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Original Paper

Cardioprotection Generated by Aerobic Exercise Training is Not Related to the Proliferation of Cardiomyocytes and Angiotensin-(1-7) Levels in the Hearts of Rats with Supravalvar Aortic Stenosis

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Key Words
Cardiac dysfunction • Physical training • Cell cycle • Renin-angiotensin system • Cardiac remodeling

Abstract
Background/Aims: The beneficial effect of aerobic exercise training (ET) on cardiac remodeling caused by supravalvar aortic stenosis (AS) has been demonstrated in experimental studies; however, the mechanisms responsible for improving cardiac function are not entirely understood. We evaluated whether ET-generated cardioprotection in pressure-overloaded rats is dependent on cardiomyocyte proliferation, increased angiotensin-(1-7) (Ang-1-7) levels, and its receptor in the myocardium. ***Methods:*** Eighteen weeks after ascending AS surgery, Wistar rats were randomly assigned to four groups: sedentary control (C-Sed), exercised control (C-Ex), sedentary aortic stenosis (AS-Sed) and exercised aortic stenosis (AS-Ex) groups. The moderate treadmill exercise protocol was performed for ten weeks. The functional capacity was assessed by treadmill exercise testing. Cardiac structure and function were evaluated by echo-

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cardiogram. Cardiomyocyte proliferation was evaluated by flow cytometry. Expression of cell cycle regulatory genes as CCND2, AURKB, CDK1, and MEIS1 was verified by RT-qPCR. Cardiac and plasma angiotensin I (Ang I), angiotensin II (Ang II), and Ang-(1-7) levels were analyzed by high-performance liquid chromatography (HPLC). The angiotensin-converting enzyme (ACE) activity was assessed by the fluorometric method and protein expression of AT1 and Mas receptors by Western blot. ***Results:*** The AS-Ex group showed reduced left ventricular wall relative thickness and improved ejection fraction; also, it showed decreased gene expression of myocyte cell cycle regulators, ACE, Ang I, Ang II and Ang II/Ang-(1-7) ratio levels compared to AS-Sed group. However, ET did not induce alterations in Ang-(1-7) and cardiac *Mas* receptor expression and myocyte proliferation. ***Conclusion:*** Aerobic exercise training improves systolic function regardless of myocyte proliferation and Ang-(1-7)/*Mas* receptor levels. However, the ET negatively modulates the vasoconstrictor/hypertrophic axis (ACE/Ang II) and decreases the expression of negative regulatory genes of the cell cycle in cardiomyocytes of rats with supravalvular aortic stenosis.

Introduction

Cardiovascular diseases constitute the leading cause of global mortality and are a major contributor to reduced quality of life, and even with the significant advance in cardiovascular biomedicine in recent years, heart diseases still represent a relevant public health problem, due to high prevalence and high financial costs [1, 2]. These indicators point out the importance of understanding the molecular mechanisms involved in the genesis of these pathologies for the development of possible new therapies.

In response to physiological or pathological stress, the heart undergoes genetic changes that trigger molecular, cellular, and protein alterations, which result in modifications in the geometry, size, and function of the heart, a process called cardiac remodeling (CR) [3]. Among the experimental models used to induce CR, AS stands out for inducing left ventricular hypertrophy, functional disorder, and, consequently, heart failure (HF) [4–6]. Several pathophysiological mechanisms may contribute to the development and progression of HF, such as the loss of myocyte numbers due to necrosis, apoptosis, and autophagy [7–9]. Recent studies have shown that there is a possibility of stimulating the proliferation of cardiomyocytes and, hence, the regeneration of cardiac tissue [10–13]; this event, in contrast to cell death, could trigger improvement in cardiac performance [14].

There is evidence in the literature that aerobic ET promotes cardiomyocyte proliferation in animal models of heart disease by stimulating signaling pathways that alter the genes involved in the cell cycle control system [15–17]. In addition, other studies have shown that ET increases the myocardial Ang-(1-7) peptide levels in several experimental models of heart disease [18–20]. This peptide, an essential functional component of the renin-angiotensin system (RAS), exerts protective actions via receptor G-protein-coupled receptor *Mas*, such as; vasodilation by NO, antioxidant defense, decreased myocardial hypertrophy and fibrosis, and improved cardiac function acting in a counter-regulatory manner to the actions of Ang II, which play an important role in pathological CR [21, 22]. In fact, *Mas*-deficient mice presented marked cardiac dysfunction, indicating the cardioprotective role of Ang-(1-7) [23]. However, it is unknown the influence of ET on cardiomyocyte proliferation and the RAS in the experimental model of ascending AS.

Different authors show that ET induces cardioprotection in animals with CR due to AS [24–26]; however, the mechanisms responsible for improving cardiac performance were not evaluated. Due to evidence that ET promotes myocyte multiplication and increases Ang-(1-7) levels in heart disease animals [15–19], this study aimed to evaluate for the first time the hypothesis that cardioprotection generated by ET in rats with pressure overload is dependent of cardiomyocyte proliferation and increased levels of Ang-(1-7) and its *Mas* receptor in the myocardium.

Materials and Methods

Animals and experimental design

Male Wistar rats (70-90 g, 3 weeks of age) from the central laboratory of Botucatu Medical School were kept in collective cages in a climate-controlled environment with food and water *ad libitum*. Two experimental groups were formed initially: operated control (C-Sed) and supravalvar aortic stenosis (AS-Sed). After 18 weeks of surgery, when rats with AS presented ventricular dysfunction confirmed by echocardiography, they were redistributed into two similar groups for cardiac structure and function and randomized for the presence or absence of ET for 10 weeks; the same procedure was performed on C-Sed group. Within this context, in the second stage, the experiment was composed of four groups: C-Sed, sedentary control group (n=20); C-Ex, exercised control group (n=20); AS-Sed, sedentary aortic stenosis group (n= 19) and AS-Ex, exercised aortic stenosis group (n= 15) (Fig. 1).

AS was surgically induced, as previously described [4, 27]. Briefly, rats were anesthetized with a mixture of ketamine hydrochloride (50 mg/kg i.p.) and xylazine hydrochloride (10 mg/kg, i.p.), and the heart was exposed through median thoracotomy. Then a stainless-steel clip (0.60 mm internal diameter) was placed in the ascending aorta, approximately 3 mm from its root. During surgery, the rats received 1 mL of warm saline intraperitoneally and were manually ventilated with positive pressure, with 100% oxygen. After the procedure, rats were kept warm until full consciousness was regained. Analgesia procedure consisted of intraperitoneal administration of carprofen (5mg/kg body weight) and was maintained until the disappearance of evidence of pain. Control animals were submitted to the same procedure but without the constriction of the aorta.

Rats were anesthetized with a mixture of ketamine hydrochloride (60 mg/kg i.p.) and xylazine hydrochloride (10 mg/kg, i.p.) and then euthanized by decapitation. Heart tissue was dissected, and then the left ventricle (LV) was removed and immediately freeze-clamped at the temperature of liquid nitrogen. Blood samples were collected, and the serum was separated by centrifugation at 1620 g for 10 min at 4°C.

This study was approved by the Ethics Committee on Animal Research of Botucatu Medical School, São Paulo State University (1133/2015-CEUA), and all protocols were conducted following the Guide for the Care and Use of Laboratory Animals published by the National Research Council (2011).

