Universidade Federal do Rio Grande do Sul

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Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

Influência do exercício físico na prevenção de alterações no status oxidativo, metabolismo energético e mecânica respiratória observadas em ratos submetidos à lesão pulmonar

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Dedico esta dissertação às três pessoas

mais importantes da minha vida: Mãe, Pai e Mana!

As três pessoas que não mediram esforços para

me apoiar em todas as minha decisões,

sempre com muita dedicação e amor,

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"Que nossos esforços desafiem as impossibilidades.

Lembrai-vos que as grandes proezas da história

foram conquistadas do que parecia impossível."

(Charles Chaplin)

Sumário

RESUMO..... VIII

ABSTRACT..... IX

LISTA DE ABREVIATURAS..... X

LISTA DE FIGURAS..... XII

TABELA..... XV

I. INTRODUÇÃO.....1

1. Definição da síndrome da angústia respiratória aguda (SARA).....1

2. Fisiopatologia.....2

3. Estresse oxidativo.....4

4. Metabolismo energético.....6

5. Mecânica respiratória.....7

6. Tratamento da lesão pulmonar.....9

7. Exercício Físico.....11

8. Exercício Físico e Lesão Pulmonar.....13

Objetivo geral.....14

Capítulo I 14

Capítulo II 15

III. RESULTADOS E METODOLOGIA.....16

Capítulo I 19

Capítulo II 30

IV. DISCUSSÃO.....65

V. CONCLUSÕES.....77

VI. PERSPECTIVAS 81

VII. REFERÊNCIAS BIBLIOGRÁFICAS..82

Resumo

A síndrome da angustia respiratória é caracterizada por uma inflamação bilateral, infiltrado intersticial e insuficiência respiratória. Acredita-se que o exercício físico seja benéfico na prevenção de doenças pulmonares. O objetivo deste estudo foi avaliar os efeitos do exercício físico sobre alguns parâmetros de estresse oxidativo, metabolismo energético e alterações na mecânica respiratória (complacência, elastância e resistência do sistema respiratório) no pulmão de ratos submetidos à lesão pulmonar. O efeito protetor do exercício físico também foi avaliado sobre às alterações da barreira alvéolo-capilar (contagem total de células, atividade da LDH e proteína total) e o conteúdo de fosfolipíedeos no lavado broncoalveolar, bem como sobre o infiltrado inflamatório e o edema no parênquima pulmonar**.** Os animais foram submetidos a dois meses de exercício físico moderado, realizado 3 vezes por semana. Transcorrido o período de exercício, a lesão pulmonar foi induzida através da injeção intratraqueal de LPS (100 μg/100g de peso corporal) e doze horas após foram submetido à avaliação da mecânica respiratória e/ou foram mortos para a coleta do material. Foram avaliados parâmetros de estresse oxidativo [formação de espécies reativas, lipoperoxidação, dano oxidativo a proteínas, atividade de enzimas antioxidantes superóxido dismutase (SOD) catalase (CAT) glutationa peroxidase (GPX), níveis de nitritos, capacidade antioxidante total (TRAP), conteúdo de glutationa (GSH)] e fator nuclear kappa-beta (NF-κβ/p65) no pulmão de ratos submetidos à lesão pulmonar. Parâmetros de metabolismo energético também foram avaliamos [succinato desidrogenase (SDH), complexo II e citocromo *c* oxidase e níveis de ATP]. Resultados mostraram um aumento nos níveis de espécies reativas, lipoperoxidação, danos a proteínas, níveis de nitritos, bem como no NF-κβ/p65 no pulmão de ratos submetidos a lesão pulmonar. O exercício físico foi capaz de prevenir o aumento das espécies reativas, os níveis de nitritos o NF-κβ/p65; e parcialmente preveniu o dano às proteínas. As atividades da SOD e CAT não foram alteradas nos animais submetidos à lesão pulmonar. No entanto, no grupo lesão pulmonar e exercício houve um aumento na atividade dessas enzimas. A atividade da GPX e G6PD, níveis de GSH e TRAP estão diminuídos nos animais submetidos à lesão pulmonar; o exercício preveniu totalmente tais alterações. Resultados mostraram que os animais submetidos à lesão pulmonar apresentaram uma diminuição na atividade das enzimas da cadeia transportadora de elétrons e nos níveis de ATP e formação de edema pulmonar. Também observamos uma redução da complacência pulmonar e um aumento da resistência alveolar. O exercício preveniu totalmente a diminuição nas atividades do complexo II e SDH e a formação do edema, bem como preveniu parcialmente a resistência alveolar. Ratos submetidos à lesão pulmonar aguda apresentaram um aumento na contagem total de células, na atividade da LDH nas proteínas e uma diminuição nos fosfolipídeos no lavado broncoalveolar; exercício preveniu parcialmente somente o aumento na

atividade da LDH. Nossos achados sugerem que o exercício físico pode ter um importante papel na prevenção de algumas alterações presentes na lesão pulmonar.

Abstract

Acute respiratory distress syndrome is characterized by pulmonary inflammation, interstitial infiltrates and respiratory failure. It is believed that exercise is beneficial in preventing lung diseases. The objective of the present study was evaluated the effects of physical exercise on some parameters of oxidative stress, energy metabolism and respiratory mechanics (dynamic and static compliance, elastance and respiratory system resistance) in the lungs of rats submitted to lung injury. The protective effect of physical exercise was also evaluated with regard to the alterations in the alveolar-capillary barrier (total cell count, activity of LDH and total protein) and phospholipids content in the bronchoalveolar lavage, as well as on the inflammatory infiltration and edema in the pulmonary parenchyma. The animals were submitted to two months of moderate physical exercise three times per week. After this period of physical exercise, lung injury was induced by intratracheal instillation of LPS (100μg/100g body weight) and twelve hours after the injury were performed the respiratory mechanical and/or were killed and collected material. It was evaluated oxidative stress parameters [formation of reactive species, lipid peroxidation, oxidative damage to protein, antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), nitrite levels, total antioxidant capacity (TRAP), glutathione content (GSH)] and nuclear factor-kappa Beta (NF-κβ/p65) in lung of rats submitted to lung injury. Parameters of energy metabolism were also evaluating [succinate dehydrogenase (SDH), complex II and cytochrome *c* oxidase, and ATP levels]. Results showed an increase in reactive species production, lipid peroxidation, oxidative damage to protein, as well as nitrite levels, as well NF-κβ/p65 in lung of rats submitted to lung injury. Physical exercise was able to totally prevent the increase in reactive species, nitrite levels and NF-κβ/p65, but partially prevented the damage to protein. The activities of SOD and CAT were not changed in animals submitted to lung injury. However in exercise–lung injury rats group the activities of these enzymes were increased. The activity of GPx, GSH content and TRAP were decreased in lung of rats submitted to lung injury. Physical exercise totally prevented such effects. Results showed that rats subjected to lung injury presented a decrease in activities of the enzymes of the electron transport chain and ATP levels as well as presented the pulmonary edema formation. It was also observed decreased lung dynamic and static compliance and increased in respiratory system resistance. Physical exercise was able to totally prevent the decrease in SDH and complex II activities and the pulmonary edema formation, as well partially prevented the respiratory system resistance. Rats subjected to lung injury presented an increase in total cell, activity of LDH, total protein and phospholipids content in bronchoalveolar lavage; exercise only partially prevented the increase in the activity of LDH. Our findings suggest that exercise may have an important role as protector in some alterations presents in lung injury.

Lista de abreviaturas

ATP- adenosina trifosfato

CAT – Catalase

DNA – ácido desoxirribonucléico

FiO_2 - Fração inspirada de oxigênio

G6PD - Glicose-6-fosfato desidrogenase

GSH - Glutationa (forma reduzida)

GPx- Glutationa peroxidase

H_2O_2 - Peróxido de hidrogênio

H_2DCF - 2',7'-diclorofluoresceína

IL-8- interleucina 8

IL-1 interleucina 1

i-NOs - Óxido nítrico sintase induzível

LPS- lipopolissacarídeo

LBA - Lavado broncoalveolar

LDH - Lactato desidrogenase

NO - Óxido nítrico

NOs - Óxido nítrico sintase

O2 - Oxigênio

O2-. - Radical superóxido

NF-κβ - Fator nuclear kappa beta

OH . - Radical hidroxil

ONOO- - Peroxinitrito

PaO2 - Pressão parcial de oxigênio arterial

PEEP - Pressão positiva expiratória final

SARA - síndrome da angústia respiratória aguda

SDH – Succinato desidrogenase

SOD - Superóxido dismutase

TBARS - Substâncias reativas ao ácido tiobarbitúrico

TNF-α - fator de necrose tumoral alfa

TRAP- potencial antioxidante total

VO_{2max}- volume por consumo de oxigênio máximo

Lista de figuras

INTRODUÇÃO

FIGURA 1. Mecanismo de lesão pulmonar.....3

FIGURA 2. Defesas Antioxidantes enzimáticas.....5

FIGURA 3. Avaliação da mecânica ventilatória - FlexiVent 9

FIGURA 4. Representação esquemática do mecanismo pelo qual as espécies reativas ativam a expressão das enzimas antioxidantes.....12

MATERIAIS E RESULTADOS

FIGURA 5. Indução da lesão pulmonar.....16

FIGURA 6. Esteira adaptada para ratos.....18

FIGURA 7. Linha de tempo dos procedimentos experimentais.....18

CAPÍTULO I – Artigo 01

FIGURA 1. The timeline of experimental procedures…….....31

FIGURA 2. Effect of lung injury and/or exercise on reactive species production.....33

FIGURA 3. Effect of lung injury and/or exercise on thiobarbituric acid reactive substances, carbonyl content and sulfhydryl content in lung of rats…….…….33

FIGURA 4. Effect of lung injury and/or exercise on total radical-trapping antioxidant potential and reduced glutathione content in lung of rats.....34

FIGURA 5. Effect of lung injury and/or exercise on enzymatic antioxidant defense superoxide dismutase, catalase and glutathione peroxidase in lung of rats….....34

FIGURA 6. Effect of lung injury and/or exercise on nitrite levels and NF-κβ/p65…….35

FIGURA 7. Histopathologic findings after lipopolysaccharide instillation..…….36

FIGURA 8. Summary of the effect of lung injury on oxidant–antioxidant status present in the lung……37

CAPÍTULO II – Artigo 02

FIGURA 1. The timeline of experimental procedures.....59

FIGURA 2. Effect of lung injury and/or exercise on SDH and complex II in lung of rats…….....60

FIGURA 3. . Effect of lung injury and/or exercise on cytochrome *c* oxidase and ATP levels in lung of rats…….....61

FIGURA 4. Effect of lung injury and/or exercise on dynamic compliance static complicance respiratory system elastance and respiratory system resistance in lung of rats.....62

FIGURA 5. Effect of lung injury and/or exercise on wet/dry weight ratio in lung of rats…….63

FIGURA 6. Summary of the effect of lung injury on alteration in energetic metabolism…….....64

CONCLUSÃO

FIGURA 8. Resumo das alterações encontradas no pulmão de ratos submetidos à lesão pulmonar.....81

Tabelas

MATERIAIS E RESULTADOS

CAPÍTULO I – Artigo 01

TABELA 1. Effect of ALI and/or Exercise on total cell count, lactate dehydrogenase activity and total protein concentration in bronchoalveolar lavage…….35

CAPÍTULO I – Artigo 02

TABELA 1. Effect of lung injury and/or exercise on phospholipids content in bronchoalveolar lavage fluid…….....65

I. INTRODUÇÃO

1. Definição síndrome da angústia respiratória aguda (SARA)

A lesão pulmonar aguda e a síndrome da angústia respiratória aguda (SARA) são caracterizadas pela deficiência respiratória, por uma inflamação bilateral e infiltrado intersticial, consequente do aumento da permeabilidade da membrana alvéolo-capilar, levando a um quadro de insuficiência respiratória aguda. A SARA foi descrita primeiramente em 1967, quando Ashbaugh e colaboradores descreveram 12 pacientes com angustia respiratória aguda, cianose, diminuição na complacência pulmonar e infiltrado pulmonar difuso, evidenciado por avaliação radiográfica do tórax (Ashbaugh et al., 1967). Em 1988 foi proposta uma definição mais ampla que pudesse quantificar a deficiência respiratória através do uso de um escore de injúria pulmonar baseado em quatro parâmetros: 1) pressão expiratória, 2) a razão entre a pressão parcial de oxigênio arterial (PaO_2) e a fração de oxigênio inspirado (FiO_2), 3) a complacência pulmonar, 4) o grau de infiltração pulmonar evidenciado por exame radiográfico de tórax (Murray et al., 1988). Embora esse escore tenha sido amplamente utilizado para quantificar a severidade da lesão pulmonar em pesquisas e ensaios clínicos, ele não pode ser usado como parâmetro de prognóstico nas primeiras 24 a 72 horas após o início da SARA, fato esse que limitou o seu uso na prática clínica (Doyle et al., 1995; Zilberberg and Epstein, 1998). Em 1994, uma nova definição foi apresentada pelo "*American-European Consensus Conference Committee*"(Bernard et al., 1994), a qual reconheceu que a severidade do dano pulmonar poderia ser uma variável. Desse modo os pacientes com hipoxemia menos severa (definida pela razão $PaO_2/FiO_2 \leq 300$) seriam considerados com lesão pulmonar aguda, e

pacientes com hipoxemia mais severa (razão ≤ 200) seriam considerados com SARA. Esta definição apresentou-se mais simples e de fácil aplicação na prática clínica (Artigas et al., 1998; Bernard et al., 1994). No entanto, ao longo dos anos surgiram algumas críticas sobre tal definição. A falta de critérios específicos para determinar a fase aguda (a partir da relação PaO_2/FiO_2), bem como a variabilidade para interpretar a presença de edema e infiltrado radiológico pulmonar (Meade et al., 2000; Rubenfeld et al., 1999) levaram pesquisadores a revisar essa definição. As questões levantadas através da experiência de profissionais, a fim de facilitar o diagnóstico e tratamento, foram apresentadas no "*American-European Consensus Conference*" em Berlin, em 2011. A nova definição tem focado a viabilidade, confiabilidade, validade e avaliação objetiva da SARA (Ferguson et al., 2012). A definição propôs três categorias mutuamente exclusivas de SARA com base no grau de hipoxemia: leve (relação PaO_2/FiO_2 entre 201 e 300), moderada (101-200) e grave (menor que 100). Uma alteração importante na definição foi baseada na eliminação da expressão "lesão pulmonar aguda" e a inclusão dos três níveis de severidade com base na medida da PaO_2/FiO_2 com pelo menos 5 cm de H_2O de PEEP. E assim os critérios de Berlim foram criados para proporcionaram uma melhora na capacidade de predição de mortalidade do paciente com SARA (Ferguson et al., 2012; Ranieri et al., 2012).

2. Fisiopatologia

Em relação à fisiopatologia da lesão pulmonar podemos observar a presença de dano difuso da parede alveolar e do endotélio vascular. As consequências agudas do dano incluem o aumento da permeabilidade vascular

e o infiltrado alveolar, perda da capacidade de difusão e um distúrbio na secreção da substância surfactante. A lesão pulmonar é o resultado de uma cascata de eventos onde há uma secreção elevada de mediadores pró-inflamatórios e uma diminuição na secreção de compostos anti-inflamatórios (Fan et al., 2001).

Os sinais que levam a uma ativação descontrolada da resposta inflamatória ainda não são muito bem compreendidos. Estudos demonstram que 30 minutos após a lesão observa-se uma síntese elevada de interleucina 8 (IL-8) pelos macrófagos que é um ativador dos neutrófilos, além da liberação de outras citocinas como interleucina 1 (IL-1), interleucina 1 beta (IL-1 β) e o fator de necrose tumoral alfa (TNF-α), que estimulam o recrutamento de neutrófilos do sistema vascular para o interior do alvéolo. Os neutrófilos ativados liberam uma variedade de produtos que amplificam o dano aos tecidos e mantém a cascata inflamatória, entre eles radicais livres, proteases, fator ativador de plaquetas e leucotrienos (Fan et al., 2001). Neste contexto, estudos pré clinicos mostram um elevado número de neutrófilos no interstício pulmonar e nos alvéolos (Lee and Downey, 2001; Ware and Matthay, 2000).

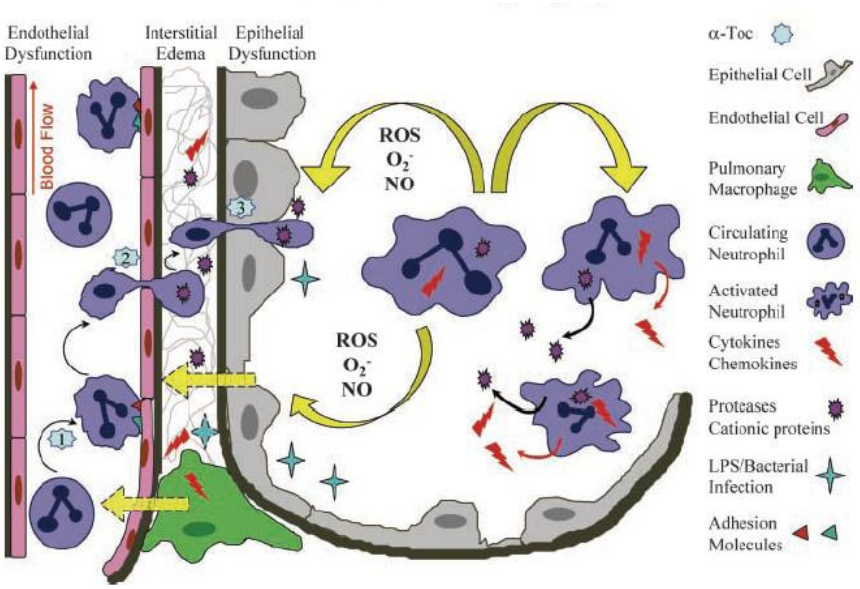

Figura 1: Mecanismo de lesão pulmonar (Chow et al., 2003).

3. Estresse oxidativo

O desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes é denominado de estresse oxidativo (Halliwell and Gutteridge, 2007). As espécies reativas são formadas em nosso organismo em condições fisiológicas e participam de funções importantes, como a fagocitose, sinalização celular, regulação de proteínas e a plasticidade sináptica (Halliwell and Gutteridge, 2007; Ward and Peters, 1995). Entretanto, em condições patológicas pode haver aumento da produção de oxidantes e/ou diminuição dos níveis de antioxidantes, favorecendo a ocorrência do estresse oxidativo (Halliwell and Gutteridge, 2007). Nessas condições as espécies reativas podem oxidar diversas biomoléculas, como os lipídios, proteínas e o DNA. O dano oxidativo aos lipídios, ou lipoperoxidação, ocorre quando as radicais livres reagem com lipídios insaturados presentes nas membranas celulares, ocasionando modificações na permeabilidade e na fluidez da membrana, além de causar dano a proteínas transmembrana, como enzimas, receptores e canais iônicos (Halliwell and Gutteridge, 2007). As principais espécies reativas são superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio H_2O_2, hidroxil (•OH), óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$). Uma fonte importante de espécies reativas é o sistema de transporte de elétrons mitocondrial, sendo seu principal sítio de formação o complexo citocromo ubiquinona (Southorn and Powis, 1988; Tyler, 1975). Na mitocôndria, a citocromo oxidase promove a redução completa de uma molécula de oxigênio (O_2) em uma molécula de água (H_2O) e, para isso, são necessários quatro elétrons. Contudo, nem sempre o O_2 origina H_2O diretamente. O O_2^- é o primeiro intermediário formado a partir da redução incompleta do O_2 molecular na formação da H_2O, e a partir dele podem se

Formar outras radicais livres como o •OH e o H_2O_2 (Duthie et al., 1992; Halliwell and Gutteridge, 2007).

A fim de evitar os efeitos danosos das espécies reativas, o nosso organismo dispõe de mecanismos eficientes para a detoxificação desses agentes oxidantes, conhecidos como defesas antioxidantes. Essas podem ser divididas em enzimáticas e não enzimáticas. As principais enzimas antioxidantes são a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX). As defesas antioxidantes não enzimáticas incluem principalmente a glutationa (GSH), riboflavina, tiamina, polifenóis, bilirrubina, entre outras (Halliwell and Gutteridge, 2007; Salvador and Henriques, 2004).

