UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

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AVALIAÇÃO DO EFEITO ANTI-INFLAMATÓRIO DA DIETILCARBAMAZINA EM MODELO MURINO DE INFLAMAÇÃO CRÔNICA E HIPERTENSÃO PULMONAR INDUZIDA POR MONOCROTALINA

RECIFE

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Tese de doutorado apresentada ao programa de Pós-graduação em Ciências Biológicas da Universidade Federal de Pernambuco para a obtenção do título de Doutora em Ciências Biológicas.

Área de concentração: Biologia Celular e Molecular

Orientadora: Dra. Christina Alves Peixoto

RECIFE

2016

Catalogação na fonte
Elaine Barroso
CRB 1728

Ribeiro, Edlene Lima
Avaliação do efeito anti-inflamatório da Dietilcarbamazina em modelo murino de inflamação crônica e hipertensão pulmonar induzida por monocrotalina/ Edlene Lima Ribeiro– Recife: O Autor, 2016.

86 folhas : il., fig., tab.
Orientadora: Christina Alves Peixoto
Tese (doutorado) – Universidade Federal de Pernambuco.
Centro de Ciências Biológicas. Ciências Biológicas, 2016.
Inclui referências e anexo

1. Hipertensão pulmonar 2. Citocinas 3. Apoptose I. Peixoto, Christina Alves (orientadora) II. Título

616.24 CDD (22.ed.) UFPE/CCB-2016-100

AGRADECIMENTO

Acredito que estou concluindo mais uma etapa na minha vida acadêmica. Após algum tempo percebemos que objetivos são alcançados e novos necessitam ser traçados. Esse momento para mim é um objetivo concluído que inicialmente parecia ser impossível, mas se tornou possível.

Agradeço à Deus e a minha família que me deram forças.

A minha orientadora Christina Peixoto pela confiança e apoio.

A minha amiga Maria da Conceição, sem ela nada disse teria iniciado.

A toda equipe do laboratório de ultraestrutura em especial a Fabiana, Amanda, Amandona, Bruna e Mari que estavam ao meu lado sempre, seja dia de Natal, ano novo, aniversário sempre podia cotar com todas. Muito Obrigada! Vocês são demais!

A toda equipe do biotério de experimentação Laurimar, Rodrigo e Eduardo.

A Bianca Torres e Patrícia Martins por terem me acolhido e me ajudado em etapas cruciais do meu projeto.

Esse é um momento especial, pois entrei no laboratório com técnica e agora estou saindo com o título de Doutor, amizades e muito conhecimento. Agradeço de coração pela oportunidade de conviver com todos durante esse tempo, cada um me fez aprender algo que sempre levarei comigo.

Obrigada!

RESUMO

A dietilcarbamazina (DEC) é um fármaco filaricida mais amplamente utilizado no tratamento e controle da filariose, e tem mostrado um potencial para tratamanto de doenças inflamatórias como resultado de sua interferência no metabolismo do ácido araquidônico, assim como inibindo fatores de transcrição e induzindo células inflamatórias à apoptose em doenças pulmonares. As doenças pulmonares principalmente as crônicas afetam milhares de pessoas em todo o mundo, representando um sério problema de saúde pública. Dentre as mais comuns podemos citar a asma, doença obstrutiva crônica, apneia obstrutiva do sono, doenças pulmonares ocupacionais, fibrose pulmonar e hipertensão pulmonar (HP), doenças graves e progressivas, que podem levar à incapacidade e à morte. Estima-se que a incidência anual de hipertensão pulmonar esteja em torno de um a dois casos por milhão de habitante, sendo uma doença grave e incapacitante e com sobrevivência histórica média de três a cinco anos após diagnóstico. Estudos demonstram que a HP apresenta cunho inflamatório, com presença no tecido pulmonar de infiltrados inflamatórios perivascular de macrófagos e linfócitos. O uso de modelo animais para avaliar o progresso da HP é extremamente importante, devido a sua complexidade, uma vez que se torna inviável o uso de material humano para tais análises, visto que, na maioria dos casos a doença é diagnosticada quando se encontra em estado avançado. Outro fato importante é a procura de novos potenciais fármacos, na busca de uma melhor qualidade de vida e quem sabe a cura da HP. Inicialmente investigamos o desenvolvimento da HP via inflamação em camundongos C57BL/6J com uma injeção intraperitoneal de monocrotalina (600 mg/kg) uma vez por semana durante 4 semanas (total de quatro doses) estabelecendo os grupos MCT_7, MCT_{14}, MCT_{21}, MCT_{28} com o objetivo de compreender os padrões de reações teciduais e moleculares. A análise dos resultados mostraram que a monocrotalina promoveu alterações teciduais, aumento de citocinas inflamatórias de fatores de crescimento e do estresse oxidativo desempenhando um papel importante no desenvolvimento da HP em camundongos. Outro aspecto que avaliamos foi a ação da DEC sobre as alterações teciduais, deposição de colágenos e apoptose em modelo experimental de HP, estabelecendo os grupos MCT_{28} e MCT_{28}/DEC. Observamos que a DEC reduziu as alterações teciduais, deposição de colágeno e aumentou os fatores relacionados a apoptose demonstrando resultados bastante satisfatórios.

Palavras chaves: Hipertensão pulmonar, dietilcarbamazina, citocinas, fatores de crescimento, apoptose.

ABSTRACT

The diethylcarbamazine (DEC) is a filaricide drug most widely used in the treatment and control of filariasis, and it has shown potential for treatment inflammatory diseases as a result of their interference with arachidonic acid metabolism, as well as inhibiting transcription factors and inducing inflammatory cells to apoptosis in lung diseases. Pulmonary diseases, especially chronics, affect thousands of people around the world, representing a serious public health problem. Among the most common pulmonary diseases there are asthma, chronic obstructive lung disease, obstructive sleep apnea, occupational lung disease, lung fibrosis and pulmonary hypertension (PH). They are severe and progressive disease, and can lead to disability and death. It is estimated that the annual incidence of pulmonary hypertension is around one to two cases per million inhabitants, and this is a serious disease, debilitating, and the historical median survival is of three to five years after diagnosis. Studies show that PH has inflammatory nature, and inflammatory perivascular infiltrates of lymphocytes and macrophages are present in the lung tissue. These injuries of the HP include medial hypertrophy of the arteries and pulmonary arterioles due to excessive proliferation of smooth muscle cells and fibrosis. The use of animal models to evaluate the PH progress is extremely important due to its complexity, since it is not feasible using human material for such analysis since, in most cases, the disease is diagnosed when it in advanced state. Another important fact is the search for new potential drugs to afford a better quality of life and perhaps the cure of PH. Initially we investigated the PH through developing inflammation in C57BL/6J mice with an intraperitoneal injection of monocrotaline (600 mg/kg) once a week for 4 weeks (total of four doses) setting the MCT7, MCT14, MCT21, MCT28 groups with order to understand the patterns of tissue and molecular reactions. The analysis of the results showed that the monocrotaline promoted tissue changes, increased inflammatory cytokine growth factors and oxidative stress plays, an important role in the development of PH in mice. Another aspect that we evaluated was the action of DEC on the tissue changes, collagen deposition and apoptosis in PH experimental model, establishing the MCT28 group and MCT28/DEC group. We noted that DEC reduced tissue changes, collagen deposition and increased factors related apoptosis demonstrating satisfactory results. Based on these results an aspect that we will also assess the DEC's action in chronic inflammation, noting that cytokines, transcription factors may be involved in the development and resolution of chronic lung disease.

Keywords: Pulmonary hypertension, diethylcarbamazine, cytokines, growth factors, apoptosis.

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LISTA DE ABREVIATURAS E SIGLAS

ALK1 – Activina receptor-like kinase 1

APAF-1 – Apoptotic Peptidase Activating Factor 1

BAX – BCL2 associado a proteína X

BCL2 – Linfoma de célula B2

BMPR-2 – Proteína morfogenética do osso

C3 – Caspase 3

C8 – Caspase 8

C9 – Caspase 9

CAV1 – Caveolina 1

COX-2 – Ciclooxigenase 2

COL-1α – Colágeno tipo 1 alfa

CY3 – Citocromo 3

DAPI – 4',6-diamidino-2-phenylindole

DDI – Domínio de morte intracelular

DISC – Complexo de sinalização indutor de morte

DEC – Dietilcarbamazina

DPOC – Doença pulmonar obstrutiva crônica

ECG - Eletrocardiograma

ENG – Endoglin

eNOS – Sintase de óxido nítrico endotelial

ET-1 – Endotelina 1

ET_A – Endotelina A

ET_B – Endotelina B

FADD – Proteína adaptadora de morte associada ao Fas

FDA – Food and Drug Administration

HAP – Hipertensão arterial pulmonar

HP – Hipertensão pulmonar

IL-1 – Interleucina 1

IL-17 – Interleucina 17

IL-6 – Interleucina 6

iNOS – Sintase óxido nítrico induzível

IκBα – Proteína inibitória do NFκB

KCNK3 – Potassium channel subfamily K member 3

LBA - Lavado bronquioalveolar

MCT – Monocrotalina

NFκB – Fator nuclear kappa B

NK – Natural killer

NO – Óxido Nítrico

NOS – Sintase óxido Nítrico

PASMC – Células musculares lisas das artérias pulmonares

PDE-5 – Fosfodiesterase-5

PMN – Polimorfonucleares

SMAD9 – Gene SMAD9

TGF-β – Fator transformador de crescimento beta

TNF-α – Fator de necrose tumoral alfa

TNFR1 – Receptor 1 do fator de necrose tumoral

TNFR2 – Receptor 2 do fator de necrose tumoral

VEGF – Fator de crescimento endotelial vascular

SUMÁRIO

1- INTRODUÇÃO

As doenças pulmonares crônicas afetam milhões de pessoas em todo o mundo, representando um sério problema de saúde pública. Dentre as mais comuns podemos citar a asma, doença pulmonar obstrutiva crônica (DPOC), apnéia obstrutiva do sono, doenças pulmonares ocupacionais, fibrose pulmonar e hipertensão pulmonar (HP), doenças graves e progressivas, que podem levar à incapacidade e à morte.

Estima-se que a incidência anual da hipertensão pulmonar esteja em torno de um a dois casos por milhão de habitante, sendo uma doença grave incapacitante e com sobrevivência histórica média de três a cinco anos após diagnóstico (SANTANA, 2006). Estudos demostram que a HP apresenta cunho inflamatório, com presença no tecido pulmonar de infiltrados perivasculares com macrófagos e linfócitos. As lesões teciduais da hipertensão incluem a hipertrofia da camada média das artérias pulmonares, devido a excessiva proliferação de células musculares lisas e fibrose.

O uso de modelo animal para avaliar o progresso da HP é extremamente importante, devido à sua complexidade, uma vez que se torna inviável o uso de material humano para tais análises, visto que, na maioria dos casos a doença é diagnosticada quando se encontra em estado avançado. Durante as últimas décadas, dois modelos em roedores têm sido fundamentais para a investigação da hipertensão pulmonar humana: o modelo de exposição hipóxia crônica e o da monocrotalina (MCT). Embora os mecanismos de remodelação vascular induzida por hipoxia sejam compreendidas até certo ponto, as lesões obstrutivas encontrados em pacientes humanos com HP grave não se desenvolvem nestes modelos de roedores. O modelo MCT continua a ser um modelo frequentemente utilizado para a investigação da HP, uma vez que oferece simplicidade técnica, reprodutibilidade e baixo custo em comparação com outros modelos de HP (GOMEZ-ARROYO et al., 2012).

Os processos inflamatórios que envolvem fatores celulares, quimiocinas, citocinas, fatores de transcrição, fatores de crescimento e resistência à apoptose, têm papel importante no remodelamento vascular característico da HP. Porém, em que ponto esses fatores convergem ou podem ser ativados ou inibidos para que a doença não progrida é ainda uma incógnita. Um melhor entendimento dessas vias inflamatórias permitirá o desenvolvimento de terapêutica direcionada para a inibição do desenvolvimento da HP, pois as terapias utilizadas atualmente tem caráter paliativo e não definitivo justamente por se desconhecer a modulação dos principais fatores envolvidos na HP, o que poderia acrescentar qualidade de vida e uma melhor sobrevida desses pacientes.

As terapias utilizadas atualmente melhoram os sintomas do paciente, a capacidade de exercício e hemodinâmica pulmonar, entretanto a cura para a HP permanece indefinida e há uma necessidade permanente de tratamentos mais eficazes (O'CONNELL et al., 2013). Além disso, apesar de toda sua importância no contexto da saúde pública mundial e dos inegáveis avanços no diagnóstico e nas terapias, a compreensão da fisiopatogênese da HP, reconhecidamente de origem multifatorial, ainda é incipiente.

Relatórios clínicos e experimentais descreveram resultados favoráveis com a utilização de dietilcarbamazina (DEC) em doenças pulmonares (QUETO et al., 2010; RIBEIRO et al., 2014). A DEC é um derivado da piperazina apresentando potencial anti-inflamatório, levando a diminuição de células e citocinas inflamatórias, fatores de transcrição e regulação nos fatores pró e anti- apoptóticos (SANTOS et al., 2014).

Santos e colaboradores (2014) demonstraram que DEC inibe o fator nuclear de transcrição kappa B (NF-kB) que é um regulador chave de genes pró-inflamatórios, tais como: fator de necrose tumoral alpha (TNF-α), interleucina-1 beta (IL-1β), Sintase de óxido nítrico induzível (iNOS) e ciclooxigenase-2 (COX-2). Estudos demostram ainda que o NFκB está presente em muitas formas de HP em seres vivos e humanos atuando na regulação de múltiplos genes associados com resposta inflamatória, controle do crescimento celular e apoptose implicando na progressão da doença (PRICE et al., 2012; RABONOVITCH et al., 2014). Dietilcarbamazina também é capaz de reduzir edema, número de células polimorfonucleares (PMN), produção de nitrito e expressão de citocinas pró-inflamatórias em modelo de lesão pulmonar aguda induzida por carragenina, demonstrando que este fármaco apresenta potencial para o tratamento de inflamações pulmonares agudas (RIBEIRO et al., 2014).

2- REVISÃO DA LITERATURA

2.1 INFLAMAÇÃO E DOENÇAS PULMONARES CRÔNICAS

A inflamação é definida como uma série complexa da interação entre fatores solúveis e células que podem surgir em resposta a uma lesão traumática, infecciosa, tóxica ou autoimune, sendo essencial para a saúde, protegendo o hospedeiro contra infecções ou danos sistêmicos e contribuindo para restauração da homeostase (PRINCE et al., 2012; LEVY & SERHAN, 2014).

Em geral, as principais características do processo inflamatório são o aumento da permeabilidade vascular ocasionando edema e um grande influxo de PMNs, especialmente neutrófilos. Além de elevada produção de mediadores inflamatórios que amplificam a intensidade do processo ao estimular a quimiotaxia de células inflamatórias e a consequente produção de novos mediadores no local da inflamação (SERHAN et al., 2008).

Na maioria dos casos, a inflamação aguda é autolimitada e os eventos moleculares e celulares durante o seu curso são bem sucedidos em limitar o prejuízo e restaurar o tecido afetado. Porém, processos alternativos incluem a formação de abcessos, fibrose, ou a conversão em inflamação crônica, como observado em várias doenças pulmonares, incluindo asma e DPOC. Além disso, no pulmão, a inflamação exacerbada pode comprometer significativamente as trocas gasosas, portanto, é necessário regular a gravidade e duração da inflamação através de um mecanismo eficaz de resolução (LEVY & SERHAN, 2014).

A liberação de mediadores inflamatórios é essencial na consolidação da inflamação. Uma das substâncias que vem recebendo destaque é o óxido nítrico (NO), que é um gás solúvel sintetizado a partir da metabolização do aminoácido L-arginina, em uma reação catalisada pela enzima óxido nítrico sintase (NOS) (GHOSH & ERZURUM, 2011). Existem três isoformas conhecidas da enzima NOS, duas são produzidas de forma constitutiva: no endotélio (eNOS) e nos neurônios (nNOS). Uma terceira isoforma é sintetizada durante o processo inflamatório, correspondendo à forma induzida (iNOS) (POBER & SESSA, 2007).

A síntese de NO pela iNOS é mediada principalmente pelos macrófagos sob estimulação de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF-α) e interleucina (IL) - 1 beta (IL-1β), sendo esse um importante mecanismo responsável pelo aumento da permeabilidade vascular, o que consequentemente facilita a formação do edema e a infiltração de células inflamatórias no tecido lesionado (POBER & SESSA, 2007).

Além das citocinas serem importantes mediadores na manutenção da homeostase do organismo, seu papel na resposta inflamatória é vital para a coordenação e regulação da

atividade da resposta imunológica. Uma vez que o estímulo inflamatório é desencadeado, a síntese e a liberação dessas proteínas são iniciadas por células, tais como: neutrófilos, macrófagos, células endoteliais, fibroblastos e células dendríticas (GILROY et al., 2004).

No elenco de citocinas que se destacam no processo inflamatório, o TNF-α apresenta um papel imunomodulador central, sendo essa condição evidenciada clinicamente em diversas doenças de caráter inflamatório crônico, como por exemplo: asma, psoríase, artrite reumatoide e lúpus eritematoso sistêmico (BERRY et al., 2007; TINCANI et al., 2007). A síntese de TNF-α ocorre em macrófagos residentes do tecido lesionado, os quais sinalizam o dano tecidual pela secreção desta citocina, o que resulta em diferentes tipos de respostas celulares, tais como, recrutamento de leucócitos, adesão e ativação celular, indução da síntese de proteínas de fase aguda, apoptose de leucócitos e proliferação de fibroblastos. Tais efeitos ocorrem pela ligação do TNF- α aos seus receptores tipo 1 e 2 (TNFR 1 e TNFR2), iniciando uma via de sinalização intracelular relacionada com a ativação do NF-kB, um fator de transcrição responsável pela síntese de mediadores pró- inflamatórios, como IL-6, IL-8 e o próprio TNF-α (BERRY et al., 2007; WONG & TERGAONKAR, 2009).

Uma nova citocina que vem se destacando é a IL-17 devido sua ação regulatória sob a resposta imune inata, além de contribuir na patogênese de doenças infecciosas e autoimunes (REYNOLDS et al., 2010). A IL-17 apresenta um grau de homologia de 63% na sequência de aminoácidos entre camundongos e humanos, o que permite a utilização de modelos experimentais murinos para investigar o mecanismo de ação desta citocina na patogênese de doenças de caráter inflamatório, em que ocorre aumento do número de neutrófilos. Isso porque a função da IL-17 está relacionada ao recrutamento e a ativação dessas células no sítio inflamatório (ALCORN et al., 2010). Além dos linfócitos T, já foi demonstrada que a secreção de IL-17 pode ocorrer em outros tipos celulares, tais como neutrófilos, mastócitos e células natural killer (NK) (PAPPU et al.,2011). Nas doenças pulmonares em humanos, a IL-17 tem sido associada à infiltração de neutrófilos nas vias aéreas de indivíduos com asma, DPOC e HP (ALCORN et al., 2010; PARK & LEE, 2010).

2.2 HIPERTENSÃO PULMONAR

A hipertensão pulmonar (HP) é uma doença incapacitante, decorrente de uma série de patologias que afetam o coração, a drenagem venosa pulmonar, a circulação arterial pulmonar, o interstício e parênquima pulmonar, sendo caracterizada pela elevação sustentada da pressão arterial pulmonar média (25 mm/Hg em repouso ou para mais de 30 mm/Hg durante o exercício) e da resistência arterial pulmonar, que resulta em arteriopatia pulmonar e

disfunção ventricular direita. O prognóstico é variável e depende da gravidade dos transtornos hemodinâmicos e da resposta ao tratamento, sendo a sobrevida histórica média de cinco anos após diagnóstico (GALIÉ et al., 2013; YAVUZ et al., 2013).

A classificação da etiologia da HP foi instituída a partir de 1973, cuja primeira reunião aconteceu devido a uma epidemia de HP em consequência da utilização do aminorex, um fármaco anorexígeno. Em 1998 na França, um novo encontro aconteceu criando categorias de acordo com semelhanças patogênicas, características clínicas e terapêuticas. Em 2003, 2008 e 2013 poucas mudanças ocorreram na classificação da HP (MONTANI et al., 2013; GALIÉ et al., 2013). A mais recente classificação da HP encontra-se descrita na tabela 1.

