UNIVERSIDADE DO PORTO

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CARDIOPROTECTIVE EFFECTS OF EXERCISE PRECONDITIONING IN AN EXPERIMENTAL MODEL OF LEFT VENTRICULAR DYSFUNCTION SECONDARY TO PULMONARY ARTERIAL HYPERTENSION

Dissertação apresentada com vista à obtenção do grau de mestre em Atividade Física e Saúde, da Faculdade de Desporto da Universidade do Porto ao abrigo do Decreto de Lei nº.74/2006 de 24 de Março, orientada pelo Professor Doutor José Alberto Ramos Duarte e co-orientada pelo Professor Doutor Daniel Moreira Gonçalves

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To my family

To you, for all support and love

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Resumo

O exercício físico regular exerce um importante papel na saúde humana global, protegendo, atrasando ou melhorando contra diversas doenças como a obesidade, a diabetes e as doenças cardiovasculares. Sabe-se também que o exercício físico proporciona um fenótipo cardioprotetor permitindo responder melhor contra vários insultos cardíacos, tais como isquemia-reperfusão, infarto do miocárdio, a cardiotoxicidade induzida pela doxorrubicina ou sobrecarga de pressão aguda. A hipertensão arterial pulmonar (HAP) afeta diretamente o ventrículo direito, mas a disfunção do ventrículo esquerdo também já foi descrita recentemente em pacientes com HAP, estando associada com atrofia do ventrículo esquerdo e/ou ativação neuro-humoral. Vendo os potenciais benefícios do exercício físico na fisiologia cardíaca encontrados em vários modelos experimentais, nós projetamos este estudo para analisar os efeitos cardioprotetores hipotéticos do exercício de pré-condicionamento no ventrículo esquerdo (VE) em um modelo animal de hipertensão arterial pulmonar induzida por monocrotalina (MCT). O estudo foi realizado com 115 ratos Wistar machos, submetidos à três intervenções, mostrado aqui em três fases. A primeira fase foi realizada com sessenta ratos, separados aleatoriamente em dois grupos experimentais: sedentário (SED, n=35; manteve-se com o movimento limitado ao espaço da gaiola durante 4 semanas) e exercício (EX n= 25; exercitaram em uma esteira rolante, 5 dias/semana, 60 minutos/dia, a 25 metros/minuto, durante 4 semanas). Depois de terminar durante quatro semanas, alguns animais receberam uma injeção subcutânea de monocrotalina (MCT, 60 mg/kg) e outros o mesmo volume de veículo (V, 1 mL/kg de soro fisiológico), originando os seguintes grupos: i) SED+MCT (n=25), ii) SED+V (n=10), iii)

EX+MCT (n=15) e iv) EX+V (n=10). Depois disso, todos os animais mantiveram-se sedentário por mais quatro semanas. Numa segunda fase, foi desenvolvido o estudo de sobrevivência com 40 animais submetidos aos respectivos protocolos experimentais (SED+V, n=5; SED+MCT, n=15; EX+V, n=5; EX+MCT, n=15). A terceira fase foi realizada com 20 animais, onde foi realizado o teste de tolerância ao esforço. No final da primeira fase, os animais foram submetidos a uma avaliação hemodinâmica do VE em condições basais e isovolumétricas, e amostras do VE foram preparadas para análise de microscopia ótica (área da secção transversal de cardiomiócitos e deposição de colagénio) e endotelina (ET-1). Descobrimos que em condições basais, a função sistólica (pressão sistólica de pico e dP/dtmax) e diastólica (dP/dtmin e Tau) foram comprometidos no grupo SED+MCT, mas não no EX+MCT (P<0.05). Sob condições isovolumétricas, SED+MCT mostraram deterioração adicional nos mesmos parâmetros, mas estas alterações foram impedidas no grupo EX+MCT (P <0.05). Esta melhora dos parâmetros hemodinâmicos foi observada juntamente com a prevenção da atrofia dos cardiomiócitos, do aumento da fibrose, e com a normalização de níveis de ET-1 de mRNA (P<0.05). O exercício de pré-condicionamento também melhorou a tolerância ao exercício, exercendo um impactado positivo nos índices de sobrevivência. É importante destacar que as melhorias foram observadas após quatro semanas da última secção de treino, destacando que o fenótipo de proteção promovida pelo exercício físico foi mantido por vários dias.

Palavras chave: Exercício físico; Cardioproteção; Monocrotaline; Atrofia dos cardiomiócitos; Endotelina-1; Taxa de sobrevivência; Tolerância ao exercício.

Abstract

Regular physical exercise exerts an important role in overall Human health, protecting, delaying or improving against some diseases like obesity, diabetes mellitus and cardiovascular diseases. It is also known that exercise training provides a cardioprotective phenotype allowing an improved response against several cardiac insults such as ischemia-reperfusion, myocardial infarction, cardiotoxicity induced by doxorubicin or acute pressure overload. Pulmonary arterial hypertension (PAH) directly affects the right ventricle but the left ventricle dysfunction was recently described in PAH patients, which is associated with left ventricle (LV) atrophy and/or neurohumoral activation. Seeing that potential benefits of exercise training on cardiac physiology were found in many experimental models, we designed our study to analyze the hypothetical cardioprotective effects of exercise preconditioning on LV in a rat model of PAH induced by monocrotaline (MCT). The study was designed with 115 male Wistar rats, submitted for three interventions, showed here with three phases. The first phase was done with 60 rats were randomly separated in two experimental groups: sedentary (SED; n=35; remained with movement confined to the cage's space during 4 weeks) and exercise (EX; n=25; exercised on a treadmill, 5 days/week, 60 minutes/day, at 25 meters/minute, during 4 weeks). After ending this 4-week period, some animals from each group received one subcutaneous injection of monocrotaline (MCT; 60 mg/kg) or an equal volume of vehicle (V; 1 mL/kg of saline), originating the following groups: i) SED+MCT (n=25), ii) SED+V (n=10), iii) EX+MCT (n=15) and iv) EX+V (n=10). Afterwards, all animals remained sedentary for additional 4 weeks. Next, animals were submitted to LV hemodynamic evaluation in baseline and isovolumetric

conditions, and LV samples were prepared for light microscopy analysis (cardiomyocyte cross sectional area and collagen deposition) and endothelin (ET-1). In a second phase, we developed the survival study with 40 animals submitted to the respective experimental protocols (SED+V, n=5; SED+MCT, n=15; EX+V, n=5; EX+MCT, n=15). The third phase was performed with 15 animals (SED+V, n=5; SED+MCT, n=5; EX+MCT, n=5) for the exercise tolerance test. We found that in baseline conditions, systolic (peak systolic pressure and dP/dtmax) and diastolic function (dP/dtmin and Tau) were compromised in SED+MCT but not in EX+MCT ($P<0.05$). Under isovolumetric conditions, SED+MCT showed additional deterioration in the same parameters, but these alterations were prevented in EX+MCT ($P<0.05$). This improved hemodynamic profile was paralleled with prevention of cardiomyocytes atrophy, fibrosis, and with normalization of ET-1 mRNA levels ($P<0.05$). Exercise preconditioning also enhanced exercise tolerance and positively impacted survival. Of note, these improvements were observed 4 weeks after the cessation of exercise training, highlighting that the protective phenotype promoted by exercise training is maintained for several days.

Key words: Exercise preconditioning; Cardioprotection; Left ventricular dysfunction; Pulmonary arterial hypertension

List of Abbreviations

ADP: deoxyribonucleic acid

Akt: protein kinase B

Ang-II: angiotensin

ATP: adenosine triphosphate

CaMK: calcium calmodulin-dependent protein kinase

CRP: C-reactive protein

DOX: doxorubicin

ET-1: endothelin-1

FFR: force-frequency relationships

HSP: heat shock protein

IGF-1: insulin-like growth factor

IL-6: interleukin-6

I-R: ischemia-reperfusion

KATP: ATP-sensitive potassium

LVD: left ventricle dysfunction

LTCC: L-type calcium channels

LV: left ventricle

MCT: monocrotaline

MI: myocardial infarction

mitoKATP: mitochondrial inner membrane

MHC: myosin heavy chain

MnSOD: manganese superoxide dismutase

mTOR: mammalian target of rapamycin

NCX: sodium/calcium exchanger

•NO: nitric oxide

PAH: pulmonary arterial hypertension

PI3K: phosphoinositide 3-kinase

PLN: phospholamban

ROS: reactive oxygen species

RV: right ventricle

RVF: right ventricular failure

RyR: ryanodine receptor

SERCA2a: sarco/endoplasmic reticulum Ca2+-ATPase

sarcKATP: sarcolemmal membrane

SOD: superoxide dismutase

SR: sarcoplasmic reticulum

TNF: tumor necrosis factor

Thr17: p-phospholamban

1. GENERAL INTRODUCTION

The high physical activity demands imposed to humans and animals in the ancient times have favored the phylogenetic development of a phenotype towards the optimization of aerobic metabolic pathways in order to conserve energy for a potential future food deficiency (4). Moreover, in the ontogenetic point of view, it is known that large periods of high-intensity physical activity induce favorable cardiovascular adaptions to support the metabolic demands of the working skeletal muscles under high exigency conditions (9). Specifically on the heart, the beneficial effect of exercise training seen from animal models indicates an increased oxidative capacity of the myocardium (7), an enhanced cardiomyocyte survival (14), and attenuating left ventricular remodeling (19). Exercise training has been shown as an important preventive measure for cardiovascular diseases, and the benefits are well reported (2, 21). Several human epidemiological studies indicate a significant association of moderate and vigorous exercise training with a reduced incidence of cardiovascular events in healthy individuals (11, 18). Also, animal studies provide evidence of a cardioprotective phenotype induced by exercise training that allows an improved response against several insults such as ischemia-reperfusion (6, 22), myocardial infarction (5, 8), cardiotoxicity induced by doxorubicin (1, 15) or acute pressure overload (20) .

