t-test.

Exercise Training

The 9-week training protocol included a 90-minute swimming session a day, 5 days a week. The rats were preconditioned to swim 6 consecutive days before initiating the main protocol. The duration of swimming was progressively increased by 15 minutes at each session, to achieve a duration of 90 minutes on the sixth day. Previously to the study, we carried out a pilot study with healthy animals to examine the efficacy of this protocol to increase physical fitness (Figure 1B). The functional fitness was evaluated by using a motorised treadmill coupled with a gas analyser (Panlab, Harvard Bioscience Company, MA, USA). Before the physical test, rats were introduced to running as previously described [22]. Each rat underwent a 2-minute warm-up period at 25 cm/s, following which the running speed was increased by 9 cm/s every 2 minutes till the rats were exhausted. Since the detection of a plateau in the maximal VO_2 is difficult in rats, VO_2peak was used as marker for cardiorespiratory fitness. The VO_2peak was expressed in $ml.kg^{-1}.min^{-1}$.

MI Model

Following the exercise training protocol, the surgical procedure to induce chronic MI was performed in both trained and non-trained rats according to a well-established technique [23]. Briefly, under anesthaesia (ketamine 50 mg/kg and xylazine 10 mg/kg, intraperitoneal) and artificial ventilation (Harvard Rodent Ventilator, Model 863; Harvard Apparatus, Holliston, MA), a left thoracotomy was performed. The heart was exteriorised and the coronary artery ligated with 6-0 polypropylene. The heart was quickly returned to its position and the thorax immediately closed. Sham rats were submitted to a similar procedure, with the exception of coronary occlusion.

ADSC Isolation and Transplantation

The ADSCs were obtained from the abdominal adipose tissue of male Fisher-344 rats and cultured as previously described [24]. A total 1×10^6 ADSCs was suspended in 100 μL culture medium (DMEM, Dulbecco's modified Eagle's medium), and injected into the myocardium at three points along the border zone by using a 30-gauge needle (BD Ultrafine, NJ, USA) following the first echocardiography analysis. Sham and sMI groups received injections of DMEM alone.

Echocardiography

Forty-eight hours following either coronary occlusion or sham surgical procedure, rats were anaesthetised as described above (ketamine-xylazin mixture). Transthoracic echocardiogram was performed to determine the size of the MI by using a 12 MHz transducer (Sonos-5500, Hewlett-Packard, MA, USA). The MI size was evaluated on transverse two-dimensional view of the LV and reported as percentage of the left ventricular perimeter [25]. Only rats bearing infarct sizes ≥37% of LV were included in the experimental groups. This cut-off MI size was shown to have negative impact on survival probability, in which highest values under a receiver operating characteristic curve were MI size ≥37% [26]. The MI was defined as presence of a segment with increased echogenicity and/or modification in myocardial thickening or systolic movement (hypokinesia, akinesia, or dyskinesia). A second echocardiographic examination was carried out 4 weeks post-infarction to evaluate the scar length, end diastolic (LVAd) systolic (LVAs) LV areas and shortening fractional (FS: LVAd − LVAs/LVAd × 100), as the LV function parameter [27]. Diastolic function was not evaluated owing to the fusion of the A and E waves.

In Vitro Myocardial Performance

Following the second echocardiography, posterior papillary muscles were immediately removed and placed in a tissue bath containing modified Krebs–Henseleit solution (130 mM NaCl, 5.0 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, 20 U insulin and 20 mM Hepes, bubbled with 100% O_2, and maintained at 29 °C, pH 7.4) [28]. The muscles were loaded to contract isometrically for 15 minutes and stretched to the apices of their length–tension curves. Data were recorded through the use of AcqKnowledge 3.5.7 software (Biopac Systems Inc., Goleta, CA, USA) to determine maximal developed tension (DT), maximal rate of tension increase (+dT/dt) and decrease (−dT/dt), and resting tension (RT).