1. Hipertensão arterial pulmonar (HAP)	1.1 Idiopatica
	1.2 Herdada 1.2.1. BMPR2 1.2.2 ALK1 1.2.3 ENG 1.2.4 SMAD9 1.2.5 CAV1 1.2.6 KCNK3 1.2.7 desconhecido
	1.3 Induzida por drogas e toxinas
	1.4 Associada com: 1.4.1 Doenças do tecido conectivo 1.4.2 Infecção por HIV 1.4.3 Hipertensão portal
	1.5 Doença cardíaca congênita
	1.6 Esquistossomose
	1.7 Anemia hemolítica crônica
	1.8 HP persistente do recém-nascido
2. Hipertensão pulmonar por doença do coração esquerdo	2.1 Disfunção sistólica
	2.2 Disfunção diastólica
	2.3 Valvulopatia
3. Hipertensão pulmonar por doença pulmonar e/ou hipóxia	3.1 Doença pulmonar obstrutiva crônica
	3.2 Doença pulmonar intersticial
	3.3 Distúrbios respiratórios durante o sono

	3.4Outras doenças pulmonares com padrões restritivos e obstrutivos misto
	3.5 Hipoventilação alveolar
	3.6 Exposição crônica a grandes altitudes
	3.7 Anormalidades do desenvolvimento
4. Hipertensão pulmonar por doença trombótica e/ou embólica crônica	
5. Hipertensão pulmonar por mecanismo pouco esclarecidos e/ou multifatorial	5.1 Desordens hematológicas: Desordem mieloproliferativa, esplenectomia.
	5.2 Desordens sistêmicas: Sarcoidose, Histiocitose de células de Langerhans, linfangioleiomiomatose, neurofibromatosis, vasculitis.
	5.3 Desordens metabólicas: doença de armazenamento de glicogênio, doença de Gaucher, distúrbios da tireóide
	5.4 Outros: Obstrução tumoral, mediastinite fibrosante, insuficiência renal crônica

Tabela 1: Classificação da hipertensão pulmonar (segundo, WHO 2013).

O dignóstico para a PH é bastante complexo, ao longo dos últimos anos, o conhecimento na área de hipertensão pulmonar evoluiu de forma consistente e significativa. Novos algoritmos diagnósticos e de tratamento foram desenvolvidos com base no resultado de diversos estudos clínicos que evidenciaram a utilidade de novas ferramentas, assim como a eficácia de novos medicamentos e combinações de medicamentos. Pacientes que apresentam dispneia aos esforços, dor precordial, tontura e/ou síncope e sinais de insuficiência cardíaca direita sem causa evidente devem ser avaliados para HP. Vários são os exames não invasivos podem ser utilizados para a avaliação inicial dos pacientes com suspeita de HP, com grande espectro de sensibilidade e especificidade. A radiografia do tórax (ressonância magnética) e o eletrocardiograma (ECG) são exemplos (HOETTE et al., 2010).

A estratégia de diagnóstico utilizado atualmente para PH leva em consideração uma série de observações: 1) a detecção de algum agente que possa estar relacionado com uma vasculopatia pulmonar; 2) descoberta da presença de HP; 3) classificação do tipo de PH; 4) a confirmação da presença de PH; e 5) a determinação de uma categoria de tratamento adequado (tabela 2).

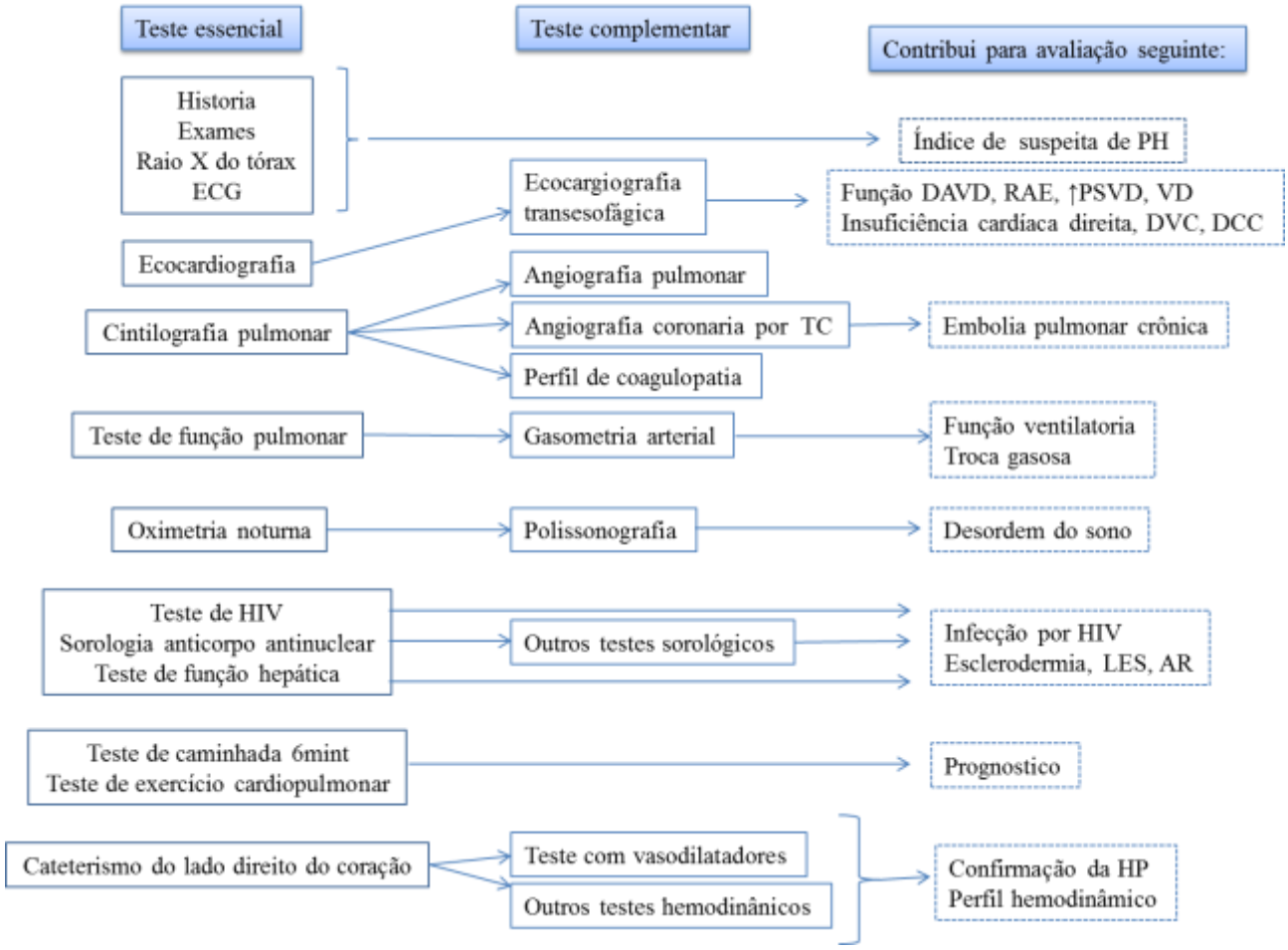


Tabela 2: Abordagem de diagnostico para HP: diretrizes gerais para a avaliação da hipertensão pulmonar. Uma vez que a suspeita de PH pode surgir de várias formas. As sequências de testes podem variar. ECG: eletrocardiografia; HIV: vírus da imunodeficiência humana; TC: Tomografia computadorizada; DAVD: Displasia arritmogênica do ventrículo direito; RAE: Alargamento atrial direito; PSVD: Pressão sistólica ventricular direita; VD: ventrículo direito; DVC: Doença da válvula cardíaca; DCC: Doença cardíaca congênita; LES: Lupus eritematoso sistêmico; AR: Artrite reumatoide. (Segundo Mclaughlin et al., 2009)

Sabe-se hoje que a hipertensão pulmonar, em algumas de suas formas, está inquestionavelmente vinculada ao caráter genético, por outro lado, uma série de fatores podem deflagrar o aparecimento da hipertensão pulmonar em qualquer indivíduo pelos

chamados fatores de riscos que incluem o uso crônico de alguns medicamentos, infecções por vírus e doenças de origem inflamatória. Ficando claro, que a HP constitui uma síndrome com várias entidades de etiopatogenia aparentemente diversa, algumas com denominadores comuns, mas indubitavelmente de interesse multidisciplinar (LOPES et al., 2005).

A incapacidade do sistema respiratório em fornecer oxigenação, ventilação ou suprimir necessidades metabólicas do organismo, estabilidade alveolar inadequada e atalectasias, pode levar a uma série de problemas com injúria pulmonar aguda, síndrome da angústia respiratória aguda e hipertensão pulmonar.

Os exatos mecanismos moleculares na patogênese da hipertensão pulmonar são pouco conhecidos. Mutações genéticas no receptor-2 da proteína morfogenética do osso (BMPR2, membro da superfamília do fator de crescimento transformador beta – TGF-β) têm sido investigadas como possível desencadeador ou regulador dos mecanismos fisiopatológicos da HP. Por outro lado, tem sido demonstrado que o gene que codifica a proteína transportadora da serotonina (5-hidroxitriptamina) está relacionado com a exacerbação da resposta hipertensiva (LOPES et al., 2005).

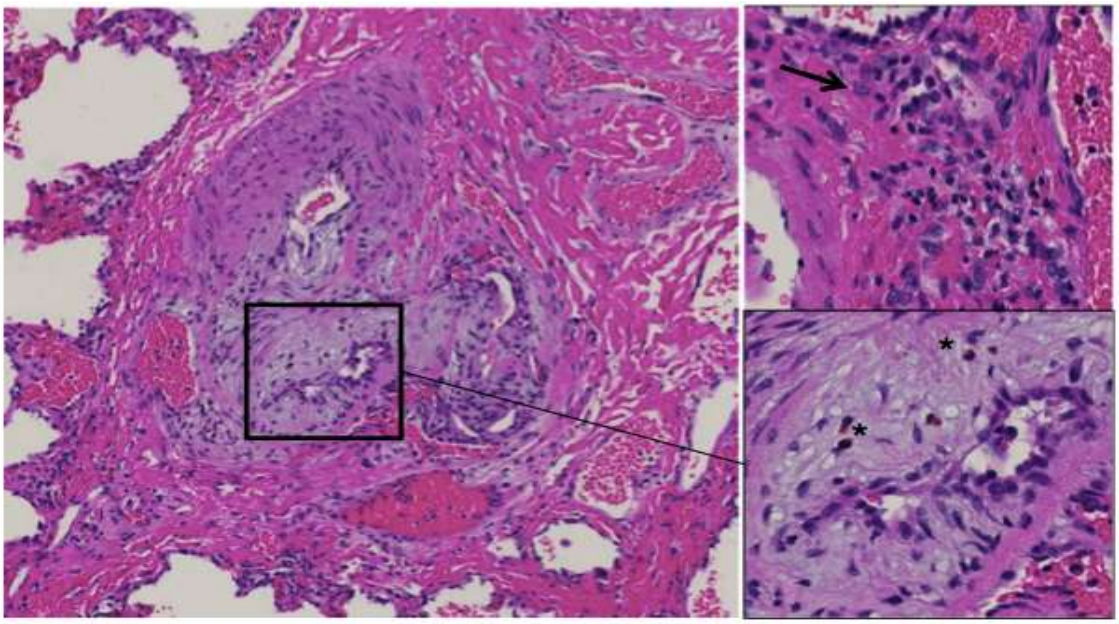
A HP está frequentemente associada à presença de doenças pulmonares intersticiais, que se cronificam e se transformam em fibrose. A disfunção endotelial é o elemento-chave desta manifestação fisiopatológica. As alterações celulares do endotélio incluem modificações funcionais, e alterações de estímulos anti-proliferativos, em alguns tipos de lesão (COOL et al., 1999).

As alterações funcionais vasculares incluem diminuição da produção de substâncias vasodilatadoras e antiproliferativas (prostaciclinas e óxido nítrico) e aumento da produção de substâncias vasoconstritoras e mitógenos (como a endotelina-1) (RUBIN et al., 2001). A lesão endotelial inicial resulta em recrutamento de mediadores vasoativos locais tais como citocinas e fatores de crescimento, promovendo um estado pró-coagulante, e consequente obstrução vascular. Além disso, alterações nos canais de potássio da musculatura lisa da circulação pulmonar também parecem envolvidos no início e/ou progressão da HP (RICACHINEVSKY & AMANTÉA, 2006).

Os estímulos iniciais ou a injúria que resulta em proliferação endotelial anormal não são bem conhecidos, mas acredita-se que a hipóxia, o estresse mecânico e a inflamação estejam envolvidos. A injúria parece não só alterar a proliferação celular e a apoptose, mas também a homeostase do endotélio, incluindo o processo de coagulação e a produção de fatores de crescimento e de agentes vasoativos (SANTANA, 2006).

As lesões teciduais da HP são caracterizadas por mudanças em todos os componentes da parede arterial, começando com hipertrofia media, devido à proliferação excessiva de células musculares lisas das artérias pulmonares (PASMCs), alterações proliferativas e fibróticas na íntima e espessamento da adventícia, além de infiltrado inflamatório perivascular e lesões trombóticas. De fato, estudos demonstram que a inflamação encontra-se presente em muitas formas de HP em seres humanos e animais, e que o fator de transcrição nuclear kappa B (NF-κB) atua regulando múltiplos genes associados à resposta inflamatória, controle de crescimento celular e apoptose, implicados na progressão da HP (HOSOKAWA et al. 2013) (figura 1).

Figura 1: Lesão plexiforme em tecido de paciente com hipertensão arterial pulmonar: Lesão vascular com fibrose, infiltrado perivascular com plasmócitos.

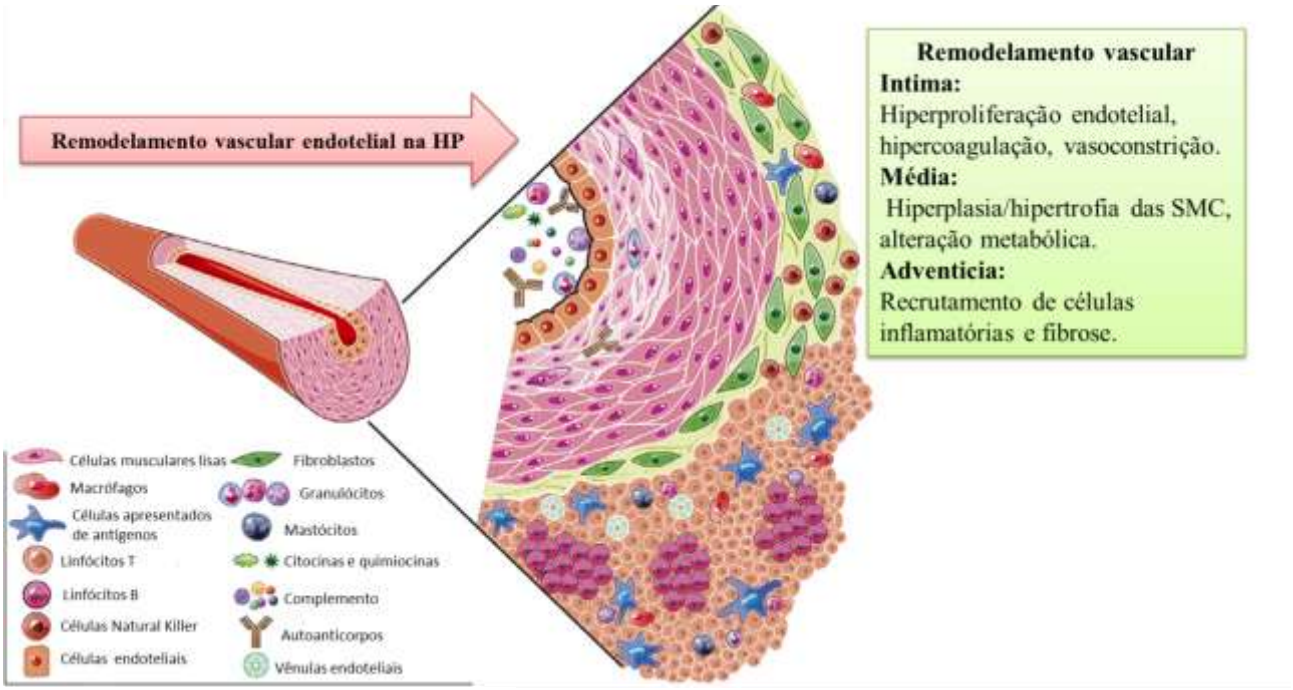


Fonte: Groth, 2014

O fato de que a inflamação precede a remodelação vascular em HP experimental sugere que a imunidade alterada é possivelmente uma de suas causas, em vez de uma consequência da doença vascular. Além do aumento de células imunitárias no infiltrado perivascular e intravascular, os níveis circulantes de certas citocinas e quimiocinas estão anormalmente elevadas. Estas incluem a interleucina (IL)-1β, IL-6, IL-8, proteína quimioatractiva de monócitos-1 (MCP-1), fractalcina, CCL5 / RANTES, e o fator de necrose tumoral alfa (TNF-α). Algumas dessas citocinas e quimiocinas se correlacionam com uma

piora da evolução clínica em pacientes com HP e, portanto, podem servir como biomarcadores de progressão da doença. Além disso, foi demostrado que a IL-1β e o TNF-α, têm sido relacionados a um acumulo de proteínas da matriz extracelular tais como fibronectina, observadas nas lesões de HP, e outros como a IL-6 tem sido relacionado com a proliferação de células musculares lisas (RABINOVITCH et al. 2014) (figura 2).

Figura 2: Alterações vasculares pulmonares na hipertensão arterial pulmonar: Ilustração baseada em analise histológica mostrando uma única camada de células endoteliais com neointima excêntrica (rosa claro) com células inflamatórias, autoanticorpos e células musculares lisas. A camada muscular media é expandida. Camada adventícia abundante com presença de fibroblastos, macrófagos, células apresentadoras de antígenos, mastócitos, células inflamatórias, linfócitos T e B.

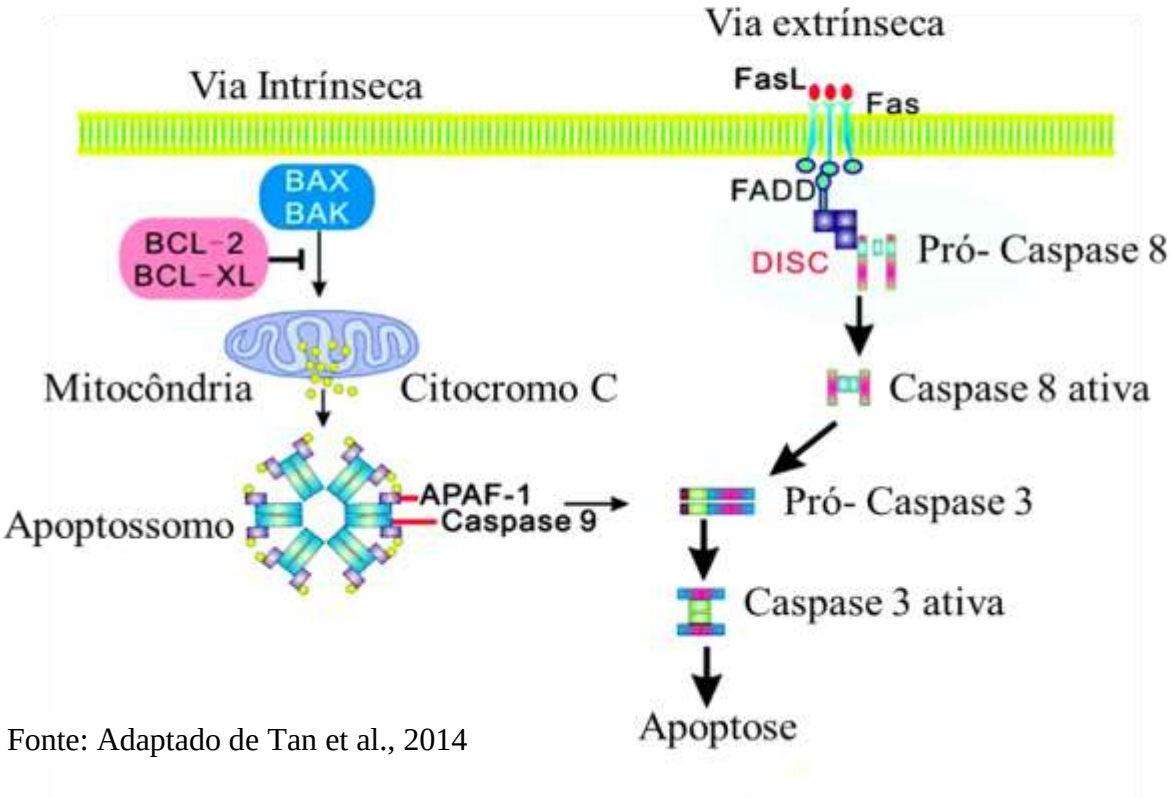


Fonte: Rabinovitch et al., 2014

O aumento da proliferação celular e resistência à apoptose das células do músculo liso da artéria pulmonar (PASMCs) têm sido considerados como fatores importantes no desenvolvimento da HP. A apoptose é um programa de morte celular extremamente regulado e de grande eficiência, que requer a interação de inúmeros fatores. As alterações morfológicas observadas são consequência de uma cascata de eventos moleculares e bioquímicos específicos e geneticamente regulados (SARASTE, et al., 2000).

