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IMPACTO DO MIRTILO SOBRE PARÂMETROS FUNCIONAIS E DE ESTRESSE OXIDATIVO EM TECIDO PULMONAR DE RATOS SUBMETIDOS À HIPERTENSÃO ARTERIAL PULMONAR

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel(a) em Biomedicina.

Orientador: Prof. Dr. Alex Sander da Rosa Araujo
Coorientador: Me. Patrick Türck

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RESUMO

A hipertensão arterial pulmonar (HAP) é uma patologia que se caracteriza inicialmente por aumento da resistência e redução gradativa do lúmen vascular pulmonar, levando ao aumento dos níveis pressóricos pulmonares e progressiva insuficiência cardíaca direita. Sabe-se que esses eventos são agravados pela presença do estresse oxidativo. Nesse contexto, a utilização de antioxidantes naturais como o mirtilo (*blueberry*) pode representar uma alternativa terapêutica. O objetivo deste trabalho foi avaliar o efeito do extrato de mirtilo sobre parâmetros funcionais e de estresse oxidativo no pulmão de ratos induzidos à hipertensão pulmonar. No presente estudo, ratos machos Wistar foram submetidos à HAP pela administração de monocrotalina (60 mg/kg I.P). Durante 5 semanas os animais foram tratados com extrato de mirtilo nas doses de 50, 100 e 200 mg/kg via gavagem. No 35º dia de protocolo experimental foram realizados o ecocardiograma e o cateterismo dos animais, com consequente eutanásia e coleta dos pulmões para posteriores análises bioquímicas. Os resultados encontrados mostram que o tratamento com o extrato de mirtilo aumentou a razão E/A do fluxo pela tricúspide (todas as doses testadas) e diminuiu a pressão média da artéria pulmonar (dose de 100 mg/kg) dos animais em relação ao grupo monocrotalina. Analisando-se o tecido pulmonar, o tratamento foi capaz de diminuir a concentração de espécies reativas totais e a atividade da enzima NADPH oxidase (todas as doses testadas) em relação ao grupo monocrotalina. Além disso, animais tratados com o extrato apresentaram aumento da atividade da enzima superóxido dismutase (SOD) e restabeleceram o conteúdo de sulfidrilas (dose de 100 mg/kg) em comparação aos animais monocrotalina. A expressão da proteína Nrf2, importante ativador de genes que transcrevem para enzimas antioxidantes, se mostrou diminuída nos pulmões dos animais monocrotalina. Por outro lado, o tratamento com extrato de mirtilo (dose de 100 mg/kg) restabeleceu a expressão de Nrf2 a nível dos animais do grupo controle. Nossos dados mostram que de fato há uma ruptura na homeostasia redox no pulmão acometido pela HAP, relacionada a perdas hemodinâmicas. O uso do extrato de mirtilo promoveu a retomada do balanço redox, principalmente modulando enzimas (NADPH oxidase e SOD) que participam intimamente do metabolismo do óxido nítrico, importante vasodilatador. A melhora redox foi acompanhada de importantes benefícios funcionais, sugerindo que o consumo de alimentos ricos em antioxidantes, como o mirtilo, pode ser eficaz em conferir proteção e retardar o desenvolvimento da HAP.

Palavras-chave: Mirtilo. Hipertensão Pulmonar. Estresse Oxidativo. Monocrotalina.

ABSTRACT

Pulmonary arterial hypertension (PAH) is a pathology characterized initially by increased resistance and gradual reduction of pulmonary vascular lumen, leading to increased pulmonary pressure levels and progressive right heart failure. It is known that these events are aggravated by the presence of oxidative stress. In this context, the use of natural antioxidants such as blueberry may represent a therapeutic alternative. The objective of this work was to evaluate the effect of the blueberry extract on functional parameters and oxidative stress in the lung of rats induced to pulmonary hypertension. In the present study, male Wistar rats were subjected to PAH by the administration of monocrotaline (60 mg/kg I.P). For 5 weeks the animals were treated with blueberry extract at doses of 50, 100 and 200 mg/kg via gavage. On the 35th day of the experimental protocol, echocardiography and catheterization of the animals were performed, with consequent euthanasia and lung collection for subsequent biochemical analysis. The results showed that treatment with the blueberry extract increased the tricuspid E/A ratio (all doses tested) and decreased the mean pulmonary artery pressure (dose of 100 mg/kg) of the animals in relation to monocrotaline group. Analyzing lung tissue, the treatment was able to decrease the concentration of total reactive species and the activity of NADPH oxidase enzyme (all doses tested) in relation to the monocrotaline group. In addition, animals treated with the extract showed an increase in the activity of superoxide dismutase enzyme (SOD) and reestablished the content of sulfhydryls (dose of 100 mg/kg) in comparison to monocrotaline animals. Expression of the Nrf2 protein, an important activator of genes that transcribe to antioxidant enzymes, has been shown to be decreased in the lungs of monocrotaline animals. On the other hand, the treatment with blueberry extract (dose of 100 mg/kg) reestablished the expression of Nrf2 to the level of control group. Our data show that in fact there is a rupture in redox homeostasis in the lung affected by PAH, related to hemodynamic losses. The use of the blueberry extract promoted the recovery of the redox balance, mainly modulating enzymes (NADPH oxidase and SOD) that participate closely in the metabolism of nitric oxide, an important vasodilator. The redox improvement was accompanied by important functional benefits, suggesting that the consumption of foods rich in antioxidants, such as blueberry, may be effective in conferring protection and delaying the development of PAH.

Keywords: Blueberry. Pulmonary Hypertension. Oxidative Stress. Monocrotaline.

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LISTA DE ABREVIATURAS

EO	Estresse Oxidativo
ERO	Espécies Reativas de Oxigênio
ERN	Espécies Reativas de Nitrogênio
HAP	Hipertensão Arterial Pulmonar
HP	Hipertensão Pulmonar
H_2O_2	Peróxido de Hidrogênio
IC	Insuficiência Cardíaca
MCT	Monocrotalina
Nrf2	NF-E2-related fator 2
$NO^{\bullet}$	Óxido Nítrico
$O_2^{\bullet -}$	Ânion Radical Superóxido
PMAP	Pressão Média na Artéria Pulmonar
RL	Radicais Livres
RVP	Resistência Vascular Pulmonar
SOD	Superóxido Dismutase
VE	Ventrículo Esquerdo
VD	Ventrículo Direito

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1. INTRODUÇÃO

1.1 HIPERTENSÃO PULMONAR

A hipertensão pulmonar (HP) é uma comorbidade comum a muitas doenças cardíacas e pulmonares, para as quais a condição do leito vascular pulmonar frequentemente é a principal determinante das manifestações clínicas e do curso clínico da doença. A circulação pulmonar é caracterizada por apresentar uma baixa pressão, ser altamente distensível, com alta capacidade e baixa resistência ao fluxo sanguíneo, sendo as pressões sistólica e diastólica na faixa de 15 a 25 mmHg e 5 a 10 mmHg, respectivamente, em indivíduos normais na posição supina (BARST, 2010). Essa doença pode ocorrer como um acontecimento independente ou associado a alterações cardiopulmonares, estando relacionada a altos níveis de morbidade e mortalidade (KIELY et al., 2013).

A HP tem sido caracterizada em indivíduos que apresentam uma pressão média na artéria pulmonar (PMAP) $\geq$ 25 mmHg em repouso. Pessoas com pressões entre 21 e 24 mmHg devem ser monitoradas cuidadosamente, particularmente quando apresentarem risco para o desenvolvimento da hipertensão arterial pulmonar (situações em que há casos hereditários da doença na família, por exemplo) (HOEPER et al., 2013).

Descrita como uma doença progressiva que advém de alterações vasculares, a HP ocasiona redução gradativa do lúmen, resultando em um aumento da resistência vascular pulmonar (RVP) com elevação inapropriada dos níveis pressóricos. Em seu processo final, por sobrecarga do ventrículo direito (VD) pode haver disfunção e falência deste ventrículo (AUSTIN et al., 2013).

Durante os últimos 20 anos, inúmeros estudos com o objetivo de promover intervenções benéficas na HP foram realizados, entretanto, poucos realmente contribuíram de uma forma efetiva com a conduta clínica para o manejo dessa doença (GURTU; MICHELAKIS, 2015). Sendo assim, a HP continua sendo uma condição altamente incapacitante, sem cura e de redução da expectativa de vida (GURTU; MICHELAKIS, 2015). Contribui para isso um atraso de cerca de dois anos do início dos sintomas até o diagnóstico (BADESCH et al., 2010). Além disso, devido às complicações desenvolvidas, internações ocorrem com frequência e de forma prolongada (VAILLANCOURT et al., 2014).

Tabela 1 – Classificação clínica da hipertensão pulmonar

I. Hipertensão arterial pulmonar
II. Hipertensão pulmonar associada a doenças do coração esquerdo
III. Hipertensão pulmonar associada com doenças pulmonares e/ou hipoxemia
IV. Hipertensão pulmonar devido à trombose crônica e/ou embólica
V. Hipertensão pulmonar com mecanismos multifatoriais não claros

Fonte: Adaptação de (SIMONNEAU et al., 2013).