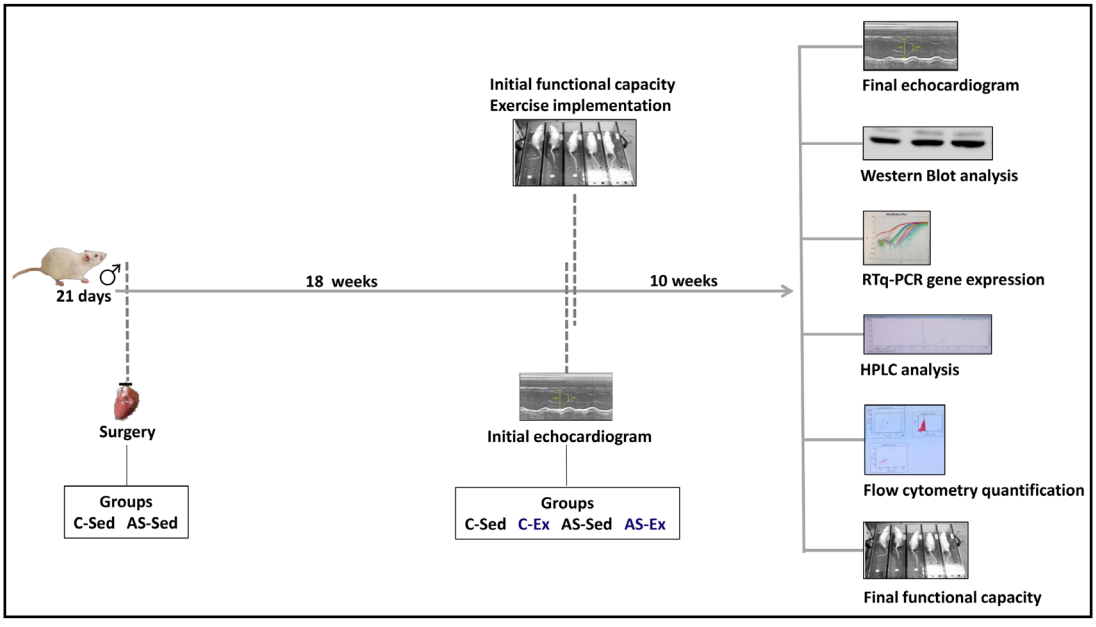

Fig. 1. Schematic representation of the experimental design. C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group.

Treadmill exercise testing (TET)

The functional capacity assessed before and after the ET period was estimated by maximal speed, total time, and distance run evaluated using graded TET as described previously [28–30]. TET was performed on a motorized treadmill for rats (AVS Projects – São Carlos, SP, Brazil) after one week of adaptation to a treadmill environment under low speed (5m/min/day). Briefly, TET began at 6 m/min and increased by 3 m/min every 3 min until exhaustion. Exhaustion was defined by no maintenance at the proposed speed. Furthermore, TET results were used to prescribe the ET protocol.

Table 1. Exercise training protocol. ES, exhaustion speed; TET, treadmill exercise training

Moments (week)	Training progression	
	Duration (minutes)	Intensity (ES)
Adaption		
1st TET		
1 st	10	50%
2 nd	12	50%
3 rd	14	50%
2nd TET		
4 th	16	50%
5 th	18	50%
6 th	20	50%
7 th	20	50%
3rd TET		
8 th	20	50%
9 th	20	50%
10 th	20	50%
4 th TET		

Exercise training protocol

The ET protocol was modified from previously published [26, 31] (Table 1). Briefly, rats were exercised five days/week (Monday to Friday) during 10 weeks (wks), at 50% of the maximal speed, achieved during the TET. The test was performed before the first week of training to initial exercise prescription and at the end of the third and seventh weeks to adjust the running speed. Exercise duration from the first to the sixth week was progressively added two min per week until 20 min/day and then remained constant from sixth to the tenth week. Animals received low-voltage electrical stimulation, during the training.

Echocardiographic study

Echocardiograms 18 and 28 weeks post-surgery were performed before euthanasia using commercially available echocardiography (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with a 5–11.5 MHz multifrequency probe was used as previously described [32]. All examinations were evaluated blindly by a cardiologist and specialist in echocardiography. Rats were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (1 mg/kg), according to previous studies [33, 34]. A two-dimensional parasternal short-axis view of the LV was obtained at the level of the papillary muscles [35]. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral valve leaflets, and at the level of the aortic valve and left atrium. M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. The same observer manually measured all LV structures according to the leading-edge method of the American Society of Echocardiography [36]. Measurements reported are the average of at least five cardiac cycles from the M-mode tracings. The cardiac structure was evaluated by diastolic diameter (LVDD), diastolic posterior wall thickness (DPWT), left atrium (LA), aortic diameter (AO), and LA/AO ratio. LV function was assessed based on posterior wall shortening velocity (PWSV), ejection fraction (EF), mesocardial fraction shortening (MFS), early and late diastolic mitral inflow velocities (E and A waves), E/A ratio, E-wave deceleration time (EDT), and isovolumetric relaxation time (IVRT). Additionally, was evaluated the tissue Doppler imaging (TDI) of systolic (s'), early (*e'*), and late (*a'*) diastolic velocity of the mitral annulus (arithmetic average of the lateral and septal walls) and E/*e'* ratio [36, 37]. The heart rate (HR) was also evaluated.

Characterization of heart failure

During euthanasia, we assessed the occurrence of clinical and pathological HF features in-vivo (tachypnea) and post-mortem (ascites, pleural effusion, atrial thrombi, and liver congestion) in the AS-Sed and AS-Ex groups. Two blind observers performed a subjective evaluation of the HF features during the experiment.

Post-mortem morphological analysis

To evaluate cardiac hypertrophy, the heart was removed, and the atriums (AT), left and right (RV) ventricles were separated and weighed. Tibia length was measured and used to normalize total heart weight (HW), AT, RV, LV in relation to tibia.

Cardiomyocyte proliferation analysis

In order to evaluate the percentage of DNA in the cell cycle phases (Sub G1: apoptotic; G1: cell growth; S: DNA synthesis; G2/M: mitosis), cardiomyocytes were isolated according to the technique of enzymatic dissociation of the tissue described by Mitra et al. [38]. The cardiomyocytes were collected in tubes and centrifuged at a rate of 450g for 5 min at room temperature. The supernatant was discarded, then the cell viability was evaluated by Trypan Blue, and cardiomyocyte concentration was adjusted to $1x10^4$ cells/mL. The cardiomyocytes were incubated with 1 U/ml RNase A (no DNase) and propidium iodide (PI) solution (0.1% sodium citrate, 0.1% Triton X-100 and 50 mg/L PI) for 30 min at room temperature in the dark. Thereafter, the samples were analyzed on a flow cytometer (FACSCanto II, BD Biosciences, Trenton, New Jersey, USA). The acquisition of the sample was performed using the CellQuest software, and ten thousand events were acquired for each sample.

RNA isolation and mRNA quantification

In order to evaluate the regulatory genes of the cell cycle control system, Real Time Quantitative PCR (RT-q-PCR) was performed. Isolated cardiomyocytes were homogenized in Trizol, and RNA was isolated according to the manufacturer's instructions (Invitrogen Life Technologies, USA). The concentration of total RNA was quantified using Nano-Drop Spectrophotometer (Nano-Drop Technologies, USA). The absorbance values of samples were acquired at 260 nm and expressed as ng/μL. All samples showed an absorbance of approximately 2.0. The cDNA was synthesized from 1 μg of total RNA using reverse transcriptase High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with the primers listed in Table 2. Real-time quantification of the target genes was performed by qPCR analysis using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA), and qRT-PCR reactions were run using StepOnePlus Real-Time PCR Systems, (Applied Biosystems). GAPDH gene was used as housekeeping controls to normalize the relative fluorescence signal of the target genes. Each heart sample was analyzed in duplicate. Relative quantities of target gene expression were compared after normalization using the expression values of internal controls [change in threshold cycle (ΔCT)]. Fold change was calculated using the differences in ΔCT values between the two samples (ΔΔCT) and the equation $2^{-\Delta\Delta CT}$. The results are expressed in fold change.

