PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

MESTRADO EM CIÊNCIA ANIMAL

INFLUÊNCIA DO TREINAMENTO AERÓBIO PREVENTIVO NA REMODELAÇÃO CARDÍACA DE RATOS COM HIPERTENSÃO ARTERIAL PULMONAR

ANA KARÊNINA D. DE ALMEIDA SABELA

Presidente Prudente – SP

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Exame geral de Defesa de Dissertação apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal.

Área de Concentração: Fisiopatologia Animal

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ANA KARÊNINA DIAS DE ALMEIDA SABELA

INFLUÊNCIA DO TREINAMENTO AERÓBIO PREVENTIVO NA REMODELAÇÃO CARDÍACA DE RATOS COM HIPERTENSÃO ARTERIAL PULMONAR INDUZIDA PELA MONOCROTALINA

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Presidente Prudente, 29 de setembro de 2015.

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A persistência é o menor caminho do êxito”.

(Charles Chaplin)

LISTA DE ABREVIAÇÕES

µm²	- micrômetro quadrado
ANOVA	- Análise de variância
AT	- átrios
Ca^{2+}	- cálcio
cDNA	- Ácido Desoxirribonucleico complementar
COBEA	- Colégio Brasileiro de Experimentação Animal
DPOC	- Doença pulmonar obstrutiva crônica
g	- gramas
h	- horas
HAP	- Hipertensão Arterial Pulmonar
HE	- Hematoxilina e eosina
IAM	- Infarto Agudo do Miocárdio
IC	- Insuficiência cardíaca
kDa	- quilodalton
km/h	- quilômetro por hora
LL	- Limiar de lactato
mg/kg	- miligrama por quilo
MHZ	- megaherts
min	- minutos
mmol/L	- milimol por litro
ms	- milissegundo
NaCl 9%	- cloreto de sódio à 9% (solução salina)
ºC	- graus célsius

PCF - peso corporal final

PLB - Fosfolamban

RNAm - Ácido Ribonucleico mensageiro

RT-qPCR - Transcrição reversa da reação em cadeia da polimerase em tempo real

RyR - Receptor de rianodina

S - Grupo Sedentário

sem - semanas

SM - Grupo Sedentário Monocrotalina

SERCA2a - Ca2+ ATPase do retículo sarcoplasmático

T - Grupo Treino

TAC Pulm - Tempo de Aceleração da Artéria Pulmonar

TEJ - Tempo de Ejeção do Ventrículo Direito

TM - Grupo Treino Monocrotalina

ua - unidade arbitrária

VD - Ventrículo direito

VE - Ventrículo esquerdo

VMÁXPulm - Velocidade do Sangue no Tronco da Artéria Pulmonar

vs - versus

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TREINAMENTO AERÓBIO PREVENTIVO EXERCE EFEITO CARDIOPROTETOR EM RATOS COM HIPERTENSÃO ARTERIAL PULMONAR

TREINO PRÉVIO NA HIPERTENSÃO PULMONAR

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Resumo

A Hipertensão Arterial Pulmonar (HAP) é uma doença crônica, que acarreta sobrecarga ao ventrículo direito e o efeito do treinamento preventivo na remodelação cardíaca nesta condição ainda é desconhecido. O estudo teve por objetivo avaliar a influência do treinamento preventivo na hipertrofia, função cardíaca e na expressão gênica de proteínas do trânsito de cálcio em ratos com HAP induzida por monocrotalina. Foram utilizados 32 ratos Wistar machos, distribuídos em 4 grupos: sedentário controle (S); treino controle (T); sedentário monocrotalina (SM); treino monocrotalina (TM). O protocolo de treino preventivo foi realizado em esteira por 13 semanas, 5vezes/semana. Houve 2 semanas de adaptação com aumento gradual velocidade/tempo. A velocidade do treinamento físico da terceira à décima semanas foi aumentado gradativamente de 0,9km/h à 1,1km/h por 60min. Então, foi aplicada monocrotalina (60mg/Kg) que induziu HAP e realizada análise do limiar de lactato para determinar as velocidades de treino. O TM nas 2 semanas seguintes foi 0,8km/h-60min e T 0,9km/h-60min; na última semana ambos com a mesma velocidade e duração 0,9km/h-60min. A função cardíaca foi avaliada por ecocardiograma, a hipertrofia ventricular por análise histomorfométrica e a expressão gênica por RT-qPCR. A função cardíaca direita avaliada pela pico de velocidade de fluxo foi de SM=75,5cm/s vs. TM=92,0cm/s (p=0,001) e hipertrofia ventricular foi SM=106,4µm² vs. TM=77,7µm² (p=0,004). Houve diminuição da expressão gênica da rianodina, S=1,12ua vs. SM=0,60ua (p=0,02) sem alterações desta com o treinamento. Desta forma, concluímos que o treinamento físico prévio exerce efeito cardioprotetor ao ventrículo direito em ratos modelo monocrotalina.

Palavras Chaves: Disfunção Ventricular, Monocrotalina, Exercício

Introdução

A Hipertensão Arterial Pulmonar (HAP) é uma doença crônica caracterizada por um aumento gradativo da resistência vascular pulmonar e da pressão na artéria pulmonar, que acarreta sobrecarga ao ventrículo direito (VD), levando a um quadro de remodelação cardíaca patológica caracterizada por hipertrofia, disfunção ventricular e insuficiência do VD (Humbert *et al*. 2004; Ochiai *et al*. 2008; Zapata-Sudo et al. 2012; Mocumbi *et al*. 2015; Talati *and* Hemmes 2015).Dados sobre a prevalência exata da HAP são desconhecidos, e seu número real pode ser subestimada (Mocumbi *et al*. 2015). A doença apresenta prognóstico ruim, com sobrevida média de 2,8 anos (D'Alonzo *et al*. 1991; Montani *et al*. 2013; Mocumbi *et al*. 2015).

A disfunção VD na evolução da HAP precede a Insuficiência Cardíaca (IC) e esta ocorre quando há alterações no relaxamento e/ou contração do músculo cardíaco sem promover retenção hídrica ou intolerância ao exercício (Cohn *et al*. 2000; Pacagnelli *et al*. 2014). Esta disfunção do VD pode estar relacionada com os mecanismos moleculares envolvendo o trânsito de cálcio (Opie 1998; Fernandes *et al*. 2015). Diversas proteínas, como receptor de rianodina (RyR), fosfolamban (PLB) e Ca^{2+} ATPase do retículo sarcoplasmático (SERCA2a), regulam a homeostase do cálcio no músculo cardíaco e são fundamentais para o seu adequado funcionamento (Opie 1998; Lima Leopoldo *et al*. 2013). A maioria dos estudos se concentram no ventrículo esquerdo (VE), entretanto a função do VD é fundamental para a sobrevida em condições fisiopatológicas como na HAP (Risgaard *et al*. 2014; La Gerche *and* Claessen 2015).

Os efeitos benéficos do exercício aeróbio crônico sobre a remodelação do VD em animais com HAP induzida pela monocrotalina estão descritos (Handoko *et al*. 2009, Colombo *et al*. 2013), entretanto, busca na literatura não apontou nenhum estudo que avaliasse os efeitos que o treinamento aeróbio preventivo exerce na remodelação VD induzida por HAP. Apesar de estudos apontarem que o treinamento físico aeróbio crônico preventivo ao infarto agudo do miocárdio promoveu melhora da função cardíaca e sobrevida (Waard *and* Duncker 2009; Bozi *et al*. 2013).

Para nosso conhecimento, não há estudos que avaliaram os efeitos que o treinamento aeróbio preventivo exerce sobre a remodelação VD induzida por HAP. O objetivo do estudo foi testar a hipótese de que o treinamento aeróbio realizado de forma preventiva na HAP induzida por monocrotalina em ratos amenize a hipertrofia VD, melhore a função do VD por alterar genes do trânsito de cálcio.

MATERIAS E MÉTODOS

Aprovação Ética

Todos os protocolos experimentais que foram utilizados neste estudo estão em conformidade com os princípios de cuidados com animais de laboratório formulado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e de acordo com o *Guide for the Care and Use of Laboratory Animals* publicado pelo *National Research Council* (Clark *et al*, 1997). Todos os procedimentos utilizados foram aprovados pelo Comitê de Ética em Pesquisa da Universidade do Oeste Paulista – UNOESTE (número 1838 e 1839).

Animais

Para realização desse estudo foram utilizados trinta e dois ratos Wistar machos, com 2 meses de idade e peso médio de 206 ± 16,35 g provenientes do Biotério Central da Universidade do Oeste Paulista – UNOESTE de Presidente Prudente – São Paulo. Foram mantidos no Laboratório de Experimentação Animal da mesma instituição em gaiolas plásticas com dimensão 41x34x16 cm (3 animais/gaiolas), à temperatura de 21 à 23ºC e umidade relativa do ar de 50% a 60%, com ciclos de luminosidade de 12h (claro /escuro) com início do ciclo claro as 7h. Os ratos receberam ração de forma controlada (Supralab, Alisul®, Brasil) e água *ad libitum*.

Os animais foram distribuídos de forma aleatória em 4 grupos experimentais com 8 animais cada denominados: grupo sedentário controle (SC), grupo sedentário monocrotalina (SM) e grupos submetidos ao protocolo de treinamento: grupo treino controle (TC) e grupo treino monocrotalina (TM).

Desenho Experimental

Para realização do estudo, inicialmente, os animais dos grupos T e TM foram submetidos a um protocolo de treinamento aeróbio em uma esteira por 11 semanas com frequência de 5 dias na semana. Inicialmente os animais passaram por 2 semanas de adaptação ao treinamento e, a seguir, foram submetidos a 8 semanas de treinamento (Machado *et al*. 2006).

No início da 11ª. semana, os ratos dos grupos S e T foram submetidos a uma injeção intraperitoneal de cloreto de sódio 9% (solução salina), enquanto que os dos grupos SM e TM a injeção de monocrotalina. Vinte e quatro horas após a injeção os animais dos grupos T e TM deram continuidade ao treinamento aeróbio por mais 3 semanas (Colombo et al. 2013). Para ajuste da

carga e continuidade do treinamento os animais foram submetidos a um teste para análise do limiar de lactato (Carvalho et al. 2005).

Após 2 semanas do término do exercício os animais passaram por uma avaliação ecocardiográfica que identificou disfunção ventricular direita. Após a avaliação ecocardiográfica, os animais foram pesados, eutanasiados com dose intraperitoneal de pentobarbital sódico (50 mg/Kg), o coração foi retirado, dissecado, separado em átrios (AT), VD e VE e pesados. Foram realizadas as avaliações: anatômica, histológica e expressão gênica do cálcio cardíaco.

