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Original Paper

Adjustments in β-Adrenergic Signaling Contribute to the Amelioration of Cardiac Dysfunction by Exercise Training in Supravalvular Aortic Stenosis

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Key Words
Aortic bandage • Pressure overload • Heart failure • Physical training • Papillary muscle • β-adrenergic pathway

Abstract
Background/Aims: Aortic stenosis-induced chronic pressure overload leads to cardiac dysfunction and congestive heart failure. The pathophysiological mechanisms of the myocardial impairment are multifactorial and include maladaptive β-adrenergic signaling. Exercise training (ET) has been used as a non-pharmacological therapy for heart failure management. The present study tested the hypothesis that exercise training attenuates diastolic dysfunction through β-adrenergic signaling preservation. ***Methods:*** Wistar rats were submitted to ascending aortic stenosis (AS) surgery, and after 18 weeks, a moderate aerobic exercise training protocol was performed for ten weeks. ***Results:*** ET attenuated diastolic dysfunction, evaluated by echocardiogram and isolated papillary muscle (IPM) assay. Also, ET reduced features of heart failure, cross-sectional cardiomyocyte area, and exercise intolerance, assessed by treadmill exercise testing. The β2 adrenergic receptor protein expression was increased in AS rats independently of exercise. Interestingly, ET restored the protein levels of phosphorylated

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phospholamban at Serine 16 and preserved the β-adrenergic receptor responsiveness as visualized by the lower myocardial compliance decline and time to 50% tension development and relaxation during β-adrenergic stimulation in the IPM than untrained rats. Additionally, AS rats presented higher levels of TNFα and iNOS, which were attenuated by ET. ***Conclusion:*** Moderate ET improves exercise tolerance, reduces heart failure features, and attenuates diastolic dysfunction. In the myocardium, ET decreases the cross-sectional area of the cardiomyocyte and preserves the β-adrenergic responsiveness, which reveals that the adjustments in β-adrenergic signaling contribute to the amelioration of cardiac dysfunction by mild exercise training in aortic stenosis rats.

Introduction

Heart failure is a syndrome with an important morbidity and mortality impact on the population worldwide and is an outcome of exposure to chronic stress, such as pressure overload [1]. Chronic pressure overload is characterized by time-dependent injury in myocardial deformation, gradual decline in diastolic and systolic functions, and heart failure (HF) installation [2]. In this scenario, an increase in β-adrenergic system activity is the most significant adaptation to the maintenance of cardiac performance to preserve the adequate nutrient and oxygen supply to tissues and organs [3–5]. In contrast, long term sustained sympathetic activity results in adverse effects on myocardium structure and function [6, 7]. Cardiotoxicity generated by the persistent increase of circulating catecholamines is induced by several mechanisms [6, 8–10], such as maladaptive β-adrenergic signaling [11, 12]. β-adrenergic signaling desensitization, characterized by uncoupling and downregulation of receptors, markedly reduces cardiac myofilament responsiveness to endogenous or exogenous agonists leading to contractile reserve depletion [13–17], heart failure (HF) [12, 18, 19] and death [7, 20]. Therefore, β-blockers are mandatory for the treatment of cardiac disease associated with sympathetic hyperactivity. In addition, enhanced expression of myocardial tumor necrosis factor-α (TNF-α) accompanied by overproduction of nitric oxide (NO) may play an additional role in the pathogenesis of HF by altering the β-adrenergic responsiveness to stimulation [21].

Ascending aortic stenosis in young rats has been used to study persistent and chronic pressure overload [22, 23]. Cardiac dysfunction induced by this experimental model occurs gradually, resembling the development of the disease in humans. Besides, aortic stenosis has particular pathophysiology with substantial hemodynamic disturbance, which generates severe dysfunction [2].

Exercise training (ET) has been prescribed as adjuvant therapy for cardiac disease and a remarkable attenuator of cardiovascular risk and disease progression [24–28]. Indeed, ET counteracts sympathetic hyperactivity in cardiac disease in humans [29] and animal models [30–32]. Our group has evaluated the effect of ET in rats with supra-valvar aortic stenosis--induced HF [33–35]; however, the beneficial effect of exercise during the transition from cardiac dysfunction to heart failure in aortic stenosis rats remain controversial so far [36, 37]. Furthermore, the mechanisms underlying the benefits of ET in this experimental model still require elucidation.

In the present study, we aimed to evaluate the effects of ET on the cardiac function and the β-adrenergic signaling pathway in the advanced stage of supra-valvar aortic stenosis remodeling in rodent heart. We hypothesized that the preservation of the β-adrenergic signaling contributes to the attenuation of cardiac dysfunction by a moderate exercise training program.

Materials and Methods

Animals

Twenty-one-day-old male *Wistar* rats (70-90 g) were obtained from the Central Animal House, Botucatu Medical School, Unesp. Rats were housed in collective polypropylene cages in a climate-controlled environment at 23°C (± 3°C) with a reverse 12:12-h light/dark cycle and free access to food and water. All experiments and procedures were supervised by a veterinarian, performed according to the Brazilian Guide for the Care and Use of Laboratory Animals and approved by Botucatu Medical School Animal Research Ethics Committee (protocol number 1192/2016).

Experimental design

Initially, rats underwent either supra-valvar aortic stenosis (SVAS) or Sham surgery. After 18 weeks, the animals were redistributed to be kept sedentary (Sham, n = 20 and AS, n = 28) or submitted to exercise training (ShamT, n = 18, and AST, n = 32) for 10 weeks. All Animals had *in vivo* cardiac function evaluated after training exercise period (i.e., 28 weeks after surgery) and posteriorly, rats were submitted to euthanasia for additional functional, histological, and molecular studies (Fig. 1). Rats were anesthetized with an intraperitoneal administration of ketamine hydrochloride (60 mg/Kg) and xylazine hydrochloride (10 mg/kg) and then euthanized by decapitation.

Supra-valvar aortic stenosis

Supra-valvar aortic stenosis was surgically induced, as described previously [38]. Rats (70-90 g) were anesthetized with intraperitoneal ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg), and the heart was exposed via a median thoracotomy. Then, a silver clip (0.60 mm internal diameter) was placed on the ascending aorta at approximately 3 mm from its root. During the surgery, the rats received 1 ml of warm saline solution intraperitoneal and manually ventilated with positive pressure on 100% oxygen. After the procedure, animals 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. Sham animals underwent the same procedure but without the constriction of the aorta.