1.2 HIPERTENSÃO ARTERIAL PULMONAR (HAP)

Definição

A hipertensão arterial pulmonar (HAP) é uma síndrome patológica que leva a um aumento sucessivo na RVP e a manifestações clínicas como dispnéia, fadiga, dores no peito, edema periférico e palpitações, sua origem pode ocorrer de diversas maneiras: idiopática, hereditária, associada a drogas e toxinas ou ser decorrente de condições associadas com a HAP (como, por exemplo, infecção por HIV, doenças do tecido conjuntivo, hipertensão portal, entre outras causas) (CHAN; LOSCALZO, 2008). Os sinais mais comuns que levam ao indício de HAP são geralmente atribuíveis ao transporte de oxigênio prejudicado, anormalidade das trocas gasosas e a redução do débito cardíaco (GROEPENHOFF et al., 2013). Outras características da HAP são a fibrose intimal das pequenas artérias pulmonares e a oclusão arterial pulmonar (CHAN; LOSCALZO, 2008; LEHRMAN et al., 2002). Mudanças na túnica íntima incluem dano endotelial, invasão da íntima por células tipo miofibroblastos, aumento da deposição de matriz extracelular levando à fibrose e, muitas vezes, obstrução do lúmen vascular em decorrência das lesões plexiformes, esse quadro pode aumentar ainda mais a resistência vascular em indivíduos com a patologia (SCHERMULY et al., 2011). Outro aspecto bem proeminente presente na HAP é a proliferação de células musculares lisas, as quais se estendem até pequenas artérias pulmonares não muscularizadas da região dos ácinos pulmonares (HUMBERT et al., 2004). Essas mudanças estruturais reforçam a noção de que a

HAP é uma doença vasoproliferativa, determinada por estímulos mitogênicos, resultando em um fenótipo resistente a apoptose na doença (CHIN; RUBIN, 2008).

Os efeitos patológicos gerados no VD resultante das doenças que afetam a função e/ou estrutura dos pulmões, caracterizam o denominado *Cor Pulmonale*. A HAP, por sua vez, é sempre um mecanismo patológico subjacente a este quadro clínico (SHUJAAT; MINKIN; EDEN, 2007). O *cor pulmonale* se origina da sobrecarga de pressão ao VD e é caracterizado por hipertrofia, dilatação e insuficiência do VD secundária à hipertensão pulmonar. No *cor pulmonale* crônico pode ocorrer também a compressão da câmara ventricular esquerda em decorrência do VD hipertrofiado, o que pode levar à regurgitação tricúspide com espessamento fibroso da valva. Comumente a HAP é o elo entre as mudanças funcionais e estruturais do VD e a patologia do pulmão. Aproximadamente 20% dos casos de pacientes com insuficiência cardíaca (IC) apresentam *Cor pulmonale* (CHAOUAT et al., 2008; FAHRAMAND; HILL; SINGAL, 2004).

Quanto maior a pós-carga imposta ao VD, induzida pelas alterações na resistência vascular pulmonar total, maior será a tensão desenvolvida pelo VD durante a sístole, desencadeando um mecanismo compensatório frente a essa situação. A pós-carga está aumentada em qualquer doença associada à HAP, na maioria das vezes, levando à hipertrofia cardíaca (MACIEL, 2001).

Além disso, a HAP é uma doença de difícil diagnóstico, que requer investigações invasivas e significativa experiência no cuidado com os pacientes. Assim, as diretrizes atuais recomendam que os pacientes devam ser avaliados por centros especializados para diagnóstico e tratamento de doenças pulmonares (BARST et al., 2009; GAINE; RUBIN, 1998). Uma vez que haja suspeitas de HAP, o intuito é de confirmar ou excluir o diagnóstico e, se presente, estabelecer a etiologia, analisar a severidade e decidir as estratégias subsequentes para o tratamento (MCLAUGHLIN et al., 2009). Nos últimos anos, a comunidade científica tem se direcionado no sentido de definir um tratamento efetivo para a HAP. As opções terapêuticas para tratamento da HAP existentes no momento são: oxigenioterapia, anticoagulantes ou agentes anti-plaquetários, fármacos vasodilatadores e opções cirúrgicas em situações específicas (GURTU; MICHELAKIS, 2015; MEYER et al., 2004).

A análise criteriosa do eletrocardiograma pode sugerir algumas etiologias para a HAP, como estenose mitral, cardiopatias congênitas e miocardiopatias (CASSERLY; KLINGER, 2009; DROMA et al., 2002). O cateterismo cardíaco direito é necessário para avaliar a gravidade da insuficiência hemodinâmica e para um diagnóstico definitivo de HAP. Os

seguintes parâmetros geralmente são avaliados: pressão do átrio direito (PAD); pressão arterial pulmonar (PAP) sistólica, diastólica e média; débito e índice cardíaco; resistência vascular pulmonar (RVP) e resistência vascular sistêmica (BADESCH et al., 2009). Algumas medidas utilizando a ecocardiografia Doppler também têm sido correlacionadas com o aumento da pressão arterial pulmonar (KITABATAKE et al., 1983).

Epidemiologia e fisiopatologia

A incidência estimada da HAP varia regionalmente no mundo, se mostrando, por exemplo, de 15 casos por milhão em adultos na França a 25 casos por milhão em adultos na Escócia (HUMBERT et al., 2006; PEACOCK et al., 2007). A exata prevalência da doença também é desconhecida, podendo ser largamente subestimada. Nos Estados Unidos, cerca de 200 mil hospitalizações anualmente tem a HP como causa primária ou secundária. Estima-se uma prevalência na Europa de 0,3 – 6% (MOCUMBI; THIENEMANN; SLIWA, 2015).

Estudos recentes mostram médias significativas de sobrevivência relativas à doença, como um estudo retrospectivo realizado no Japão, em que os pacientes apresentaram média de sobrevivência de 14,9 ± 0,8 anos com taxas de sobrevivência em 1, 5 e 10 anos de 98%, 96% e 78% respectivamente. Essa melhora foi relacionada com uma maior taxa de prescrições de drogas para tratamento da HAP (OGAWA; EJIRI; MATSUBARA, 2014). Mudanças na epidemiologia da HP podem ter sido influenciadas por fatores independentes da patologia em si, como modificações na sua classificação, maior precisão no diagnóstico e maior acesso aos tratamentos. Entre os pacientes, se observa um maior número de mulheres acometidas pela doença em comparação com homens, sendo a razão dessa predominância ainda desconhecida (HUMBERT et al., 2006; MCGOON et al., 2013). A apresentação clínica não parece ser diferente entre os sexos, porém, a idade média de diagnóstico da doença se mostra menor em mulheres. Uma vez estabelecido o quadro da doença, homens apresentam maior risco de mortalidade apesar do mesmo tratamento (MANES et al., 2012).

Mesmo com avanços de divulgação terapêutica e diagnóstica, ainda há carência de informações básicas a respeito da epidemiologia da doença (CHIN; RUBIN, 2008), o que dificulta a distribuição do conhecimento.

As diferentes categorias da HP (ver Tabela 1) diferem na sua causa, porém todas são caracterizadas por vasoconstrição pulmonar excessiva junto de um processo de remodelamento vascular anormal que usualmente afeta todas as camadas do vaso e resulta em severa perda de área de secção transversa (CHEMLA et al., 2002).

Devido a sua fisiopatologia ser multifatorial, seu mecanismo patogênico preciso é desconhecido (RABINOVITCH, 2008). Contudo, sugere-se que essa resposta seja desencadeada por um sinal inflamatório inicial, levando à superexpressão de substâncias vasoconstritoras como a endotelina-1, e a produção cronicamente prejudicada de vasodilatadores, tais como as prostaciclinas e o óxido nítrico ($NO^{\bullet}$) (LIAO, 2013; VAILLANCOURT et al., 2014). Este desequilíbrio entre vasodilatadores e vasoconstritores, promove um aumento da espessura da camada muscular lisa e remodelamento da membrana basal (NEWBY, 2006). Além disso, relata-se uma deposição excessiva de trombina relacionada a um estado pró-coagulante, colaborando para o aumento da RVP resultante da lesão endotelial, com formação de trombose intravascular local (SCHULMAN; GROSSMAN; OWEN, 1993). Por ser formado por uma parede fina e sofrer um aumento de pressão em seu lúmen, há um aumento do estresse de parede no VD, com consequente prejuízo à perfusão do miocárdio (MCCRORY et al., 2013).

Não sendo capaz de sustentar esta sobrecarga de pressão por longo prazo, há diminuição da força de contração cardíaca por modificações estruturais e funcionais dos cardiomiócitos, e o VD dilata (HEIN et al., 2003). Por esta dilatação subsequente, há novamente aumento do estresse de parede com necessidade de maior disponibilidade de oxigênio e, simultaneamente diminuição da perfusão do VD, seguindo um ciclo vicioso que leva à diminuição da capacidade funcional (CASSERLY; KLINGER, 2009). Esta está fortemente relacionada com a fração de ejeção do VD, levando pacientes com HAP a apresentarem redução da fração de ejeção deste ventrículo (DROMA et al., 2002). Os mecanismos que levam da hipertrofia ventricular à dilatação não estão bem definidos, porém, acredita-se que se relacionem a modificações nas proteínas reguladoras do miocárdio, ativação neuro-hormonal, estresse oxidativo e nitrosativo, ativação do sistema imune, apoptose, entre outros (BOGAARD et al., 2009).

A HAP é uma doença caracterizada também por remodelamento vascular proliferativo, alterações estruturais e funcionais no VD (BOGAARD et al., 2009), aumento da produção de espécies reativas de oxigênio (ERO) pelos cardiomiócitos (LIAO, 2013) e não somente por vasoconstrição. Infelizmente, as terapias utilizadas atualmente promovem apenas uma melhora da qualidade de vida (MCLAUGHLIN et al., 2009) e não demonstram efeitos significativos sobre a reversão dessa doença (GURTU; MICHELAKIS, 2015).

