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CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITO DA SUPLEMENTAÇÃO DE N-ACETILCISTEÍNA E DO EXERCÍCIO FÍSICO SOBRE OS MARCADORES DE ESTRESSE OXIDATIVO PULMONAR INDUZIDO PELA EXPOSIÇÃO AGUDA AO CARVÃO MINERAL

Ricardo Aurino de Pinho

Orientador: Dr. José Cláudio Fonseca Moreira

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“Que estranha é a sina que cabe a nós, mortais! Cada um de nós está aqui para uma temporada; com que propósito não se sabe [...] Os ideais que tem iluminado meu caminho, e repetidamente me tem renovado a coragem para enfrentar a vida com ânimo, são a Bondade, a Beleza, e a Verdade [...] O único homem que está isento de erros é aquele que não arrisca acertar”

ALBERT EINSTEIN
The World as I see it (1931)

AGRADECIMENTOS

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ABREVIATURAS

ERO: espécies reativas de oxigênio

O_2: oxigênio molecular

$O_2^{\bullet -}$: ânion superóxido

H_2O_2: peróxido de hidrogênio

H_2O: água

$OH^{\bullet}$: radical hidroxil

e^-: elétron

H^+: hidrogênio

Fe^{2+}: ferro II

Fe^{3+}: ferro III

CAT: catalase

GPX: glutationa peroxidase

PNM: neutrófilos polimorfonucleares

GSH: glutationa reduzida

AMP: monofosfato de adenosina

ATP: trifosfato de adenosina

GSSG: glutationa oxidada

SOD: superóxido dismutase

TBA: ácido tiobarbitúrico

TBARS: substâncias reativas ao ácido tiobarbitúrico

MDA: malondealdeídeo

LDH: lactato dehidrogenase

BAL: lavado bronco-alveolar

NAC: N-acetilcisteína

DFX: deferoxamina

ERN: espécies reativas de nitrogênio

XO: xantina oxidase

RESUMO

O objetivo geral dessa tese foi investigar os efeitos terapêuticos da N-acetilcisteína (NAC) e do exercício físico aeróbico sobre os possíveis danos oxidativos pulmonares de ratos Wistar após exposição ao carvão mineral. Os animais (200-250g) receberam, por instilação traqueal, carvão mineral (3mg/0,5ml salina) ou somente solução salina 0,9%. Após o período de instilação os animais foram imediatamente mortos por decapitação ou suplementados com NAC (20mg/kg de peso corporal/dia, i.p) mais deferoxamina (DFX) (20mg/kg de peso corporal/semana, i.p) ou ainda submetidos a um programa de exercício físico progressivo (até 17 $m.min^{-1}$, 10% de inclinação, 50 $min.dia^{-1}$). Os pulmões foram cirurgicamente removidos e armazenados em solução de formol a 10% para análise histopatológica ou imediatamente congelado à –70°C para posteriores análises bioquímicas. Em alguns casos, antes do congelamento, foi realizado o lavado bronco-alveolar. A capacidade antioxidante total foi avaliada a partir da técnica de quimiluminescência gerada pelo luminol. Outras análises bioquímicas foram realizadas a partir das atividades das enzimas catalase, superóxido dismutase, citrato sintase e lactato dehidrogenase. Os danos oxidativos em lipídios e proteínas foram avaliados respectivamente pelos níveis de espécies reativas ao ácido tiobarbitúrico e pelos danos em proteínas, quantificados pela determinação de grupamentos carbonila. Os resultados histopatológicos mostraram uma infiltração linfocitária seguida por infiltração crônica caracterizada por agregados de macrófagos, sugerindo que a resposta a essas alterações são mediadas por radicais livres. Constatou-se ainda que a administração da NAC associada ou não com a DFX reduziram de forma similar a resposta inflamatória e o dano oxidativo após exposição ao carvão mineral e também que o exercício físico de baixa a moderada intensidade contribui para a redução dos danos oxidativos. Nossos resultados portanto sugerem que a utilização de agentes terapêuticos como a suplementação de antioxidantes e do exercício físico pode amenizar os efeitos deletérios induzidos pela exposição ao carvão mineral.

Palavras-chave: radicais livres, estresse oxidativo, carvão mineral, antioxidantes, NAC, exercício físico

ABSTRACT

The objective of this work was to investigate the therapeutic effects of N-actylcysteine (NAC) and aerobic physical exercise on the lungs of rats exposed to coal dust. The animals (200-250g) received, by intratracheal instillation, mineral coal dust (3mg/0.5ml saline) or only saline solution 0.9% (0.5ml). After the instillation the animals were killed by decapitation, were supplemented with NAC (20mg/kg of body weight/day, i.p) plus DFX (20mg/kg of body weight/week) or were still submitted to a progressive training program (until 17m/min^{-1}, inclination 10%, 50min/day^{-1}). The lungs were surgically removed to the determination of histological and biochemical parameters. The total radical-trapping antioxidant parameter was estimated by luminol chemoluminescence emission. Other biochemical analyses were obtained by catalase, superoxide dismutase, citrate synthase and lactate dehidrogenase enzyme activities. Oxidative damages in lipids and proteins were respectively evaluate by the level of thiobarbituric acid reactive species and by protein damage, quantified by carbonyl groups determination. The histological results demonstrated a lymphocyte infiltration followed by a chronic infiltration characterized by macrophages aggregates and these responses were mediated by free radicals. We also observed that the administration of NAC alone or in association with DFX reduced to a similar degree the inflammatory response and the oxidative damage in rats exposed to coal dust and that also the exercise physical reduced oxidative damages. Our results suggest that therapeutic agents as antioxidants supplementation and of the physical exercise may attenuate the deleterious effects induced by the mineral coal exposure.

Key words: free radical, oxidative stress, mineral coal, antioxidants, NAC, physical exercise

CAPÍTULO I

INTRODUÇÃO

I.1 Exposição a poeiras de carvão mineral e doenças pulmonares

A inalação de poeiras minerais provoca distúrbios orgânicos significativos, principalmente, as de ordem respiratória, tais como: infecções, pneumoconiose e bronquite crônica ocupacional. Essas situações levam a um estado de morbidez, invalidez precoce, diminuição da expectativa de vida e conseqüente mortalidade. A quantidade, o tamanho da partícula, o tipo de mineral presente e tempo de exposição influenciam a severidade e progressão da doença, desde a formação de pequenos agregados de macrófagos repletos de carvão disseminado pelo parênquima pulmonar até o desenvolvimento de fibrose nodular ou difusa pulmonar irreversível.

Embora o próprio carvão não seja um agente fibrogênico, outros elementos agregados em sua composição, durante a extração, como a sílica e o ferro, por exemplo, depois de inalados, podem alterar tanto a estrutura física quanto a fisiologia pulmonar.

É bem verdade que com o avanço tecnológico na extração de carvão e com uma maior conscientização dos trabalhadores e empregadores na adoção de comportamentos preventivos, a incidência de doenças respiratórias, como a pneumoconiose, tem diminuído consideravelmente nos últimos anos. No entanto, existem milhares de seres humanos que necessitam de atendimento primário urgente para amenizar os efeitos deletérios da doença já adquirida.

O principal mecanismo de indução de dano pulmonar por depósito de partículas industriais é, provavelmente, mediado por ativação de macrófagos e recrutamento de células polimorfonucleares (PMN). Esse processo ativa

mediadores inflamatórios, como citocinas, quimocinas e espécies reativas de oxigênio (ERO), que provocam efeitos deletérios sobre o tecido pulmonar. (Dalal et al., 1995; Zhang et al., 1999; Mossman, 2003).

Estudos recentes têm sugerido que, após a exposição a partículas industriais, principalmente asbestos e sílica, ocorrem alterações bioquímicas pulmonares. Entretanto, são poucos os estudos que reportam essas alterações por partículas de carvão mineral.

As intervenções terapêuticas de rotina, especialmente as ações farmacológicas, têm contribuído significativamente para a diminuição da gravidade do problema, mas apresentam limitações, isto é, a intervenção farmacológica impede o avanço da doença e ameniza seus efeitos degenerativos, mas não é suficiente para garantir uma vida mais saudável. Acredita-se que o exercício físico regular orientado pode ser um agente auxiliar no restabelecimento da capacidade cardiorrespiratória. É importante fazer com que os indivíduos retornem suas atividades de rotina com maior eficiência e menos desconforto físico.

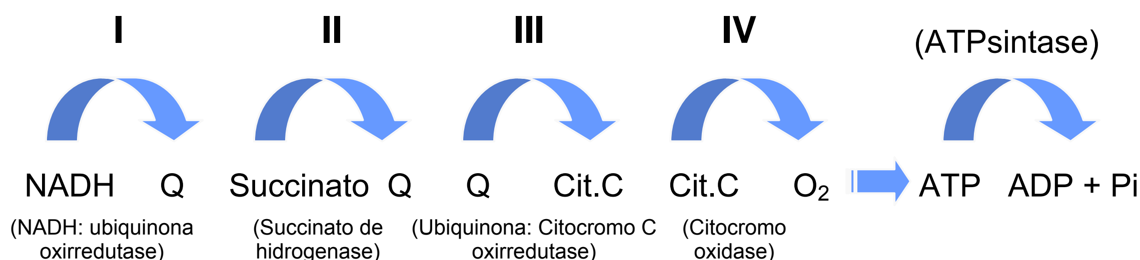
I.2 Espécies reativas de oxigênio

Sob condições fisiológicas normais, a maioria das espécies reativas de oxigênio (ERO) é produzida na cadeia respiratória mitocondrial, onde 90-95% do oxigênio consumido é reduzido à água. Entretanto, as ERO podem ser geradas em outros eventos bioquímicos na célula, por exemplo, em processos inflamatórios, na grande quantidade de metabolização de gordura pela Beta-oxidação, degradação da xantina a ácido úrico e auto-oxidação de

catecolaminas. Embora esses processos sejam normais para a vida das células, a produção excessiva de ERO pode induzir danos a biomoléculas entre elas ácidos nucléicos, proteínas e lipídeos que, em grande extensão, podem levar à morte celular (Halliwell e Gutteridge, 1999).