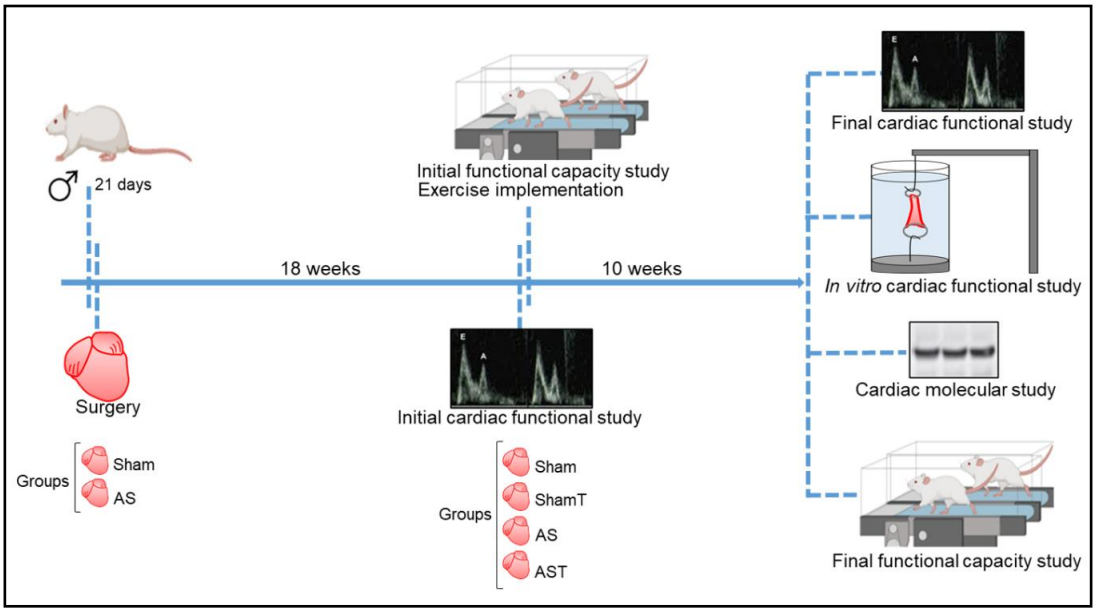

Fig. 1. Schematic representation of the experimental design. Sham, untrained control group; ShamT, trained control group; AS, untrained aortic stenosis group; AST, trained aortic stenosis group.

Treadmill exercise testing (TET) and exercise training protocol

Exercise tolerance assessed before and after the ET period was estimated by maximal speed, total time, and distance run achieved using graded TET as described previously [39, 40]. TET was performed on a motorized treadmill for rats (AVS Projetos – São Carlos, SP, Brasil) after one week of adaptation to the treadmill environment under low speed (5m/min/day). Briefly, TET began at 6 m/min and increased by 3 m/min every 3 minutes until exhaustion. Exhaustion was defined as non--maintenance of the race at the proposed speed. Furthermore, TET results were used to prescribe the exercise training protocol. The ET protocol was modified from those previously published [35, 41] (Table 1). Briefly, rats were exercised for ten weeks, five days/week (Monday to Friday) 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 minutes per week until 20 min/day and then remained constant from sixth to the tenth week. During the training, animals received low-voltage electrical stimulation.

Table 1. Exercise protocol. ES: exhaustion speed; TET: treadmill exercise testing

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

Echocardiographic study

In-vivo cardiac function and morphology were measured at 18 and 28 weeks post-surgery via echocardiography (Vivid S6, General Electric Medical Systems, Tirat Carmel, Israel) using a 5.0 ± 11.5 MHz multi-frequency transducer, in according to previous studies [23, 42]. All examinations were performed 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). A two-dimensional parasternal short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles [43]. 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. All LV structures were manually measured by the same observer according to the leading-edge method of the American Society of Echocardiography [44]. 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), relative posterior wall thickness (RWT), left atrium (LA), aortic diameter (AO) and LA/AO ratio. LV function was evaluated by heart rate (HR), posterior wall shortening velocity (PWSV), ejection fraction (EF), mesocardial fraction shortening (MFS), early and late diastolic mitral inflow velocities (E and A waves), and 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 [28, 44].

HF features and cardiac morphological evaluation

At euthanasia, was observed the occurrence of heart failure features *in vivo* (tachypnea) and *post-mortem* (ascites, pleural effusion, atrial thrombi, and liver congestion) in the AS and AST groups. Blind two observers carried out a subjective evaluation of HF signals throughout the experiment.

The atria (AT) and left (LV) and right (RV) ventricles were separated and weighed to determine cardiac hypertrophy. Tibia length was measured and used to normalize total heart weight (HW), and its separate components by the ratios HW/tibia, AT/Tibia, RV/tibia LV/tibia.

The histological evaluation of the myocardium, involving the myocyte transverse diameter, was performed on samples of the LV. After being fixed in a solution of 10% formol saline, the fragments were embed-

ded in paraffin blocks [22]. 4 µm sections, collected in histology slides, were stained with Picrosirius-red. At least 200 cells from the ventricular subendocardial layer were measured in each sample. The histological sections were projected at a magnification of 40 times, with the aid of a microscope (Leica DM LS) coupled to a video camera that projected the images on an IBM PC, which was equipped with the image analyzer software Image Pro-plus (Media Cybernetics, Silver Spring, Maryland, USA).

Myocardial mechanical performance and β-adrenergic stimulation

The isolated papillary muscle (IPM) function was carried in according to the protocol described previously [45, 46]. The following basal parameters were measured from isometric contraction at 2.5 mmol/L calcium concentration: peak developed tension (DT, g/mm^2), resting tension (RT, g/mm^2), time to peak tension (TPT, ms), maximum rate of tension development (+dT/dt, g/mm^2/s) and maximum rate of tension decline (-dT/dt, g/mm^2/s). All parameters were normalized by the cross-sectional area of the papillary muscle.

To evaluate the activity of the β-adrenergic system, was determinate the time to 50% developed tension (TPT_{50}, ms), and time from peak tension to 50% relaxation (RT_{50}, ms) in IPM preparation under stimulation with a non-selective β-agonist (isoproterenol). Isoproterenol (10^{-6} mol/L) (Sigma-Aldrich, St Louis, MO, USA) was added to the bath at 1.0 mmol/L calcium concentration.

