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GÉSSICA LUANA ANTUNES

ALTERAÇÕES NEUROINFLAMATÓRIAS E MODULAÇÃO DA VIA COLINÉRGICA ANTI-INFLAMATÓRIA EM UM MODELO EXPERIMENTAL DE ASMA

Porto Alegre
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Dissertação de Mestrado apresentada à Escola de Medicina da PUCRS para obtenção de título de Mestre em Pediatria e Saúde da Criança

Prof. Dra. Aline Andrea da Cunha

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Dissertação de Mestrado apresentada ao Curso de Pós-Graduação em Medicina/Pediatria e Saúde da Criança da Pontifícia Universidade Católica do Rio Grande do Sul, como parte dos requisitos necessários à obtenção do título de Mestre em Pediatria e Saúde da Criança

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“*A Morte é terrivelmente final, ao passo que a vida está cheia de possibilidades.* ”

Tyrion Lannister

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RESUMO

INTRODUÇÃO: A asma resulta da inalação continua dos alergenos, levando a inflamação crônica das vias aéreas. Tipicamente, a síntese de imunoglobulina E (IgE), recrutamento eosinofílico, superprodução de muco e hiper-reatividade de células musculares lisas são impulsionadas pelas interleucinas (IL) IL-4, IL-5 e IL-13, produzidos por linfócitos Th2, tendo como resultado final a obstrução das vias aéreas. Estudos têm demonstrado que além da inflamação pulmonar a asma pode resultar em alterações no sistema nervoso central (SNC) e neuroinflamação, caracterizada pela liberação de citocinas pró-inflamatórias, como interleucina 1 beta (IL-1β) e fator de necrose tumoral α (TNF-α) e pelo aumento do tamanho mitocondrial. Além disso, sabe-se que a asma está frequentemente associada com ansiedade. Para minimizar esses danos são necessários mecanismos que regulem a resposta imune. Nesse sentido, Tracey e colaboradores, descreveram a via colinérgica anti-inflamatória, a qual regula a inflamação por meio do nervo vago e da ligação da acetilcolina (ACh) ao receptor nicotínico de acetilcolina alpha7 (α7nAChR). Desde a descoberta da via em questão, diversos estudos têm demonstrado seu potencial terapêutico em diversas patologias.

OBJETIVO: Os artigos científicos que compõe essa dissertação tiveram como objetivo identificar as alterações neuroinflamatórias que podem levar a ansiedade em animais submetidos a um modelo experimental de asma (artigo 1). Avaliar os efeitos da modulação da via colinérgica anti-inflamatória através do tratamento com neostigmina sobre o estresse oxidativo e inflamação das vias aéreas em um modelo experimental de asma (artigo 2). Avaliar os efeitos da modulação da via colinérgica anti-inflamatória através do tratamento com neostigmina sobre o estresse oxidativo no córtex cerebral de animais submetidos a um modelo experimental de asma (artigo 3).

METODOLOGIA: Para indução de um modelo experimental de asma, camundongos BALB/cJ foram sensibilizados com injeção subcutânea de ovalbumina (OVA) nos dias 0 e 7 do protocolo, seguido por três desafios intranasais com OVA nos dias 14, 15 e 16. O grupo controle recebeu apenas solução salina tamponada com fosfato de Dulbecco (DPBS). Nos artigos 2 e 3 para avaliar os efeitos da via colinérgica anti-inflamatória, os animais foram

tratados com um inibidor da acetilcolinesterase - Neostigmina (Normastig) (80 µg/Kg) por via intraperitoneal 30 minutos depois de cada um dos 3 desafios com OVA. No 17º dia de protocolo os animais foram eutanasiados para obtenção das amostras e desenvolvimento das técnicas descritas, conforme os objetivos de cada um dos artigos científicos.

RESULTADOS: No artigo científico 1, observamos que a inflamação das vias aéreas promoveu importantes alterações no encéfalo dos animais, verificamos formação de estresse oxidativo e presença de disfunção mitocondrial, resultando na alteração da atividade de importantes enzimas, como a Na^+,K^+-ATPase e acetilcolinesterase (AChE). Além disso, demonstramos aumento de citocinas pró-inflamatórias (IL-1β, lL-9, IL-13 e eotaxina). Em resposta a esses danos, demonstramos através de técnica de microPET utilizando o ^{18}F-fluorodesoxiglicose (^{18}F-FDG) que ocorre aumento no metabolismo de glicose em estruturas do eixo hipotálamo-pituitária-adrenal (HPA), levando a hiperatividade do eixo através da deficiência de *feedback* negativo, indicado pela diminuição dos receptores de glicocorticoide. Por fim, em resposta a esses danos também demonstramos que ocorre um aumento no imunoconteúdo de de BDNF no encéfalo. Por fim, verificamos que os animais submetidos a um modelo experimental de asma apresentam um aumento do comportamento de ansiedade. Assim, propomos, pela primeira vez, que a inflamação no tecido pulmonar medeia alterações neuroquímicas, neurometabólicas e neuroinflamatórias dentro do cérebro, que levam ao fenótipo de ansiedade na asma. No artigo cientifico 2, demonstramos que a ativação farmacológica da via colinérgica anti-inflamatória com neostigmina diminuiu significativamente a liberação de citocinas pró-inflamatórias e atenuou o estresse oxidativo e, consequentemente diminuiu o recrutamento eosinofílico e a hipersecreção de muco, levando a uma melhora nos parâmetros de mecânica ventilatória. Por fim, no artigo 3, observou-se que o tratamento com neostigmina foi capaz de reduzir o recrutamento eosinofílico nas vias aéreas e preveniu a formação de estresse oxidativo no córtex cerebral dos animais submetidos a um modelo experimental de asma.

CONCLUSÃO: Os resultados encontrados nesta dissertação demonstram que a inflamação nas vias aéreas em animais submetidos a um modelo experimental de asma promove diversas alterações neuroquímicas, neurometabólicas e neuroinflamatórias que levam ao fenótipo de ansiedade. Uma dessas importantes alterações encontradas é a alteração na atividade da AChE, descrita

como um importante componente da via colinérgica anti-inflamatória que regula continuamente os níveis de ACh. Nesse sentido, utilizando a neostigmina, a qual inibe a ação da AChE nas junções neuromusculares e não tem efeito direto sobre a AChE no SNC, visto que a mesma não ultrapassa a barreira hematoencefálica (BHE), demonstramos que o tratamento com neostigmina além de controlar a resposta inflamatória no pulmão também foi capaz de atenuar o estresse oxidativo no córtex cerebral de animais submetidos a um modelo de asma. Acreditamos, que esses resultados possam ser explicados pela redução da inflamação nas vias aéreas, que diminui o subsequente sinal do nervo vago ao SNC e desta forma, reduz as alterações neuroquímicas e neuroinflamatórias. Assim, acreditamos que nossos resultados possam contribuir para essa imensa lacuna que existe entre asma e transtornos emocionais, como a ansiedade e propomos que a asma deve ser olhada não apenas como uma inflamação pulmonar localizada.

Palavras-chave: asma; via colinérgica anti-inflamatória; neostigmina; neuroinflamação; estresse oxidativo.

ABSTRACT

INTRODUCTION: Asthma results from the continuous allergens inhalation, leading to airways inflammation. Immunoglobulin E (IgE) synthesis, eosinophilic recruitment, mucus overproduction and muscle cell hyperreactivity are typically driven by interleukins (IL) IL-4, IL-5, and IL-13, secreted by Th2 lymphocytes, resulting in airways obstruction. Asthma is often associated with anxiety. In fact, asthma not only induced lung inflammation but also results in neuroinflammation, characterized by the release of proinflammatory cytokines, such as IL-1β and tumor necrosis factor α (TNF-α) and an increase in mitochondrial size. To minimize these damages are required mechanisms that regulate the immune response. In this sense, Tracey and colleagues described the cholinergic anti-inflammatory pathway (CAP), that regulates the inflammation through of the vagus nerve and binding of acetylcholine (ACh) to α7 nicotinic acetylcholine receptor (α7nAChR). Diverse studies have demonstrated its therapeutic potential in diverse pathologies.

OBJECTIVE: The scientific articles that compose this dissertation had as objective to identify the neuroinflammatory changes that can lead to anxiety in animals submitted to an experimental asthma model (article 1). To evaluate the modulation effects of cholinergic anti-inflammatory pathway with neostigmine over oxidative stress and airway inflammation in an experimental model of asthma (article 2). To evaluate the modulation effects of cholinergic anti-inflammatory pathway with neostigmine over oxidative stress in the cerebral cortex in an experimental model of asthma (article 3).

METHODS: For experimental asthma model induction, the animals were sensitized by subcutaneous injections of ovalbumin (OVA), on days 0 and 7, followed by three intranasal challenges with OVA on days 14, 15, and 16 of the protocol. The animals of the control group received only DPBS in the sensitization and intranasal challenges. In articles 2 and 3 to evaluate the CAP effects, on days 14, 15, and 16 after 30 minutes of OVA challenge, mice received 80 µg/kg of neostigmine intraperitoneally. On 17º day mice were euthanized for sample obtain and development the described techniques, according to the objectives for each of the scientific articles.

RESULTS: In the scientific article 1, we observed that the airways inflammation promoted important alterations in mice brain, we verified the oxidative stress

formation and presence of mitochondrial energy dysfunction, resulting in activity alteration of the important enzymes, such as Na^+,K^+-ATPase and acetylcholinesterase (AChE). In addition, we also demonstrated increased proinflammatory cytokines (IL-9, IL-13, eotaxina, and IL-1β,). In response to these damages we demonstrate through microPET technique using ^{18}F-fluorodeoxyglucose (^{18}F-FDG) that increase glucose metabolism occurs in in structures of the hypothalamic-pituitary-adrenal (HPA) axis, leading to axis hyperactivity through the negative feedback deficiency, indicated by a decrease in glucocorticoid receptors. Finally, in response to these damages we also demonstrate that upregulation occurs at BDNF levels. In addition, we demonstrated that animals submitted to an experimental model of asthma present an increase in anxiety behavior. Thus, we propose, for the first time, that airway inflammation mediates critical neuroinflammatory changes within the brain, which lead to the anxiety phenotype in asthma. In the scientific article 2, we demonstrated that the CAP pharmacological activation with neostigmine significantly decreased the release of proinflammatory cytokines and attenuated oxidative stress and consequently decreased eosinophilic recruitment and mucus hypersecretion, leading to improved parameters of ventilatory mechanics. Finally, in article 3, it was observed that treatment with neostigmine was able to reduce eosinophilic recruitment in the airways and decreased the formation of oxidative stress in the cerebral cortex of animals submitted to an experimental model of asthma.

CONCLUSION: The results found in this dissertation demonstrate that the inflammation in the airways in animals submitted to an experimental model of asthma promotes several neuroinflammatory changes that lead to the anxiety phenotype. One of these important alterations is the change in AChE activity, described as an important component of the cholinergic anti-inflammatory pathway that continuously regulates ACh levels. In this sense, using neostigmine, which inhibits the action of AChE in the neuromuscular junctions and does not have a direct effect on AChE in the CNS, since it does not exceed the blood brain barrier (BBB), we demonstrated that neostigmine treatment besides controlling the inflammatory response in the lung was also able to attenuate the oxidative stress in the cerebral cortex of animals submitted to an asthma model. We believe that these results can be explained by the reduction of inflammation in the

airways, which decreases the subsequent signal from the vagus nerve to the CNS and thereby reduces neuroinflammation. Thus, we believe that our results may contribute to this huge gap between asthma and emotional disorders such as anxiety and we propose that asthma should be looked at not just as localized lung inflammation.

Keywords: asthma; cholinergic anti-inflammatory pathway; neostigmine, neuroinflammation, oxidative stress

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ACh – Acetilcolina

AChE – Acetilcolinesterase

AKT – Proteína quinase B

ACTH - Hormônio adrenocorticotrófico

BDNF – Fator neurotrófico derivado do cérebro

CAT – Catalase

CD – Célula dendrítica

cDNA – Ácido desoxirribonucleico complementar

CeMBE – Centro de modelos biológicos experimentais

ChAT – Acetiltransferase

CHT1 – Transportador de colina de alta afinidade

CRH - Hormônio Liberador de Corticotrofina

EROs – Espécies reativas de oxigênio

GAPDH – Gliceraldeido-3-fosfato desidrogenase

GATA3 – Fator de transcrição

GPx – Glutationa peroxidase

GR – Receptor de glicocorticoide

GSH – Glutationa reduzida

GSSG – Dissulfeto de glutationa

H_2O_2 – Peróxido de hidrogênio

HO – Heme oxigenase

HPA – Hipotálamo-pituitária-adrenal

IgE – Imunoglobulina E

IL – Interleucina

JAK2 – Janus quinase 2

mRNA – Ácido ribonucleico mensageiro

NFkB – Fator nuclear kappa B

Nrf2 – Fator nuclear eritroide 2

$O_2^{\bullet -}$ – Ânion superóxido

OH^- – Radical hidroxil

OVA – Ovalbumina

PCR – Reação em cadeia de polimerase

PKA – Proteína quinase A

RPM – Rotação por minuto

SNC – Sistema nervosa central

SOD – Superóxido dismutase

STAT – Transdutor e ativador de transcrição

Th – Célula T auxiliar

TNF – Fator de necrose tumoral

VAChT – Transportador vesicular de acetilcolina

α7nAChR – Receptor nicotínico de acetilcolina alpha7

SUMÁRIO

1 INTRODUÇÃO

A asma é uma doença inflamatória crônica mediada por linfócitos Th2, com aumento da secreção de interleucinas (IL) IL-4, IL-5 e IL-13, que promovem a síntese de imunoglobulina E (IgE) o recrutamento e ativação eosinofílica, produção de espécies reativas de oxigênio (EROs) e hiperplasia de células caliciformes e, tem como resultado final a obstrução das vias aéreas. Ao longo dos anos, pesquisadores têm focado em estudos com objetivo de elaborar estratégias medicamentosas que controlem os sintomas da doença e previnam o risco de futuras exacerbações. Contudo, atualmente não há cura para a asma e apesar dos esforços da comunidade científica na busca de tratamentos para a doença, a morbidade da mesma continua a ser um problema de saúde pública muito comum e grave.[1]

Além do sofrimento físico causado pela resposta imune exacerbada também ocorre diversos prejuízos psicológicos e transtornos mentais, especialmente ansiedade. Muitos estudos relatam que os transtornos de ansiedade ocorrem tanto em crianças quanto em adultos, levando a insucessos como escolar e no trabalho, respectivamente. Sabe-se que poucas doenças crônicas geram um impacto tão significativo quanto a asma e, que quando somado a ansiedade apresentam um impacto substancial na qualidade de vida dos pacientes.[2-4] Contudo, os mecanismos que levam ao fenótipo de ansiedade nesses pacientes permanecem desconhecidos. Assim, torna-se evidente a necessidade de mais estudos que busquem elucidar esses mecanismos, tratamentos que busquem tanto o controle dos sintomas clássicos e que previnam o risco de futuras exacerbações, bem como visem os aspectos psicológicos desses pacientes.

A via colinérgica anti-inflamatória foi descrita por Tracey e colaboradores como um mecanismo neural que é capaz de controlar a inflamação através do nervo vago e da ligação da acetilcolina (ACh) ao receptor nicotínico de acetilcolina alpha7 (α7nAChR).[5, 6] Estudos experimentais e clínicos já comprovaram seu potencial terapêutico em diversas patologias.[7-10] Neste sentido, a modulação da via colinérgica anti-inflamatória pode fornecer importantes *insights* para o desenvolvimento de novas estratégias terapêuticas para o tratamento da asma.

Durante minha iniciação científica no laboratório de Respirologia Pediátrica, além de participar de estudos experimentais também auxiliei em projetos clínicos desenvolvidos no ambulatório de Pneumologia Pediátrica do Hospital São Lucas (HSL) da PUCRS. Em reuniões do nosso grupo de pesquisa eram comuns relatos de pesquisadores sobre alterações emocionais em pacientes asmáticos e o impacto da asma na qualidade vida dos mesmos. De fato, em 2017 um estudo (dados ainda não publicado) realizado no ambulatório de Pneumologia Pediátrica do HSL, demonstrou que transtornos emocionais, especialmente ansiedade são comuns em crianças com asma, evidenciando que os aspectos psicológicos necessitam de maior atenção nesses pacientes. Outros estudos científicos também já demonstraram essas associações tanto em crianças quanto em adultos. Contudo, pouco se sabe sobre os mecanismos que levam a esses transtornos de ansiedade. Por isso, está dissertação se propõe em investigar essas possíveis alterações no SNC através de um modelo experimental de asma.

Assim, esta dissertação é composta por três artigos científicos. No artigo científico 1, identificamos as alterações no encéfalo que levam ao comportamento de ansiedade. Com os resultados encontrados no artigo 1, verificamos alteração na atividade da AChE, a qual é expressa em neurônios e junções neuromusculares, e é responsável por manter continuamente os níveis de ACh, a qual foi descrita como um componente essencial da via colinérgica anti-inflamatória. Assim, resolvemos investigar os efeitos da modulação farmacológica da via colinérgica anti-inflamatória com neostigmina em um modelo experimental de asma, resultando nos artigos científicos 2 e 3. Inicialmente, avaliamos os efeitos do tratamento com neostigmina sobre a inflamação das vias aéreas (artigo cientifico 2), visto que a droga não ultrapassa a barreira hematoencefálica (BHE) e não tem efeito direto sobre o SNC. Finalmente, como a modulação farmacológica da via foi capaz de controlar a inflamação nas vias aéreas, no artigo científico 3 investigamos os efeitos da modulação da via com neostigmina sobre o estresse oxidativos no córtex cerebral. Desta forma, essa dissertação busca investigar as possíveis alterações neuroquímicas, neurometabólicas e neuroinflamatórias que podem levar a ansiedade, bem como a modulação farmacológica da via colinérgica anti-inflamatória na inflamação em um modelo experimental de asma.

2 REVISÃO DE LITERATURA

2.1 EPIDEMIOLOGIA

A asma é uma doença respiratória crônica, caracterizada por sintomas variados de sibilo, falta de ar, aperto no peito, tosse e por limitação variável do fluxo aéreo expiratório. Esses episódios são uma consequência da obstrução ao fluxo aéreo pulmonar generalizado e variável, reversível espontaneamente ou com tratamento. É uma doença influenciada tanto por fatores genéticos quanto ambientais, sendo considerada uma doença heterogênea e, atualmente, tem sido reconhecida como uma síndrome, por sua complexidade em apresentar diferenças quanto a gravidade da doença, comorbidades, fenótipos e resposta ao tratamento. O diagnóstico clínico da asma é sugerido pela avaliação dos sintomas, função pulmonar e resposta ao tratamento. Atualmente, os tratamentos para a doença visam o manejo sintomático e a profilaxia das crises, com objetivo de diminuir os sintomas e evitar novas exacerbações. Ainda não existe cura para asma e apesar dos avanços na evolução do tratamento sua morbidade continua elevada.[1]

A asma acomete pessoas de todas as idades e, atualmente cerca de 300 milhões de indivíduos no mundo são portadores da doença, com um índice de mortalidade de 250.000 pessoas por ano. Além disso, estima-se que até o ano de 2025 mais de 100 milhões de pessoas também irão desenvolver a doença no mundo. No Brasil, o Departamento de Informática do Sistema Único de Saúde (DATASUS) registrou 160 mil hospitalizações em todas as idades, este dado colocou a asma como a quarta causa de internação.[11] Assim, não existe dúvida, do ponto de vista médico e de saúde pública, de que a asma se trata de uma doença altamente prevalente e com elevados custos tanto pessoais quanto econômicos no Brasil. Dentre os diversos fenótipos da doença, o fenótipo de asma alérgica é o mais facilmente identificado, o qual inicia na infância ou no início da adolescência e está associado a uma história passada e/ ou familiar de doenças alérgicas como rinite e eczema, e o exame de escarro induzido nesses pacientes revela a inflamação eosinofílica das vias aéreas.[1]

2.2 INFLAMAÇÃO DAS VIAS AÉREAS

A inflamação é um mecanismo importante de resposta do sistema imune e participa do complexo sistema de defesa do organismo, iniciado tanto por fatores endógenos (necrose tecidual), como por fatores exógenos (agentes físicos, químicos e/ou estímulos biológicos) e tem como objetivo a destruição e a eliminação do agente infecioso. Assim, uma resposta insuficiente pode levar a imunodeficiência que resulta em infecções e câncer. Por outro lado, uma resposta exacerbada pode levar a maior morbidade em doenças como a artrite reumatoide, doença de Crohn, diabetes, aterosclerose, doença de Alzheimer e asma.[12] No caso de muitas doenças crônicas pulmonares, como a asma, a inflamação é o aspecto central, onde o processo inflamatório tem como resultado as manifestações clínico-funcionais características da doença. A partir da sensibilização a alergenos específicos, diversas células e mediadores inflamatórios participam da resposta imunológica e tecidual na asma alérgica, destacando-se os eosinófilos, mastócitos, neutrófilos, macrófagos, células dendríticas (CDs), linfócitos e, como mediadores inflamatórios, destacam-se as espécies reativas de oxigênio (EROs) e citocinas.

2.2.1 Estresse oxidativo na asma

O recrutamento e atividade dessas células características da resposta inflamatória, especialmente dos eosinófilos, produzem as EROs. As principais EROs distribuem-se em dois grupos: o grupo dos radicais livres, os quais destacam-se o ânion superóxido ($O_2^{\bullet -}$) e o radical hidroxila ($OH^{\bullet}$), e os pertencentes ao grupo dos compostos não radicalares, o qual inclui o peróxido de hidrogênio (H_2O_2). As espécies reativas são produtos do metabolismo celular e, em níveis moderados, têm efeito benéfico e estão envolvidas em diversas funções fisiológicas. Por outro lado, em maiores concentrações podem causar danos às biomoléculas, incluindo lipídeos, proteínas e DNA.[13]

Para neutralizar esses radicais, o organismo possui mecanismos de defesas enzimáticas e não enzimáticas. Dentre estas defesas enzimáticas, destacam-se a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Como defesas não enzimáticas podemos citar a glutationa reduzida (GSH) os polifenóis e as vitaminas A, C, e E.[14] A SOD é uma

metaloenzima que catalisa a dismutação do $O_2^{\bullet-}$, formando H_2O_2. Uma molécula do H_2O_2 formada é reduzida a H_2O e a outra é oxidada a O_2 através da ação da enzima CAT. A GPx também atua decompondo o H_2O_2 por meio do acoplamento de sua redução a H_2O com a concomitante oxidação da GSH ao dissulfeto de glutationa (GSSG). Assim, juntas essas enzimas evitam o acúmulo de $O_2^{\bullet-}$ e de H_2O_2 e a consequente produção de $OH^{\bullet}$, contra o qual não existe nenhum sistema enzimático de defesa.[13]

Quando há um desequilíbrio entre a produção de espécies reativas e estas defesas antioxidantes, ou seja, um aumento na formação de EROs e/ou uma diminuição nas defesas antioxidantes – como na asma, ocorre o estresse oxidativo. Sabe-se que as EROs produzidas por células presentes nas vias aéreas, contribuem para a exacerbação da doença, por meio do aumento da contração da musculatura lisa, hiper-responsividade brônquica e da hipersecreção de muco.[15-17]

2.2.2 Citocinas pró-inflamatórias na asma

Citocinas desempenham um importante papel na comunicação e ativação celular. A maioria das células do sistema imunológico respondem as citocinas, aumentando a síntese das mesmas e amplificando a cascata inflamatória. As citocinas podem ser produzidas por várias células inflamatórias, tais como mastócitos, eosinófilos e as células T.[18] As células T são consideradas chave para a amplificação da resposta imune e exacerbação da doença. Na parte central da resposta imune alérgica de um indivíduo encontram-se as células $TCD4^+$ de memória. As células apresentadoras de antígenos, como DCs e macrófagos, iniciam a resposta imune adaptativa contra os antígenos, através da ativação destas células T. Essas células podem diferenciar-se em diferentes fenótipos, como linfócitos Th1, linfócitos Th2, linfócitos Th17 e linfócitos T regulatórios (Treg), onde cada subpopulação celular possui funções efetoras distintas. As citocinas classicamente relacionadas a fisiopatogenêse da asma alérgica são as secretadas pelo perfil Th2.[19-21] Esses linfócitos se diferenciam através de contato com alérgenos e são característicos de doenças alérgicas.

Células Th2 são programadas para produzir citocinas específicas como IL-4, IL-5 e IL-13, entre outras, que sustentam a inflamação aguda e persistente nos brônquios de indivíduos com asma. Através do transdutor e ativador de transcrição (STAT6), a sinalização de IL-4 regula o fator de transcrição (GATA-3), promovendo uma retroalimentação positiva para o perfil Th2. Além disso, a IL-4 promove a troca de isotipo e produção de IgE.[21] Em um estudo com animais deficientes em IL-4 foi observado que o desenvolvimento da inflamação alérgica induzida por um antígeno foi significativamente menor em relação aos animais do tipo selvagem.[22] A sinalização da IL-5 é crítica para a expansão, diferenciação e recrutamento eosinofílico. [23] A importância da IL-5 sobre os eosinófilos é evidenciada em camundongos que superexpressam essa citocina, os quais aumentam expressivamente o número de eosinófilos.[24] O papel crítico da IL-5 também já foi demonstrado em ensaios clínicos, um anticorpo monoclonal humanizado (Mepolizumab) com potentes efeitos na neutralização de IL-5, reduziu a contagem de eosinófilos no sangue, tecidos periféricos e medula óssea em pacientes.[25, 26] Portanto, a IL-5 modula a inflamação eosinofílica importantemente. A importância da IL-13 na exacerbação da asma também já foi demonstrada, animais deficientes em IL-13 não foram capazes de produzir hiper-reatividade brônquica e muco, mesmo na presença de IL-4 e IL-5. Assim, a IL-13 possui importante efeito sobre o epitélio e células musculares lisas, por meio da produção de muco pela diferenciação e hiperplasia das células epiteliais secretoras e induz hiper-responsividade brônquica.[27]

As citocinas secretadas pelo perfil Th2 determinam em grande parte a gravidade e a persistência das exacerbações. Contudo, outras citocinas pró-inflamatórias também podem contribuir durante a resposta inflamatórias nas vias aéreas. Recentemente, Faiz e colaboradores (2018) demonstraram que a IL-1β também está associada a produção de muco nos pulmões.[28] Fator de necrose tumoral (TNF-α) induz liberação de outras citocinas pró-inflamatórias, aumentando os danos as células pulmonares.[29] No pulmão, a IL-9 também é expressa por eosinófilos e estimula a proliferação de células T ativadas além de aumentar a produção de IgE pelas células B.[30]

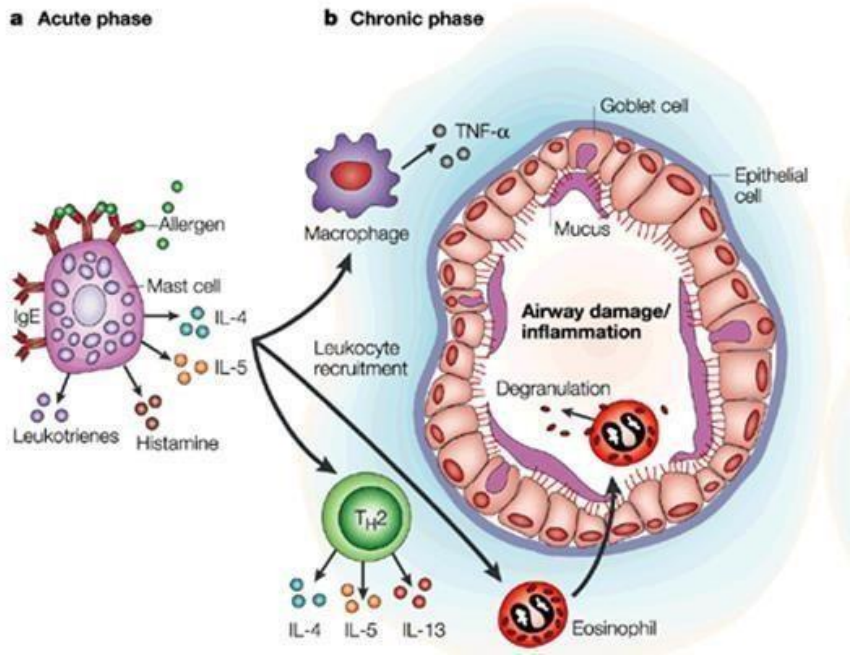

Figura 1: Visão geral do processo inflamatório nas vias aéreas. A reação alérgica inicia com a degranulação dos mastócitos que estão com IgE associados em sua superfície. A ligação do antígeno com IgE leva a liberação imediata de mediadores inflamatórios, como histamina e leucotrienos. A asma alérgica resulta da inalação continua dos alergenos, levando a inflamação crônica das vias aéreas mediada por eosinófilos, o qual degranula, libera componentes citotóxicos e produz as EROs. Tipicamente, a síntese de IgE, o recrutamento e a atividade eosinofílica, superprodução de muco e hiper-reatividade de células musculares lisas são impulsionadas pelas citocinas IL-4, IL-5 e IL-13, produzidos por linfócitos Th2, tendo como resultado final a obstrução das vias aéreas. Adaptado de Gern.[31]

2.3 INFLAMAÇÃO NAS VIAS AÉREAS E NEUROINFLAMAÇÃO

Durante muitos anos o SNC foi descrito como “órgão imunológicamente privilegiado”, devido à dificuldade de acesso de células do sistema imune periférico ao seu interior. A presença da BHE a qual controla a passagem de moléculas e íons e protege o cérebro de toxinas e patógenos, foi considerada um ponto chave na qualificação do SNC como um sítio privilegiado imunologicamente.[32] Contudo, apesar da estabilidade da BHE, durante inflamações crônicas, como na esclerose múltipla pode ocorrer ruptura da BHE e neuroinflamação.[33]

A neuroinflamação é caracterizada pelo aumento da liberação de citocinas pró-inflamatórias, influxo de células T, recrutamento de leucócitos e lesão tecidual. Uma vez que células T e leucócitos atravessam BHE, produzem

mediadores inflamatórios como as espécies ROS e espécies reativas de nitrogênio (RNS) que completam a degradação da BHE e alimentam a inflamação e o dano tecidual.[33, 34] Durante injúrias ao SNC as microglias, que constituem 10% do total de células da glia e são consideradas macrófagos residentes do cérebro, representam a primeira linha de defesa do sistema imune no SNC. Outra importante célula da glia que constitui o sistema de defesa no SNC são os astrócitos.[35] Juntas, essas células desempenham um papel neuroprotetor no SNC. Por outro lado, produção de citocinas pró-inflamatórias e espécies reativas, fazem parte do progresso da resposta orquestrada por células do SNC amplificando a neuroinflamação.[36]