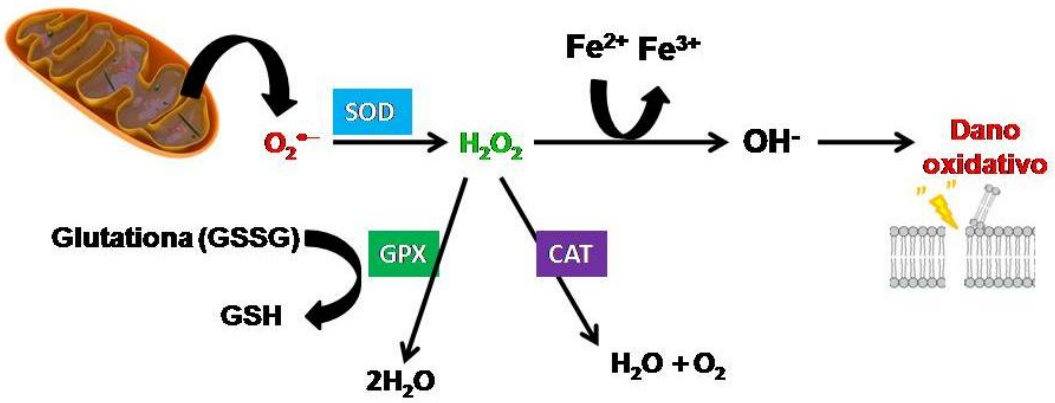

Figure 2. Defesas Antioxidantes enzimáticas.

O pulmão é um órgão que está continuamente exposto a O_2 (cerca de 21 %) além da exposição a poluentes, toxinas e micro-organismos (Quinlan et al., 2002). Ele é órgão vulnerável à inflamação que pode gerar espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) (Lang et al., 2002). Na lesão pulmonar observa-se uma alteração no balanço oxidantes/antioxidantes, sendo essa uma característica muito importante na sua fisiopatologia. Estudos mostram que pacientes com lesão pulmonar

apresentam concentrações elevadas de enzimas antioxidantes e concentrações elevadas principalmente de H_2O_2, bem como de produtos de peroxidação de fosfolipídeos de membrana (Bowler et al., 2003; Gessner et al., 2003). Dentre essas alterações observa-se um aumento na produção de $NO^{\bullet}$ que em situações patológicas é citotóxico, pois interage com o O_2^- e forma um dos mais potentes e deletérios radicais livres, o $ONOO^-$ (Bowler et al., 2003; Gessner et al., 2003). Ritter e colaboradores (2006), verificaram a indução de estresse oxidativo em pulmão de ratos submetidos à lesão pulmonar através da injeção intratraqueal do lipopolissacarídeo (LPS), evidenciado pelo aumento nos níveis de substâncias reativas ao ácido tiobarbotúrico (TBARS) e um desequilíbrio entre as defesas antioxidantes enzimáticas SOD e CAT. Além disso, um estudo clínico demonstrou um aumento nos níveis da CAT e uma diminuição nos níveis da GSH no lavado broncoalveolar (LBA) de pacientes com lesão pulmonar (Pacht et al., 1991).

4. Metabolismo energético

A mitocôndria é a organela responsável pela respiração celular, por meio da oxidação dos produtos do metabolismo. As mitocôndrias executam várias funções celulares, incluindo a produção de energia em forma de adenosina trifosfato (ATP), regulação do cálcio intracelular, ativação de vias de morte celular (apoptose e necrose), sensoriamento do oxigênio e sinalização (Reddy, 2006). A geração de energia mitocondrial é um sistema complexo que depende da demanda metabólica, que é aumentada durante períodos de atividades de alta intensidade e de estresse (Servais et al., 2003; Singer, 2007). A matriz mitocondrial contém enzimas envolvidas no ciclo de Krebs, porém a succinato

desidrogenase (SDH) fica ligada à membrana interna. Também se encontra na membrana interna da mitocôndria os complexos enzimáticos envolvidos no transporte de elétrons e na fosforilação oxidativa (Devlin, 2011). O gradiente eletroquímico formado pelo bombeamento de prótons durante a cadeia respiratória mitocondrial é utilizado como força motriz para a ATP sintase, formar ATP (fosforilação oxidativa). Disfunções mitocondriais podem afetar o metabolismo, aumentar a formação de radicais livres, induzir apoptose e contribuir para o desequilíbrio na formação de ATP (Brooks et al., 2008; Saikumar et al., 1998; Schild et al., 1997; Sommer et al., 2011). Estudos têm demonstrado que deficiências no funcionamento normal da cadeia respiratória mitocondrial levam a disfunções celulares (Heales et al., 1999). Nesse contexto, estudos sugerem que anormalidades na mitocôndria estão envolvidas na fisiopatologia do envelhecimento, doenças neurodegenerativas, câncer e diabetes (Beal, 2005; Maurer et al., 2000; Reddy, 2008; Trigulova, 1978), bem como no desenvolvimento de doenças inflamatórias (Raby et al., 2007).

5. Mecânica respiratória

A respiração é um processo cíclico que envolve trabalho mecânico dos músculos respiratórios para a movimentação do sistema respiratório. A mecânica do sistema respiratório é subdividida em dois sistemas elásticos: os pulmões e a parede torácica (West, 2002). O tecido pulmonar e tórax são constituídos fibras elásticas, cartilagens, células epiteliais e endoteliais, glândulas, nervos, vasos sanguíneos e linfáticos que possuem propriedades elásticas que permitem o retorno do sistema ventilatório à sua forma original

após sofrer deformação por uma força aplicada sobre eles (Romero et al., 1998).

A elasticidade é uma propriedade da matéria que permite ao corpo retornar a sua forma original após sofrer deformação por uma força aplicada sobre ele. Define-se elastância do sistema respiratório como a razão entre a variação de pressão aplicada ao sistema respiratório e a variação correspondente do volume, representando a capacidade de retração elástica do sistema respiratório (Zin et al., 1989). A elastância representa o inverso da complacência.

A complacência é definida como, a alteração de volume por unidade de pressão através do pulmão. Uma complacência pode estar reduzida devido ao aumento de tecido fibroso no pulmão, por edema alveolar, o qual evita a inflação de alguns alvéolos, presença de áreas de atelectasias. A complacência pode ser avaliada quando tórax e pulmões encontram-se em movimento (complacência dinâmica) ou em um determinado ponto entre a inspiração e a expiração (complacência estática) (West, 2002).

Além das propriedades elásticas dos tecidos pulmonares, os pulmões ainda apresentam um importante fator que contribui para a sua característica elástica, chamado tensão superficial. A tensão superficial ocorre no interior dos alvéolos devido a uma interface ar-líquido revestindo toda a parede interna dos alvéolos (Quintero and Wright, 2002; West, 2002). Essa tensão pode ser reduzida pela ação de uma substância produzida pelos pneumócitos tipo II, o surfactante que é formado basicamente de fosfolipídeos (King, 1985; Rau et al., 2003). Esta caracteristica do surfactante é importante para aumentar complacência e reduzir do trabalho respiratório e diminui a tendência ao colapso das unidades

alveolares ao final da expiração. Sem o surfactante o pulmão torna-se rígido, com baixa complacência, áreas de atelectasias e alvéolos cheios de transudado, característico do paciente com SARA (Abraham et al., 2000; Ashbaugh et al., 1967; Ware and Matthay, 2000).

Durante a movimentação do sistema respiratório, quando ocorre fluxo de gás, um elemento adicional ao elástico precisa ser vencido pela pressão motriz: a resistência. Resistência das vias aéreas representa a diferença de pressão entre os alvéolos e a boca por unidade de fluxo. A resistência pulmonar pode ser dividida em dois subcomponentes a resistência das vias aéreas que depende do fluxo de ar no interior dos pulmões e a resistência tecidual que é determinada pelas perdas energéticas. A resistência das vias aéreas pode ser influenciada pela geometria da árvore brônquica, pelo volume pulmonar, pela complacência das vias aéreas entre outros (West, 2002).

Para avaliação da mecânica ventilatória em modelos animais um dos equipamentos utilizados é o Flexi Vent.

Figura 4. Avaliação da mecânica ventilatória – FlexiVent.

Para que ocorra ventilação, a força de contração muscular deve vencer forças de oposição tais como: forças elásticas devidas às propriedades elásticas do tecido pulmonar, tensão superficial alveolar e propriedades elásticas da parede torácica; forças resistivas, devido ao fluxo de gás através das vias aéreas, a resistência viscosa dos tecidos que compõem o pulmão e a parede torácica, e as forças inerciais dos gases e das estruturas tóraco-pulmonares deslocadas na ventilação (D'Angelo et al., 1994; Hildebrandt, 1970; Milic-Emili, 1997; West, 2002).

6. Tratamento da lesão pulmonar

O tratamento da lesão pulmonar tem sido o suporte ventilatório com pressão positiva e altas concentrações de oxigênio inspirado (Delclaux et al., 2000; Meade et al., 2008; Mercat et al., 2008; Pipeling and Fan, 2010). Embora essas medidas sejam de grande importância na sobrevida dos pacientes, estudos mais recentes mostram que a própria ventilação mecânica, contribui para o dano pulmonar e possivelmente para a disfunção de múltiplos órgãos desses pacientes (Kavanagh and Slutsky, 1999; Slutsky, 1999). O importante no tratamento é conhecer a doença de base, para buscar alternativas de resolução do quadro (Frank et al., 2006).

Alguns estudos promissores avaliam o efeito de antioxidantes sobre estresse oxidativo presente na lesão pulmonar. Entre estes antioxidantes destacam-se a N-acetilcisteína (Aggarwal et al., 2012; Atis et al., 2006; Fan et al., 2000; Ritter et al., 2006; Victor et al., 2003), α-tocoferol e vitamina C (Nathens et al., 2002), e também as enzimas antioxidantes como a catalase e superóxido dismutase (Arita et al., 2007; Supinski et al., 1993). Embora alguns

resultados tenham sido positivos na diminuição do estresse oxidativo, algumas intervenções realizadas em estudos clínicos demonstraram resultados controversos em relação à diminuição do dano pulmonar e ao tempo de ventilação mecânica (Domenighetti et al., 1997; Jepsen et al., 1992; Pontes-Arruda et al., 2006).

Pelo menos para o nosso conhecimento, existem poucos estudos relacionando o papel protetor do exercício físico regular sobre as alterações, principalmente de estresse oxidativo, presente na lesão pulmonar.

7. Exercício Físico

O exercício pode ser definido como qualquer atividade planejada estruturada que leva a um aumento no gasto de energia e freqüência cardíaca. Há diferentes modalidades de exercício em relação à intensidade temos o exercício aeróbio e anaeróbio. Há evidências de que o exercício físico regular previne o desenvolvimento de muitas doenças (Blair et al., 2001), entre elas doenças cardiovasculares, hipertensão (Lenz and Monaghan, 2008), diabetes mellitus (Gill and Cooper, 2008), síndrome metabólica (Blaha et al., 2008), Alzheimer (Rolland et al., 2008) e outras doenças que podem estar relacionadas com inflamação sistêmica (Petersen and Pedersen, 2005). Durante o exercício físico ocorre aumento significativo na produção de ATP, o qual está associado a aumento do fluxo mitocondrial, e aumento no consumo de O_2, fazendo com que ocorra à ativação de vias metabólicas específicas que resultam na formação de espécies reativas (Servais et al., 2003). Acredita-se que esse estímulo seja capaz de disparar adaptações em resposta a uma maior produção dessas espécies reativas (Davies et al., 1982; Ji et al., 2006;

Powers et al., 2010; Powers et al., 2011; Radak et al., 2008). Há evidências mostrando o exercício regular aumenta as atividades das enzimas antioxidantes e os níveis de espécies reativas. As espécies reativas produzidas durante o exercício podem agir como moléculas de sinalização estimulando a expressão de enzimas antioxidantes (Gomes et al., 2012; Radak et al., 2008). Uma forma de adaptação e proteção contra o estresse oxidativo pode ser pela via do fator nuclear kappa beta (NF-kβ). O NF-kβ é um factor de transcrição que reside numa forma inativa no citoplasma, ligado ao inibidor da proteína inibitória I-kB (Baeuerle and Baltimore, 1988b). Através do estímulo gerado pelas espécies reativas formadas durante a sessão de exercício o NF-kβ é ativado e translocado para o núcleo onde induz a expressão de genes de enzimas antioxidantes (Baeuerle and Baltimore, 1988a; Hollander et al., 2001; Ji et al., 2004; Ji et al., 2006; Leaf et al., 1999; Meyer et al., 1994)

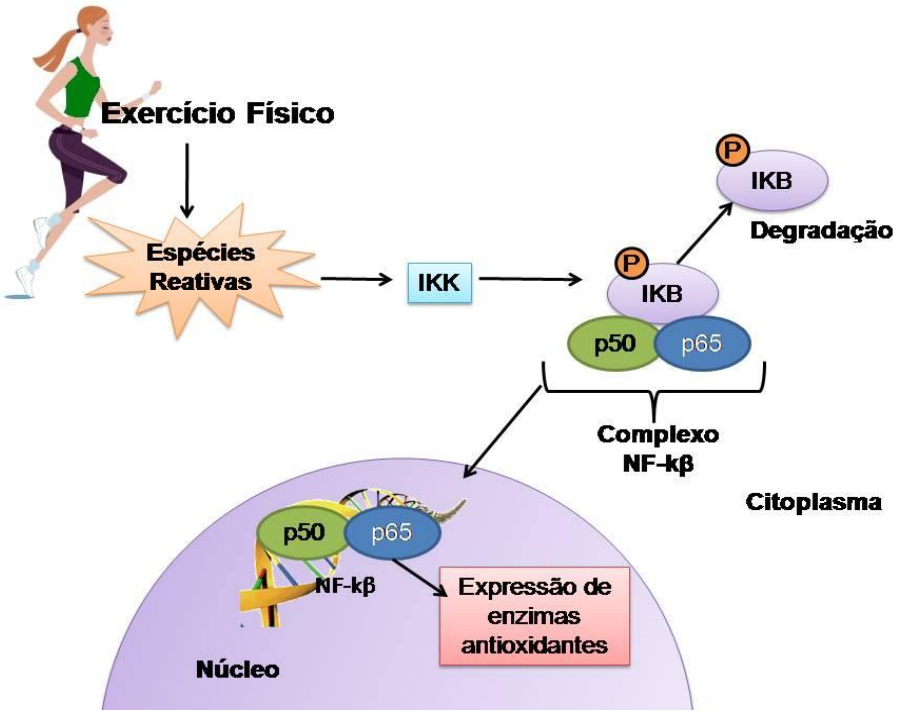

Figura 4. Representação esquemática do mecanismo pelo qual as espécies reativas ativam a expressão das enzimas antioxidantes.

Exercício Físico e Lesão Pulmonar

Dados da literatura sugerem que o exercício pode prevenir o desenvolvimento de doenças pulmonares (Chen et al., 2007; Mussi et al., 2008; Vieira et al., 2008). Acredita-se que o exercício físico possui um efeito anti-inflamatório, que se dá em grande parte pelo aumento da liberação de citocinas anti-inflamatórias como a interleucina 10 (IL-10) e de antagonistas de receptores de IL-1, além da diminuição da liberação de interleucina IL-1β e do TNF-α. Esses achados foram demonstrados em um modelo de lesão por isquemia e reperfusão no pulmão (Mussi et al., 2008). Outro achado interessante desse estudo foi demonstrado pelo aumento na atividade da SOD observada nos animais que foram submetidos ao exercício antes da indução da lesão.

Ramos e colaboradores (2009) mostraram que a prática de exercício físico previne a inflamação pulmonar e o aumento nos níveis de óxido nítrico exalado, que pode promover um aumento de radicais livres e induzir a liberação de citocinas e quimiocinas. Outro estudo realizado por Pinho e colaboradores (2006), demonstrou que o exercício físico é capaz de reverter danos oxidativos, avaliados através dos níveis de TBARS e de oxidação de proteínas em pulmão de ratos submetidos à lesão por inalação de carvão mineral.

OBJETIVOS

Objetivo geral

Considerando que existe uma relação entre a produção de espécies reativas e a lesão pulmonar, e que é muito importante identificar estratégias que possam reparar ou diminuir essa lesão, e que o exercício físico tem se mostrado benéfico na prevenção de doenças pulmonares, o objetivo geral do presente estudo foi avaliar a influência do exercício físico sobre alguns parâmetros de estresse oxidativo, metabolismo energético e processo inflamatório bem como na mecânica respiratória de pulmão de ratos submetidos a um modelo experimental de lesão pulmonar atraves da injeção intratraqueal de LPS.

Objetivos específicos

Os objetivos específicos estão subdivididos em dois capítulos, que serão apresentados na forma de artigos científicos, como seguem:

Capítulo I

- Determinar alguns parâmetros de estresse oxidativo [aumento de espécies reativas através da oxidação do 2',7'-diclorofluoresceína (DCF) lipoperoxidação através do método de TBARS, carbonilas proteícas, sulfidrilas, atividades das enzimas antioxidantes SOD, CAT, GPX, conteúdo de GSH, capacidade antioxidante total (TRAP) e níveis de nitritos] no tecido pulmonar de ratos submetidos à lesão pulmonar com ou sem exercício físico;

- Avaliar o imunoconteúdo do NF-κβ no tecido pulmonar de ratos submetidos à lesão pulmonar com ou sem exercício físico;
- Determinar a contagem de leucócitos totais no lavado broncoalveolar de animais submetidos à lesão pulmonar com ou sem exercício físico;
- Determinar a atividade da enzima lactato desidrogenase (LDH) e a concentração de proteínas no lavado broncoalveolar de animais submetidos à lesão pulmonar com ou sem exercício físico;
- Verificar a presença de infiltrado inflamatório através da análise histopatológica do tecido pulmonar nos animais submetidos à lesão pulmonar com ou sem exercício físico;

Capítulo II

- Verificar as possíveis alterações do metabolismo energético, através da avaliação da succinato desidrogenase (SDH), complexo II, citocromo *c* oxidase e níveis de ATP em tecido pulmonar de animais submetidos à lesão pulmonar com ou sem exercício físico;
- Verificar o efeito da lesão pulmonar sobre as possíveis alterações na mecânica respiratória, através da avaliação da complacência dinâmica e estática, elastância e resistência do sistema respiratório no pulmão de ratos; submetidos à lesão pulmonar com ou sem exercício físico;
- Analisar o conteúdo de fosfolipídeos (fosfatidilcolina, fosfosfatidiletanolamina e fosfatidilglicerol) no lavado broncoalveolar nos animais sumetidos à lesão pulmonar com ou sem exercício físico;
- Avaliar o a formação de edema no pulmão de ratos submetidos à lesão pulmonar com ou sem exercício físico.

II. MÉTODOS E RESULTADOS

Para os capítulos I e II foi desenvolvido um estudo experimental controlado com utilizando ratos Wistar, machos, provenientes do biotério do Departamento de Bioquímica, ICBS, UFRGS. Os animais foram mantidos com alimentação padrão livre e ciclo de 12 h claro-escuro em salas climatizadas (20°C + 1°C). Os cuidados com os animais seguiram as diretrizes governamentais oficiais conforme a Federação da Sociedade Brasileira para Biologia Experimental aprovadas pelo Comitê de Ética da Universidade Federal do Rio Grande do Sul (nº19322).

1. Modelo de LPA

Os animais foram anestesiados com uma mistura de ketamina (90mg/kg) e xilazina (10mg/Kg) por via intraperitoneal e a lesão pulmonar foi induzida através de injeção intratraqueal de lipopolissacarídeo (LPS) (*E. coli* 055:B5, Sigma Chemical, St Louis, MO) na dose de 100 μg/100g de peso corporal (da Cunha et al., 2011b; Ritter et al., 2006).

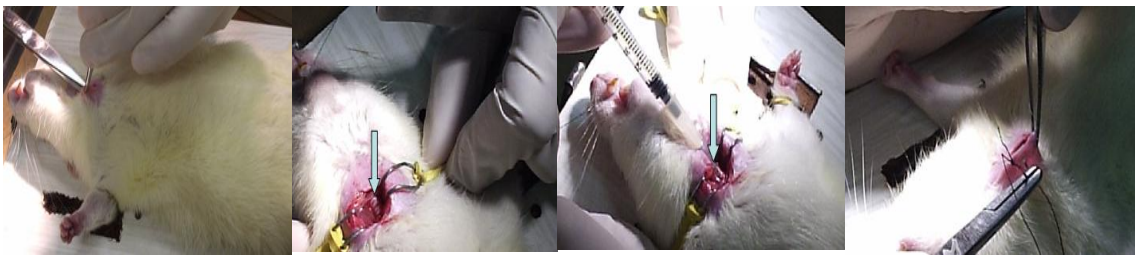

Figura 5. Indução da lesão pulmonar.