Protein expression

The protein expression of β1 adrenergic receptor (β1AR), β2 adrenergic receptor (β2AR), Adenylate cyclase (AC), total PKA (tPKA), and phosphorylated PKA at Threonine 197 ($pPKA^{Thr197}$), total phospholamban (PLB), and phosphorylated PLB at Serine 16 (PLB^{Ser16}) and Threonine 17 (PLB^{Thr17}) was performed by Western Blot technique. In order to verify the influence of the nitric oxide pathway on β-adrenergic receptors responsiveness, we also evaluated the myocardial expression of inducible NO synthase (iNOS). LV samples were rapidly frozen in liquid nitrogen and subsequently homogenized in a solution containing cold RIPA buffer (Amresco LLC, Solon, OH) and protease (Sigma-Aldrich, St. Louis, MO) and phosphatase (Roche Diagnostics, Indianapolis, IN) inhibitors. The homogenate was centrifuged at 12,000g for 20min at 4°C, and the supernatant was collected. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Scientific, Wilmington, DE, USA). Samples (50µg) were subjected to SDS-PAGE in 10% polyacrylamide gel, and after that, were electrotransferred to the nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blotted membrane was blocked with 5% nonfat dry milk (20 mmol/L Tris-HCl pH 7.4, 137 mmol/L NaCl and 1% Tween 20) for 2 h at room temperature and then incubated overnight at 4-8°C with primary antibody against β1AR (1:1000; Abcam, Cambridge, MA, USA), β2AR (1:500; Abcam), AC 5/6 isoforms (1:300; Novus Biologicals, Littleton, CO, USA), tPKA (1:200; Abcam), $pPKA^{Thr197}$ (1:1000; Cell Signaling, Danvers, MA, USA), PLB (1:1000; Abcam), PLB^{Ser16}, PLB^{Thr17} (1:1000; Badrilla, Leeds, West Yorkshire, UK), and iNOS (1:1000, BD Transductions Laboratories, KY, USA). The immunoblots were washed three times with TBS-T and incubated for 1.5 h with peroxidase-conjugated anti-rabbit secondary antibody (1:5000 – 1:10000; Abcam), then washed three times again with TBS-T and incubated with ECL (Enhanced Chemi-Luminescence, Amersham Biosciences, Piscataway, NJ) for chemiluminescence detection in a western blot detection system (Image Quant™ LAS 40– GE Healthcare Life Sciences, Chalfont, UK), and quantified by densitometry using Image J Analysis software. We used enriched membrane fraction [47] for the detection of βARs and AC since the number of these proteins in the membrane is crucial to intracellular signaling. Targeted bands were normalized to the expression of cardiac GAPDH (1:2000; Santa Cruz, Dallas, TX, USA).

Myocardial TNFα measurement

Frozen LV samples (~ 50mg) VE were homogenized in 1.0mL of Phosphate-Buffered saline (PBS) pH 7.4 cold solution ULTRA-TURRAX® T25 basic IKA® Werke Staufen/Germany and centrifuged at 800g at 4°C for 10 min. The supernatant was used for the analysis of the tumoral necrosis factor-alpha (TNF-α) using the enzyme-linked immunosorbent assay (ELISA) method (R&D System, Minneapolis, MN, USA). The results were corrected by protein concentration.

Statistical analysis

Data are expressed as mean ± standard deviation. Kolmogorov-Smirnov normality test was used to test data normal distribution. Two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test

was used to analyze differences in echocardiogram, cardiac morphology, protein expression, and baseline IPM variables between groups. Two-way ANOVA for repeated measures followed by Bonferroni *post hoc* was performed to test the impact of exercise on functional capacity parameters between before and after ET protocol and on mechanical myocardial performance between baseline and isoproterenol addition in the IPM. Goodman test was used to test differences in heart failure signals between AS and AST groups. The statistical analyses were performed using SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA, USA). Differences between groups or moments were accepted as statistically significant at a $p < 0.05$.

Results

Exercise tolerance testing

Functional capacity was assessed by exhaustion speed, total time, and total distance during TET (Fig. 2). The initial test (18th post-surgery) showed that AS rats displayed exercise intolerance when compared with Sham. ET improved the functional capacity of trained groups in both intergroup and intragroup comparison.

Heart failure features

Pressure overload triggered HF in both AS groups (Fig. 3). Pleural effusion and tachypnea were the most evident HF features in both AS groups. AST rats presented a significant reduction in the frequency of the HF features. The survival rate did not differ between AS and AST groups.