Durante a respiração celular, na cadeia transportadora de elétrons (figura I), o oxigênio molecular é completamente reduzido à água. A molécula de oxigênio pode aceitar um total de quatro elétrons para ser reduzida a duas moléculas de água, mas pode, também, ser reduzida por um elétron por vez, levando à produção de ERO (Matsuo e Kaneko, 2001), mais especificamente de Radicais Livres de Oxigênio (RLO). Dois a cinco por cento do oxigênio utilizado nesse processo são desviados para a formação desses radicais.

Figura I: exemplo esquemático da cadeia transportadora de elétrons



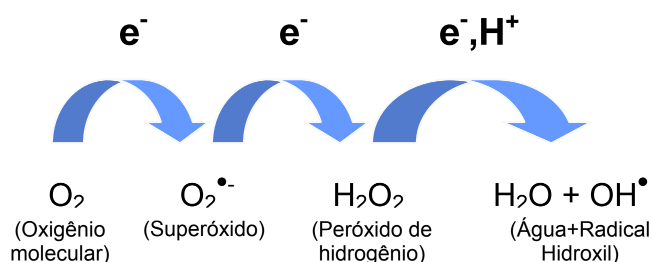
Por definição, segundo Halliwell e Gutteridge (1999), RLO são moléculas ou fragmentos moleculares reativos que contêm um elétron não pareado em seu orbital mais externo. Tendem a extrair elétrons de outras moléculas para alcançar um estado quimicamente mais estável.

A geração de RLO nem sempre é prejudicial ao organismo, pelo contrário, é necessária em vários processos biológicos: sinalização celular, contração muscular e sistema imune (Matsuo e Kaneko, 2001). Por exemplo, quando as células são agredidas por algum agente estressor (que também

pode ser RLO), elas acabam produzindo RLO para combater esses agentes. O grande problema é quando os níveis totais gerados de RLO forem maiores que a capacidade de defesa, podendo ocorrer danos celulares significativos.

A Figura II exemplifica a redução do oxigênio molecular e a formação das principais ERO: superóxido (O_2•-), peróxido de hidrogênio (H_2O_2) e radical hidroxil (OH•). Essas substâncias são capazes de reagir com qualquer tipo de molécula orgânica, extraindo elétrons e gerando novos radicais livres em cadeias citotóxicas. O radical hidroxil, segundo Ames et al. (1993), é o mais potente dos radicais e sua ação resulta na formação de peróxido de lipídeos e radicais orgânicos.

Figura II: Formação de ERO



Os complexos I e III da cadeia respiratória são locais conhecidos para a produção de superóxido e H_2O_2 e isso é causado pela transição de um e^- da NADH e FADH para ubiquinona, formando semiquinona (QH^o), e o outro e^- forma o superóxido. O superóxido é rapidamente reduzido pela superóxido dismutase (SOD) mitocondrial a H_2O_2. Quando um metal é catalisado pela reação de Fenton (Fe) ou Haber-weis (Cu) entre a dismutação de superóxido para H_2O_2, é formado o hidroxil (Matsuo e Kaneko, 2001).

A produção de RLO ocorre em cascatas em três fases: 1) iniciação: quando duas moléculas se condensam e formam um radical; 2) Progressão: quando o radical se liga a outra molécula qualquer (lipídeos, proteínas, núcleos), gerando um novo radical e 3) Término: quando a cascata de geração de radical acaba. Isso ocorre por "scavengers" ou elementos antioxidantes, que, ao se ligarem ao RL, formam novos radicais menos reativos e estes se esgotam ao reagirem com outros antioxidantes (Halliwell e Gutteridge, 1999).

I.3 Espécies reativas de oxigênio e poluentes atmosféricos

Após a inalação de poluentes atmosféricos, a produção de ERO e ERN (espécies reativas de nitrogênio) pode aumentar significativamente (Castranova et al., 2002). Estudos como os de MacNee e Rahman (2001), Vallyathan et al. (1998) e Zhang et al. (1999) têm demonstrado que a interação do sistema imune pulmonar e o estresse oxidativo podem estar diretamente relacionados com o desenvolvimento de várias doenças pulmonares.

O aumento na quantidade de fagócitos no pulmão, induzido pela exposição ao carvão, causa uma produção local de ânion superóxido ($O_2^{\bullet}$-) e peróxido de hidrogênio (H_2O_2). Na presença de ferro, o H_2O_2 é convertido em radical hidroxil pela reação de Fenton (Vallyathan et al., 1998). De acordo com Kim et al. (2000), a quantidade de ferro em partículas minerais pode ser o principal mediador de toxicidade e dos danos oxidativos sobre o pulmão. Adicionalmente, o superóxido é também um inibidor da alfa-1-antitripsina, uma importante glicoproteína que inibe a ação de várias proteases, incluindo elastases. Esse desequilíbrio antiproteases-proteases pode levar à destruição

tecidual e ao enfisema pulmonar (Lemaire e Quellet, 1996; Olszewer, 1999; MacNee e Rahman, 2001).

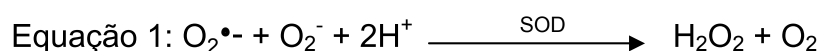
A resposta pulmonar ao processo inflamatório e à excessiva produção de ERO leva a lesões endoteliais, recrutamento de neutrófilos, peroxidação lipídica, danos em DNA (Mossman e Churg, 1998; Zhang et al., 1999), produção de TNF-α e interleucina -1β e formação de peroxinitrito (Blackford et al., 1997; Discroll et al., 1995; Dorger et al., 2002, Tsuda et al., 1997). Além disso, a produção de ERO induzida pela exposição ao carvão pode aumentar o dano pulmonar por inativação de antiproteases, rompimento de membranas, danos alveolares e fibroses (Discroll et al., 1995; Tsuda et al., 1997; Wang et al., 1999). Zhang e Huang (2002) sugerem que essas alterações podem aumentar pelo conteúdo de ferro presente nas poeiras industriais.

I.4 Sistema de defesa antioxidante

É possível que um eficiente sistema de defesa tenha condições de minimizar o dano oxidativo pulmonar induzido pelo depósito de partículas minerais. De acordo com Halliwell e Gutteridge (1999), esse sistema de defesa pode atuar de forma associada ou independente por duas vias:

I.2.1 - Ativação de enzimas antioxidantes: as principais enzimas antioxidantes incluem a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPX) que são ativadas normalmente durante o metabolismo celular, porém, suas atividades podem aumentar em função da presença de ERO.

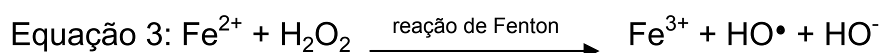
A SOD constitui a primeira linha de defesa enzimática contra a produção intracelular de radicais livres, catalisando a dismutação do O_2•- (Hollander et al., 2000). Está presente na matriz mitocondrial (Mn-SOD), no citosol (CuZn-SOD) e no meio extracelular. Embora o O_2•- não seja altamente danoso, pode extrair elétrons de diversos componentes celulares causando reações em cadeia de radicais livres (Halliwell e Gutteridge, 1999). O produto resultante da reação catalisada pela SOD é o H_2O_2 que deve ser retirado do meio o mais rápido possível (equação 1).



Equação 1: $O_2^{\bullet -} + O_2^{-} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$

A enzima Catalase catalisa a degradação do H_2O_2. Na reação, uma das moléculas de peróxido de hidrogênio é oxidada a oxigênio molecular e a outra é reduzida à água (equação 2) (Chance et al., 1979). Está localizada, principalmente, no peroxissoma, entretanto, outras organelas como as mitocôndrias podem conter alguma atividade da CAT. A catálise do H_2O_2 é importante, pois, na presença de Fe^{+2}, leva à formação de radical hidroxil (HO•) (reação de Fenton), altamente reativo e danoso às biomoléculas (equação 3).

Equação 2: $2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O$

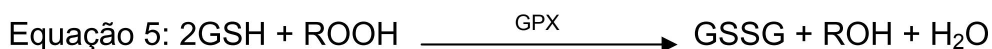


Equação 3: $Fe^{2+} + H_2O_2 \xrightarrow{\text{reação de Fenton}} Fe^{3+} + HO^{\bullet} + HO^{-}$

A GPX é uma enzima selênio-dependente que catalisa a redução do H_2O_2 (equação 4) e hidroperóxidos orgânicos (ROOH) (equação 5) para H_2O e

álcool, usando a glutationa (GSH) como doador de elétrons (Flohe, 1982). Está localizada tanto no citosol quanto na matriz mitocondrial.

Equação 4: $2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$


Equação 5: $2GSH + ROOH \xrightarrow{GPX} GSSG + ROH + H_2O$

I.2.2 - Antioxidantes biológicos não-enzimáticos: são constituídos por antioxidantes hidrossolúveis que incluem glutationa (GSH), ácido ascórbico e ácido úrico e antioxidantes lipossolúveis que incluem alfa-tocoferol, ubiquinóis e carotenóides.

Segundo Halliwell e Gutteridge (1999), a glutationa reduzida é um dos mais abundantes agentes antioxidantes biológicos, atua na conversão de dissulfidas para tióis e serve como um substrato para a GPX e glutationa S-transferase. A GSH, em sua forma reduzida, tem um importante papel no mecanismo de defesa do pulmão contra ataques de radicais livres (Smith et al., 1994; Zhang et al., 1999) e, dependendo do nível na célula, a GSH pode ser uma fonte importante contra respostas inflamatórias causadas por partículas industriais (Zhang et al., 1999).

O nível intracelular de GSH é regulado pelo equilíbrio entre sua utilização e síntese. Embora a GSH seja essencial para a função normal da célula, muitos órgãos não realizam a sua ressíntese e, portanto, precisam importá-la de fontes extracelulares (Deneke e Fanburg, 1989).

Tem sido proposto, em diversos estudos (Sprong et al., 1998; Paterson et al, 2003; Ritter et al, 2004), que uma das formas de alterar o conteúdo de GSH, na célula, é através da suplementação de N-acetilcisteína (NAC). A NAC

é um doador de grupos tióis que atua como precursora da cisteína intracelular, indispensável para a síntese de GSH, assegurando-lhe sua função normal na proteção celular. É considerado um importante agente terapêutico, utilizada comumente na prática clínica por manter a capacidade antioxidante pulmonar.

