

UNIVERSIDADE FEDERAL DE VIÇOSA

**Effects of blueberry extract and resistance training on the heart and lung
structure and function in rats with experimental severe pulmonary arterial
hypertension**

Luciano Bernardes Leite
Doctor Scientiae

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Thesis submitted to the Physical Education Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Emily C. Carlo Reis
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"Great achievements are not made by impulse, but by a sum of small achievements."(Vincent van Gogh).

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"The greatest glories in life are not in never falling, but in rising every time we fall."
(Nelson Mandela)

ABSTRACT

LEITE, Luciano Bernardes, D.Sc., Universidade Federal de Viçosa, March, 2026.
Effects of blueberry extract and resistance training on the heart and lung structure and function in rats with experimental severe pulmonary arterial hypertension
. Adviser: Antonio Jose Natali. Co-advisers: Emily Correna Carlo Reis and Pedro Miguel Gomes Forte.

The overall objective of this study was to evaluate the effects of blueberry extract and moderate-intensity resistance training (RT), either alone or in combination, on the heart and lung structure and function in a model of severe pulmonary arterial hypertension (PAH) induced by monocrotaline (MCT) in rats. In study 1, we evaluated the effects of blueberry extract and resistance training on lung and right ventricle (RV) function and structure in severe experimental PAH. Male Wistar rats were induced PAH by a single intraperitoneal injection of MCT (60 mg/kg). One day later, the animals started receiving blueberry extract (100 mg/kg daily by gavage) and were submitted to a three-week low-intensity resistance exercise protocol (ladder climbing - 15 climbs, 60s interval, 60% load, one session/day, 5 days/wk). After these treatments and echocardiographic assessment animals were euthanized and lung and RV samples were collected for mechanical, morphological, biochemical, and molecular analyses. Both blueberry extract and RT attenuated RV dysfunction (i.e., TAPSE) and pulmonary artery resistance (i.e., AT/ET) observed over the development of severe PAH. In addition, single RV myocyte mechanical function (i.e., shortening and velocities of shortening and relaxation) was maintained in treated rats. Moreover, the applied treatments mitigated oxidative imbalance [i.e., lung (SOD, CAT, GST, NO); RV (SOD, CAT, GST, NO and PC)] and structural remodeling [i.e., lung (i.e., alveolar septum, pulmonary alveoli, arteriole's diameter, and total collagen); RV (total collagen and extracellular matrix)]. Furthermore, the interventions promoted partial or complete preservation of calcium regulatory proteins' expression (i.e., RyR2, p-PLBSer16 and SERCA2a). In conclusion, blueberry extract and RT, either alone or in combination, alleviate cardiopulmonary oxidative imbalance and adverse structural remodeling, and sustain RV myocyte contractility and expression of calcium regulatory proteins and thus conserve pulmonary and RV function in severe PAH induced by monocrotaline. In study 2 we tested whether regular administration of blueberry extract and low-intensity RT, either alone or in combination, during the development of MCT induced severe PAH in rats, protect the left ventricle (LV) from redox dysregulation and pathological remodeling.

Groups of seven male Wistar rats were formed for the experiment: sedentary control; sedentary hypertensive; sedentary hypertensive blueberry; exercise hypertensive; and exercise hypertensive blueberry. PAH was experimentally induced through a single intraperitoneal administration of MCT at a dose of 60 mg/kg. One day after injection, the blueberry groups started receiving a daily dose of blueberry extract (100 mg/kg) by gavage, while the exercise groups initiated a three-week program of RT (Ladder climbing; 15 climbs carrying 60% of maximum load; one session/day; 5 times/week). Echocardiographic evaluations were conducted 23 days after injection, and the rats were euthanized the next day to harvest LV tissue. Separately, blueberry extract and RT mitigated augments in pulmonary artery resistance, LV tissue's redox dysregulation (i.e., increased PC levels) and detrimental remodeling (i.e., reduced inflammation), and reductions in ejection fraction (EF) and fractional shortening (FS) caused by PAH. The combination of treatments prevented reductions in EF and FS, as well as the development of a D-shaped LV. In conclusion, blueberry extract and moderate-intensity resistance training administered during the development of MCT-induced severe PAH in rats prevented LV redox dysregulation and pathological remodeling, thereby preserving its function. Together, these findings reinforce the potential of blueberry extract and resistance training, especially when combined, as promising non-pharmacological strategies in the management of severe PAH.

Keywords: oxidative stress; cardiac remodeling; non-pharmacological strategies; pulmonary hypertension; heart failure.

RESUMO

LEITE, Luciano Bernardes, D.Sc., Universidade Federal de Viçosa, março de 2026. **Efeitos do extrato de mirtilo e do treinamento resistido sobre a estrutura e a função do coração e dos pulmões em ratos com hipertensão arterial pulmonar grave experimental.** Orientador: Antonio Jose Natali. Coorientadores: Emily Correna Carlo Reis e Pedro Miguel Gomes Forte.

O objetivo geral deste estudo foi avaliar os efeitos do extrato de mirtilo e do treinamento resistido (TR) de intensidade moderada, isoladamente ou em combinação, sobre a estrutura e função cardíaca e pulmonar em um modelo de hipertensão arterial pulmonar (HAP) grave induzida por monocrotalina (MCT) em ratos. No estudo 1 foram testados os efeitos do extrato de mirtilo e do treinamento de resistência sobre a função e a estrutura do pulmão e ventrículo direito (VD) em hipertensão arterial pulmonar (HAP) experimental grave. Neste estudo foram avaliados o estresse oxidativo, a expressão de proteínas regulatórias de cálcio, a função mecânica de miócitos e o remodelamento do VD, assim como o remodelamento pulmonar. No estudo 2, foi investigado se a administração regular de extrato de mirtilo e o TR de baixa intensidade, isoladamente ou em combinação, durante o desenvolvimento da HAP induzida MCT em ratos, protege o ventrículo esquerdo (VE) da desregulação redox e do remodelamento patológico. Neste estudo foram avaliados a função, o remodelamento estrutural e a desregulação redox do VE. Para tal, ratos machos Wistar tiveram a HAP induzida por uma única injeção intraperitoneal de MCT (60 mg/kg). Um dia depois, os animais começaram a receber extrato de mirtilo (100 mg/kg diariamente por gavagem) e foram submetidos a um protocolo de exercício de resistência de baixa intensidade de três semanas (escalada em escada - 15 subidas, intervalo 60s, 60% de carga, uma sessão/dia, 5 dias/semana). Após os tratamentos e a avaliação ecocardiográfica, os animais sofreram eutanásia por decapitação e amostras de pulmão, VD e VE foram coletadas para análises mecânicas, morfológicas, bioquímicas e moleculares. Os resultados mostram que tanto o extrato de mirtilo quanto o TR atenuaram a disfunção do VD (ex., TAPSE) e a resistência da artéria pulmonar (ex., TA/TE), observadas durante o desenvolvimento de HAP grave. Além disso, a função mecânica de miócitos do VD (ex., encurtamento e velocidades de encurtamento e relaxamento) foi mantida nos ratos tratados. Ademais, os tratamentos aplicados mitigaram o desequilíbrio oxidativo [i.e., pulmão (SOD, CAT, GST, ON); VD (SOD, CAT, GST, NO e PC)] e a remodelação estrutural [i.e., pulmão (i.e., septo alveolar, alvéolos pulmonares, diâmetro de arteríola e colágeno total); VD (colágeno total e matriz extracelular)].

As intervenções promoveram a preservação parcial ou completa da expressão de proteínas regulatórias do cálcio (i.e., RyR2, p-PLBSer16 e SERCA2a) no VD. No VE, o extrato de mirtilo e o TR mitigaram a desregulação redox, evidenciada pelo aumento dos níveis de proteína carbonilada (PC), bem como o remodelamento adverso, incluindo inflamação. Ademais, preveniram reduções na fração de ejeção (FE) e no encurtamento fracional (FS) causadas pela HAP. A combinação de tratamentos impediu reduções na EF e na FS, bem como o desenvolvimento da forma de D (ex. D-shaped) do VE. Em conclusão, o extrato de mirtilo e o TR, isoladamente ou em combinação, atenuam o desequilíbrio oxidativo e o remodelamento estrutural adverso do VD e pulmão, além de preservar a contratilidade dos miócitos do VD e a expressão de proteínas regulatórias de cálcio, contribuindo para a manutenção da função pulmonar e do VD na HAP grave induzida por MCT. Adicionalmente, essas intervenções previnem a desregulação redox e o remodelamento patológico do VE, preservando sua função. Em conjunto, esses achados reforçam o potencial do extrato de mirtilo e do TR, especialmente quando combinados, como estratégias não farmacológicas promissoras no manejo da HAP grave.

Palavras-chave: estresse oxidativo; remodelação cardíaca; estratégias não farmacológicas; hipertensão pulmonar; insuficiência cardíaca.

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LIST OF ABBREVIATIONS

ANOVA – Analysis of Variance

AT – Acceleration Time

AT/ET – Ratio between Acceleration Time and Ejection Time

BW - body weight

Ca²⁺ – Calcium Ion

CAT – Catalase

CDNB – 2,4-Dinitrochlorobenzene

CP – Carbonylated Protein

DNPH – 2,4-Dinitrophenylhydrazine

EF – Ejection Fraction

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

EH – Exercise Hypertensive

EHB – Exercise Hypertensive Blueberry

EHMS – Exercise Hypertensive Blueberry Survival

EHS – Exercise Hypertensive Survival

ELISA – Enzyme-Linked Immunosorbent Assay

eNOS – Endothelial Nitric Oxide Synthase

ET – Ejection Time

FS – Fractional Shortening

GAE – Gallic Acid Equivalents

GAPDH – Glyceraldehyde-3-phosphate Dehydrogenase

GST – Glutathione S-Transferase

H&E – Hematoxylin and Eosin

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC – High-Performance Liquid Chromatography

LV – Left Ventricle

MCT – Monocrotaline

MDA – Malondialdehyde

mPAP – Mean Pulmonary Arterial Pressure

NaCl – Sodium Chloride

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NCX – Sodium-Calcium Exchanger

ND – Not Detected

NIH – National Institutes of Health
NO – Nitric Oxide
O₂ – Oxygen
PA – Pulmonary Artery
PAGE – Polyacrylamide Gel Electrophoresis
PAH – Pulmonary Arterial Hypertension
CP – Protein Carbonyl
PH – Pulmonary Hypertension
PLB – Phospholamban
p-PLB – Phosphorylated Phospholamban
p-PLB(Ser16) – Phosphorylated Phospholamban at Serine 16
PTFE – Polytetrafluoroethylene
PVR – Pulmonary Vascular Resistance
RIPA – Radioimmunoprecipitation Assay
ROS – Reactive Oxygen Species
RT – Resistance Training
RV – Right Ventricle
RVF – Right Ventricular Failure
RyR2 – Ryanodine Receptor Type 2
SC – Sedentary Control
SDS – Sodium Dodecyl Sulfate
SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM – Standard Error of the Mean
SERCA2 – Sarcoplasmic Reticulum Ca²⁺-ATPase
SH – Sedentary Hypertensive
SHB – Sedentary Hypertensive Blueberry
SHMS – Sedentary Hypertensive Blueberry Survival
SHS – Sedentary Hypertensive Survival
SOD – Superoxide Dismutase
TAPSE – Tricuspid Annular Plane Systolic Excursion
TBS-T – Tris-buffered Saline with Tween 20
TP – Total Protein
Tris-HCl – Tris(hydroxymethyl)aminomethane Hydrochloride
USA – United States of America

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1. INTRODUCTION

Pulmonary hypertension (PH) comprises a heterogeneous and multifactorial group of cardiopulmonary disorders characterized by a sustained increase in mean pulmonary arterial pressure (mPAP) above 20 mmHg at rest, reflecting a pathological elevation in pulmonary vascular load and progressive impairment of cardiopulmonary hemodynamics [1-4]. Clinically, PH is classified into five major categories according to etiology and pathophysiological mechanisms: (1) pulmonary arterial hypertension (PAH); (2) PH associated with left heart disease; (3) PH secondary to lung diseases and/or chronic hypoxia; (4) PH related to chronic thromboembolic or other pulmonary artery obstructions; and (5) PH with unclear and/or multifactorial mechanisms [5]. Although these clinical groups differ in their underlying causes, all forms of pulmonary hypertension share a common pathophysiological hallmark characterized by increased pulmonary vascular resistance with consequent right ventricular (RV) pressure overload, ultimately leading to functional limitation, reduced exercise capacity, progression to right heart failure, and increased mortality [6-8].

Among the clinical subtypes of PH, PAH is a rare disease at the population level [1, 9, 10]. Based on the most recent Global Burden of Disease Study, the age-standardised prevalence of PAH is estimated at 2.28 cases per 100,000 inhabitants, corresponding to approximately 192,000 individuals living with the disease worldwide, with around 22,000 deaths attributed to PAH annually [11]. The disease disproportionately affects females and older individuals, and its prevalence increases with advancing age, reflecting important demographic patterns in its global distribution [11]. Although estimates suggest some improvement in disease burden over time, PAH remains a rare but severe condition associated with substantial morbidity and mortality [11, 12]. Contemporary registry data indicate that survival is still limited, with estimated mortality rates of approximately 8%, 16%, and 21% at 1, 2, and 3 years, respectively, even in the modern treatment [13]. In Brazil, epidemiological data remain limited and are largely based on estimates, suggesting that approximately 5,000 to 8,000 individuals may be living with PAH, although these figures are likely underestimated due to underdiagnosis and limited national registries [14].

PAH is characterized by various pathological changes, including pulmonary artery remodeling, inflammation, and fibrosis, which can lead to vascular lumen obstruction and the formation of complex lesions known as plexiform lesions [15, 16]. Concurrently, there is an increase in oxidative stress in the lung, evidenced by a

decrease in antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), as well as a reduction in nitric oxide (NO) [19, 21]. In addition, an increase in pro-inflammatory enzymes, such as malondialdehyde (MDA), is observed [19, 21]. The consequent lesions impair pulmonary circulation, resulting in increased pulmonary vascular resistance (PVR) [17]. The increase in PVR augments the pulmonary artery resistance, which in turn raises the afterload on the RV that over time develops hypertrophy and dilation. These changes in the RV reduce stroke volume and impair overall cardiopulmonary function [18] as it directly impacts pulmonary parenchyma.

Additionally, adverse remodeling of the RV affects the dynamics of the left ventricle (LV) due to ventricular interdependence. In patients with PAH, the LV often assumes a characteristic "D" shape, compromising diastolic filling and reducing end-diastolic volume [22-25]. These changes weaken LV function by influencing its segmental function and twist, resulting in a reduced stroke volume and limiting its ability to tolerate physical exertion, further aggravating the functional impairment in PAH patients [22, 26]. Such alterations due to PAH frequently lead to symptoms such as shortness of breath during physical activities, fatigue, lethargy, fluid retention, lower limb edema, and episodes of pre-syncope or syncope. Additionally, ventilatory inefficiency and muscle mass loss worsen exercise intolerance, impair functional capacity, and negatively impact the quality of life of patients [27].

Due to the complexity of PAH, experimental models have been widely used over the years to deepen the understanding of the disease. Animal models (chronic hypoxia, monocrotaline [MCT] injury, MCT associated with pneumonectomy, Sugen 5416 with hypoxia, and knockout mice) are employed to study the cellular and molecular mechanisms involved in PAH, as well as to aid in identifying potential therapeutic targets [28]. The most common model is the MCT-induced PAH. When metabolized by the liver, MCT produces dehydromonocrotaline, a toxic metabolite that causes progressive hypertension due to pulmonary mononuclear vasculitis due to endothelial dysfunction in response to disruptions in fundamental elements regulating the redox homeostasis in the lung as MCT increases oxygen reactive species (ROS) and elevate the production of oxidants [e.g., superoxide, hydrogen peroxide, and NO] and/or decrease the antioxidant defenses [e.g., CAT, SOD, and glutathione S-transferase (GST)] [7, 13, 21]. Such imbalance in association with other stimuli (e.g., increased transmural pressure, shear stress, hypoxia, mediators like angiotensin II, endothelin 1, growth factors, and inflammatory cytokines) leads to endothelial

dysfunction and hence vasoconstriction [5, 21]. This process results in pathological lung remodeling, including, inflammation, thickening of alveolar walls, increased connective tissue and collagen content, reduced alveolar number, and decreased blood vessel lumen [29, 30]. These pulmonary structural and metabolic (i.e., redox homeostasis disruption) changes affect the RV as the pulmonary artery resistance increases and overload the RV leading to hypertrophy and dilation, accompanied by cellular injury and apoptosis, inflammation, extracellular matrix remodeling (i.e., increased collagen deposition and ultimately fibrosis), contributing thus to the functional deterioration of the RV [15, 31]. In this model, the LV is also affected. The LV is often "D" shaped, compromising its ejection fraction and fractional shortening [28, 34] thus weakening LV function which may limit its ability to tolerate physical exertion.

At the cellular level, both RV and LV myocytes often exhibit abnormalities in excitation-contraction coupling, with lower amplitude and slower times in the intracellular Ca^{2+} transients, resulting in contractile dysfunction and reduced responsiveness to increases in contractile demand [19, 28, 32-34]. This dysfunction is associated with disturbances in the expression and activity of Ca^{2+} regulating proteins, such as phospholamban (PLB), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a), ryanodine receptor type 2 (RyR2), and sodium-calcium exchanger channel (NCX) [27, 29] in response to excessive ROS production (21-23). Given this scenario, the heart and pulmonary parenchyma are the most affected by PAH, undergoing significant structural and functional damage that compromises patients' quality of life.

