LEÔNCIO LOPES SOARES

EFFECTS OF RESISTANCE EXERCISE TRAINING ON THE STRUCTURE AND FUNCTION OF CARDIAC, PULMONARY AND SKELETAL MUSCLE TISSUES IN RATS WITH EXPERIMENTAL PULMONARY ARTERIAL HYPERTENSION

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Physical Education, to obtain the title of *Doctor Scientiae*.

Advisor: Antônio José Natali

Co-advisors: Emily Correna Carlo Reis
Mariana Machado Neves

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Assent:

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Author

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To all those who encouraged, supported and welcomed me...

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ABSTRACT

SOARES, Leôncio Lopes, D.Sc., Universidade Federal de Viçosa, December 2022. **Effects of resistance exercise training on the structure and function of cardiac, pulmonary and skeletal muscle tissues in rats with experimental pulmonary arterial hypertension**. Advisor: Antônio José Natali. Co-advisors: Emily Correna Carlo Reis and Mariana Machado Neves.

The general objective of this work was to evaluate the effects of low- to moderate-intensity resistance exercise training (RT) on the structure and function of pulmonary, cardiac and skeletal muscle tissues in the stable pulmonary artery hypertension (PAH) model induced by monocrotaline (MCT) in rats. In the first study we focused on evaluating the effects of RT on the structure and oxidative stress of the lung and biceps brachii, as well as on the function, structure, single myocyte contractility and gene and protein expression in the right ventricle (RV). Male Wistar rats were randomly divided into groups: sedentary hypertension until failure; exercise hypertension until failure; sedentary control; exercise control; sedentary hypertension; and exercise hypertension. PAH was induced by two MCT injections (20 mg/kg, with 7 days interval). After the first MCT injection, animals in the exercise groups were submitted to a low- to moderate-intensity RT protocol (Ladder climbing; 55-65% of the maximal carrying load), 5 times/week, during the experimental period. Echocardiographic examination and physical effort tolerance test were carried out at specific time points of the experimental period. After euthanasia, lung, heart, and biceps brachii were dissected, weighed, and processed for histological, single myocyte, and biochemical analysis. The results show that RT improved survival and physical effort tolerance (i.e., Maximum carrying load), mitigated the pulmonary artery resistance increase (i.e., TA/TE), and preserved cardiac function (i.e., Fractional shortening, ejection fraction, stroke volume and TAPSE). In addition, RT counteracted oxidative stress (i.e., CAT, SOD, GST, MDA and NO) and adverse remodeling in lung (i.e., Collapsed alveoli) and in biceps brachii (i.e., atrophy and total collagen) tissues. Moreover, RT retarded RV adverse remodeling (i.e., hypertrophy, extracellular matrix, collagen types I and III, and fibrosis) and impairments in single RV myocyte contractility (i.e., amplitude and velocity to peak and relaxation). Furthermore, RT improved the expression of gene (i.e., miRNA 214) and regulatory proteins of the intracellular Ca^{2+} cycling (i.e., PLB^{ser16}) as well as of pathological (i.e., α/β-MHC, and Foxo3) and physiological (i.e., Akt, p-Akt, mTOR, p-mTOR, and Bcl-xL) hypertrophy pathways in RV tissue. In conclusion, along with

survival and physical effort tolerance enhancement, low- to moderate-intensity RT during the development of stable MCT-induced PAH postpones pulmonary artery resistance increases and prevents RV dysfunction, RV adverse remodeling and myocyte contractility deterioration in rats. In the second study, we investigated whether low- to moderate-intensity RT is beneficial to left ventricle (LV) and LV myocyte contractile functions in such model of stable PAH induced by MCT. Following the experimental design of the first chapter, the results showed that in conjunction with the improvements in survival and physical effort tolerance, RT mitigated the LV and cardiomyocyte contractility dysfunctions promoted by MCT by preserving the ejection fraction and fractional shortening, the amplitude of shortening, and the velocities of contraction and relaxation in cardiomyocytes. Resistance exercise training also prevented increases in LV fibrosis and type I collagen caused by MCT and maintained the type III collagen and myocyte dimensions reduced by MCT. In conclusion, low- to moderate-intensity RT benefits LV and cardiomyocyte contractile functions in rats during the development of stable MCT-induced PAH. Taken together, these results are of clinical relevance insofar as it indicates that low- to moderate-intensity RT may contribute positively to the health and survival of individuals with stable PAH.

Keywords: Pulmonary hypertension. Heart failure. Ventricular dysfunction. Physical exercise. Exercise tolerance. Adverse remodeling. Isolated cardiomyocytes.

RESUMO

SOARES, Leôncio Lopes, D.Sc., Universidade Federal de Viçosa, dezembro de 2022. **Efeitos do treinamento físico resistido na estrutura e função dos tecidos cardíaco, pulmonar e muscular esquelético em ratos com hipertensão arterial pulmonar experimental**. Orientador: Antônio José Natali. Coorientadores: Emily Correna Carlo Reis e Mariana Machado Neves.

O objetivo geral deste trabalho foi avaliar os efeitos do treinamento físico resistido (TR) de baixa a moderada intensidade na estrutura e função dos tecidos pulmonar, cardíaco e muscular esquelético no modelo de hipertensão arterial pulmonar (HAP) estável induzida por monocrotalina (MCT) em ratos. No primeiro estudo nos concentramos em avaliar os efeitos do TR na estrutura e estresse oxidativo do pulmão e bíceps braquial, bem como na função, estrutura, contratilidade de cardiomiócitos isolados e expressão gênica e proteica no ventrículo direito (VD). Ratos Wistar foram divididos aleatoriamente em grupos: sedentário hipertenso até a falha; exercitado hipertenso até a falha; sedentário controle; exercitado controle; sedentário hipertenso; e exercício hipertenso. A HAP foi induzida por duas injeções de MCT (20 mg/kg, com intervalo de 7 dias). Após a primeira injeção de MCT, os animais dos grupos de exercício foram submetidos a um protocolo de TR de baixa a moderada intensidade (subida de escada; 55-65% da carga máxima carregada), 5 vezes/semana, durante o período experimental. O exame ecocardiográfico e o teste de tolerância ao esforço físico foram realizados em momentos específicos do período experimental. Após a eutanásia, pulmão, coração e bíceps braquial foram dissecados, pesados e processados para análise histológica, de cardiomiócitos isolados e bioquímica. Os resultados demonstram que o TR melhorou a sobrevivência e a tolerância ao esforço físico (ou seja, carga máxima carregada), mitigou o aumento da resistência da artéria pulmonar (ou seja, TA/TE) e preservou a função cardíaca (ou seja, fração de encurtamento, fração de ejeção, volume sistólico e TAPSE). Além disso, a RT neutralizou o estresse oxidativo (ou seja, CAT, SOD, GST, MDA e NO) e a remodelação adversa nos tecidos do pulmão (ou seja, alvéolos colapsados) e do bíceps braquial (ou seja, atrofia e colágeno total). Além disso, a TR retardou a remodelação adversa do VD (ou seja, hipertrofia, matriz extracelular, colágeno tipos I e III e fibrose) e prejuízos na contratilidade de miócitos isolados do VD (ou seja, amplitude e velocidade para pico e relaxamento). Além disso, a TR melhorou a expressão gênica (ou seja, miRNA 214) e proteínas reguladoras do ciclo intracelular

de Ca^{2+} (ou seja, PLB^{ser16}), bem como de fatores patológicos (ou seja, α/β-MHC e Foxo3) e fisiológicos (ou seja, Akt , p-Akt, mTOR, p-mTOR e Bcl-xL) no tecido do VD. Em conclusão, juntamente com o aumento da sobrevivência e tolerância ao esforço físico, a TR de baixa a moderada intensidade durante o desenvolvimento de HAP induzida por MCT estável adia o aumento da resistência da artéria pulmonar e previne a disfunção do VD, remodelamento adverso do VD e deterioração da contratilidade dos miócitos em ratos. No segundo estudo, investigamos se a TR de baixa a moderada intensidade é benéfica para a função do ventrículo esquerdo (VE) e contratilidade dos miócitos do VE nesse modelo de HAP estável induzida por MCT. Seguindo o desenho experimental do primeiro capítulo, os resultados mostraram que em conjunto com as melhorias na sobrevivência e tolerância ao esforço físico, o TR atenuou as disfunções da contratilidade do VE e dos cardiomiócitos promovidas pelo MCT ao preservar a fração de ejeção e a fração de encurtamento, a amplitude de encurtamento, e as velocidades de contração e relaxamento em cardiomiócitos. O treinamento de força também preveniu o aumento da fibrose do VE e do colágeno tipo I causados pelo MCT e manteve as dimensões do colágeno tipo III e dos miócitos reduzidos pelo MCT. Em conclusão, o TR de intensidade baixa a moderada beneficia as funções contráteis do VE e dos cardiomiócitos em ratos durante o desenvolvimento de HAP estável induzida por MCT. Em conjunto, esses resultados são de relevância clínica na medida em que indicam que o TR de baixa a moderada intensidade pode contribuir positivamente para a saúde e sobrevivência de indivíduos com HAP estável.

Palavras-chave: Hipertensão pulmonar. Insuficiência cardíaca. Disfunção ventricular. Exercício físico. Tolerância ao exercício. Remodelamento adverso. Cardiomiócitos isolados.

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LIST OF ABBREVIATIONS

Akt – Protein kinase B

ANF– Atrial natriuretic factor

ANOVA – Analysis of variance

AT – Acceleration time

ATPase – An enzyme that catalyzes the hydrolysis of ATP for energy production

Bcl-xL– B-cell lymphoma-extra large

BW – Body weight

Ca^{2+} – Calcium ion

CaCl2 – Calcium chloride

cDNA – Complementary DNA

CO – Cardiac output

CSA – Cross-sectional area

DNA- Deoxyribonucleic acid

EC – Exercise control

EF – Ejection fraction

EGTA – Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

EH – Exercise hypertension

EHF – Exercise hypertension until failure

ET – Ejection time

FLB – Phospholamban

Foxo3 – Forkhead box O

FPS – Frames per second

FS – Fractional shortening

GAPDH – Gliceraldeído-3-fosfato desidrogenase

H – Horas

Hz – Hertz

IGF-1 – Insulin-Like Growth Factor-1

KCl – Potassium chloride

LV – Left ventricle

LV + SW – Left ventricle plus septum weight

MAPKs – Mitogen Activated Protein Kinases

MCT – Monocrotaline

Mg – Milligram

mg/kg – Milligram per kilogram

mg/ml – Milligram per milliliter

$MgCl_2$ – Magnesium chloride

MHz – Megahertz

min – Minutes

miRNA – microARNA

mM – Millimolar

mmHg – Millimeters of mercury

mRNA – Messenger RNA

mTOR – Mammalian target activities of rapamycin

NaCl – Sodium Chloride

NaH_2PO – Sodium phosphate monobasic monohydrate

NaOH – Sodium hydroxide

NCX – Sodium–calcium exchanger channel

NFAT – Nuclear factor of activated T-cells

NF-kβ – Nuclear factor kappa B

NO – Nitric oxide

O_2 – Oxygen

PAH – Pulmonary arterial hypertension

PCR – Polymerase chain reaction

PH – Pulmonary hypertension

pH – Hydrogen potential

PI3K – Phosphatidylinositol 3-kinase

PKB – Protein kinase B

pl – Picoliters

PVR – Pulmonary vascular resistance

r.c.l – Resting cell length

RNA – Ribonucleic acid

RT – Resistance exercise training

RV – Right ventricular

RVW – Right ventricle weight

RVW: LV + SW – Fulton Index

RyR2 – Ryanodine receptor type 2

SC – Sedentary control

SEM – Standard error of mean

^{Ser} – Serine

SERCA2a – Sarco/endoplasmic reticulum Ca^{2+}-ATPase

SH – Sedentary hypertension

SHF – Sedentary hypertension until failure

SD – Standard deviation

TAPSE – Tricuspid annular plane systolic excursion

^{Thr} – Threonine

TNF-α – Tumor necrosis fator alpha

TNF-α – Tumor necrosis factor alpha

TWEAK – Tumor necrosis factor inducing receptors

VO_{2max} – Maximum oxygen volume

x^2 – Chi-square test

α-MHC – Alpha myosin heavy chain

β-MHC – Beta myosin heavy chain

µg – Microgram

µl – Microliter

µm – Micrometer

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1. INTRODUCTION

Pulmonary hypertension (PH) is a rapidly progressing disease that affects 15 to 50 people per million in the world and is most often fatal (1). Pulmonary hypertension was previously classified as primary or secondary, according to the absence or presence of a causal agent or identified risk factors. Currently, a clinical classification is established to individualize different categories of PH according to pathological findings, hemodynamic characteristics, and similar treatments. This classification is related to the five main etiologies, including left heart disease, respiratory disease and/or hypoxia, chronic thromboembolic disease, unclear multifactorial mechanisms, and pulmonary arterial hypertension (PAH) (2, 3).

Pulmonary arterial hypertension, the most frequent subtype of PH, is a progressive disease of the pulmonary vascular system. Defined as pulmonary vasculopathy, PAH is characterized by mean resting pulmonary arterial pressure above 20 mmHg and pulmonary capillary pressure below 15 mmHg (3, 4). This disease can be caused by several factors, including chronic shear stress, hypoxia, autoimmune phenomena, viral infections, drugs and toxins, or genetic alterations, which results in luminal narrowing. Such framework arises from the proliferation and remodeling of endothelial smooth muscle cells, in situ thrombosis, resistance to apoptosis, inflammation and fibrosis mediated by a dramatic remodeling of the extracellular matrix of pulmonary arteries and arterioles, which results in increased pulmonary vascular resistance (PVR) and ultimately heart failure (5).

The chronic increase in PVR leads to an increase in right ventricular (RV) afterload. This overload generates an adaptive response or adverse RV remodeling, which results in hypertrophy associated with increased passive tension in the sarcomeres, collagen deposition in the extracellular matrix and fibrosis in the

myocardium, inflammation, oxidative stress and cell apoptosis and, consequently, dilation and contractile dysfunction (6). This state leads to RV failure as structural and functional damage progresses (7). Heart failure is the leading cause of death among patients with PAH (8, 9).

The adverse RV remodeling caused by PAH, affects the left ventricle (LV) dynamics because of the direct ventricular interaction. In this framework the left ventricle dynamics are damaged by the interventricular septum flattening (10, 11) as it faces impaired early diastolic filling, reduced end-diastolic volume and adverse remodeling (10, 12-14). Therefore, PAH patients exhibit reduced stroke volume (10).

The structural and functional changes in the small circulation and the damages and dysfunctions in the ventricles of patients with PAH, are associated with impaired functional capacity as a result of intolerance to physical effort, since the ability of the RV to increase stroke volume during physical effort is impaired. Thus, cardiac output (CO) is compromised due to RV and LV dysfunction and less systemic oxygen supplementation is evidenced, which in turn affects skeletal muscles, contributing to a decrease in type I fibers, an increase in anaerobic metabolism, increased oxidative stress and inflammation, suppression of hypertrophic signaling pathways, atrophy and impaired muscle contraction, which contributes to the reduction the physical work tolerance in PAH patients (15).

All these central and peripheral damages caused by PAH, often leads the patients to present shortness of breath during physical exertion, fatigue, lethargy, fluid retention, edema in the lower limbs and pre-syncope/syncope. In addition, ventilatory inefficiency and loss of muscle mass contribute to intolerance to physical exertion, impaired functional capacity and consequent decline in the quality of life of patients (16).

Animal models [chronic hypoxia; monocrotaline (MCT) injury; MCT and pneumonectomy; Sugen 5416 and hypoxia and knockout mice] have been used to study PAH and understand the cellular and molecular mechanisms involved in the pathology of the disease, as well as for the discovery of therapeutic targets. In the most used model, the model of severe PAH induced by monocrotaline (MCT; single injection of 60 mg/kg body mass), there is endothelial dysfunction followed by an increase in pulmonary artery resistance which, in turn, leads to pathological remodeling of the lung, i.e., oxidative stress, inflammation, enlargement of the alveoli wall, increase in connective tissue and collagen content, and reduction in number of alveoli and reduction of blood vessel lumen (17, 18). These changes in the small circulation leads to increased pulmonary resistance and, consequently, RV overload. In this case, the RV undergoes hypertrophy and dilation associated with increased collagen deposition in the extracellular matrix, inflammation, apoptosis and fibrosis, which contributes to the deterioration of its function (4, 19). At the cellular level, RV myocytes commonly exhibit excitation-contraction coupling anomalies, 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 (20, 21). These alterations in the excitation-contraction coupling in PAH, in turn, are associated with disturbances in the expression and activity of Ca^{2+} regulatory proteins, such as phospholamban (FLB), SERCA2a (sarcoplasmic reticulum Ca^{2+} ATPase), ryanodine receptor type 2 (RyR2) and sodium–calcium exchanger channel (NCX) (16, 17). These pulmonary and cardiovascular changes reduce blood flow and oxygen supplementation to skeletal muscle, which in turn leads to an increase in the formation of reactive oxygen species and disruption of cellular redox status, favoring inflammation and tissue damage, thus impairing muscle functionality (22-24).

