

**UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE
PORTO ALEGRE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA
REABILITAÇÃO**

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**Efeitos do Exercício Físico sobre
Danos Pulmonares e Sistêmicos
Induzidos pela Exposição à Fumaça
de Cigarro em Ratos**

UFCSPA
Universidade Federal de Ciências da Saúde
de Porto Alegre

Porto Alegre

2018

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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Reabilitação da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

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Porto Alegre

2018

Catálogo na Publicação

Bohrer Scherer, Naira Helena

Efeitos do exercício físico sobre danos pulmonares e sistêmicos induzidos pela exposição à fumaça de cigarro em ratos / Naira Helena Bohrer Scherer. -- 2018.

87 p. : 30 cm.

Dissertação (mestrado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Reabilitação, 2018.

Orientador(a): Pedro Dal Lago ; coorientador(a): Gisele Lovatel.

1. exercício físico. 2. exposição à fumaça de cigarro. 3. danos pulmonares. 4. danos sistêmicos. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

**Efeitos do Exercício Físico sobre Danos Pulmonares e
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Ratos**

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2018

Aos meus pais, que sempre me apoiaram e estiveram ao meu lado em todas as minhas decisões.

AGRADECIMENTO

Agradeço, primeiramente, a Deus por ter me dado força e guiado meus passos durante esta caminhada.

Agradeço à minha família, meu pai Ilton Scherer e minha mãe Marta Scherer, dois educadores nas quais sempre me serviram como fonte de inspiração, e à minha irmã Nádia Scherer. Agradeço imensamente por todo apoio, por toda compreensão e pelo incentivo de sempre. Amo muito vocês.

Agradeço ao meu orientador Prof. Dr. Pedro Dal Lago pela oportunidade em integrar um grupo de pesquisa tão renomado como o GPIC, obrigada por todo o seu suporte quando foi preciso, por seus ensinamentos e também por incentivar seus alunos a crescerem cada vez mais.

À Dra. Gisele Lovattel que me co-orientou neste trabalho e que se tornou, em tão pouco tempo, muito mais do que uma co-orientadora, mas sim uma amiga que não mediu esforços em me ajudar em tudo o que fosse preciso. Obrigada pela disponibilidade de sempre, pelas trocas de experiências e pelas palavras de apoio e incentivo. Carregarei comigo não somente os aprendizados da área científica, mas um exemplo de ser humano que leva a humildade, simplicidade e a generosidade na essência.

Aos colegas do GPIC, em especial a Me. Júlia Perini, ao Me. Lucas Capalonga e ao Me. Giuseppe Potrick que me auxiliaram com os procedimentos do laboratório. Agradeço à aluna de iniciação científica Bárbara Schneider pelo empenho e dedicação prestados, mesmo por um curto período de tempo.

E por fim, a todos aqueles que contribuíram de alguma maneira, para a realização deste trabalho: Dra. Rosane Gomez, Me. Solange Bandiera, Joana Fish, Bruna Luivizon, Luana Redel, Lucas Ribeiro, Dra. Alessandra Peres e Me. Gilson Pires Dorneles.

“Acima de tudo, não perca o desejo de prosseguir.”

Soren Kierkegaard

RESUMO

Introdução: O tabagismo é considerado um grave problema de saúde pública em todo o mundo. A fumaça do cigarro contém diversas substâncias tóxicas para o organismo que quando inalada, ativa uma cascata inflamatória intracelular, alterando a homeostase e induzindo, ao longo do tempo, comprometimentos celulares e funcionais, a nível pulmonar e sistêmico, que estão diretamente associados ao desenvolvimento de doenças crônicas. O exercício físico é uma alternativa de tratamento não farmacológica, devido suas propriedades antioxidantes e anti-inflamatórias diminui o risco de doenças crônicas, além de melhorar a força muscular e a capacidade cardiopulmonar. Entretanto, ainda não está claro o seu efeito sobre os danos induzidos pela fumaça do cigarro quando ainda não se tem um processo patológico instalado.

Objetivos: Avaliar o efeito protetor do exercício físico sobre danos pulmonares e sistêmicos induzidos pela exposição à fumaça de cigarro (EFC) em ratos.

Metodologia: Foram utilizados 22 ratos wistar machos (300-400g; 90 dias), divididos em três grupos experimentais: controle (CTRL; n=8), fumante sedentário (F-Sed; n=7) e fumante exercício (F-Ex; n=7). Os animais do grupo CTRL não foram expostos a fumaça de cigarro. Já os animais do grupo F-Sed e F-Ex foram expostos a fumaça de cigarro duas vezes ao dia, por quatro semanas. Os animais foram submetidos a teste de capacidade funcional (distância percorrida (m), tempo para exaustão (s), velocidade máxima (m/min) e consumo máximo de oxigênio com análise de gases em esteira acoplada à caixa metabólica para ratos, realizado em dois momentos (pré-EFC e exercício e após EFC e exercício). Após o período de EFC foram coletados dados sobre a função ventricular esquerda através de ecocardiografia; avaliação da mecânica ventilatória por meio de um ventilador mecânico específico para ratos (Flexy Vent, Scireq) e coleta de tecidos e plasma.

Resultados: Houve perda de peso e inibição do apetite nos animais do grupo F-Sed e F-Ex comparado ao grupo CTRL (-9 ± 15.6 e -12 ± 16.9 vs 46 ± 27.6 ; $p<0,05$). Além disso, houve comprometimento da mecânica pulmonar evidenciado pelo aumento da resistência no sistema respiratório dos grupos F-Sed e F-Ex em relação ao grupo CTRL (0.09 ± 0.01 e 0.10 ± 0.008 vs 0.06 ± 0.008 cmH₂O.s/mL; $p<0.05$). O exercício físico aumentou a distância percorrida dos animais do grupo F-Ex comparado ao grupo CTRL e ao grupo F-Sed (395.5 ± 128.1 vs 191.47 ± 34.8 e 156.7 ± 36.3 m; $p<0.05$), o tempo de corrida (977.3 ± 247.7 vs 645.2 ± 163.1 e 571.7 ± 85.2 s; $p<0.05$) e a velocidade máxima alcançada ($38\pm 2,7$ vs 24.3 ± 4.17 e 23 ± 2.7 m/min; $p<0.05$). Os níveis pulmonares de interleucina (IL)-1 β foram significativamente mais elevados no grupo F-Sed em comparação ao grupo CTRL e ao grupo F-Ex (40.34 ± 5.22 vs 21.9 ± 6.87 e 22.03 ± 8.18 pg/ml; $p<0,05$). Os níveis plasmáticos do fator de necrose tumoral-alfa (TNF- α) foram mais baixos no grupo F-Ex em comparação ao grupo F-Sed (19.37 ± 18.38 vs 45.51 ± 20.48 pg/ml; $p<0,05$). Os níveis de TNF- α no coração foram menores no grupo F-Ex em comparação ao grupo CTRL (6.59 ± 2.92 vs 25.56 ± 16.55 pg/ml; $p<0,05$). Houve uma redução nos níveis de TBARS no pulmão do grupo F-Ex em comparação ao grupo F-Sed (3.70 ± 1.10 vs 5.86 ± 1.33 mmol/mg; $p<0.05$).

Conclusão: Apesar do exercício físico ter demonstrado efeito sobre alterações celulares induzidas pela EFC em ratos, ainda não é possível afirmar que esse

exerça função protetora aos comprometimentos pulmonares e sistêmicos induzidos pelo cigarro.

Palavras-chave: Exposição à fumaça de cigarro; Exercício físico; Inflamação; Dano; Doenças Pulmonares; Capacidade Funcional.

ABSTRACT

Introduction: Smoking is considered a serious public health problem throughout the world. Cigarette smoke (CS) contains several substances toxic to the body that, when inhaled, activate an intracellular inflammatory cascade, altering homeostasis and inducing, over time, cellular and functional impairments, pulmonary and systemic that are directly associated with the development of chronic diseases. Physical exercise is an alternative non-pharmacological treatment because of its antioxidant and anti-inflammatory properties, it decreases the risk of chronic diseases, besides improving muscle strength and cardiopulmonary capacity. However, its effect on cigarette smoke damage is still unclear when a pathological process is not yet in place.

Objectives: To evaluate the protective effect of physical exercise on lung and systemic damages induced by cigarette smoke exposure in rats. **Methods:** Twenty-two male Wistar rats (300-400g, 90 days) divided into three experimental groups: control (CTRL; n=8), smoke-sedentary (S-Sed; n = 7); and smoke-exercise (S-Ex; n=7). The animals in the CTRL group were not exposed to cigarette smoke. The animals of the S-Sed and S-Ex groups were exposed to cigarette smoke twice a day for four weeks. The animals were submitted to a functional capacity test (distance traveled (m), time for exhaustion (s), maximum speed (m/min) and maximal oxygen uptake with a gas analysis on a treadmill coupled to the rat metabolic box, performed in after and before CS period data were collected on left ventricular function through echocardiography, evaluation of ventilatory mechanics by means of a mechanical ventilator specific for rats (Flexy Vent, Scireq) and collection of tissues and plasma. **Results:** There was weight loss and inhibition of appetite in the S-Sed and S-Ex groups compared to the CTRL group (-9 ± 15.6 and -12 ± 16.9 vs. 46 ± 27.6 ; $p < 0.05$). In addition, there was impairment of pulmonary mechanics evidenced by increased resistance in the respiratory system of the S-Sed and S-Ex groups in relation to the CTRL group (0.09 ± 0.01 and 0.10 ± 0.008 vs. 0.06 ± 0.008 cmH₂O.s/mL; $p < 0.05$). Physical exercise increased the distance covered from the S-Ex group compared to the CTRL group and the S-Sed group (395.5 ± 128.1 vs. 191.47 ± 34.8 and 156.7 ± 36.3 m; $p < 0.05$), running time (977.3 ± 247.7 vs. 645.2 ± 163.1 and 571.7 ± 85.2 s; $p < 0.05$) and the maximum velocity achieved (38 ± 2.7 vs. 24.3 ± 4.17 and 23 ± 2.7 ; $p < 0.05$). Pulmonary levels of interleukin (IL) -1 β were significantly higher in the S-Sed group compared to the CTRL group and the S-Ex group (40.34 ± 5.22 vs. 21.9 ± 6.87 and 22.03 ± 8.18 pg/ml; $p < 0.05$). Plasma levels of tumor necrosis factor-alpha (TNF- α) were lower in the S-Ex group compared to the S-Sed group (19.37 ± 18.38 vs. 45.51 ± 20.48 pg/ml; $p < 0.05$). TNF- α levels in the heart were lower in the S-Ex group compared to the CTRL group (6.59 ± 2.92 vs. 25.56 ± 16.55 pg/ml; $p < 0.05$). There was reduction in the lung of the S-Ex group in compared to the S-Sed group (3.70 ± 1.10 vs. 5.86 ± 1.33 mmol/mg; $p < 0.05$). **Conclusion:** Although physical exercise has demonstrated an effect on the cellular changes induced by the CS in rats, it is still not possible to affirm that it exerts a protective function to the pulmonary and systemic compromises induced by the cigarette.

Key words: Cigarette smoking exposure; Exercise; Inflammation; Damage; Lung diseases; Functional Capacity.

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LISTA DE ABREVIATURAS E SIGLAS

AECs	Epitélio das Células Alveolares
CAT	Catalase
CO ₂	Dióxido de Carbono
DAMPs	Padrões Moleculares Associados a Danos
DPOC	Doença Pulmonar Obstrutiva Crônica
DCVs	Doenças Cardiovasculares
FC	Frequência Cardíaca
GOLD	Global Initiative for Chronic Obstructive Lung Diseasev
GPx	Glutaciona Peroxidase
GSH	Glutaciona Reduzida
GSSG	Glutaciona Oxidada
IAM	Infarto Agudo do Miocárdio
IL-1 β	Interleucina-1 beta
IL-6	Interleucina-6
IL-8	Interleucina-8
LDL	Lipoproteína de baixa densidade
MMP-1	Metaloproteases-1
MMP-2	Metaloproteases-2
MMP-9	Metaloproteases-9
MMP-12	Metaloproteases-12
MMP-14	Metaloproteases-14
mTOR	Mammalian Target of Rapamycin
nAChRs	Receptores Nicotínicos de Acetilcolina
NF- κ B	Fator de transcrição nuclear kappa B
NPY	Neuropeptídeo Y
O ₂	Oxigênio
OMS	Organização Mundial da Saúde
ON	Óxido Nítrico
PA	Pressão Arterial
PCR	Proteína C Reativa
PEmáx	Pressão Expiratória Máxima

P _{Imáx}	Pressão Inspiratória Máxima
ROS	Espécies Reativas de Oxigênio
SNC	Sistema Nervoso Central
SNS	Sistema Nervoso Simpático
SOD	Superóxido Dismutase
TBARS	Ácido tiobarbitúrico
TNF- α	Fator de Necrose Tumoral-alfa
TLRs	Receptores do tipo Toll
UPS	Sistema Ubiquitina Proteassoma
VEF ₁	Volume Expiratório Forçado no Primeiro Segundo
VO _{2máx}	Consumo Máximo de Oxigênio

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1 INTRODUÇÃO

O hábito de fumar ainda é uma prática comum em nossa sociedade, mesmo com políticas que incentivam a diminuição do consumo de cigarro. A Organização Mundial da Saúde (OMS) estima que mais de um bilhão de indivíduos façam uso do cigarro mundialmente (WHO, 2016). Isso representa um grave problema de saúde pública, já que o tabagismo é um importante fator de risco para o desenvolvimento de doenças crônicas.

A fumaça do cigarro, contém mais de 4700 substâncias tóxicas ao organismo (VAN DE VAART et al., 2004; TUDER et al., 2012). Esta quando inalada induz comprometimentos deletérios nas vias aéreas e também danos sistêmicos, que se iniciam com o aumento de um processo inflamatório e de estresse oxidativo. A longo prazo, é possível identificar aumento nos riscos de doenças cardiovasculares (DCVs), como a aterosclerose e o infarto agudo do miocárdio (IAM) e doenças pulmonares, como a doença pulmonar obstrutiva crônica (DPOC) (NEMMAR et al., 2013; GARCIA-LUCIO et al., 2018) nas quais culminam em um elevado índice de morbidade e mortalidade na população fumante (MacNEE et al., 2005; HU et al., 2014; CARTER et al., 2015).

Dentre os constituintes tóxicos presentes na fumaça do cigarro, destaca-se a nicotina e o monóxido de carbono como os principais envolvidos na indução dos danos pulmonares, bem como aos comprometimentos sistêmicos decorrentes da inalação desses produtos (VAN DE VAART et al., 2004; TUDER et al., 2012). Inicialmente, a fumaça do cigarro quando inalada induz danos à membrana das células epiteliais das vias aéreas favorecendo a proliferação de células e mediadores inflamatórios, além do aumento de espécies reativas de oxigênio (ROS) no pulmão e no sangue (MADANI et al., 2018). Estudos experimentais demonstram que a exposição crônica à fumaça do cigarro, em um período de seis meses, além de induzir ativação de neutrófilos e macrófagos com consequente aumento de ROS e citocinas pró-inflamatórias no tecido pulmonar, também demonstram lesão parenquimatosa e danos alveolares e intersticiais, resultando em aspectos e características

histológicas semelhantes ao enfisema pulmonar (WORTHAM et al., 2016; KODE et al., 2006). Além disso, marcadores inflamatórios extrapulmonares também foram identificados em outros estudos, propondo que o cigarro induz um processo de inflamação pulmonar e sistêmica com desequilíbrio na resposta antioxidante, mensurado pelos elevados níveis de marcadores inflamatórios e de estresse oxidativo no sangue e no tecido muscular (AGUSTI et al., 2012; SINDEN et al., 2010, KRUGER et al., 2018).

Os efeitos a curto prazo da exposição à fumaça do cigarro, em um período de 30 dias ou mais, também têm sido relatados e demonstram resultados semelhantes. Estudos evidenciam que em um tempo inferior à vinte e quatro semanas observa-se inflamação pulmonar evidenciada pelo influxo de neutrófilos e linfócitos e aumento da permeabilidade epitelial, no entanto, não se observa danos à estrutura pulmonar, o que indica que a fumaça do cigarro induz um processo inflamatório primário que está envolvido diretamente na patogênese da doença pulmonar (NEMMAR et al., 2012; ITOH et al., 2014). Nesse mesmo período de tempo, também tem sido relatado a presença de prejuízos extrapulmonares que envolvem comprometimento ao endotélio vascular, função cardíaca, cérebro, e ainda, ao tecido musculoesquelético (NEMMAR et al., 2013; SINDEN et al., 2010).