Pulmonary arterial hypertension (PAH) is characterized by progressive pulmonary vascular remodeling, imposing an increased overload to the right ventricle (RV). Although initially adapting by developing, among others, RV hypertrophy, it rapidly progresses to ventricular failure and premature death (3,

10, 13). PAH selectively overloads the RV, but it is known that left ventricle dysfunction (LVD) may also be present in some forms of PAH, leading to a decrease in LV preload, and low cardiac output states (12). Contrarily to RV, the left ventricle (LV) has received less attention in the context of this disease. Mechanisms underlying LVD remain poorly understood but LV atrophy (12) and/or neurohumoral activation (17) have been reported to play a role. Indeed, the chronic ET-1 overactivity in heart disease has been associated with slower relaxation and impaired contractility, favoring the accumulation of fibrosis (16).

The benefits of exercise training to improve overall health and protect, delay or improve cardiovascular diseases are well understood, but, the exact mechanisms behind the cardioprotective effects of exercise training have not yet been fully explained, as well as the impact of this on LVD, secondary HAP. Therefore, the objective of this study was to analyze, in a rat model of PAH induced by monocrotaline (MCT), the hypothetical cardioprotective effects of a previous exercise training on LV, trying to identify the underlying mechanisms involved.

1.1 STRUCTURE OF THE DISSERTATION

This dissertation is presented according the Scandinavian model, being divided in four sections:

Section 1: This chapter constitutes the general introduction to the topic, highlighting the relevance of the study and its objectives.

Section 2: This chapter, entitled "Acute and chronic mechanisms underlying prior exercise-induced cardioprotection: potential role in left ventricular

dysfunction secondary to pulmonary arterial hypertension" review the mechanisms involved in cardioprotection induced by the exercise training as well as the heart repercussions of the arterial pulmonary hypertension.

Section 3: This chapter is an experimental manuscript entitled **"**Exercise preconditioning prevents left ventricular dysfunction and maladaptive remodeling secondary to pulmonary arterial hypertension in rats"**.** It constitutes the experimental part of the dissertation, presenting the material and methods used, the obtained results and their discussion.

Section 4: The main conclusions of the dissertation are presented in this chapter.

The bibliography references supporting concepts, theories, and/or methods are presented at the end of each chapter.

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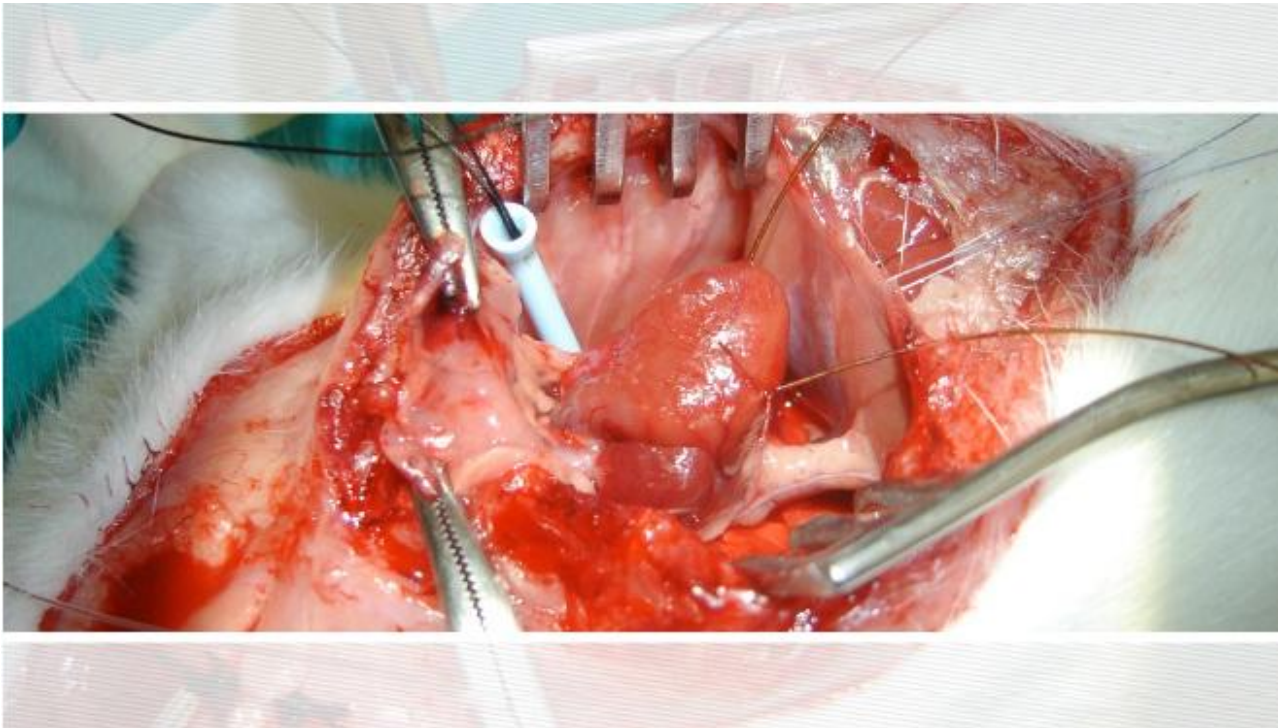
Acute and chronic mechanisms underlying prior exercise-induced cardioprotection: potential role in left ventricular dysfunction secondary to pulmonary arterial hypertension

ABSTRACT

Growing body of evidence supports the notion that exercise training is capable to provide a protective phenotype. Epidemiological studies suggest a significant association of moderate and vigorous exercise training with a reduced incidence of cardiovascular events and all-cause mortality. Animal studies provide direct evidence that exercise preconditioning confers cardiac protection against cardiac insults such as ischemia-reperfusion, myocardial infarction, doxorubicin cardiotoxicity and acute pressure overload. Moreover, to date, the only practical and sustainable countermeasure capable of promoting protection against cardiac harmful stimuli seems to be the regular practice of endurance exercise. The mechanisms responsible for give the cardioprotector phenotype is still unclear. In this context, this document intents to review the mechanism behind cardioprotection exercise-induced against some pathological stimuli, including left ventricular dysfunction secondary to pulmonary arterial hypertension. While cardioprotection afforded by acute exercise has been associated with the increased expression and activity of a few mediators such as ion channels (ex: ATP-sensitive potassium channels), enzymes (ex: manganese superoxide dismutase (MnSOD)) or chaperones (HSP's), the chronic effect of exercise training seems to induce more profound alterations in the heart. Specifically, cardiac protection conferred by chronic exercise can be

due to morphological remodeling, intrinsic and extrinsic alterations to cardiomyocytes, and improved anti-inflammatory, anti-neurohumoral and anti-oxidative status.

PAH have complex pathophysiology that comprises increased pulmonary vascular resistance and pulmonary vascular remodeling leading to progressive right ventricular failure. Of note, although PAH selectively overloads the right ventricle, left ventricular dysfunction (LVD) also manifests in the course the disease. Potential mechanisms to explain cardiac dysfunction leading to RVF include maladaptive cardiomyocyte remodeling, neurohumoral activation inflammation, and oxidative stress, among others. It was already shown that exercise training protects cardiac function and prevents the activation of maladaptive mechanisms in the presence of chronic and acute LV pressure overload. In addition, exercise training is known to positively modulate oxidative stress, inflammation, neurohumoral activation and endothelial dysfunction in LV heart failure and hypertension, all of which were implicated in the genesis and progression of PAH. This evidence suggests that exercise training may have the potential to confer cardiac protection against LVD secondary to PAH.