Historically, pulmonary arterial hypertension has been associated with a poor prognosis [35, 36]. In recent decades, advances in the understanding of disease mechanisms, supported by evidence from both clinical studies and experimental models, have driven the development of targeted pharmacological therapies [19]. Current treatments primarily aim to modulate pulmonary vascular tone and remodeling and to reduce RV overload, thereby addressing key hemodynamic alterations characteristic of PAH [37]. These pharmacological strategies have led to meaningful improvements in clinical outcomes and survival; however, PAH remains a progressive and life-limiting condition [38]. However, in recent years, studies have shown that non-pharmacological approaches, such as physical exercise [21, 28, 31, 33, 34, 39, 40] and natural antioxidants [18, 41-44], can provide significant benefits for individuals with PAH.

In this context, clinical studies [45–47] and experimental investigations [19, 21, 34, 48] consistently demonstrate that physical exercise training induces favorable adaptations in individuals with PAH, particularly with respect to the morphology and function of right-sided cardiopulmonary structures, including the RV and pulmonary system, across organ, cellular, and molecular levels. In this context, previous studies conducted by our group have shown that aerobic [29, 48], resistance [19, 28], and combined exercise training [21, 34] attenuate LV and RV dysfunction and improve cardiopulmonary function in rats with MCT-induced PAH. Collectively, these findings support physical exercise as a relevant and effective adjuvant strategy to mitigate functional and structural impairments associated with PAH.

Regarding natural antioxidants, accumulating evidence indicates that these compounds can contribute to the prevention and management of cardiovascular diseases, thereby promoting improved life expectancy and quality of life [49-51]. Although relatively few clinical studies have directly evaluated the effects of antioxidant supplementation in patients with PAH, emerging data suggest potential therapeutic benefits. For instance, the administration of beetroot juice, a dietary source rich in inorganic nitrate, has been shown to increase pulmonary NO bioavailability and improve pulmonary vascular responses in patients with PAH [52]. In addition, supplementation with C vitamin and iron has been reported to improve clinical outcomes and functional status in a patient with PAH [53].

In experimental models of MCT-induced PAH, a growing body of evidence demonstrates that several antioxidant and bioactive compounds exert protective effects on cardiopulmonary structure and function. Interventions using genistein [54, 55], L-arginine [56], naringenin [57], ellagic acid [58], capsaicin [59], xanthohumol [60], blueberry [18, 43], quercetin [61], and beetroot juice [62] have been associated with improvements in hemodynamic, structural, and functional cardiopulmonary parameters, contributing to the attenuation of disease-related impairments.

Among these compounds, blueberry (*Vaccinium* L.), a native species of the American continent, has received particular attention due to its high content of anthocyanins and other polyphenolic constituents with potent antioxidant and anti-inflammatory properties [63]. Anthocyanins, which are abundantly present in blueberries, belong to the flavonoid class of polyphenols and are considered among the most effective antioxidant compounds found in fruits and vegetables [64]. In MCT-induced PAH model, blueberry supplementation has been shown to improve RV

systolic and diastolic function, reduce mPAP, and enhance redox balance in both heart and lung [18,43].

Despite these promising findings, the potential protective effects of blueberry supplementation combined with structured physical training remain largely unexplored. Therefore, given the limited number of studies addressing the combined effects of blueberry extract and resistance training in PAH, investigations focusing on their impact on cardiopulmonary morphology and function, as well as on the molecular and cellular mechanisms underlying these adaptations, may provide relevant clinical and scientific insights.

2. AIMS

2.1. General

To evaluate the effects of blueberry extract and low- to moderate-intensity resistance training on the heart and lung structure and function in a model of severe MCT-induced PAH.

2.1. Specifics

To investigate whether blueberry extract and resistance exercise training during the development of monocrotaline-induced pulmonary arterial hypertension (PAH) in rats affect:

- a) Pulmonary artery and ventricular hemodynamics;
- b) Morphometry of the lungs and ventricles;
- c) Oxidative stress biomarkers in cardiac and pulmonary tissues;
- d) Contraction amplitude, as well as contraction and relaxation velocities of isolated RV cardiomyocytes;
- e) Expression of calcium-regulating proteins in the RV.

To achieve these objectives, two experimental studies were conducted, as described below. In the first study, we evaluated the effects of blueberry extract and resistance exercise training on the RV and lungs. In the second study, we investigated the effects of these interventions on the LV.

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4. STUDY 1

Blueberry extract and resistance training in severe pulmonary arterial hypertension: Effects on lung and right-ventricle function and structure

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Abstract

This study evaluated the effects of blueberry extract and resistance training on lung and right ventricle (RV) function and structure in severe experimental pulmonary arterial hypertension (PAH). Male Wistar rats were induced PAH by a single intraperitoneal injection of monocrotaline (60 mg/kg). One day later, the animals started receiving blueberry extract (100 mg/kg daily by gavage) and were submitted to a three-week low-intensity resistance exercise protocol (ladder climbing - 15 climbs, 60s interval, 60% load, one session/day, 5 days/wk). After these treatments and echocardiographic assessment animals were euthanized and lung and RV samples were collected for mechanical, morphological, biochemical, and molecular analyses. Both blueberry extract and resistance training attenuated RV dysfunction (i.e., TAPSE) and pulmonary artery resistance (i.e., AT/ET) observed over the development of severe PAH. In addition, single RV myocyte mechanical function (i.e., shortening and velocities of shortening and relaxation) was maintained in treated rats. Moreover, the applied treatments mitigated oxidative imbalance [i.e., lung (SOD, CAT, GST, NO); RV (SOD, CAT, GST, NO and PC)] and structural remodeling [i.e., lung (i.e., alveolar septum, pulmonary alveoli, arteriole's diameter, and total collagen); RV (total collagen and extracellular matrix)]. Furthermore, the interventions promoted partial or complete preservation of calcium regulatory proteins' expression (i.e., RyR2, p-PLBSer16 and SERCA2a). In conclusion, blueberry extract and resistance training, either alone or in combination, alleviate cardiopulmonary oxidative imbalance and adverse structural remodeling, and sustain RV myocyte contractility and expression of calcium regulatory proteins and thus conserve pulmonary and RV function in severe PAH induced by monocrotaline.

Keywords: RV dysfunction; oxidative imbalance; adverse remodeling; myocyte contractility; calcium regulatory proteins

1. INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare and progressive disease that involves sustained augments of pulmonary vascular pressure and pulmonary artery resistance [1-3]. This chronic process results in right ventricle (RV) overload, causing hypertrophy, failure, and eventually death [1, 4, 5]. Over the disease's development, oxidative stress and inflammation are decisive, which makes its prediction difficult [6-9]. The imbalance between excessive generation of reactive oxygen species (ROS) and insufficient antioxidant defense causes cardiopulmonary cells and tissue deterioration and dysfunctionality [10-14]. The concomitant augment of inflammation aggravates such framework thus worsening the disease pathology and favoring pulmonary and RV dysfunctions [15, 16]. These complications actively contribute to the well-known chronic fatigue and impaired quality of life in PAH patients [17].

Despite advancements in conventional treatments for PAH, there are limitations in preventing disease progression and high mortality and morbidity persist [18, 19]. Therefore, non-pharmacological and combined strategies are required to lessen PAH adverse outcomes, like pulmonary artery pressure and RV overload. In this sense, physical exercise [20-23] and natural antioxidants [24-26] have potential as adjuvant components in PAH management. Recent evidence provided by our research group [27-32] and elsewhere [9, 33, 34] indicates that aerobic, resistance, and combined exercise training can preserve cardiac and pulmonary function and structure by counteracting oxidative stress and inflammation, resulting in improved exercise capacity and survival in rats with MCT-induced PAH. Likewise, blueberry extract, which is rich in polyphenols and anthocyanins, can protect cardiac and pulmonary tissues by preventing oxidative imbalance and attenuating functional and structural damage in this model [35-37].

Given the therapeutic potential of combining non-pharmacological strategies in the management of severe PAH and considering that approaches involving natural antioxidants and resistance exercise remain limited particularly regarding integrated cardiopulmonary responses this study aimed to advance current knowledge by investigating not only cardiac but also pulmonary alterations. Specifically, we evaluated the effects of blueberry extract and resistance exercise training on the structure and function of RV and lungs, as well as cardiomyocyte contractility, oxidative stress, and calcium-handling proteins in the model of severe PAH-induced MCT.

2. METHODS

2.1. Experimental design

Male Wistar rats (~200 g, 6 weeks of age) were randomly distributed into five experimental groups: sedentary control (SC, n=7), sedentary hypertensive (SH, n=7), sedentary hypertensive blueberry (SHB, n=7), exercise hypertensive (EH, n=7), and exercise hypertensive blueberry (EHB, n=7). Animals were housed in transparent polycarbonate cages (4 per cage) under controlled environmental conditions ($23 \pm 1^\circ\text{C}$, ~60% relative humidity, 12/12-hour light/dark cycle), with free access to water and standard rodent chow. All experimental procedures were conducted in accordance with the Ethical Principles in Animal Experimentation and were approved by the Ethics Committee on the Use of Animals of the Federal University of Viçosa (protocol no. 11/2022).

2.2. PAH induction

The animals in the hypertensive groups received a single dose of 60 mg/kg of monocrotaline (MCT, Sigma-Aldrich, St. Louis, MO, USA) via intraperitoneal injection, prepared in saline solution (140 mM NaCl, pH 7.4) [38]. Control group animals received the same volume of saline solution (140 mM NaCl, pH 7.4) without MCT.

2.3. Administration and phenolic profile of the blueberry extract

Throughout the experimental period, animals in the SHB and EHB groups received a daily oral dose of blueberry extract (100 mg/kg body weight) by gavage. This dosage was selected based on prior research conducted by our group, which demonstrated its therapeutic potential in experimental models of severe PAH [35, 36, 39, 40]. The extract was previously analyzed for its phenolic composition, revealing a total content of 38.89 mg gallic acid equivalents per gram. Among the identified phenolic compounds, the anthocyanidins cyanidin (219.21 $\mu\text{g/mL}$) and malvidin (128.68 $\mu\text{g/mL}$) were particularly prominent.

2.4. Resistance training protocol

All animals were adapted to a resistance training protocol (i.e. Ladder climb), as described by Hornberger and Farrar [41]. During a one-week familiarization period, the rats were exposed to the protocol, which consisted of climbing an 80° inclined grid with devices attached to their tails, but without any load. To encourage climbing, a gentle

tap was applied to the animals' tails, and after each climb, they rested for 60 seconds in a cage (20 × 20 × 20 cm). This procedure was conducted five times per week. After adaptation, the experimental groups performed a maximum load test before PAH induction and on days 14 and 21 post-induction. The test began with an initial load of 75% of body weight, and 30 grams were added in each further climb, interspersed with 120-second intervals, until the animal could no longer complete the climb.

Resistance training was initiated 24 hours after MCT administration, using a vertical ladder adapted for rats. Based on individual maximum load test, the animals underwent resistance training sessions, five times per week (Monday to Friday) for approximately 3 weeks. Each session consisted of 15 climbs, with 60s intervals between them [42]. The training intensity was adjusted to 60% of the maximum load obtained in the tests, which is classified as moderate and is proven beneficial for animals with severe PAH, according to studies conducted by our group [29, 31, 43].

2.5. Echocardiography

Echocardiographic assessments were conducted on day 23 following MCT administration. Animals were anesthetized with isoflurane (3% induction, 1.5% maintenance; oxygen flow: 1 L/min) and positioned in lateral decubitus. Images were acquired in M-mode (120 frames/s) using a MyLab™30 ultrasound system (Esaote, Genoa, Italy) equipped with 10 MHz transducers, with scan speed adjusted to 200 mm/s based on heart rate. Pulmonary artery flow was assessed by pulsed Doppler to calculate the RV acceleration time (AT) and ejection time (ET), with the AT/ET ratio serving as an indirect marker of pulmonary vascular resistance. RV systolic function was evaluated through tricuspid annular plane systolic excursion (TAPSE), obtained by placing the M-mode cursor at the lateral tricuspid annulus and measuring the systolic displacement from end-diastole to end-systole. All images were acquired in accordance with the American Society of Echocardiography guidelines and stored for subsequent analysis [44].

2.6. Sample collection

On the 24th day after MCT administration, all animals from the SC, SH, SHB, EH, and EHB groups were euthanized by decapitation. This time point was selected according to previous reports [27, 30, 32, 45], as it represents the average survival period of rats developing PAH after receiving a 60 mg/kg dose of MCT.

2.7. Lung and RV oxidative stress biomarkers

2.7.1. Tissue preparation

Frozen lung and right ventricle (RV) samples (100 mg each) were homogenized in 1 mL of phosphate buffer (pH 7.4) using a Tissue Master 125 homogenizer (OMNI) and centrifuged at 10,000 ×g (12,000 rpm) for 10 minutes at 4°C. Supernatants were collected to assess the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), as well as levels of malondialdehyde (MDA), nitric oxide (NO), and total proteins (TP). The pellets were reserved for carbonyl protein (CP) quantification. Measurements were performed using either an ELISA microplate reader (Multiskan GO, Thermo Scientific) or a UV-visible spectrophotometer (UV-Mini 1240, Shimadzu).

2.7.2. Superoxide dismutase activity

The SOD activity was determined according to the method described by Dieterich et al., [46]. In this procedure, 99 µL of potassium phosphate buffer (5 mmol/L, pH 8.0) were pipetted along with 30 µL of the sample and 15 µL of pyrogallol (100 µmol/L). The reaction mixture was then measured by absorbance at 570 nm. SOD activity was calculated in units per milligram of protein, with one unit of SOD defined as the amount required to inhibit the autoxidation rate of pyrogallol by 50%. Duplicates of blank and standard samples for SOD activity were prepared without and with pyrogallol, respectively.

2.7.3. Catalase activity

The CAT activity was measured based on an adaptation of the Hadwan method [47], using hydrogen peroxide (H₂O₂) as a substrate. For this, 100 µL of H₂O₂ (20 mmol/L) were pipetted into 5 µL of the sample. After 3 minutes, 150 µL of ammonium molybdate (32.4 mmol/L) were added to stop the reaction. Blank samples were prepared by replacing H₂O₂ with sodium and potassium phosphate buffer (50 mmol/L, pH 7.0). The reading was taken at 374 nm using a spectrophotometer. To calculate the CAT value, a standard curve was prepared with serial dilutions of H₂O₂. Test values were corrected by subtracting control values to eliminate interference from other serum compounds that could react with ammonium molybdate. CAT activity was expressed as CAT KU/mg of protein.

2.7.4. Glutathione S-Transferase Activity

The GST activity was measured based on the method of Habig et al., [48]. For this analysis, 10 μL of 2,4-dinitrochlorobenzene (CDNB) conjugated with glutathione at a concentration of 1 mmol/L was added to 970 μL of 50 mmol/L buffer, pH 7.0, along with 10 μL of reduced glutathione at 1 mmol/L and 10 μL of the test sample. After CDNB addition, the absorbance change was monitored at 340 nm over 60 seconds. The molar extinction coefficient used for CDNB was $\epsilon_{340} = 9.6 \text{ mmol/L/cm}$. One unit of GST activity was defined as the amount of enzyme that catalyzed the formation of one micromole of product per minute per milliliter. GST activity was expressed in micromol/min/g.

2.7.5. Nitric oxide production

NO production was quantified using the standard Griess reaction, as described by Tsikas [49]. Briefly, 50 μL of samples were incubated with an equal volume of Griess reagent, composed of 1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine, and 2.5% phosphoric acid, at room temperature for 10 minutes. Absorbance was measured at 570 nm using a microplate reader. NO concentrations ($\mu\text{mol/L}$) were determined from a sodium nitrite standard curve in the concentration range of 0 to 100 $\mu\text{mol/L}$.

2.7.6. Malondialdehyde determination

Lipid peroxidation was measured according to the method of Buege and Aust [50], by quantifying MDA, a product of lipid peroxidation. For this, 0.2 mL of tissue supernatant was homogenized in 0.4 mL of a solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.6% hydrochloric acid. Reactions were maintained in a water bath at 90°C for 40 minutes. After cooling on ice, 0.6 mL of butanol was added. The solution was vortexed for 2 minutes and centrifuged for 10 minutes at 9,000 g. The supernatant was used to measure MDA by absorbance at 540 nm using a microplate spectrophotometer (Multiskan GO). A standard curve with known concentrations of 1,1,3,3-tetramethoxypropane was used to determine the MDA concentration. Results were expressed in micromol/L per mg of protein.

2.7.7. Carbonylated protein determination

The determination of CP content was performed using 2,4-dinitrophenylhydrazine (DNPH) [51]. Briefly, pellets obtained from the extraction homogenates were mixed with 0.5 mL of 10 mmol/L DNPH solution diluted in 7% hydrochloric acid. The solution was stirred and incubated at room temperature in the dark, with periodic shaking, for 30 minutes. Then, 0.5 mL of chilled 10% trichloroacetic acid was added to each tube, followed by centrifugation at 5000 g for 10 minutes at 4 °C. After centrifugation, the supernatant was discarded, and the precipitate was washed three times with 1 mL of an ethyl acetate and ethanol mixture (1:1 v/v). Finally, 1 mL of 6% sodium dodecyl sulfate was added, and the tubes were vortexed to dissolve the sediment. The absorbance of the supernatant was measured at 370 nm. Results were expressed in nanomol/mg of protein, based on the molar extinction coefficient of $\epsilon_{370} = 22 \text{ mmol/L/cm}$.