I.5 Exercício físico

O exercício físico, especialmente o de elevada intensidade, pode ser um agente capaz de aumentar a produção de ERO e a atividade de enzimas antioxidantes.

Taxas metabólicas elevadas como resultado do exercício físico podem aumentar dramaticamente o consumo máximo de oxigênio (VO_2máx) em até 20 vezes em relação aos valores de repouso (Carmeli et al., 2000). Esse aumento é seguido por um concomitante aumento na produção de ERO (Alessio e Goldfarb, 1988; Liu et al., 2000). Entretanto, estudos têm demonstrado que o treinamento de “endurance” aumenta as defesas antioxidantes, assim como a capacidade oxidativa do músculo (Alessio e Goldfarb, 1988; Radák et al., 1999; Terblanche, 2000).

O estresse oxidativo tem sido associado com a diminuição da performance, fadiga, dano muscular e excesso de treinamento (overtraining). Por essa razão, alguns pesquisadores (Powers et al., 1999; Radák et al., 1999; Polidori et al., 2000; Carmeli et al., 2000) sugerem que reduzir o estresse oxidativo pode melhorar a tolerância ao exercício bem como a performance física.

Embora os benefícios do aumento no VO_2máx sejam bem estabelecidos, um paradoxo bioquímico é verificado. O aumento no consumo máximo de O_2 é essencial para a aptidão cardiovascular e performance, porém o aumento no consumo durante o exercício pode ser prejudicial.

Dependendo do tipo e intensidade do exercício, têm sido propostos vários mecanismos na geração de ERO, segundo König e Berg (2002):

1) Aumento na produção de $O_2^{\bullet}$- na cadeia respiratória.

2) Ativação Xantina Oxidase (XO): a XO catalisa a degradação do monofosfato de adenosina (AMP) durante o trabalho muscular isquêmico, levando ao aumento na produção de $O2^{\bullet}$-. Durante a isquemia, o AMP, formado do ATP (trifosfato de adenosina) pela reação da adenilato quinase, é degradado para hipoxantina. A XO é convertida e, dessa forma, reduzida para xantina desidrogenase durante a isquemia por proteases intramusculares, as quais necessitam de Ca^+. A XO converte a hipoxantina para xantina e ácido úrico usando o oxigênio molecular como receptor de elétrons, formando assim o $O_2^{\bullet}$-. Em condições aeróbicas, o oxigênio suficiente assegura que o ATP seja reposto via fosforilação oxidativa mitocondrial e que a hipoxantina/xantina sejam, primeiramente, convertidas para ácido úrico através da xantina desidrogenase. Além disso, o músculo esquelético tem baixa atividade da XO. Todavia, a XO pode ser um importante caminho quando o músculo apresentar um déficit de adenina dinucletídeo. Essa situação, teoricamente, pode acontecer em situação isquêmica, exercício isométrico, alta velocidade, déficit de O_2 e exercícios com limitação vascular de fluxo sanguíneo (Ji, 1999).

3) Ativação de neutrófilos polimorfonucleares após danos musculares induzidos por exercício: o exercício leva à formação de várias células do sistema imune

como neutrófilos, monócitos e macrófagos que são capazes de produzir ERO. Entre essas células, os neutrófilos são a maior fonte de produção de $O_2^{\bullet}$- pela reação NADPH-oxidase. Na presença de H_2O_2 e íon clorido, os neutrófilos geram ácido hipocloroso, pela atividade da mieloperoxidade.

As ERO produzidas por neutrófilos são geradas para destruir bactérias invasoras e remover tecidos danificados. A neutrofilia induzida pelo exercício ocorre como resultado da migração de neutrófilos vindos dos tecidos endoteliais (mediados por catecolaminas) e da medula óssea (mediado pelo cortisol). Isso faz com que removam proteínas e células danificadas e também células mortas. Embora isso seja uma reação desejável, quando não bem regulada, pode ser uma das causas de inflamações agudas devido a um grande aumento na produção de mediadores proinflamatórios (interleucinas 1,6,8, TNF-alfa) e prostaglandinas, levando à indução e à intensificação de processo inflamatório adicional, aumentando a produção de ERO. As interleucinas estão diretamente envolvidas na geração de ERO e são ativadores de fator transcrição NF-kB.

4) Menor homeostase do cálcio em músculos estressados: o exercício leva a uma isquemia muscular e à diminuição da homeostase do Ca^+, o que favorece a produção de ERO por reações catalisadas pela XO (Chevion et al., 2003).

Entretanto, mesmo que o exercício intenso induza a uma alteração significativa na produção de ERO, estudos recentes mostram que o exercício físico regular de “endurance” pode tornar mais eficiente o sistema de defesa antioxidante e melhorar a capacidade oxidativa dos músculos, estabelecendo um equilíbrio entre os danos induzidos pelas ERO e os sistemas de reparos

antioxidantes (Alessio e Goldfarb, 1988; Powers et al., 1999; Radák et al., 1999; Liu et al., 2000; Terblanche, 2000; Carmeli et al., 2000).

1.6 Objetivos

Diante das evidências de que a inalação de partículas industriais altera a resposta oxidativa pulmonar e favorece o surgimento de doenças, pressupõe-se que a suplementação de NAC e a prática regular de exercício físico possam amenizar esses efeitos. A partir de tais pressupostos foram elaborados os seguintes objetivos:

1) Avaliar as alterações oxidativas pulmonares em ratos pela exposição ao carvão mineral e seu envolvimento nas reações redox e na produção de radicais livres.
2) Avaliar se a administração de NAC e DFX é capaz de atenuar o dano oxidativo pulmonar em ratos posterior à exposição aguda ao carvão mineral.
3) Estabelecer um protocolo de exercício em esteira para ratos e avaliar a associação entre exercício físico, dano oxidativo e defesas enzimáticas antioxidantes.
4) Avaliar os efeitos do exercício físico regular sobre os parâmetros de estresse oxidativo pulmonar induzido pela inalação de carvão mineral em ratos.

1.7 Organização da Tese

A tese está organizada em capítulos, onde o Capítulo I, na forma de introdução, apresenta uma revisão de literatura referente ao tema. Os resultados estão distribuídos nos quatro capítulos subseqüentes (II, III, IV, V), apresentados na forma de artigos, dos quais um já foi publicado e outros três foram submetidos à publicação. O capítulo II apresenta resultados referentes à determinação de um modelo animal de dano pulmonar induzido pela exposição ao carvão mineral. Esses resultados estão publicados na Environmental Research, 196: 290-297, 2004. O capítulo III avalia o efeito da NAC sobre a resposta oxidativa pulmonar após exposição ao carvão mineral (artigo submetido à Environmental Research). O capítulo IV estabelece um modelo animal de treinamento físico em esteira (artigo submetido à Redox Report). E, finalmente, o capítulo V associa o treinamento físico em esteira com os parâmetros de danos oxidativos em animais expostos ao carvão mineral (artigo submetido à Revista Brasileira de Medicina do Esporte). Uma discussão abrangendo todos os artigos e as conclusões gerais estão apresentadas respectivamente nos capítulos VI e VII. As referências bibliográficas apresentadas nos itens que não àqueles referentes aos artigos estão presentes no final dessa tese.

CAPÍTULO II

LUNG OXIDATIVE RESPONSE AFTER ACUTE COAL DUST EXPOSURE

Ricardo A. Pinho, MSc[1,2*]; Fernanda Bonatto, BSc[1]; Michael Andrades, BSc[1]; Mário Luis C. Frota Jr., BSc[1]; Cristiane Ritter, M.D.[1,3]; Fábio Klamt, MSc[1], Felipe Dal-Pizzol, PhD[1,3]; Jane M. Uldrich-Kulczynski, PhD[4]; José Cláudio F. Moreira, PhD[1]

[1]Laboratório de Estresse Oxidativo/Departamento de Bioquímica/UFRGS
[2]Laboratório de Fisiologia e Bioquímica do Exercício/UNESC
[3]Laboratório de Fisiopatologia Experimental/UNESC
[4]Hospital de Clínicas de Porto Alegre

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ABSTRACT

Coal dust exposure can induce an acute alveolar and interstitial inflammation and that can lead to chronic pulmonary diseases. The objective of this study was to describe the acute and later effects of acute coal dust exposure in lung parenchyma and the involvement of ROS in coal dust effects. *Fort*y-eight male Wistar rats (200-250mg) were separated in four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation. Gross mineral coal dust (3mg/0,5ml saline) was administered directly in the lung of the treatment group by intratracheal instillation. Control animals received only saline solution (0,5ml). Lipid peroxidation was determined by the quantity of thiobarbituric acid reactive species, oxidative damage to protein was obtained by the determination of carbonyl groups, the total radical-trapping antioxidant parameter was estimated by luminol chemoluminescence emission, catalase activity was measured by the rate of decrease in hydrogen peroxide and superoxide dismutase activity was assayed by inhibition of adrenaline auto-oxidation. Histological evaluation of coal dust treated rats demonstrated an inflammatory infiltration after 48 hours of the exposure. Initially, this was a cellular infiltration suggestive of lymphocyte infiltration with lymphoid hyperplasia that remains until 7 days after induction. This initial response was followed by a chronic inflammatory infiltration characterized by aggregates of macrophages 30 days after induction. This inflammatory response tends to resolve 60 days after induction, similar to control animals. During both acute and chronic phase of lung inflammation we observed a decrease in the TRAP in the lung of coal dust exposed animals when compared to control animals. We also observed an activation of superoxide dismutase 60 days after coal dust

exposition. TBARS were increased 60 days after coal dust exposure and protein carbonyl groups increased all times after coal dust exposure (48 hours, 7days, 30 days and 60 days). These data suggested a biphasic inflammatory response and the involvement of oxidative damage in coal dust induced lung damage.

Key-words: oxidative stress, free radicals, lung, coal dust

INTRODUCTION

During recent years, many studies have shown that lung function is markedly altered after deposition of industrial particles, such as asbestos and silica. However, few studies have reported lung alterations in the presence of other particles, such as coal dust. Although that coal is not a fibrogenic agent, there are other mineral dusts present during its extraction, mainly silica, capable of altering lung morphology.