Histomorphometry

The heart was perfused and fixed according to standard protocol [29]. Samples were cut into 3-μm-thick sections and stained with periodic acid-Schiff. Capillary density was evaluated in 10 randomised micrographs (40 × magnification) by using a computerised acquisition system (Leica Imaging Systems, Bannockburn, IL, USA).

Apoptosis Assay

The LV tissue, which was fixed in 10% paraformaldehyde, was embedded in paraffin to obtain 6-μm-thick sections. The sections were prepared and stained by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method by using *in situ* detection kits as recommended by the manufacturer (Roche Applied Science, Germany). The analyses were carried out as described in detail [30].

Immunoblotting

Proteins were extracted from the LV remote area as previously described by us [31]. Homogenate protein samples of 30 μg were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gel. Separated proteins were transferred onto hydrophobic polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences; Piscataway, NJ, USA), and the transfer efficiency was examined with 0.5% Ponceau S. The membranes were soaked in a blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.5) for 1 hour at room temperature and then incubated overnight at 4 °C with primary antibodies: rabbit anti-Akt_1 (1:5000 dilution; Abcam, Cambridge, MA, USA); rabbit anti-phosphoSer473-Akt1 (1:5000 dilution; Abcam, Cambridge, MA, USA); goat anti-VEGF (1:1,000; Abcam, Cambridge, MA, USA); and anti-GAPDH (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After overnight incubation, membranes were washed five times and then incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat secondary antibodies (1:2000; Invitrogen, San Diego, CA, USA). Membranes were finally washed five times with blocking buffer and then rinsed twice in PBS. Bound antibody was detected by using chemiluminescence reagent for 1 minute. The bands were imaged by using ImageScanner LAS4000 mini (GE HealthCare, Little Chalfont, UK, USA) and quantified by using ImageJ software (Bethesda, MD, USA).

Statistical Analysis

Shapiro-Wilk test was used to verify normality data. Levene test was applied to assess the equality of variances. Results were presented as mean ± standard error of mean. The comparisons between the groups were made by one-way ANOVA (*post-hoc:* Newman–Keuls). A *p-value* of ≤ 0.05 was regarded as statistically significant.

Results

Figure 1B shows the maximum aerobic performance relative changes between the assessment before starting the exercise and following the completion of the observation period, evidencing the improve in trained group. During the follow-up, the body weight did not differ between groups (SHAM: 184 ± 5; sMI: 173 ± 8; sADSC: 178 ± 1; tADSC: 184 ± 2).

Figure 2 shows the cardiac morphologic and functional analysis that was performed 4 weeks following MI. There were no differences in wall thickness (Figures 2A and 2B) and heart rate (Figure 2C) between the groups. Figure 2 also shows infarction scar size for sMI, sADSC, and tADSC groups analysed 4 weeks following coronary occlusion. The data demonstrated a smaller scar length in the ADSC-treated rats compared to the sMI group (Figure 2D). In all MI groups, LV areas were higher than SHAM animals, suggesting myocardial remodelling (Figures 2E and 2F). The FS was lower in the sMI and sADSC groups when compared to that in the SHAM and tADSC animals (Figure 2G). Exercise training before ADSC transplantation appeared to prevent LV systolic dysfunction. All MI groups exhibited lower maximal developed tension (DT) and maximal rate of tension increase (+dT/dt) when compared to SHAM animals (Figures 2I and 2J). However, myocardial dysfunction does not appear to occur in the ADSC-treated rats. The DT and +dT/dt values in the tADSC group were not significantly different from the sham group. Figure 2L shows that the only significant difference regarding resting tension (RT) was found between the sMI and SHAM groups.