Assim, até o presente momento, a busca sobre os efeitos da exposição à fumaça do cigarro tanto a curto como a longo prazo, estão claros e compreendidos. Entretanto, observa-se que a maioria dos estudos tem se detido a pesquisar os efeitos da exposição prolongada à fumaça do cigarro e sobre os mecanismos fisiológicos envolvidos no tratamento de doenças crônicas relacionadas ao tabaco (WANG et al., 2018).

O exercício físico aeróbico é uma alternativa de tratamento não farmacológica, que induz respostas adaptativas em vias sensíveis ao redox, atuando sobre diversos órgãos e tecidos e diminuindo o risco de doenças crônicas, além de melhorar a força muscular e a capacidade cardiopulmonar devido suas propriedades antioxidantes/anti-inflamatórias já evidenciadas podendo ser uma estratégia terapêutica importante sobre os danos induzidos pelo cigarro (GOMES et al., 2016 MALEKIA, et al., 2018).

Foi demonstrado que o exercício físico regular diminui os níveis circulantes de citocinas pró-inflamatórias e de marcadores de estresse oxidativo, além de aumentar citocinas anti-inflamatórias e as defesas antioxidantes em diferentes patologias (FARINHA et al., 2015; PETERSEN et al., 2005; MALEKIA et al., 2018). Na DPOC, um estudo com indivíduos fumantes demonstrou que o exercício físico regular de baixa a alta intensidade, diminuiu as internações hospitalares decorrentes da exacerbação da doença, bem como diminuiu as taxas de mortalidade na população estudada (GARCIA et al., 2006). Outros estudos também observaram efeitos positivos do exercício físico sobre a função pulmonar e na diminuição do risco da DPOC em fumantes ativos, demonstrando que aqueles indivíduos que praticavam exercício de moderada a alta intensidade tiveram uma redução no declínio da função pulmonar e um risco menor de desenvolver DPOC em relação àqueles que praticavam exercício de baixa intensidade (GARCIA, et al., 2007). Além disso, estudos experimentais também demonstram efeitos positivos do exercício físico na diminuição da inflamação das vias aéreas e nos danos pulmonares estruturais em ratos expostos à fumaça crônica do cigarro (TOLEDO et al., 2012; MENEGALI et al., 2009; KRUGER et al., 2018)

Observa-se que muitos estudos têm se detido a investigar os efeitos do exercício físico sobre os danos pulmonares e sistêmicos induzidos pela fumaça do cigarro quando já se tem uma doença crônica estabelecida. Assim, torna-se importante o estudo e compreensão dessa ferramenta como uma estratégia de prevenção, aplicada quando ainda não há a instalação de um quadro patológico irreversível, visando minimizar os comprometimentos induzidos pelo cigarro. Até o presente momento, poucos estudos avaliaram os efeitos protetores do exercício físico sobre as alterações celulares e funcionais induzidas pela exposição à fumaça do cigarro, há uma lacuna na literatura para tentar compreender o mecanismo fisiológico pelo qual o exercício físico atua.

Assim, levando em consideração que os modelos experimentais de tabagismo por inalação à fumaça do cigarro, demonstram ser ideais em reproduzir os efeitos do cigarro em ratos e proporcionam uma investigação mais acurada sobre os aspectos fisiopatológicos envolvidos na progressão de doenças em um rápido período de tempo, o objetivo deste estudo foi investigar

o efeito protetor do exercício físico em danos pulmonares e sistêmicos induzidos pela exposição à fumaça de cigarro em ratos.

2 REVISÃO DE LITERATURA

2.1 TABAGISMO

2.1.1 Epidemiologia, Prevalência e Mortalidade

O consumo do cigarro é antigo, começou a ser fabricado no ano de 1840 e durante essa época era associado à sinônimo de elegância e poder, além de ser muito apreciado em diferentes países. Em 1964, a *Surgeon General's Report* divulgou o primeiro relatório sobre os riscos do cigarro para a saúde humana e, mesmo existindo evidências consistentes sobre os problemas de saúde provocados pelo uso do cigarro, seu consumo anual por adulto aumentou entre as décadas de 1970 a 1990 (Figura 1). A partir desse período, políticas de controle de tabaco passaram a ser implementadas em todo o mundo, e com isso, a partir da década de 90 observou-se um maior cuidado em relação ao consumo de cigarro.

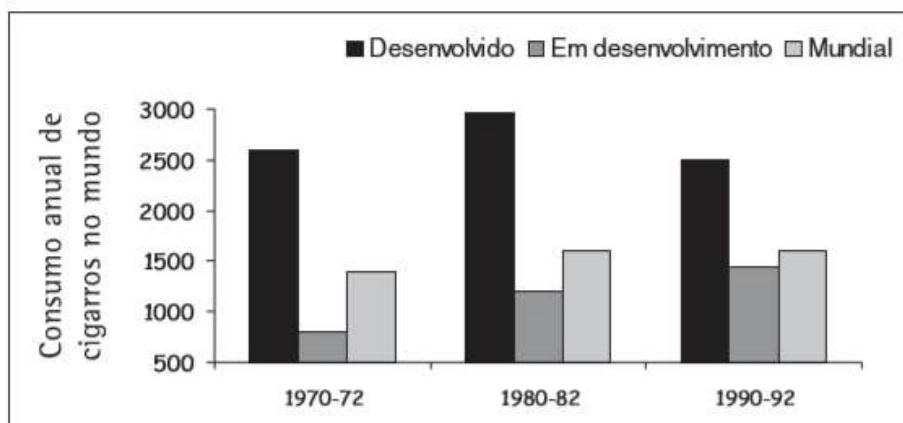


Figura 1 – Consumo anual de cigarros por adulto no mundo entre as décadas de 1970 a 1990. **Fonte:** ARAUJO et al., 2004.

Atualmente, o uso do cigarro está representado como uma epidemia global com um forte impacto social associado e mesmo sendo considerado pela OMS como a principal causa de morte evitável no mundo, o consumo do cigarro ainda é comum em nossa sociedade. Estima-se que um terço da população mundial adulta seja fumante e destes, cerca de 47% são do sexo masculino e 12% são mulheres (BRASIL, 2014). O tabagismo está associado a uma alta prevalência de mortalidade, sendo responsável por mais de cinco

milhões de mortes/ano em todo o mundo, só no Brasil são mais de 200mil mortes/ano em decorrência do cigarro (BRASIL 2014). Desses, 85% das mortes estão relacionadas por DPOC (bronquite e enfisema), 30% por diferentes tipos de câncer, dentre eles, o de pulmão, boca, laringe, faringe, esôfago, pâncreas, rim, bexiga, colo de útero, estômago e fígado; 25% por DCVs (angina e IAM) e ainda 25% por doenças cerebrovasculares, como o acidente vascular cerebral (WHO, 2011). Além disso, homens fumantes entre 45 e 54 anos de idade possuem uma probabilidade maior de morrer por IAM do que os não fumantes da mesma faixa etária (BRASIL, 2003). A OMS estima que o número de fumantes até 2030 passará de 1,2 bilhões para 1,6 bilhões, aumentando também o número mundial de mortes anuais.

No Brasil, cerca de 60% dos homens e 33% das mulheres adultas fumam cigarro e isso ainda está diretamente relacionado com as menores condições socioeconômicas e os menores níveis educacionais (MONTEIRO et al., 2007). E independentemente do sexo, a frequência de fumantes é maior entre adultos de 25 a 65 anos, no entanto, o consumo de cigarro entre adolescentes vem aumentando nas últimas décadas (BRASIL 2014).

Além disso, os altos custos para o sistema de saúde atribuível ao tabagismo estão diretamente associados às doenças tabaco-relacionadas. Em 2011 os custos totais para o sistema de saúde foram de R\$ 23.374.477.024 sendo que a maior parte destes custos foi com DCVs seguidas por DPOC (PINTO et al., 2015). Evidências epidemiológicas apontam uma relação de causalidade entre o tabagismo e diversas doenças crônicas, das quais se destacam as respiratórias, cardiovasculares e as neoplasias, aumentando a taxa de hospitalização e o tempo de internação hospitalar nessa população (TANNI et al., 2010). Um estudo de carga tabágica no Brasil analisou mais de 2.000 doenças e destas, 821 estavam associadas ao cigarro (PINTO et al., 2015). Vale ainda ressaltar que não somente o fumo ativo, mas também o passivo está associado com um aumento considerável sobre os riscos de doença na população fumante, pesquisas apontam que o tabagismo passivo aumenta em 30% o risco para câncer de pulmão e 24% o risco para IAM, além disso, sete não fumantes morrem por dia em consequência do fumo passivo (INCA, 2011).

2.1.2 Componentes Tóxicos da Fumaça de Cigarro

A fumaça de cigarro contém mais de 4700 substâncias tóxicas heterogêneas com um alto nível de radicais livres e moléculas oxidativas, que quando inaladas provocam efeitos deletérios ao organismo. A fumaça de cigarro pode ser dividida em duas fases: uma fase particulada e outra gasosa (DA SILVA, 2004). Dentre as principais substâncias encontradas na fase gasosa, tem-se o monóxido de carbono, amônia, cetonas, formaldeído, acetaldeído, acroleína que representam cerca de 60% da composição da fumaça de cigarro. Já a fase particulada é formada pela mistura de nicotina e alcatrão (TALHOUT et al., 2012). Esses componentes tóxicos estão presentes tanto na fumaça principal, ou “mainstream” correspondente ao fumo ativo, ou seja, é a fumaça inalada pelo fumante como também pela fumaça lateral, ou “sidestream” correspondente ao fumo passivo, que provem da queima da ponta do cigarro (DA SILVA, 2004; CHENG et al., 2016). Apesar de se observar a presença dessas substâncias tóxicas em ambas formas da fumaça, há evidências mostrando que a fumaça lateral apresenta níveis mais elevados de monóxido de carbono, nicotina, alcatrão e amônia (PAKHALE et al., 1997; RUBENSTEIN et al., 2004) sendo mais prejudicial para a saúde humana.

Diversos estudos tem objetivado investigar a relação desses componentes com o risco de doenças tabaco-relacionadas. Alguns estudos demonstram que o alcatrão é o principal produto carcinogênico do cigarro. Isso porque ele contém, dentre outras substâncias, benzopireno que é um hidrocarboneto potentemente carcinogênico (DA SILVA, 2004). Já a nicotina, quando inalada, atua sobre o SNC liberando catecolaminas o que causa vasoconstrição e ativação do sistema nervoso simpático (SNS) que em resposta a isso, ocorre aumento da pressão arterial (PA) e frequência cardíaca (FC). A inalação de nicotina também favorece a agregação plaquetária e o aumento nos riscos de trombose. Já o monóxido de carbono possui alta afinidade com a hemoglobina, os altos níveis de carboxihemoglobina no sangue induz um quadro de hipóxia que compromete a oferta de oxigênio aos tecidos (ZEVIN et al., 2001). Além disso, tanto a nicotina como o monóxido de carbono, causam danos ao endotélio vascular, diminuindo a produção de Óxido

Nítrico (ON) e aumentando os níveis circulantes de lipoproteínas de baixa densidade (LDL), em decorrência disso tem-se o aumento nos riscos de aterosclerose e IAM (ZEVIN et al., 2001; SMITH et al., 2001).

2.1.3 Mecanismo Fisiológico de Dependência da Nicotina

Parar de fumar é muito difícil para a maioria dos fumantes devido a dependência física e psíquica provocada pelos produtos tóxicos derivados do tabaco, dentre eles o principal associado à dependência destaca-se a nicotina.

A exposição à fumaça de cigarro, tanto em sua forma ativa quanto passiva quando inalada percorre as vias aéreas até os pulmões e de lá do epitélio alveolar para a corrente sanguínea. A nicotina, principal constituinte tóxico da fumaça de cigarro, interage com diversos receptores em diferentes tecidos, porém é no cérebro que essa interação com receptores específicos leva a dependência associada ao tabagismo (KIRCHENCHTEJN e CHATKIN, 2004).

Rapidamente, em um período de 7 a 14 segundos a nicotina atinge todas as áreas do cérebro (tálamo, hipotálamo, hipocampo, tronco cerebral e *nucleus accumbens*) chegando até o córtex onde age no sistema mesolímbico-dopamínico (MEIRELLES, 2009). A partir daí, a nicotina é disponibilizada a diversos neurônios do sistema nervoso central (SNC), interagindo com receptores colinérgicos presentes nas membranas de muitos neurônios, já que apresenta estrutura molecular semelhante a da acetilcolina (BALFUR, 1982). Essa interação, ocorre por meio de receptores nicotínicos de acetilcolina (nAChRs), distribuídos nas regiões periféricas e centrais do cérebro que liberam neurotransmissores (ROSEMBERG, 2004; ARAUJO et al., 2004). A exposição crônica a nicotina aumenta a densidade dos receptores nicotínicos e altera a fisiologia do SNC (ARAUJO et al., 2004).

Dentre os neurotransmissores liberados no SNC por ação da nicotina destaca-se a dopamina, serotonina, beta-endorfinas, acetilcolina, noradrenalina, vasopressina, glutamato e ácido gama-aminobutírico (GABA) que exercem atividades específicas diferentes no cérebro (ARAUJO et al.,

2004; MEIRELLES, 2009). A dopamina é o neurotransmissor que está diretamente associado à dependência de nicotina, pois, é o principal neurotransmissor que produz sensação de prazer e euforia (REINA, 2003).

2.1.4 Efeitos Pulmonares do Tabagismo

A exposição à fumaça de cigarro quando inalada exerce efeitos inflamatórios no trato respiratório que danificam o epitélio pulmonar, aumentando o estresse oxidativo e levando a comprometimentos pulmonares imunológicos, estruturais e funcionais. Ruznack et al. (2000) observaram em culturas de células humanas do epitélio pulmonar que a exposição a fumaça de cigarro induz dano na membrana celular e aumento da permeabilidade da mucosa. Em decorrência a isso, o estado imunológico fica comprometido o que favorece a invasão de patógenos agressores que causam infecção e contribuem para o aumento no processo inflamatório (MADANI, 2018). Além disso, outras evidências também demonstram que as substâncias tóxicas do tabagismo alteram a “clearance” mucociliar favorecendo a retenção de muco e predispondo ao aumento de infecções nas vias aéreas (TOHOKARU, 1980; BEHR, 2002).

Os mecanismos fisiológicos para explicar os efeitos da fumaça do cigarro no processo inflamatório pulmonar baseiam-se na ideia de que oxidantes presentes na fumaça de cigarro geram danos às membranas das células alveolares epiteliais (AECs), alterando a sinalização inflamatória em resposta à exposição à fumaça do cigarro. Estas células aumentam a ativação do fator de transcrição nuclear kappa B (NF- κ B), uma via importante na regulação e secreção de citocinas inflamatórias, que uma vez hiperativada aumenta a produção dessas citocinas e metaloproteases no meio intracelular (PARMENTIER et al., 2000; JIMENEZ et al., 2000).

Além disso, danos celulares liberam para o meio extracelular padrões moleculares associados a danos (DAMPs) que aumentam a expressão de receptores do tipo Toll (TLRs) fagocitados por macrófagos (BROZ et al., 2016). Diante disso, ocorre aumento no número de macrófagos e níveis aumentados de metaloproteases (MMP-1, MMP-2, MMP-9, MMP-12 e MMP-14) e citocinas

pró-inflamatórias, como Fator de Necrose Tumoral-alfa (TNF- α) e Interleucinas (IL) tais como: IL-1, IL-1 β , IL-6, IL-8 recrutando neutrófilos e células imunes para o interstício pulmonar (WORTHAM et al., 2016; CROTTY et al., 2015; MADANI et al., 2018) (Figura 2).