O detalhe do treinamento aeróbio e delineamento experimental (Figura 1) estão descritos abaixo.

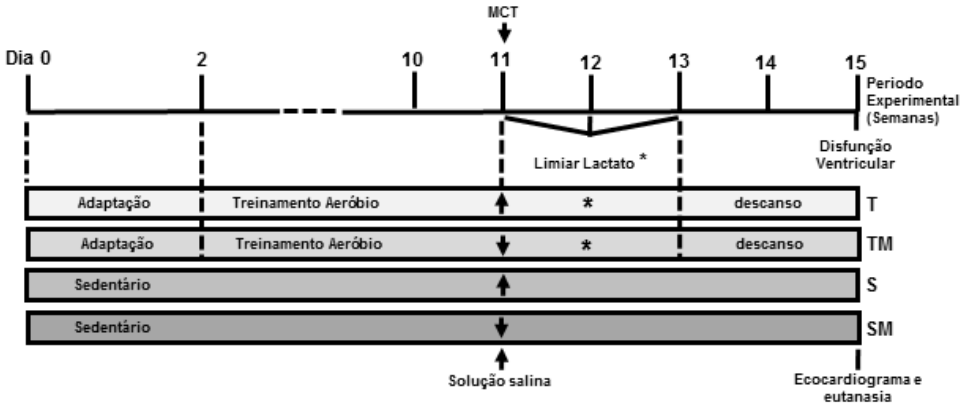

Figura 1. Esquema do treinamento aeróbio e delineamento experimental. Sedentário Controle (S); Sedentário monocrotalina (SM); Treino controle (T); Treino monocrotalina (TM); MCT: monocrotalina.

Protocolo de Treinamento

Os animais dos grupos T e TM foram submetidos a um protocolo de treinamento aeróbio realizado em esteira adaptada para roedores (SEBRAE, Presidente Prudente, SP, Brasil). O treinamento foi realizado por 13 semanas com frequência de 5 vezes semanais, sendo 10 semanas antes da aplicação de monocrotalina (2 semanas iniciais de adaptação e 8 de treinamento) e 3 semanas após a injeção de monocrotalina (Colombo *et al*. 2013).

No período de adaptação o tempo e velocidade da esteira foram aumentados gradativamente, com início do treino à 0,6 km/h em 15 minutos no primeiro dia e término com velocidade de 0,9 km/h em 45 minutos na segunda semana (Rodrigues *et al*. 2007). Após a adaptação iniciou-se o treinamento físico aeróbio por mais 8 semanas com aumento gradativo da velocidade (Machado *et al*. 2006). Em todas as sessões foi realizado um período de 5 minutos de aquecimento e desaquecimento com velocidade de 0,6 km/h.

No início da 11ª. semana de treino os animais dos grupos T (receberam solução salina) e grupo TM (receberam monocrotalina) foram submetidos 24 horas após um teste para avaliar a velocidade do treino correspondente ao limiar de lactato. Esta velocidade foi ajustada após a realização de novos testes para avaliar o limiar de lactato que ocorreu na 11° e 12° semanas. O exercício na 11° semanas foi para os grupos TM=0,8km/h e T=0,9km/h por 60min e na última semana ambos os grupos realizaram o treino a 0,9km/h por 60min (Souza *et al*. 2014).

Limiar do Lactato

Para determinar o limiar do lactato e a velocidade que o limiar do lactato ocorreu, os animais dos grupos T e TM foram submetidos ao teste de exercício incremental em esteira rolante para modelos experimentais.

O protocolo usado foi adaptado a partir descrição prévia de Carvalho *et al*. 2005 e foi realizado 24 hrs após a injeção da monocrotalina, no início da 11ª e final da 12ª e 13ª semanas para ajustar a velocidade do treino. O teste iniciou com 2 minutos de aquecimento a 0,5 km/h, seguida de 5 minutos de descanso. Após este período a velocidade foi aumentada para 0,7 km/h por 3 minutos, com aumento progressivo de 0,2 km/h a cada 3 minutos com 0% de inclinação,

até o lactato atingir o valor de 1mmol/L, comparado com o valor inicial, ou até a exaustão (Bech *et al*. 1990; Svedah *and* Macintosh 2003). A exaustão foi definida como o momento que os ratos não poderiam mais manter a corrida por 3 minutos. Após o aumento de cada carga, os animais foram removidos manualmente do treinamento por 1 minuto para o sangue ser coletado. Amostras de sangue foram coletadas da cauda dos animais utilizando o aparelho Lactimetro Accutrend Plus (Roche®, Portugal). O aparelho foi calibrado conforme especificações do fabricante. O cálculo para estipular a velocidade máxima foi realizado com uma média aritmética de todas as velocidades de cada grupo experimental ao atingirem o limiar de lactato ou a exaustão. (Souza *et al*. 2014).

O limiar de lactato foi definido com a velocidade de corrida que poderia ser mantida sem um aumento do lactato de 1,0 mmol/L acima da concentração lactato no sangue com a velocidade anterior (Ferreira *et al*. 2007).

Indução da Disfunção Ventricular Direita

No início da 11ª. semana nos animais do grupo SC e TC foi realizado administração intraperitoneal de solução salina (NaCl 0,9%), para que todos os animais fossem submetidos ao mesmo grau de estresse. O protocolo para indução da disfunção VD foi realizado nos animais dos grupos SM e TM, com injeção de uma única dose intraperitoneal da monocrotalina (Sigma Chemical, St Louis, MO, USA) na proporção 60mg/kg em 1mol/L em tampão HCl ph 7.0 com 1 mol/L de NaOH (Souza-Rabbo *et al*. 2008).

Controle de Ração

Há evidencias que a monocrotalina reduz a ingestão de alimentos. Para controlar para este efeito, os animais foram separados em gaiolas individuais para dosar o consumo diário de ração. Os ratos tratados com monocrotalina (grupos SM e TM) comeram livremente a ração oferecida. Nos grupos S e T, os ratos foram alimentados apenas com a quantidade de ração consumida no dia anterior pelos os ratos tratados com monocrotalina. O controle de avanço foi realizado após a administração da monocrotalina, uma vez que os animais, que receberam a droga, começou apresentar um quadro de remodelação cardíaca patológica, diminuindo sua ingestão de alimentos devido à disfunção do VD (Lopes et al. 2008).

Avaliação ecocardiográfica

A avaliação ecocardiográfica foi realizada utilizando um ecocardiógrafo comercialmente disponível (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipado com uma sonda multifreqüencial 5-11,5 MHz. Os ratos foram anestesiados por injeção intraperitoneal de cetamina (50 mg / kg) e xilazina (0,5 mg / kg). Fluxo da artéria pulmonar foi obtida por Doppler pulsado, e a velocidade pulmonar tempo de aceleração (PVAT), tempo de ejeção pulmonar (PET), e velocidade de pico de fluxo foram medidos (PFV). (Martinez et al 2011;. Eguchi et al 2014). PVAT é um indicador do grau de hipertensão pulmonar, em outras palavras, quanto maior o nível de pressão da artéria pulmonar sistólica, menor é o valor de PVAT. O PET é um parâmetro relacionado com a função sistólica e grau HAP. PFV está relacionado com a função sistólica do VD (Dabestani et al. 1987, al. Lawrence et 2010).

Avaliação dos Parâmetros Anatômicos

O coração foi retirado, dissecado em átrios (AT), ventrículos direito e esquerdo + septo ventricular e pesados. Os parâmetros anatômicos foram normalizados pelo peso corporal final (AT/PCF, VD/PCF e VE/PCF) e foram utilizados como índice de hipertrofia. O pulmão e fígado também foram retirados, pesados, onde foram acondicionados em estufa por 48h. Após isso, foram pesados novamente para cálculo da relação peso úmido/peso seco que foi utilizado para avaliar sinais de insuficiência cardíaca (Carvalho *et al*. 2010).

Histologia e Análise Histomorfométrica

Amostras de tecido cardíaco foram fixadas em solução de formol tamponado a 10% por um período de 48 horas. Após fixação, o tecido foi incluso em blocos de parafina, obtendo-se a seguir cortes histológicos coronais de 4 micrômetros. Os cortes histológicos foram corados em lâmina com solução Hematoxilina-Eosina (HE) para aferição de áreas da secção transversa dos cardiomiócitos, empregando-se microscópio LEICA (modelo DM750, Alemanha), que envia imagens digitais a computador dotado de sistema de análise de imagens Leica Application Suíte LAS 4.2.0 (Media Cybernetics, Silver Spring, Maryland, USA) (Gomes *et al*. 2009; Oliveira-Júnior *et al*. 2010).

As imagens foram obtidas por meio de microscópio óptico binocular. Todas as imagens foram capturadas por câmara de vídeo no aumento de 400x (objetiva 40x). A seleção das imagens para captura e digitalização foi feita visualmente. A morfometria dessas imagens obtidas e digitalizadas foi realizada utilizando-se software apropriado para tal fim. De cada um dos quatro cortes obtidos do VD de cada animal foram realizadas capturas de campos diferentes, escolhidos de acordo com o local onde se pudessem visualizar mais

células em corte transversal. Foram mensuradas cinquenta células por ventrículo analisado. Os cardiomiócitos selecionados estavam seccionados transversalmente e apresentavam forma redonda, núcleo visível no centro da célula e localizavam-se na camada subendocárdica da parede muscular do VD. Esse cuidado visou uniformizar ao máximo o conjunto de cardiomiócitos dos diferentes grupos. As áreas seccionais médias obtidas para cada grupo foram utilizadas como indicador do tamanho celular (Oliveira-Júnior *et al*. 2013).