1. Introduction

Growing body of evidence supports the notion that exercise training is capable to provide a protective phenotype. Two lines of evidence strongly support this concept. First, a wide array of human epidemiological studies suggest a significant association of moderate and vigorous exercise training with a reduced incidence of cardiovascular events and all-cause mortality (94) in persons involved in regular physical exercise. Second, numerous animal studies provide direct evidence that exercise preconditioning confers cardiac protection against cardiac insults such as ischemia-reperfusion (I-R) (47, 61, 88, 106, 108, 121), myocardial infarction (MI) (28, 31, 45, 46), doxorubicin cardiotoxicity (DOX) (5, 70, 76) and acute pressure overload (99). Moreover, to date, the only practical and sustainable countermeasure capable of promoting protection against cardiac harmful stimuli seems to be the regular practice of endurance exercise (107). For instance, and regarding an ischemic event, there are two preconditioning agents, the prior ischemia and adenosine receptor agonist, which were showed to promote delayed preconditioning against subsequent ischemia. However, if continuously administered, these treatments are no longer effective (17). Also, humans rarely know when an ischemic event will occur and so, they will not be able to precondition themselves previously (17). On the other hand, 1 week of exercise training was shown to protect the heart against the deleterious effects of ischemia-reperfusion induced 9 days after the cessation of training (88).

Despite the benefits of exercise are widely recognized, the underlying mechanisms remain poorly comprehended. In this sense, diverse experimental models have been used to test the ability of the exercised heart to deal with

different stressors as well as to decipher the molecule(s) responsible for such protective effect. This review is written to try understanding the mechanism behind cardioprotection exercise-induced against some pathological stimuli, including left ventricular dysfunction secondary to pulmonary arterial hypertension.

2. Mechanisms underlying cardiac protection induced by exercise preconditioning

The mechanisms underlying the cardioprotective phenotype induced by exercise training are only now starting to be comprehended and seem to include an unknown variety of mediators. Improving our understanding of the molecular basis for exercise-induced cardioprotection will play an important role in developing optimal exercise interventions. When analyzing the available studies dedicated to understand the mechanisms of prior exercise-induced cardiac protection, we felt the need to differentiate between acute and chronic exercise protocols' effects. While cardioprotection afforded by acute exercise has been associated with the increased expression and activity of a few mediators such as ion channels (ex: ATP-sensitive potassium channels), enzymes (ex: manganese superoxide dismutase (MnSOD)) or chaperones (HSP's), the chronic effect of exercise training seems to induce more profound alterations in the heart. In the next section of this review, we will summarize the main mediators that have been implicated in prior exercise-induced cardiac protection. We do not intend to individually highlight any of them since we believe that cardiac protection can be explained by multiple factors and can even be stress-specific. Therefore, we start from the view-point that exercise-

induced cardiac protection is the sum of a myriad of intricate molecular networks, that, in the case of chronic exercise, can act together with functional and structural adaptations.

2.1- Cardiac protection conferred by acute exercise

2.1.1- Heat shock proteins

Heat shock proteins are a group of proteins that play an important role in the maintenance of cellular homeostasis against potentially lethal stimuli (29). They were originally identified on the basis of their induction by hyperthermia but there is a wide range of stimuli that can up-regulate them in different cells, including cardiomyocytes (85, 127). Of note, it seems that members of the 70-kDa family (particularly HSP72) are the heat shock proteins most responsible for cardiac protection. Experimental evidences indicate that cardiac over-expression of HSP72 is sufficient to protect the heart against the damaging effects of ischemia (69) and chronic heart failure (120). The mechanism by which HSP72 provides protection is not totally clear. Beside its role in protein synthesis, folding, transport, and degradation, it is though that HSP72 may play a role in augmenting myocardial anti-oxidant capacity as well as preventing apoptosis (85, 103, 107). In this sense, the observation that exercise improved myocardial tolerance to I-R was associated with increased myocardial HSP72 (33, 106), grounded the rational that they could mediate the exercise-induced cardiac protection. However, some experiments demonstrated that the protective effect of exercise on cardiac muscle is independent of HSP's. In fact, it was shown that exercise training in a cold environment improved myocardial performance after I-R without elevation of myocardial levels of HSP72 (61, 108,

121), HSP10, HSP40, HSP60, HSP73 or HSP90 (61). Similar data was obtained in response to DOX, where the acute exercise was also shown to provide protection (73, 76, 132, 133) but, independent from myocardial HSP72 levels (76). Overall, these studies suggest that exercise-induced cardioprotective phenotype is not dependent on increased myocardial levels of HSP72.

2.1.2- Anti-oxidants

Oxidative stress is caused by an unbalance between the production of reactive oxygen (ROS) or nitrogen species and the antioxidant capacity and repair ability of cell (55) . The overall result of this impairment is cellular damage to macromolecules such as deoxyribonucleic acid (AND), proteins and lipids (4). Cells have developed highly complex antioxidant systems that work all together to protect our body from the noxious effects of oxidative stress. The most efficient enzymatic antioxidants comprise glutathione peroxidase, catalase, and superoxide dismutase (SOD) (55). Exercise seems to increase several key antioxidative enzymes such as MnSOD, glutathione peroxidase, and catalase (52). Of note, it seems that the duration and intensity of exercise protocols are determinant factors since the expression of some of these enzymes may show some variability according to the features of the protocol (55, 107). This ability to augment MnSOD with exercise appears to be maintained in the aged heart

Several studies have shown that overexpression or administration of exogenous antioxidants results is cardioprotection, suggesting that it may play an important protective role (44, 111, 124), with a particular role recognized to MnSOD. The strongest evidence that directly link increases in myocardial

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antioxidants and acute exercise-induced cardioprotection implicates a contributory role for MnSOD. In a very elegant study, French and coworkers (47) employed antisense oligonucleotide techniques to silence MnSOD genes and thus prevent exercise-induced increases in myocardial MnSOD activity. With this approach they showed that acute exercise-induced increase in MnSOD is essential to achieve the full protection against IR-induced arrhythmias and infarction (47). However, it should be noted that authors from the same group also reported that protection against myocardial stunning seems to work even in the absence of MnSOD (89), supporting the concept that cardioprotection is multifactorial and, eventually, model specific.

2.1.3- ATP-dependent potassium channels

Located throughout the body in metabolically active tissues, the ATP-sensitive potassium (KATP) channels were first discovered in the cardiomyocyte sarcolemma, where they are abundantly expressed (75). There is one family of KATP channels in the sarcolemmal membrane (sarcKATP), and another in the mitochondrial inner membrane (mitoKATP) (44). These channels are normally inhibited (closed) by intracellular concentration of adenosine triphosphate (ATP) but certain conditions such as exercise, stress, severe ischemia, and gain-of-function genetic mutations may cause them to open (137). In general terms, KATP activation (opening) reduces calcium entry and preserves energy stores that would otherwise be depleted, therefore providing cardioprotection (17, 137). Several investigations support a protective role for both the sarcKATP and the mitoKATP isoforms through distinct mechanisms (2, 16, 57, 102, 109, 136). Specifically concerning to the exercise-induced

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cardioprotective phenotype, their contribution remains relatively uninvestigated but some evidence supports their relevance. Short-term exercise training (1-5 days) increased sarcKATP channel subunit expression in the heart and their blockade resulted in increased infarct size, suggesting that the exercise-acquired resistance to myocardial ischemia-reperfusion injury is dependent on sarcKATP activity (22). Regarding to mitoKATP channel, available data does not support their participation on exercise-induced cardioprotection against IR-induced myocardial infarction (16). However, they seem to play an important role in preventing short-duration IR-mediated arrhythmias (109). Additional work is required to clarify the role that sarcKATP and mitoKATP channels play in exercise-induced cardioprotection.

2.2- Mechanisms underlying cardiac protection induced by chronic exercise

Hemodynamic overload due to long-term exercise training typically involves both left and right ventricles, inducing morphological, functional and electrical changes that are globally referred to as the "athletic or athletes" heart (26, 39, 40, 49, 59). Although it is generally assumed that exercise training provides cardiac favorable adaptations, we are only now starting to understand the limits of such adaptation, with some data supporting the notion that even the exercise benefits may be dose-dependent (14). We recognize the importance of this topic but it will not be considered for the purpose of this review. What we would like to highlight is what are the compensatory adaptations that occur with chronic exercise training that translate into improved cardiac function, allowing the heart to respond more efficiently to the daily hemodynamic demands as well

as to more demanding and injurious insults.