Ao nível molecular, a morte programada da célula pode ser dividida em três partes: iniciação, execução e rescisão. O início da apoptose pode ocorrer por diversos estímulos, tais como retirada de fatores de crescimento, radiação ultravioleta, fármacos quimioterapêuticos e sinais oriundos de receptores de morte (KRUEGER et al., 2001). O processo de apoptose é regulado pela ativação de proteínas intracelulares ricas em serina (caspases) que promovem a clivagem do DNA (MATUTE-BELLO et. al., 2005). As caspases envolvidas na apoptose foram classificadas de acordo com seu mecanismo de ação em: iniciadoras (caspase -8 e -9) ou efetoras (caspase-3, -6 e -7). As iniciadoras ativam as executoras que, posteriormente, induzem a destruição de proteínas estruturais chaves ocasionando a morte celular (KUIDA et al., 1998). Existem duas vias de ativação apoptótica que podem ser distinguidas pelas moléculas adaptadoras e caspases iniciadoras envolvidas (Figura 3).

Figura 3: Via da apoptose: Na via extrínseca, a ligação entre o receptor FAS e o seu ligante recruta a proteína adaptadora FADD, que por sua vez recruta caspase 8. Já na via intrínseca, a proteína pró-apoptótica Bax estimula a liberação de citocromo c no citosol a partir da mitocôndria, levando a ativação da caspase 9. Esta via pode ser inibida pela proteína Bcl-2. As vias tem um ponto em comum: a ativação da caspase 3, efetora da apoptose.



Fonte: Adaptado de Tan et al., 2014

A via intrínseca, através de um desequilíbrio na membrana mitocondrial ou a extrínseca, através de sinais enviados a partir de receptores de membrana específicos. Na via extrínseca a apoptose é desencadeada por sinais extracelulares que se ligam a um receptor de morte. Essa família de receptores caracteriza-se por possuir uma sequência de repetições extracelulares ricas em cisteína e conta com seis membros, entre eles o receptor de TNF-α e o Fas (CD95), sendo que este último é o mais bem estudado na apoptose. Esses receptores contém ainda um domínio de morte intracelular (DDI) que é essencial para a transdução do sinal apoptótico. O sinal é transmitido através da formação do complexo indutor de sinalização de morte (DISC) (KRUEGER et. al., 2001; KUWANO, 2007).

O receptor Fas é ativado pelo seu ligante (FasL) (também conhecido por CD95L) na superfície de diversas células, incluindo macrófagos e linfócitos T citotóxicos (MARTIN et al., 2005). A ligação Fas-FasL recruta a proteína adaptadora com domínio de morte associado ao Fas (FADD), que funciona como um canal de sinalização formando o DISC e resultando na dimerização e ativação da caspase-8 que por sua vez, pode clivar a pró-caspase 3, que em seguida sofre auto-catálise tornando-se uma caspase efetora, iniciando o processo de apoptose (McILWAIN et al., 2013; LIOU et al., 2014). No caso da via intrínseca a apoptose pode ser desencadeada por qualquer estímulo que gere estresse celular, como: drogas, radiação, agentes infecciosos, espécies reativas de oxigênio e hipóxia. Essa via é ativada quando há uma despolarização na membrana da mitocôndria e um desequilíbrio entre as proteínas regulatórias Bax (pró-apoptótica) e Bcl-2 (anti-apoptótica), favorecendo a liberação do citocromo c para o citosol. O citocromo c tem um papel muito bem estabelecido no transporte de elétrons, porém também induz a ativação de caspases e apoptose. Neste caso, a caspase iniciadora é a caspase-9, que quando ativada pelo citocromo c torna-se um dímero e se liga a proteína adaptadora APAF1. A caspase-9 ativa a caspase- 3 promovendo a apoptose (McILWAIN et al., 2013; LIOU et al., 2014).

A apoptose desempenha um papel crucial no desenvolvimento de lesões plexiforme existentes na patogênese da HP, posteriormente a injuria das células endotelial associadas à proliferação das células musculares lisas facilita a remodelação vascular levando ao estreitamente da luz do vaso, aumento da resistência vascular pulmonar, aumento da pressão arterial pulmonar e insuficiência cardíaca direita. O desequilíbrio entre a morte celular e a proliferação ocorre em todas as fases da remodelação vascular pulmonar e patogênese da HP, e envolve todos os tipos de células do sistema vascular, incluindo a células endoteliais, as células musculares lisas e fibroblastos (JIN & CHOI, 2012).

Grande parte do foco da pesquisa histórica da HP foi sobre o papel nocivo da disfunção endotelial e, em particular, sobre a importância do desequilíbrio entre vasodilatadores e vasoconstritores endoteliais e seus derivados. Estes esforços levaram ao desenvolvimento clínico e aprovação de terapias para a HP atualmente disponíveis, como analogos das prostaciclina, inibidores de encotelina, inibidores de fosfodiesterase-5 e estimulador de guanilato ciclase. Em geral, as estratégias de desenvolvimento de novos medicamentos para HP caem em duas grandes categorias: a identificação de agentes que atuam, em uma ou mais vias, tendo uma maior eficácia e tolerabilidade do que os tratamentos correntemente em uso; ou os que são caracterizadas por uma maior atividade anti-proliferativa que visam à inibição da fibrose da íntima, da hipertrofia do músculo liso e da proliferação associada à HP (O'CONNELL et al., 2013).

2.3 TRATAMENTO DA HIPERTENSÃO PULMONAR

O tratamento da HP é complexo e de alto custo, assim como exige uma equipe multidisciplinar. Embora não haja atualmente nenhuma cura para a hipertensão pulmonar, existem opções de tratamento disponíveis e mais estão em testes. Os tratamentos incluem terapias médicas convencionais utilizando algumas vias de adminitração como oral, inalada, intravenosa e subcutânea. Dependendo da gravidade do HP, transplante de coração ou de pulmão pode também ser uma opção.

O tratamento dos pacientes com HP se dá de acordo com o diagnóstico e sua classificação clínica. Inicialmente esses pacientes recebem orientações com algumas medidas de suporte. Eles são orientados a não realizarem exercícios físicos, receber vacinação, pois infecções são causas importantes de morbidade e mortalidade, além do acompanhamento especial do uso de alguns medicamentos, como de anticoncepcionais e de anticoagulantes. A maioria dos testes para o tratamentos da HP encontra-se restrito aos pacientes enquadrados no grupo 1 (tabela 1), todavia nem todos os subgrupos de pacientes com HP têm resposta terapêutica comprovadas com o uso das medicações especificas atualmente disponíveis. Segundo a associação de hipertensão pulmonar as classes de medicamentos específicos que são aprovados para o uso em HP são os seguintes:

As terapias médicas convencionais

- Bloqueadores dos Canais de Cálcio (CCB) - Ajuda na diminuição da pressão arterial (apenas apropriado para uma pequena minoria de pacientes que demonstram uma resposta favorável ao teste de vasodilatador no momento do cateterismo cardíaco).
- Digoxina - Assiste o bombeamento do coração

- Diuréticos
- Oxigênio - inalado pelo paciente através de uma cânula nasal ou máscara facial
- Varfarina (Coumadin®) - anticoagulante

Análogos das prostaciclinas

- Treprostinil (Orenitram®) – Oral
- Treprostinil (Tyvaso™) – Inalado
- Treprostinil (Remodulin®) – Intravenoso ou subcutâneo
- Iloprost (Ventavis®) – Inalado
- Epoprostenol (Flolan®) – Intravenoso
- Selexipag (Uptravi®) – Oral (aprovado pela FDA em dezembro 2015)

Inibidores dos receptores de endotelinas

- Ambrisentan (Letairis®) - Oral
- Bosetan (Tracleer®) - Oral
- Macitentan (Opsumit®) - Oral

Inibidores de fosfodiesterase-5

- Sildenafil (Revatio®) - Oral
- Tadalafil (Adcirca®) - Oral

Estimulador de guanilato ciclase

- Riociguat (Adempas®) - Oral

Apesar do arsenal terapêutico para o tratamento de HP ter aumentado muito nos últimos anos, uma parcela significativa dos pacientes não apresenta melhora ou evolui com piora clínica durante a monoterapia, sendo necessário o uso de terapia combinada, que se mostra segura e eficaz em alguns casos.

Esses medicamentos têm aumentado a sobrevida dos pacientes e abrandado a gravidade dos casos, melhorando muito a qualidade de vida, e elevando a sobrevida de três para até doze anos. Antes do advento desses fármacos, a hipertensão pulmonar era responsável por 12% das indicações de transplantes de pulmão no mundo. Hoje, esse índice caiu para 2% (CARAMORI et al.; WAXMAN et al., 2013).

O uso dos prostanóides no tratamento da HP vem sendo utilizado a bastante tempo, sendo a prostaciclina uma molécula importante envolvida em vasodilatação e inibição da agregação plaquetaria, inflamação e proliferação de células musculares lisas.

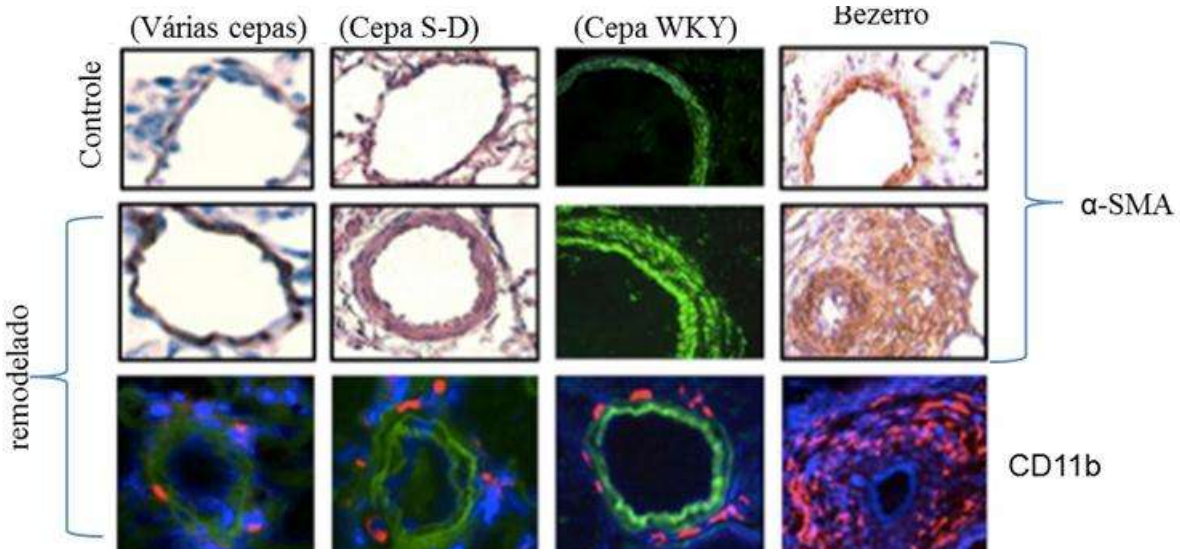
Em 2001, os antagonistas dos receptores de endotelina foram os primeiros medicamentos aprovados pelo FDA (U. S. Food and Drug Administration) para o tratamento da HP, sendo a endotelina um potente vasoconstritor e mitógeno de células do músculo liso, encontrada em níveis elevados na HP e que contribui para a progressão da doença. Mais recentemente, os inibidores de fosfodiesterase-5 (PDE-5) foram introduzidos como medicamento de escolha no tratamento da HP (WOLFSON, et al., 2014).

2.4 MODELOS DE HIPERTENSÃO PULMONAR

Inúmeros modelos animais de HP estão atualmente disponíveis e os mais utilizados são o modelo de hipóxia crônica e modelo de monocrotalina (MCT). Estes modelos animais são utilizados há muito tempo e sem dúvida, contribuíram para uma melhor compreensão do processo de hipertensão pulmonar (STENMARK et al., 2009; GOMEZ-ARROYO et al. 2012).

A hipóxia crônica é frequentemente utilizada para induzir HP em uma variedade de espécies animais (Figura 4). Este modelo é útil porque é muito previsível e reprodutível em camundongos, entretanto as complexas lesões encontradas em humanos com HP grave não se desenvolvem bem neste modelo (STENMARK et al., 2009; VONK-NOORDEGRAAF et al., 2013).

Figura 4: Hipóxia induzindo remodelamento vascular e perivascular em diferentes animais: Espessamento (média e adventícia) e acumulo de células inflamatórias. S-D: Sprague Dawley; WKY: Wistar Kyoto; CD11b: marcador para leucócitos; α-SMA: alpha actina do musculo liso.



Fonte: Stemmark et al., 2009

No modelo de MCT, ocorre uma lesão endotelial, seguida de intensa inflamação perivascular culminando no desenvolvimento de hipertensão pulmonar grave em ratos. As células inflamatórias envolvidas consistem principalmente de macrófagos derivados da medula, células dendríticas imaturas (DCs), e uma minoria de linfócitos (SAHARA et al., 2007; PERROS et al., 2007).

Para exercer seu efeito tóxico, a MCT necessita ser ativada pelo sistema enzimático citocromo p450 dos hepatócitos, que ativa compostos chamados dehidroalcalóides ou pirróis (MATTOCKS et al. 1986). Esses compostos funcionam como agentes alquilantes (inativa enzimas e proteínas) de macromoléculas celulares, iniciando uma toxicidade aguda ou crônica. Efeitos nefrotóxicos, cardiotóxicos, fetotóxicos e carcinogênicos também estão relacionados a MCT (MEDEIROS et al., 2000).

No modelo de hipertensão pulmonar, a MCT é injetada uma única vez por via subcutânea ou intraperitoneal e torna-se metabolicamente ativada, pela enzima citocromo p450 sendo chamada de MCT-pirrol (COOPER & HUXTABLE 2009). A MCT-pirrol é pneumotóxica, provocando danos nas células endoteliais da artéria pulmonar (GOMEZ-ARROYO et al., 2012). Outras características de remodelação vascular pulmonar induzida por MCT é a hiperplasia da camada média das artérias pulmonares, edema intersticial, inflamação da camada adventícia e eventualmente fibrose, resultando no aumento da resistência vascular pulmonar por hipertrofia do ventrículo direito (HANDOKO et al., 2009). Em camundongos, a monocrotalina apresenta uma variação na sua toxicidade, uma vez que a conversão de MCT em MCT-pirrol não acontece de maneira eficiente sendo necessário a conversão química *in vitro* deste composto e sua aplicação intravenosa (jugular) (DUMITRASCU et al., 2008).

Pouco se sabe sobre quais os mecanismos celulares, bioquímicos e moleculares que estão envolvidos no desenvolvimento da HP induzida por monocrotalina, e portanto, é de extrema importância caracterizar o desenvolvimento do processo inflamatório durante o estabelecimento da HP, uma doença de etiopatogenia diversa (RUITER et al., 2013).

2.5 DIETILCARBAMAZINA

A dietilcarbamazina é um fármaco filaricida que tem sido utilizado com sucesso por autoridades de saúde pública como uma ferramenta-chave para a eliminação da filariose linfática em vários países. É um derivado da piperazina sintetizada como 1- dietilcarbamil-4-metilpiperazina e preparada na forma de cloridrato, citrato ou fosfato. Apresenta-se na forma

de pó branco, muito solúvel em água, estável, mesmo em condições de umidade e temperatura elevadas (DREYER & NORÕES 1997). Atualmente é produzida pelo laboratório farmacêutico federal Farmanguinhos e distribuída sem custo para a população, por órgãos responsáveis pelo controle da filariose no Brasil, inclusive nos postos de saúde na Região Metropolitana do Recife.

Apresenta vantagens como baixa toxicidade, intenso efeito microfilaricida e possibilidade de administração oral. É rapidamente absorvida pelo trato gastrointestinal e atinge o pico da sua concentração plasmática entre uma e três horas após a ingestão (HAWKING et al., 1979). De acordo com Ilondu e colaboradores (2000), ela está quase ausente na urina, plasma e saliva de humanos após 24h da ingestão. Já em camundongos, estudos toxicológicos e farmacológicos indicaram que após 3h o composto é completamente excretado pelo rim (HARNED et al., 1948).

O esquema terapêutico da DEC utilizado para filariose consiste em um tratamento de 12 dias, na dose de 6mg/kg/dia (WHO, 1995). As reações adversas mais comumente encontradas são: náuseas, vômito, dores abdominais, diarréias, dores de cabeça, febre, sono e mialgia que se estendem por um ou mais dias. No entanto, essas reações relacionam-se claramente à carga parasitária (PARTONO et al., 1981).

O mecanismo de ação da DEC ainda é pouco conhecido. De acordo com Maizels e Denham (1992) este fármaco alteraria o metabolismo do ácido araquidônico nas microfilárias e nas células endoteliais do hospedeiro, interferindo nas vias da ciclooxigenase e da lipooxigenase, e assim bloqueando a produção de leucotrienos, prostraglandinas e tromboxanos.

Diversos estudos demonstraram que a DEC é altamente eficaz no tratamento de doenças pulmonares: Boggild e colaboradores (2004) trataram pacientes com eosinofilia pulmonar tropical por 21 dias com DEC e observaram uma melhora acentuada de seus sintomas respiratórios. Stenmark e colaboradores (1985) observaram que DEC melhorou a pressão sistólica, número de PMN no lavado bronquioalveolar (LBA), diminuiu número de leucócitos ativados e os níveis de prostaglandinas em hipertensão pulmonar induzida por monocrotalina.

Queto e colaboradores (2010) concluíram que a DEC tem importante ação no bloqueio da inflamação eosinofílica pulmonar em camundongos sensibilizados com ovalbumina por diminuir a expressão de citocinas e reduzir o número de células inflamatórias no LBA e no tecido pulmonar. Estudo realizado por Florêncio, Saraiva e Peixoto (2005) demonstrou que

após 12 dias de tratamento com DEC os pneumócitos do tipo II apresentaram uma ativação do metabolismo de surfactante pulmonar. O aumento da síntese e secreção de surfactante diminui a tensão superficial dos alvéolos, reduzindo os esforços musculares dos movimentos respiratórios. Pesquisas também apontaram que DEC atenuou a sintomatologia da asma por bloquear a produção de leucotrienos (ZUO et al., 2004).

Estudos recentes utilizando diferentes modelos experimentais de inflamação também têm indicado que DEC inibe o NF-kB que é um regulador chave de genes pró- inflamatórios, tais como TNF-α, IL-1β, iNOS e COX-2 (SILVA et al., 2014). Ribeiro e colaboradores (2014) observaram redução do edema bem como do número de células PMNs, diminuição da produção de nitrito e da expressão de mediadores inflamatórios, além da inibição da expressão do NF-kB em modelo de LPA induzida por carragenina, demonstrando que a DEC é um fármaco com potencial terapêutico para doenças pulmonares agudas.

Além de atividades anti-inflamatórias, DEC já tem sido relacionada também como pró-apoptótica. Estudos ultraestruturais mostraram danos morfológicos drásticos em microfilárias incluindo a presença de grandes vacúolos, lise da cromatina e do citoplasma, além de corpos de extrusão a partir da membrana plasmática, características indicativas de apoptose (PEIXOTO et al., 2004), que foram confirmados por outros testes, como PCR e TUNEL (PEIXOTO et al., 2008). Queto et al. (2010) relataram que a DEC não teve nenhum efeito sobre animais deficientes de CD95L, o ligante para o receptor de indução de apoptose CD95 (Fas). Estes resultados confirmaram que a DEC pode possivelmente atuar como um indutor de apoptose como anteriormente demonstrado por estudos ultraestruturais e moleculares em microfilárias de *W. bancrofti* além de estudos ainda não publicados demonstranto que a DEC induz apoptose em células inflamatórias em modelo de ALI (PEIXOTO et al., 2008).

Diante do exposto, sugerimos a hipótese de que DEC pode atuar como fármaco anti-inflamatório diminuindo a expressão de proteínas inflamatórias, remodelação vascular, indução de apoptose contribuindo para resolução da inflamação crônica e hipertensão pulmonar.

3- OBJETIVOS

3.1 Objetivo geral:

Caracterizar o processos patológicos teciduais e moleculares envolvido no desenvolvimento da hipertensão induzida por monocrotalina em camundongos C57BL/6J e a ação da DEC sobre esses processos.

3.2 Objetivos específicos:

Caracterizar o envolvimento de células, apoptose, citocinas e fatores de transcrição na etiopatogenia da hipertensão pulmonar em camundongos C57BL/6J e o efeito do tratamento com DEC através dos seguintes parâmetros:

- Avaliar as modificações histopatológicas na formação de lesões plexiformes no tecido pulmonar durante desenvolvimento da HP.
- Caracterizar as células inflamatórias que podem estar envolvidas no desenvolvimento da HP como os neutrófilos, macrófagos, linfócitos T e linfócitos B através de seus marcadores específicos (GRO, F4/80, CD40L e CD24 respectivamente).
- Avaliar a proliferação celular através da expressão do fator de crescimento vascular endotelial (VEGF), proteína morfogenética do osso tipo II (BMPR2) e Fator de crescimento transformador beta (TGF-β), bem como pela presença de α-actina do músculo liso (α -SMA), em cada fase do desenvolvimento da HP.
- Caracterizar as proteínas inflamatórias, interleucina 1β (IL-1β), Interleucina 6 (IL-6), Interleucina 17 (IL-17) e marcador endotelial endotelina 1, A e B.
- Avaliar a via de sinalização da inflamação através da expressão de NFkB, NFkB-P, IkBα, IkBα-p.
- Avaliar o estresse oxidativo pelas enzimas óxido nítrico sintase endotelial e induzível (eNOS e iNOS), bem como os níveis de óxido nítrico.
- Caracterizar o processo de apoptose pela avaliação da, FADD, caspase 8, caspase 3, caspase 9, citocromo 3, BAX e BCL2.
- Avaliar o perfil hemodinâmico através da ecocardiografia.