Despite that, the model of MCT lung injury in rodents using a single injection of 60 mg/kg body mass induces severe PAH in a subacute process, which is limited to simulate human chronic PAH (25). In this sense, Zhuang et al. (2018) (26) demonstrated that 40 mg/kg MCT divided into two injections of 20 mg/kg with an interval of seven days better mimics chronic PAH with those common changes in the structure and function of pulmonary arteries and right ventricle observed in humans. However, cell mechanics, as well as the roles of intracellular Ca^{2+} regulatory proteins and its regulatory gene expression at the post-transcriptional level [i.e., microRNAs (miRNAs)] in this model is not known. In addition, to our knowledge, to date, the morphological changes, and changes in the redox state in pulmonary and skeletal muscle tissues in this PAH model are unknown.

Due to the clinical characteristics and prognosis of the disease, treatments for PAH have evolved considerably over the last decade as a result, in part, of advances in the knowledge of the pathobiology of the disease in humans and in animal models. The therapies available for the treatment of PAH aim, due to severe hemodynamic disturbances, to reduce pulmonary arterial pressure and consequently the afterload in the RV (27). Taking into account that RV failure is the most common cause of death in patients with PAH, and given the restrictions in the treatment of RV dysfunction (28), studies on strategies for the maintenance of cardiac function are needed. In this sense, there is growing evidence in humans (29-31) and in animal models (32-34), that aerobic exercises (i.e., 60-80% VO_{2max}) and intermittent (High-intensity interval training and voluntary running) promote beneficial effects to individuals with PAH, as it improves the quality of life, functional capacity, physical effort tolerance, ventilatory efficiency and overall cardiac function (35, 36). However, the effects of resistance exercise training (RT) in patients with PAH are poorly understood.

The RT is an activity in which each effort is performed against a specific opposition force generated by the resistance (37). It is commonly prescribed for skeletal muscle hypertrophy, strength and power development, and is recognized and recommended as an important beneficial tool for health (38), as it promotes beneficial effects to various body systems and may be an effective treatment for various clinical conditions (39). Consequently, the American Heart Association and the American College of Sports Medicine approves the inclusion of RT as an integral part of an exercise program for health promotion and disease prevention (38, 40). Regarding PAH, combined exercise interventions including aerobic, resistance and specific inspiratory muscle training proved safe for these patients and yielded significant improvements in muscle power, exercise capacity and survival (41-43). Recently, our group found that combined training (44) (aerobic and resistance training) and previous resistance training (45), that is, performed before the development of PAH, contributes to the prevention of increased resistance in the pulmonary artery and pathological lung remodeling, prevention of cardiac function and adverse cardiac remodeling, as well as for the prevention of skeletal muscle atrophy and for better tolerance to physical effort. Despite that, the impact of RT in the structure and function of pulmonary, cardiac, and skeletal muscle tissues, during the development of PAH in this model is unclear.

2. OBJECTIVES

2.1. General

The aim of this study was to evaluate the effects of low- to moderate-intensity RT on the function and structure of pulmonary, cardiac and skeletal muscle tissues in the stable PAH model induced by MCT.

2.2. Specific

To test whether resistance exercise training performed during the development of stable PAH induced by monocrotaline in rats affects:

a) The survival;
b) The tolerance to physical effort;
c) The hemodynamics of the pulmonary artery and ventricles;
d) The morphometry of the lung, ventricles and skeletal muscle tissues;
e) The oxidative stress biomarkers in the lung and skeletal muscle tissues;
f) The contractility and intracellular calcium transient in ventricular myocytes;
g) The gene expression of fetal gene and miRNAs in the right ventricle; and
h) The expression of calcium regulatory proteins and hypertrophy/atrophy signaling pathways in the right ventricle.

To reach these objectives, two experimental studies were carried out and are described below. In the first one, the effects of RT in lung, biceps brachii and RV were evaluated; and in the second, the effects of RT on the LV were examined.

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STUDY 1

Resistance exercise training offsets damages in the structure and function of pulmonary, right ventricle, and skeletal muscle tissues in stable pulmonary artery hypertension model.

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Abstract

Aim: To test the effects of low- to moderate-intensity resistance exercise training (RT) on the structure and function of pulmonary, right ventricle (RV), and skeletal muscle tissues in the stable pulmonary artery hypertension (PAH) model.

Methods: After the first dose of monocrotaline (MCT; 20 mg/kg), male rats were submitted to a RT program (Ladder climbing; 55-65% of carrying maximal load), 5 times/week. Seven days later rats were injected with another MCT dose (20 mg/kg). Physical effort tolerance test and echocardiographic examination were carried out at specific time points of the experimental period. After euthanasia, lung, heart, and biceps brachii were dissected, weighed, and processed for histological, single myocyte, and biochemical analysis.

Results: RT improved survival and physical effort tolerance (i.e., Maximum carrying load), mitigated the pulmonary artery resistance increase (i.e., TA/TE), and preserved cardiac function (i.e., Fractional shortening, ejection fraction, stroke volume and TAPSE). In addition, RT counteracted oxidative stress (i.e., CAT, SOD, GST, MDA and NO) and adverse remodeling in lung (i.e., Collapsed alveoli) and in biceps brachii (i.e., atrophy and total collagen) tissues. Moreover, RT delay RV adverse remodeling (i.e., hypertrophy, extracellular matrix, collagen types I and III, and fibrosis) and impairments in single RV myocyte contractility (i.e., amplitude and velocity to peak and relaxation). Furthermore, RT improved the expression of gene (i.e., miRNA 214) and regulatory proteins of the intracellular Ca^{2+} cycling (i.e., PLB^{ser16}) as well as of pathological (i.e., α/β-MHC, and Foxo3) and physiological (i.e., Akt, p-Akt, mTOR, p-mTOR, and Bcl-xL) hypertrophy pathways in RV tissue.

Conclusion: Along with survival and physical effort tolerance enhancement, low- to moderate-intensity RT during the development of stable MCT-induced PAH postpones

pulmonary artery resistance increases and prevents RV dysfunction, RV adverse remodeling and myocyte contractility deterioration in rats. These results are of clinical relevance insofar as it indicates that low- to moderate-intensity RT may contribute positively to the health and survival of individuals with stable PAH.

Keywords: Pulmonary hypertension, exercise tolerance, adverse remodeling, isolated cardiomyocytes.

1. Introduction

PULMONARY ARTERY HYPERTENSION (PAH) is a disease characterized by a progressive increase in pulmonary vascular resistance which leads to right ventricular adverse remodeling, dysfunction and hence failure (1-3). Changes in the pulmonary vascular resistance occurs due to pulmonary vascular remodeling that is secondary to uncontrolled growth of endothelial and smooth-muscle cells and fibroblasts, induced by shear stress, hypoxia, autoimmune phenomena, viral infections, drugs and toxins, or genetic alterations, which results in inflammation and luminal narrowing (4). In response to an increase in pulmonary vascular resistance, the right ventricle (RV) undergoes hypertrophy, dilatation, collagen deposition, fibrosis, metabolic shifts and failure as pulmonary hypertension progresses (4). The functional changes in the small circulation are associated with impaired functional capacity as a result of intolerance to physical effort, since the ability of the RV to increase stroke volume during physical effort is impaired (5). In addition, low systemic oxygenation leads to increased anaerobic metabolism, increased oxidative stress and inflammation, suppression of hypertrophic signaling pathways, and atrophy of skeletal muscles, which contributes to the reduction the physical work tolerance in PAH patients (6).

In the model of severe PAH induced by monocrotaline (MCT; single injection of 60 mg/kg body mass), there is endothelial dysfunction followed by an increase in pulmonary artery resistance which, in turn, leads to pathological remodeling of the lung, i.e., oxidative stress, inflammation, enlargement of the alveoli wall, increase in connective tissue and collagen content, and reduction in number of alveoli and reduction of blood vessel lumen (7, 8). These changes in the small circulation leads to increased pulmonary resistance and, consequently, RV overload. In this case, the RV undergoes hypertrophy and dilation associated with increased collagen deposition in the extracellular matrix, inflammation, apoptosis and fibrosis, which contributes to the deterioration of its function (9, 10). At the cellular level, RV myocytes commonly exhibit excitation-contraction coupling anomalies, 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 (11, 12). These pulmonary and cardiovascular changes reduce blood flow and oxygen supplementation to skeletal muscle, which in turn leads to an increase in the formation of reactive oxygen species and disruption of cellular redox status, favoring inflammation and tissue damage, thus impairing muscle functionality (13, 14).

Despite that, the model of MCT lung injury in rodents using a single injection of 60 mg/kg body mass induces severe PAH in a subacute process, which is limited to simulate human chronic PAH (15). In this sense, Zhuang et al. (2018) (16) demonstrated that 40 mg/kg MCT divided into two injections of 20 mg/kg with an interval of seven days better mimics chronic PAH with those common changes in the structure and function of pulmonary arteries and right ventricle observed in humans. In this model, we observed excitation-contraction coupling impairments in left ventricle (LV) myocytes as well as the presence of fibrosis, augmented pulmonary artery

resistance, and reduced physical effort tolerance (17). Although these mechanical and structural factors are fundamental for the cardiac pump functional stability (18, 19), the roles of intracellular Ca^{2+} regulatory proteins as well as its regulatory gene expression at the post-transcriptional level [i.e., microRNAs (miRNAs)] in this model is not known. In addition, to our knowledge, to date, the morphological changes, and changes in the redox state in pulmonary and skeletal muscle tissues in this PAH model are unknown.

Regarding the available therapies to treat PAH, reductions in pulmonary artery pressure and, consequently, in RV afterload are crucial (20). Considering that RV failure is the most common cause of death in patients with PAH, and in face of restrictions in the treatment of RV dysfunction (21), studies on strategies for the maintenance of cardiac function are needed. In this sense, there is growing evidence in humans (22-24) and in animal models (25-27), that aerobic exercises promote beneficial effects to individuals with PAH, as it improves the quality of life, functional capacity, physical effort tolerance, ventilatory efficiency and overall cardiac function (28, 29). Although beneficial effects of aerobic (i.e., 60-80% VO_{2max}) and intermittent (High-intensity interval training and voluntary running) physical exercise have been shown on the health of patients with PAH (7, 9, 12, 30), the effects of resistance exercise training (RT) in patients with PAH are poorly understood.

Low- to moderate-intensity RT has been used to compose exercise programs to promote health and prevent cardiovascular diseases (31, 32). Regarding PAH, combined exercise interventions including aerobic, resistance and specific inspiratory muscle training proved safe for these patients and yielded significant improvements in muscle power, exercise capacity and survival (33-35). In MCT-induced PAH, we reported that RT prolonged survival, enhanced the physical effort tolerance, and mitigated the LV and cardiomyocyte contractility dysfunctions and prevented the

increases in left ventricle fibrosis and type I collagen (17). Despite that, the impact of RT in the structure and function of the pulmonary, RV, and skeletal muscle, in this model is unclear.

Therefore, we thought to evaluate the effects of low- to moderate-intensity RT on the function and structure of pulmonary, RV and skeletal muscle tissues in the stable PAH model induced by MCT.

2. Methods

2.1. Study design

Male Wistar rats aged 7 weeks [Body weight (BW): ~ 200 g] were housed in transparent polycarbonate cages, 4 animals per cage, and were randomly divided into four groups: sedentary control (SC, n = 8), sedentary hypertensive (SH, n = 8), exercised control (EC, n = 8) and exercised hypertensive (EH, n = 8). Additionally, to assess survival, 12 animals were divided into two groups: sedentary to failure (SHF, n = 6) and exercised to failure (EHF, n = 6). 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. After the first injection of MCT, 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 (nº 02/2019 – ATTACHMENT 1) in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. Induction of pulmonary artery hypertension

Animals from SH, EH, SHF and EHF groups received twice intraperitoneal injections of 20 mg/kg MCT (Sigma-Aldrich, St. Louis, MO, EUA) dissolved in saline

(140 mM NaCl; pH 7.4) on day 0 and day 7, respectively. Likewise, control animals (SC and EC) received the same volume of saline solution (140 mM NaCl; pH 7.4) on day 0 and day 7 (16).

2.3. Maximum carrying load test and resistance exercise training

The animals of all groups (SC, SH, EC, EH, SHF and EHF) were adapted to a resistance training (RT) protocol, as described by Hornberger and Farrar, 2004 (36). Briefly, the rats were familiarized for one week with the RT protocol which consisted of climbing stairs (1.1 x 0.18 m, 2-cm spacing between the steps of the grid, 80º of inclination) with devices fixed on their tails without load. Initially, the animals were encouraged to climb by applying a stimulus (pinching fingers) on their tail to initiate the movement. At the top of the stairs, there is a cage (20 x 20 x 20 cm) where they rested for 60 seconds. These procedures were performed five times a week.

After adapting to the RT protocol, all animals in the experimental groups (SC, SH, EC, EH, SHF and EHF) performed the maximum carrying load test before PAH induction, and on the 14th, 21st and 28th days after PAH induction. The test consisted of an initial load of 75 % of body weight, which was progressively increased by an additional 15 % of body weight in the subsequent climbs (120 seconds interval between each climb) until the animal can no longer climb (37). The maximum carrying load was used as an index of physical effort tolerance.

After the first maximum carrying load test and the first injection of MCT, animals from the EC, EH and EHF groups were submitted to the RT program five times a week for four weeks. The training intensity was 55-65% calculated from the exercise tolerance test, following the recommendations for patients with cardiovascular diseases (31). Each training session consisted of fifteen climbs with intervals between

each climb of 60 seconds. The training load was adjusted after the tolerance tests (14th and 21st days).

2.4. Animal survival

The survival curve was evaluated in animals in the SHF and EHF groups until death. The median survival time represented the time after MCT treatment when more than 50% of the group reached the heart failure end point.

2.5. Echocardiography

The echocardiographic evaluation was performed on the 14th, 21st and 28th days after the first injection of MCT in animals from SC, SH, EC and EH groups. The animals were anesthetized (Isoflurane 1.5% dissolved in 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. Tricuspid annular plane systolic excursion (TAPSE) was evaluated and used as an indicator of right ventricular function. To evaluate LV function, the following parameters were assessed: LV ejection fraction (EF); and fractional shortening (FS), and stroke volume was calculated from these variables. The images were collected according to the

recommendations of the American Society of Echocardiography and stored for further analysis (38).

2.6. Sample collection

Animals from all groups (SC, SH, EC and EH) were euthanized by decapitation on the 30th day after the first injection of MCT, date that represents the median survival of animals in the SHF group. After euthanasia, the heart, ventricles and lungs were dissected, weighed, and processed for the analyzes of interest, as described below.

2.7. Histological analyzes

The histological analyzes of the RV were performed as previously described (39). Briefly, immediately after collection, fragments of the RV were fixed on the Karnovsky fixator (paraformaldehyde 4% and glutaraldehyde 4% in 0.1M phosphate buffer, pH 7.4) for 24 hours. Then, the fragments were dehydrated in ethanol, clarified in xylol and embedded in paraffin. Blocks were cut into 5 µm-thick sections, mounted on histological slides and stained with Hematoxylin & Eosin to measure myocytes, extracellular matrix and the cross-sectional area (CSA), Sirius Red to count collagen fibers and Masson's trichrome for cardiac fibrosis count. 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 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) and images of slides stained with Hematoxylin & Eosin and Masson's trichrome were obtained using a light microscope (Olympus AX-70, Tokyo, Japan) connected to a digital camera (Olympus Q Color-3, Tokyo, Japan). The quantification of myocytes, extracellular matrix, collagen types and

cardiac fibrosis was performed using a specific color identification tool using the Image-pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). Myocyte CSA was measured using a specific tool (manual measurement in software image pro-plus 4.5).

2.8. Antioxidant enzymes and oxidative stress markers

Frozen samples of biceps brachii and lung (100 mg) were homogenized in 1 mL phosphate buffer solution (PBS) pH 7.4 and centrifuged at 15,000 xg for 10 min at 4°C. The supernatant was used to determine the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), and oxidative metabolites, including NO and malondialdehyde (MDA), as described earlier (40). These analyzes were performed in an ELISA microplate reader (Multiskan GO, Thermo Scientific) or a spectrophotometer (UV-Mini 1240, Shimadzu).