2.2.1- Cardiac growth, cardiomyocyte hypertrophy and hyperplasia

Endurance exercise training can induce hypertrophy at the organ as a whole but also at the cellular level. Morphometric studies have shown that this hypertrophy includes proportionate increases of cardiac myocytes and coronary vasculature with no change in the proportion of extracellular collagen (40). Growth of cardiomyocytes is dependent on the initiation of several events in response to an increase in functional load, including activation of signaling pathways, changes in gene expression, increases in the rate of protein synthesis, and the organization of contractile proteins into sarcomeric units (8). Activation of the insulin-like growth factor (IGF)-1/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) signaling pathway is considered a hallmark of adaptive growth of cardiomyocytes, typical from normal postnatal development or exercise training (8, 38, 79, 101). Activation or restoration of this pathway has been associated with enhanced contractile function and improved calcium kinetic (20, 48, 79, 97), enhanced angiogenesis, glucose uptake, proliferation and anti-apoptotic effect (9, 13, 21). Cardiac hyperplasia, i.e. the addition of new cardiomyocytes, is today recognized to occur in the adult heart under different cardiac stressful conditions. Of note, exercise training was shown to activate cardiac stem cells to differentiate into new cardiomyocytes (41, 81, 90, 126) as well as to induce cardiomyocyte proliferation (13). Increased cardiac regeneration ability by prior exercise training may help to explain decreased maladaptive remodeling and improved survival, several weeks after myocardial infarction induction (28, 31,

45, 46).

2.2.2- Contractile improvements of cardiomyocyte

The ability of the heart to eject blood is highly dependent on myocardial shortening velocity, a propriety largely determined by its myosin heavy chain (MHC) isoforms composition (60). Improvement of cardiac function has been constantly associated with a coordinate increase in alpha- and a decrease in beta-MHC in the rat heart (65, 71, 83). Exercise training generally induces an up-regulation of alpha-MHC in rats (74, 110). Enhancement of contractility has also been associated with enhanced calcium handling. Depolarization of the cardiomyocyte membrane leads to entrance of calcium to the cytosol through the opening of L-type calcium channels (LTCC), triggering further calcium release from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR). Intracellular calcium then binds to troponin C in the myofilaments and initiates contraction (10, 77, 129). Subsequent relaxation is dependent of calcium detachment from troponin C, which is recaptured into the SR by sarco/endoplasmic reticulum Ca2+-ATPase (SERCA2a) or extruded from the cell by the sarcolemmal sodium/calcium exchanger (NCX). Exercise was shown to increase the expression and activity of SERCA2a, but not total phospholamban (PLN) (78, 129). This up-regulates the SERCA2a/PLN ratio and therefore allows SERCA2a to increase the rate of calcium uptake. Increased phosphorylation status of PLN at p-phospholamban (Thr17) residue mediated by exercise-induced activation of calcium calmodulin-dependent protein kinase (CaMK) II and by Akt was shown to contribute to increase SERCA activity (42, 80). Akt also seems to regulate LTCC stability, thus

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influencing cardiomyocyte calcium entry, handling and contractility (42). Moreover, exercise seems to increase contractility by increasing myofilament responsiveness to calcium (130).

2.2.3- Increased capillarization

Exercise training is associated with adaptations in the coronary microvasculature including increased arteriolar densities and/or diameters. As the heart remodels in response to exercise training, concomitant capillary growth is thought to guarantee that capillary density and perfusion remains normal (40, 86, 128). Exercise training also alters the distribution of coronary vascular resistance so that more capillaries are recruited, resulting in an increase in the permeability-surface area product (40). This may represent an important mechanism to explain why prior exercise training confers protection to IR injury by granting collateral perfusion. Exercise training also increases nitric oxide (˙NO) production and K+ channel activity in coronary resistance vessels, which may provide a greater intrinsic capacity of local vascular control mechanisms to regulate blood flow to collateral-dependent myocardium and thereby contribute to the improved perfusion observed after exercise training (40).

2.2.4- Anti-inflammatory, anti-neurohumoral and anti-oxidative effect

Persons who exercise on a regular basis show a reduction in systemic inflammation (mitogen-stimulated inflammatory cytokine production, skeletal

muscle inflammatory protein content, adipokine production, and serum levels of C-reactive protein (CRP) (54, 118). In addition, it seems that exercise training is able to reduce the local expression of TNF-alpha, IL-1-beta, IL-6, and iNOS in the skeletal muscle of chronic heart failure (HF) patients (51). Regarding neurohumoral activation, exercise training was shown to reduce the circulating levels of angiotensin (Ang-II), aldosterone, vasopressin and natriuretic peptides in HF patients (25, 131). This positive effect was also observed in relation to endothelial function, with regular exercise promoting an increase in nitric oxide bioavailability and number of endothelial progenitor cells (113), as well as decreasing oxidative stress and protein oxidation (131). These overall adaptations may be important in the modulation of maladaptive hypertrophic signaling (7) and explain how exercise training prior to permanent coronary artery ligation protected cardiac function, decreased maladaptive remodeling and improved survival, several weeks after myocardial infarction induction (28, 31, 45, 46).

3. Pulmonary Arterial Hypertension

3.1- Epidemiology and Pathophysiology

Pulmonary arterial hypertension (PAH) is a multi-factorial disease with genetic background and environmental stress as principal components. It is defined as a mean pulmonary artery pressure greater than or equal to 25 mmHg at rest and a mean pulmonary-capillary wedge pressure lesser than or equal to 15 mmHg (53). This disease is rare, with an incidence of approximately 2.4 cases per million diagnosed per year, and a prevalence of approximately 15 cases per million diagnosed per year (68). Two-thirds of PAH patients are

women, with 41-50 years of age at the time of diagnose (122). In the absence of treatment, the survival rate was estimated as low as 2.8 years after diagnosis (135).

The pathophysiology of PAH is complex and includes increased pulmonary vascular resistance and pulmonary vascular remodeling leading to progressive right ventricular failure (RVF) (93). Potential mechanisms to explain cardiac dysfunction leading to RVF include maladaptive cardiomyocyte remodeling (11, 23, 104), neurohumoral activation (43, 66), inflammation (18), and oxidative stress (111), among others (10). Of note, although PAH selectively overloads the right ventricle, left ventricular dysfunction (LVD) also manifests in the course the disease (24, 34, 82, 123). In fact, decreased left ventricular systolic and diastolic dysfunction was reported in human and animals settings of chronic pulmonary hypertension, including PAH (3, 15, 23, 27, 36, 82, 95, 125). If the RV response to PAH remains poorly comprehended, even less is known about LVD. In the next section, we will review some of the mechanisms that have been implicated in LVD secondary to PAH.

3.2- Mechanisms underlying LV dysfunction secondary to PAH

A few mechanisms have been proposed to explain LV dysfunction in PAH, including ventricular interdependence and impaired LV filling (123), LV atrophy (63), intrinsic LV myocardial abnormalities (84, 91) and extracellular matrix remodeling (23, 125).

3.2.1- Ventricular Interdependence

The phenomenon by which the RV directly influences LV diastolic filling is known as direct ventricular interaction (92, 123). In patients with RV chronic pressure overload, echocardiographic studies have shown altered LV filling dynamics (82, 92). Altered LV filling dynamics seems to be caused by massive RV enlargement as well as flattening and leftward displacement of the interventricular septum (92). Specifically, these structural alterations compress the LV, distorting its cavity geometry (decreased LV end-diastolic volume and abnormal eccentricity index) (92). As a consequence, early diastolic filling is impaired and LV filling is redistributed toward late diastole, impairing LV filling which, according to Frank-Starling's law, reduces stroke volume (123). Of note, normalization of interventricular septal motion as well as improved venous return to the left atrium normalized LV diastolic and systolic function (95). Finally, it was suggested that LV is affected only in the presence of RV dilation and failure, since mild RV pressure overload in the absence of RV failure does not affect LV structure and function (32, 63).

3.2.2- LV atrophy

LV unloading may cause atrophic remodeling that is associated with diastolic and systolic dysfunction (63, 115). LV atrophic remodeling has been noted in both human and animal settings of chronic pulmonary hypertension and thus may contribute to LV pathophysiology (63, 119). Loss of cardiac mass can be due to either atrophy (a reversible process) or cellular death the cardiomyocytes (115). Currently it remains unknown which of the two processes is responsible for the loss of myocardial mass in the unloaded LV. Although some evidence point to some contribution of cardiomyocyte apoptosis (23),

atrophy is considered the main mechanism (63). Contrarily to the pathways regulating protein synthesis in the heart, protein degradation is poorly studied. It would be interesting to assess the role of the major pathways regulating protein degradation in LV atrophic remodeling secondary to PAH, namely the calcium-dependent calpain system, lysosomal proteolysis and autophagy, and the ubiquitin proteasome system (6).