The essential mechanism of coal dust induced lung damage is likely to be mediated by macrophage activation and recruitment of polymorphonuclear cells. This cell activation induces the release of inflammatory mediators, such as reactive oxygen species (ROS), (Dalal et al., 1995). According to Mossman (2003), the lung is a primary target for inhaled oxidants generated naturally during the combustion of oxygen and after inhalation of atmospheric pollutants.

In last years attention has been given to ROS generation in lung damage (Castranova et al., 2002, Cho et al., 1999, Shukla et al., 2003, Zhang et al., 1999). Excessive production of ROS induces molecular damage or cell death, which could lead to several physiologic and pathological processes.

Recently, several studies have demonstrated that the interaction of the lung immune system and oxidative stress could be related to the development of several lung diseases. (MacNee and Rahman, 2001; Vallyathan et al., 1998; Zhang et al., 1999). The inhalation of fibrogenic particles activates alveolar phagocytic cells and increases superoxide anion production. According to MacNee and Rahman (2001) this increase leads to the inhibition of alfa-1-antitrypsin, an elastase inhibitor, and this imbalance could be important for the disruption of lung elastic recoil. In addition, activated macrophages attract polymorphonuclear cells, which increases alveolitis and promotes an increase in elastase activity, with the destruction of connective tissue (Lemaire and Quellet, 1996).

Several authors demonstrated that ROS such as superoxide anion, hydrogen peroxides and hydroxyl radicals are important mediators of silica-induced lung toxicity (Borges et al., 2001, Cho et al., 1999, Mosmann and Churg, 1998). The proinflammatory properties of ROS in the lung includes endothelial cell damage, formation of chemostatic factors, recruitment of neutrophils, lipid peroxidation and oxidation, DNA damage (Mossman and Churg, 1998; Zhang et al., 1999), release of TNF-α and IL-1β and formation of peroxynitrite (Blackford et al., 1997; Discroll et al., 1995; Dorger et al, 2002, Tsuda et al., 1997). ROS production could enhance lung damage induced by coal dust exposure leading to inactivation of antiproteases, basal membrane disruption, alveolar cell damage and lung fibrosis (Discroll et al., 1995; Tsuda et al., 1997; Wang et al., 1999). This is reinforced by the suggestion that bioavailable iron content in coal may be responsible for the observed regional

differences in the prevalence and severity of pneumoconiosis (Zhang and Huang, 2002).

There are several animal models in the literature used to elucidate mechanisms involved in the effects of silica and asbestos in lung damage, as well the role of ROS in this damage (Castranova et al., 2002; Dorger et al., 2002). However, there are no studies that demonstrate the differences between acute and late responses to a single exposure of coal dust. Thus, the main objective of this study is to determine the ability of coal dust in the participation of redox reactions that generate free radicals.

MATERIAL AND METHODS

Coal dust preparation: 1kg of gross coal was collected from Carboniferous Cooperminas located in the municipal district of Criciuma/Santa Catarina/Brazil. Samples of 300 grams were triturated in a mill of spheres for 3 hours, at a frequency of 25hz. The coal was analyzed by the Laboratory of Analyses of Soil and Fertilizers of the Soil and Fertilizer Analysis Laboratory at the Universidade do Extremo Sul Catarinense/Criciuma/SC/Br, presenting the following mineralogical characteristics: copper (0,003%), iron (2,480%), zinc (0,003%) and silica (27,3%). The coal dust used in the experiments presented diameter less than 15 μm.

***Coal dust exposure**: Fort*y-eight male Wistar rats (200-250g) were used in the experimental protocol. The animals were anesthetized with ketamin (80mg/kg of body weight, i.p.). Gross mineral coal dust (3mg/0.5ml saline) was administered directly in the lung by intratracheal instillation. This dose was determined in

dose response pilot studies (1mg, 2mg, 3mg and 5mg/0.5 ml saline), and we cold only find pulmonary alterations in doses above 3mg. Control animals received only saline solution 0,9% (0.5ml). The animals were separated into four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation. After each instillation period the animals were killed by decapitation and lungs were quickly removed, and stored at -80°C for oxidative stress analysis (n= 6 each group). In a separate cohort of animals lungs were fixed in formaldehyde for histological analysis (n=3 each group). All procedures were performed in accordance with the “Guiding Principles in the Care and uses of Animals” (Olert et al., 1993) and were approved by the local ethic committee.

Pathological analysis*:* Histologic assessment was performed in a blind fashion and judged by three independent observers to assure that the presented data are representative. We analyzed, under both low- and highpower fields, ten samples in each lobe.

Total radical-trapping antioxidant parameter (TRAP): TRAP measurement has been developed as previously described by Tsal et al. (2000). Briefly, the reaction was initiated by adding luminol and ABAP in a glycine buffer that resulted in a steady luminescence emission. The addition of organ homogenate (150μg of protein) decreases the luminescence to the same proportion as the sample concentration of non-enzymatic antioxidants. Luminescence was measured in a scintillation counter.

Tthiobarbituric acid reactive species (TBARS)**:** As an index of lipid peroxidation we used the formation of TBARS during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1ml of trichloroacetic acid 10% and 1ml of thiobarbituric acid 0.67%,

subsequently they were heated in a boiling water bath for 30 minutes. TBARS were determined by the absorbance at 532nm and were expressed as malondialdehyde equivalents (nm/mg protein).

Protein carbonyls: The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described by Levine et al. (1990). Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and reacted with DNPH. After the samples were redissolved in 6M guanidine hydrochloride, carbonyl contents were determined from the absorbance at 370nm using a molar absorption coefficient of 22,0000 Molar^{-1}.

Catalase (CAT) and superoxide dismutase (SOD) activity: In order to determine CAT activity, organ systems were sonicated in a 50mM phosphate buffer and the resulting suspension was centrifuged at 3000g for 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240nm (Aebi 1984). SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described (Bannister and Calaberese, 1987).

Protein Determination: The amount of proteins in the assays of catalase, SOD, TBARS and TRAP was assayed using the Lowry technique (Lowry et al., 1951) and protein carbonyl by Broadford assay (Braford, 1976).

Statistical Analysis: Data is expressed as mean and the statistical method was assessed by an analysis of variance (ANOVA), followed by Tukey post hoc test. Where applicable, an unpaired Student's test was used. The level of established significance used for all the statistical tests will be of $p < 0.05$. The

software used for analysis of the data was “Statistical Package for the Social Sciences (SPSS) version 10.0 for Windows”.

RESULTS

Histopathological analysis: 48 hours after coal dust exposure there is a markedly inflammatory infiltration with predominance of lymphocytes with lymphoid hyperplasia, and some neutrophils (data not shown) that remains until 7 days after induction (figure I). This initial response was followed by a chronic inflammatory infiltration characterized by aggregates of macrophages presents 30 days after induction (figure I). The presence of brown pigmentation in macrophages cytoplasm suggests coal dust phagocytosis. This inflammatory response tends to resolve 60 days after induction, similar to control animals (data not shown). This characterizes a biphasic inflammatory process. An initial phase of lymphoid infiltration with some neutrophil recruitment, followed by a second phase of macrophage response.

Total antioxidant capacity (TRAP): During both acute and chronic phase of lung inflammation we observed a decrease in the TRAP in the lung of coal dust exposed animals when compared to control animals. This indicates a consumption of non-enzymatic antioxidant defenses (figure II). The addition of exogenous coal dust in control lungs did not alter TRAP values (data not shown).

Antioxidant enzymes activities: pulmonary antioxidant defenses are widely distributed and include both enzymatic and nonenzymatic systems. SOD and catalase are one of the major enzymatic antioxidant defenses in lung tissue. These defenses are generally up regulated in the presence of oxidative stress. We observed significant variations only in SOD activity 60 days after coal exposure. This could be related to the occurrence of oxidative damage demonstrated above (figure IIIa and IIIb).

Oxidative damage parameters: we evaluated the formation of TBARS and protein carbonylation as an index of oxidative damage. Despite the cessation of inflammatory response, TBARS were increased 60 days after coal dust exposure in relation to control. This suggests a time-dependent relation to oxidative stress and coal dust exposure (figure IVa). In contrast, protein carbonyl groups increased every time after coal dust exposure (48 hours, 7days, 30 days and 60 days) when compared to the control group (figure IVb). These results indicated that acute coal dust exposure induces chronic oxidative damage in rat lung, and to some degree this is independent of inflammatory response.

DISCUSSION

The objective of this study was to evaluate the possible involvement of ROS in acute and later coal dust effects. Earlier studies have reported a toxic effect of inorganic dusts such silica and asbestos and their relation to ROS.

The mechanisms involved in the development of pulmonary damage after coal dust inhalation is not well defined. Silica is a ubiquitous occupational fibrogenic agent, present in coal dust in variable proportions, capable of inducing inflammation, release of proteases, and eventual lung scarring (Parkes, 1984, Castranova and Vallyathan, 2000; Castranova et al., 2002; Ding et al., 2002). Mossman (2003) suggested that the lung is a primary target for inhaled oxidants which generate naturally after inhalation of environmental pollutants, and lung inflammation is the source of reactive oxygen species.

In a recent study, chronic environmental silica exposure induces pulmonary damage, inflammation and alveolar type II epithelial cell activity rapidly increasing to a significantly elevated but stable new level for the first 41 days of exposure and increased at a steep rate thereafter (Castranova et al. 2002). In this work we have demonstrated a two-step inflammatory response to acute coal dust (figure I). In an acute phase we observed a lymphocyte infiltration and some neutrophil recruitment followed by a chronic phase of macrophage predominance. There are several factors that are thought to modify wound healing and, ultimately, the degree of parenchyma fibrosis. First, the type of inflammatory response may modulate tissue injury, fibrosis, or both during the evolution of pulmonary fibrosis. Our results suggest that inflammatory response in fibrosis secondary to coal dust is thought to closely resemble a Th2-type immune response. In murine models of lung disease, animals whose response to tissue injury is predominantly of the Th2 type are more prone to pulmonary fibrosis after lung injury than those with a predominantly Th1 response (Gross and Hunninghake. 2001). We expected a more prominent

neutrophil infiltration after coal dust exposure but we are not able to detect this. Further studies will address this unexpected finding.