2.9. mRNA and miRNA expressions

The relative gene expression of ANF (atrial natriuretic factor), skeletal α-actin, α-MHC (myosin heavy chain), β-MHC and miRNA-1 and -214 were analyzed by real-time polymerase chain reaction (real-time PCR) as previously described (40). Frozen RV samples (100 mg) were homogenized in Trizol and ribonucleic acid (RNA) was isolated, according to the manufacturer's instructions (Invitrogen Life Technologies, Strathclyde, UK). After extraction, the total RNA concentration was quantified using a NanoDrop Spectrophotometer (Nano Drop Technologies, USA) and checked for integrity by EtBr-agarose gel electrophoresis.

Primers, for real-time PCR, were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). DNA sequence was obtained from GenBank, and primers were made in separate exons to distinguish by size PCR products derived from

cDNA from those derived from genomic DNA contaminants. The mRNA expression of pathological markers of cardiac hypertrophy were assessed by oligonucleotides primers as follows: for ANF,5′-CTT CGG GGG TAG GAT TGA C-3′ and 5′-CTT GGG ATC TTT TGC GAT CT-3′; skeletal α-actin, 5′-ACC ACA GGC ATT GTT CTG GA-3′ and 5′-TAA GGT AGT CAG TGA GGT CC-3′; α-MHC,5′-CGA GTC CCA GGT CAA CAA G-3′ and 5′-AGG CTC TTT CTG CTG GAC C-3′; and β-MHC,5′-CAT CCC CAA TGA GAC GAA G-3′ and 5′-AGG CTC TTT CTG CTG GAC A-3′.

Real-time quantification of the target genes was performed with a SYBRgreen PCR Master Mix, (Applied Biosystem, PE, Foster City, CA) using ABI PRISM 7700 Sequence Detection System (Applied Biosystem). The expression of cyclophilin A (5′-AAT gCT ggA CCA AAC ACA AA-3′ and 5′-CCT TCT TTC ACC TTC CCA AA-3′) was measured as an internal control for sample variation in real-time reaction. An aliquot of the real-time reaction reaction was used for 50-cycle PCR amplification in the presence of SYBRgreen fluorescent dye, according to a protocol provided by the manufacturer (Applied Biosystems).

For the quantification of miRNAs, cDNA was synthesized from total RNA using miRNA-1 and -214 specific primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, CA, USA). The 15 μl reactions obtained by the TaqMan MicroRNA Reverse Transcription Kit protocol (Applied Biosystems, CA, USA) were incubated in a Thermal Cycler for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C.

The 20 μl PCR included 1.33 μl RT product, 10 μl TaqMan Universal PCR master mix II (2 ×), 7.67 μl nuclease-free water and 1 μl of primers and probe mix of the TaqMan MicroRNA Assay protocol. The reactions were incubated in a 96-well optical plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for

1 min. Samples were normalized by evaluating U6 expression. Each RV sample was analyzed in duplicate. Relative quantities of target miRNA expressions of sedentary rats vs. trained rats were compared after normalization to the values of reference gene (ΔCT). Fold-changes in miRNA expression were calculated using the differences in ΔCT values between the samples against mean of all control samples (ΔΔCT) and equation $2^{-\Delta\Delta CT}$. Results were expressed as % of control.

2.10. Protein expression

The expression of Akt, phospho-Akt, mTOR, phospho-mTOR, Foxo3a, Bcl-xL, Phospholamban, Phospho-Phospholamban, SERCA2 ATPase and NCX1 proteins was determined using Western blotting as previously described (40). Frozen fragments of the right ventricle (100 mg) were homogenized in RIPA buffer, composed of 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% sodium dodecyl sulphate, 1% NP-40 plus protease and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich, St. Louis, MO; PhosSTOP 1:10; Roche, Basel, Switzerland). Insoluble heart tissues were removed by centrifugation at 3,000 *g*, 4°C, 10 min. Samples were loaded and subjected to SDS-PAGE in 8-10% polyacrylamide gels. After electrophoresis, proteins were electro-transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Equal loading of samples (40 µg) and even transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5% nonfat dry milk, 10 mM Tris·HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and incubated with specific antibodies overnight at 4 °C. Detection of the labelled proteins was achieved using the Odyssey® Fc Imaging System (LI-COR; Lincoln, NE, USA).

The following primary antibodies were used: Akt (#9272), phospho-Akt (S473) (#9271), Bcl-xL (#2764), mTOR (#2972), phospho-mTOR (Ser2448) (#2971), α-Tubulin (#2144) and GAPDH (#97166), all of them were from Cell Signalling Technology (Danvers, MA, USA). Phospholamban (#PA5-85268), Phospho-Phospholamban (Ser16, Thr17) (#711401), SERCA2 ATPase (#MA3-919) were from Invitrogen Life Technologies (Strathclyde, UK). NCX1 (#151608) and Foxo3a (#12162) were from Abcam (Cambridge, UK). For all primary antibodies were used at 1:1000 dilution, the secondary antibodies used were IRDye® 680RD or 800CW (1:15,000 LI-COR, Lincoln, NE, USA). Bands were analyzed with Image J software (ImageJ based on NIH Image).

2.11. Isolation of right ventricular myocytes

Myocytes from the RV were enzymatically isolated as previously described (41). 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 37º C. The isolated cells were stored at 5° C until use. Isolated myocytes were used within 2 to 3 hours after isolation.

2.12. Measurement of cardiomyocyte contractility and intracellular Ca^{2+} transient

The contractile function 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 (42). 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) It was evaluated. The cell departure velocity and return velocity were measured as the maximal rate of sarcomere shortening and relaxation, respectively (43). All these parameters were obtained 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 1Hz-stimulation with a single twitch were analyzed.

The intracellular Ca^{2+} transient was measured in as previously described (44). 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 (Fura2340/380 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).

2.13. Statistics

The normality of the data was tested using the Shapiro-Wilk test. Survival data were compared using the Kaplan-Meier curve analysis by the Log-rank test. Differences between groups and between different points of time for the variables of physical exercise, body weight, TA/TE, TAPSE were tested using repeated measures analysis of variance (ANOVA *two-way* repeated measures) followed by the Tukey's post hoc test. Organ weight and cellular parameters were tested using analysis of variance (ANOVA two-way) followed by the Tukey's post hoc test. Pearson's correlation was used to relate variables studied and their magnitude determined as correlation (r): insignificant (0.0-0.3); small (0.3-0.5); moderate (0.5-0.7); strong (0.7-0.9) and very strong (> 0.9) (45). 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. Survival

All animals in the SHF and EHF groups presented signs of heart failure and died. However, animals in EHF group had a longer median survival time (37 days) than those in SHF group (28 days; $P < 0.05$), indicating RT benefit. The body weight was lower ($P < 0.05$) in SHF (227.00 ± 5,21 g) than in EHF (294.70 ± 9.62 g) group.

3.2. Physical effort tolerance, skeletal muscle remodeling and oxidative stress

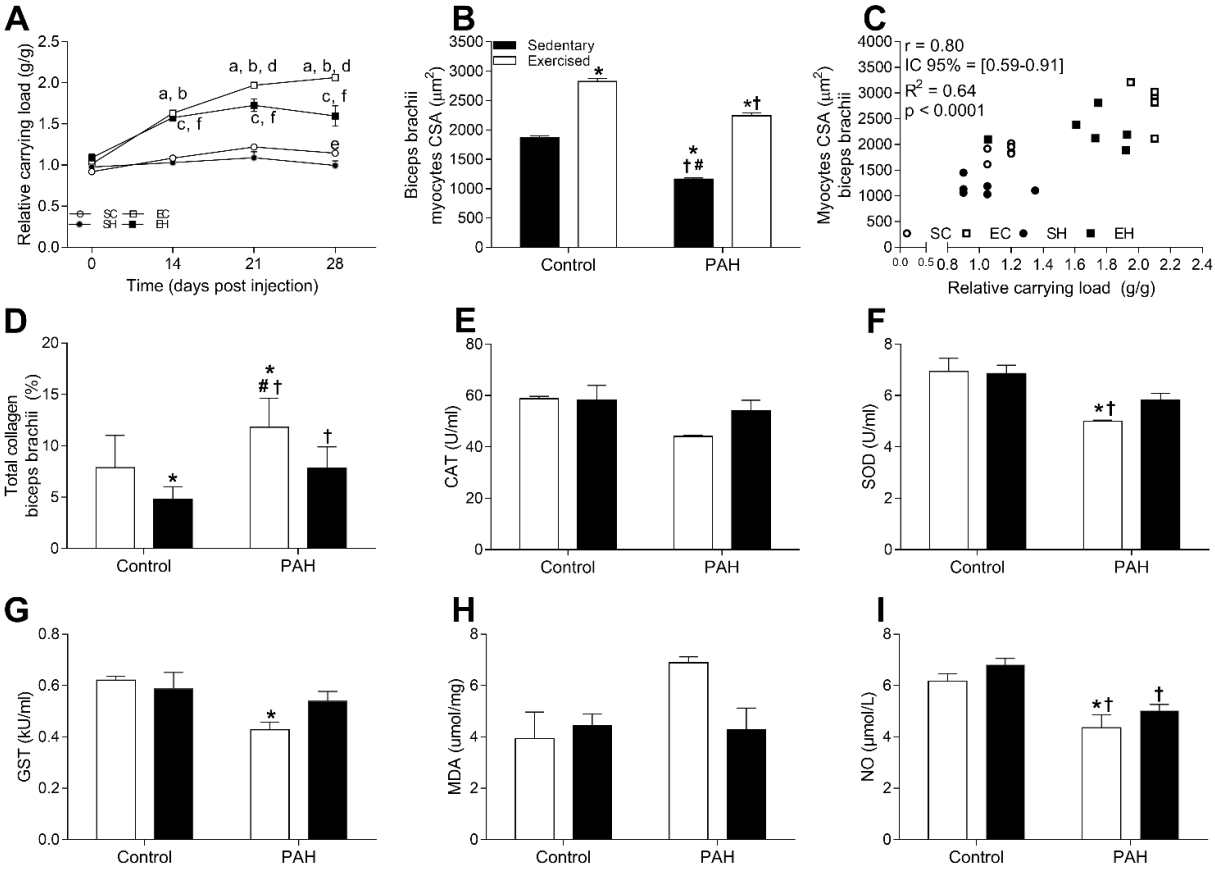


Figure 1. Effects of resistance exercise training and PAH on physical effort tolerance, skeletal muscle remodeling and oxidative stress. (A) Relative maximum load in physical effort tolerance

tests (maximum load/body weight ratio) evaluated pre-injection of MCT and 14, 21 and 28 days after MCT injection. (B) Cross-sectional area (CSA) of the biceps brachii. (C) Correlation between CSA and relative maximum load of the last physical effort tolerance test (28th day). (D) Percentage of total collagen in the biceps brachii. (E-I) Oxidative stress biomarkers, CAT (catalase), SOD (superoxide dismutase), GST (glutathione s-transferase), MDA (malondialdehyde) and NO (nitric oxide). Data are mean ± SEM of 6-8 rats in each group. SC, sedentary control; SH, sedentary hypertensive; EC, exercised control; EH, exercised hypertensive. $^{a}P < 0.05$, EC *vs.* SC; $^{b}P < 0.05$, EC *vs.* SH; $^{c}P < 0.05$, EH *vs.* SH; $^{d}P < 0.05$, EC *vs.* EH; $^{e}P < 0.05$, SC *vs.* SH; $^{f}P < 0.05$, EH *vs.* SC. *P<0.05 vs. SC; †P<0.05 vs. EC; $^{\#}$P<0.05 vs. EH. Two-way ANOVA followed by the Tukey's post hoc test was used for test differences between groups for all variables. The relationship between CSA and relative maximum load was tested by Pearson's correlation.

Figure 1A shows the relative load carrying in the physical effort tolerance tests. Animals in the EC group had a higher relative load than: SC and SH on the 14th day and; SC, SH and EH on the 21st e 28th. Animals in the EH group showed a higher relative carrying load compared to the SC and SH groups in all tests after MCT application (14th, 21st and 28th day). Animals in the SC group had a higher relative carrying load compared to animals in the SH group only in the last physical effort tolerance tests (28th day).

Figures 1B and 1D show that animals from the SH group had lower CSA, and higher percentage of total collagen ($p < 0.05$) compared to the other experimental groups (SC, EC and EH). On the other hand, MCT-treated animals that exercised (EH) showed similar values to the control group, suggesting an effect of exercise in delaying skeletal muscle damage in animals with PAH. In addition, figure 1C shows that there

is strong correlation (r = 0.80) between CSA and relative maximum load, suggesting an important contribution of muscle mass to physical effort tolerance.

Figures 1E-I present biomarkers of oxidative stress in the biceps brachii of rats with PAH submitted to resistance physical training. Sedentary animals treated with MCT had lower values of SOD, GST and NO compared to the control groups (Fig. 1H, I and K, respectively). On the other hand, animals that received MCT that were submitted to the exercise program, showed intermediate values of these parameters, between control animals and animals with PAH sedentary. These findings show an important role of resistance exercise in delaying the oxidative imbalance in the skeletal muscle present in PAH, which in turn may be associated with the prevention of pathological remodeling in this tissue and maintenance of tolerance to physical exertion.

3.3 Pulmonary artery resistance, cardiac function and hemodynamics

Figure 2D shows that animals in the SH group showed greater pulmonary artery resistance (i.e., lower TA/TE ratio) than animals in the other groups (SC, EC and EH) on the 21st and 28th days after first MCT-injection; animals in the EH group showed greater pulmonary artery resistance only on the 28th day compared to animals in the SC group. As for the RV function (Fig. 2E), the results showed that trained animals (EC and EH) had higher TAPSE values than animals in the SC and SH groups on the 14th and 21st days. There were no differences in TAPSE between SC and SH animals on the 14th and 21st days. However, on the 28th day, animals in the SH group had lower TAPSE values than animals in the other groups (SC, EC and EH). These results suggest a detrimental effect of MCT on the cardiac function of the animals in the SH group and a beneficial effect of the TR in delaying the impairments in the cardiac

function, since there was no difference in the values of TAPSE between the SC, EC and EH groups in the 28th day ($p > 0.05$).

Regarding left ventricular function (Fig. 2F, and 2G), although the data show a lower ejection and shortening fraction for the animals in the SH groups on the 21st and 28th days after the first MCT-injection, these differences were not statistically significant. Statistically significant differences were only observed in stroke volume (fig. 2H), on the 21st and 28th day, where animals from the SH group had lower values compared to the control groups (SC and EC). On the other hand, an effect of exercise was observed for both left heart function and stroke volume, since there were no differences in these parameters between the animals in the EH and SC groups.

The echocardiographic evaluation also showed that the flattening of the interventricular septum, named D-shaped left ventricle in animals from SH and EH group (Fig. 2C), which suggests right ventricle pressure overload, characteristic in PAH. Such morphological change was greater in SH than in the SC and EC groups, while the EH presented intermediate values between SC, EC and SH (Fig. 2I), suggesting an effect of resistance exercise in delaying damage.