3.2.3- Intrinsic LV myocardial abnormalities

LV dysfunction in PAH can also be explained, at least partially, to abnormalities intrinsic to cardiomyocytes. For instance, it has been shown that LV muscle strips from MCT-treated rats exhibited negative force-frequency relationships (FFR) (91). The FFR is an important intrinsic regulatory mechanism of cardiac contractility. While the normal myocardium increases developed force with higher frequencies of stimulation showing normal contractile reserve, the failing or dysfunctional myocardium loses this reserve (91). Of note, ET-1 blockade after MCT injection restored the positivity of LV myocardium FFR, suggesting a possible direct detrimental action of ET-1 overexpression in the LV myocardium of PH rats (91). Cardiac ET-1 overexpression has been associated with slower relaxation and impaired contractility through dysfunctional Ca2+ homeostasis and myosin heavy chain (MHC) isoform switch (71, 72). Accordingly, LV from MCT-treated rats was shown to express greater beta (23) and reduced alpha myosin heavy-chain isoform (63), as well as diminished SERCA2a (63).

3.2.4- Extracellular matrix remodeling

Extracellular matrix composition and fibrillar collagen content were also suggested to be involved in LVD that accompanies severe PH (3, 23, 125). Accumulation of fibrillar collagen (fibrosis) affects cardiac stiffness, promotes arrhythmias and impairs the diffusion of oxygen to cardiomyocytes increasing the susceptibility for heart failure development (35, 64). Collagen is synthesized by myofibroblasts and TGF-beta is its most important activator (87). Neurohumoral factors such as ET-1, Ang II and aldosterone, as well as inflammatory mediators [e.g. interleukin-6 (IL-6), tumor necrosis factor (TNF)-alpha] are also involved on activation of myofibroblasts (58). Both neurohumoral and inflammatory mediators (produced locally in LV or released systemically from the RV and lungs) are increased in PAH and therefore may promote a pro-fibrotic environment that can help to explain the increased LV myocardial stiffness (3, 23, 37).

3.3- Exercise training in PAH

For a long time, exercise training was discouraged for PAH patients to avoid provoking PAH symptoms, notably exercise-induced syncope, and due to the risk of sudden cardiac death (34). Recent evidence demonstrates that exercise training is safe, improves exercise tolerance and functional class, provided cardiovascular improvements, reduced lactate production, improved ventilatory reserve, and increased leg blood flow during progressive exercise (30, 96). Our group recently showed that exercise preconditioning protects the RV (98) while others have shown no deleterious effects of exercise when performed after the establishment stable PAH (62, 96). As far as we could address, no study addressed the impact of exercise preconditioning in LVD in

PAH. It was already shown that exercise training protects cardiac function and prevents the activation of maladaptive mechanisms in the presence of chronic (12, 48) and acute (99) LV pressure overload. In addition, exercise training is known to positively modulate oxidative stress, inflammation, neurohumoral activation and endothelial dysfunction in LV heart failure and hypertension (1, 25), all of which were implicated in the genesis and progression of PAH. Based on these data, we anticipate that exercise preconditioning may have a positive impact in LVD secondary to PAH. This is of particular importance if we consider that establishing a clinical diagnosis early in the course of the disease is very hard given the nonspecific nature of the symptoms and the subtleness of the signs on the initial moments of the disease (114). The average time interval between the first symptoms detection and the establishment of the diagnosis was recently estimated to be 27 month (117). In this context, the eventual preventive role of exercise might even assume greater relevance in familial PAH (FPAH), where the disease can develop at an earlier age and assume more severe manifestations in familial members (134). In this case, individuals at risk would eventually benefit from the preconditioning protection recognized to exercise.

4. Monocrotaline as a model to induce RV and LV dysfunction

MCT is a pyrrolizidine alkaloid, derived from Crotalaria spectabilis that can be administrated by intraperitoneal (60 mg/kg), subcutaneous (60 mg/kg), or intravenous injection (1–5 mg/kg). Although the MCT rat model has contributed to a better understanding of vascular remodeling in PH, the underlying basic mechanisms of MCT-induced PH are still unclear. MCT is

converted to its bioactive pyrrolic derivative in the liver by the cytochrome P450 3A (112) that has a half-life of ~3 s in aqueous media and primarily affects the pulmonary arterial bed because lungs are the first major vascular bed after the liver (105). Nonetheless, MCT can injury other sites like the liver or the kidney, which clearly represent an important limitation of this model (19, 116). In the lung, endothelial cells are the site of first damage of MCT. Medial hypertrophy in smaller arteries is present from day 12-14 which is accompanied by a rise in pulmonary artery pressure and both parameters increase progressively with the development of the disease RV hypertrophy developed later than medial thickening and is present only at day 21 postinjection (50). These changes are accompanied by increase in RV systolic and diastolic pressures and ultimately by RV failure (67). MCT model has also been shown to induce LV atrophy, dysfunction, and maladaptive remodeling (23, 63, 91). Summarizing, MCT is a simple model that induce alterations with some similarities with human PAH, such as hemodynamic repercussions, histological changes and high mortality. On the contrary, it diverges from human PAH in the precocious loss of endothelial barrier and in the inflammatory adventitial proliferation (100). However, it is frequently utilized since it offers technical simplicity, reproducibility, and low cost compared with other models of PAH, and is one of the most used models to study pharmacologic therapies (56).

5. Conclusions

It is clear that exercise preconditioning provides a cardioprotective phenotype that confers cardiac protection against several cardiac insults. The mechanisms behind this phenotype are unclear and may differ according to the duration of exercise protocol (acute vs. chronic) and may be stimuli-specific. Little is known about the impact of exercise training in cardiac function in PAH. Given that chronic exercise modulates the main pathologic pathways underlying the disease, it is possible that it can act in multiple targets, preventing both RV and LV dysfunction.

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Experimental Study

Exercise preconditioning prevents left ventricular dysfunction and maladaptive remodeling secondary to pulmonary arterial hypertension in rats

ABSTRACT

Exercise training can provide a cardioprotective phenotype that allows an improved response against several insults such as ischemia-reperfusion, myocardial infarction, cardiotoxicity induced by doxorubicin or acute pressure overload. Pulmonary arterial hypertension (PAH) directly affects the right ventricle but left ventricle dysfunction (LVD) was recently described in PAH patients, which is associated with left ventricle (LV) atrophy and/or neurohumoral activation. The objective of our study was to analyze the hypothetical cardioprotective effects of exercise preconditioning on LV in a rat model of PAH induced by monocrotaline (MCT). The study was designed with 115 male Wistar rats, submitted for three interventions, showed here with three phases. The first phase was done with 60 rats were randomly separated in sedentary (SED; 4 weeks sedentary) and trained groups (EX; running sessions of 60 mim/day, 5 days/week, at 25 m/min, during 4 weeks). After, animals were injected with MCT (60mg/kg; SED+MCT and EX+MCT) or the same volume of vehicle (SED+V and EX+V). Afterwards, all animals remained sedentary for additional 4 weeks. Next, animals were submitted to LV hemodynamic evaluation in baseline and isovolumic conditions, and LV samples were prepared for light microscopy analysis (cardiomyocyte cross sectional area and collagen deposition) and endothelin (ET-1). In a second phase, we developed the survival study with 40 animals submitted to the respective experimental

protocols (SED+V, n=5; SED+MCT, n=15; EX+V, n=5; EX+MCT, n=15). The third phase was performed with 15 animals to assess their exercise tolerance. We found in baseline conditions, systolic (peak systolic pressure and dP/dtmax) and diastolic function (dP/dtmin and Tau) were compromised in SED+MCT but not in EX+MCT ($P<0.05$). Under isovolumic conditions, SED+MCT showed additional deterioration in the same parameters, but these alterations were prevented in EX+MCT ($P<0.05$). This improved hemodynamic profile was paralleled with prevention of cardiomyocytes atrophy and fibrosis, and with normalization of ET-1 mRNA levels ($P<0.05$). Exercise preconditioning also enhanced exercise tolerance and positively impacted survival. Of note, these improvements were observed 4 weeks after the cessation of exercise training, highlighting that the protective phenotype promoted by exercise training is maintained for several days.

Key words: Exercise training; Cardioprotection; Monocrotaline; Cardiomyocytes atrophy; Endothelin 1; Survival rate; Exercise tolerance

INTRODUCTION

Exercise training has been shown as an important preventive measure for cardiovascular disease, and the benefits for a human health are well reported (2, 34). Several human epidemiological studies indicate a significant association of moderate and vigorous exercise training with a reduced incidence of cardiovascular events in healthy individuals (15, 28). Also, animal studies provide evidence of a cardioprotective phenotype induced by exercise training that allows an improved response against several insults such as ischemia-reperfusion (11, 38), myocardial infarction (9, 12), cardiotoxicity induced by doxorubicin (1, 24) or acute pressure overload (31) .

Pulmonary arterial hypertension (PAH) is characterized by progressive pulmonary vascular remodeling, imposing an increased overload to the right ventricle (RV). Although initially adapting by developing, among others, RV hypertrophy, it rapidly progresses to failure and premature death (3, 13, 22). PAH selectively overloads the RV, but it is known that left ventricle dysfunction (LVD) may also manifests in some forms of PAH, leading to a decrease in LV preload, and low cardiac output states (17). Contrarily to RV, the left ventricle (LV) has received less attention in the context of this disease. Mechanisms underlying LVD remain poorly understood but LV atrophy (17) and/or neurohumoral activation (27) have been reported to play a role. Chronic ET-1 overactivity in heart disease has been associated with slower relaxation and impaired contractility, favoring the accumulation of fibrosis (26).

