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Voluntary running counteracts right ventricular adverse remodeling and myocyte contraction impairment in pulmonary arterial hypertension model

Leôncio Lopes Soares[a], Filipe Rios Drummond[b], Leonardo Mateus Teixeira Rezende[a], Alexandre Jaime Lopes Dantas Costa[a], Tiago Ferreira Leal[a], Meilene Ribeiro Fidelis[a], Mariana Machado Neves[b], Thales Nicolau Prímola-Gomes[a], Miguel Araujo Carneiro-Junior[a], Emily Correna Carlo Reis[c], Antônio José Natali[a,*]

[a] Universidade Federal de Viçosa, Departamento de Educação Física, Viçosa, Brazil
[b] Universidade Federal de Viçosa, Departamento de Biologia Geral, Viçosa, Brazil
[c] Universidade Federal de Viçosa, Departamento de Medicina Veterinária, Viçosa, Brazil

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ABSTRACT

Aim: Analyze the effects of voluntary running during the development of pulmonary arterial hypertension (PAH) induced by monocrotaline (MCT) on the right ventricle (RV) structure, RV myocyte contractility and intracellular Ca^{2+} transient in rats with MCT-induced PAH.

Main methods: Male Wistar rats were housed sedentary or with free access to a running wheel after MCT or saline injection for until HF or median end-point day of HF in sedentary animals (24 days). Echocardiographic examination and exercise tolerance test were carried out at specific time points of the experimental period. After euthanasia, the heart was dissected, weighed and processed for either histological or single myocyte contractility and intracellular Ca^{2+} transient analyzes.

Key findings: Voluntary running delayed the onset of HF (29 days) and the increase in pulmonary artery resistance, and improved exercise tolerance. In the median end-point day of HF, exercise retarded RV adverse remodeling (i.e. increase in extracellular matrix and collagen content). At this stage, exercise also delayed impairments in cell contractile function (i.e. amplitude and times to peak and to half relaxation) and intracellular calcium cycling (i.e. amplitude and times to peak and to half decay) in RV single myocytes.

Significance: Along with HF onset delay and physical effort tolerance enhancement, voluntary running during the development of PAH postpones pulmonary artery resistance increases, RV adverse remodeling and myocyte contractility and intracellular calcium cycling deterioration in rats. Therefore, self-paced intermittent exercise of high intensity may contribute positively to the health and survival of individuals with PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance which leads to right ventricular adverse remodeling, dysfunction and hence failure [1]. The functional capacity and tolerance to physical effort also are gradually reduced in PAH patients since the ability of their RV to increase stroke volume during exercise is impaired [2,3]. At the tissue level, in the model of progressive PAH induced by monocrotaline (MCT), the right ventricle (RV) undergoes hypertrophy and dilation associated with increased collagen deposition in the extracellular matrix, inflammation, apoptosis and fibrosis, which contributes to the impairment of RV function [4,5]. At the cellular level, RV myocytes commonly exhibit excitation-contraction coupling abnormalities [6,7], in which contractility and the intracellular Ca^{2+} transient often displays smaller amplitude and slower timecourses, leading to contractile dysfunction and reduced ability to respond to increased contractile demands [8]. It is acknowledged that these mechanical and structural factors are fundamental for the cardiac pump [9,10] and RV functional stability in heart failure [7,11].

The available therapies for the treatment of PAH comprise supportive (anticoagulants, diuretics and supplementary oxygen) and disease-targeted (vasodilators and antiproliferative agents) therapies, aiming at reducing the pulmonary arterial pressure and hence the afterload in the RV [12]. Therapeutic strategies capable of maintaining cardiac function are of clinical relevance, and one such strategy is physical exercise.

* Corresponding author. Av. P. H. Rolfs, s/n, Viçosa, Minas Gerais, Post code: 36570900, Brazil.
E-mail address: anatali@ufv.br (A.J. Natali).

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Despite the limitations imposed by PAH to physical exertion, there is growing evidence in humans [13–15] and in animal models [16–18], that physical exercise can promote beneficial effects to individuals with PAH.

Regarding exercise mode, it is recognized that it may influence study outcomes. For instance, continuous aerobic exercise (i.e. treadmill running) of different intensities (i.e. 60–80% VO_{2max}) was shown to diminish collagen deposition, fibrosis and inflammation [4,19] in the myocardium of rats with MCT-induced PAH, which positively impacted ventricular function. Despite that, low-intensity treadmill running (i.e. 50% VO_{2max}) was detrimental (i.e. increased pulmonary vascular adverse remodeling) to rats in this model [20]. In patients with left ventricular heart failure, the greatest benefits of exercise are seen with high-intensity training [21]. Even in MCT-induced PAH, high-intensity interval training in an electric treadmill [i.e. 2 min at 85–90% VO_2 reserve (~14 m/min) interspersed with 3 min at 30% VO_2 reserve (~5 m/min), for 30 min], but not low-intensity continuous training [i.e. 50% VO_2 reserve (~9 m/min) for 60 min], decreased RV systolic pressure, hypertrophy and fibrosis in rats [18]. However, with enforced regimes, the loss of control over locomotion and use of a reinforcement stimulus can trigger stress responses [22–24]. Moreover, continuous running is an unnatural mode of locomotion for rodents [22,24]. Furthermore, stress has a negative effect on heart failure prognosis [25,26], and enforced exercise is not used with humans.

In this way, it is important that we test voluntary exercise in our animal PAH models, since this more closely resembles how patients exercise, and previous studies have examined mostly forced exercise (e.g. treadmill running) which has known associated stress responses [22–24]. Though normal rats in voluntary running can attain speeds of ~45 m/min, the running is intermittent and carried out in short bursts [27]. Our group demonstrated that voluntary running performed during the development of MCT-induced PAH improved RV myocyte contractile function in rats and they kept exercising when heart failure signs developed [16], though the effects on the intracellular Ca^{2+} dynamics and RV structure and function have yet to be determined. Therefore, the aim of this study was to examine the effects of voluntary running during the development of MCT-induced PAH on the RV structure, and on the RV myocyte contractility and intracellular Ca^{2+} transient in rats.

2. Methods

2.1. Study design and voluntary running

Male Wistar rats [Body weight (BW): ~200 g] were housed individually in transparent polycarbonate cages (Model 80859S, Lafayette Instrument Company, Indiana, USA) equipped with stainless steel wheels of activity (diameter: 14" (35.56 cm), width: 4.3" (10.92 cm) of free access. The number of spins was recorded using digital magnetic counter (Cycle Computer-AS820 – Assize (Machine Motors LTDA; Camaquã – RS, Brazil), which allowed the calculation of the daily distance traveled and maximum running speed. These animals were randomly divided into three experimental groups: exercised control (EC, n = 12), exercised failure (EF, n = 12) and exercised median end-point (EM, n = 12). Because voluntary running is known to delay the onset of heart failure [16], animals from EM group had the parameters evaluated in the mean survival (± 1 day) of SF animals. Such strategy allowed us to point out the effects of voluntary running performed during the development of MCT-induced PAH at the same time point of non-exercised animals (SF group). All exercised animals were introduced to the running wheels 2 days before treatment with monocrotaline (MCT) or saline. Another group of rats of the same strain and weight did not have access to the running wheel and were housed in transparent polycarbonate cages, 4 animals per cage, and were divided into two groups: sedentary control (SC, n = 12) and sedentary failure (SF, n = 12).