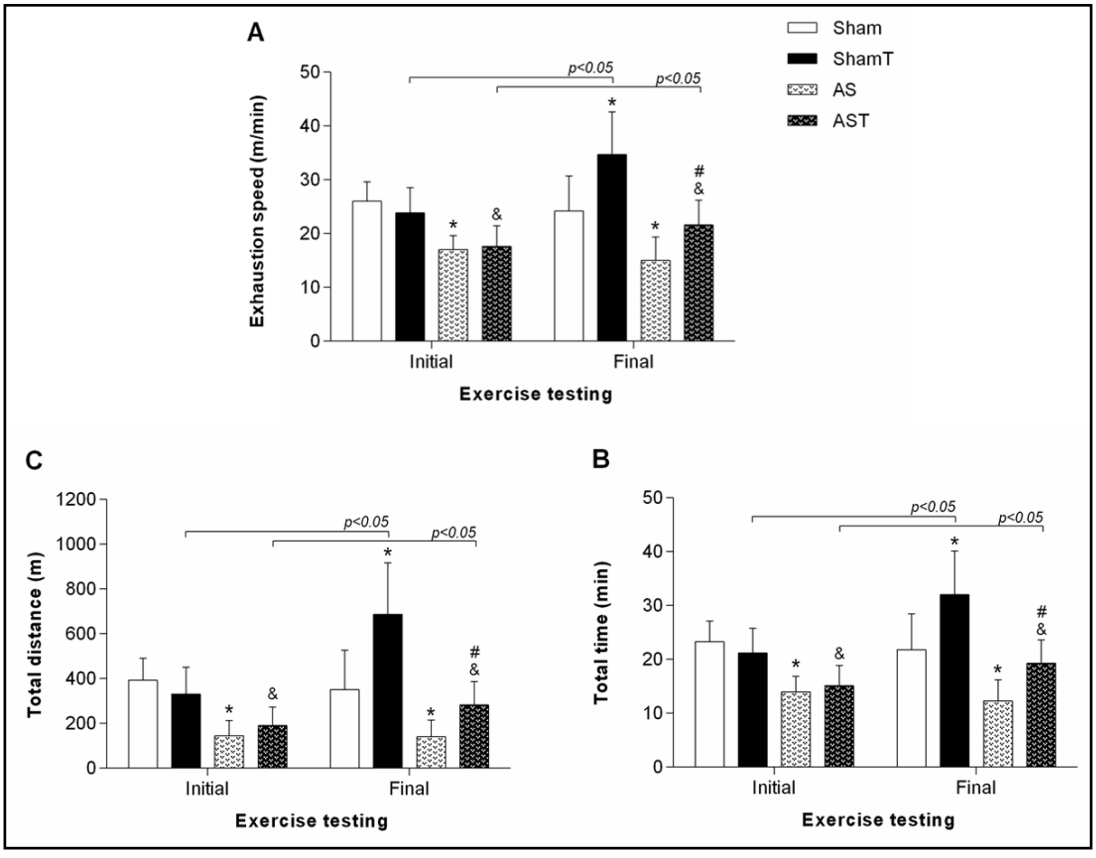

Fig. 2. Exercise tolerance evaluated by a treadmill exercise test. Exhaustion speed (A), total time (B), and distance (C) evaluated in treadmill exercise testing. Data expressed as mean ± SD. p <0.05. * vs. Sham; & vs. ShamT; # vs. AS. (Initial test, n= 18-32 each group and final test, n= 15-22 each group).

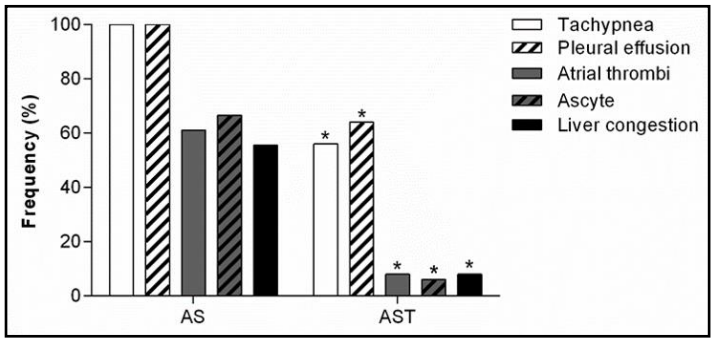

Fig. 3. Clinical-pathological heart failure features frequency. Data expressed as relative frequency. * p<0.05 vs. AS. (n = 16-26 each group).

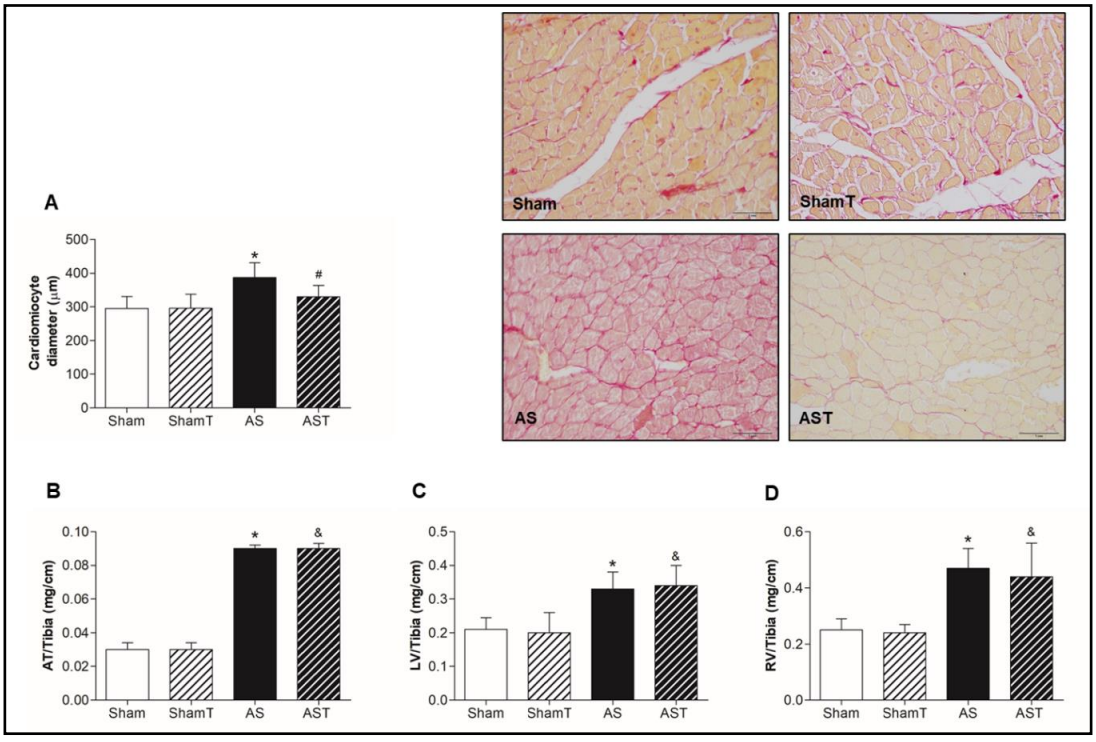

Fig. 4. Microscopic and macroscopic cardiac morphology. Representative images panel and myocardial morphometry (A); Atria to tibia ratio (B); left ventricle to tibia ratio (C), and right ventricle to tibia ratio (D). Data expressed as mean ± SD. p<0.05. * vs. Sham; # vs. AS. (n = 7 each group).

Cardiac morphology assessment

Aortic stenosis led to the hypertrophic remodeling of all cardiac chambers, and the exercise program did not attenuate the heart hypertrophy. However, the LV myocyte diameter was lower in AST than AS group. There was no difference between Sham and ShamT groups, both in the macroscopic and microscopic analysis (Table 2 and Fig. 4).