Figure 3 shows the results of the apoptosis and myocardial capillarity analysis performed 4 weeks post-infarction. The number of apoptotic cells in the border zone (Figure 3B) and remote area (Figure 3C) of infarction were found to be greater in the sMI group. On the other hand, both groups of ADSC-treated rats, sADSC, and tADSC, showed similar apoptosis levels as the SHAM group. As shown in Figure 3D, the sMI group had a lower capillary density in the border zone (Figure 3E) and remote area (Figure 3F) of infarction. ADSC therapy appeared to blunt this reduction of capillary density in the border zone, while increasing the capillarity in the remote area when compared with the sMI group. The positive effects of ADSCs at the border zone were enhanced in rats with prior exercise training. The capillary density was significantly higher in the tADSC group compared to both sMI and sADSC groups.

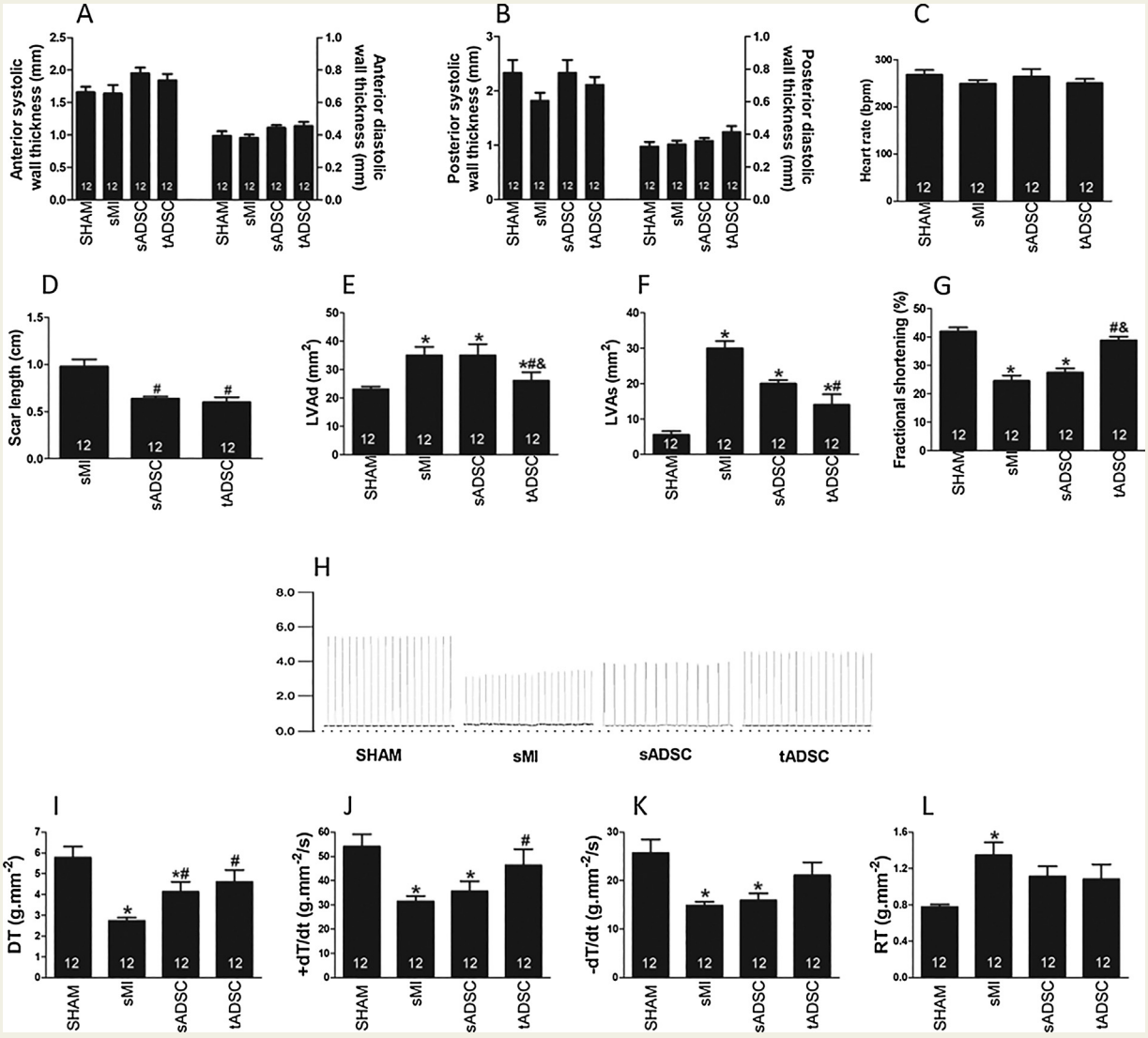

Figure 2 Exercise preconditioning enhances ADSC effects on left ventricular (LV) function and *in vitro* myocardial performance. Experiments were performed 4 weeks after MI. Data include analyses of nine animals in each group. *(A)* Anterior systolic wall thickness; *(B)* Anterior diastolic wall thickness; *(C)* Heart rate; *(D)* scar size; *(E)* end-diastolic LV area (LVAd); *(F)* end-systolic LV area (LVAs); *(G)* LV fractional shortening (SF); *(H)* representative image of a typical register for an isometrically contracting papillary muscle. Data obtained at muscle lengths corresponding to 100% of Lmax from Sham, sMI, sADSC and tADSC groups as described in Methods; *(I)* developed tension; *(J)* maximum positive time derivative of developed tension (+dT/dt); *(K)* maximum negative time derivative of developed tension (−dT/dt); *(L)* resting tension (RT). Numbers within the bar graphs indicate the number of rats per group. *$p < 0.05$ *vs.