2. Grupos Experimentais

Os animais foram divididos em 4 grupos experimentais:

- **Grupo 1:** Injeção intratraqueal de solução salina e sedentário (sham sedentário);
- **Grupo 2:** Injeção intratraqueal de solução salina exercício (sham exercício);
- **Grupo 3:** Indução de lesão pulmonar e sedentário (lesão pulmonar);
- **Grupo 4:** Indução de lesão pulmonar e exercício (lesão pulmonar exercício).

Doze horas após a indução da lesão pulmonar os ratos foram anestesiados com uma mistura de ketamina (80mg/kg) e xilazina (12mg/Kg) por via intraperitoneal para a coleta do LBA e do tecido pulmonar para a análise dos diferentes parâmetros avaliados nessa dissertação.

3. Treinamento físico

No 25° dia de vida, os animais foram habituados ao aparelho para minimizar o estresse da novidade. O exercício em esteira começou no dia 30º de vida e terminou no dia 90º. O treinamento consistiu de 20 minutos de corrida em esteira adaptada para roedores (INBRAMEDTK01, Porto Alegre, Brasil) três vezes por semana, durante dois meses. Um protocolo de exercício moderado intensidade foi usado (Ben et al., 2009; Cechetti et al., 2007), isto é, a intensidade do exercício foi fixada em 60% do consumo de oxigénio máximo do animal (VO_{2max}) (Brooks and White, 1978). A estimativa de pico de consumo de oxigênio VO_{2max} foi então realizada em todos os animais antes do treino. Aproximadamente 24 horas após a última sessão de exercício, os ratos foram submetidos à lesão pulmonar.

Figure 6. Esteira adaptada para ratos.

A linha de tempo dos procedimentos experimentais é mostrada na Figura 7.

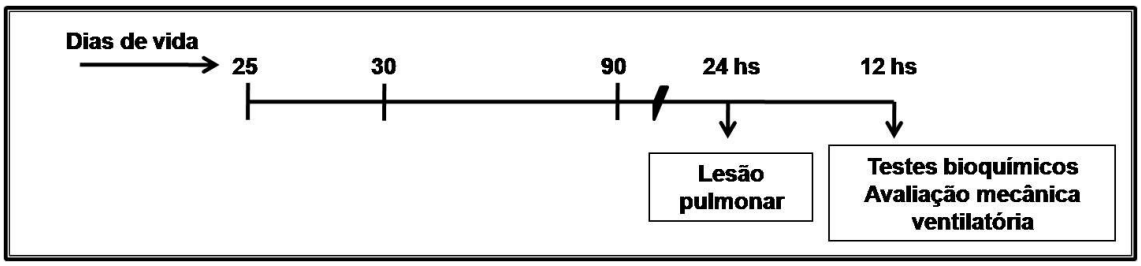

Figura 7. Linha de tempo dos procedimentos experimentais.

III. ARTIGOS CIENTÍFICOS

CAPÍTULO I

Effect of exercise on oxidative stress induced by experimental lung injury

Maira J. da Cunha, Aline A. da Cunha, Andrea G. K Ferreira, Maurício E. Baladão, Luiz E. B Savio, Carlos L. Reichel, Adriana Kessler, Carlos A. Netto and Angela T. S. Wyse

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The effect of exercise on the oxidative stress induced by experimental lung injury

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ABSTRACT

Aim: The effects of physical exercise on oxidative stress parameters and immunocontent of NF-κβ/p65 in lung of rats submitted to lung injury, as well as its possible protective effect on the changes in the alveolar-capillary barrier (total cell count, lactate dehydrogenase and total protein) in the bronchoalveolar lavage fluid (BALF) and the inflammatory infiltration in the pulmonary parenchyma were evaluated.
Main methods: Wistar rats were submitted to two months of physical exercise and after this period, lung injury was induced by intratracheal instillation of lipopolysaccharide (dose of 100 μg/100 g body weight). Twelve hours after injury, the animals were sacrificed and lung and BALF were collected.
Key findings: Results showed an increase in reactive species production, lipid peroxidation, oxidative damage to protein, as well as in nitrite levels and NF-κβ/p65 immunocontent in lung of rats submitted to lung injury. Physical exercise was able to totally prevent the increase in reactive species, nitrite levels and NF-κβ/p65 immunocontent, but partially prevented the damage to protein. Superoxide dismutase and catalase were not changed in lung injury group, but the activities of these enzymes were increased in lung injury plus exercise group. Non-enzymatic antioxidant capacity, glutathione content and glutathione peroxidase were decreased and exercise totally prevented such effects. Rats subjected to lung injury presented an increase in total cell, lactate dehydrogenase and total protein; exercise partially prevented the increase in lactate dehydrogenase.
Significance: These findings suggest that physical exercise may prevent, at least partially, the oxidative damage caused by experimental lung injury, suggesting that exercise may have an important role as protector in this condition.

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Introduction

Acute respiratory distress syndrome (ARDS) is a type of acute diffuse lung injury that affects both clinical and surgical patients. The pulmonary injury can be caused by gastric aspiration, polytrauma, pancreatitis, hemorrhagic shock, severe burns, oxygen toxicity and surgery involving cardiopulmonary bypass (Hagiwara et al., 2008; da Cunha et al., 2011).

The ARDS syndrome is characterized by progressive hypoxemia and pulmonary edema (Bernard et al., 1994). Its physiopathology involves destruction of the pulmonary capillary endothelium and alveolar epithelium (Windsor et al., 1993; Sutcliffe, 1994) accomplished by neutrophil, macrophages and erythrocyte accumulation, as well as hyaline membranes and protein-rich fluid in the alveolar spaces (Ware and Matthay, 2000). These inflammatory cells transmigrate across the endothelium and epithelium into the alveolar space and release a variety of proinflammatory compounds, including proteolytic enzymes, reactive oxygen species and nitrogen species and inflammatory cytokines. Thereafter, a vicious cycle is perpetuaded by the recruitment of additional inflammatory cells that in turn produce more cytotoxic mediators, ultimately leading to prominent injury to the alveolar-capillary membrane and respiratory failure (Windsor et al., 1993; Lee and Downey, 2001). It is believed that a key aspect of this pulmonary inflammatory response is mediated by increased levels of reactive species (Chow et al., 2003; Cross and Eiserich, 2004; Lang et al., 2002). In this framework experimental studies demonstrate that rats subjected to pulmonary injury present an increase in lipid peroxidation, oxidative damage to the protein and disrupted antioxidant defenses in the lung (Ritter et al., 2006; da Cunha et al., 2011).

At the moment, no specific treatment for ARDS is available, although recent progress has been made in this direction; then the search for new therapeutic strategies is necessary. However, interventions in animal models of lung injury aiming to reduce the production and/or the effects of reactive species present controversial results (Weinbroum et al., 2000; Leme et al., 2002; Koksel et al., 2004; Ritter et al., 2006).

Clinical studies have shown that regular physical exercise may be able to prevent development of pathologic conditions, including

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pulmonary and cardiovascular diseases, diabetes mellitus, metabolic syndrome and those associated with systemic inflammation (Blair et al., 2001; Pinho et al., 2007; Blaha et al., 2008; Gill and Cooper, 2008; Lenz and Monaghan, 2008; Vieira et al., 2008). Experimental evidence indicates that regular exercise is able to increase antioxidant defenses in heart, muscle and liver of rats (Venditti and Di Meo, 1997) and to prevent the lipid peroxidation and oxidative damage to protein present in lung injury (Pinho et al., 2006, 2009). However, few studies have investigated the effects of physical exercise in the development of lung injury. At least to our knowledge, there are no studies focusing on the protective role of regular exercise on the oxidative stress that is triggered by lung injury. Therefore, the present study was designed to evaluate whether a moderate physical exercise program would be able to prevent the induction of oxidative stress by measuring reactive species production, thiobarbituric acid reactive substances, carbonyl content, sulfhydryl content, total radical-trapping antioxidant potential, glutathione content, nitrite levels, activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and immunocontent of nuclear factor-kappaB (NF-κβ/p65) in the lung of rats subjected to lung injury. In addition, the disruption of the alveolar-capillary barrier was evaluated by parameters, such as total cell counts, lactate dehydrogenase activity (LDH) and total protein in the bronchoalveolar lavage fluid (BALF). Finally, the histological analyses were performed in order to verify the presence of inflammatory infiltrate in the pulmonary parenchyma.

Material and methods

Animals and reagents

Twenty-eight male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were randomly divided into four groups: (1) sham (sedentary and isotonic saline instillation, n = 7), (2) exercise (exercise and isotonic saline instillation, n = 7), (3) lung injury (sedentary and lipopolysaccharide (LPS) instillation, n = 7) and (4) exercise plus lung injury (exercise and LPS instillation, n = 7). Animals were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1 °C), with free access to water and commercial protein chow. Animal care followed the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 80-23, revised 1996) and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul (no 19322). All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Physical exercise training

On the 25th day of life, the animals were habituated to the apparatus to minimize novelty stress. The treadmill exercise started on the 30th day of life and finished on the 90th day. Training consisted of 20 min of running sessions on an adapted motorized rodent treadmill (INBRAMEDTK01, Porto Alegre, Brazil), three times per week (Cechetti et al., 2007). A moderate intensity exercise protocol was used (Cechetti et al., 2007; Ben et al., 2009), *i.e.*, exercise intensity was set at 60% of the animal's maximal oxygen uptake (Brooks and White, 1978). The estimation of oxygen uptake (VO_2) peak was then carried out in all animals before training, until exhaustion, as follows: each rat ran on a treadmill at a low initial speed, followed by increases in speed of 5 m/min every 3 min until the point of exhaustion (*i.e.*, failure of the rats to continue running); the time to fatigue (in min) and workload (in m/min) was taken as indexes of capacity for exercise, which was taken as VO_2 max. Animals from the control group (not exercised) were transported to the experimental room, handled exactly as the experimental ones and maintained in the turned off treadmill for 20 min. Rats were adapted to the treadmill by gradually increasing the running speed of up to 36 m/min (Cechetti et al., 2007; Ben et al., 2009) and were submitted to lung injury approximately twenty-four hours after the last exercise.

The timeline of experimental procedures is shown in Fig. 1.

Experimental procedures

Rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), intraperitoneally (IP), and lung injury was induced by intratracheal instillation of LPS (*Escherichia coli* 055:B5; Sigma Chemical, St. Louis, MO) at a dose of 100 μg/100 g body weight (da Cunha et al., 2011). Twelve hours after the LPS instillation, rats were anesthetized and BALF was collected three times after LPS instillation and withdrawn with 5 mL of phosphate-buffered saline. The final BALF lavage volume retrieved was approximately 15 mL. The BALF was then centrifuged (1000 *g* for 10 min) and the cell pellet was dissolved in 500 μL saline, and then diluted with *Turk's* solution and the total cell count was determined using a "*Neubauer*" counting chamber to confirm the lung injury induction (Asti et al., 2000). In order to estimate the alveolar cell injury and the alveolar–capillary membrane alterations, BALF LDH activity and total protein were determined. Subsequently, the rats were killed by decapitation and lung tissue was collected. One lung sample was isolated and fixed in a 4% formalin solution for histopathological analysis (see below) and the other was isolated and tissue homogenates were immediately stored at −70 °C until assaying for oxidative stress.

Tissue preparation

Lung tissue was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenate was centrifuged at 750 *g* for 10 min at 4 °C; the pellet was discarded and the supernatant was immediately separated and used for the measurements.

2′7′ dichlorofluorescein fluorescence assay

Reactive species production was measured according to the method of LeBel and colleagues (1992) and based on the oxidation of 2′7′-dichlorofluorescein (H_2DCF). The sample was incubated in a medium containing 100 μM 2′7′-dichlorofluorescein diacetate (H_2DCF-DA) solution. The reaction produces the fluorescent compound dichlorofluorescein (DCF) which is measured at $\lambda_{em} = 488$ nm and $\lambda_{ex} = 525$ nm; results were represented as nmol DCF/mg protein.

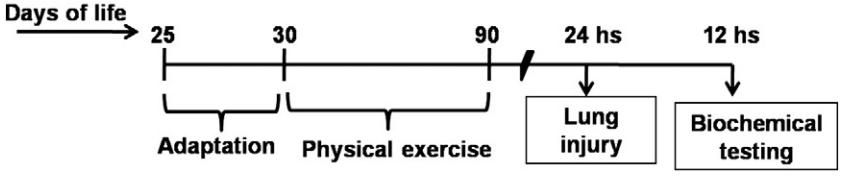

Fig. 1. The timeline of experimental procedures.

Thiobarbituric acid-reactive substances (TBARS)

TBARS, an index of lipid peroxidation, were measured according to Ohkawa and colleagues (1979). The sample was incubated in a medium contained 8.1% SDS, 20% acetic acid and 0.8% thiobarbituric acid. The mixture was vortexed and the reaction carried out in a boiling water bath for 1 h. The resulting pink stained TBARS were determined spectrophotometrically at 535 nm. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard and the results were represented as nmol TBARS/mg protein.

Protein carbonyl content

Oxidatively-modified proteins present an enhancement of carbonyl content. In this study, protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine, forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Results were represented as protein carbonyl content (nmol/mg protein).

Sulfhydryl content

This parameter was performed according to Aksenov and Markesbery (2001). The oxidation of free thiols in the sample leads to the formation of disulfide bonds. The 5,5′ dithio-bis(2-nitrobenzoic acid) (DTNB), color reagent is not reduced by the thiols oxidized, generating a yellow derivative (TNB), read spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to the protein. The results were represented as nmol TNB/mg protein.

Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,20-azo-bis-(2-amidinopropane) (ABAP) thermolysis in a Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA) (Evelson et al., 2001; Lissi et al., 1992). The time taken by the sample to maintain low chemiluminescence is directly proportional to the antioxidant capacity of the tissue. Results are represented as nmol Trolox/mg protein.

Superoxide dismutase assay (SOD)

The SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity was then indirectly assayed at 420 nm (Marklund, 1984). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were represented as SOD units/mg protein.

Catalase assay (CAT)

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

Glutathione peroxidase assay (GPx)

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

Reduced glutathione content (GSH)

This method is based on the reaction of GSH with the fluorophore *o*-phtalaldeyde (OPT) after deproteinizing the samples, and was measured according to Browne and Armstrong (1998). The sample was incubated in a medium contained sodium phosphate buffer pH 8.0, OPT 1 mg/mL (prepared in methanol). Subsequently, fluorescence was measured at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 350$ nm in a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). A calibration curve was also performed with a commercial GSH solution, and the results are represented as μmol GSH/mg protein.

Nitrite assay

Nitrite levels were measured using the Griess reaction; the sample was incubated in a medium contained Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The absorbance was measured at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards (Green et al., 1982).

Cellular fractionation for nuclear NF-κβ/p65 subunit

Lung was homogenized in hypotonic lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 1 mM sodium orthovanadate plus protease inhibitor cocktail). Lung homogenate was then lysed with 18 μL 10% IGEPAL. The homogenate was centrifuged (14,000 ×*g*, 30 s, 4 °C), and the nuclear pellet was resuspended in 200 μL ice-cold hypertonic extraction buffer (10 mM HEPES (pH 7.9), 0.40 M NaCl, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 1 mM sodium orthovanadate, 0.25 mM EDTA, 25% glycerol plus protease inhibitor cocktail). After 40 min of intermittent mixing, extracts were centrifuged (14,000 ×*g*, 10 min, 4 °C), and supernatants containing nuclear protein were secured (Zanotto-Filho et al., 2009). Aliquots were taken for protein determination and, for electrophoresis analysis, were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8.

Western blotting for NF-κβ/p65

Nuclear fraction was used for NF- κβ/p65 subunit according to the protocol described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE. Protein samples were separated by 10% SDS-PAGE (50 μg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% bovine serum albumin (BSA). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (TTBS) and then incubated overnight at 4 °C in blocking solution containing the antibody: anti-NF-κβ/p65

(1:1000; Santa Cruz Biotechnology). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated antirabbit IgG diluted 1:1000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS. Anti-β-actin was used as an internal control since its level is not affected by the experimental treatments. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemiluminescence was detected using X-ray films that were scanned and analyzed using the Optiquant Software (Packard Instruments).

Lactate dehydrogenase activity (LDH)

LDH activity in BALF was determined by a colorimetric assay with commercially available kits (Labtest Diagnostic ®, Brazil).

Histopathological analysis

The lungs were immediately removed after decapitation and placed in formalin. Histological slides from the samples were prepared using standard coloration techniques (hematoxylin and eosin). All samples were analyzed based on a qualitative determination of the presence of diffuse alveolar infiltrate. The analysis of inflammatory infiltrates was carried out by a pathologist blinded to the experimental groups and regions of sampling. Six samples for each experimental group were analyzed.

Protein determination

Protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Science (SPSS) software running on a PC-compatible computer. Data were analyzed by two way ANOVA followed by Tukey *post hoc* test, and lung injury and exercise were considered as interfering factors. Descriptive statistical data are expressed as means ± SD; differences of $p<0.05$ are considered as significant.

Results

Initially, we investigated the effects of lung injury and/or exercise on reactive species production (DCF levels) in the lungs of rats. As depicted in Fig. 2, two way ANOVA showed a significant interaction for lung injury *versus* exercise in this parameter [$F(1,24)=8.50$, $p<0.01$]. In addition, the *post hoc* Tukey test revealed a significant increase in reactive species production in rats submitted to lung injury ($p<0.01$), and this effect was significantly prevented by exercise.

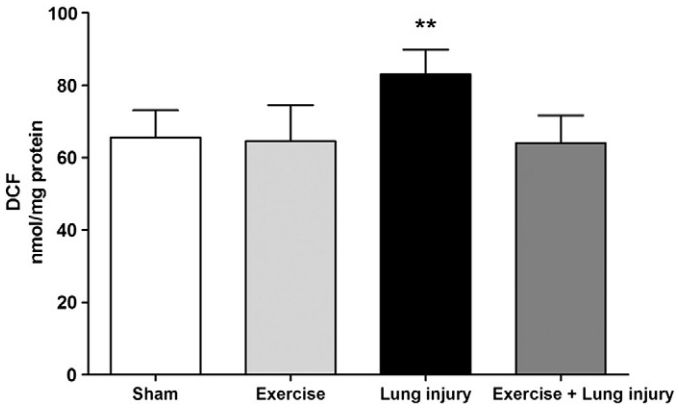

Fig. 2. Effect of lung injury and/or exercise on reactive species production. Data are expressed as mean ± SD for 7 animals in each group. Different from other groups, $^{**}p<0.01$ (two way ANOVA followed by Tukey test).

Next, we evaluated the effects of lung injury and/or exercise on lipid peroxidation, as measured by TBARS levels and damage to protein measured by carbonyl and sulfhydryl content in lungs of rats. Two-way ANOVA revealed a significant main effect in the lung injury-rats group (TBARS levels [$F(1,24)=17.63$, $p<0.001$], carbonyl content [$F(1,24)=12.90$, $p<0.01$] and sulfhydryl content [$F(1,24)=11.13$, $p<0.01$]). Subsequent *post hoc* Tukey test showed a significant increase in TBARS levels and carbonyl and decrease in sulfhydryl content in the lung of rats submitted to lung injury ($p<0.05$). Interestingly, exercise did not prevent the increase in TBARS levels, and partially prevented the increase in carbonyl and the decrease in sulfhydryl, contents (Fig. 3).