Angiotensin-converting enzyme (ACE) activity assay

The ACE activity was determined in serum and cardiac tissue by using fluorescent substrates [39]. Frozen cardiac tissue samples were homogenized in 0.1 M Tris-HCl buffer pH 7.0, containing 50 mM NaCl and centrifuged at 1,000g for 10 min. The assays were performed at 37°C in 0.1 M Tris-HCl buffer pH 7.0, containing 50 mM NaCl and 10 mM ZnCl2, and captopril 0.5 mL as an inhibitor in negative samples. The hydrolysis rate of the intramolecularly quenched fluorogenic substrate Abz-FRK-(Dnp) P-OH (10 uM) incubated with aliquots of homogenate and serum for 30 min at 37°C was assessed to obtain ACE enzymatic activity (420 nm LEM and 320 nm lex, read in 90 cycles). The ACE activity was expressed as $uF.min^{-1}.mg^{-1}$ of cardiac tissue protein concentration, or $uF.min^{-1}.mL^{-1}$ of serum.

High-performance liquid chromatography

Quantification of Ang I, Ang II and Ang-(1-7) in plasma and myocardium were performed using the High-Performance Liquid Chromatography (HPLC, Shimadzu System, Kyoto, Japan), as previously demonstrated by Fernandes et al. [40].

Table 2. Primers sequences

Gene	Primers sequence
CCND2	F 5´- TGTGCATGTTCCTAGCTTCC -3´ R 5´- TCCACTTCAGCTTACCCAAC -3´
AURKB	F 5' - GTTTCATCGTGGCGCTAAAG - 3' R 5' – CTCTGCTGGTCATAGAAGTAGTTG - 3'
CDK1	F 5'- ATCTTCGAGTTCCTGTCCATG -3' R 5'- GACAAAACACAATCCCCTGG -3'
MEIS1	F 5'- AGTTGGCACAAGATACGGGAC -3' R 5'- CCATCGGGGTTATAAGGTGTCC -3'
GAPDH	F 5'- CTACCCACGGCAAGTTCAAC -3' R 5'- CCAGTAGACTCCACGACATAC -3'

The LV was weighed and homogenized in 100 mM sodium phosphate buffer pH 7.2, 340 mM sucrose, and 300 mM NaCl, containing protease inhibitor cocktail (1:100, Sigma-Aldrich, MO, USA). The samples were centrifuged at 10, 000g, 4°C, 20 min. The extraction of angiotensins was held in Oasis C18 columns (Waters, MA, USA) previously activated with methanol (5 mL), tetrahydrofuran (5 mL), hexane (5 mL), methanol (5 mL) and water (10 mL). After activation, the samples were applied to the columns, washed with water, and eluted in ethanol/acetic acid/water in the proportion of 90%/4%/6%. The eluted fractions were lyophilized and resuspended in 500 uL of mobile phase A (5% acetonitrile in 0.1% orthophosphoric acid) and filtered with a 0.22 mm membrane for analysis by HPLC. The angiotensin of each sample was separated on a reversed-phase column ODS Aquapor 300 (250 x 4.6 mm), 7µ (PerkinElmer's Browlee Columns) using the gradient from 5–35% of mobile phase B (95% acetonitrile in 0.1% phosphoric acid) under a flow of 1.5 mL/min for 40 min. The angiotensins were identified by comparing them with the retention time of standard angiotensins. Results were expressed as pmol/g of tissue.

Western blot analysis

Cardiac proteins were extracted by homogenization in RIPA buffer containing protease (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase (Roche Diagnostics, Indianapolis, IN, USA) inhibitors using a bead beater homogenizer (Bullet Blender®, Next Advance, Inc., NY, USA), followed by centrifugation (12, 000g, 4°C, 20 min). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). A total of 25µg protein lysate was resolved by SDS-PAGE and transferred to nitrocellulose membrane (Armsham Biosciences, Piscataway, NJ, USA). The blotted membrane was blocked with 5% non-fat dry milk in TBS-T (20 mmol/L Tris-HCl pH 7.4, 137 mmol/L NaCl and 0.1% Tween 20) for 1h at room temperature (RT) and incubated overnight at 4 - 8°C with the following primary antibodies: Anti-Angiotensin II Receptor Type-1 (1:1000, Novus Biologicals, Littleton, CO, USA) and Anti-Angiotensin-(1-7) *Mas* Receptor (1:5000, Alomone Labs, Jerusalem, Israel). Then, blots were incubated for 1.5h at RT with secondary antibody (Abcam Cambridge, MA, USA) followed by chemiluminescence detection with SuperSignal® West Pico kit (Thermo Scientific) using ImageQuant™ LAS 4000 (GE Healthcare). Quantification of band intensities was performed using ImageJ software (NIH). Results were normalized to the β-actin (1:1200, Cell Signaling, Danvers, MA, USA).

Statistical analysis

The data were tested for normality using the Shapiro-Wilk test and were expressed as mean ± SEM or median and percentiles. Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to analyze differences in protein and gene expression, enzyme activity, and angiotensin levels between groups. Mann-Whitney test was used to evaluate cardiac mass analysis. Two-way ANOVA for repeated measures followed by Bonferroni post hoc was performed to test the impact of exercise training on functional capacity parameters and cardiac function and structure between groups before and after TE protocol. Kruskal-Wallis and Dunn were used to analyzed cell cycle. Goodman test was used to test differences in heart failure signals between AS-Sed and AS-Ex groups. The level of significance considered was 5%. Statistical analyzes were performed in Sigma Plot 12.0 (Systat Software, Inc.), and the graphs were generated in GraphPad Prisma 8 (GraphPad Software Inc.).

Results

Effects of exercise training on functional capacity and features of heart failure

Our results showed that AS-Ex and C-Ex animals had significant improvement ($p < 0.05$) in exhaustion velocity, total time and distance compared to their respective sedentary groups, showing that ET improved functional capacity (Fig. 2).

The AS-Ex group presented attenuation of the clinical and pathological heart failure features concerning the AS-Sed group, showing a significant decrease ($p < 0.05$) in the occurrence of ascites, tachypnea, pleural effusion, left atrial thrombus and liver congestion (Fig. 3). It is noteworthy that the survival rate did not differ between the AS-Sed and AS-ET groups (data not shown).