Table 2. Cardiac macroscopic morphology post-mortem. LV, left ventricle; RV, right ventricle; AT, atria. Data expressed as mean ± SD. p<0.05. * vs. Sham; & vs. ShamT; # vs. AS. (n = 15-22 each group)

Parameter	Groups			
	Sham	ShamT	AS	AST
Heart (g)	1.32 ± 0.15	1.23 ± 0.28	2.35 ± 0.29*	2.25 ± 0.43&
Heart/Tibia	0.29 ± 0.01	0.28 ± 0.06	0.53 ± 0.06*	0.52 ± 0.10&
LV (g)	0.95 ± 0.12	0.88 ± 0.27	1.47 ± 0.22*	1.49 ± 0.28&
LV/Tibia	0.21 ± 0.02	0.20 ± 0.06	0.33 ± 0.05*	0.34 ± 0.06&
RV (g)	0.25 ± 0.04	0.24 ± 0.03	0.47 ± 0.07*	0.44 ± 0.12&
RV/Tibia	0.05 ± 0.007	0.05 ± 0.007	0.11 ± 0.02*	0.10 ± 0.03&
AT (g)	0.12 ± 0.02	0.12 ± 0.02	0.43 ± 0.08*	0.40 ± 0.12&
AT/Tibia	0.03 ± 0.004	0.03 ± 0.004	0.09 ± 0.02*	0.09 ± 0.03&

Echocardiographic assessment

Before exercise program, an initial echocardiogram was performed to ensure the homogeneity between groups (data not shown). After the experimental period (28th-week post-surgery), the echocardiographic analysis (Table 3) showed that both AS groups presented left ventricular concentric hypertrophy and left atrium dilatation. ET did not change cardiac structure variables in AS rats. Heart rate was similar among all groups. Systolic and diastolic function were significantly impaired either in AS or AST when compared with Sham controls, as shown by EF, MFS, PWSV and TDI *s'*. However, the AST rats exhibit lower diastolic dysfunction than AS, as shown by higher A and TDI *a'* and lower E/A ratio. ShamT presented a significant improvement in EF, MFS, and PWSV parameters compared to Sham. These results suggest that ET improves systolic function in healthy rats, and attenuates diastolic dysfunction in advanced cardiac remodeling associated with AS.

Table 3. Echocardiographic assessment at 28th-week post surgery. BW, body weight; HR, heart rate; LVDD, left ventricle diastolic diameter; DPWT, diastolic posterior wall thickness; LA/Ao, left atrium to aorta ratio; EF, ejection fraction; MFS, mesocardial shortening fraction; PWSV, posterior wall shortening velocity; EDT, E-wave deceleration time; IVRT isovolumetric relaxation time; S': tissue Doppler imaging (TDI) of systolic; E/A, E wave to A wave ratio; 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. Data expressed as mean ± SD. $p<0.05$. * vs. Sham; & vs. ShamT; # vs. AS. (n = 15-22 each group)

Variables	Groups			
	Sham	ShamT	AS	AST
BW (g)	537 ± 57.0	523 ± 48.9	485 ± 37.1*	483 ± 47.2&
HR (bpm)	306 ± 41	329 ± 43	311 ± 35	310 ± 34
LVDD (mm)	7.65 ± 0.64	7.65 ± 0.38	7.97 ± 0.53	8.29 ± 0.99&
DPWT (mm)	1.66 ± 0.13	1.66 ± 0.14	2.97 ± 0.23*	2.84 ± 0.40&
RWT	0.43 ± 0.04	0.43 ± 0.03	0.75 ± 0.08*	0.70 ± 0.14&
LA (mm)	4.97 ± 0.32	4.95 ± 0.24	8.31 ± 0.84*	7.85 ± 1.19&
LA/Ao	1.27 ± 0.12	1.24 ± 0.09	1.97 ± 0.16*	1.92 ± 0.29&
EF (%)	90.4 ± 3.38	93.3 ± 1.12*	87.6 ± 5.73*	87.6 ± 3.57&
MFS (%)	25.8 ± 2.96	29.5 ± 2.90*	24.9 ± 4.34	24.0 ± 2.55&
PWSV (mm/s)	57.7 ± 5.32	63.9 ± 7.23*	40.6 ± 8.95*	42.1 ± 9.69&
s' (average, cm/s)	5.51 ± 0.32	5.85 ± 0.41	3.98 ± 0.36*	4.05 ± 0.77&
IVRT (ms)	22.6 ± 3.95	22.1 ± 2.53	13.3 ± 5.78*	14.1 ± 6.26&
EDT (ms)	49.7 ± 5.80	45.3 ± 4.87	31.3 ± 12.4*	31.7 ± 10.6&
E wave (cm/s)	81.9 ± 11.7	88.3 ± 13.9	132 ± 28.8*	133 ± 27.4&
A wave (cm/s)	56.1 ± 9.60	60.0 ± 11.6	24.5 ± 16.9*	36.8 ± 16.4&#
E/A	1.48 ± 0.19	1.49 ± 0.14	5.73 ± 1.01*	4.35 ± 1.86&#
e' (average, cm/s)	6.18 ± 1.06	7.16 ± 1.22	6.10 ± 1.64	6.05 ± 1.24
a' (average, cm/s)	4.48 ± 0.83	4.88 ± 0.79	3.01 ± 0.52*	3.79 ± 1.42&#
E/e'	13.5 ± 2.34	12.4 ± 1.37	22.7 ± 7.12*	22.9 ± 6.15&

Myocardial function assessment

Myocardial mechanical performance was assessed by the IPM technique (Table 4 and Fig. 5). The comparison between Sham and AS showed that pressure overload impaired the myocardial mechanical performance in DT, RT, and speed efficiency myocardial response (+dT/dt and TPT). In support of *in vivo* diastolic measurements, AST displayed significantly lower RT value than AS rats, which indicates a positive effect ET in myocardial compliance preservation.

Myocardial responsiveness to stimulation of β-adrenoceptors

The effects of inotropic stimulation on the IPM function are shown in Fig. 6. No difference was observed between Sham and ShamT groups. AS group presented higher time to 50% developed tension (Fig. 6-A) and time from peak tension to 50% relaxation (Fig. 6-B) when compared with Sham rats at baseline or under isoproterenol 10^{-6} (M). Interestingly, AST rats presented greater myocardial performance than AS and displayed reduced time to 50% tension development and relaxation under β-agonist stimulation.

Protein expression

Myocardial β-adrenergic signaling downstream components are shown in Fig. 7. Myocardial protein levels of β1AR, AC, PKA, and pPKA were not affected by either pressure overload or ET. However, the β2AR protein level was increased in AS rats independently of ET. In spite of increased β2AR protein level and no changed β1AR, AC, PKA, and pPKA protein levels AS rats displayed reduced levels of pPLB at both Ser^{16} and Thr^{17} residues. Interestingly, ET restored the protein levels of pPLB at Ser^{16} residue. To assess the role of the cytokine-NO pathway on β-adrenergic responsiveness we added the protein level quantification of iNOS and TNFα. Both the iNOS and TNFα expression were shown higher in the AS group, while AST did not differ from their respective control (Fig. 7-C and -D).