Expressão gênica relativa de reguladores do Ca^{2+} intracelular

O RNA total foi extraído do tecido do VD (tecido a fresco) utilizando-se Trizol (Invitrogen), tratado em seguida com DNAse de acordo com orientação do fabricante. A integridade do RNA foi avaliada por eletroforese. O kit High Capacity cDNA Reverse Transcription (Applied Biosystems, CA, EUA) foi usado para a síntese de DNA complementar (cDNA) a partir de 1000 ng de RNA total. Utilizou-se RT-PCR para medir quantitativamente os níveis relativos de RNAm de RyR (Rn01470303_m1), PLB (Rn01434045_m1) e SERCA2a (Rn00568762_m1). Para tal, utilizaram-se Taq Man Universal PCR Master Mix (Applied Biosystems, CA, EUA), conforme as instruções do fabricante, e o sistema de detecção Applied Biosystems Step One Plus. Todas as amostras foram avaliadas em duplicata. As condições de ciclagem foram as seguintes: ativação da enzima a 50°C por 2 minutos; desnaturação a 95°C por 10 minutos; amplificação dos produtos de cDNA por 40 ciclos de desnaturação a 95°C por 15 segundos; e anelamento/extensão a 60°C por 1 minuto. A expressão gênica foi quantificada em relação aos valores do grupo S e após normalização por um controle interno β-actina (ACTB, Rn00667869_m1), sendo determinada pelo

método 2-ΔΔCt, como anteriormente descrito (Livak *and* Schmittgen 2001; Lima Leopoldo *et al*. 2013).

Análise estatística

Para análise da normalidade foi utilizado o teste de Shapiro Wilk. Os dados foram expressos em média ± desvio padrão, mediana e valores mínimo e máximo. Para comparação entre os grupos foram utilizados o teste não paramétrico de Kruskal-Wallis seguido de pós teste de Dunn's (PVAT; PET; átrios; fígado úmido/seco; fosfolamban) ou teste paramétrico ANOVA (Oneway), seguido pelo pós teste de Tukey (PFV; VD; VE; pulmão úmido/seco; análise histológica e histomorfométrica; rianodina; Serca2a). Foi considerado significante o valor de $p< 0,05$. O software utilizado foi o Graph Pad Prism®.

RESULTADOS

Avaliação ecocardiográfica

Os resultados da avaliação ecocardiográfica do VD mostram que os animais que foram tratados com monocrotalina apresentavam disfunção VD caracterizada pela diminuição de 23% da velocidade máxima da artéria pulmonar (S vs. SM, p=0,001) e diminuição de 30% do tempo de aceleração da artéria pulmonar (S vs. SM, p=0,005). O treinamento físico foi capaz de normalizara velocidade máxima da artéria pulmonar no grupo TM com aumento de 28% (SM vs. TM, p=0,001) (Figura 2).

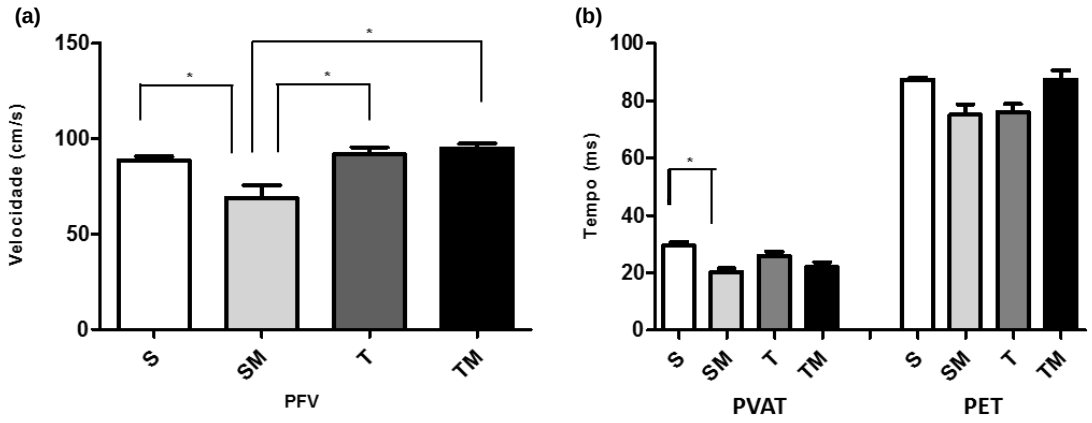

Figura 2. Ecocardiograma: (a) Velocidade do Pico de Fluxo (PFV); (b) Velocidade Pulmonar Tempo de Aceleração (PVAT) e do Tempo de Ejeção Pulmonar (PET). Sedentário Controle (S); Sedentário Monocrotalina (SM); Treino Controle (T); Treino Monocrotalina (TM); cm/s: centímetro por segundo; ms: milisegundo. * *p*<0,05.

Avaliação dos Parâmetros Anatômicos

A tabela 1 e figura 3 mostram os parâmetros anatômicos dos grupos S, SM, T e TM. Após 35 dias, os animais que receberam a monocrotalina do grupo SM e TM apresentaram sinais de disfunção cardíaca no exame pós morte, tais como: hipertrofia atrial e ventricular direita: átrios de 43% (S vc. SM, *p*=0,001) e do VD de 46% (S vs. SM, *p*=0,0004), sem derrame pleural e congestão hepática. Os animais dos grupos controles S e T não apresentaram alterações.

Tabela 1. Dados anatômicos (peso) do pulmão e fígado, expressos em média ± desvio padrão, mediana, mínimo e máximo e *p*-valor.

	GRUPOS				
VARIÁVEIS	**S**	**SM**	**T**	**TM**	***p*-valor**
Pulmão	4,45 ± 0,72	4,84 ± 0,32	4,72 ± 0,33	4,04 ± 1,07	
úmido/seco (g)	4,81 [3,22 - 5,05]	4,90 [4,17 - 5,12]	4,87 [4,07 - 5,08]	4,57 [2,65 - 5,26]	0,13
Fígado	4,37 ± 2,99	3,36 ± 0,18	03,29 ± 0,29	3,32 ± 0,04	
úmido/seco (g)	3,19 [2,89 - 0,48]	3,27 [3,21 - 3,74]	3,23 [2,80 - 3,83]	3,32 [3,25 - 3,38]	0,14

Sedentário Controle (S); Sedentário Monocrotalina (SM); Treino Controle (T); Treino Monocrotalina (TM); g:gramas. *p*<0,05.

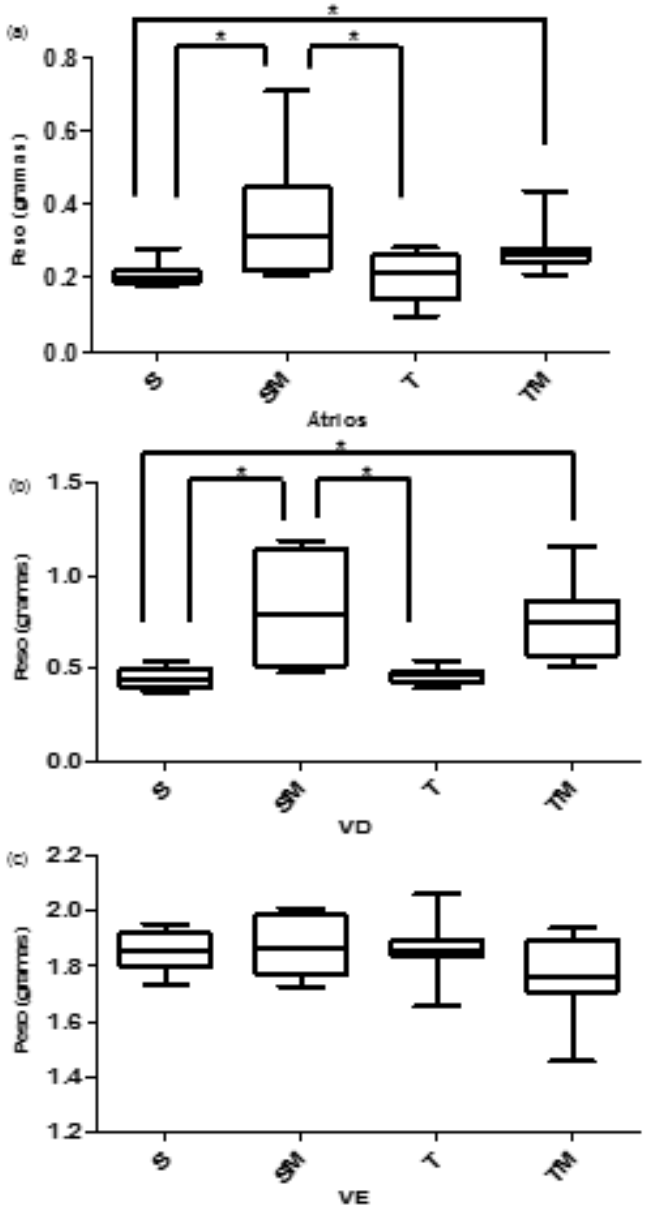

Figura 3. Box Plot dos Parâmetros Anatômicos: (a) átrios; (b) VD (ventrículo direito); (c) VE (ventrículo esquerdo). Sedentário Controle (S); Sedentário Monocrotalina (SM); Treino Controle (T); Treino Monocrotalina (TM). *p<0,05.

Análise Histológica e Histomorfométrica

Houve aumento de 41% da área seccional dos cardiomiócitos indicando hipertrofia ventricular direita nos animais do grupo monocrotalina (S vs. SM, p=0,0001) e o exercício físico foi capaz de amenizar a hipertrofia

nestes animais, valores observados na diminuição de 21% dos cardiomiócitos (SM vs. TM, *p*=0,0001) (Figura 4).

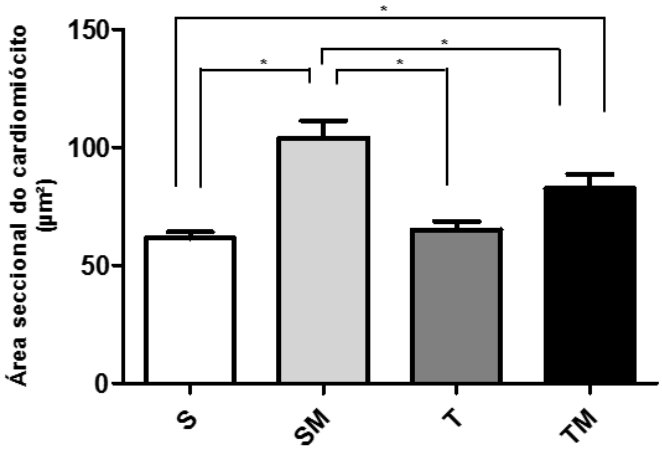

Figura 4. Análise Histomorfométrica: valores expressos em média e desvio padrão referente ao tamanho dos cardiomiócitos mensurados em µm². Sedentário Controle (S); Sedentário Monocrotalina (SM); Treino Controle (T); Treino Monocrotalina (TM); * *p*<0,05.

Expressão gênica relativa de reguladores do Ca^{2+} intracelular

Foi observada diferença estatística apenas no gene Rianodina, com diminuição de 46% (S vs. SM, *p*=0,02) (Figura 5).