O cérebro se caracteriza por ser um dos órgãos mais ativos metabolicamente, no entanto, possui reservas energéticas extremamente pequenas em relação a sua alta taxa metabólica. A glicose é o principal substrato energético utilizado pelo SNC e a fosforilação oxidativa é particularmente importante para cérebro, pois é responsável por fornecer mais de 95% do ATP sintetizado.[37] Contudo, a formação em excesso de espécies reativas também pode gerar disfunção energética mitocondrial. Na doença de Alzheimer, uma importante doença neurodegenerativa, é encontrada principalmente uma redução na atividade do complexo IV (citocromo c oxidase).[38] Catarina e colaboradores, demonstraram em modelo experimental de sepse, aumento da produção de EROs e diminuição da CAT no cérebro, bem como hipometabolismo de glicose, evidenciado pelo microPET marcado com com o radiofármaco ^{18}F-fluoro-2-desoxi-D-glicose (^{18}F- FDG).[39] Além disso, Wyse e colaboradores já demonstraram que estresse oxidativo gerado pelo excesso de EROs altera a atividade de importantes enzimas do SNC, como a AChE.[40] A AChE é responsável por controlar continuamente os níveis de ACh, a qual regula o sistema imunológico e a produção de citocinas, levando ao controle da inflamação.[12] A AChE controla os níveis de ACh através da hidrólise em acetato e colina, sendo esta última recaptada pelo neurônio por meio de uma Na^+, K^+-ATPase. No entanto, doenças degenerativas, como epilepsia e isquemia cerebral, têm sido associadas à diminuição da atividade da Na^+, K^+-ATPase.[41, 42]

Citocinas também desempenham um importante papel durante a neuroinflamação. De todas as citocinas liberadas no SNC a IL-1β é considerada um importante marcador de neuroinflamação, visto que sua expressão já foi

demonstrada em diversas doenças do SNC.[34] Além disso, experimentos farmacológicos demonstraram que administração de IL-1 β e TNF-α em ratos e camundongos induz "comportamento doentio" (*sickness behavior*). Interessantemente, os sintomas nesse comportamento doentio, tais como letargia, sonolência, fadiga, falta de interesse, falta de apetite e menor concentração, é similar a muitos sintomas descritos na síndrome depressiva.[43] Além desse "comportamento doentio" as citocinas também podem ativar o eixo hipotálamo-hipófise-adrenal (HPA). A administração de LPS ou de citocinas como IL-1(α ou β) e TNF-α provocam direta ou indiretamente efeitos psicológicos e comportamentais, bem como ativação do eixo HPA.[43]

Em consequência a esses eventos estressores, o eixo hipotálamo-hipófise-adrenal (HPA) responde ao estímulo a fim de restabelecer a homeostase corporal. O núcleo paraventricular hipotalâmico estimula a produção e secreção de dois neuropeptídios, o hormônio liberador de corticotrofina (CRH) e a arginina vasopressina, os quais atingem o lobo anterior da pituitária e ativam a secreção de hormônio adrenocorticotrófico (ACTH). Na circulação periférica o ACTH estimula a secreção dos glicocorticoides, epinefrina e noraepinefrina pelo córtex da adrenal.[44] Um importante mecanismo regulatório que inibe a liberação exacerbada dos hormônios pituitários-adrenais durante a ativação do eixo HPA é o *feedback* negativo. A inibição do eixo HPA realizada pelo *feedback* negativo é mediada pelos glicocorticoides e por seus receptores no hipocampo, hipotálamo e na pituitária. Este fenômeno exercido pelos glicocorticóides sobre a secreção de ACTH e de CRH limita a duração da exposição total do organismo aos próprios glicocorticoides, inibindo deste modo seus efeitos imunossupressores. Os receptores envolvidos nesta ação inibitória sobre eixo HPA incluem o receptor mineralocorticóide (MR), que responde a níveis basais de glicocorticóides, e o receptor para glicocorticoide (GR) que responde a concentrações mais altas, devido a diferenças na sensibilidade destes receptores a estes hormônios.[45]

O eixo HPA pode influenciar o fator neurotrófico derivado do cérebro (BDNF). O BDNF é secretado pelas células gliais, como os astrócitos e tem um papel importante na neurogênese e plasticidade neuronal. Contudo, doenças neuroendócrinas têm sido relacionadas com alterações nos níveis de BDNF.[46]

Zhuang e colaboradores, observaram alteração nos níveis de BDNF no hipocampo de animais submetidos a um modelo experimental de asma.[47]

De fato, algumas alterações neuroinflamatórias já foram observadas em animais submetidos a um modelo de asma. Xia e colaboradores, demonstraram em modelo experimental que a asma não apenas induz inflamação periférica, mas também resulta no aumento dos níveis de IL-1β e TNF-α no hipocampo e no córtex pré-frontal.[48] Guo e colaboradores demonstraram, pela primeira vez, que em um modelo experimental de asma com OVA ocorre prejuízo no potencial de longa duração (LTP) e alterações em sinapses de neurônios das regiões CA1 e CA3 do hipocampo, bem como um aumento do tamanho mitocondrial nessas regiões.[49] Esse aumento do tamanho da mitocôndria pode estar diretamente relacionado com a produção de EROs pelo processo inflamatório. Além disso, ressonância magnética funcional em pacientes com asma, demonstrou aumento da atividade cerebral, particularmente em regiões do córtex.[50]

2.3.1 Alterações emocionais em pacientes com asma

Em 1993 Bergel reconheceu que além do potencial alérgico, existe também um potencial psicológico na asma.[51] Ainda, neste mesmo ano, Torre e Ortega, relataram que estímulos emocionais são capazes de provocar alterações no calibre das vias aéreas.[52] Contudo, os mecanismos que levam a essas alterações não eram claros. Sabe-se que asma não é causada por emoções, porém em 1996 Gerlero e colaboradores, observaram que as mesmas podem servir de gatilhos, desencadeando crises de asma e influenciando diretamente na intensidade e frequência das mesmas e, assim, demonstraram que o fator emocional colabora em grande parte para a exacerbação da doença.[53] Desde então, muitos autores têm demonstrado que asma e emoções negativas frequentemente co-ocorrem.

Adolescentes com sintomas da doença não controlados, apresentam risco duas vezes maior de desenvolver ansiedade e depressão em comparação aos indivíduos que apresentam sintomas controlados da doença.[54] Ansiedade é preditivo de pior manejo da asma, apresentando piores resultados em relação ao tratamento do paciente. Alguns dados clínicos demonstram relação entre nível de ansiedade e a gravidade da obstrução das vias aéreas avaliado pelo teste de função pulmonar.[55] A ansiedade aumenta o senso de vulnerabilidade do

paciente, levando a identificação de situações corriqueiras como risco iminente. Desta forma, pacientes asmáticos e ansiosos podem ter percepção destorcida de seus sintomas, aumentando a busca por consultas médicas e em emergências.

Asma, por si só, possui um impacto significativo na vida do paciente e, quando somado a ansiedade afeta diretamente a qualidade de vida dos mesmos. É reconhecido pela comunidade científica que pacientes asmáticos podem apresentar sintomas de depressão e, principalmente ansiedade, contudo os mecanismos que levam a esses transtornos emocionais ainda não são claros. Assim, considerando o impacto gerado nos pacientes se faz necessário estudos que busquem compreender essa lacuna entre asma e ansiedade para direcionar esforços visando o tratamento completo dos pacientes.

2.4 VIA COLINÉRGICA ANTI-INFLAMATÓRIA

Um importante avanço na investigação em neurociência, é a descoberta da comunicação entre o sistema nervoso e o sistema imunológico. Borovikova e colaboradores, descreveram a via colinérgica anti-inflamatória como sendo um desses caminhos pelo qual esses sistemas interatuam, a via é responsável por transmitir informações sobre o estado imunológico periférico para o sistema SNC com objetivo de regular a inflamação.[5] O nervo vago – responsável pela inervação parassimpática, inerva os principais órgãos, como, baço, fígado, rins, intestino e pulmões.[12]

No pulmão, a inervação tem sua predominância em vias aéreas proximais, o nervo surge no tronco cerebral e se dirige até os pulmões através de suas fibras, fazendo sinapses com os gânglios embutidos ou expressos na parede das vias aéreas, traqueias e brônquios para dar origem a fibras pós-ganglionares e inervar as glândulas submucosas, vasos sanguíneos e musculo liso das vias aéreas. Sabe-se que as citocinas pró-inflamatórias como a IL-1β ativam as fibras aferentes do nervo vago, as quais servem de sensor para a inflamação.[56] Esta informação é transmitida ao SNC, que estimula o nervo vago eferente a produzir ACh, a qual é o principal neurotransmissor parassimpático e é considerada um elemento chave no controle da resposta imune.[57]

A ACh é sintetizada no terminal nervoso colinérgico a partir do substrato de acetil-CoA e colina, uma ação catalisada pela enzima acetiltransferase

(ChAT), e posteriormente transportada para o interior das vesículas sinápticas pelo transportador vesicular de acetilcolina (VAChT). As vesículas sinápticas liberam, por exocitose, ACh na fenda sináptica, que ao ser liberada interage com os receptores muscarínicos e nicotínicos. Após a dissociação dos respectivos receptores, a ACh é hidrolisada pela ação da enzima AChE em colina e acetato. A colina é recaptada pelo transportador de colina de alta afinidade (CHT1) para síntese de novas moléculas de acetilcolina.[57]

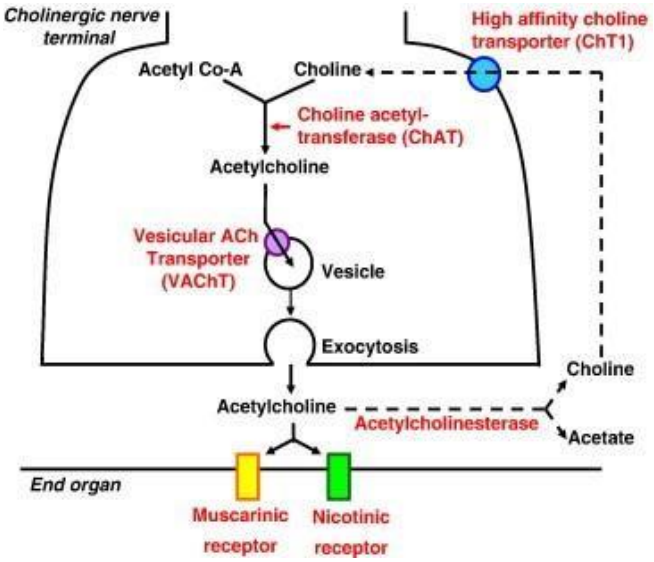

Figura 2: Síntese de acetilcolina e componentes do sistema colinérgico. A acetilcolina (ACh) é sintetizada a partir do substrato de acetil-CoA e colina, por ação da acetiltransferase (ChAT), e posteriormente transportada para o interior das vesículas sinápticas pelo transportador vesicular de acetilcolina (VAChT). As vesículas sinápticas liberam, por exocitose, ACh na fenda sináptica, que ao ser liberada interage com os receptores muscarínicos e/ou nicotínicos. A atividade e permanência da ACh na fenda sináptica são reguladas por hidrólise em colina e acetato catalisada pela acetilcolinesterase (AChE). A colina é recaptada pelo transportador de colina de alta afinidade (CHT1) para síntese de novas moléculas de ACh.[57]

Vários dos componentes colinérgicos têm sido identificados em diversas células do sistema imune, como células epiteliais, endoteliais, linfócitos, eosinófilos, neutrófilos e macrófagos. Os eosinófilos, característicos de pacientes com asma alérgica, expressam receptores de ACh e, sabe-se que a acetilcolina desempenha seu afeito anti-inflamatório através da ligação com o α7nAChR, o qual exerce um papel chave no controle da inflamação periférica. [58] Tracey e colaboradores, demonstraram que animais *knockout* para α7nAChR

não são capazes de inibir a síntese e liberação de TNF em um modelo de endotoxemia.[6] A ligação da ACh ao α7nAChR desencadeia uma série de sinalizações intracelulares por meio de fosfatases e cinases. A ligação da ACh ao α7nAChR ativa Janus Quinase 2 (JAk2), o qual fosforila o transdutor de sinal e ativador de transcrição 3 (STAT-3), inibindo a translocação do fator nuclear kappa B (NFkB) para o núcleo. [59] Além disso, uma cascata de sinalização separada envolve a ativação da proteína quinase (AKT) e proteína quinase A (PKA), permitindo a translocação nuclear do fator de transcrição (Nrf2), que impulsiona a expressão da heme oxigenase 1 (HO-1), a qual exerce uma importante função antioxidante.[60]

Tracey e colaboradores, demonstraram em um ensaio clínico, tais propriedades da via, onde a estimulação elétrica do nervo vago através de um implante em pacientes com artrite reumatoide inibiu a liberação de TNF e melhorou a gravidade da doença. Ainda, utilizando o mesmo dispositivo, um resultado similar foi encontrado em pacientes com epilepsia, a produção das citocinas pró-inflamatórias TNF, IL-1β e IL-6 foi inibida.[8] As propriedades anti-inflamatórias da via também já foram testadas em diversos modelos animais de inflamação, tais como sepse, pancreatite e artrite reumatoide.[7,9,10] Recentemente, Kanashiro e colaboradores demonstraram que a modulação farmacológica da via com neostigmina, um inibidor reversível da enzima AChE, em modelo experimental de artrite, diminuiu o recrutamento de neutrófilos e melhorou os sintomas clínicos da doença inflamatória. [7]

3 JUSTIFICATIVA

Asma caracteriza-se por ser uma doença inflamatória crônica das vias aéreas, na qual o processo inflamatório tem como resultado as manifestações clínico-funcionais características da doença. Sabe-se que é uma doença altamente prevalente, apresenta elevados custos para os sistemas de saúde e está fortemente associada com transtornos emocionais, especialmente ansiedade. Este último aspecto impacta tanto no manejo da asma, bem como diretamente na qualidade de vida dos pacientes. De fato, alguns estudos têm demonstrado que a asma não apenas induz inflamação no pulmão, mas também resulta em aumento dos níveis de citocinas pró-inflamatórias e aumento do tamanho mitocondrial em regiões do hipocampo, possivelmente pela formação em excesso de EROs. Para minimizar esses danos são necessários mecanismos que regulem a imunidade inata e adaptativa. Neste sentido, a via colinérgica anti-inflamatória, a qual controla a inflamação através do nervo vago e da ligação da ACh ao α7nAChR, pode fornecer importantes *insights* para a redução das alterações neuroinflamatórias. Desde a descoberta da via em questão, diversos estudos têm testado seu potencial terapêutico em diversas patologias e, atualmente seu potencial é reconhecido pela comunidade científica. Considerando a importância da ACh para o controle da inflamação inibidores da enzima AChE têm sido utilizados com intuito de aumentar os níveis de ACh disponível e sua consequente ligação ao α7nAChR. Kanashiro e colaboradores, demonstraram que a ativação farmacológica da via colinérgica anti-inflamatória com neostigmina (inibidor da acetilcolinesterase) diminuiu o recrutamento de neutrófilos e melhorou os sintomas clínicos em modelo experimental de artrite. Além disso, inibidores de AChE também estão sendo utilizados para prevenção de danos cerebrais, o tratamento com galantamina aumentou a sobrevivência neuronal e a atividade antioxidante em animais submetidos a um modelo de isquemia por hipóxia neonatal. Assim, torna-se um importante agente farmacológico a ser investigado com objetivo de reduzir as alterações neuroquímicas e neuroinflamatórias que podem levar a alterações comportamentais.

4 OBJETIVOS

O objetivo dessa dissertação é investigar as alterações neuroinflamatórias e o efeito da modulação farmacológica da via colinérgica anti-inflamatória em um modelo experimental de asma. O objetivo geral bem como os objetivos específicos estão subdivididos em artigos 1, 2 e 3 e serão apresentados na forma de artigos científicos como seguem.

4.1 Artigo Científico 1

4.1.1 Objetivo Geral

Identificar as alterações neuroinflamatórias que podem levar ao comportamento de ansiedade em um modelo experimental de asma.

4.1.2 Objetivos Específicos

- Avaliar a contagem total e diferencial de células no lavado broncoalveolar (LBA);

- Analisar os níveis de EROs (medida da fluorescência da diclorofluoresceína – DCF, os níveis de ERNs (nitritos), lipoperoxidação (medida das substâncias reativas ao ácido tiobarbitúrico – TBARS) e os possíveis danos oxidativos em proteínas (medido pelos níveis de carbonila no encéfalo;

- Avaliar os níveis das defesas antioxidantes (SOD e CAT) no encéfalo;

- Avaliar os níveis das defesas antioxidantes (GPx, GSH e grupamentos tióis (SH)) no encéfalo;

- Investigar parâmetros de metabolismo energético mitocondrial (succinato desidrogenase, complexo II e complexo IV) no encéfalo;

- Investigar a atividade das enzimas Na^+,K^+-ATPase no encéfalo;

- Avaliar os níveis de citocinas (IL-4, IL-5, IL-9, IL-13, eotaxina e IL-1β,) no encéfalo;

- Avaliar o imunoconteúdo do BDNF por Western Blotting e atividade da AChE no encéfalo;

- Investigar o metabolismo de glicose através do microPET marcado com o radiofármaco ^{18}F-fluoro-2-desoxi-D-glicose (^{18}F-FDG);

- Avaliar a expressão gênica por PCR em tempo real dos receptores de glicocorticoides no hipotálamo;

- Investigar o comportamento de ansiedade através do teste do labirinto em cruz elevado.

4.2 Artigo Científico 2

4.2.1 Objetivo Geral

Investigar os efeitos da modulação farmacológica da via colinérgica anti-inflamatória com neostigmina na inflamação das vias aéreas em um modelo experimental de asma.

4.2.2 Objetivos Específicos

- Verificar alguns parâmetros de mecânica ventilatória (resistência, elastância e complacência das vias aéreas);
- Avaliar a contagem total e diferencial de células no LBA e as alterações histopatológicas no tecido pulmonar, através da presença de infiltrado inflamatório e muco;
- Verificar os níveis de citocinas (IL-4, IL-5, IL-13, ILβ, TNF-α, IL-10) no tecido pulmonar;
- Analisar os níveis de EROs (medida da fluorescência da diclorofluoresceína - DCF) e das defesas antioxidantes (SOD, CAT e GPx) no tecido pulmonar;
- Determinar o imunoconteúdo da enzima AChE e do fator nuclear kappa beta (NFκβ) por imunofluorescência no tecido pulmonar;
- Avaliar o imunoconteúdo da enzima AChE e da proteína quinase (AKT) por Western Blotting no tecido pulmonar.

4.3 Artigo Científico 3

4.3.1 Objetivo Geral

Investigar os efeitos do tratamento com neostigmina sobre parâmetros de estresse oxidativos no córtex cerebral de camundongos submetidos a um modelo de asma.

4.3.2 Objetivos específicos

- Investigar a contagem de leucócitos no LBA e a presença de infiltrado inflamatório no tecido pulmonar;
- Verificar os níveis de EROs (medida da fluorescência da diclorofluoresceína - DCF) no córtex cerebral;
- Avaliar os níveis das enzimas antioxidantes (SOD, CAT e GPx) no córtex cerebral;
- Determinar a atividade da enzima Na^+,K^+-ATPase no córtex cerebral;
- Mensurar a atividade da enzima AChE no córtex cerebral.

5 MATERIAIS E MÉTODOS

5.1 Considerações éticas

Os estudos que compõem esta dissertação foram submetidos à Comissão Científica do Instituto de Pesquisas Biomédicas e ao Comitê de Ética para Uso de Animais (CEUA), no qual recebeu aprovação com número de registro 7934. Todos os procedimentos adotados, durante as técnicas envolvendo os animais, seguiram aqueles preconizados pela Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL), utilizando o menor número de animais e com protocolos de anestesia e eutanásia adequados. [61]

5.1.2 Local de desenvolvimento do projeto

Os experimentos foram realizados no Laboratório de Respirologia Pediátrica do Centro Infant (Escola de Medicina) no Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

5.1.3 Animais

O trabalho foi realizado com camundongos fêmeas BALB/cJ, entre 6-8 semanas, provenientes do Centro de Modelos Biológicos Experimentais (CeMBE) da PUCRS. Os animais foram mantidos em ambientes de temperatura controlada (24±2ºC), ciclo claro/escuro de 12 horas, com livre acesso a água e alimento.

5.1.4 Desenho experimental utilizado nos artigos científicos

Para indução de um modelo experimental de asma, camundongos BALB/cJ foram sensibilizados com injeção subcutânea de ovalbumina (OVA) nos dias 0 e 7 do protocolo, seguido por três desafios intranasais com OVA nos dias 14, 15 e 16. O grupo controle recebeu apenas DPBS. [62] Nos artigos 2 e 3 para avaliar os efeitos da via colinérgica anti-inflamatória, os animais foram tratados com neostigmina (Normastig) (80 µg/Kg) [63] por via intraperitoneal 30 minutos depois de cada um dos 3 desafios com OVA. No 17º dia de protocolo os animais foram eutanasiados para obtenção das amostras e desenvolvimento das técnicas descritas, conforme o objetivo para cada um dos artigos científicos que

compõem esta Dissertação. Uma ilustração do desenho experimental dos estudos é apresentada na figura abaixo.

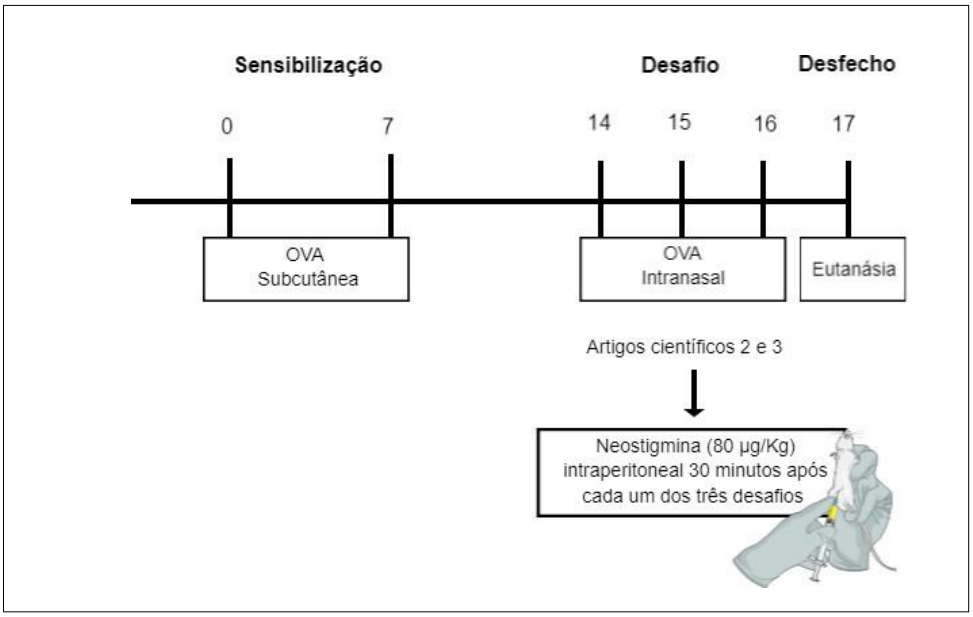

Figura 3: Visão geral do protocolo experimental utilizado nos artigos. Nos artigos 1, 2 e 3 os animais foram submetidos ao protocolo de indução de asma com OVA. Os animais do grupo controle receberam apenas DPBS. Nos artigos 2 e 3 para avaliar os efeitos da via colinérgica anti-inflamatória os animais receberam 80 µg/Kg de neostigmina 30 minutos após cada um dos três desafios com OVA.

5.2 METODOLOGIA REFERENTE AO ARTIGO CIENTÍFICO 1

5.2.1 Coleta do lavado broncoalveolar (LBA)

Os animais foram previamente anestesiados com uma mistura de cetamina 0,4mg/g e xilazina 0,2 mg/g e em seguida eutanasiados através de exanguinação por punção cardíaca. O LBA foi coletado através da injeção e aspiração de 1 mL de PBS contendo 2% de soro fetal bovino (SFB), por 2 vezes no pulmão, através da cânula intratraqueal.

5.2.2 Contagem total e diferencial de células

A amostra do LBA foi centrifugada (420 g, por 4 minutos). O sobrenadante do LBA foi coletado e armazenado e o pellet de células foi ressuspendido com 350 µL de PBS contendo 2% de SFB para a realização da contagem total e diferencial de células. A contagem total de células (CTC) e o

cálculo da viabilidade celular nesta suspensão, foram realizadas através do método de exclusão com azul de tripan em câmara de Neubauer (Boeco, Germany). Lâminas para a citologia diferencial das células inflamatórias foram preparadas com 80 μL da suspensão, em citocentrífuga (FANEM, São Paulo, Mod. 218). As lâminas foram fixadas em ar ambiente e coradas com corante panótico rápido (Laborclin®). Os tipos celulares observados ao microscópio óptico foram expressos em contagem absoluta, após a contagem de 400 células.

5.2.3 Produção de espécies reativas de oxigênio (EROs)

A produção de EROs foi determinada através da técnica descrita por Lebel e colaboradores. O método se baseia na oxidação do 2'7' diclorofluoresceína (H2DCF) que ao final da reação forma um composto fluorescente que foi medido em espectrofotômetro em 488 nm de excitação e 525 nm de emissão. Os resultados foram expressos por DCF nmol/mg de proteína.[64]

5.2.4 Determinação dos níveis de nitritos

A dosagem foi baseada no método descrito por Griess, onde o nitrito reage com a sulfanilamida e N-1-naftiletilenodiamina (NEED), formando um composto corado que é mensurado em espectrofotômetro em 540nm.[65]

5.2.5 Determinação das substâncias reativas ao ácido tiobarbitúrico (TBARS)

O TBARS, índice de lipoperoxidação, foi determinado de acordo com o método descrito por Ohkawa e colaboradores. A técnica consiste na adição de 20 µL de uma solução de dodecil sulfato de sódio 8,1%, 600 µL de ácido acético 20%, pH 3,5 e 600 µL de ácido tiobarbitúrico 0,8% em 200 µL de homogeneizado de tecido. Em seguida, o tubo foi aquecido em banho de água fervente por 60 min. Depois de resfriada, a mistura foi centrifugada a 1000g por 10 minutos e a camada orgânica foi retirada para determinação em espectrofotômetro em 535nm.[66]

5.2.6 Níveis de carbonilas

Os níveis de carbonilas foram mensurados baseado na reação de proteínas carboniladas com dinitrofenilhidrazina, formando dinitrofenilhidrazona,

medido espectrofotometricamente a 370 nm. Os resultados foram representados como níveis de proteína carboniladas (nmol/mg proteína).[67]

5.2.7 Níveis de sulfidrilas (SH)

Este parâmetro foi medido de acordo com o método de Aksenov e Markesbery. A oxidação de tióis livres na amostra leva à formação de ligações dissulfureto. O reagente de cor 5,5-ditiobis (ácido 2-nitrobenzóico) (DTNB) não é reduzido pelos tióis oxidados, gerando um derivado amarelo (TNB), lido espectrofotometricamente a 412 nm. Os resultados foram representados como nmol TNB/mg de proteína.[68]

5.2.8 Atividade da superóxido dismutase (SOD)

A atividade da enzima antioxidante SOD foi determinada de acordo com Marklund. Esse método é baseado na capacidade de auto-oxidação do pirogalol, um processo altamente dependente de $O_2^{\bullet -}$, que é substrato para a SOD. A inibição da auto-oxidação desse composto ocorre na presença da SOD, cuja atividade pode ser indiretamente medida em espectrofotômetro a 412 nm. A atividade da SOD foi representada em unidades/mg de proteína.[69]

5.2.9 Atividade da catalase (CAT)

A atividade da CAT foi baseada na medida da diminuição do consumo de H_2O_2 em 240 nm, em uma reação contendo H_2O_2 20 mM com 0,1 de Triton X-100, fosfato de potássio 10 mM, pH 7,4. Uma unidade de CAT é definida em 1 μM de H_2O_2 consumido por minuto. A atividade da CAT é representada em unidades/mg de proteína.[70]

5.2.10 Atividade da glutationa peroxidase (GPx)

A Atividade da GPx foi determinada de acordo com Wendel e colaboradores, usando tert-butil-hidroperóxido como substrato. O decaimento do NADPH foi monitorado a 340 nm em espectrofotômetro com controle de temperatura. A atividade da GPx será representada em unidades/mg de proteína.[71]

5.2.11 Atividade da glutationa reduzida (GSH)

GSH foi mensurado de acordo com Browne e Armstrong. Este método baseia-se na reação do GSH com o fluoróforo o-ftalaldeído (OPT). Todas as amostras foram incubadas em tampão de fosfato de sódio contendo pH 8,0, OPT 1 mg/mL (preparado em metanol). Subsequentemente, mediu-se a fluorescência a λem = 420 nm e λex = 350 nm num Leitor de Microplacas SpectraMax M5 / M5 (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, EUA). Os resultados são representados como nmol/mg de proteína.[72]

5.2.12 Atividade da succinato desidrogenase e complexo II

As atividades da succinato desidrogenase (SDH) e complexo II (succinato-2,6-dicloroindofenol-oxidoredutase) foram mensuradas pelo método de Fischer e colaboradores. Este método é baseado na diminuição da absorbância devido à redução de 2,6-dicloroindofenol (DCIP) a 600 nm com 700 nm como comprimento de onda de referência ($\mathring{a}$ = 19.1 mM^{-1} cm^{-1}) na presença de metanosulfato de fenazina (PMS). A mistura reacional consistindo em 40 mM de fosfato de potássio, pH 7,4, 16 mM succinato e 8 µM DCIP foi pré-incubada com 40 a 80 µg de proteína homogeneizada a 30°C por 20 minutos. Posteriormente, para a atividade do complexo II, azida sódica 4 mM e rotenona 7 µM foram adicionados. A reação foi iniciada pela adição de 40 µM de DCIP em um volume final de 1,2 ml e foi monitorada por 5 min.[73]

5.2.13 Atividade do complexo IV (citocromo c oxidase)

A atividade da citocromo c oxidase foi medida de acordo com Rustin e colegas. Foi mensurado seguindo o decréscimo na absorbância devido a oxidação do citocromo c previamente reduzido a 550 nm com 580 nm. A atividade foi medida a 25 ° C durante 10 minutos. Os resultados foram expressos em nmol/min mg de proteína.[74]

5.2.14 Atividade da enzima acetilcolinesterase (AChE)

Foi determinada de acordo com Ellman e colegas. Ao homogeneizado do tecido (50 µL) foi adicionado o tampão fosfato 30 mM, pH 7,5 contendo 1 Mm de DTNB a 25ºC, ficando em incubação por 3 minutos. A velocidade de hidrólise

da ACh foi monitorada através da formação de DTNB, que foi medida durante 3 min (intervalos de 30 segundos) a 412 nm.[75]

5.2.15 Atividade da enzima Na^+,K^+-ATPase

O tecido foi homogeneizado na proporção de 1:10 (massa/volume) de solução de sacarose a 0,32 mM contendo HEPES 5 mM e EDTA 1 mM, pH 7,4 e centrifugado a 3000g por 10 minutos a 4 °C. O sobrenadante foi imediatamente separado para a medição. A mistura reacional para o ensaio continha 5,0 mM de MgCl2, 80,0 mM de NaCl, 20,0 mM de KCl e 40,0 mM de Tris-HCl, pH 7,4, num volume final de 170 mL. A reação foi iniciada pela adição de ATP. Os controles foram realizados sob as mesmas condições com a adição de 1,0 mM de ouabaína. A atividade foi calculada pela diferença entre os dois ensaios. [42] O fosfato inorgânico liberado (Pi) foi medido pelo método de Chan e colaboradores.[76] A atividade da enzima foi expressa em nmol Pi liberado por min por mg de proteína.