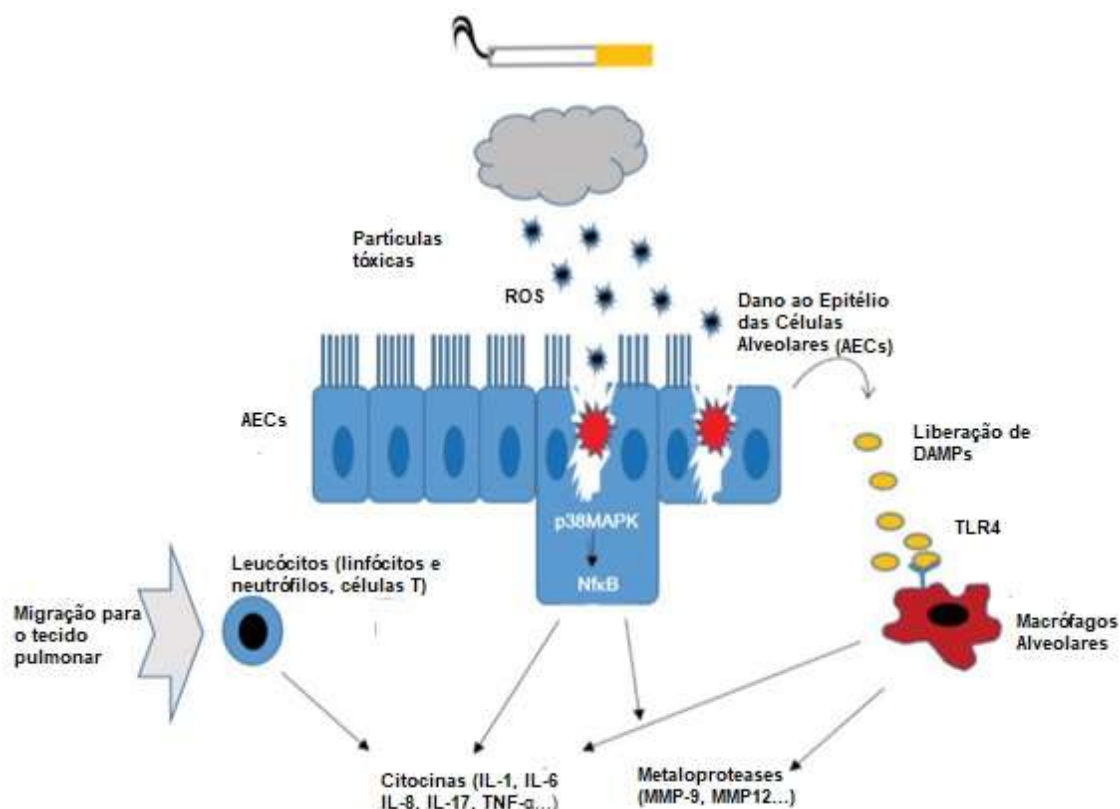


Figure 2. Mecanismo de dano inflamatório nas vias aéreas causado pelo cigarro. **Fonte:** MADANI et al., (2018).

Espécies reativas de oxigênio liberadas por leucócitos e macrófagos envolvidas no processo inflamatório, induzem aumento no estresse oxidativo e um desequilíbrio oxidante/antioxidante (RAHMAN et al., 1996). Em estudos com modelos experimentais, como o de ITOH et al. (2014) que avaliaram a exposição aguda à fumaça do cigarro, em um período de dez dias, os autores observaram que dentro desse período já é possível observar um aumento no número de neutrófilos, macrófagos e linfócitos, além de demonstrar um aumento no estresse oxidativo observados em amostras de lavado bronco-

alveolar de ratos (ITOH et al., 2014). Outros estudos demonstraram que em indivíduos fumantes e em animais expostos a fumaça crônica do cigarro, ocorre aumento da peroxidação lipídica e aumento na degradação de proteínas da matriz extracelular (VAN DE VAART et al., 2004). Além disso, há aumento da oxidação de tióis e da oxidação do DNA (FREI et al., 1991; PARK et al., 1998). Marcadores do estresse oxidativo e de dano ao DNA, estão também significativamente elevados em portadores com DPOC (CEYLAN et al., 2006).

O pulmão apresenta um sistema de defesa com elevada atividade de enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT) e glutathione peroxidase (GPx) (HALLIWELL E GUTRIDGE, 1999). Foi demonstrado que o cigarro aumenta os níveis de glutathione oxidada (GSSG) e diminui a glutathione reduzida (GSH), enzimas importantes envolvidas no sistema antioxidante pulmonar (PARK et al., 1998). Assim, elevadas concentrações de agentes oxidantes no pulmão diminuem a capacidade antioxidante, o que contribui para o dano às células, favorecendo ainda mais a resposta inflamatória (COMHAIR e ERZURUM, 2002; VALENÇA e PORTO, 2008; MENEGALI et al., 2009; AYDIN et al., 2015).

Outro mecanismo envolvido no processo inflamatório é o desequilíbrio protease/antiprotease nos pulmões (MACNEE, 2005). Esse processo induz o predomínio da atividade de enzimas proteolíticas nas vias aéreas que degradam componentes elásticos do pulmão e favorecem anormalidades ventilatórias (TORRES, 2004). Uma possível explicação para isso baseia-se na teoria de que os oxidantes presentes na fumaça de cigarro desencadeiam uma deficiência funcional de α 1-antitripsina que ocorre pela oxidação de metionina em seu sítio ativo (BIETH, 1982; EVANS, 1992). Com a exposição crônica a fumaça do cigarro, tem-se um ciclo vicioso de todos esses efeitos. Há o predomínio de um processo inflamatório pulmonar crônico, capaz de modificar a estrutura fisiológica pulmonar com destruição alveolar e redução da função pulmonar (FÄHNDRICH et al., 2011).

2.1.4.1 Tabagismo e DPOC

A DPOC é a quarta principal morte nos Estados Unidos e deve se tornar a terceira causa de mortalidade em todo o mundo até 2020 (HOYERT, 2012; VIEGI, 2006). O tabagismo é o principal fator de risco para o desenvolvimento de DPOC e essa relação já está bem estabelecida na literatura. Trata-se de uma doença progressiva que se caracteriza por obstrução não totalmente reversível do fluxo aéreo e que afeta, predominantemente, o parênquima pulmonar e as vias aéreas periféricas (BARNES, 2014). Seu desenvolvimento está associado à resposta inflamatória pulmonar em decorrência à exposição a gases ou partículas tóxicas, principalmente, do cigarro. E os mecanismos citados, anteriormente, estão diretamente envolvidos na fisiopatologia dessa doença.

As diretrizes da Iniciativa Global para Doença Pulmonar Obstrutiva Crônica (GOLD) são geralmente usadas para classificar a gravidade das limitações do fluxo aéreo e são classificadas como: leves ($VEF_1 \geq 80\%$ do previsto: GOLD 1), moderado ($50\% \leq VEF_1 < 80\%$ do previsto: GOLD 2), severo ($30\% \leq VEF_1 < 50\%$ do previsto: GOLD 3) ou muito grave ($VEF_1 < 30\%$ do previsto: GOLD 4) (GOLD 2014). Existem duas manifestações distintas de DPOC: a bronquite crônica e o enfisema pulmonar. A primeira, a bronquite crônica, se apresenta com tosse produtiva por pelo menos três meses consecutivos (HOYERT, 2012; SMITH, 2006). Já o enfisema se caracteriza por destruição alveolar, o que resulta em hiperinsuflação pulmonar e uma troca gasosa prejudicada que leva ao aumento de dióxido de carbono (CO_2) e redução de oxigênio (O_2) no sangue (BOUKHENONA, 2018). Por isso, nos casos mais avançados da doença é comum a presença de dispneia e intolerância aos esforços que levam a inatividade física em indivíduos com DPOC (KENYON, 1997; GEA, 2015; MAZZARIN, 2018). O sedentarismo é um importante preditor de mortalidade em indivíduos com DPOC e, por isso, as diretrizes da Sociedade Americana Torácica e da Sociedade Respiratória Europeia destacam a importância do exercício no tratamento da DPOC (SPRUIT et al. 2001).

Evidências apontam que em um período de quatro à seis meses de exposição à fumaça de cigarro, ocorre o desenvolvimento de enfisema em camundongos, ratos e coelhos (SHAPIRO et al., 2000). Esses estudos apontam que a fumaça do cigarro induz destruição da parede alveolar pela interação de apoptose, estresse oxidativo e desequilíbrio protease/antiprotease. Dessa interação ocorre o enfisema, o que leva à perda progressiva da função pulmonar devido à destruição do parênquima pulmonar e da inflamação crônica.

2.1.4.1.1 *Comprometimentos na mecânica ventilatória*

Os prejuízos na mecânica ventilatória podem ser classificados em comprometimentos pulmonares elásticos e resistivos. A redução do fluxo aéreo, principal alteração funcional no DPOC, pode ser justificada, respectivamente, pela redução e aumento nesses comprometimentos.

Alguns fatores contribuem para essas alterações. Barnes et al. 2014, aponta que o aumento na resistência das vias aéreas ocorre porque há espessamento epitelial, formação de folículos linfoides e deposição de colágeno que reduzem o diâmetro interno bem como a capacidade de distensão nas vias aéreas. Além disso, outros fatores como o aumento de muco e alterações na arquitetura pulmonar podem contribuir para a redução do fluxo aéreo e ao aumento da resistência nas vias aéreas (LANGE et al., 1990; BARNES et al., 2004). Já em relação à redução dos componentes elásticos, é possível que essa alteração ocorra por destruição do parênquima pulmonar e das fibras elásticas. Isso diminui a elastância pulmonar gerando um menor fluxo expiratório (TANAKA et al., 2012). Assim, modificações na mecânica ventilatória influenciam, diretamente, a função pulmonar.

A função pulmonar é um comprometimento comum em pacientes com DPOC. Normalmente, esses indivíduos apresentam fraqueza muscular respiratória e baixo volume expiratório forçado no primeiro segundo (VEF_1), que se configura como uma medida importante no desfecho e avaliação do curso clínico da doença (LEEMING, 2017; NASHIMURA, 2012; MONTES, 1997). Os principais achados relacionados a isso demonstram que,

microscopicamente, observa-se um padrão miopático associado à atrofia generalizada das fibras do tipo IIX nos músculos da respiração (DECRAMER, 2001). Além disso, em pacientes mais graves há fraqueza muscular diafragmática associada, que se deteriora progressivamente (MONTES, 1997). Outros prejuízos também foram relatados e incluem redução nas pressões inspiratória e expiratória máxima (PI_{máx} e PE_{máx}) e redução na capacidade residual funcional (MONTES, 1997; DECRAMER, 1997).

2.1.4.1.3 *Disfunção muscular*

A disfunção muscular é outro comprometimento importante do tabagismo para pacientes com DPOC (GOSKER et al., 2003). Essa pode envolver tanto os músculos respiratórios quanto os periféricos causando consequências significativas para esses indivíduos, pois, além de diminuir a tolerância ao exercício também reduz a qualidade de vida (DECRAMER, 1997; MATHUR et al., 2014). Estudos apontam que há uma relação do TNF- α com a fraqueza muscular periférica, acredita-se que essa citocina esteja envolvida no processo de atrofia que induz, posteriormente, à caquexia. O aumento no estresse oxidativo também está relacionado com fadiga muscular e facilitação da proteólise muscular (BARNES et al., 2014).

2.1.5 Efeitos Sistêmicos do Tabagismo

Frequentemente, os efeitos do tabagismo vão além das manifestações exclusivamente pulmonares. É muito comum de se observar perda de peso em fumantes, associação com doenças cardiovasculares, distúrbio muscular periférico entre outros (AUGUSTI, 2001).

A fumaça do cigarro uma vez inalada atinge os pulmões e por meio da corrente sanguínea chega a outros tecidos causando efeitos deletérios à célula e estrutura tecidual. Como já mencionado anteriormente, o monóxido de carbono possui uma alta afinidade à hemoglobina e por isso, há elevados níveis de carboxihemoglobina no sangue, um composto estável que pode ser mensurado em fumantes e que induz um quadro de hipoxemia (ARONO, 1971). Tem sido sugerido que fumantes ativos apresentam níveis superiores ao

dobro de carboxihemoglobina no sangue quando comparados aos não fumantes. A falta de oxigênio à célula leva a um predomínio do metabolismo anaeróbio com excessiva produção de oxidantes ocasionando lesão em diferentes tecidos (TORRES et al., 2004). Estudos em humanos demonstram um aumento significativo no número de neutrófilos e no estresse oxidativo no sangue de fumantes. Resultados semelhantes a esses também foram identificados em modelos animais e em células *in vitro* (SHOJI, 1995; CAVARRA, 2001).

Evidências apontam que a exposição à fumaça do cigarro leva a um processo de inflamação sistêmica crônica que se apresenta com estresse oxidativo sistêmico, ativação de células inflamatórias circulantes e aumento nos níveis de citocinas pró-inflamatórias como TNF- α e outros como Proteína C-reativa (PCR) e IL-8 e IL-6 no sangue de indivíduos fumantes e com DPOC (AUGUSTI, 2001; EIDI, 2001). No estudo de Ardite et al. (2006) os autores avaliaram os efeitos da exposição aguda e crônica à fumaça de cigarro. Os animais foram expostos em um período de 2, 4 e 6 meses. Com objetivo de avaliar os efeitos sistêmicos, no músculo e no plasma, em diferentes tempos de exposição, os autores demonstraram que o cigarro induz um efeito oxidativo transitório e repetido que resulta em prejuízos metabólicos sistêmicos.

2.1.5.1 Tabagismo e danos ao sistema cardiovascular: alterações hemodinâmicas, estruturais, morfológicas e funcionais do coração.

Nos últimos anos, têm sido crescente a investigação sobre a relação entre tabagismo e o risco de doenças cardiovasculares. Evidências até o presente momento apontam que fumantes possuem um risco maior de desenvolver aterosclerose, hipertensão e ainda IAM quando comparados a não fumantes (TILTON, 2013). A inflamação crônica gerada em decorrência à exposição à fumaça de cigarro torna-se um fator importante no desenvolvimento dessas comorbidades cardíacas (DE SÁ, 2014).

Os efeitos do cigarro para o sistema cardiovascular envolvem desde danos ao endotélio vascular até alterações hemodinâmicas, morfológicas, estruturais e funcionais do coração. Foi demonstrado que a exposição crônica

à fumaça de cigarro em animais, pode resultar em cardiomiopatia com progressiva e irreversível deterioração da função cardíaca associada a fibrose intersticial (ARMANI et al., 2009; MINICUCCI et al., 2009) além de favorecer processos inflamatórios nas células endoteliais gerando aterogênese precoce (VAYSSIER, 2001). Conforme Messner e Bernhard (2014) o processo de aterogênese se inicia pela redução da biodisponibilidade do óxido nítrico (NO) e também pelo aumento da expressão de moléculas de adesão que causam disfunção endotelial. Além disso, o tabagismo induz aumento da aderência de plaquetas e macrófagos provocando o desenvolvimento de um ambiente pró-coagulante e inflamatório (MESSNER et al., 2014).

A vasoconstrição é um dos fatores que induz alterações hemodinâmicas, como o aumento na pressão arterial, em fumantes (CASTARDELI et al., 2005). A nicotina está diretamente associada à esse processo. Quando inalada, libera catecolaminas e por meio de estimulação alfa-adrenérgica (α -adrenérgica) causa vasoconstrição em grandes artérias, como a artéria coronária e aumento do tônus em vasos de resistência (TAKAHIRO et al., 1998). Além de danificar as paredes dos vasos sanguíneos, tornando-os menos elásticos (JÚNIOR; TOLEDO; MELO, 2005). Resumidamente, evidências demonstram que o tabagismo aumenta a pós-carga cardíaca, promove um status pró-trombótico, reduz a fibrinólise, altera o perfil dos lipídios circulantes, promove infiltração de neutrófilos no miocárdio, altera a função das células T e ainda causa danos de DNA no miocárdio (TILTON et al., 2013; DAS et al., 2012; IZZOTI et al., 2008).

Em modelos experimentais, Castardeli et al. (2005) observaram alterações morfológicas do ventrículo esquerdo. Os autores submeteram animais por quatro meses de exposição à fumaça de cigarro. Como resultado, demonstraram que o átrio esquerdo encontrava-se maior e, além do mais, também observaram aumento no diâmetro diastólico e sistólico do ventrículo esquerdo resultando em remodelação cardíaca, quando comparados aos animais não expostos ao tabagismo. Em um período de exposição menor, PAIVA et al. (2003) demonstraram que a exposição a fumaça de cigarro durante 30 dias não causou alterações no músculo papilar isolado em ratos,

entretanto, os autores observaram um aumento no diâmetro sistólico final do ventrículo esquerdo e diminuição da fração de ejeção e de encurtamento sistólico quando comparado ao grupo que não foi exposto a fumaça de cigarro. Estudos em humanos corroboram com esses achados, demonstrando que fumantes possuem maior massa ventricular esquerda em comparação com não fumantes (GIDDING et al., 2005).