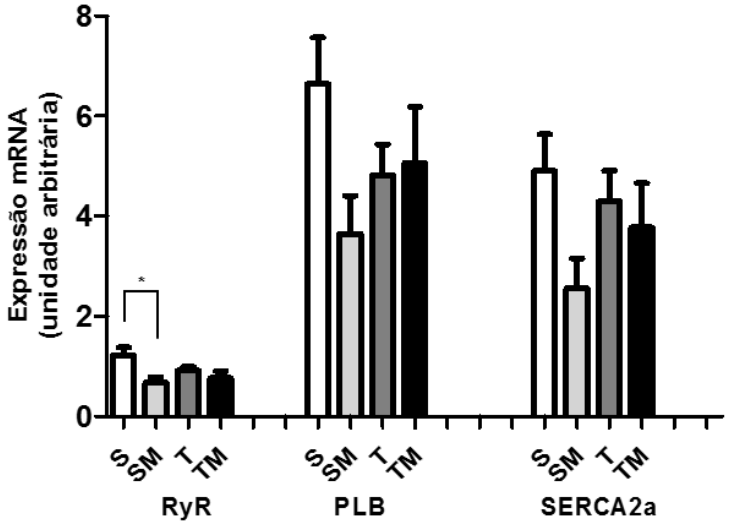

Figura 5. Expressão Gênica das proteínas do cálcio cardíaco: RyR, PLB e Serca2a. Valores mensurados em unidade arbitrária. Todas as análises dos genes foram normalizados pela β-Actina.

Sedentário Controle (S); Sedentário Monocrotalina (SM); Treino Controle (T); Treino Monocrotalina (TM); RyR: Rianodina; PLB: Fosfolamban; SERCA2a: Serca 2a. $p<0,05$: *S vs. M.

DISCUSSÃO

O principal achado deste estudo aponta que o treinamento aeróbio preventivo exerceu efeito cardioprotetor ao VD, demonstrado por diminuição da hipertrofia do VD e melhora funcional ventricular sem alterar a expressão gênica das proteínas envolvidas no trânsito de cálcio cardíaco. Pelo nosso conhecimento esse é o primeiro estudo que avalia o efeito cardioprotetor do treinamento aeróbio prévio em ratos com HAP.

O modelo experimental utilizado neste estudo para induzir HAP foi pela monocrotalina, o qual é bastante utilizado para promover disfunção VD e IC (Lopes *et al*. 2008; Souza-Rabbo *et al*. 2008; Gomez-Arroyo *et al*. 2012; Pereira *et al*. 2013; Colombo *et al*. 2013; Alencar *et al*. 2014). Neste modelo experimental a remodelação VD é caracterizada por hipertrofia do VD, piora funcional e evolução para IC direita (Handoko *et al*. 2009; Maarman *et al*. 2013).

No presente estudo, os animais evoluíram para quadro de HAP e disfunção VD sem IC como demonstrado pelas alterações ecocardiográficas, hipertrofia no VD, aumento da área do cardiomiócito sem apresentar retenção hídrica, o que confirma a eficácia do modelo escolhido para promover a disfunção VD. A presença da disfunção ventricular e não da IC direita pode ser em decorrência da indução da HAP em uma fase mais tardia, uma vez que os estudos induzem mais precocemente (Souza-Rabbo *et al*. 2008; Zapata-Sudo *et al*. 2012; Colombo *et al*. 2013). A idade, o peso, a dose de monocrotalina e o número de dias que os ratos são mantidos depois da injeção de monocrotalina,

foram determinantes nos ratos com disfunção VD, sem IC. Estudos relatam que a monocrotalina na dose de 60 ou 80 mg/Kg pode leva ao desenvolvimento da IC progressivamente e pode ser fatal entre 3-6 semanas (Hessel *et al*. 2006; Handoko *et al*. 2009; Ruiter *et al*. 2013).

A HAP tem sido bastante estudada uma vez que ocasiona limitações funcionais, perda na capacidade de exercício e piora na qualidade de vida (Zafrir 2013; Mocumbi *et al*. 2015; Sahni *et al*. 2015). Os avanços no tratamento medicamentoso e a implementação do exercício físico melhoram o prognóstico, a qualidade de vida e a capacidade funcional em pacientes, e, em modelos experimentais animais os efeitos benéficos do treinamento na remodelação cardíaca direita estão sendo evidenciados (Mereles *et al*. 2006; Handoco *et al*. 2009; Natali *et al*. 2015). No passado, a prática de exercício físico por pacientes com HAP foi contra-indicada devido ao risco de morte súbita. No entanto, a aplicação de exercício foram re-examinadas nestes pacientes devido a evidência dos seus efeitos benéficos (Zafrir 2013). No entanto, não há informações que demonstrem como o treinamento aeróbio preventivo atua nesta condição.

Poucos estudos avaliam a influência do exercício preventivo na remodelação cardíaca, e os que avaliam esse aspecto enfatizam o VE (Portes *and* Tucci 2006; Veiga *et al*. 2011; Veiga *et al*. 2013). Estudos que abordam treinamento preventivo foram realizados em modelos experimentais de ratos com infarto agudo do miocárdio que realizaram natação e mostraram resultados controversos. Embora um estudo demonstrou que o exercício aeróbio preventivo tenha sido eficaz para proteger o coração (Portes *and* Tucci 2006), há pesquisas que não evidenciaram benefícios deste exercício nesta condição. (Veiga *et al*. 2011; Veiga *et al*. 2013). Os protocolos de treinamento

preventivo no infarto agudo do miocárdio foram realizados em média de 8 semanas, sendo este o período utilizado no nosso estudo (Freimann *et al*. 2005; Bozi *et al*. 2013; Veiga *et al*. 2013).

Estudos que utilizaram treinamento físico em ratos com HAP já instalada no modelo da monocrotalina evidenciaram melhora na sobrevida, na disfunção vascular pulmonar, retardando a progressão da HAP, com melhora hemodinâmica, mas sem influenciar na hipertrofia do VD. (Souza-Rabbo *et al*. 2008; Colombo *et al*. 2013; Natali *et al*. 2015).

Nosso estudo demonstrou como aspecto importante de cardioproteção do treino aeróbio preventivo a melhora funcional e da hipertrofia ventricular. A função cardíaca avaliada pelo ecocardiograma foi demonstrada pelo aumento da contratilidade do VD, evidenciada pelo parâmetro PFV , sem alterar o PVAT que é um indicador de gravidade da HAP (Greenberg 2001; Rudski, et al. 2010).

Logo, o treinamento aeróbio melhorou a função do VD sem alterar a gravidade da HAP, o que pode ser justificado por ações diretas do exercício no VD, sem diminuir a HAP, mecanismos não estudados mas que já foram elucidados em estudos anteriores (Ryan and Archer 2014; Talati and Hemmes 2015). Outras pesquisas utilizando este modelo avaliaram a função por meio do cateterismo (Colombo *et al*. 2013), entretanto o ecocardiograma também avalia a função cardíaca e não é uma conduta invasiva (Eguchi *et al*. 2014; Alencar *et al*. 2014). Um dos mecanismos que podem estar envolvido com a disfunção VD são as proteínas do trânsito de cálcio, onde temos receptor de rianodina (RyR), fosfolamban (PLB) e Ca^{2+}ATPase do retículo sarcoplasmático (SERCA2a) (Opie 1998; Lima-Leopoldo *et al*. 2013). A piora da função sistólica no nosso estudo pode estar relacionada com a expressão gênica da RyR. A RyR é uma

proteína tetramérica, que tem peso molecular de 565 kDa sendo predominante no músculo cardíaco (Yano *et al*. 2008). Quando o canal de RyR2 é aberto, o cálcio é liberado do retículo sarcoplasmático para o citoplasma o que permite o sistema acoplamento excitação/contração cardíaco (Meissner 1994; Eisner 2014). Alterações na RyR2 favorecem arritmias e IC (Marx *et al*. 2000; Eisner 2014), e mesmo o treinamento físico preventivo não alterando a expressão desta, outras condutas que melhorem o trânsito de cálcio na HAP podem ser consideradas. Outros mecanismos moleculares podem estar envolvidos na disfunção contrátil, como a endotelina-1 (ET-1), peptidio nutriurético atrial (ANP) e o fator de crescimento semelhante a insulina (IGF-1) (Loennechen *et al*. 2001; Wisloff *et al*. 2002; Fontoura *et al*. 2014), fatores neuro-humorais, o sistema nervoso simpático por meio dos receptores adrenérgicos (α1a, β1 e β2) e o sistema endócrino via angiotensina II (Ang II) (Dai *et al*. 2011). Da mesma forma os mediadores de fibrose (colágeno I, colágeno III, e TGF-β1 (Yan *et al*. 2011), mecanismos neuromoduladores circulatórios (óxido nítrico) (Xiao *et al*. 2012) e a expressão da α e β-MHC (Fernandes *et al*. 2011) também podem estar envolvidos. Outro aspecto a ser considerado é que o aumento da atividade física tem efeitos conhecidos sobre o conteúdo mitocondrial, respiração mitocondrial e utilização do substrato, que pode muito bem ter efeitos sobre a função VD de ratos monocrotalina (tratada ou não) (Piao et al 2010; Ryan et al 2014; Talati e Hemnes 2015). Mais estudos são necessários para investigar esses outros mecanismos moleculares.

Estudos prévios demonstraram que a redução da hipertrofia cardíaca está diretamente associada com a diminuição da espessura da artéria pulmonar e do volume intersticial, resultando na diminuição da resistência vascular pulmonar e consequente redução da pós carga VD (D'Alonso *et al*.

1991; Talati *and* Hemnes 2015). Tal mecanismo poderia explicar o efeito atenuador do treinamento aeróbio prévio sobre a hipertrofia cardíaca durante condições de HAP. A hipertrofia foi avaliada por meio da mensuração da área dos cardiomiócitos no VD. Diferentemente dos resultados de Colombo *et al*. 2013 que realizou o treinamento após a aplicação da monocrotalina, em nosso estudo, o treinamento preventivo amenizou a hipertrofia do VD. Colombo *et al*, 2013, entretanto, avaliou a hipertrofia por meio do parâmetro anatômico, e não mensurou a área do cardiomiócito. A diminuição da hipertrofia ventricular no nosso estudo pode estar relacionada a modificações moleculares envolvendo vias de sinalização como a da proteína GSK -3β, já avaliada neste modelo (Colombo *et al*. 2013). Outras vias podem estar relacionados com a hipertrofia ventricular: mTOR, FOXO3a, FOXO1, calcineurina, mindin, AKT, receptor proliferação ativado de peroxisoma (PPAR delta) (Jucker *et al*. 2007; Yan *et al*. 211; Colombo *et al*. 2013).