5.2.16 Níveis de citocinas

Para mensurar os níveis de citocinas, o tecido coletado no 17º dia de protocolo foi homogeneizado com PBS. Os níveis de IL-1β, lL-4, IL-5, IL-9, IL-13 e eotaxina foram mensuradas por beads magnéticas utilizando um kit de ensaio Multiplex (MILLIPLEX®, Germany). Os níveis das citocinas foram mensurados no equipamento MagPix (MILLIPLEX®, Germany) e os resultados foram analisados através do xPONENT® 4.2 (MILLIPLEX®, Germany).

5.2.17 Western Blotting

Para a análise do imunoconteúdo, o tecido foi macerado em uma solução de CHAPS (10mM Tris-HCl, pH 7.5, 1mM $MgCl_2$, 1mM EDTA pH 8.0, 0.1 mM PMSF, 5 mM B- mercaptoetanol, 0.5 % CHAPS e 10% glicerol) e centrifugado a 17.900 g, 4 °C durante 60 minutos para remoção do material insolúvel. Quantidades iguais de proteínas foram submetidas à eletroforese SDS-PAGE. Em seguida, as proteínas foram transferidas para membranas de nitrocelulose, e as mesmas foram corada com solução de Ponceau (Sigma-Aldrich, St. Louis, USA) para a confirmação da transferência. As membranas foram lavadas com Tris-HCl, pH 7.4, NaCl and 0.05% Tween (TTBS), seguido de uma incubação em

solução de bloqueio com TTBS contendo 5% de albumina de soro bovino (BSA). Após a incubação, as membranas foram lavadas novamente e incubadas overnight a 4°C com a solução de bloqueio contendo os respectivos anticorpos: anti-BDNF (1:200) e anti-β-actin (1:2000) (Cell signaling, Danvers, EUA). Após a incubação overnight, as membranas foram lavadas e incubadas por 2 horas com o anticorpo secundário (conjugado com HRP) anti-IgE (1:2000) (Santa Cruz Biotechnology, Santa Cruz, USA). A quimioluminescência de BDNF foi detectada utilizando um sistema de imagem (Fujifilm, LAS-3000) e quantificadas através do programa ImageJ.

5.2.18 ^{18}F-FDG-microPET

O metabolismo de glicose cerebral foi analisado com tomografia por emissão de pósitrons (PET) utilizando o ^{18}F-fluorodesoxiglicose (^{18}F-FDG). Os animais foram escaneados antes e depois da indução do modelo experimental de asma alérgica. Camundongos foram anestesiados individualmente utilizando uma mistura de isoflurano e oxigênio (3%-4% de indução e 2%-3% de manutenção) e 1 mCi de ^{18}F-FDG foi injetado via intraperitoneal. Os animais foram mantidos na gaiola em placa de aquecimento a 36 ºC por 30 minutos para captação do ^{18}F-FDG. Após esse período, os camundongos foram posicionados no microPET Triumph ™ (LabPET-4, TriFoil Imaging, Northridge, CA, EUA) sob anestesia inalatória. Cada imagem reconstruída foi normalizada espacialmente no PMOD v3.5 (PMOD Technologies, Zurique, Suíça). O metabolismo da glicose foi expresso como valores padronizados de captação (SUVs).[39]

5.2.19 Expressão gênica

Foi realizada a expressão gênica dos receptores de glicocorticoides. O RNA celular total foi extraído pelo método de Trizol (ThermoFisher - Scientifc) segundo as instruções do fabricante. O RNA foi ressuspendido em 20 µL de água livre de nuclease (Ambion®) e convertido em ácido desoxirribonucleico complementar (cDNA) (GoScript™ Reverse Transcription System Protocol - Promega), de acordo com o protocolo indicado pelo fabricante. A concentração final de cDNA foi analisada por método fluorimétrico (Qubit® - ThermoFisher – Scientifc) a partir de *kit* comercial (Qubit® dsDNA HS Assay - ThermoFisher – Scientifc). A expressão gênica foi realizada em PCR quantitativo de tempo real

(Step One Plus - Applied Biosystems) utilizando 16 ng de cDNA. As amostras foram preparadas em duplicata e a expressão relativa de mRNA foi calculada pelo método Delta-Delta Ct (ΔΔCt) adotando gliceraldeido-3-fosfato desidrogenase (GAPDH) como gene endógeno de referência. Um controle negativo para cada primer foi utilizado em cada placa para verificar possível contaminação. As medidas dos reagentes para a amplificação foram calculadas baseadas na incorporação do marcador fluorescente SYBR® Green (Applied Biosystems) na dupla fita de cDNA para cada reação de amplificação. O conjunto de *primers* específicos foram: GAPDH (direto 5'GGGGAGCCAAAAGGGTCATC 3'; reverso 5'GACGCCTGCTTCACCACCTTCTTG3') e GR (direto 5' GGAATAGGTGCCAAGGGTCT3'; reverso 5'GAGCACACCAGGCAGAGTTT3').

5.2.20 Teste do Labirinto em Cruz-Elevado (LCE)

Para avaliação comportamental de ansiedade foi utilizado o teste de LCM, o qual consiste em um labirinto com dois braços abertos (30 cm x 5 cm) e dois braços fechados (30 cm x 5 cm x 15 cm) conectados através de uma plataforma central (5 cm × 5 cm). Cada animal foi colocado na plataforma central de frente para o braço fechado por 10 minutos para a livre exploração. O comportamento ansioso foi investigado pela preferência do animal pelos braços fechados, utilizando o software ANY-maze.

5.2.21 Quantificação de proteínas

A concentração de proteínas totais foi determinada de acordo com método de Bradford, utilizando BSA como padrão.[77]

5.3 METODOLOGIA REFERENTE AO ARTIGO CIENTÍFICO 2

5.3.1 Mecânica ventilatória

Os animais foram anestesiados com uma mistura de cetamina 0,4mg/g e xilazina 0,2 mg/g e em seguida traqueostomizados. O animal foi mantido em um ventilador (FlexiVent, SCIREQ), por 5 minutos antes do início do teste, após a administração de pancurônio intraperitoneal (1mg/Kg). Foram realizadas seis medidas de técnica de oscilação forçada, durante pausa do respirador (6 segundos). Os dados foram analisados em software específico (flexiWare), onde

resistência das vias aéreas e propriedades elásticas (viscosidade e elasticidade) do pulmão foram mensuradas através da impedância pulmonar (R_{aw}, G, H).[78]

5.3.2 Coleta do lavado broncoalveolar (LBA)

O protocolo de coleta do LBA é apresentando no item 5.2.2.

5.3.3 Contagem total e diferencial de células

A contagem total e diferencial de células foi realizada conforme descrito no item 5.2.3.

5.3.4 Análise histopatológica do tecido pulmonar

A presença de infiltrado inflamatório foi avaliada conforme descrito no item 5.2.4.

5.3.5 Níveis de citocinas

Os níveis das citocinas foram mensurados conforme descrito no item 5.2.16. Para os níveis de IL-1β, TNF-α e IL-13 utilizou-se um kit de ensaio Multiplex (MILLIPLEX®, Germany) e para os níveis de IL-4, IL-5 e IL-10 foi utilizado um kit de ensaio Multiplex (Thermo Fisher Scientific, Waltham, USA).

5.3.6 Produção de espécies reativas de oxigênio (EROs)

A produção de EROs foi mensurada de acordo com o item 5.2.3.

5.3.7 Atividade da superóxido dismutase (SOD)

A atividade da SOD foi realizada seguindo o protocolo do item 5.2.8.

5.3.8 Atividade da catalase (CAT)

Realizado conforme item 5.2.9.

5.3.9 Atividade da glutationa peroxidase (GPx)

Foi mensurada de acordo com item 5.2.10

5.3.10 Imunofluorescência

Após desparafinização, cortes histológicos do tecido pulmonar foram tratados com 10 mM de citrato de sódio ($Na_3C_6H_5O_7$) por 10 minutos e em 0.3% de peróxido de hidrogênio (H_2O_2) em metanol (CH_3OH) por 20 minutos. Após esse processo, o tecido foi incubado em solução de bloqueio com PBS contendo 10% de BSA durante 30 minutos. Em seguida, os tecidos histológicos foram incubados com os anticorpos: anti-AChE e anti-NFκB p65 (1:500) (Thermo Fisher Scientific, Waltham, USA) por 40 minutos, seguido da incubação com o anticorpo secundário anti-Rabbit FITC (1:500) durante 40 minutos. O núcleo celular foi corado utilizando Hoechst (1:2000). As imagens foram obtidas em microscópio confocal Zeiss LSM 5.

5.3.11 Western Blotting

O imunoconteúdo de AChE e AKT foi mensurado seguindo o protocolo descrito no item 5.2.17, utilizando anticorpos específicos anit-AChE (1:200) (Thermo Fisher Scientific, Waltham, USA) e anti-AKT (1:500) (Cell signaling, Danvers, EUA).

5.3.12 Quantificação de proteínas

A quantificação foi realizada conforme item 5.2.21.

5.4 METODOLOGIA REFERENTE AO ARTIGO CIENTÍFICO 3

5.4.1 Coleta do lavado broncoalveolar (LBA)

A coleta do LBA foi realizada seguindo o mesmo protocolo descrito no item 5.2.1.

5.4.2 Contagem total e diferencial de células

Foi realizada de acordo com o item 5.2.2.

5.4.3 Análise histopatológica do tecido pulmonar

A presente análise já foi descrita no item 5.3.4

5.4.4 Produção de espécies reativas de oxigênio (EROs)

A formação de EROs foi mensurada conforme o item 5.2.3.

5.4.5 Atividade da superóxido dismutase (SOD)

O protocolo utilizado está descrito no item 5.2.8.

5.4.6 Atividade da catalase (CAT)

Para mensurar a atividade da CAT foi seguido o protocolo do item 5.2.9.

5.4.7 Atividade de glutationa peroxidase (GPx)

Foi avaliada conforme o item 5.2.10

5.4.8 Atividade da enzima acetilcolinesterase (AChE)

Foi mensurada de acordo com o item 5.3.14.

5.4.9 Atividade da enzima Na^+,K^+-ATPase

O protocolo para avaliação da atividade desta enzima está apresentado no item 5.3.15.

5.5 Análise Estatística

Os dados foram expressos através de média±desvio padrão (SD). Os resultados foram analisados utilizando o software SPSS (Chicago, IL, EUA). No artigo 1 foi utilizado teste *t* de Student para a comparação entre os dois grupos Nos artigos 2 e 3 para a avaliação das diferenças entre os grupos experimentais foi utilizado ANOVA de uma via seguida do pós-teste de *Tukey*. As diferenças foram consideradas significativas quando $*P<0,05$.

6 CONCLUSÕES

Os resultados apresentados nesta dissertação, de acordo com os artigos científicos, permitem concluir que:

6.1.1 Artigo científico 1

- A inflamação nas vias aéreas em um modelo experimental de asma aumenta a contagem total de células e o número absoluto de eosinófilos, macrófagos, neutrófilos no LBA;

- A inflamação nas vias aéreas promove aumento da produção de EROs no encéfalo;

- O processo inflamatório nas vias aéreas em um modelo experimental de asma induz danos proteicos avaliado pelos níveis de carbonilas no encéfalo;

- A inflamação nas vias aéreas altera os níveis das enzimas antioxidantes (CAT, GPx e GSH) no encéfalo;

- O processo inflamatório nas vias aéreas promove disfunção no metabolismo energético mitocondrial através da alteração na atividade da SDH e do complexo IV no encéfalo;

- A inflamação nas vias aéreas aumenta a atividade da enzima AChE;

- O processo inflamatório nas vias em um modelo experimental de asma diminui a atividade da enzima Na^+,K^+-ATPase;

- A inflamação nas vias aéreas induz liberação de citocinas pró-inflamatórias (IL-1β, IL-9, IL-13 e eotaxina) no encéfalo;

- O processo inflamatório nas vias aéreas aumenta o metabolismo de glicose no hipotálamo, hipocampo, amígdala e estriado, demonstrado pelo microPET utilizando o ^{18}F-FDG;

- A inflamação nas vias aéreas induz hiperatividade do eixo HPA através da deficiência de *feedback* negativo, evidenciado pela diminuição dos receptores de glicocorticoide no hipotálamo;

- A inflamação nas vias aéreas aumenta o imunoconteúdo de BDNF no encéfalo;

- Asma induz comportamento de ansiedade avaliado pelo teste de LCE nos animais.

6.1.2 Artigo científico 2

- A modulação da via colinérgica com neostigmina diminui a contagem total de células, o número absoluto de macrófagos, linfócitos, neutrófilos e eosinófilos, bem como a atividade da enzima EPO no LBA;

- O tratamento com neostigmina diminui o infiltrado inflamatório e a hiperplasia de células caliciformes no tecido pulmonar;

- A modulação da via colinérgica com neostigmina diminui os níveis de IL-4, IL-5, IL-13, IL-1β e TNF-α no tecido pulmonar;

- O tratamento com neostigmina reduz os níveis de EROs e aumenta os níveis de CAT no tecido pulmonar;

- Neostigmina aumenta o imunoconteúdo de AKT e NFκB p65 no tecido pulmonar;

- A modulação da via colinérgica com neostigmina melhora parâmetros (da mecânica ventilatória pulmonar.

6.1.3 Artigo científico 3

- A modulação da via colinérgica com neostigmina diminui a contagem total de células e o número absoluto de leucócitos no LBA;

- O tratamento com neostigmina reduz o infiltrado inflamatório no tecido pulmonar;

- A modulação da via colinérgica com neostigmina foi capaz de diminuir a produção de EROs no córtex cerebral;

- A modulação da via colinérgica com neostigmina diminui os níveis de CAT e aumentou a relação SOD/CAT no córtex cerebral;

- O tratamento com neostigmina não alterou a atividade das enzimas AChE e Na^+,K^+-ATPase no córtex cerebral.

6.2 Conclusão Geral

Os resultados encontrados nesta dissertação demonstram que a inflamação nas vias aéreas em animais submetidos a um modelo experimental de asma promove diversas alterações neuroquímicas, neurometabólicas e neuroinflamatórias que levam ao fenótipo de ansiedade. Interessantemente, investigando essas possíveis alterações no SNC, verificamos alteração na atividade da AChE, a qual é expressa em neurônios colinérgicos e junções neuromusculares e é responsável pela degradação rápida da ACh, descrita como componente essencial da via colinérgica anti-inflamatória. Assim, resolvemos investigar a modulação farmacológica da via colinérgica anti-inflamatória em um modelo experimental de asma. Inicialmente, investigamos o efeito da modulação da via sobre a inflamação das vias aéreas, por se tratar de uma droga que não ultrapassa a BHE e, nossos resultados demonstram que a modulação farmacológica da via colinérgica anti-inflamatória com neostigmina foi capaz de diminuir a liberação de citocinas pró-inflamatórias e atenuar o estresse oxidativo e, consequentemente reduzir o recrutamento eosinofílico e a hipersecreção de muco, levando a melhora de parâmetros da mecânica ventilatória. Por fim, curiosamente, mesmo a neostigmina não tendo efeito direto sobre a atividade da AChE no SNC, visto que não atravessa BHE, demonstramos que o tratamento com neostigmina além de controlar a resposta inflamatória no pulmão também foi capaz de atenuar o estresse oxidativo que contribui em grande parte para a neuroinflamação no córtex cerebral dos animais. Acreditamos, que esses resultados possam ser explicados pela redução da inflamação nas vias aéreas, que diminui o subsequente sinal do nervo vago ao SNC e desta forma, reduz as alterações neuroquímicas e neuroinflamatórias. Por isso, acreditamos que nossos resultados possam contribuir para essa imensa lacuna que existe entre asma e ansiedade e propomos que asma deve ser olhada não apenas como uma inflamação pulmonar localizada.

7 PERSPECTIVAS

Nossos resultados demonstram que a inflamação nas vias aéreas em animais asmáticos induz diversas alterações neuroquímicas, neurometabólicas e neuroinflamatórias que levam ao comportamento de ansiedade. Verificamos que o tratamento com neostigmina foi capaz de diminuir a inflamação nas vias aéreas e reduzir o estresse oxidativo no córtex desses animais. Contudo, considerando os diversos processos envolvidos durante a neuroinflamação se faz necessário mais estudos que investiguem o efeito da modulação farmacológica com neostigmina sobre as demais alterações encontradas nesta dissertação. Desta forma, em um estudo ainda em andamento, em animais submetidos a um modelo de asma, está sendo avaliado:

- Níveis de citocinas (IL-5, IL-13, IL-1β e TNF-α) no córtex cerebral, hipocampo e hipotálamo;
- Marcadores intracelulares por Western Blotting (AChE, AKT, BDNF, JAK, NFκB p65, pSTAT-3) no córtex cerebral, hipocampo e hipotálamo;
- Expressão gênica (receptores de glicocorticoide) no córtex cerebral, hipocampo e hipotálamo;
- Metabolismo de glicose (microPET marcado com ^{18}F-FDG);
- Comportamento de ansiedade através do teste do LCE.

O estudo mencionado está em andamento e será finalizado até dezembro. Assim, não foi apresentado nesta dissertação.

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ANEXO I

CARTA DE APROVAÇÃO CEUA

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 7934 Porto Alegre,21 de junho de 2017

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "Papel da via anti-inflamatória colinérgica no processo neuroinflamatório e neuroquímico em animais tratados com inibidor de acetilcolinesterase e submetidos a modelos experimentais de doenças respiratórias" coordenado por PAULO MARCIO CONDESSA PITREZ.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Duração do Projeto: 21/06/2017 - 21/12/2018

Nº de Animais	Espécie
144	Camundongo
Total de Animais: 144	

Atenciosamente,

Comissão de Ética no Uso de Animais(CEUA)

ANEXO II

ARTIGO CIENTÍFICO 1

Artigo em fase de submissão

Revista: Molecular Neurobiology

Fator de impacto: 5.076

Asthma induces neuroinflammation and anxiety-like behavior in mice

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ABSTRACT

Asthma is inflammatory chronic disease orchestrated by the $CD4^+$ T helper 2 (Th2) cells and the infiltration of leukocytes, especially eosinophils. The brain is highly sensitive to peripherals inflammation that can induce disruption of blood-brain barrier (BBB) and neuroinflammation. Neuroinflammation can induces emotional alterations. In fact, many asthmatic patients present anxiety disorders. However, pathogenic mechanism of asthma development by physiological stress is unknown. In this context, the aim of this study is to investigate the neurochemical alterations that lead to neuroinflammation and anxiety-like behavior in an experimental model of asthma. Female BALB/cJ mice were submitted to asthma model induced by ovalbumin (OVA). Our results showed that airway inflammation in asthma model induces neuroinflammation and oxidative stress in the brain. The oxidative stress formation resulted in proteins damage, mitochondrial dysfunction, and compromise of the Na^+,K^+-ATPase activity. Neuroinflammation increased BDNF immunocontent and probably decreased choline recycling for the ACh formation, which was demonstrated by high AChE activity in asthmatic mice. In addition, we observed that asthma induces an increase in glucose uptake and reduces the mRNA levels of the glucocorticoid receptor in hypothalamus, leading to hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis by a deficiency in feedback inhibition. In conclusion, we demonstrate, for the first time, that inflammation in lung tissue mediates neurochemical and neurometabolic changes within of the brain, which leads to anxiety phenotype in asthma.

Keywords: asthma, neuroinflammation, mitochondrial dysfunction, oxidative stress, microPET, anxiety-like behavior

INTRODUCTION

Asthma is a major and ever-increasing health problem that currently affects 300 million people in the world. Allergic asthma is a prototype of type 2 immunity, orchestrated by an aberrant adaptive Th2 cells immune response to airborne allergens. Th2 lymphocytes classically produce specific cytokines such as interleukins (IL) IL-4, IL-5, and IL-13 which induces B cells to produce of immunoglobulin E (IgE), mucus overproduction and infiltration of leukocytes, especially eosinophils in the airway, and oxidative stress by increased levels of reactive oxygen species (ROS), causing bronchial hyperreactivity (BHR) and creates breathing difficulties [1].

Activated central nervous system (CNS)-antigen-reactive T helper cells recognize their target antigen through CNS vessel-associated antigen-presenting cells (APCs). These Th cell produce others cytokines have a direct impact on the integrity of the BBB as well as in CNS-resident cells, such as astrocytes and microglia [2]. The BBB is a special barrier with endothelial junctions of high integrity, which restrict the entry of ions, macromolecules and harmful substances from the circulation. Despite the stability of this barrier, during chronic inflammation diseases, such as multiple sclerosis, occurs disruption this BBB [3]. Chemokines produced by both invading cells and tissue-resident cells fuel the chronic influx of leucocytes across the BBB. Once pathogenic Th cells and newly recruited leukocytes break through the basement membrane and complete the BBB breakdown, produce reactive oxygen species (ROS), reactive nitrogen species (RNS), and cytokines such as IL‑ 1, which further fuel the inflammation and tissue destruction [2]

Neurons are highly vulnerable to the harmful effects of these reactive species due to its high metabolic rate, the predominance of fatty acids with a tendency to peroxidation, high intracellular concentrations of transition metals capable of catalyzing the formation of ROS, and low levels of antioxidants [4]. ROS generated in mitochondria has been reported extensively in the literature and the electron transport chain (ETC) has been acknowledged for a long time as one of the main intracellular sites of oxygen formation [5]. Oxidative stress indicates an excess of reactive species accompanied by a compromised intrinsic antioxidant defense. BBB breakdown mediated by oxidative stress is a common phenomenon in neurological diseases, including amyotrophic lateral sclerosis, multiple sclerosis, and stroke [6-8].

Cytokines as well as oxidative stress are principal biomarkers of neuroinflammation. During neuroinflammation, typically occurs release of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). This cytokines also play an important role in orchestrating sickness behavior [9,10]. Indeed, epidemiological studies have strongly associated asthma with anxiety and depression symptoms, which directly affect patients quality of life [11-13]. Particularly, increasing evidence suggests that anxiety disorders and asthma frequently co-occur. Stressor events, such anxiety is known to result in increased serum IgE while simultaneously influencing the immune system by shifting responses towards the Th2 type. Thus, asthma and anxiety disorders can combine into persistent escalations of further complications over the course of life. Many studies have relates that anxiety disorders occur in both children and adults, leading to as school failure, academic underachievement and unemployment [14-16]. Many studies have demonstrates association between anxiety and HPA axis change. Interestingly, the existing literature links the HPA axis activation to altered brain-derived neurotrophic factor (BDNF) expression in

the brain [17], suggesting that variants in stress-related genes may potentially modulate BDNF that is involved in plasticity, neuronal survival, formation of new synapses, dendritic branching, and modulation of excitatory and inhibitory neurotransmitter profiles [18].

Previous studies have shown that asthma induces neuroinflammatory changes. Xia and colleagues demonstrated that asthma not only induced lung inflammation, but also results in an increased in the IL-1β and TNF-α levels in the hippocampus and prefrontal cortex of mice [19]. Recently, Caulfield and colleagues showed that persistent lung inflammation coincides with changes in brain gene expression that are associated with emotion and stress regulation [20]. However, many of the mechanisms involved in these CNS changes and anxiety disorder remain unclear. In this context, the aim of this study is to investigate the neurochemical alterations that lead to neuroinflammation and anxiety-like behavior in an experimental model of asthma.

MATERIAL AND METHODS

Mice and experimental groups

BALB/cJ mice were provided by the Center for Experimental Biological Models (CeMBE). The animals were fed with a balanced chow diet with access to water *ad libitum*, housed in cages and maintained on a 12/12-h light/dark cycle. Animals used in this study were female and ranged from 6 to 8 weeks of age.

Induction of experimental asthma model

Female were randomized in two groups: (1) DPBS group: control group and (2) OVA group: animals submitted an experimental model of asthma mice. The animals were scanned using ^{18}F-FDG-microPET before (baseline) induction of airway inflammation. Next, female were sensitized by two subcutaneous injections of 20 µg ovalbumin (OVA) (Grade V, Sigma-Aldrich, St. Louis, USA), diluted (200 µL) in Dulbecco's phosphate-buffered saline (DPBS), on days 0 and 7, followed by three intranasal challenges with 100 µg of OVA, diluted in DPBS (50 µL), on days 14, 15, and 16 of the protocol. The animals of negative control received only DPBS in the sensitization and intranasal challenges. On day 16, twelve hours after the intranasal challenge with OVA the mice were submitted to the anxiety behavioral test. On day 17 of the protocol, to perform the scans of the ^{18}F-FDG MicroPET each animal was individually anesthetized with isoflurane and medical oxygen for quantification of cerebral glucose metabolism. The protocol of the study is illustrated in Figure 1.

Total and differential cells count from bronchoalveolar lavage (BAL)

BAL was performed after the ^{18}F-FDG MicroPET scan, animals were euthanized through cardiac puncture. Trachea was cannulated with a blunt needle, and was injected two consecutive flushes of DPBS in the lung of mice.

Next, BAL was centrifuged at 420g, for 5 minutes, at 4 °C and the pellet was resuspended in 350 µL of PBS. Total cells count (TCC) were determined by the trypan blue exclusion test, with a Neubauer chamber (BOECO, Hamburg, Germany). For differential cytology slides, BAL suspension was centrifuged through a cytospin (FANEM, São Paulo, Brazil), and slides were stained with Hematoxylin and Eosin (H&E) (Panótico Rápido - Laborclin, Brazil). Four hundred cells were counted under light microscopy BMX 43 (Olympus, Tokyo, Japan).

Brain preparation for oxidative stress

To evaluate oxidative stress parameters, after euthanasia the brain was removed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenates were centrifuged at 750 g for 10 minutes, at 4 ºC. The pellet was discarded and the supernatant was immediately separated and used for the analysis described in the study.

2′7′-Dichlorofluorescein fluorescence assay

Reactive oxygen species (ROS) production was based on the oxidation of 2′7′-dichlorofluorescein (H2DCF), according to the method of LeBel and colleagues. The sample was incubated in a medium containing 100 µM of 2′7′-dichlorofluorescein diacetate (H2DCF-DA) solution. The reaction produces the fluorescent compound dichlorofluorescein (DCF), which is measured at λ_{em} = 488 nm and λ_{ex} = 525 nm. [21]. Results were represented as nmol DCF/mg protein

Nitrite assay

The levels of nitrite were measured by the Griess reaction. All the samples was incubated in a medium containing Griess reagent (1:1 mixture of

1% sulfanilamide in 5% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride in water). Nitrite concentration was calculated using sodium nitrite standards and the absorbance was measured at a wavelength of 543 nm [22].

Thiobarbituric acid-reactive substances (TBARS)

TBARS, an index of lipid peroxidation, were measured according to the method of Ohkawa and colleagues. All sample was incubated in a medium (8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, and 0.8% thiobarbituric acid - TBARS) and the mixture was vortexed, and the reaction was carried out in a boiling water bath for 1 hour. The resulting pink-stained TBARS were determined spectrophotometrically at 535 nm [23]. A calibration curve was generated using 1,1,3,3 tetramethoxypropane as a standard, and the results were represented as nanomole of TBARS/milligram of protein

Protein carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content. The protein carbonyl contente was assayed by amethod based on the reaction of protein carbonyls with dinitrophenylhydrazine, forming dinitrophenylhydrazone, measured spectrophotometrically at 370 nm [24]. Results were represented as protein carbonyl content (nmol/mg protein).

Superoxide dismutase (SOD) activity

The SOD activity assay is based on the capacity of pyrogallol to autoxidize. This process highly dependent on superoxide, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence

of SOD, whose activity was then indirectly assayed at 420 nm [25]. The results were represented as SOD units/mg protein.

Catalase (CAT) activity

Activity of the CAT is based on the disappearance of H_2O_2 at 240 nm, in a reaction medium (20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/ml), using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). [26]. One CAT unit is defined as 1 µmol of hydrogen peroxide consumed per minute and the results were presented as CAT units/mg protein.

Glutathione peroxidase (GPx) activity

The GPx activity was measured using tert-butyl-hydroperoxide as substrate. NADPH disappearance was monitored at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH [27]. One GPx units defined as 1 µmol of NADPH consumed per minute. The specific activity is presented as GPx units/mg protein.

Reduced glutathione content (GSH)

This method is based on the reaction of GSH with the fluorophore O-phthalaldehyde (OPT). All sample was incubated in a médium containing sodium phosphate buffer pH 8.0, OPT 1 mg/mL (prepared in methanol). Subsequently, fluorescence was measured at λ em=420 nm and λ ex=350 nm in a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies,

Sunnyvale, CA, USA) [28]. A calibration curve was also performed with a commercial GSH solution, and the results are represented as nmol/mg protein.

Sulfhydryl content

The oxidation of free thiols in the sample leads to the formation of disulfide bonds. The 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) color reagent is not reduced by the thiols oxidized, generating a yellow derivative (TNB), read spectrophotometrically at 412 nm. [29]. The results were represented as nmol TNB/mg protein.