1.3 MODELO EXPERIMENTAL POR MONOCROTALINA

O modelo animal mais utilizado para mimetizar a HAP é gerado por meio da administração de monocrotalina (MCT) (POLONIO et al., 2012), advinda de plantas do gênero *Crotalaria*. Estas são denominadas plantas invasoras que se adaptam a distintos ambientes, as Crotalárias são facilmente encontradas em plantações de grãos e em pastagens (TOKARNIA; DÖBEREINER; PEIXOTO, 2000). A MCT é um alcalóide pirrolizidínico presente em uma grande quantidade de plantas, como nas sementes de *Crotalaria spectabillis*. A hepatotoxicidade dos alcalóides pirrolizidínicos está associada à presença de insaturação entre os carbonos 1 e 2 que na presença de oxidases leva à formação de pirróis, que se associam a grupos nucleofílicos de macromoléculas, como sulfidrila, hidroxila e grupos amino de enzimas, globulinas, hemoglobinas, bem como purinas e pirimidinas, formando ligações cruzadas irreversíveis com o ácido desoxirribonucléico (DNA) e ácido ribonucléico (RNA), acarretando efeitos citotóxicos, mutagênicos e carcinogênicos (COMINI et al., 1996; SANTOS et al., 2004).

A biotransformação hepática é responsável pela sua toxicidade, sendo a dehidroxilação, realizada pelo complexo enzimático citocromo P-450, uma das reações químicas envolvidas com esse processo. Após a sua dehidroxilação, o metabólito ativo dehidromonocrotalina, além de atuar no fígado, promove alterações estruturais e funcionais nos pulmões e na vasculatura pulmonar (DAICHO et al., 2009; FARHAT et al., 1993). As principais vantagens da aplicabilidade da MCT são a facilidade e rapidez de reproduzir o modelo, bem como, mimetizar os sintomas da síndrome (DOGGRELL; BROWN, 1998).

O seu mecanismo de ação pulmonar ainda não está totalmente elucidado. No entanto, sabe-se que os glóbulos vermelhos do sangue aumentam significativamente o transporte dos metabólitos da MCT do fígado para o pulmão, onde são liberados no endotélio pulmonar (PAN et al., 1991). Então, por meio do desencadeamento de uma resposta inflamatória com resposta hipertrófica e hiperplásica das células musculares lisas, (GOMEZ-ARROYO et al., 2012) desequilíbrio entre substâncias vasodilatadoras e vasoconstritoras e elevação da pressão arterial pulmonar, a MCT torna-se uma potencial produtora de lesão das células endoteliais, levando ao surgimento progressivo de HAP (MEYRICK, 1980).

Figura 1 – Imagem da *Crotalaria spectabillis.*

Fonte: disponível na web em: http://www.tropical.theferns.info/image.php?id=Crotalaria+spectabilis

O uso de modelos animais tem contribuído extensivamente para o atual entendimento da fisiopatologia e para a investigação de tratamentos experimentais na HP (MAARMAN et al., 2014). Levantam-se por vezes preocupações com os modelos animais não apresentarem a completa severidade da HP observada em humanos em respeito a parâmetros histológicos e hemodinâmicos. Apesar dos modelos animais não recapitularem completamente a severidade da HP em humanos, eles podem ser muito bem correlacionados com formas mais suaves e moderadas da doença (MAARMAN et al., 2014).

Em geral, a MCT tem sido utilizada em ratos na dose de 60-80 mg/kg administrada via subcutânea ou intraperitoneal (GOMEZ-ARROYO et al., 2012). A resposta à MCT varia entre as espécies por conta das diferenças na farmacocinética da MCT, envolvendo a degradação e a formação hepática do seu derivado pirrólico (BARMAN; ZHU; WHITE, 2009). Dentro de horas após a exposição do animal à MCT, já se observa danos no endotélio vascular. Na primeira semana, pode-se perceber um aumento do dano endotelial, com presença de infiltrado inflamatório e edema. Na segunda semana já se observa o aumento da pressão arterial pulmonar (AUSTIN et al., 2013). Em contraste com o modelo animal de hipóxia crônica para mimetizar a HAP, por exemplo, os efeitos da injeção de MCT foram fortemente associados ao número de dias para introduzir a HP. Alguns relatos anteriores revelaram que o início do aumento das pressões arteriais pulmonares e do remodelamento vascular é adiado até uma a duas semanas após a dose inicial. Além disso, a dose utilizada de

MCT não parece ter um efeito tão grande quanto o período de indução (SZTUKA; JASIŃSKA-STROSCHEIN, 2017).

Comumente, o modelo MCT é caracterizado por hipertrofia significativa e disfunção do VD, alguns autores apresentaram animais com pressões sistólicas do VD de 80 mmHg após cinco semanas, e isso foi associado a uma baixa taxa de sobrevida. Além disso, após quatro semanas da injeção de MCT, a pressão sistólica média do VD foi de 65 mmHg. Considera-se que tal elevação provoca miocardite, que afeta tanto o VD quanto o ventrículo esquerdo (VE) (SZTUKA; JASIŃSKA-STROSCHEIN, 2017). Assim, para estudo da HAP, o modelo de MCT continua sendo utilizado com frequência, uma vez que ele oferece simplicidade técnica, reprodutibilidade e baixo custo quando comparado com outros modelos (GOMEZ-ARROYO et al., 2012).

1.4 O PAPEL DO ESTRESSE OXIDATIVO NA HAP

O estresse oxidativo (EO) não está envolvido somente na IC, mas em várias doenças cardiovasculares, como na HAP. O EO representa uma situação onde, a produção das ERO seja superior à capacidade do organismo de neutralizar os efeitos dessas moléculas. Os radicais livres (RL) e as ERO causam modificações nos lipídios, proteínas e DNA, alterando sua conformação e funções (HALLIWELL; GUTTERIDGE, 1999; LLESUY, 2002; TSUTSUI; KINUGAWA; MATSUSHIMA, 2009). Responsáveis pela respiração celular acoplada à fosforilação oxidativa para a formação de ATP (adenosina trifosfato) e sustentação da vida dos seres aeróbicos, as mitocôndrias são as principais formadoras de RL (SILVA; FERRARI, 2011). Por meio dos processos metabólicos oxidativos naturais do organismo, uma adição sequencial de elétrons leva à formação destes RL, que são caracterizados por conter número ímpar de elétrons em sua última camada eletrônica (WILSON; PIERCE; CLANCY, 2001). Desemparelhados na sua órbita externa, muitos deles apresentam-se com alta reatividade e instáveis, tendendo a interagir com outras moléculas na sua proximidade, por meio da captação (atuando como agentes oxidantes) ou da cedência (atuando como agentes redutores) de elétrons ou átomos de hidrogênio (FERREIRA; MATSUBARA, 1997).

Em condições fisiológicas, processos enzimáticos e não enzimáticos rotineiros levam à formação de ERO e espécies reativas de nitrogênio (ERN). Em baixas concentrações, as ERO são moléculas com reatividade moderada que promovem uma resposta celular adaptativa,

resultando em citoproteção (SAMJOO et al., 2013) e sobrevivência celular (TSUTSUI; KINUGAWA; MATSUSHIMA, 2009).

O equilíbrio entre a geração das ERO e sua neutralização é mantido por vários mecanismos interligados e complexos, e uma disfunção de qualquer um destes por estímulos endógenos ou exógenos, pode levar à alteração na sinalização redox, aumentando os níveis intracelulares de ERO e consequentemente levando ao EO (TRACHOOTHAM et al., 2008; TSUTSUI; KINUGAWA; MATSUSHIMA, 2009). A interação com lipídeos é denominada lipoperoxidação, causando modificações na permeabilidade e fluidez das membranas. Quando atingem as proteínas, são formadas proteínas oxidadas com perda estrutural e funcional (REZNICK; PACKER, 1994). Já é bem reconhecido que o EO contribui para o desenvolvimento e progressão de diversas doenças vasculares incluindo a modulação da inflamação na HAP (MONTANI et al., 2014).

Um dos principais radicais livres discutidos na HAP é o $NO^{\bullet}$. De maneira geral, há um consenso que a sinalização desencadeada pelo $NO^{\bullet}$ na HAP está prejudicada (TABIMA; FRIZZELL; GLADWIN, 2012). Há indícios de que a maior produção de ERO, principalmente do ânion radical superóxido ($O_2^{\bullet-}$) e peróxido de hidrogênio (H_2O_2), possa estar aumentada na HAP em consequência de uma maior ativação das enzimas NADPH oxidases, as quais utilizam o NADPH como doador de elétrons para formação $O_2^{\bullet-}$ e H_2O_2 (DEMARCO et al., 2008; NISBET et al., 2010).