All animals were kept in a room with controlled temperature (~22 °C) and ~60% relative humidity, under a 12/12 h light/dark cycles, and had free access to water and standard rodent chow. The distance and duration of running were recorded daily. After the MCT injection, the body weight (BW) was recorded once a week in the first three weeks and daily from the third week on until euthanasia. The Ethic Committee in Animal Use from Federal University of Viçosa approved the experimental protocol (protocol nº 81/2016) in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. Induction of pulmonary arterial hypertension

Animals from EF, SF and EM groups received a single intraperitoneal injection of 60 mg/kg BW of MCT (Sigma-Aldrich, St. Louis, MO, EUA) dissolved in saline (140 mM NaCl; pH 7.4) to induce PAH and heart failure [28]. Control animals (EC and SC) received the same volume of saline solution (140 mM NaCl; pH 7.4).

2.3. Physical effort tolerance test

The total exercise time until fatigue (TTF) was used as an index of physical effort tolerance. A protocol proposed by Koch and Britton [29], was adapted to measure exercise time until fatigue [30,31]. Succinctly, prior to the start of the physical training program, the animals were placed on an electric treadmill (AVS Projects®, SP, Brazil) for adaptation (10 min/day, 0° inclination, 5 m/min) for 3 days. Forty-eight hours after the adaptation period, the test was performed, where the animals started the exercise at 5 m/min, 0° inclination. Increases of 3 m/min were made every 3 min until fatigue. Fatigue was determined and the test was interrupted when the animal could no longer keep pace with the speed of the treadmill and remained for 10 s on the shock grid (0.28 mA) at the back of the treadmill rather than run [17]. The test was performed in all animals one week prior to the injection. On the 21st post injection the test was repeated in all animals; and on the 27th day after injection the test was repeated in animals from EC, EF, SC and SF groups.

2.4. Echocardiography

The echocardiographic evaluation was performed on the 22nd and 28th days after injection in animals from EC, EF, SC and SF groups, and on the 24th day in animals from EM group. The animals were anesthetized (Isoflurane 1.5% and 100% oxygen in a constant flow of 1L/min; Isoflurane, BioChimico, RJ, Brazil). The images were obtained while the animals remained in the lateral decubitus position. Two-dimensional studies with a fast sampling rate of 120 fps in M mode were performed using the MyLabTM30 ultrasound system (Esaote, Genoa, Italia) and 11 MHz nominal frequency transducers. The two-dimensional transthoracic echocardiography and M-mode was obtained at a scanning speed of 200 mm adjusted according to heart rate. Pulmonary artery flow was obtained by pulsatile Doppler. The acceleration (AT) and ejection (ET) times in the pulmonary artery were evaluated and its ratio (AT/ET) was calculated. The images were collected according to the recommendations of the American Society of Echocardiography and stored for further analysis [32].

2.5. Animal survival

Animals from SF and EF groups had their survival time registered at the euthanasia day, which took place upon the presence of heart failure. Heart failure was inferred based on the presentation of 10 g or more loss of BW overnight and/or dyspnea, cyanosis and lethargy by the animal. The median survival time represented the time after MCT treatment when more than 50% of the group reached the inferred heart failure end point.

2.6. Sample collection

Animals from EF and SF groups were euthanized by decapitation upon showing inferred heart failure, and on equivalent days for their respective controls (EC and SC). Animals from EM group were euthanized in median end-point (± 1 day) of the animals from SF group (i.e. on the 24th day after MCT injection). After euthanasia, the heart, ventricles and lungs were dissected, weighed and processed for analyzes of interest, as described below.

2.7. Histological analyzes

The histological analyzes of the RV were performed as previously described [33]. Briefly, immediately after collection, the RV was fixed in 10% formaldehyde for 48 h. Then, it was dehydrated in ethanol, clarified in xylol and embedded in paraffin. Blocks were cut into 5 μm-thick sections that were mounted on histological slides and stained with hematoxylin & eosin to count myocytes and extracellular matrix; or Sirius Red to count collagen fibers. To avoid repeated analyzes of the same histological area, the sections were evaluated in semi-series, using one in every 10 sections. Digital images from hematoxylin-eosin stained slides were captured using a light microscope (Olympus BX-50, Tokyo, Japan), whereas images from Sirius Red stained slides were obtained using a polarized light microscope (Olympus AX-70, Tokyo, Japan) connected to a digital camera (Olympus Q Color-3, Tokyo, Japan). For quantification of extracellular matrix, myocytes and collagen, a grid with 266 intersections was superimposed to the slides, and the intersections in specific tissue were counted and then the percentage was calculated. For all measures, twelve random images from each animal were used. All these measurements were performed using Image-pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). The myocyte cross-sectional area (CSA) was measured using a specific tool (manual measurement) in the software Image-pro Plus.

2.8. Isolation of myocytes

Myocytes from the RV were enzymatically isolated as previously described [34]. In short, after euthanasia, the heart was rapidly dissected, blotted dry and weighed. The heart as then attached to a Langendorff-retrograde perfusion system via aorta and perfused with Tyrode solution containing (in mM): 130 NaCl, 1.43 MgCl2, 5.4 KCl, 0.75 CaCl2, 5.0 Hepes, 10.0 glucose, 20.0 taurine and 10.0 creatine, pH 7.4 until for about 5 min. The Tyrode solution was thus exchanged to Tyrode solution containing EGTA (0.1 mM) for 5 min. Thereafter, the heart was perfused with Tyrode solution containing 1 mg/ml collagenase type II (Worthington, USA) and 0.1 mg/ml protease (Sigma-Aldrich, USA) for about 12 min. Following, the ventricles of the digested heart were removed, blotted dry and weighed. The entire RV also was separated, weighed and cut into small fragments. Such fragments were placed into a conical flask containing the enzymatic solution (collagenase and protease). The cells were mechanically separated by shaking the flask for 5 min. Thereafter, the dispersed cells were separated from the non-dispersed tissue by filtration. After centrifugation the resulting cells were suspended in Tyrode solution. The non-dispersed tissue was again subjected to the mechanical dispersion process. The solutions used in the isolation procedure were oxygenated (O_2 100% - White Martins, Brazil) and maintained at 37 °C. The isolated cells were stored at 5 °C until use. Isolated myocytes were used within 2 to 3 h after isolation.

2.9. Measurement of myocyte contractility and intracellular Ca^{2+} transient

The contractions of RV myocytes were measured by using an edge detection system (Ionoptix, Milton, USA) mounted on an inverted microscope (Nikon Eclipse - TS100, Japan) as previously described [30]. In summary, myocytes were accommodated in a bath on the stage of the inverted microscope and superfused with a Tyrode solution containing (in mM): 137 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.5 $MgCl_2$, 5 HEPES, 5.6 glucose, e 1.8 $CaCl_2$, (pH 7,4) at 37 ± 1 °C. Myocytes were externally stimulated (40 V, 5 ms) to contract at progressive frequencies (1, 3, 5 and 7 Hz) using an electric field stimulator (Myopacer, Ionoptix, Milton, USA). Individual myocytes were then visualized on a PC monitor using a CCD camera (Myocam, Ionoptix, Milton, USA) and the images of cell contractions were recorded. From the records, the amplitude of cell shortening (% of resting cell length) and the times to peak and to half relaxation of shortening were measured using software (IonWizard 6.3; Ionoptix, Milton, USA). Only myocytes that had clear, regular striated (sarcomere) pattern and did not spontaneously contract in the absence of external stimulation and responding to a 1 Hz-stimulation with a single twitch were analyzed.