We hypothesized that repeated episodes of acute lung injury induce by coal dust could ultimately leads to fibrosis, with loss of lung function. We cannot ascertain if the lymphocyte infiltration of a Th2 type, but further studies will address this issue. This lymphocyte initial response could induce a chronic phase of macrophage activation that could initiate the fibrotic response.

We have demonstrated that acute exposure to coal dust could induce a long-term oxidative stress. This sustained response could be associated with lung inflammation, and lung fibrosis. The increase in the amount of phagocytes in the lung, induced by coal dust exposure causes local production of $O_2^{\bullet -}$ which can result in H_2O_2 formation. In the presence of iron the H_2O_2 are converted to the hydroxyl radical by the reaction of Fenton (Vallyathan et al., 1998). According to Kim et al. (2000) surface iron on a mineral particle may be a major mediator of mineral-dust-induced toxicity, because iron on the surface of the particle acts as a Fenton catalyst to produce hydroxyl radical from H_2O_2. In addition, $O_2^{\bullet -}$ is an inhibitor of alfa-1-antripsina, an important glycoprotein which it inhibits the action of several proteases, including elastase. This induces an imbalance of proteases and antiproteases that can lead to destruction of tissue and lung emphysema (Olszewer, 1999).

Current evidence suggests a central role for alveolar macrophages in lung fibrosis. Alveolar macrophages are a potential source of ROS (Zhang et al., 1999) and could contribute to lung damage after coal dust exposure. Coal dust particles can be phagocytosed by pulmonary macrophages, and activated macrophages can initiate a cascade of ROS production that could lead to cell

damage. Several mineral dust fibers are capable of activating the respiratory burst of phagocytes (Mossman and Churg, 1998). In addition, coal dust has been shown to contain iron that can stimulate hydroxyl radical formation, lipid peroxidation and oxidative DNA damage. In this way, inhalation of coal dust for prolonged periods can cause lung fibrosis.

Lung inflammation and consequent fibrosis are the two main pathological events verified after the silica exposure, although the mechanisms of this process are still not well understood (Zhang et al. 1999). According to Churg (1996) the amount of inflammatory cells can increase the level of oxidants spontaneously during the evolution of the inflammatory process. Recently the role of oxidative stress in lung damage after the silica exposure and other fibrogenics substances has been established (Borges et al., 2001; Castranova et al., 2002; Cho et al., 1999; Vallyathan et al., 1998). The activation of immune system in response to silica and other dust fybrogenic, the production of ROS leads to an oxidative explosion when the fibers are phagocytized for macrophages or other epithelials alveolar cells and fibroblasts.

According to Mossman (2003) lung phagocytes such as alveolar and interstitial macrophages are classically regarded as effector cells of oxidative stress because of their capacity to metabolize many xenobiotics and generate ROS through an oxidative response.

In order to access the non-enzymatic antioxidant status after acute exposure to the coal dust the TRAP assay was performed. TRAP has provided a sensitive tool to quantify combined non-enzymatic antioxidant capacity of plasma or tissues. Among those nonenzymatic antioxidants, it is well known that glutathione plays an important role in the defense mechanism of the lung

(Zhang et al., 1999). We observed that the animals exposed to coal dust exhaust the system earlier in relation to the group control (figure II). Previous studies have shown the involvement of the glutathione as nonenzymatic defense depends on the level it meets the lesion. Ghio et al (1994) showed that the glutathione decrease in lung silicotic tissues in rats at the early stage and Yamano et al. (1995) showed an increase at the late stage. In another study, Smith et al. (1994) and Zhang et al. (1999) suggest that cellular glutathione level may also be a source of oxidant protection against inflammation and inhalation of mineral dusts, respectively.

The unaltered activity of antioxidants enzyme after exposure to coal dust (figure IIIa and IIIb) suggests that the enzymatic system is not the principle cause for lung oxidative defense. However, Mossman et al. (1999) demonstrated that catalase administration reduces both inflammation and fibrosis in a silicosis animal model. Based on this, we believe that the increase in SOD activity 60 days after coal dust exposure could reflect an adaptation against oxidative stress. Probably, during coal dust exposure the inflammatory cells produce products superoxide which are known as the major inductor of SOD activation.

Silica is generally considered a dominant factor in coal mine dust's cytotoxic and fibrogenic potential. Silica-free coal dust could also exhibit oxygen-radical generating potential we don't know. According to Dalal et al. (1995) it has proposed that fibrogenic dust exerts its toxic effects through oxygen-radical initiated chain reactions leading to lipid peroxidation. Free radical trigger lipid peroxidation chain reactions by abstracting a hydrogen atom from a side-chain methylene carbon which results in carbon-centered lipid

radical. This reacts with O_2 to give a peroxyl radical that subsequently propagates a chain reaction, which transforms polyunsaturated fatty acids (Halliwel, 1998).

We also determined lipid peroxidation as measurement of cellular damage induced by coal dust. The results show that after 60 days of exposure to coal dust the rats induced an increase in TBARS levels (figure IVa) in relation to the control group. The presence of iron in the coal dust sample is a measure of the potential of the dust for cellular injury through hydroxyl radical generation. According to Dalal et al. (1995) the content of lipid peroxidation induced by coal dust has a fairly good correlation with the amount of surface iron. In a recent study Zhang and Huang (2002) analyzed twenty-nine coal samples from three coal mine regions and were tested in the human lung epithelial Type II A549 cells. The results suggest that low molecular weight chelators bound iron, a fraction of bioavailable iron in the cells released from coals, ferritin, and lipid peroxidation were significantly higher in cells treated with various coals than in control cells, this was parallel to the prevalence of coal workers' pneumoconiosis in these coal mine regions.

Oxygen radical can modify amino acid side chain from protein aggregates, cleave peptide bands and make proteins more susceptible to proteolytic degradation. In the process, some carboxyl residues are converted to carbonyl derivates (Halliwel 1998). The results (figure IVb) show an increased protein carbonilation level, suggesting that the exposure to coal dust also damage proteins, producing carbonylated proteins.

To summarize, the present study evaluated the role of coal dust as an inductor of oxidative damage in the lung. It is believed that acute coal dust

exposure may lead to chronic lung oxidative damage. This indicates that the use of antioxidants could have prophylactic and therapeutic value, but further studies will address this issue.