2.7.8. Total protein

Total protein determination was performed according to the method described by Lowry et al., [52], using bovine serum albumin as a standard. The obtained total protein concentrations were used to standardize the results of CAT, SOD, MDA, and PC.

2.8. Isolation of right ventricular myocytes

RV myocytes were isolated using a protocol previously described [38]. After euthanasia, the heart was quickly dissected, dried, and weighed. The heart was then connected to a Langendorff retrograde perfusion system via the aorta, using Tyrode solution with the following components in millimoles (mM): 130 NaCl, 1.43 MgCl_2 , 5.4 KCl, 0.75 CaCl_2 , 5.0 HEPES, 10.0 glucose, 20.0 taurine, and 10.0 creatine, adjusted to pH 7.4, for about 5 minutes. Following this step, the solution was replaced with Tyrode containing EGTA (0.1 mM) for another 5 minutes. The next step involved perfusion with Tyrode containing 1 mg/ml of type II collagenase (Worthington, USA) and 0.1 mg/ml of protease (Sigma-Aldrich, USA) for approximately 12 minutes. The RV was separated and cut into small fragments, which were placed in a conical flask containing the enzymatic solution (collagenase and protease). To facilitate cell separation, the flask was shaken for 5 minutes, and the dispersed cells were isolated from non-dispersed tissue by filtration. After centrifugation, the resulting cells were

suspended in Tyrode solution. The remaining tissue was subjected to the mechanical dispersion process again. Throughout the procedure, the solutions used were oxygenated (100% O₂ - White Martins, Brazil) and maintained at 37°C. The isolated cells were stored at 5°C and used within 2 to 3 hours after isolation.

2.9. Measurement of cellular contractility

Cardiomyocyte contractions were assessed using the length variation technique, with an edge-detection system (Ionwizard, Ionoptix, USA) coupled to an inverted microscope (Nikon Eclipse – TS100, Japan) with an oil immersion objective lens (S Fluor, 40x, Nikon, USA), as previously described [53]. In summary, isolated myocytes were placed in an experimental chamber mounted on the inverted microscope and immersed in a perfusion buffer solution (HEPES-buffered solution; see Item 4.11.2) maintained at 37°C. The myocytes were externally stimulated at a frequency of 5 Hz (10 Volts, 5 ms duration) using an electrical stimulator (Myopacer, Field Stimulator, Ionoptix, USA). Myocyte images were captured by a camera (Myocam, Ionoptix, USA) attached to the inverted microscope, with an image detection software (Ionwizard, Ionoptix, USA - frequency of 240 Hz). Contraction and relaxation movements of the myocytes were recorded by the edge-detection system (Ionwizard, Ionoptix, USA) and stored for later analysis of mechanical properties. Only myocytes in good condition, with well-defined edges (left and right) and sarcomeric striations, relaxed at rest, and without involuntary contractions, were used. The analyzed variables included contraction amplitude, contraction velocity, and relaxation velocity.

2.10. Protein expression

The expression of type 2 ryanodine receptors (RyR2), Phospholamban (PLB), Phospho-Phospholamban, sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA2) and sodium/calcium exchanger (NCX1) proteins was determined by Western blotting, as previously described [54]. Frozen RV fragments (100 mg) were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 1% NP-40) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich; PhosSTOP, Roche). After centrifugation (3,000 g, 4°C, 10 min), supernatants were subjected to SDS-PAGE (8–10% gels), and proteins were transferred to nitrocellulose membranes. Equal protein loading (40 µg) was confirmed with 0.5% Ponceau S staining. Membranes were blocked (5% milk in TBS-T) for 2 h at room temperature,

then incubated overnight at 4°C with primary antibodies. Primary antibodies included α -Tubulin (#2144) and GAPDH (#97166, Cell Signaling), RyR2 (#TA5-87416), PLB (#PA5-85268), p-PLB (Ser16, Thr17) (#711401), SERCA2 (#MA3-919, Invitrogen), and NCX1 (#151608, Abcam), all at 1:1000. Secondary antibodies (IRDye® 680RD or 800CW, LI-COR) were used at 1:15,000. Bands were visualized using the Odyssey® Fc Imaging System and quantified with ImageJ (NIH).

2.11. Lung and RV histological analysis

Histological analyses of lung and right ventricle (RV) followed previously established procedures [55-57]. Briefly, tissue fragments were fixed in Karnovsky solution (4% paraformaldehyde and 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 24 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections (5 μ m) were obtained and stained with hematoxylin and eosin for assessment of cellular and extracellular components, and with Sirius Red for collagen fiber quantification. To avoid duplicate analysis, sections were evaluated in semi-series, with one section analyzed every 10 sections.

Images were acquired using a digital camera (Olympus Q Color-3, Tokyo, Japan) coupled to a light or polarized microscope (Olympus AX-70, Tokyo, Japan) according to the staining method. Quantitative analyses were performed using ImageJ® software (National Institutes of Health, USA). In the RV, the relative proportions of cardiomyocytes (cytoplasm and nucleus), extracellular matrix, and total collagen were determined using a point-counting approach (i.e., superimposed grid with 266 intersection points). In the lung, morphometric parameters, including alveolar septa and pulmonary alveoli, were quantified using the same approach and expressed as percentage area.

Pulmonary arteriole remodeling was additionally assessed in hematoxylin and eosin-stained sections. For each lung, nine representative fields (200 $\times$ magnification) were analyzed in a blinded manner. Arteriolar wall thickness and lumen diameter were measured, and the lumen-to-wall thickness ratio was calculated as an index of vascular remodeling [36].

2.12. Statistical analysis

Data were initially subjected to the Shapiro-Wilk test to assess normality. For variables with parametric distribution, one-way analysis of variance (ANOVA) was

used for group comparisons, followed by Tukey's post hoc test when applicable. For nonparametric data, the Kruskal-Wallis test was used, followed by Dunn's post hoc test as needed. Quantitative data are presented as mean $\pm$ standard deviation (SD), while qualitative data are expressed as percentages. A significant level of 5% ($p < 0.05$) was adopted for all analyses. Detailed information on the statistical tests applied, as well as sample sizes (i.e., n for animals and cells), are provided in the respective tables and figures. All statistical analyses were performed using GraphPad Prism® software, version 10.4.0.

3. RESULTS

3.1. PAH establishment indicators and effects of treatments

By the end of the experimental period, rats in the SH group had lower AT/ET compared with those in the SC group (Table 1). However, the treated groups (SHB, EH, and EHB) exhibited higher values than the SH group. Likewise, TAPSE was lower in the SH, SHB, and EH groups compared with the SC group. Even so, exercised rats (EH and EHB) showed higher values than those in the SH group, and the value of the EHB group was higher than that of the SHB group. In addition, exercised animals presented higher maximum carrying load than the non-exercised ones.

Regarding weights, rats with PAH had lower body weight compared with the control group. The heart weight of SH animals was greater compared with those of SC, SHB, EH, and EHB rats. The RV weight was superior in the hypertensive groups compared with that of the SC group. Nonetheless, treated rats (SHB, EH and EHB) displayed lower values than SH animals. The Fulton's index was higher in the SH group than in the SC group, however treated groups showed values compared with the SH group. The lung weight was bigger in the SH and SHB groups than in the SC group, whereas the EH and EHB groups' values were not different from that of the SC group. Similar results were observed for the lung weight to tibial length ratio. The tibial length was not different between groups. The heart weight to tibial length ratio was greater in the SH group than in the SC group, while treated groups presented lower values compared with the SH group. Similar results were observed for the RV weight to tibial length.

Table 1. Animal physical and functional parameters.

	SC	SH	SHB	EH	EHB
Initial body weight, g	201.30 ± 5.37	200.90 ± 6.64	201.10 ± 5.03	201.90 ± 3.56	200.40 ± 3.38
Final body weight, g	294.30 ± 19.92	258.50 ± 17.55*	263.50 ± 17.14*	259.60 ± 13.86*	289.00 ± 15.03*
Heart weight, g	0.97 ± 0.07	1.40 ± 0.09*	1.04 ± 0.12 [#]	1.01 ± 0.08 [#]	0.99 ± 0.15 [#]
RV weight, g	0.16 ± 0.02	0.37 ± 0.03*	0.27 ± 0.06 [#]	0.23 ± 0.05 [#]	0.22 ± 0.04 [#]
Fulton's index, g/g	0.97 ± 0.07	1.40 ± 0.09*	1.04 ± 0.12 [#]	1.00 ± 0.08 [#]	0.99 ± 0.15 [#]
Lung weight, g	1.84 ± 0.30	3.10 ± 0.73*	3.09 ± 0.56*	2.68 ± 0.61	2.62 ± 0.83
Tibial length, mm	37.77 ± 0.68	36.03 ± 1.10	36.06 ± 0.87	35.77 ± 1.22	36.23 ± 1.12
Heart weight/tibial length, mg/mm	0.029 ± 0.00	0.038 ± 0.00*	0.029 ± 0.00 [#]	0.026 ± 0.00 [#]	0.025 ± 0.00 [#]
RV weight/tibial length, mg/mm	0.004 ± 0.00	0.010 ± 0.00*	0.008 ± 0.00 [#]	0.007 ± 0.00 [#]	0.006 ± 0.00 [#]
Lung weight/tibial length, mg/mm	0.05 ± 0.00	0.09 ± 0.02*	0.09 ± 0.02*	0.07 ± 0.01	0.07 ± 0.02
AT/ET, ms	0.60 ± 0.02	0.22 ± 0.01*	0.39 ± 0.03 [#]	0.45 ± 0.02 [#]	0.44 ± 0.03 [#]
TAPSE, mm	2.57 ± 0.12	1.55 ± 0.07*	1.89 ± 0.08*	2.02 ± 0.01 [#]	2.26 ± 0.11 ^{#†}
Initial maximum carrying load, g/g	1.11 ± 0.11	1.05 ± 0.08	1.01 ± 0.13	0.99 ± 0.08	0.98 ± 0.08
Final maximum carrying load, g/g	1.26 ± 0.13	1.00 ± 0.10	1.30 ± 0.19	1.74 ± 0.12 [#]	1.74 ± 0.08 [#]

Data are means ± SD of seven rats in each group. SC, sedentary control. SH, sedentary hypertensive. SHB, sedentary hypertensive blueberry. EH, exercise hypertensive. EHB, exercise hypertensive blueberry. AT/ET, pulmonary artery flow acceleration time (AT) to ejection time (ET) ratio. BW, body weight. Fulton's Index, right ventricle weight to interventricular septum + left ventricle weight ratio. RV, right ventricle. TAPSE, tricuspid annular plane systolic excursion. One-Way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. SC; [#]P<0.05 vs. SH; [†]P<0.05 vs. SH.

3.2. Lung oxidative stress

The activity of SOD was lower in the SH group than in the other groups (Fig. 1A). In addition, CAT activity and NO levels were reduced in the SH group compared with the SC group (Fig. 1 B and D). The isolated treatments (SHB and EH) did not prevent these reductions, whereas the combined treatment avoided them (SH < EHB). Regarding GST activity, lower values were found in the SH, SHB, and EH groups compared with the SC group (Fig. 1C), whereas the combined treatment avoided such reduction (SH < EHB). Concerning the PC levels (Fig. 1F), rats in the SH group exhibited greater values than those in the SC group, however, no effects of treatments were observed. No differences between groups were found for the MDA levels (Fig. 1E).

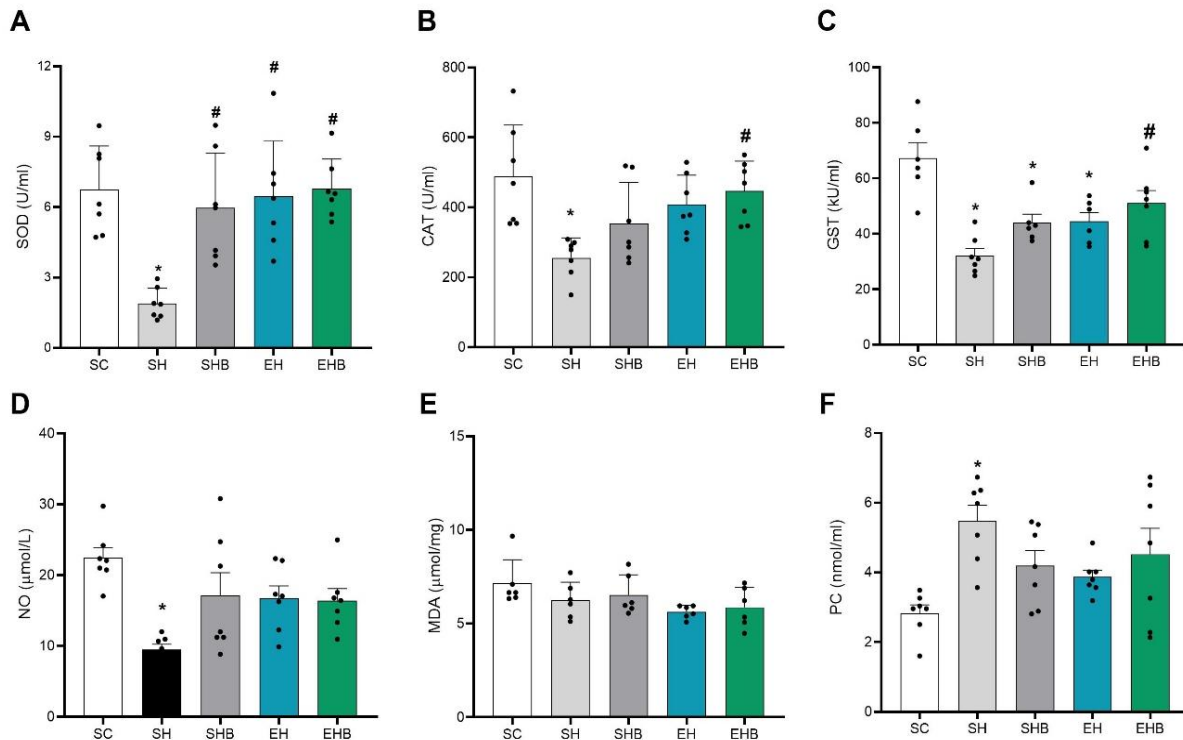


Figure 1. Effects of blueberry extract and resistance training on lung oxidative stress biomarkers. (A) SOD (superoxide dismutase), (B) CAT (catalase), (C) GST (glutathione S-transferase), (D) NO (nitric oxide), (E) MDA (malondialdehyde), (F) PC (protein carbonyl). SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary

hypertensive blueberry; EH, exercise hypertensive; EHB, exercise hypertensive blueberry. The data are expressed as means $\pm$ SD of five to seven rats per group. One-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$ vs. SC; # $P < 0.05$ vs. SH.

3.3. RV oxidative stress

The rats in the SH group showed decreased SOD (Fig. 2A), CAT (Fig. 2B), and GST (Fig. 2C) activities compared with those in the SC group. However, the treatments, either isolated (SHB and EH groups) or in combination (EHB group), prevented such reduction to a similar extent. The levels of NO were also lower in the SH group than in the SC and EHB groups (Fig. 2D). Concerning MDA levels (Fig. 2E), it were higher in the SH group than in the SC group. As for the CP levels (Fig. 2F), rats in the SH group showed increased values compared with those in the SC group, while this elevation was similarly attenuated by both interventions, either alone (SHB and EH groups) or combined (EHB group).