* SHAM; $^{\#}p < 0.05$ *vs.* sMI; $^{\&}p < 0.05$ *vs.* sADSC.
Abbreviations: ADSC, adipose-derived stem cells; LV, left ventricular; MI, myocardial infarction.

To determine the molecular effects of exercise on cell therapy, we evaluated the Akt1, phosphoSer473Akt1, and VEGF protein expression acutely after cell therapy. The protein oexpression was analysed either 1 hour or 7 days following ADSC transplantation. No significant differences were found in total Akt_1 expression (Figures 4B and 4F), but phosphoSer473Akt1 levels were significantly higher only in the tADSC group (Figures 4C and 4G). There was no difference in VEGF expression between the groups at 1 hour following ADSC transplantation (Figure 4D). However, after 7 days, the expression of cardiac VEGF was lower in rats with MI than in sham and tADSC groups (Figure 4H).

Discussion

Limiting the infarct size is a common means of attenuating cardiac remodelling and improving cardiac function following an MI [32]. Thus, the development or recruitment of new blood vessels is the target of many therapeutic approaches [33]. Currently, SC therapy is considered a suitable modality to improve cardiac function in the infarcted heart [7].

Reduced perfusion adjacent to the infarcted area may be associated with scar expansion and impaired cardiac remodelling [34]. Our findings are consistent with previous studies [35], and demonstrate that ADSC transplantation into the