2.1.6 Modelos Experimentais de Tabagismo

Os modelos experimentais de tabagismo demonstram boa acurácia para avaliar os efeitos fisiopatológicos da inalação à fumaça do cigarro. Assim, existem na literatura diferentes protocolos com que os animais são expostos ao cigarro, variando desde os equipamentos utilizados até o tempo e a quantidade de cigarros. Além disso, é possível encontrar alguns estudos que não utilizam o método de inalação à fumaça e sim, a instilação pulmonar de enzimas elastolíticas como a Papaína, por exemplo, que é capaz de induzir enfisema pulmonar em cerca de um mês (SHAPIRO et al., 2000), no entanto, apesar de gerar danos pulmonares em um período de tempo relativamente curto, seu uso se torna limitante para avaliar e mimetizar os reais efeitos sistêmicos decorrentes da inalação à fumaça do cigarro. Assim, o método por inalação a fumaça de cigarro mostra-se ser mais consistente, além de ser bem aceito e utilizado na literatura.

Esse método foi descrito por Le Mesurier et al., (1981) e consiste de um equipamento gerador de fumaça de cigarro em uma câmara hermeticamente fechada, onde os animais são confinados e expostos à fumaça por corpo inteiro. O tempo de exposição entre esses modelos é variável, podendo variar de quatro dias até oito meses de inalação à fumaça (NEMMAR et al., 2013; KRUGER et al., 2018). Apesar disso, o que já se sabe é que dentro das primeiras 24h de exposição à fumaça de cigarro, já é possível observar o aumento de neutrófilos e macrófagos no tecido pulmonar e no lavado broncoalveolar, sem induzir dano pulmonar funcional e estrutural (VAN DE VAART et al., 2014). Para isso, já está bem estabelecido que um tempo de exposição à fumaça do cigarro superior a seis meses é capaz de induzir enfisema pulmonar em modelo animal, sendo capaz de representar aspectos

semelhantes às alterações ocorridas em seres humanos. Arruda et al. (2017) avaliaram diferentes tempos de exposição a fumaça do cigarro (4, 8 e 12 semanas) e demonstraram que nos três primeiros meses ocorreu aumento no infiltrado inflamatório pulmonar, mas, apenas após 12 semanas é que se observou aumento dos espaços aéreos, sugerindo que a partir desse período os danos pulmonares estruturais começam a ser mais evidentes.

Além do tempo de exposição que pode variar, a forma como o cigarro é queimado também pode ser diferente entre os estudos. Dessa forma, pode-se dizer que existem dois métodos distintos: o “mainstream” e o “sidestream” (CHENG et al., 2016). O primeiro corresponde ao fumo ativo – “mainstream” que se refere à fumaça inalada pelo fumante, sendo aquela que passa pelo filtro do cigarro. Já o segundo corresponde ao fumo passivo – “sidestream”, a fumaça inalada é derivada da ponta do cigarro (CHENG et al., 2016). Alguns estudos buscaram investigar as diferenças entre esses dois métodos e demonstraram que os componentes da fumaça “sidestream” apresentam-se mais insaturados e menos oxigenados (SCHRAMM et al, 2011). Ademais, há maior concentração de nicotina e monóxido de carbono na fumaça proveniente da ponta do cigarro e, por isso, esta seria mais tóxica em relação a fumaça “mainstream” (CHENG et al., 2016).

O modelo de exposição à fumaça de cigarro utilizado no presente estudo utiliza o método de inalação “sidestream”. Esse modelo, inicialmente, reproduzido por Pereira et al. (2007) que analisaram a inalação de partículas de ar poluente sobre as respostas inflamatórias em pulmões de ratos e, foi, posteriormente, modificado por Gomez et al. (2015) que avaliaram a exposição a fumaça de cigarro por 30 dias no hipocampo de ratos wistar, consiste da inalação a fumaça de cigarro por meio de uma câmara hermeticamente fechada conectada a uma bomba à vácuo com um fluxo constante de 10L/min, que mantem o cigarro aceso e conduz a fumaça para interior da caixa. Esse modelo além de fácil reprodutibilidade e baixo custo possibilita uma investigação acurada dos efeitos do tabagismo de forma sistêmica e por isso, foi utilizado no presente estudo.

2.2 EXERCÍCIO FÍSICO AERÓBICO

2.2.1 Evidências dos Efeitos Anti-inflamatórios e Anti-oxidantes

O exercício físico aeróbico regular tem sido demonstrado como uma opção terapêutica não farmacológica que atua como estratégia de prevenção e tratamento para diversas doenças metabólicas e cardiorrespiratórias (GLEESON et al., 2011). Sugere-se que os efeitos benéficos do exercício ocorram pela melhora na força e resistência muscular (PINHO et al., 2007), aumento no consumo máximo de oxigênio (McARDLE et al., 1998) e melhora na capacidade cardiorrespiratória, além de atuar como um potente anti-inflamatório e antioxidante (MENEGALI, et al., 2009; YU et al., 2012). As respostas adaptativas ao exercício dependem de vários fatores que correspondem ao tempo de exercício, intensidade e duração (GARBER et al., 2011).

Evidências demonstram que o exercício físico exerce seus efeitos de regulação imunológica ativando vias de sinalização anti-inflamatórias sendo capaz de reverter prejuízos sistêmicos (Figura 3) (BOWEN et al., 2017; EMMONS et al., 2017; GLEESON et al., 2011; PEDERSEN et al., 2017). Observa-se a expressão reduzida de receptores do tipo Toll (TLRs) em monócitos e macrófagos o que resulta na inibição na produção de citocinas pró-inflamatórias após a realização do exercício (FLYNN et al., 2006). Durante e após o exercício físico agudo ocorre um aumento transitório de IL-6 pela contração muscular, em resposta a isso os níveis circulantes de citocinas anti-inflamatórias, como a IL-10 e IL-1RA, se elevam (GLEESON et al., 2011). A IL-1RA, especificamente, é um limitante dos efeitos da citocina pró-inflamatória IL-1 β e, por isso, tem um importante papel na regulação anti-inflamatória induzida pelo exercício (PEDERSEN, et al., 2017).

A ação antioxidante do exercício tem início com um aumento agudo do metabolismo oxidativo após a prática imediata de exercício, isso produz um ambiente pró-oxidativo, mas, que não causa danos à estrutura celular. Quando o exercício é repetido, o organismo possui a capacidade em se adaptar frente a esse estresse reduzindo enzimas oxidativas que geram danos celulares (LI et

al., 2002). Nesta perspectiva, dada sua importante ação anti-inflamatória e antioxidativa, estudos tem se detido a investigar os efeitos do exercício sobre os danos causados pelo tabagismo. Hassel et al. (2016), avaliaram os efeitos do exercício físico na função ventricular direita em modelo animal DPOC. Os autores observaram que os animais exercitados e expostos a fumaça de cigarro tiveram um $VO_{2\text{pico}}$ maior do que os sedentários também tabagistas. Além disso, a função sistólica no ventrículo direito apresentou-se diminuída e através do exercício físico esta redução foi atenuada.

Com o objetivo de avaliar os efeitos do exercício físico regular de moderada intensidade em camundongos expostos a fumaça crônica do cigarro, Toledo et al. (2012) expuseram os animais durante 24 semanas e após esse período submetem os camundongos a oito semanas de exercício físico contínuo. Os principais achados foram redução na elastância pulmonar no grupo treinado, redução de ROS no lavado broncoalveolar e 8-isoprostano no tecido pulmonar. Também observaram redução na IL-10 e na CuZn superoxide dismutase (CuZnSOD) demonstrando assim que o exercício físico de moderada intensidade atenua o desenvolvimento da DPOC induzida pela exposição à fumaça crônica de cigarro. Dentro desse mesmo contexto, Yu et al. (2012) avaliaram os efeitos do exercício físico realizado em um período de oito semanas após quatro semanas de exposição a fumaça de cigarro. Os autores observaram que nos animais expostos à fumaça de cigarro houve aumento significativo da permeabilidade capilar bronco-alveolar, infiltração de células inflamatórias, espessamento epitelial e ativação de NF- κ B em amostras do tecido pulmonar. No grupo treinado os autores demonstraram que o exercício foi capaz de inibir NF- κ B reduzindo a produção de mediadores inflamatórios.

Recentemente, Krüger et al. (2015) avaliaram os efeitos do exercício físico sobre a inflamação e perda de massa muscular em ratos expostos a fumaça de cigarro durante oito meses. Os animais foram exercitados em um período de dois meses sendo que o protocolo de exercício iniciou-se no sexto mês de exposição ao cigarro. Os resultados do estudo demonstram que ao final do período de experimentação os animais fumantes tiveram uma redução no consumo máximo de oxigênio $VO_{2\text{máx}}$ sendo revertido pelo exercício físico no grupo treinado. A expressão de células imunes apresentou-se aumentadas

no grupo fumante sedentário e reduzidas no grupo fumante exercício. Além do mais, a fumaça de cigarro induziu aumento de citocinas inflamatórias avaliadas no plasma e no tecido muscular inibiu a sinalização da via mTOR, uma quinase importante com função de crescimento celular. O exercício físico reverteu esses prejuízos e ainda melhorou a estrutura, capacidade metabólica oxidativa, inativando o sistema ubiquitina proteasoma (UPS) e estimulação da expressão de fatores de crescimento no quadríceps e tríceps sural.

O efeito protetor do exercício físico realizado antes da exposição a fumaça do cigarro também tem sido relatado. Nesi et al. (2016) submetem camundongos à natação em um período de oito semanas. Após, os animais foram expostos a fumaça do cigarro por dois meses. Os autores observaram que a exposição à fumaça do cigarro induziu alterações histológicas nos pulmões e o treinamento físico impediu essa progressão, atenuando a infiltração de macrófagos nos espaços aéreos, melhorando os aspectos das fibras elásticas e ainda melhora no mecanismo de defesa antioxidante.

Em humanos com DPOC, tem sido demonstrado efeitos satisfatórios do exercício físico em programas de reabilitação cardiopulmonar (LACASSE et al., 2006; McCARTHY et al., 2015). Para determinar o impacto da reabilitação na qualidade de vida e capacidade máxima de exercício em pacientes com DPOC, Lacasse et al., 2007 realizaram uma meta-análise sobre estudos que abordassem um protocolo de treinamento físico por um período mínimo de quatro semanas. Foram selecionados 31 ensaios clínicos randomizados que preencheram os critérios de inclusão. Os autores apontam que a reabilitação alivia a dispneia e a fadiga, melhora a função emocional e aumenta o controle dos pacientes sobre sua condição. Essas melhorias são moderadamente grandes e clinicamente significativas. Além disso, a reabilitação constitui um componente importante no manejo da DPOC.

Assim, tendo em vista os efeitos sistêmicos inflamatórios e oxidativos do tabagismo em diferentes tecidos corporais, sugere-se que o exercício físico, dado seus benefícios evidenciados, possa ser capaz de modular e minimizar os prejuízos induzidos pelo cigarro, por isso torna-se importante o estudo de seus efeitos sobre os danos induzidos pela exposição à fumaça do cigarro em ratos.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito protetor do exercício físico sobre os danos pulmonares e sistêmicos induzidos pela exposição à fumaça de cigarro em ratos wistar.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito protetor do exercício físico sobre a capacidade funcional (distância, tempo, velocidade e $VO_{2máx}$) em ratos expostos a fumaça de cigarro.

- Avaliar o efeito protetor do exercício físico sobre os níveis plasmáticos, pulmonares e cardíacos de mediadores inflamatórios (TNF- α e IL-1 β) em ratos expostos a fumaça de cigarro.

- Avaliar o efeito protetor do exercício físico sobre os níveis plasmáticos, pulmonares e cardíacos de Catalase (CAT) em ratos expostos a fumaça de cigarro.

- Avaliar o efeito protetor do exercício físico sobre os níveis plasmáticos, pulmonares e cardíacos de Glutathione Peroxidase (GPx) em ratos expostos a fumaça de cigarro.

- Avaliar o efeito protetor do exercício físico sobre os níveis plasmáticos, pulmonares e cardíacos de peroxidação lipídica (TBARS) em ratos expostos a fumaça de cigarro.

- Avaliar o efeito protetor do exercício físico sobre a mecânica ventilatória em ratos expostos a fumaça de cigarro.

- Avaliar o efeito protetor do exercício físico sobre a função ventricular esquerda em ratos expostos a fumaça de cigarro.

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4 ARTIGO

Protective effect of physical exercise on damage induced by exposure to cigarette smoke in rats

(A ser submetido ao periódico *Experimental Physiology*
Fator de Impacto: 2.91; Qualis A2).

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New findings

- **What is the central question of this study?**

Exposure to cigarette smoke induces pulmonary and systemic damage and physical exercise may be a treatment strategy used in attempting to reduce such damage and prevent the development of chronic diseases.

- **What is the main finding and its importance?**

We show the acute effects of exposure to cigarette smoke over a period of 30 days induce functional and cellular compromises at the pulmonary level that do not compromise functional capacity in rats, suggesting that these are primary manifestations involved in the pathogenesis of diseases. Physical exercise has a protective effect on cellular and functional alterations, however it is still not possible to affirm that it exerts a protective function to the pulmonary and systemic compromises induced by the cigarette.

Abstract

Smoking exposes the individual to toxic substances contained in cigarette smoke leading to pulmonary and systemic impairments que a longo prazo induzem doenças crônicas. Exercise can be a strategy of prevention and treatment reducing the damage caused by the cigarette. The objective of this study was to evaluate the protective effect of exercise on lung and systemic damages induced by exposure to cigarette smoke. Twenty-two male Wistar rats were exposed to cigarette smoke for 30 days and assigned to three experimental groups: Control (CTRL; n=8): not exposed to cigarette smoke and sedentary; Smoking sedentary (S-SED; n=7): exposed to cigarette smoke and sedentary; and Smoking-Exercise (S-EX; n=7): trained and exposed to cigarette smoke. After four weeks, the exposure to cigarette smoke induced loss body weight (P=0.001) and reduced food intake (P=0.049). There was an increase in respiratory system resistance in animals exposed to cigarette smoke (P=0.001). Lung levels of interleukin-1 β increased in the S-SED group (P=0.004) and were reduced in S-EX (P=0.006). Exercise improved functional capacity in the S-EX group with increased at a distance traveled (P=0.000), time until exhaustion (P=0.008) and maximum velocity (P=0.002). In addition, there was a decrease in levels of the TNF- α in plasma (P=0.048) and of the heart (P=0.009) in the S-EX group. TBARS levels in the lung of the S-EX decreased compared S-SED (P=0.044). Although physical exercise has demonstrated an effect on the cellular changes induced by the CS in rats, it is still not possible to affirm that it

exerts a protective function to the pulmonary and systemic compromises induced by the cigarette.

Keywords: Tobacco; physical exercise; functional capacity; inflammation.

Introduction

Cigarette smoking is a habit present in our society, despite public policies to reduce tobacco consumption. The World Health Organization (WHO) estimates that more than a billion people worldwide are smokers. This is a serious public health issue since smoking is a significant risk factor for chronic diseases, including chronic obstructive pulmonary disease (COPD) and cardiovascular disease such as acute myocardial infarction (García-Lucio et al., 2018; Nemmar et al., 2013). Cigarette smoke contains more than 4,700 compounds that are toxic to the human body (Tuder & Petrache, 2012; van der Vaart, Postma, Timens, & Ten Hacken, 2004). When inhaled, it induces local and systemic deleterious effects, such as increased inflammatory response and oxidative stress (van der Vaart et al., 2004), protease-antiprotease imbalance (MacNee, 2005), increased risk for atherosclerosis (Benowitz & Burbank, 2016), along with pulmonary function decline and exercise intolerance (Hu, Zhao, Ma, Wang, & Zheng, 2014), which culminates in elevated mortality and morbidity rates in the smoking population (Carter et al., 2015).