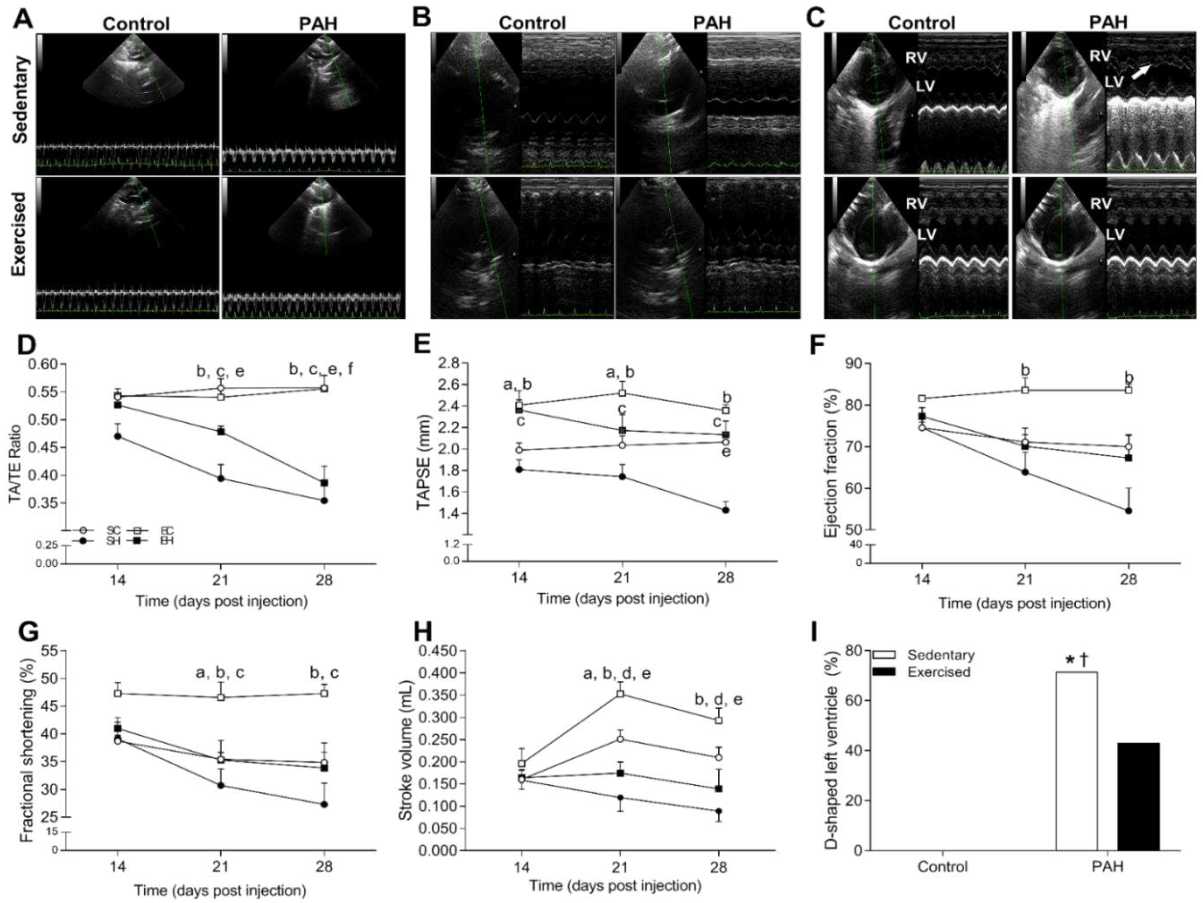


Figure 2. Effects of resistance exercise training and PAH on pulmonary artery resistance and cardiac function. (A) Representative images of pulmonary artery flow. (B) Representative images of Tricuspid annular plane systolic excursion (TAPSE). (C) Representative images of LV echocardiography. (D) Ratio of flow acceleration time to ejection time (AT/ET) in the pulmonary artery measured 14, 21 and 28 days after first injection of MCT. (E) TAPSE measured 14, 21 and 28 days after first injection of MCT. (F) LV ejection fraction. (G) LV fractional shortening. (H) Stroke volume. (I) D-shaped left ventricle on the 28th day. Data are mean ± SEM of 8 rats in each group. SC, sedentary control; SH, sedentary hypertensive; EC, exercised control; EH, exercised hypertensive. $^{a}P < 0.05$, EC *vs.* SC; $^{b}P < 0.05$, EC *vs.* SH; $^{c}P < 0.05$, EH *vs.* SH; $^{d}P < 0.05$, EC *vs.* EH; $^{e}P < 0.05$, SC *vs.* SH; $^{f}P < 0.05$, EH *vs.* SC; *P<0.05 vs. SC; †P<0.05 vs. EC. Two-way ANOVA followed by the Tukey's post hoc test was used for test differences between groups.

3.4. Pulmonary remodeling and oxidative stress

Figures 3A-E present the histological results of lung remodeling in experimental animals with PAH. Among the evaluated parameters, animals in the SH group had a lower percentage of alveoli (Fig. 3A) and a higher percentage of collapsed alveoli (Fig. 3C). However, the resistance exercise program delayed the losses in the percentage of alveoli (Fig. 3A) and prevented the increase in the percentage of collapsed alveoli (Fig. 3C). As for the biomarkers of oxidative stress in this tissue (Fig. 3G-K), it was observed that animals in the SH group had lower values for CAT, SOD, GST and NO and higher values for MDA compared to animals in the control groups (SC and EC). Nonetheless, for all these parameters, animals with PAH that exercised (EH) showed intermediate values between control animals and animals from the SH group, suggesting a beneficial effect of resistance physical exercise in delaying the damages in the pulmonary oxidative balance present in PAH.

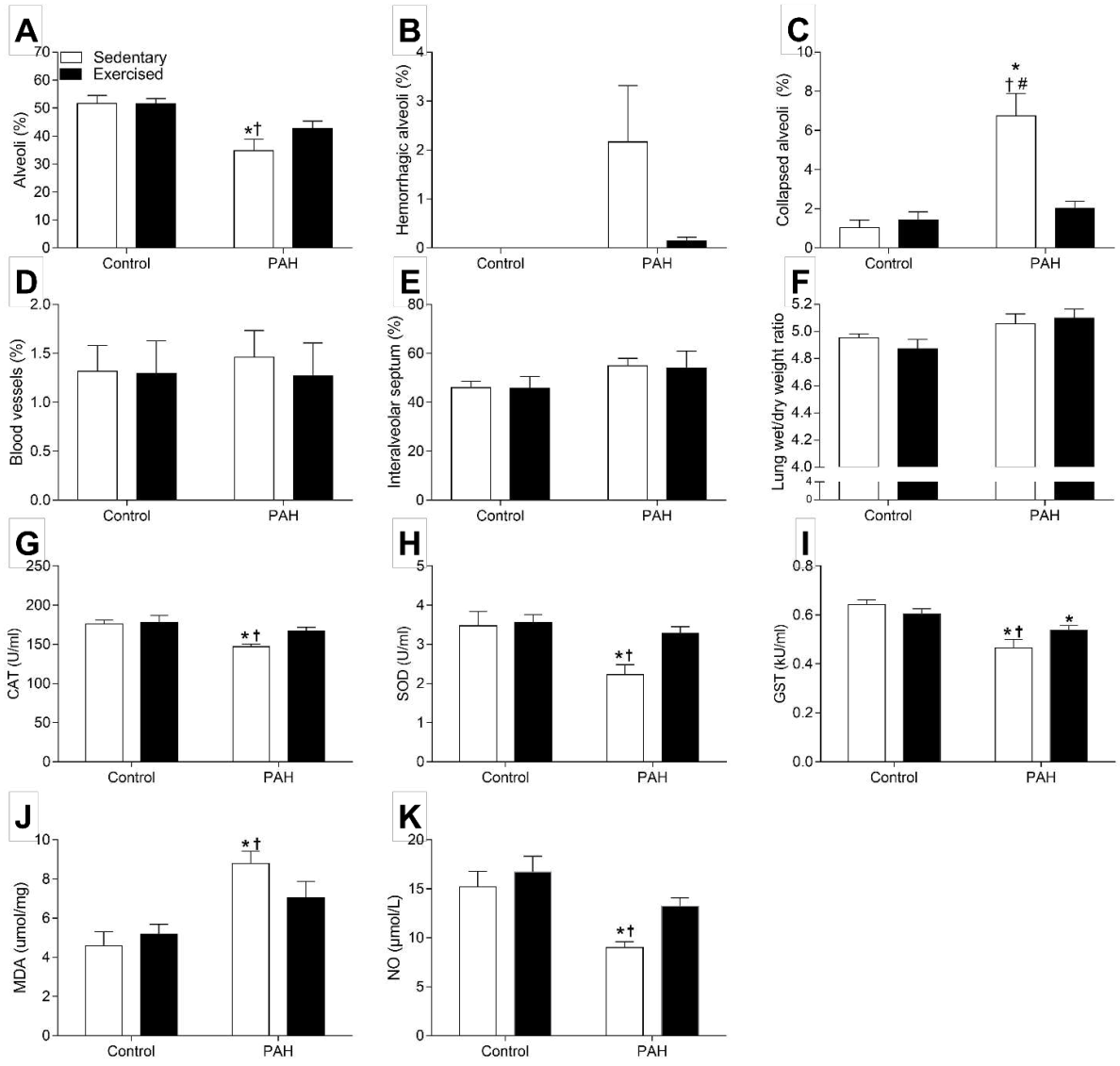


Figure 3. Effects of resistance physical training and PAH on pulmonary remodeling, edema and oxidative stress. (A) percentage of alveoli. (B) percentage of hemorrhagic alveoli. (C) percentage of collapsed alveoli. (D) Blood vessels. (E) Interalveolar septum. (F) Lung water content (G-K) Oxidative stress biomarkers, CAT (catalase), SOD (superoxide dismutase), GST (glutathione s-transferase), MDA (malondialdehyde) and NO (nitric oxide). Data are mean ± SEM of 6-8 rats in each group. SC, sedentary control; SH, sedentary hypertensive; EC, exercised control; EH, exercised hypertensive. *P<0.05 vs. SC; †P<0.05 vs. EC; #P<0.05 vs. EH. Two-way ANOVA followed by the Tukey post hoc test was used for test differences between groups for all variables.

3.5. Right ventricular remodeling

Table 1- Body weight, heart weight, right and left ventricle weight, and ratios.

	SC	EC	SH	EH
BW initial, g	204.1± 4.168	207.3 ± 2.562	207.3 ± 5.873	206.9 ± 6.231
BW final, g	296.6 ± 5.873	308.6 ± 5.719	232.4 ± 12.35*†	259.7 ± 8.352*†
ΔBW, g	92.5 ± 5.148	101.4 ± 7.221	25.13 ± 11.72*†	52.86 ± 11.23*†
Heart, g	1.01 ± 0.02	1.10 ± 0.02	1.22 ± 0.05*	1.25 ± 0.04*
RVW, g	0.17 ± 0.00	0.20 ± 0.00	0.34 ± 0.01*†	0.32 ± 0.02*†
LV + SW, g	0.69 ± 0.01	0.71 ± 0.02	0.59 ± 0.02*†	0.66 ± 0.02
Atrios, g	0.14 ± 0.01	0.19 ± 0.03	0.28 ± 0.03*	0.26 ± 0.03
RVW: LV + SW, g/g	0.25 ± 0.01	0.28 ± 0.01	0.57 ± 0.04*†	0.48 ± 0.04*†

Data are mean ± SEM of 8 rats in each group. SC, sedentary control; EC, exercised control; SH, sedentary hypertensive; EH, exercised hypertensive. RVW, right ventricle weight; LV + SW, left ventricle plus septum weight; RVW: LV + SW, Fulton Index. *P<0.05 vs. SC; †P<0.05 vs. EC. Two-way ANOVA followed by the Tukey post hoc test.

Table 1 shows the average organ weight values of the experimental animals. Animals with PAH (SH and EH) presented mean values of final BW, ΔPC, heart, RV and fulton index different from their controls (SC and EC). In addition, there was an effect of PAH on the reduction of left ventricular mass (LV + SW in the SH < SC and EC group) and increase in atrial mass (weight of the atria in the SH > SC group). However, the RT program was able to delay these changes in the left heart and atria, since animals in the EH group showed LV + SW and atria values intermediate between the control animals (SC and EC) and the animals in the SH group, as there were no differences between these groups.

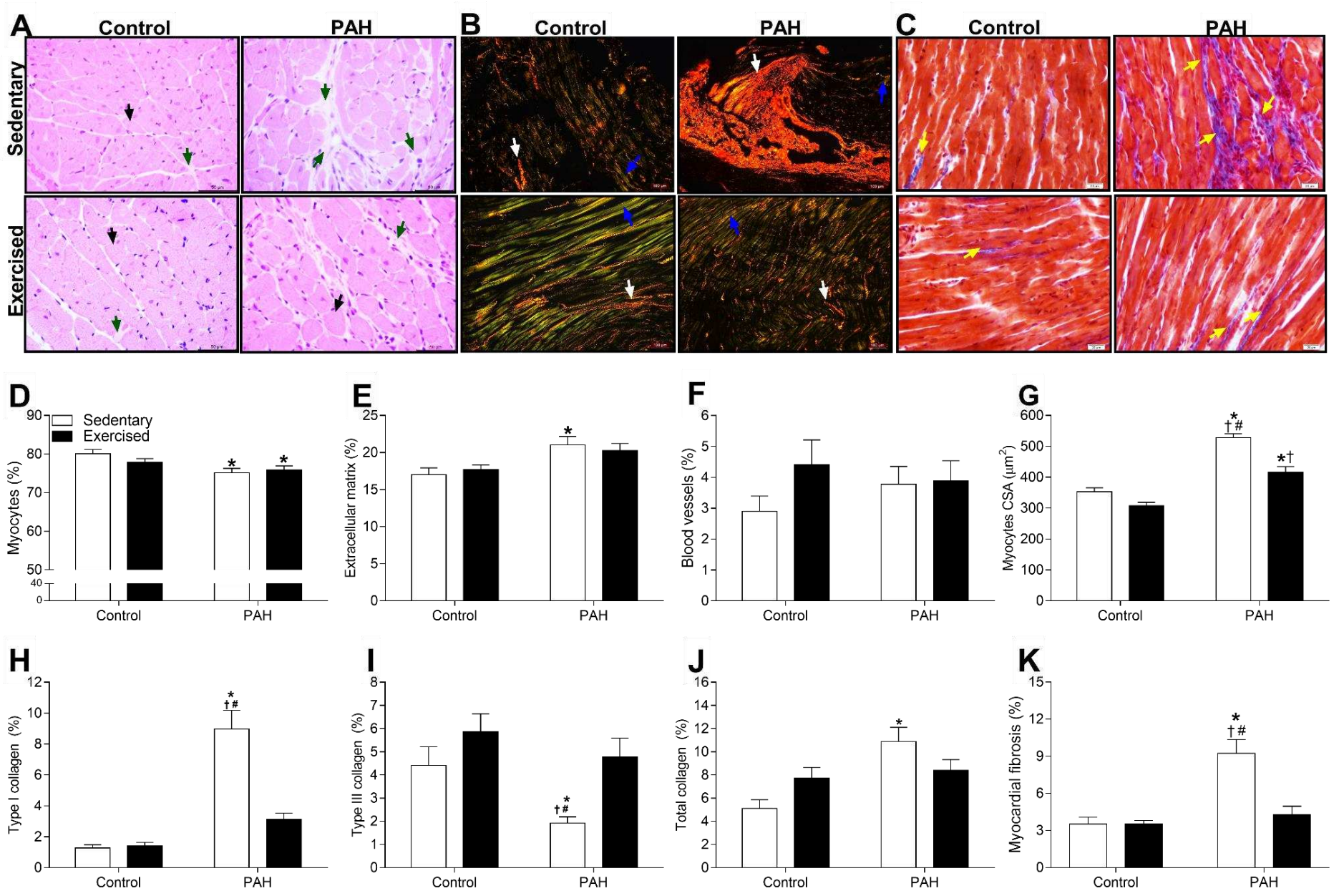

A
Control
PAH
Sedentary
Exercised
B
Control
PAH
C
Control
PAH
D
Sedentary
Exercised
Myocytes (%)
Control
PAH
E
Extracellular matrix (%)
F
Blood vessels (%)
G
Myocytes CSA (μm²)
H
Type I collagen (%)
I
Type III collagen (%)
J
Total collagen (%)
K
Myocardial fibrosis (%)

Figure 4- Effects of resistance exercise training and PAH on right ventricle remodeling. Representative photomicrographs of right ventricle tissue stained with Hematoxylin & Eosin staining (A), Sirius Red staining (B) and Masson's trichrome (C). Black arrow indicates cardiomyocytes; Green arrow indicates extracellular matrix; White arrows indicates type I collagen; Blue arrow indicates type III collagen and; Yellow arrows indicate cardiac fibrosis. (D) Percentage of cardiomyocytes. (E) Percentage of extracellular matrix. (F) Blood vessel percentages. (G) Cross-sectional area of cardiomyocytes (CSA). (H) Percentage of type I collagen. (I) Percentage of type III collagen. (J) Percentage of total collagen. (K) Percentage of cardiac fibrosis. Data are mean ± SEM of 8-10 images per animal in each group (n = 5 rats in each group). *P<0.05 vs. SC; †P<0.05 vs. EC; #P<0.05 vs. EH. Two-way ANOVA followed by the Tukey post hoc test.

Figure 4 presents the percentage of monocytes, extracellular matrix and blood vessels, RV myocyte CSA, percentage of type I, type III and total collagen and fibrosis in the RV of the experimental animals. The animals in the SH and EH group had a lower percentage of cardiomyocytes compared to the SC group (Fig. 4D). Furthermore, animals in the SH group had a higher percentage of extracellular matrix compared to animals in the SC group (Fig. 4E). There were no significant differences between groups in the percentage of blood vessels (Fig. 4F). When assessing CSA (Fig. 4G), animals in the SH and EH group showed higher values compared to the control groups (SC and EC) showing the effect of PAH. However, animals in the EH group showed lower CSA values compared to SH, evidencing on the other hand an effect of the RT in delaying the progression of the disease.