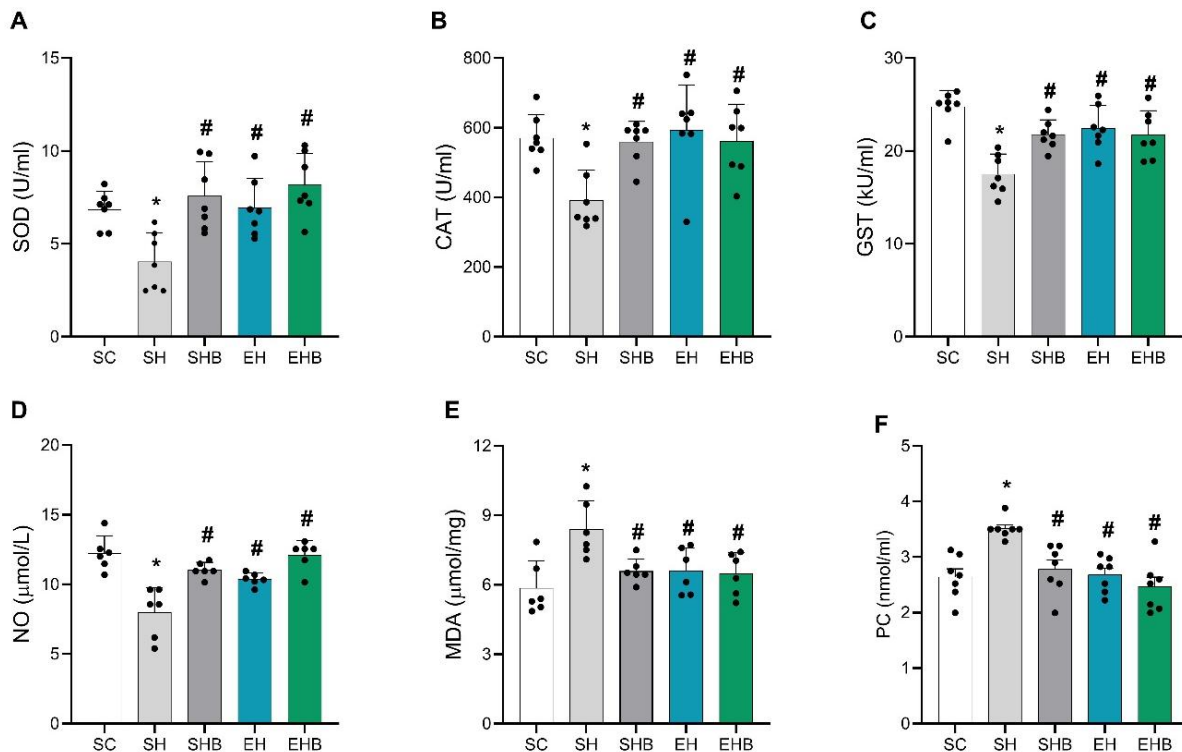


Figure 2. Effects of blueberry extract and resistance training on right ventricular oxidative stress biomarkers. (A) SOD (superoxide dismutase), (B) CAT (catalase), (C)

GST (glutathione S-transferase), (D) MDA (malondialdehyde), (E) NO (nitric oxide), (F) PC (protein carbonyl). SC, sedentary control. SH, sedentary hypertensive. SHB, sedentary hypertensive blueberry. EH, exercise hypertensive. EHB, exercise hypertensive blueberry. Data are means $\pm$ SD of five to seven rats per group. One-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$ vs. SC; # $P < 0.05$ vs. SH.

3.4. Single RV myocyte contractile function

Myocytes from rats in the SH group showed reduced contraction amplitude compared with those from the SC group (Fig. 3A). However, both treatments, either alone (SHB and EH groups) or combined (EHB group), prevented such reduction to a similar extent. The departure (Fig. 3B) and return (Fig. 3C) velocities of shortening were lower in myocytes from hypertensive rats (SH, SHB, EH, and EHB groups) than in those from the SC group. Nevertheless, the employed treatments either isolated (SHB and EH groups) or combined (EHB groups) resulted in higher velocities compared with those observed in the SH group.

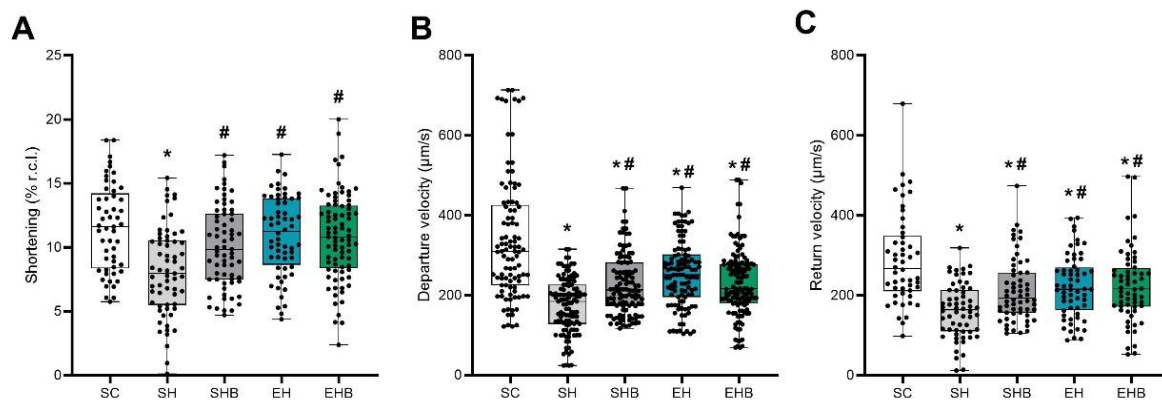


Figure 3. Effects of blueberry extract and resistance training on contractility in single right-ventricular myocytes stimulated at 5Hz. (A) Amplitude of shortening. (B) Departure velocity of shortening. (C) Return velocity of shortening. SC, sedentary control. SH, sedentary hypertensive. SHB, sedentary hypertensive blueberry. EH, exercise hypertensive. EHB, exercise hypertensive blueberry. Data are shown as box-and-whisker plots (median, interquartile range; whiskers = minimum–maximum) overlaid with individual cell values (10 cells per animal; 5–6 animals per group). Between-group

differences were assessed with the Kruskal–Wallis test followed by Dunn’s multiple comparisons. * $P < 0.05$ vs. SC; # $P < 0.05$ vs. SH.

3.7. Expression of Ca^{2+} handling proteins

Concerning the expression of proteins involved in Ca^{2+} handling, the levels of RyR2 (Fig. 4A) and p-PLB^{ser16} (Fig. 4C) were lower in the SH group compared with the SC, EH, and EHB groups. No differences between groups were observed in the expression of PLBt (Fig. 4B) or NCX (Fig. 4E). Regarding SERCA2a expression, the SH group showed reduced levels compared with that of the SC group (Fig. 6D).

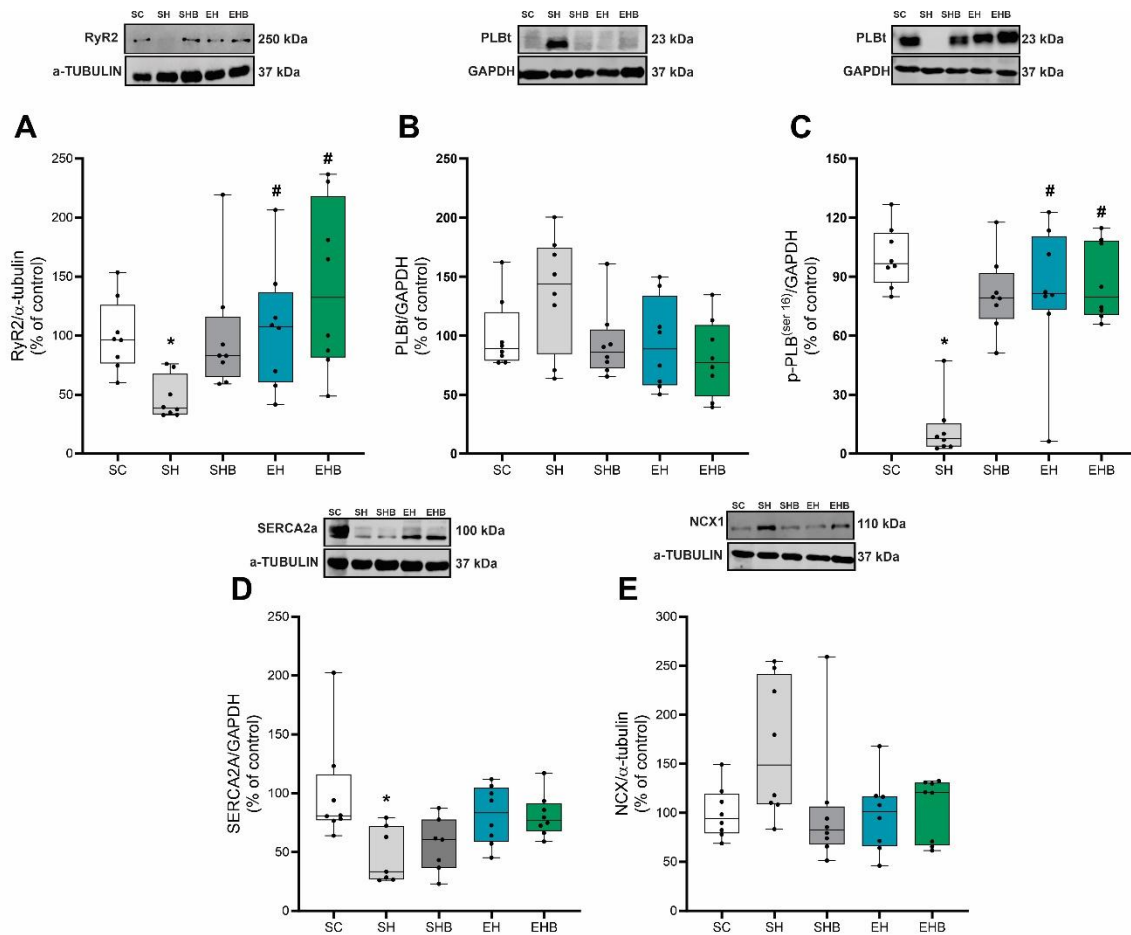


Figure 4. Effects of blueberry extract and resistance training on the expression of Ca^{2+} -handling proteins in the right ventricle. (A) Ryanodine receptor type 2 (RyR2). (B) Total phospholamban (PLB_t). (C) Phospholamban phosphorylated at Ser¹⁶ (p-PLB^{Ser16}). (D) Sarcoplasmic reticulum calcium ATPase type 2a (SERCA2a). (E) $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX1). SC, sedentary control. SH, sedentary hypertensive. SHB,

sedentary hypertensive blueberry. EH, exercise hypertensive. EHB, exercise hypertensive blueberry. Data are shown as box-and-whisker plots (median, interquartile range; whiskers = minimum–maximum) with individual animal values (n = 7–8 per group), expressed as % of SC (control). Group differences were tested with the Kruskal–Wallis test followed by Dunn's post hoc comparisons. *P<0.05 vs. SC; #P<0.05 vs. SH.

3.5. Lung remodeling

The lung representative photomicrographs (Fig. 5A) reveal maintained pulmonary architecture in the SC group, characterized by thin septa, open alveoli, and absence of inflammatory signs. In contrast, the SH group displayed disrupted microarchitecture, with thickened alveolar septa and inflammatory infiltrates. However, these structural alterations were attenuated in all treated groups (SHB, EH, and EHB) that showed better alveolar preservation, reduced septal thickening, and less inflammation. In addition, in the lung sections in figure 5B it can be observed that the SH group displays thickened arteriole wall compared with the SC group, while such change was lessened in the treated groups. Moreover, lung sections in figure 5C show augmented collagen fibers in the SH group than in the control group, and that this increase was attenuated in the treated groups. The quantitative analysis indicated that hypertensive rats (SH, SHB, EH, and EHB groups) had higher percentage of alveolar septum (Fig. 5D) and lower percentage of pulmonary alveoli (Fig. 5E) than the control ones (SC group). The diameter-to-wall thickness ratio of pulmonary arterioles was lower in rats from the SH group than in those from the SC group (Fig. 5F). Nevertheless, all treatments attenuated this reduction, partially restoring the diameter-to-wall thickness ratio, although values remained lower than those observed in the SC group. Finally, total collagen was higher in rats from the SH group compared with the control ones (SC group) (Fig. 5G). However, all treatments prevented such increase.

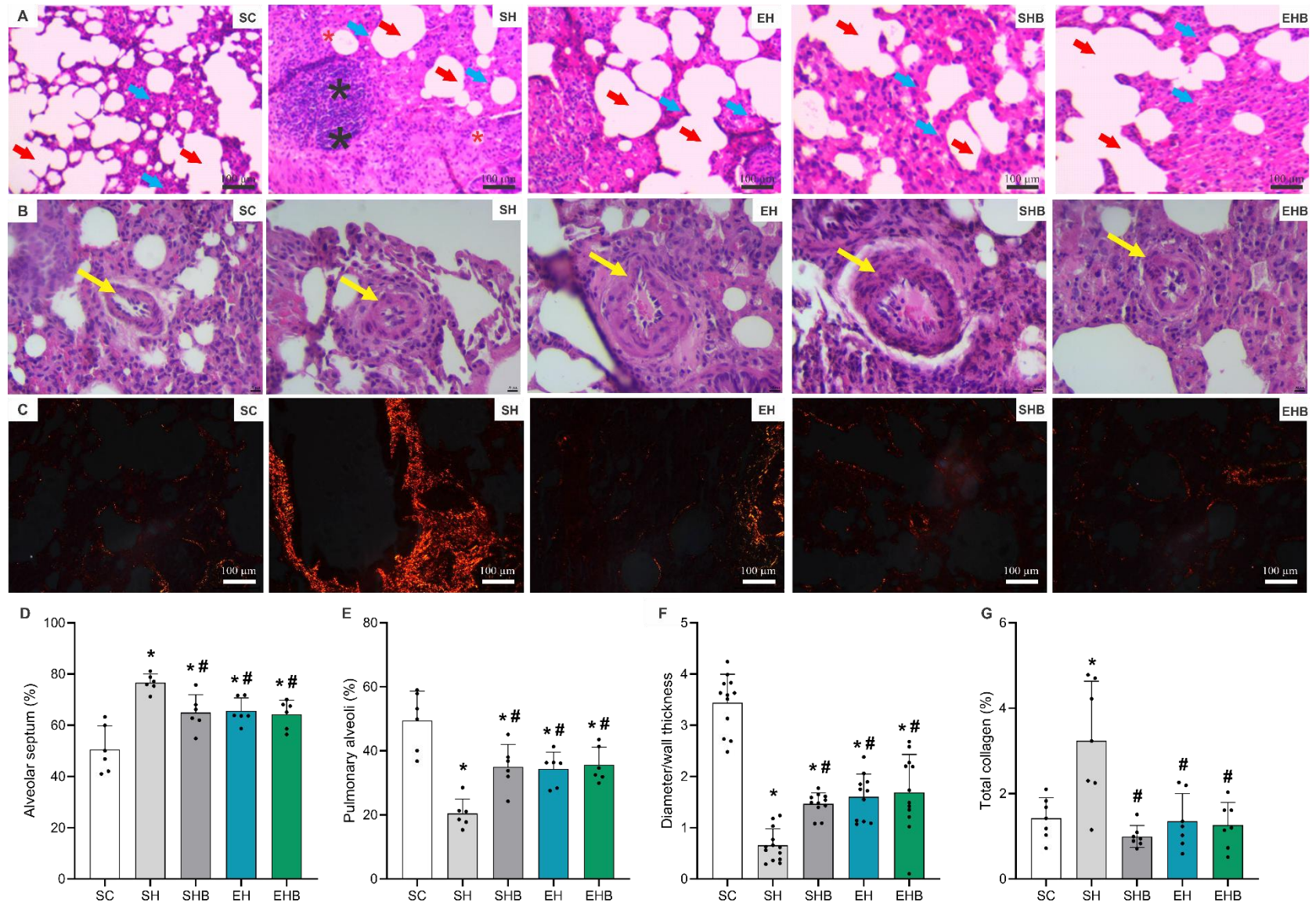


Figure 5. Effects of blueberry extract and resistance training on lung histomorphometry. (A) Representative photomicrograph of lung tissue stained with hematoxylin and eosin (scale bar: 100 μ m). (B) Representative photomicrographs of pulmonary arterioles stained with hematoxylin and eosin (scale bar: 100 μ m). (C) Representative photomicrographs of lung tissue stained with picrosirius red (scale bar: 100 μ m). Blue arrows: alveolar septum; red arrow: alveolus; red asterisk: hemorrhagic alveolus black asterisk: inflammatory foci; Yellow arrow: pulmonary arteriole. SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary hypertensive blueberry; EH, exercise hypertensive; EHB, exercise hypertensive blueberry. Data are expressed as mean $\pm$ SD of 10 images per animal in each group (7 animals per group). One-way ANOVA followed by Tukey's post hoc test. *P < 0.05 vs. SC; #P < 0.05 vs. SH.

3.6. RV remodeling

Cardiac sections from the SC group (Fig. 6 A and B) shows preserved RV morphology, with organized fibers, central nuclei, and homogeneous cytoplasm, without inflammatory infiltration or extracellular matrix accumulation. In contrast, SH animals present disorganized fibers, reduced cardiomyocyte density, inflammatory foci, and marked extracellular matrix and collagen deposition. These changes were attenuated in the treated groups (SHB, EH, and EHB), that display better conservation of tissue architecture and less fibrosis compared with that of the SH group, with no prominent inflammatory infiltration observed. The quantitative analysis shows that rats in the SH group had a lower percentage of cardiomyocytes (Fig. 6C), and higher percentages of extracellular matrix (Fig. 6D) and total collagen (Fig. 6E) than those in the SC group. However, all treated groups showed increased proportions of cardiomyocytes and reduced extracellular matrix and total collagen compared with the SH group.