Succinate dehydrogenase and Complex II activities

The activities of succinate/phenazine oxidoreductase (succinate dehydrogenase, SDH) and complex II (succinate-2,6-dichloroindophenol-oxidoreductase) were assayed by the method of Fischer and colleagues. This method is based on the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength (*å* = 19.1 mM^{-1} cm^{-1}) in the presence of phenazine methosulfate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 µM DCIP was preincubated with 40 to 80 µg homogenate protein at 30 °C for 20 min. Subsequently, for complex II activity, 4 mM sodium azide and 7 µM rotenone were added. The reaction was initiated by addition of 40 µM DCIP in a final volume of 1.2 mL and was monitored for 5 minutes [30].

Complex IVactivity

The activity of cytochrome c oxidase was measured according to Rustin and colleagues. Was measured by following the decrease in absorbance due to oxidation of previously reduced cytochrome C at 550 nm with 580 nm. The reaction buffer (10 mM potassium phosphate, pH 7.0, and 0.6 mM *n*-dodecyl-β-D-maltoside, and 2-4 µg homogenate protein, and the reaction was initiated with addition of 0.7 µg) reduced cytochrome c. The activity was measured at 25 °C for 10 min [31]. The results were expressed as nmol/min mg of protein.

Na^+,K^+-ATPase activity

To measure the Na^+,K^+-ATPase activity, the brain was homogenized with 10 volumes of 0.32 mM sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 3000 g RPM for 10 minutes at 4 °C. The pellet was discarded and the supernatant was immediately separated for the measurement. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in a final volume of 170 µL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. The activity was calculated by the difference between the two assays, as previously described [32]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [33]. Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

Cytokine (IL-4, IL-5, IL-9, IL-13, eotaxin, and IL-1β) levels

Brain was homogenized in PBS 1X and IL-4, IL-5, IL-9, IL-13, IL-1β, and eotaxin levels were measured by multiplex assay using a Milliplex MAP mouse kit (MILLIPLEX®, Millipore, Germany) according to the manufacturer's

recommendations. Results were analyzed using the xPONENT® Solutions software (MILLIPLEX®, Millipore, Germany).

Western blot for BDNF protein

Brain was homogenized in CHAPS solution (10mM Tris-HCl, pH 7.5, 1mM $MgCl_2$, 1mM EDTA pH 8.0, 0.1 mM PMSF, 5 mM B- mercaptoethanol, 0.5% CHAPS and 10% glycerol). The extracts were centrifuged at 17.900 g, 4 °C, for 60 minutes to remove insoluble material. Equal amounts of proteins were separated by electrophoresis (SDS-PAGE). After running the gel, the proteins were transferred to nitrocellulose membrane and stained with Ponceau S solution (Sigma-Aldrich, St. Louis, USA) for transfer confirmation. The blot was then washed (Tris-HCl, pH 7.4, NaCl and 0.05% Tween - TTBS), followed by 30 minutes incubation in blocking solution TTBS containing 5% BSA. After incubation, the blot was washed again with TTBS and then incubated overnight at 4 °C in blocking solution containing the antibodies: anti-BDNF (1:200) (Thermo Fisher Scientific, Waltham, USA), and anti-β-actin (1:2000) (Cell signaling, Danvers, EUA). The blot was then washed, incubated for 2 hours with horseradish peroxidase-conjugated (HRP-conjugated) anti-IgG (1:2000) (Santa Cruz Biotechnology, Santa Cruz, USA). The chemiluminescence was detected by a gel documentation system (Fujifilm, LAS-3000) that was scanned and band intensities were quantified through ImageJ software.

Acetylcholinesterase (AChE) activity assay

The AChE activity was determined according to the method of Ellman and colleagues with modifications. Brain was homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 minutes at 1000

g. The supernatants were used for the enzymatic AChE analyses. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 300 µL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) at 25 °C. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2-3 minutes (intervals of 30 s) [34].

^{18}F-FDG microPET scan

The mice were anesthetized individually using a mixture of isoflurane and oxygen (3%-4% induction and 2%-3% maintenance), and 1 mCi of ^{18}F-FDG was administered through intraperitoneal injection. The animals were returned to the home cage for a 30 minutes period of conscious tracer uptake and were placed on a heat plate to maintain the body temperature at approximately 36 °C. After the uptake period, the mice was placed in a head-first prone position and scanned with the Triumph™ microPET (LabPET-4, TriFoil Imaging, Northridge, CA, USA) under inhalatory anesthesia. Each reconstructed microPET image was spatially normalized into a ^{18}F-FDG template in PMOD v3.5 Fusion Toolbox (PMOD Technologies, Zurich, Switzerland). The glucose metabolism was expressed as standardized uptake values (SUVs) [35].

Quantification of mRNA levels by real-time PCR

Total RNA was extracted from the hypothalamus tissue using the Trizol method (Thermo Scientific) according to the manufacturer's protocol. The RNA was reconstituted in 20 µl of nuclease-free water and reverse transcribed using Promega RT kit (GoScript ™ Reverse Transcription System Protocol - Promega). The final concentration of cDNA was measured by the fluorometric method

(Qubit® - ThermoFisher - Scientific) from a commercial kit (Qubit® dsDNA HS Assay - Thermo Scientific). mRNA expression analysis was performed in a PCR (Step One Plus - Applied Biosystems) machine, based on the incorporation of the SYBR® Green fluorescence marker (Applied Biosystems) into the double cDNA ribbon for each amplification reaction. A total of 16 ng of cDNA were prepared in duplicate and a negative control for each primer was used on each plate to check for possible contamination. The relative expression of mRNA was calculated by the ΔΔCt method adopting GAPDH as the reference endogenous gene. The following primers were used for real-time PCR: GAPDH (forward 5' GGGGAGCCAAAAGGGTCATC 3'; reverse 5' GACGCCTGCTTCACCACCTTCTTG 3') and GR (forward 5' GGAATAGGTGCCAAGGGTCT 3'; reverse 5' GAGCACACCAGGCAGAGTTT 3').

Elevated plus-maze (EPM) test

The plus maze apparatus is a pharmacologically validated anxiety measure in rodents [36] and consisted of a black painted Plexiglas, with two open arms (30cm x 5cm) and two closed arms (30cm x 5cm x 15cm) connected via a central platform (5cm × 5cm). Each animal was placed in the central square facing a closed arm for 10 minutes of free exploration. The time spent in the open or closed arms was analyzed using a video-tracking software ANY-maze.

Protein determination

Protein concentration was measured by the method of Lowry et al. using bovine serum albumin as standard [37].

Statistical analyses

Data are presented as mean ± SD. Results were analyzed using Statistical Package for the Social Sciences, version 20.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed with Student's *t*-test. Differences were considered significant at $*p < 0.05$.

RESULTS

OVA-induced airway inflammation

First, we confirmed asthma model induction in mice by BAL cellularity. Our results showed that mice submitted to asthma model with OVA had a significant increase in TCC ($P < 0.001$) when compared to the control group (Fig. 2a). Moreover, the OVA induced a significant decrease in macrophages ($P < 0.001$), and increase in neutrophils ($P < 0.01$), and especially of the eosinophils ($P < 0.001$) when compared to the control group (Fig. 2b-f).

Oxidative stress parameters in brain of asthmatic mice

We investigated whether the airway inflammation could induce change in oxidative stress parameters. Initially, we evaluated free radicals in brain of mice. The nitrite levels did not alter between the studied groups as illustrated in Fig. 3a. On the other hand, we investigated ROS production, indicated by DCF formed from the oxidation of H2DCF. Animals of the OVA group had an increased in ROS formation ($P < 0.001$) when compared to the control group (Fig. 3b). Then, we also evaluated the effects of airway inflammation on lipid peroxidation, as measured by TBARS levels and protein damage was measured by carbonyl content. Airway inflammation induced with OVA did not alter TBARS levels between the studied groups (Fig. 3c). However, carbonyl levels had an increased in OVA group ($P < 0.01$) when compared to the control group (Fig. 3d).

We also evaluated the enzymatic antioxidant defenses (SOD, CAT and GPx) and non-enzymatic antioxidant (GSH) in brain of mice. As can be seen in Fig. 4a SOD activity did not alter between the studied groups. However, we observed a significant increased in CAT activity in OVA group ($P < 0.05$) when compared to the control group (Fig. 4b). In addition, GPx activity was decreased

($P < 0.01$) in mice brain (Fig. 5a). Furthermore, the significant decreased in non-enzymatic antioxidant GSH ($P < 0.05$) also observed in brain of mice subjected to asthma when compared to control group (Fig. 5b). Furthermore, Fig. 5c shows that OVA group significantly decreased sulfhydryl content compared to the DPBS group.

Asthma model alters energetic mitochondrial metabolism in brain of mice

We also evaluated the activity of SDH, an enzyme of the tricarboxylic acid cycle located in the inner mitochondrial membrane where it can directly transfer electrons into the electron transport chain (ETC). Results showed that asthma significantly decreased SDH activity ($P < 0.05$) in mice brain (Fig. 6a). We analyzed complex II of the ETC, but activitie did not alter between studied groups (Fig. 6b). On the other hand, asthmatic mice significantly decreased the complex IV activity ($P < 0.01$) in the brain of asthmatic mice (Fig. 6c).

Asthma promoted decrease in Na^+,K^+-ATPase activity in the brain of mice

Next, we investigated the effect of asthma on Na^+,K^+-ATPase and AChE activities in brain of mice. Figure 7 shows that mice subjected to an asthma model present a significantly reduced the Na^+,K^+-ATPase activity ($P < 0.001$) in brain.

Asthma induced neuroinflammation in brain of mice

We measured the pro-inflammatory cytokines levels (IL-4, IL-5, IL-9, IL-13, and IL-1β), that contribute to the classic clinical symptoms of asthma. In addition, we also measured the eotaxin levels which directly involved in the eosinophils recruitment. Figure 8a, b shows that IL-4 and IL-5 levels did not alter between the studied groups. In contrast, the IL-9, IL-13 and eotaxin levels were

increased ($P < 0.001$, $P < 0.05$, $P < 0.01$, respectively) in asthma brain when compared to the control group (Fig. 8c-e). Interestingly, the IL-1β levels also significantly increased ($P < 0.01$) in brain of asthmatic mice (Fig. 8f).

Asthma increased BDNF immunocontent and AChE activity in brain of mice

In addition, we evaluated the immunocontent of the BDNF. The BDNF is not just a growth factor, but that BDNF expression and signaling are intricately enmeshed with a number of regulatory pathways including sex steroids, glucocorticoids and inflammation [38]. Figure 9a demonstrates that asthmatic mice significantly increased the BDNF immunocontent ($P < 0.01$) in brain when compared to control group. In addition, was also evaluated AChE in brain of mice. Figure 9b shows that asthma group had an increase in AChE activity when compared to the control group.

Asthma increased glucose brain metabolism by micro-PET

We observed that airways inflammation promoted several changes in the whole brain of animals submitted to asthma model. Thus, we investigate brain glucose metabolism using positron emission tomography (PET) with ^{18}F-Fluorodeoxyglucose (^{18}F-FDG), which measures the glucose uptake in tissues. Figure 10 shows that ^{18}F FDG SUVs significantly increased in hypothalamus ($P < 0.05$), amygdala ($P < 0.05$), hippocampus ($P < 0.05$), and striated ($P < 0.05$) in animals submitted to asthma model compared to the control group.

Asthma reduced glucocorticoid receptors in hypothalamus of mice

Psychological stress is recognized as a key factor in the exacerbation of asthma. This event leads to eosinophilic airway inflammation through activation

of the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system (ANS). This is followed by the secretion of stress hormones into the blood, including glucocorticoids, epinephrine, and norepinephrine, which enhance Th2 and type 17 T-helper (Th17)-type asthma profiles in humans and rodents [39]. In this context, we investigated the levels of glucocorticoids receptors in the hypothalamus of mice. As can be seen in Figure 11a the allergic airway inflammation group had a significantly decreased in glucocorticoid receptor mRNA expression ($P < 0.05$) when compared with the control group.

Inflammation of the airway caused anxiety-like behavior in asthmatic mice

Anxiety behavior has significant impacts on asthma associated dyspnea, hypoxia, and acute exacerbation as well as patients quality of life and health care costs [40]. Anxiety behavior was estimated based on the time spent in the arms. We did not observe significant differences ($P > 0.05$) in the exploration time of the open arms (Fig. 11b). However, we observed that mice submitted to allergic airway inflammation had an increased in the exploration time of the closed arms ($P < 0.05$) when compared to the control group (Fig. 11c).

DISCUSSION

Asthma is a chronic inflammatory airway disease that affects 300 million people worldwide. The clinical symptoms of allergic asthma are wheezing, coughing, and chest tightness and are a consequence of a dysregulated type 2 inflammation in the lung, orchestrated by cytokines such as IL-4, IL-5, IL-9, and IL-13. Anxiety does not permit that the patient to tolerate symptoms of asthma [13] and directly affect patients quality of life. Moreover, anxious patients seeking medical or emergency care due to asthma attack may not have signs of decreased lung function [41]. The present study was designed to clarify the link between asthma and neuroinflammation and anxiety disorders.

First, we confirmed that the experimental model of asthma induction by TCC and differential cells count, especially of eosinophils. Initially, we evaluated the production of free radicals by ROS and RNS that are products of normal cellular metabolism. However, in high concentrations, they can be either harmful to living systems and lead to tissue damage. We demonstrated that the damage caused by production of free radicals in brain of asthma possibly occurs through that production of ROS but not RNS. The ROS production can induce damage to DNA, lipids, and proteins [42]. Lipid peroxidation (as observed by TBARS) did not alter in all groups studied. On the other hand, we demonstrated that protein oxidation (as observed by carbonyl content) had an increase in the brain of animals submitted to asthma model.

The antioxidant defenses are responsible for removing ROS and to maintain homeostasis cellular. Thus, we evaluated the enzymatic antioxidant defenses (SOD, CAT and GPx). SOD enzyme is important to detoxify the excess of O_2 formed, but the activity did not alter between the studied groups. On the

other hand, CAT activity had an increase in asthma group, which may be a compensatory effect in SOD levels. GPx removes H_2O_2 and other peroxides by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH), a thiol-containing tripeptide [43]. In our study, GPX activity decreased this parameter. Based on these data, we investigated the non-enzymatic antioxidant (GSH) levels in the brain of our model. We observed that asthmatic mice decreased GSH levels in the brain. In this context, GSH/GSSG homeostasis is one of the most important antioxidant defenses. This system uses GSH as a substrate in the detoxification of peroxides such as H_2O_2, a reaction that involves GPx [44]. In addition, GPX activity in brain of asthmatic mice was reduced.

In addition, we have shown that asthma group significantly decreased sulfhydryl content. Oxidation of sulfhydryl groups of proteins causes conformational changes, protein unfolding, and degradation [45]. Therefore, it might be expected that protein sulfhydryl are the main targets of ROS-mediated attack, reducing their content. In fact, brain tissues have unique characteristics that make them especially susceptible to damage, mainly due to its high oxygen consumption, high iron, and polyunsaturated fatty acid side-chains contents, and low antioxidant levels [46].

Mitochondria represent antoher source for intracelular oxidant production. In contrast, oxidative stress impairs mitochondrial function by inducing structural changes [47]. Mitochondrial membrane phospholipids are extremely susceptible to ROS-induced lipid peroxidation due to its high content of unsaturated fatty acids. However, oxidative damage is not limited to phospholipids, protein and DNA are also important structural targets, which leads to dysfunctional respiratory chain complex and increased concentration of

reactive species, leading to a vicious circle of reactive species production and enhancing mitochondrial damage [42]. Our results showed a significantly decreased in the activity of SDH (that converts succinate to fumarate in the citric acid cycle) and in the activity complex IV in asthmatic mice. Most evidence suggests that mitochondrial oxidants are formed predominantly at complex I or complex II of the cytochrome chain when electrons initially derived from NADH or FADH2 can react with oxygen to produce superoxide anion [48].

Consistent with our current study, numerous evidences relate neuroinflammatory diseases to a decrease in the energetic metabolism. Maurer and collegues showed a decreased of the complex IV activity in Alzheimer disease [49]. Thus, in allergic asthma the oxidative stress leads to a decrease in the activity of citric acid cycle enzyme (SDH), as well as mitochondrial complex, possibly compromising the synthesis of adenosine triphosphate (ATP).

In this context, more than a third of the ATP consumed is used to pump ions by Na^+,K^+-ATPase. In the brain, this enzime it plays a key role in being responsible for the maintenance of ionic gradients and the propagation of the nerve impulse [50]. In our study, asthmatic mice reduced Na^+,K^+-ATPase activity in brain, compared to the control group. Corroborating with our results, degenerative diseases, such as epilepsy and cerebral ischemia has been associated with the decreased in Na^+,K^+-ATPase activity [51,32].

Our results demonstrated that oxidative stress induces many neurochemical alterations, contributing in part to neuroinflammation in our asthma model. Inflammation is a normal response of the organismo to infection, injury, and trauma. Within the brain, this response can be elicited from the residente cells or it can be induced with infiltration of imune cells from the

periphery [52]. Thus, we investigated the cytokines (IL-4, IL-5, IL-9, IL-13, eotaxin, and IL-1β) levels in brain of asthmatic mice. In our study, we observed that asthmatic mice increased IL-9, IL-13, and eotaxin levels in mice brain. In the lung, IL-9 is also expressed by eosinophils and stimulates the proliferation of activated T cells, enhances the production of IgE by B cells [53]. IL-13 contributes to the bronchial hyperreactivity and for goblet cell metaplasia, which clogs the airway lumen. Eotaxin is produced in the lungs during inflammatory response and is a potent chemoattractant for eosinophils [1]. Therefore, these results suggest that peripheral production of cytokines in the lung cross the BBB and also contributes to neuroinflammation in asthma disease. Furthermore, we also showed significantly increased in the IL-1β, a cytokine associated with mucus production in lung [54] and with sickness behaviour in neuroinflammatory diseases [9]. In fact, we observed anxiety behavior in asthmatic mice by EPM test. These results are in agreement with other studies showing that during sepsis, the peripheral proinflammatory cytokines and ROS increasing permeability of these barriers, contributing in large part to the neuroinflammatory process [55].

Neurotrophin (NT) family is classically considered to consist of four polypeptides of comparable structure and function: nerve growth fator (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4)[56]. Particularly BDNF and their receptors have now been shown to be expressed, albeit to different extents, in different lung compartments: nerves, imune cells, bronchial and alveolar epithelium, smooth muscle, fibroblasts and vascular endothelium [57]. Increased expression of epitelial BDNF has been reported in mouse models of allergic airway hyperresponsiveness [58]. In this context, we investigated the

BDNF immunocontent by Wester blott. We have shown that asthmatic mice increases BDNF immunocontent when compared to the control group.

The relevance of BDNF effects in the airways lies in recente recognition that circulating BDNF levels as well as local receptor expression are both increased in asthma and that there is clinical evidence for increased BDNF levels in both sputum and BAL of patients with chronic inflammatory airway disease [59-61].

Certainly, such increases may be a result of enhanced BDNF secretion by resident airway cells, as well as by immune cells that also happen to expression BDNF and it is receptors [57]. In addition, increased BDNF may then promote airway irritability via the nerves, modulate epithelium-derived bronchodilator response, or increased airway smooth muscle (ASM) [Ca+2]i and force responses of human ASM to agonists such as ACh [62] especially in the presence of inflammatory cytokines [63].

Furthermore, acetylcholine (ACh) is able to regulate the immune system and cytokine production, leading to inflammation control [64]. The AChE is an important enzyme that regulates the ACh levels in the synaptic cleft, hydrolyzing into acetate and choline. The choline is recycled into the cell by Na^+,K^+-ATPase for reuse [65]. In this study, we demonstrate a significant increased in the AChE activity in the brain of asthmatic animals. Thus, during asthma ACh is degraded by high AChE activity and probably the choline recaptation is impaired by decreased in Na^+,K^+-ATPase activity, these changes contribute to the pro-inflammatory environment by decrease ACh formation.

The role of physiological stress and emotional factors in asthma exacerbation has garnered much attention [66]. However, the pathogenic mechanism of asthma development by physiological stress is unknown. In this context, we investigated for the first time in vivo brain imaging the glycolytic metabolism through the ^{18}F-FDGmicroPET technique. Glucose constitutes the major source of energy in the brain, with it is utilization rate correlating with neuronal activity and recently with astrocytes activity [67]. In addition, high glucose has been shown to increase release of pro-inflammatory mediators from various immune cells [68]. Our study is the first experimental PET imaging study of alterations in cerebral glucose metabolism in asthmatic mice. We demonstrated that asthma increased ^{18}F-FDG absorption in the hippocampus, hypothalamus, and amygdala when compared to the control group.

Notably, in individuals with asthma, potential changes in the brain could occur as a consequence of hypoxia or inflammation that resulted from asthma [69]. It was found that 13 of 21 people with asthma had brain magnetic resonance imaging abnormalities [70]. Guo and colleagues have found that mice treated with the allergen OVA from the first weeks of life (sensitization phase) and challenged until mid-early adulthood display a spatial learning and memory deficit [71]. In addition, in agreement with our results, anxiety predisposition may exacerbate inflammatory symptoms, resulting in more severe or persistente asthma, and/or more frequente recollection of these symptoms [72]. Research indicates that anxiety can be brought on from experiencing a chronic health challenge and associated adverse medical events [73]. Additionaly, parental anxiety can influence a child, putting them at increased risk for developing and anxiety

disorder [74]. This bi-directional relationship between asthma and internalizing disorders require further study to elucidate causal directionality and mechanism. Several epidemiological studies have demonstrated an association between psychological stress and asthma development [75,76]. In response to stress, such anxiety the hypothalamic-pituitary-adrenal (HPA) axis hypothalamic paraventricular nucleus (PVN) stimulates production and secretion of two neuropeptides, corticotrophin-releasing hormone (CRH) and arginine vasopressin that reach the anterior lobe of the pituitary and activate adrenocorticotropic hormone (ACTH) secretion [77]. When released into the peripheral circulation, ACTH stimulates the secretion of epinephrine, noraepinephrine, and glucocorticoids by the adrenal cortex. Ke and collegues demonstrated that anxiety disorders are associated with hyperactivity of the HPA axis [78]. This hyperactivity is due, in particular, to the deficiency in feedback inhibition by a decrease of glucocorticoid receptors, leading to hypercortisolemia. Interestingly, our results demonstrated reduced mRNA levels of the glucocorticoid receptor in the hypothalamus of the asthma mice, suggesting a deficiency in feedback inhibition in asthma. In addition, we verified a significant increase in glucose uptake by the ^{18}F-FDGmicroPET technique in hippocampus, hypothalamus, and amygdala, which is associated with hypothalamic-pituitary-adrenal (HPA) axis.

On the other hand, glucocorticoid evokes defensive responses to stress that help to maintain homeostatic balance, including regulatory effects on imune-system activities. By affecting the imune system, glucocorticoid can potentially enhance Th2 immune responses through inhibiting the development of Treg cells essential for respiratory tolerance [79,80].

In this sense, we have previously showed that the airway inflammation in asthma model induces oxidative stress and neuroinflammation in brain of mice. The oxidative stress formation results in proteins damage, mitochondrial dysfunction and leads to the generation of more ROS in a vicious self-destructive cycle with compromise of the Na^+,K^+-ATPase activity. In addition, the neuroinflammation increased BDNF immunocontent and probably decreased choline recycling for the ACh formation, which was demonstrated by high AChE activity in asthmatic mice. Also, we verified that asthma induces an increase in glucose uptake and reduces the mRNA levels of the glucocorticoid receptor in hypothalamus leading to hyperactivity of the HPA axis by a deficiency in feedback inhibition. Finally, these results could explain why many asthmatic patients development of anxiety disorders. However, more studies are needed to clarify which pathways could be acting in our experimental model.

In conclusion, we demonstrate, for the first time, that inflammation in lung tissue mediates neurochemical and neurometabolic changes within of the brain which leads to anxiety phenotype in asthma. Understand the mechanisms of emotional aspects in asthma is essential for the efficient management of disease and so that the patients' well-being and quality of life.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was conducted under ethical standards, following the recommendations of the Brazilian Society of Laboratory Animal Science (SBCAL), using fewer animals and adequate management of pain and suffering, during the study procedures and euthanasia. All protocols and procedures for

handling of the mice were reviewed and approved by the Ethical Committee on the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul, Brazil (CEUA 7934).

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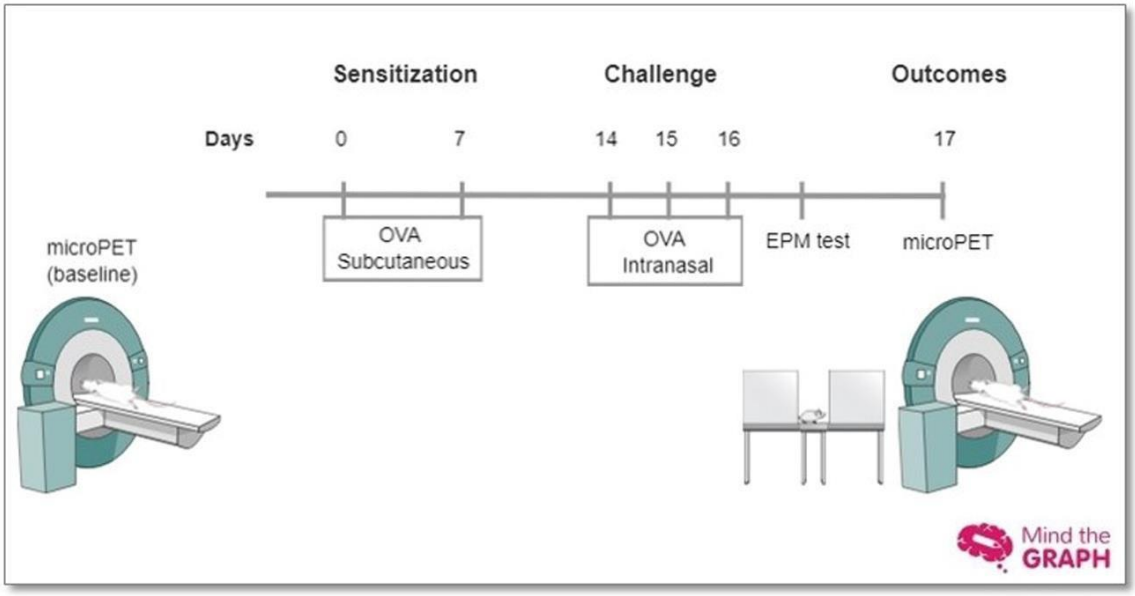

Fig. 1 Protocol used to induce an experimental model of asthma. OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

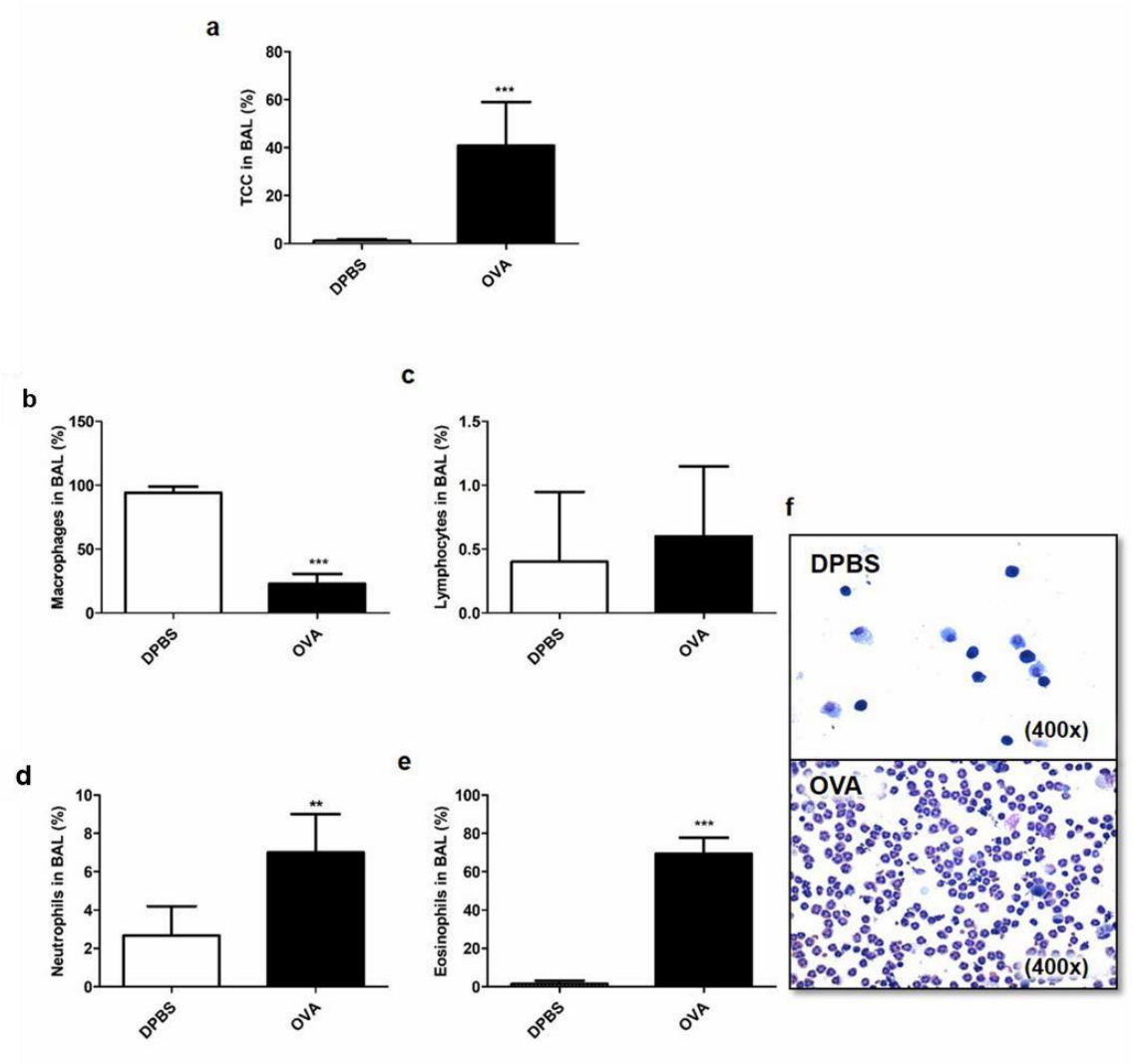

Fig. 2 Effect of airway inflammation with ovalbumin in cellularity from BAL. (a) total cells count, (b) macrophages, (c) lymphocytes, (d) neutrophils, (e) eosinophils and (f) representative image of the differential cells count (H&E, 400x magnification). Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p <$ 0.001, **$p <$ 0.01 (Student's *t* test). BAL: bronchoalveolar lavage; OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