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5- ARTIGO I- Characterization of a mice model of monocrotaline-induced pulmonary hypertension through inflammation mediators and endothelial markers

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Submetido a revista:

Pulmonary Pharmacology and Therapeutics

ISSN: 1094-5539

Characterization of a mice model of monocrotaline-induced pulmonary hypertension through inflammation mediators and endothelial markers

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Abstract:

Monocrotaline (MCT) is a pyrrolizidine alkaloid produced by the *Crotalaria* genus. Its ingestion induces pulmonary hypertension (PH) in various animal species, and is associated with right ventricular hypertrophy and an increase in the medial thickness of the pulmonary trunk and the small pulmonary arteries. The aim of the present study was to investigate the pathogenic progress of a mice model of monocrotaline-induced PH through analysis of inflammation mediators and endothelial markers analysis. C57/BL6 male mice were used in all experiments. An MCT solution intraperitoneal injection (600mg/kg) was administered once per week. Five groups (n=10) were studied: Sham; MCT_7; MCT_{14}; MCT_{21}, and MCT_{28}. Echocardiography analysis was performed and lung tissues were collected for light microscopy (haematoxylin-eosin and Masson's trichrome staining), immunohistochemistry (GRO, F4/80, CD40L, CD24, VEGF, BMPR2, TGFβ1, αSMA, IL-1β, IL-6, MCP-1, IL17 and iNOS), western blot (IL1 β, IL6, MCP-1, eNOS, endothelin 1, endothelin A receptor,

endothelin B receptor, IL17, VEGF and BMPR2) and Rt-PCR (iNOS and TGF-β). MCT enhanced pulmonary artery pressure, mainly by increasing pulmonary vascular resistance, with no impairment of left ventricular function Monocrotaline-induced PH lung sections exhibited alveolar exudates, interstitial edema, inflammatory cellular infiltrates and emphysema. Deposition of collagen fibers and αSMA staining around the pulmonary arteries were evident the on 28th day, confirming fibrosis and arterial muscularization. There was a significant increase in the nitrite and nitrate levels of the pulmonary tissue in the MCT groups, in comparison with the sham group. A significant expression ($p<0.05$) of F4/80, CD40L, CD24, VEGF, TGFβ1, αSMA, IL-1β, IL-6, MCP-1, IL17, iNOS, eNOS were found in the MCT groups. Contrastingly, MCT groups exhibited a reduced expression of TGF-1β ($p<0.05$). Endotelin-1 and the ET-B receptor expression increased significantly in the MCT28 group in comparison with the other groups. These findings characterize the evolution of the inflammatory process, with the participation of cells, cytokines, growth factors and oxidative stress in a PH model induced by MCT in mice.

1- INTRODUCTION

Pulmonary hypertension (PH) is a progressive condition defined by a mean pulmonary artery pressure greater than 25 mmHg, leading to a chronic elevation of pulmonary vascular resistance, right ventricular failure, and early death [1]. A common histological finding is the presence of complex, multicellular vascular lesions which obstruct and obliterate the pulmonary arterioles [2].

It is known that some forms of pulmonary hypertension are unquestionably linked to genetic character. At the same time, a number of factors can trigger the onset of pulmonary hypertension in an individual by a number of known risk factors including the chronic use of some medications, viral infections and inflammatory based diseases. PH is a syndrome of various entities with seemingly diverse etiologies, some of which are common denominators, and is therefore of multidisciplinary interest [3].

The lesions resulting from PH are characterized by changes in the components of the arterial wall, beginning with medial hypertrophy due to the excessive proliferation of smooth muscle cells of pulmonary arteries (PASMCs), proliferative and fibrotic changes in the thickening of the intima and adventitia, perivascular inflammatory infiltrates, and thrombotic lesions. In fact, studies have shown that inflammation occurs in many forms of PH in humans and animals, and that the nuclear transcription factor kappa B (NF-kB) regulates multiple

genes associated with inflammatory response, cell growth control and apoptosis, which are implicated in the progression of the disease [4].

The fact that inflammation precedes vascular remodeling in an experimental PH model suggests that impaired immunity is probably a cause, rather than a consequence of vascular disease. Besides the increase of immune cells in the intravascular and perivascular infiltrate, circulating levels of certain cytokines and chemokines are abnormally high. These include interleukin (IL)1β, IL-6, IL-8, chemoattractant protein-1 monocytes, fractalkine, CCL5 / RANTES, and tumor necrosis factor (TNF)α. Some of these cytokines and chemokines correlate with a worse clinical outcome in patients with PH, and can therefore serve as biomarkers of disease progression. Furthermore, it was demonstrated that IL-1β and TNF-α are associated with a extracellular matrix accumulation of proteins such as fibronectin, observed in PH and other injuries, while IL-6 has been associated with the proliferation of smooth muscle cells [5].

Animal models are important tools for the study of the pathogenic mechanisms of PH, and for the development of new therapeutic strategies. Established models of PH include chronic exposure to hypoxia, monocrotaline (MCT), monocrotaline pyrrole (MCTP) and increased pulmonary blood flow in various mammals [1, 6].

MCT model is considered, by some to be a toxic model, with it being suggested that MCT rats die from hepatic veno-occlusive disease with liver failure instead of right ventricle failure [7]. MCT is a plant-derived alkaloid found in the plant *Crotalaria spectabilis.* Its ingestion induces pulmonary hypertension in various animal species, associated with right ventricular hypertrophy and an increase in the medial thickness of the pulmonary trunk and small pulmonary arteries [8]. Pulmonary endothelial injury caused by toxins, reactive oxygen species, autoimmune activity, and shear stress are known to lead to severe PH [9]. MCT is known to cause pulmonary endothelial injury and pulmonary hypertension in humans and rats [1, 10] but has little effect on mice [11]. To exert a toxic effect MCT, needs to be activated by the cytochrome p450 enzyme system of hepatocytes, which activates compounds called dehydro alkaloids or pyrroles (MCTP) [2]. MCT produces a variation in toxicity in mice, because as the MCT-pyrrole conversion is not efficient. As a result *in vitro* chemical conversion is necessary, and its intravenous administration promotes high mortality [6].

Perivascular inflammation is common in the remodeling of blood vessels, both in animal models and in human PH. However, it is unclear whether such inflammatory processes

are integral to the initiation and propagation of vascular remodeling, or are just bystander effects [1].

Studies in literature on the evolution of the inflammatory process have shown that the participation of cells, cytokines, growth factors and oxidative stress in PH models induced by MCT in mice is not well understood. While these are known to be present, in order to cause the resulting damage, the evolutionary process which can lead to permanent damage is not known.

The aim of the present study was to examine pulmonary endothelial injury caused by MCT in mice by measuring inflammation and endothelial markers of the development of pulmonary hypertension.

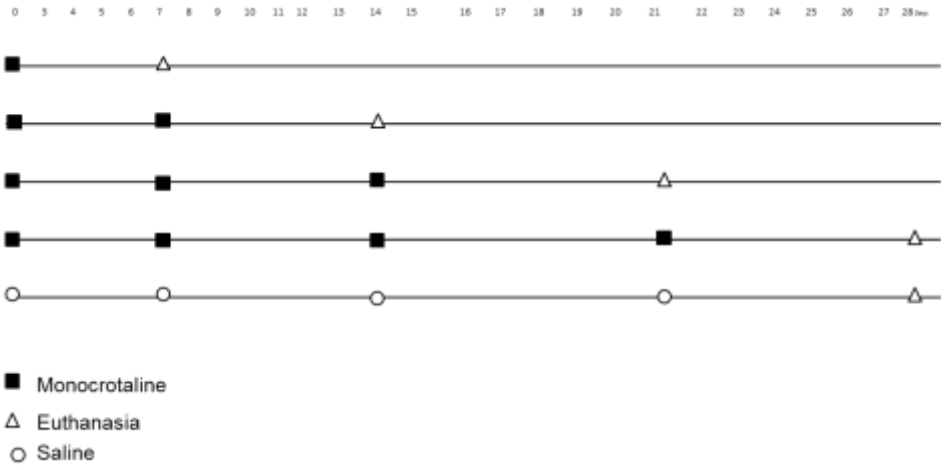
2- MATERIALS AND METHODS

2.1 Animals

Fifty male C57BL/6 mice, weighing 26-30 g, were used in all experiments. The mice were examined to determine their health status and acclimated to the laboratory environment of 23-24°C. They were kept in a 12/12 h day/night cycle photoperiod. The animals were housed in metal cages and fed a standard diet with water ad libitum. All experimental procedures were approved by the Ethics Committee for Animal Experimentation (FIOCRUZ/CPqAM).

2.2 Experimental Design

Mice received a intraperitoneal (i.p) injection of MCT (600mg/kg Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline solution and administered once per week (7, 14, 21 and 28 days). Five groups (n=10) were studied: Sham; MCT_7; MCT_{14}; $MCT_{21,}$ and MCT_{28}.



2.3 Determination of right ventricular hypertrophy (RVH), pulmonary artery and left ventricular function

Echocardiography analysis was performed using a VisualSonic Vevo770, Toronto, Canada) on day 28. During the procedure, isofluorane/O2 administration was administered using a facemask to keep the mice lightly anesthetized, with heart rates in the range of 300-350 bpm. The right ventricle was visualized from the right parasternal long axis view with a 704 RMV scanhead. The right ventricular wall thickness was measured from images produced in M-mode, using the depth interval (mm) generic measurement tool (Vevo770 v3.0 software, VisualSonics). Doppler flow images were recorded from the left parasternal long axis view with a 707 B scanhead pointing slightly towards the left shoulder to visualize the pulmonary artery. Volume was measured at the level of the pulmonary valve, and several indices of pulmonary artery blood flow (velocity time integral, peak and mean pressure gradient and peak and mean velocity) were assessed using the pulmonary valve protocol measurement tool [12].

2.4 Euthanasy

The animals were euthanized after echocardiography analysis by CO2. The lungs were collected and processed for further analysis.

2.4 Histopathological analysis

Lung fragments were washed twice in PBS, pH 7.2, and fixed in Bouin Solution (1% saturated picric acid, formaldehyde, and 40% glacial acetic acid) for 8 hours, dehydrated in an increasing ethanol series, cleared in xylene, and embedded in purified paraffin. Tissue sections of 5 μm were cut using a microtome, deparaffinized with xylene, stained with haematoxylin-eosin, and then Masson's trichrome stain was performed according to a previously described method [13]. Slides were histopathologically evaluated using a semi-quantitative scoring method: lung injury was graded from 0 (normal) to 4 (severe) in four categories: interstitial inflammation, inflammatory cell infiltration, congestion, and edema. The total lung injury score was calculated by adding up the individual scores of each category [14].

2.5 Immunohistochemical Localization

The paraffin sections of lung tissue were mounted onto slides. After being deparaffinized, the tissues were incubated overnight at 4°C with primary antibody GRO (1:100 cat. ab17882), F4/80 (1:100 cat. ab6640), CD40L (1:100 cat. ab65854), CD24 (1:50 cat. st19651), VEGF (1:100 cat. ab1316), BMPR2 (1:100 cat. ab96826), TGFβ1 (1:50 cat.

st146), αSMA (1:100 cat. ab5694), IL-1β (1:100 cat. ab9722), IL-6 (1:50 cat. eb14-7061), MCP-1 (1:100 ab7202), IL17 (1:50 cat. st7927) and iNOS (1:100 cat 3523). The antigen-antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB + Kit, Peroxidase), using 3,3-diaminobenzidine as a chromogen. Imaging was performed by light microscopy. Five pictures at the same magnification were quantitatively analyzed using the Gimp 2.8 software program (GNU Image Manipulation Program, UNIX platforms) [15].

2.6 Western Blot Analysis

The lungs were pulverized in liquid nitrogen and the total proteins were extracted using an extraction cocktail (10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate (NaVO4), 10 mM of aprotinin, and 100 mM Tris(hydroxymethyl)aminomethane, pH 7.4). Western blotting and the subsequent quantification of each blot were performed as previously described [14]. The primary antibodies for IL1 β, IL6, MCP-1, eNOS, endothelin 1 (ET1), endothelin A (ET_A), endothelin B (ET_B) were from obtained Abcam (CA, USA), IL17, VEGF, BMPR2 were obtained the from Santa Cruz Biotechnologies (Santa Cruz, CA) and secondary antibodies for β-actin and α-tubulin were from Sigma-Aldrich (USA).

2.7 Measurement of nitrite–nitrate concentration

The total nitrite concentration in the lung tissue was measured as previously described [16]. Griess colorimetric reaction was used to measure nitric oxide, involving the detection of nitrite (NO_2) and the oxidation of NO in the lung tissue. In duplicate, 50 μl of the samples was added to a 96-well ELISA plate, followed by the same volume of Griess reagent, which was composed of 1% sulfanilamide, diluted in 2.5% H_3PO_4 (solution A) and N-1-naphtyl-ethtylenodiamina, also diluted in 2.5% H_3PO_4 (solution B). To prepare the standard curve, a solution of sodium nitrite in an initial concentration of 100 μM was serially diluted in PBS. After incubation for 10 min in the dark, a reading was taken from the spectrophotometer at 490 nm. The absorbance of different samples was compared with the standard curve and the results were expressed as mean ± standard error of the duplicate, using the GraphPad Prism program (v. 6.0).

2.8 RNA extraction and quantitative real-time polymerase chain reaction (q RT-PCR)

Total RNA from mouse tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The forward and reverse primers used for each gene were as follows: 5′-

GTTCTCAGCCCAACAATACAAGA-3′ and 5′-GTGGACGGGTCGATGTCAC-3′ for iNOS; 5’-AAAATCAAGTGTGGAGCAAC-3’ and 5’-CCACGTGGAGTTTGTTATCT-3’ for TGF-β; 5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTGAGGTCA-3′ for GAPDH (endogenous control). All reactions were performed in triplicate and included the following: 1 μL of cDNA; 5 μM of each primer; 2x SYBR Green PCR Master Mix (Applied Biosystems); and water added to give a final volume of 25 μL. The relative amount of mRNA was determined using the comparative threshold (Ct) method by normalizing target cDNA Ct values to those of GAPDH. Fold increase ratios were calculated relative to control (basal conditions) for each group using the formula 2e-ΔΔCt [15].

2.9 Data Analysis

GraphPad Prism software (version 6) was used for statistical analysis. Data were expressed as mean ± standard deviation. Differences between the control and treatment groups were analyzed using analysis of variance (ANOVA), prior to the performance of Tukey’s post hoc test or the Student's t-test. Probability values less than 0.05 were considered significant.

3 - RESULTS

3.1 Monocrotaline induced pulmonary hypertension

Doppler analysis at the pulmonary valve level, recorded by ultrasonography, demonstrated that MCT at 600mg/kg induced a significant increase in the pulmonary arterial blood flow gradient and systolic velocity (Fig. 1 A- E). A significant increase of the chamber size of the RV, as indicated by the end-diastolic dimension, was identified after MCT treatment (Fig. 1H). There was no significant alteration in LV function, represented by ejection fraction and LV chamber size, between the groups (Fig. 1F-G).

3.2 Histopathologic evaluation

Although histological analysis of the sham group did not reveal any morphological changes, the MCT7 group presented alveolar exudate, interstitial edema with thickening of the alveolar septum and inflammatory cell infiltration. The MCT14 group presented inflammatory infiltration with the presence of activated macrophages, plexiform lesions of the pulmonary arteries and emphysema. Interestingly, in the majority of arteries the adventitia layer was completely invaded by inflammatory cells, and, in some case, dissolved (Fig. 2A).

In the MCT21 and MCT28 groups there was an increase in these injuries, as well as thickening of the medial layer of the pulmonary arteries (Fig. 2A). The MCT28 group displayed a decrease in inflammatory infiltrates in comparison with the MCT7, MCT14 and MCT21 groups (Fig. 2B).

Masson's trichrome staining revealed a significant increase in collagen deposition in the pulmonary interstitium and around the arteries and vessels of the MCT7, MCT14 and MCT21 groups. Additionally, the MCT28 group displayed collagen deposition around the bronchioles. There was no Masson staining in some adventitia areas due to the lesions caused by the inflammatory infiltrates, as described in the histopathogical analyses (Fig. 2C). Immunolabeling of the lung sections with α-smooth muscle actin revealed a significant decrease in the MCT14 and MCT21 groups, compared to the sham group, possibly due to the countless inflammatory infiltrate foci around the arteries and vessels. On the other hand, the MCT28 group displayed a significant increase of α-smooth muscle actin labeling around the arteries, vessels and bronchioles, confirming the muscularization process (Fig. 2D).

3.3 Inflammatory cells and cytokines

The expression of inflammatory cell markers such as neutrophil (GRO), macrophage (F4/80), Lymphocyte B (CD24) and lymphocyte T (Cd40L) in the lung tissue was evaluated by immunohistochemical analysis. There was a significantly greater infiltration of neutrophilic cells in tissue sections obtained from mice from the MCT14 group than from mice from other groups (Fig. 3A). The expression of monocyte and macrophage labeling, detected by F4/80, was greater in the MCT14, MCT21 and MCT28 groups than in the sham group. However, there was a significant decrease of mononuclear cells in the MCT28 group than in the MCT 14 and MCT21 groups (Fig. 3B). The CD24 marker for B-lymphocytes was detected in all groups treated with MCT, when compared with the sham group (Fig. 3C). At the same time, the marker for T-lymphocyte, CD40L, was significantly greater in the MCT21 and MCT28 groups when compared to the other groups (Fig. 3D). Interleukin-1 (IL-1) is a central mediator of innate immunity and inflammation, whereas IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory actions. IL-6 is often induced together with IL-1 in many alarm conditions, and circulating IL-6 plays an important role in the induction of acute phase reactions. The MCT7 and MC14 groups displayed significant IL-1 and IL6 immunolabeling, confirmed by western blot analysis (Fig. 4A-B and I). The Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) is a key chemokine for the regulation of the migration and infiltration of monocytes/macrophages. Immunohistochemical analysis for

MCP-1 revealed macrophages around plexiform injuries and significant expression from the 14th day onwards, data confirmed by western blot analysis (Fig. 4 C and I). Interleukin 17 (IL-17) is expressed by a distinct type of T cell, T helper 17 cells, and certain other lymphocytes. The MCT14 and MCT28 groups presented significant immunolabeling for IL-17, which was confirmed by protein expression analysis (Figure 4D and I).

3. 4 Analysis of cell proliferation markers

To determine the effect of monocrotaline on the PH mouse model, gene expression, protein level and immunostaining of Vascular endothelial growth factor (VEGF), Transforming growth factor beta (TGF-β) and Bone morphogenetic protein receptor type II (BMPR2) ware evaluated in the lung tissue.

VEGF immunostaining was observed throughout the lung tissue, mostly in the pulmonary arteries of the MCT28 group. VEGF expression was significantly higher in the MCT21 and MCT28 groups than in the sham group (Fig. 5A and G).

TGF-β is a family of multifunctional molecules that regulate many cellular functions, including cell growth and differentiation, and are also potent modulators of extracellular matrix synthesis. The alveolar macrophages, bronchial epithelium and vessels were significantly labeled by anti-TGFβ in the MCT14, 21 and 28 groups (Fig. 5B). In contrast, molecular analysis of t TGF-β gene expression was significantly enhanced in the MCT7 group, compared to the sham group (Fig. 5I).

Bone morphogenetic protein receptor II (BMPR2) is a component of the heteromeric vascular smooth muscle cell BMPR receptor, a member of the transforming growth factor-β family. All groups that received monocrotaline revealed a decrease of BMPR2 immunostaining around the arteries, vessels and bronchioles when compared with the sham group (Fig.5C), whereas western blot analysis revealed a decrease in BMPR2 on the 28^{th} day, compared to the sham group (Fig 5G).

3.5 Expression of endothelial markers and oxidative stress in MCT-treated lungs

NO is an important biological mediator in the living organism, and is synthesized from L-arginine using NADPH and molecular oxygen. However, the overproduction of NO, which is catalyzed by iNOS, a soluble enzyme and active in its dimeric form, is cytotoxic. Immunohistochemistry for iNOS in the lung sections revealed an increase in all the monocrotaline-groups (Fig. 6A). Corroborating these results the levels of nitric oxide in the lung tissue increased in all groups that received monocrotaline (Fig. 6G). Contrastly, the

evaluation of iNOS gene expression revealed no significant difference between the groups (Fig.6 H). The eNOS isoform is predominantly expressed in the endothelial cells and regulates basal pulmonary and peripheral vasomotor tone. Interestingly, the MCT14, MCT21 and MCT28 groups displayed higher eNOS expression in the lung tissue (Fig. 6B and E).