Animals in the SH group had a higher percentage of type I collagen and a lower amount of type III collagen compared to the other groups (Fig. 4H and 4I). When evaluating total collagen (Fig. 4J), animals in the SH group had a higher proportion compared to animals in the SC group ($p < 0.05$). Corroborating the collagen data, figure 4K shows that animals in the SH group had a higher percentage of cardiac fibrosis compared to animals in the SC, EC and EH groups, showing damage to the RV. In contrast, hypertensive animals that exercised (EH), did not show changes in the percentage of fibrosis (EH = SC and EC), indicating a beneficial effect of TR in preventing the progression of tissue damage.

3.6. Right ventricular gene and protein expression

Table 2 presents the results of gene expression of fetal genes and miRNAs in the right ventricle of rats with PAH submitted to a RT program. Animals with PAH showed higher expression of ANF and skeletal α-actin compared to control animals

(SC and EC), suggesting greater pathological hypertrophy and cardiac injury. The physical exercise program was not able to reverse or delay this dysfunction. In addition, when the α/β-MHC ratio was evaluated, animals in the SH group showed a lower ratio, suggesting damage in the sarcomeric units. However, the animals in the EH group showed intermediate values between the animals in the SC and SH groups, suggesting an effect of exercise in delaying losses in these parameters.

When evaluating miRNAs-1 and -214 in the right ventricle, it was observed that there was an effect of exercise on miRNA-1 expression, with no effect of hypertension. However, for miRNA-214 there was an effect of PAH, as it was increased in the animals of the SH group compared to the SC group. We also observed an effect of exercise in delaying the increase in miRNA214 in the animals of the EH group, since the animals of this group presented intermediate values between the control groups (SC and EC) and animals of the SH group.

Table 2- Gene expression of fetal gene and miRNAs in the right ventricle.

% of control	SC	EC	SH	EH
ANF	100 ± 21.14	110.4 ± 13.55	815.1 ± 153.8*†	811.6 ± 101.4*†
Skeletal α-actin	100 ± 08.67	83.87 ± 12.75	347.0 ± 43.33*†#	597.9 ± 77.07*†
α/β-MHC	100 ± 16.68	239.6 ± 19.39*	15.56 ± 2.192*†	51.65 ± 14.7†
miRNA-1	100 ± 19.00	223.3 ± 26.77*	92.54 ± 9.363	136.1 ± 43.52*
miRNA-214	100 ± 39.41	191.4 ± 40.39	319.4 ± 54.84*	276.7 ± 71.83

Data are mean ± SEM of 8 rats in each group. SC, sedentary control; EC, exercised control; SH, sedentary hypertensive; EH, exercised hypertensive. ANF, Atrial Natriuretic Factor; α/β-MHC, alpha/beta myosin heavy chain; miRNA-1, microRNA-1; miRNA-1, microRNA-214. *P<0.05 vs. SC; †P<0.05 vs. EC; #P<0.05 vs. EH. Two-way ANOVA followed by the Tukey post hoc test.

Figure 5 shows the results of protein expression of the physiological hypertrophy signaling pathway. There was an effect of PAH on the reduction of Akt, p-Akt, mTOR, p-mTOR and Bcl-xL (SH < SC, EC e EH). On the other hand, the physical training program was able to prevent a reduction in these same parameters, since animals with PAH that exercised showed values similar to animals in the control groups (EH = SC e EC). In addition, animals that exercised had lower Foxo3 values (EC and EH < SC and SH).

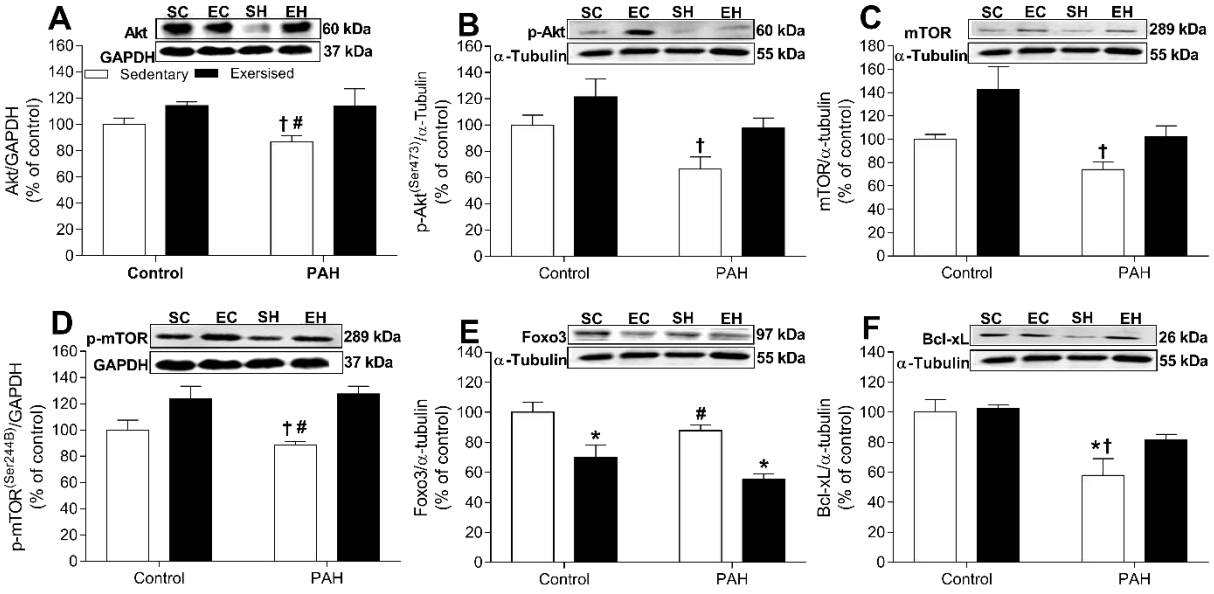


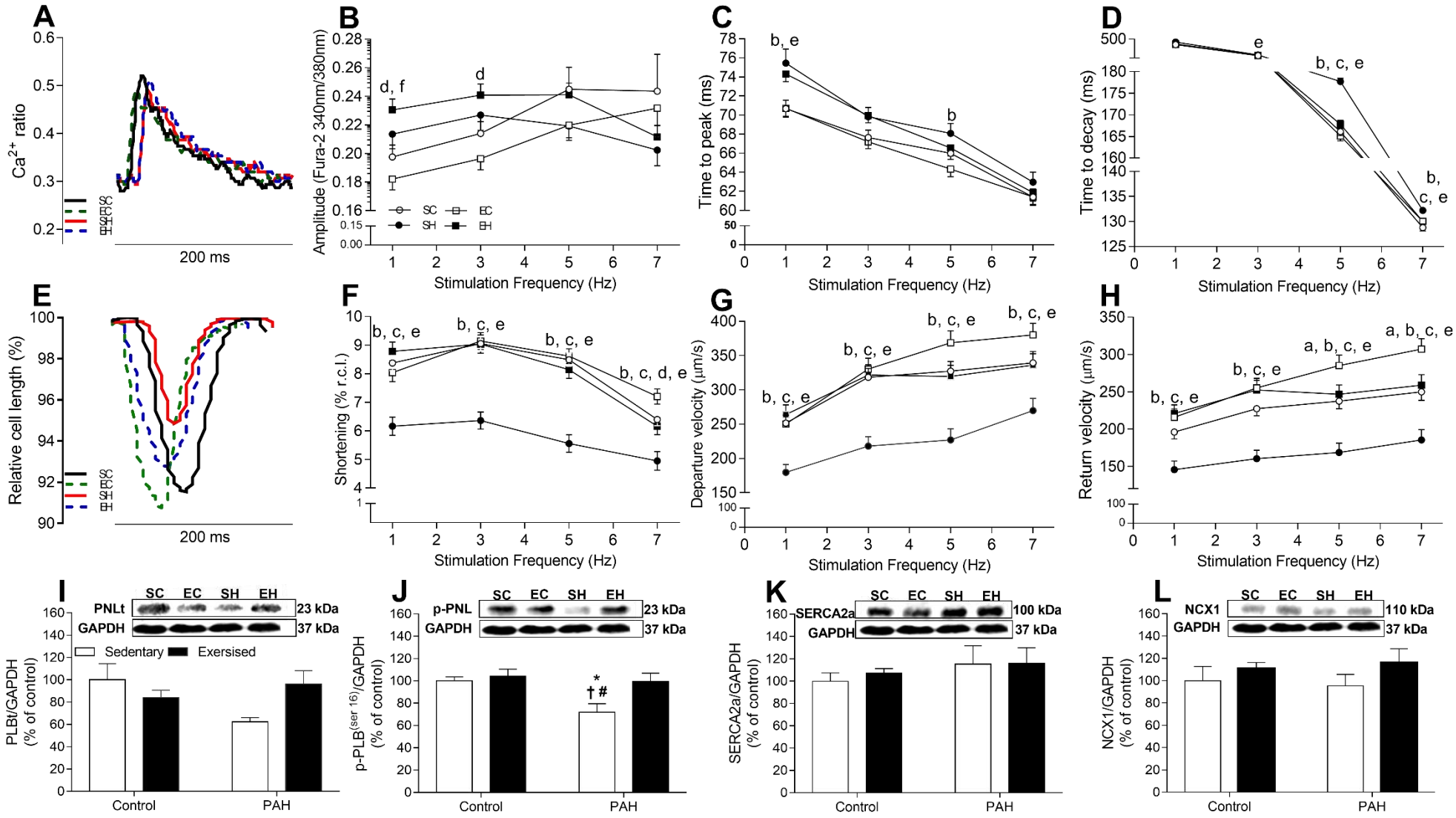
Figure 5. Effect of resistance physical training and PAH on the physiological hypertrophy pathway in the right ventricle. (A) Akt (protein kinase B) expression. (B) p-Akt (phosphorylated Akt) expression. (C) mTOR (mammalian target of rapamycin) protein expression. (D) p-mTOR (phosphorylated mTOR). (E) Foxo3a (Forkhead Box O3) protein expression. (F) Bcl-xL (B-cell lymphoma-extra-large) protein expression. Values are means ± SEM of 5-7 rats in each group. SC, sedentary control; SH, sedentary hypertensive; EC, exercised control; EH, exercised hypertensive. *P<0.05 vs. SC; †P<0.05 vs. EC; #P<0.05 vs. EH. Two-way ANOVA followed by the Tukey's post hoc test was used for test differences between groups.

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

Animals in the EH group showed higher amplitude of the intracellular Ca^{2+} transient compared to the control groups (SC and EC) at frequencies of 1 and 3 Hz (Fig. 6B). There was no difference in this parameter between the groups in the frequencies of 5 and 7 Hz. When evaluating the time to peak of the intracellular Ca^{2+} transient, animals from the SH group presented longer time compared to the control groups (SC and EC) in the stimulations of 1 and 5 Hz, with no differences between the experimental groups in the frequencies of 3 and 7 Hz (Fig. 6C). It was also observed (Fig. 6D) that animals in the SH group showed lower time to decay of the intracellular Ca^{2+} transient at 3 Hz (vs. SC) and 5 and 7 Hz (vs. SC, EC and EH) frequencies.

Regarding the contractility of RV, myocytes from SH animals showed lower amplitude of shortening at all stimulation frequencies (1-7 Hz) compared the SC, EC and EH groups (Fig. 6F). Regarding the shortening speed (departure velocity), the myocytes of animals in the SH group showed lower speed for the peak compared to animals in the SC, EC and EH groups at all evaluated stimulation frequencies (Fig. 6G). Likewise, when assessing the speed of relaxation (Return velocity), animals in the SH group showed lower speed than animals in the SC, EC and EH groups at all evaluated stimulation frequencies. In addition, animals in the EC group showed greater relaxation speed compared to the SC group at the frequencies of 5 and 7 Hz (Fig. 6G).

On the expression of proteins that regulate the intracellular Ca^{2+} transient PLB, p-PLB^{ser16}, SERCA2a and NCX1 (Fig. 6I, 6J, 6K and 6L, respectively) there was a significant difference only in the protein expression of p-NLP (Fig. 6J), where animals from the SH group showed a reduction in values compared to the other groups (SC, EC and EH).


A
B
C
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J
K
L
Ca2+ ratio
200 ms
Amplitude (Fura-2 340nm/380nm)
Stimulation Frequency (Hz)
Time to peak (ms)
Time to decay (ms)
Relative cell length (%)
Shortening (% r.c.l.)
Departure velocity (μm/s)
Return velocity (μm/s)
SC
EC
SH
EH
PNLt
GAPDH
23 kDa
37 kDa
Sedentary
Exersised
PLBt/GAPDH (% of control)
Control
PAH
p-PNL
p-PLB(ser 16)/GAPDH (% of control)
SERCA2a
100 kDa
SERCA2a/GAPDH (% of control)
NCX1
110 kDa
NCX1/GAPDH (% of control)

Figure 6. Effects of resistance exercise training and PAH on right ventricular myocyte intracellular Ca^{2+} transient and contractility. (A) Typical traces of the intracellular Ca^{2+} transient (stimulation 5 Hz). (B) Amplitude of the intracellular Ca^{2+} transient. (C) Time to peak of the intracellular Ca^{2+} transient. (D) Time to decay of the intracellular Ca^{2+} transient. (E) Typical cell shortening traces (stimulation 5 Hz). (F) Amplitude of shortening. (G) Departure velocity. (H) Return velocity. (I) Total phospholamban (PLBt). (J) Phospholamban phosphorylated to serine 16 (PLB^{ser16}). (K) Sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a). (L) Na^{+}/Ca^{2+} exchanger (NCX1). Values are means ± SEM of 6-10 cells per animal in each group (n = 8 rats in each group). SC, sedentary control; SH, sedentary hypertensive; EC, exercised control; EH, exercised hypertensive. $^{a}P < 0.05$, EC *vs.* SC; $^{b}P < 0.05$, EC *vs.* SH; $^{c}P < 0.05$, EH *vs.* SH; $^{d}P < 0.05$, EC *vs.* EH; $^{e}P < 0.05$, SC *vs.* SH; $^{f}P < 0.05$, EH *vs.* SC. $^{*}P<0.05$ vs. SC; $^{\dagger}P<0.05$ vs. EC; $^{\#}P<0.05$ vs. EH. Two-way ANOVA followed by the Tukey's post hoc test was used for test differences between groups.

4. Discussion

In the present study we examined the effects of low- to moderate-intensity RT on the structure and function of pulmonary, RV and skeletal muscle tissues in rats with stable MCT-induced PAH. Our findings evidenced that the applied RT enhanced physical effort tolerance and survival, prevented pulmonary artery resistance increases, along with pulmonary and skeletal muscle detrimental remodeling and oxidative stress. Moreover, RT mitigated RV dysfunction, adverse remodeling and RV myocyte contractility impairment mediated by prevention of reductions in p-PLB protein and α/β-MHC gene expressions. Furthermore, RT increased miRNA-1 and delayed the increase in miRNA-214 gene expression.

The exercise regimen employed during the development of PAH was able to increase the tolerance to physical effort (EC and EH), measured at different time points (14th, 21st and 28th days after the first MCT injection). Resistance exercise training is commonly prescribed for skeletal muscle hypertrophy and strength development, and prevents muscle wasting and dysfunction (46) since it stimulates the insulin-like growth factor/phosphoinositide 3-kinases/protein kinase B/mammalian target of rapamycin (IGF-1/PI3K/AKT/mTOR) signaling pathway which is essential for muscle growth, development, and regeneration (47). In fact, in the present study there was an increase in the CSA of the biceps brachii in exercised rats (EC and EH) while in sedentary PAH animals (SH) the CSA reduced, such that there was a good and statistically significant correlation of the CSA with the relative carrying load. These results suggest a beneficial effect of RT to prevent the loss of muscle mass, possibly by activating the signaling IGF-1/PI3K/AKT/mTOR pathway. Furthermore, increases in total collagen and oxidative stress observed in the biceps brachii of SH rats was not present in EH

animals, suggesting an important role of RT in the maintenance of tissue structure and quality.

Other factors that may have contributed to the enhancement of tolerance to physical exertion in EH animals throughout the experimental protocol. For example, the employed RT mitigated damages in pulmonary structures (i.e., reduction of pulmonary alveoli and increase in collapsed alveoli), in addition to prevention of oxidative imbalance in this tissue, which was altered in SH animals. Moreover, the prevention of the increase in pulmonary artery resistance (i.e., AT/ET) and RV adverse remodeling (i.e., delay in CSA increase, prevention of type I collagen increase, prevention of type III collagen reduction, and prevention of fibrosis) and dysfunction (i.e., TAPSE), as well as LV dysfunctions (i.e., maintenance of systolic volume and prevention of intraventricular septum flattening) possibly influenced the tolerance to physical exertion of EH animals.