Algumas limitações devem ser consideradas. Primeiramente, os animais do grupo TM continuaram a realizar o treinamento por mais 3 semanas para impedir os efeitos do destreinamento que eles teriam com a interrupção do treino. Outro aspecto a ser considerado é que este treinamento mesmo sendo continuado por 3 semanas após a aplicação da monocrotalina, ele é preventivo pois o treinamento foi realizado anteriormente à insuficiência cardíaca, no estágio de disfunção ventricular. Outra limitação foi a implicações biológicas da técnica de análise da expressão do RNAm e seu impacto sobre as vias de sinalização que não podem ser determinadas com confiança usando somente a análise de RT-qPCR. Além disso, quando se avalia a expressão do RNAm de forma isolada não está relacionada à expressão da atividade proteica e estas estão intrinsecamente ligadas. Desta forma, se a disfunção cardíaca

ocasionada pela HAP reflete um efeito direto ou indireto sobre a mudança no trânsito de cálcio, e se isto está relacionado com a modulação da transcrição ou fatores pós transcricionais, não podemos distinguir. Diante disto, estudos futuros precisam ser realizados para melhor compreensão sobre os mecanismos moleculares envolvidos com a HAP, o treinamento aeróbio prévio e seus efeitos no VD.

CONCLUSÃO

O treinamento aeróbio preventivo promoveu efeito cardioprotetor, diminuindo a hipertrofia e melhorando a função do coração (VD) dos ratos com disfunção VD induzidos por monocrotalina, sem modificar os genes do trânsito de cálcio.

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PREVENTIVE AEROBIC TRAINING EXERTS A CARDIOPROTECTIVE EFFECT IN RATS WITH PULMONARY ARTERIAL HYPERTENSION

PRIOR TRAINING IN PULMONARY HYPERTENSION

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Abstract

Pulmonary Arterial Hypertension (PAH) is a chronic disease which causes overload to the right ventricle. The effect of preventive training on cardiac remodeling in this condition is still unknown. This study aimed to evaluate the influence of preventive training on hypertrophy, heart function and gene expression of calcium transport proteins in rats with monocrotaline-induced PAH. Thirty-two male Wistar rats were randomly divided into 4 groups: sedentary control (S); trained control (T); sedentary monocrotaline (SM); trained monocrotaline (TM). The preventive training protocol was performed on a treadmill for 13 weeks, 5 times/week. The first two weeks were adopted for adaptation to training with gradual increases in speed/time. The speed of the physical training from the third to tenth weeks was gradually increased from 0.9km/h to 1.1km/h for 60 minutes. Next, monocrotaline was applied (60mg/Kg) to induce PAH and lactate threshold analysis performed to determine the training speeds. The training speed of the TM group in the following two weeks was 0.8km/h-60min and the T=0.9km/h-60min; in the final two weeks both groups trained at the same speed and duration 0.9km/h, 60 min. Cardiac function was assessed through echocardiography, ventricular hypertrophy through histomorphometric analysis and gene expression through RT-qPCR. Right cardiac function assessed through the peak flow velocity was SM=75.5cm/s vs. TM=92.0cm/s (p=0.001) and ventricular hypertrophy was SM=106.4μm² vs. TM=77.7μm² (p=0.004). There was a decrease in the gene expression of ryanodine was S=1.12au vs. SM=0.60au (p=0.02) without alterations due to training. Thus, we conclude that prior physical training exerts a cardioprotective effect on the right ventricle in the monocrotaline rat model.

Key words: Ventricular dysfunction, Monocrotaline, Exercise

Introduction

Pulmonary Arterial Hypertension (PAH) is a chronic disease characterized by a progressive increase in pulmonary vascular resistance and pulmonary artery pressure, which causes overload to the right ventricle (RV), leading to a framework of pathological cardiac remodeling characterized by hypertrophy, ventricular dysfunction and RV insufficiency (Humbert *et al*. 2004; Ochiai *et al*. 2008; Zapata-Sudo *et al.* 2012; Mocumbi *et al*. 2015; Talati *and* Hemmes 2015). Data on the exact prevalence of PAH is unknown, and the true figure could be underestimated (Mocumbi *et al*. 2015). The disease presents a poor prognosis with a mean survival of 2.8 years (D'Alonzo *et al*. 1991; Montani *et al*. 2013; Mocumbi *et al*. 2015).

In the development of PAH, RV dysfunction precedes heart failure (HF) and this occurs when there are alterations in the relaxation and/or contraction of the heart muscle without promoting fluid retention or exercise intolerance (Cohn *et al*. 2000; Pacagnelli *et al*. 2014). This RV dysfunction may be related to the molecular mechanisms involved in calcium transport (Opie 1998; Fernandes *et al*. 2015). Several proteins such as ryanodine receptors (RyR), phospholamban (PLB) and Ca^{2+} ATPase of the sarcoplasmic reticulum (SERCA2a), regulate calcium homeostasis in the heart muscle and are essential for its proper functioning (Opie 1998; Lima Leopoldo *et al*. 2013). The majority of studies focus on the left ventricle (LV), even though RV function is fundamental to survival in pathophysiological conditions such as PAH (Risgaard *et al*. 2014; La Gerche *and* Claessen 2015).

The beneficial effects of chronic aerobic physical exercise on RV remodeling in animals with PAH induced by monocrotaline have been described

(Handoko *et al*. 2009, Colombo *et al*. 2013), however, a search in the literature did not reveal any studies which assessed the effects that preventive aerobic training exerts on RV remodeling induced by PAH. Although studies suggest that chronic aerobic physical training applied in a preventive manner in acute myocardial infarction promoted improvement in cardiac function and survival (Waard *and* Duncker 2009; Bozi *et al*. 2013).

To our knowledge there are no studies evaluating the effects that preventive aerobic training exerts on RV remodeling induced by PAH. The aim of the present study was to test the hypothesis that physical aerobic training carried out preventively on PAH induced by monocrotaline in rats would ease RV hypertrophy and improve RV function by altering calcium transport genes.

MATERIALS AND METHODS

Ethics Approval

All experimental protocols used in this study were in accordance with the principles of laboratory animal care formulated by the Brazilian College of Animal Experimentation (COBEA) and according to the *Guide for the Care and Use of Laboratory Animals* published by the *National Research Council* (Clark *et al*, 1997). All procedures were approved by the Ethics Committee of the University of Western São Paulo – UNOESTE (numbers: 1838 and 1839).

Animals

To conduct this study, thirty-two male Wistar rats were used, two months of age and average weight of 206 ± 16.35g, from the Central Animal Facility of the University of Western São Paulo – São Paulo. The animals were kept in the

Animal Experimentation Laboratory of the same institution, in plastic cages with dimensions of 41x34x16 cm (3 animals/cage) at a temperature of 21 to 23°C and relative humidity of 50% to 60%, with luminosity cycles of 12h (light/dark) starting with the light cycle at 7am. The rats received food in a controlled manner (Supralab, Alisul®, Brazil) and water *ad libitum*.

The animals were randomly distributed into 4 experimental groups of 8 animals each, denominated: sedentary control group (S), sedentary monocrotaline (SM) and the groups undergoing the training protocol: trained control group (T) and trained monocrotaline group (TM).

Experimental Design

To conduct the study, the animals in the T and TM groups were submitted to a aerobic training protocol on a treadmill for 13 weeks with a frequency of 5 days a week. The animals underwent two weeks of adaptation to training followed by eleven weeks of training (Machado *et al*. 2006). At the beginning of the 11^{th} week, the rats of the C and T groups were subjected to an intraperitoneal injection of saline, while the SM and TM groups received an injection of monocrotaline. Twenty-four hours after the injection, the animals of the T and TM groups continued aerobic training for another 3 weeks (Colombo *et al.* 2013). For load adjustment and continuity of training, the animals underwent a test for lactate threshold analysis (Carvalho *et al.* 2005).

Two weeks after completing the exercise protocol, an echocardiographic evaluation was performed which identified right ventricular dysfunction. After the echocardiographic evaluation, the animals were weighed and then euthanized with an intraperitoneal dose of sodium pentobarbital (50 mg/Kg). The heart was removed, dissected and the atria (AT), RV and LV separated and weighed.

Anatomical, histological and gene expression of cardiac calcium evaluations were performed.

Details of the aerobic training and experimental design (Figure 1) are described below.

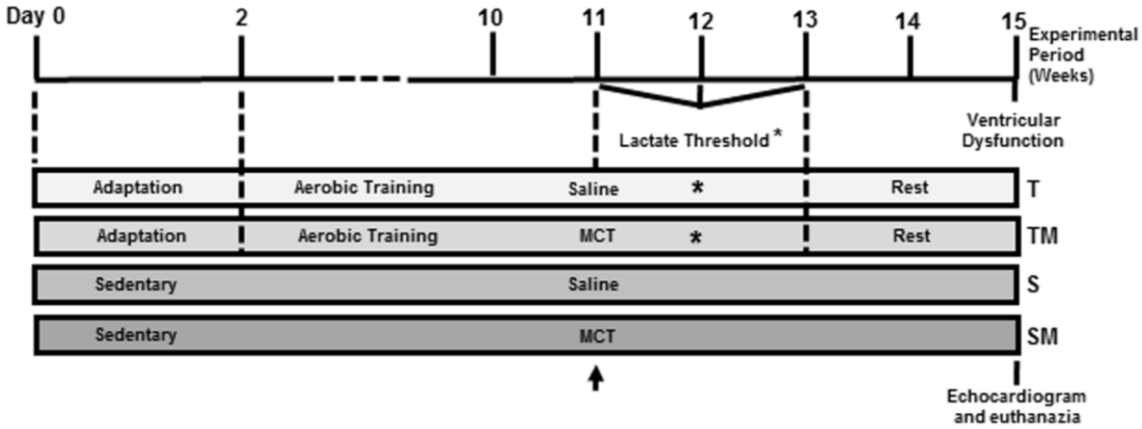

Figure 1. Scheme of the aerobic training and experimental design. Sedentary Control (S); Sedentary monocrotaline (SM); Trained control (T); Trained monocrotaline (TM); MCT: monocrotaline.