Devido a constante produção de ERO, as células possuem formas de minimizar seus efeitos agressores por meio de antioxidantes (HALLIWELL; GUTTERIDGE, 1999). Os sistemas de proteção endógena, denominados reservas antioxidantes, podem ser enzimáticos ou não enzimáticos (FERREIRA; DUARTE, 2007). Os tióis são antioxidantes não enzimáticos que carregam grupos sulfidrilas (-SH) compostos por um átomo de enxofre e um de hidrogênio, que estão ligados a um átomo de carbono. Na presença de oxidantes, os tióis originam uma reação de oxidação formando pontes dissulfeto, reduzindo o composto oxidado (EREL; NESELIOGLU, 2014). Reservas enzimáticas incluem, por exemplo, a enzima superóxido dismutase (SOD) (MCCORD; FRIDOVICH, 1969). A redução monovalente do oxigênio molecular leva à formação do $O_2^{\bullet-}$ (POLJSAK, 2011). Esta molécula não consegue atravessar facilmente as membranas, ficando normalmente no compartimento no qual é produzido, sendo este geralmente a matriz mitocondrial. A SOD atua dismutando o $O_2^{\bullet-}$ em um não radical, o H_2O_2, este mecanismo é importante uma vez que o H_2O_2 pode estimular diversas vias de sinalização e contribuir para a regulação do tônus da vasculatura e modulação da proliferação celular (MURPHY et al., 2011). A expressão de muitas enzimas antioxidantes

é regulada por fatores de transcrição redox-sensíveis como o Nrf2 (NF-E2-related fator 2). O Nrf2 é considerado o regulador chave da resposta celular antioxidante, induzindo a transcrição de centenas de genes com ação citoprotetora, incluindo enzimas de fase II e antioxidantes. O Nrf2 também produz citoproteção por mecanismos de desintoxicação, levando ao aumento da excreção de xenobióticos orgânicos e metais tóxicos, além de desempenhar funções anti-inflamatórias e melhora da função mitocondrial (PALL; LEVINE, 2015).

Obter um entendimento mais abrangente das vias de sinalização redox e dos mecanismos de ação dos pró-oxidantes e antioxidantes é importante para o desenvolvimento de estratégias terapêuticas na prevenção de doenças cardiovasculares (TRACHOOTHAM et al., 2008). Sugere-se que as ERO atuem como segundos mensageiros intracelulares, mediando tanto a hipertrofia adaptativa quanto a mal-adaptativa (BAE; LEE; KANG, 2005). Assim, o EO tem importante papel na diferenciação, crescimento e apoptose celular, atuando sobre o remodelamento do miocárdio através da modulação de proteínas (GIORDANO, 2005).

Diversos trabalhos já mostraram os efeitos benéficos de terapias ou intervenções que têm as ERO e ERN como alvos no estudo da HAP (WUNDERLICH et al., 2008). Assim, novos ensaios visando a entender melhor a importância das ERO e ERN e os efeitos da ruptura homeostática do EO na HAP precisam ser realizados, bem como a busca de novas terapias que possam atuar sobre esse quadro e melhorar o prognóstico e a sobrevida de pacientes com HAP.

1.5 EXTRATO DE MIRTILO

O aumento da produção de espécies reativas ou a ineficiência das defesas antioxidantes, causadas pelo EO, estão envolvidas no desenvolvimento e progressão da HAP. Tentativas de utilizar antioxidantes sintéticos para bloquear ou atenuar os efeitos negativos das ERO têm apresentado, em muitos casos, resultados negativos (COZMA, 2004). Assim, tem crescido a atenção para o uso de produtos naturais (AHMET et al., 2009).

A maioria das plantas apresenta mecanismos para a detoxificação de RL, os quais são produzidos durante processos metabólicos normais (PRIOR; CAO, 2000). Em particular, pequenas frutas do tipo *berry* têm demonstrado altos conteúdos de compostos antioxidantes, incluindo antocianinas e compostos fenólicos. Esses metabólitos funcionam de maneira a proteger a planta contra reações fotodinâmicas ao diminuir a produção de ERO, sugere-se

também, que apresentem efeitos protetivos contra diversas doenças em humanos (ELKS et al., 2011). As *blueberries* (*Vaccinium spp*.) apresentam umas das maiores capacidades antioxidantes entre frutas e vegetais testados até hoje, contendo antocianinas, pró-antocianidinas, ácidos fenólicos e flavonóis (ZHAO et al., 2017).

Segundo a Pesquisa Nacional de Educação em Saúde e Nutrição (NHANES, EUA), são consumidos nos Estados Unidos em média 12,5 mg de antocianinas por dia/pessoa, principalmente de *strawberries*, *blueberries*, *blackberries* e *raspberries*, pois são as frutas com componentes antioxidantes mais comumente consumidas no país. As *berries* contribuem significativamente para a dieta humana e o seu consumo tem mostrado efeitos positivos na saúde cardiovascular (CUTLER; PETERSEN; BABU, 2016).

Figura 2 – Imagem representativa da *Vaccinium spp*.

Fonte: disponível na web em: <https://www.medicalnewstoday.com/articles/287710.php>

Em modelo animal de dano cardíaco causado por administração de ciclofosfamida, o uso de um extrato de *blueberries* rico em antocianinas reduziu a hipertrofia cardíaca e atenuou a redução na fração de ejeção do VE. No mesmo trabalho se observou aumento da atividade da enzima SOD e aumento dos níveis de glutationa reduzida no tecido cardíaco de ratos que receberam o tratamento com o extrato (LIU et al., 2015). A suplementação de *blueberries*, além de melhorar o equilíbrio redox, parece atenuar a produção de citocinas pró-inflamatórias e proteger o miocárdio de ratos contra isquemia (AHMET et al., 2009).

Outro estudo mais recente comparou o efeito de um consumo de *berries* de curta duração (quatro semanas) com uma duração relativamente maior (sete semanas) sobre a contração vascular e reatividade aórtica em ratos. Neste estudo, as quatro semanas de tratamento tiveram influência limitada na reatividade vascular, no entanto, sete semanas de consumo de *berries* reduziram significativamente a contração máxima e melhoraram a

sensibilidade dos vasos, sugerindo que um tratamento mais prolongado pode ser necessário para melhorar a função vascular. Por outro lado, alguns estudos em humanos sugeriram que o tempo de tratamento mais curto é suficiente para obterem-se os efeitos vasculares benéficos (CUTLER; PETERSEN; BABU, 2016). Além disso, um grande estudo prospectivo que examinou as dietas de 93.600 mulheres norte-americanas relatou uma correlação negativa significativa entre o consumo habitual de *blueberries* e o risco de infarto do miocárdio. Neste estudo, o consumo regular de *blueberries* e *strawberries*, duas das fontes mais ricas de antocianinas nas dietas ocidentais modernas, foi associado com uma redução de 34% no risco de infarto do miocárdio em comparação com o consumo infrequente desses frutos (CUTLER; PETERSEN; BABU, 2016). É bem conhecido que as *blueberries* são grandes fontes de antocianinas (MOŽE et al., 2011) e diversos estudos mostram que estas ativam sistemas antioxidantes celulares, com benefícios que conduzem para a proteção cardiovascular (ZAFRA-STONE et al., 2007).

O uso do mirtilo (*Vaccinium myrtillus*) diminuiu a lipoperoxidação e inibiu a redução da atividade da SOD, bem como atenuou mudanças patológicas em modelo de cardiotoxicidade induzida por doxorrubicina (ASHOUR et al., 2011). Adicionalmente, a avaliação dos efeitos da suplementação com mirtilo por dez semanas sobre a função endotelial e a pressão arterial em ratos alimentados com dieta hiperlipídica, mostrou que a suplementação com mirtilo reduziu a pressão arterial sistêmica em 14% e melhorou a disfunção endotelial. Evidências recentes mostram que *berries* exercem efeitos vasculares benéficos ao atuarem em múltiplos alvos, tais como ativação da via de sinalização óxido nítrico sintase endotelial (eNOS), óxido nítrico ($NO^{\bullet}$), modificação da sinalização redox, melhora do estado inflamatório e da reatividade vascular, melhora da dislipidemia e redução da pressão arterial, além de propriedades anti-aterogênicas (CUTLER; PETERSEN; BABU, 2016; ZHAO et al., 2017).

Até o momento, nenhum estudo verificou o papel da suplementação de *berries*, incluindo o mirtilo, sob parâmetros oxidativos em análise de tecido pulmonar, em modelo animal de HAP.

1.6 JUSTIFICATIVA

Como estudos recentes vêm retratando a capacidade antioxidante da suplementação com *blueberries*, o extrato de mirtilo pode melhorar ou atenuar as descritas alterações observadas na vasculatura pulmonar em modelo de HAP induzida por MCT.

1.7 OBJETIVOS

1.7.1 Objetivo geral

Avaliar o efeito do extrato de mirtilo sobre o tecido pulmonar no modelo de hipertensão arterial pulmonar. Nesse contexto, enfoca-se na sua atuação sobre o estado redox do tecido.

1.7.2 Objetivos específicos

Verificar os efeitos do tratamento com extrato de mirtilo em ratos com hipertensão arterial pulmonar:

a) realizando a análise do estado redox celular: verificando a lipoperoxidação, mensurando a atividade das enzimas superóxido dismutase e NADPH oxidases, conteúdo de sulfidrilas, nitritos totais e espécies reativas de oxigênio totais; avaliando a expressão de Nrf2 por Western Blot.
b) analisar parâmetros relacionados com a função cardíaca realizando a ecocardiografia e o registro de pressão ventricular direita.