The increase in pulmonary artery resistance triggers the adverse remodeling in the RV (48) and, consequently, in the LV (49), since RV-LV hemodynamics are altered. Increase in pulmonary artery resistance has been associated with an unbalance between vasoconstrictors (e.g., endothelin-1; thromboxane) and vasodilators (e.g., nitric oxide - NO; prostanoids), where vasoconstrictors augments (50) contributing thus to vascular stiffening and remodeling. Therefore, reductions in pulmonary artery resistance and pulmonary vascular resistance are crucial for reducing RV overload and hence adverse remodeling, which is associated with the maintenance of cardiac function (48). Then, it is conceivable that the right heart hypertrophy evidenced in hypertensive animals (i.e., RVW, Fulton Index and CSA) is due to the chronic pulmonary artery resistance increase. Although the employed RT prevented impairments in RV function, it did not prevent the increase in pulmonary artery

resistance, such increase was postponed. It is important to note that, although RV myocyte CSA was higher in EH animals compared to SC and EC animals, RV myocyte CSA in EH animals was lower than in SH animals. Thus, these results indicate that the delay in the pulmonary artery resistance increases evidenced in EH animals delay the overload in the RV and contributes to the prevention of precocious heart failure.

We observed that the RV of sedentary animals with PAH (SH group) had less myocytes, more extracellular matrix, more type I collagen, less type III collagen, and more fibrosis than those of other groups. These changes in the RV are characteristic in PAH model (12, 51) and were associated with increases in 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 (tumor necrosis factor inducing receptors), and oxidative stress, inflammation and apoptosis (9). More important, our exercised animals with PAH (EH group), presented RV structural parameters like those in animals from SC and EC groups (i.e., extracellular matrix, Type I and Type III collagen and cardiac fibrosis). These results are interesting, as it shows a beneficial effect of RT in preventing adverse cardiac remodeling and explains in part the prevention of RV dysfunction (i.e., TAPSE) observed in exercised animals treated with MCT. Type III collagen fibers form thin networks and exhibit high distensibility, while type I collagen fibers are thicker than type III and have low distensibility when subjected to mechanical stress (52) and is a precursor of cardiac fibrosis (53). Thus, it indicates that the preservation of the RV function in EH animals is explained, in part, by the prevention of damages to the myocardium structure. Differently, SH animals displayed harmful changes in the RV structure (i.e., higher percentage of type I collagen and fibrosis) and function (i.e., lower TAPSE) values compared to those of other groups.

Regarding RV contractile function at the cellular level, it was observed that although the amplitude of the intracellular Ca^{2+} transient in single myocytes from SH animals was not different from those from SC, EC and EH animals, the cell shortening (contraction amplitude) in SH animals was smaller than that in animals from the other groups at all stimulation frequencies. In addition, myocytes from SH animals showed longer time to peak of the intracellular Ca^{2+} transient compared to those from SC and EC animals at the frequencies of 1 and 5Hz; and a longer time to decay of the intracellular Ca^{2+} compared those from SC, EC and EH animals at the stimulation frequencies of 3, 5 and 7Hz, which in turn led to lower velocities of contraction and relaxation in myocytes from SH animals compared to from the other groups at all stimulation frequencies. These changes in isolated RV contractile function are characteristic of acute MCT-induced PAH (single MCT dose of 60 mg/kg) (12, 25), and were associated with damages to intracellular Ca^{2+} transient (7, 12). In fact, although no significant differences were found in the amplitude of the intracellular Ca^{2+} transient in this model of stable PAH (two MCT doses of 20 mg/kg), the times to peak and decay of the intracellular Ca^{2+} transient were prolonged in RV myocytes isolated from SH animals.

Reductions in RV cell shortening are associated with decreased sensitivity of myofilaments to Ca^{2+} and with reduced intracellular Ca^{2+} (54, 55). Since we did not find differences in the amplitude of the intracellular Ca^{2+} transient, it is possible that the reduction in cell shortening observed in SH animals is related to lower sensitivity of myofilaments to Ca^{2+}. Reductions in velocity of shortening, in turn, is linked to a slow release of Ca^{2+} from the sarcoplasmic reticulum (SR) via ryanodine receptor 2 (RyR2), while the reduced relaxation velocity is connected mainly with a slow reuptake of Ca^{2+} from the cytosol into the SR via SERCA2a (Ca^{2+}-ATPase of the SR, type 2a) when

phosphorylated by PLB (Phospholamban) (56). Indeed, previous studies have shown that SERCA2a and RyR2 proteins are reduced in the RV of rats with acute MCT-induced PAH (26, 57). Differently, our results show that single myocytes from sedentary animals with stable PAH had a significant reduction in p-PLB, without significant changes in PLBt, SERCA2a or NCX1.

The contractility of cardiomyocytes can also be affected by sarcomeres' proteins, such as myosin [α and β myosin heavy chain (MHC)] and actin. For example, overload induced by hypertension results in an increase in β-MHC which has low ATPase activity and slow rate of sarcomere shortening; and decreased α-MHC which has high ATPase activity and fast rate of sarcomere shortening (58). Indeed, our results showed a reduction in the gene expression of the α/β-MCP ratio in the RV of SH animals, in agreement with others (16, 59, 60).

The RT program used here, nevertheless, neutralized the impairments of intracellular Ca^{2+} transient and contractile function in RV myocytes caused by PAH. Single myocytes from rats in the EH group presented times to peak and to decay of intracellular Ca^{2+} transient and velocity of shortening like those from control groups (SC and EC). We further observed that the RT program prevented deterioration in the expression of Ca^{2+} regulatory proteins (i.e., pPLB) and delayed the reduction in α/β-MHC ratio (gene expression) in the RV of these animals. Such effects help explain the prevention of deterioration in the contractile function RV myocytes in rats with PAH.

Complementing this framework referring to RV function and structure, we analyzed the gene expression miRNA-1 and miRNA-214. Studies indicated that increase in miRNA-214 is associated with cardiac fibrosis (61) and pathological RV hypertrophy (62). Moreover, mitochondrial ribosomal protein L17 (Mrpl17), B-cell lymphoma 2 (BCl-2) and Protein kinase B (Akt) are targets of miRNA-214 (63). In this

sense, as these proteins and genes regulate the cell cycle and protein synthesis, we believe that the increase in miR-214 observed the RV of SH rats is an anti-apoptotic response to pressure overload. In contrast, the employed RT program offset such deleterious effect observed in the stable MCT-induced PAH model. Regarding miRNA-1, although we did not see an effect of PAH on its reduction, increases were observed in animals that exercised (EC and EH), which differs from other studies that show a reduction in miRNA-1 in rats submitted to aerobic physical training protocols (Running and Swimming) (64). Unlike the results of the present study, miRNA-1 was decreased in the RV of PAH model (62). miRNA-1 reduction is associated with arrhythmias and pathological hypertrophy through activation of the Ca^{2+}/calmodulin signaling pathway (65, 66). We suggest that future studies should establish the relationship between resistance physical training and miRNA-1 to elucidate the findings of the present study.

Furthermore, our results showed that the physiological RV hypertrophy pathway was down-regulated in SH rats (i.e., Akt, p-Akt, mTOR, p-mTOR, and Bcl-xL), which is in agreement with a previous study (67), and that RT program employed here was able to delay this negative regulation in this stable PAH model. Indeed, resistance and aerobic exercise training up-regulates protein synthesis through physiological hypertrophy pathway PI3K/AKT/mTOR (68, 69). We believe that the activation of this pathway during physical exercise plays an important role in counterbalancing the cellular apoptosis pathway. In addition, curiously, animals with PAH (SH and EH) showed a reduction in Foxo3a levels, although we did not observe an effect of PAH on Akt levels. It is well known that Foxo3a is controlled by Akt and is associated with pathological hypertrophy by activating transcription factors linked to oxidative stress, apoptosis and cell atrophy (70). Thus, future studies to evaluate other signaling

pathways that regulate Foxo3 and its interaction with PAH and physical exercise are warranted.

To summarize, the improvements caused by RT at the tissue, cellular and molecular levels presented herein are related to the reestablishment of pulmonary artery resistance and prevention of damages to the structure and function of pulmonary, RV and skeletal muscle tissues. Thus, the enhancement in the physical effort tolerance and survival promoted by RT is mediated by these benefits.

This study has limitations. First, the speed of climbing is not controlled in this model. Second, the duration of the training period is limited by the effects of MCT. Despite that, our results showed positive effects of the low- to moderate-intensity RT on the structure and function of pulmonary, RV and skeletal muscle tissues in rats with stable MCT-induced PAH.

Finally, we have chosen to employ a low- to moderate-intensity (55-65% of a maximum load) and high-volume RT program as it is indicated for patients with cardiovascular diseases (31) and other chronic diseases (71). Although currently high-load exercises are prescribed to diverse populations and have beneficial effects (72-75), we were able to show in the present study that a short period of low- to moderate-intensity RT is able to generate important adaptations delaying or preventing structural and functional damages imposed by PAH. Therefore, we believe that this type of exercise training is applicable to patients with PAH without leading the patients to the high stress of high-intensity exercise training, which warrants further investigations.

5. Conclusion

Along with survival and physical effort tolerance enhancement, low- to moderate-intensity RT during the development of stable MCT-induced PAH postpones

pulmonary artery resistance increases and prevents RV dysfunction, RV adverse remodeling and myocyte contractility deterioration in rats. These results are of clinical relevance insofar as it indicates that low- to moderate-intensity RT may contribute positively to the health and survival of individuals with stable PAH.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Acknowledgements

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STUDY 2

Resistance exercise training mitigates left ventricular dysfunctions in pulmonary artery hypertension model

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Abstract

Background: The right ventricular hypertrophy and dilation observed in pulmonary artery hypertension (PAH) damages the left ventricle (LV) dynamics by flattening the interventricular septum.

Objective: To investigate whether low- to moderate-intensity resistance exercise training (RT) is beneficial to LV and cardiomyocyte contractile functions in rats during the development of monocrotaline (MCT)-induced PAH.

Methods: Male Wistar rats (Body weight: ~ 200 g) were used. To assess the time to potential heart failure onset (i.e., end point), rats were divided into sedentary hypertension until failure (SHF, n=6) and exercise hypertension until failure (EHF, n=6) groups. To test RT effects, rats were divided into sedentary control (SC, n = 7), sedentary hypertension (SH, n=7) and exercise hypertension (EH, n=7) groups. PAH was induced by two MCT injections (20 mg/kg, with 7 days interval). Exercise groups were submitted to a RT protocol (Ladder climbing; 55-65% of carrying maximal load), 5 times/week. Statistical significance was assumed at $P < 0.05$.

Results: RT prolonged the end point (~25 %), enhanced the physical effort tolerance (~ 55%) and mitigated the LV and cardiomyocyte contractility dysfunctions promoted by MCT by preserving the ejection fraction and fractional shortening and the amplitude of shortening and the velocities of contraction and relaxation in cardiomyocytes. RT also prevented the increases in left ventricle fibrosis and type I collagen caused by MCT and maintained the type III collagen and myocyte dimensions reduced by MCT.

Conclusion: Low- to moderate-intensity RT benefits LV and cardiomyocyte contractile functions in rats during the development of MCT-induced PAH.

Keywords: Left ventricle fibrosis, monocrotaline, myocyte contractility, physical effort tolerance, pulmonary hypertension

1. Introduction

Increases in the pulmonary vasculature resistance, mainly caused by endothelial dysfunction, leads to pulmonary arterial hypertension (PAH) (1). The chronic pulmonary vasculature resistance overloads the right ventricle resulting in pathological remodeling (2), and dysfunction because of hypertrophy and dilation (1). Such remodeling affects the left ventricle (LV) dynamics because of the direct ventricular interaction. In this framework the left ventricle dynamics are damaged by the interventricular septum flattening (3, 4) as it faces impaired early diastolic filling, reduced end-diastolic volume and adverse remodeling (3, 5, 6). Therefore, PAH patients exhibit reduced stroke volume (3) and physical effort tolerance which negatively impacts their quality of life and survival (7).

The pharmacological therapies aim to reduce pulmonary artery pressure and hence the overload to the right ventricle and thus the maintenance of the cardiac function (8). It has been reported that patients with PAH may maintain the cardiac function by non-pharmacological means such as practicing physical exercise regularly (9, 10). In experimental the model of monocrotaline (MCT)-induced severe PAH, for example, previous and early aerobic exercise have been shown to promote cardiovascular benefits, such as mitigation of right ventricular hypertrophy, dysfunction, and adverse remodeling (11-16). Our group (17, 18) reported recently that voluntary running (i.e. intermittent high-intensity exercise) postpones heart failure commencement, and lightens right ventricle adverse remodeling and myocyte dysfunction (i.e. myocyte contractility and intracellular Ca^{2+} cycling deterioration) in this model. Furthermore, we also determined that moderate-intensity continuous aerobic exercise prevents right ventricle adverse remodeling and myocyte contractility and Ca^{2+} cycling impairments (19).

The use of low- to moderate-intensity resistance exercise training (RT) has been recommended to compose exercise programs to promote health and prevent cardiovascular diseases (20, 21) including those related to left ventricular dysfunction (22). Regarding PAH, combined exercise interventions including aerobic, resistance and specific inspiratory muscle training proved safe for these patients and yielded significant improvements in muscle power, exercise capacity and survival (23-25). Despite that, while aerobic exercise has been found to prevent left ventricular systolic and diastolic dysfunction in both baseline and isovolumic conditions (26) in MCT-induced PAH, the impact of RT in the left ventricular dysfunction in this model is unknown.

While the animal models have supported the discovery of new therapies and the understanding of PAH pathophysiology, the model of MCT lung injury in rodents using the injection of 60 mg/kg body mass induces severe PAH in a subacute process, which is limited to simulate human chronic PAH (27). In this sense, Whang et al. (28) demonstrated that 40 mg/kg MCT divided into two injections of 20 mg/kg with an interval of seven days better mimics chronic PAH with those common changes in the structure and function of pulmonary arteries and right ventricle observed in humans. Therefore, in the present study, we used this model in rats to test whether low- to moderate-intensity RT might prove beneficial to LV and myocyte contractile functions during the development of MCT-induced PAH. We hypothesized that low- to moderate-intensity RT is beneficial to LV and myocyte contractile functions in rats during the development of MCT-induced PAH.

2. Methods

2.1. Experimental design and PAH induction

After the definition of the sample size (29), thirty-three male Wistar rats [Body weight: ~200 g] were obtained from the animal house at the Federal University of Viçosa-MG, Brazil. The animals were housed in transparent polycarbonate cages, 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 commercial chow.

To assess the time to potential heart failure onset, 12 animals (~ 200 g) were divided into two groups, by using simple randomization: sedentary hypertension until failure (SHF, n = 6); exercise hypertension until failure (EHF, n = 6). After the MCT injections, rats from SHF and EHF groups were euthanized when showed previously validated external clinical signs of potential heart failure onset (e.g., weight loss, dyspnea, piloerection) and could no longer feed properly, climb the ladder (EHF group) or even move in the cage (30-37), which was considered the end point.

To test whether RT is beneficial during the development of PAH, 21 animals (~ 200 g) were divided into groups by using blocked randomization: sedentary control (SC, n = 7); sedentary hypertension (SH, n = 7); and exercise hypertension (EH, n = 7). Animals from SH, EH and SC groups were euthanized at the median end point day (± 1 day) of the SHF animals (i.e., 28 days). The median time to potential heart failure onset represented the moment after MCT treatment when more than 50% of the group reached the end point day. The animals in the exercise groups were submitted to RT while those in sedentary groups were maintained in their cages.

To induce PAH, animals from the SHF, EHF, SH and EH groups received intraperitonially 2 MCT (Sigma-Aldrich, USA) injections of 20 mg/kg, with 7 days

interval to induce right ventricular failure (28). Control animals received equivalent volume injections of saline.

Experiments were conducted in accordance with international procedures for animal research (Scientific Procedures; Act 1986). All protocols were reviewed and approved by the Institutional Ethics Committee (protocol number 02/2019 - ATTACHMENT 1).

2.2. Resistance training and maximal load test

The animals were familiarized to the RT protocol (adapted from Hornberger and Farrar (38)) for one week before the first MCT or saline injection, with no additional load. RT consisted of climbing a ladder (1.1 m high; 80º inclination) with 2 min resting intervals, being the load based on a maximal carrying load test. The maximal carrying load test was performed before MCT or saline injection (time 0) and on the 14th, 21st and 28th after injections. The test consisted of ladder climbing with initial load of 75 % of body weight, which was progressively increased by an additional 15 % in the subsequent climbs until the animal could no longer climb (39). Load was fixed on the rat's tail and climbs were interspersed with 2 min resting intervals. The maximal carrying load was used as the physical effort tolerance index.