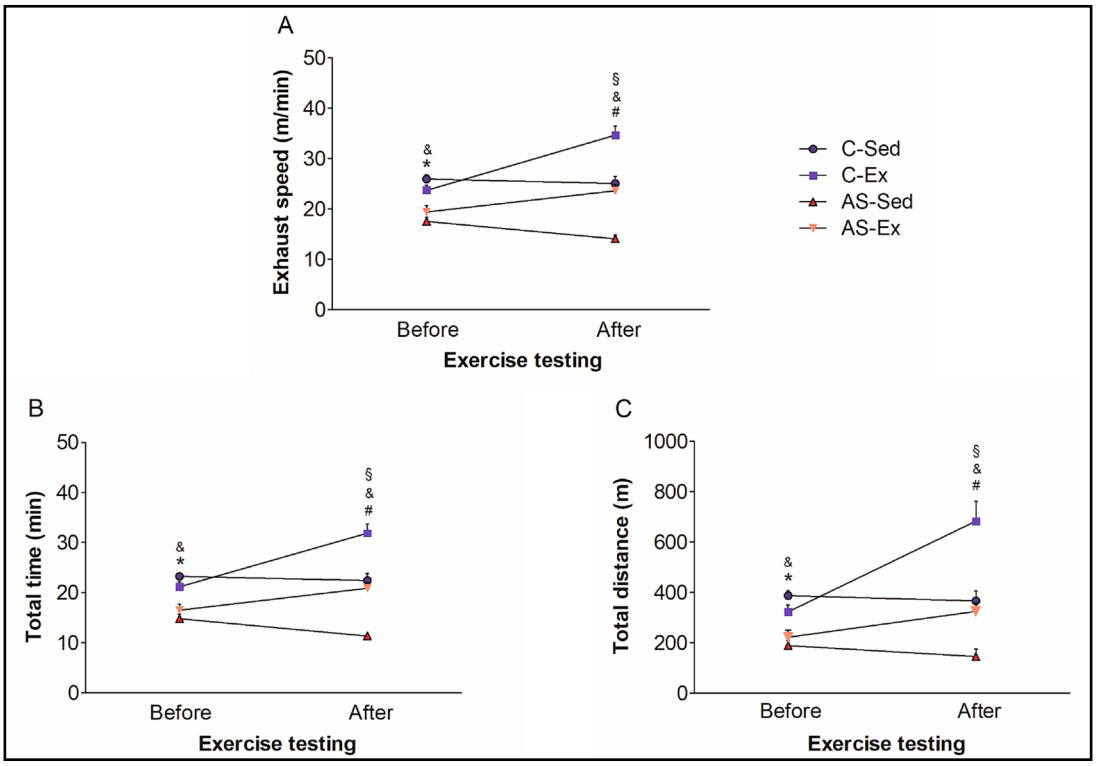

Fig. 2. Functional capacity of animals after 10 weeks of exercise training. Exhaustion speed (A), total time (B), and total distance (C) evaluated in treadmill exercise testing. C-Sed, sedentary control group; C-Ex, exercised control group; AS-Sed, sedentary aortic stenosis group; AS-Ex, exercised aortic stenosis group; n= 15-20 per group. Data are mean ± SEM; Two-way repeated-measures ANOVA and Bonferroni. p<0,05. * AS-Sed vs. C-Sed; # AS-Ex vs. AS-Sed; & AS-Ex vs. C-Ex; § C-Ex vs. C-Sed.

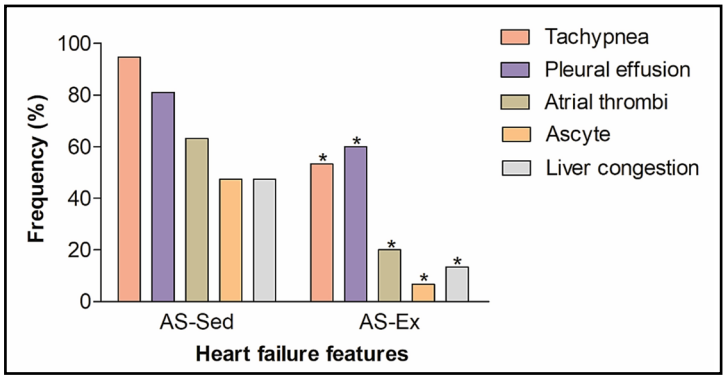

Fig. 3. Frequency of heart failure features in the aortic stenosis rats. AS-Sed, sedentary aortic stenosis; AS-Ex, exercised aortic stenosis; n= 15-19 per group. Data expressed as relative frequency. Goodman test. p<0.05. * vs. AS-Sed.

Echocardiographic data before and after exercise training

Before the ET protocol, AS groups (AS-Sed and AS-Ex) were homogeneous in cardiac structure and function. The AS-Sed group showed concentric left ventricular hypertrophy and systolic and diastolic dysfunction. When we compared the different groups over time (28 wks *vs.* 18 wks), we found that the AS-Sed group decreased RWT and increased LVDD and LA; in the C-Ex group, there was an increase in EF, PWSV, a` and a decrease in TRIV. According to our results, the AS-Ex 28 wks group did not show changes in CR over time, indicating a beneficial effect of ET. After 10 weeks of training protocol, the AS-Ex group presented a decrease in the LA, increased EF, and MSF in relation to the AS-Sed group (p<0.05). Also, the C-Ex animals showed significant improvement (p<0.05) in EF, MSF, and PWSV when compared to C-Sed animals. The C-Sed group demonstrated no changes after 10 weeks (Table 3).

Table 3. Echocardiographic data before and after exercise training. HR, Heart rate; LVDD, left ventricular diastolic diameter; DPWT, diastolic posterior wall thickness; RWT, relative wall thickness; LA/Ao, left atrium to aorta ratio; EF, ejection fraction; MSF, mesocardial shortening fraction; PWSV, posterior wall shortening velocity; EDT, E-wave deceleration time; IVRT, isovolumetric relaxation time; s', tissue Doppler imaging (TDI) of systolic; Mitral E, Early diastolic mitral inflow; E/A, Ratio between early- (E-) to-late (A) diastolic mitral inflow; e', TDI of early diastolic velocity of mitral annulus; a', TDI of end diastolic velocity of mitral annulus; E/e', E wave to e' ratio. C-Sed, sedentary control group; C-Ex, exercised control group; AS-Sed, sedentary aortic stenosis group; AS-Ex, exercised aortic stenosis group; n= 15-20 per group. Data are mean ± SEM; Two-way repeated-measures ANOVA and Bonferroni. p<0,05. * vs. C-Sed; # vs. AS-Sed; & vs. C-Ex; § vs. C-Sed; † vs. AS-Sed 18 wks; Δ vs. C-Ex 18 wks