The intracellular Ca^{2+} transient was measured in as previously described [35]. In brief, myocytes were loaded with calcium sensitive indicator fura-2 AM (ThermoFisher, Waltham, USA; 3 mM for 10 min) for whole cell epi-fluorescence at 37 ± 1 °C (Ionoptix, Milton USA) during external field stimulation (40 V, 5 ms) to contract at progressive frequencies (1, 3, 5 and 7 Hz) using an electric stimulator (Myopacer, Ionoptix, Milton, USA). The same inverted microscope and experimental bath and superfusion solution described above for myocyte contraction were used for the measurement of intracellular Ca^{2+} transient. The ratio of the emitted fluorescence at 510 nm in response to excitation at 340 and 380 nm (340 nm/380 nm ratio) was our index of intracellular Ca^{2+}. From the obtained records, the amplitude (Fura-2340/380 ratio) and the times to peak and to half decay of the intracellular Ca^{2+} transient were obtained using software (IonWizard 6.3; Ionoptix, Milton, MA, USA).

2.10. Statistics

The normality of the data was tested using the Kolmogorov-Smirnov test. The daily distance traveled data (on representative days 1, 8, 15 and 22) were compared using repeated measures analysis of variance (ANOVA) and Pearson's correlation was used to evaluate the relationship between total distance traveled, TTF, average speed in the voluntary running and survival. Survival data were compared using the Kaplan-Meier curve analysis by the Log-rank test. Data on cell parameters, body weight and organ weight were compared using ANOVA one-way or Kruskall-Wallis, according to the distribution of data, followed by appropriate post-hoc for multiple comparisons. The statistical test used is specified in the tables and figures. Data are presented as mean ± SEM. $P < 0.05$ was considered for statistically significant differences. All analyzes were performed using GraphPad Prism, version 6.01 (San Diego, CA, USA).

3. Results

3.1. Physical effort tolerance

Details of daily voluntary running and physical effort tolerance test are presented in Fig. 1. There was no significant difference between groups of the average daily running distance traveled (~2–3 km/day) on representative days (days 1, 7, 14 and 21) (Fig. 1 A). The average speed of voluntary running is showed in Fig. 1B. Such speed progressed to and stabilized around 35 m/min over the experimental period, but no significant difference between groups was found on representative days. Regarding the mean TTF, there was no difference between groups before MCT injection (Fig. 1C). Likewise, at this point the mean maximum speed reached during the physical effort tolerance test (~30 m/min) was not different between groups (Fig. 1D). However, on the 21st day after MCT injection, the mean TTF of SF group was not different from that of SC group, but lower than that of EM and EF groups (Fig. 1E). At this point, the mean maximum speed followed the same pattern (Fig. 1F). In addition, on the 27th day after MCT injection

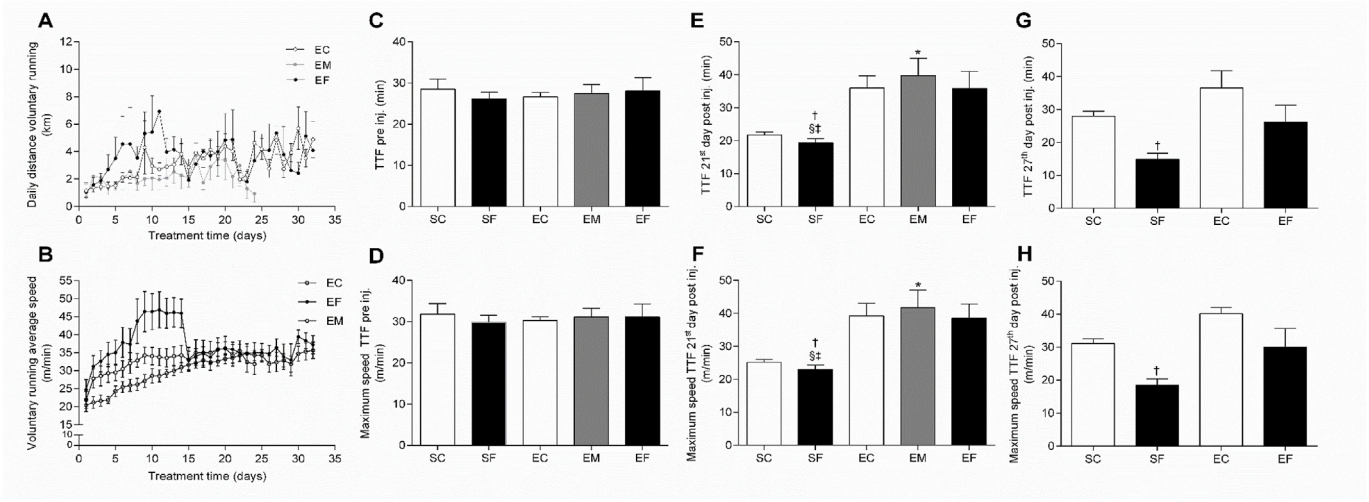

Fig. 1. Effect of voluntary running on physical effort tolerance. (A) Traveled distance of voluntary running. (B) Average speed of voluntary running. (C) Total exercise time until fatigue (TTF) measured prior to monocrotalin (MCT) injection. (D) Maximum speed in the physical effort tolerance test prior to MCT injection. (E) TTF measured 21 days after MCT injection. (F) Maximum speed in the physical effort tolerance test measured 21 days after MCT injection. (G) TTF measured 27 days after MCT injection. (H) Maximum speed in the physical effort tolerance test measured 27 days after MCT injection. Data are mean ± SEM of 7 rats in each group. SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. *P<0.05 vs. SC; †P<0.05 vs. EC; §P<0.05 vs. EF; ‡P<0.05 vs. EM. One-way repeated measures ANOVA followed by the Tukey post hoc test to compare representative days 1, 8, 15, and 22 (Panels A and B); One-way ANOVA followed by the Tukey post hoc test for between group comparisons (Panels C, D, E, F, G and H).

(Fig. 1G) only animals from EC group showed a higher TTF, compared to that of SF animals. Again, the mean maximum speed (Fig. 1H) at this time point followed the mean TTF pattern.

We also calculated the mean change in wheel running distance between week one and final week for EF (8.77 ± 8.84 km), EC (9.95 ± 4.42 km), and EM (8.42 ± 4.80 km) rats. There was no statistical difference between groups, and it did not correlate with other endpoints measured. Likewise, the mean change in wheel running speed between week one and final week for EF (0.19 ± 0.32 m/min), EC (0.63 ± 0.13 m/min), and EM (0.19 ± 0.11 m/min) rats was not statistically different between groups, and no correlation with other endpoints measured was found.