The lung non-enzymatic antioxidant defense was evaluated by TRAP and GSH content. As can be observed in Fig. 4a two way ANOVA revealed a significant effect in the lung injury-rats group [$F(1,24)=6.53$, $p<0.05$] and exercise group [$F(1,24)=8.18$, $p<0.01$]

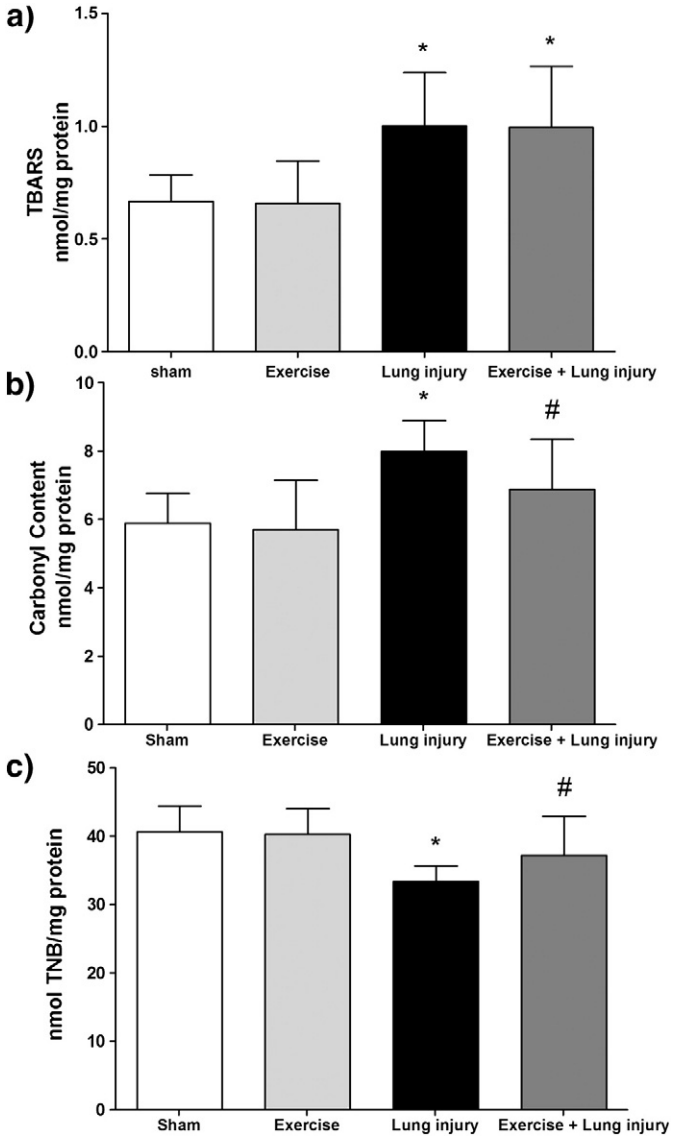

Fig. 3. Effect of lung injury and/or exercise on thiobarbituric acid reactive substances (a), carbonyl content (b) and sulfhydryl content (c) in lung of rats. Data are expressed as mean ± SD for 7 animals in each group. Different from other groups, $^{*}p<0.05$; # $p>0.05$ compared with sham and lung injury group (two way ANOVA followed by Tukey test).

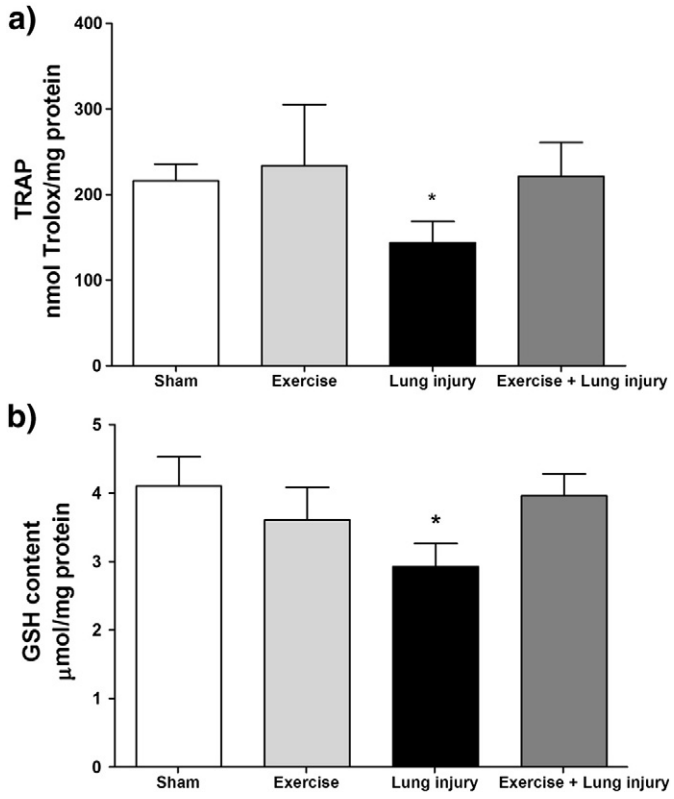

Fig. 4. Effect of lung injury and/or exercise on total radical-trapping antioxidant potential (a) and reduced glutathione content (b) in lung of rats. Data are expressed as mean ± SD for 7 animals in each group. Different from other groups, *$p<0.05$ (two way ANOVA followed by Tukey test).

by TRAP. Subsequent *post hoc* revealed a significant reduction of TRAP in rats submitted to lung injury ($p<0.05$) and this effect was significantly prevented by exercise. Furthermore, two-way ANOVA showed a significant interaction for lung injury *versus* exercise [$F(1,24) = 26.07$, $p<0.001$] on GSH content (Fig. 4b). The *post hoc* test indicated a decrease in GSH content in rats submitted to lung injury ($p<0.05$). Exercise *per se* did not alter this parameter, but it prevented the effect of lung injury.

The lung enzymatic antioxidant defense was also evaluated by the activities of SOD, CAT and GPx in the lung of rats submitted to lung injury and/or exercise. As can be observed in Fig. 5a, two way ANOVA showed a significant interaction of lung injury *versus* exercise on SOD activity [$F(1,24) = 14.87$, $p<0.01$]. The *post hoc* Tukey test showed that SOD activity was not altered in animals submitted to lung injury ($p>0.05$), but was increased in the exercise-plus-lung injury group ($p<0.001$). As regards to CAT activity (Fig. 5b), two way ANOVA demonstrated a significant effect in the lung injury-rat group [$F(1,24) = 6.67$, $p<0.05$] and in the exercised-rat group [$F(1,24) = 38.96$; $p<0.001$]. Subsequent *post hoc* Tukey tests revealed that CAT activity was not altered in animals submitted to lung injury; however it was increased in the animals in the exercise group ($p<0.05$) and exercise-plus-lung injury group ($p<0.001$). With regard to GPx activity, Fig. 5c shows a significant interaction of lung injury *versus* exercise [$F(1,24) = 5.80$, $p<0.05$]. The *post hoc* test revealed a significant decrease in GPx activity in rats submitted to lung injury ($p<0.001$), and physical exercise prevented this effect.

Nitrite levels in the lung of rats submitted to lung injury and/or exercise were also investigated. Two way ANOVA showed a significant interaction of lung injury *versus* exercise on nitrite levels [$F(1,24) = 33.49$, $p<0.001$]. As can be seen in Fig. 6a, nitrite levels were increased in rats subjected to lung injury ($p<0.001$), when compared to the control; physical exercise prevented this effect.

We also investigated the NF-κβ/p65 immunocontent in the lung of rats submitted to lung injury and/or exercise. Two-way ANOVA shows a significant interaction of lung injury *versus* exercise on NF-κβ/p65 [$F(1,24) = 8.23$, $p<0.05$]. The *post hoc* test showed an increase in NF-κβ/p65 immunocontent in rats submitted to lung injury ($p<0.01$) and exercise prevented this effect (Fig. 6b).

The disruption of the alveolar–capillary barrier was evaluated by parameters such as total cell count, LDH activity and total protein in BALF. Two way ANOVA (Table 1) showed a significant effect in the lung injury-rat group (total cell count [$F(1,24) = 176.52$, $p<0.001$], LDH activity [$F(1,24) = 27.61$, $p<0.001$) and total protein [$F(1,24) = 46.12$, $p<0.001$]. The Tukey test showed a significant increase in total cell count, protein concentration ($p<0.001$) and LDH activity ($p<0.01$) in the BALF of rats submitted to lung injury when compared to the control group. Exercise was able to partially prevent the increase in LDH, but did not prevent the increase in total cell count and total protein.

Qualitative histological analysis of lung tissue was done in order to verify the presence of inflammatory infiltrates and so validate the lung injury model and assess effect of exercise. Fig. 7 shows that

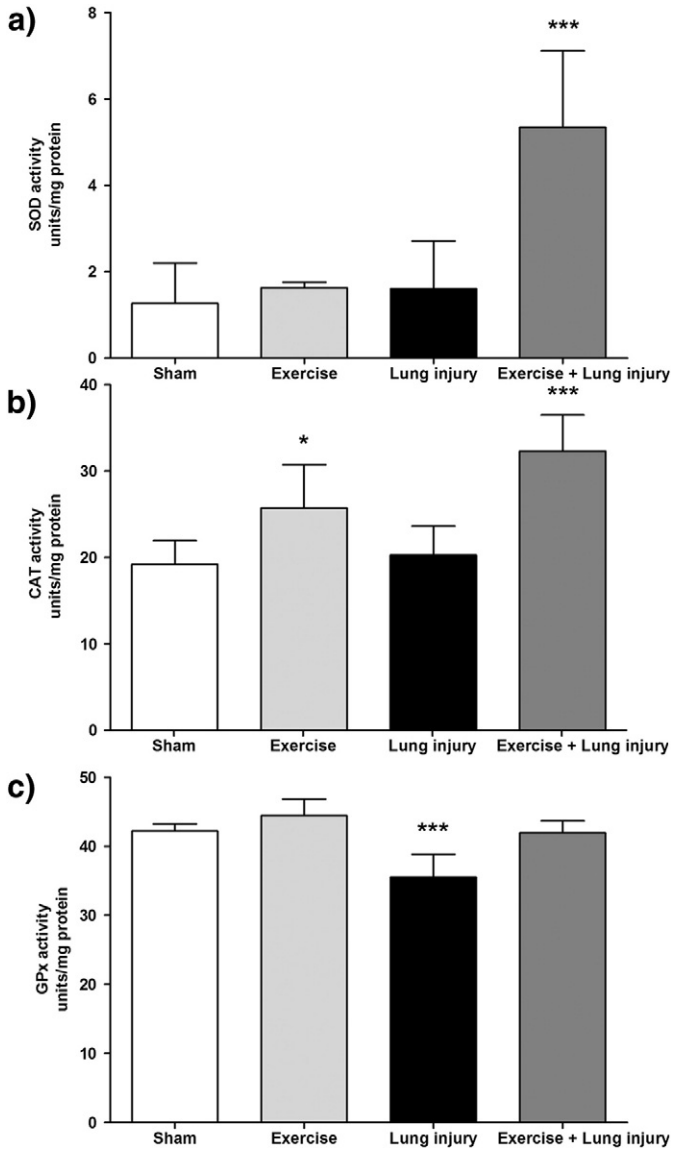

Fig. 5. Effect of lung injury and/or exercise on enzymatic antioxidant defense superoxide dismutase (a), catalase (b) and glutathione peroxidase (c) in lung of rats. Data are expressed as mean ± SD for 7 animals in each group. Different from other groups, *$p<0.05$, ***$p<0.001$ (two way ANOVA followed by Tukey test).

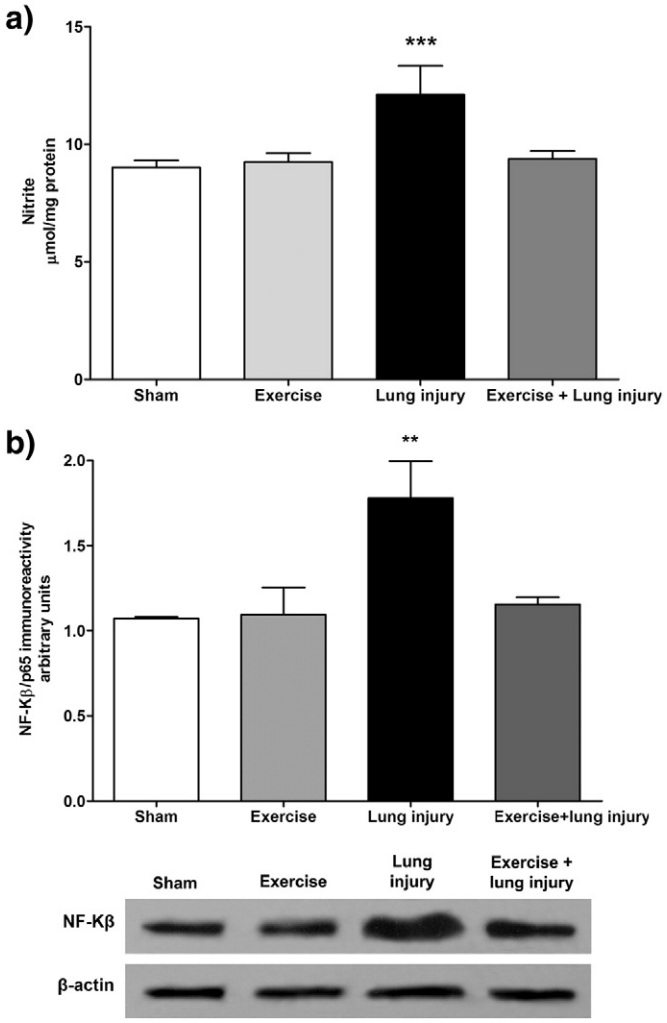

Fig. 6. Effect of lung injury and/or exercise on nitrite levels (a) and NF- κβ/p65 (b). β-**actin was used** as **loading control**. Data are expressed as mean ± SD for 7 animals in each group. Different from other groups, ***$p<0.001$; **$p<0.01$ (two way ANOVA followed by Tukey test). NF-κβ/p65: nuclear factor kappa B.

rats submitted to lung injury presented a higher inflammatory infiltrate in the lungs (Fig. 7c), when compared to the control group (Fig. 7a). Exercise *per se* neither affected this parameter (Fig. 7b) nor was not able to prevent it (Fig. 7d).

Discussion

ARDS is characterized by a disturbance of the alveolar–capillary barrier, which results in fluid accumulation and impaired gas exchange (Ware and Matthay, 2000). It is known that oxidative stress plays important roles in pulmonary injury, particularly in triggering endothelial damage and inflammatory edema (Hagiwara et al., 2008). It is believed that physical exercise can ameliorate the vascular function by stimulating the $NO^{\bullet}$ synthesis by the endothelial cells (Woodman et al., 1997; Kingwell, 2000), which improves the defense systems against the action of reactive species and induces an adaptive response (Venditti and Di Meo, 1997; Pinho et al., 2006, 2009). In addition, physical exercise has been shown to prevent the development of many diseases, such as pulmonary diseases and others associated with systemic inflammation (Petersen and Pedersen, 2005; Pinho et al., 2007; Vieira et al., 2008). However, there are no studies investigating the role of regular physical exercise as a protector against oxidative stress present in lung injury. Present work was designed to evaluate whether a moderate physical exercise program would be able to prevent the induction of oxidative stress in the lungs of rats subjected to experimental lung injury. The protective effect of physical exercise on disturbances of alveolar–capillary barrier was also evaluated by total cell count, LDH activity and total protein in BALF, as well as in the infiltrate in the pulmonary parenchyma (histological analyses).

Our results revealed that lung injury increased the production of reactive species (as observed by DCF levels) and induced lipid peroxidation (as observed by TBARS) in the lungs of rats. Additionally, we also observed an increase in carbonyl and decrease in sulfhydryl content. These results suggest that oxidative stress may be an important contributor to development of lung injury by disruption of protein and lipid integrity. Physical exercise was able to prevent the production of reactive species (totally) and the protein damage (partially), but not prevent the lipid peroxidation induced by lung injury. With regard to the protective effect of exercise, our results are in agreement with other studies that have shown that the response to oxidative stress induced by H_2O_2 was significantly lower in the heart of trained rats than in sedentary rats (De Angelis et al., 1997). In this context, studies suggest that the ability of the lung tissue to withstand the effects caused by free radicals, for example by increasing the antioxidant defenses, may be more important than preventing lipid peroxidation (Oh-ishi et al., 1997; Pinho et al., 2006).

Studies have demonstrated that a single bout of exercise can increase the production of reactive species, causing damage to lipids, proteins and DNA (Davies et al., 1982; Powers and Lennon, 1999; Radak et al., 1999; Ji et al., 2006). On the other hand, it has been suggested that the molecular basis for the beneficial effect of regular physical exercise may be related to the increase in reactive species while causing molecular damage and inducing adaptive stress responses (Powers et al., 2010, 2011; Radak et al., 2008; Gomes et al., 2012). Once the adaptive properties result from the cumulative effects of repeated exercise bouts, the initial signal for the stimulation leading to the long-term modulation must occur after each exercise bout (Ji et al., 2006). One way of adaptation and protection against oxidative stress is the NF-κβ signaling pathway. On the direct binding of H_2O_2 or through other reactive species, NF-κβ is translocated to the nucleus to enhance transcription of genes for antioxidant enzymes such as mitochondrial SOD and nitric oxide synthase (Meyer et al., 1994; Wan et al., 1994; Hollander et al., 2001; Ji et al., 2006).

Since the protective effect of exercise could play a beneficial role on imbalance between oxidants and antioxidants involved in the disease related to oxidative stress, such as ARDS, and the mechanisms of biological adaptation is still largely unknown in the lung, we evaluated the effect of lung injury and physical exercise on immunocontent of NF-κβ/p65. Our results showed an increase in immunocontent of NF-κβ/p65 in animals submitted to lung injury, while the exercise

Table 1
Effect of lung injury and/or exercise on total cell count, lactate dehydrogenase activity and total protein concentration in bronchoalveolar lavage.

	Sham	Exercise	Lung injury	Exercise lung injury
Total cell ($\times 10^5$ cells/µL)	10.99 ± 1.33	11.85 ± 0.83	110 ± 15.75 ***	117.24 ± 11.17***
Lactate dehydrogenase activity (U/L)	518 ± 28.87	510.76 ± 29.06	677.11 ± 42.81 **	606.84 ± 22.47#
Total protein (mg/mL)	34.42 ± 3.47	43.99 ± 5.73	118 ± 15.77***	102 ± 29.08***

Data are expressed as mean ± S.D. for 7 animals in each group. Different from other groups, **$p<0.01$, ***$p<0.001$; #$p>0.05$ compared with sham and lung injury group (two way ANOVA followed by Tukey test).

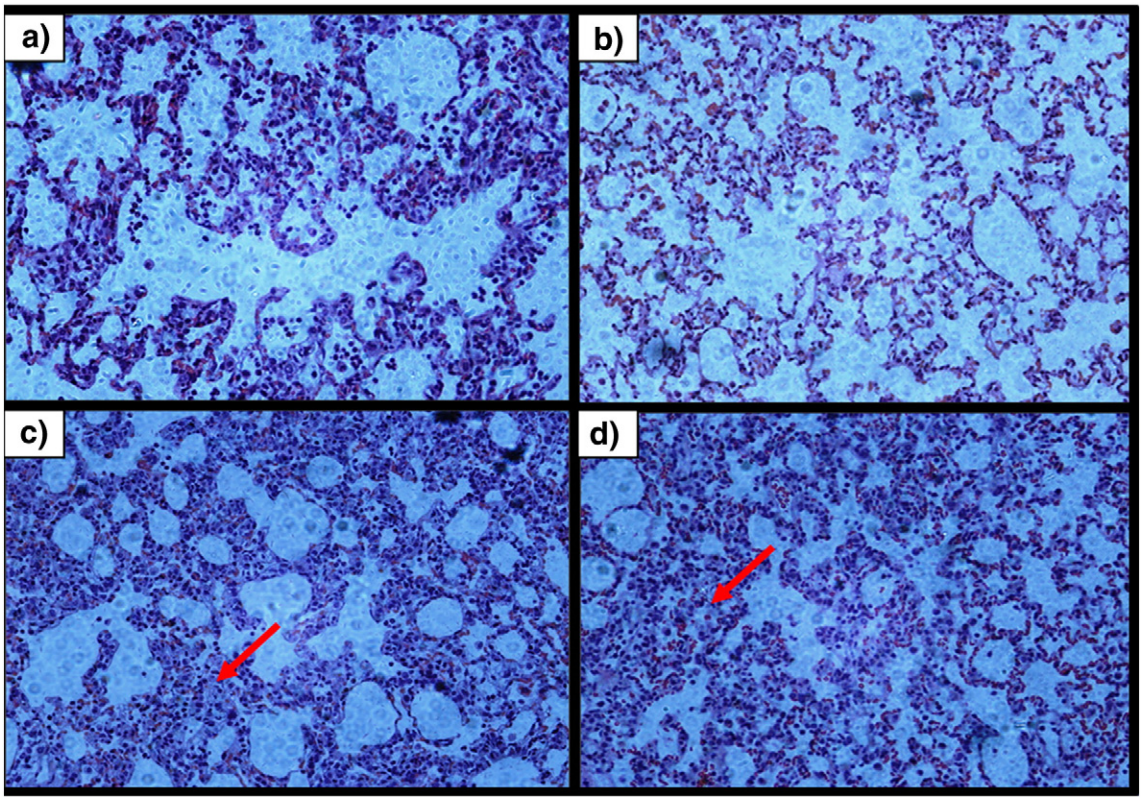

Fig. 7. Histopathologic findings after lipopolysaccharide instillation. Sham: sedentary operated isotonic saline instillation (a); exercise: operated isotonic saline instillation (b); lung injury: sedentary and lipopolysaccharide instillation (c); lung injury plus exercise (d). Twelve hours after lipopolysaccharide administration, the lung was removed for histopathologic analysis. Representative illustration from 6 animals each group (H&E, 20× magnification).

prevented this effect. Considering that NF-κβ may be activated by a variety of stimulants, including free radicals, proinflammatory cytokines, LPS and other (Baeuerle and Baltimore, 1988), the effect of physical exercise in normalizing the NF-κB levels in lung of rats submitted to lung injury, suggests a potent anti-inflammatory action through the inhibition of NF-κB activation, which in turn may be underlie by decreasing in reactive species generation observed in the lung injury-exercise rat group (as described below).