Parameters	Groups before exercise training (18 weeks)				Groups after exercise training (28 weeks)			
	C-Sed	C-Ex	AS-Sed	AS-Ex	C-Sed	C-Ex	AS-Sed	AS-Ex
HR (bpm)	324 ± 13.8	322 ± 8.26	310 ± 11.0	297 ± 8.56	303 ± 9.48	327 ± 9.88	308 ± 9.34	305 ± 7.33
LVDD (mm)	7.29 ± 0.12	7.42 ± 0.10	7.51 ± 0.23	7.50 ± 0.25	7.68 ± 0.15	7.61 ± 0.08	8.03 ± 0.20†	7.89 ± 0.23
DPWT (mm)	1.65 ± 0.03	1.64 ± 0.03	3.02 ± 0.05*	3.00 ± 0.04&	1.65 ± 0.03	1.65 ± 0.03	3.00 ± 0.06*	2.79 ± 0.11&
RWT	0.45 ± 0.01	0.44 ± 0.01	0.82 ± 0.03*	0.81 ± 0.03&	0.43 ± 0.01	0.43 ± 0.01	0.76 ± 0.03*†	0.71 ± 0.03&
LA (mm)	4.88 ± 0.06	4.94 ± 0.06	7.91 ± 0.26*	8.14 ± 0.28&	4.95 ± 0.06	4.92 ± 0.05	8.49 ± 0.22*†	7.63 ± 0.40#&
LA/AO	1.26 ± 0.02	1.26 ± 0.02	2.00 ± 0.07*	2.00 ± 0.06&	1.27 ± 0.03	1.24 ± 0.02	1.92 ± 0.11*	1.86 ± 0.09&
EF	0.93 ± 0.01	0.92 ± 0.01	0.88 ± 0.01*	0.89 ± 0.01&	0.91 ± 0.01	0.93 ± 0.01§Δ	0.88 ± 0.01*	0.91 ± 0.01#&
MFS (%)	27.6 ± 0.72	28.5 ± 1.15	23.9 ± 1.10*	26.6 ± 1.01&	25.9 ± 0.52	29.2 ± 0.66§	24.8 ± 0.66	26.7 ± 0.84#&
PWSV (mm/s)	59.7 ± 1.47	58.2 ± 1.21	39.9 ± 1.96*	40.4 ± 1.96&	59.5 ± 1.29	65.7 ± 1.37§Δ	40.0 ± 2.69*	44.8 ± 2.96&
s' (cm/s)	5.72 ± 0.11	5.88 ± 0.08	4.06 ± 0.12*	4.29 ± 0.16&	5.63 ± 0.10	5.95 ± 0.09	4.34 ± 0.15*	4.37 ± 0.23&
IVRT (ms)	23.1 ± 0.78	23.5 ± 0.84	16.0 ± 1.43*	14.7 ± 1.09&	23.1 ± 1.00	21.8 ± 0.51Δ	14.1 ± 1.54*	14.9 ± 1.27&
EDT (ms)	46.9 ± 1.20	45.7 ± 1.05	33.7 ± 3.03*	36.2 ± 3.23&	48.6 ± 1.38	44.8 ± 1.11	31.7 ± 2.99*	35.1 ± 3.75&
E wave (cm/s)	82.8 ± 1.50	87.3 ± 2.81	129 ± 6.24*	124 ± 8.10&	84.3 ± 2.30	87.8 ± 3.36	137 ± 6.86*	130 ± 6.95&
A wave (cm/s)	57.4 ± 1.67	60.8 ± 2.24	39.2 ± 5.34*	42.0 ± 7.76&	59.1 ± 1.92	60.4 ± 2.71	32.7 ± 4.38*	42.4 ± 6.52&
E/A	1.46 ± 0.03	1.45 ± 0.03	4.39 ± 0.52*	4.42 ± 0.63&	1.44 ± 0.04	1.47 ± 0.03	5.01 ± 0.38*	4.22 ± 0.58&
e' (cm/s)	6.51 ± 0.23	6.86 ± 0.31	5.95 ± 0.34	5.38 ± 0.29&	6.40 ± 0,22	7.15 ± 0.27	6.52 ± 0.42	6.08 ± 0.44&
a' (cm/s)	5.01 ± 0.29	4.58 ± 0.20	3.87 ± 0.42*	4.15 ± 0.49&	4.40 ± 0.17	4.93 ± 0.20Δ	3.61 ± 0.30	4.24 ± 0.52
E/e'	13.0 ± 0.43	13.0 ± 0.49	22.7 ± 1.35*	23.6 ± 1.54&	13.3 ± 0.41	12.3 ± 0.32	22.5 ± 1.78*	22.8 ± 1.81&

Post-mortem cardiac morphological analysis

The data of the *post-mortem* cardiac macroscopic structure of all groups studied are presented in Table 4. The AS-Sed group showed significant changes (p <0.05) in all cardiac variables analyzed when compared to the C-Sed group. The groups submitted to ET did not present a statistical difference in relation to the respective controls, showing that ET did not alter the post-mortem cardiac morphology in AS.

Table 4. Cardiac mass analysis. LV, left ventricle weight; RV, right ventricle weight; AT, atrium weight; Tibia, tibia length. C-Sed, sedentary control group; C-Ex, exercised control group; AS-Sed, sedentary aortic stenosis group; AS-Ex, exercised aortic stenosis group; n = 10 per group. Data are mean ± SEM or median and percentiles; Two-way ANOVA and Bonferroni or Mann-Whitney. p<0,05. * vs. C-Sed; & vs. C-Ex

Parameters	Groups			
	C-Sed	C-Ex	AS-Sed	AS-Ex
Heart (g)	1.44 ± 0.072	1.22 ± 0.104	2.28 ± 0.132*	2.20 ± 0.206&
LV (g)	1.05 ± 0.059	0.86 ± 0.101	1.46 ± 0.077*	1.42 ± 0.137&
RV (g)	0.26 ± 0.012	0.25 ± 0.014	0.43 ± 0.036*	0.40 ± 0.043&
AT (g)	0.13 ± 0.009	0.12 ± 0.007	0.39 ± 0.044*	0.37 ± 0.051&
Heart/Tibia (g/cm)	0.32 ± 0.015	0.28 ± 0.022	0.50 ± 0.027*	0.51 ± 0.046&
LV/Tibia (g/cm)	0.23 ± 0.012	0.19 ± 0.022	0.32 ± 0.015*	0.33 ± 0.031&
RV/Tibia (g/cm)	0.05 (0.05-0.06)	0.05 (0.04-0.06)	0.09 (0.08-0.10)*	0.09 (0.07-0.13)&
AT/Tibia (g/cm)	0.03 ±0.002	0.03 ± 0.002	0.09 ± 0.010*	0.08 ± 0.011&

Proliferation of cardiomyocytes

In order to verify whether ET causes cardiomyocyte proliferation, DNA content was analyzed in the SubG1, G1, S, and G2/M phases of the cell cycle. Our data revealed that there was no statistical difference between all groups in the four phases that constitute the cell cycle (Table 5).

Regulatory genes of the cell cycle

We evaluated the expression of crucial genes that regulate the cell cycle control system (Fig. 4). The expression of cyclin D2 (CCND2), cyclin-dependent kinase 1 (CDK1), aurora kinase B (AURKB) genes (Fig. 4A-C) were significantly higher in AS-Sed in relation to C-Sed ($p<0.05$) and the AS-Ex group showed lower mRNA expression of the same genes than AS-Sed group ($p<0.05$). Concerning the Meis homeobox 1 (MEIS1) gene (Fig. 4D), the AS-Ex group showed a significantly decreased expression compared to the AS-Sed and C-Ex groups.

Cardiac and plasma Ang I, Ang II, Ang-(1-7) and Ang II/Ang-(1-7) ratio levels

The AS-Sed group presented a significant decrease in the cardiac concentration of the Ang I compared to the C-Sed group. The ET was effective in reducing the cardiac and plasmatic concentrations of Ang I in relation to their respective controls (Fig. 5A and B). Regarding the Ang II (Fig. 5C and D), higher concentrations were detected in the myocardium of AS-Sed group compared to the C-Sed group ($p<0.05$), however, a decrease in the plasma and cardiac concentrations of Ang II in the AS-Ex group was observed when compared to the AS-Sed group ($p<0.05$). The cardiac concentrations of Ang-(1-7) did not differ among groups; however,

Table 5. Analysis of DNA content and distribution in the phases of the cell cycle (%). Sub G1, apoptotic; G1, cell growth; S, DNA synthesis; G2/M, mitosis. C-Sed, sedentary control group (n=8); C-Ex, exercised control group (n=6); AS-Sed, sedentary aortic stenosis group (n=9); AS-Ex, exercised aortic stenosis group (n=6). Data are median and percentiles; Kruskal-Wallis and Dunn. p<0,05

Groups	Cell cycle phases			
	SubG1	G1	S	G2/M
C-Sed	28.3 (11.2-34.3)	49.5 (24.6-64.1)	7.63 (3.80-14.9)	17.9 (1.35- 44.2)
C-Ex	30.3 (7.26-48.1)	51.1 (26.3-62.1)	6.10 (2.56-14.5)	13.3 (1.15- 52.6)
AS-Sed	29.1 (23.9-47.6)	46.9 (38.5-63.7)	7.11(3.70-16.9)	2.94 (1.20-19.6)
AS-Ex	40.3 (10.9-52.5)	40.6 (30.4-57.8)	11.0 (2.62-25.4)	9.06 (0.55-34.5)
P value	>0.05	>0.05	>0.05	>0.05