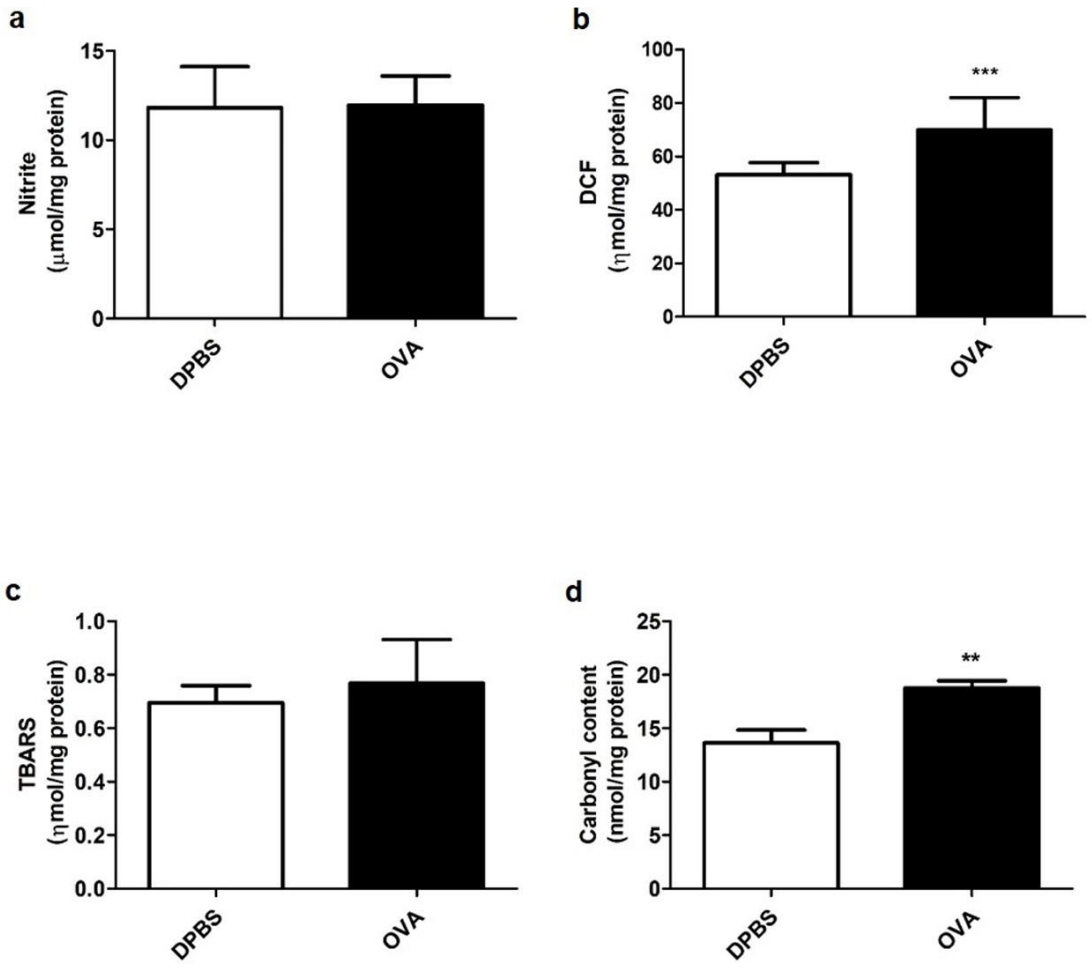

Fig. 3 Effect of airway inflammation on oxidative stress parameters in the brain of asthmatic mice. (a) nitrite levels, (b) reactive species production by DCF, (c) lipid peroxidation by TBARS, and (d) protein damage by carbonyl contente. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p < 0.001$, **$p < 0.01$ (Student's t test). DPBS: Dulbecco's phosphate-buffered saline; OVA: ovalbumin; DCF: dichlorofluorescein; TBARS: thiobarbituric acid-reactive substances

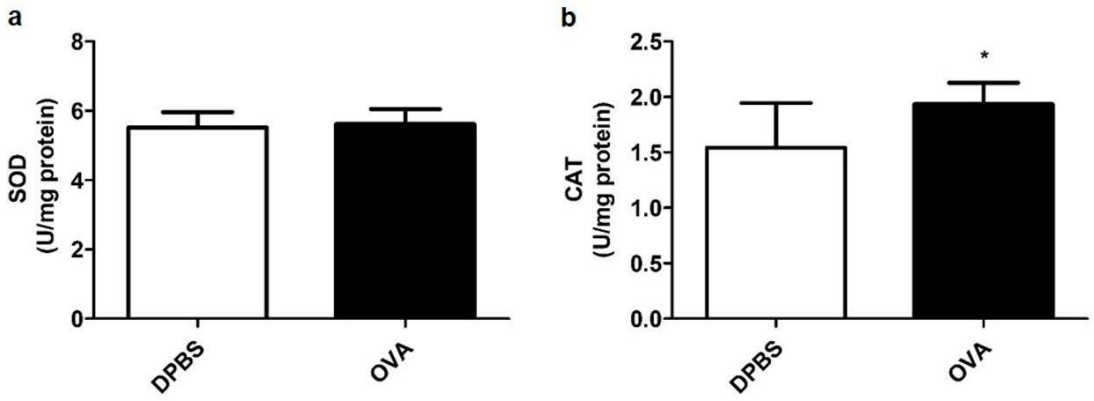

Fig. 4 Airway inflammation effect over SOD and CAT activities parameters in the brain of asthmatic mice. (a) SOD activiti and, (b) CAT activity. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group *$p < 0.05$ (Student's *t* test). DPBS: Dulbecco's phosphate-buffered saline; CAT: catalase; OVA: ovalbumin; SOD: superoxide dismutase

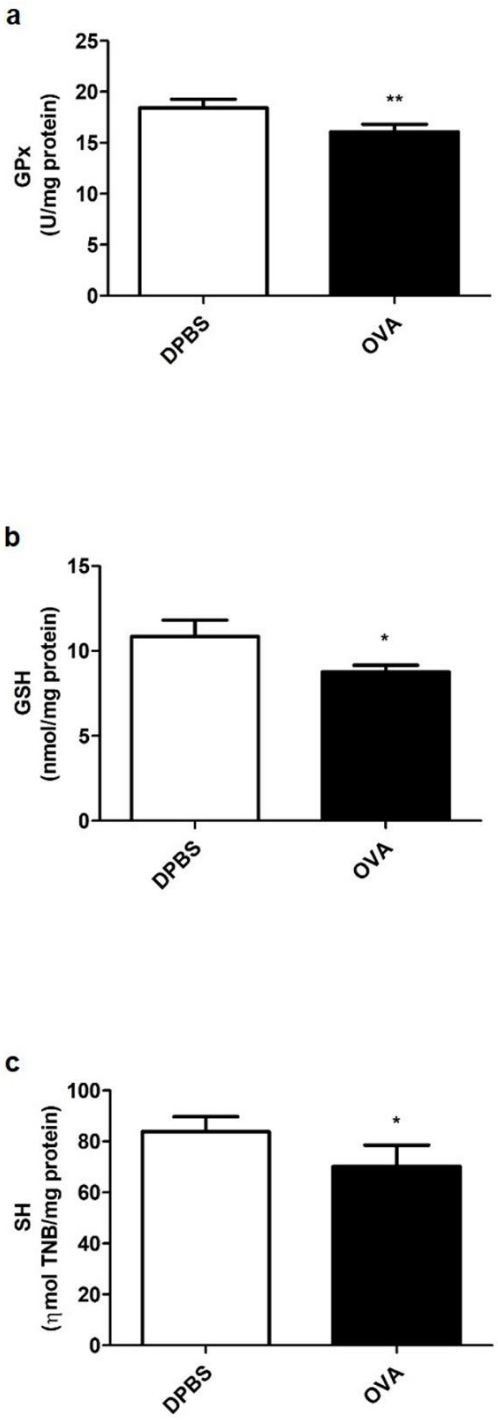

Fig. 5 Effect of airway inflammation over GPx, GSH and SH levels in the brain of asthmatic mice. (a) GPx activity, (b) GSH activity and, (c) SH levels. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group $^{**}p < 0.01$, $^{*}p < 0.05$ (Student's *t* test). DPBS: Dulbecco's phosphate-buffered saline; GPx: glutathione peroxidase; GSH: reduced glutathione; OVA: ovalbumin; SH: sulfhydryl

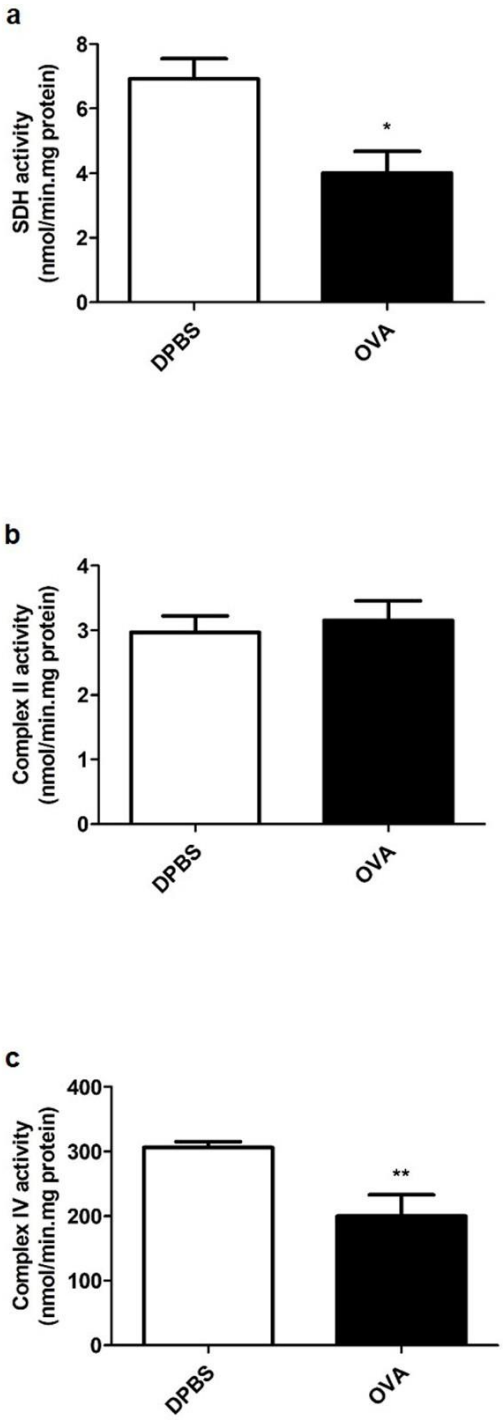

Fig. 6 Airway inflammation effect over energetic mitochondrial metabolism in brain of mice. (a) succinate dehydrogenase, (b) complex II activities and, (d) complex IV activities. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group $^{**}p < 0.01$, $^{*}p < 0.05$ (Student's *t* test). DPBS: Dulbecco's phosphate-buffered saline; OVA: ovalbumin

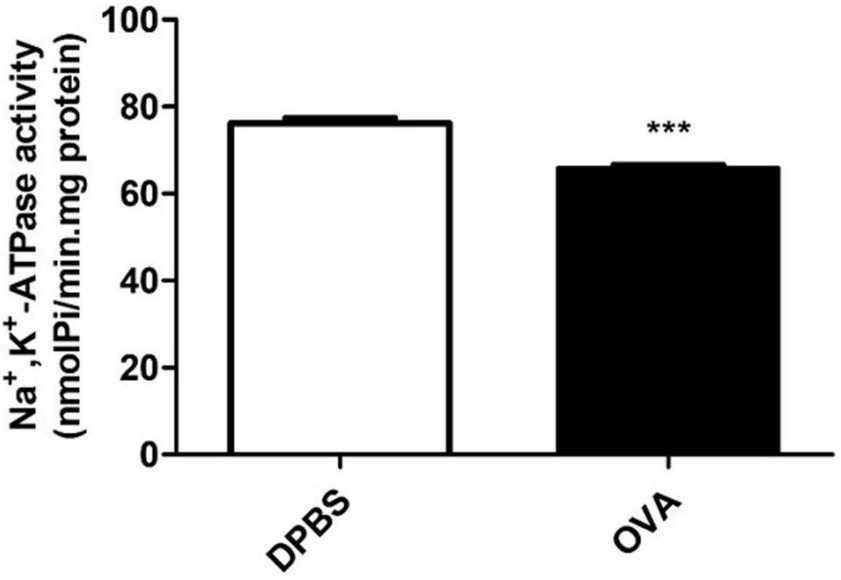

Fig. 7 Airway inflammation decreased activity of Na^+,K^+-ATPase in mice brain. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p <$ 0.001 (Student's *t* test). DPBS: Dulbecco's phosphate-buffered saline; OVA: ovalbumin

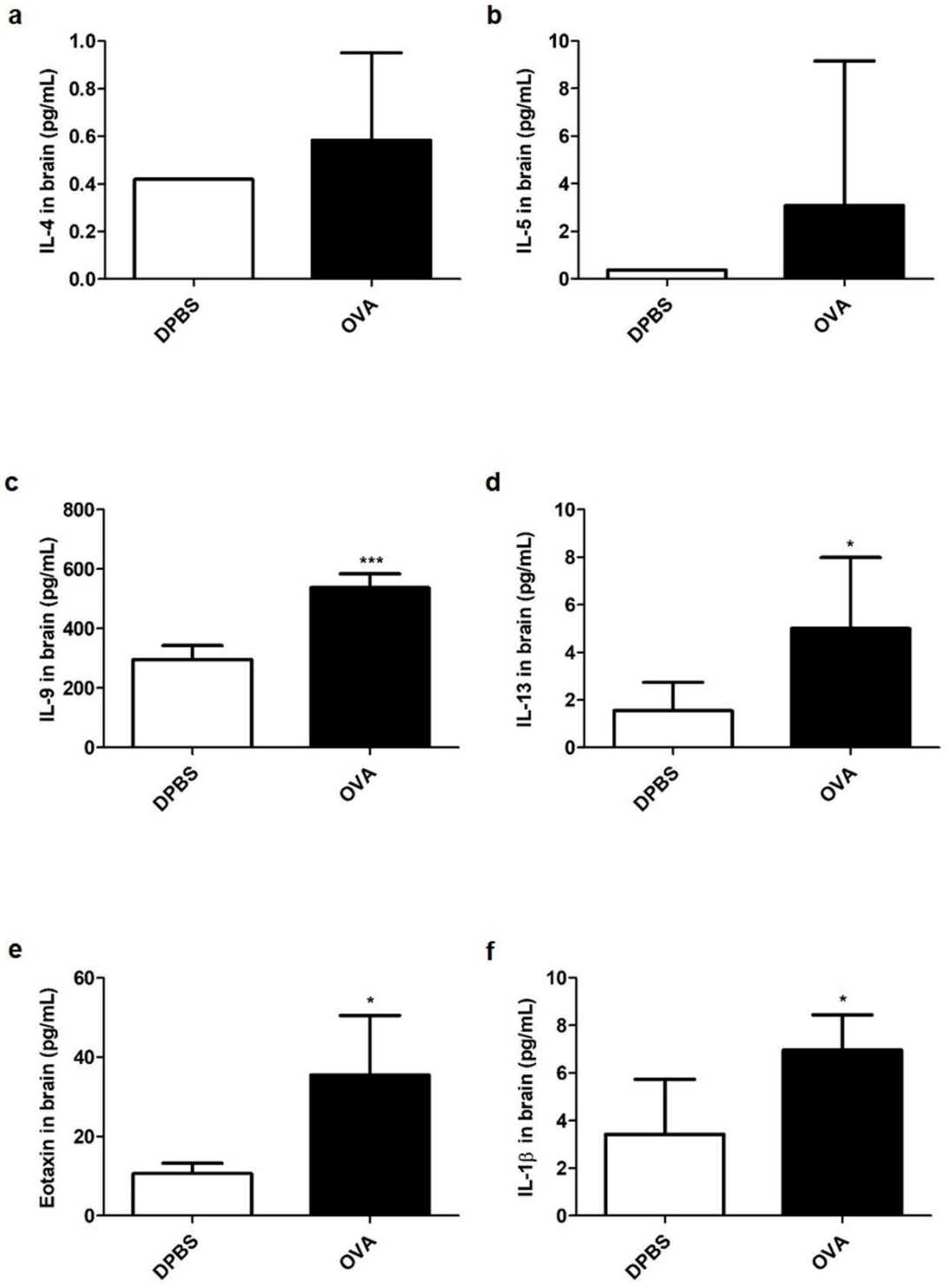

Fig. 8 Inflammation in the airway promoted the release of cytokines pro-inflammatory in mice brain. (a) IL-4, (b) IL-5, (c) IL-9, (d) IL-13, (e) eotaxin levels, and (f) IL-1β. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p < 0.001$, *$p < 0.05$ (Student's *t* test). DPBS: Dulbecco's phosphate-buffered saline; OVA: ovalbumin

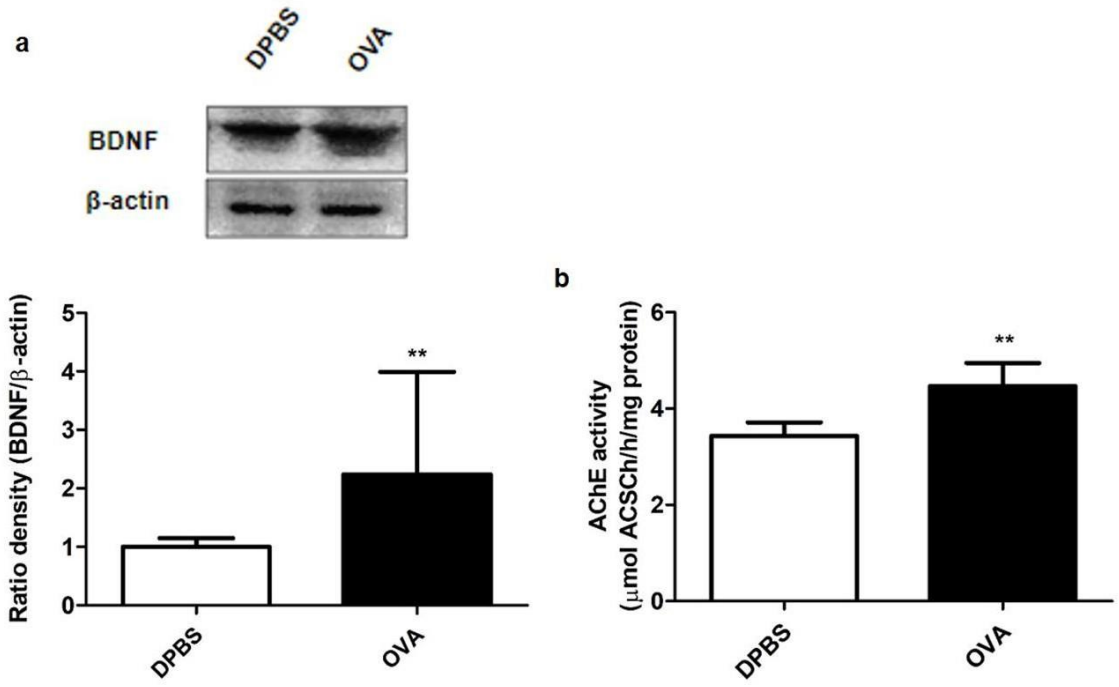

Fig. 9 Airway inflammation increased BDNF immunocontent and increased AChE activity in mice brain. (a) immunocontent of BDNF and, (b) AChE activity. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group $^{**}p < 0.01$ (Student's *t* test). AChE: acetylcholinesterase; DPBS: Dulbecco's phosphate-buffered saline; OVA: ovalbumin

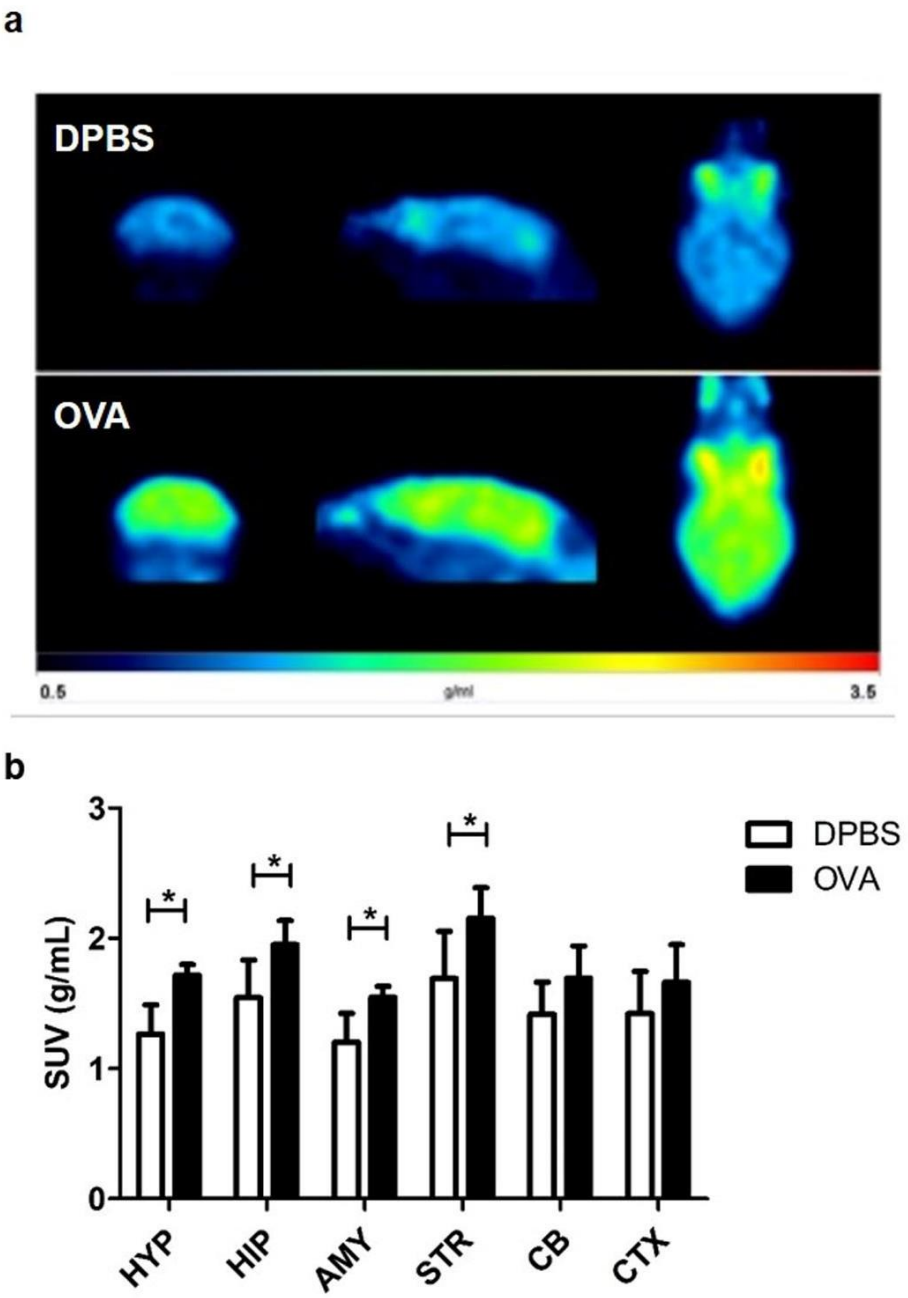

Fig. 10 Inflammation in the airway increased glucose uptake by the ^{18}F-FDGmicroPET. (a) glucose uptake in the brain illustrated in coronal (left), sagittal (middle), and transverse (right) views. This image shows a high uptake of ^{18}F-FDG in the OVA group compared to the control group, (b) the graph shows glucose metabolism expressed by standard uptake values (SUVs). Results are expressed as mean ± SD, for four-six animals in each group. Different from DPBS group *p < 0.05 (Student's t test). AMY: amygdala; CB: cerebellum; CTX: cortex DPBS: Dulbecco's phosphate-buffered saline; HIP: hippocampus; HYP: hypothalamus; OVA: ovalbumin; STR: striated

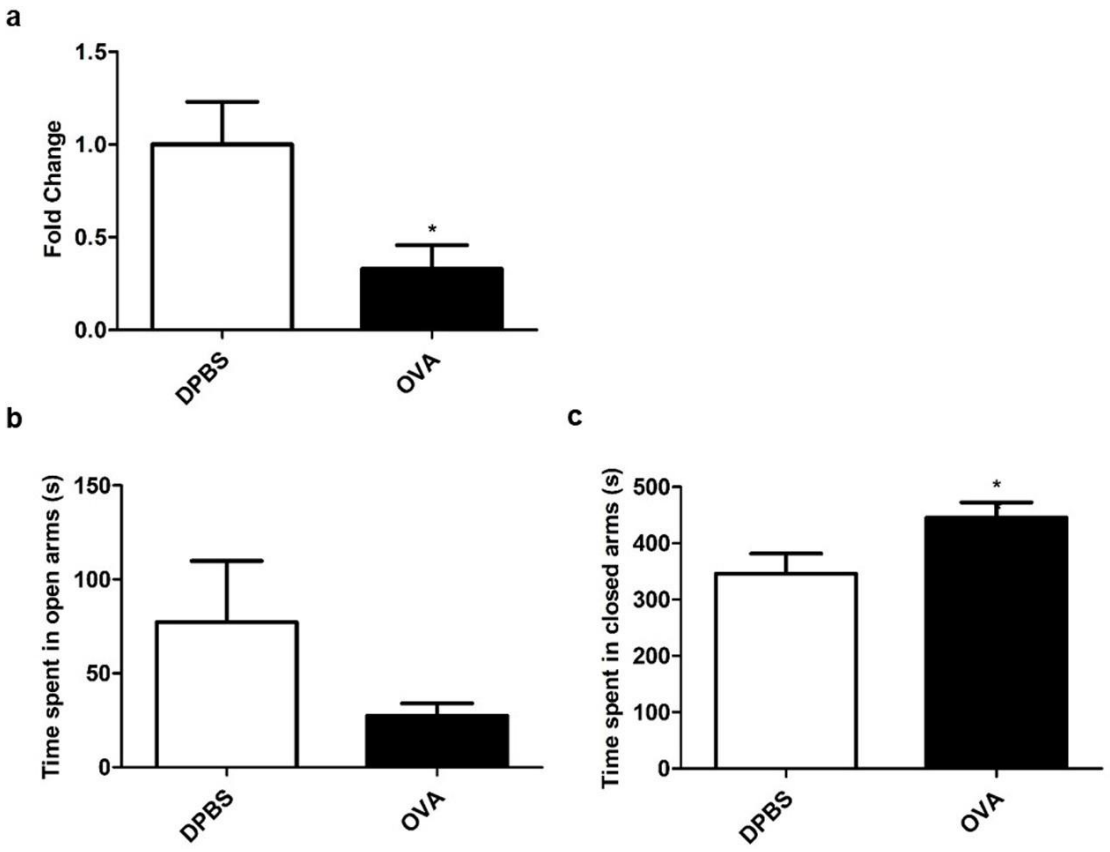

Fig. 11 Effect of airway inflammation over glucocorticoid receptors in hypothalamus and over anxiety-like behavior in asthmatic mice. (a) mRNA levels of glucocorticoid receptor (b) time in open arms (c) time in closed arms. Results are expressed as mean ± SD, for eleven-fourteen animals in each group. Different from DPBS group *$p < 0.05$ (Student's *t* test). DPBS: Dulbecco's phosphate-buffered saline; OVA: ovalbumin

ANEXO III

ARTIGO CIENTÍFICO 2

Artigo submetido

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Submission Confirmation

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Thank you for your submission

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Authors	Antunes, Géssica Silveira, Josiane Kaiber, Daniela Luft, Carolina da Costa, Mariana Marques, Eduardo Ferreira, Fernanda Breda, Ricardo Wyse, Angela Terezinha Stein, Renato Pitrez, Paulo Marcio Cunha, Aline
Date Submitted	05-Oct-2018

Cholinergic anti-inflammatory pathway attenuates oxidative stress and airway inflammation in an experimental model of eosinophilic pulmonary response

Running title: CAP in asthma

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ABSTRACT

Asthma is a highly prevalent chronic disease. During asthma exacerbation, immune cells infiltrate the airways, produce pro-inflammatory cytokines and inflammatory mediators, such as reactive oxygen species (ROS). Recent advances in science reveal that the cholinergic anti-inflammatory pathway (CAP) inhibit the cytokines production and controls inflammation. In this sense, the objective of this study is to evaluate the CAP pharmacological activation effects in the oxidative stress and airway inflammation in an experimental model of eosinophilic pulmonary response. Female BALB/cJ were submitted to a model of eosinophilic pulmonary response by ovalbumin (OVA) and treated with neostigmine (acetylcholinesterase inhibitor). Neostigmine treatment decreased eosinophil recruitment, oxidative stress and pro- inflammatory cytokines levels in the lung of mice. Our results demonstrated, for the first time, that pharmacological activation of the CAP by neostigmine attenuates the oxidative stress and airway inflammation in an experimental model of eosinophilic pulmonary response.

Keywords: asthma, airway inflammation, oxidative stress, cholinergic anti-inflammatory pathway, neostigmine.

INTRODUCTION

Asthma is a major health problem that currently affects 300 million people in the world. The clinical symptoms of allergic asthma are wheezing, coughing, and chest tightness, which is caused by a combination of events in the airways such as bronchial hyperreactivity (BHR), remodeling of the airways, with narrowing (Deckers, De Bosscher, Lambrecht, & Hammad, 2017). These clinical symptoms of allergic asthma are a consequence of a chronic inflammatory lung, orchestrated by adaptive $CD4^+$ T helper 2 (Th2) cell immune to airborne allergens. T helper 2 (Th2) lymphocytes classically produce specific cytokines such as interleukins 4, 5 and 13 (IL-4, IL-5 and IL-13) which induces B cells to produce of immunoglobulin E (IgE), mucus overproduction and infiltration of neutrophils, macrophages, lymphocytes and especially eosinophils in the airway (Haspeslagh et al., 2018). Moreover, Th2 cells have plasticity and, in a response to the environmental influences, can secrete other pro-inflammatory cytokines of different profiles, including T helper 1 (Th1) and T helper 17 (Th17), contributing to the pulmonary inflammatory response (Manni et al., 2014; McKinley et al., 2008).