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Figures

Figure I

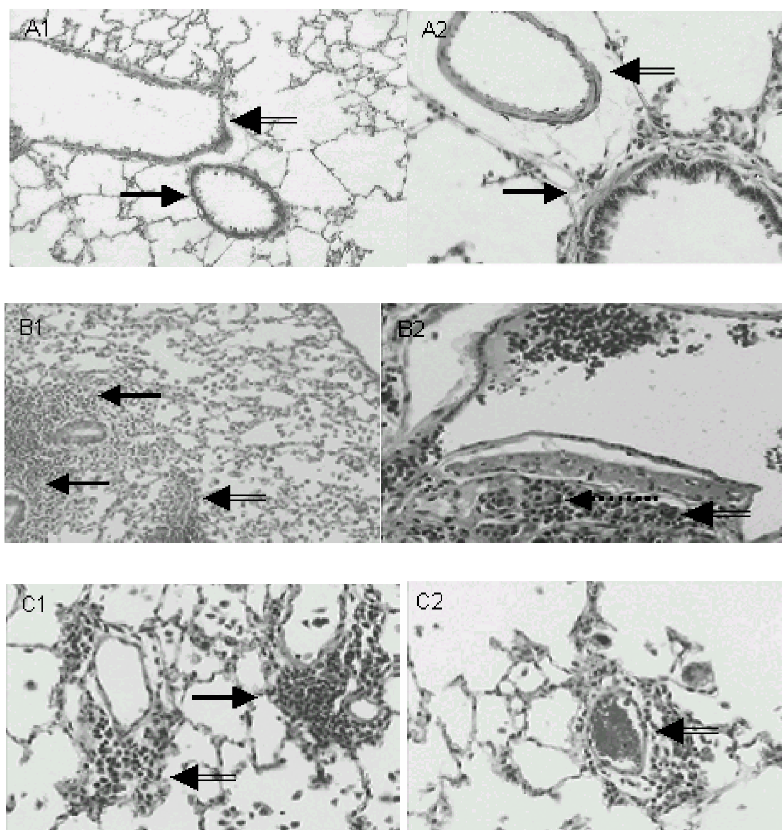


Figure II

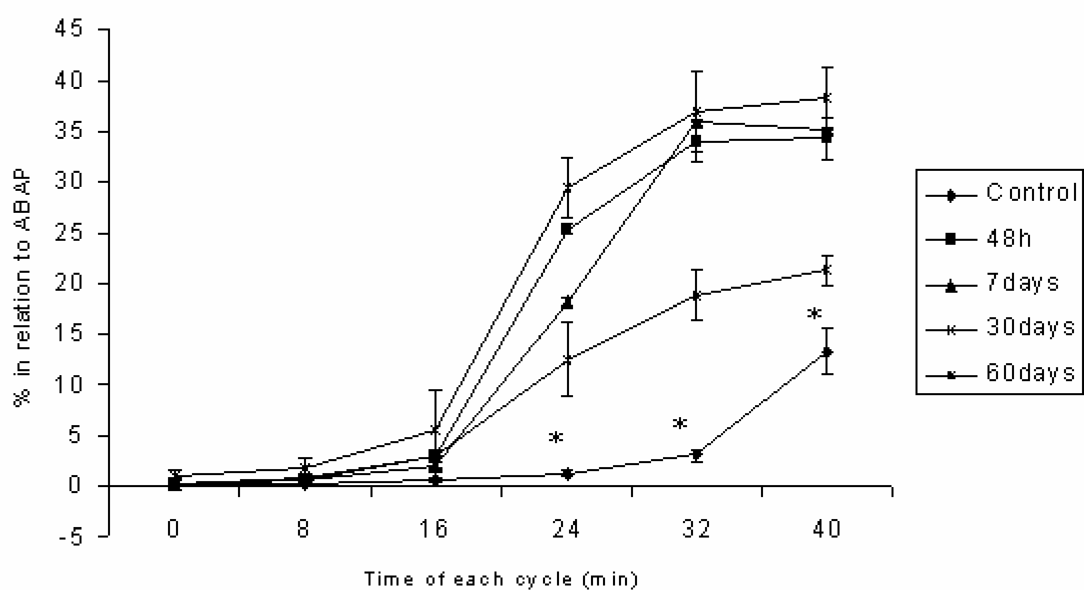


Figure IIIA

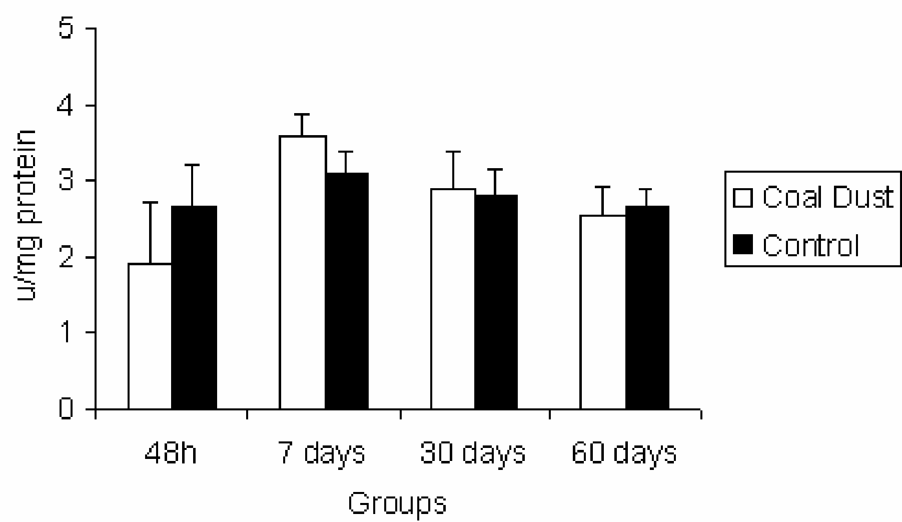


Figure IIIB

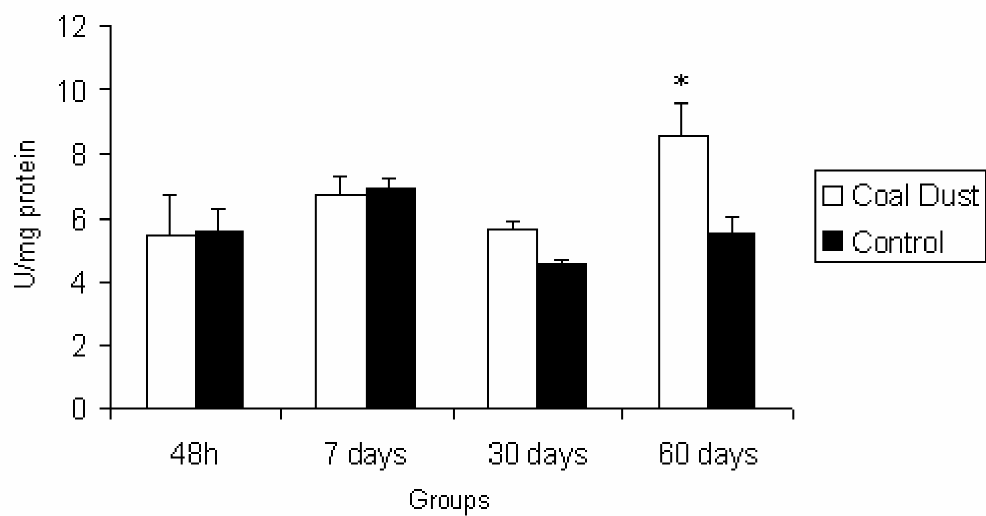


Figure IVA

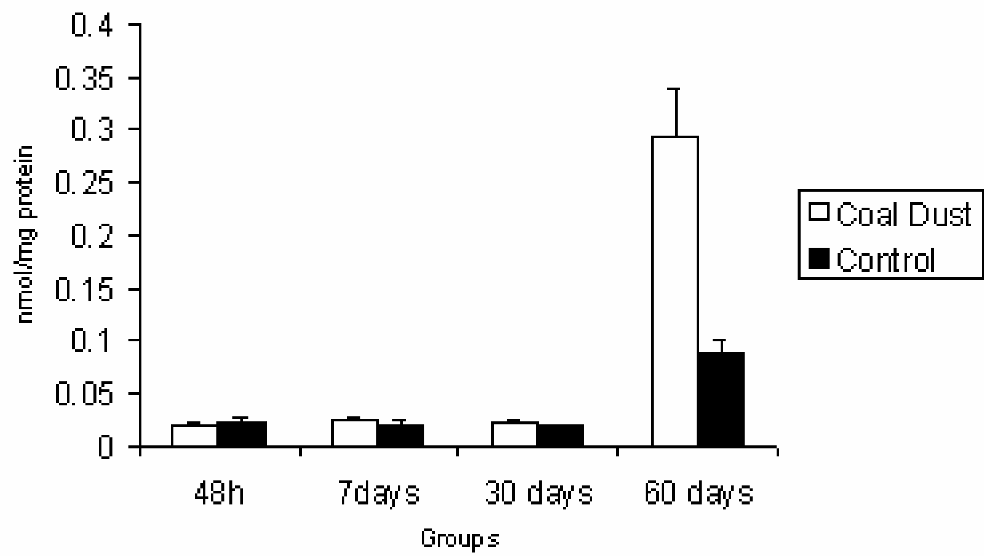
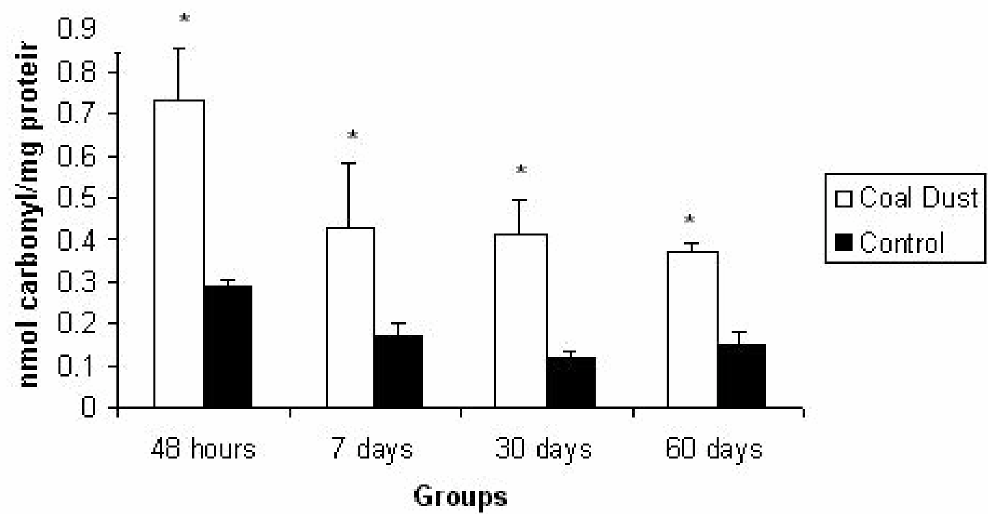


Figure IVB



Legends

Figure I: Morphologic alterations by the accumulation of coal dust in lung tissue of rats after acute exposure (7 days and 30 days). Gross mineral coal dust (3mg/0,5ml saline) was administered directly in the lung by intratracheal instillation. Control animals received only saline solution 0,9% (0,5 ml). Cuts were made in sheets of 4μm and stained with hematoxiline-eosine.
A1 and A2: Control: peribronchial tissue (black arrow), perivascular tissue (double black arrow) and interstitials septs without important alterations (200X, 400X - HE).
B1 and B2: 7 days: peribronchial (black arrow) and perivascular (double black arrow) inflammatory infiltrated and macrophages in the alveolar light, phagocyting brown material (dotted arrow) (100X, 200X - HE).
C1 and C2: 30 days: inflammatory infiltrated perivascular (black arrow) and intra-alveolar giant cell (dotted black arrow) with presence of macrophages (double black arrow). (200X - HE).

Figure II: Total antioxidant capacities of the lung of rats after dust coal acute exposure were determinate according material and methods. The results were expressed of ABAP during a time for each cycle. Animals (*Fort*y-eight male Wistar rats-200-250g) were separated in four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation, with controls respective. The control groups no show level of significance different (data not shown). To compare the data was used the group it controls of 48 hours as representative group of the others. Gross mineral coal dust (3mg/0,5ml saline) was administered directly in the lung by intratracheal instillation. Control animals received only saline solution 0,9% (0,5 ml). A significance level of $^*p<0,05$ was considered, in relation to groups.

Figure IIIA: Catalase activity in lung of rats after coal dust acute exposure was determinated according material and methods. The results were expressed in unit per milligram of protein according time of exposure. Animals (*Fort*y-eight male Wistar rats-200-250g) were separated in four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation, with controls respective. Gross mineral coal dust (3mg/0,5ml saline) was administered directly in the lung by intratracheal instillation. Control animals received only saline solution 0,9% (0,5 ml). A significance level of $^*p<0,05$ was considered, in relation control.

Figure IIIB: SOD activity in lung of rats after coal dust acute exposure was determinated according material and methods. The results were expressed in unit per milligram of protein according time of exposure. Animals (*Fort*y-eight male Wistar rats-200-250g) were separated in four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation, with controls respective. Gross mineral coal dust (3mg/0,5ml saline) was administered directly in the lung by intratracheal instillation. Control animals received only saline solution 0,9% (0,5 ml). A significance level of $^*p<0,05$ was considered, in relation control.

Figure IVA: Lipid peroxidation in lung of rats after coal dust acute exposure was determinated according material and methods. Results were expressed in nmol

of TBARS per milligram of protein according time of exposure. Animals (*Fort*y-eight male Wistar rats-200-250g) were separated in four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation, with controls respective. Gross mineral coal dust (31mg/0,5ml saline) was administered directly in the lung by intratracheal instillation. Control animals received only saline solution 0,9% (0,5 ml). A significance level of *p<0,05 was considered, in relation control.