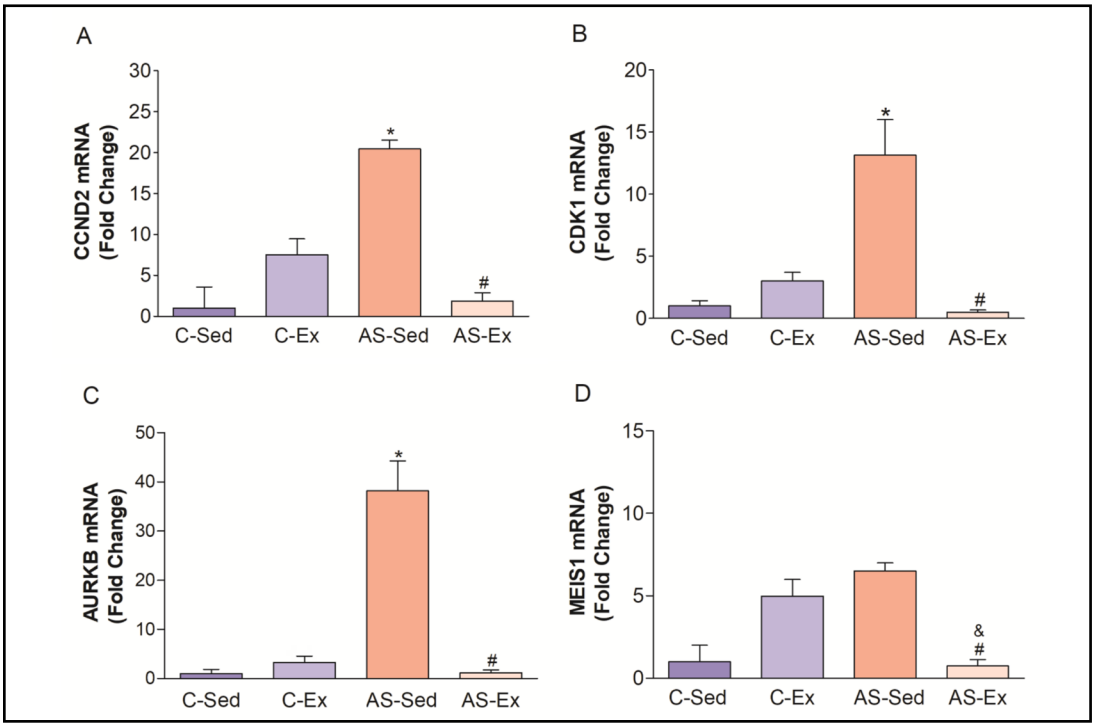

Fig. 4. Regulating genes of the cardiomyocyte cell cycle control system. CCND2 (A); CDK1 (B); AURKB (C); MEIS1 (D). C-Sed, sedentary control group (n=3); C-Ex, exercised control group (n=6); AS-Sed, sedentary aortic stenosis group (n=4); AS-Ex, exercised aortic stenosis group (n=5). Data are mean ± SEM; Two-way ANOVA and Bonferroni. p<0,05. * vs. C-Sed; # vs. AS-Sed; & vs. C-Ex.

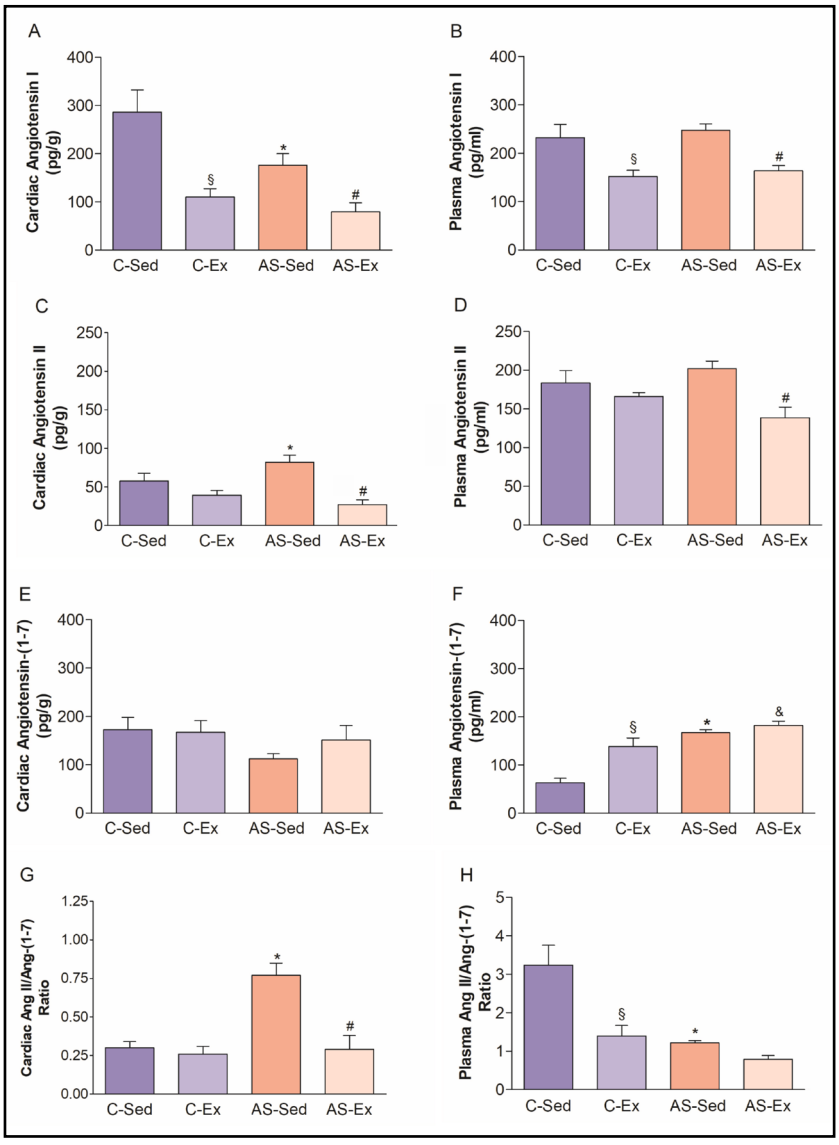

Fig. 5. The cardiac and plasmatic renin-angiotensin system (RAS) in sedentary and exercise-trained. Cardiac angiotensin I (A); Plasma angiotensin I (B); Cardiac angiotensin II (C); Plasma angiotensin II (D); Cardiac angiotensin-(1-7) (E); Plasma angiotensin-(1-7) (F); Cardiac angiotensin II/ang-(1-7) ratio (G); Plasma angiotensin II/ang-(1-7) ratio (H). C-Sed, sedentary control group; C-Ex, exercised control group; AS-Sed, sedentary aortic stenosis group; AS-Ex, exercised aortic stenosis group; n = 10 per group. Data are mean ± SEM; Two-way ANOVA and Bonferroni. $p<0,05$. * vs. C-Sed; # vs. AS-Sed; & vs. C-Ex; § vs. C-Sed.

increased plasma levels were observed in AS-Sed and C-Ex groups in relation to C-Sed and AS-Ex compared to C-Ex groups (Fig. 5E and F). When we evaluated the ratio of tissue angiotensin II / Ang- (1-7), the AS-Sed group increased compared to the C-Sed group; in addition, the exercise protocol in the AS-EX group was able to reduce this proportion compared to AS-Sed. In plasma, the AS-Sed and C-Ex groups decreased their ratio compared to the C-Sed group (Fig. 5G and H).