3.2. Animal survival

Fig. 2 shows the survival of animals from SF and EF groups. Although all animals in these groups showed signs of heart failure, the animals in the EF group had a longer average survival (29 days) than those in the SF group (24 days; $p < 0.05$). Moreover, significant correlation ($p < 0.05$) was observed between survival and total distance traveled ($r = 0.75$), TTF ($r = 0.75$) and average speed ($r = 0.54$).

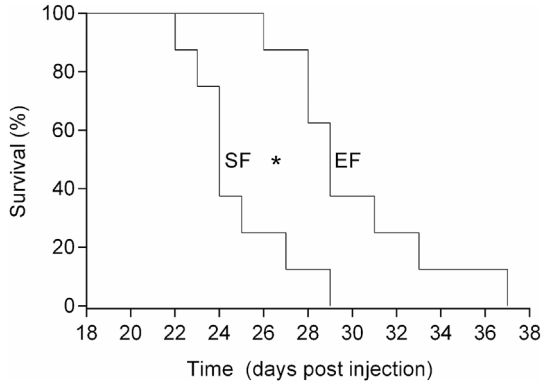

Fig. 2. Effect of voluntary running on survival measured on days of onset of signs of heart failure. SF, sedentary failure. EF, exercise failure. *P<0.05, Kaplan-Meier Curve, followed by the Log-rank post-hoc test.

3.3. Pulmonary artery resistance

Fig. 3 shows the echocardiographic data. The AT/ET was lower ($p < 0.05$) in animals from SF group, compared to that of rats from SC and EC groups, measured on the 21st day after MCT injection (Fig. 3B). Likewise, when measured on the 24th day after MCT injection, the AT/ET values were lower in animals from SF and EF groups, compared to those of rats from SC and EC groups (Fig. 3C). The AT/ET values from EM group were not different from those of the other groups.

3.4. Right ventricular adverse remodeling

Table 1 exhibits the data for whole animal and organ parameters. The initial body weight (BW) was not different between groups. The final BW as well as the BW gain of animals injected with MCT did not differ from those from their respective controls. Animals from SF and EF groups had greater RV weight (RVW) than those in rats from their respective control groups. In addition, the RVW of EM group was higher than that of SC. Likewise, the Fulton Index (RVW to left ventricular + septum weight ratio) of animals from SF and EF groups was higher than those in rats from their respective control groups and EM group. The lung weight and its ratio to BW were also higher in animals from SF and EF groups than those in their respective control groups.

Fig. 4 displays the RV histological data. Animals from SF and EF groups had a lower percentage of myocytes (indicated by white arrows in panel A; and mean data in panel B) and higher percentages of extracellular matrix (indicated by black arrows in panel A; and mean data in panel C), compared to those in animals from respective control groups (SC and EC). In addition, RV myocytes in animals from SF and EF groups exhibited larger CSA (Fig. 4D) than those in rats from respective control groups (SC and EC). Thus, voluntary running was able to delay these changes, since animals from EF group showed values better than those for rats in the SF group.

Fig. 5 displays the interstitial collagen in the RV. Animals from SF and EF groups had a higher percentage of interstitial collagen, compared to those in animals from respective control groups (SC and EC) (Fig. 5). Voluntary running was able to positively affect these changes, since animals from EM group had values lower than those from SF group, and both of these groups were harvested at approximately the

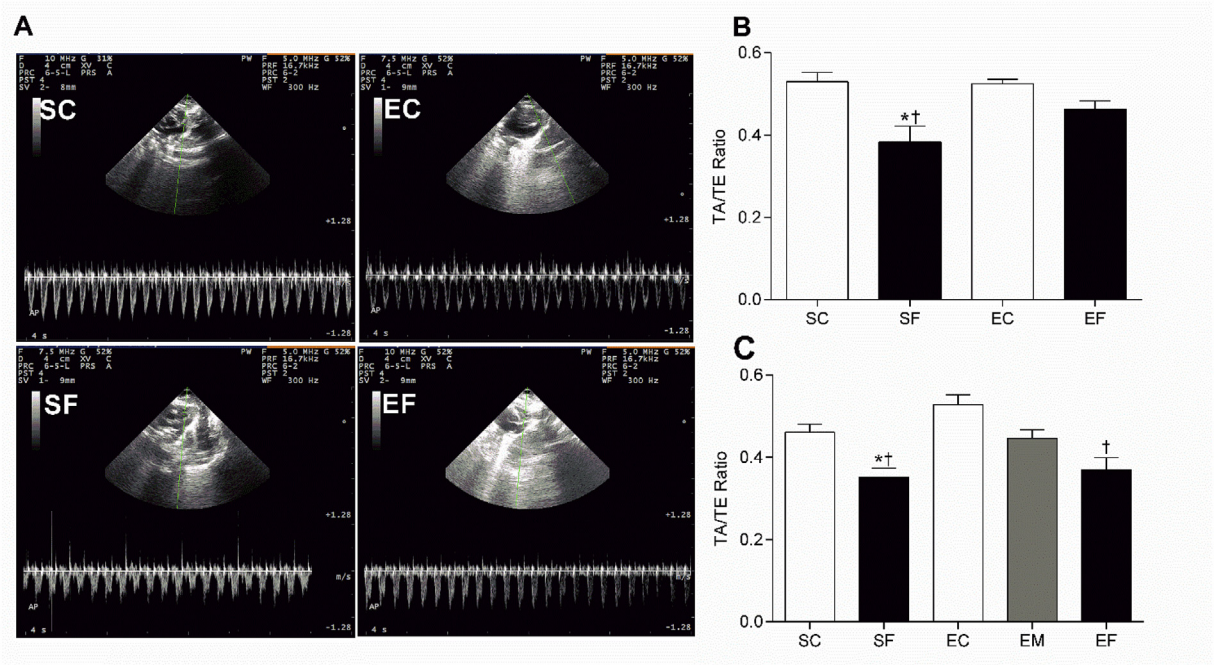

Fig. 3. Effect of voluntary running on the pulmonary artery resistance. (A) Representative images of pulmonary artery flow. (B) Ratio of flow acceleration time to ejection time (AT/ET) in the pulmonary artery measured 22 days after injection of MCT. (C) AT/ET measured 24 days (EM group) and 28 days (other groups) after injection of MCT. SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. Data are mean ± SEM of 5–7 rats in each group. One-way ANOVA followed by the Tukey post hoc test: *P< 0.05 vs. SC; †P<0.05 vs. EC.

same time point (i.e. 24 days).

3.5. Right ventricular myocyte contractility and intracellular Ca^{2+} transient impairments

Data for RV myocyte contractile function are presented in Fig. 6. Myocytes from SF and EF animals showed lower amplitude of shortening at the stimulation frequencies of 3, 5 and 7 Hz, compared to that from their respective control (SC and EC) and EM groups (Fig. 6B).

Data are mean ± SEM of 12 images per animal in each group (n = 4 rats in each group). SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. One-way ANOVA followed by the Tukey post hoc test: *P<0.05 vs. SC; †P<0.05 vs. EC; §P<0.05 vs. EF; ‡P<0.05 vs. EM. White arrow indicate myocyte; Black arrow indicate extracellular matrix.