TRAP is a useful estimate of non-enzymatic antioxidants and assesses the total potential of the main antioxidants found in the lung tissue (for example, glutathione, uric acid, ascorbic acid, and α-tocopherol) (Evelson et al., 2001). In the present study, we showed that lung injury decreased TRAP and GSH content, suggesting that the TRAP reduction observed in the animals submitted to lung injury can be due to a decrease in GSH content. A substantial lack of the antioxidant, GSH, in the respiratory tract of ARDS patients may enhance the risk for oxidant injury. The delicate balance between oxidant and antioxidant systems, however, is of major importance for maintaining normal lung function and structure (Bunnell and Pacht, 1993). Interestingly, our study showed that physical exercise prevents the decrease in GSH content, which could explain why the TRAP values return to normal. Our results suggest that physical exercise could be recommended for the restoration of normal GSH content, since it can increase the activities of enzymes involved in GSH synthesis (Sen et al., 1992; Kretzschmar and Muller, 1993; Ji et al., 2006).

With regard to the enzymatic antioxidant defenses that are responsible for removing reactive species (Halliwell and Gutteridge, 2007), our results showed that activities of SOD and CAT in the lung of rats subjected to lung injury were not altered. Interestingly, the lung injury-exercise group was able to increase the activities of SOD and CAT in the lung, when compared to lung injury and/or the control group, in contrast to exercise that *per se* increased CAT activity. In this context, it has been shown that rats submitted to lung injury induced by ischemia and reperfusion did not present any alteration in their serum antioxidant enzymes, but rats subjected to injury plus exercise present an increase in SOD activity, when compared with sedentary rats (Mussi et al., 2008). Based on this, the authors suggested that physical exercise can increase the expression of antioxidant enzymes. Other studies also show that physical exercise, at a moderate intensity, promotes upregulation of antioxidant enzyme expression (Powers and Lennon, 1999; Chang et al., 2004; Linke et al., 2005; Ordonez et al., 2006; Toledo et al., 2012). It is probable that the increase in antioxidant enzymes could lead to lower reactive species production. In our study, the lack of activation of antioxidant enzymes caused by lung injury could lead to accumulation of reactive species, which may join to form hydroxyl radicals ($OH^{\bullet}$), a powerful free radical with the ability to rapidly remove electrons from other molecules (Del Maestro, 1980; Yu, 1994). Our results showed that physical exercise prevented the increased DCF levels induced by lung injury. The CAT detoxifies the H_2O_2 formed and H_2DCF that can be oxidized to DCF in the presence H_2O_2. A hypothesis for the decreased DCF levels observed in lung injury-exercise group may be due to the fact that CAT activity was increased by exercising.

As discussed earlier, CAT reduces H_2O_2 to H_2O, removing a key intermediate in the formation of $OH^{\bullet}$. It is has been suggested that the lungs have a lower CAT content, as compared to other antioxidant enzymes (Heffner and Repine, 1989; Repine and Parsons, 1994; Halliwell and Gutteridge, 2007). GPx also removes H_2O_2 and other peroxides by coupling its reduction to H_2O with oxidation of GSH, using selenium as a cofactor. The inhibition of GPx activity in the animals submitted to lung injury observed in our study could be associated within the decrease in GSH, since the activity of this enzyme

depends on the regeneration of GSH. We also observed that physical exercise prevents GPx and GSH alterations.

Clinical and preclinical studies have demonstrated that $NO^{\bullet}$ levels are increased in the BALF of ARDS patients and experimental lung injury (Sittipunt et al., 2001; da Cunha et al., 2011). In the present study, we observed that rats subjected to lung injury present an increase in nitrite levels and that exercise was able to prevent such effect. We believe that a possible activation of macrophages leads to the increase of this radical and could contribute to endothelial or parenchyma injury, increasing the microvascular permeability, which could result in lung injury. However, we cannot discard the fact that $NO^{\bullet}$ bioavailability may be reduced by the increase in $O_2^{\bullet -}$, since SOD activity was altered in animals submitted to lung injury, which can react with $NO^{\bullet}$ to yield the potent oxidant peroxynitrite ($ONOO^-$), capable of oxidizing proteins, lipids and nucleic acids, causing cellular damage (Beckman et al., 1990; Beckman and Koppenol, 1996). Studies have also demonstrated that the harmful effects of elevated levels of $NO^{\bullet}$ in ARDS are related to the induction of chemokines and cytokines and to the formation of most harmful reactive species (Sittipunt et al., 2001; Vanoirbeek et al., 2006). Our findings demonstrate that exercise decreases the nitrite levels, which is in agreement with studies that demonstrate that physical exercise inhibits the elevation of the pulmonary $NO^{\bullet}$ levels in animals submitted to lung injury (Ramos et al., 2009). The authors suggested that the decrease in $NO^{\bullet}$ levels could be involved in anti-inflammatory effects caused by exercise.

The increases in the total cell counts in the BALF and infiltrate, observed in the lung parenchyma, confirm the inflammatory process in rats submitted to lung injury, when sacrificed 12 h after injury. In agreement with this result, Ritter et al. (2006) showed an increase in total cell count in BALF analysis at 12 h after intratracheal LPS injection. In our study, exercise was not found to prevent the increase in the inflammatory process in the BALF and pulmonary parenchyma that was caused by the lung injury model. In disagreement with our results, previous studies have demonstrated that animals presubmitted to six weeks of aerobic conditioning, obtained through low-intensity swimming training, demonstrate attenuated lung neutrophil inflammation, at 24 h after lung injury, suggesting that physical exercise may have a long-term effect (Ramos et al., 2009). The lack of results in our study could be due to the fact that 12 h after injury is insufficient for the organisms that decrease the inflammatory process present in lung injury, since it has been shown that regular physical exercise promotes an acceleration of repair processes in inflammation, interferes in several steps of inflammatory processes and increases the phagocytic ability of the cells (Fehr et al., 1989; Nieman, 1998).

In order to confirm the lung injury, we also investigated the influence of lung injury and/or exercise on the total protein concentration and LDH activity in BALF. Results showed that lung injury increased LDH activity and protein concentration in BALF. These results are in agreement with other studies that showed that the total protein concentration and LDH activity in BALF were increased in animals submitted to lung injury (Dal-Pizzol et al., 2006; da Cunha et al., 2011). The concentration of protein in BALF increases rapidly after lung injury and the protein concentrations reach maximal values at between 6 and 12 h (Ritter et al., 2006). The increased protein concentration in BALF may indicate an increase in permeability of the alveolar capillary membrane (Lenz et al., 1999). Physical exercise did not alter the changes in the protein concentration caused by lung injury, but it was able to partially prevent the increase in LDH activity, suggesting that there was injury in animals subjected to lung injury. It is suggested that the prevention of injury can be explained, at least in part, by the decreased oxidative stress observed in our study. Although our results show significant inflammatory changes that are present in pulmonary injury, our results do not demonstrate the degree of extension of the lesion. The ratio of partial pressure of oxygen (PaO2) to fractional concentration of oxygen (FiO2) is a clinical measure of lung oxygenation and is frequently used to describe the extent of lung dysfunction;

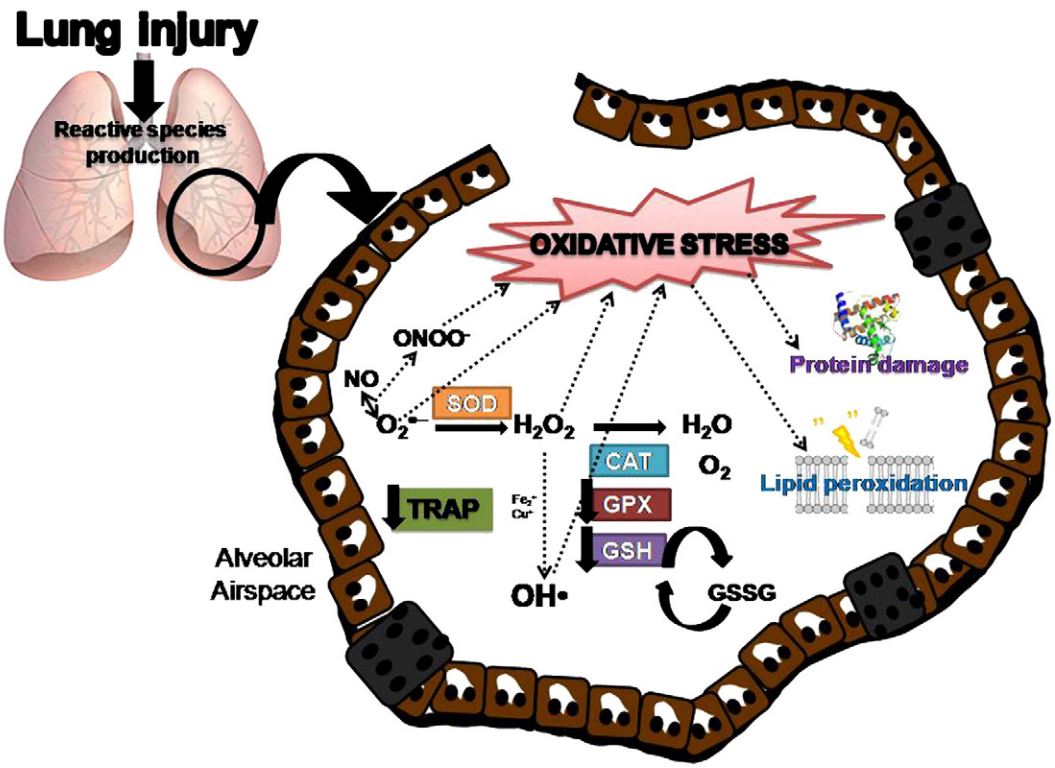

Fig. 8. Summary of the effect of lung injury on oxidant–antioxidant status present in the lung, highlighting those processes that were quantified throughout the investigations. SOD (superoxide dismutase) CAT (catalase), GPx (glutathione peroxidase), GSH (reduced glutathione) and TRAP (total radical-trapping antioxidant potential).

therefore, this is a limitation in our study. However, other studies have showed that the PaO2/FiO2 ratio was significantly decreased in lung of rats submitted to lung injury by LPS instillation (DiRocco et al., 2006; Xie et al., 2012). Fig. 8 summarizes the oxidant–antioxidant status present in the lung, highlighting the processes that were here quantified.

Conclusion

In summary, our findings show that physical exercise prevents some alterations in oxidative parameters, such as reactive species production, GPX activity, GSH content and nitrite levels, as well as the increase in NF-κβ/p65 immunocontent caused by experimental lung injury. Based on these findings, we suggest that exercise is able to protect against the oxidative damage caused by lung injury; however, the mechanisms and biological significance of these findings require further scrutiny to verify the effects of exercise in this pathological condition and the possible benefits of its use to prevent the oxidative stress present in ARDS.

Conflict of interest statement

The authors declare that they have no competing interests.

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CAPÍTULO II

Experimental lung injury promotes alterations in energy metabolism and respiratory mechanical in the lungs of rats: prevention by exercise

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Status: a ser submetido

Experimental lung injury promotes alterations in energy metabolism and respiratory mechanical in the lungs of rats: prevention by exercise

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Abstract

OBJECTIVE: In the present study we investigated the effects of lung injury on energy metabolism (succinate dehydrogenase, complex II, cytochrome *c* oxidase and ATP levels), respiratory mechanics (dynamic and static compliance, elastance and respiratory system resistance) in the lungs of rats, as well as on phospholipids in bronchoalveolar lavage fluid. The protective effect of physical exercise on the alterations caused by lung injury, including lung edema was also evaluated.

DESIGN: Prospective experimetal study

SETTING: Research laboratory

INTERVENTIONS: Wistar rats were submitted two months of physical exercise. After this period the lung injury was induced by intratracheal instillation of lipopolysaccharide.

MEASUREMENTS AND MAIN RESULTS: Adult Wistar rats were submitted to two months of physical exercise and after this period the lung injury was induced by intratracheal instillation of lipopolysaccharide in dose 100µg/100 g body weight. The sham group received isotonic saline instillation. Twelve hours after the injury was performed the respiratory mechanical or the rats were decapitated and samples collected. Results showed that rats subjected to lung injury presented a decrease in activities of the enzymes of the electron transport chain and ATP levels in lung, as well as the formation of pulmonary edema. A decreased lung dynamic and static compliance, as well as an increase in respiratory system resistance and a decrease in phospholipids content were observed. Physical exercise was able to totally prevent the decrease in succinate dehydrogenase and complex II activities and the formation of

pulmonary edema. It also partially prevented the increase in respiratory system resistance, but did not prevent the decrease in dynamic and static compliance, as well as in phospholipids content.

CONCLUSIONS: These findings suggest that the mitochondrial dysfunction may be one of the important contributors to lung damage and that physical exercise may be beneficial in this pathology although it did not prevent all changes present in lung injury.

Key words: Lung injury; Physical exercise; Respiratory mechanical; Energy metabolism.

Introduction

Acute respiratory distress syndrome (ARDS) is characterized by progressive hypoxemia, respiratory failure associated with acute pulmonary inflammation, which affects both clinical and surgical patients (1). Several conditions can elicit lung injury, including gastric aspiration, polytrauma, pancreatitis, hemorrhagic shock, severe burns, oxygen toxicity and surgery involving cardiopulmonary bypass (2-3). Its physiopathology involves destruction of the pulmonary capillary endothelium and alveolar epithelium (4-5) accomplished by neutrophil, macrophages and erythrocytes accumulation, protein-rich fluid in the alveolar spaces. Besides that, there is a decreased surfactant production by type II epithelial cells associated with diminished compliance of the lung. The clinical syndrome of lung injury results in multiple pathophysiological changes that may cause a severe respiratory dysfunction (6).

Although there are several studies trying to unravel the mechanisms that may be involved in lung injury, the exact mechanisms underlying the alterations present in pulmonary injury are still not completely understood. Dysfunction of the mitochondria affects metabolism function, increases radical formation, and induces apoptosis, which may contribute to cellular imbalance (ATP) (7-11). Increasing evidence suggests that abnormalities in mitochondria are involved not only in aging, neurodegenerative diseases, cancer, diabetes, and several other mitochondrial diseases (12-15), but also in the development of inflammatory diseases (16), however the precise connection between mitochondrial abnormalities and ARDS is not known.

At the moment there is not a specific treatment to prevent and/or reverse the complications present in lung injury, although recent progress has been made in this direction. Therefore, the search for new therapeutic strategies is necessary to control the effects caused by lung injury. Clinical studies have shown that regular physical exercise may be able to prevent the development of many pathologies, including pulmonary and cardiovascular diseases, diabetes mellitus, metabolic syndrome and those associated with systemic inflammation (17-22). During the exercise a significant increase in ATP production occurs, which is associated with increased mitochondrial flow (23). It is possible that the increased activity of the complexes of the electrons transport chain caused by exercise can be sufficient to reduce oxidative stress markers (24). In this context, experimental evidence indicates that regular exercise prevents some oxidative stress parameters caused by lung injury (25-26).

Since, to our knowledge, there are no studies that show any association between lung injury and energy metabolism, the present study was designed to evaluate the possible changes in some parameters of energy metabolism (succinate dehydrogenase (SDH), complex II, cytochrome *c* oxidase activities and ATP levels) and respiratory mechanics (dynamic and static compliance, elastance and respiratory system resistance) in the lungs of rats submitted to the lung injury, as well as on phospholipids analysis in bronchoalveolar lavage fluid (BALF). The effect of exercise on these possible changes and edema present in the lung injury was also investigated.

Material and Methods

Animals and reagents

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Adult Wistar rats were divided into four group: (1) sham (isotonic saline instillation) and (2) (exercise and isotonic saline instillation), (3) lung injury (sedentary and LPS instillation) and (4) exercise plus lung injury (exercise and LPS instillation). Animals were maintained on a 12h light/12h dark cycle at a constant temperature (22 ± 1°C), with free access to water and commercial protein chow. Animal care followed the NIH ‘‘Guide for the Care and Use of Laboratory Animals’’ (NIH publication no. 80-23, revised 1996) and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul (nº19322). All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Physical exercise training

On the 25th day of life, the animals were habituated to the apparatus to minimize novelty stress. The treadmill exercise started on the 30th day of life and finished on the 90th day. Training consisted of 20 min of running sessions on an adapted motorized rodent treadmill (INBRAMEDTK01, Porto Alegre, Brazil), three times per week (27). A moderate intensity exercise protocol was used (27-28), *i.e.*, exercise intensity was set at 60% of the animal’s maximal oxygen uptake (29). The estimation of oxygen uptake (VO_2) peak was then carried out in all animals before training, until exhaustion, as follows: each rat ran on a treadmill at a low initial speed, followed by increases in speed of 5 m/min every 3 min until the point of exhaustion (i.e., failure of the rats to

continue running); the time to fatigue (in min) and workload (in m/min) were taken as indexes of capacity for exercise, which was taken as VO_2 max. Animals from the control group (not exercised) were transported to the experimental room, handled exactly as the experimental ones and maintained in the turned off treadmill for 20 min. The animals were adapted to the treadmill by gradually increasing the running speed of up to 36m/min (27-28). Approximately twenty-four hours after the last exercise session, rats were submitted to lung injury.

The timeline of the experimental procedures is shown in Fig. 1.

Experimental procedures

Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), intraperitoneally (IP), and lung injury was induced by intratracheal instillation of LPS (Escherichia coli 055:B5; Sigma Chemical, St. Louis, MO) at a dose of 100 µg/100 g body weight (30). Twelve hours after the LPS instillation, one part of the rats were anesthetized for the assessment of respiratory mechanical (see below) and de others part of rats were killed by decapitation and the right lung tissue was isolated and homogenized immediately to determine the energetic metabolism assays and the left lung tissue was weighed and dried in an oven at 80 °C for 48 h. The Wet/Dry lung weight ratio was calculated by dividing the wet weight by the dry weight. The BALF was collected three times and withdrawn with 5 mL of phosphate-buffered saline and the final BALF lavage volume retrieved was approximately 15 mL. The BALF was then centrifuged (1000 *g* for 10 min) and the cell pellet was dissolved in 500µL saline, and then diluted with Turk's solution and the total cell

count determined using a “Neubauer” counting chamber to confirm the lung injury induction (31). The supernatant was used for phospholipids analysis as explained below.

Tissue preparation

For determination of complex activity, the lung was homogenized with a Teflon-glass homogenizer in five volumes of ice-cold sucrose buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl), pH 7.4. The homogenate was centrifuged at 800×g and the pellet was discarded. The supernatant was centrifuged at 10.000×g for 10 min. After the pellet was resuspended in sucrose buffer and centrifuged at 10.000 again. The pellet was resuspended with Seth buffer and used to enzymatic assay.

Succinate dehydrogenase and Complex II activities

Immediately before the assay, the samples were frozen and thawed three times to break mitochondrial membranes. The activity of succinate: DCIP oxiredutase (complex II) were measured in homogenates following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength (å=19.1 mM-1cm-1) in the presence of phenazine methasulphate (PMS), according to Fischer and colleagues (32). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 µM DCIP was pre-incubated with 40-80 µg homogenate protein at 30°C for 20 min. Subsequently, for complex II activity, was added 4 mM sodium azide in order to inhibit the activity of complex

I and 7 µM rotenone to inhibit the complex IV activity. The reaction was initiated by addition of 40 µM DCIP and was monitored for 5 min.

Cytochrome c oxidase activity

The activity of cytochrome c oxidase was measured according to Rustin and colleagues (33). Enzymatic activity was measured by following the decrease in absorbance due to oxidation of previously reduced cytochrome c at 550 nm with 580 nm as reference wavelength (å=19 .1 mM-1cm-1). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM n-dodecyl-α-D-maltoside, 2-4 µg homogenate protein and the reaction was initiated with addition of 0.7 µg reduced cytochrome c. The activity of cytochrome c oxidase was measured at 25°C for 10 min.