Cardiac and plasma ACE activity

Fig. 6A shows that there was an increase in ACE cardiac activity in the AS-Sed and AS-Ex groups compared to their respective controls. Interestingly, the AS-Ex group presented decreased cardiac and plasma ACE activity compared to AS-Sed (Fig. 6B).

Renin-angiotensin system receptors

AT1 expression was lower in animals with stenosis compared to their respective controls ($p<0.05$), and there was no difference between AS groups (Fig. 7A). *Mas* receptor expression was similar among the groups (Fig. 7B).

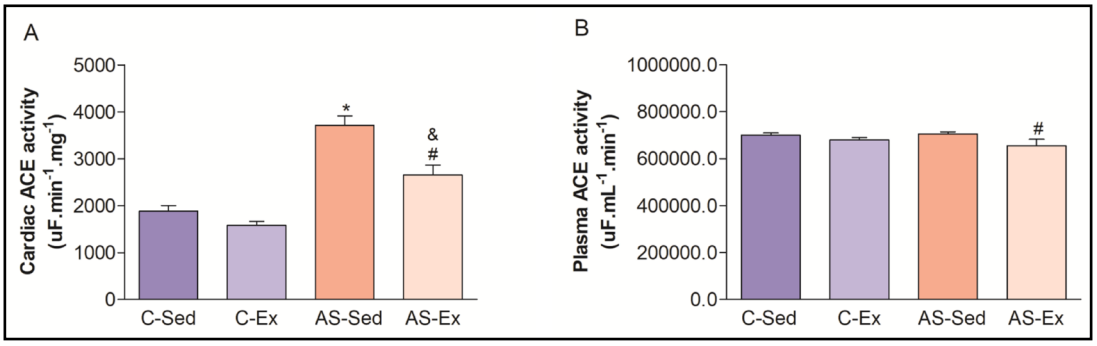

Fig. 6. Cardiac and plasma renin-angiotensin system activity. Cardiac ACE activity (A); Plasma ACE activity (B). C-Sed, sedentary control group; C-Ex, exercised control group; AS-Sed, sedentary aortic stenosis group; AS-Ex, exercised aortic stenosis group; n = 10 per group. Data are mean ± SEM; Two-way ANOVA and Bonferroni. p<0,05. * vs. C-Sed; # vs. AS-Sed; § vs. C-Sed.

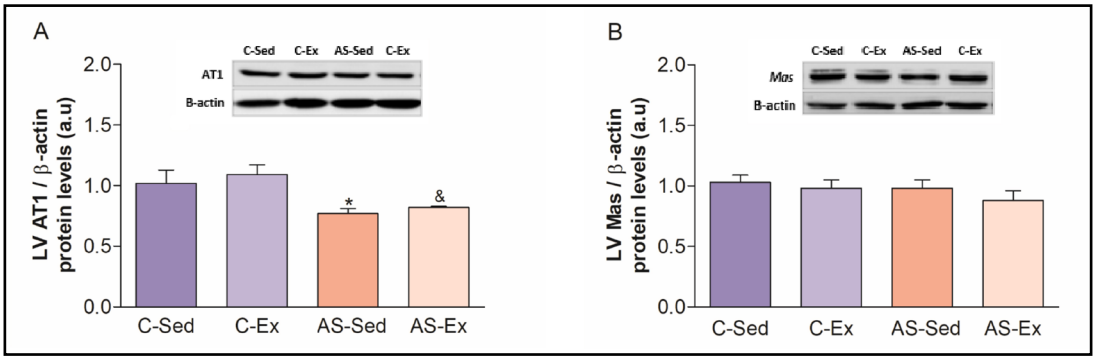

Fig. 7. Protein expression of AT1 receptor and Mas receptor assessed by Western blot. AT1 receptor (A); Mas receptor (B). C-Sed, sedentary control group; C-Ex, exercised control group; AS-Sed, sedentary aortic stenosis group; AS-Ex, exercised aortic stenosis group; n = 7 per group. Data are mean ± SEM, Two-way ANOVA and Bonferroni. p<0,05. * vs. C-Sed; & vs. C-Ex.

Discussion

The findings of this study show that ET improved the systolic function, regardless of the cardiomyocytes proliferation and increased Ang-(1-7) levels and *Mas* receptor. The ET also negatively modulated the vasoconstrictor/hypertrophic axis of the RAS. Furthermore, this is the first study showing that exercise decreases the gene expression of negative regulators of the cardiomyocyte cell cycle.

Several experimental models have been applied to study CR caused by pressure overload [41–43]. Our group has used a well-standardized model of ascending stenosis in young rats, which promotes early left ventricular hypertrophy. The pressure overload initially is mild and progressively increases as animals grow, causing ventricular dysfunction, and showing initial clinical and pathological features of HF after 18 weeks [4, 5]. Several pathophysiological mechanisms can contribute to the dysfunction and progression of HF, from alterations in the biology of cardiomyocytes, the geometry of the chamber, and myocardium such as loss of myocytes by necrosis, apoptosis, and autophagy [3, 7, 9, 44]. Pathological hypertrophy may be due to the decrease in capillary density, inflammatory response, oxidative stress, and the exacerbation of the RAS, especially [7, 45].

In the scope of non-drug therapies for cardiovascular diseases, stands out the exercise training, a programmed and systematized activity that attenuates ventricular failure in individuals with pathological CR [46]. Similar to previous results in our group [24–26], the ET, low volume and moderate intensity, improved the functional capacity of AS animals and

reduced the intensity and frequency of HF signals. In contrast, other researchers did not observe changes in the characteristics of HF after a similar exercise protocol, perhaps due to differences in the magnitude of the progression from dysfunction to HF [33, 47]. The ET also improved systolic performance; however, the mechanisms related to this benefit are not fully understood in the literature. A possible mechanism for this improvement is that ET reduces oxidative stress and increases NO production by neuronal nitric oxide synthase (nNOS), both associated with β-3 adrenergic receptor activation [48]. In this work, we investigated the participation of other factors, such as the proliferation of myocytes and the Ang-(1-7) axis.

Throughout life, there is a natural loss of cardiomyocytes, which can regenerate; however, this process is limited and insufficient to recover the injured myocardium [49]. It is known that the loss of cardiomyocytes is one of the reasons that lead to deterioration of cardiac function [44]; in this regard, it would be interesting to use strategies to stimulate the proliferation of myocytes in order to regenerate the injured tissue. Elegantly, researchers showed some of these strategies, such as the use of stem cells, inhibition of cell cycle regulatory genes, and stimulation of signaling pathways [12, 50, 51].

Since investigations with different heart diseases have shown that ET can stimulate cardiac regeneration [15–17, 52], one of our goals was to evaluate whether the proliferation of cardiomyocytes can be stimulated by exercise training and participate in the mitigation of cardiac dysfunction. Our data revealed that ET did not stimulate myocytes to re-enter the cell cycle. Unlike our results, a recent study showed that 8 weeks of aerobic ET in adult mice induced cardiomyogenesis in healthy and infarcted hearts [52]. Several factors can probably be related to this contradictory result, such as the method used to evaluate the cell cycle, which was different from the study that used the nucleus marking [53] or incorporation of N-thymidine [52]; sample size; the volume of exercise training used; the intensity of cardiac aggression; and period analyzed. Thus, further studies are needed to clarify the interaction between exercise and myocyte proliferation during the transition from compensated LV hypertrophy to HF, using other methods of cell cycle analysis.