2. ARTIGO CIENTÍFICO

BLUEBERRY EXTRACT IMPROVES OXIDATIVE STRESS IN LUNGS FROM RATS WITH PULMONARY ARTERIAL HYPERTENSION

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a pathology characterized by a gradual lumen reduction and increased resistance of pulmonary vasculature, culminating in pulmonary pressure increase. These processes involve disturbances in lung redox state homeostasis and cause progressive right heart failure. In this context, the use of natural antioxidants such as blueberry may represent a therapeutic approach. In our study, we evaluated the effect of a blueberry extract (BB) on functional parameters and oxidative stress in the lung of rats induced to PAH. Male Wistar rats were submitted to PAH by the administration of monocrotaline (60 mg/kg I.P). Animals were treated with BB at doses of 50, 100 and 200 mg/kg via gavage for 35 days. BB (100 mg/kg) increased the E/A ratio and decreased the mean pulmonary artery pressure (MPAP) of animals. Moreover, BB increased the activity of superoxide dismutase (SOD), restored sulfhydryl content and decreased NADPH oxidase activity and total reactive species concentration. In addition, BB was also able to restore expression of Nrf2 in lungs of treated animals. These results show intervention with BB mitigated functional PAH outcomes through improvement of pulmonary redox state.

Keywords: Blueberry. Pulmonary Hypertension. Oxidative Stress. Monocrotaline.

1. INTRODUCTION

Described as a progressive disease that results from vascular disturbances, pulmonary arterial hypertension (PAH) is characterized primarily by an increase in pulmonary vascular resistance and gradual reduction of the lumen, with consequent inappropriate elevation of pressure levels. In its final process, due to right ventricular (RV) overload, there may be dysfunction and failure of this ventricle [1]. Pulmonary hypertension has been characterized in individuals with a mean pulmonary artery pressure ≥ 25 mmHg at rest [2]. Since PAH's pathophysiology is multifactorial, its precise pathogenic mechanism is unknown [3]. However, it is suggested that PAH is triggered by an initial inflammatory signal, [4] leading to overexpression of vasoconstricting molecules and chronically impaired production of vasodilators, promoting an increase in smooth muscle layer thickness and remodeling of the basement membrane [5,6]. In addition to the imbalances between vasodilators and vasoconstrictors already mentioned, there is an important contribution of oxidative stress (OS) in PAH, which may cause structural and functional damage to lipids, proteins and DNA [7].

One of the major free radicals discussed in this pathology is nitric oxide ($NO^{\bullet}$). In general, there is a consensus that the signaling triggered by $NO^{\bullet}$ in PAH is impaired [8]. There are indications that the higher production of reactive oxygen species (ROS), especially the superoxide radical anion ($O_2^{\bullet-}$), may be increased in PAH as a consequence of a higher activation of NADPH oxidase enzymes [9,10]. Endogenous protection systems, called antioxidant reserves, may be enzymatic or non-enzymatic [11]. As an example of an enzymatic antioxidant, we can mention the enzyme superoxide dismutase (SOD) [12], which acts by disrupting $O_2^{\bullet-}$ in a non-radical, the hydrogen peroxide (H_2O_2) [13]. The thiols act as non-enzymatic antioxidants, which carry sulfhydryl groups (-SH), and, in the presence of oxidants, give rise to an oxidation reaction, forming disulfide bonds and reducing the oxidized compound [14].

Obtaining a more comprehensive understanding of the redox signaling pathways and the mechanisms of action of pro-oxidants and antioxidants is important for the development of therapeutic strategies in the prevention of cardiovascular diseases [15]. Thus, it is essential to search for new trials in order to understand better the effects of homeostatic rupture of OS on PAH, as well as the search for new therapies that may act on this condition and improve the prognosis and survival of patients with PAH. In this context, it is known the growth of attention to the use of natural antioxidant products in diseases that involve increase of OS [16]. In particular, small fruits of the berry type have demonstrated high content of

antioxidant compounds, including anthocyanins and phenolic compounds [17]. The blueberry (*Vaccinium spp.),* features one of the highest antioxidant capacities among fruits and vegetables tested to date [18]. It is well known that blueberries are great sources of anthocyanins [19] and several studies show that these activate cellular antioxidant systems and inhibit the expression of genes linked to pro-inflammatory proteins [20]. Evidence suggests that berries have beneficial vascular effects by acting on multiple targets, including reducing blood pressure, improving oxidative parameters and vascular reactivity [18,21].

Therefore, we aimed to evaluate the effects of the blueberry extract on right ventricular functional parameters and in lung tissue redox state using the monocrotaline-induced animal model of PAH.

2. METHODOLOGY

2.1 Animals

Male Wistar rats weighing approximately 200 grams were provided from the Center for Reproduction and Experimentation of Laboratory Animals (CREAL) of the Federal University of Rio Grande do Sul (UFRGS). The animals were kept in polypropylene boxes (340 x 200 x 410 mm), three or four animals per cage. All animals were maintained under standard environmental conditions: controlled temperature (20-25°C), light-dark cycle of 12 hours and relative humidity of 70%. Water and commercial feed were offered "*ad libitum*". The animals were randomly assigned to the groups (n = 60 animals) and weight gain was measured weekly.

2.2 Ethical Considerations

The project was referred to the Ethics Committee on Animal Use (CEUA) of the Federal University of Rio Grande do Sul, and the experiments only started after its approval. All procedures in this study are in accordance with Law 11.794 and the Guidelines for the Care and Use of Animals for Scientific and Educational Purposes (DBCA) of National Council for Control of Animal Experimentation (CONCEA). The number of the project approved by the University's CEUA is 32192.

2.3 Experimental protocol and procedures

The blueberry extract was purchased from Active Pharmaceutica - Brazil (Bilberry – Vaccinium myrtillus L. Extract Standardized with 25% anthocyanidins) and kept refrigerated (4°C) and protected from light. For the experiments, the blueberry extract was solubilized in water purified in a Milli-Q system (Millipore Corporation, MA, EUA). The extract solutions were prepared daily and ultra-pure water was used as control. Treatment with blueberry extract (*Vaccinium myrtillus*) was performed daily for five weeks (two weeks pretreatment prior to the induction of PAH with monocrotaline and three weeks after monocrotaline injection). Three different doses were tested: 50, 100 and 200 mg/kg/day (MCT + BB50, MCT + BB100 and MCT + BB200 groups, respectively) Treatment was administered orally through the gavage method, respecting the maximum volume of 1 mL of solution for each 250g of body weight. Selected doses were based on studies that verified the effect of blueberry treatment on heart damage using different animal models [16,17,22,23].

For the induction of pulmonary arterial hypertension, monocrotaline (MCT) rats received a single intraperitoneal dose of 60 mg/kg monocrotaline (Crotaline – C2401 Sigma Aldrich, MI, EUA) [24]. Control rats (CTR) received a single dose of intraperitoneal saline solution (0.9% NaCl). The animals of the CTR and MCT groups received treatment with water vehicle solution, also by gavage.

At the end of the 35-day protocol, echocardiography followed by right ventricular catheterization were performed to assess cardiac function after anesthetizing animals with ketamine (90 mg/kg, intraperitoneal) and xylazine (10 mg/kg, intraperitoneal) simultaneously. Subsequently, the animals were euthanized under anesthetic effect by manual guillotine decapitation in an isolated environment. Finally, the lungs were collected and kept at -80ºC until redox state biochemical analyses.

2.4 Echocardiography

Animals were anesthetized, submitted to trichotomy of the thoracic region and placed in lateral decubitus position. Images were obtained through two-dimensional mode and pulsed Doppler (Philips HD7 Ultrasound System), using an S12-4 (Philips) transducer. E/A ratio of blood flow through the tricuspid was evaluated [25].

2.5 Hemodynamic evaluation

Animals were anesthetized, submitted to trichotomy of the right jugular region and the surgical field disinfected. The jugular was dissected and isolated. Blood flow was blocked by use of a metal occluder. With obstructed flow, an incision was made in the vessel's upper wall to allow the introduction of a polyethylene (PE-50) catheter filled with saline solution (0.9% NaCl). The catheter was inserted into the right ventricle and its position was determined by the observation of the characteristic ventricular pressure waveform. Immediately after this initial recording, followed by 5 minutes of stabilization, right ventricular diastolic and systolic pressures were recorded. Analog pressure signals were digitized (Windaq-Data Acquisition System, PC) with a sampling rate of 1000 Hz, expressed in mmHg [26]. The following formula was used to estimate the mean pulmonary artery pressure (MPAP): MPAP (mmHg) = 0.61 × RV systolic pressure + 2 mmHg [27].

2.6 Tissue preparation

Lungs were immediately stored in liquid nitrogen after removal and stored at -80^{0}C until analyses. Lung homogenization was performed for 40 seconds with Ultra-Turrax (OMNI Tissue Homogeneizer, OMNI International, USA) in the presence of 1.15% KCl (5 mL/g tissue) and 100 mmol/L phenyl methyl sulfonyl fluoride (PMSF). Then, the homogenates were centrifuged for 20 minutes at 10000 g at 4°C. The supernatant was removed and stored at -80°C for further analyses of the redox state. Proteins were quantified by the method described by Lowry et al. [28].

2.7 Total nitrite content

Nitrites concentration in samples were measured using the Griess reaction principle. Final staining was detected spectrophotometrically at 548 nm and results expressed in mM NO_2^- [29].

2.8 Activity of NADPH Oxidase

Activity of the NADPH oxidase enzyme was determined spectrophotometrically by observing the consumption of NADPH at 340 nm over a 10 minute interval. For this assay, 25 µL of lung homogenate was used in the presence of 925 µL of sodium phosphate buffer 100

mM adding up 50 μL of NADPH 1 mg/mL (N1630 Sigma Aldrich, MI, EUA) [30]. Results were expressed as nmol NADPH/min/mg protein.