ATP determination

Immediately after the rats had been sacrificed, the lung was dissected out and frozen in liquid nitrogen. Each lung was weighed and homogenized in 5 mL of 0.1 M NaOH (to inactivate cellular ATPases activity). Samples were assayed using the ATP lite Luminescence ATP detection assay system (Perkin Elmer, Waltham, MA, USA), according to Witt and colleagues (34). Chemiluminescence was measured using the Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet tissue weights in grams and expressed as µmol/g.

Protein determination

Protein concentration was measured by the method of Lowry and colleagues (35), using bovine serum albumin as standard.

Assessment of Respiratory Mechanics

Twelve hours after LPS instillation, the rats were anesthetized again, as described above, tracheostomized and a rigid-type cannula (2-mm ID) was inserted into the trachea and firmly tied in place. The cannula was connected to a small animal ventilator (flexi Vent, SCIREQ, Montreal, PQ, Canada). Rats were mechanically ventilated at a breath rate of 90 breaths/min, with a tidal volume (VT) of 10 ml/kg, using 5 cmH2O of positive end-expiratory pressure (PEEP) established by a water column. In addition, the breath rate was set above the normal (120 breaths/min) to suppress spontaneous breathing when measuring respiratory mechanics (36-37). Rats were allowed to stabilize on the ventilator for 5 min before the measurements were taken. Impedance of the respiratory system was measured following a forced oscillation. Impedance was measured during 16s volume perturbation signal. Before taking the measurements of each rat, calibration signals were collected by applying the volume oscillation through the tracheal cannula, at first with the cannula completely closed and then with it open to atmosphere (38).

The perturbation of 16s is composed of sinusoids with mutuallyprime frequencies ranging from 0.25 to 19.625 Hz, which were chosen to avoid harmonic distortion (39). The amplitudes of the sinusoids decreased hyperbolically with frequency. The constant-phase model described by Hantos and colleagues (39) was used to partition impedance into components representing the mechanical properties of the airway and parenchyma. Using

the force oscillatory technique and a constant phase the dynamic and static compliance, respiratory system elastance and respiratory system resistance were evaluated using the Salazar-Knowles equation as described by the flexi Vent manufacturer (40) .

Phospholipids analysis

The extraction of lipids from the BALF was performed with chloroform:methanol (2:1 v/v) (41). The obtained organic phase was dried under a stream of nitrogen, the dry lipid extract was dissolved in 0.2 mL of chloroform and the individual phospholipid classes were separated by thin layer chromatography on silica gel 60 plates (Merck) using a solvent system containing chloroform/methanol/isopropanol/0.25% KCl/triethylamine (30:9:25:6:18,v/v). Phospholipid bands were visualized with Comassie-Blue and the chromatographic bands were quantified by scanning densitometry.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Science (SPSS) software with a PC-compatible computer. Data were analyzed by two way ANOVA followed by Tukey *post hoc* test. Lung injury and physical exercise were considered as interfering factors. Descriptive statistical data are expressed as means ± SD; differences of $p < 0.05$ are considered as significant.

Results

Energy Metabolism

Firstly, we investigated the effects of lung injury and/or physical exercise on parameters of energy metabolism in the lung of rats (SDH, complex II, cytochrome *c* oxidase activities and ATP levels). Two way ANOVA showed significant effect of lung injury versus exercise on SDH [$F(1,20)= 12.98$, $p<0.01$], complex II [$F(1,20)=20.15$, $p<0.001$] and ATP levels [$F(1,20)= 11.63$, $p<0.01$]. Cytochrome *c* oxidase activity two way ANOVA showed a significant main effect in the lung injury-rats group [$F(1,20)=61.02$, $p<0.001$] and exercise group [$F(1,20)=6.08$, $p<0.05$]. Subsequent post hoc Tukey test showed a significant decrease in SDH ($p<0.01$), complex II ($p<0.01$), cytochrome *c* oxidase ($p<0.001$) and ATP levels ($p<0.05$) in the lung of rats submitted to lung injury. Exercise prevented the decrease in SDH and complex II activities caused by lung injury (Figure 2), but did not prevent the decrease in cytochrome *c* oxidase and ATP levels (Figure 3). As can be observed exercise *per se* increase only the ATP levels, being that other parameters studies were not altered by exercise.

Respiratory Mechanics

We also evaluated the effects of lung injury and/or exercise on respiratory mechanics in rats. Two way ANOVA show a significant main effect in the lung injury-rats group on dynamic compliance [$F(1,16)=12.67$, $p=0.01$], static compliance [$F(1,16)=36.86$, $p<0.001$] and respiratory system resistance [$F(1,16)=14.01$, $p<0.01$]. The respiratory system elastance did not alter by lung injury and/or exercise. The *post hoc* test, revealed a significant decrease in the compliance dynamic ($p<0.05$) and compliance static ($p<0.01$), as well as caused a significant increase in the airway resistance ($p<0.05$) in rats submitted

to lung injury. Exercise per se did not alter these parameters, but totally prevented the decrease in compliance (dynamic and static), partially prevented the increase in respiratory system resistance (Figure 4).

Pulmonary edema

We also investigated the pulmonary edema in the lung of rats submitted to lung injury and/or exercise. Two way ANOVA showed a significant interaction of lung injury *versus* exercise on lung wet/dry weight ratio [$F(1,24)=5.85$, $p<0.05$]. As can be seen in Figure 5, lung wet/dry weight ratio was increased in rats subjected to lung injury ($p<0.001$), when compared to the control; physical exercise per se did not alter this parameters, but prevented the pulmonary edema caused by lung injury.

Phospholipids analyses

The phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol) were evaluated in the lung of rats submitted to lung injury and/or exercise. Two way ANOVA show a significant interaction of lung injury *versus* exercise on phosphatidylcholine [$F(1,16)= 6.09$, $p<0.05$], and a significant effect in lung injury-rats on total phospholipids [$F(1,16)=4.70$, $p<0.05$]. Subsequent *post hoc* test revealed a significant reduction in phosphatidylcholine and phospholipids total in lung of rats submitted to lung injury ($p<0.05$); physical exercise did not prevent such changes (Table1). Exercise per se did not alter phospholipids content.

Discussion

ARDS is characterized by a disturbance of the alveolar capillary barrier which results in respiratory failure, pulmonary inflammation and bilateral pulmonary infiltrates consistent with edema (2, 6). Although some studies show an association between mitochondria dysfunction and pulmonary inflammatory diseases (16), the mechanisms involved are not well understood. The main objective of the present study was to investigate the possible changes in energy metabolism (SDH, complex II, cytochrome *c* oxidase activities and ATP levels) and ventilatory mechanics (dynamic and static compliance, respiratory system elastance and respiratory system resistance) in lungs of rats submitted to experimental lung injury. Phospholipids analysis was evaluated in BALF. We have also investigated the effect of exercise on possible changes caused by experimental lung injury, as well as on the edema present in the lung.

Initially, we evaluated the effect of lung injury on the respiratory chain enzymes. Results showed that SDH, complex II and cytochrome *c* oxidase activities were significantly reduced, suggesting that lung injury compromised the respiratory chain function in lung of rats. It is well known that mitochondrial oxidative phosphorylation system generates free radicals and that the complexes of electron transport chain are vulnerable to damage by free radicals (42). Thereby, it is plausible to suggest that the inflammatory cells in the airway increased the production of reactive species which could induce the inhibition of mitochondrial enzyme activities presented in our study (11, 43). According to our findings, several studies show that oxidative stress can play an important role in lung injury, particularly in triggering endothelial damage and inflammatory edema (2, 30, 44).

Mitochondrial activity accounts for 90% of total body oxygen consumption and is responsible, in most cell types, for 90% of adenosine triphosphate (ATP) generation (45-47). The cytochrome *c* oxidase is the terminal and rate-limiting enzyme of the mitochondrial respiratory chain, catalyzing the transfer of electrons from cytochrome c to molecular oxygen (48). Thus, an inhibition in the activity of this enzyme can lead to incomplete reduction of oxygen and consequently impair the ATP synthesis (49-52). In this context, we showed that the lung injury reduced significantly the ATP levels in lung of rats. The decrease in ATP generation may compromise normal cellular metabolic activity, leading to biochemical and physiologic organ dysfunction (53).

It has been shown that physical exercise prevents the development of many diseases, such as pulmonary and other diseases associated with systemic inflammation. Furthermore, it increases the defense systems against the action of reactive species and induces an adaptive response to oxidative stress (25, 54-55). In the present study, we observed that physical exercise was able to prevent the reduction of SDH and complex II activities caused by lung injury. Since in exercise prevented the increase in reactive species, nitrite levels, nuclear factor-kappa β (NF-κβ), damage to protein, as well as the decrease in antioxidant defenses (26), we believe that physical exercise could protect the lung tissue from oxidative insult caused by experimental lung injury,

Since lung injury is accompanied by impaired respiratory mechanics and edema formation (56-57), which contribute to the respiratory failure (58), we have extended our investigation to evaluate the effect of experimental lung injury on dynamic and static compliance, elastance, respiratory system resistance and edema formation. Results show that lung injury decreases the

dynamic and static compliance and increases the respiratory system resistance. We also demonstrated a pulmonary edema formation in lung of rats submitted to lung injury, suggesting that air flow may be decreased in airways in lungs with interstitial fluid accumulation and may be contributing to the decrease in compliance and respiratory system resistance observed in this experimental model.

Surfactant forms a lipid film at the air/water interface and thus reduces the surface tension (59-60), protecting the alveoli against collapse at end-expiration and precluding alveolar edema. It is composed mainly by phospholipids, of which phosphatidylcholine represents 70-80% (61-62). Considering that the alterations in respiratory mechanics and edema formation presented in our study may be related to dysfunction in the surfactant, we also investigated the effects of lung injury on surfactant through phospholipid analysis (phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol). Results showed a decrease in phosphatidylcholine in the BALF of rats submitted to lung injury. One possible reason proposed for this decrease in phospholipids may be the destruction of the air-water interface by alveolar edema, phospholipid degradation by phospholipases, degradation of surfactant proteins by proteases (63-64), damage by reactive species and decreased synthesis of surfactant components by damaged type II cells (65).

Since physical exercise prevents some alterations of the energy metabolism and oxidative stress we extended our investigation, and evaluated the influence of physical exercise on edema, respiratory mechanical, and phospholipids alterations caused by lung injury. Results show that physical exercise totally inhibited pulmonary edema formation and partially prevented the

increase in respiratory system resistance. Nevertheless, it did not prevent the alterations in dynamic and static compliance and phosphatidylcholine. Changes in resistive forces and tissue viscoelastic properties are a major characteristic of lung injury and seem to be associated with alveolar barrier disruption, alveolar edema, surfactant dysfunction, collapse and alveolar inflammation (66). In our study, there was no significant effect of exercise on the changes in surfactant phospholipid. The decrease of respiratory system resistance in lung injury associated with physical exercise is likely related to the decrease in pulmonary edema formation.

In summary, our findings showed that lung injury can induce alterations in mitochondrial function which play an important role in the damage to lung tissue (Figure 6 summarizes the alterations present in the lung of rats submitted to lung injury). The mechanisms by which lung injury elicited these alterations are not clear, however we suggest that oxidative stress present in this injury could be responsible for respiratory chain impairment, which probably limits ATP production and increases reactive species production in a vicious cycle. Physical exercise training inhibits lung edema and pulmonary energy metabolism (SDH and complex II), but does not prevent the impairment in respiratory mechanics and the decrease in phospholipids content. These data suggest that mitochondrial dysfunction may be involved in the changes present in lung injury and physical exercise may be beneficial in this pathology, although it did not prevent all changes present in lung injury.

Acknowledgments

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Legends of figures

Figure 1. The timeline of experimental procedures.

Figure 2. Effect of lung injury and/or exercise on SDH (a) and complex II (b) in lung of rats. Data are expressed as mean ± SD for 6 animals in each group. Different from other groups, $^{**}p < 0.01$ (two way ANOVA followed by Tukey test).

Figure 3. Effect of lung injury and/or exercise on cytochrome *c* oxidase (a) and ATP levels (b) in lung of rats. Data are expressed as mean ± SD for 6 animals in each group. Different from other groups, $^{*}p < 0.05$; $^{***}p < 0.001$ (two way ANOVA followed by Tukey test).

Figure 4. Effect of lung injury and/or exercise on (a) dynamic compliance (b) static complicance (c) respiratory system elastance and (d) respiratory system resistance in lung of rats. Data are expressed as mean ± SD for 5 animals in each group. Different from other groups, $^{*}p < 0.05$; $^{**}p < 0.01$; # $p>0.05$ compared to sham and lung injury (two way ANOVA followed by Tukey test).

Figure 5. Effect of lung injury and/or exercise on (a) wet/dry weight ratio in lung of rats. Data are expressed as mean ± SD for 8 animals in each group. Different from other groups, $^{***}p < 0.001$ (two way ANOVA followed by Tukey test).

Figure 6. Summary of the effect of lung injury on alteration in energetic metabolism, highlighting those processes that were quantified throughout the investigations SDH (succinate dehydrogenase) complex II, cytochrome c oxidase and ATP levels.

FIGURE 1.

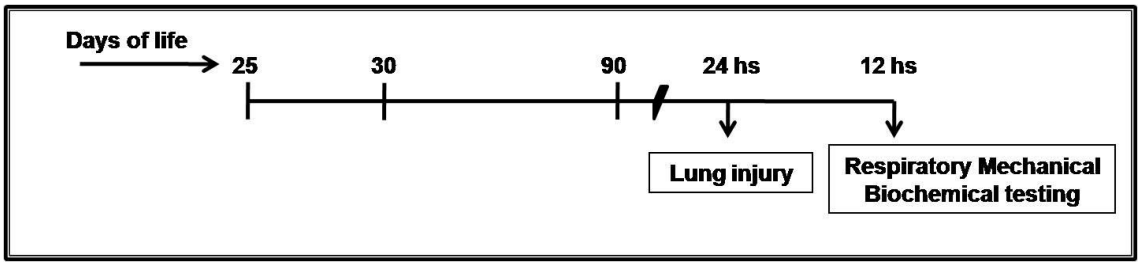
Days of life
25
30
90
24 hs
12 hs
Lung injury
Respiratory Mechanical
Biochemical testing

FIGURE 2.

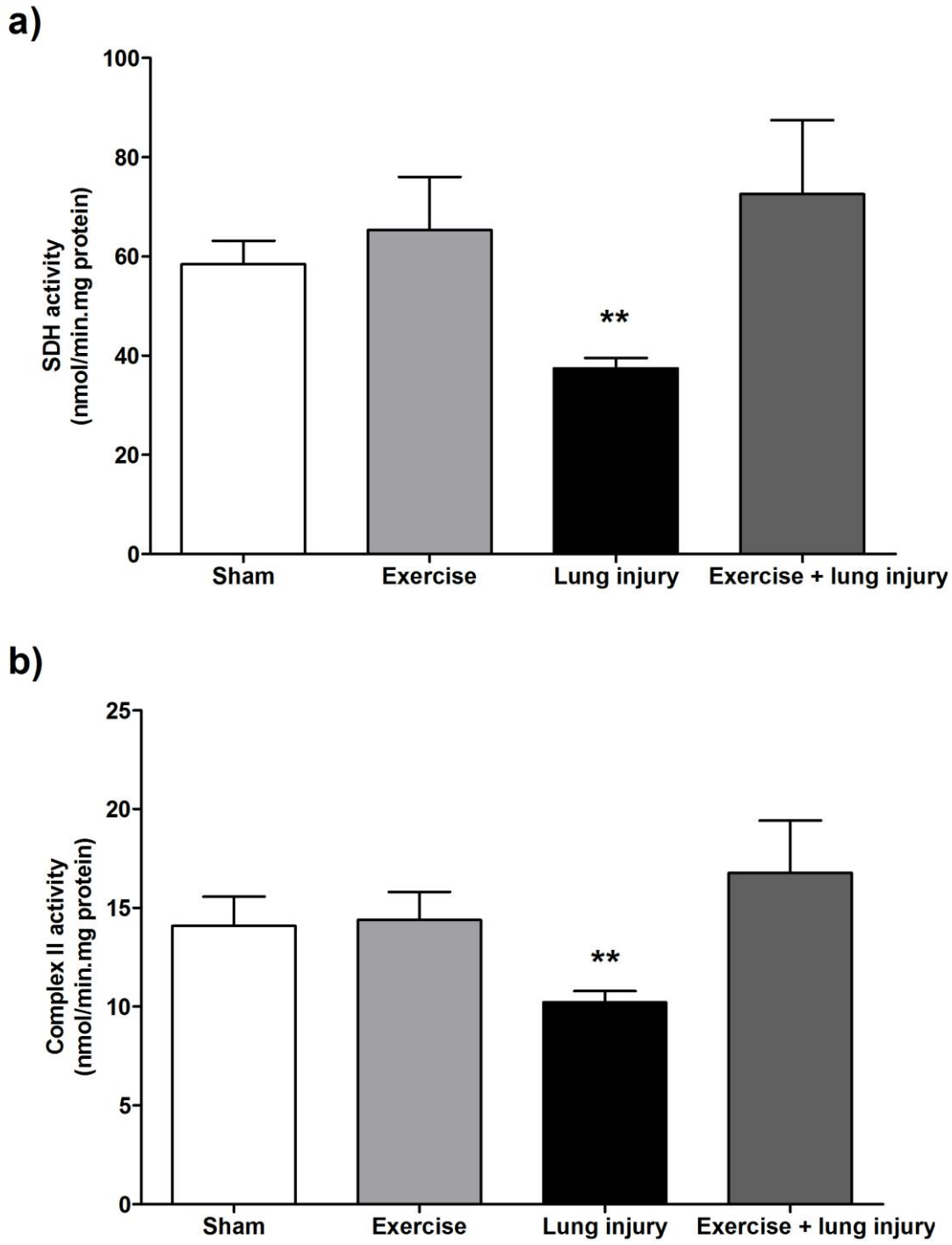
a)
SDH activity
(nmol/min.mg protein)
100
80
60
40
20
0
**
Sham
Exercise
Lung injury
Exercise + lung injury
b)
Complex II activity
(nmol/min.mg protein)
25
20
15
10
5
0
**
Sham
Exercise
Lung injury
Exercise + lung injury

FIGURE 3.

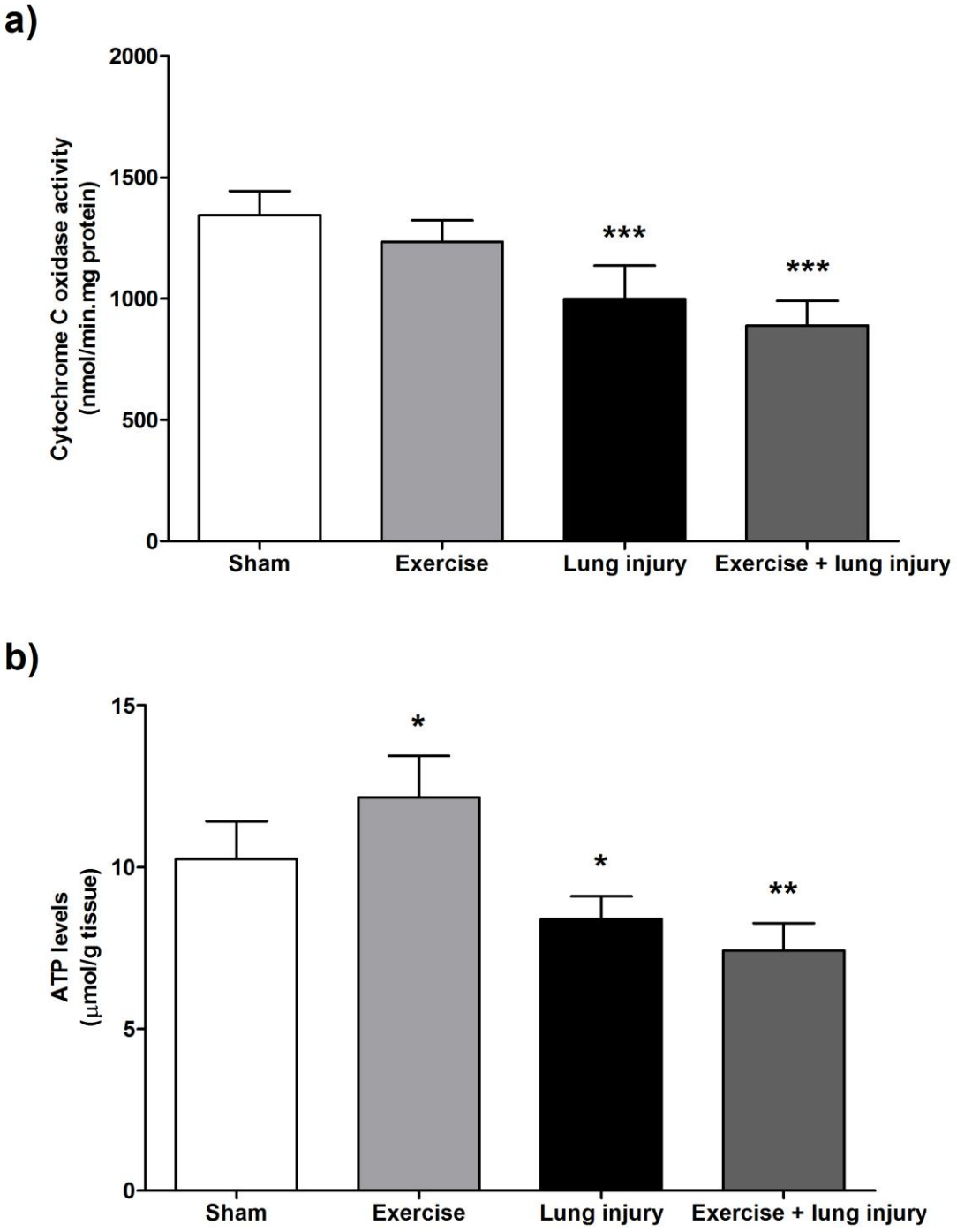
a)
Cytochrome C oxidase activity (nmol/min.mg protein)
2000
1500
1000
500
0
***
***
Sham
Exercise
Lung injury
Exercise + lung injury
b)
ATP levels (µmol/g tissue)
15
10
5
0
*
*
**
Sham
Exercise
Lung injury
Exercise + lung injury

FIGURE 4.