Different cigarette-induced lung impairments have in common the presence of inflammatory cells and reactive oxygen species (ROS), involved in the progression of pulmonary tissue lesions. Acute effects of exposure to tobacco smoke can be observed within hours of inhalation; there is an increased influx of neutrophils, macrophages, lymphocytes, and increased epithelial permeability in the lung which initially do not induce structural damage (Kode, Yang, & Rahman, 2006; Toledo et al., 2012). On the other hand, chronic exposure to tobacco smoke, in a period longer than three months, has been shown to induce pulmonary emphysema. In addition to the increased number of neutrophils and macrophages, resulting in increased levels of pro-inflammatory cytokines (Wortham et al., 2016) and ROS in the airway with enhanced airway resistance, parenchymatous lesion with alveolar and interstitial damage is also

observed (Kode et al., 2006), as well as loss of pulmonary elastic component (Toledo et al., 2012).

Inflammatory biomarkers such as TNF- α and IL-1 β are involved in the inflammatory cascade induced by smoking. In fact, an inflammatory response is present not only in the lung but also at a systemic level, with an imbalanced antioxidative response, since elevated levels of these markers were observed in both the lung and the bloodstream (Agustí & Faner, 2012; Sinden & Stockley, 2010). Extrapulmonary effects of smoking were also identified in vascular endothelium, heart, liver and skeletal muscle (Nemmar et al., 2013; Sinden & Stockley, 2010). However, there is still a gap in literature in attempting to understand the cellular and functional changes involved in the pathogenesis of chronic diseases.

In addition, it is important to think of intervention strategies within a period of time when there is still no irreversible structural and functional cellular damage. Therefore, several treatment strategies that present preventive and therapeutic effects against the damage caused by smoking have been proposed. Among them, aerobic exercise is a non-pharmacological alternative treatment that has been investigated for its antioxidant and anti-inflammatory effects (Maleki et al., 2018). Exercise induces adaptive responses in pathways that are sensitive to the redox state, thus acting on multiple organs and tissues, reducing the risk of chronic diseases, and improving muscular strength and cardiopulmonary capacity (Gomes et al., 2016). Regular exercise can reduce the circulating levels of pro-inflammatory cytokines and oxidative stress markers, while increasing anti-inflammatory cytokines and antioxidants in distinct pathologies (Farinha et al., 2015; Maleki et al., 2018; Petersen & Pedersen, 2005). In COPD, a cohort study demonstrated that regular physical exercise, from low to high intensity, reduced hospital admissions due to disease exacerbation and decreased the mortality rate in the studied population (J Garcia-Aymerich, Lange, Benet, Schnohr, & Anto, 2006). Other studies also observed positive effects of exercise on pulmonary function and COPD risk reduction in active smokers, demonstrating that those individuals who performed moderate to high-intensity training had reduced decline of pulmonary function and a lower risk of developing COPD in comparison with those who

performed the low-intensity training (Judith Garcia-Aymerich, Lange, Benet, Schnohr, & Antó, 2007). Furthermore, experimental studies observed that exercise reduced airway inflammation and oxidative damage in rats chronically exposed to cigarette smoke (Krüger et al., 2018; Menegali et al., 2009; Toledo et al., 2012). However, few studies have investigated the effects of physical exercise on smoking-induced damage before disease onset, when manifestations are still reversible. Thus, considering the high prevalence of smoking in the population, the study of prevention strategies is increasingly relevant to mitigate the multiple impairments that result from chronic disease.

Therefore, this study aimed to investigate the protective effect of physical exercise against health damage induced by exposure to cigarette smoke in Wistar rats. We hypothesized that within four weeks of exposure to cigarette smoke there would be pulmonary and systemic impairments, and that physical exercise would be able to reverse or reduce them.

Methods

Ethical Approval

All the procedures outlined in this study were approved by the Research Ethics Committee of UFCSPA (protocol 200/16). The investigation followed the ethical guidelines established by the Guide for the Care and Use of Experimental Animals published by the National Institute of Health (NIH publication no. 85-23, revised in 1996).

Animals

Twenty-two male Wistar rats (350 to 400 g; 90 days of age) obtained from the Laboratory Animals Breeding Unit of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) were housed under standard conditions (controlled food and water ad libitum; 12:12h light-dark cycle; 22°C).

Experimental design

Rats were assigned to three experimental groups: Control (CTRL; n=8), not exposed to cigarette smoke and sedentary; Smoking sedentary (S-SED;

n=7), exposed to cigarette smoke and sedentary; and Smoking-Exercise (S-EX; n=7), trained and exposed to cigarette smoke. The Smoking-Exercise group was always submitted to the exercise protocol following the first session of exposure to cigarette smoke; the rats were allowed to rest for 60 minutes or more, then exposure to cigarette smoke was restarted. After four weeks of exposure to cigarette smoke and physical exercise, 24h, 48h and 72h after the last session of both protocols, the animals were submitted to functional capacity assessment, echocardiography and respiratory mechanics were then killed by decapitation and performed tissue collection (Figure 1).

Cigarette Smoke exposure protocol

The animals of the S-SED and S-EX groups were exposed to sidestream smoke of a commercial brand cigarettes (0,8 mg of nicotine, 10 mg of tar, and 10 mg of carbon monoxide per cigarette). The exposure period consisted of four hours/day, two hours in the morning and two hours in the afternoon, five days a week, for four weeks. Exposure to tobacco smoke was performed in sealed glass chambers (50 × 30 × 30 cm) with an airflow of 10 L/min kept constant by a vacuum pump, according to the model previously proposed by Pereira, Heck, Saldiva, & Rhoden (2007). In these chambers, animals (n = 5/chamber) were exposed to the tobacco smoke or to the ambient air, according to their groups. Initially, the animals went through a period of habituation to the cigarette smoke, which consisted in burning six cigarettes per day on the first three days. After this period, this rate was increased to 12 cigarettes per day until the end of the experiment. Animals were weighed periodically and were monitored twice a day for health status.

Treadmill physical exercise protocol

Initially, the rats underwent an adaptation period on the treadmill (0% incline) maintaining the running exercise at a speed of 10 m/min, for 5 min on the first and second days, and for 10 min on the third, fourth and fifth days. After this period, the training consisted of 45 min of running five times a week at a speed of 10 m/min, which progressively increased up to 15 m/min (0% incline)

(Emter et al., 2008) until the end of the training, along four weeks of physical exercise.

Functional Capacity Evaluation

The functional capacity test was performed in two moments: pre-and-post intervention. Initially, the animals were habituated to the apparatus (3 min/day, 10 m/min, 3 x/week). Twenty-four hours from the last exposure to tobacco smoke and physical exercise protocol, rats underwent functional capacity test out based on an incremental speed protocol of exercise until time to animal exhaustion, as previously described (Batista, Santos, Oliveira, Seelaender, & Costa Rosa, 2007). Thereafter, initial speed was stated in 10 m/min and an increment of 5 m/min was applied at every 3-min interval until the animal reached exhaustion. Animal exhaustion was established as the time at which the animal was unable to run for at least 15 s, even while receiving an electrical shock (1.5 μ A). Each test day, the gas analyzer was calibrated with a known gas mixture. The distance covered, time until exhaustion and maximum velocity were defined as the maximal distance, time and velocity at the end of the protocol. The highest values of extracted data measured at the last step were taken as a measure of VO_{2max} .

Echocardiography

Forty-eight hours after the last day of cigarette smoke exposure protocol and aerobic training, rats underwent non-invasive cardiac function evaluation using a commercially available echocardiograph (GE Vivid I; GE Medical Systems, Israel). This equipment has an 8–13-MHz electronic transducer and was used by a trained operator with experience in small animal echocardiography. All echocardiographic evaluations were performed by the same researcher. Echocardiographic examination followed the recommendations of the American College of Echocardiography (Lang et al., 2005). The rats were anesthetized with isoflurane (2%), and positioned in the

lateral decubitus (45° angle). An ultrasound transmission gel was applied on the previously shaved chest, and M-mode tracings were derived from a 2D mode obtained from parasternal short-axis views of the left ventricle (LV) at three levels: basal (at the tip of the mitral valve leaflets), middle (at the papillary muscle level) and apical (distal to the papillary muscle but before the cap of the cavity). For cardiac structural parameters, the following structural variables were measured: interventricular septum in diastole (IVSd, mm), interventricular septum in systole (IVSs, mm), left ventricular end-diastolic diameter (LVEdD, mm), left ventricular end-systolic diameter (LVEsD, mm), left ventricular posterior wall in diastole (LVPWd, mm) and left ventricular posterior wall in systole (LVPWs, mm). The measurements obtained were the means of at least three cardiac cycles at each of the three levels, and the final value for each rat was the mean of all three described plans. Then, the following secondary variables of left ventricular systolic function were obtained: end-diastolic volume (LVEdV (mL)=1.047(LVEdD)³ and end-systolic volume (LVEsV (mL)=1.047(LVEsD)³) by cubic or ellipsoid model (Miranda, Leal-Junior, Marchetti, & Dal Corso, 2013); left ventricular ejection fraction (EF (%))=[(LVEdV -LVEsV)-LVEdV]×100), left ventricular fractional shortening (FS (%))=[(LVEsD-LVEsD)/LVEdD]×100). Left ventricular diastolic function was guided by an adapted protocol previously reported for rats (Nozawa et al., 2006). Briefly, mitral diastolic inflow measurement by pulsed Doppler was obtained from the four-chamber view, and the sample volume was positioned at the tip of the mitral valve to obtain the mitral diastolic flow velocity, which was used to measure the peak E and A wave velocities (cm/s) and the ratio between them (E/A ratio). Additionally, the heart rate was calculated using an average of three consecutive cycle intervals.

Assessment of respiratory mechanics

The rats were anesthetized with ketamine (90mg/kg ip.) and xilazina (12mg/kg ip.) and tracheostomized. A rigid-type cannula (2-mm ID) was inserted into the trachea and tied firmly in place. The cannula was connected to a small animal ventilator (FlexiVent, SCIREQ, Montreal, QC, Canada). Rats

were mechanically ventilated at a breath rate of 90 breaths/min, with a tidal volume of 10 mL/kg using 5 cmH₂O positive end-expiratory pressure established by a water column (Jaenisch et al., 2011). Respiratory mechanics variables were determined as previously described (Hantos, Daroczy, Suki, Nagy, & Fredberg, 1992; Jaenisch et al., 2011).

Tissue preparation

Immediately after the end of evaluation of the ventilatory mechanics the animals were killed by decapitation and the tissues were collected for biochemical analysis. The lung and heart were dissected out and instantaneously placed in liquid nitrogen and stored at -80°C until biochemical assays. The blood samples were centrifuged at 3500 rpm for 10 minutes and the plasma was obtained for biochemical assays. For assays of cellular oxidative state, the lung and heart were homogenized in 10 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4) containing ethylenediamine tetraacetic acid (EDTA, 2 mM) and phenylmethylsulfonyl fluoride (PMSF, 1mM) in a Teflon-glass homogenizer. The homogenate was centrifuged at 3500 rpm for 10 min and the supernatant was used for the assays.

Determination of the inflammatory cytokines levels

The levels of TNF- α and IL-1 β were determined using Rat ELISA Assay kits (Colorimetric Detection, catalog number 88- 7340; 88-6010 eBioscience Ready-SET-Go, USA; 555198, BD OptEIA, USA, respectively) according to the manufacturer's instructions. Briefly, 96-well plates were coated with specific monoclonal antibody. The plates were then blocked for non-specific binding using Assay Diluent. The samples were homogenized with specific kit lysis buffer, lysates were centrifuged, and the supernatant and standard curve were incubated with the capture antibody followed by detection antibody. The plates were incubated with detection enzyme Avidin-HRP followed by Substrate Solution. The Stop Solution was added and the absorbance was measured on a microplate reader (450 nm). The protein concentration of each sample was measured by the Lowry method (Peterson, 1977). The cytokines levels were expressed as pg/ml.

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was evaluated by thiobarbituric acid reactive substances (TBARS) test. Aliquots of samples were incubated with 10% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was heated (30 min) on a boiling water bath. Afterwards, n-butanol was added and the mixture was centrifuged. The organic phase was collected to measure fluorescence at excitation and emission wavelengths of 515 and 553 nm, respectively. 1,1,3,3-tetramethoxypropane, which is converted to malondialdehyde, was used as the standard.

Activity of antioxidant system

Glutathione peroxidase (GPx) activity was measured spectrophotometrically. The assay mixture consisted of tris buffer (pH 7.6), 3 mM NADPH, 20 mM cumene hydroperoxide, 40 mM reduced glutathione (GSH), and 10 U/mL GR. The activity was calculated using the molar extinction coefficient for NADPH ($6.22 \mu\text{mol}^{-1} \times \text{cm}^{-1}$ at 340 nm). Catalase (CAT) activity was measured in homogenates incubated in ethanol (10%) and triton-X (10%) as previously described (Aebi, 1984). The CAT activity was analyzed through the determination of the rate of H_2O_2 degradation at 240nm in 10mM potassium phosphate buffer (pH 7.0) at 25°.

Protein concentration

Protein concentration was determined by the Coomassie brilliant blue G-250 dye binding method using bovine serum albumin as a standard curve.

Statistical analysis

Mean values and standard deviation (\pm SD) were calculated for all the analyzed data. The Shapiro-Wilk normality test was performed. To compare the effects between groups, one-way ANOVA was performed, followed by the

Tukey post-hoc test, and the Kruskal-Wallis test was used to assess differences between nonparametric distributions. For paired analyses, the paired t-test was employed to verify differences between normal distributions, and the Wilcoxon test was performed for non-parametric samples. In all tests, $P < 0.05$ was considered statistically significant. Data analysis was performed with SPSS software version 22 (IBM SPSS Statistic 22).

Results

Body weight and food intake

After four weeks of exposure to cigarette smoke, the S-SED and S-EX groups lost body weight when compared to the CTRL group (CTRL: 46 ± 27.6 g vs. S-SED: -9 ± 15.6 g and S-EX: -12 ± 16.9 g; $P = 0.001$); however, there was no difference between the trained and sedentary groups ($P = 0.469$; Fig. 2A). Similarly, regarding food intake, the animals exposed to cigarette smoke consumed a smaller amount of food in relation to the CTRL group (CTRL: 6.5 ± 14 vs. S-SED: -11 ± 11.3 and S-EX: -8.7 ± 7 ; $P = 0.049$; Fig. 2B). No differences were observed between the S-SED and S-EX groups regards food intake (Fig. 2b; $P = 0.772$).

Echocardiography

Table 1 shows the values for the non-invasive assessment of left ventricular function. There was a difference in the heart rate (HR) of the S-SED group compared with the CTRL group (CTRL: 392.6 ± 34.5 ; S-SED: 329.4 ± 27.7 and S-EX: 356.2 ± 35.2 ; $P = 0.005$). There was no significant difference between groups in the variables of interventricular septum in diastole (IVSd); interventricular septum in systole (IVSs); left ventricular end-diastolic diameter (LVEdD); left ventricular end-systolic diameter (LVEsD); left ventricular posterior wall in diastole (LVPWd); left ventricular posterior wall in systole (LVPWs); left ventricular ejection fraction (EF); left ventricular fractional shortening (FS); relative wall-thickness (RWT); maximal early diastolic peak velocity (E) and late peak velocity (A) ($P > 0.05$).

Functional Capacity

Figure 3 shows the pre-and post-intervention values for distance, time until exhaustion, maximum velocity reached, and maximum oxygen consumption (VO_{2max}) in the CTRL, S-SED and S-EX groups. All the variables in the pre-intervention period did not show difference between groups ($P>0.05$).

In the S-EX group, there was an increase in the distance covered (Pre-S-EX: $203\pm 101.3m$ vs. Post-S-EX: $395\pm 128.1m$; $P=0.025$; Fig.3A), the time to exhaustion (Pre-S-EX: $725.5\pm 240.1s$ vs. Post-S-EX: $977\pm 247.7s$; $P=0.003$; Fig.3B) and the maximum velocity (Pre-S-EX: $27.1\pm 6.9m/min$ vs. $38\pm 2.7m/min$; $P=0.039$; Fig. 3C) when compared to the pre-intervention. The CTRL and S-SED groups showed no difference in functional capacity in the post- compared to pre-intervention period in the distance covered ($P>0.05$; Fig. 3A; 3B; 3C; 3D).