2.9 SOD activity

Determination of SOD activity was based on the inhibition of the reaction of the superoxide radical anion with pyrogallol. Buffer solution (Tris-base 50 mmol/L, EDTA 1 mmol/L, pH 8.2), pyrogallol 24 mmol/L (in 10 mmol/L hydrochloric acid) and catalase 30 μmol/L were used. A standard curve with known concentrations of SOD (Superoxide Dismutase from bovine, lyophilized powder, ≥ 1500 units/mg protein, Sigma Aldrich, MI, EUA) was employed. SOD activity can be determined by measuring the rate of formation of the oxidized pyrogallol, which leads to the formation of a colored product detected spectrophotometrically at 420 nm. Results were expressed as U SOD/mg protein [31].

2.10 Sulfhydryl content

Sulfhydryl content represents a non-enzymatic antioxidant defense, which is inversely correlated with oxidative damage to proteins. For the assay, 0.1 mM DTNB was added to 15 μL of lung homogenates, which were incubated for 30 min at room temperature in the dark, as described [32]. Absorbance (TNB formation) was measured spectrophotometrically at 412 nm (Anthos Zenyth 200 RT, Biochrom, UK) and results were expressed as nmol TNB/mg protein.

2.11 Total reactive species

Total reactive species quantification is based on the measurement of fluorescence produced by the oxidation of DCFH-DA (2,3-dichlorofluorescein diacetate) by reactive species present in the sample. A standard curve with known concentrations of DCF was used. Fluorescence was measured using excitation (480 nm) and emission (535 nm) wavelengths. Results were expressed as pmol of DCF/mg protein [33].

2.12 Lipid peroxidation

Lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS). Lung homogenate (50 µL) was mixed with 0.75 mL of 20% (w / v) TCA, and then centrifugation was performed at 3500 rpm for 10 minutes. Thiobarbituric acid [0.67% (m / v)] 0.75 mL was added to an aliquot (0.5 mL) of the supernatant and the mixture was heated in a boiling water bath for 15 min. Absorbance was measured at 535 nm and results were expressed as nmol of TBARS/mg protein [34].

2.13 Western Blot

Electrophoresis and protein transfer were performed as described by Laemmli [35]. 200 µg of protein from lung homogenates were subjected to sodium dodecyl sulfate (SDS-PAGE) monodimensional polyacrylamide gel electrophoresis in a batch system using 8% (w/v) separation gel. Separated proteins were transferred through membrane electrophoresis using modified Towbin buffer containing 20 mmol/L Tris, 150 mmol/L glycine, 20% (v/v) methanol, 0.02% (w/v) SDS (pH 8,3) in a cooled Bio-Rad transfer unit. Afterwards, nonspecific protein sites were blocked by 1h incubation in 5% (w/v) blocking solution of skim milk and 0.1% (w/v) Tris-saline buffer. Immunodetection was processed using the following primary antibody: Nrf2 (57kDa) (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibody was detected using rabbit peroxidase-conjugated, anti-rabbit secondary antibody and the membranes were developed using chemiluminescent detection reagents. Molecular weights of the bands were determined using a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). Results were normalized by the Ponceau method [36].

2.14 Statistics

The means and standard deviations were calculated for each of the measures performed and for each of the groups studied. For parametric and non-parametric results, the comparisons were made through one-way ANOVA, complemented with the Tukey post-test or Dunn post-test, respectively. The differences were considered significant when statistical analysis showed $P<0.05$. GraphPad Instat 6.01 for Windows software was used as statistical analysis tool.

3. RESULTS

3.1 Functional parameters

Regarding the functional parameters measured after the 35-day experimental protocol, we observed that E/A ratio (right cardiac function parameter) was reduced in MCT group in comparison to CTR group. On the other hand, all groups treated with the blueberry extract (MCT + BB50, 100 and 200) were different from CTR and MCT groups (*P<0,0001). Interestingly, animals treated with the dose of 100 mg/kg (MCT + BB100) showed the closest results to the reestablishment of this parameter compared to CTR group (Figure 1). Regarding MPAP, all groups showed a higher value of pressure compared to CTR group. However, MCT + BB100 group reduced this measure compared to the MCT group (*P<0,0001), indicating an attenuation in MPAP as a consequence of treatment with blueberry extract after PAH induction (Figure 2).

3.2 Measures of oxidative damage

There was an increase in total reactive oxygen species in MCT group in relation to CTR group. On the other hand, all groups treated with blueberry extract were able to decrease this measure in comparison to MCT group (*P = 0,0002) (Figure 3). When evaluating NADPH oxidase activity, there was an increase in MCT group compared to CTR group. However, all treated groups decreased NADPH oxidase activity in relation to MCT group (*P = 0,0003) (Figure 4). Regarding the lipid damage measured by TBARS, we did not find differences between the groups (*P = 0,6532) (Figure 5).

3.3 Total nitrite content

No differences were observed between groups (*P=0,9648) (Figure 6).

3.4 Enzymatic and non-enzymatic antioxidants

Evaluating the redox state, regarding SOD activity, all groups were different from CTR group. However, the group treated with the dose 100 mg/kg blueberry extract (MCT + BB100) was different from MCT group (* P<0,0001), recovering enzyme activity (Figure 7). Concerning the sulfhydryl content, MCT and MCT + BB50 groups showed lowered levels compared to CTR (*P=0,0008). MCT + BB100 and MCT + BB200 were not different from CTR group, indicating the reestablishment of this antioxidant system in these treated groups (Figure 8).

3.5 Expression of Nrf2 by Western Blot

MCT group showed reduced Nrf2 expression in comparison to CTR group (*P=0,0021). Treatment with blueberry extract at the dose of 100 mg/kg (MCT + BB100) was able to reestablish the expression of this factor in the lung tissue of animals, not differing in relation to CTR group (Figure 9).

4. DISCUSSION

The E/A ratio (tricuspid flow) in echocardiographic parameters is an important measure of right ventricular diastolic function, mainly because of its importance in detecting pathologies, [25] so that this ratio tends to decrease in the PAH disorder. In the present study we found a reduction in this ratio in MCT animals, which was attenuated in all treated groups, suggesting the blueberry's ability to partially improve this parameter. Other studies also report functional improvements after administration of antioxidants in different protocols of hypertensive disorders [17,37]. As PAH is a syndrome that leads to an excessive increase in pulmonary pressure levels, it is important that an intervention that can regulate and reduce this pressure to avoid more severe vascular consequences is performed. In our study we found a relative improvement in mean pulmonary artery pressure (MPAP) after administration of 100 mg/kg of blueberry extract, indicating that blueberry was able to alleviate vasoconstriction and pulmonary vascular remodeling after PAH.

Oxidative stress (OS) is a result of the imbalance between increased levels of reactive oxygen species (ROS) and nitrogen (RNS) associated with a low activity of antioxidant mechanisms. Increased OS can induce damage to the cellular structures and potentially

destroy tissues [38]. In this way, the enhancement of antioxidant mechanisms for the maintenance of cellular integrity is of prime importance.

A recent study using anthocyanin-enriched blueberries extract evaluated the activity of SOD enzyme in cyclophosphamide-induced lung injured rats [39]. This study showed an improvement in the activity of this enzyme in the animals treated with the extract, similar to what we found in our work in the animals treated with the dose of 100 mg/kg, reflecting in an important antioxidant potential presented by blueberries. Interestingly, another study on pulmonary toxicity evaluated treatment with extract of a berry fruit, *Carissa opaca*, in which SOD activity was also restored in the lung of treated animals [40].

Due to the fundamental role of SOD in modulating the concentration of superoxide radical anion ($O_2^{\bullet-}$) in the vasculature, impairment in this enzyme has been associated with pathological conditions involving vascular dysfunction such as diabetes and hypertension. It is believed that OS, especially by the production of $O_2^{\bullet-}$, induces endothelial dysfunction. In addition, excess $O_2^{\bullet-}$ can directly antagonize the actions of nitric oxide ($NO^{\bullet}$) by a direct chemical interaction. Endothelium-derived $NO^{\bullet}$ controls the extent of vascular smooth muscle relaxation, inhibits platelet aggregation, and attenuates neutrophil adhesion to the endothelium. SOD, by opposing the inactivation of vascular $NO^{\bullet}$ may be crucial for the maintenance of blood vessel tonus [41].

$NO^{\bullet}$ is also a free radical, able to diffuse freely and with limited action because of its short life. It is important to note that the deleterious radical from $NO^{\bullet}$ is peroxynitrite ($ONOO^-$), which is a result from the reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$. Peroxynitrite is a powerful oxidant for all types of molecules and is, therefore, highly cytotoxic. $ONOO^-$ is unstable and can be converted into new active species, such as the hydroxyl radical ($^{\bullet}OH$), which does not have an enzymatic antioxidant system capable of neutralizing it [38]. $NO^{\bullet}$ is also an important signaling molecule that modulates various physiological processes [41].

Although our work did not show differences in total nitrite concentrations between the experimental groups, we suggest that there is a lower probability of $ONOO^-$ formation in the treated groups, since we observed the decrease of total reactive species in all groups that received the blueberry extract. Thus, there may be a higher availability of $NO^{\bullet}$ to the lung tissue of these animals. As discussed earlier, the SOD enzyme assists in controlling the level of $O_2^{\bullet-}$ available to react with $NO^{\bullet}$, contributing to preserve the physiological functions of this molecule [41].