The endothelin-1 (ET-1) protein is produced primarily by the vascular endothelial cells, and is characterized as a powerful vasoconstrictor and smooth muscle mitogen. Endotelin-1 protein expression increased significantly in the MCT28 group compared with the other groups. While there was no significant difference in ET-A receptor expression between the groups, ET-B receptor expression was significantly enhanced on the 28^{th} day after monocrotaline-induction, confirming the ET-1 expression data (Fig. 6E).

4- DISCUSSION

Alkaloids are molecules that may have hepatoxic, neurotoxic, mutagenic and pneumotoxic effects [17]. The alkaloid monocrotaline induces PH and is used as a research tool in an attempt to understand the multifactorial mechanisms of disease. In the present study, it was observed that inflammatory cells, cytokines, growth factors and oxidative stress play an important role in the development of pulmonary hypertension induced by monocrotaline in mice.

One of the processes involved in the development of pulmonary hypertension is an increase RV overload, leading to RVH and RV failure [18]. Studies have shown that MCT treatment induced RVH in C57Bl-6 wild-type mice and was associated with an increase in myocytes in cross-sectional areas [19]. These findings were confirmed in the present study by Doppler analysis, demonstrating that MCT enhances pulmonary artery pressure, mainly by increasing pulmonary vascular resistance, with no impairment of left ventricular function.

Some investigators have suggested that the increases in pulmonary artery pressure and vascular remodeling are caused by early and often dramatic accumulation of mononuclear inflammatory cells in the adventitial sheath of the small intra-acinar vessels [20]. MCT induces proinflammatory reaction and invites leukocyte infiltration, which may play a critical role in the pathogenesis of PH mediated by RVH. Cytokines and chemokines are predominantly produced by inflammatory cells of the innate immune system, but can also be produced by any of the cellular components of the vascular wall or adventitia [1, 21]. Upregulation and the increased production of cytokines represent an intrinsic or innate stress response against cell injury in PH [19]. The present study characterized the inflammatory cells types during the PH development, and identified a majority of polymorphonuclear cells

in the early phase (until the 14th day), and the presence of mononuclear cells in the late phase (until the 28th day), promoting intense tissue destruction, mainly around the pulmonary arteries.

Recent investigations have provided evidence that both the pulmonary vascular cells and the inflammatory cells are important local sources of chemokines that can lead to pulmonary vascular remodeling in PH [22, 23]. Patients with idiopathic pulmonary arterial hypertension (iPAH) have elevated serum levels of cytokines, which may act as biomarkers both for the diagnosis and clinical outcome of patients with pulmonary hypertension [1, 24].

This data suggests that the increased levels of such mediators may be a mutual pattern of PH pathology per se, and are not restricted to one particular subtype. IL-1 is primarily secreted by macrophages and endothelial cells, and stimulates the expression of adhesion molecules and chemokines. Elevated IL-1β has been found in the serum of patients with iPAH. In turn, rats with monocrotaline-induced PAH were shown to have elevated levels of IL-1β in the bronchoalveolar lavage fluid, and treatment with an IL-1 receptor inhibitor attenuated both PAH and RVH [25].

It was observed that IL-1β increased until the 14th day after the induction of MCT, corroborating the results obtained by Gillespie et al (1989) [26]. These authors demonstrated that the increase of IL-1β plays an important role in the development of PH. MCT administration was associated with an initial phase of pulmonary edema on the 7th day until the 14th day coinciding temporally with the development of lung injury, inflammation and/or PH. These observations indicated that the increase of bronchoalveolar fluid content and presence of IL-1β are temporally related to the development of lung injury induced by monocrotaline.

Cytokine IL-6 is often induced together with the proinflammatory cytokines TNF-α and IL-1β in many alarm conditions. Circulating IL-6 plays an important role in the induction of acute phase reactions, modulating several aspects of inflammation, some cytokine responses and inflammatory tissue infiltration [27]. Additionally, it plays an important role in PH, being consistently greater in the serum and lungs of patients with iPAH [28]. Interestingly, in monocrotaline-induced PH, IL-6 levels were coincident with IL-1β levels, peaking in the early stages of lung inflammation.

Inflammation with an increase in the activity of pathogenic T cells and macrophages is a hallmark of different types of pulmonary hypertension, particularly when associated with autoimmunity, chronic obstructive pulmonary disease, helminth parasite infections and

idiopathic pulmonary hypertension [29]. The right heart develops structural changes as well as metabolic and molecular reprogramming [30]. IL-17is expressed by T helper 17 cells and plays a key regulatory role in adaptive immune defense and inflammatory diseases [31]. Monocrotaline-induced PH revealed that on the 28th day there was a significant increase in IL-17 pulmonary levels, as well as a significant accumulation of T-cells. These results suggest that IL17, as the result of the production of activated T-cells, contributes to the development of PH. Park et al (2014) [32] showed that the combined neutralization of interleukin 13 (IL-13) and IL-17 significantly ameliorated the increase in right ventricular systolic pressure, the circumferential muscularization of pulmonary arteries, and molecular change in the right ventricle. Although Th-17 cells are the major source of IL-17, Brodlie et al (2011) [33] described elevated IL-17 in the airways of patients with advanced cystic fibrosis lung disease and PH, being the first description of neutrophils as a potential source of this key cytokine in the human airways.

MCP-1 is a chemoattractant for monocytes, lymphocytes, and basophils. It is produced by a number of cells in response to inflammatory stimuli, including macrophages, lymphocytes, basophils, epithelial cells, endothelial cells, fibroblasts, osteoblasts, and bone marrow stromal cells. Inflammatory stimuli for MCP-1 release include IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ, TGF-β, while mounting evidence suggests that MCP-1 is involved in inflammatory disorders of the lungs [34]. Studies with (NEMO)-binding domain (NBD) peptide, which can block the activation of the IκB kinase (IKK) complex, significantly reduced the number of proliferating cells and reduced injury-induced neointimal formation. These effects were associated with a significant reduction of NF-κB activation and monocyte chemotactic protein-1 expression in the carotid arteries of rats treated with peptide [35]. Other studies have demonstrated that the signaling cascade from fibroblast growth factor 2 (FGF2) to plasminogen activator inhibitor 1 (PAI-1) and monocyte chemotactic protein-1 (MCP-1) via the nuclear transcription factor nuclear factor kappaB (NF-kB) plays a critical role in the progression of PAH [4]. Confirming these previous results, the present study revealed MCP-1 labelling of macrophages around plexiform injuries and a significant protein expression from the 14th day, followed by a deposition of collagen and α-SMA.

Growth factors and inflammatory mediators are implicated in the abnormal proliferation and migration of pulmonary vascular cells. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are potent mitogens and chemoattractants for endothelial cells, smooth muscle cells, and fibroblasts [36]. It has been postulated that in

lungs with PAH, endothelial cells in plexiform lesions express gene encoding for proteins involved in angiogenesis, in particular, vascular endothelial growth factor (VEGF) and those involved in VEGF receptor-2 (VEGFR-2) signaling [37]. These findings are consistent with the concept that plexiform lesions develop via a process resembling angiogenesis.

Other studies have shown that VEGF expression can be stimulated in PH via TGF-β1. Mata-Greenwood et al (2005) [38] demonstrated that an earlier increase in TGF-β1 expression in the small pulmonary arteries of shunted lambs preceded VEGF upregulation. According to these authors, VEGF expression is dependent on TGF-β1-mediated activation of the NAD(P)H oxidase complex and increased generation of ROS. Additionally, TGF-β1 promotes the activation, proliferation, and differentiation of fibroblasts into α-smooth muscle actin (α-SMA)-expressing myofibroblasts that secrete excessive amounts of extracellular matrix (ECM) components (collagen and fibronectin). The accumulation and persistence of myofibroblasts is believed to contribute to the development of fibrosis [39]. In the present study, the high levels of TGF-β correlate with the pulmonary α-SMA and collagen deposition in monocrotaline-induced PH.

BMPR2 mutations have been identified in patients diagnosed with familiar PAH. Moreover, BMPR2 mutations have been also identified in some patients with idiopathic PAH [40]. BMPR2-mutant mice develop inflammation, endothelial injury, and persistent pulmonary hypertension [9]. In the late phase of monocrotaline-induced PH, decreased levels of BMPR2 expression were detected, consistent with PH development.

Another factor is the dysfunction or injury of the pulmonary vascular endothelial cells, resulting in the aberrant production of endothelium-derived mediators such as endothelin-1 and NO, which play a role in the pathogenesis of PH [40]. An increase in NO levels, as well as eNOS, endothelin 1 and endothelin B, was observed after induction with monocrotaline, findings which are consistent with endothelial injury. Interestingly, lung tissue from patients with idiopathic pulmonary arterial hypertension displayed increased eNOS activation and PKG nitration and reduced caveolin-1 expression. This data shows that the loss of caveolin-1 leads to hyperactive eNOS and subsequent tyrosine nitration–dependent impairment of PKG activity, which results in PH [41].

According to the findings of the present study, it can be concluded that the intraperitoneal administration of monocrotaline promotes tissue remodeling, as well stimulating the patterns of pro-inflammatory cytokines and growth factors, and therefore, constitutes an effective experimental mice model of PH.

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Legends

Fig.1: **Echocardiographic analysis of SHAM and MCT28 group**. A: Velocity time integral (VTI, cm); B-C: mean and peak gradient (mmHg); D-E: mean and peak velocity (mm/s) F: Ejection fraction (%); G: Left ventricular (LV) area (mm^2) at diastole; H: right ventricular (RV) area (mm^2) at diastole. n=6 (SHAM), n=10 (MCT28). Data is expressed as means ± SD from independent mice. The letter at the top of the columns represents the groups where there was a significant difference with the cited group: a - SHAM; b – MCT28 ($p < 0.05$).

Fig. 2: **MCT-induced lung injury in mice**. A- B: Representative images of H&E staining demonstrating alveolar exudate, interstitial edema with thickening of the septum alveolar, infiltrates of inflammatory cells with the presence of activated macrophages and plexiform lesions of pulmonary arteries and emphysema in MCT groups. Histological analysis of the sham group did not reveal any morphological changes. C: Representative images of Masson's trichrome staining demonstrating significantly increased collagen deposition in the pulmonary interstitium and around the arteries and vessels and also around the bronchioles. D: Immunohistochemical localization of αSMA labeling around the arteries, vessels and bronchioles. E: The slides were histopathologically evaluated using a semi quantitative scoring method. F-G: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. The letter at the top of the columns represents the groups where there was a significant difference with the cited group (a - SHAM; b – MCT7; c- MCT14; d- MCT21; e- MCT28 ($p < 0.05$).

Fig. 3: **MCT-induced migration of inflammatory cells in the lung tissue of mice**. A: Immunohistochemical localization of GRO, note expressive neutrophilic cells in the initial phase (MCT14); B: Immunohistochemical localization of F4/80, labeled macrophages were detected from the 14^{th} day; C: Immunohistochemical localization of CD24, labeled B cells were present from the initial to the final stage; D: Immunohistochemical localization of CD40L, showing that T cells were significantly increased in the final phase. E-H: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. The letter on the top of the columns represents the groups where there was a significant difference with the cited group (a - SHAM; b – MCT7; c- MCT14; d- MCT21; e- MCT28 ($p < 0.05$).

Fig. 4: **MCT-induced inflammatory cytokines in the lung tissue of mice.** Immunohistochemical localization for IL-1β and IL-6 (A and B respectively) mainly around the vessels and arteries. Densitometric analysis showed similar pattern of expression of both cytokines, being intensely expressed in the MCT7 and MCT14 groups. Immunohistochemical localization for MCP-1 and IL-17 (C and D respectively) showed an increased expression in the MCT14, MCT21 and MCT28 groups. E-H: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. I-J: Representative Western blotting showing protein expression for IL-1β, IL-6, MCP-1 and IL17 in the SHAM, MCT7, MCT14, MCT21 and MCT28 groups. β-actin was used as an internal loading control. Data is expressed as mean ± S.D. from n = 3 mice for each group. ND: not detected. The letter on the top of the columns represents the groups where there was a significant difference with the cited group (a - SHAM; b- MCT7; c- MCT14; d- MCT21; e- MCT28 ($p < 0.05$).

Fig. 5: **Cell proliferation markers in the lung tissue of mice after MCT treatment**. A: Immunohistochemical localization for VEGF mainly around the vessels and arteries, with expression gradually increasing, peaking in the MCT28 group. B: Immunohistochemical localization for TGF-β showed an increase in levels in groups MCT7, MCT14, MCT21 and MCT28. C: Immunohistochemical localization for BMPR-2 showed a decrease in levels in groups MCT7, MCT14, MCT21 and MCT28. D-F: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. G-H: Representative western blotting protein expression for VEGF and BMPR2 confirmed the immunohistochemical analysis. α- tubulin was used as an internal loading control. Data is expressed as mean ± S.D from n = 3 mice for each group. I: Relative expression of mRNA TGF-β showed a significant increase in the MCT7 group. Data is expressed as mean ± S.D. from n = 5 mice for each group. The letter on the top of the columns represents the groups where there was a significant difference with the cited group (a - SHAM; b- MCT7; c- MCT14; d- MCT21; e- MCT28 ($p < 0.05$).

Fig. 6: **Expression of endothelial markers and oxidative stress in the lung tissue of mice after MCT treatment.** A: Immunohistochemical localization for iNOS showed an increase in levels in groups MCT7, MCT14, MCT21 and MCT28. B: Immunohistochemical localization for eNOS showed increased levels in MCT21 and MCT28 groups. C-D: Quantitative

densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. E -F: Representative Western blotting showing protein expression for eNOS, ET1, ET_A and ET_B in SHAM, MCT7, MCT14, MCT21 and MCT28 groups. α- tubulin was used as an internal loading control. Data is expressed as mean ± S.D. from n = 3 mice for each group. G: Assessment of NO in lung tissue through the measure of total nitrite metabolites. Data is expressed as mean ± S.D. from n = 10 mice for each group. H: Relative mRNA expression of iNOS. Data is expressed as mean ± S.D. from n = 5 mice for each group. ND: not detected. The letter on the top of the columns (a - SHAM; b- MCT7; c- MCT14; d- MCT21; e- MCT28 ($p < 0.05$).

Figure 1:

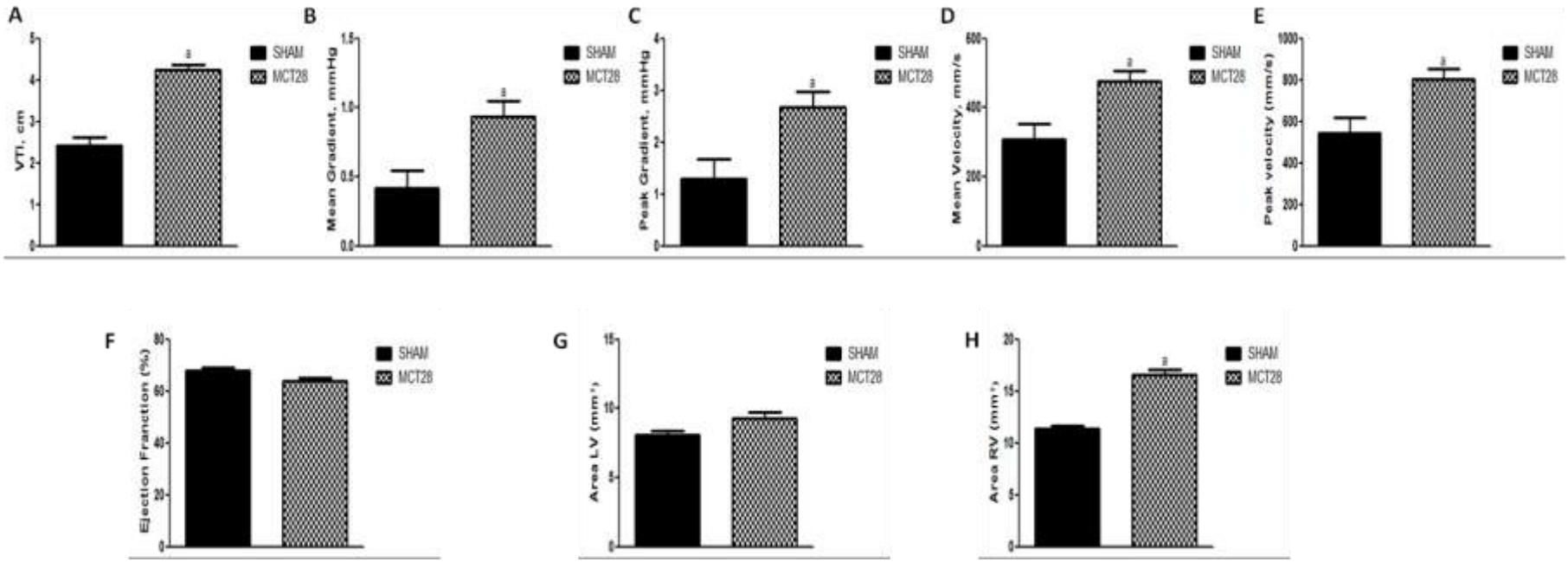

A
VTI, cm
SHAM
MCT28
B
Mean Gradient, mmHg
C
Peak Gradient, mmHg
D
Mean Velocity, mm/s
E
Peak velocity (mm/s)
F
Ejection Frandtion (%)
G
Area LV (mm²)
H
Area RV (mm²)

Figure 2:

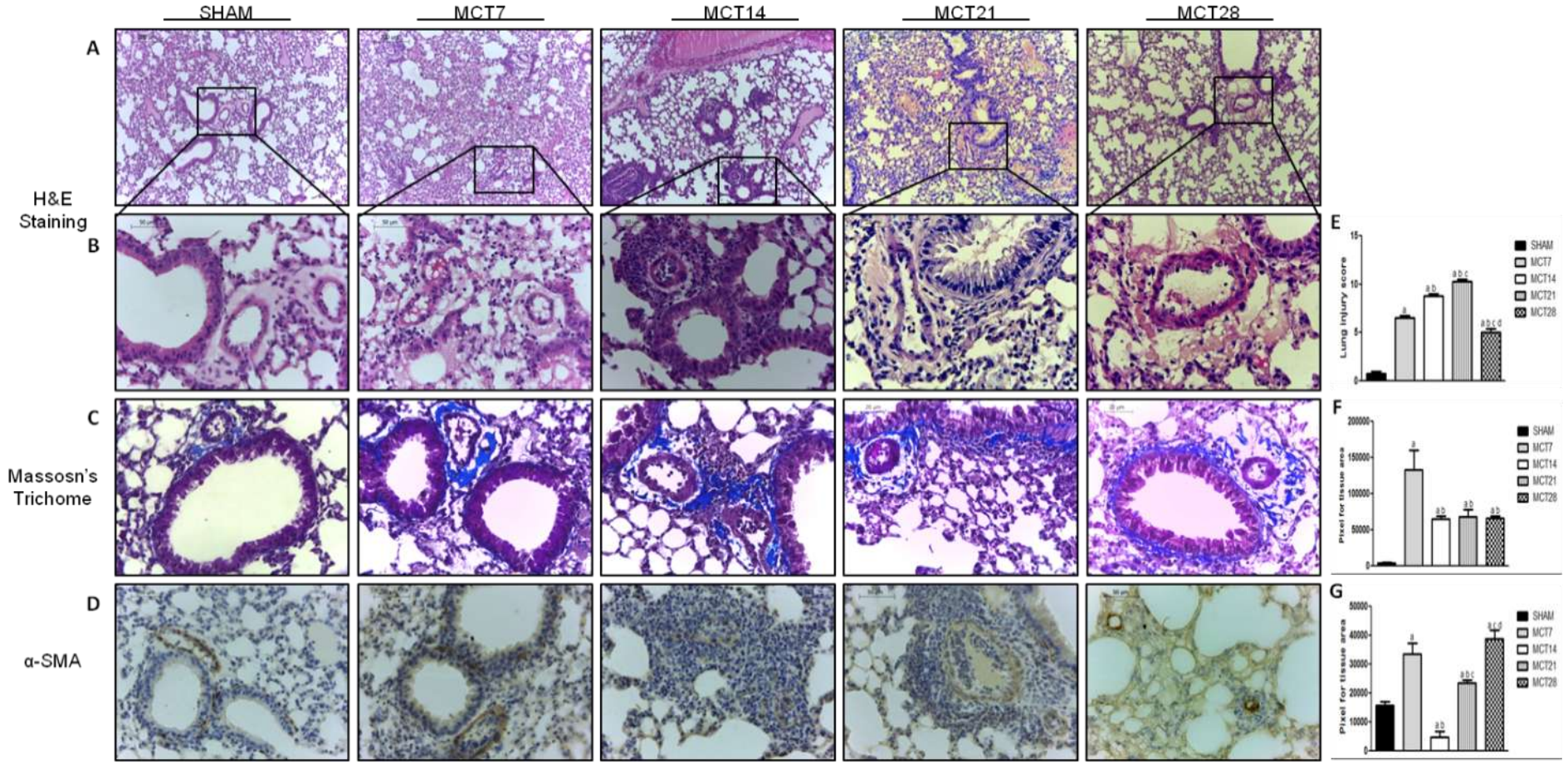
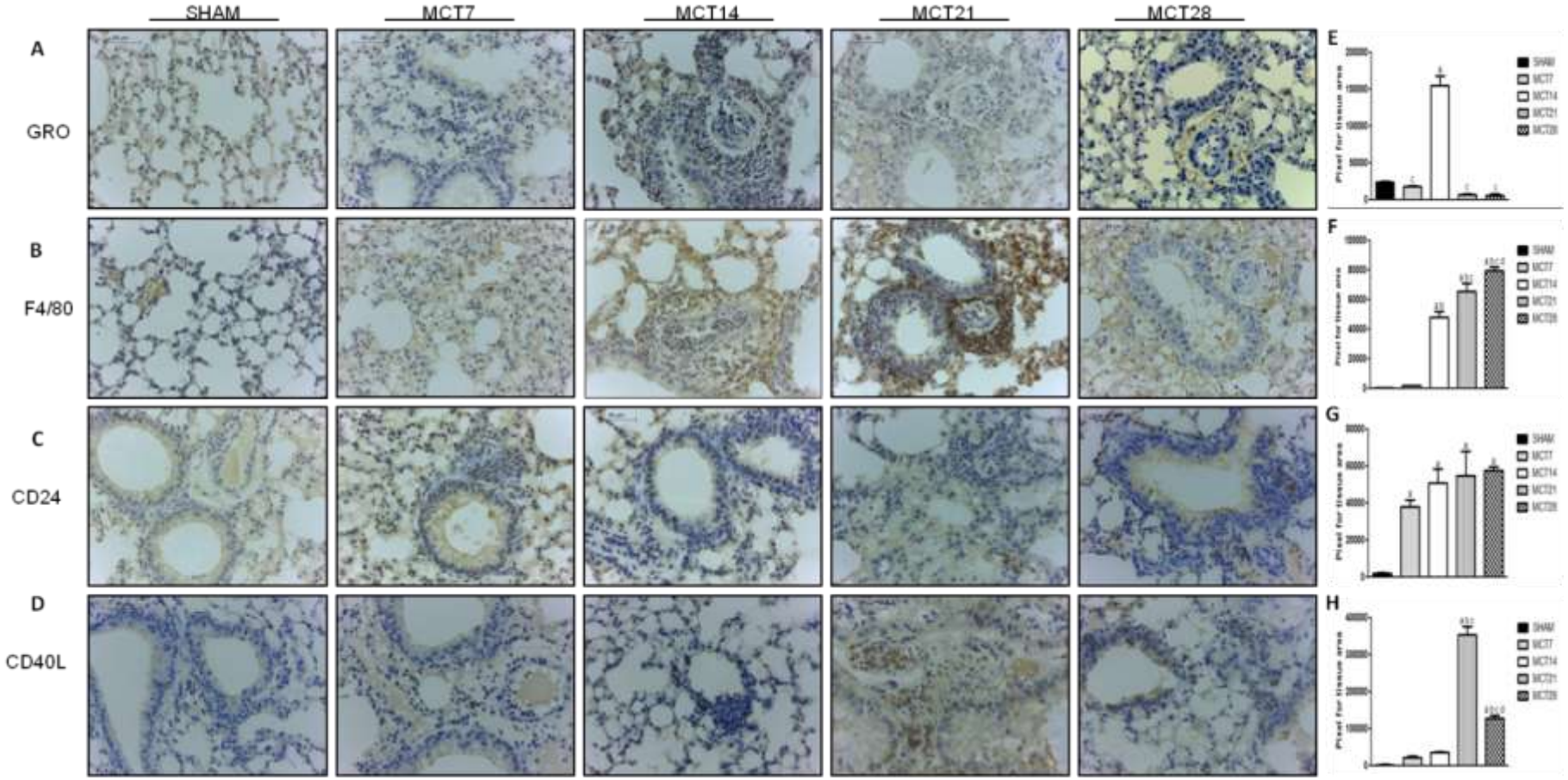