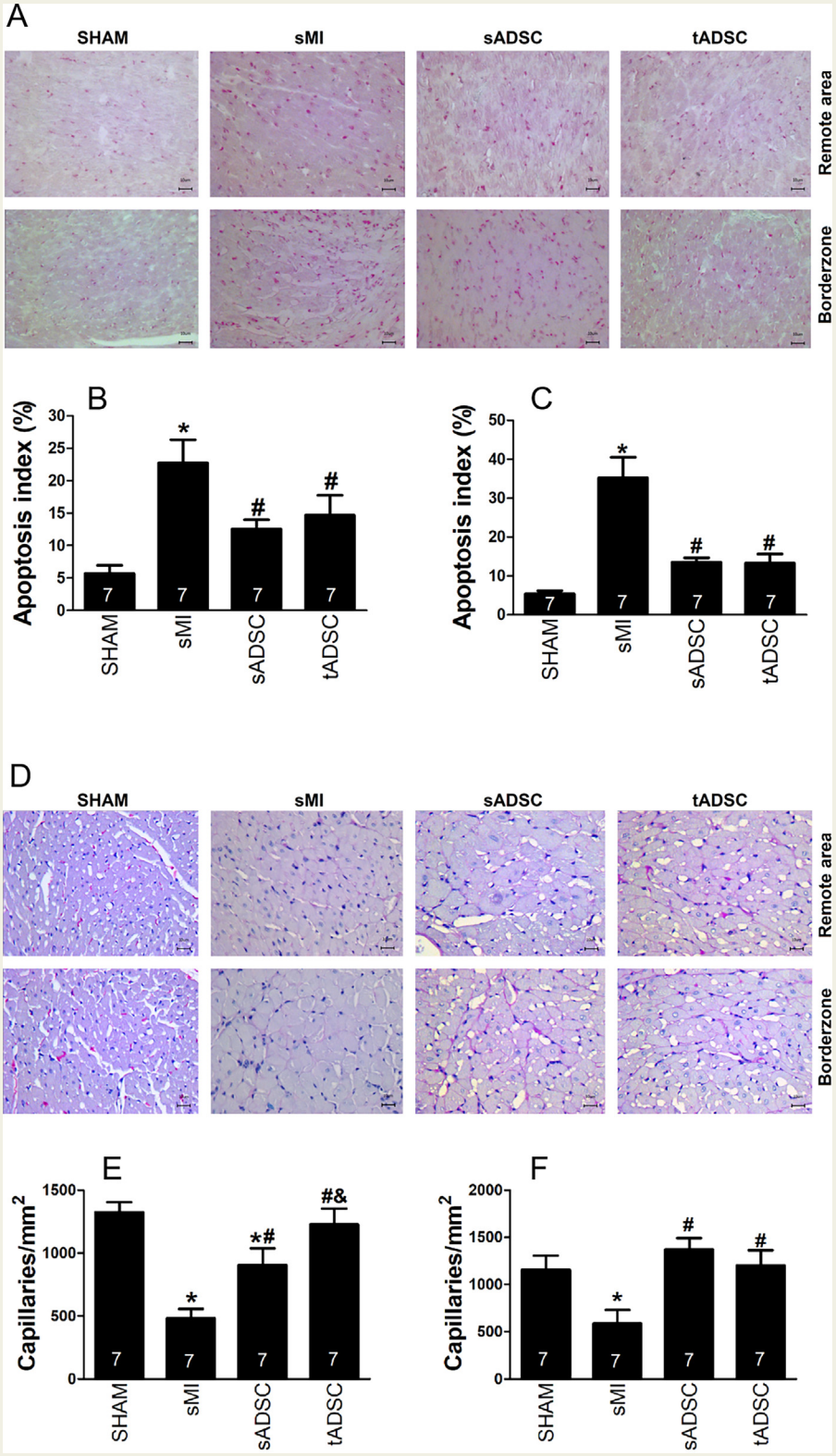

Figure 3 Apoptotic cardiomyocytes and myocardial capillarity in experimental groups. Experiments were performed 4 weeks after inducing MI. *(A)* Representative TUNEL photomicrographs; *(B)* relative apoptosis in the remote area (%); *(C)* relative apoptosis in the border zone (%); *(D)* Representative photomicrographs of periodic acid-Schiff staining; *(E)* Capillarity in the border zone; *(F)* Capillary density in the remote area. Numbers within the bar graphs indicate the number of rats per group. *$p < 0.05$ *vs.* SHAM; $^{\#}p < 0.05$ *vs.* sMI; $^{\&}p < 0.05$ *vs.* sADSC. Magnification ×40.
Abbreviations: ADSC, adipose-derived stem cells; MI, myocardial infarcation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