Training Protocol

The animals in the T and TM groups underwent an aerobic training protocol performed on a treadmill adapted for rodents (SEBRAE, Presidente Prudente, SP, Brazil). The training was performed for 13 weeks with a frequency of 5 times per week, consisting of 10 weeks prior to the application of the monocrotaline (2 weeks of adaptation and 8 of training) and 3 weeks after the injection of monocrotaline (Colombo *et al*. 2013).

During the adaptation period, the training time and treadmill speed were increased gradually, starting at 0.6 km/hr for 15 minutes on the first day and ending at a speed of 0.9 km/hr for 45 minutes at the end of the second week (Rodrigues *et al*. 2007). After the adaptation, the aerobic physical training was performed for an additional 8 weeks with gradual increases in intensity

(Machado *et al*. 2006). A 5 minute warm-up and cool-down period was included in every session at a speed of 0.6 km/h.

At the beginning of the 11th week of training, 24 hours after the animals of the T (saline) and TM (monocrotaline) groups had received the injections; a test was carried out to assess the velocity of the training corresponding to the lactate threshold. This speed was adjusted after conducting further tests to assess the lactate threshold in the 11th and 12th weeks. The exercise intensity in the 11th week was 60 minutes at 0.8km/h for the TM group and 0.9km/h for the T group. In the final week, both groups underwent training at 0.9km/h for 60 minutes (Souza *et al*. 2014).

Lactate Threshold

To determine the lactate threshold and the speed at which the lactate threshold occurred, the animals in the T and TM groups were submitted to an incremental exercise test on a treadmill for experimental models.

The protocol used was adapted from that previously described by Carvalho *et al*. 2005 and was carried out 24 hrs after the administration of monocrotaline and at the beginning and end of the 11th 12th and 13th weeks, to adjust the training speed. The test began with a two minute warm-up at 0.5 km/h, followed by five minutes of rest. After this the speed was increased to 0.7 km/h for 3 minutes with gradual increases of 0.2 km/h every 3 minutes with a 0% slope, until the lactate reached a value of 1 mmol/L compared to the initial value, or until exhaustion (Bech*et al*. 1990; Svedah *and* Macintosh 2003). Exhaustion was defined as the moment that the rats could no longer keep running for 3 minutes. After each load increase, the animals were manually removed from the training for 1 minute for blood to be collected. Blood samples

were collected from the tail of the animal using an Accutrend Plus lactimeter (Roche®, Portugal). The device was calibrated according to the manufacturer's specifications. The calculation to stipulate the maximum speed was performed using an arithmetic mean of all speeds from each experimental group to reach lactate threshold or exhaustion (Souza *et al*. 2014).

The lactate threshold was defined as the running speed which could be maintained without an increase in lactate of 1.0 mmol/L above the blood lactate concentration of the previous speed (Ferreira *et al*. 2007).

Induction of Right Ventricular Dysfunction

At the beginning of the 11th week, saline (NaCl 0.9%) was administered intraperitoneally to the animals in the S and T groups, in order to ensure that all animals were subjected to the same degree of stress. The protocol for induction of RV dysfunction was performed in the animals of the SM and TM groups, with an intraperitoneal injection of a single dose of monocrotaline (Sigma Chemical, St Louis, MO, USA) in the proportion of 60mg/kg in 1 mol/L HCl buffer pH 7.0 with 1 mol/L of NaOH (Souza-Rabbo *et al*. 2008).

Feed Control

Monocrotaline has been shown to reduce food intake; to control for this effect, the animals were separated into individual cages to dose the daily feed intake. The rats treated with monocrotaline (SM and TM groups) were allowed to eat freely from a supply of standard rat cubes. In the groups S and T, the rats were diet-matched to the treated rats by allowing them only the amount of food consumed on the previous day by the treated rats. The feed control was performed after the administration of monocrotaline, since the animals, which

received the drug, began the framework of pathological RV remodeling, decreasing their feed intake due to RV dysfunction (Lopes et al. 2008).

Echocardiographic evaluation

Echocardiographic evaluation was performed using a commercially available echocardiograph (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with a 5-11.5 MHz multifrequencial probe. Rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (0.5 mg/kg). Pulmonary artery flow was obtained by pulsed Doppler, and the time to peak flow velocity (pulmonary velocity acceleration time - PVAT), pulmonary ejection time (PET), and peak flow velocity were measured (PFV). (Martinez *et al*. 2011; Eguchi *et al*. 2014). PVAT is an indicator of the severity of pulmonary hypertension, the higher the level of systolic pulmonary artery pressure, the smaller is the value of PVAT. PET is a parameter related to the systolic function and PAH degree. PFV is related to RV systolic function (Dabestani *et al*. 1987, Lawrence *et al*. 2010)

Evaluation of Anatomical Parameters

The heart was removed, dissected into the atria (AT), right (RV) and left ventricles (LV) and ventricular septum and weighed. The anatomical parameters were normalized by the final body weight (AT/FBW, RV/FBW and LV/FBW) and were used as the hypertrophy index. The lungs and liver were also removed, weighed and stored in an oven for 48h. Next they were weighed again to calculate the wet/dry weight ratio which was used to evaluate signs of cardiac failure (Carvalho *et al*. 2010).

Histology and Histomorphometric Analysis

Cardiac tissue samples were fixed in 10% buffered formaldehyde solution for 48 hours. After fixation, the tissue was embedded in paraffin blocks to obtain coronal histological sections of 4 micrometers. The histological sections were stained on slides with hematoxylin-eosin solution (HE) to measure the cross-sectional areas of the cardiomyocytes, using a LEICA microscope (model DM750, Germany), which sent digital images to a computer equipped with the analysis system of the Leica images Application Suite LAS 4.2.0 (Media Cybernetics, Silver Spring, Maryland, USA) (Gomes *et al*. 2009; Oliveira-Júnior *et al*. 2010).

The images were obtained using a binocular optical microscope. All images were captured by video camera at 400x magnification (objective 40x). The selection of images to capture and digitization were performed visually. The morphometry of the images obtained and digitalized was accomplished using software appropriate for the purpose. For each of the four slices obtained from the RV of each animal, captures were performed in different fields, chosen according to the area where more cells could be viewed in cross-section. Fifty cells were measured per ventricle analyzed. The cardiomyocytes selected were transversely sectioned and presented a round shape and visible nucleus in the center of the cell and were located in the subendocardial layer of the muscular wall of the RV. This precaution was aimed at standardizing the maximum cardiomyocytes in the different groups. The average cross-sectional areas obtained for each group were used as an indicator of cell size (Oliveira-Júnior *et al*. 2013).

Gene expression relative to regulators of the intracellular Ca^{2+}

The total RNA was extracted from the RV tissue (fresh tissue) using Trizol (Invitrogen), then treated with DNAse according to the manufacturer's instructions. The RNA integrity was assessed through electrophoresis. A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) was used for the synthesis of complementary DNA (cDNA) from 1000 ng of total RNA. RT-PCR was used to quantitatively measure the relative levels of mRNA to RyR (Rn01470303_m1), PLB (Rn01434045_m1) and SERCA2a (Rn00568762_m1). To this end, TaqMan Universal PCR Master Mix was used (Applied Biosystems, CA, USA), according to the manufacturer's instructions, and the detection system Applied Biosystems StepOne Plus. All samples were analyzed in duplicate. The cycling conditions were as follows: enzyme activation at 50°C for 2 minutes; denaturation at 95°C for 10 minutes; amplification of the cDNA products for 40 cycles of denaturation at 95°C for 15 seconds; and annealing/extension at 60°C for 1 minute. The gene expression was quantified in relation to the values of the S group and after normalization by a β-actin internal control (ACTB, Rn00667869_m1), being determined by the 2-ΔΔCt method as previously described (Livak *and* Schmittgen 2001; Lima Leopoldo *et al*. 2013).

Statistical analysis

For analysis of normality, the Shapiro-Wilk test was used. Data are expressed as mean ± standard deviation, median, and minimum and maximum values. For comparison between groups the nonparametric Kruskal-Wallis was used followed by Dunn's post-test (PVAT; PET; atria; wet/dry liver; phospholamban) or the ANOVA parametric test (Oneway), followed by the

Tukey post test (PFV; RV; LV; wet/dry lung; histological and histomorphometric analysis; ryanodine; SERCA2a). A p value<0.05 was considered significant. The software used was GraphPadPrism®.

RESULTS

Echocardiographic evaluation

The results of the echocardiographic evaluation of the RV showed that the animals treated with monocrotaline presented RV dysfunction characterized by decrease 23% the maximal velocity of the pulmonary artery (S vs. SM, *p*=0.001) and decrease 30% pulmonary artery acceleration time (S vs. SM, *p*=0.005). The physical training normalized the maximal velocity of the pulmonary artery in the TM group, increase 28% (SM vs. TM, p=0.001) (Figure 2).

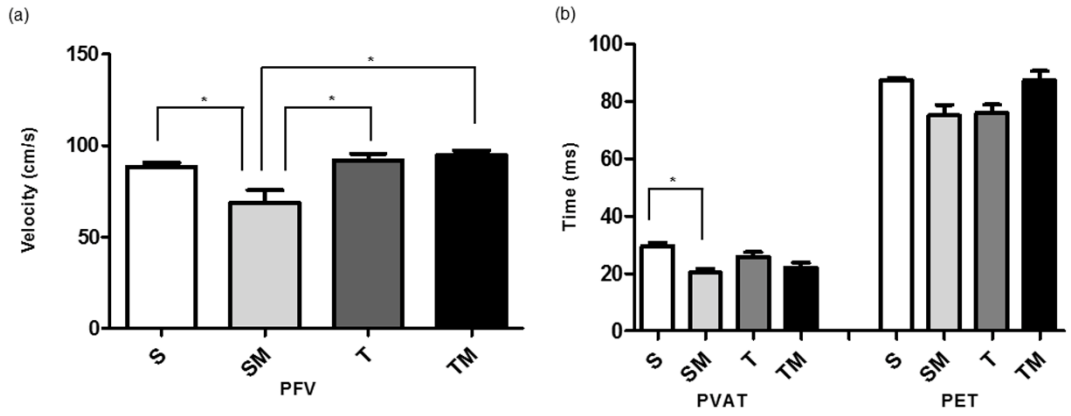

Figure 2. Echocardiogram: values expressed as mean and standard deviation. (a) Peak Flow Velocity (PFV). (b) Pulmonary Velocity Acceleration Time (PVAT) and Pulmonary Ejection Time (PET). Sedentary Control (S); Sedentary Monocrotaline (SM); Trained Control (T); Trained Monocrotaline (TM); cm/s: Centimeters per second; ms: Millisecond. * p <0.05.