Figure IVB: Protein carbonilation in lung of rats after coal dust acute exposure was determinated according material and methods. Results were expressed in nmol of Carbonyl per milligram of protein according time of exposure. Animals (*Fort*y-eight male Wistar rats-200-250g) were separated in four groups: 48 hours, 7 days, 30 days and 60 days after coal dust instillation, with controls respective. Gross mineral coal dust (31mg/0,5ml saline) was administered directly in the lung by intratracheal instillation. Control animals received only saline solution 0,9% (0,5 ml). A significance level of *p<0,05 was considered, in relation control.

CAPÍTULO III

COMBINATION N-ACETYLCYSTEINE AND DEFEROXAMINE REDUCE PULMONARY OXIDATIVE STRESS AND INFLAMMATION IN RATS AFTER COAL DUST EXPOSURE

Ricardo A. Pinho[1e2], Paulo C. L. Silveira[2], Luciano A. Silva[2], Felipe Dal-Pizzol[1e3], José C. F. Moreira[1]

[1]Centro de Estudos em Estresse Oxidativo/Departamento de Bioquímica/UFRGS
[2]Laboratório de Fisiologia e Bioquímica do Exercício//UNESC
[3]Laboratório de Fisiopatologia Experimental/UNESC

Submetido a Environmental Research

ABSTRACT

Coal dust inhalation induces oxidative damage and inflammatory infiltration on lung parenchyma. Thus, the aim of this study was to determine whether N-acetylcysteine (NAC) administered alone or in combination with deferoxamine (DFX) significantly reduced the inflammatory infiltration and oxidative damage in the lungs of rats exposed to coal dust. Forty-two male Wistar rats (200-250mg) were exposed to the coal dust (3mg/0,5ml saline, 3days/week, during 3 weeks) by intratracheal instillation. The animals were randomly divided into three groups: saline 0.9% (n=8), supplemented with NAC (20mg/kg of body weight/day, i.p) (n=8) and supplemented with NAC (20mg/kg of body weight/day, i.p) plus DFX (20mg/kg of body weight/week) (n=8). Control animals received only saline solution (0,5ml). Lactate dehidrogenase activity and total cell number were determinated in the bronchoalveolar lavage fluid (BALF). In the lung parenchyma were determined lipid peroxidation, oxidative protein damage, catalase and superoxide dismutase activity was measured by the rate of decrease in hydrogen peroxide and superoxide dismutase activities. As expected the intratracheal instillation of coal dust in the lung of rats leads to an inflammatory response and induces significant oxidative damage. The administration of NAC alone or in association with DFX reduced to a similar degree the inflammatory response and the oxidative stress in rats exposed to coal dust.

Key-words: oxidative stress, free radicals, lung, coal dust, NAC, supplementation

INTRODUCTION

A number of studies have shown that the parameters of oxidative damage to pulmonary function are altered following inhalation of industrial particles like asbestos (Dörger et al., 2002), silica (Castranova et al., 2002) and coal (Pinho et al., 2004). However, few have reported the effects of the supplementation/addition of antioxidants on these alterations, more specifically, on the oxidative stress markers induced by acute exposure to coal. According to Mossman (2003), following the inhalation of atmospheric pollutants, the lungs are the first targets affected by the production of oxidants during the combustion of oxygen. Tao et al. (2003) suggested that this biochemical response is mediated by the activation of macrophages and the recruitment of polymorphonuclear cells, which induce an increase in inflammatory mediators and the formation of reactive oxygen species (ROS).

Reactive oxygen species like superoxide anion and hydrogen peroxide are capable of causing tissue damage and inflammation. These species are normally produced in the organism during cellular metabolism, though when the production is excessive it may lead to molecular damage or cellular death by reacting with cellular components including nucleic acids, proteins and membrane lipids. This process, known as oxidative stress, may be reversed by an efficient defence system with the activation of antioxidant enzymes, endogenous antioxidants or by antioxidant supplementation.

Several studies (Castranova et al., 2002, Cho et al., 1999, Shukla et al., 2003, Zhang et al., 1999) have shown the direct relation between the inhalation

of industrial particles and the development of various pulmonary diseases, mediated by inflammatory processes and the excessive production of ROS.

N-acetylcysteine (NAC) is considered an important therapeutic agent and is commonly used in clinical practice as it presents properties capable of maintaining the lungs oxidant capacity. NAC is a thiol donor that acts as an intracellular cysteine precursor, increasing the production of glutathione (GSH). It is suggested that the potential effect of NAC reduces H_2O_2, altering the pulmonary oxidant-antioxidant balance (Repine at al, 1997).

We hypothesise that the accumulation of hydrogen peroxide in the presence of iron during the inflammatory process induced by the acute exposure to coal may lead to the formation of the hydroxyl radical. Furthermore, the use of NAC alone may have limitations and present pro-oxidant effects, due to the facility with which it interacts with iron (Ritter et al., 2004). Given this, the use of deferoxamine (DFX), an iron chelate, may improve response to the use of NAC. Thus, the main aim of this study was to verify whether NAC administered alone or in combination with DFX significantly reduced the inflammatory indicators and oxidative damage in the lungs of rats exposed to coal dust.

MATERIAL AND METHODS

Coal dust preparation: 1kg of gross coal was collected from Carboniferous Cooperminas located in the municipal district of Criciuma/Santa Catarina/Brazil. Samples of 300 grams were triturated in a mill of spheres for 3 hours, at a frequency of 25hz. The coal was analyzed by the Laboratory of Analyses of Soil

and Fertilizers of the Soil and Fertilizer Analysis Laboratory at the Universidade do Extremo Sul Catarinense/Criciuma/SC/Br, presenting the following mineralogical characteristics: copper (0,003%), iron (2,480%), zinc (0,003%) and silica (27,3%). The coal dust used in the experiments presented diameter less than 15 μm.

Coal dust instillation: *Fort*y-two male Wistar rats (200-250g) were used in the experimental protocol. The animals were kept on a 12-h light/dark cycle in a conventional, nonbarrier rodent housing unit in polycarbonate cages. Tap water and standard rodent laboratory diet were supplied *ad libitum*. The animals were anesthetized with ketamin (80mg/kg of body weight, i.p) and xylazine (20mg/kg de body weight, i.p). Gross mineral coal dust (3mg/0.5ml saline) or only saline solution 0,9% (0.5ml) was administered directly in the lung by intratracheal instillation (3days/week, during 3 weeks), adapted at Pinho et al. (2004). The animals were randomly divided into tree groups with controls respective: saline (n=8) e control (n-6), supplemented with NAC (20mg/kg of body weight/day, i.p) (n=8) and control (n=6) and supplemented with NAC (20mg/kg of body weight/day, i.p) and DFX (20mg/kg of body weight/week) (n=8) and control (n=6).

Bronchoalveolar lavage (BAL): Thirty days after the first instillation, the animals were anesthetized with a ketamine/xylazine overdose. Bronchoalveolar lavage was conducted as published previously Dörger *et al., (*1997). Briefly, after cannulation of the trachea, the thorax was opened, and the lungs of rats were lavaged with ten, 5-ml aliquots of PBS. Pooled samples were centrifuged at 300x*g* for 10 min, and the cell pellets were washed twice and resuspended.

After BAL, the animals were killed by decapitation and lungs were quickly removed, and stored at -80°C for oxidative stress analysis.

All procedures were performed in accordance with the “Guiding Principles in the Care and uses of Animals” (Olert et al., 1993) and were approved by the local ethic committee.

***Cell number total**:* A total cell number presence in BAL was obtained with a hemacytometer Chamber, immediately after the obtaining of the sample. BAL cells were adjusted to a concentration of $5x10^5$ cells/ml in PBS.

***Lactate dehidrogenase activity (LDH)**:* LDH was determinate in BAL with aid of specific kit obtained by Labtest Diagnóstica SA, Brazil. The reading was made starting from enzymatic system with kinetic method, according to the technical orientations observed in the bull of the referred kit.

Catalase (CAT) and superoxide dismutase (SOD) activity: In order to determine CAT activity, organ systems were sonicated in a 50mM phosphate buffer and the resulting suspension was centrifuged at 3000g for 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240nm (Aebi 1984). SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, absorbance at 480nm as previously described (Bannister and Calaberese, 1987).

***Thiobarbituric acid reactive species (TBARS)*:** As an index of lipid peroxidation we used the formation of TBARS during an acid-heating reaction as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1ml of trichloroacetic acid 10% and 1ml of thiobarbituric acid 0.67%, subsequently they were heated in a boiling water bath for 30 minutes. TBARS

were determined by the absorbance at 532nm and were expressed as malondialdehyde equivalents (nm/mg protein).

Protein carbonyls: The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described by Levine et al., (1990). Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and reacted with DNPH. After the samples were redissolved in 6M guanidine hydrochloride, carbonyl contents were determined from the absorbance at 370nm using a molar absorption coefficient of 22.0000 $Molar^{-1}$.

Protein Determination: The amount of proteins in BAL and in the assays of catalase, SOD, TBARS and protein carbonyl was assayed using the Lowry technique (Lowry et al., 1951).

Statistical Analysis: Data is expressed as mean and the statistical method was assessed by an analysis of variance (ANOVA), followed by Tukey post hoc test. The level of established significance used for all the statistical tests will be of $p <0.05$. The software used for analysis of the data was “Statistical Package for the Social Sciences (SPSS) version 10.0 for Windows”.

Reagents: Thiobarbituric acid, CAT, SOD, dinitrophenylhidrazine, adrenaline, hydrogen peroxide were purchased from Sigma Chemical (St. Louis, MO). 2,2'-azobis (2-methylpropionamidine) dihydrochloride was purchased from Aldrich Chemical (Milwaukee, WI). NAC was purchased from Zambon Labotatórios Farmacêuticos (Brazil). DFX was purchased from Novartis (Brazil).