Regarding the timecourse of shortening, myocytes from SF and EF animals presented higher time to peak than those from respective control groups at all stimulation frequencies assessed (Fig. 6C). Compared to EM group, myocytes from SF rats presented longer time to peak at 1, 3, 5 and 7 Hz, whereas myocytes from EF rats showed longer time to peak only at 1, 3 and 5 Hz. Likewise, myocytes from SF and EF animals exhibited longer time to half relaxation (Fig. 6D), compared to those of myocytes from rats in respective control (SC and EC) and EM groups at the frequencies of stimulation of 1, 3 and 5 Hz. It was also observed that at the frequency of 3 Hz, myocytes from SF animals presented longer time to half relaxation than those from EF rats.

Figure 6E shows the proportion of cells responding to the stimulation frequency of 7 Hz. It was found that the proportion of myocytes able to entrain 7 Hz was smaller in the failing groups (SF and EF), than in their respective controls (SC and EC).

Fig. 7 shows data for the intracellular Ca^{2+} transient in RV myocytes. The amplitude was higher in EF group than in EC, EM and SF groups, at the frequencies of 1 and 3 Hz (Fig. 7B). When stimulated at 1 Hz, the amplitude was greater in myocytes from EC and EM groups than that in cells from SC group. At the stimulation frequency of 5 Hz, the amplitude in myocytes from EF group was higher ($p<0.05$) than those in myocytes from EM, SC and SF groups; while the amplitude in EC group was higher than in SC group. When stimulated at 7 Hz, cells from animals in EM, SC and EF groups presented greater amplitude, when compared to that in SF group.

Concerning the timecourse of the intracellular Ca^{2+} transient (Fig. 7C), at the stimulation frequencies of 1 and 3 Hz myocytes from

Table 1
Effect of voluntary running on whole animal and organ parameters.

	SC	EC	SF	EF	EM
BW initial, g	229.85 ± 5.69	227.42 ± 7.35	229.71 ± 6.09	222.14 ± 5.97	222.71 ± 8.15
BW final, g	306.00 ± 12.34	324.14 ± 10.66	273.16 ± 14.26†	284.00 ± 7.40	290.66 ± 11.27
Δ BW, g	76.14 ± 15.26	96.71 ± 8.38	43.45 ± 14.69†	61.86 ± 6.59	67.95 ± 7.00
RVW, g	0.25 ± 0.01	0.29 ± 0.02	0.39 ± 0.02*	0.50 ± 0.04*†	0.40 ± 0.03*
LV + SW, g	0.89 ± 0.03	0.93 ± 0.07	0.92 ± 0.07	0.98 ± 0.05	1.08 ± 0.03
RVW: LV + SW, g/g	0.27 ± 0.01	0.32 ± 0.03	0.43 ± 0.03*	0.51 ± 0.03*†‡	0.37 ± 0.03
LW, g	1.54 ± 0.12	1.63 ± 0.07	3.18 ± 0.21*†‡	3.52 ± 0.29*†‡	2.32 ± 0.12
LW: BW, mg/g	4.05 ± 0.08	5.05 ± 0.14	11.67 ± 0.92*†‡	12.55 ± 1.27*†‡	7.96 ± 0.46*

Data are mean ± SEM of 5–7 rats in each group. SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. BW, body weight; RVW, right ventricle weight; LV + SW, left ventricle plus septum weight; LW, lung weight; *P<0.05 vs. SC; †P<0.05 vs. EC; ‡P<0.05 vs. EM. One-way ANOVA followed by the Tukey post hoc test.

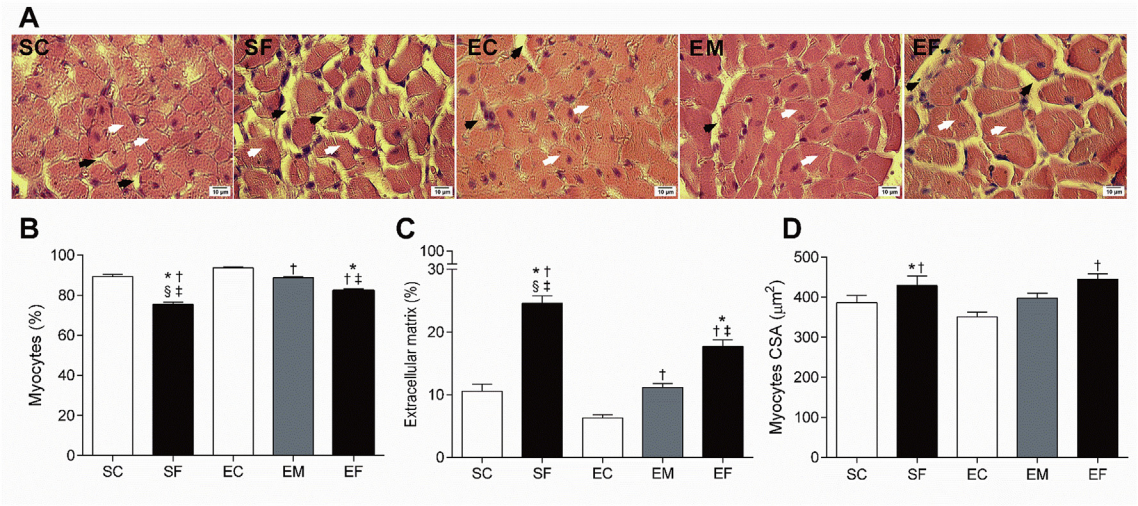

Fig. 4. Effect of voluntary running on right ventricle remodeling. (A) Representative photomicrographs of right ventricle tissue (Hematoxylin & Eosin staining). (B) Percentage of cardiomyocytes. (C) Percentage of extracellular matrix. (D) Cross-sectional area of cardiomyocytes (CSA).

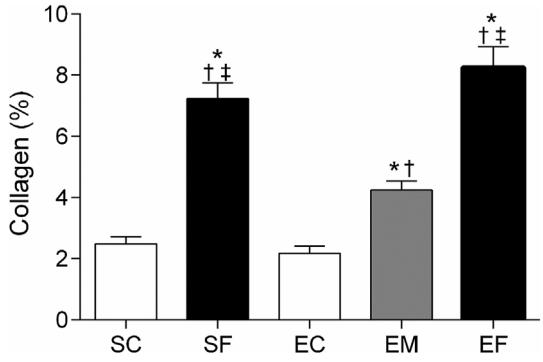

Fig. 5. Effect of voluntary running on interstitial collagen in the right ventricle. Data are mean ± SEM of 12 images per animal in each group (n = 4 rats in each group). SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. One-way ANOVA followed by the Tukey post hoc test: *P<0.05 vs. SC; †P<0.05 vs. EC; ‡P<0.05 vs. EM.

failure groups (SF and EF) exhibited higher time to peak, compared to those in myocytes from their respective controls (SC and EC), and those in myocytes from EM group. When stimulated at 5 and 7 Hz, the time to peak was higher in myocytes from EF group, compared to that in myocytes from EC group.

As for the time to half decay of the intracellular Ca^{2+} transient (Fig. 7D), cells from failure animals (SF and EF groups) displayed higher values at stimulation frequencies of 1, 3 and 5 Hz, than those in myocytes from their respective control (SC, EC) and from EM groups. Furthermore, when stimulated at 1 Hz, myocytes from SC group had higher time, compared to that of myocytes from EC and EM groups.