The group S-EX, in the post-intervention, there was an increase in the distance covered (CTRL: $191.47\pm 34.8m$; S-SED: $156.7\pm 36.3m$; S-EX: $395.5\pm 128.1m$; $P=0.000$; Fig. 3A), in the time for exhaustion (CTRL: $645.2\pm 163.1s$; S-SED: $571.7\pm 85.2s$; S-EX: $977.3\pm 274.7s$; $P=0.008$; Fig. 3B) and maximum velocity reached (CTRL: $24.3\pm 4.17m/min$; S-SED: 23.0 ± 2.7 ; S-EX: 38 ± 2.7 ; $P=0.002$; Fig. 3C) compared to the CTRL and S-SED group. There was no changes in VO_{2max} in different experimental groups (CTRL: 30.59 ± 10.6 ; S-SED: 20.86 ± 5.55 ; S-EX: 27.03 ± 5.96 ; $P>0.05$; Fig. 3D).

Respiratory Mechanics

The respiratory system resistance (R_{sr}) was higher in the S-SED and S-EX groups compared with CTRL group (CTRL: 0.06 ± 0.008 ; S-SED: 0.09 ± 0.01 ; S-EX: 0.10 ± 0.008 ; $P=0.001$; Fig. 4A); physical exercise did not influence this response (S-SED vs. S-EX: $P=0.127$). There was no significant difference between the experimental groups in the respiratory system elastance (CTRL: 1.98 ± 0.5 ; S-SED: 1.60 ± 0.1 ; S-EX: 1.45 ± 0.3 ; $P=0.552$; Fig. 4B), tissue resistance (G_{ti}) (CTRL: 0.52 ± 0.27 ; S-SED: 0.40 ± 0.02 ; S-EX: 0.38 ± 0.10 ; $P=0.688$; Fig. 4C), and tissue elastance (H_{ti}) (CTRL: 1.71 ± 0.18 ; S-SED: 1.78 ± 0.08 ; S-EX: 1.64 ± 0.20 ; $P=0.265$; Fig. 4D).

Inflammatory Cytokines, Stress Oxidative and Antioxidant Enzymes Activity

Figure 5 shows the levels of Interleukin (IL-1 β) and Tumour Necrosis Factor (TNF- α) in lung, plasma and heart in the experimental groups. In the lung, there was an increase in the levels of IL-1 β in the S-SED group compared with the CTRL group (CTRL: 21.9 \pm 6.87; S-SED: 40.34 \pm 5.22; S-EX: 22.03 \pm 8.18; P=0.006; Fig. 5A). The physical exercise decrease the levels of IL-1 β in the lung on the S-EX group compared to S-SED (P=0.006). In plasma, there was decrease in the levels of TNF- α in the S-EX group compared with S-SED (CTRL: 34.81 \pm 13.4; S-SED: 45.51 \pm 20.48; S-EX: 19.37 \pm 18.38; P=0.048; Fig. 5E), and in heart there was a decrease in TNF- α levels in the S-EX group compared with the CTRL group (CTRL: 25.56 \pm 16.55; S-SED: 15.13 \pm 7.01; S-EX: 6.59 \pm 2.92; P=0.009; Fig. 5F).

There were no differences in the levels of catalase activity (CAT) in the lung (CTRL: 0.20 \pm 0.07; S-SED: 0.22 \pm 0.009; S-EX: 0.21 \pm 0.12; P=0.965; Fig. 5A), plasma (CTRL: 0.02 \pm 0.014; S-SED: 0.03 \pm 0.007; S-EX: 0.06 \pm 0.019; P=0.109; Fig. 5B) and heart (CTRL: 0.14 \pm 0.063; S-SED: 0.21 \pm 0.098; S-EX: 0.17 \pm 0.056; P=0.393; Fig. 5C). The levels of the Glutathione Peroxidase (GPx) presented no differences between the experimental groups in the lung (CTRL: 48.15 \pm 18.52; S-SED: 39.98 \pm 33.78; S-EX: 57.22 \pm 13.08; P=0.544; Fig. 6A), in the plasma (CTRL: 39.82 \pm 15.79; S-SED: 36.52 \pm 10.97; S-EX: 23.06 \pm 6.51; P=0.108; Fig. 6B) and heart (CTRL: 62.06 \pm 26.34; S-SED: 68.7 \pm 6.62; S-EX: 65.06 \pm 23.11; P=0.921; Fig. 6C).

There was a decrease in the levels of lipid peroxidation (TBARS) in the lung of the S-EX group compared with the S-SED group (CTRL: 3.83 \pm 0.82; S-SED: 5.86 \pm 1.33; S-EX: 3.70 \pm 1.10; P=0.044; Fig. 8A).

Discussion

In this study, physical exercise exerted a protective effect against damages induced by exposure to cigarette smoke. The smoke exposure protocol induced a significant weight loss, associated with reduced food intake. There is evidence that pulmonary disease may trigger inflammatory processes and cause weight loss in affected individuals (Albanes, Jones, Micozzi, & Mattson, 1987; Degens, 2010; Hussain et al., 2012; Ypsilantis et al., 2013).

Experimental studies showed that within a short period of exposure to tobacco smoke, appetite loss followed by weight loss can be observed in smoking animals (Chen et al., 2005; Tsuji et al., 2013). Smoking is suggested to alter energetic homeostasis, and despite its mechanisms not being fully understood, nicotine is recognized as the main tobacco compound that acts on control and regulation of appetite and satiety in the hypothalamus. Specific nicotine receptors have been shown to inhibit the synthesis of neuropeptide Y (NPY), a peptide with an orexigenic effect that controls food intake (Chen et al., 2007; Jo, Talmage, & Role, 2002). Moreover, nicotine affects plasma leptin levels. High leptin levels in the hypothalamus regulate NPY synthesis and, therefore, satiety is increased and appetite is reduced, which could explain the weight loss observed in this study. Our results demonstrate that physical exercise over a four-week period did not modify this mechanism, since appetite and weight loss were the same between groups exposed to cigarette smoking. This finding corroborates recent studies, demonstrating that exercise for four weeks does not prevent weight loss in animals exposed to tobacco (Toledo-Arruda et al., 2017). However, over a period of at least eight weeks, a protective effect of exercise has been observed for these variables (Toledo-Arruda et al., 2017; Toledo et al., 2012).

Furthermore, our results demonstrate that a four-week period of exposure to tobacco smoke did not induce functional capacity impairments. Studies employing longer protocols, over eight and twelve weeks, observed worsening functional capacity at the end of the exposure period (Toledo-Arruda et al., 2017; Toledo et al., 2012). Hence, the decline in functional capacity seems to be associated with the duration of smoke exposure, requiring periods of over four weeks to set in. In patients with COPD, there is an association between pulmonary function decline and intolerance to physical exercise (Belman, 1993; Wright & Churg, 1990). In this study, we did not observe impairments in pulmonary elastance, since the smoking exposure model did not induce emphysema. However, despite the absence of functional manifestations, a pro-inflammatory state and increased airway resistance were evidenced after four weeks of continuous exposure, indicating that these are the primary mechanisms involved in the pathogenesis of pulmonary disease. Therefore, we

suggest that cigarette smoke exposure induces pulmonary damage in a progressive manner, which initially does not lead to a decline in functional capacity.

Additionally, we demonstrated that the mechanism by which smoke causes increased airway resistance may be related to epithelial lesions, since there was cellular damage induced by lipid peroxidation in the lungs of rats exposed to tobacco smoke. This finding is in agreement with Barnes (2014), who showed that increased airway resistance is associated with epithelial thickening and collagen deposition, thus leading to reduced internal diameter and impaired distension of the airway. An additional mechanism includes the activation of inflammatory markers, which seem to play distinct roles in the pathogenesis of pulmonary disease. Recently, it was shown that the exposure to cigarette smoke over a period of three months induced a significant increase in TNF- α levels in the pulmonary tissue of rats (Toledo-Arruda et al., 2017). Thus, it is possible that this marker has a role in more advanced stages of lung inflammation (Takabatake et al., 2000), since we did not observe increased TNF- α levels in the lungs of animals exposed to smoke. However, IL-1 β levels are elevated early, participating in damage induction and in the activation of the inflammatory cascade. This finding is similar to those of other studies, which also found increased IL-1 β levels in the pulmonary tissue of animals exposed to cigarette smoke, which can be characterized by a cigarette-induced inflammasome (Eltom et al., 2014; Kang et al., 2007).

We observed that physical exercise prevented the progression of lung epithelial damage, as evidenced by the reduction in oxidative damages to membrane lipids. In addition, we demonstrated that exercise was able to reverse the smoking-induced increase in the levels of pulmonary IL-1 β and plasma concentration of TNF- α . The increased concentration of these interleukins is associated with tissue damage; thus, decreased concentrations represent a protective effect of exercise against physical and functional damages observed with chronic smoking. A similar result was found in other studies, which demonstrate that aerobic exercise may have anti-inflammatory activity in the lung and other tissues. Krüger et al. (2018) observed that physical

exercise reduced expression of TNF- α and IL-1 β in skeletal muscle tissue after chronic exposure to cigarette smoke. These effects may be partially mediated by the release of anti-inflammatory cytokines such as IL-10 and IL-1RA, which play an important role in regulating the expression of IL-1 β e TNF- α (Chen, Hsieh, Yang, Hsu, & Lin, 2007). An additional mechanism involved in the regulation of these cytokines by exercise is the attenuation of NF- κ B pathway activation. This pathway is an important modulator of inflammatory response in the pathogenesis of chronic inflammatory lung diseases. According to Yu et al. (2012), exercise prevents the activation of the NF- κ B pathway and reduces the expression of Toll-like receptors (TLRs), which in turn reduces the production of cytokines by macrophages and T helper 1 (Th1) cells, demonstrating its the anti-inflammatory effects.

At the systemic level, we did not find increased concentrations of pro-inflammatory cytokines. In this regard, results from other studies vary; Krüger et al. (2018) observed increased plasmatic levels of inflammatory markers in animals following eight months of exposure to cigarette smoke, whereas Toledo et al. (2012) did not detect alterations after a six-month exposure period. Thus, a systemic level increase of the pro-inflammatory state is suggested to occur when an irreversible, chronic lesion has already been established.

Our results demonstrate that a four-week period of physical exercise is able to improve the functional capacity of animals exposed to cigarette smoke; indeed, studies have shown that different exercise protocols may improve functional capacity by distinct pathways. Such effects are due to increased lactate levels, which result in metabolic alterations in the muscle tissue, and the predominance of the oxidative mechanism. Exercise reduces the activation of the Ubiquitin-Proteasome System (UPS) and stimulates the expression of IGF-1 (insulin-like growth factor 1), followed by the activation of Akt (protein kinase B) and mTOR (mammalian target of rapamycin) pathways in the muscle tissue (Krüger et al., 2018). However, we did not observe an improvement in VO_{2max} in the animals that performed exercise. This finding may be associated with other factors such as intensity, since high-intensity exercise has been shown to improve VO_{2max} in animals exposed to cigarette smoke (Bowen et al., 2017). Although VO_{2max} was not increased, the low-intensity exercise protocol

employed in our study was able to improve the functional capacity of trained animals.

Conclusion

Our data indicates that physical exercise may exert a protective and anti-inflammatory effect reducing the damage induced by exposure to cigarette smoke. Our model of tobacco smoke exposure induced impairments in respiratory mechanics, as evidenced by the increased Rsr, without impairments to the functional capacity of exposed animals. Hence, this time period represents a window for the establishment of therapeutic and preventive strategies, considering that smoking-induced alterations are present, but the damages are not fully established and can be reversed.

Competing Interests

The authors declare that there are no conflicts of interest.

Authors Contributions

All authors contributed to:

The conception or design of the work

The acquisition, analysis, or interpretation of data for the work

The drafting the work or revising it critically for important intellectual content.

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

None.

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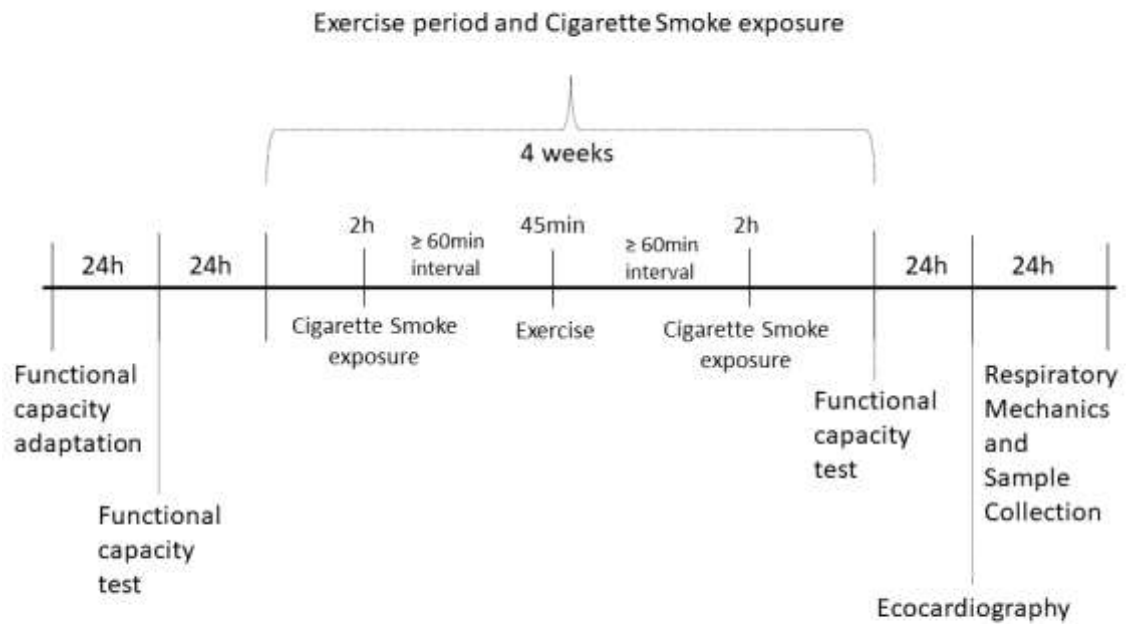
FIGURE LEGENDS**Figure 1.** Experimental design.

Figure 2. Body Weight Variation and Food Intake Variation. **a) Body Weight;** **b) Food Intake.** Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post-hoc tests; CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7). *P<0.05 vs. CTRL.