Although we have not found any influence of ET on the proliferation of cardiomyocytes, surprisingly, it decreased the gene expression of negative cell cycle regulators in cardiomyocytes from the AS-Ex group. The positive regulatory genes of the cell cycle are expressed in the embryonic phase, a period in which the mitotic activity of the cardiomyocyte is high. In adulthood, there is an inversion of the behavior of these genes, which become negative regulators [54–56]. One of these genes was MEIS1, which Mahmoud and cols [12]. showed that its deletion resulted in the proliferation of neonatal myocytes and reactivation of mitosis in these cells in adult mouse hearts. Exercise training also promoted a decrease in the expression of the CCND2, AURKB, and CDK1 genes in animals with heart disease, another attempt to re-enter the cell cycle. We believe, therefore, that ET may act as a trigger at the genic level to stimulate cardiomyocytes proliferation.

Another objective of this study was to evaluate the RAS, focusing on the role of Ang-(1-7), which counter regulates the actions of Ang II [57]. The RAS plays an essential role in cardiovascular physiology by regulating blood pressure and electrolyte balance [58]. However, under pathological conditions, the effects of RAS can trigger RC [59], mainly by altering catabolism and increasing inflammation, oxidative stress [60, 61], and apoptosis [62]. Our results showed that while levels of Ang II and ACE activity were elevated in the myocardium, Ang I decreased, and Ang-(1-7)/*Mas* receptor did not change in the AS group, showing the predominance of the vasoconstrictor/hypertrophic axis over the antihypertrophic vasodilator axis. This effect of the classic RAS axis was corroborated by the behavior of the angiotensin II/-(1-7) ratio, which was shown to be elevated in the AS-Sed group. The results of Ang I, and Ang II and ACE are in agreement with other previous studies [63–65]. Filho et al. showed that SHR rats did not demonstrate differences in cardiac Ang-(1-7) levels compared to controls [18]. Unlike our results, the literature shows decreased *Mas* receptor expression in the heart of rats with aortic constriction [66]. AS animals also showed reduced AT1 receptor protein expression. The behavior of this receptor in chronic HF remains controversial; some authors found decreased levels in humans [67, 68], while other studies evidenced expres-

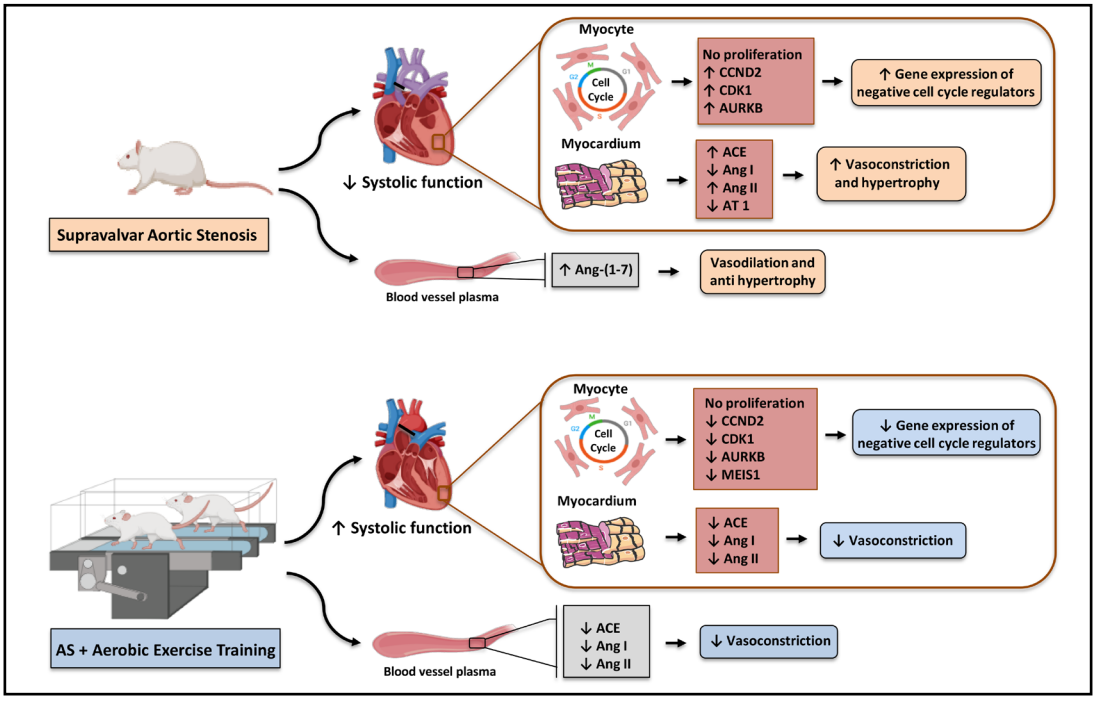

Fig. 8. Overview of the effects of aerobic exercise training on the proliferation of cardiomyocytes and the cardiac renin-angiotensin system in rats with supravalvular aortic stenosis. AS, aortic stenosis; ACE, angiotensin-converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; AT 1, receptor; Ang-(1-7), angiotensin-(1-7); CCND2, cyclin D2; CDK1, cyclin-dependent kinase 1; MEIS1, Meis homeobox 1; AURKB, aurora kinase B;,↑, increase;↓, decrease. Note: Variables that do not change significantly have not been included in the figure.

sion unchanged [69]. The authors who verified a reduction in the AT1 density, suggest that the increase in the concentration of Ang II is responsible for this behavior [68].

Regarding the effect of ET on RAS, our findings showed that it was effective in decreasing the myocardial and plasmatic Ang II, Ang I, and ACE activity, previously corroborating studies [18, 70–72]. Gomes-Santos et al. [72] showed a similar result in skeletal muscle of rats with HF by myocardial infarct; the authors suggest that the reduction of Ang II by ET is due to the decrease in Ang I cleavage, confirmed by our results that also showed a decrease in the levels of myocardial Ang I and ACE. This reduction of cardiac levels of Ang II could reduce the deleterious effects on the myocardium, such as cardiac hypertrophy and fibrosis, improving heart function. The reduced plasma Ang II may be associated with an improvement in the signs of HF seen in animals with heart disease since it is associated with hydro saline retention and peripheral vasoconstrictor effect [73–75].

We hypothesized that ET could cause a change in the RAS in the myocardium towards the Ang-(1-7)/*Mas* axis. Interestingly, exercise did not alter the myocardial and plasma Ang-(1-7) concentrations, different from other studies that found the increase in this peptide in animals with heart disease [18, 21, 64, 65, 76]. We believe that in the experimental model used, which presents a robust cardiac remodeling, ET attempts to regulate the vasoconstrictor axis (ACE/Ang II) that is increased and acts less expressively on the vasodilator axis (Ang-(1-7)/*Mas*), that has not been changed. However, the Ang II/Ang-(1-7) ratio was decreased by exercise training, indicating an attenuation of the effects of the vasoconstrictor/hypertrophic axis. There was also no change in the expression of the *Mas* receptor after ET. Since there was no modification in Ang-(1-7), we believe that the organism does not need to change the *Mas* receptor.

To better visualize the effects of aerobic exercise on the proliferation of cardiomyocytes and the cardiac RAS of rats with AS, we made a portrait placing the main molecular changes, as can be seen in Fig. 8.

Conclusion

Our results show that ET improves systolic function, regardless of myocyte proliferation and Ang-(1-7) and *Mas* receptor levels. However, the ET negatively modulates the vasoconstrictor/hypertrophic axis (ACE/Ang II) and decreases the expression of negative regulatory genes of the cell cycle in cardiomyocytes of rats with supravalvular aortic stenosis.

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Disclosure Statement

The authors report no relationship that could be construed as a conflict of interest.

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