4. Discussion

In the present study we examined the effects of voluntary running during the development of PAH on the RV structure and on the RV myocyte contractility and intracellular Ca^{2+} transient in rats with MCT-induced PAH. Our findings evidence that along with physical effort tolerance enhancement (i.e. increased TTF) and inferred HF onset delay (i.e. increased survival), voluntary running postponed pulmonary artery resistance increases (i.e. reduced AT/ET), RV adverse remodeling (i.e. reduced myocyte CSA; % of extracellular matriz and collagen) and RV myocyte contractility (i.e. increased shortening amplitude; reduced times to peak and to half relaxation) and intracellular calcium cycling deterioration (i.e. reduced times to peak and to half decay).

The exercise regime employed during the development of PAH was able to increase tolerance to physical effort in animals with PAH, measured 21 days after MCT injection at different time points of PAH development (i.e. EM and EF). This finding might be explained by the beneficial effects of exercise training at the level of skeletal muscle [27] that prevents muscle wasting and dysfunction [36,37], and improves RV cardiac function [38]. Rats with MCT-induced PAH had their aerobic capacity and hemodynamics improved by an 8-week high-intensity interval training (HIIT) mainly due to favorable pulmonary vascular endothelial adaptation (i.e. greater lung endothelial nitric oxide synthase) to the pulsatile HIIT stimulus [18]. In the present study, the average speed of voluntary running observed was ~35 m/min, which is far higher than that used in the HIIT by Brown et al. [18] (i.e. 14 m/min). Although we did not monitor the exercise bout duration and interval between bouts, rats are known to run voluntarily in wheels in short bursts (~60 to 120 s) interspersed with intervals [27]. Therefore, considering that voluntary running is a natural mode of locomotion for rodents, free wheel running may actually be advantageous when compared to the HIIT training regimen since HIIT utilizes forced exercise whereas free wheel running does not.

Furthermore, admitting that the beneficial effects of exercise training on skeletal muscle was not measured here, the positive effects of exercise training on pulmonary vascular reactivity [39] or on the efficacy of pulmonary gas exchange and preventing hypoxemia during the exercise test [40] also has to be considered. In this way, the reduced pulmonary artery resistance (i.e. increase in AT/ET ratio) found in EM and EF animals, may infer a more effective transfer of blood from RV to pulmonary circulation. In fact, physical exercise has been shown to improve cardiac function, ventilatory efficiency and cardiorespiratory fitness in individuals with PAH [13,41,42].

Although unexpected, healthy and sick animals had no difference in TTF and maximum speed at the 21st day after MCT injection. It has to be considered that the effects of MCT are progressive and being so the running capacity of sick animals was not affected since in this model the signs of heart failure are normally observed from the 21st day after injection on. In fact, the running capacity of sick animals reduced from the 21st to the 27th day after injection [SF: 21st vs 27th ($p = 0.0006$); EF: 21st vs 27th ($p = 0.003$)] which did not occur within healthy rats. Although the positive effects of exercise did not persist until the 27th day after MCT injection when the signs of inferred heart failure were present in SF rats, one important finding here was that voluntary running postponed the appearance of heart failure in EF animals.

Indeed, alongside such increased tolerance, MCT treated animals who had access to voluntary wheel had the onset of inferred heart

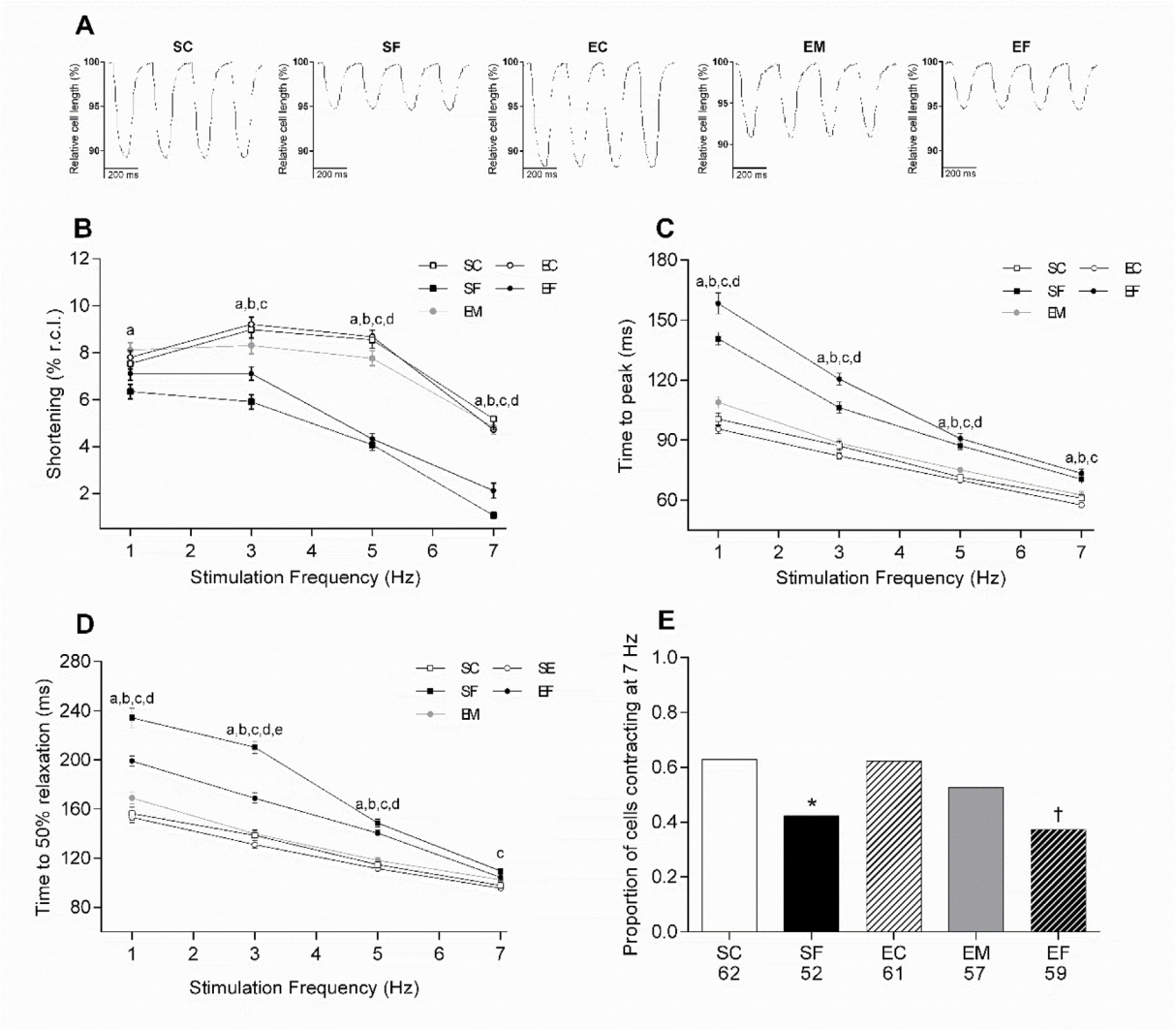

Fig. 6. Effect of voluntary running on right ventricular myocyte contractility. (A) Typical cell shortening traces (stimulation 5 Hz). (B) Amplitude of shortening. (C) Time to peak. (D) Time to 50% relaxation. (E) Proportion of cells responding to stimulation at 7 Hz. SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. Values are means ± SEM of 6–10 cells per animal in each group (n = 7 mice in each group). Kruskall-Wallis, followed by the Dunn's post hoc test: $p < 0.05$, [a]EM *vs.* SF; [b]EC vs. EF; [c]SC vs. SF; [d]EM vs. EF; [e]EF vs. SF; [f]EC vs. SC; [g]EM vs. SC. Panel E: X^2 test, $^*p<0.05$ *vs. SC;* $^\dagger p < 0.05$ *vs.* EC. Number of cardiomyocytes that responded to 1, 3 and 5 Hz are shown in panel E.