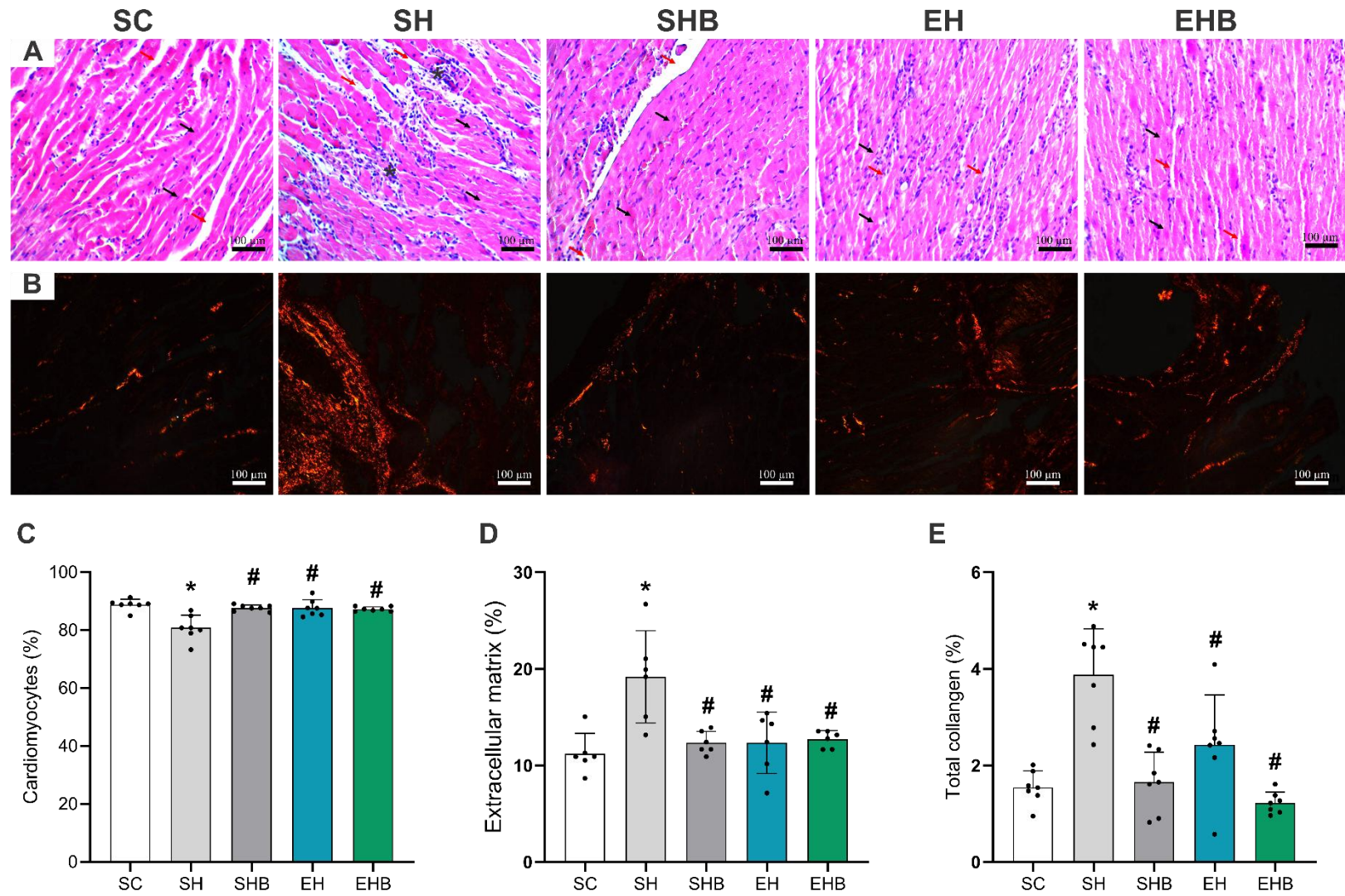


Figure 6. Effects of blueberry extract and resistance training on right ventricular remodeling. (A) Representative photomicrographs of tissue stained with hematoxylin and eosin (scale bar: 100 μ m). (B) Representative photomicrographs of tissue stained with picrosirius red (scale bar = 100 μ m). (C) Percentage of cardiomyocytes. (D) Percentage of extracellular matrix. (E) Percentage of total collagen. Black arrows: cardiomyocytes; red arrow: extracellular matrix; asterisks: inflammatory foci. SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary hypertensive blueberry; EH, hypertensive exercise; EHB, exercise hypertensive blueberry. Data are means $\pm$ SD of seven rats in each group. One-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$ vs. SC; # $P < 0.05$ vs. SH.

4. DISCUSSION

We used a model of severe PAH in rats to evaluate the effects of blueberry extract and resistance exercise training on pulmonary and RV function and structure. Both treatments applied during the development of PAH, either alone or in combination, mitigated cardiopulmonary oxidative imbalance and adverse structural remodeling. Moreover, it sustained right ventricular myocyte contractility and expression of calcium cycling regulatory proteins. These benefits therefore alleviated pulmonary and right ventricle dysfunction.

The echocardiographic analyses confirmed that the injected dose of monocrotaline markedly increased the pulmonary artery resistance (i.e., reduced AT/ET ratio) and reduced RV systolic function (i.e., TAPSE) in hypertensive rats which are typically observed in the disease establishment [27, 30, 32, 58]. Such RV dysfunction was followed by impaired RV single cardiomyocyte contractility, characterized by reduced amplitude of shortening and slower velocities of shortening and relaxation.

In line with these functional disturbances, we observed that the induced PAH caused a pronounced oxidative imbalance (i.e., decreased SOD, CAT, GST and NO) and elevated oxidative damage (i.e., increased MDA and PC) in both lung and RV. In addition, the lung of these rats exhibited inflammatory infiltrate and hemorrhagic alveoli, followed by alveolar septal thickening and increased collagen deposition, along with reduced alveolar density and arteriole diameter. Likewise, the RV of hypertensive rats showed structural changes like reduced proportion of myocytes, expansion of extracellular matrix, and collagen accumulation. Moreover, the RV presented downregulation of Ca^{2+} regulatory proteins (i.e., RyR2 and SERCA2a). These changes are strongly associated with PAH progression [10-14, 30, 35]. More important, our results indicate that both interventions attenuated these impairments, and the observed functional benefits are believed to be associated with metabolic, mechanical and structural adaptations to the applied treatments. Initially, it is noteworthy that the resistance training protocol used here improved the rats' exercise tolerance (i.e., maximum carrying load), although blueberry extract either alone or combined with resistance training did not affect exercise tolerance. This improvement may be

attributed not only to the preservation of central parameters (i.e., lung and heart) but also to peripheral adaptations to resistance training, including maintenance of skeletal muscle integrity and enhanced respiratory efficiency [29, 59].

Regarding the cardiopulmonary functional benefits of the used treatments (i.e., AT/ET and TAPSE), it is conceivable that the preservation of NO bioavailability provided by the treatments has acted, since resistance exercise enhances eNOS activity and antioxidants can potentiate this pathway [60, 61]. Indeed, our results shows the maintenance of NO levels in lung and RV tissues in treated groups, suggesting a direct role in sustaining ventricular performance. Moreover, the interventions attenuated the lung and RV pro-oxidant profile, with exercise showing the greatest capacity to preserve antioxidant activity. Since oxidative stress activates fibroblasts and apoptotic pathways [62-64], these biochemical adaptations provide a mechanistic explanation for the prevention of pathological remodeling observed in both lung and RV tissues. Really, the RV of treated rats displayed attenuated extracellular matrix and collagen accumulation and preserved architecture. Similarly, the interventions mitigated the structural changes and improved the pulmonary oxidative profile. Altogether, our data support the concept that pathological remodeling in PAH emerges as the final consequence of oxidative imbalance, and that interventions capable of restoring these upstream processes can substantially mitigate cardiopulmonary damage [30, 35].

Concerning the impaired contractility of single RV myocytes and the downregulation of Ca^{2+} handling proteins, the employed treatments attenuated the reduction in cellular mechanical function, as well as the expression of RyR2 and p-PLB(Ser16), indicating partial preservation of calcium release and reuptake. These findings align with previous studies showing that resistance training improves cardiomyocyte contractile function as well as intracellular calcium dynamics by upregulating regulatory proteins [29]. It is noteworthy that our resistance exercise protocol improved the antioxidant defense in the RV of trained rats and thus attenuated oxidative stress which positively impacts on the disruption of Ca^{2+} handling [11, 62, 63]. Notably, this study demonstrates for the first time that blueberry extract directly contributed to the preservation of RV myocyte function, despite having no direct impact

on the expression of Ca^{2+} regulatory proteins. It is reasonable, nevertheless, that the antioxidant properties of blueberry have favored RV redox regulation which positively influenced intracellular Ca^{2+} dynamics [10, 11].

Finally, despite its promising findings, this study has limitations, and its results should be interpreted with caution. First, in the exercise protocol employed it was not feasible to precisely control the animals' climbing speed which limits the accurate determination of exercise intensity and total workload. Second, natural variability in the chemical composition of blueberry extracts affected by cultivation conditions, harvesting, and processing may influence the reproducibility and consistency of outcomes when this compound is used as an intervention. Third, the treatments' duration was constrained by the MCT-induced PAH time course. This short period may limit the generalizability of long-term adaptations. And fourth, only male rats were used, which limits the applicability of our findings, thus warranting future studies on sex-specific responses to the employed treatments in severe PAH.

5. CONCLUSION

In conclusion, blueberry extract and resistance training, either alone or in combination, alleviate cardiopulmonary oxidative imbalance and adverse structural remodeling, and sustain RV myocyte contractility and expression of proteins involved in Ca^{2+} cycling and thus conserve pulmonary and right ventricle function in severe PAH induced by monocrotaline. These findings reinforce the relevance of integrating non-pharmacological strategies involving natural antioxidants and physical exercise in mitigating cardiopulmonary dysfunctions and structural damage associated with severe PAH that could have significant implications for the disease's management and prognosis.

6. FUNDING

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funding agencies were not involved in the study's design, data collection, analysis, or interpretation, the writing of the manuscript, or the decision to submit it for publication.

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8. CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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5. STUDY 2

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Blueberry extract and resistance training prevent left ventricular redox dysregulation and pathological remodeling in experimental severe pulmonary arterial hypertension

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Abstract:

Objective: To investigate whether regular administration of blueberry extract and low-intensity resistance exercise training (RT), either alone or in combination, during the development of monocrotaline (MCT)-induced severe pulmonary arterial hyper-tension (PAH) in rats, protect the left ventricle (LV) from redox dysregulation and pathological remodeling. **Methods:** Groups of seven male Wistar rats were formed for the experiment: sedentary control; sedentary hypertensive; sedentary hypertensive blueberry; exercise hypertensive; and exercise hypertensive blueberry. PAH was experimentally induced through a single intraperitoneal administration of MCT at a dose of 60 mg/kg. One day after injection, the blueberry groups started receiving a daily dose of blueberry extract (100 mg/kg) by gavage, while the exercise groups initiated a three-week program of RT (Ladder climbing; 15 climbs carrying 60% of maximum load; one session/day; 5 times/week). Echocardiographic evaluations were conducted 23 days after injection, and the rats were euthanized the next day to harvest LV tissue. **Results:** Separately, blueberry extract and RT mitigated augments in pulmonary artery resistance, LV tissue's redox dysregulation (i.e., increased PC levels) and detrimental remodeling (i.e., reduced inflammation), and reductions in ejection fraction (EF) and fractional shortening (FS) caused by PAH. The combination of treatments prevented reductions in EF and FS, as well as the development of a D-shaped LV. **Conclusion:** Blueberry extract and moderate-intensity resistance training administered during the development of MCT-induced severe PAH in rats prevented LV redox dysregulation and pathological remodeling, thereby preserving its function.

Keywords: Pulmonary artery resistance; exercise training; left ventricle; redox state; ejection fraction; adverse remodeling; blueberry extract

1. INTRODUCTION

Pulmonary arterial hypertension (PAH) is primarily a right heart disease [1], and disruptions in redox homeostasis—characterized by increased reactive species production alongside weakened antioxidant defenses—are considered key contributors to its development [2]. However, in patients with severe PAH, mortality has been independently associated with left ventricular (LV) global longitudinal strain [3]. In this context, LV dysfunction has been observed in these patients, marked by impaired LV filling and a reduction in end-diastolic volume [4-7]. These alterations are thought to stem from changes in LV geometry, such as the development of a D-shaped configuration [7, 8], driven by right ventricular (RV) hypertrophy, dilation, and interventricular septal flattening [9]. These structural modifications affect LV segmental function and torsion, ultimately leading to a reduced systolic volume [10-14].

The rat model of monocrotaline (MCT)-induced severe PAH (i.e., 60 mg/kg) has contributed over the years to the understanding of the disease's development and potential therapy proposals. The involvement of oxidative stress in the development of right ventricular (RV) dysfunction is evident in this model, with MCT inducing elevated production of reactive oxygen species (ROS) and triggering proapoptotic pathways [7, 15, 16]. These mechanisms disrupt calcium cycling by affecting its regulatory proteins, ultimately leading to contractile impairment [7, 17, 18]. Recently, our research group demonstrated that LV dysfunction also occurs in rats with severe PAH induced by MCT, as redox homeostasis was disrupted [i.e., reduced catalase (CAT) and superoxide dismutase (SOD); and increased malondialdehyde (MDA)], and adverse functional and structural remodeling were observed [7]. Therefore, under the condition of oxidative stress, potential therapies that stimulate the increase of cellular antioxidant capacity are of interest [19].

In this background, alternative therapeutic strategies like the use of natural antioxidants and physical exercise have been investigated. For instance, our group showed that blueberry (*Vaccinium myrtillus*) extract, which is rich in polyphenols, especially anthocyanins, restored the redox state and contributed to attenuating pulmonary pressure on the lungs of rats with MCT-induced severe PAH [20]. Likewise, blueberry extract lessened the RV detrimental remodeling, lipid peroxidation, and nicotinamide

adenine dinucleotide phosphate (NADPH) oxidase activity and increased CAT activity in this model [21]. Moreover, blueberry (*Vaccinium* spp.) and has been proven to reduce LV fibrosis and inflammation in a cardiac injury model due to improvements in redox balance [22]. Regarding physical exercise, we have demonstrated that aerobic and combined (i.e., Resistance plus aerobic) types are beneficial to mitigate oxidative stress, as well as adverse remodeling contractile parameters in both RV and LV in rats with MCT-induced severe PAH, which resulted in the maintenance of cardiac function and physical exercise tolerance [7, 23].

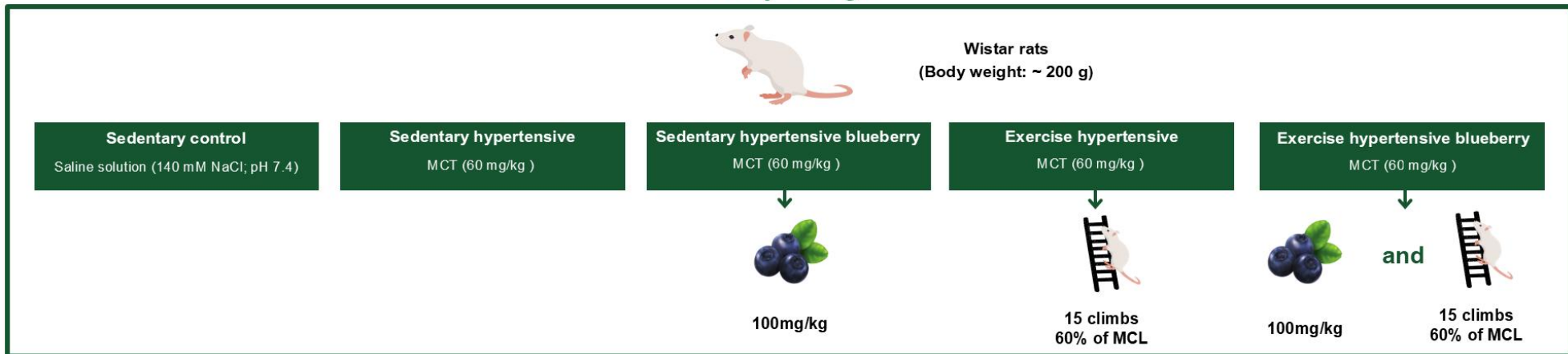
Therefore, considering that approaches involving natural antioxidant fruits and resistance exercise training (RT) had sparsely been explored in severe PAH, especially aiming at the LV, we thought to investigate whether regular administration of blue-berry extract and moderate-intensity RT, either alone or in combination, during the development of MCT-induced severe PAH in rats, protect the LV from redox dysregulation and pathological remodeling.

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats (Body weight: ~ 200 g) were housed in transparent polycarbonate cages, four animals per cage. The animals were divided into five experimental groups, with seven animals per group: sedentary control (SC), sedentary hypertensive (SH), sedentary hypertensive blueberry (SHB), exercise hypertensive (EH), and exercise hypertensive blueberry (EHB). All animals had free access to tap water and commercial chow, and environmental conditions were kept constant [temperature of $23 \pm 1^\circ\text{C}$, light/dark regime of 12/12 hours, and relative humidity of approximately 60%]. This study was conducted in compliance with ethical standards and was approved on July 8, 2022, by the Ethics Committee on the Use of Animals at the Federal University of Viçosa, under protocol number 11/2022. Figure 1 presents a flowchart of the study design with its respective analyses.