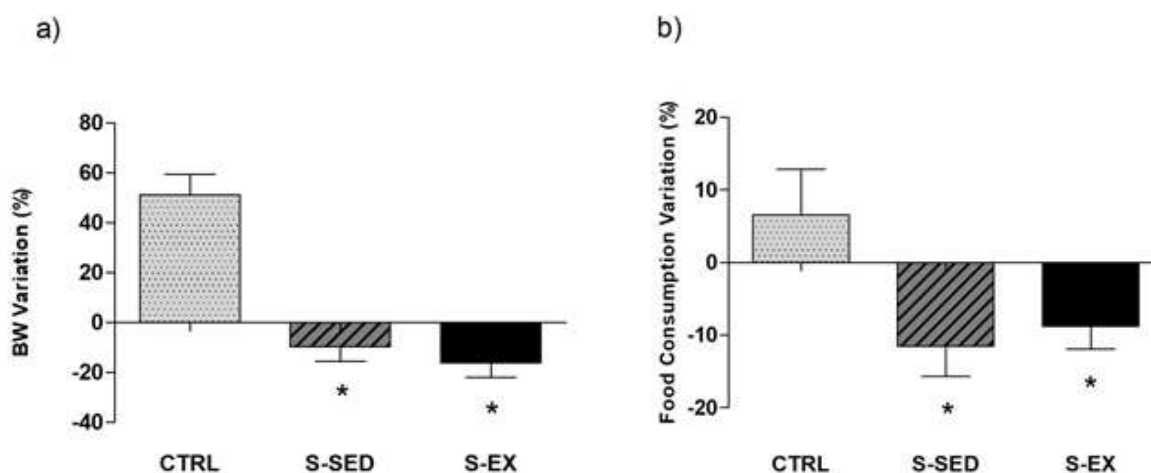


Table 1. Echocardiography and hemodynamic evaluation of experimental

	CTRL	S-SED	S-EX	P
HR, bpm	392.6 \pm 34.5	329.4 \pm 27.7*	356.2 \pm 35.2	0.005
IVSd, mm	1.6 \pm 0.2	1.9 \pm 0.3	1.7 \pm 0.1	0.232
IVSs, mm	3.0 \pm 0.3	3.2 \pm 0.2	3.0 \pm 0.2	0.109
LVEdD, mm	8.7 \pm 0.7	9.0 \pm 0.4	9.0 \pm 0.4	0.571
LVEsD, mm	6.4 \pm 0.4	6.4 \pm 0.5	6.8 \pm 0.4	0.234
LVPWd, mm	1.5 \pm 0.2	1.4 \pm 0.2	1.5 \pm 0.3	0.857
LVPWs, mm	1.6 \pm 0.4	1.6 \pm 0.5	1.5 \pm 0.3	0.698
EF, %	62.2 \pm 3.7	63.2 \pm 4.6	59.0 \pm 3.9	0.094
FS, %	27.7 \pm 2.3	28.4 \pm 2.9	25.7 \pm 2.4	0.420
RWT	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0	0.969
E, cm/s	0.4 \pm 0.6	0.5 \pm 0.8	0.4 \pm 0.0	0.516
A, cm/s	0.2 \pm 0.8	0.2 \pm 0.1	0.1 \pm 0.0	0.108
E/A	2.0 \pm 0.7	1.9 \pm 0.4	2.5 \pm 0.5	0.223

HR, heart rate; **IVSd**, interventricular septum in diastole; **IVSs**, interventricular septum in systole; **LVEdD**, left ventricular end-diastolic diameter; **LVEsD**, left ventricular end-systolic diameter; **LVPWd**, left ventricular posterior wall in diastole; **LVPWs**, left ventricular posterior wall in systole; **EF**, left ventricular ejection fraction; **FS**, left ventricular fractional shortening; **RWT**, relative wall-thickness; **E**, maximal early diastolic peak velocity; **A**, late peak velocity. Values are expressed as means \pm SD. Groups were

compared with One-way ANOVA (IVSd, IVSs, LVEdD, LVEsD, LVPWd, LVPWs, RWT, EF %, FS %, E cm/s, E/A) and Kruskal-Wallis (A cm/s). * $P < 0.05$ vs. CTRL.

Figure 3. Functional capacity evaluation pre-and post-intervention. **a) Running Distance (m, meters); b) Time to exhaustion (s, seconds); c) Maximal velocity (m/min, meters per minute); d) Maximal oxygen uptake (VO_{2max}).** Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post-hoc tests (Running Distance and Time to exhaustion) and Kruskal-Wallis tests (Maximal velocity); CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7). * $P < 0.05$ vs. CTRL; # $P < 0.05$ vs. S-SED; $\Delta P < 0.05$ vs. S-EX PRE.

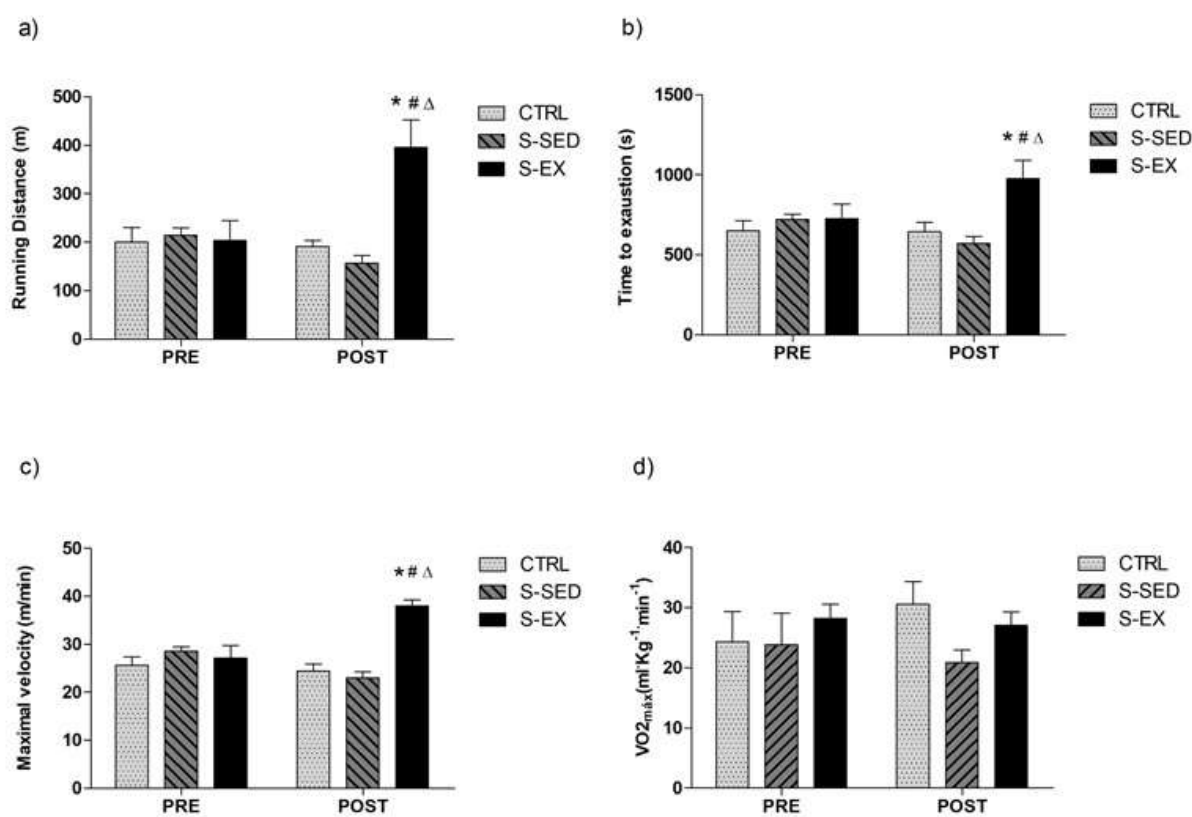


Figure 4. Assessment of respiratory mechanics. **a) Respiratory system resistance (Rrs); b) respiratory system elastance (Ers); c) Tissue resistance (Gti); d) Tissue elastance (Hti) in studied groups.** Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post hoc and Kruskal-Wallis tests. CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7). * $P < 0.05$ vs. CTRL.

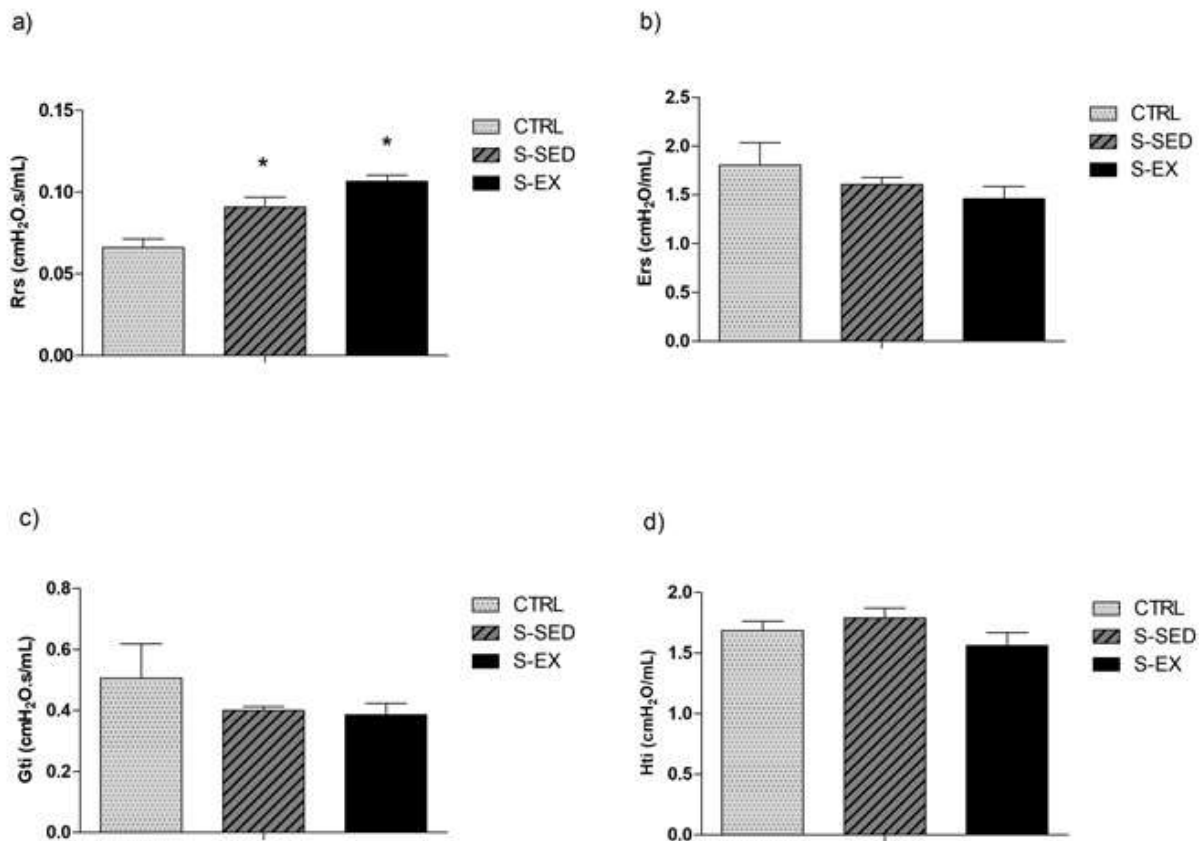


Figure 5. Quantification of inflammatory markers in Lung, Plasma and Heart. **Interleukin (IL)-1 β** : a) in Lung; b) in Plasma; c) in Heart; and **Tumour necrosis factor (TNF- α)**: d) in Lung; e) in Plasma; f) in Heart. Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post-hoc and Kruskal-Wallis tests. CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7). *P<0.05 vs. CTRL; #P<0.05 vs. S-SED.

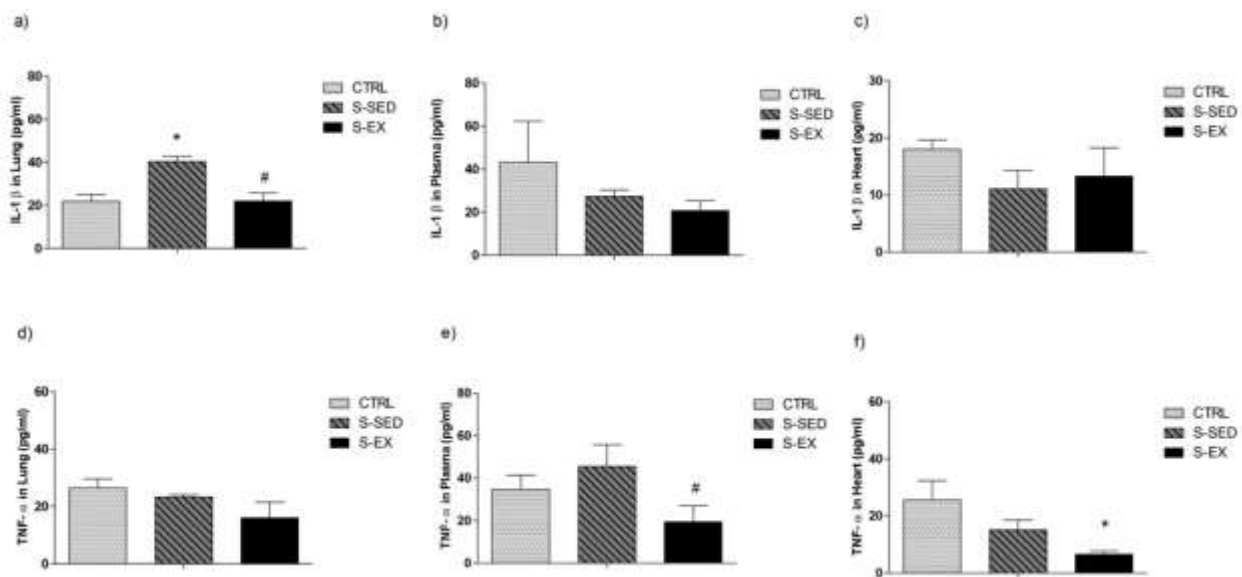


Figure 6. Catalase activity. a) in Lung; b) in Plasma; c) in Heart. Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post-hoc. CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7).

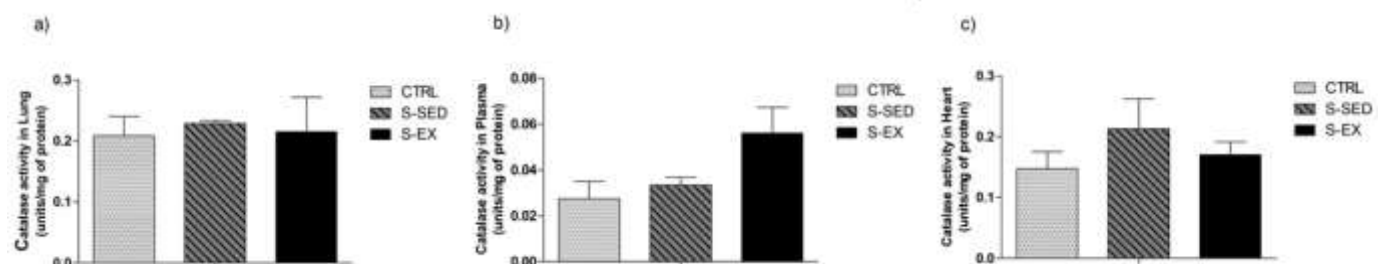


Figure 7. Glutathione Peroxidase (GPx) Activity. **a) in Lung; b) in Plasma; c) in Heart.** Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post-hoc and Kruskal-Wallis tests. CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7).

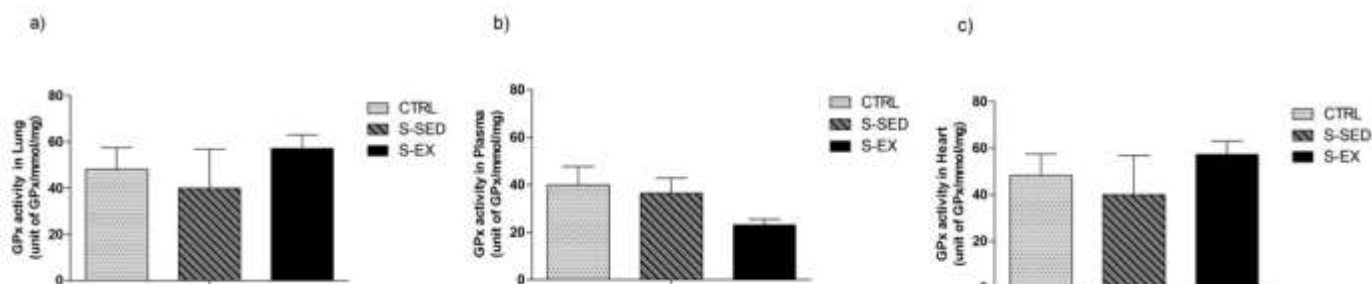
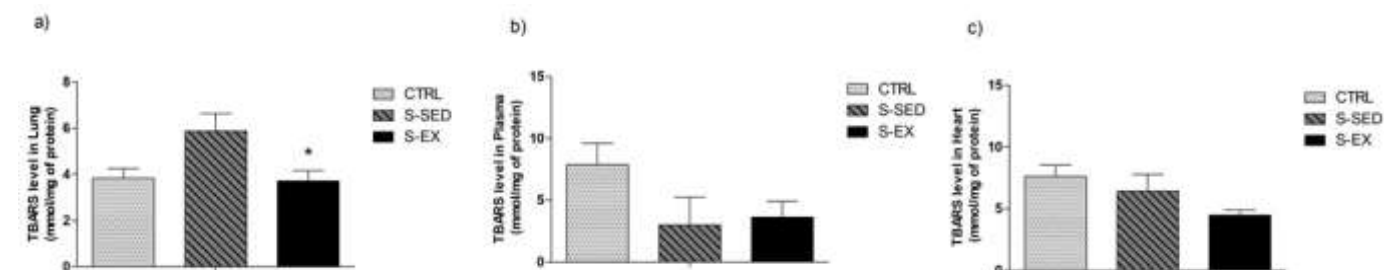


Figure 8. Lipid peroxidation (TBARS). **a) in Lung; b) in Plasma; c) in Heart.** Values are means \pm SD. Groups were compared with one-way ANOVA and Tukey post-hoc tests. CTRL, control (n=8); S-SED, smoking sedentary (n=7); S-EX, smoking exercise (n=7). *P<0.05 vs. S-SED.