Exercised animals were submitted to a RT program, 5 times/week during the experimental period until the day before euthanasia, totaling twenty exercise sessions. RT load was 55-65% of the maximal carrying load, following the recommendations for patients with cardiovascular diseases (20). Each training session consisted of 15 climbs interspersed with 60 s interval, being training load adjusted after the maximal carrying load tests (14th and 21st days).

2.3. Echocardiography and sample collection

The echocardiographic evaluations were performed on the 28th day after the first MCT injection. The animals were anesthetized (Isoflurane 1.5% and 100% oxygen in a constant flow of 1L/min; Isoflurane, BioChimico, Brazil) and 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 (40). To evaluate LV function, the following parameters were assessed: LV ejection fraction (EF); and fractional shortening (FS). To characterize the PAH, the tricuspid annular plane systolic excursion (TAPSE) was determined.

At the median end point day (± 1 day) of the SHF animals, animals from SH, EH and SC groups were euthanized. After euthanasia, animals from SC, SH and EH groups had the heart, ventricles and lungs dissected, weighed, and processed for analyzes of interest, as described below. The right tibia was dissected, and its length measured.

2.4. Histomorphometry

The histological analyzes of the LV were performed as previously described (41, 42). Briefly, immediately after collection, fragments of the LV were fixed on the Karnovsky fixator (paraformaldehyde 4% and glutaraldehyde 4% in 0.1M phosphate buffer, pH 7.4) for 24 hours. Then, the fragments were dehydrated in ethanol, clarified in xylol and embedded in paraffin. Blocks were cut into 5 µm-thick sections, mounted

on histological slides, and stained with Hematoxylin & Eosin to measure the cross-sectional area (CSA), or with Sirius Red to count collagen fibers and or with Masson's trichrome for cardiac fibrosis count. 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 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) and images of slides stained with Hematoxylin & Eosin and Masson's trichrome were obtained using a light microscope (Olympus AX-70, Tokyo, Japan) connected to a digital camera (Olympus Q Color-3, Tokyo, Japan). The quantification of collagen types and cardiac fibrosis was performed using a specific color identification tool using the Image-pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). Myocyte CSA was measured using a specific tool (manual measurement in software image pro-plus 4.5).

2.5. Isolation of left ventricle myocytes

The heart was attached to a Langendorff-retrograde perfusion system and single LV myocytes isolated as previously described (18). In brief, the heart perfused system via aorta with Tyrode solution containing (in mM; Sigma-Aldrich, USA): 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 exchanged to Tyrode solution containing EGTA (0.1 mM) for 6 min. Then, 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. Then, the LV of the digested heart was removed and cut into small fragments which 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. The dispersed cells were separated from the non-dispersed tissue by filtration by centrifugation. The isolated cells were stored at 5° C until use. Isolated myocytes were used within 2 to 3 hours after isolation. The solutions used in the isolation procedure were oxygenated (O_2 100% - White Martins, Brazil) and maintained at 37º C.

2.6. Single myocyte contractile function

The contractile function of LV myocytes was measured by using an edge detection system (Ionoptix, Milton, USA) mounted on an inverted microscope (Nikon Eclipse - TS100, Japan) as previously described (19). Myocytes were placed in a bath on the stage of an inverted microscope and superfused with a Tyrode's solution containing in mM (Sigma-Aldrich, USA): 137 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.5 $MgCl_2$, 5 HEPES, 5.6 glucose 1.8 $CaCl_2$, pH 7.4 with 5N NaOH, at 37˚ C. Only myocytes exhibiting clear, regular striation (sarcomere) pattern, no spontaneous contraction in the absence of external stimulation and responding to 1Hz stimulation with a single twitch were tested. Myocytes were stimulated (Myopacer, Ionoptix, Milton, USA) to contract at a progressive stimulation frequency (1, 3, 5 and 7 Hz) using external electrodes and the resultant cell shortening measured via analysis of a video image of the cell using Ionoptix camera and software (Ionoptix, Milton, MA, USA). Cell shortening was expressed as % of resting cell length.

The myocyte length and width were obtained from the video image of the cell; and the cell volume was calculated as previously described (43).

2.7. Statistical analysis

The normality of the data was tested using the Shapiro-Wilk test. Data are presented as mean ± SD for continuous variables with normal distribution and median

accompanied by the interquartile range for continuous variables without normal distribution. The end point, ventricular remodeling parameters and contractile parameters of isolated cardiomyocytes showed a non-normal distribution, while exercise parameters, body and organ weight and cross-sectional area and isolated cell morphometry presented normal distribution. The end point was tested by the Kaplan-Meier curve analysis by the Log-rank test. Maximum carrying load, body weight, LV function, organ weight and single cell parameters were tested by one-way analysis of variance (ANOVA) or Kruskal-Wallis followed by the Dunn's post hoc test. Maximum carrying load was tested by one-way repeated measures ANOVA. ANOVAs were followed by pairwise Tukey correction test. The Person's Chi-squared test (X^2) was used to assess the proportion of animals that presented intraventricular septum flattening. Statistical significance was assumed at $P < 0.05$. Data description, numbers of rats and myocytes are given in the table and figure legends. All analyzes were performed using GraphPad Prism, version 6.01 (San Diego, CA, USA).

3. Results

3.1. Potential heart failure onset and physical effort tolerance

Figure 1A illustrates that animals from EHF group performed the resistance exercise protocol during the development of PAH until presenting signs of potential heart failure onset. The maximal carrying load increased progressively until day 21 and afterwards it decreased to the initial level on the 35th day after the first MCT injection. All animals from both SHF and EHF groups presented signs of potential heart failure onset - end point (Fig. 1B), however, animals in EHF group had a longer median end point time (37 days) than those in SHF group (28 days), indicating resistance exercise benefit.

Hypertensive rats from EH group improved their tolerance to physical effort (Fig. 1C) throughout the experiment. The maximum carrying load in the EH group was higher in days 21 and 28 than in day 0. Moreover, these animals had higher maximum carrying load on the 14th, 21st and 28th day, compared to those in SC and SH groups.

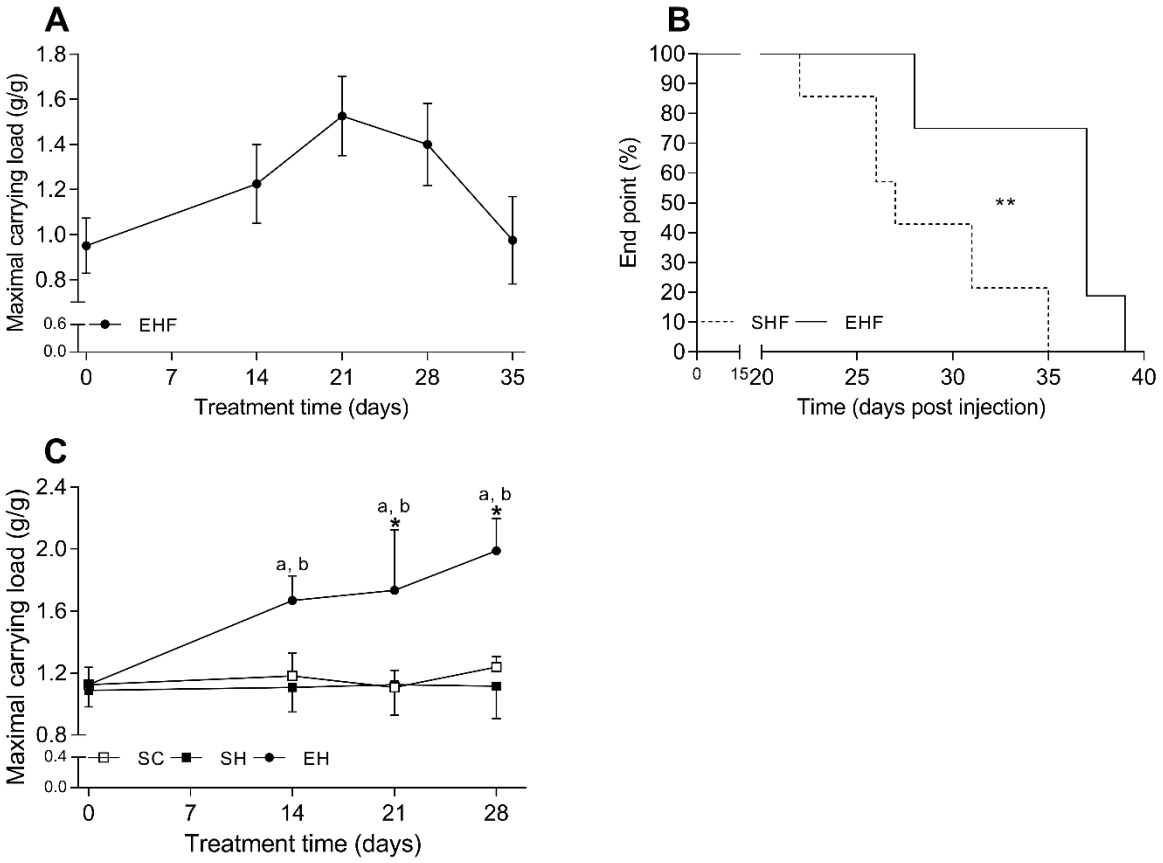

Figure 1. Effect of resistance training on potential heart failure onset (end point) and physical effort tolerance. (A) Relative maximal carrying load of hypertensive animals until failure, determined by the maximal carrying load normalized to body weight, at pre-injection (day 0) and on the 14th, 21st, 28th and 35th day after the first monocrotaline injection. (B) End point, measured in days to present signs of potential heart failure onset, was significantly shorter in sedentary hypertension until failure (SHF, n = 6) than in exercise hypertension until failure (EHF, n = 6) rats. $^{**}P < 0.01$, Kaplan-Meier curve analysis by the Log-rank test. (C) Relative maximal carrying load of control, hypertensive sedentary and exercise animals, determined as in panel A. Exercise hypertension (EH, n=7) rats exhibited higher carrying load gain than sedentary control (SC, n=7) and sedentary hypertension (SH, n=7) from the 14th day on.

Repeated measures ANOVA followed by Tukey correction test. [a]*P* < 0.05 *vs.* SH; [b]*P* < 0.05, *vs.* SC; [*]*P* < 0.05 vs. before MCT injection.

3.2. Left ventricular function and morphology

The echocardiographic evaluation showed flattening of the interventricular septum, named D-shaped left ventricle in animals from SH and EH group (Fig. 2A), which suggests right ventricle pressure overload, characteristic in PAH. Such morphological change was greater in SH than in SC group, while EH presented intermediate values (Fig. 2D). Regarding the left ventricle function, no difference between group for ejection fraction (Fig. 2B) and fractional shortening (Fig. 2C) was found. Despite that, it is important to note that 3 out of 7 animals in the SH group had ejection fraction < 50% and 3 out of 7 had fractional shortening < 25%, indicative of left ventricular failure. On the other hand, none of the exercised animals (EH), in the same period, showed ejection fraction < 50% and only 1 out of 7 had fractional shortening < 25%.

The presence of PAH in animals from SH group was characterized also by the TAPSE values. Animals from SH group exhibited lower TAPSE values (1.43 ± 0.23) than those from SC group (2.06 ± 0.17) and EH (2.13 ± 0.36).

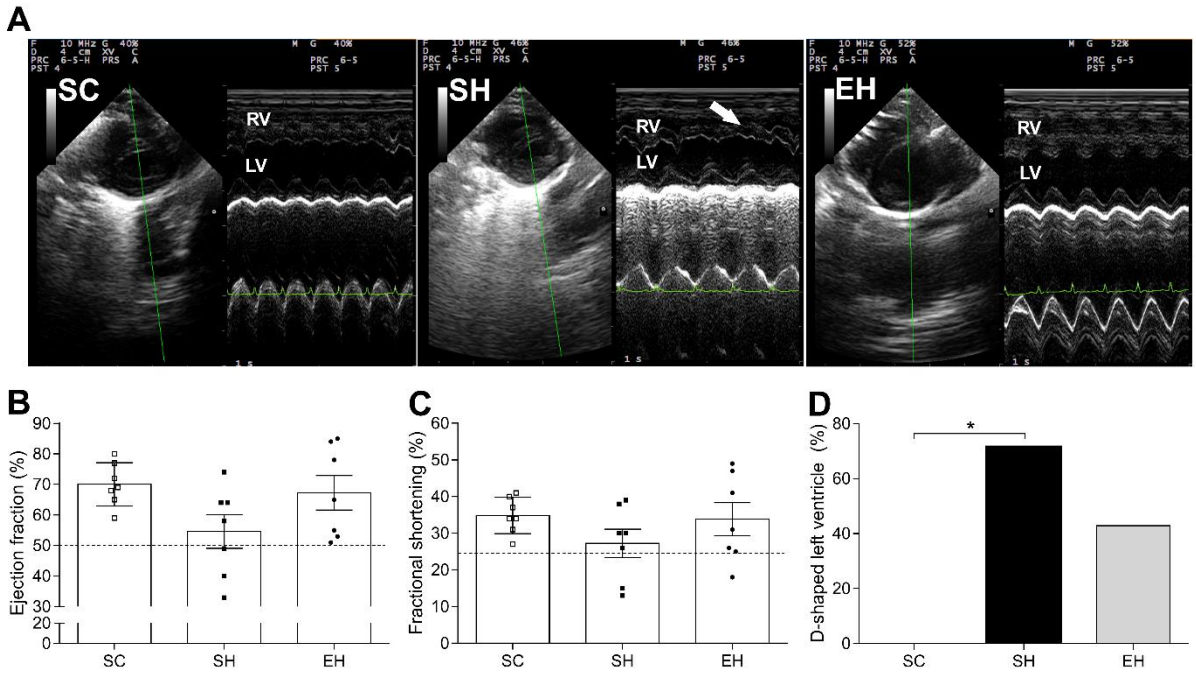

Figure 2. Effect of resistance exercise training on left ventricular function assessed on the 28th day after the first monocrotaline injection. (A) Representative echocardiograph images. (B) Ejection fraction. (C) Fractional shortening. (D) D-shaped left ventricle. Values are means ± SD (n = 7 rats in each group). SC, sedentary control; SH, sedentary hypertension; EH, exercise hypertension. Dotted line indicates limits for the classification of impaired function. Panel B and C: One-Way ANOVA followed by the Tukey's post hoc test. Panel D: Pearson' s Chi-squared test (x^2 test). $^*P < 0.05$.

Animals from SH group presented lower body weight than those from SC and EH groups (Table 1). Despite no difference between group for heart weight, animals from SH and EH groups had higher right ventricle (RV) weight and right ventricle-to-tibia ratio than animals in the SC, which indicates right ventricle hypertrophy. While lung weight and lung weight-to-tibia ratio were higher in SH and EH than in SC groups, no difference between groups for LV weight and LV weight-to-tibia ratio was found. Regarding left ventricle myocyte dimensions, SH group presented lower length, width and volume than SC group. EH group presented intermediate values between SC and

SH groups. Animals of the SH groups exhibited lower CSA compared to animals in the SC and EH groups. On the other hand, there was no difference in the CSA of animals in the EH group compared to animals in the SC group, suggesting a beneficial effect of the resistance exercise program used here on preventing adverse left ventricle remodeling.

Table 1. Effect of resistance exercise training on body and organ weights.

	SC	SH	EH
Final BW. (g)	298.6 ± 19.01	276.3 ± 19.87*	303.7 ± 20.98†
Heart weight (g)	1.23 ± 0.11	1.30 ± 0.18	1.28 ± 0.12
RV weight (g)	0.33 ± 0.04	0.42 ± 0.03*	0.44 ± 0.05*
LV weight (g)	0.74 ± 0.10	0.65 ± 0.07	0.70 ± 0.07
Lung weight (g)	1.65 ± 0.28	2.77 ± 0.41**	2.38 ± 0.33**
Ratio of RV weight to tibia length (g/cm)	0.09 ± 0.01	0.11 ± 0.00*	0.11 ± 0.01*
Ratio of LV weight to tibia length (g/cm)	0.20 ± 0.02	0.17 ± 0.02	0.18 ± 0.02
Ratio of lung weight to tibia length (g/cm)	0.45 ± 0.10	0.73 ± 0.11**	0.63 ± 0.09*
Myocyte length (μm)	132.3 ± 19.09	122.5 ± 19.86**	129.2 ± 21.42
Myocyte width (μm)	46.12 ± 10.08	41.75 ± 9.95*	43.64 ± 9.50
Myocyte volume (pL)	46.24 ± 3.97	38.71 ± 3.18**	42.62 ± 3.61
Myocyte CSA (μm^2)	462.1 ± 21.86	400.5 ± 43.34*	492.2 ± 66.56†

Data are mean ± SD of 7 rats and 10 cells in each group. SC. sedentary control; SH. sedentary hypertension; EH. exercise hypertension. BW. body weight; RV. right ventricle; LV. left ventricle; $^{*}P < 0.05$ vs. SC; $^{**}P < 0.01$ vs. SC; $^{\dagger}P < 0.05$ vs. SH. One-way ANOVA followed by the Tukey post hoc test.