SHAM
MCT7
MCT14
MCT21
MCT28
A
H&E Staining
B
C
Massosn's Trichome
D
α-SMA
E
Lung injury score
F
Pixel for tissue area
G
Pixel for tissue area
SHAM
MCT7
MCT14
MCT21
MCT28

Figure 3:



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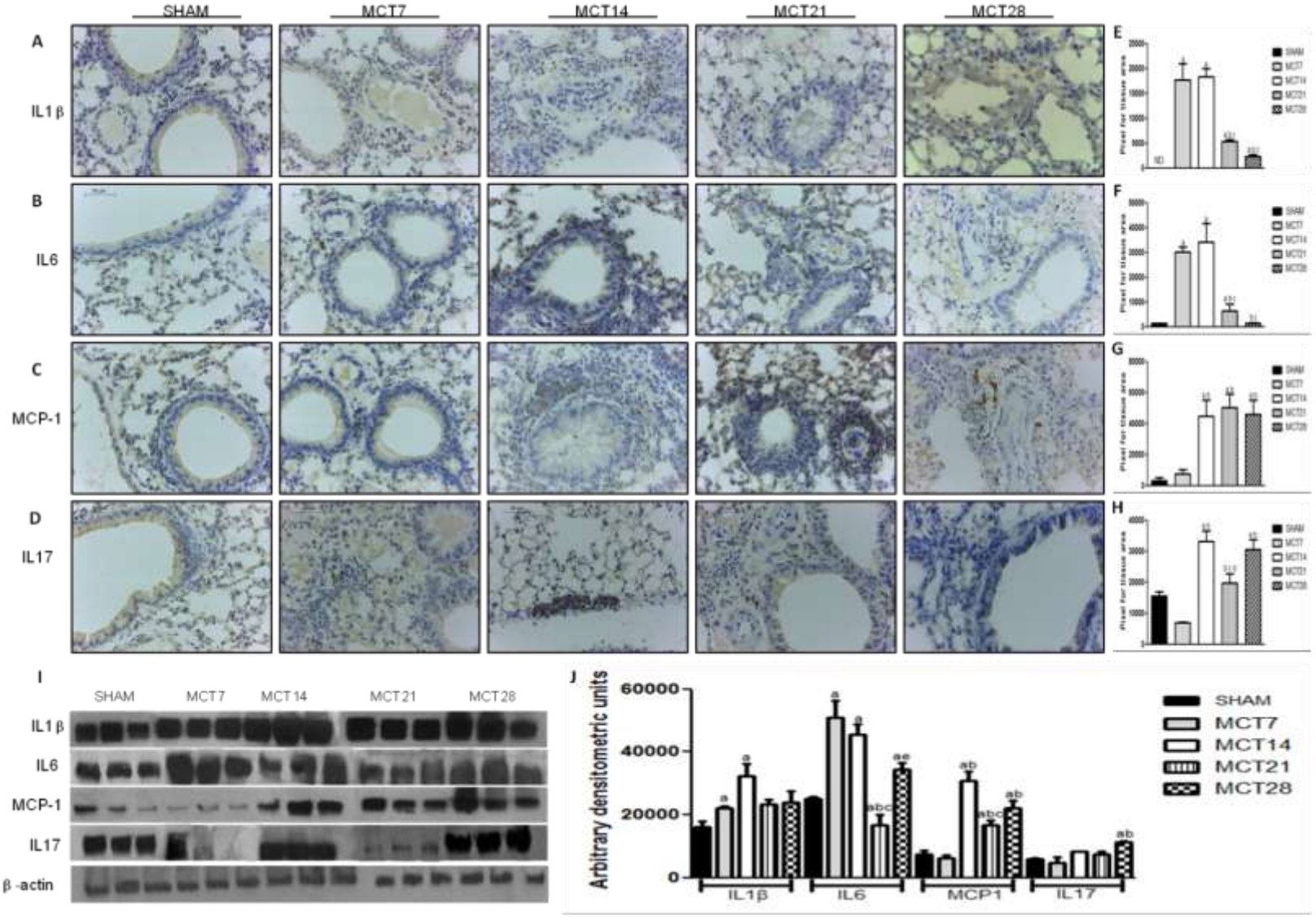

SHAM
MCT7
MCT14
MCT21
MCT28
A
IL1β
B
IL6
C
MCP-1
D
IL17
E
F
G
H
I
SHAM
MCT7
MCT14
MCT21
MCT28
IL1β
IL6
MCP-1
IL17
β -actin
J
Arbitrary densitometric units
60000
40000
20000
0
IL1β
IL6
MCP1
IL17
SHAM
MCT7
MCT14
MCT21
MCT28

Figure 5:

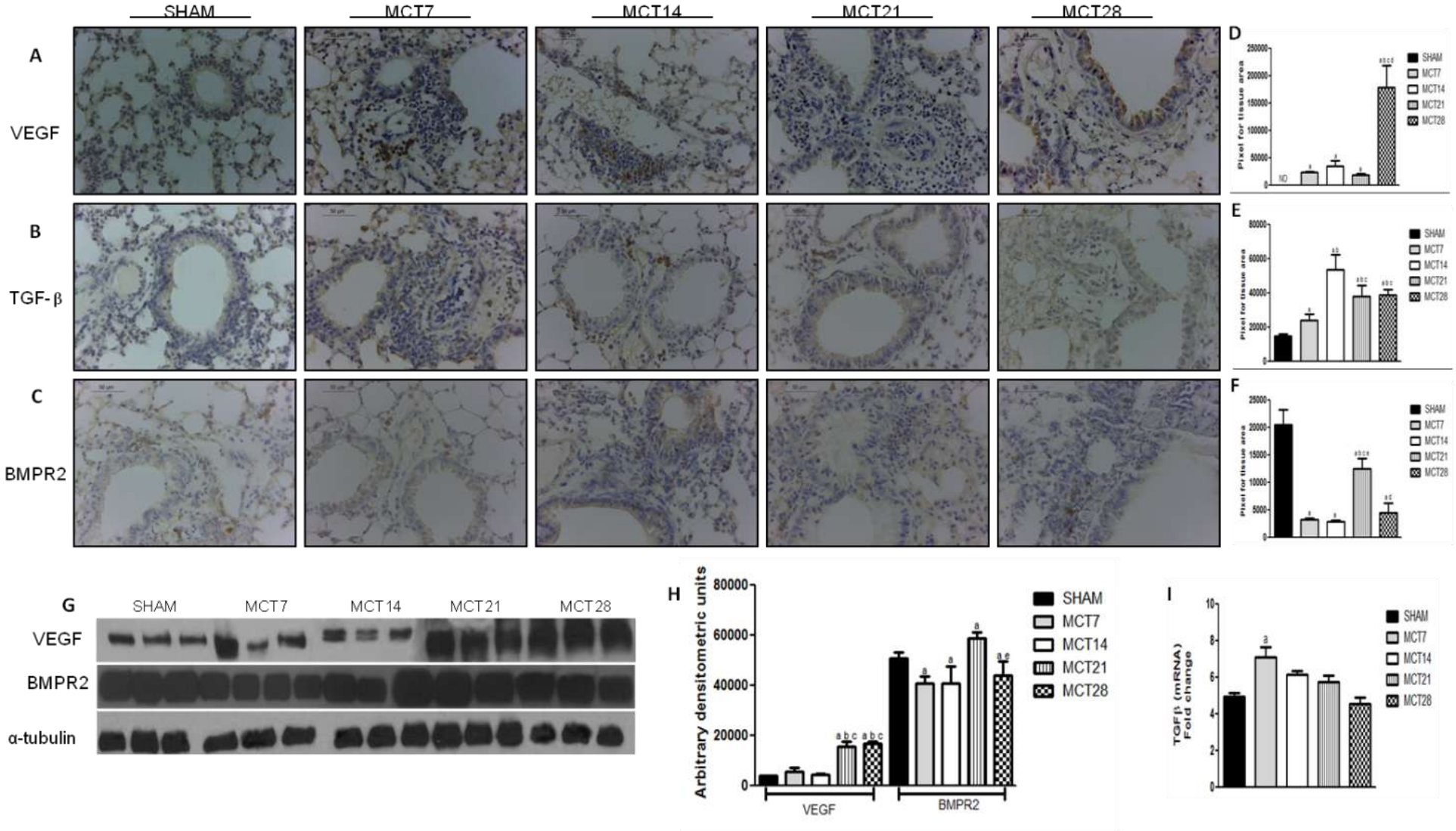
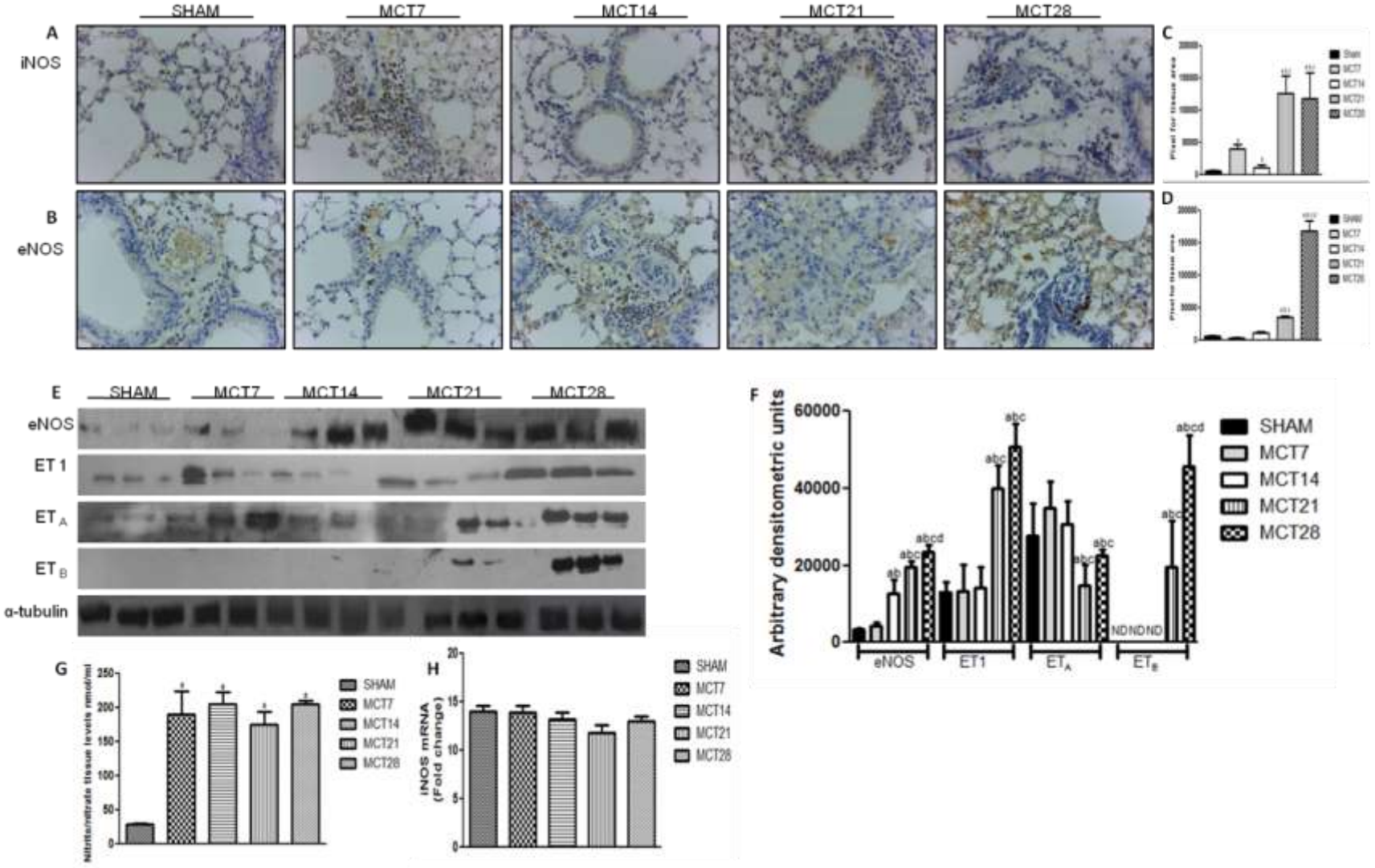

SHAM
MCT7
MCT14
MCT21
MCT28
A
VEGF
B
TGF-β
C
BMPR2
D
E
F
G
VEGF
BMPR2
α-tubulin
H
Arbitrary densitometric units
VEGF
BMPR2
I
TGFβ (mRNA) Fold change

Figure 6:


SHAM
MCT7
MCT14
MCT21
MCT28
A
iNOS
B
eNOS
C
D
E
eNOS
ET 1
ET A
ET B
α-tubulin
F
Arbitrary densitometric units
60000
40000
20000
0
eNOS
ET1
ET A
ET B
ND
SHAM
MCT7
MCT14
MCT21
MCT28
G
Nitrite/nitrate tissue levels nmol/ml
H
iNOS mRNA (Fold change)

6- ARTIGO 2: Diethylcarbamazine: a potential drug for pulmonary hypertension?

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Este artigo será submetido para a revista:
European Journal of Pharmacology

ISSN: 0014-2999

Diethylcarbamazine: a potential drug for pulmonary hypertension?

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Abstract

Pulmonary hypertension (PH) is a progressive and lethal disease. Current therapeutic alternatives may improve symptoms and reduce the severity of the hemodynamic parameters. Diethylcarbamazine (DEC) is a drug used against lymphatic filariasis. However, many studies have described anti-inflammatory activities and pro-apoptotic effect of DEC. We demonstrate the potential effects of DEC on monocrotaline (MCT)-induced pulmonary hypertension. C57BL/6 male mice were used in all experiments. An MCT solution intraperitoneal injection (600mg/kg) was administered once per week and 50 mg/kg body weight of DEC, adjusted according to the body weight of mice for 28 days. Three groups (n=10) were studied: Cont; $MCT_{28,}$ and MCT_{28}/DEC. Echocardiography analysis was performed and lung tissues were collected for light microscopy (haematoxylin-eosin and Masson's trichrome staining), immunohistochemistry (αSMA, FADD, caspase 8, caspase 3, BAX, BCL2, cytochrome C and caspase 9) western blot (FADD, caspase 8, caspase 3, BAX, BCL2, cytochrome C and caspase 9) and Rt-PCR (COL-1α and αSMA). Echocardiography analysis demonstrated an increased in the pulmonary arterial blood flow gradient, velocity in systole and area RV at MCT28 group, after treatment with DEC a significant reduction of these parameters. Lung sections exhibited remarkable changes in the pulmonary arteries, arterioles and in the pulmonary parenchyma, as well as congestion and atelectasis at MCT28 group. The treatment

with DEC reduced the lung damage. Deposition of collagen fibers and αSMA staining around the pulmonary arteries were evident the on MCT28 group after treatment with DEC there was a reduction of these levels. Western blot analysis revealed a decrease in BMPR2 on the MCT28 group, compared to the control group. DEC in the group of level BMPR2 increased significantly returning to its normal level. Also observed an increase in VEGF levels the MCT28 group when compared with the control group. DEC in the group of level VEGF decreased significantly. We also observed a change in markers of extrinsic and intrinsic pathway in MCT28 group compared to the control group. After treatment with DEC these levels returned to the baseline. Results of this study indicate that DEC attenuates a PH in a model experimental induced-monocrotaline, probably by inhibiting a series of markers involved in the proliferation / cell death.

Key words: Diethylcarbamazine , monocrotaline, hypertension, pulmonary apoptosis.

1- INTRODUCTION

Pulmonary hypertension (PH) is a life-threatening progressive disorder associated with abnormally elevated pulmonary pressures and right heart failure [2], and is an example of disease that result from complex interactions between an individual's genetic make-up and the surrounding environment [1]. The initial pathological events are related to dysregulation of pulmonary artery and smooth muscle proliferation cells. Several lines of evidence suggested an impaired regulation of pulmonary arterial smooth can induce muscle proliferation [3]. Increased proliferation and decreased apoptosis of pulmonary arterial smooth muscle could mediate thickening of the pulmonary vasculature, which subsequently would lead to reduced inner diameter and increased pulmonary vascular resistance.

Recent studies have proposed that endothelial cell (EC) apoptosis and apoptosis resistant proliferation play crucial roles in the development of featured plexiform lesions in the pathogenesis of PH. Subsequently, EC injury associated smooth muscle cell (SMC) proliferation facilitates vascular remodeling and eventually leads to narrowed vascular lumen, increased pulmonary vascular resistance, increased pulmonary arterial pressure, and right heart failure [4]. The imbalance between cell death and proliferation occurs in every stage of pulmonary vascular remodeling and pathogenesis of PH, and involves every cell type in the vasculature including, but not limited to ECs, SMCs, and fibroblasts [5].

Intriguingly, PH pathogenesis involves both inappropriate apoptosis and over proliferation. Apoptosis in ECs, after initial environmental insults, has been recognized as one of the crucial events that trigger the pulmonary vascular remodeling in PH [6]. Despite extensive studies, the detailed cellular and molecular mechanisms on how the transition from initial apoptosis to apoptosis resistant proliferation of ECs and SMCs remains unclear [4].

MCT model is considered, by some to be a toxic model, being suggested that MCT rats die from hepatic veno-occlusive disease with liver failure instead of right ventricle failure [7]. MCT is known to cause pulmonary endothelial injury and pulmonary hypertension in humans and rats [8, 9] but has little effect on mice [10].