Table 4. Baseline condition of isolated papillary muscle. DT, maximum developed tension; RT, resting tension; + dT/dt, peak of the positive tension derivate; - dT/dt, peak of the negative tension derivate; CSA, cross-sectional area of the papillary. Data expressed as mean ± SD. p<0.05. * vs. Sham; & vs. ShamT; # vs. AS. (n = 15-22 each group)

Variables	Groups			
	Sham	ShamT	AS	AST
DT (g/mm²)	7.12 ± 1.42	6.35 ± 1.60	5.18 ± 1.23*	5.70 ± 1.31
RT (g/mm²)	0.64 ± 0.14	0.56 ± 0.16	0.88 ± 0.23*	0.75 ± 0.17&#
+ dT/dt (g/mm²/s)	78.6 ± 18.3	71.9 ± 20.7	45.0 ± 13.2*	54.0 ± 13.8&
- dT/dt (g/mm²/s)	24.7 ± 4.40	24.3 ± 5.20	23.3 ± 4.92	25.9 ± 6.90
TPT (ms)	183 ± 10.7	179 ± 20.6	216 ± 22.3*	208 ± 18.0&
CSA (mm²)	1.10 ± 0.21	1.10 ± 0.20	1.17 ± 0.25	1.21 ± 0.27

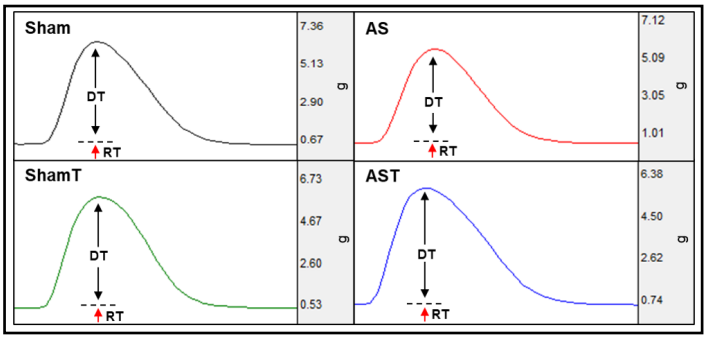

Fig. 5. Illustrative papillary muscle recordings during isometric contraction. DT: peak of developed tension (g); RT: resting tension (g).

Discussion

In this study, we aimed to investigate the role of a moderate ET program to reduce cardiac dysfunction following pressure overload and to clarify how ET modulates β-adrenergic signaling in a severe HF model associated with SVAS. We showed that ET 1) improved exercise tolerance, 2) attenuated diastolic dysfunction, myocardial mechanical performance, and peripheral manifestations of heart failure and 3) reduced β-adrenergic signaling impairment in the SVAS model.

In sustained pressure overload associated with AS, the early compensatory hypertrophic process becomes maladaptive leading to cardiac functional deterioration and pathological remodeling related to disorders at the molecular and cellular levels [48]. Here, we demonstrated that SVAS induced cardiomyocyte hypertrophy, which was attenuated by ET, as visualized by histological analysis. Echocardiographic data showed a concentric pattern of hypertrophy and systolic and diastolic ventricular dysfunction in AS rats; this cardiac performance impairment was confirmed by the myocardial mechanical study performed in isolated papillary muscles.

For this study, the training was started when rats had ventricular dysfunction, but no signs of HF. The training protocol was adapted from previous studies with the same experimental model [33–36]. Consistent with the earlier investigations [34, 36, 49], both trained groups presented higher functional capacity than sedentary Sham and AS groups. These data strengthen the efficacy of the exercise protocol used to improve exercise tolerance in normal or pathological conditions.