3.3. Left ventricular adverse remodeling

Figure 3 shows data on LV collagen fibers and fibrosis. Hypertensive animals (SH and EH) presented a higher percentage of type I collagen compared to animals in the control group (SC) (Fig. 3A). However, animals in the EH group had a lower percentage of type I collagen compared to animals in the SH group, showing the protective effect of RT on the progression PAH. In addition, animals in the EH group exhibited a higher percentage of type III collagen, than those in the sedentary animals (SC and SH) (Fig. 3B). Hypertensive animals (SH and EH) had a higher percentage of total collagen, compared to animals in the control group (Fig. 3C). Concerning LV fibrosis (Fig. 3D), animals in the SH group presented a higher percentage, compared to the those in the SC and EH groups. There was no difference between the percentage of fibrosis in animals from EH and SC groups, showing a beneficial effect of resistance exercise on the prevention of pathological cardiac remodeling.

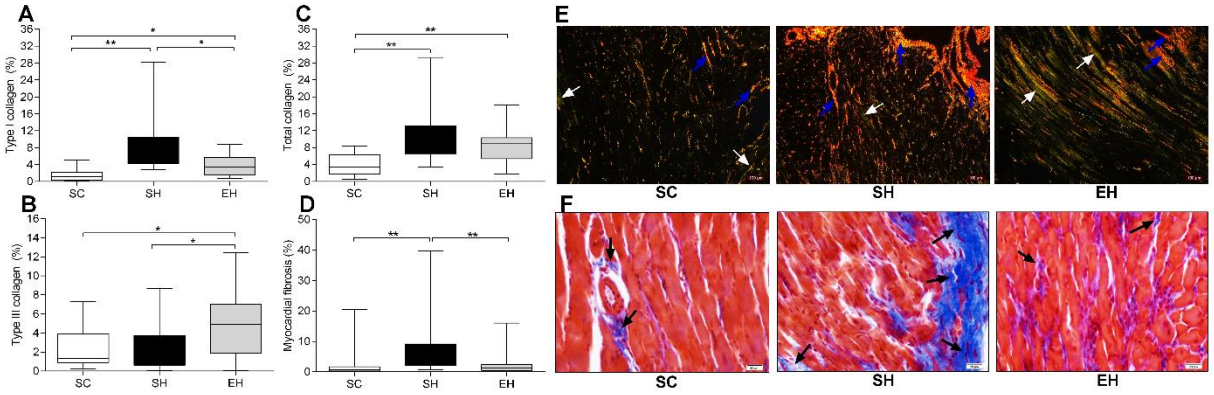

Figure 3. Effect of resistance exercise training on left ventricle remodeling. (A) Percentage of type I collagen. (B) Percentage of type III collagen. (C) Percentage of total collagen. (D) Percentage of fibrosis in the LV. (E) Representative photomicrographs of LV tissue stained with Sirius Red; (F) Representative photomicrographs of LV tissue stained with Masson's trichrome. Blue arrow indicates type I collagen; White arrow indicates type III collagen; Black arrow indicates cardiac fibrosis. Values are presented as median accompanied by the interquartile range of 10 images per animal in each group (n = 5 rats in each group). SC,

sedentary control; SH, sedentary hypertension; EH, exercise hypertension. Kruskal-Wallis, followed by the Dunn's post hoc test: * $P < 0.05$, and ** $P < 0.01$.

3.4. Single myocyte contractile function

Under electrical stimulation, myocytes from SH animals showed a positive contraction-frequency relationship at the frequencies of 1, 3 and 5 Hz; and a lower magnitude of shortening than those from SC and EH groups (Table 2). Such difference has lost its statistical difference from 5 to 7Hz, where the contraction-frequency relationship became negative. In addition, the departure velocity (an index of contraction velocity) was slower in cells from SH group than in those from SC group over the range of 1-7 Hz. However, when compared to the EH, the lower speed was found only at 1, 3 and 7 Hz. Likewise, the return velocity (an index of relaxation velocity) was slower in SH group than in SC group. When compared to the EH, the slower speed was found only at 1 and 3 Hz.

Table 2. Effect of resistance exercise training on left ventricular myocyte contraction and relaxation

	SC	SH	EH
	Median (IQR 25%-75%)	Median (IQR 25%-75%)	Median (IQR 25%-75%)
Shortening (% r.c.l.)			
S.F. (1 Hz)	7.69 (5.74-9.42)	5.26 (3.23-7.08)*	7.89 (5.80-9.30)†
S.F. (3 Hz)	8.02 (5.47-10.14)	6.08 (3.73-8.29)*	7.70 (6.59-10.11)†
S.F. (5 Hz)	8.16 (6.06-10.15)	6.74 (4.83-8.78)*	8.26 (6.25-10.20)†
S.F. (7 Hz)	7.32 (4.86-9.33)	6.04 (4.37-8.01)	6.95 (5.41-8.90)
Departure velocity			
S.F. (1 Hz)	262.9 (191.8-330.3)	189.2 (106.1-266.8)*	250.7 (179.1-307.0)†
S.F. (3 Hz)	317.7 (222.6-411.2)	250.2 (129.3-332.6)*	288.5 (226.4-416.1)†
S.F. (5 Hz)	365.8 (246.7-473.2)	303.3 (175.5-417)*	342.4 (254.7-467.3)
S.F. (7 Hz)	369.8 (284.4-472.9)	322.2 (209.7-367.9)*	344.9 (293.1-469.5)†
Return velocity			
S.F. (1 Hz)	229.0 (158.2-282.5)	143.6 (76.53-220.7)*	206.5 (148.4-274.1)†
S.F. (3 Hz)	254.6 (177.2-321.3)	191.9 (97.98-254.2)*	241.3 (159.2-323.8)†
S.F. (5 Hz)	273.3 (218.4-354.5)	236.1 (126.9-279.6)*	247.6 (178.4-353.6)
S.F. (7 Hz)	285.2 (226.9-362.6)	234.3 (153.5-293.8)*	260.5 (202.9-356.9)

Data are presented as median accompanied by the interquartile range (IQR) of 10 cells per animal in each group (n = 7 rats in each group). % r.c.l., percentage of resting cell length; S.F., stimulation frequency. SC, sedentary control; SH, sedentary hypertension; EH, exercise hypertension. $^{*}P < 0.05$ vs. SC; $^{**}P < 0.01$ vs. SC; $^{\dagger}P < 0.05$ vs. SH. Kruskal-Wallis, followed by the Dunn's post hoc test.

4. Discussion

The present study examined whether low- to moderate-intensity RT might prove beneficial to LV and myocyte contractile functions in rats during the development of

MCT-induced PAH. Our findings reveal for the first time that rats treated with MCT (Two MCT injections of 20 mg/kg, with 7 days interval) climbed the ladder during the development of PAH and increased progressively their tolerance to physical effort. Our index of physical effort tolerance, the maximal carrying load, was progressively higher in EH group compared to SH and SC groups throughout the experiment. This model of RT was efficient in increasing muscle strength in another rat model of hypertension (44). The increase in body weight and maximal carrying load observed here suggests a protective effect of RT against skeletal muscle loss and dysfunction. This is an interesting finding since sarcopenia, intolerance to physical effort and lethargy are reported characteristics of this PAH model (13, 42, 45, 46). Muscle power has been shown to be improved in PAH patients in response to combined exercise (Aerobic + Resistance) programs (23-25). Moreover, the increase in muscle strength is important for hypertensive individuals as it lightens the cardiovascular overload during their daily life activities and has been associated with protection against all-cause mortality (47).

The RT program used in the present study expanded the time until the animals exhibited the signs of potential heart failure onset (i.e., end point). Although there is no study on the effects of the RT model on such end point of rats with MCT-induced PAH, prolonged end point of rats injected with MCT has been reported in response to voluntary running by our group (17, 18); and extended survival in response to treadmill running was reported by others (13, 48), more markedly when started at the early stages of the disease. Enhanced survival has also been observed in PAH patients submitted to combined exercise (Aerobic + Resistance) interventions (23-25).

Our RT regime benefited the LV functional and structural parameters in MCT-injected rats. Regarding left ventricular function, echocardiography showed that 42,86 % of sedentary rats injected with MCT (SH group) had ejection fraction below 50% and

28,57 % presented fractional shortening below 25%, which indicates left ventricular dysfunction. Nevertheless, in exercised animals (EH group) the presence of left ventricular disfunction was lower than in sedentary rats (SH group), thus suggesting a protective role of resistance exercise. These findings are in line with changes caused by the employed RT in the LV tissue. For instance, RT increased the percentage of type III collagen while reduced the percentage of type I collagen and fibrosis in rats with MCT-induced PAH, thus showing the protective effect of such exercise regime against left ventricular dysfunction and adverse remodeling leading to mitigation of the PAH progression.

The organ parameters showed that MCT-injected sedentary rats (SH group) exhibited higher right ventricle (i.e. right ventricle weight, Fulton's index and right ventricle weight/tibia length ratio) and lung (i.e. Lung weight, lung weight/tibia length ratio) values than the control group (SC). Despite no change in whole LV weight and LV to tibia length ratio, single myocyte length, width and volume were decreased by MCT (SC > SH). However, RT prevented such cell dimension reduction (EH = SC), which indicates the maintenance of the left ventricular mass and suggests the protective effect of the applied RT program against the left ventricular adverse remodeling.

Along with reduction in myocyte dimensions, MCT induced single myocyte contractile dysfunction. Myocytes from SH group had lower shortening and longer contraction and relaxation velocities than those in SC group. More important, RT mitigated the contractile dysfunction as these cell parameters in EH group were similar to those in SC group, indicating thus improvements in the contractile function in myocytes from EH group relative to those from SH group. The calcium regulatory proteins (i.e., Ryanodine receptor 2; Phospholamban and Sarcoplasmic reticulum

ATPase 2a) manage the force and timecourse of cardiomyocyte contraction and are reported downregulated in the right ventricle of MCT-treated rats (13, 15). Whether the employed RT regime increases the expression and activity of these proteins warrants further investigations, though such exercise effect has been found in normotensive healthy rats (49, 50).

Taken together, our results indicate that the RT employed during the development of MCT-induced PAH was beneficial to left ventricle and myocyte contractile structure and function, which resulted in enhanced tolerance to physical effort and time to potential heart failure onset of the animals.

Upon resistance exercise recommendations for patients with cardiovascular diseases (20), we used low- to moderate-intensity. Considering that high-intensity exercise is reported to promote highest benefits in patients with heart failure (51) and that MCT-injected rats exercised with progressive load until the median end point time of the sedentary ones (28 days), it might be possible to experimentally increase exercise intensity using reward techniques to enlarge the resistance exercise effects.

Finally, this study has limitations. First, the speed of climbing is not controlled in this model. Second, the duration of the training period is limited by the effects of MCT. Despite that, our results showed positive effects of the resistance exercise program on both the time to potential heart failure onset, physical effort tolerance and LV dysfunction.

5. Conclusion

Our findings show that along with the increase in the time to potential heart failure onset and in the physical effort tolerance, low- to moderate-intensity resistance exercise mitigates the development of left ventricular dysfunctions in the MCT-induced

PAH model. Therefore, low- to moderate-intensity RT is beneficial to left ventricular and myocyte contractile functions in this model. These results are of clinical relevance as it supports the health benefits of resistance exercise to individuals with cardiopulmonary disease, including PAH. We suggest that low- to moderate-intensity resistance exercise is tested in PAH patients.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Acknowledgements

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FINAL CONSIDERATIONS

Taken together, our results reveal that low- to moderate-intensity RT during the development of MCT-induced stable PAH:

- ✓ At the systemic level, it delays the increase in pulmonary artery resistance, prevents ventricular dysfunctions, and adverse remodeling in pulmonary, cardiac and skeletal muscle tissues, which contributes to increases in tolerance to physical exertion and survival.
- ✓ At the cellular level, it delays deteriorations in single ventricular myocyte contractile function; and
- ✓ At the molecular level, it delays changes in gene and protein expression of myocardial damage markers, regulators of the intracellular Ca^{2+} cycling, and of cell cycle.

Future studies should focus on evaluating different resistance training intensities and their effects on systemic, cellular and molecular parameters in the MCT-induced stable PAH to provide further information about this training method. In addition, studies evaluating the combined effects of physical training and other therapies such as drug and nutraceuticals may provide important information concerning the treatment of PAH.

ATTACHMENT 1 – Ethical approval

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº02/2019, intitulado "**Efeitos do treinamento físico resistido sobre a musculatura cardíaca e esquelética em ratos com hipertensão arterial pulmonar**", coordenado pelo professor Antônio José Natali do Departamento de Educação Física, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 16/04/2019, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 02/2019, named **"Effects of resistive physical training on the cardiac and skeletal muscles in rats with pulmonary arterial hypertension"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on April 16, 2019 valid for 12 months.

Prof.ª Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV

ATTACHMENT 2 – First page of the published article (Study 2)

Original Article

Resistance Exercise Training Mitigates Left Ventricular Dysfunctions in Pulmonary Artery Hypertension Model

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Abstract

Background: The right ventricular hypertrophy and dilation observed in pulmonary artery hypertension (PAH) damages the left ventricle (LV) dynamics by flattening the interventricular septum.

Objective: To investigate whether low- to moderate-intensity resistance exercise training (RT) is beneficial to LV and cardiomyocyte contractile functions in rats during the development of monocrotaline (MCT)-induced PAH.

Methods: Male Wistar rats (Body weight: ~ 200 g) were used. To assess the time to potential heart failure onset (i.e., end point), rats were divided into sedentary hypertension until failure (SHF, n=6) and exercise hypertension until failure (EHF, n=6) groups. To test RT effects, rats were divided into sedentary control (SC, n = 7), sedentary hypertension (SH, n=7), and exercise hypertension (EH, n=7) groups. PAH was induced by two MCT injections (20 mg/kg, with 7 days interval). Exercise groups were submitted to an RT protocol (Ladder climbing; 55-65% of carrying maximal load), 5 times/week. Statistical significance was assumed at $P < 0.05$.

Results: RT prolonged the end point (~25 %), enhanced the physical effort tolerance (~ 55%), and mitigated the LV and cardiomyocyte contractility dysfunctions promoted by MCT by preserving the ejection fraction and fractional shortening, the amplitude of shortening, and the velocities of contraction and relaxation in cardiomyocytes. RT also prevented increases in left ventricle fibrosis and type I collagen caused by MCT, and maintained the type III collagen and myocyte dimensions reduced by MCT.

Conclusion: Low- to moderate-intensity RT benefits LV and cardiomyocyte contractile functions in rats during the development of MCT-induced PAH.

Keywords: Heart Failure; Pulmonary Hypertension; Rats; Physical Conditioning, Animal/methods; Myocytes, Cardiac; Ventricular Dysfunction, Left; Exercise.

Introduction

Increases in the pulmonary vasculature resistance, mainly caused by endothelial dysfunction, leads to pulmonary arterial hypertension (PAH).[1] The chronic pulmonary vasculature resistance overloads the right ventricle (RV), resulting in pathological remodeling,[2] and dysfunction because of hypertrophy and dilation.[1] Such remodeling affects the left ventricle (LV) dynamics because of the direct ventricular interaction. In this framework, the left ventricle dynamics are damaged by the interventricular septum flattening,[3,4] as it faces impaired early diastolic filling, reduced end-diastolic volume, and adverse remodeling.[3,5,6] Therefore, PAH patients exhibit reduced stroke volume[3] and physical effort tolerance, which negatively impacts their quality of life and survival.[7]

Pharmacological therapies aim to reduce pulmonary artery pressure and the overload to the RV, thereby maintaining the cardiac function.[8] It has been demonstrated that patients with PAH may maintain the cardiac function by non-pharmacological means, such as practicing regular physical exercise.[9,10] In the experimental model of monocrotaline (MCT)-induced severe PAH, for example, previous and early aerobic exercise have been shown to promote cardiovascular benefits, such as mitigation of right ventricular hypertrophy, dysfunction, and adverse remodeling.[11-16] Our research group[17,18] recently reported that voluntary running (i.e. intermittent high-intensity exercise) postpones the onset of heart failure, and lightens RV e adverse remodeling and myocyte dysfunction (i.e. myocyte contractility and intracellular Ca^{2+} cycling deterioration) in this model. Furthermore, our study also demonstrated that moderate-intensity continuous aerobic exercise prevents

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