Activation of eosinophils during asthma exacerbation release cytotoxic mediators, such as eosinophil peroxidase (EPO) and produces reactive oxygen species (ROS) (Dworski, 2000). Under physiological conditions, ROS is neutralized by antioxidant defense systems, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). However, in inflammatory diseases, such as asthma, occurs an imbalance between the ROS production and antioxidant defense, generating oxidative stress (da Cunha et al., 2016). In asthma the oxidative stress stimulates pulmonary function

impairment, mast cell degranulation, airway remodeling, and mucus secretion by epithelium, aggravating pulmonary inflammation. (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Nesi et al., 2017; Zhang, Deng, Zhang, Zhang, & Bai, 2018).

Inflammation is essential for the maintaining immunological homeostasis. However, the exaggerated immune response usually results in tissue injury and numerous pathologies (Gwilt, Donnelly, & Rogers, 2007). To minimize this damage is necessary regulatory mechanisms that modify innate and adaptive immunity. In this context, Borovikova and colleagues described neuroimmune mechanism termed cholinergic anti-inflammatory pathway (CAP) that controls cytokine production through the vagus nerve and acetylcholine (ACh), leading to inflammation control (Borovikova et al., 2000). This neural circuit requires the interaction of acetylcholine with the α7 subunit of the nicotinic acetylcholine receptor (α7nAChR) expressed in immunes cells, such as eosinophils (McGovern & Mazzone, 2014). Pinheiro and colleagues demonstrated, in knockout mice, that long-term vesicular acetylcholine transporter (VAChT) deficiency induced inflammation, remodeling, and hyperresponsiveness by an increase of nuclear factor kappa B (NFκB p65) and inhibition of Janus Kinase-2 (JAK2) expression (Pinheiro et al., 2015).

Recently, Kanashiro and colleagues showed that acetylcholinesterase (AChE) inhibition by neostigmine ameliorated the clinical symptoms of experimental arthritis, suggesting that AChE inhibition reinforces the cholinergic anti-inflammatory response by increasing ACh (Kanashiro et al., 2016). Therefore, AChE inhibition represents a clinically relevant therapeutic target for the treatment of chronic inflammatory diseases, such as asthma. In this context,

we investigated the CAP modulation effect by neostigmine on oxidative stress and airway inflammation in an experimental model of eosinophilic pulmonary response.

METHODS

Animals and experimental groups

Female adult BALB/cJ mice (6-8 weeks old) were acquired for all experiments (CeMBE, PUCRS). The animals were fed with a balanced chow diet with access to water *ad libitum*, housed in cages and maintained on a 12/12-h light/dark cycle. Female were randomized in three groups: (1) DPBS group: control group (2) OVA group: animals submitted an experimental model of eosinophilic pulmonary response (3): OVA + neostigmine group: animals submitted an experimental model of eosinophilic pulmonary response and treated with neostigmine (acetylcholinesterase inhibitor).

Sensitization, airway challenge and drug treatment

The animals were sensitized by two subcutaneous injections of 20 µg ovalbumin (OVA) (Grade V, Sigma-Aldrich, St. Louis, USA), diluted (200 µL) in Dulbecco's phosphate-buffered saline (DPBS), on days 0 and 7, followed by three intranasal challenges with 100 µg of OVA, diluted in DPBS (50 µL), on days 14, 15, and 16 of the protocol. The animals of negative control received only DPBS in the sensitization and intranasal challenges. To evaluate the CAP effects, on days 14, 15 and 16, after 30 minutes of OVA challenge, mice received 80 µg/kg of neostigmine (Normastig, União Química, São Paulo, Brazil) intraperitoneally (Hofer et al., 2008). The protocol of the study is illustrated in Figure 1.

Assessment of respiratory mechanics

On day 17 of the protocol, animals were anesthetized by intraperitoneal injection of a solution of ketamine (0.4 mg/g) and xylazine (0.2 mg/g) and paralyzed with a solution of pancuronium bromide (1 mg/kg, i.p.). After were

tracheostomized, by a steel cannula (18-gauge, 0.838 mm inner diameter and 12.7 mm length) and connected to a mechanic ventilator (Flexi Vent, SCIREQ, Montreal, PQ, Canada). Lung mechanic was measured using a modification of the forced oscillation technique (FOT). Animals were placed under default ventilation with a positive end-expiratory pressure (PEEP) of 3 cmH_20, at a frequency of 150 breaths per minute, and displaced volume of 10 mL/kg. The respiratory system impedance Zrs was measured as the load impedance on the wave tube. A three parameters model with constant phase tissue impedance was fitted to the Zrs data to obtain measures of Raw, the Newtonian resistance (Raw), tissue damping (G), and tissue elastance (H) (Mori et al., 2017).

Bronchoalveolar lavage (BAL)

BAL was performed after the respiratory mechanics, was injected two consecutive flushes in the lung with 1 mL of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) through the previously inserted tracheal cannula.

Total and differential cells count from BAL

BAL was centrifuged at 420g, for 5 minutes, at 4°C and the supernatant was collected for analysis of EPO activity through colorimetric assay (Silveira et al., 2002). The pellet was resuspended in 350 µL of PBS containing 2% FBS. Total cells count (TCC) were determined by the trypan blue exclusion test, with a Neubauer chamber (BOECO, Hamburg, Germany). For differential cytology slides, BAL suspension was centrifuged through a cytospin (FANEM, São Paulo, Brazil), and slides were stained with hematoxylin and eosin (H&E) (Panótico

Rápido - Laborclin, Brazil). Four hundred cells were counted under light microscopy BMX 43 (Olympus, Tokyo, Japan).

Histopathologic analysis in lung tissue

After euthanasia the lungs were perfused with 10% buffered formalin on a gravity column (20 mmHg), removed and the specimens were embedded in paraffin blocks, cut into 5 µm sections and stained with H&E (Cytological Products Soldan, Brazil) for assess peribronchial and perivascular infiltrate. For identification of mucus-secreting goblet, sections were stained with alcian blue (InLab, Brazil). Images of the sections were captured through a BMX 43 microscope equipped with a digital camera DP73 (Olympus, Tokyo, Japan). For the inflammatory infiltrate quantification, ten measurements (µm) were performed in each of the evaluated regions using the imaging software CellSens Standard Olympus. At least 5 fields were evaluated for each animal and the mean was calculated for analysis.

Measurement of lung cytokines

To determine cytokine levels, lung was collected from mice on day 17 of protocol and homogenized in PBS. Multiple soluble cytokines: interleukin 1 beta (IL-1β), tumor necrosis factor α (TNF-α) and IL-13 were simultaneously measured using a Multiplex Assay kit (MILLIPLEX®, Millipore, Germany). For IL- 5, IL-4 and IL-10 were used ProcartaPlex (Thermo Fisher Scientific, Waltham, USA). Cytokines were measured by MagPix (MILLIPLEX®, Millipore, Germany) and the results were analyzed through the software xPONENT® 4.2 (MILLIPLEX®, Millipore, Germany). All cytokines in lung homogenate were expressed in pg of cytokines/mg of total protein.

Oxidative stress in lung tissue

To evaluate oxidative stress parameters, after euthanasia the lung tissue was removed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The lung homogenates were centrifuged at 750g for 10 minutes, at 4ºC. The pellet was discarded and the supernatant was immediately separated and used for the analysis described in the study.

Reactive oxygen species (ROS) activity in lung tissue

ROS quantification was based on the oxidation of 2′7′-dichlorofluorescein (H2DCF), according to the method of LeBel and colleagues (LeBel, Ischiropoulos, & Bondy, 1992). The sample was incubated in a medium containing 100 µM of 2′7′-dichlorofluorescein diacetate (H2DCF-DA) solution. The reaction produces the fluorescent compound dichlorofluorescein (DCF), which is measured at λ_{em} = 488 nm and λ_{ex} = 525 nm; results were represented as nmol DCF/mg protein.

Superoxide dismutase (SOD) activity in lung tissue

SOD activity is based on the capacity of pyrogallol to autoxidize. This process highly dependent on superoxide, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity was then indirectly assayed at 420 nm (Greenwald, 2018). The results were represented as SOD units/mg protein.

Catalase (CAT) activity in lung tissue

The CAT activity is based on the disappearance of H_2O_2 at 240 nm, in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/ml (Aebi, 1984), using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). Each CAT unit is defined as 1 µmol of hydrogen peroxide consumed per minute and the results were presented as CAT units/mg protein.

Glutathione peroxidase (GPx) activity in lung tissue

The GPx activity was evaluated using tert-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. Each GPx units defined as 1 µmol of NADPH consumed per minute. The specific activity is presented as GPx units/mg protein.

Immunofluorescence

After deparaffinization, the lung sections were sequentially treated with 10 mM sodium citrate ($Na_3C_6H_5O_7$) for 10 minutes and 0.3% hydrogen peroxide (H_2O_2) in methanol (CH_3OH) for 20 minutes and rinsed thoroughly with PBS. Sections were blocked with 10% bovine serum albumin (BSA) in PBS for 30 minutes and incubated with anti-AChE and anti-NFκB p65 (1:500) (Thermo Fisher Scientific, Waltham, USA) for 40 minutes, followed incubation with secondary antibody anti-rabbit FITC (1:500) for 40 minutes. The cellular nuclei

were stained using (Hoechst) (1:2000). Confocal images were taken in a Zeiss LSM 5 Exciter microscope.

Western blot analysis

Lung tissue was homogenized in CHAPS solution (10mM Tris-HCl, pH 7.5, 1mM $MgCl_2$, 1mM EDTA pH 8.0, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5 % CHAPS and 10% glycerol). The extracts were centrifuged at 17.900g, 4°C, for 60 minutes to remove insoluble material. Equal amounts of proteins were separated by electrophoresis (SDS-PAGE). After running the gel, the proteins were transferred to nitrocellulose membrane and stained with Ponceau S solution (Sigma-Aldrich, St. Louis, USA) for transfer confirmation. The blot was then washed with Tris-HCl, pH 7.4, NaCl and 0.05% Tween (TTBS), followed by 30 minutes incubation in blocking solution TTBS containing 5% BSA. After incubation, the blot was washed again with TTBS and then incubated overnight at 4°C in blocking solution containing the antibodies: anti-AchE (1:200) (Thermo Fisher Scientific, Waltham, USA), anti-AKT (1:500) and anti-β-actin (1:2000) (Cell signaling, Danvers, EUA). The blot was then washed, incubated for 2 hours with horseradish peroxidase-conjugated (HRP-conjugated) anti-IgG (1:2000) (Santa Cruz Biotechnology, Santa Cruz, USA). The chemiluminescence was detected by a gel documentation system (Fujifilm, LAS-3000) that was scanned and band intensities were quantified through ImageJ software.

Protein determination

The total protein concentration was determined by Bradford, using bovine serum albumin as standard (Bradford, 1976).

Ethics statement

This study was conducted under ethical standards, following the recommendations of the Brazilian Society of Laboratory Animal Science (SBCAL), using fewer animals and adequate management of pain and suffering, during the study procedures and euthanasia. This study was approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul (CEUA, 7934).

Statistical analysis

Data are presented as mean±SD. Results were analyzed using Statistical Package for the Social Sciences, version 20.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test. Differences were considered significant at $*p < 0.05$.

RESULTS

Neostigmine treatment ameliorated parameters of respiratory mechanics in an experimental model of eosinophilic pulmonary response

We investigated the CAP modulation effect by neostigmine on parameters of respiratory mechanics. We showed that the resistance (Raw) did not alter in groups studied (Figure 2A). However, tissue damping (G) increased in the OVA group. On the other hand, neostigmine-treated group had a decreased this parameter (Figure 2B). A similar effect was seen for tissue elastance. The animals submitted to the asthma model with OVA showed an increase in tissue elastance and improvement of this parameter in animals treated with neostigmine (Figure 2C).

Treatment with neostigmine decreased inflammatory cells from BAL in an experimental model of eosinophilic pulmonary response

Firstly, we investigated the neostigmine treatment effect on total cells count in BAL of mice. We observed that the OVA group had a significant increased in TCC when compared with the control group. This increased found in the OVA group was reduced in the group treated with neostigmine (Figure 3A). Moreover, the OVA group presented a significant increased of macrophages, neutrophils, lymphocytes, and eosinophils when compared to the control group. Also, treatment with neostigmine was able to reduce the number of macrophages, neutrophils, lymphocytes, and eosinophils in BAL when compared to the OVA group (Figure 3B-F). In addition, we demonstrated that OVA significantly increased the EPO activity when compared to the control group. On the other

hand, we showed that the neostigmine treatment decreased EPO activity in BAL when compared with OVA group (Figure 3G).

Neostigmine treatment reduced inflammatory infiltrate and goblet cells hyperplasia in lung tissue in an experimental model of eosinophilic pulmonary response

Next, lung sections were stained with H&E and we observed that only induction with OVA was able to cause inflammation in the lung (Figure 4A). Histological quantification of the lung sections demonstrated that in comparison with the control group, OVA-induced asthma had an increased in the peribronchial and perivascular inflammatory cells, but neostigmine treatment prevented the peribronchial and perivascular inflammation when compared to the OVA group (Figure 4B-C). Another characteristic of asthma is an increase in goblet cells and the mucus overproduction, as shown in Figure 4D. However, the treatment with neostigmine was also able to reduced goblet cells in the lungs of animals (Figure 4D).

Treatment with neostigmine decreased the pro-inflammatory cytokines levels in lung tissue in an experimental model of eosinophilic pulmonary response

Next, we evaluated the acetylcholinesterase inhibitor effect on the cytokine levels in lungs of mice. We measured the Th2 cytokines levels (IL-4, IL-5 and IL-13), which contribute to the classic clinical symptoms of allergic asthma, and other pro-inflammatory cytokines (IL-1β and TNF-α) that may also contribute to disease exacerbation. In addition, we also measured regulatory cytokine IL-10. The concentrations of IL-4, IL-5, IL-13, IL-1β and TNF-α levels were

significantly increased in BAL in OVA group (Figure 5A-E). Interestingly, the treatment with neostigmine decreased classical cytokines IL-4, IL-5 and IL-13 levels (Figure 5A-C), when compared to the OVA group. IL-1β and TNF-α were also reduced in animals treated with neostigmine when compared to the OVA group (Figure 5D, E), demonstrating a therapeutic role of the cholinergic anti-inflammatory pathway in the pulmonary inflammation control. However, IL-10 levels did not alter between the groups studied (Figure 5F).

Neostigmine improved oxidative stress in an experimental model of eosinophilic pulmonary response

We investigate if neostigmine could prevent the formation of oxidative stress induced by eosinophil recruitment in the lungs of mice. First, we evaluated the effect of neostigmine on ROS production in lungs, indicated by DCF formed from the oxidation of H2DCF. Animals of the OVA group had an increased production of ROS when compared to the control group. On the other hand, we observed that the neostigmine treatment was decreased the ROS formation, compared to the OVA group (Figure 6A). We also tested the neostigmine effect on the enzymatic antioxidant defenses (SOD, CAT and GPx) in the lung of mice. As can be seen in Figure 6 we observed a significant reduced in the SOD, CAT and GPx activity in OVA group when compared to the DPBS group (B-D). We demonstrated that the neostigmine did not alter SOD (B) and GPx activity (D) when compared to the OVA group. However, neostigmine-treated mice increased CAT activity compared to the OVA group (C).

Treatment with neostigmine did not alters AChE immunocontent, but decreased NFκB immunocontent and increased AKT immunocontent in lung tissue

Finally, we investigated CAP components in the lung tissue. We performed the AChE immunocontent through Western blot and we demonstrated an increase in OVA group when compared to the DPBS group. However, a reduction in neostigmine-treated group did not observed in comparison to OVA group (Figure 7A). We performed immunofluorescence staining and we observed the same immunocontent increase in OVA group when compared to the DPBS group (Figure 7B). We also performed NFκB p65 immunofluorescence. Results showed that allergic asthma significantly increased NFκB p65 immunocontent when compared to the DPBS group, but neostigmine treatment reverted the NFκB p65 immunocontent when compared to the OVA group (Figure 7C). Finally, we investigated the AKT immunocontent by Western blot in lung tissue. We did not observe alteration between OVA and DPBS group. On the other hand, neostigmine-treated group increased the AKT immunocontent when compared to the OVA group (Figure 7D).

DISCUSSION

Asthma is a chronic inflammatory lung disease that affects all age groups and presents a high prevalence, morbidity, and mortality in the world with a relevant impact on global public health care (Eder, Ege, & von Mutius, 2006; Lai et al., 2009). In Brazil, approximately 5 patients die from asthma daily and according to the DATASUS has more than 120,000 asthma-related hospitalizations per year (Prietsch, Zhang, Catharino, Vauchinski, & Rodrigues, 2012). The disease can be controlled by a combination of an inhaled corticosteroid and a short-or long-acting β2-adrenergic agonist. Another treatment option is the muscarinic cholinergic receptor antagonist that results in bronchodilation and decreased mucus production (FitzGerald et al., 2015). However, the nicotinic receptor (α7), described as an important CAP component, plays an important role in the inflammation control when activated by acetylcholine. The protective effects of CAP activation have been reported in several inflammatory diseases, such as ALI, sepsis, colitis, pancreatitis, and rheumatoid arthritis (Ghia, Blennerhassett, El-Sharkawy, & Collins, 2007; Su et al., 2007; van Maanen, Stoof, LaRosa, Vervoordeldonk, & Tak, 2010; van Westerloo et al., 2006; Wang et al., 2004). More importantly, Koopman e colleagues demonstrated positives results when extend the preclinical data to the clinic, showing that vagus nerve stimulation inhibits TNF and attenuates disease severity in rheumatoid arthritis patients (Koopman et al., 2016), demonstrating CAP clinical importance. In this sense, the major findings of the present study were that CAP pharmacological activation attenuated oxidative stress and lung inflammation in a murine model of asthma.

First, we analyzed TCC and we observe a decreased BAL. In the differential cells count it was evident the decreased on cells infiltrate, especially of eosinophils in the group treated with neostigmine. In addition, we found an increased in EPO activity in BAL. However, the treatment with neostigmine reduced EPO activity, what is directly associated with a decreased recruitment eosinophilic. Indeed, experimental studies also have demonstrated that CAP activation promote the reduction of cells recruitment in inflammatory diseases (Kanashiro et al., 2016). Our histological analysis corroborated with these findings. We demonstrated an intense peribronchial and perivascular infiltrate in the OVA group. However, neostigmine-treated mice showed a decreased peribronchial and perivascular infiltrates. In addition to the eosinophil infiltration in airways, mucus hypersecretion also tightly contributes to the disease exacerbation (Haspeslagh et al., 2018). In histological sections of lung tissue stained with alcian blue, it is possible to observe that treatment with neostigmine also decreased mucus hypersecretion. In allergic asthma eosinophil infiltration of the airways and mucus overproduction contributes to the impaired respiratory mechanics (Lambrecht & Hammad, 2015). In this context, we decided to evaluate the neostigmine effect on respiratory mechanics parameters. We observed that airway resistance did not alter in groups studied. Mori *et al.* also found no change in this parameter in an asthma model (Mori et al., 2017). We suppose that this result is possible because it is an acute model of disease and we did not use adjuvant. On the other hand, we demonstrated that neostigmine significantly decreased tissue damping (which represents the resistance of the small airways) and tissue elastance (which assesses the stiffness of the lung parenchyma) compared to the OVA group.

Studies suggest that ACh attenuates inflammation by a direct effect in pro-inflammatory cytokines (Hofer et al., 2008; Kox et al., 2012). We evaluated the levels of Th2 cytokines (IL-4, IL-5 and IL-13) which strongly contribute to disease exacerbation. Asthmatic mice presented higher levels of this cytokines when compared to the DPBS group. IL-4 induces IgE production by B cells and is necessary for the development of adaptive Th2 immunity. Eosinophilia in lung tissue is driven by IL-5 and the IL-13 contribute to the bronchial hyperreactivity and for goblet cell metaplasia, which clogs the airway lumen (Lambrecht & Hammad, 2015). We showed that asthmatic mice treated with neostigmine decreased these cytokines. This reduced in pro-inflammatory cytokines levels supports the found results in decreased eosinophil infiltration and mucus hypersecretion of the airways. Although asthma is classically associated with eosinophilia and Th2 cytokines, in some cases it may occur a mixed Th1 and Th17 cytokine. In this context, we also measured the levels of IL-1β and TNF-α. In response to allergens, cell can produce IL-1β, a strong pro-inflammatory signaling molecule, associated with mucus production (Faiz et al., 2018). TNF-α induces the release of more inflammatory cytokines, exacerbating lung cell damage and also contributing to cells migration into allergic lung tissue in asthmatic mice (Sun et al., 2017). Neostigmine treatment also reduced the levels of IL-1β and TNF-α, demonstrating the CAP anti-inflammatory potential. However, we did not observe an increase of IL-10 in the treated group when compared to the OVA group. It is possible because CAP has a direct effect on pro-inflammatory cytokines. Furthermore, other authors also have not been able to show that cholinergic anti-inflammatory system acts in anti-inflammatory cytokines, such IL-10, corroborating with our results (Pinheiro et al., 2015).

Also, we demonstrated a massive eosinophils infiltration in the airway and an increased EPO activity in the OVA-induced asthma group that contributes to ROS formation. Recently, Mishra and colleagues (2017) demonstrated that oxidative stress-mediated damage may aggravate airway inflammation by inducing diverse pro-inflammatory mediators, increasing bronchial hyperresponsiveness, vascular permeability and mucus hypersecretion (Mishra, Banga, & Silveyra, 2017). We verified an increase in ROS production through DCF fluorescence assay in OVA group. However, the treatment with neostigmine decreased ROS formation in asthmatic mice. During oxidative stress, the homeostasis cellular depends on protective antioxidant mechanisms. In the lung, the major enzymatic antioxidants are SOD, CAT and GPx (Rahman, Biswas, & Kode, 2006). In this context, we also evaluated the neostigmine effects on enzymatic antioxidant systems. SOD enzyme is necessary to detoxify the excess of O_2^- formed, which would justify the decrease found in the OVA group. The detoxification caused by SOD generate H_2O_2 that requires the CAT enzyme to neutralize and GPx removes H_2O_2 and other peroxides by coupling its reduction to H_2O. In the same way as SOD, the CAT and GPx enzymes was also decreased in the OVA group, possibly because they are being consumed in this inflammatory process. Our results showed that the treatment with neostigmine did not alter SOD and GPx activity. On the other hand, we demonstrated CAT activity increased in the lung tissue. We believe that the DCF formed was decreased in the lungs of animals treated with neostigmine because ROS are being neutralized by the antioxidant enzyme, shown by the CAT activity increased in this present study. We demonstrated, for the first time, the antioxidant effect of

CAP pharmacological activation in an experimental model of eosinophilic pulmonary response.

We evaluated the CAP effects in an experimental model of eosinophilic pulmonary response by neostigmine, a peripheral cholinesterase inhibitor. In this way, we investigated neostigmine treatment effect on AChE immunocontent in lung tissue. We verified by immunofluorescence microscopy and Western blot that AChE immunocontent increased in the OVA group. However, the immunocontent did not decrease in mice treated with neostigmine. We believed that this result may be due to the time of lung tissue collection. Analyzes twenty-four hours after the last administration of neostigmine may have hidden possible effects in the immunocontent due to the half-life of the drug, being approximately one hour. Interestingly, even with a short half-life, our results showed a beneficial effect on oxidative stress and in airway inflammation by CAP activation.

Acetylcholine release acts via α7nAChR present on immune cells. α7nAChR activation triggers the JAK-2-STAT-3 pathway that inhibits the nuclear translocation of NFκB. In a model of septic renal inflammation pharmacological α7nAChR activation was showed to attenuate through suppressing NFκB (Chatterjee et al., 2012). Nuclear fator-kappa B induces the expression of various pro-inflammatory genes, including those encoding cytokines and plays a critical role in the activation and differentiation of innate immune cells and inflammatory T cells. Like this, deregulated NFκB activation can contributes to the asthma exacerbation (Liu, Zhang, Joo, & Sun, 2017). Thus, we demonstrated that treatment with neostigmine decreased the NFκB immunocontent in the lung of mice, corroborating with the cytokines results that decreased in asthmatic mice treated with neostigmine. Recently, Kim and colleagues (2018) described a

molecular mechanism whereby α7nAChR, in proximal tubular cells, protects the kidney from ischemia-reperfusion injury by increasing heme oxygenase (HO)-1 expression levels via phosphoinositide 3-kinase (PI3K)/Akt and protein kinase C (PKC) signaling (Kim et al., 2018). Heme oxygenase (HO)-1 is a protein that can protect against cell injury from oxidative stress and inflammatory responses (An et al., 2012). We evaluated the AKT immunocontent in lung and observed an increased in animals treated with neostigmine, suggest activation of the via PI3K/Akt and protein PKC. Thus, this result supports the antioxidant effect found in pharmacological activation with neostigmine.

The limitation of the present study is that we have not analyzed expression of α7nAChR in the lung of mice. However, Tracey and colleagues, showed that electrical stimulation of the vagus nerve inhibits TNF synthesis in wild-type mice, but fails to inhibit TNF synthesis in α7-deficient mice (Wang et al., 2003), demonstrating that the pathway depends specifically on this receptor for inhibiting cytokine synthesis. Our results strongly suggest activation α7nAChR which explains the pro-inflammatory cytokine levels reduced and of oxidative stress that leads to control of the pulmonary inflammatory response. In addition, we also evaluated signaling mediators of α7nAChR and showed changes in these components expression.

In conclusion, the decreased in eosinophil recruitment, reduced mucus hypersecretion, and ameliorated tissue damping and elastance after the neostigmine administration were associated with CAP capacity in suppressing the pro-inflammatory cytokines levels and attenuating oxidative stress. Thus, to summarize our results we showed in Fig. 8 the CAP pharmacological activation effects that lead to control of airway inflammatory response. We propose, for the

first time, that the CAP pharmacological activation is a promising therapeutic target for asthma treatment.

Author Contributions

GLA designed the study, acquired data, analysis and interpretation of data and wrote the manuscript. JSS, DBK, CL, MSC, RVB, EPM and FSF acquired data, revised the article, and approved the final version. ATSW and PMCP revised the article, and approved the final version. AAC supervised the study, designed the work, revised the article, and approved the final version.

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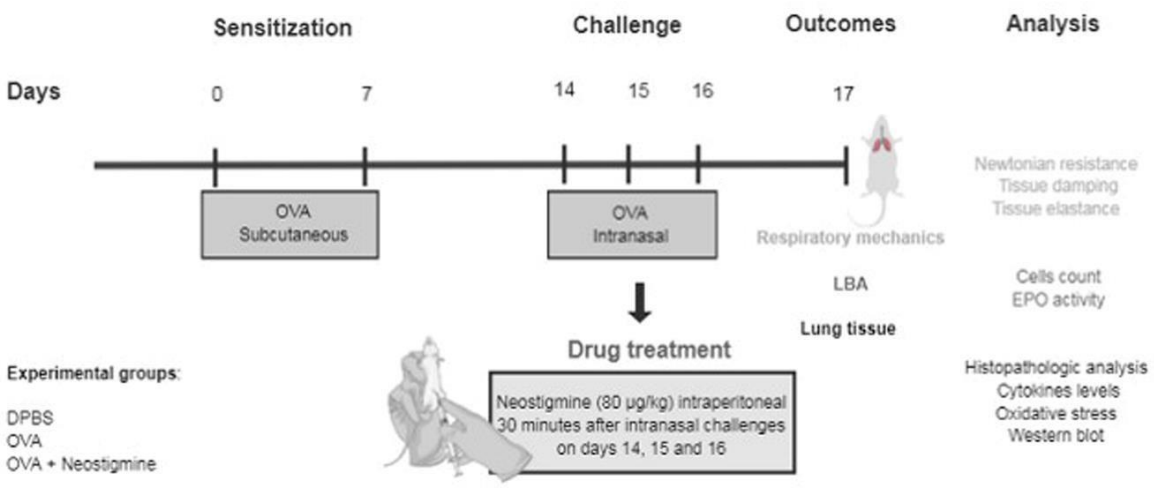

Figure 1: Protocol used to induce an experimental model of eosinophilic pulmonary response and treatment with neostigmine. OVA: ovalbumin.

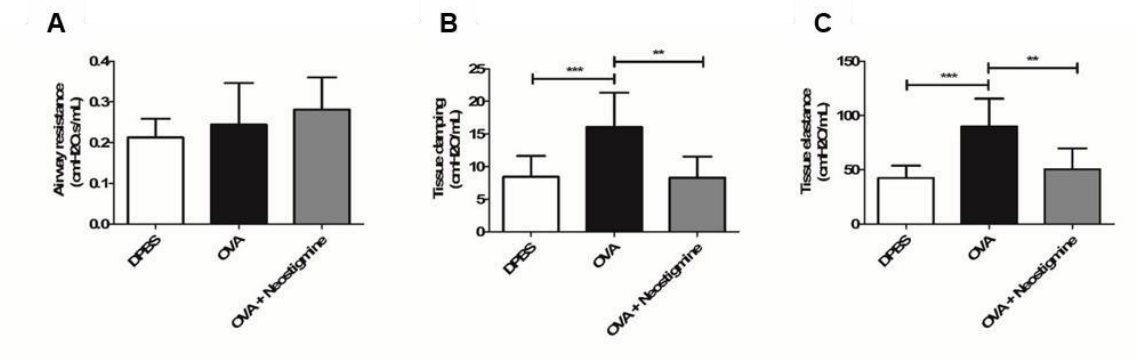

Figure 2: Neostigmine treatment ameliorated parameters of respiratory mechanics. (A) Airway resistance (Raw), (B) tissue damping and (C) tissue elastance. Results are expressed as mean ± SD, for eight animals in each group. Different from DPBS group ***$p<0.001$, different from OVA group **$p<0.01$ (One-way ANOVA followed by Tukey test).