Oxidases (such as NADPH oxidase) transfer an electron from a donor molecule to oxygen, thereby generating $O_2^{\bullet-}$ [38]. Vascular NADPH oxidases have been identified as

capable of initiating the generation of ROS, which may result in dysfunction and cellular death. Although no specific role has been assigned to each form of the enzyme, it has been established that both its expression and activity are regulated positively in the vasculature of hypertensive individuals, as well as being linked to the development of macro and microvascular diseases [42]. Therefore, it is important to reduce oxidases in favor of reducing the oxidative stress of the tissue, particularly attenuating the generation of superoxide. In our study we showed a decrease in NADPH oxidase activity in the animals of all treated groups, a result that can also be related to the relative improvement of the SOD activity (in the treated group at the dose of 100 mg/kg) and the reduction of total reactive species. Moreover, this finding is also consistent with that found by other authors with the use of different extracts [37,43].

Sulfydryl compounds are sensitive to changes in the concentration of free radicals, since the free -SH groups can be oxidized in the presence of OS. Thus, they are used as indicators of non-enzymatic antioxidant activity [44]. In this way, thiol compounds are characterized as a barrier to OS. In the present study we verified a restoration of sulfhydryl content in groups treated with the doses of 100 and 200 mg/kg, indicating a reestablishment of the antioxidant capacity in the lung of treated animals, similar to that found by other authors in therapy with potential antioxidants in different tissues [43,45].

The transcriptional factor Nrf2 plays a central role in redox response. Its activation occurs basically due to its translocation to the cell nucleus, culminating in the transcription of genes of several enzymes with antioxidant properties [46]. The capacity of natural antioxidants compounds in activating the Nrf2 pathway has been reported, which resulted in repair of redox balance in several disorders [47]. Our work showed that blueberry extract (100 mg/kg dose) was able to restore the expression of this factor in the lung tissue of treated animals, showing that blueberry supplementation contributes both to the decrease of ROS and to the improvement of antioxidant signaling, resulting in improved redox balance.

Interestingly, unlike previously reported by other authors, [22,39,40] there was no difference between groups regarding lipid damage in our study. We suggest that there are possibly other pathways operating more actively in the disease, such as inflammatory and/or apoptotic pathways [48] or that blueberry treatment has no influence on lipid peroxidation parameters in the lung. However, further studies are needed for a more accurate explanation of this result.

Eventually, with this study, it was possible to observe that in fact there is the presence of an imbalance in redox homeostasis in lungs after PAH, associated to functional losses.

Structural changes in pulmonary arteries observed in MCT-induced pulmonary hypertension are similar to the characteristics of human pulmonary hypertension at marked medial wall thickening levels [49]. Thus, the use of animal models contributes extensively to a better understanding of PAH and helps in the study of possible new therapies. The extract of blueberry was able to reverse oxidative damage in the lungs, possibly in its vasculature, and we suggest that these effects were attributed mainly to the use of the dose of 100 mg/kg, modulating enzymes that participate in the metabolism of nitric oxide, leading to vascular improvement and resumption of the redox balance.

5. CONCLUSION

We conclude that blueberry extract, especially at the dose of 100 mg/kg, was able to attenuate vascular and oxidative alterations caused by PAH. The improvement in the redox balance seems to be associated with the extract's ability to enhance right ventricular functional. Thus, we suggest that regular consumption of foods rich in antioxidants, such as blueberry, may attenuate or defer the development of diseases that compromise redox homeostasis, as we shown for PAH.

FINANCIAL SUPPORT

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

APPENDIX – Figures

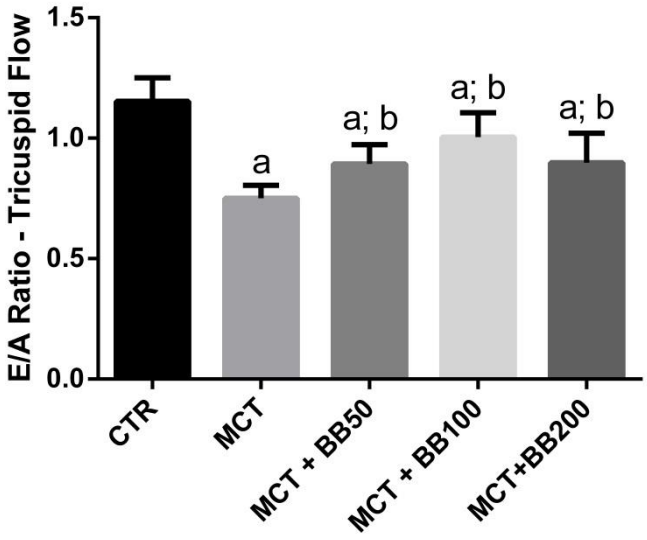

Figure 1: Effect of blueberry extract (BB) on E/A ratio in control rats (CTR) or with pulmonary arterial hypertension (MCT), treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (*P<0,0001); a- different from CTR; b- different from MCT.

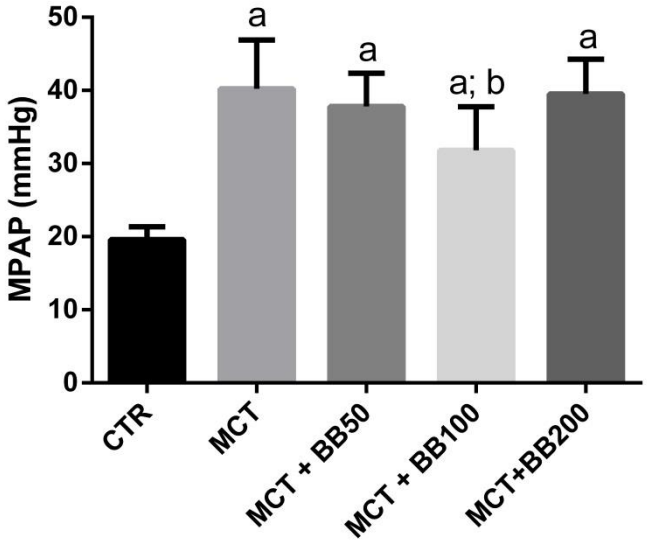

Figure 2: Effect of blueberry extract (BB) on mean arterial pressure (MPAP) in control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (*P<0,0001); a- different from CTR; b- different from MCT.

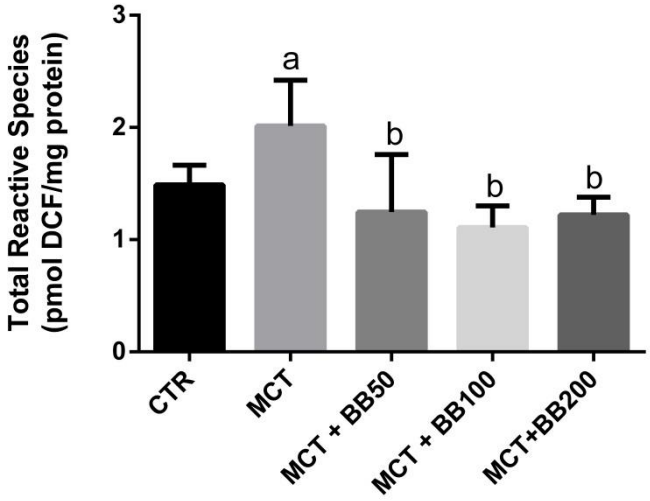

Figure 3: Effect of blueberry extract (BB) on the concentration of total reactive species in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (*P=0,0002); a- different from CTR; b- different from MCT.

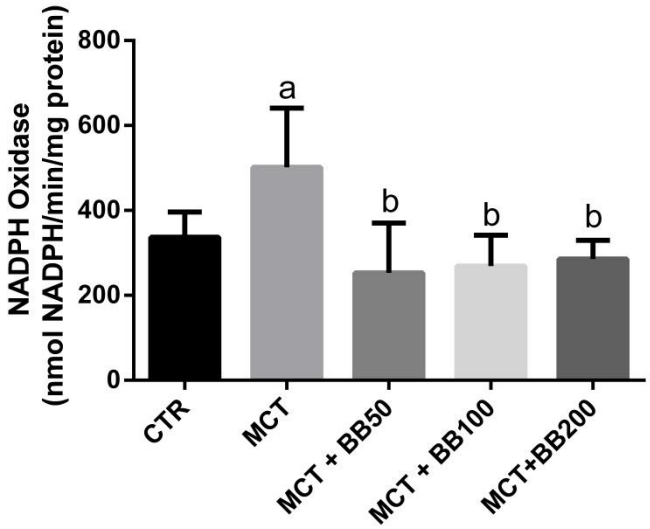

Figure 4: Effect of blueberry extract (BB) on the activity of NADPH oxidase in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (*P=0,0003); a- different from CTR; b- different from MCT.

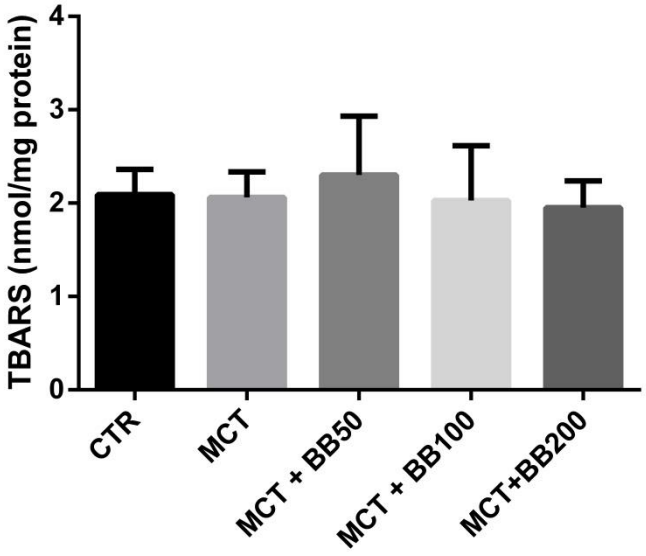

Figure 5: Effect of blueberry extract (BB) on lipoperoxidation (TBARS) in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (**P*=0,6532).