Physical activity is known to improve overall health and protect, delay or improve many common cardiovascular diseases. However, the impact of

exercise training on LVD, secondary HAP is still unknown. Seeing that potential benefits of exercise training on cardiac physiology were found in many experimental models, we designed our study to analyze the cardioprotective effects of exercise preconditioning on LV in a rat model of PAH induced by monocrotaline (MCT).

MATERIALS AND METHODS

The study was designed with 115 male Wistar rats, submitted for three interventions, showed here with three district phases.

Phase 1:

Animal models and experimental design

All experimental procedures involving animal care and sacrifice were performed according to the Portuguese law on animal welfare and specifications of the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals, and approved by local ethics committee (University of Porto). Following one week of quarantine after arrival, 60 male Wistar rats (age= 4 weeks; Charles River Laboratories, Barcelona), were housed in groups of 5 rats per cage, maintained in an inverted 12h light/dark cycle, in an environment with controlled temperature of 22°C, and had free access to food. After that, they were randomly separated into two experimental groups: sedentary (SED; n=35; remained with movement confined to the cage's space during 4 weeks) and exercise (EX; n=25; exercised on a treadmill 5 days/week during 4 weeks). After ending this 4-week period, some animals from each group received one subcutaneous injection of monocrotaline (MCT; 60 mg/kg, Sigma, Barcelona, Spain) or an equal volume of vehicle (V; 1 mL/kg of saline), originating the following groups: i) SED+MCT (n=25), ii) SED+V (n=10), iii) EX+MCT (n=15) and iv) EX+V (n=10).

Exercise training protocol: Animals were acclimated to the treadmill during one week. Running speed and exercise duration were progressively increased until animals reached a maximum of 25 meters/minute (estimated work rate of 70% maximum oxygen consumption (25) during 60 min, at the end of the week. Then, animals exercised during 4 weeks, 5 days per week, 60 minutes/day, at 25 meters/minute, with no grade.

Hemodynamic evaluation

Procedure: At day 28-29 after MCT or vehicle administration, animals were prepared for left ventricular hemodynamic evaluation with a pressure-volume catheter. Rats were anaesthetized by inhalation with a mixture 4% sevoflurane with oxygen, intubated for mechanical ventilation (Harvard Small Animal Ventilator- Model 683). The right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) and the heart was exposed by a median sternotomy and the pericardium was widely opened. LV hemodynamic function was measured with conductance catheter (FTS-1912B-8018, Scisense) inserted by apical puncture on the LV cavity, along the ventricular long axis. The catheter was connected to MVP-300 conductance system through interface cable (PCU-2000 MPVS, FC-MR-4, Scisense), coupled to PowerLab16/30 converter (AD Instruments) and a personal computer for data acquisitions. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made with respiration suspended at the end of expiration under steady-state conditions, during preload reductions (inferior vena cava occlusion) and in response to isovolumetric contractions

induced by ascending aortic occlusion. Parameters from conductance catheter were recorded at a sampling rate of 1000 Hz and analyzed with Millar conductance data acquisition and analysis software (PVAN3.5).

Hemodynamic parameters: The following parameters were recorded: heart rate (HR), peak systolic pressure (Pmax), minimum pressure (Pmin), end-diastolic pressure (EDP), peak rate of pressure rise (dP/dtmax), peak rate of pressure fall (dP/dtmin), constant time of isovolumetric pressure decay (Tau), cardiac output (CO) and maximal elastance (Emax). To assess intrinsic myocardial function, preload-recrutable stroke work (PRSW), end-diastolic pressure–volume relation (EDPVR) and systolic elastance (Ees) were determined from pressure–volume loops recorded during transient preload reduction by gently pulling the inferior vena cava with a silk suture previously placed around it.

Conductance calibration: Parallel conductance values were obtained by the injection of approximately 100 □l of 10% NaCl into the right atrium. Calibration from relative volume units (RVU) conductance signal to absolute volumes (□l) was undertaken using a previously validated method of comparison to known volumes in Perspex wells (33).

Animal sacrifice and tissue harvesting

After collecting all hemodynamic data, the animals were sacrificed by exsanguination, the heart was excised and weighed, and samples from LV+septum (LV+S) were dissected, washed in cold PBS (pH 7.2) and weighted

together. Heart weight (HW) and LV+S weight were normalized to tibia length. Then, the LV was separated from the septum and divided in samples that were prepared for light microscopy (LM) analysis (cardiomyocyte cross sectional area and collagen deposition) and endothelin (ET)-1 mRNA quantification by RT-PCR, following routine procedures.

Histological analysis

Cubic pieces coming from intermediate cardiac region from LV were fixed (4% paraformaldehyde) by diffusion during 24 hours and subsequently dehydrated through graded ethanol solutions, cleared in xylene and mounted in paraffin. Transverse 6µm thick sections were cut, and used for assessing cardiomyocyte cross-sectional area (CSA) and fibrous tissue accumulation.

Left ventricular fiber cross-sectional area (CSA): Left ventricle sections were stained with hematoxylin and eosin as previously described (31). All the images were analyzed with ImageJ software (NIH, Bethesda, MD). CSA was determined in 5 animals per group, 10 pictures per animal, totalizing around 1000 cardiomyocytes per group.

Assessment of fibrous tissue accumulation: In order to determine the amount of cardiac fibrosis, LV sections were stained with Picrosirius red and quantified. Paraffin sections were dewaxed and hydrated, stained 4-µm-slide with Picrosirius red for 1 hour and 30 min, followed by two washes with acidified water. Finally, they were dehydrated in 80, 90 and 100% of ethanol, cleared in xylene and mounted in a resinous medium. Images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) for quantification of the

percentage area covered by collagen and muscle tissue. For quantitative comparisons, random microscopic fields (magnification of x400) were considered and 10 representative images per animal were obtained, from 5 animals per group. Areas of reparative and perivascular fibrosis were excluded.

Relative quantification of mRNA

Two-step real-time RT-PCR was performed as previously described (19). Briefly, after total mRNA extraction (no. 74124; Qiagen), standard curves were obtained for each gene correlating (R ≥ 0.98) the mRNA quantities in graded dilutions of a rat cardiac tissue sample with the respective threshold cycles (second derivative maximum method). Equal amounts of mRNA from every sample underwent three separate two-step real time RT-PCR experiments for each gene, using SYBR green as marker (no. 204143; Qiagen). GAPDH was used as internal control and results are relative to the mean obtained for the SED+V group and normalized for GAPDH (fold increase). All the analysis was performed in duplicates. Specific PCR primer pairs for the studied genes are presented in Table 1.

Table 1: Primers used in mRNA quantification by real-time RT-PCR

Gene	Sequence 5'-> 3'
GAPDH	**fw:** TGG CCT TCC GTG TTC CTA CCC **rev:** CCG CCT GCT TCA CCA CCT TCT
ET-1	**fw:** CGG GGC TCT GTA GTC AAT GTG **rev:** CCA TGC AGA AAG GCG TAA AAG

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ET-1: endothelin-1; fw: forward; rev: reverse.

Exercise tolerance test

For the exercise tolerance test, 15 animals were divided in 3 groups: 5 animals to SED+V, 5 to SED+MCT and 5 to EX+MCT. The test consisted in running on a treadmill with a constant speed of 20 m/min at 0° slop until exhaustion. The exercise tolerance of the rats was determined by the time from start until exhaustion, where exhaustion was established when the rats accepted the electric stimulus 3 consecutive times as opposed to running. The maximal running time achieved in the control group (SED+V; 110 minutes) was used as criteria to satisfactorily finish the test.

Phase 3:

Survival study

For the survival study, 40 animals were submitted to the respective experimental protocols (SED+V, n=5; SED+MCT, n=15; EX+V, n=5; EX+MCT, n=15). After that, animals remained sedentary with movement confined to the cage's space until day 42 after MCT injection, which was considered the study endpoint.

Statistical Analysis

The Shapiro-Wilk test was used to investigate within-group normality for a given variable. One-way ANOVA with Tukey´s posthoc test to compare all

groups was used for normally distributed data. Kruskal-Wallis test followed by Dunns test was used for non-normal data. For survival analysis, Kaplan–Meier analysis and the Gehan–Breslow test was performed, and pairwise comparisons were made using the Holm–Sidak method. All results are presented as mean ± sandard deviation (SD). Differences were considered significantly when $P<0.05$. Statistical analysis was performed with Graph Pad Prism software (version 5.0).