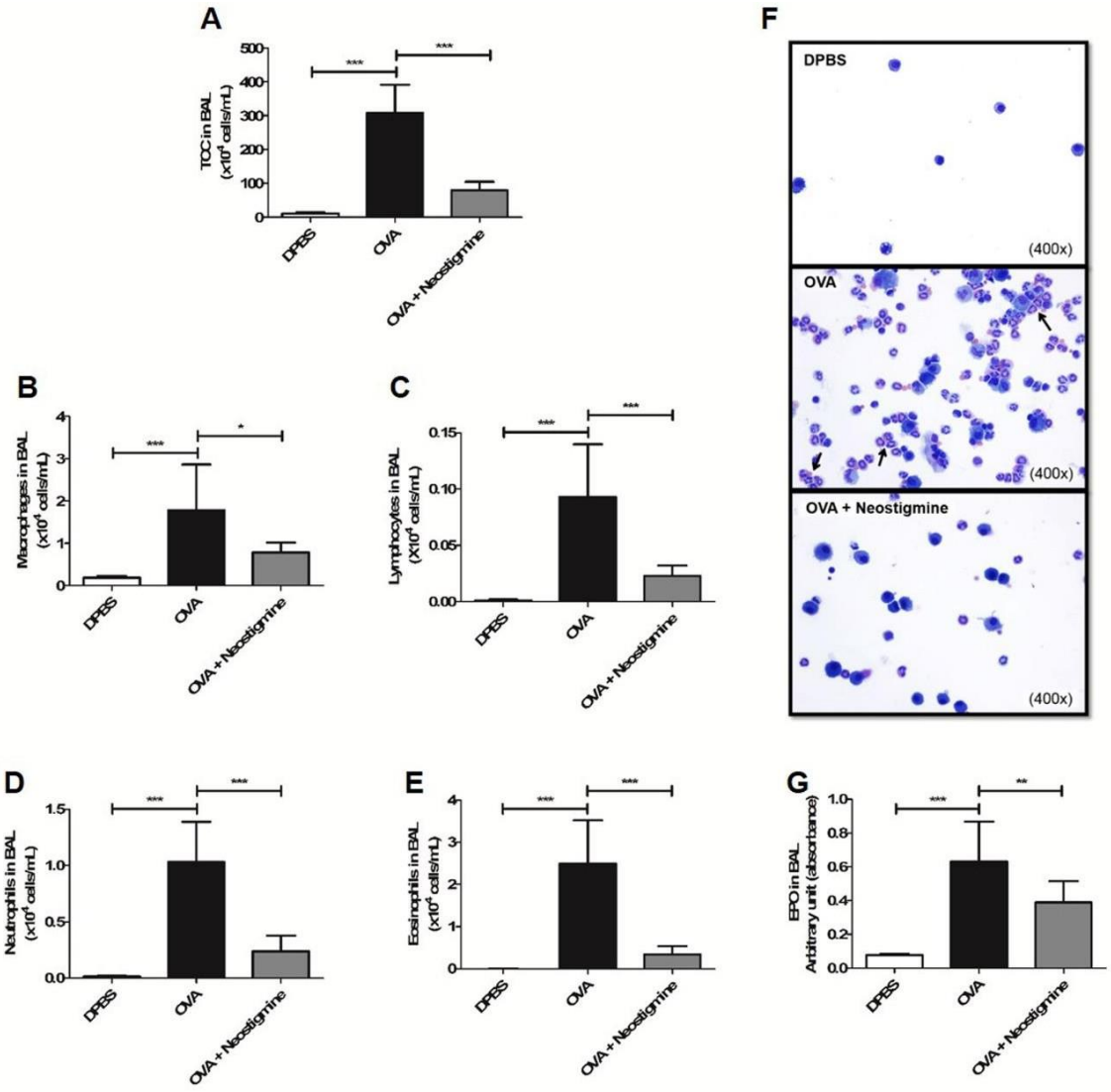

Figure 3: Mice treated with neostigmine decreased cells count and EPO activity in BAL. (A) Total cells count, (B) absolute macrophages count, (C) absolute lymphocytes count, (D) absolute neutrophils count, (E) absolute eosinophils count. (F) Representative image of the differential cells count (H&E, 400x magnification) and (G) EPO activity. Results are expressed as mean ± SD, for eight animals in each group. Different from DPBS group ***$p<0.001$, different from OVA group *$p < 0.05$, **$p < 0.01$ and ***$p<0.001$ (One-way ANOVA followed by Tukey test). BAL: bronchoalveolar lavage; EPO: eosinophil peroxidase; OVA: ovalbumin.

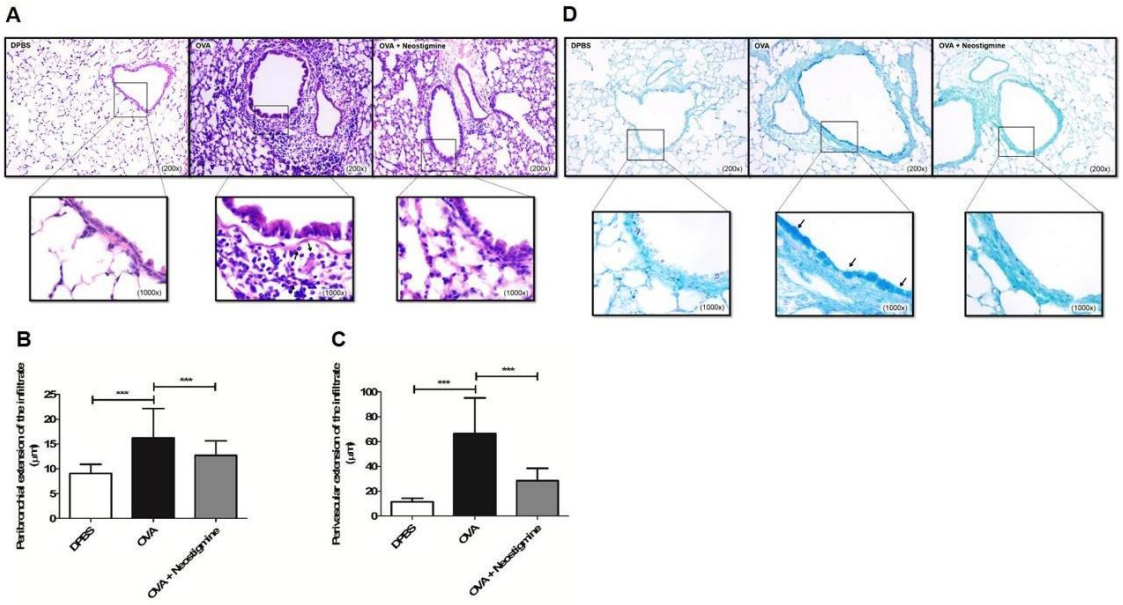

Figure 4: Mice treated with neostigmine reduced inflammatory infiltrate and goblet cells in the lung. (A) Representative lung sections stained with H&E (200x and 1000x magnification). Histological quantification of (B) peribronchial and (C) perivascular infiltrate. (D) Representative lung sections stained with alcian blue (200x and 1000x magnification). Results are expressed as mean ± SD, for eight animals in each group. Different from DPBS group ***$p<0.001$, different from OVA group ***$p<0.001$ (One-way ANOVA followed by Tukey test).

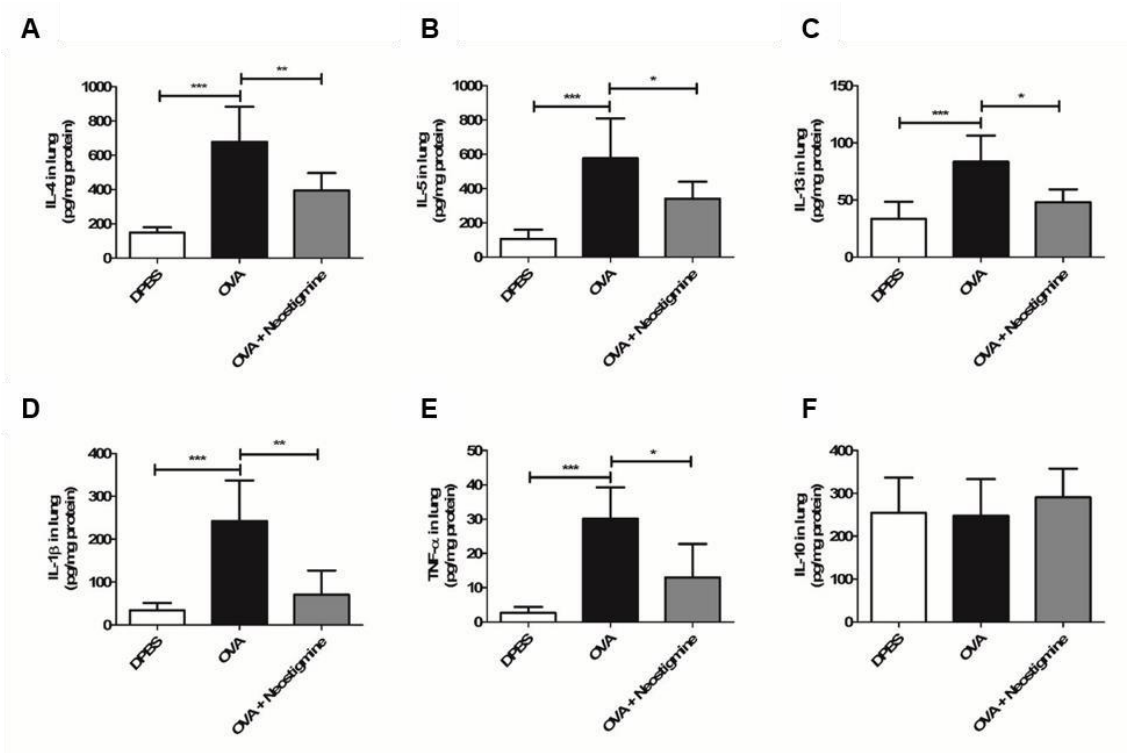

Figure 5: Mice treated with neostigmine decreased the pro-inflammatory cytokines levels in lung. (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-1β, (E) TNF-α and (F) IL-10 levels. Results are expressed as mean ± SD, for eight animals in each group. Different from DPBS group ***$p<0.001$, different from OVA group *p<0.05, **$p<0.01$ (One-way ANOVA followed by Tukey test).

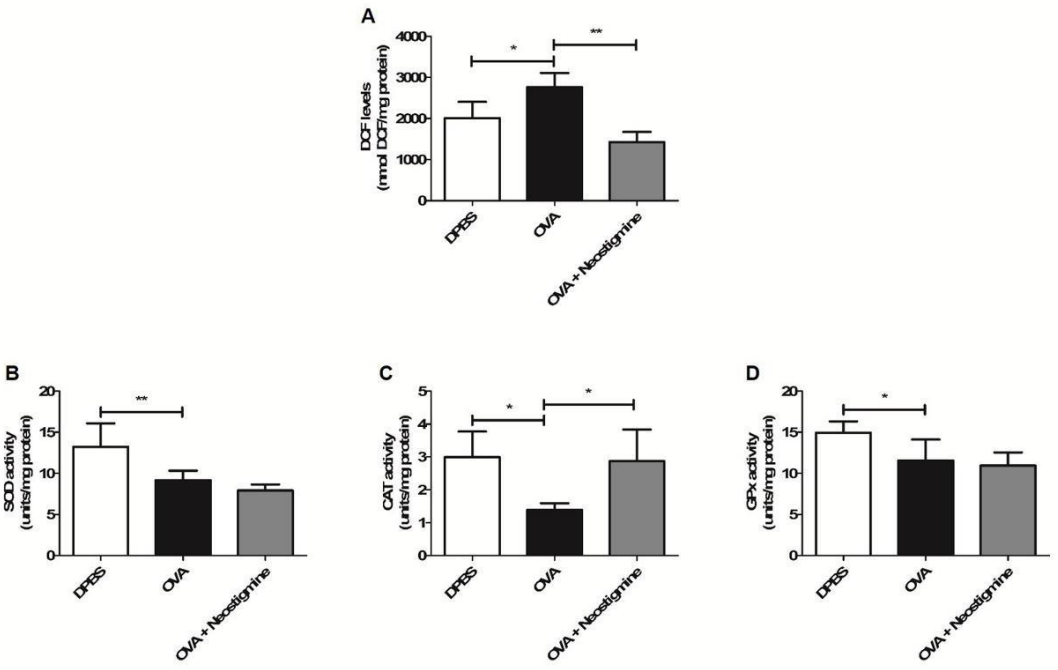

Figure 6: Neostigmine treatment reduced oxidative stress in the lung. (A) Reactive species production by DCF, (B) superoxide dismutase, (C) catalase and (D) glutathione peroxidase activity. Results are expressed as mean ± SD, for eight animals in each group. Different from DPBS group *p<0.05, **p<0.01, different from OVA group *p<0.05, **p<0.01 (One-way ANOVA followed by Tukey test).

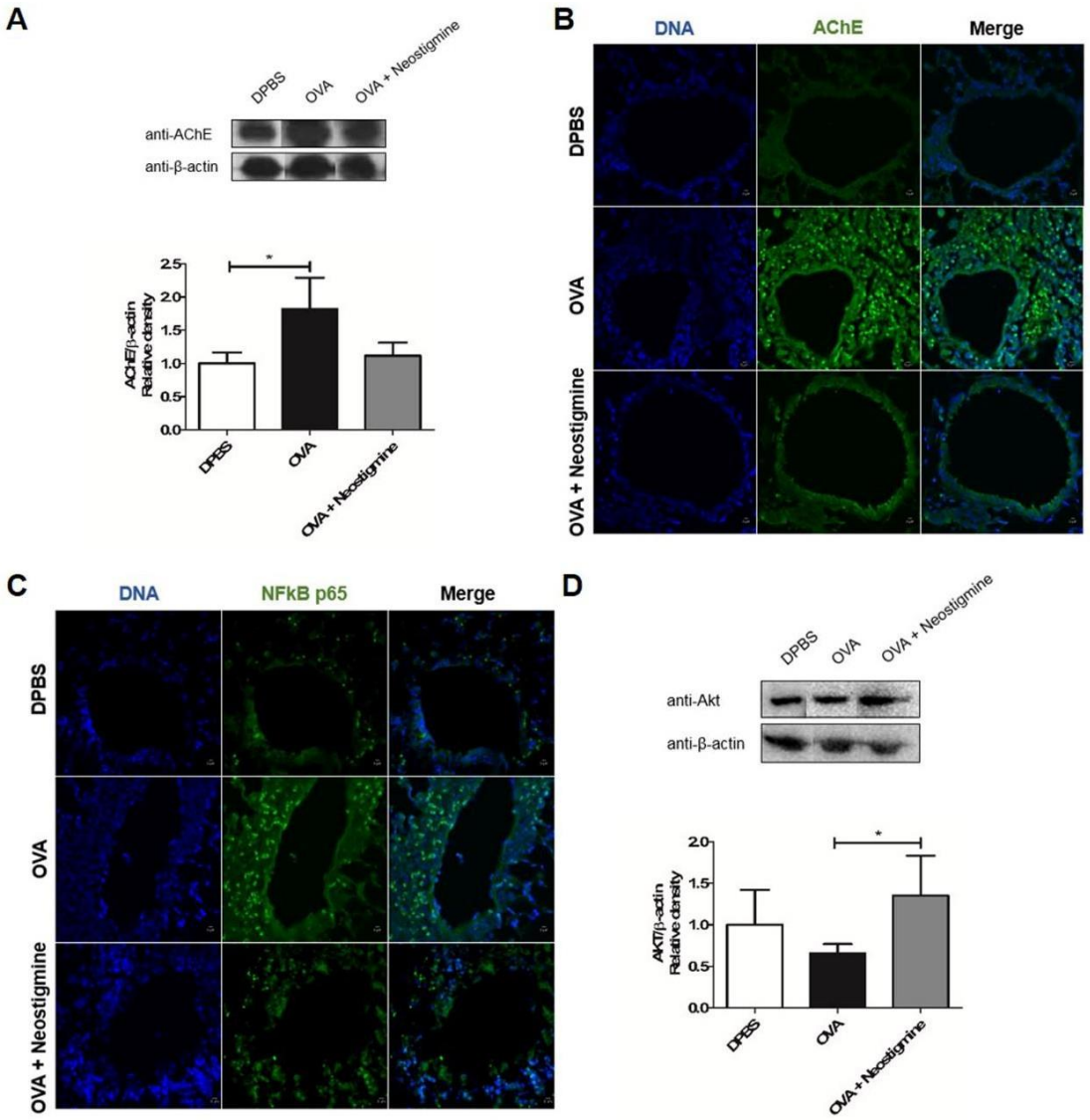

Figure 7: Neostigmine effect in the CAP components immunocontent. (A) AChE analyzed by Western blot, (B) AChE analyzed by immunofluorescence staining, (C) NFκB p65 analyzed by immunofluorescence staining and (D) AKT analyzed by Western blot. Results are expressed as mean ± SD, for six animals in each group. Different from DPBS group *p<0.05, different from OVA group *p<0.05 (One-way ANOVA followed by Tukey test).

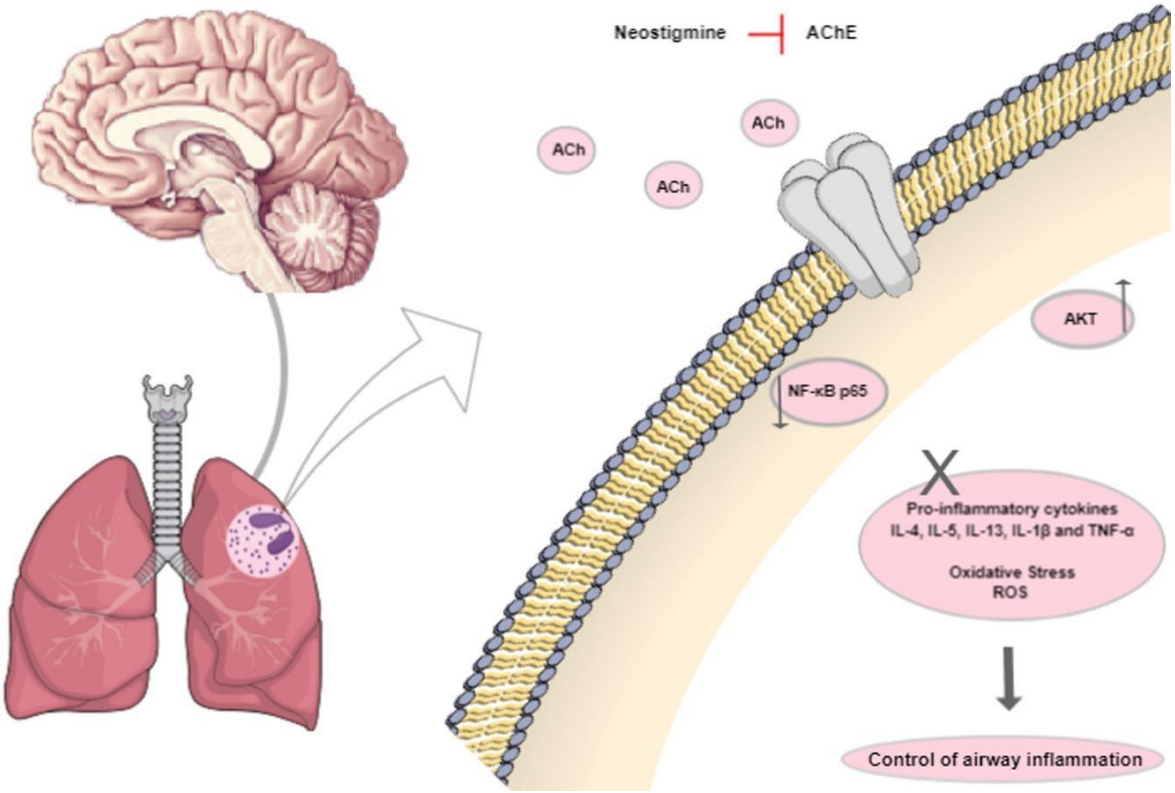

Figure 8: Summary of the CAP pharmacological activation effects that lead to control of airway inflammatory response. ACh: acetylcholine; AChE: acetylcholinesterase; NFκB p65: nuclear factor kappa; ROS: reactive oxygen species; AKT: protein kinase.

ANEXO IV

ARTIGO CIENTÍFICO 3

Artigo submetido

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Neostigmine treatment induces neuroprotection against oxidative stress in cerebral cortex of asthmatic mice

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Abstract

During chronic inflammatory disease, such asthma, leukocytes can invade the central nervous system (CNS) and together with CNS-resident cells, generate excessive reactive oxygen species (ROS) production and disbalance in the antioxidant system, causing oxidative stress that contributed a large part to neuroinflammation. In this sense, the aim of this study is to investigate the effects of neostigmine treatment on oxidative stress in cerebral cortex of asthmatic mice. Female BALB/cJ mice were submitted to asthma model induced by ovalbumin (OVA). Control group received only Dulbecco's phosphate-buffered saline (DPBS). To evaluate neostigmine effects, mice received 80 µg/kg of neostigmine intraperitoneally 30 minutes after each OVA challenge. Our results showed, for the first time, that neostigmine treatment (an acetylcholinesterase inhibitor that no crosses the BBB) was able to reduce leukocyte recruitment in the lung and thereby prevented oxidative stress in mice cerebral cortex through decreased ROS production and upregulation in the antioxidant system. These results support the communication between the peripheral immune system and the CNS, and suggest that acetylcholinesterase inhibitors, such as neostigmine, should be further studied as possible therapeutic strategies for neuroprotection in asthma.

Keywords: asthma, oxidative stress, neostigmine, acetylcholinesterase inhibitor, neuroprotection.

Introduction

Asthma is a chronic inflammatory airway disease that affects 300 million people worldwide. Allergic asthma is clinically defined by variable airway obstruction that causes recurrent periods of shortness of breath, chest tightness, wheezing, and coughing. These clinical symptoms are a consequence of a dysregulated inflammation orchestrated by adaptive $CD4^+$ T helper 2 (Th2) that classically produce specific cytokines such as interleukins 4, 5, and 13 (IL-4, IL-5, and IL-13) which induces B cells to produce immunoglobulin E (IgE), mucus overproduction, and neutrophils, macrophages, lymphocytes, and eosinophils infiltration in the airway, as well as increased levels of reactive oxygen species (ROS) (Deckers et al. 2017).

Very common and relevant clinically many patients with asthma present mood disorders, especially anxiety and depression (Daniel et al. 2012; Goodwin et al. 2003; Kohlboeck et al. 2013). So it does make sense to pay attention to the brain neuroinflammation in patients with allergic asthma. According to the literature, studies have shown that in chronic inflammatory disease, leukocytes can invade the central nervous system (CNS) parenchyma and promoted a drastic loss of blood-brain barrier (BBB) integrity, causing neuroinflammation (Becher et al. 2017).

During neuroinflammation, ROS generated by invading and CNS-resident cells have been strongly implicated in tissue damage. Evidence shows which excessive ROS production is a major mechanism causing damage in multiple sclerosis (MS), with oxidization in DNA, lipids, and proteins reported in CNS lesions, cerebrospinal fluid, and plasma of MS patients (Haider et al. 2011;

Hunter et al. 1985; Lu et al. 2000). In sepsis, a severa inflammatory disease, was demonstrated that ROS production played an important role in the progression of neuroinflammation and brain dysfunction (Danielski et al. 2017). In fact, ROS formation is involved in many damage process studies show that Na^+,K^+-ATPase activity can be inhibited by ROS in diverse disease (Lees 1993). Recently the excessive ROS production also has reported during neuroinflammation in asthma disease (Duan et al. 2018). Under normal conditions, ROS is neutralized by antioxidant defense systems, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). However, defects in antioxidant mechanisms and excessive ROS production leads to oxidative stress and tissue damage.

Recent studies demonstrated that acetylcholinesterase inhibitors are an important tool for therapeutic intervention in diverse diseases. Inhibition of acetylcholinesterase by physostigmine and neostigmine reduced neuroinflammation and degeneration in the cortex and hippocampus of a surgery stress rat model (Kalb et al. 2013). Recently, Odorcyk and colleagues showed that galantamine administration upregulates the antioxidant enzyme catalase in rats submitted to neonatal hypoxia ischemia (Odorcyk et al. 2017). Thus, taking into account the oxidative stress importance for the neuroinflammation, the aim of the present study is to investigate the effects of neostigmine treatment on oxidative stress in cerebral cortex of asthmatic mice.

Materials and Methods

Animals and experimental groups

This study was performed with female BALB/cJ mice (6-8 weeks old) was obtained from the Center for Experimental Biological Models (CeMBE, PUCRS). The animals were fed with a balanced chow diet with access to water *ad libitum*, housed in cages and maintained on a 12/12-h light/dark cycle. Female mice were randomized in three groups: DPBS: control; OVA: animals submitted an experimental model of asthma; and OVA+Neostigmine: animals submitted an experimental model of asthma and treated with neostigmine (acetylcholinesterase inhibitor).

Sensitization, airway challenge and neostigmine treatment

The animals were sensitized by two subcutaneous injections of 20 µg ovalbumin (OVA) (Grade V, Sigma-Aldrich, St. Louis, USA), diluted (200 µL) in Dulbecco's phosphate-buffered saline (DPBS), on days 0 and 7, followed by three intranasal challenges with 100 µg of OVA, diluted in DPBS (50 µL), on days 14, 15, and 16 of the protocol. The control group received only DPBS in the sensitization and intranasal challenges. To evaluate neostigmine effects on the oxidative stress in the cerebral cortex, on days 14, 15 and 16, after 30 minutes of OVA challenge, mice received 80 µg/kg of neostigmine (Normastig, União Química, São Paulo, Brazil) intraperitoneally (Hofer et al. 2008). On day 17 of the protocol, animals were anesthetized by intraperitoneal injection solution of ketamine (0.4 mg/g) and xylazine (0.2 mg/g) followed euthanasia by heart puncture exsanguination.

Bronchoalveolar lavage (BAL), lung tissue and cerebral cortex for analyzes were collected. The study protocol is illustrated in figure 1.

Bronchoalveolar lavage (BAL)

Mice was tracheostomized by a steel cannula and injected with two consecutive flushes in the lung with 1 mL of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS).

BAL cellularity

BAL was centrifuged at 420 g, for 5 minutes, at 4 °C and the pellet was resuspended in 350 µL of PBS containing 2% FBS. Total cells count (TCC) were determined by the trypan blue exclusion test, with a Neubauer chamber (BOECO, Hamburg, Germany). For differential cytology slides, BAL suspension was centrifuged through a cytospin (FANEM, São Paulo, Brazil), and slides were stained with hematoxylin and eosin (H&E) (Panótico Rápido - Laborclin, Brazil). Four hundred cells were counted under light microscopy BMX 43 (Olympus, Tokyo, Japan).

Histopathologic analysis of lung tissue

The lungs were perfused with 10% buffered formalin on a gravity column (20 mmHg), removed and the specimens were embedded in paraffin blocks, cut into 5 µm sections and stained with H&E (Cytological Products Soldan, Brazil) for assess inflammation infiltrate. Images of the sections were captured through a BMX 43 microscope equipped with a digital camera DP73 (Olympus, Tokyo, Japan). For the peribronchial and perivascular infiltrate quantification, ten

measurements (µm) were performed in each of the evaluated regions using the imaging software CellSens Standard (Olympus, Tokyo, Japan). At least 5 fields were evaluated for each animal and the mean was calculated for analysis.

Oxidative stress in the cerebral cortex

To evaluate oxidative stress parameters, after euthanasia the cerebral cortex was removed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The cerebral cortex homogenates were centrifuged at 750 g for 10 min at 4 ºC. The pellet was discarded and the supernatant was immediately separated and used for the analysis described above.

2′7′-Dichlorofluorescein oxidation activity

Reactive species production was measured by method based in the oxidation of 2′7′-dichlorofluorescein (H2DCF) (LeBel et al. 1992). The sample was incubated in a medium containing 100 µM of 2′7′-dichlorofluorescein diacetate (H2DCF-DA) solution. The reaction produces the fluorescent compound dichlorofluorescein (DCF), which is measured at λ_{em} = 488 nm and λ_{ex} = 525 nm; results were represented as nmol DCF/mg protein.

Superoxide dismutase (SOD) activity

The SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity was then indirectly assayed at 420 nm (Greenwald 2018). The results were represented as SOD units/mg protein.

Catalase (CAT) activity

The CAT activity is based on the disappearance of H_2O_2 at 240 nm, in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/ml (Aebi 1984), using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). Each CAT unit is defined as 1 µmol of hydrogen peroxide consumed per minute and the results were presented as CAT units/mg protein.

Glutathione peroxidase (GPx) activity

Was measured GPx activity by the method that using tert-butyl-hydroperoxide as substrate (Wendel 1981). NADPH disappearance was monitored at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. Each GPx units defined as 1 µmol of NADPH consumed per minute. The specific activity is presented as GPx units/mg protein.

Na^+, K^+-ATPase activity

To measure the Na^+,K^+-ATPase activity, the cerebral cortex was homogenized with 10 volumes of 0.32 mM sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 3000 g RPM for 10 min at 4 °C. The pellet was discarded and the supernatant was immediately separated for the measurement. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in a

final volume of 170 µL. The reaction was initiated by the addition of ATP. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. The activity was calculated by the difference between the two assays, as previously described (de Souza Wyse et al. 2000). Released inorganic phosphate (Pi) was measured by the method of Chan *et al*. (Chan et al. 1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

Acetylcholinesterase (AChE) activity

AChE activity was determined according to the method of Ellman and colleagues (Ellman et al. 1961) with modifications. The cerebral cortex were homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 minutes at 1,000 g. The supernatants were used for the enzymatic AChE analyses. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 300 µL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s).

Protein determination

Protein was measured according to Bradford, using bovine serum albumin as standard (Bradford 1976).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism (GraphPad Software, LA Jolla, CA, USA). The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test. Differences were considered significant at $*p < 0.05$ and results are expressed as the mean ± SD.

Results

Neostigmine treatment decreased leukocyte recruitment in the BAL of asthmatic mice

First, we investigated if neostigmine could improve the inflammatory response in the lung of mice. The OVA group had a significant increase in the TCC when compared with the control group ($P < 0.001$, Fig. 2a). However, in the group that received neostigmine is possible to observe a reduced in the TCC ($P < 0.001$, Fig. 2a). Moreover, the asthma group presented a significant increase in absolute count of macrophages ($P< 0.001$, Fig. 2b), lymphocytes ($P < 0.01$, Fig. 2c), neutrophils ($P < 0.001$, Fig. 2d), and eosinophils ($P < 0.001$, Fig. 2e) when compared to the control group. On the other hand, we showed that the neostigmine treatment was able to reduce the number of macrophages ($P < 0.05$, Fig. 2b), lymphocytes ($P < 0.01$, Fig. 2c), neutrophils ($P < 0.001$, Fig. 2d), and eosinophils ($P < 0.001$, Fig. 2e) in BAL when compared to the asthma group.