Evaluation of Anatomical Parameters

Table 1 and figure 3 presents the anatomical parameters of the S, SM, T and TM groups. After 35 days application the monocrotaline, the animals which received the drug in the SM and TM groups presented signs of RV dysfunction in the post mortem examination, such as: right atria and ventricular hypertrophy: atria increase 43% (S vs. SM, *p*=0.001) and RV increase 46% (S vs. SM, *p*=0.0004) without pleural effusion or liver congestion. The animals of the S and T control groups showed no changes.

Table 1. Anatomical data (weight) of the lung and liver, expressed as mean ± standard deviation, median minimum and maximum and *p*-value.

	GROUPS				
VARIÁBLES	**S**	**SM**	**T**	**TM**	***p*-value**
Lung					
wet/dry (g)	4.81 [3.22 - 5.05]	4.90 [4.17 - 5.12]	4.87 [4.07 - 5.08]	4.57 [2.65 - 5.26]	0.13
Liver					
wet/dry (g)	3.19 [2.89 - 0.48]	3.27 [3.21 - 3.74]	3.23 [2.80 - 3.83]	3.32 [3.25 - 3.38]	0.14

Sedentary Control (S); Sedentary Monocrotaline (SM); Trained Control (T); Trained Monocrotaline (TM); g: grams. p<0.05.

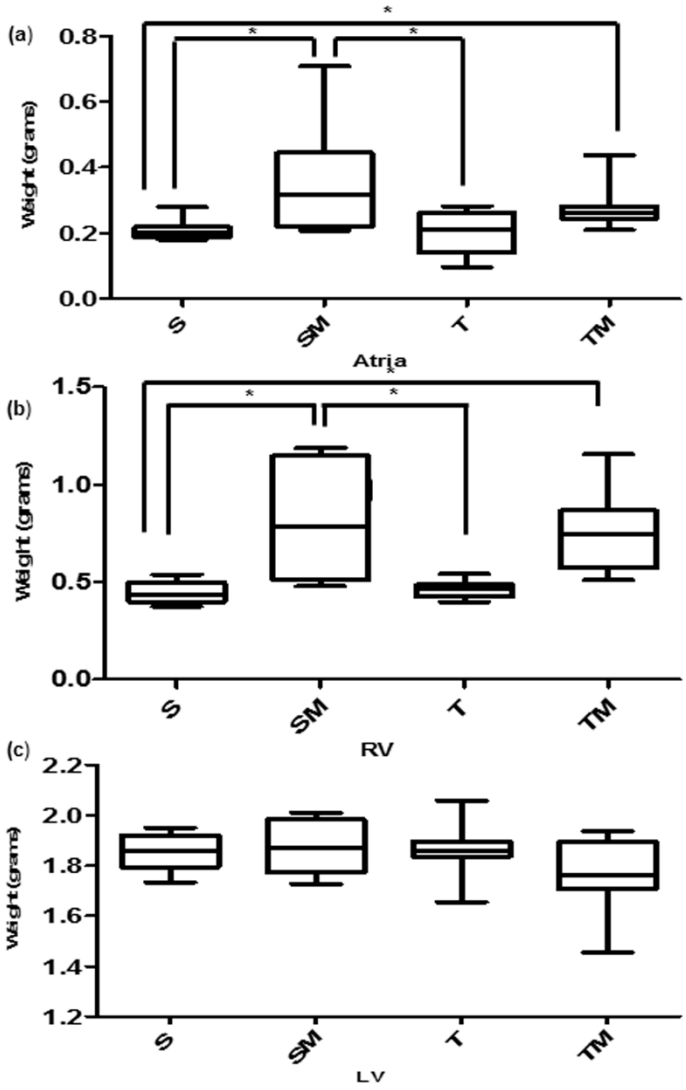

Figure 3. Box Plot corresponding to evaluation of anatomical parameters: (a) Atria; (b) RV (right ventricle); (c) LV (left ventricle). Sedentary Control (S); Sedentary Monocrotaline (SM); Trained Control (T); Trained Monocrotaline (TM). * p<0.05.

Histological and Histomorphometric Analysis

There was an increase 41% in sectional area of the cardiomyocytes, indicating right ventricular hypertrophy, in the animals of the monocrotaline group (S vs. SM, *p*=0.0001); the physical exercise was able to relieve the hypertrophy in these animals, values observed in decrease 21% the cardiomyocytes (SM vs. TM, *p*=0.0001) (Figure 4).

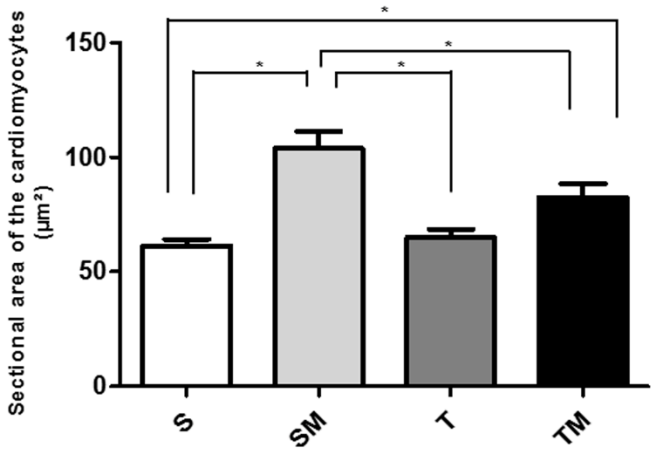

Figure 4. Histomorphometric analysis: values expressed as mean and standard deviation. Sedentary Control (S); Sedentary Monocrotaline (SM); Trained Control (T); Trained Monocrotaline (TM);* p<0.05.

Relative gene expression of Regulators of intracellular Ca^{2+}

A statistical difference was observed only in the ryanodine gene, decrease 46% (S vs. SM, p=0.02) (Figure 5).

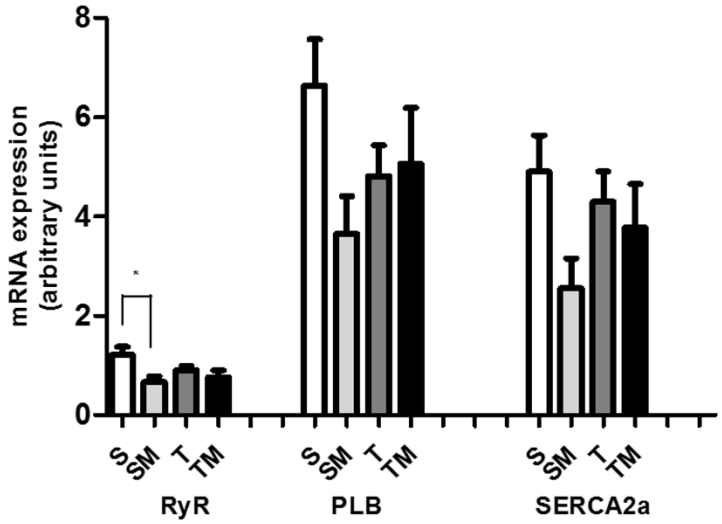

Figure 5. Gene expression of cardiac calcium proteins: Data expressed as mean ± standard deviation. RyR, PLB and Serca2a. All analyzes of the genes were normalized by β-actin. Sedentary Control (S); Sedentary Monocrotaline (SM); Trained Control (T); Trained Monocrotaline (TM); RYR: ryanodine; PLB: phospholamban; SERCA: Serca 2a. p<0.05: *S vs. M

DISCUSSION

The main finding of this study indicates that preventive aerobic training exerted a cardioprotective effect on the RV, demonstrated by decreased RV hypertrophy and ventricular functional improvement without altering the gene expression of proteins involved in cardiac calcium transport. To our knowledge this is the first study to assess the cardioprotective effect of prior aerobic training in rats with PAH.

The experimental model used in this study to induce PAH was through monocrotaline, which is widely used to promote RV dysfunction and HF (Lopes *et al*. 2008; Souza-Rabbo *et al*. 2008; Gomez-Arroyo *et al*. 2012; Pereira *et al*. 2013; Colombo *et al*. 2013; Alencar *et al*. 2014). In this experimental model, cardiac remodeling is characterized by RV hypertrophy, functional deterioration and progression to right-sided HF (Handoko *et al*. 2009; Maarman *et al*. 2013).

In the present study, the animals progressed to PAH and RV dysfunction without HF, as demonstrated by the echocardiographic alterations, RV hypertrophy, increased areas of cardiomyocytes without presenting fluid retention, which confirms the effectiveness of the chosen model to promote RV dysfunction. The presence of RV dysfunction and not right-sided HF could be due to the induction of PAH at a later stage, since other studies induce PAH at an earlier stage (Souza-Rabbo *et al*. 2008; Zapata-Sudo *et al*. 2012; Colombo *et al*. 2013). The age, weight and that the dose of monocrotaline and the number of days rats are kept after monocrotaline-injection, are greater determinants of the rats having RV dysfunction without HF. Studies have reported that a dose of monocrotaline at 60 or 80 mg/kg can lead to the development of progressive HF and can be fatal within 3-6 weeks (Hessel *et al*. 2006; Handoko *et al*. 2009; Ruiter *et al*. 2013).

PAH has been widely studied since it causes functional limitations, loss of exercise capacity and a poor quality of life (Zafrir 2013; Mocumbi *et al*. 2015; Sahni *et al*. 2015). Advances in drug treatment and the implementation of physical exercise improve the prognosis, quality of life and functional capacity of patients and in experimental animal models the beneficial effects of training on RV remodeling are highlighted (Mereles *et al*. 2006; Handoko *et al*. 2009; Natali *et al*. 2015). In the past, the practice of physical exercise by patients with PAH was contra indicated due to the risk of sudden death. However, the application of exercise has been reconsidered in these patients due to accumulating evidence of its beneficial effects (Zafrir 2013). However, there is no information demonstrating how preventive aerobic training acts on this condition.

Few studies have evaluated the influence of preventive exercise on cardiac remodeling, or the same aspect with emphasis on the LV (Portes *and* Tucci 2006; Veiga *et al*. 2011; Veiga *et al*. 2013). Studies addressing preventive training were performed in experimental models in rats with acute myocardial infarction and presented controversial results. Aerobic exercise is a non-pharmacological and effective way to protect the heart against aggression (Portes *and* Tucci 2006), however, there are studies that found no benefits to this condition (Veiga *et al*. 2011; Veiga *et al*. 2013). The preventive training protocols for acute myocardial infarction were performed for an average of 8 weeks, which was the period used in the present study (Freimann *et al*. 2005; Bozi *et al*. 2013; Veiga *et al*. 2013).