Diethylcarbamazine (DEC) is a drug used throughout the world against lymphatic filariasis. However, in recent years many studies have described other pharmacological activities of DEC. It has been established that DEC interferes with the cyclooxygenase and lipoxygenase pathways, reducing eicosanoid production, thereby acting as an anti-inflammatory drug [11]. Furthermore, DEC inhibit activation of NF-kB, suppressing target genes involved in the inflammatory response [12]. DEC also been shown to be effective in different models of pulmonary inflammation, such as tropical pulmonary eosinophilia, pulmonary hypertension, eosinophilic pulmonary inflammation and asthma [13, 14, 15, 16]. Queto et al. (2010) [15] reported that DEC was ineffective in CD95L-deficient mice. CD95L (FasL) is a ligant for the apoptosis inducing receptor CD95 (Fas). These results suggest that DEC can possibly act as an apoptosis inductor. The aim of the present study is to evaluate the cell death markers and action DEC on a monocrotaline-induced pulmonary hypertension model.

2- MATERIALS AND METHODS

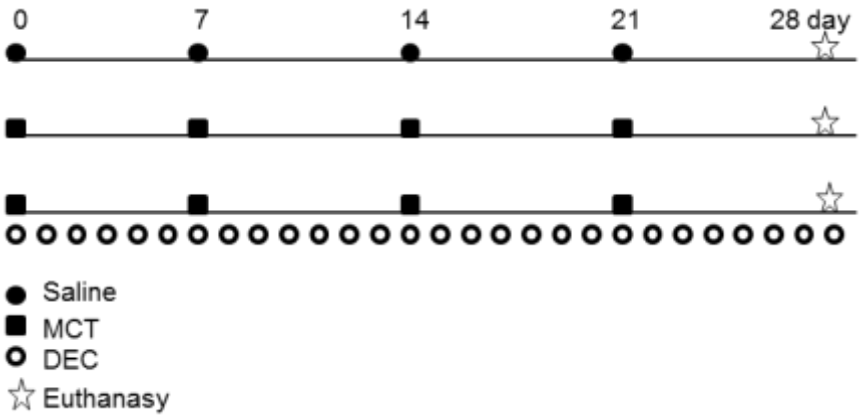
2.1 Animals

Thirty male C57BL/6 mice, weighing 26-30 g, were used in all experiments. The mice were examined to determine their health status and acclimated to the laboratory environment of 23-24°C. They were kept in a 12/12 h day/night cycle photoperiod. The animals were housed in metal cages and fed a standard diet with water ad libitum. All experimental procedures were approved by the Ethics Committee for Animal Experimentation (Prot. 63/2014 FIOCRUZ/CPqAM).

2.2 Drugs and experimental Design

Diethylcarbamazine citrate (DEC) was obtained from Sigma (St. Louis, MO, D8765) and dissolved in distilled water. The lymphatic filariasis therapeutic dose regimens recommended by the World Health Organization is 6 mg/kg/day. Considering that the total metabolism rate of a mouse is approximately seven times that of the human, the present study used 50 mg/kg body weight of DEC, adjusted according to the body weight of mice for 28 days.

Mice received a intraperitoneal (i.p) injection of MCT (600mg/kg Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline solution and administered once per week (0,7, 14 and 21 days). The mice were randomly allocated into three groups of ten (N = 10) animals each:



2.3 Determination of right ventricular hypertrophy (RVH), pulmonary artery and left ventricular function

Echocardiography analysis was performed using a VisualSonic Vevo770, Toronto, Canada) on day 28. During the procedure, isofluorane/O2 administration was administered using a facemask to keep the mice lightly anesthetized, with heart rates in the range of 300-350 bpm. The right ventricle was visualized from the right parasternal long axis view with a 704 RMV scanhead. The right ventricular wall thickness was measured from images produced in M-mode, using the depth interval (mm) generic measurement tool (Vevo770 v3.0 software, VisualSonics). Doppler flow images were recorded from the left parasternal long axis view with a 707 B scanhead pointing slightly towards the left shoulder to visualize the pulmonary artery. Volume was measured at the level of the pulmonary valve, and several indices of pulmonary artery blood flow (velocity time integral, peak and mean pressure gradient and peak and mean velocity) were assessed using the pulmonary valve protocol measurement tool [17].

2.4 Histological examination

The lung fragments were washed twice in PBS pH 7.2 and fixed in Bouin solution for 8 hours (1% saturated picric acid, formaldehyde and 40% glacial acetic acid), before being dehydrated in increasing ethanol series, cleared in xylene, embedded and included in purified paraffin (VETEC, São Paulo, SP, Brazil). Tissue sections of 5 μm were cut using a microtome (Leica RM 2125RT) deparaffinized with xylene. They were then stained with hematoxylin/eosin and studied using light microscopy [18].

2.5 Immunohistochemical Localization

The paraffin sections of lung tissue were mounted onto slides. After being deparaffinized, the tissues were incubated overnight at 4°C with primary antibody anti- α-SMA (1:100 cat. ab 5694) anti-FADD (1:50 cat. Sc 6036), anti-caspase 8 (1:50 cat. Sc5263), anti-caspase 3 (1:100 cat. Ab4011), anti-BAX (1:50 cat. Ab7977), anti-BCL2 (1:100 cat. Ab7973), anti-cytochrome C (1:50 cat. Sc13156), anti-caspase 9 (1:50 cat. sc56076). The antigen-antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB + Kit, Peroxidase), using 3,3-diaminobenzidine as a chromogen. Imaging was performed by light microscopy. Five pictures at the same magnification were quantitatively analyzed using the Gimp 2.8 software program (GNU Image Manipulation Program, UNIX platforms) [19].

2.6 RNA extraction and quantitative real-time polymerase chain reaction (q RT-PCR)

Total RNA from mouse tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The forward and reverse primers used for each gene were as follows: Collagen Type 1α (COL-1α): 5’-GAACGGTCCACGATTGCATG-3’ and 5’-GGCATGTTGCTAGGCACGAAG-3’, αSMA: 5’-ATCTGGCACCACTCTTTCTA-3’ and 5’-GTACGTCCAGAGGCATAGAG-3’, GAPDH (endogenous control): 5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTGAGGTCA-3′. All reactions were performed in triplicate and included the following: 1 μL of cDNA; 5 μM of each primer; 2x SYBR Green PCR Master Mix (Applied Biosystems); and water added to give a final volume of 25 μL. The relative amount of mRNA was determined using the comparative threshold (Ct) method by normalizing target cDNA Ct values to those of GAPDH. Fold increase ratios were calculated relative to control (basal conditions) for each group using the formula 2e-ΔΔCt [20].

2.7 Western Blot Analysis

The lungs were submerged in liquid nitrogen and the total proteins were extracted using an extraction cocktail (10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate (NaVO4), 10 mM of aprotinin, and 100 mM Tris(hydroxymethyl)aminomethane, pH 7.4). Western blotting and the subsequent quantification of each blot were performed as previously described [20]. The primary antibodies for anti-BAX, anti-Bcl2 and anti-caspase 3 were obtained from Abcam (CA, USA), anti-caspase 8, anti-FADD, anti-caspase 9 and anti-cytocrome c were obtained the from Santa Cruz Biotechnologies (Santa Cruz, CA). Secondary antibodies and β-actin were acquired from Sigma-Aldrich (USA).

2.8 Data Analysis

GraphPad Prism software (version 6) was used for statistical analysis. Data were expressed as mean ± standard deviation. Differences between the control and treatment groups were analyzed using analysis of variance (ANOVA), prior to the performance of Tukey's post hoc test or the Student's t-test. Probability values less than 0.05 were considered significant.

3- RESULTS

3.1 Effect of DEC on pulmonary hypertension

In humans, transthoracic echocardiography is an excellent noninvasive screening test for patients with symptoms or risk factors for PH by providing direct and/or indirect signs of elevated pulmonary artery pressure (PAP). Doppler analysis at the pulmonary valve level, recorded by ultrasonography on lightly anesthetized mice (heart rate 300-350 bpm) demonstrated an increased in the pulmonary arterial blood flow gradient, velocity in systole and area RV at MCT28 group, after treatment with DEC a significant reduction of these parameters (table 1).

Tissue lesions of pulmonary hypertension are characterized by changes in all components of pulmonary arterial walls. The group MCT28 exhibited remarkable changes in the pulmonary arteries, arterioles and in the pulmonary parenchyma, as well as congestion and atelectasis. The treatment with DEC reduced the lung damage (figure 1A).

3.2 Effect of DEC on pulmonary arteriole muscularization

Vascular remodeling and fibrosis are among the key pathological features in PAH. One of the main features of vascular remodeling seen in PAH is collagen deposition in the remodeled pulmonary vessels. Masson's trichrome staining revealed a significant increase in collagen deposition in the pulmonary interstitium, around the arteries, vessels and bronchioles. In contrast, after treatment with DEC there was an evident reduction of collagen (figure 1B), data also observed in the gene expression of COL-1α mRNA (figure 1E).

αSMA it is a marker of expression of a smooth-muscle phenotype, expressed by PSMCs of existing vessel walls, in both the normal and hypertensive lung, however, an increase in the αSMA expression can lead to thickening of the middle layer of the pulmonary arteries. Immunolabeling of the lung sections with αSMA revealed a significant increase in the MCT28 group, compared to the control group, mainly around the arteries and vessels, demonstrate the muscularization process. In contrast, the DEC group decreased the expression of αSMA (figure 1C). Those same results were observed in gene expression of αSMA (figure 1F).

3.3 Effect of DEC at growth factors

Studies identified the bone morphogenetic protein (BMP) pathway, a member of the TGF-superfamily of receptors, as having particular importance in PAH pathogenesis, suggesting that this pathway might be important in the pathogenesis of a variety of common clinical situations in which pulmonary hypertension is a feature. Western blot analysis revealed a decrease in BMPR2 on the MCT28 group, compared to the control group. DEC in the group of level BMPR2 increased significantly returning to its normal level (figure 2).

Models of pulmonar hypertension have been shown to be associated with increased levels of vascular endothelial growth factor (VEGF) transcripts, its dysregulation can cause increased vascular permeability and stimulating neovascularization in physiological and pathological processes. Western blot analysis revealed an increase in VEGF on the MCT28 group, compared to the control group. DEC in the group of level VEGF decreased significantly (figure 2).

3.4 Effect of DEC on expression of proteins Apoptotic Pathways

Apoptosis is triggered and modulated by two pathways. The intrinsic pathway involves the mitochondria in response to stress, such as reactive oxygen species, nutrient deprivation or DNA damage, whereas the extrinsic pathway is induced by receptor binding to proapoptotic death ligands such as tumor necrosis factor–α (TNF-α) and Fas.

The extrinsic pathway markers FADD, caspase 8 and caspase 3 were analyzed by immunohistochemistry and western blotting, in which is seen a significant reduction in the MCT group 28 of these markers is observed. In contrast, the treatment with DEC increased significantly levels of FADD, caspase 8 and caspase 3 (figure 3 A, B, C and G).

The intrinsic pathway markers BAX, cytochrome C and caspase 9 showed a reduced level expression, excepting Bcl2 that did not show significant change. The treatment with DEC increased the expression levels of BAX, cytochrome C and caspase 9, demonstrating that this drug exerts an effect on both the intrinsic as extrinsic pathway of apoptosis (figure 4 A, B, C, D and I).

4- DISCUSSION

In this study, we present evidence that MCT promotes changes in the pathway of apoptosis, also interferes with growth factors the pathobiological mechanisms of PH has been extensively studied. The PH "phenotype" is characterized by endothelial dysfunction, a decreased ratio of apoptosis/proliferation in PASMCs, and a thickened, disordered adventitia in which there is excessive activation of adventitial metalloproteases. Like cancer and atherosclerosis, PAH does not have a single cause: a "multi-hit model" is more likely (21).

Recent advances have led to increased recognition and new therapies, however there are still few therapies used for PH. The MCT model continues to be a frequently investigated model of PH, since it offers technical simplicity, reproducibility, and low cost compared with other models of PH, elucidating the pathobiology of PH continues to be critical for the design of new effective therapeutic strategies, and animal models are fundamental to achieve this objective (22). The C57BL/6J strain displayed the advantage of presenting a knockout series for several genes, which allows the function of different cytokines and growth factors in HP development.

DEC is a drug used all around the world against lymphatic filariasis, however, in recent years many studies have also described other pharmacological activities of DEC [11]. Ribeiro et al. (2014) [18] showed that DEC had an anti-inflammatory effect in acute lung injury and Santos et al. (2014) [19] showed that DEC inhibits the NF-kB pathway in murine model of acute lung injury induced by carrageenan. Interestingly, a study in 1985 tested the hypothesis that monocrotaline would activate arachidonic acid metabolism in rats. These authors described that arachidonate metabolism was activated before pulmonary hypertension developed, and that the inflammatory cells infiltration in the alveolus followed the hypertensive process. Furthermore, DEC treatment attenuated both the monocrotaline-induced

inflammatory response and the pulmonary hypertension (23). Our studies amplifies these results demonstrating that DEC would also have an action on growth factors and apoptotic.

Pulmonary hypertension is a fatal disease characterized by increased pulmonary vascular pressure because of pathological remodeling [24]. This pathology is associated with abnormal connective tissue deposition and characterized by structural and functional changes in the pulmonary vasculature, including vascular smooth muscle cell proliferation hypertrophy and excess collagen formation [25].

One of the main features of vascular remodeling seen in PAH is collagen deposition in the remodeled pulmonary vessels. Circulating levels of N-terminal propeptide of type III procollagen (PIIINP), Carboxyterminal telopeptide of type I collagen (CITP), matrix metalloproteinase 9 (MMP-9) and Tissue inhibitor of metalloproteinase 1 (TIMP) were elevated in PH patients, and these results suggest that the elevated levels were markers of disease state rather than markers of the etiology of PAH. Furthermore, circulating markers of new collagen formation, type 1 collagen degradation, elastase (MMP9) activity, and inhibition of matrix metalloproteinase by a ubiquitous inhibitor of MMP's (TIMP1) may be indicative of active vascular remodeling and reflect clinically relevant.[26].

The sequence of morphologic changes that lead to neomuscularization of the microvessels also it has been assigned and a there is a series of studies to characterize the evolving phenotype of smooth-muscle cells (PSMCs). We studied α-SMA expression, a recognized first marker of developing smooth-muscle cells [27, 28], during microvessel wall remodeling in a model experimental as well as human. We described that the DEC promoted an improvement in markers considered early in the development of morphological changes in the pulmonary arteries, however isolated cells could be used to confirm cell specifies on which DEC could be acting.

There have been a number of studies that have examined the effect of BMPR2 and VEGF to induce apoptosis of human and models experimental pulmonary artery smooth muscle cell (PASMC) [29, 30, 31]. BMPs are signaling molecular that belong to the transforming growth factor-β (TGF-β) superfamily [32]. BMPs are synthesized and secreted from a variety of cell types including pulmonary vascular smooth muscle and endothelial cells and play an important role in regulating cell proliferation, apoptosis and differentiation [29]. In the monocrotaline-induced PH, there was a decreased in levels of BMPR2 expression, consistent with PH development and after treatment with DEC significant increased levels in of BMPR2 were evident .

It is described in the literature that the reduced level of vascular endothelial growth factor (VEGF) in both hypoxia‑ and MCT‑induced PH models [33,34] VEGF has been known to confer a potent protective effect on ECs from apoptosis through the extrinsic pathway [35]. In a variety of experimental PH models, EC apoptosis has been shown to be associated with reduced levels of VEGF [33,34]. MCT28 group exhibited high levels of VEGF in protein analysis showed that there was an increase in these proteins and after DEC treatment a significant reduction of VEGF levels was observed. Another interesting fact was the action of DEC in extrinsic apoptosis pathway markers, where we observe that FADD, active caspase-8 and caspase-3 had their levels decreased in MCT28 group compared with the control group, whereas after treatment with DEC these apoptotic proteins had similar expression to the control group. Similar results were observed in the intrinsic pathway except the BCL-2 protein.

Some hypotheses are discussed in relation apoptosis and lung cells. The lung is the most vascular organ in the body and the massive pulmonary endothelial surface area is exposed nearly directly to the environment through the air we breathe [36]. Thus, it is very likely that even in healthy individuals, there are episodes of endothelial cells (EC) injury induced by environmental triggers resulting in waves of the apoptosis, but the normal reparative mechanisms involving the proliferation and migration of neighboring ECs and possibly the homing of circulating endothelial progenitor cell, are sufficient to restore the vascular continuity and maintain the integrity of the pulmonary circulation [37].

It has been demonstrated that increased PASMC proliferation and/or inhibited PASMCS apoptosis both contribute to induce pulmonary vascular medial hypertrophy. However, the precise mechanisms involved in the regulation of PASMC proliferation and apoptosis in PH are still incompletely understood [29]. Another important factor is the BMPR2 pathway may play a critical role in preventing EC apoptosis and thus maintaining the integrity of the lung microvascular, and loss of function mutations in this pathway. [37].

In conclusion the results of this study indicate that DEC attenuates a PH in a model experimental induced-monocrotaline, probably by inhibiting a series of markers involved in the proliferation / cell death, however it is necessary to assess on which cells DEC would be acting. We are the first to test this drug in HP animal model in mice and have a lot to investigate. It is probable that the use of a higher dose can bring further benefits.

ACKNOWLEDGEMENTS: The present study was supported by the following Brazilian fostering agencies: Fundação de Amparo à Ciência e Tecnologia do estado de Pernambuco (FACEPE) and Centro de pesquisas Aggeu Magalhães (CPqAM/FIOCRUZ).

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Legends

Tabela 1: **Pulmonary artery blood velocity after DEC treatment:** Velocity-time integral (VTI, cm) mean and peak gradient (mmHg) and mean and peak velocity (mm/s) of blood flow in the pulmonary artery and area of the right ventricle (mm2) were measured from Doppler waveforms acquired by ultrasound imaging. N=5. Mean±SD;

* $p<0.05$ vs SHAM

#$p<0.05$ vs MCT28

Fig. 1: **Effect of DEC treatment on histological alterations in lung after MCT-induced pulmonary hypertension.** A: Representative images of H&E staining demonstrating, interstitial edema with thickening of the septum alveolar, infiltrates of inflammatory cells with the presence of activated macrophages and plexiform lesions of pulmonary arteries and emphysema in MCT28 groups. Administration of DEC significantly attenuated the lung damage. Histological analysis of the control group did not reveal any morphological changes. **B**: Representative images of Masson's trichrome staining demonstrating significantly increased collagen deposition and MCT28 group. Administration of DEC significantly reduced collagen. **C**: Immunohistochemical localization of αSMA labeling around the arteries, vessels and bronchioles and MCT28 group. After treatment with DEC there was a reduction of immunostaining for α-SMA. **D**: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. **E** e **F**: Relative expression of mRNA COL-1α and αSMA showing a significant increase in the MCT28 group. After treatment with DEC there was a reduction of expression COL-1α and αSMA respectively. Data is expressed as mean ± S.D. from n = 5 mice for each group. The letter at the top of the columns represents the groups where there was a significant difference with the cited group (a - CONT; b – MCT28; c- MCT28/DEC ($p < 0.05$).

Fig. 2: **Effect of DEC treatment on expression of BMPR2 and VEGF**: **A**: Representative western blotting protein expression for BMPR2 and VEGF respectively. Data is expressed as mean ± S.D from n = 3 mice for each group. **B**: Quantitative densitometry analysis (ImageJ analyzed). The letter on the top of the columns represents the groups where there was a significant difference with the cited group (a - CONT; b – MCT28; c- MCT28/DEC ($p < 0.05$).

Fig. 3: **Effect of DEC treatment on the extrinsic pathway of apoptosis**: Immunohistochemical localization for FADD, C8 and C3 (**A, B** and **C** respectively). **D-F**: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. **G**: Representative Western blotting showing protein expression for FADD, C8 and C3 in the CONT, MCT28 and MCT28/DEC groups. β-actin was used as an internal loading control. Data is expressed as mean ± S.D. from n = 3 mice for each group. **H**: Quantitative densitometry analysis (ImageJ analyzed). The letter on the top of the columns represents the groups where there was a significant difference with the cited group (a - CONT; b – MCT28; c- MCT28/DEC ($p < 0.05$).

Fig. 4: **Effect of DEC treatment on the intrinsic pathway of apoptosis**: Immunohistochemical localization for BAX, BCL2, C9 and Cytocrome C (**A, B, C** and **D** respectively). **E-H**: Quantitative densitometry analysis (GIMP2 analyzed). Data is expressed as mean ± S.D. from n = 5 mice for each group. ND: not detected **I**: Representative Western blotting showing protein expression for BAX, BCL2, C9 and Cytocrome C in the CONT, MCT28 and MCT28/DEC groups. β-actin was used as an internal loading control. Data is expressed as mean ± S.D. from n = 3 mice for each group. **H**: Quantitative densitometry analysis (ImageJ analyzed). The letter on the top of the columns represents the groups where there was a significant difference with the cited group (a - CONT; b – MCT28; c- MCT28/DEC ($p < 0.05$).