Treatment with neostigmine improved leukocyte infiltrate in the lung tissue of asthmatic mice

The histological quantification of the lung sections demonstrated that, in comparison with the control group, animals submitted to a model of allergic asthma had an increased peribronchial ($P < 0.01$, Fig. 3b) and perivascular ($P < 0.001$, Fig. 3c) cells infiltrate. These results corroborate our previous finding in BAL cellularity and confirm that our asthma model was effective in inducing an allergic pulmonary response. Figure 3 also shows that neostigmine treatment reduced peribronchial ($P < 0.05$, Fig. 3b) and perivascular ($P < 0.001$, Fig. 3c) infiltration when compared to the asthma group, demonstrating the therapeutic

role of acetylcholinesterase inhibitor in control of the recruitment of inflammatory cells.

Neostigmine treatment prevented ROS production in the cerebral cortex of asthmatic mice

After confirming the treatment efficiency in control of inflammatory cells recruitment in the lung tissue, we investigate whether neostigmine can provide a protector effect in ROS production in the cerebral cortex of mice. We evaluated the neostigmine treatment in ROS production by DCF formed from the oxidation of H2DCF. We observed a significant increase in ROS production ($P < 0.05$, Fig. 4a) in the cerebral cortex of asthma group when compared to the control group. Interestingly, we verified that the neostigmine administration reverted ROS production ($P < 0.01$, Fig. 4a) in the cerebral cortex when compared with asthma group.

Neostigmine treatment altered antioxidant enzymes in the cerebral cortex of asthmatic mice

Antioxidant defenses were also measured by determination of antioxidant enzymatic activities (SOD, CAT, and GPx) in the cerebral cortex of mice submitted to asthma model. In SOD and GPx did not alter activity between the groups. However, is possible observed that CAT activity increased ($P < 0.01$, Fig. 4c) in asthma group when compared to the control group. On the other hand, Figure 4 showed that treatment with neostigmine significantly decreased CAT activity ($P < 0.001$, Fig. 4c) when compared with asthma group. SOD and CAT are oxidant-detoxifying enzymes that work in sequence converting superoxide anion ($O_2^{\bullet -}$) to water. In this sense, a ratio between SOD and CAT enzyme

activities were also analyzed. The SOD/CAT ratio was decreased ($P < 0.05$, Fig. 4e) in asthmatic mice group when compared to the control group. On the other hand, neostigmine treatment promoted SOD/CAT ratio increased ($P < 0.05$, Fig. 4e) when compared to the asthma group.

Treatment with neostigmine did not alter Na^+,K^+-ATPase activity in the cerebral cortex of asthmatic mice

We also investigated the effect of neostigmine treatment in Na^+,K^+-ATPase activity. Figure 5 shows that mice subjected to asthma model showed a significant reduction of Na^+,K^+-ATPase activity ($P < 0.001$, Fig. 5) when compared to the control group. However, this figure also showed that neostigmine did not alter the Na^+,K^+-ATPase activity when compared to the asthma group.

Neostigmine treatment did not alter AChE activity in cerebral cortex of asthmatic mice

Finally, we investigated the neostigmine treatment on AChE activity. Asthmatic mice showed a significant increase in the AChE activity ($P < 0.05$, Fig. 6) when compared to the control group. However, the neostigmine-treated group was not reduced the AChE activity when compared to the asthma group.

Discussion

Asthma is characterized by chronic inflammatory in the lung driven by leukocytes and Th2 lymphocytes. The brain is highly sensitive to peripherals inflammation that can induce disruption of BBB. Xia and colleagues showed that asthma not only induced lung inflammation, but resulted an increase of IL-1β and TNFα in the mice hippocampus and prefrontal cortex, causing neuroinflammation (Xia et al. 2014). Cytokines are important biomarkers of neuroinflammation. However, oxidative stress has been reported to be associated strongly with neuroinflammation and tissue damage. Thus, the major findings of this study were that neostigmine (an acetylcholinesterase inhibitor that no crosses the BBB) improved leukocyte recruitment in the lung and thereby prevented oxidative stress in cerebral cortex of asthmatic mice.

Initially, we investigated whether treatment with acetylcholinesterase inhibitor decreased recruitment of leukocytes in the airways. TCC showed a decrease in the number after neostigmine treatment. In the differential cells count it was evident the neostigmine ability to reduce recruitment of inflammatory cells, especially of eosinophils that the hallmark of type 2 inflammation. Indeed, our histopathologic analysis of lung tissue showed a decreased peribronchial and perivascular infiltrate in mice that received neostigmine treatment. Corroborating with our results, Kanashiro and colleagues also observed the ability of neostigmine to reduce recruitment of leukocytes in inflammatory diseases (Kanashiro et al. 2016).

Then, we began to investigate the neostigmine effects on the oxidative stress parameters in the cerebral cortex of mice submitted an allergic asthma

model. The brain tissues have unique characteristics that make them especially susceptible to free radicals damage, have a high rate of oxygen consumption, low antioxidant levels, has a high content of lipids, and presents a high metabolic activity, being, therefore, a tissue more sensitive to oxidative damages when compared with other tissues (Netto et al. 2018). Neuroinflammation has been reported to be associated with oxidative stress caused by exacerbated ROS production (Hritcu and Ciobica 2013). The ROS consist of free radicals of oxygen and associated molecules, including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet -}$) (Kumar et al. 2012). A ROS increase was observed, as measured through the DCF fluorescence assay in the OVA group, demonstrating that lung inflammation can induce oxidative damage in the cerebral cortex. In contrast, our results showed that neostigmine treatment prevented ROS formation. Thus, considering the susceptible of CNS to damage, the treatment with neostigmine may be of considered important to avoid tissue damage.

To protect against the oxidative damage, cells possess several enzymatic and nonenzymatic antioxidant defenses that interrupt the cascade of oxidative reactions, limiting the extent of oxidative damage. Given that neostigmine treatment prevented ROS production, we also evaluated the neostigmine effects on enzymatic antioxidant systems. SOD is a metalloenzyme that catalyzes the dismutation of $O_2^{\bullet -}$, forming H_2O_2. One molecule of the formed H_2O_2 is reduced to H_2O and the other is oxidized to O_2 by dismutation of CAT. GPx also acts convert H_2O_2 to H_2O.

SOD activity did not alter between the groups, indicating that the increase in the production of reactive species is not by $O_2^{\bullet -}$ formation. However, we

observed an increase in CAT activity in the OVA group, probably is a compensatory mechanism to minimize the excess production H_2O_2, since GPx activity did not alter between the groups. Furthermore, the mice treated with neostigmine showed a reduce CAT activity probably because neostigmine reduces the production of reactive species and the consequent formation of H_2O_2.

SOD and CAT work in sequence. Thus, we evaluated a ratio between SOD and CAT enzyme activities. The cerebral cortex SOD/CAT ratio was decreased in OVA group when compared to control group. Interestingly, mice submitted to allergic asthma and treated with neostigmine showed enhance in SOD/CAT ratio. Thus, we suggest that the ROS production is being neutralized through the antioxidant system by neostigmine effect. Odorcyk and colleagues showed similar results, galantamine (acetylcholinesterase inhibitor) administration promoted upregulates in the anti-oxidant system (Odorcyk et al. 2017).

Studies have been showed that ROS increase and exacerbate oxidative stress response and these processes may be associated with brain energy metabolism impairment. However, the mitochondrial ATP production is a fundamental function for cellular energy metabolism (Biasibetti-Brendler et al. 2018). About 40-50% of the ATP generated is consumed by Na^+,K^+-ATPase, a crucial enzyme that is responsible for the generation of the membrane potential necessary to maintain neuronal excitability and cellular volume control. The Na^+,K^+-ATPase is present in high concentration in cell membranes and is crucial for brain development and function (Jeremias et al. 2012). Impairment in Na^+,K^+-ATPase activity has been associated with diverse diseases, including cerebral ischemia and neurodegenerative disorders (Wyse et al. 2000; Yu 2003).

Corroborating with this results, our data also showed an impairment in Na^+,K^+-ATPase activity in the cerebral cortex of the allergic asthma group. However, neostigmine treatment was not able to revert the impairment in Na^+,K^+-ATPase activity. Machado and colleagues showed that rats subjected to hyperhomocysteinemia (Hcy) presented a significant reduction of Na^+, K^+-ATPase. However, vitamin C, *per se*, did not alter the Na^+,K^+-ATPase activity, but when administered concomitantly with Hcy, it was able to prevent the damage caused by Hcy (Machado et al. 2011), suggest that our results can be explained by a moment of neostigmine administration.

In this article we observed that neostigmine administration promoted neuroprotection by decreased ROS production and upregulation in the antioxidant system. We believe that these results are due to the mechanism of action of the drugs that act by inhibiting acetylcholinesterase and thus, upregulation acetylcholine (ACh). ACh has been recognized as an important component of the cholinergic anti-inflammatory pathway (CAP) that leads to the inflammation control (Borovikova et al. 2000; Kanashiro et al. 2016; Wang et al. 2003). In order, ACh is synthesized from choline and acetyl Co-A by choline acetyltransferase (ChAT) and pumped into storage vesicles by the vesicular acetylcholine transporter (VAChT). ACh is released by exocytosis and either binds to receptors (muscarinic and/or nicotinic) and after release, ACh is degraded by AChE to non-active choline (Gwilt et al. 2007). In this way, we observed that mice submitted to a model of asthma had an increased AChE activity when compared to the control group. On the other hand, we did not observe alterations in activity after neostigmine administration. We hypothesized that this result may be due to the time of cerebral cortex collection for analysis.

The AChE activity was measured twenty-four hours after the last administration of neostigmine. However, the half-life of the drug is approximately one hour. Interestingly, even with a short half-life, is evident that neostigmine provided neuroprotection by decreased ROS production and upregulation in the antioxidant system.

In conclusion, our data showed, for the first time, that neostigmine (an acetylcholinesterase inhibitor that no crosses the BBB) improves recruitment of leukocytes in the airways and induces neuroprotection against oxidative stress in the cerebral cortex of asthma model. Thus, we summarize our results in Figure 7. Therefore, our finding is of important clinical that supports the communication between the peripheral immune system and the CNS, and suggest that acetylcholinesterase inhibitors, such as neostigmine, should be further studied as possible therapeutic strategies for neuroprotection in asthma.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The experiments were conducted in accordance with the Brazilian Society of Laboratory Animal Science (SBCAL), using fewer animals and adequate management of pain and suffering, during the study procedures and euthanasia. This study was approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul (CEUA, 7934).

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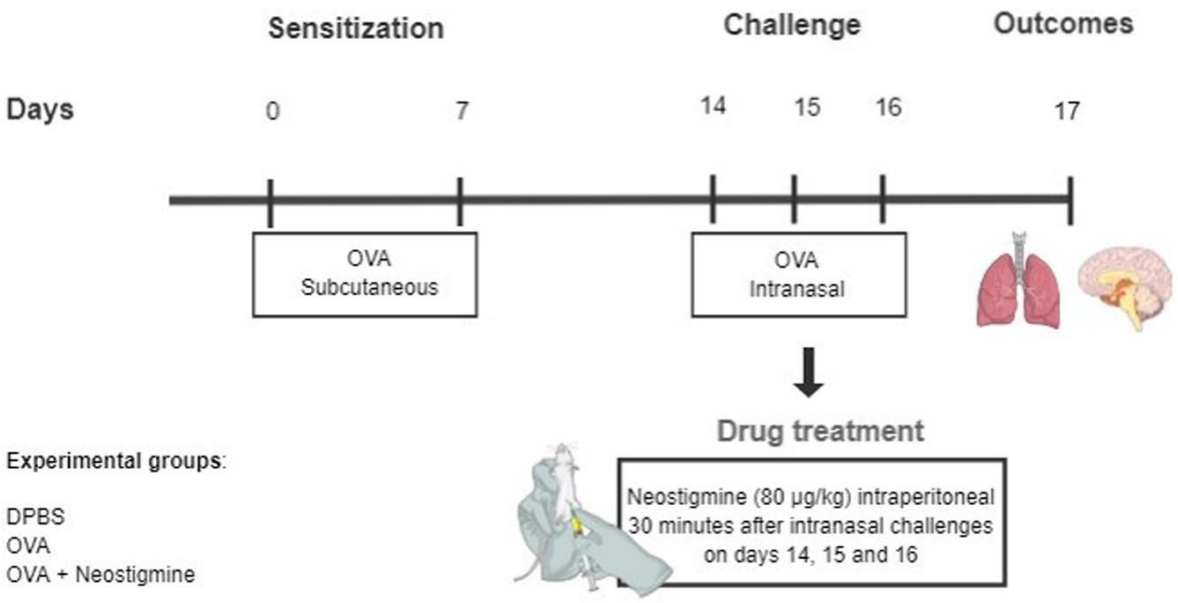

Fig. 1 Protocol used to induce an experimental model of asthma and treatment with neostigmine. OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

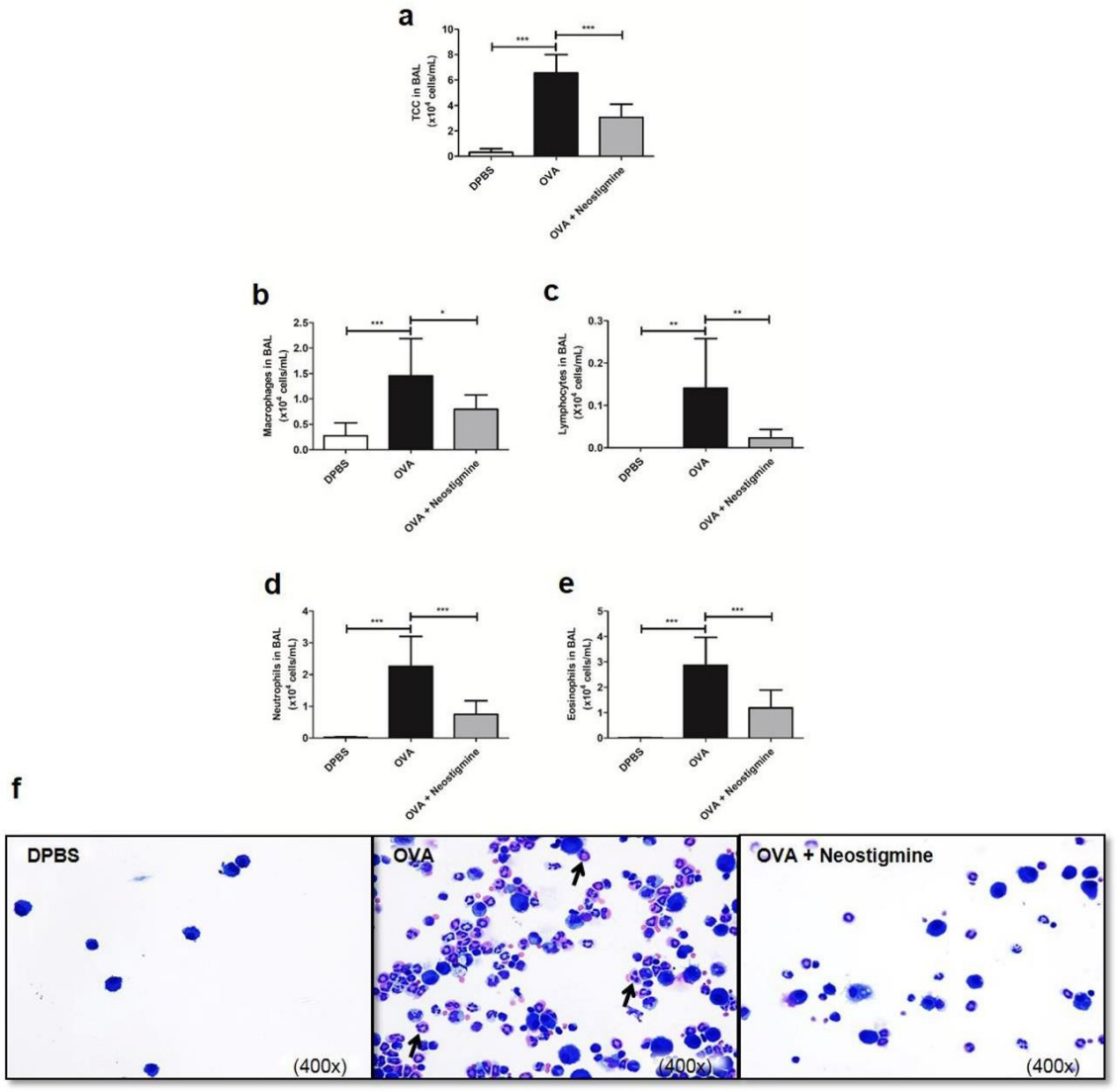

Fig. 2 Mice treated with neostigmine decreased leukocytes recruitment in the BAL. (a) absolute total cells count, (b) absolute macrophages count, (c) absolute lymphocytes count, (d) absolute neutrophils count, (e) absolute eosinophils count. (f) Representative image of the differential cells count (H&E, 400x magnification). Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p < 0.001$, **$p < 0.01$ different from OVA group *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (One-way ANOVA followed by Tukey test). BAL: bronchoalveolar lavage; OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

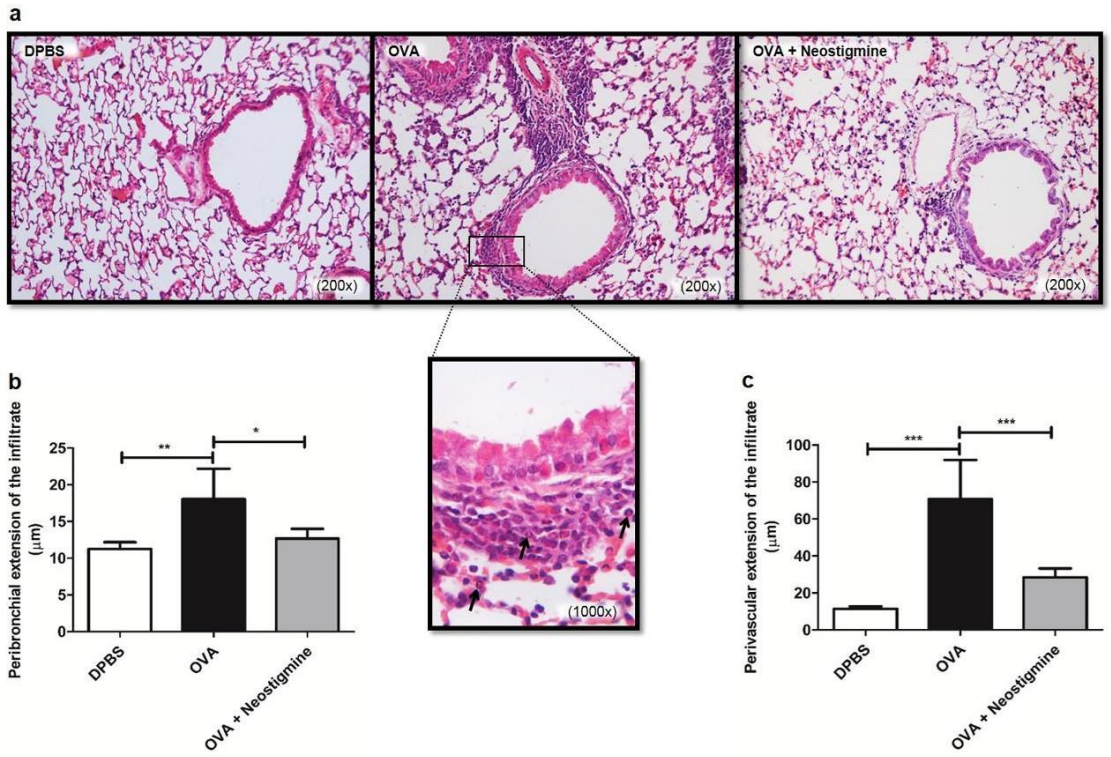

Fig. 3 Neostigmine treatment reduced leukocytes infiltrate in the lung. (a) Representative lung sections stained with H&E (200x and 1000x magnification). Histological quantification of (b) peribronchial and (c) perivascular infiltrate. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group **p<0.01, ***p<0.001, different from OVA group *p<0.05, ***p<0.001 (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

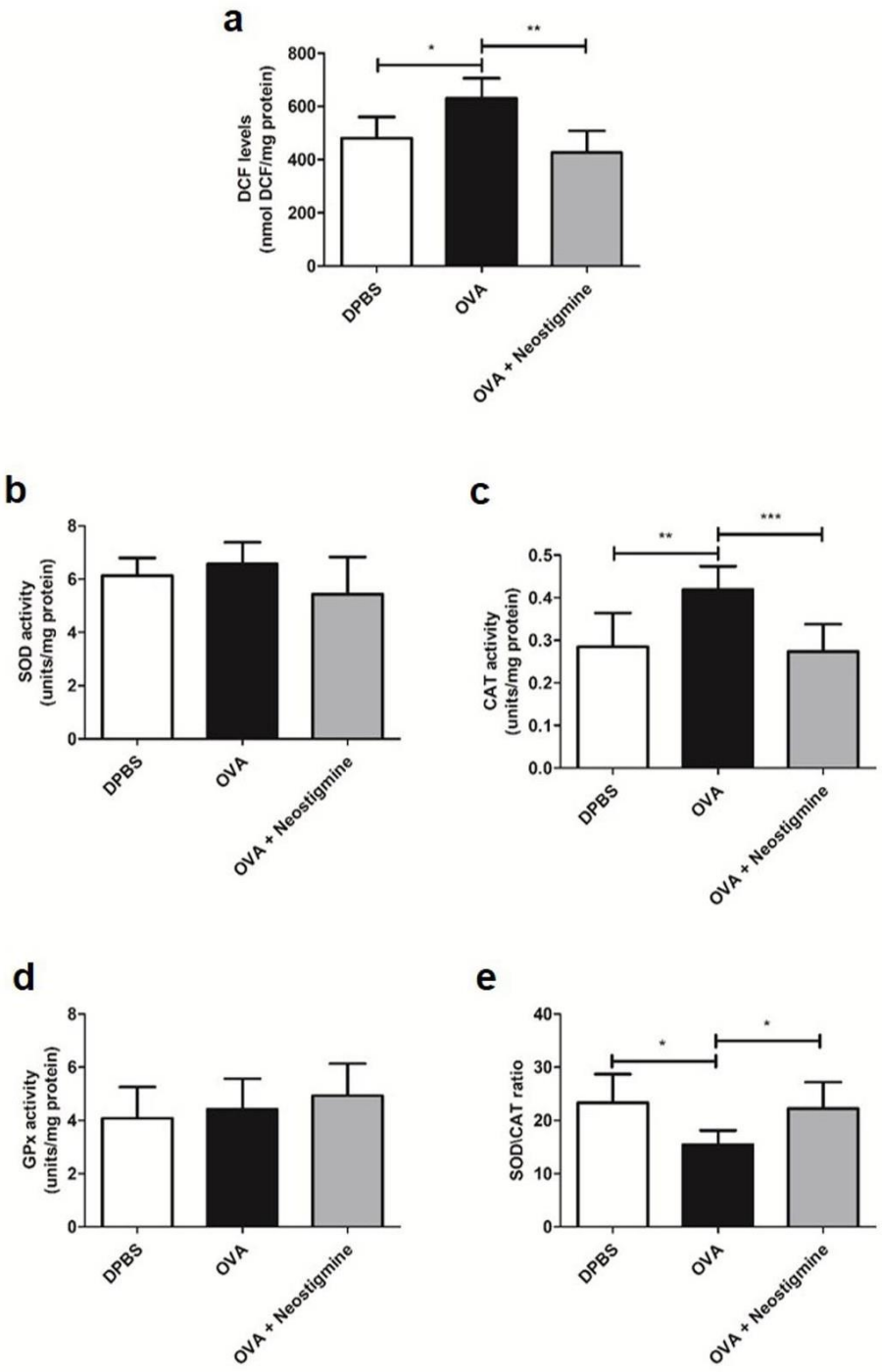

Fig. 4 Treatment with neostigmine improves oxidative stress parameters in the cerebral cortex. (a) Reactive species production by DCF, (b) superoxide dismutase activity, (c) catalase activity, (d) glutathione peroxidase activity (e) SOD/CAT ratio. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group *p<0.05, **p<0.01, different from OVA group *p<0.05, **p<0.01, ***p<0.001 (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

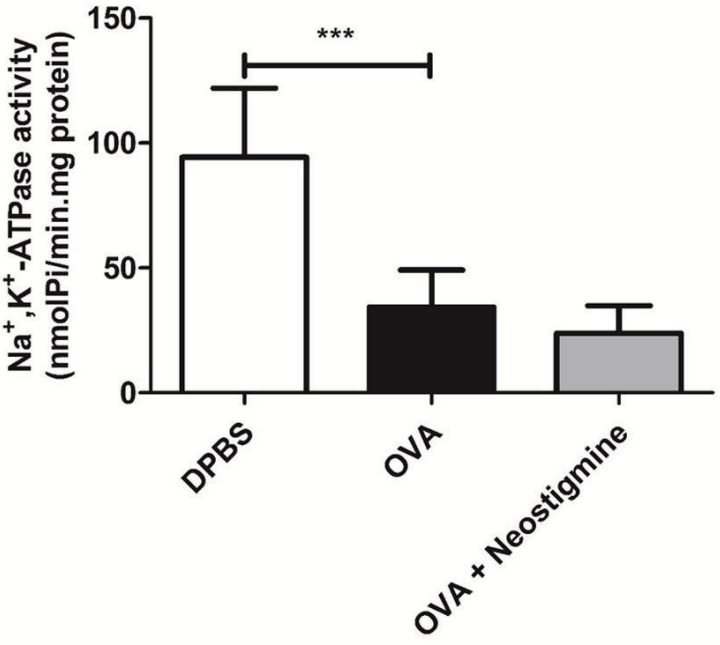

Fig. 5 Effect of neostigmine treatment on Na^+,K^+-ATPase activity in the cerebral cortex. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group ***$p<0.001$ (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

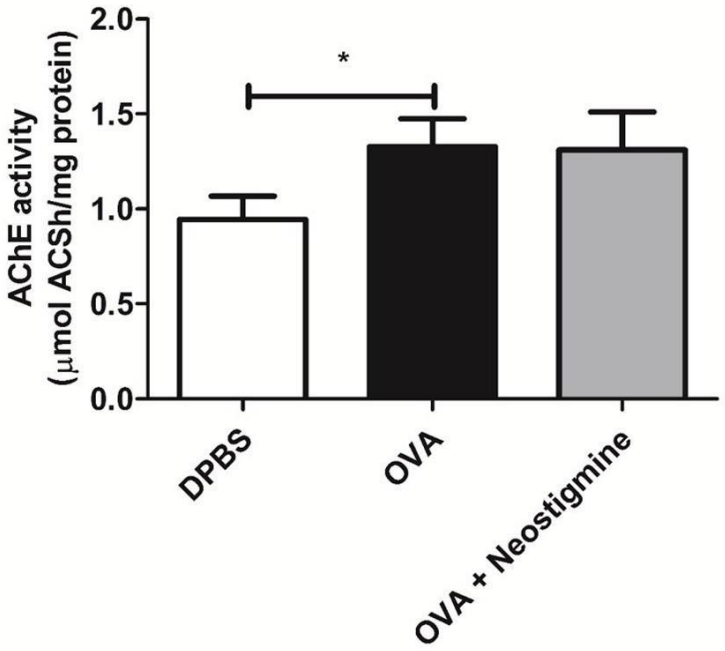

Fig. 6 Effect of treatment with neostigmine on AChE activity in the cerebral cortex. Results are expressed as mean ± SD, for six-eight animals in each group. Different from DPBS group *p<0.05 (One-way ANOVA followed by Tukey test). OVA: ovalbumin; DPBS: Dulbecco's phosphate-buffered saline

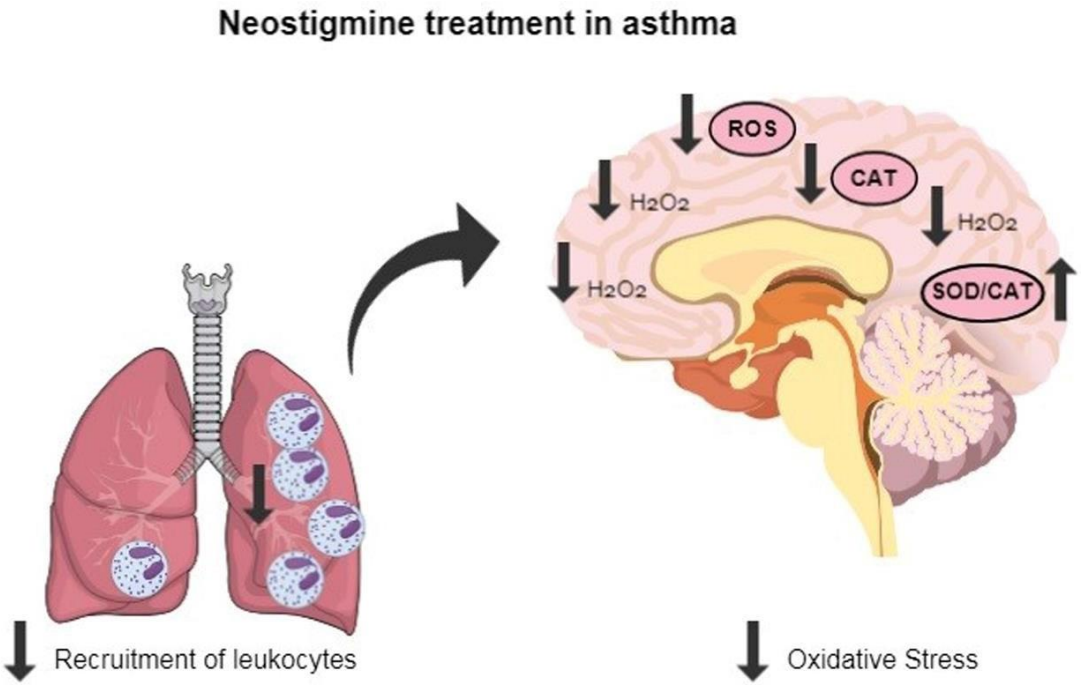

Fig. 7 Summary of the neostigmine effects that lead to neuroprotection against oxidative stress in asthmatic mice. CAT: catalase; SOD: superoxide dismutase; ROS: reactive oxygen species, H_2O_2: hydrogen peroxide