failure postponed, augmenting thus their survival (e.g. r = 0.75 between survival, voluntary running distance and TTF). The survival prognosis in humans [43] can be significantly improved by exercise training specially when started in the early stages. The amelioration in AT/ET ratio observed in our exercised rats have important predictive value in PAH for the assessment of disease severity and mortality, and may support the better prognosis that we found in EM group [44–46].

The increase in pulmonary artery resistance is a crucial factor for the adverse remodeling of the RV [5]; and has been associated with an unbalance between vasoconstrictors (e.g. endothelin-1; thromboxane) and vasodilators (e.g. nitric oxide - NO; prostanoids), where vasoconstrictors augments [47], contributing thus to vascular stiffening and remodeling. Thus, reductions in pulmonary artery resistance and PVR are fundamental for reducing RV overload and hence adverse remodeling, which is associated with the maintenance of cardiac function [5]. Our data show that the increase in pulmonary artery resistance (i.e. increased AT/ET ratio) found in animals from SF group was reduced in rats from EF and EM groups, which indicates a protective role of voluntary running. The alternate increase in shear stress typical of intermittent exercise triggers the synthesis and release of nitric oxide and other endothelial vasodilators [48], which in long-term leads to benign arterial remodeling. For instance, high-intensity interval training was reported to prevent arterial adverse remodeling and augments in pulmonary vascular resistance in experimental PAH [18].

Our results revealed that the RV of failing animals (SF and EF) displayed lower amounts of myocytes and higher amounts of extracellular matrix and collagen than those of control rats (SC and EC). Such lower percentage of myocytes is characteristic of this disease [49] and since extracellular matrix and collagen were increased in these animals it may be due to apoptosis, as demonstrated previously [18]. This finding reflects the adverse remodeling of the RV in response to the sustained increase in pulmonary vascular resistance [17,33] which leads to progressive contractile dysfunction and hence failure [1]. In fact, we demonstrated that the pulmonary artery resistance of animals from SF and EF was augmented. More important, these negative changes in the extracellular matrix were attenuated in the RV of animals from EM group. Such evidence gives support to the defensive effect of voluntary running performed during the development of MCT-induced PAH. Exercise training has been shown to oppose the RV adverse remodeling by decreasing the circulating and tissue levels of TNF-α (tumor necrosis factor alpha), NF-kβ (nuclear factor kappa B) and caspase-3, as well as the expression of TWEAK (receptors of inductor

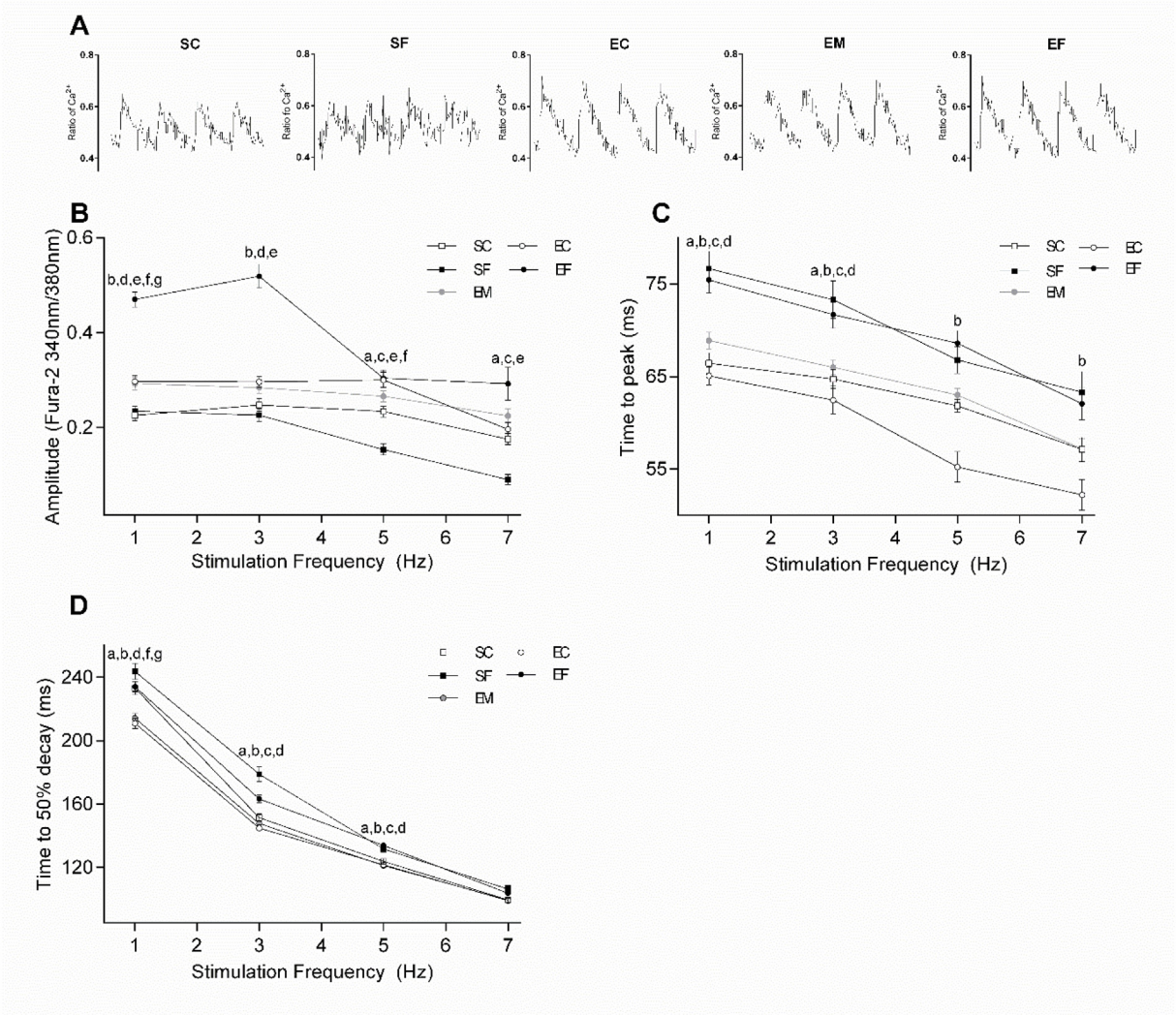

Fig. 7. Effect of voluntary running on right ventricular myocyte intracellular Ca^{2+} transient. (A) Typical traces of the intracellular Ca^{2+} transient (stimulation at 5 Hz). (B) Amplitude. (C) Time to peak. (D) Time to 50% decay. (E) Proportion of cells responding to stimulation at 7 Hz. SC, sedentary control; EC, exercised control; SF, sedentary failure; EF, exercised failure; EM, exercised median. Values are means ± SEM of 6–10 cells per animal in each group (n = 7 rats in each group). Panels B, C, D: Kruskall-Wallis, followed by the Dunn's post hoc test: $P < 0.05$, [a]EM *vs.* SF; [b]EC vs. EF; [c]SC vs. SF; [d]EM vs. EF; [e]EF vs. SF; [f]EC vs. SC; [g]EM vs. SC.

tumor necrosis factor), that are related to oxidative stress, inflammation and apoptosis in the RV of animals with experimental PAH [4].