Study design



Analysis

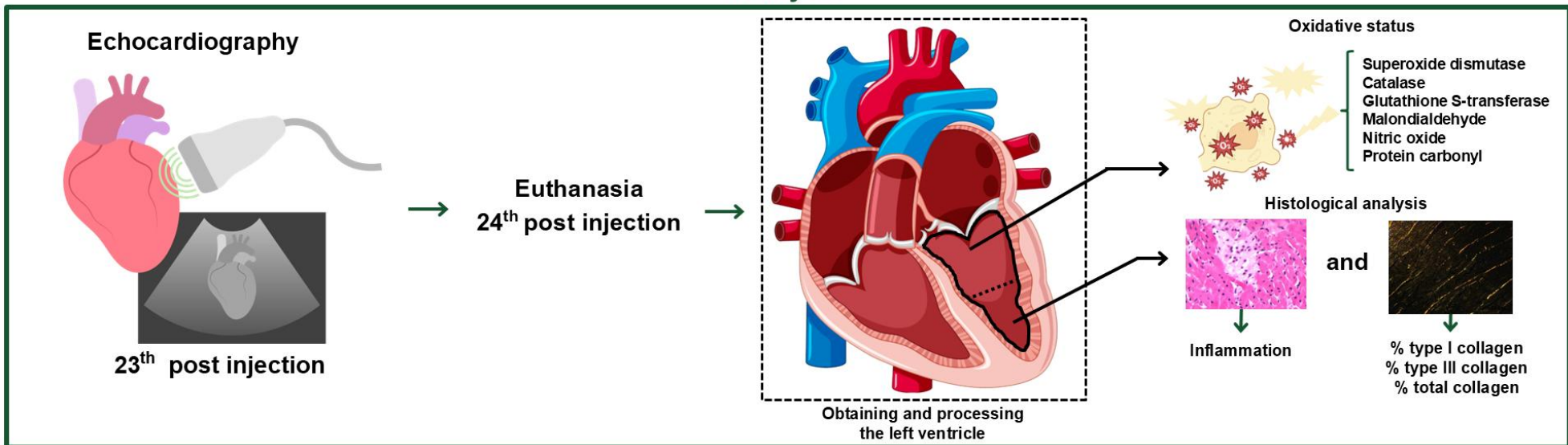


Figure 1. Flowchart of the study design with its respective analyses.

2.2. PAH induction

The animals in the SH, SHB, EH, and EHB groups were administered a single intraperitoneal injection of MCT (60 mg/kg body weight) (Sigma-Aldrich, St. Louis, USA) dissolved in a saline solution (140 mM NaCl; pH 7.4) [24]. The animals in the SC group were administered an equivalent volume of saline solution (140 mM NaCl; pH 7.4).

2.3. Preparation and characterization of blueberry extract

Freshly ripened blueberries (cultivar Bluegem, group Rabbiteye) were harvested manually by gently handpicking each berry from its stem and storing them in a sterile plastic bag stored in dry ice. The extraction process combined 200 g of fresh blueberries with 500 mL of a 50% (v/v) ethanol extraction solvent. The mixture underwent initial extraction in a cutting mill for 10 minutes, followed by sonication for 30 minutes in an ultrasound bath containing ice to avoid compound degradation. The resulting mixture was then vacuum filtered to separate the extract. The extract was further concentrated to 100 mL using a rotary evaporator.

2.4. Composition and administration of blueberry extract

The extract contained a total phenolic compound concentration of 7,795.48 mg gallic acid equivalents (GAE) per liter. After processing, the concentration of phenolics was 38.89 mg GAE/g of extract. As for the identification and concentrations of anthocyanidins, the following were observed: cyanidin (219.21 µg/mL); and malvidin (128.68 µg/mL). Other phenolic compounds (rutin, gallic acid, catechin, and epicatechin) were not detected (Table 1). Considering the composition of the extract, the rats in the SHB and EHB groups received a daily dose of blueberry extract (100mg/kg of animal body weight) administered by gavage at 7:00 a.m. during the experimental protocol. This amount was calculated based on the average amount of total polyphenols consumed by populations in North and South America [25] and has been shown to have a beneficial effect on the treatment of experimental severe PAH [20, 21].

Table 1. Blueberry extract composition.

Compound	Concentration
Total phenolic compounds (mg GAE/g)	38.89
Total flavonoids compounds	
Cyanidin (µg/mL)	219.21 ± 5,38
Malvidin (µg/mL)	128.68 ± 1,27
Rutin	ND
Gallic acid	ND
Catechin	ND
Epicatechin	ND

GAE, gallic acid equivalent; ND, not detected.

2.5. Blueberry extract analysis

The study employed high-performance liquid chromatography (HPLC) (Shimadzu HPLC-20A, Kyoto, Japan) featuring a SIL-20A autosampler, an HPLC-20AT pump, and an SPD-20AV photodiode array detector. Data acquisition was performed using Shimadzu HPLC Solution GPC software (Shimadzu, Kyoto, Japan). The stationary phase comprised a CLC-ODS column (4.6 mm × 25 cm i.d.; 5 µm) (Shimadzu, Kyoto, Japan) combined with a C18 pre-column (20 mm × 3.9 mm i.d.; 10 µm) (Waters, Milford, MA, USA). The mobile phase consisted of solvent A (ultrapure water with 0.1% trifluoroacetic acid) and solvent B (acetonitrile), applied under a gradient elution: 8% B for 5 min, increasing from 8% to 25% B over 25 min, maintaining 25% B for 5 min, and then returning to 8% B over 10 min. The flow rate was set at 0.8 mL/min, with a detection wavelength of 515 nm and an injection volume of 20 µL. Before injection, all samples were filtered through a Millipore polytetrafluoroethylene (PTFE) membrane with a 0.45 µm pore size.

2.6. Resistance exercise training

All rats underwent a two-week familiarization period with the RT model [26], during which they climbed a vertical ladder (height: 1.1 m; width: 0.18 m; step spacing: 2 cm; inclination: 80°) carrying a proportional load attached to their tail. Following this adaptation period, a maximum load test was performed. Rats began the test carrying 75% of their body weight, with 30 g added after each subsequent climb until exhaustion,

with a 120-second rest interval between climbs. After completing the maximum load test and PAH induction, the EH and EHB groups participated in a four-week RT program. This consisted of 15 climbs per session, carrying 60% of their maximum load, with 60-second rest intervals between climbs, performed once daily, five days a week (Monday to Friday). Previous research has highlighted the advantages of this protocol in the context of MCT-induced PAH [7, 27-29]. The training load was adjusted based on performance in a new maximum load test conducted 14 days after PAH induction. It is noteworthy that no signs of right ventricular failure (i.e., cyanosis, edema) have been observed either during or after the exhaustive physical effort.

2.7. Echocardiography

Echocardiographic evaluations were performed on all animals 23 days after the MCT injection. The animals were anesthetized with 1.5% isoflurane in 100% oxygen, delivered at a constant flow rate of 1 L/min (Isoflurane, Bio-Chimico, Rio de Janeiro, Brazil). Imaging was conducted with the animals positioned in lateral decubitus. Two-dimensional echocardiographic studies were carried out at a high frame rate of 120 fps in M mode, using the MyLabTM30 ultrasound system (Esaote, Genoa, Italy) with 11 MHz transducers. Transthoracic echocardiography and M-mode scans were obtained at a scanning speed of 200 mm/s, synchronized to the heart rate.

Image acquisition adhered to the guidelines of the American Society of Echocardiography and was stored for further analysis [30]. LV function was evaluated using ejection fraction (EF) and fractional shortening (FS). Pulmonary arterial hypertension (PAH) was confirmed by calculating the ratio of acceleration time (AT) to ejection time (ET). A quantitative analysis was conducted to identify the presence or absence of a flattened interventricular septum (i.e., D-shaped left ventricle) in each animal based on echocardiographic images, as used in previous studies [7, 28, 29].

2.8. Sample collection

All animals (SC, SH, SHB, EH, and EHB groups) were euthanized by decapitation on the 24th day following the MCT injection. Such time point was chosen based on prior studies [7, 28, 29], which corresponds to the average survival period for

animals with PAH induced by a 60 mg/kg dose of MCT. After euthanasia, the hearts of the animals were removed by an experienced researcher (L.L.S.), carefully dissected, and the absolute weight was obtained. Afterward, the LV was dissected and divided in half, with one portion being standardized for histological analysis and the other for enzymatic analysis (Supplementary Material). The histological procedures and enzymatic analyses are described in detail below. Also, at the time after euthanasia, the left tibia of the animals was removed and dissected, and measurements were performed to determine the heart weight/tibia length ratio.

2.9. Histological processing and histopathological analysis

Histological analyses of the LV were performed following standardized protocols [31]. LV fragments (n = 7 animals/group), obtained immediately after dissection, were fixed in Karnovsky's solution (4% paraformaldehyde and 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 24 hours. The fragments were then dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 100%), cleared in xylene, and embedded in paraffin. Ten histological sections, per animal, with a thickness of 5 μ m were obtained in semi-series using a rotary microtome, respecting intervals of 100 μ m between consecutive sections to avoid repetition of areas from adjacent regions [32, 33]. Histological sections of cardiac tissue were stained with hematoxylin and eosin (HE) for histopathological and stereological analysis and Sirius red staining (Sirius red F3B, Mobay Chemical Co., Union, New Jersey, USA) to quantify collagen fibers [32]. The slides were analyzed for the histopathological structure of the LV using an optical microscope (Olympus AX-70, Tokyo, Japan) at $\times 200$ magnification. The sections were analyzed entirely by a blinded researcher (L.O.G-E), the semi-qualitative presence of inflammation foci was analyzed according to Navarro-Hortal et al. [34]. Specifically, inflammation was assessed using a six-level scale: 0 indicated the absence of lesions, I corresponded to fewer than three foci, II to more than three foci, III to mild generalized inflammation, IV to moderate inflammation, and V to severe inflammation. Representative images of the LV were captured using a coupled digital camera (Olympus Q Color-3, Tokyo, Japan).

2.10. Quantification of collagen fibers

Sirius red sections were analyzed in ten histological fields per animal ($n = 7$ animals/group) using Image-Pro Plus 4.5® software (Media Cybernetics, Silver Spring, MD, USA) to quantify collagen fibers. A polarized filter was attached to the microscope to identify collagen fibers in Sirius red-stained sections. Pixels identified as type I (orange-red) and type III (yellowish-greenish) collagen were quantified based on the software's color histogram [33]. Results were expressed as a percentage of the total image area ($1.92 \times 10^6 \mu\text{m}^2$), with the sum of the percentages of both collagen types representing the total amount of fibers.

2.11. Evaluation of antioxidant enzyme activity and oxidative stress indicators

LV samples ($n = 6/\text{group}$) stored at -80°C ($\sim 100 \text{ mg}$) were homogenized in 1 mL of phosphate buffer (pH 7.4) and centrifuged at $10,000 \times g$ for 10 minutes at 4°C . The supernatants were used to evaluate the enzymatic activity of SOD, CAT and glutathione S-transferase, as well as to quantify byproducts of nitrosative and oxidative stress, specifically nitric oxide and MDA, along with total protein quantification. The resulting pellets were used to determine carbonylated proteins. All analyses were performed using an ELISA microplate reader (Multiskan SkyHigh, Thermo Scientific).

2.11.1. Superoxide dismutase

SOD activity was assessed based on its ability to catalyze the conversion of superoxide ($\text{O}_2^{\cdot-}$) into hydrogen peroxide (H_2O_2), following the pyrogallol auto-oxidation method [35]. The reaction mixture contained 10 μL of the sample and 170 μL of sodium phosphate buffer (pH 7.8). The reaction was initiated by adding 20 μL of pyrogallol (10 mM) and incubated at 37°C for 30 minutes. Absorbance was measured at 320 nm. SOD activity was expressed in units (U)/mg of protein, with one unit of SOD defined as the amount needed to inhibit the pyrogallol auto-oxidation rate by 50%.

2.11.2. Catalase

CAT activity was assessed by measuring the rate of hydrogen peroxide (H_2O_2) decomposition, following the method described by Aebi [36]. Briefly, 100 μL of H_2O_2

(20 mM) was added to 5 μL of the sample. After 3 minutes, 150 μL of ammonium molybdate (32.4 mM) was added to terminate the reaction. Sample blanks were prepared by replacing H_2O_2 with sodium phosphate buffer (50 mM, pH 7.4). The values obtained from the test samples were adjusted by subtracting the blank values. A standard curve was generated using serial dilutions of H_2O_2 to determine CAT activity. Absorbance was measured at 374 nm using a spectrophotometer. CAT activity was expressed in units (U)/mg of protein, with one unit defined as the amount of enzyme required to decompose 1 mmol of H_2O_2 per minute.

2.11.3. Glutathione S-Transferase

Glutathione S-transferase (GST) activity was evaluated using the method described by Habig et al. [37] measuring the formation of glutathione conjugated to 2,4-dinitrochlorobenzene (CDNB). The reaction mixture consisted of 0.1 M CDNB, 0.1 M reduced glutathione (GSH), and sodium phosphate buffer (0.1 M; pH 7.2), along with 10 μL of the sample. After the addition of CDNB, the reaction progress was monitored by measuring the absorbance change at 340 nm over 60 seconds, with readings taken at two intervals (30 and 90 seconds). The molar extinction coefficient for CDNB was $\epsilon_{340} = 9.6 \text{ mmol L}^{-1} \times \text{cm}$. GST activity was expressed in units (U)/mg of protein, where one unit of GST corresponds to the amount of enzyme that catalyzes the formation of 1 μmol of product per minute per milliliter.

2.11.4. Nitric Oxide

Nitric oxide (NO) production was quantified using the Griess reaction, following the method described by Tsikas [38]. Samples (50 μL) were incubated with an equal volume of Griess reagent (50 μL ; 1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine, and 2.5% H_3PO_4) at room temperature for 10 minutes. NO concentration was determined using a sodium nitrite standard curve (0–125 μM) and expressed in $\mu\text{mol L}^{-1}$. Absorbance was measured at 570 nm.

2.11.5. Malondialdehyde

Lipid peroxidation was assessed by quantifying total malondialdehyde (MDA) following the method of Buege and Aust [39]. Briefly, 200 μL of the sample supernatant was mixed with 400 μL of TBARS solution (15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 M HCl). The reaction mixture was incubated in a water bath at 90 °C for 40 minutes. After cooling on ice, 600 μL of butyl alcohol was added, and the solution was shaken and centrifuged at 3,500 rpm for 5 minutes. A standard curve using known concentrations of 1,1,3,3-tetramethoxypropane (TMPO) was applied to calculate MDA concentration. The formation of thiobarbituric acid-reactive substances was monitored by absorbance at 535 nm, and the results were expressed as nmol/mg of protein.

2.11.6. Carbonylated proteins

Protein oxidation was evaluated using the method of Levine et al., [40] by quantifying the carbonyl groups present in the protein structure. The concentration of carbonylated proteins (CP) was determined by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH). The pellets obtained from sample centrifugation were resuspended in 0.5 mL of DNPH solution (10 mM) diluted in 2 M HCl, vortexed, and incubated at room temperature in the dark for 30 minutes with periodic shaking. To stop the reaction, 0.5 mL of ice-cold 10% trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 5,000 g for 10 minutes at 4 °C. The supernatant was discarded, and the precipitate was washed three times with 1 mL of a 1:1 (v/v) mixture of ethyl acetate and ethanol, followed by centrifugation. Next, 1 mL of 6% sodium dodecyl sulfate (SDS) was added, and the mixture was centrifuged. The absorbance of the supernatant was measured at 370 nm. The results were expressed as nmol/mL, based on a molar extinction coefficient of $\epsilon_{370} = 22 \text{ mmol} \times \text{L}^{-1} \times \text{cm}^{-1}$.

2.11.7. Total Protein

Total protein quantification was performed using the Bradford method [41], with bovine serum albumin as the standard. Protein content was used to normalize the results for SOD, CAT, GST and MDA.

2.12. Statistical analysis

The Shapiro-Wilk test was used to assess data distribution. All data were parametric and analyzed using one-way analysis of variance (ANOVA) to identify differences between groups, followed by Tukey's post hoc test for multiple comparisons when applicable. Fisher's exact test was used to evaluate the proportion of animals exhibiting flattening of the interventricular septum. Pairwise comparisons between groups were performed as appropriate. Quantitative results are expressed as mean $\pm$ SEM, while qualitative data are presented as percentages. A significance threshold of 5% ($p \leq 0.05$) was adopted for all statistical tests. The specific tests and the number of animals analyzed for each parameter are detailed in the corresponding figures. All statistical analyses were performed using GraphPad Prism Software version 10.4.0.

3. RESULTS

3.1. Validation of pulmonary hypertension

A qualitative analysis of the echocardiographic images (Supplementary Fig. 1A) shows that all hypertensive groups (SH, SHB, EH, EHB) exhibited impairments in pulmonary artery flow dynamics, indicating hemodynamic changes compared to the SC group. However, the in treated groups (SHB, EH, and EHB) the pulmonary artery flow was visible less altered compared to SH, suggesting a protective effect of the interventions. These observations were confirmed by the quantitative analysis of the AT/ET ratio, a hemodynamic index of pulmonary arterial flow. Animals in the SH group had lower AT/ET values (0.22 ± 0.01) than those in the SC group (0.61 ± 0.02), reinforcing the pulmonary vascular impairment induced by PAH. However, the SHB (0.39 ± 0.01), EH (0.45 ± 0.02), and EHB (0.44 ± 0.02) groups showed higher AT/ET values than SH, confirming the beneficial effects of blueberry and exercise.

3.2. LV echocardiographic data

Rats in SH and SHB groups showed greater interventricular septal flattening (D-shaped LV) compared to those in the SC group (Fig. 2A). Although blueberry extract (SHB group) alone did not affect this alteration, the combination of treatments (EHB

group) prevented flattening. Moreover, animals with PAH not treated (SH group) presented reduced EF (Fig. 2B) and FS (Fig. 2C) compared to controls (SC group). Both treatments (SHB and EH groups) alleviated these reductions, and the combination of treatments (EHB group) maintained EF and FS values similar to those of the control group.