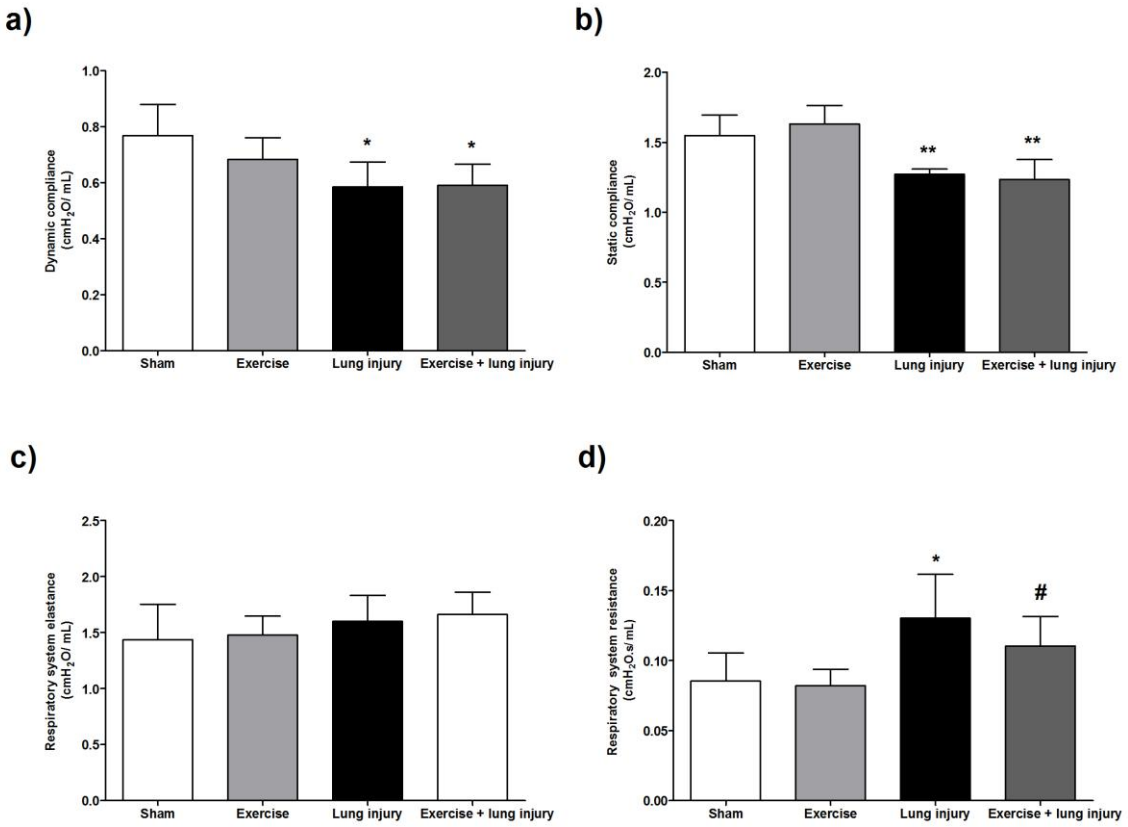
a)
Dynamic compliance (cmH2O/ mL)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Sham
Exercise
Lung injury
Exercise + lung injury
b)
Static compliance (cmH2O/ mL)
2.0
1.5
1.0
0.5
0.0
**
**
Sham
Exercise
Lung injury
Exercise + lung injury
c)
Respiratory system elastance (cmH2O/ mL)
2.5
2.0
1.5
1.0
0.5
0.0
Sham
Exercise
Lung injury
Exercise + lung injury
d)
Respiratory system resistance (cmH2O.s/ mL)
0.20
0.15
0.10
0.05
0.00
*
#
Sham
Exercise
Lung injury
Exercise + lung injury

FIGURE 5.

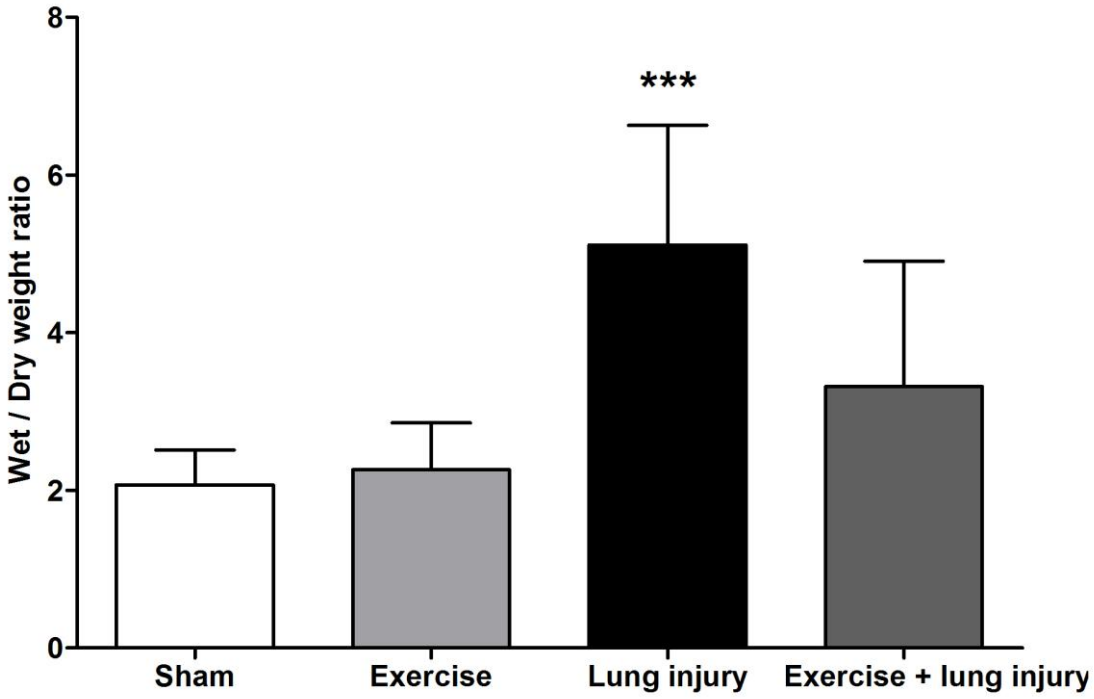
8
6
4
2
0
Wet / Dry weight ratio
***
Sham
Exercise
Lung injury
Exercise + lung injury

FIGURE 6.

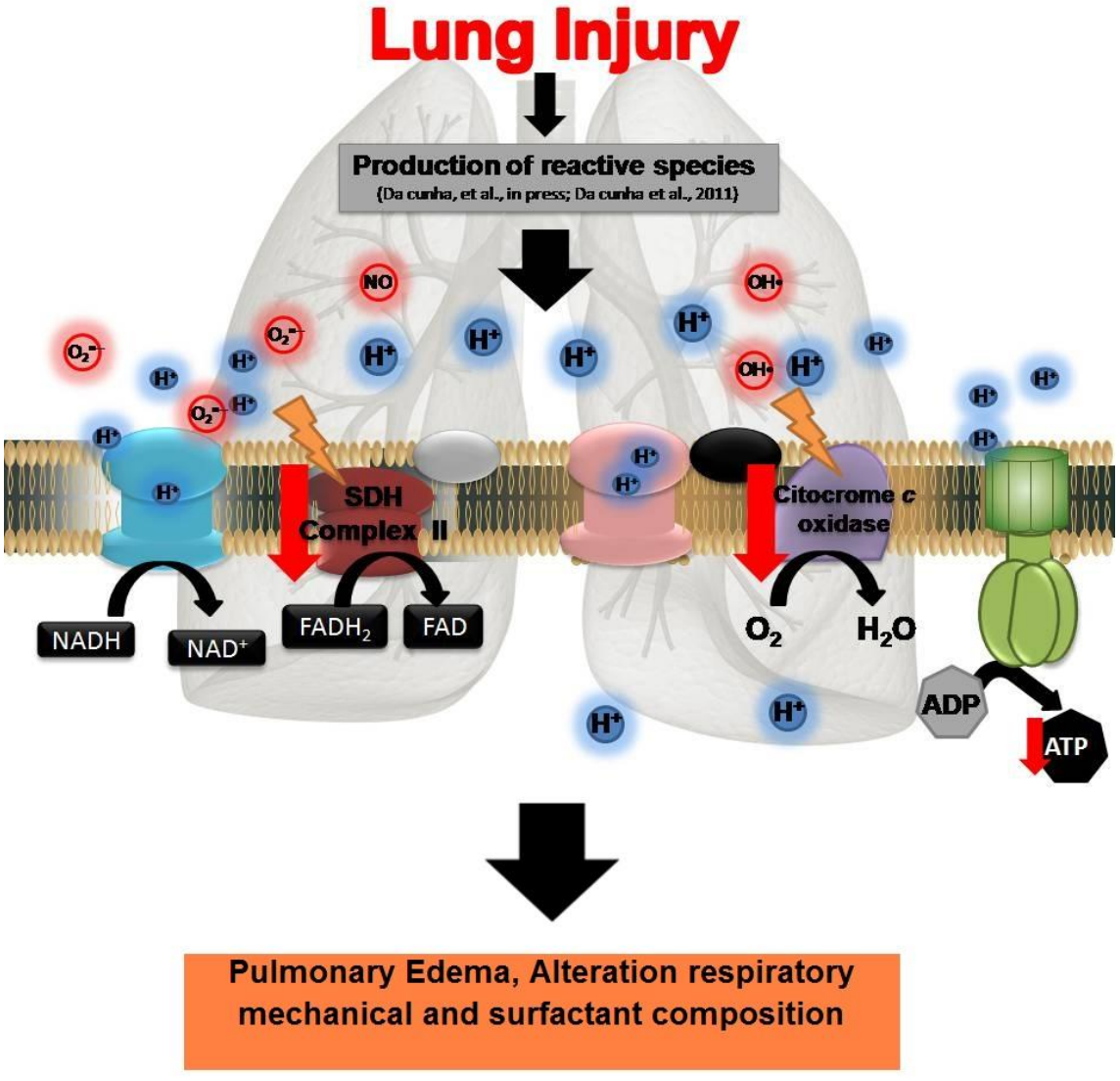
Lung Injury
Production of reactive species
(Da cunha, et al., in press; Da cunha et al., 2011)
NO
OH•
$O_2^{\bullet-}$
H+
SDH
Complex II
Citocrome c
oxidase
NADH
NAD+
$FADH_2$
FAD
O_2
H_2O
ADP
ATP
Pulmonary Edema, Alteration respiratory
mechanical and surfactant composition

Table 1. Effect of lung injury and/or exercise on phospholipids content in bronchoalveolar lavage fluid

Phospholipids	Sham	Exercise	Lung injury	Exercise + lung injury
	arbitrary units	arbitrary units	arbitrary units	arbitrary units
Phosphatidylcholine	368.53 ±69.58	300.9±39.64	239.29± 32.04*	266.10±58.63*
Phosphatidylethanolamine	31.78± 4.07	26.05±9.63	32.50±11.21	30.40±5.91
Phosphatidylglycerol	67.37±12.87	62.13±6.90	58.91±5.40	59.78±10.11
other	65.61±2.29	47.77±13.03	61.70±24.49	45.46±16.23
Total	535.34±77.86	443.33±85.04	392.41±56.16*	398.77±84.18*

Data are expressed as mean ± S.D. for 5 animals in each group. Different from other groups, *$p < 0.05$ (two way ANOVA followed by Tukey test).

IV. DISCUSSÃO

SARA é uma sindrome caracterizada por um distúrbio na barreira alvéolo-capilar, que resulta no acumulo de exsudato protéico no espaço alveolar e no intertício, dificultando as trocas gasosas e levando a um quadro de insuficiência respiratória (Ware and Matthay, 2000). Sabe-se que o estresse oxidativo desempenha um papel importante no agravamento do quadro, principalmente sobre o dano no epitélio alveolar (Hagiwara et al., 2008). Por outro lado, acredita-se que o exercício físico possa aumentar o sistema de defesa contra a ação das espécies reativas e induzir uma resposta adaptativa, bem como previnir doenças relacionadas com inflamação sistemica (Pinho et al., 2009; Pinho et al., 2006; Venditti and Di Meo, 1997).

Considerando que para o nosso conhecimento, existem poucos estudos mostando o papel do exercício físico regular como um protetor contra o estresse oxidativo presente na lesão pulmonar o objetivo da primeira parte (artigo 1) desta dissertacão foi avaliar alguns parâmetros de estresse oxidativo tais como formação de espécies reativa através da oxidação do DCF, TBARS, carbonilas, sulfidrilas, atividades das enzimas antioxidantes SOD, CAT, GPX, conteúdo de GSH, TRAP, níveis de nitritos e o imunoconteúdo do NF-κβ no tecido pulmonar de ratos submetidos à lesão pulmonar com ou sem exercício físico. O efeito protetor do exercício físico sobre as perturbações na barreira alvéolo-capilar também foi avaliado através da contagem total de células, a atividade LDH e proteínas no LBA, bem como no infiltrado no parênquima pulmonar (análise histológica).

Nossos resultados mostraram que animais submetidos à lesão pulmonar tiveram um aumento na produção de espécies reativas (observado por um aumento nos níveis de DCF) e na lipoperoxidacão (observado pelo aumento do TBARS). Além disso, observamos um aumento das carbonilas e uma diminuição do conteúdo de sulfidrilas. Esses resultados sugerem que o estresse oxidativo pode ser um importante contribuinte para o desenvolvimento da lesão pulmonar através do dano gerado aos lipídeos e as proteínas. O exercício físico foi capaz de prevenir (totalmente) o aumento na produção de espécies reativas e de prevenir (parcialmente) os danos às proteínas, no entanto, não foi capaz de prevenir a peroxidação lipídica induzida pela lesão pulmonar. Com relação ao efeito protetor do exercício, os resultados estão de acordo com outros estudos que mostram que a resposta ao estresse oxidativo induzido pelo H_2O_2 foi significativamente menor no coração de ratos treinados do que em ratos sedentários (De Angelis et al., 1997). Neste contexto, Oh-Ishi e colaboradores (1997) acreditam que mais importante que prevenir a lipoperoxidação é a capacidade do tecido pulmonar em resistir aos efeitos causados pelos radicais livres como, por exemplo, aumentando as defesas antioxidantes (Oh-ishi et al., 1997; Pinho et al., 2006).

Estudos demonstraram que uma única sessão de exercício pode levar ao um aumento na produção de espécies reativas, e assim causar danos aos lipídeos, proteínas e DNA (Davies et al., 1982; Ji et al., 2006; Powers and Lennon, 1999; Radak et al., 1999). Há relatos, sugerindo que a base molecular para o efeito benéfico do exercício físico regular pode estar relacionado com aumento de espécies reativas causando dano molecular e ao mesmo tempo induzindo uma resposta adatativa ao insulto (Aebi, 1984; Gomes et al., 2012;

Powers et al., 2010; Powers et al., 2011; Radak et al., 2008). Uma forma de adaptação e proteção contra o estresse oxidativo é atraves da via de sinalização NF-κβ. Através da ligação direta de espécies reativas, o NF-κβ é translocado para o núcleo onde ativa a transcrição dos genes para as enzimas antioxidantes (Hollander et al., 2001; Ji et al., 2006; Meyer et al., 1994; Wan et al., 1994).

Considerando que, o exercício físico pode ter papel benéfico no desequilíbrio oxidante e antioxidante presente nas doenças relacionadas com o estress oxidativo, tais como SARA, e os mecanismos de adaptação biológica gerados no pulmão são em grande parte desconhecidos, por este motivo, avaliamos o efeito da lesão pulmonar e/ou exercício físico sobre o imunoconteúdo de NF-κβ/p65. Nossos resultados mostraram um aumento na imunoconteúdo de NF-κβ/p65 em animais submetidos à lesão pulmonar, e o exercício preveniu este efeito. Considerando-se que NF-κβ pode ser ativados por uma variedade de estímulos, incluindo os radicais livres, citocinas pró-inflamatórias, LPS e outros (Baeuerle and Baltimore, 1988a), o efeito do exercício físico de normalizar os níveis de NF-κβ em pulmão de ratos submetidos a lesão pulmonar, sugere uma ação anti-inflamatória potente, através da inibição da ativação de NF-κβ, que por sua vez pode estar relacionado com a diminuição na geração de espécies reativas observada no grupo de animais submetidos lesão pulmonar mais exercício físico.

O TRAP é uma estimativa útil para avaliar a capacidade antioxidante não enzimático encontrado no tecido pulmonar (por exemplo, glutationa, ácido úrico, ácido ascórbico e α-tocoferol) (Evelson et al., 2001). No presente estudo, mostramos que os animais submetidos à lesão pulmonar tiveram diminuíção do

TRAP e o conteúdo de GSH. Esses resultados sugerem que a redução da TRAP observada nos animais submetidos à lesão pulmonar pode estar relacionada com a diminuição no conteúdo de GSH. A falta substancial de GSH no trato respiratório de pacientes com SARA pode aumentar o risco de lesão oxidativa, visto que, o equilíbrio entre oxidante e sistemas antioxidante é essecial para manter a função e a estrutura pulmonar (Bunnell and Pacht, 1993). Nosso estudo mostrou que o exercício físico previne a diminuição do conteúdo de GSH, o que poderia explicar os valores do TRAP semelhantes aos do controle. Nossos resultados sugerem que o exercício físico pode ser recomendado a fim de restaurar o conteúdo de GSH, uma vez que estudos mostram que há um aumento na atividade das enzimas envolvidas na síntese de GSH (Ji et al., 2006; Kretzschmar and Muller, 1993; Sen et al., 1992).

Em relação às defesas antioxidantes enzimáticas, que são responsáveis pela remoção de espécies reativas (Halliwell and Gutteridge, 2007), os nossos resultados mostraram que a atividade da SOD e da CAT no pulmão de ratos submetidos à lesão pulmonar não foram alterados. Curiosamente, o grupo de animais submetidos à lesão pulmonar mais exercício tiveram um aumento na atividade de ambas as enzimas, quando comparado com os grupos lesão pulmonar e/ou grupo controlo. Em contrapartida o exercício *per se* aumentou a atividade de CAT. Neste contexto, tem sido mostrado que ratos submetidos à lesão pulmonar induzida por isquemia e reperfusão não apresentaram qualquer alteração nas suas enzimas antioxidantes no soro, no entanto, os ratos submetidos à lesão mais exercício apresentam um aumento da atividade da SOD, quando comparados com ratos sedentários (Mussi et al., 2008). Com base neste resultado, os autores sugeriram que o exercício físico pode

aumentar a expressão de enzimas antioxidantes (Chang et al., 2004; Linke et al., 2005; Powers and Lennon, 1999; Toledo et al., 2012). É provável que o aumento das enzimas antioxidantes possa levar a uma menor produção de espécies reativas.