Tabela 1:

	VTI, cm	Mean Gradient, mmHg	Peak Gradient, mmHg	Mean Velocity, mm/s	Peak velocity, mm/s	Area RV (mm^2)
SHAM	2,27 ± 0,4	0,29 ± 0,1	0,84 ± 0,22	268,0 ±49,49	473,4± 86,49	11,40 ± 0,52
MCT_{28}	4,28 ± 0,35*	1,19 ± 0,22*	3,40 ± 0,69*	565,5± 42,39*	959,2± 67,87*	17,57 ± 0,97*
$MCT_{28/DEC}$	3,15 ± 0,24#	0,84 ± 0,15#	2,42 ± 0,45#	461,8± 40,48#	775,1± 75,80#	12,78 ± 1,63#

Figure 1:

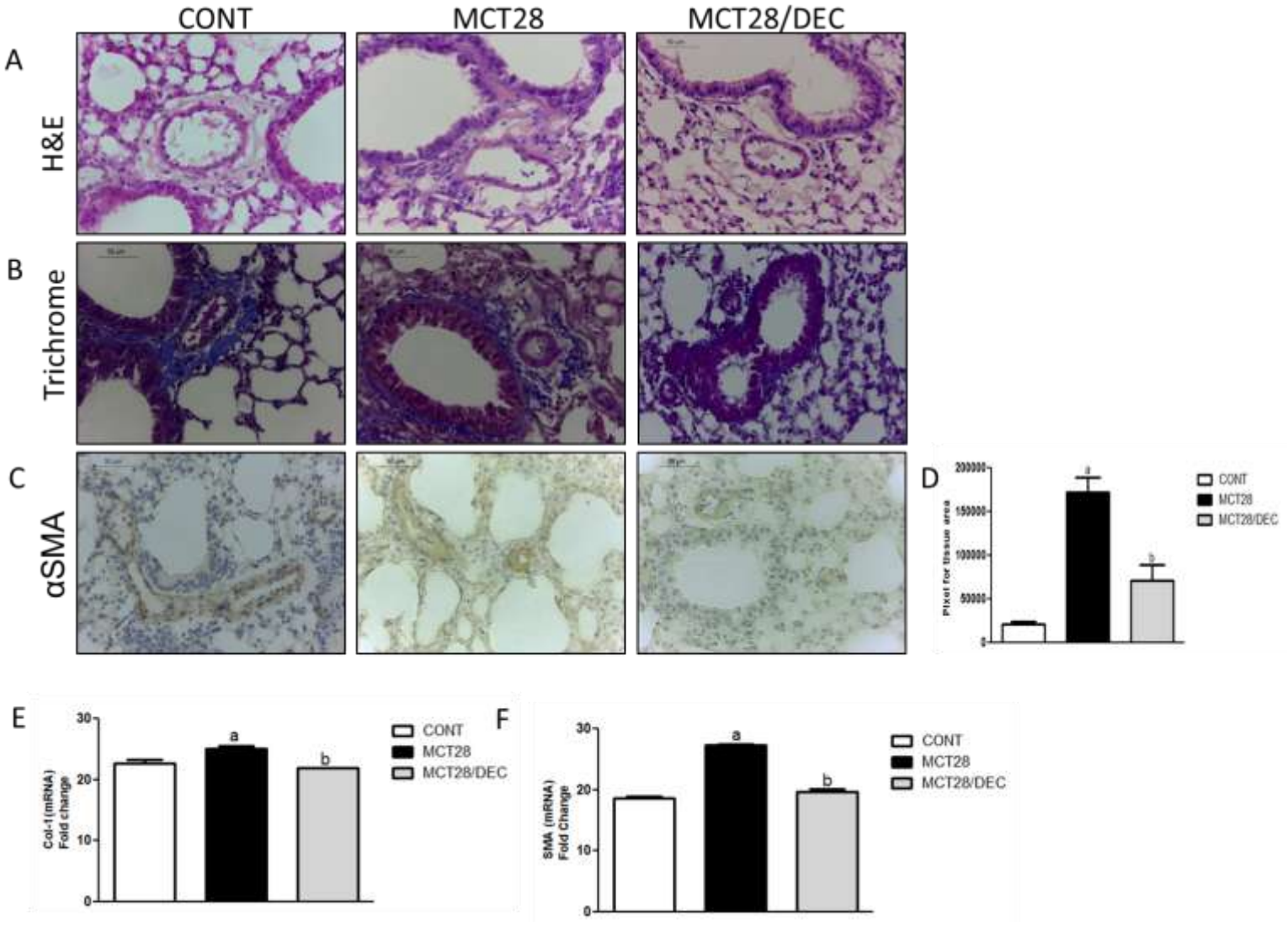

CONT
MCT28
MCT28/DEC
A
H&E
B
Trichrome
C
αSMA
D
Pixel for tissue area
CONT
MCT28
MCT28/DEC
E
Col-1(mRNA) Fold change
F
SMA (mRNA) Fold Change

Figure 2:

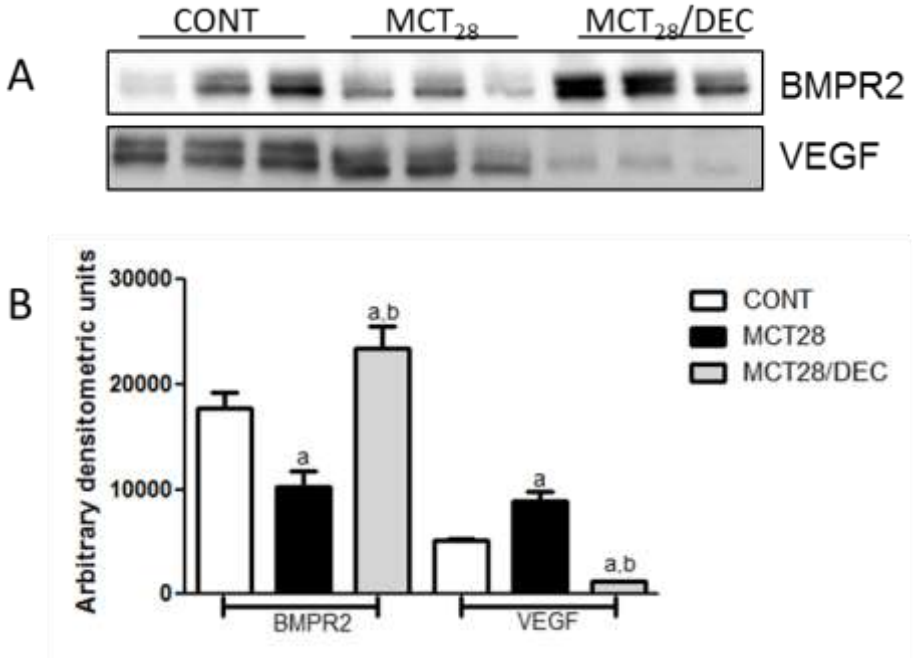

CONT
MCT28
MCT28/DEC
A
BMPR2
VEGF
B
Arbitrary densitometric units
30000
20000
10000
0
a,b
a
a
a,b
BMPR2
VEGF
CONT
MCT28
MCT28/DEC

Figure 3:

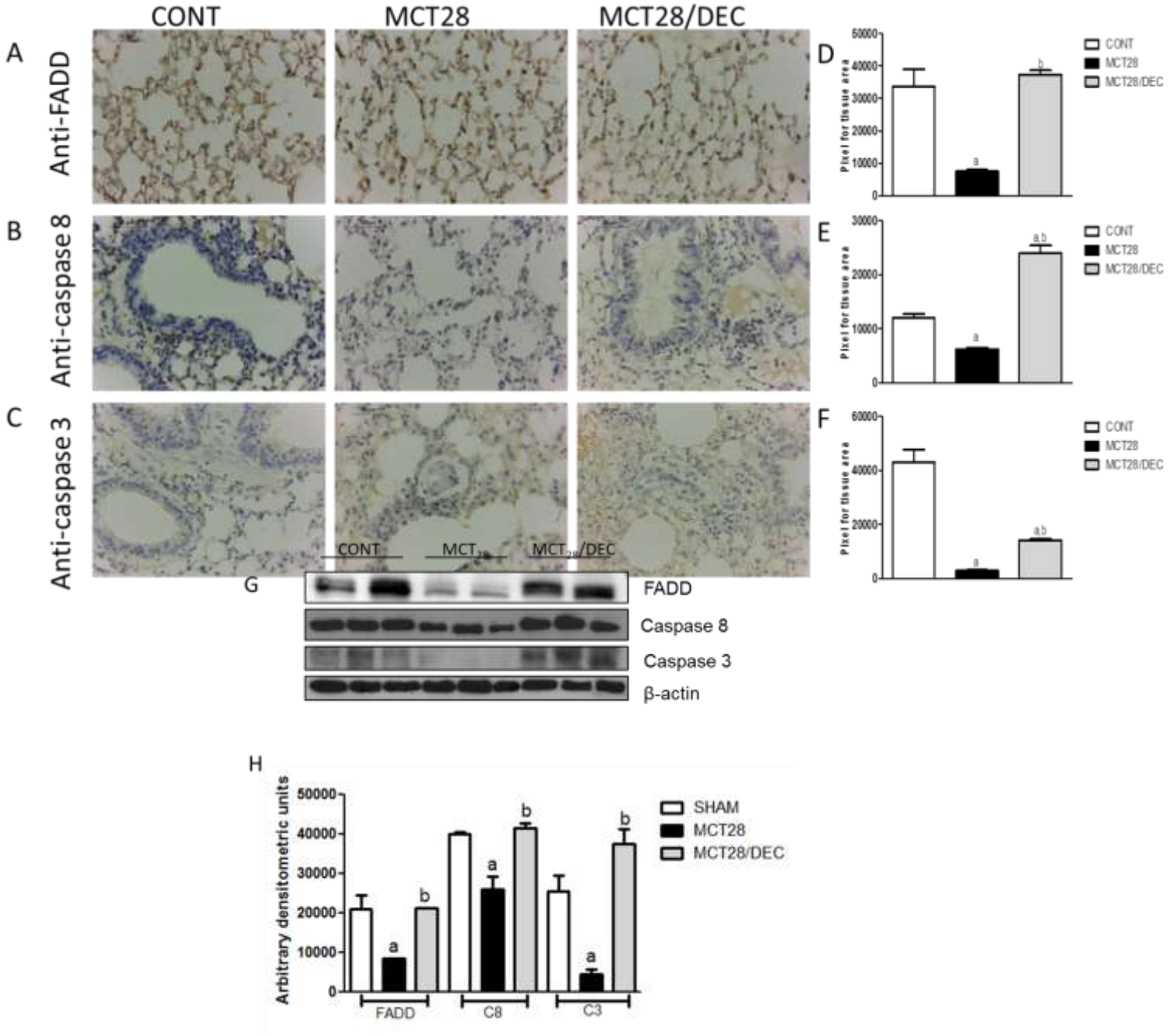
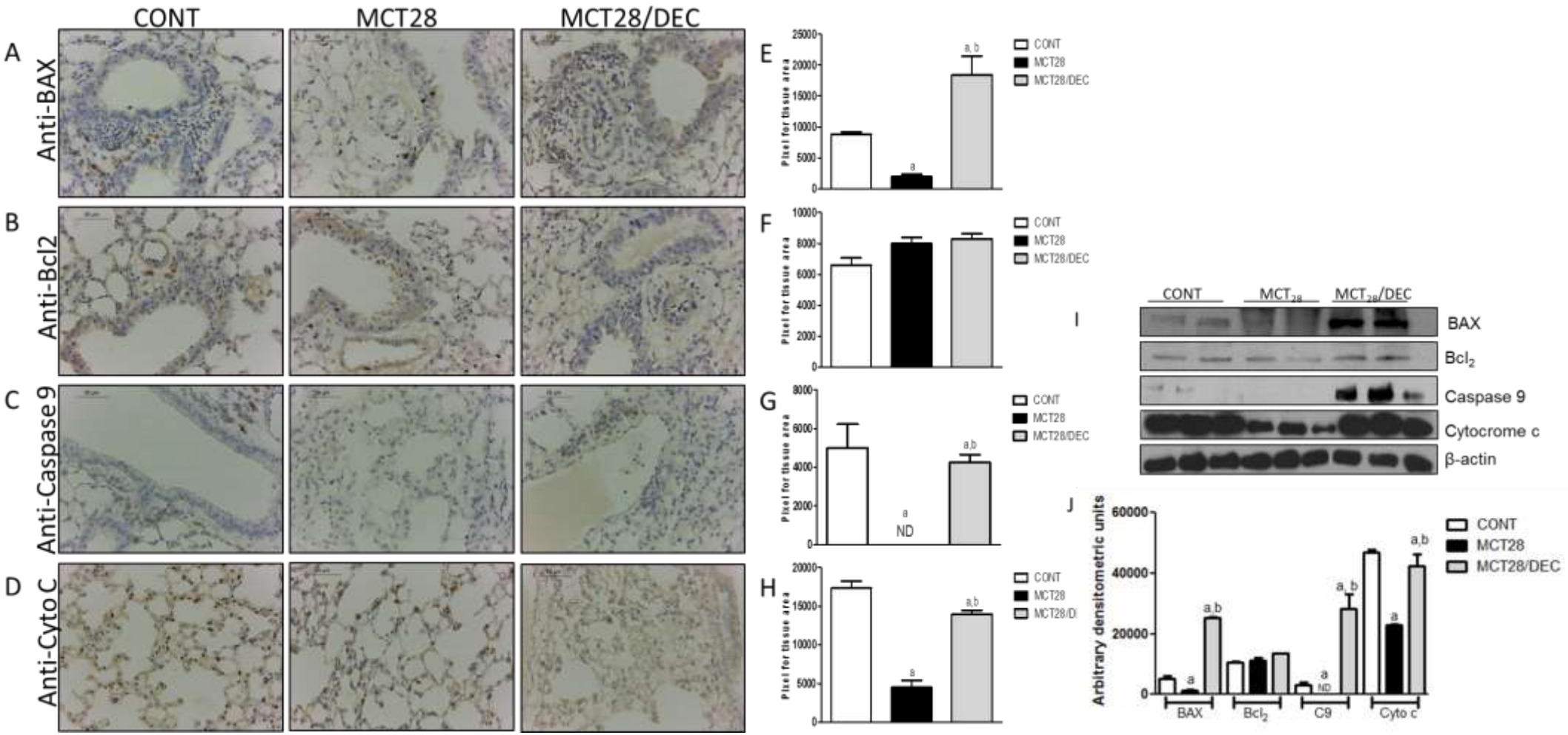

CONT
MCT28
MCT28/DEC
A
Anti-FADD
B
Anti-caspase 8
C
Anti-caspase 3
D
E
F
Pixel for tissue area
CONT
MCT28
MCT28/DEC
G
FADD
Caspase 8
Caspase 3
β-actin
H
Arbitrary densitometric units
SHAM
MCT28
MCT28/DEC
FADD
C8
C3

Figure 4:


CONT
MCT28
MCT28/DEC
A
Anti-BAX
B
Anti-Bcl2
C
Anti-Caspase 9
D
Anti-Cyto C
E
F
G
H
Pixel for tissue area
CONT
MCT28
MCT28/DEC
a
a,b
ND
I
CONT
MCT28
MCT28/DEC
BAX
Bcl2
Caspase 9
Cytocrome c
β-actin
J
Arbitrary densitometric units
BAX
Bcl2
C9
Cyto c

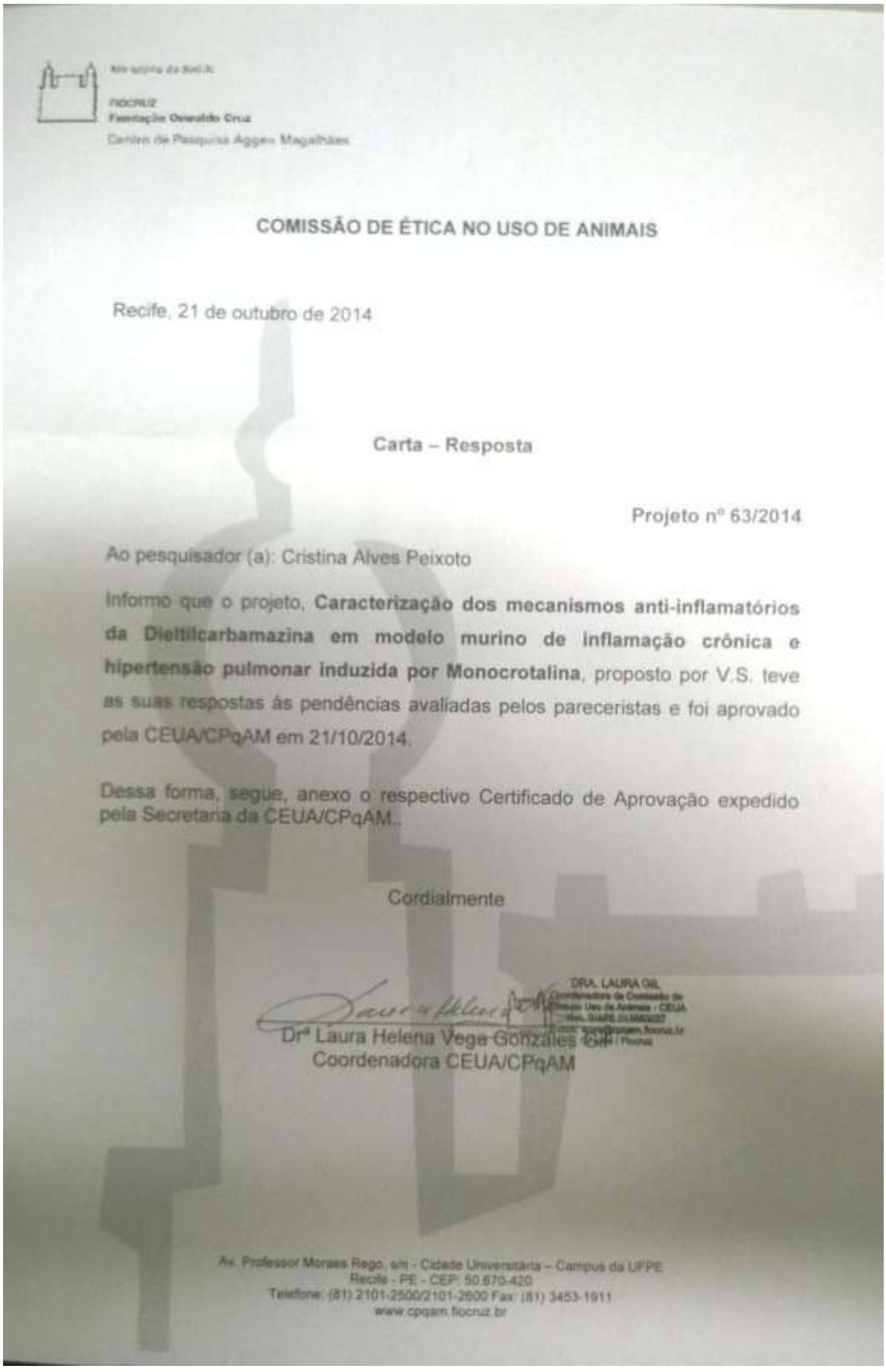
7- CONSIDERAÇÕES FINAIS

Os ensaios desenvolvidos no presente trabalho demonstram que:

1. O estudo de novos modelos experimentais para entender o processo inflamatório envolvido na hipertensão pulmonar se faz necessário devido à grande complexidade que envolve essa doença, o modelo de hipertensão pulmonar induzido por monocrotalina em camundongos é um modelo que pode promover a utilização de vários recursos genéticos na tentativa de compreender as alterações celulares e moleculares envolvidas na hipertensão pulmonar. Nós observamos que em momentos diferentes do processo inflamatório há presença de células e citocinas inflamatórias que promovem alterações teciduais importantes.
2. A utilização da dietilcarbamazina no controle e tratamento da filariose é algo utilizado há bastante tempo, entretanto esse fármaco apresenta uma ação anti-inflamatória já demonstrada cientificamente por diversos pesquisadores mostrando bastante eficaz em modelos de inflamação pulmonar, ativando ou inibindo vias importantes envolvidas no processo inflamatório, além disso, observamos que a dietilcarbamazina melhorou a hipertensão pulmonar no modelo induzido por monocrotalina, ativando a via da apoptose comprometida no processo de hipertensão pulmonar.

8- ANEXO

8.1 Parecer da comissão de ética no uso de animais



FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Recife, 21 de outubro de 2014

Carta – Resposta

Projeto nº 63/2014

Ao pesquisador (a): Cristina Alves Peixoto

Informo que o projeto, **Caracterização dos mecanismos anti-inflamatórios da Dietilcarbamazina em modelo murino de inflamação crônica e hipertensão pulmonar induzida por Monocrotalina**, proposto por V.S. teve as suas respostas às pendências avaliadas pelos pareceristas e foi aprovado pela CEUA/CPqAM em 21/10/2014.

Dessa forma, segue, anexo o respectivo Certificado de Aprovação expedido pela Secretaria da CEUA/CPqAM..

Cordialmente

DRA. LAURA GIL
Coordenadora da Comissão de Ética no Uso de Animais - CEUA

Drª Laura Helena Vega Gonzales
Coordenadora CEUA/CPqAM

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