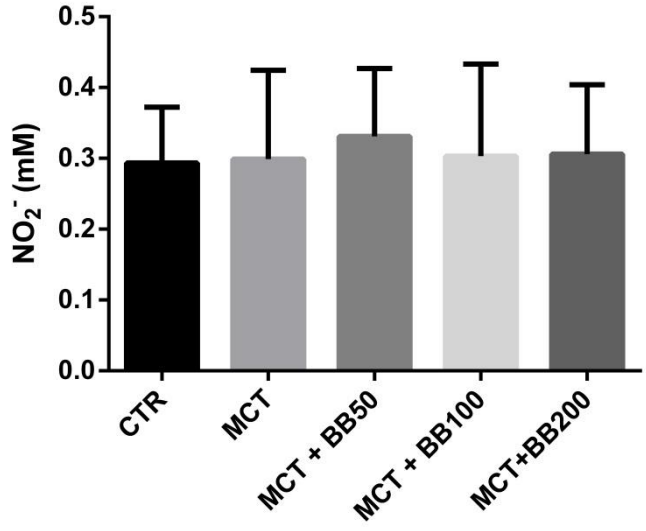

Figure 6: Effect of blueberry extract (BB) on total nitrite content in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (**P*=0,9648).

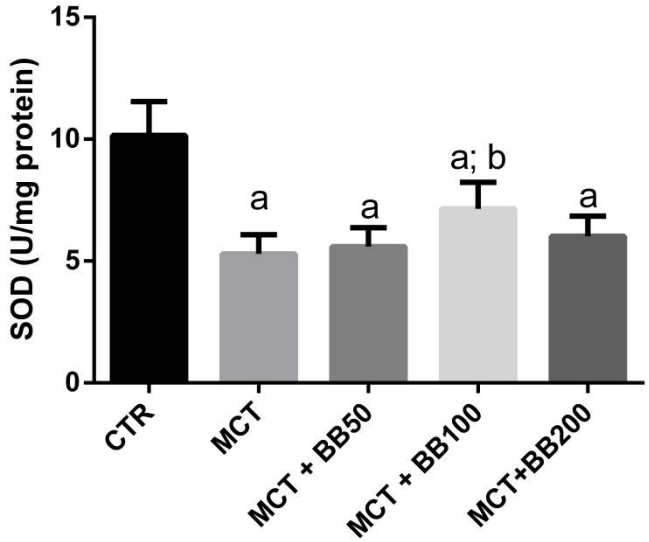

Figure 7: Effect of blueberry extract (BB) on superoxide dismutase enzyme activity (SOD) in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (**P*<0,0001); a- different from CTR; b- different from MCT.

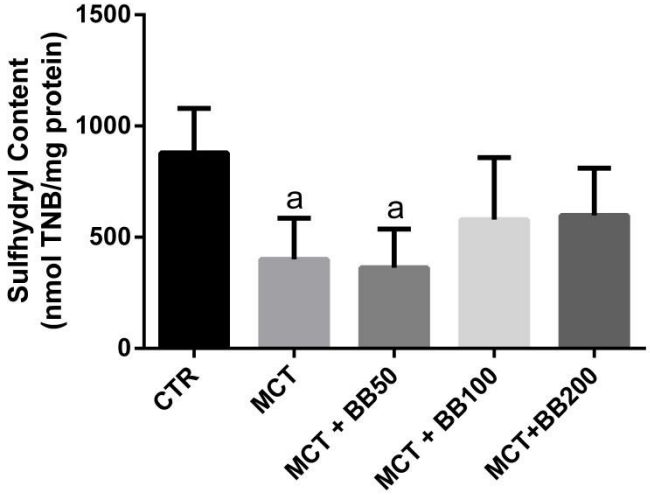

Figure 8: Effect of blueberry extract (BB) on the sulfhydryl content in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT) treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (**P*=0,0008); a- different from CTR.

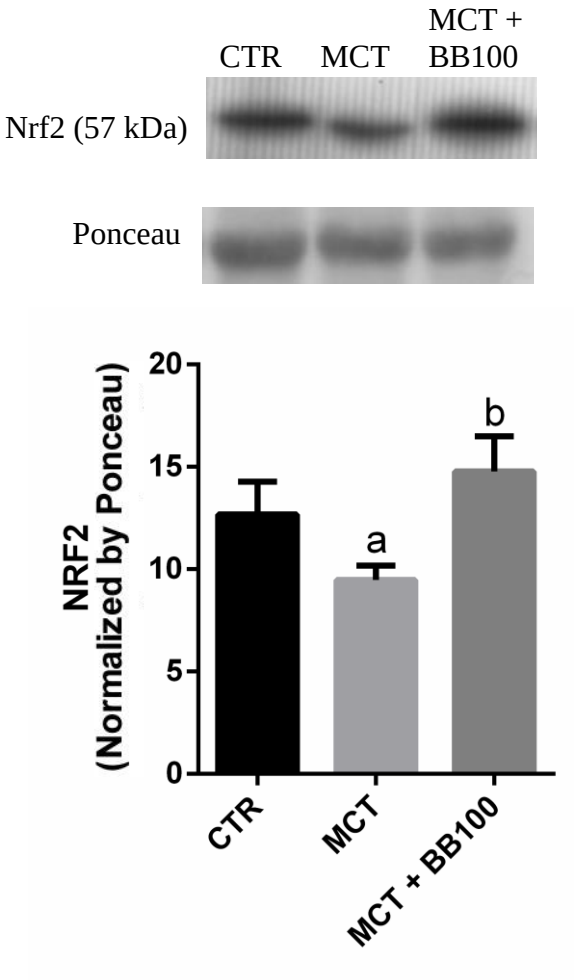

Figure 9: Effect of blueberry extract (BB) on the expression of Nrf2 in the lung of control rats (CTR) or with pulmonary arterial hypertension (MCT), treated by gavage for 35 days. Values represented as mean ± standard deviation; n = 8-12/group; one-way ANOVA followed by Tukey post-test (**P*=0,0021); a- different from CTR; b- different from MCT.

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3. CONCLUSÕES E PERSPECTIVAS

Poucos estudos se dedicam a análise do efeito de *blueberries* em danos cardiopulmonares, o que torna nossos achados interessantes e relevantes. A partir dos resultados apresentados no presente estudo, podemos inferir que a administração dose-dependente do extrato de mirtilo reduz a concentração de espécies reativas totais, bem como contribui na manutenção do equilíbrio redox e funcional do pulmão.

Desse modo, concluímos com este trabalho que o extrato de mirtilo foi capaz de atenuar algumas alterações vasculares e oxidativas ocasionadas pela hipertensão arterial pulmonar no modelo experimental proposto. Entre as perspectivas, nosso grupo de estudo pretende também verificar o efeito do mirtilo em outros tecidos, como no coração, avaliando-se juntamente as vias oxidativas, mecanismos relacionados ao metabolismo do óxido nítrico, além de outras possíveis vias ativas na doença, como vias inflamatórias e apoptóticas.

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ANEXO A – CARTA DE APROVAÇÃO CEUA

U F R G S
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 32192
Título: AVALIAÇÃO DOS EFEITOS DO EXTRATO DE MIRTILO SOBRE O VENTRÍCULO DIREITO DE RATOS COM HIPERTENSÃO ARTERIAL PULMONAR INDUZIDA POR MONOCROTALINA

Vigência: 01/12/2016 à 01/11/2020

Pesquisadores:

Equipe UFRGS:

ALEX SANDER DA ROSA ARAUJO - coordenador desde 01/12/2016
PATRICK TURCK - Aluno de Doutorado desde 01/12/2016

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 23/01/2017 - SALA 330 DO ANEXO - PRÉDIO DA REITORIA DA UFRGS/CAMPUS CENTRO/UFRGS, em seus aspectos éticos e metodológicos, para a utilização de 162 ratos Wistar machos, com 200 gramas de massa corporal, provenientes do CREAL/UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 17 de Fevereiro de 2017

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Vice Coordenador da comissão de ética

ANEXO B – NORMAS DE PUBLICAÇÃO DA REVISTA "BIOMEDICINE & PHARMACOTHERAPY"

BIOMEDICINE & PHARMACOTHERAPY

AUTHOR INFORMATION PACK

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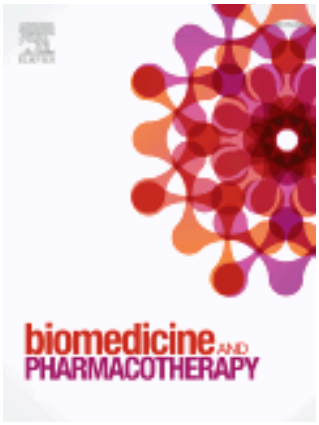

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DESCRIPTION

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The general fields of interest will include Cancer, Nutriceutics, Neurodegenerative, Cardiac and Infectious Diseases.

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