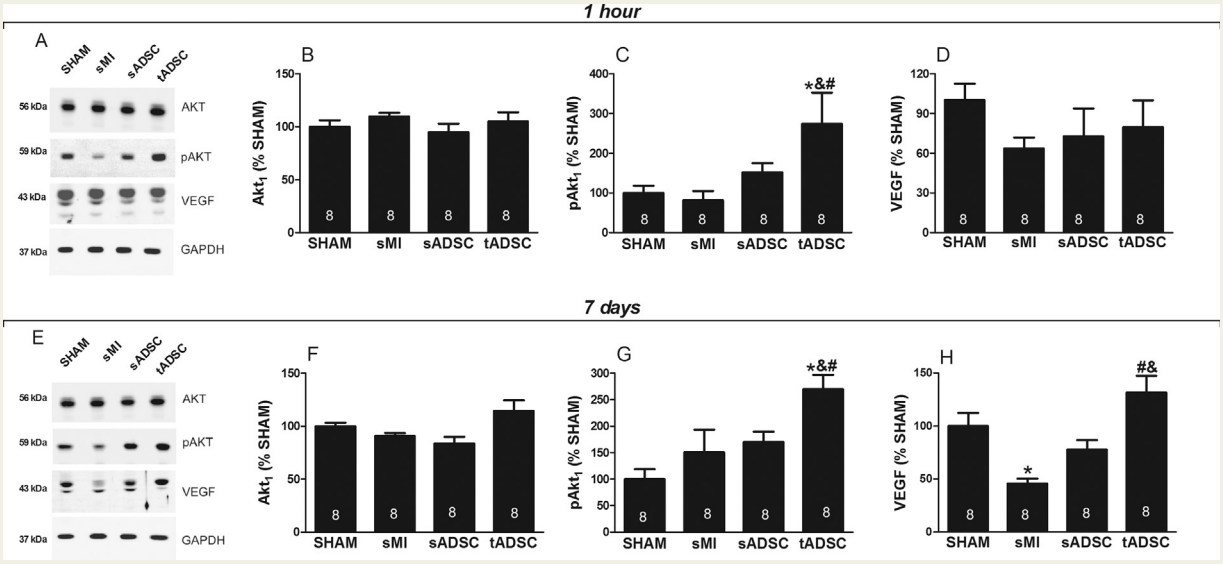

Figure 4 Exercise preconditioning promotes expression of angiogenic factors. Data are representative of six samples (remote myocardium) from each group. Panels A-D: Protein expression 1 day after ADSC transplantation. *(A)* Representative immunoblots; *(B)* Akt_1; *(C)* phosphoSer473Akt1; *(D)* VEGF. Panels E-H: Protein expression 7 days after ADSC transplantation. *(E)* Representative western blot experiments; *(F)* Akt_1; *(G)* phosphoSer473Akt1; *(H)* VEGF. $^{*}p < 0.05$ *vs.* SHAM; $^{\#}p < 0.05$ *vs.* sMI; $^{\&}p < 0.05$ *vs.* sADSC.
Abbreviations: ADSC, adipose-derived stem cells; VEGF, vascular endothelial growth factor.

myocardium strongly augments capillary density in the border zones and remote areas of infarction. These data corroborate a previous study of our group wherein bone marrow stem cells were used [36]. The increased capillary tissue is a key finding because SC therapy-related angiogenesis can result in reduced apoptosis, thus leading to structural and functional cardiac benefits following an infarction [37,38]. In fact, mechanistic revisions have shown that paracrine secretion is responsible for the angiogenesis that occurs in the treated myocardium post-SC transplantation. Thus, SC can induce angiogenesis by modulating pathways, releasing angiogenic factors (e.g. VEGF, bFGF, and PDGF) that stimulates neovessel formation [39] or by differentiating into vascular lineage [40].

We also found that the ADSCs inhibit cardiomyocyte apoptosis in both the regions. In spite of greater angiogenesis and repressed apoptosis, we did not find a significant impact on LV function with ADSC transplantation *per se*, without the addition of exercise. This apparent contradiction is not unique to our study, and it has been reported that SCs result in only modest recovery of cardiac function, despite their effects on angiogenesis and apoptosis [41]. Unfortunately, this disparity in observations is yet to be satisfactorily explained.