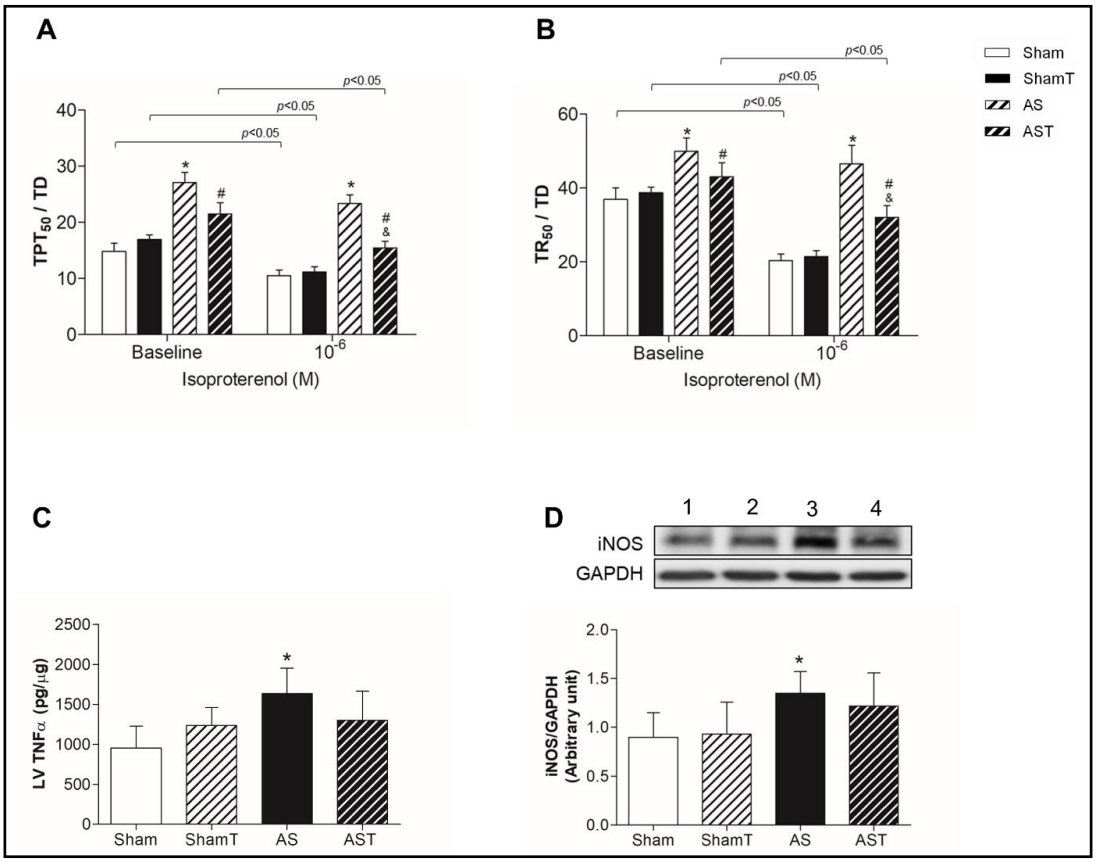

Fig. 6. β-adrenergic responsiveness. Time to 50% developed tension (TPT_{50}, ms) (A) and time from peak tension to 50% relaxation (RT_{50}, ms) (B) evaluated in isolated papillary muscle, and left ventricle (LV) TNFα (C) and inducible nitric oxide synthase (D) levels. Data expressed as mean ± SD. The numbers above representative bands represent Sham, ShamT, AS, and AST, respectively. ANOVA and Bonferroni post hoc analysis. p<0.05. * vs. Sham; # vs. AS. (n = 14-19 each group for β-adrenergic responsiveness and 07 each group for protein expression).

ET did not affect impaired systolic function in the AS rats. This result suggests that the efficacy of exercise in attenuating systolic dysfunction during HF progression depends on the degree of cardiac functional impairment. Positive effects of ET on systolic function were demonstrated when animals enroll ET before systolic dysfunction occurs [33–35], and no improvement was obtained in the present study and other [36] where animals presented systolic dysfunction before starting ET.

ET attenuated the diastolic dysfunction in AST rats, as observed in echocardiographic and myocardial mechanical evaluation. The improved diastolic function is associated with an enhanced LA contractile capacity in AST rats, shown by A and *a'* values, and a presumably reduced LV end-diastolic pressure. The reduced LV end-diastolic pressure is supported, at least in part, by the improved mechanical function observed *in vitro* in AST papillary muscles, which showed a decrease in the RT. Although the noninvasive detection of diastolic dysfunction by E/*e'* index has been widely accepted [50], this index was similar among sedentary or trained groups. In contrast, the E/A ratio showed a better diastolic performance in AST. The marked stiffness of hypertrophied muscle possibly blocked the decay in *e'* values, as demonstrated by authors who also investigated this variable in the model of SVAS [36, 37]. Therefore, for the remodeling pattern of the animals studied, the E/e' index may not be the most accurate one for evaluating the diastolic function. In this regard, the E/A ratio plays an important role, since its determinants are the initial (E) and late (A) transmitral flow velocity, including both phases of ventricular filling.

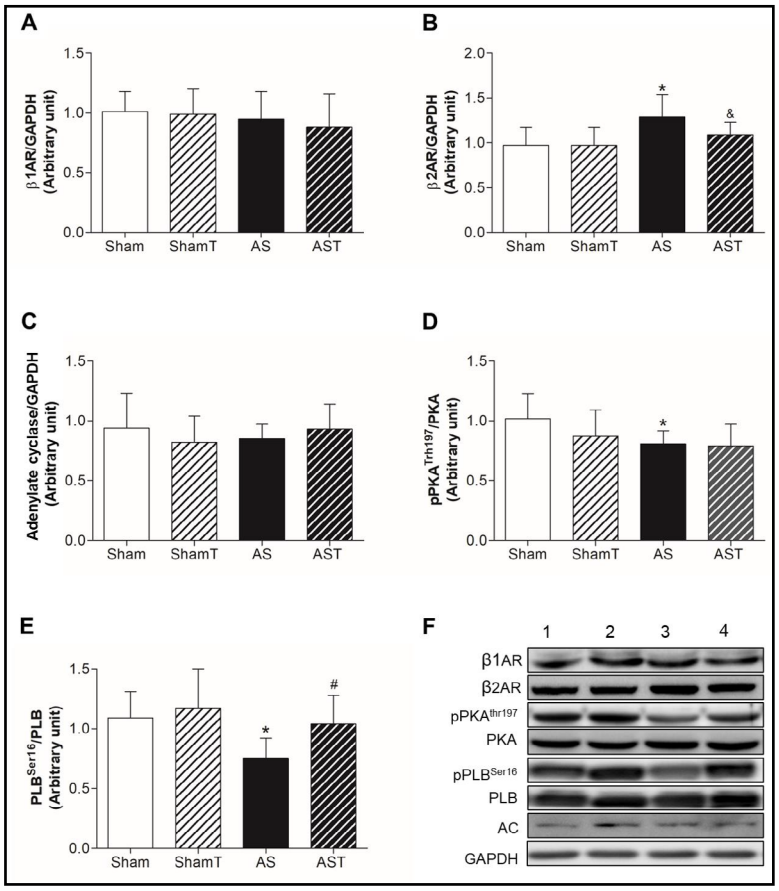

Fig. 7. Quantification and representative western blots. β1 adrenergic receptor (A), β2 adrenergic receptor (B), adenylate cyclase (C), phosphorylated protein kinase A at Thr 197 to total protein kinase A ratio (D), phosphorylated phospholamban at Ser 16 to total phospholamban (E), and representative Western blots (F). The numbers above representative bands represent Sham, ShamT, AS, and AST, respectively. Data expressed as mean ± SD. $p<0.05$. * vs. Sham; & vs. ShamT; # vs. AS. (n = 7 each group).

Besides the attenuation of left ventricular diastolic dysfunction, ET improved exercise tolerance and reduced clinical-pathological signs of heart failure. All evaluated HF features are related to extra-cardiac fluid retention caused by cardiorenal axis disturbance, in response to sympathetic nervous and the renin-angiotensin systems hyperactivation due to sustained pathological stimulus [17, 51]. The beneficial effects of ET on the control of the activation of the renin-angiotensin system [52, 53] and the sympathetic hyperactivation [52–54] are widely described in the literature, and support the hypothesis that the ET protocol employed in this study attenuated the cardiorenal derangement with consequent reduction of fluid retention in the AST group.