RESULTS

General morphometric characteristics

Table 2 summarizes the analyzed morphometric parameters. In comparison to all groups, SED+MCT presented lower BW (P<0.05) while this was prevented in EX+MCT. Heart weight was increased in both SED+MCT and EX+MCT in comparison to SED+V (P<0.05). Regarding to LV mass, this parameter was reduced in SED+MCT, even when normalized to the length of the tibia (P<0.05 vs. SED+V), while in EX+MCT this was averted.

Table 2: General morphometric characteristics

	SED+V (n=10)	EX+V (n=10)	SED+MCT (n=25)	EX+MCT (n=15)
Morphometry				
BW (kg)	0.343±0.013	0.347±0.031	0.276±0.025*	0.341±0.027†
HW (g)	0.834±0.098	0.0989±0.086*	0.945±0.107*	1.015±0.085*
HW/Tibia (g/cm)	0.225±0.011	0.244±0.026	0.264±0.025*	0.256±0.024*
LV+S (g)	0.6166±0.049	0.6663±0.072	0.5588±0.036*	0.6440±0.050†
LV/Tibia (g/cm)	0.158±0.012	0.172±0.018	0.149±0.011	0.168±0.014†

BW: body weight; LV+S: left ventricle+septum weight; LV/Tibia: left ventricle/Tibia; HW: heart weight; HW/Tibia: heart weight / tibia . Data are presented as mean±SD. *P<0.05 vs SED+V and †<0.05 vs. SED+MCT.

Histological analysis

The reduced LV mass observed in SED+MCT was accompanied by LV cardiomyocytes atrophy (figure 1). In addition, animals from SED+MCT group

also showed increased tissue fibrosis (P<0.05 vs. SED+V). All these features were prevented in EX+MCT (P<0.05 vs. SED+MCT).
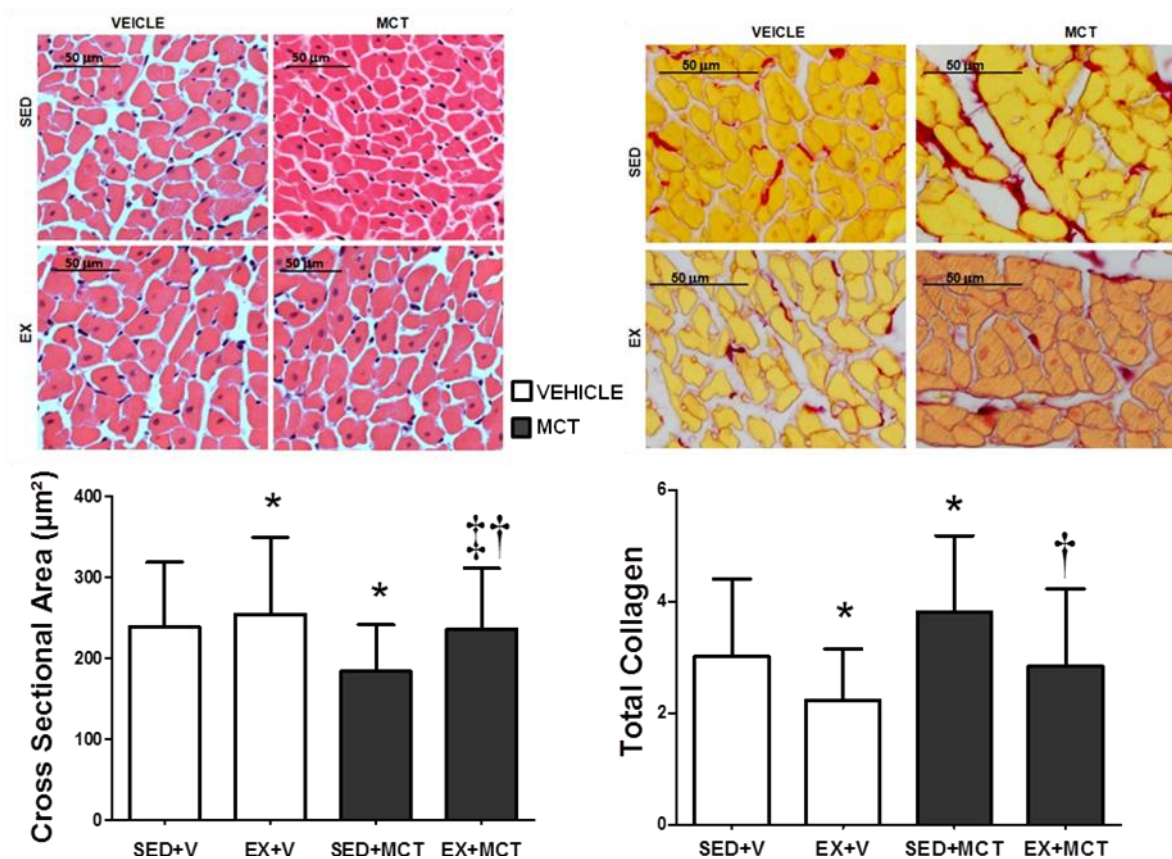

FIGURE 1: Effects of exercise training on LV CSA and fibrosis (A and B). Values are presented as mean±SD (n=5 per group). * P<0.05 vs. SED+V and † P<0.05 vs. SED+MCT.

Characterization of cardiac function – baseline and isovolumetric conditions

The results from hemodynamic evaluation are summarized in Table 3. Heart rate was significantly reduced in SED+MCT and normalized in EX+MCT group (P<0.05). Peak systolic pressure, dP/dtmax, dP/dtmin and the time constant *tau* were altered in SED+MCT group (P<0.05 vs. SED+V), while these alterations were avoided in EX+MCT (P<0.05 vs. SED+MCT), with exception of

dp/dt max. No significant modifications from vena cava occlusion derived parameters were observed.

A sudden and acute increase in pressure overload induced by total occlusion of the ascending aorta was performed in all animals in order to stress the heart. Under these conditions, systolic dysfunction was obvious in SED+MCT as shown by the decrease in dP/dtmax. Diastolic dysfunction was also present in SED+MCT as illustrated by the reduction in dP/dtmin, increased end-diastolic pressure and longer relaxation time in (P<0.05 vs. SED+V). Exercise training prevented all these alterations (P<0.05 vs. SED+MCT).

Table 3. Hemodynamic evaluation parameters

	SED+V	EX+V	SED+MCT	EX+MCT
Basal conditions				
HR (bpm)	401.5±26.2	366.3±27.6	330.3±54.1 *	371.1±33.4 †
P_{max}	115.8±14.6	119.7±13.1	86.74±24.8 *	102.6±14.3
dP/dt_{max} (mmHg/s)	7742±1495	8678 ±1407	5174±2403 *	6530±172 ‡
dP/dt_{min} (mmHg/s)	-9483±2110	-8727±1595	-3967±1885 *	-6486±159 *†‡
Tau (ms)	8.742±0.8	9.379±0.8	13.17±3.9 *	10.28±1.0 †
CO (uL/min)	54743±13427	60068±21047	34521±18191 *	49285±12034
Isovolumetric conditions				
P_{max}	179.72±11.52	215.46±39.25 *	160.08±28.32	160.42±13.26 ‡
EDP	6.07±1.32	6.39±1.42	7.72±2.61	6.23±0.80
dP/dt_{max} (mmHg/s)	7881±1182	10259±205.7 *	5252±1408 *	5703±486.3 *‡
dP/dt_{min} (mmHg/s)	-6008±1432	-7079±1220	-4027±1077 *	-4731±844.0 ‡
Tau (ms)	10.72±1.34	12.22±1.73	17.07±3.56 *	13.11±2.09 †

HR: heart rate; Pmax: maximum pressure; CO: cardiac output; dP/dtmax: peak rate of pressure rise; dP/dtmin: peak rate of pressure fall; Tau: time constant of ventricular pressure decay; EDP: end-diastolic pressure. Data are presented as mean±SD. *P<0.05 vs SED+V, †P<0.05 vs SED+MCT, ‡P<0.05 vs EX+V.

Neurohumoral activation

Due to the important role of ET-1 activation in PAH pathophysiology, the ET-1 gene expression was quantified on LV. ET-1 mRNA levels were increased in LV of SED+MCT group and normalized in EX+MCT group ($P<0.05$) (figure 2).
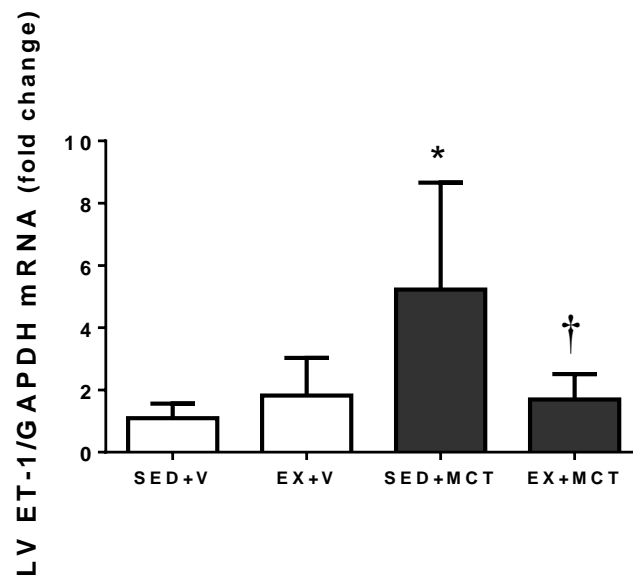

FIGURE 2: Effects of exercise training in LV ET-1 mRNA. Values are mean±SD (n=7 animals per group). * $P<0.05$ vs. SED+V and † $P<0.05$ vs. SED+MCT.