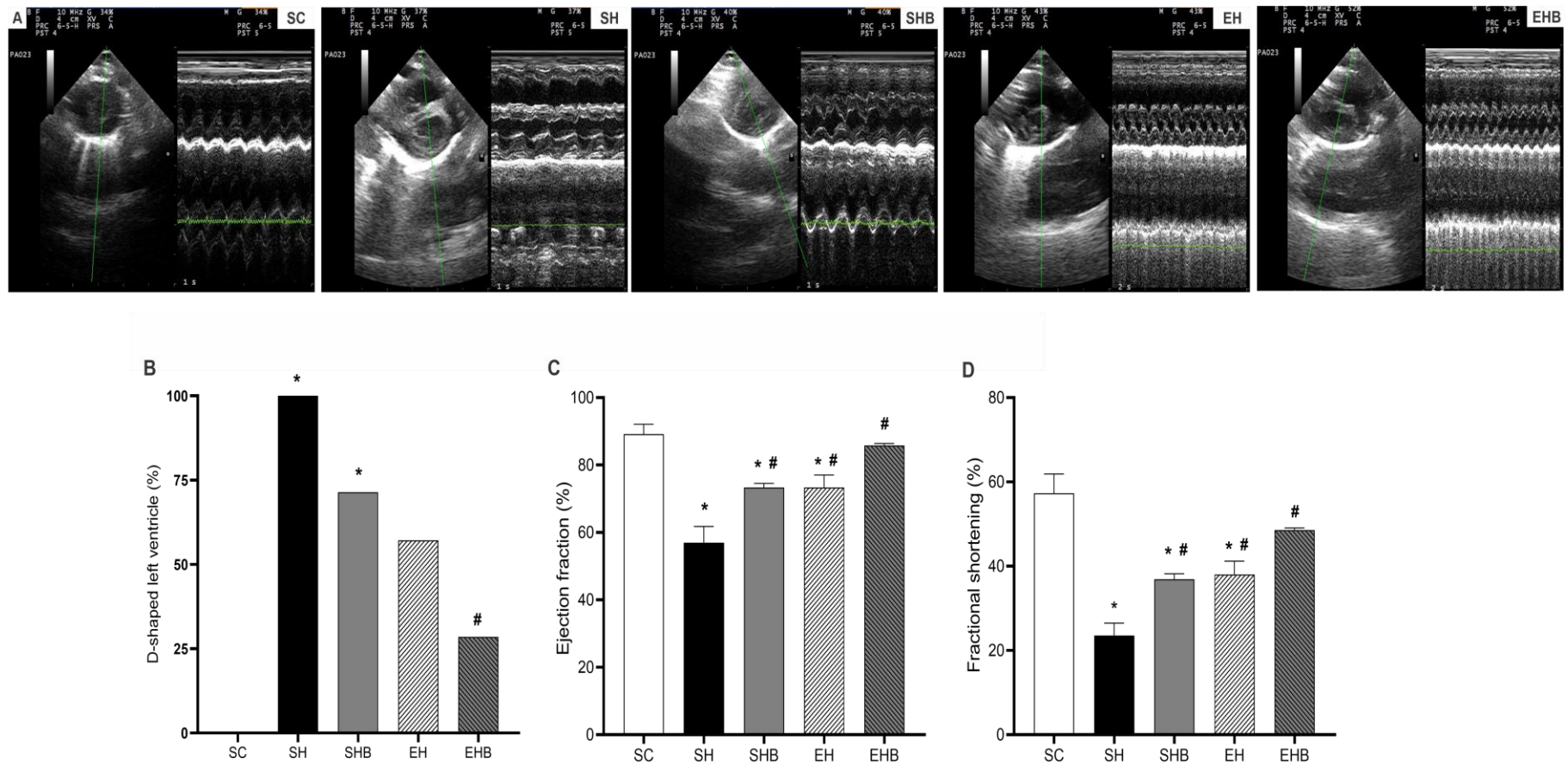


Figure 2. Effects of blueberry extract and resistance training on left ventricular function. (A) Representative echocardiograph images. (B) D-shaped left ventricle. (C) Ejection fraction. (D) Fractional shortening. The data are expressed as means $\pm$ SEM of seven rats per group. SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary hypertensive blueberry; EH, hypertensive exercise; EHB, exercise hypertensive blueberry. * $P < 0.05$ vs. SC; # $P < 0.05$ vs. SH. Panel A (Fisher's exact test). Panel B and C (One-way ANOVA followed by Tukey's post hoc test).

3.3. Morphology and adverse remodeling of the LV tissue

Animals injected with MCT not treated (SH group) showed higher heart weight (HW; Fig. 3A) and heart weight/tibia length ratio (HW/TL; Fig. 3C) than the controls (SC group). Nevertheless, both treatments, either isolated (SHB and EH groups) or in combination (EHB group), hindered these increases. There were no differences between groups in LV weight (Fig. 3B) and LV weight/tibia length ratio (Fig. 3D).

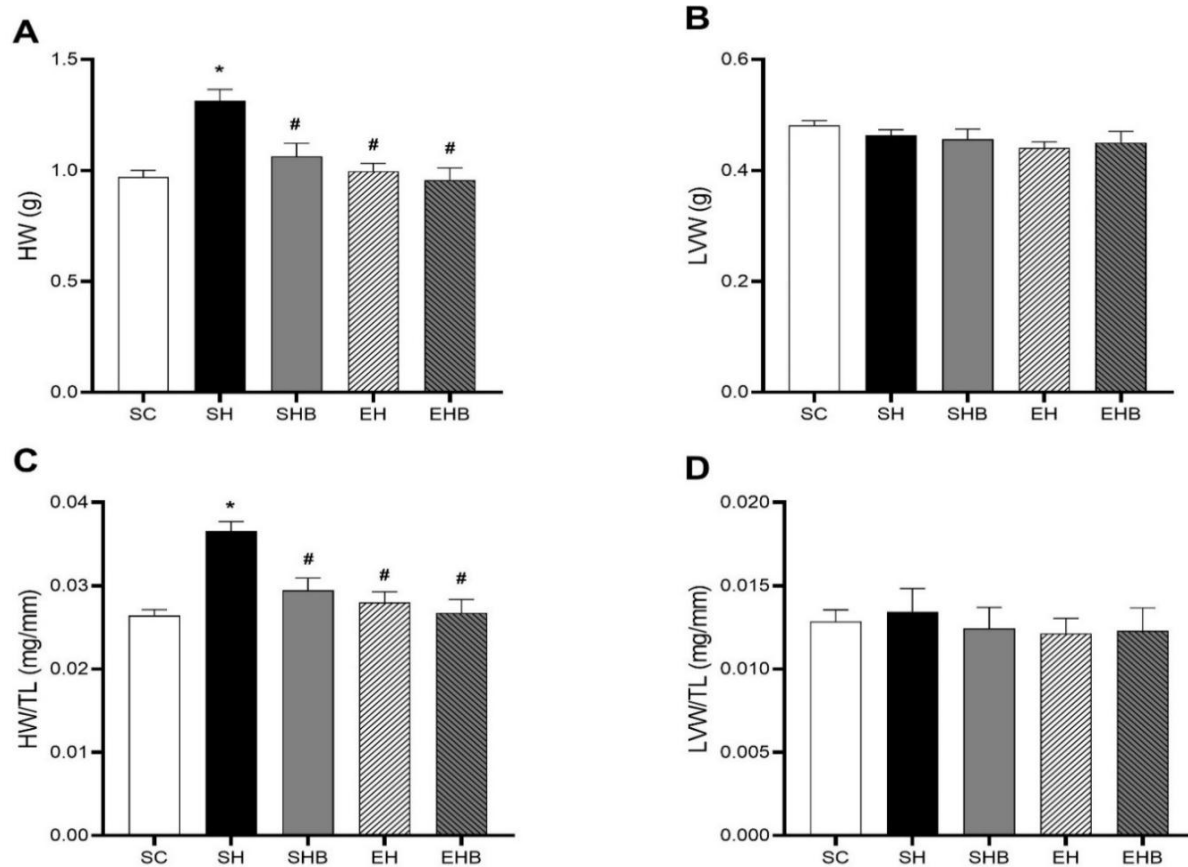


Figure 3. Effects of blueberry extract and resistance training on cardiac morphometric parameters. (A) Heart weight (HW). (B) Left ventricular weight (LVW). (C) Heart weight/tibial length (HW/TL). (D) Left ventricular weight/tibial length (LVW/TL). The data are expressed as means $\pm$ SEM of seven rats per group. SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary hypertensive blueberry; EH, hypertensive exercise; EHB, exercise hypertensive blueberry. One-way ANOVA followed by Tukey's post hoc test.

The qualitative analysis revealed that healthy animals from the SC group exhibited preserved cardiac tissue architecture (Fig. 4A, F, K and Supplementary Fig. 1B), with aligned cardiomyocytes, well-defined central nuclei, and homogeneous cytoplasm, without signs of inflammation, cell death, or abnormal extracellular matrix accumulation. In contrast, animals injected with MCT and not treated (SH group) showed disorganized cardiac muscle fibers, reduced cardiomyocyte area, and increased extracellular matrix (Fig. 4B, G, L, supplementary Fig. 1B), along with frequent cellular infiltrates (Table 2) and areas of cell death, indicating severe inflammation and structural damage.

Animals receiving isolated treatment with blueberry extract (SHB group) presented fewer inflammatory foci and lower cellular infiltrate density (Fig. 4C, H, M and supplementary Fig. 1B; Table 2), with partial preservation of muscle fibers and reduced extracellular matrix compared to the untreated animals (SH group) (Fig. 4P and Q). Similarly, animals undergoing the isolated treatment with resistance training (EH group) showed improved muscle fiber organization, reduced inflammation, and partial preservation of cardiomyocyte and extracellular matrix (Fig. 4D, I, N and supplementary Fig. 1B; Table 2). Finally, animals submitted to the combined treatment (EHB group) demonstrated more evident preservation of LV morphology, with fewer inflammatory foci and maintained muscle fiber architecture (Fig. 4E, J, O and supplementary Fig. 1B). None of the treated groups (SHB, EH, and EHB) exhibited areas of cell death (Fig. 4 and supplementary Fig. 1B), highlighting the protective effect of the interventions.

Table 2. Percentage (%) of inflammation in the left ventricle of healthy Wistar rats, hypertensive rats, and hypertensive rats treated with blueberry extract, resistance training, or their combination.

Grade	SC	SH	SHB	EH	EHB
0	2 (28.57%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
I	4 (57.14%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
II	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (28.57%)
III	1 (14.20%)	0 (0.00%)	2 (28.57%)	1 (14.20%)	1 (14.20%)
IV	0 (0.00%)	0 (0.00%)	5 (71.42%)	6 (85.71%)	4 (57.14%)
V	0 (0.00%)	7 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

Inflammation was assessed using a semiquantitative scale, where 0 represents no lesions, I represent less than three foci, II represents more than three foci, III indicates mild inflammation, and IV indicates moderate inflammation. The experimental groups are as follows: SC (sedentary control), SH (sedentary hypertensive), SHB (sedentary hypertensive treated with blueberry extract), EH (hypertensive treated with resistance training), and EHB (hypertensive treated with blueberry extract and resistance training). n = 7 animals/group.

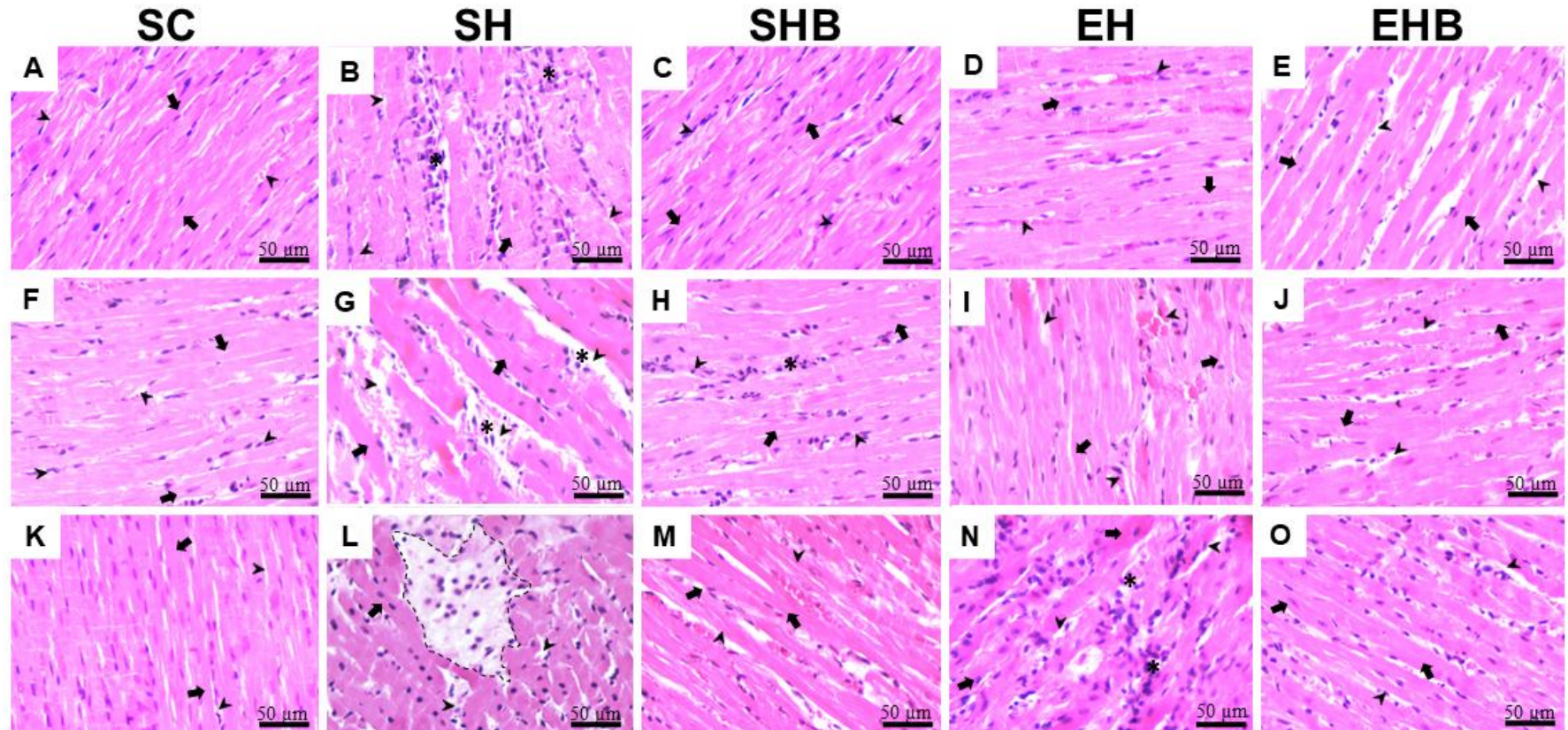


Figure 4. (A–O) Representative photomicrographs of left ventricle tissue stained with hematoxylin and eosin (scale bar: 50 µm). SC: Sedentary control; SH: Sedentary hypertensive; SHB: Sedentary hypertensive treated with blueberry extract; EH: Hypertensive treated with resistance training; EHB: Hypertensive treated with combined blueberry extract and resistance training. Black arrows: cardiomyocytes; arrowheads: extracellular matrix; asterisks: inflammatory foci; dotted regions: areas of cell death. Data are expressed as means $\pm$ SEM of seven rats per group.

Figure 5A presents representative photomicrographs of LV tissue, illustrating notable structural differences among the experimental groups. The SH group exhibits pronounced collagen deposition, as indicated by the presence of type I collagen (yellow arrows) and type III collagen (blue arrows), in contrast to the other groups, which display a more preserved myocardial architecture. Figures 5B and 5D quantitatively confirm these observations. Hypertensive untreated rats (SH group) exhibited a significantly higher percentage of type I collagen (Fig. 5B) and total collagen (Fig. 5D) compared to control animals (SC group) ($p < 0.05$), suggesting increased fibrosis. However, no significant differences were observed in type III collagen levels among the groups (Fig. 5C). Nonetheless, the protective effect of treatments, either isolated or in combination, did not reach statistical significance.