Right ventricular hypertrophy also was observed in rats with inferred heart failure - SF and EF groups (greater RVW to LV + SW ratio and myocyte CSA, compared to SC group). Such remodeling was shown to be accompanied by adverse changes in the extracellular matrix and increased pulmonary artery resistance in the present study and elsewhere [4,5]. However, in the EM group, the hypertrophic response was prevented; as was the extracellular matrix deterioration and pulmonary artery resistance increase. Therefore, again, voluntary running delayed the progression of the RV adverse remodeling in these animals, mainly by reducing the pulmonary artery resistance playing thus a protective role.

We observed that the amplitude of shortening was reduced in RV myocytes from inferred HF animals (SF and EF), compared to controls (SC and EC), whereas the times to peak and to half relaxation of shortening were prolonged. The reduction in cell shortening amplitude is associated with decreased sensitivity of the myofilaments to Ca^{2+} and reduced intracellular Ca^{2+} [7,50]. The prolonged time to peak of shortening, in turn, is due to a slow release of Ca^{2+} from the sarcoplasmic reticulum (SR) via ryanodine receptor 2 (RyR2), while the longer time to half relaxation is caused mainly by a slow reuptake of Ca^{2+} from the cytosol into the RS via SERCA2a (Ca^{2+}-ATPase of the SR) that is phosphorylated by PLB (phospholamban) [51]. Indeed, previous studies have shown that SERCA2a and RyR2 proteins are reduced in the RV of rats with MCT-induced PAH [17,52].

Furthermore, RV myocytes from rats with inferred HF (SF and EF) showed a negative contraction-frequency relationship (3, 5 and 7 Hz). This phenomenon has been observed in this model of PAH [16], which is accompanied by a fall in the amplitude of the intracellular Ca^{2+} transient [7,11]. These findings suggest the inability of these myocytes to respond to increased contractile demands, a characteristic of heart failure [7]. For example, a lower proportion of cells responding to high stimulation frequency (i.e. 7 Hz) in these animals, in relation to control ones, was observed here and elsewhere [16].

On the other hand, voluntary running was demonstrated to counteract the myocyte contractile function impairment by PAH. In this sense, RV myocytes in animals from EM group presented shortening and times to peak and to half relaxation similar to those from control groups (SC and EC). A possible explanation is that exercise prevented the deterioration of the calcium regulatory proteins in the RV of these animals. For example, previous studies have shown increased expression of SERCA2a in the RV of rats with MCT-induced PAH submitted to moderate intensity aerobic training on a treadmill [17]. Despite that, no

equivalent effect of aerobic exercise training on SERCA2a, RyR2 and PLB was found by others [52]. Then, further investigations are needed to reveal the effects of voluntary running on the Ca^{2+} regulatory proteins in cardiomyocytes of animals with MCT-induced PAH. Furthermore, the proportion of cells responding to the stimulation frequency of 7 Hz was intermediate between the control and failure groups. These results reinforce the protective role of voluntary running against the progression of the PAH-induced functional damage in the right heart.

Concerning the intracellular Ca^{2+} transient, as expected, data for the intracellular Ca^{2+} transient followed those of cell shortening. RV myocytes from inferred HF group (SF) exhibited lower amplitude (5 and 7 HZ), as well as prolonged times to peak (1, 3, 5 and 7 Hz) and to half decay (1, 3 e 5 Hz), compared to control groups. These results indicate the disturbance of the intracellular Ca^{2+} regulatory proteins caused by PAH in these animals [8,17], as mentioned above. Moreover, the negative contraction-frequency relationship was also observed here.

Like in cell shortening, myocytes from EM group displayed the parameters of the intracellular Ca^{2+} transient comparable to those from control groups. This benefit of exercise reflects a better RV contractile function. It appears thus that voluntary running prevented the deterioration of the calcium regulatory proteins induced by PAH in these animals. Indeed, the RV of rats with MCT-induced PAH undergoing moderate-intensity aerobic exercise showed greater increased of SERCA2a [17].

It is worse to note that myocytes from EF group showed higher amplitude of the intracellular Ca^{2+} transient than those from SC and EM groups at the stimulation frequencies of 1 and 3 Hz, which are below those physiological ones for rats. Indeed, at physiological frequencies (i.e. 5 and 7 Hz), such alteration has disappeared. Since the higher amplitude of the intracellular Ca^{2+} transient at 1 and 3 Hz did not result in greater cell shortening, it suggests reduction in the myofilament sensitivity to Ca^{2+}. Although it is unexpected, it warrants further studies inasmuch as up to date there is no reports on that.

Finally, the improvements caused by exercise in the tissue and cellular parameters presented herein are associated with the re-establishment of pulmonary artery resistance in the attenuation of its adverse remodeling. Therefore, although the RV function has not been evaluated, we believe that the enhancement in exercise tolerance and survival promoted by voluntary running is mediated by the improvement in the RV tissue structure and cellular contractile function, which positively reflects in the RV function.

4.1. Study limitations

This study has limitations. First, we did not monitor the exercise bout duration and interval between bouts. Despite that, voluntary running is known to be performed in short bursts (~60 to 120 s) interspersed with intervals [27]. Second, our echocardiographic examination was limited, thus the development of pulmonary hypertension could not be confirmed by the measurement of RV hemodynamics. However, we believe that the enhancement in exercise tolerance and survival promoted by voluntary running is mediated by the improvement in the RV tissue structure and cellular contractile function, which positively reflects in the RV function; and third, the exercised animals were housed individually in order to have free access to the running wheel, different from sedentary animals who were housed in groups. We acknowledge that solo housing is stressful to rats and mice. Nevertheless, such stress appears to affect especially female animals as reviewed by Beery and Kaufer (2015) [53]. Therefore, we believe that in the present study the isolation of rats with free access to running wheels had minimum impact on their physiology.

5. Conclusion

In conclusion, along with inferred HF onset delay and physical effort tolerance enhancement, voluntary running during the development of PAH postpones pulmonary artery resistance increases, RV adverse remodeling and myocyte contractility and intracellular calcium cycling deterioration in rats. We believe such results are of clinical relevance insofar as it indicates that self-paced exercise of high intensity may contribute positively to the health and survival of individuals with PAH. Furthermore, these results give support to the prescription of high-intensity interval training to individuals with cardiopulmonary disease.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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