Exercise tolerance

SED+MCT showed lower exercise tolerance when compared with SED+V has shown by their reduced running time to exhaustion ($P<0.05$). Exercise training promoted a significant improvement in exercise tolerance in MCT trained group (Figure 3), with all animals reaching 110 minutes that was set as the test end-point ($P<0.05$ vs. SED+MCT).
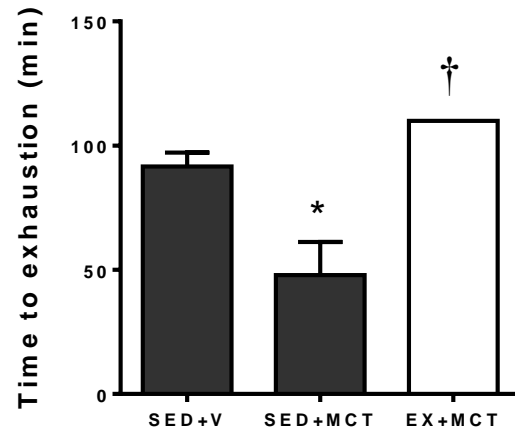
FIGURE 3: Impact of exercise training on exercise tolerance. Values are mean±SD (n=5 animals per group).* $P < 0.05$ vs. SED+V and † $P < 0.05$ vs. SED+MCT

Survival study

Figure 4 illustrates the survival rate. We found a decreased survival rate in all animals treated with MCT, with 25% survival in EX+MCT and 13% in SED+MCT at day 42 after MCT. Of note, survival rate from EX+MCT was significantly improved in comparison to SED+MCT. In control groups, no deaths have occurred.
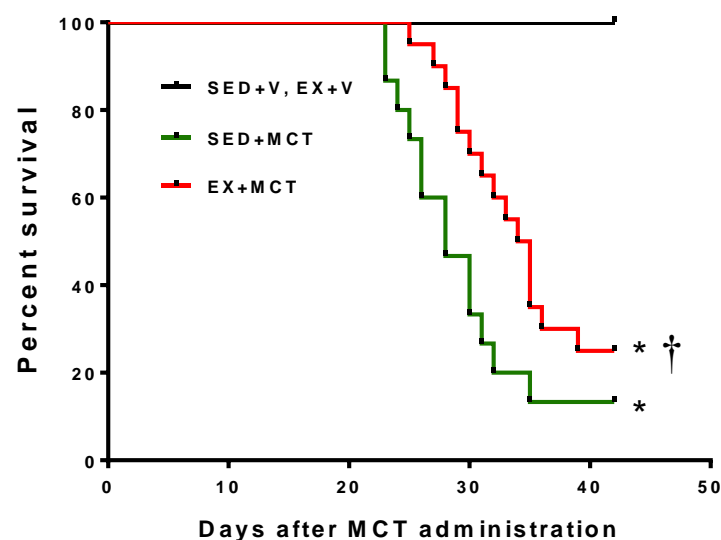

FIGURE 4: Impact of exercise training on survival: exercise training delayed mortality. MCT: monocrotaline. *$P < 0.05$ vs. SED+V and † $P < 0.05$ vs. SED+MCT.

DISCUSSION

The present study found that aerobic exercise preconditioning exerts a positive impact on left ventricle dysfunction secondary to pulmonary arterial hypertension, protecting from functional impairments in baseline and under stress conditions. These benefits were paralleled with prevention of cardiomyocytes atrophy and fibrosis, and with normalization of ET-1 mRNA levels. Exercise preconditioning also enhanced exercise tolerance and positively impacted survival. Of note, these improvements were observed 4 weeks after the cessation of exercise training, highlighting that the protective phenotype promoted by exercise training is maintained for several days.

In order to study the cardioprotective phenotype afforded by exercise training, we submitted previously exercised animals to experimental MCT-induced PAH. This experimental model is widely used to study therapeutic targets to PAH as well as a model of RV failure (5, 17, 20, 21, 23, 27, 36). We show that prior exercise training restored cardiac output and prevented alterations in several indexes of LV contractility and relaxation. Exercise training also prevented the fall of LV Pmax and heart rate, which have been previously related with the severity of the disease and reduced responsiveness to sympathetic stimulation, respectively (27). Of note, exercise-induced cardiac protection was observed even when the LV was submitted to an additional acute stressful stimulus. Recently, our group showed that exercise preconditioning protects the RV (32) while others have shown no deleterious effects of exercise when performed after the establishment stable PAH (16, 30). We now extend these benefits to the LV, which was the main focus of the

present work. This is of main importance since although PAH selectively overloads the RV, who ultimately fails, LV function is also affected (5, 17, 27).

The improved LV hemodynamic response in EX+MCT group was accompanied by the prevention of LV mass and cardiomyocyte atrophy, fibrosis and ET-1 mRNA. LV mass and cardiomyocyte atrophy can be attributed to decreases in both diastolic and systolic loading of the LV through a mechanism of ventricular interdependence (10). Contrarily to SED+MCT, EX+MCT showed almost normal loading conditions (improved cardiac output and LV Pmax) that probably contributed to prevent LV atrophy. Although cardiomyocyte death may also be responsible for the loss of some myocardial mass in the unloaded LV (5), atrophy is thought to be the main responsible (17). Extracellular remodeling is another hallmark in cardiac remodeling and has also been described in this experimental model (5). Accumulation of fibrosis has a negative impact on cardiac function, affecting cardiac stiffness, promoting arrhythmias and impairing the diffusion of oxygen to cardiomyocytes (18). Exercise preconditioning was able to prevent its increase. This could be due to the anti-neurohumoral proprieties of exercise training. Indeed, we found lower levels of ET-1 mRNA in LV from exercised animals. ET-1 is known to favor the accumulation of fibrosis by activating myofibroblasts (14, 35, 39). Moreover, ET-1 was implicated in the modulation of LV function since its blockade with chronic administration of Bosentan prevented LV functional deterioration in PAH [22]. In this sense, exercise-induced inhibition of ET-1 would also explain the preserved LV function that was found in EX+MCT.

Patients with PAH show compromised exercise capacity which is associated with a poor quality of life (6). Therefore we tested our animals and

observed reduced exercise tolerance in SED+MCT and improvement in EX+MCT. Both human and animal studies show that exercise increases exercise capacity in stable PAH (16, 30). However, it should be noted that in our work, this was observed four weeks after the last training session. Exercise intolerance is classically attributed to the poor functional status due to persistent cardiac and respiratory impairment (7, 37). But compelling evidences suggests that skeletal muscle abnormalities may also limit physical capacity and exercise tolerance (8). Since EX+MCT did not show body weight loss, it seems reasonable to assume that their skeletal muscle mass was preserved.

Analyzing all this alteration on functional and structural parameters, it could be the explication for caridoprotective effects of moderate exercises training in LV dysfunction, secondary to PAH, being an important tool in PAH prevention. Together with these findings, we also found that moderate aerobic exercise training improves survival in rats, in an experimental model of HAP. This data is important information since the death rate in patients with pulmonary hypertension is extremely high (29). Our findings are agreement with other studies that showed the increase in survival on training animals (4, 31).

CONCLUSION

In conclusion, our findings suggest that exercise preconditioning can prevent against LVD secondary to PAH induced by MCT, improves exercise tolerance and survival. Our data also highlights that cardioprotection can be afforded for several weeks after the end of the last training session. Mechanisms underlying exercise-induced protection can be related to the prevention of LV atrophy, fibrosis and neurohumoral activation.

ACKNOWLEDGMENTS

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4. MAIN CONCLUSION

Considering the overall findings supported by our study, the main conclusions that must be highlighted are:

1. Exercise preconditioning improved exercise tolerance and prevented from cardiac dysfunction.

2. Systolic and diastolic parameters were ameliorated in response of exercise training.

3. This improve was associated with preventing LV atrophy, fibrosis and regularization in ET-1 mRNA levels.

4. Exercise training also improved survival.

5. The cardioprotective effects of exercise training seem to persist for several weeks after exercise cessation.