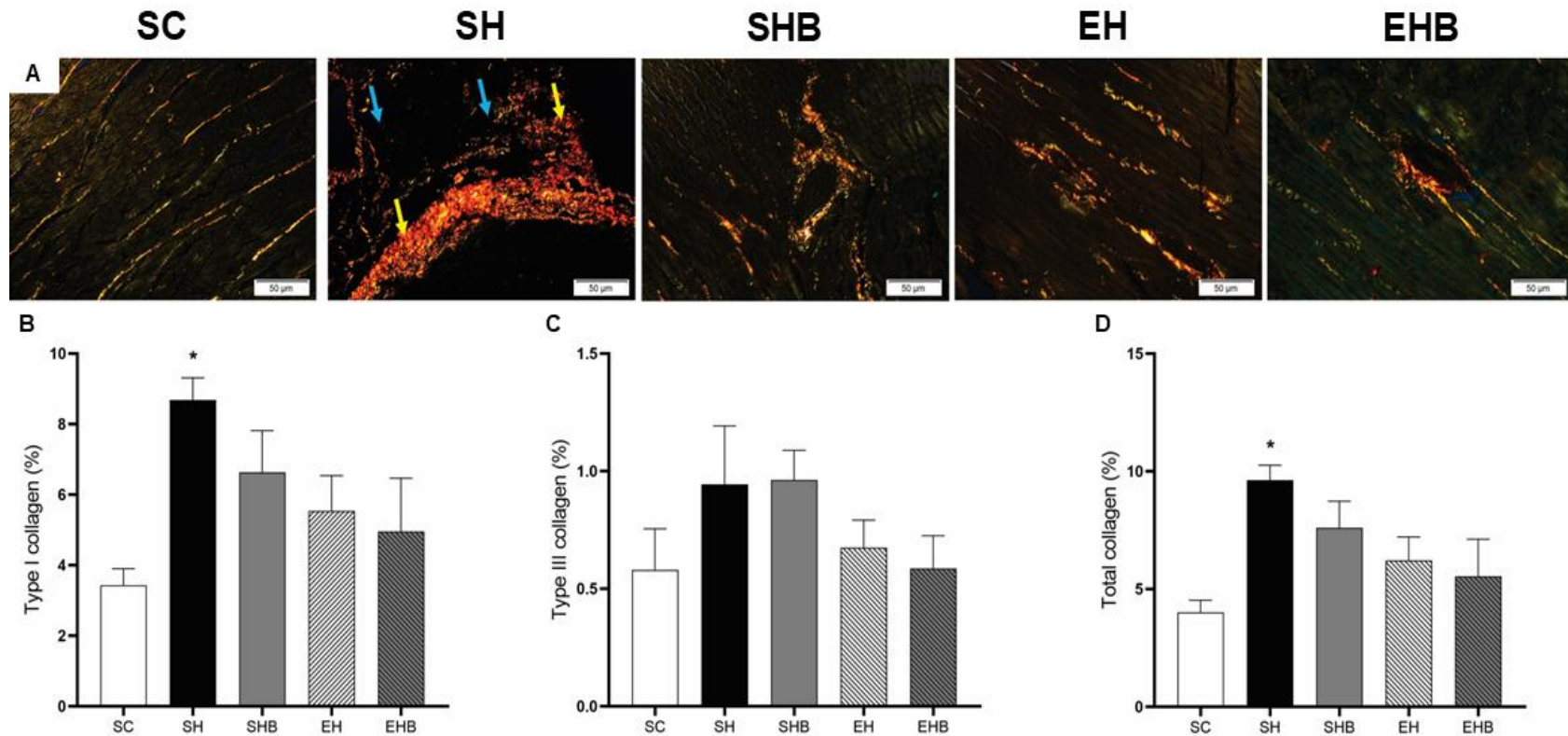


Figure 5. Effects of blueberry extract and resistance training on left ventricular collagen deposition. (A) Representative photomicrographs of LV tissue stained with Sirius red. (B) Percentage of type I collagen. (C) Percentage of type III collagen. (D) Percentage of total collagen. The data are expressed as means $\pm$ SEM of seven rats per group. SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary hypertensive blueberry; EH, hypertensive exercise; EHB, exercise hypertensive blueberry. One-way ANOVA followed by Tukey's post hoc test. *P < 0.05 vs. SC.

3.4. Oxidative stress markers

The reduction in SOD activity caused by MCT injection did not reach statistical significance (Fig. 6A), nor did the effects of treatments. The rats in SH group showed lower CAT activity than those in SC (Fig. 6B). While isolated treatments (SHB and EH groups) did not affect significantly such dysregulation, the combined treatments avoided such reduction (SH < EHB), which suggests a synergistic action of treatments. The activity of GST was also decreased by MCT injection (SH < SC), however, no effects of treatments were observed (Fig. 6C). The concentrations of NO were not changed by MCT, and no significant effect of treatments were observed (Fig. 6D). Regarding MDA (Fig. 6E), MCT increased its concentrations (SH > SC); however, no significant protective effects of treatments were found. The rats in SH group showed higher PC levels, as compared to those in SC group (Fig. 6F). Nevertheless, both treatments, either isolated (SHB and EH groups) or combined (EHB group), protected the hypertensive rats from such increases to a similar extent.

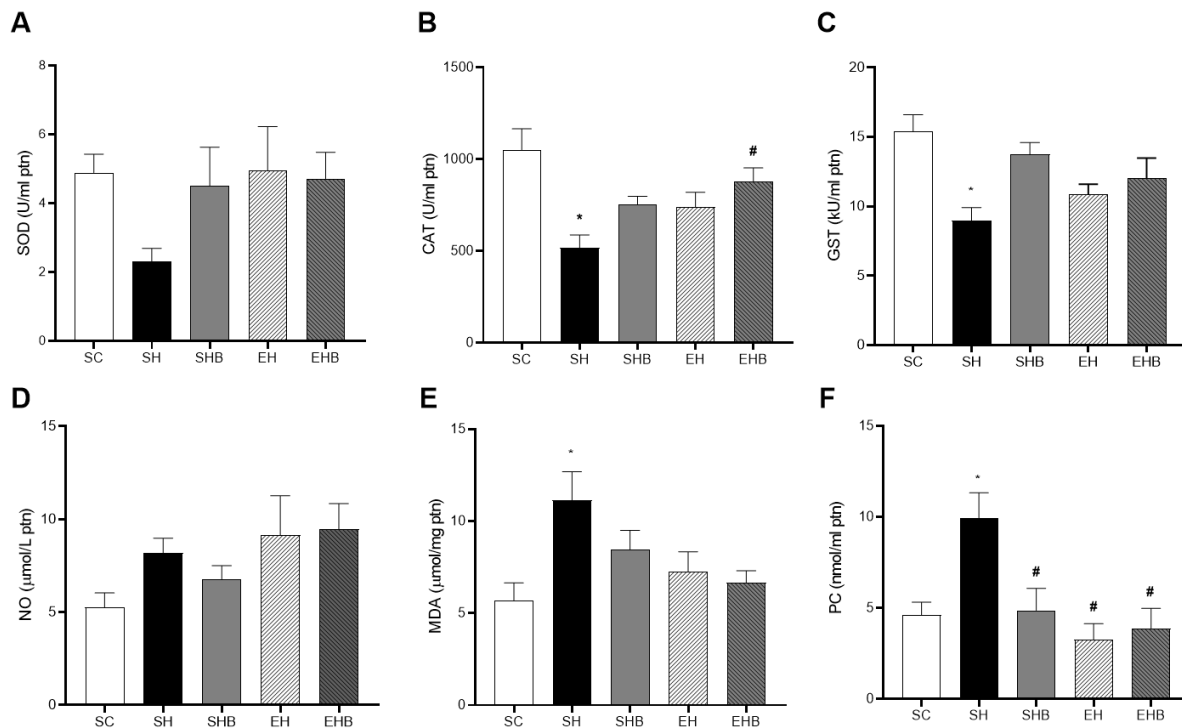


Figure 6. Effects of blueberry extract and resistance training on left ventricular oxidative stress biomarkers. (A) CAT (catalase), (B) SOD (superoxide dismutase), (C) GST (glutathione S-transferase), (D) MDA (malondialdehyde), (E) NO (nitric oxide), (F) PC

(protein carbonyl). The data are expressed as means $\pm$ SEM for five or seven rats per group. SC, sedentary control; SH, sedentary hypertensive; SHB, sedentary hypertensive blueberry; EH, hypertensive exercise; EHB, exercise hypertensive blueberry. One-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$ vs. SC; # $P < 0.05$ vs. SH.

4. DISCUSSION

This study investigated whether regular administration of blueberry extract and moderate-intensity RT, either alone or in combination, during the development of MCT-induced severe PAH in rats, protect the LV from redox dysregulation and pathological remodeling. We found that both treatments effectively prevented increases in pulmonary artery resistance, LV deformation, redox dysregulation, and adverse remodeling, thereby preserving LV function.

The MCT injection used in the present study increased the pulmonary artery resistance which directly imposes an overload to the RV and flattens the interventricular septum. Consequently, it imposed LV's morphological, structural, and functional damages. To illustrate, both EF and FS were reduced in rats from SH group. Such effects have been revealed in a previous study on this model [7]. It is reasonable that such effects are caused by morphological, structural, and redox balance changes. In fact, our hypertensive rats who were not treated had D-shaped LV and cardiac hypertrophy, which can negatively influence LV segmental function and torsion and thus decrease the systolic volume [42, 43]. In addition, these sedentary hypertensive rats exhibited augmented LV tissue ECM, type I, and total collagen, along with evidence of inflammation (i.e., increased cellular infiltrate and cell death). Moreover, these rats' LV tissue presented lower activities of CAT and GST, while the concentrations of MDA and PC were higher, as compared to control animals. Such LV tissue redox dysregulation was reported previously by our group [7] and is known to be induced by MCT [44, 45] as it increases the cardiopulmonary production of ROS and activates proapoptotic pathways [6, 18]. Oxidative stress induces fibroblast activation, thus accumulating collagen [46], and damages DNA, proteins, and lipids of cardiac cells causing cell death [47] and augmenting, therefore, fibrosis. MCT also leads to LV

overexpression of endothelin-1, tenascin-C, and beta-myosin heavy chain as well as angiotensin-converting enzyme upregulation [44]. Together, these changes lead to impairments in the LV filling and end-diastolic volume [12-14] as they diminish the LV tissue's elasticity. Furthermore, increased ROS can directly affect the calcium cycling regulatory proteins [48, 49] and impair LV myocyte contractile function as recently reported by our group [7, 28].

More importantly, the employed treatments prevented the increases in pulmonary artery resistance as well as interventricular septum flattening which protected the LV of hypertensive rats from the harmful changes caused by MCT. For instance, administration of either blueberry extract or RT alone mitigated the EF and FS reductions. Notably, the combination of treatments induced even greater protection and maintained these LV functional parameters to control levels. It is conceivable that such protective effects are based on morphological, structural, and redox changes promoted by the treatments. To illustrate, blueberry extract and RT, either isolated or combined, avoided the cardiac hypertrophy promoted by MCT. The singular protective effects of blueberry and other antioxidants (i.e., quercetin, L-arginine, naringenin, ellagic acid, and beet Juice) on cardiac hypertrophy in this model of severe PAH have been demonstrated [50-54], whereas the protective effect of combined exercise (i.e., RT plus aerobic exercise) was recently reported by our group [7]. We also demonstrated the beneficial effects of RT alone on LV morphology in rats with stable PAH [28]. Nonetheless, the present study reveals for the first time the beneficial effect of combining blueberry extract and RT against pathological LV remodeling in severe MCT-induced PAH.

At the LV tissue level, the structural damages induced by MCT were counteracted by the treatments. For instance, the qualitative analysis showed that the treatments, whether applied individually or in combination, similarly prevented inflammation and extracellular matrix accumulation, and promoted better tissue organization with preservation of cardiomyocytes in the LV. Regardless of no treatment's significant effect against the augmented amount of LV collagen, the observed protection against the increase in extracellular matrix and inflammation may be due to the maintenance of redox balance in response to the treatments. Indeed, the

combined treatments avoided the reduction in the CAT activity showing a synergistic action of blueberry extract and RT, since no single effects were found. Furthermore, the increase in PC levels was halted by both treatments, whether administered individually or in combination, with comparable efficacy. Previous studies on MCT-induced severe PAH have shown that single treatments with blueberry extract [21] and combined exercise training [7] can reduce oxidative stress and prevent redox dysregulation in RV and LV tissues, respectively.

The beneficial effects of blueberry on RV tissue's redox status in the studied model seem to be related to the attenuation of increases in pro-oxidant enzymes (i.e., NADPH oxidase), though no change in the levels of $O_2^{\cdot-}$ was found [21]. Such a study also showed that blueberry extract elevated CAT activity, though it neither restored SOD nor changed GPx activity, which resulted in improved pulmonary artery resistance (e.g., AT/ET) and RV functional parameters (e.g., CO). Concerning LV, the blueberry anthocyanins-enriched extract was shown to be efficient in reducing fibrosis, inflammation, and LV dysfunctions in a model of cyclophosphamide-induced cardiac injury. These effects were associated with protection against redox dysregulation since it increased the levels of SOD and GSH and reduced those of MDA [22].

Regarding physical exercise, the beneficial effects of aerobic exercise training on maintaining the systemic and heart redox regulation in PAH by improving the antioxidant defense are well established [4, 55]. Nevertheless, RT approaches involving its antioxidant effects have rarely been explored, especially in PAH models and aimed at the LV tissue. Recently, our group examined its effects on the LV of rats with MCT-induced stable PAH [45] and found that RT mitigated the MCT damages to LV contractile function by reducing LV collagen and fibrosis. We also demonstrated that combined exercise (RT + aerobic exercise) provides protection to the LV against structural and functional damage induced by MCT in a model of severe PAH, by maintaining the activities of CAT and SOD and preventing the increase in MDA levels [7], thus avoiding redox dysregulation.

Our results show that blueberry extract and RT mitigated MCT-induced redox dysregulation, reducing pulmonary artery resistance and thus protecting against detrimental LV structural and functional remodeling during severe PAH development.

While the MCT-induced PAH model mirrors key aspects of human PAH, physiological and metabolic differences between rats and humans must be considered. Nonetheless, the observed protective effects suggest promising therapeutic potential for further clinical investigations.

Lastly, the findings of our study should be interpreted with some caution, as there are a few limitations. First, we cannot completely rule out the direct effects of MCT on the LV, which may have influenced the observed improvements in LV function. Second, the chemical composition of blueberry extracts can vary depending on factors like growing, harvesting, and processing, which may affect the consistency of results when using this type of intervention. Third, protease inhibitors were not used during sample processing, which may have led to partial protein degradation and, consequently, influenced the accuracy of enzymatic activity values. Lastly, although hemodynamic impairment was confirmed by echocardiography on day 23, we did not perform serial assessments to monitor the progression of pulmonary hypertension, nor were we able to calculate cardiac efficiency due to limited access to advanced imaging techniques. These aspects should be considered when interpreting the functional findings. Future studies are encouraged to analyze the effects of physical training and blueberry extract, either alone or in combination, on the progression of pulmonary hypertension and its impact on LV function.

5. CONCLUSIONS

Our findings indicate that blueberry extract and moderate-intensity resistance training, when administered during the progression of MCT-induced severe PAH in rats, prevent oxidative imbalance and structural deterioration in the LV. These protective effects contribute to the maintenance of LV function, highlighting the potential of these interventions in mitigating cardiac impairments associated with PAH.

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8. CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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5. FINAL CONSIDERATIONS

Taken together, our results demonstrate that blueberry extract and moderate-intensity resistance training, administered alone or in combination during the development of MCT-induced severe PAH:

- ✓ At the functional level, attenuate pulmonary vascular impairment and preserve RV and LV performance, contributing to the maintenance of overall cardiopulmonary function;
- ✓ At the structural level, mitigate heart and lung adverse remodeling thus preventing pathological changes;
- ✓ At the cellular level, preserve RV myocyte contractile function and contribute to the maintenance of myocardial functional integrity;
- ✓ At the molecular level, attenuate redox imbalance and pathological signaling pathways in the RV, including the preservation of calcium-handling protein expression and redox homeostasis.

Future studies are warranted to investigate different exercise intensities, blueberry extract doses, and its long-term effects on functional, structural, cellular, and molecular outcomes in PAH models both in male and female rats.

Finally, exploring the integration of exercise training and nutraceutical strategies with pharmacological therapies may provide important insights into more comprehensive approaches for PAH management.

ATTACHMENT 1 – Ethical approval

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 11/2022, intitulado **“Efeitos do exercício resistido ou terapia com extrato de mirtilo nas funções cardiopulmonares, marcadores inflamatórios e células do sistema imune em modelo experimental de 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/MCTIC, 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/MCTIC, portanto sendo aprovado por esta Comissão em 08/07/2022, com validade de 2 anos

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 11/2022, named **“Effects of resistance exercise or blueberry extract therapy on cardiopulmonary functions, inflammatory markers and immune system cells in an experimental model of pulmonary arterial hypertension”**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTIC, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTIC therefore being approved by the Committee on July 08, 2022 valid for 2 years.



Prof. Fabrício Luciani Valente
Coordenador
Comissão de Ética no Uso de Animais – CEUA/UFV

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



Article

Blueberry Extract and Resistance Training Prevent Left Ventricular Redox Dysregulation and Pathological Remodeling in Experimental Severe Pulmonary Arterial Hypertension

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Abstract: Objective: To investigate whether the regular administration of blueberry extract and low-intensity resistance exercise training (RT), either alone or in combination, during the development of monocrotaline (MCT)-induced severe pulmonary arterial hypertension (PAH) in rats protect the left ventricle (LV) from redox dysregulation and pathological remodeling. Methods: Groups of seven male Wistar rats were formed for the experiment: sedentary control; sedentary hypertensive; sedentary hypertensive blueberry; exercise hypertensive; and exercise hypertensive blueberry. PAH was experimentally induced through a single intraperitoneal administration of MCT at a dose of 60 mg/kg. One day after injection, the blueberry groups started receiving a daily dose of blueberry extract (100 mg/kg) by gavage, while the exercise groups initiated a three-week program of RT (ladder climbing; 15 climbs carrying 60% of maximum load; one session/day; 5 times/week). Echocardiographic evaluations were conducted 23 days after injection, and the rats were euthanized the next day to harvest LV tissue. Results: Separately, blueberry extract and RT mitigated augments in pulmonary artery resistance, LV tissue redox dysregulation (i.e., increased PC levels) and detrimental remodeling (i.e., reduced inflammation), and reductions in ejection fraction (EF) and fractional shortening (FS) caused by PAH. The combination of treatments prevented reductions in EF and FS, along with the development of a D-shaped LV. Conclusions: blueberry extract and moderate-intensity resistance training