The novelty of this study is in its demonstration of the positive impact of exercise training on ADSC therapy. We demonstrated that prior exercise enables the restoration of capillary density in the border zone and the improvement of LV function with ADSC therapy, as illustrated by the greater SF. We also demonstrated that ADSC therapy associated with exercise had a greater impact on myocardial inotropism. Our findings of similar DT, RT, and +dT/dt data between the sham group and the tADSC group, in the analysis of papillary muscle function, indicate a synergistic action of exercise and ADSCs in restoring myocardial performance.

Exercise can regulate tissue survival factors such as Akt [34]. In our study, there was a greater phosphoSer473Akt1 (an active form) expression in the early and late stages following exercise training. We presumed that Akt signalling may blunt apoptosis, increase angiogenesis, and improve LV as well as *in vitro* myocardial performance. The benefits resulting from Akt and phosphoAkt overexpression following SC therapy are reported by several studies [42,43]. Here, phosphoSer473Akt1 was augmented only in the tADSC group, supporting our assumption regarding the role of exercise in Akt signalling. We also demonstrated that myocardial VEGF expression was not altered in untreated rats in the first 1 hour after infarction. Zhao et al. [44,45] demonstrated up-regulation of VEGF after ischaemia that returned to the baseline level by the second day. Our findings also indicate that the angiogenic potential is limited, as noted by a low VEGF level, 7 days following infarction. Interestingly, it appears that exercise has an important role in establishing higher VEGF levels, 7 days following the induction of infarction in rats that underwent SC transplantation. Higher VEGF expression is linked to higher post-infarction myocardial capillarity [46], and it appears that the combination of ADSC therapy with prior exercise is associated with improved VEGF expression, thus enhancing capillary density, and resulting in improved tissue perfusion. Unfortunately, we have not analysed the impact of this higher capillarity on myocardial perfusion. It is possible that future studies may use scintigraphy or contrast studies to correlate myocardial capillarity with myocardial perfusion.

Based on our demonstration of enhanced phosphoSer473Akt and VEGF expression in the first week of infarction in rats with prior exercise training, exercise training may be considered as a promising strategy for improving the myocardial microenvironment before ADSC transplantation. It is known that the hostile microenvironment following an MI, with reduced myocardial oxygen and nutrients, decreases the survival of ADSCs [7]. Interventions that enhance myocardial oxygenation are associated with increased SC engraftment and better functional cardiac recovery post-infarction [47]. Therefore, the molecular changes evoked by exercise training may contribute to a more powerful ADSC action on cardiac remodelling. Our study demonstrated a marked ADSC effect on capillary density, LV function and myocardial inotropism in trained rats compared to untrained rats, thus implying that exercise training may increase the myocardial rooming of transplanted SC [15].

Conclusion

The results of the present study illustrate a beneficial role of exercise training preconditioning on the myocardial microenvironment for ADSC transplantation following MI. Thus, ADSC-treated rats that were preconditioned with exercise training exhibited greater phosphoSer473Akt/VEGF signalling, neovascularisation, inhibition of apoptosis, and improved LV and *in vitro* myocardial performance. Our findings hold translational significance to humans in understanding how exercise training could be useful in potentiating the effects of ADSCs in the myocardial. Thus, exercise can be considered as an approach that precedes cell therapy in the treatment of MI.

Limitations

Although this study has shown that prior aerobic exercise training could positively affect the action of the SC in the infarcted myocardium, some limitations need to be considered. From a clinical perspective, the data in this study are applicable for people currently in exercise training, in whom they can experience a MI and following SC therapy. Additionally, our animals can be seen as having a high level of physical fitness (Figure 1B); thus, future research should examine if SC therapy may be of limited practical benefit for those that suffer a MI and have a low to moderate physical fitness level.

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Conflict of Interest

The authors declare that they have no competing interest. All authors have read and approved the final article.

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