Studies that used physical training in rats with PAH, previously installed through the monocrotaline model, demonstrated improvements in survival and pulmonary vascular dysfunction, slowing the progression of PAH, with

hemodynamic improvement, but without influencing RV hypertrophy (Souza-Rabbo *et al*. 2008; Colombo *et al*. 2013; Natali *et al*. 2015).

Our study demonstrated, as an important cardioprotective aspect of preventive aerobic training, improvement in function and ventricular hypertrophy. Cardiac function was assessed through echocardiography, demonstrated by the increase in RV contractility, evidenced by the PFV parameter, without changing the PFV which is an indicator of severity of PAH (Greenberg 2001; Rudski, et al. 2010). Soon PHYSICAL TRAINING improved RV function without changing the PAH severity, which can be justified by some direct actions of the training in the RV, without decreasing the PAH, mechanisms have not been studied but have been elucidated in previous studies (Ryan and Archer 2014; Talati and Hemmes 2015). Other studies using this model evaluated cardiac function through catheterization (Colombo *et al.* 2013); however echocardiography also evaluates cardiac function and is not an invasive method (Eguchi *et al*. 2014; Alencar *et al*. 2014).

One of the mechanisms that may be involved in RV dysfunction is calcium transport proteins, which include ryanodine receptor (RyR), phospholamban (PLB) and Ca^{2+} ATPase of sarcoplasmic reticulum (SERCA2a) (Opie 1998; Lima-Leopoldo *et al*. 2013). The decline in systolic function in the present study may be related to the gene expression of RyR. RyR is a tetrameric protein which has a molecular weight of 565 kDa being predominant in cardiac muscle (Yano *et al*. 2008). When the RyR2 channel is open, calcium is released from the sarcoplasmic reticulum into the cytoplasm allowing the cardiac excitation/contraction coupling system (Meissner 1994; Eisner 2014). Alterations in RyR2 promote arrhythmias and HF (Marx *et al*. 2000; Eisner 2014), and even though preventive aerobic training does not alter this

expression, other approaches to improve calcium transport in PAH may be considered. Other molecular mechanisms may be involved in the contractile dysfunction, such as endothelin-1 (ET-1), atrial natriuretic peptide (ANP), insulin-like growth factor (IGF-1) (Loennechen *et al*. 2001; Wisloff *et al*. 2002; Fontoura *et al*. 2014), neurohumoral factors, the sympathetic nervous system through the adrenergic receptors (α1a, β1 and β2) and the endocrine system via angiotensin II (Ang II) (Dai *et al*. 2011). Likewise mediators of fibrosis (collagen I, collagen III, and TGF-β1 (Yan *et al.* 2011), circulatory neuromodulatory mechanisms (nitric oxide) (Xiao *et al.* 2012) and expression of MHC-α e β (Fernandes *et al.* 2011) may also be involved. Another aspect to be considered is that the increase in physical activity has known effects on mitochondrial content, mitochondrial respiration and substrate utilization, which may very well have effects on the RV function of monocrotaline rats (treated or untreated) (Piao et al. 2010; Ryan et al. 2014; Talati *and* Hemnes 2015). Further studies are necessary to investigate these molecular mechanisms.

Previous studies have demonstrated that reduction in cardiac hypertrophy is directly associated with thinning of the pulmonary artery and interstitial volume, resulting in decreased pulmonary vascular resistance and consequent reduction in RV afterload (D'Alonso *et al*. 1991; Talati *and* Hemnes 2015). Such a mechanism could explain the attenuating effect of the prior exercise training on cardiac hypertrophy during PAH. The hypertrophy was evaluated by measuring the area of cardiomyocytes in the RV. Differently from the results of Colombo *et al*. 2013 who conducted training after the application of monocrotaline, in the present study, aerobic training mitigated the RV hypertrophy. Colombo *et al*, 2013, however, evaluated the hypertrophy by means of anatomical parameters, and did not measure the area of

cardiomyocytes. The decrease in ventricular hypertrophy in the present study may be related to molecular changes involved in signaling pathways such as the protein GSK -3β, already evaluated in this model (Colombo *et al*. 2013). Other pathways may be related to ventricular hypertrophy: mTOR, FOXO3a, FOXO1, calcineurin, mindin, AKT and peroxisome proliferator-activated receptors (PPAR delta) (Jucker *et al*. 2007; Yan *et al*. 211; Colombo *et al*. 2013).

Some limitations should be considered. Primarily, the animals from TM group kept on training for more three weeks to prevent the effects of detraining they would have to interrupt their training. Another aspect to be considered is that this training even being conducted for three weeks after the application of monocrotaline, it is preventive because the training was performed prior to heart failure since the animals were in a stage of ventricular dysfunction. Another limitation was the biological implications of the analysis technique of mRNA expression and its impact on signaling pathways which cannot be reliably determined using only analysis of RT-qPCR. In addition, when assessing mRNA expression in isolation, it is not related to the expression of protein activity, and these are intrinsically linked. Thus, if the RV dysfunction caused by PAH reflects a direct or indirect effect on the change in calcium transport, and if this is related to the modulation of transcriptional or post transcriptional factors we would be unable to distinguish this. Thus, future studies are needed to better understand the molecular mechanisms involved in PAH, prior physical training and its effects on RV.

CONCLUSION

Preventive aerobic training exerted a cardioprotective effect, decreasing hypertrophy and improving heart function in the rats with monocrotaline-induced RV dysfunction, without modifying the calcium transport genes.

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ANEXO 1 - APROVAÇÃO ÉTICA

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PROBIC - Programa de Bolsas de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"ANÁLISE MORFOLÓGICA E FUNCIONAL DO TREINAMENTO PREVENTIVO REALIZADO EM RATOS COM INSUFICIÊNCIA CARDÍACA INDUZIDA PELA MONOCROTALINA"**, cadastrado na Coordenadoria Central de Pesquisa (CCPq) sob o número nº **1838** e tendo como participante(s) **FRANCIS LOPES PACAGNELLI (responsável), CARLOS ALEXANDRE SANT'ANNA DE OLIVEIRA (técnico Participante), ANA KARENINA DIAS DE ALMEIDA SABELA (discente), MARIANA FERNANDES PELLOSI (discente),** foi avaliado e **APR. COM RECOMENDAÇÃO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Presidente Prudente, 10 de Agosto de 2015.

Prof. Dr. Jair Rodrigues Garcia Jr.
Coordenador Científico da CCPq

Profª Ms. Adriana Falcó de Brito
Coordenadora da CEUA - UNOESTE

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UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PROBIC - Programa de Bolsas de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"AVALIAÇÃO DO TREINAMENTO PREVENTIVO REALIZADO EM RATOS COM INSUFICIÊNCIA CARDÍACA INDUZIDA PELA MONOCROTALINA NA HOMEOSTASIA DO CÁLCIO CARDÍACA"**, cadastrado na Coordenadoria Central de Pesquisa (CCPq) sob o número nº **1839** e tendo como participante(s) **FRANCIS LOPES PACAGNELLI (responsável), INES CRISTINA GIOMETTI (docente), ANA KARENINA DIAS DE ALMEIDA SABELA (discente), NAIR KARINA DE OLIVEIRA (discente),** foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Presidente Prudente, 13 de Agosto de 2015.

Prof. Dr. Jair Rodrigues Garcia Jr.
Coordenador Científico da CCPq

Profª Ms. Adriana Falco de Brito
Coordenadora da CEUA - UNOESTE

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ANEXO 2 - AUTHOR GUIDELINES: INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

Author Guidelines

International Journal of Experimental Pathology is published as an online-only journal from 2014

Ethics

The *International Journal of Experimental Pathology* (IJEP) encourages its contributors and reviewers to adopt the standards of the International Committee of Medical Journal Editors(ICMJE). More information on various issues relating to Publication Ethics are dealt with in the relevant sections below and are outlined in full in the separate document Ethical Policies of the International Journal of Experimental Pathology. Submitted work must comply with these policies, which are based on the Best Practice Guidelines on Publication Ethics: a Publisher's Perspective(Graf C, Wager, E, Bowman A et al. Int J ClinPract 2007;61[s152]:1-26) and the Committee on Publication Ethics (COPE) guidelines on good publication and comply with their Code of Conduct. IJEP is a member of the Committee on Publication Ethics elf Submission is considered on the conditions that papers are previously unpublished, and are not offered simultaneously elsewhere; that all authors (defined below) have read and approved the content, and all authors have also declared all competing interests; and that the work complies with Ethical Policies of the Journal, and has been conducted under internationally accepted ethical standards after relevant ethical review.

Animal and Human Studies

Manuscripts describing studies involving animals should comply with local/national guidelines governing the use of experimental animals and must contain a statement indicating that the procedures have been approved by the appropriate regulaendntory body. Manuscripts concerned with human studies must contain statements indicating that informed, written consent has been obtained, that studies have been performed according to the Declaration

of Helsinki, and that the procedures have been approved by a local ethics committee. If individuals might be identified from a publication (e.g. from images) authors must obtain explicit consent from the individual.

Disclosures

Authors are required to disclose financial interests (e.g. employment, significant share ownership, patent rights, consultancy, research funding, etc.) in any company or institution that might benefit from their publication. All authors must provide details of any other potential competing interests of a personal nature that readers or editors might consider relevant to their publication. Upon acceptance for publication, all authors should supply this information, to the Journal's Editorial Assistant, Biljana Nikolic at b.nikolic@ucl.ac.uk . All **sources of funding must be disclosed** in the Acknowledgments section of the paper. List governmental, industrial, charitable, philanthropic and/or personal sources of funding used for the studies described in the manuscript and attribute the funding to appropriate authors.

Examples:

• This work was supported by a grant from the National Institutes of Health, USA (DKxxxx to AB).

• This work was supported by the Crohn's and Colitis Foundation of Canada (grant to AB and CD).

• This work was supported by a grant from Big Pharma Inc. (to AB) and equipment was donated by Small Pharma Inc. EF received a graduate studentship award from the University of xxxxx.

Authorship

All authors must fulfil the following three criteria:

• Substantial contributions to research design, or the acquisition, analysis or interpretation of data,

• Drafting the paper or revising it critically, and

• Approval of the submitted and final versions

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