We presently assessed the expression of proteins involved in β-adrenergic signaling to test whether ET would improve cardiac function by reestablishing expression levels of proteins downstream of the β-adrenergic signaling pathway. The adaptive protection mechanism against catecholamine-induced cardiotoxicity notably reduces the ability of the failing heart to respond to inotropic stimulation and aggravates the disease prognosis [17, 55, 56]. Deficient myocardial responsiveness to β-adrenergic agonist stimulation appears to be related to impairments in tissue contractile properties and the specific inefficiency of intracellular β-adrenergic signaling [17, 55, 57]. The failing heart presents selective downregulation of the β1AR subtype population and unchanged or, mostly, augmented β2AR subtype myocardial density that results in an adaptive negative inotropic response, aiming to reduce the system hyperstimulation [14, 17, 55]. In contrast, our results showed that despite evidence of HF, the level of β1AR subtype protein in the membrane was maintained. This finding is particularly intriguing since extensive literature points to a reduction in β1AR levels in failing hearts. Therefore, at least two considerations should be made as 1) AS rats had mild systolic dysfunction, which would be incompatible with a change in β1AR expression pattern, and 2) immunoblot is not the most accurate method for quantifying membrane adre-

noceptor levels. Thus, further studies with a more reliable method to determine changes in β-adrenergic receptor density, such as radioligand binding, are needed to clarify the β1AR behavior in this model.

The expression of the β2AR subtype was increased in both sedentary and trained AS rats. Since β2AR subtype is a counter-regulatory of β1AR overstimulation [12], β2AR overexpression indicates β1AR hyperactivity. Under exacerbated stimulation, the coupling of β2AR to inhibitory G protein ($G_{\alpha\text{-}i}$), becomes more prominent, resulting in negative inotropic response [58] and augmented expression of $G_{\alpha\text{-}i}$ protein is observed in heart failure [59]. Therefore, the augmented β2AR level expresses a compensatory mechanism for countering the negative impact of β1AR overstimulation.

The desensitization of β-adrenergic receptor signaling comprises not only the modified myocardial receptors but a set of changes in other critical components of the β-adrenergic pathway, such as AC and levels and activity of PKA [17]. In this study, no differences were observed in AC and PKA protein levels among groups. However, AS rats had lower PKA phosphorylation status, assessed by the $pPKA^{Thr197}$/PKA ratio, in parallel to reduced PLB^{Ser16}. ET prevented PLB phosphorylation decline, which is recognized as a positive indicator of calcium reuptake status. A likely mechanism for this exercise outcome is the modulation of PKA activity. However, although AST showed an unchanged PKA phosphorylation level, we did not perform reliable measurements of the activity of this kinase; therefore, the role of PKA in PLB phosphorylation in this experimental context remains to be elucidated. On the other hand, HF is found to be accompanied by high level and activity of protein phosphatase [60, 61], as a consequence of βAR overstimulation [62]. Thus, the higher expression of the phosphorylated PLB in the AST in comparison to the AS group may also be related to the restoration of the balance between phosphorylation and dephosphorylation, ultimately leading to an improved diastolic function. This is a plausible explanation for the maintenance of PLB phosphorylation since PKA phosphorylation did not differ between AS and AST, and phosphorylation is an important step of kinase activation. For this study, we did not examine the calcium handling mechanistically, and the PLB phosphorylation study aimed to evaluate the β-adrenergic system phosphorylation capacity. Additional studies should look at cAMP production to improve knowledge regarding the β-adrenergic activity.

To assess the functional status of β-adrenergic signaling and to test its molecular disturbance over the cardiac mechanical performance, a non-selective β-agonist was used in the IPM study. The AS group did not respond significantly to β-agonist stimulation, while the AST group showed a positive response in both strength development and relaxation phases. Previously, it has been shown in studies with human hearts [63, 64] and with rodent hearts [65–68] that the responsiveness of the β-adrenergic system is improved by exercise training even regardless of the changes in myocardial receptor density [69], which is in line with our findings. Thus, the downstream signaling of β-adrenergic receptors had a proper cascade transduction signaling.

The increased mechanical response to the agonist in the trained group could be due to a number of mechanisms, including an increase in β-receptor affinity and improved receptor-adenylate cyclase coupling [71], as well as an increase in G_s amount [70]. In addition, increased levels of cardiac G-protein coupled receptor kinase-2 (GRK-2) is tightly related to receptor dysfunction by attenuating the intracellular G protein signaling, and extensive literature documented a positive effect of exercise in reducing GRK-2 levels in failing hearts, as reviewed [71]. The β-adrenergic signaling reestablishment following exercise may have been responsible for preserved activation of PLB, a target of this phosphorylation system [72, 73], which contributed to the positive myocardial contractile responsiveness in the AST. Indeed, the major regulator of PLB activity is the β-adrenergic system since PLB phosphorylation at serine 16 regulates SERCA2a operation, which may hamper or not recapturing Ca^{2+} from the sarcoplasmic reticulum [74].

Here, we also showed that the AS group presented increased TNF-α and iNOS, which were prevented by ET in the AST group compared to their respective control. A remarkable characteristic of HF is the inotropic hyporesponsiveness to β-adrenergic stimulation often

related to reduced density of membrane receptors [5]. However, enhanced expression of TNF-α accompanied by overproduction of NO may play an additional role in the pathogenesis of HF [21]. On the other hand, previous studies have demonstrated the influence of exercise in reducing pro-inflammatory markers such as TNF-α [75, 76].

A vast literature has shown that myocardial β-adrenergic hyporesponsiveness may have been mediated by hyperstimulation of iNOS [77, 78], which is activated mostly by inflammation and cytokine, where TNF-α enrolls part. Therefore, our results suggest that impaired myocardial responsiveness to inotropic stimulation is also, in part, mediated by enhanced NO production in the myocardium. ET prevented significative iNOS overexpression and was involved with a lower impairment in the myocardial contractility than in the sedentary group. Based on these observations, the data suggest a potential role of cytokine and NO on β-adrenergic responsiveness in the pressure overload condition. Additional studies are required to identify other potential mechanisms underlying the beneficial impact induced by exercise on cardiac function and refine the knowledge about the relationship between exercise and β-adrenergic signaling in the pressure overload condition. Furthermore, more significant on cardiac function results should be achieved with early exercise implementation, which remains to be tested in this experimental model.

This study demonstrates a significant clinical relevance since revealing a beneficial impact of short sessions of moderate ET on cardiac function and functional capacity and, specifically, on β-adrenergic signaling. Since ET delays HF progression, it can be a useful adjunct to the management of this syndrome in conjunction with β-blockers, improving the clinical status of heart disease patients, including those with aortic stenosis.

Conclusion

Moderate exercise training attenuates cardiac dysfunction and β-adrenergic receptor responsiveness decline, which supports the hypothesis that adjustments in the β-adrenergic signaling contributes to attenuation of cardiac dysfunction by exercise training in aortic stenosis rats.

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Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

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