No nosso estudo, a falta de ativação de enzimas antioxidantes causada pela lesão pulmonar pode levar ao aumento de espécies reativas, incluindo a formação do •OH, um radical livre poderoso que possui a capacidade de remover rapidamente elétrons de outras moléculas e que não possui uma enzima antioxidante capaz de neutralizá-lo (Del Maestro, 1980; Yu, 1994). Nossos resultados mostraram que o exercício físico impediu o aumento nos níveis de DCF induzido pela lesão pulmonar. A CAT detoxifica o H_2O_2 formado, o H_2DCF por sua vez, pode ser oxidado a DCF na presena H_2O_2. Uma hipótese para a diminuição dos níveis de DCF observados no grupo lesão pulmonar e exercício, pode ser devido ao fato de que a atividade da CAT está aumentada.

Como discutido anteriormente, a CAT reduz H_2O_2 em H_2O, removendo um intermediário chave na formação de •OH. Estudos mostram que os pulmões têm um menor concentracão de CAT, em comparação com outras enzimas antioxidantes (Halliwell and Gutteridge, 2007; Heffner and Repine, 1989). A GPx também remove o H_2O_2 formando H_2O, no entanto para isso faz-se necessário a oxidação de GSH e também a atuação do selénio como cofactor. A inibição da atividade da GPx, em animais submetidos à lesão pulmonar, observada no nosso estudo poderia ser associado com diminuição de GSH, uma vez que a atividade desta enzima depende da regeneração de GSH. Observou-se também que o exercício físico previne totalmente alterações da GPx e GSH.

Estudos clínicos e pré-clínicos têm demonstrado que os níveis de NO• estão aumentados no LBA dos pacientes com SARA e nos animais submetidos à lesão pulmonar experimental (da Cunha et al., 2011a; Sittipunt et al., 2001). No presente estudo, observamos que os ratos submetidos à lesão pulmonar apresentam um aumento dos níveis de nitritos e o exercício foi capaz de diminuir seus niveis. O NO• produzido em quantidade excessiva pode ligar-se ao O_2^- e formar um dos mais potentes radicais livres o $ONOO^-$, desencadeador de vários danos celulares incluindo dano aos lipídeos (Beckman et al., 1990; Beckman and Koppenol, 1996). Estudos demonstram que os efeitos nocivos dos níveis elevados de NO• em pacientes com SARA estão relacionados com a indução de quimiocinas e citocinas e com a formação de espécies reativas mais prejudiciais (Sittipunt et al., 2001; Vanoirbeek et al., 2006). Nossos resultados mostram que o exercício diminui os níveis de nitritos, corroborando com outro estudo que demonstra que o exercício físico inibe a elevação dos níveis NO• pulmonar em animais submetidos à lesão pulmonar (Ramos et al., 2009). Esses autores sugerem que a diminuição dos níveis de NO• pode estar relacionado com efeitos anti-inflamatórios causados pelo exercício

O aumento na contagem de total de celulas no LBA e o infiltrado inflamatório no parênquima pulmonar confirmam a presença de um processo inflamatório nos animais submetidos à lesão pulmonar. Esses achados estão de acordo com dados da literatura que mostraram um aumento na contagem total de células no LBA, 12 horas após a injeção intratraqueal de LPS (Ritter et al., 2006). O exercício não foi capaz de prevenir o processo inflamatório, observado no LBA e no parênquima pulmonar, causado pela lesão pulmonar. Por outro, lado estudos anteriores demonstraram que os animais pré-

submetidos a seis semanas de condicionamento aeróbico, obtidos através de baixa intensidade de treinamento de natação, demonstrar uma diminuição no processo inflamatório no pulmão, no entanto foram analisadas 24 horas após a indução da lesão pulmonar, sugerindo que o exercício físico pode ter um efeito em longo prazo (Ramos et al., 2009). A falta de resultados positivos em nosso estudo, em relação ao exercício físico pode estar relacionada ao fato dos animais terem sido mortos 12 horas após a lesão. Acreditamos que o intervalo de tempo utilizado em nosso estudo tenha sido insuficiente para que o organismo dos animais pudesse reagir e assim diminuir o processo inflamatório presente na lesão pulmonar, uma vez que tem sido demonstrado que o exercício físico regular promove uma aceleração dos processos de reparação na inflamação, interfere em várias etapas de processos inflamatórios e aumenta a capacidade fagocítica das células (Fehr et al., 1989; Nieman, 1998).

A fim de confirmar a eficácia do modelo, também investigamos a atividade da LDH e a concentração de proteínas no LBA. Os resultados mostraram que a lesão pulmonar aumentou a atividade LDH e a concentração de proteínas no LBA nos animais submetidos à lesão pulmonar e/ ou exercício. Esses resultados estão de acordo com outros estudos que mostraram que a concentração de proteína total e atividade da LDH no LBA estão aumentadas em animais submetidos à lesão pulmonar (da Cunha et al., 2011a; Dal-Pizzol et al., 2006). A concentração de proteína no LBA aumenta rapidamente após a lesão pulmonar e as concentrações de proteínas podem atingir valores máximos de entre 6 e 12 horas (Ritter et al., 2006). O aumento nas proteínas encontrado no LBA é um indicativo de edema, causado pelo aumento da permeabilidade da membrana alvéolo capilar, que por sua vez, faz com que

ocorra um influxo de proteínas do meio vascular para o interstício e para o interior do alvéolo (Lenz et al., 1999). O exercício físico não foi capaz de prevenir as alterações na concentração de proteína causada pela lesão pulmonar, entretanto, foi capaz de impedir parcialmente o aumento da atividade de LDH, sugerindo que houve uma diminuição da lesão. Acreditamos que a prevenção de lesão pulmonar evidenciada pela atividade da LDH pode ser explicada, pelo menos em parte, pela diminuição de alguns parâmetros de estresse oxidativo observado no presente estudo.

Estudos mostram que há uma associação entre a disfunção mitocondrial e doenças inflamatórias pulmonares (Raby et al., 2007), no entanto os mecanismos envolvidos não estão completamente elucidados. Assim, na segunda parte desta dissertacão (artigo 2) avaliamos o efeito da lesão pulmonar sobre as enzimas da cadeia respiratória (SDH, complexo II, citocromo c oxidase e niveis de ATP). Resultados mostraram que a atividade da SDH, complexo II e citocromo C oxidase foram significativamente reduzidas nos animais submetidos à lesão pulmonar. Sugerindo que essa lesão pode comprometer a função da cadeia respiratória no pulmão de ratos. O sistema de fosforilação oxidativa mitocondrial gera espécies reativas e sabe-se que, os complexos da cadeia de transporte de eletrons são vulneráveis aos danos causados pelas espécies reativas (Dudkina et al., 2008). Assim, é plausível sugerir que o processo inflamatório gerado no tecido pulmonar aumentou a produção de espécies reativas e induziu a inibição da atividade das enzimas mitocondriais apresentada em nosso estudo (Aguilera-Aguirre et al., 2009; Mabalirajan et al., 2008). De acordo com os nossos resultados, estudos mostram que o estresse oxidativo desempenha um importante papel na lesão

pulmonar, principalmente no desencadeamento de lesão endotelial e do edema inflamatório (da Cunha et al., 2011a; Hagiwara et al., 2008; Ritter et al., 2006).

A atividade mitocondrial consome cerca de 90% de oxigênio do corpo e sendo responsável, na maioria dos tipos celulares, por 90% da geração de ATP (Crouser, 2004; Fink, 2002; Singer and Brealey, 1999). O citocromo c oxidase é uma enzima terminal limitante da cadeia respiratória mitocondrial, catalisando a transferência de eletrons do citocromo c para o O_2 (Capaldi, 1990). Assim, uma inibição da atividade dessa enzima pode levar à redução incompleta do oxigênio e, consequentemente, diminuir a síntese de ATP (Bose et al., 1992; Gupta et al., 2001; Milatovic et al., 2001). Neste contexto, nossos resultados mostraram que a lesão pulmonar reduziu significativamente os níveis de ATP em pulmão de ratos. Essa diminuição na produção de ATP pode comprometer a atividade metabólica celular normal, levando a disfunções celulares (Porta et al., 2006).

Como já foi relatado anteriormente, acredita-se que o exercício fisico melhore o sistema de defesa contra a ação de espécies reativas e induza uma resposta adaptativa ao estresse oxidativo (Pinho et al., 2009; Pinho et al., 2006; Venditti and Di Meo, 1997). No presente estudo, observou-se que o exercício físico foi capaz de impedir a redução na atividade da SDH e complexos II causada pela lesão pulmonar. Acreditamos que o exercício físico pode proteger o tecido pulmonar de insulto oxidativo causado pela lesão pulmonar, uma vez que nossos resultados demonstraram que o exercício físico impediu o aumento nos níveis de espécies reativas e de nitritos, a ativação do NF-κβ, bem como o evitou o dano à proteína e a redução das defesas antioxidantes.

A lesão pulmonar é acompanhada pela alteração na mecânica respiratória e também pela formação de edema (Ferguson et al., 2012; Gattinoni et al., 1991), o que contribui para o quadro de insuficiência respiratória (Gregory et al., 1991). Baseado nestas evidencias extendemos nossa investigação para avaliar o efeito da lesão pulmonar experimental sobre a complacência dinâmica e estática, elastância, resistência do sistema respiratório bem como sobre a formação de edema (peso úmido e peso seco). Nossos resultados mostram que a lesão pulmonar diminui a complacência dinâmica e estática e aumentar a resistência do sistema respiratório. Também observamos a formação de edema pulmonar em ratos submetidos à lesão pulmonar, o que sugere que o fluxo de ar pode estar reduzido nas vias aéreas devido ao acumulo de fluido intersticial podendo contribuir para a diminuição da complacência e aumento da resistência do sistema respiratório observado neste modelo experimental.

O surfactante forma uma película de lípideo na interface ar/água e, portanto, reduz a tensão superficial (Goerke, 1998; Perez-Gil and Keough, 1998), e protege contra o colapso dos alvéolos no final da expiração. O surfactante é composto principalmente por fosfolípidos, dos quais fosfatidilcolina representa cerca 70-80% do total (Taeusch et al., 2005; Veldhuizen and Possmayer, 2004). Considerando-se que as alterações na mecânica respiratória apresentados em nosso estudo podem estar relacionadas à disfunção do surfactante, resolvemos investigar o efeito lesão pulmonar sobre surfactante através da análise por cromatografia dos fosfolípidos (fosfatidilcolina, fosfatidiletanolamina e fosfatidilglicerol). Os resultados mostraram uma diminuição na fosfatidilcolina no LBA de ratos

submetidos à lesão pulmonar. Uma das possíveis razões para a diminuição de fosfolípidos causadas por lesão pulmonar pode ser a destruição da interface ar-água pelo edema alveolar, que provavelmente foi causado pela degradação de fosfolípidos pelas fosfolipases, degradação de proteínas pelas proteases (Baker et al., 1999; Greene et al., 1999), danos por espécies reativas e a diminuição da síntese de surfactante pelas células do tipo II (Rodriguez-Capote et al., 2006).

Como o exercício físico previniu algumas alterações do metabolismo energético e estresse oxidativo, extendemos nossas investigação, e avaliamos a influência do exercício físico sobre as alterações causadas pela lesão pulmonar, tais como formação do edema pulmonar, mecânica respiratória e o conteúdo de fosfolipídios. Os resultados mostram que o exercício físico inibiu a formação do edema pulmonar (relação peso úmido e peso seco) e preveniu parcialmente o aumento da resistência do sistema respiratório, mas não preveniu as alterações na complacência dinâmica e estática e a diminuição da fosfatidilcolina.

Alteração de forças resistivas e propriedades viscoelásticas do tecido são características importantes da lesão pulmonar e parecem estar associados com o rompimento de barreira alveolar, edema alveolar, disfunção do surfactante, colapso alveolar e inflamação (Santos et al., 2006). Em nosso estudo, não houve efeito significativo do exercício sobre a diminuição dos fosfolipídeos. A diminuição de resistência do sistema respiratório causada pela lesão pulmonar mais exercício físico está provavelmente relacionado com a diminuição da formação de edema pulmonar.

Em suma, os resultados obtidos nesta dissertação demonstraram que lesão pulmonar experimental promoveu estresse oxidativo e alterações no metabolismo energético no pulmão de ratos, bem como, alterou a mecânica ventilatória. O exercício físico, por sua vez, preveniu alguns parâmetros de estresse oxidativo e metabolismo energético, contudo não preveniu as alterações de mecânica ventilatória. Com base nesses resultados, podemos sugerir que o exercício pode ter um papel protetor contra o dano pulmonar causado pela lesão pulmonar, no entanto, os mecanismos e importância biológica desses achados necessitam de mais estudos para avaliar os efeitos do exercício nesta condição patológica e o possível benefício de utilizá-lo como estratégia terapeutica não farmacológica para prevenir algumas alterações presentes na SARA e melhorar a qualidade de vida dos pacientes afetados.

V. CONCLUSÕES

O nosso modelo de lesão pulmonar experimental em ratos wistar adultos:

- Induziu um aumento de espécies reativas e de dano a lipídeos e proteínas bem como promoveu uma diminuíçãs nas defesas antioxidantes enzimáticas e não enzimaticas (GPX, GSH, TRAP);
- Aumentou os níveis de nitritos e no immunoconteúdo do NF-KB;
- Induziu um processo inflamatório (contagem total de células e pela presença de um infiltrado inflamatório no parênquima pulmonar).
- Promoveu um influxo de proteínas para interior do alvéolo (edema) bem como, aumentou a atividade da LDH (morte celular);
- Provocou alterações no metabolismo energético através da diminuição das enzimas da cadeia transportadora de elétrons (SDH, complexo II, citocro c oxidase e níveis) e dos níveis de ATP;
- Promoveu alterações na mecânica ventilatória (diminuição na complacência e aumento na resistência do sistema respiratório);
- Alterou o conteúdo de fosfolipídeos no LBA bem como promoveu a formação do edema pulmonar avaliado através do peso úmido e peso seco.

O exercício físico em ratos wistar adultos:

- Preveniu o aumento nas espécies reativas, e parcialmente o dano às proteínas, bem como preveniu totalmente a diminuição na atividade da GPX e a diminuição nos níveis de GSH e do TRAP causado pela lesão pulmonar;
- Aumentou a atividade das enzimas antioxidantes enzimáticas SOD e CAT nos animais submetidos à lesão pulmonar

- Previniu o aumento dos níveis de nitritos e do imunocnteúdo do NF-Kβ causado pela lesão pulmonar;
- Previniu parcialmente o dano pulmonar evidenciado através atividade da LDH;
- Peveniu a diminuição das enzimas das enzimas do metabolismo energético SDH e complexo II no pulmão de ratos submetidos à lesão pulmonar;
- Impediu a formação do edema avaliado através do peso úmido e peso seco;
- Aumentou *per se* a atividade da CAT e os níveis de ATP.

Conclusão geral

Nossos resultados mostraram que a lesão pulmonar pode induzir alterações na função mitocondrial. Os mecanismos pelos quais a lesão pulmonar provocou tais alterações não estão claros, no entanto, sugerimos que o estresse oxidativo presente nesse modelo pode estar prejudicando a cadeia respiratória, o que provavelmente limita a produção de ATP e aumenta ainda mais a produção de espécies reativas em um ciclo vicioso (Figura 8 resume os resultados). O treinamento de exercícios físicos inibiu a formação do edema pulmonar e preveniu agumas alterações de estesse oxidativo e metabolismo energético. Esses achados sugerem que exercício físico pode ser benéfico na prevenção de algumas alterações presentes na lesão pulmonar e pode representar estratégia terapêutica não farmacológica, entretando mais estudos fazem-se necessários para melhor elucidar os mecanismos de ação

relacionados aos benefícios da atividade fisica regular para pacientes com SARA.

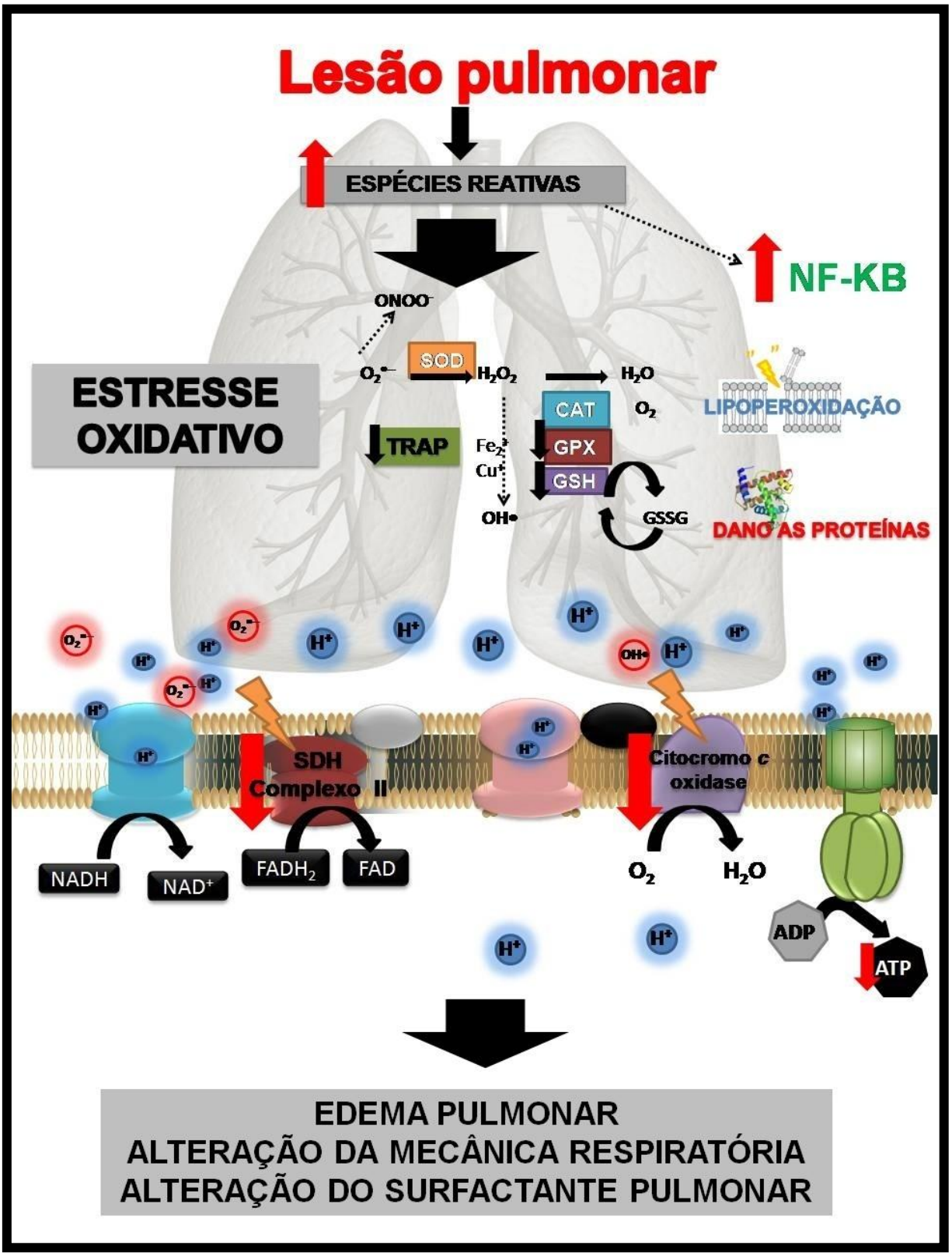

Figura 8. Resumo das alterações encontradas no pulmão de ratos submetidos a lesão pulmonar analisadas neste estudo. SOD (superóxido dismutase) CAT (catalase), GPx (glutationa peroxidase), GSH (glutationa), TRAP (capacidade antioxidante total), fator nuclear kappa-beta (NF-κβ) e SDH (succinato desidrogenase

VI. PERSPECTIVAS

- Avaliar o efeito da lesão pulmonar e do exercício físico sobre o immunoconteúdo e a expressão das enzimas antioxidantes (SOD, CAT e GPX);
- Avaliar efeito da lesão pulmonar e do exercício físico sobre o imunoconteúdo da iNOS;
- Avaliar as alterações presentes na lesão pulmonar aguda 48h, 3 dias, 1 semana e 1 mês após a sua indução;
- Avaliar se exercício físico previne as alterações na lesão pulmonar em diferentes tempos (48h, 3 dias e 1 semana após a indução da lesão);
- Avaliar o efeito do exercício físico após a lesão pulmonar;
- Investigar as possíveis alterações neuroquímicas, neuroinflamatórias e comportamentais no córtex e hipocampo de ratos submetidos ao modelo experimental de lesão pulmonar em diferentes tempos (12h, 48h, 3dias, 1 semana e 1 mês apos a indução)

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