ANEXOS

ANEXO A

Título do Apêndice



Information for Authors

Papers should fulfill our [scope](#) and embrace the journal's orientation of translation and integration. Footnotes are not acceptable.

You can prepare your manuscript using [Overleaf](#) and submit directly from Overleaf to *Experimental Physiology*. For information more click [here](#).

Papers should be written as clearly and concisely as possible and figures and tables should be kept to the minimum necessary to illustrate the hypothesis being tested and support the conclusions. There are no absolute restrictions on the length of Research submissions but 6,000 words including legends and references is normally sufficient. Where there are clear and compelling reasons, a more extensive manuscript will be considered subject to the agreement of the Editor.

Title Page

The title page should include Title/Authors/Addresses/Additional information as listed below.

Title. The title should normally contain no more than 150 characters (including spaces). Include the species, tissue, organ or system if this is important in the context of the findings. Avoid specialist abbreviations if possible. For abbreviations and symbols that are acceptable in the title see [List 1](#). Titles should be drafted carefully to indicate broadly what the paper is about to all *Experimental Physiology* readers, including those who are not specialists in the field.

For suggestions on how to optimize your title for search engines see <http://www.wiley.com/legacy/wileyblackwell/pdf/SEOforAuthorsLINKSrev.pdf>

Authorship. The Editorial Board endorses the general principles set out in Guidelines on Good Publication Practice produced by the Committee on Publication Ethics ([COPE](#)). The author submitting a manuscript must confirm that all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed, and that all authors have approved the final version of the paper. Each author should take responsibility for a particular section of the study and have contributed to writing the paper (see [Author contributions](#)). Acquisition of funding or the collection of data does not alone justify authorship; these

contributions to the study should be listed in the Acknowledgements.

Additional information such as 'X and Y have contributed equally to this work' may be added as a footnote on the title page.

If authorship of a manuscript changes during the publication process, notification of the change, signed by all authors must be sent to the [Publications Office](#).

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Additional information. The following information is also required on the title page:

- A running title not exceeding 70 characters and spaces for page headings.
- Three key words for use in the reviewing process.
- The total number of words in the paper, excluding references and figure legends.
- The total number of references.
- The name and postal and email addresses for the corresponding author.
- The Subject Area as assigned in the [online submission form](#) for the print and online Table of Contents.

New Findings

Authors are required to complete two 'New findings' boxes on the submission form using no more than 100 words in total distributed between the answers to two questions.

For Research articles:

- What is the central question of this study?
- What is the main finding and its importance?

For Case Studies:

- What is the main observation in this case?
- What insights does it reveal?

For Review articles (including Hot Topics, Symposium Reports and Lectures):

- What is the topic of this review?
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This information will influence editorial handling of the manuscript.

Please include your New Findings questions and answers in your submitted manuscript file as bullet points above the Abstract where they will be published if your article is accepted for publication. Please ensure that your entry is grammatically correct and does not include any undefined abbreviations.

Please see New Findings examples:

['Altered ion transport by thyroid epithelia from CFTR^{-/-} pigs suggests mechanisms for hypothyroidism in cystic fibrosis'](#) (click on title for Abstract)

What is the central question of this study?

Subclinical hypothyroidism is associated with cystic fibrosis although the mechanisms through which gene mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) produce this effect have not been previously explored.

What is the main finding and its importance?

Using well-differentiated primary thyroid epithelial cells cultured from CFTR^{-/-} pigs, we show the requirement for CFTR in cAMP-stimulated Cl⁻ secretion. Knockout of CFTR also promotes increased Na⁺ absorption without increased expression of the epithelial sodium channel. These functional alterations lead to several models for CFTR function in thyroid and suggest mechanisms for cystic fibrosis-induced changes in thyroid function.

What is the central question of this study?

Activation of angiotensin-converting enzyme 2, resulting in production of angiotensin-(1-7) and stimulation of its receptor (Mas), exerts beneficial actions in a number cardiovascular diseases. A potential role for angiotensin-(1-7) in cerebral ischaemic stroke has not previously been reported.

What is the main finding and its importance?

We show that pharmacological activation of angiotensin-converting enzyme 2 or central administration of angiotensin-(1-7) attenuates cerebral damage induced by middle cerebral artery occlusion in rats. This role of the angiotensin-(1-7) has not previously been reported and identifies a potential new therapeutic target in ischaemic strokes.

Abstract

This should be in one unnumbered paragraph that accurately reflects the contents of the paper and makes clear the physiological significance of the work, the problem addressed, the nature of the results, and the principal conclusions; authors are expected to conclude the summary by explaining the conceptual novelty and the broader physiological importance of their work. Results should be presented quantitatively where appropriate, together with the statistical significance, and the conclusions indicated. References may not be cited. Since the Abstract may be used by abstracting services, a limit of 250 words is recommended. It must not exceed 5 % of the text (excluding references and figure legends), with an absolute maximum of one printed page.

See [Guidelines](#) for optimizing the Title and Abstract of your article for search engines.

Introduction

The Introduction should make the background and the object of the research clear, indicate the justification for the work and be understandable to the non-specialist. Reference to the

authors' previous work is desirable only if it has a direct bearing on the subject of the paper; an extensive historical review is not appropriate.

Methods

Please read our policies regarding [Animal Experiments](#) and [Human Experiments](#).

A detailed explanation of the journal's principles and guidelines on animal experimentation, which includes a checklist for authors, is given in 'Principles and standards for reporting animal experiments in *The Journal of Physiology* and *Experimental Physiology*' by David Grundy, *Experimental Physiology*. doi: 10.1113/EP085299.

Methods should be described once only and should not appear in the legends to figures and tables. Details should be sufficient to allow the work to be repeated by others.

The Methods section should start with a paragraph headed 'Ethical Approval'. Animal studies must meet a minimum set of requirements. A checklist outlining these requirements and detailing the information that must be provided in the paper can be found [here](#). Please note that ether is not an acceptable anaesthetic as it no longer complies with the principle of best practice.

Authors should demonstrate in their Methods section that their experiments comply with the policies and regulations set out in the editorial.

If experiments were conducted on humans, authors must provide confirmation that informed consent was obtained, preferably in writing, that the studies conformed to the standards set by the latest revision of the Declaration of Helsinki (or the version that was in place at the time of the experiments), and that the procedures were approved by a properly constituted ethics committee, which should be named. If the research study was registered (clause 35 of the Declaration of Helsinki) the registration database should be indicated, else the lack of registration should be noted as an exception (e.g. the study conformed to the standard set by the Declaration of Helsinki, except for registration in a database). More information can be found [here](#).

Authors must include the reference number from their Institutional Ethics Committee Approval form or if no number has been provided, give details of who has issued the 'Letter of approval' and on what date.

Authors working on isolated tissues, including primary cell cultures, must state whether the donor animal was anaesthetised or killed, and give details of the relevant procedures. Where tissues are obtained from an abattoir or similar establishment the method of killing need not be specified unless scientifically important.

Ethical information must be included for each manuscript. It is not sufficient to refer to previous publications for details, unless the paper is one of a series published in the same issue.

Where appropriate, lists of solutions, chemicals and equipment, and an explanation of data handling procedures may be given as separate headed paragraphs. The maker's name should be given for all non-standard chemicals, apparatus and equipment. Please see below for more information on the use of antibodies. Materials known by a trade name, e.g. Perspex, have the initial letter as a capital. The Latin names as well as the common name of non-mammalian species should be given.

Please state access codes for data deposition (e.g. protein/DNA/RNA sequences, macromolecular structures) and where relevant, indicate where computer source codes can be obtained.

All articles that use antibodies must provide the following information:

- The source of antibodies, including company name, distributor, developer.
- A representative full blot detailing the validation of each antibody used in the study for protein specificity, excluding loading controls. Lanes on the blot should be labelled to note the nonspecific and specific bands and the positive and negative controls. Exposure time should be indicated.
- Proof for antibody specificity. If specificity data have already been published, authors need not repeat the experiment, but reference where the validation has been published previously. This should be within a peer-review publication and not a manufacturer's site.

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- Antibodies: "Sections were stained with a NeuN antibody (Millipore Cat# MAB377, Lot#307 RRID:AB_2298772)".
- Cell Lines: "Subjects include the following cell line obtained from the CLS cell repository, CLS Cat# 300384/p699_HeLa_S3, RRID:CVCL_0058".
- Organisms: "Subjects in this study were Tlx3 transgenic mice obtained from MMRRC (RRID:MMRRC_036547-UCD)".
- Software tools: "...terminals were mapped with a computer-assisted mapping program (Neurolucida, v10, RRID:SCR_001775)".

Please find all of the antibodies, organisms, cell lines and software tools that you have used in the course of this study and add the RRID for each in the methods section. If you need help with either finding a resource or registering a new resource, the help desk on SciCrunch is staffed and you should hear back within one business day.

Please note, searching the website by catalogue or stock number is usually most effective in quickly retrieving the correct resource.

Results

Presenting the data. Data may be better presented graphically than in tables. Where possible, graphs should show individual values, rather than solid bars indicating a mean value. If a plot of individual values obscures some of the data presented, a box and whisker plot can be used to show mean or median, and 95% confidence intervals or quartiles.

Describing the samples. To characterise a normally distributed sample, report the mean (SD), and the number of samples (n). Use an appropriate number of significant figures. If samples are not normally distributed, use median and quartile values, or transform the data to obtain a more normal distribution. When describing the precision of estimation of a mean value, 95% confidence limits of the mean should be used, rather than the SEM.

Clearly indicate any inclusion or exclusion criteria. State whether the experimenter was 'blinded' to the experimental conditions, if applicable.

Comparing data. To express a difference between a control group and treated group, state the mean values, the estimated difference between the measurements, and the confidence limits of this difference. Since a common significance level for P is taken to be 0.05, the common confidence limits used are the 95% intervals.

Do not state that there is no difference between samples unless you are confident that the test has sufficient power to allow this conclusion. If necessary, indicate the power of the test, to distinguish absence of evidence from evidence of absence.

Statistical tests. Tests of significance should be specified on each occasion and in full, e.g. Student's paired t test. Use the exact P value, to the appropriate number of decimals, particularly when values are near a stated limit. For example, $P = 0.051$ and $P = 0.049$ have very similar implications.

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Please note that *Experimental Physiology* will ask authors at the revised manuscript stage to submit complete, original western blot images if they are not included in the manuscript. These should be uploaded as 'Supporting information for review process only'. Please ensure that in the original gel image provided, the relevant blots are highlighted and that these images are correctly labelled .e. Fig 1A, Fig 2C etc. This is to ensure that the original gel images can be directly compared to the manuscript figures, which should also be marked with figure labels.

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Experimental Physiology is committed to encouraging the free exchange of scientific information and to promote progress in all areas of physiological research. *Experimental Physiology* requires, as a condition for publication, that nucleic acid and protein sequences, microarray data and data obtained using high throughput sequencing techniques, which support the results in the paper, should be archived in an appropriate public database, 'Nucleic acid and protein sequences and microarray data must be deposited in an appropriate public data base (see below) and must be accessible without restriction from the date of publication.

An entry name or accession number, together with a direct link, must be included within the Methods section in the final version of the manuscript. Microarray data should be MIAME compliant (for guidelines, see <http://www.mged.org/Workgroups/MIAME/miame.html>). FGED (The Functional Genomics Data) Society: MIAME (Minimum Information About a Microarray Experiment). The public repositories ArrayExpress at the EBI (UK), GEO at NCBI (US) and CIBEX at DDBJ (Japan) are designed to accept, hold and distribute MIAME compliant microarray data. Exceptions may be granted at the discretion of the Editor, e.g. for sensitive information that might compromise the anonymity of human subjects.

Examples of suitable databases:

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EMBL (<http://www.ebi.ac.uk>),
DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>)
Protein Data Bank Japan (PDBj, <http://www.pdbj.org>)
Protein Databank (PDB, <http://www.rcsb.org/pdb/>)
Worldwide Protein Data Bank (wwPDB, <http://www.wwpdb.org>)
UniProtKB/Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html)

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Discussion

The Discussion, which follows the Results section, should be separate from it. The assumptions involved in making inferences from the experimental results should be stated. The Discussion should not merely recapitulate the results. Authors should provide a succinct conclusion to their work and are encouraged to conclude the Discussion by expressing an opinion on the relevance of the results in the context of work cited in the paper.

In appropriate circumstances an Appendix or a Theory section may be accepted where, for example, it is necessary to derive mathematical results required in the paper.

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The paper should conclude with a list of the papers and books cited in the text. Authors should avoid an excessive number of references. Normally about 50 should be adequate. The order of references is strictly alphabetical, regardless of chronology.

References should be prepared according to the *Publication Manual of the American Psychological Association* (6th Edition). This means in text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text. For example, (Jones, 1998). The complete reference list should appear alphabetically by name at the end of the paper.

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Journal Article

Example of reference with 2 to 7 authors

Beers, S.R., & De Bellis, M.D. (2002). Neurophysiological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry*, 159, 483-486. <https://doi:10.1176/appi.ajp.159.3.483>

Ramus, F., Rosen, S., Dakin, S.C., Day, B.L., Castellote, J.M., White, S., & Frith, U. (2003). Theories of developmental dyslexia: Insights from a multiple case study of dyslexic adults. *Brain*, 126(4), 841-865. <https://doi:10.1093/brain/awg076>

Example of reference with more than 7 authors

Rutter, M., Caspi, A., Fergusson, D., Horwood, L.J., Goodman, R., Maughan, B., ... Carroll, J. Sex differences in developmental reading disability: New findings from 4 epidemiological studies. *Journal of the American Medical Association*, 291(16), 2007-2012. <https://doi:10.1001/jama.291.16.2007>

Book Edition

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

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Unpublished material may be referred to sparingly in the text, by giving the authors' initials and names followed by 'unpublished observations' or 'personal communication'; such citations should not appear in the list of references. References cited as being 'in press' must have been accepted for publication, and the name of the journal or publisher included in the reference list.

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Preprints deposited in bioRxiv should be cited using their digital object identifier (DOI). Example: Author AN, Author BT. 2013. My article title. bioRxiv <https://doi.org/10.1101/123456>

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All manuscripts must contain a statement regarding Competing interests, Author contributions and Funding. If you have no competing interests or have not receiving funding, statements to this effect must be included. This will be published under a section entitled Additional Information at the end of the paper.

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If your submitted article has been published on a not for profit subject based preprint server (e.g. bioRxiv) please provide a foot note stating that this article was first published as a preprint and provide full citation using the digital object identifier (DOI). Example: This article first appeared as a preprint: Author AN, Author BT. 2013. My article title. bioRxiv <https://doi.org/10.1101/123456>

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All manuscripts must contain a statement regarding Competing interests. If there are no competing interests, a statement to this effect must be included. Authors should disclose any conflict of interest in accordance with [Journal Policy](#).

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Author contributions This section must state the laboratory where the experiments were performed and list the contribution (in words) of each author (using their initials) to the following aspects of the study:

- 1. Conception or design of the work
- 2. Acquisition, analysis, or interpretation of data for the work
- 3. Drafting of the work or revising it critically for important intellectual content

All authors must have contributed to 1 or 2 AND 3.

It must also confirm that all authors:

- 4. Approved the final version of the manuscript
- 5. Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

and that

- 6. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Contributors who do not meet the above criteria for authorship should not be listed as authors, but have their contribution (such as acquisition of funding; general supervision of a research group or general administrative support; and writing assistance, technical editing, language editing, and proofreading) listed in the Acknowledgements.

Additional information such as 'X and Y have contributed equally to this work' may be added as a footnote on the title page.

Funding

Authors must indicate all sources of funding, including grant numbers. If authors have not received funding, this must be stated.

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Acknowledgements

Acknowledgements should be the minimum consistent with courtesy. The wording of acknowledgements of scientific assistance or advice must have been seen and approved by the persons concerned.

Tables

Tables should be used sparingly. They should be referred to in the text by arabic numerals, e.g. Table 3. Each table should have its own self-explanatory title. The same information should not be presented in both tabular and graphical forms. Tables will be processed as text and therefore should NOT be submitted as figures.

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