RESULTS

Total cell number and total protein concentration: BAL was performed in order to verify the cellular infiltration into the alveolar spaces following tracheal coal instillation and daily supplementation of NAC and NAC+DFX. Table 1 shows the existence of significant differences in the total quantity of cells in the supplemented groups in relation to the saline group (Saline = 4.83 ± 0.51 x10^5; NAC = 3.94 ± 0.31 x10^5; NAC+DFX = 2.9 ± 0.33 x$10^{5,}$ p<0.05*). The total protein concentration in the fluid obtained from the LBA was chosen as an indicator of hyperpermeability induced by exposure to coal. Table 1 shows that only the protein concentration in the group treated with NAC+DFX presented values significantly lower than those from the saline group (saline = 405.13 ± 37.46μg/ml; NAC+DFX = 226.35 ± 24.17μg/ml, p<0.05*).

Lactate dehydrogenase activity (LDH): LDH activity in the fluid obtained from the LBA following coal instillation and daily supplementation of NAC and NAC+DFX or 0.9% saline solution was determined as the cell membrane integrity indicator. According to figure I, the results show significant differences in LDH activity in the groups supplemented with NAC and NAC+DFX in relation to the group that received only saline solution (saline = 185.96 ± 26.38 U/ml; NAC = 100.76 ± 15.03 U/ml; NAC+DFX = 80.99 ± 6.82 U/ml, p<0.05*). The presence of coal on the lung tissue increases the LDH activity of the saline group when compared to the control group (saline = 185.96 ± 26.38 U/L; control 73.99 ± 5.86 U/L, $p<0.05^{\#}$).

Oxidative damage parameters: TBARS formation and protein carbonylation were evaluated in order to obtain the index of oxidative damage resulting from exposure to coal. The results show, according to figure IIA, a increased in the values of TBARS in the presence of coal on the lung tissue (coal = 0.649 ± 0.107 nmol/mg protein; control = 0.328 ± 0.082 nmol/mg protein, $p<0.05^{\#}$) and a significant reduction is seen in the values of TBARS following supplementation of NAC and NAC+DFX in comparison to the saline group (saline = 0.60 ± 0.13 nmol/mg protein; NAC = 0.36 ± 0.058 nmol/mg protein; NAC+DFX = 0.28 ± 0.04 nmol/mg protein, p<0,05*).

In relation to the oxidative damage in proteins, the results show, according to figure IIB, that the exposure to coal induces significant carbonylation in proteins (coal = 0.195 ± 0.042 nmol/mg protein; control = 0.103 ± 0,029 nmol/mg protein, $p<0.05^{\#}$), although this damage is diminished significantly with NAC supplementation (saline = 0.20 ± 0.049; NAC = 0.10 ± 0.017, p<0.05*). This is not seen when NAC is used in combination with DFX.

SOD and Catalase activity: The pulmonary antioxidant defences include enzymatic and non-enzymatic systems. SOD and catalase are the most important antioxidant enzymes responsible for the oxidative balance in the lungs, which are generally regulated by oxidative stress. According to figure IIIA, the presence of coal on the lung tissue increases the SOD activity of the saline group when compared to the control group (saline = 24.33 ± 1.58 U/mg protein; control 13.67 ± 0.938 U/mg protein, $p<0.05^{\#}$). The results show a significant difference in SOD activity in the groups supplemented with NAC and NAC+DFX in relation to the group that received only saline solution (saline =

24.33 ± 1.58 U/mg protein; NAC = 14.39 ± 0.866 U/mg protein; NAC+DFX = 11.10 ± 0.719 U/mg protein, $p<0.05^*$).

The figure IIIB, show a significant increase in the catalase activity following exposure to coal compared to the control group (saline = 4.13 U/mg protein ± 0.232; control = 2.44 ± 0.191 U/mg protein, $p<0.05^{\#}$) although these values were reduced with NAC and NAC+DFX supplementation (NAC = 2.90 units/mg protein ± 0.223; NAC+DFX = 2.74 units/mg protein ± 0.102, $p<0.05^*$).

DISCUSSION

While the physiological mechanisms of lesions from the inhalation of industrial particles may not be well established, evidence demonstrates that the pulmonary response to occupational and atmospheric pollutants, like coal, provokes a chain inflammatory response, resulting in an increase in pro-inflammatory factors, extracellular matrix synthesis and fibroblasts proliferation (Blackford et al., 1997; Vallyathan et al., 2000; Saldiva et al., 2002; Tao et al., 2003). However, several authors have suggested that the use of antioxidants reduces the inflammatory response and the effects generated by the ERO in animals (Repine at al, 1997; Sprong et al., 1998; Thiemermann, 2003; Ritter et al., 2004).

As indicators of the inflammatory response and the alteration in the permeability of the pulmonary epithelium, the total number of cells and proteins in the lavage bronchial alveolar (BAL) of rats following instillation of coal dust and concomitant supplementation of NAC and DFX was verified. In table I, it can be seen that the animals that received supplement of NAC or NAC+DFX

showed a significant reduction in these indicators when compared to the saline group. Although we have not characterized the cellular types, it is suggested, based upon evidence present in other studies (Discroll et al., 1995; Vallyathan et al., 2000; Dörger, et al., 2002), that this significant increase in the total number of cells and proteins present in BAL from rats exposed to coal may result from the increase in the number of polymorphonuclear leukocytes, mainly lymphocytes and neutrophils.

According to MacNee et al., (1993), the first phase in the recruitment of neutrophils in the air spaces is the uptake of these cells by the pulmonary microcirculation. This permits the adherence of the neutrophils to the capillary endothelium and the migration through the alveolar membrane to the intersticium and air spaces of the lungs. While passing through the pulmonary microcirculation the neutrophils may be activated by mediated like cytokines, alveolary macrophages and by endothelial and epithelial cells (Tao et al., 2003).

In recent research carried out by our group (Pinho et al., 2004), we showed the two possible phases characterise the inflammatory response in the lungs following acute exposure to coal. A phase marked by lymphocyte infiltration and neutrophil influx and another characterised by the predominance of alveolar macrophages.

Although the high protein levels found in the BAL already suggested greater cell membrane permeability and integrity, it was decided to assess the LDH activity. As seen in figure I, the group exposed only to coal, presented a significant increase LDH in comparison with the control. Moreover, the results show that the LDH activity in the groups supplemented with NAC and NAC+DFX, was significantly reduced. The data indicate that exposure to coal

dust provokes alveolar lesion in the lungs of rats. Blackford et al., (1997) suggests that one of the effects on the lung tissue following the instillation of coal is the breaking of the membranes, which. In the presence of ROS, facilitates an acute inflammatory response (Discroll et al., 1995).

The significant reduction of inflammatory markers and cellular permeability following supplementation of NAC and DFX may be directly related to the antioxidant properties attributed to NAC. Blackwell et al., (1996) suggest that the mechanism that reduces the inflammatory process with the use of NAC includes the inhibition of the production of ROS and the decrease of both NF-kB activation and the expression of pro-inflammatory cytokines.

This effect is important in guaranteeing the enhanced pulmonary integrity, in the failure of which the production of oxidants may increase the inflammatory response by the inactivation of antiproteases, rupturing membranes, damaging alveolars and fibroses (Discroll et al., 1995; Tsuda et al., 1997; Wang et al., 1999). Recently, in vitro experiments have shown that thiol antioxidants like NAC block epithelial and macrophage cell inflammatory mediators through a mechanism involving synthesis of GSH and the reduction of NF-kB activation (Parmentier et al., 1999, Rhoden et al., 2004).

Besides these effects, the pulmonary inflammatory response associated with the excessive production of ROS leads to endothelial lesions, neutrophil recruitment, lipid peroxidation, enzyme inactivation, DNA damage (Mossman and Churg, 1998; Zhang et al., 1999, Vallyathan et al., 2000), production of TNF-α and interleukin-1β and peroxynitrite formation (Blackford et al., 1997; Discroll et al., 1995; Dörger et al, 2002, Tsuda et al., 1997). Zhang and Huang (2002) suggested that these alterations may be augmented by the iron content

present in industrial dusts. Nevertheless, it is possible that the administration of NAC alone may contribute to the production and release of other oxidative mediators, probably due to the facility with which it integrates with iron (Ritter et al., 2004); thus, the use of DFX can contribute to assuaging these effects.

The oxidative capacity of industrial particles is primarily attributed to the presence among its constituents of transition metals that include preferentially Fe, Cu, Zn, Mn among others. Some of these metals can catalyse Fenton Chemistry and produce ROS (Tao et al., 2003).

The ROS also stimulate the release of alveolar macrophages and polymorphonuclear leukocytes during phagocytosis of coal dust and other inorganic minerals and it has been proposed that the production of ROS by mineral particles is generally increased, depending on the redox property and the iron concentration in these particles (Dalal et al., 1995). These ROS production factors may be greater than the antioxidative defence capacity and lead to oxidative stress.

The increased in the quantity of phagocytes in the lung, induced by the exposure to coal, cause local production of superoxide anion ($O_2^{\bullet}$-) and hydrogen peroxide (H_2O_2). In the presence of iron, the H_2O_2 is converted into the radical hydroxyl by the Fenton Reaction (Vallyathan et al., 1998). According to Kim et al., (2000) the quantity of iron in the mineral particles may be the main mediator of toxicity and of oxidative damage in the lungs. Dalal et al., (1995), suggest that it is possible to diminish the oxidative effects produced by the exposure to mineral particles with the use of iron chelates such as DFX, though chelates such as EDTA or DETAPAC, can produce the opposite effect,

stimulating the production of the radical hydroxyl (Halliwell and Gutteridge, 1999).

DFX is widely employed in the treatment of several haematological diseases as it permits stability in the iron levels. As iron is essential for normal cell function, continuous and prolonged administration of DFX may cause have harmful effects on the organism (Halliwell and Gutteridge, 1999).

As shown in a previous study (Pinho et al., 2004), exposure to coal dust through tracheal instillation leads to a significant increase in the oxidative stress parameters through the carbonylation of proteins and peroxidation of lipids. In the present study, our results show that the administration of NAC in isolation and in combination with DFX significantly reduce these parameters (Figure IIA and IIB). Furthermore, the use of NAC and DFX also re-established balanced activity of the CAT (Figure IIIA) and SOD (Figure IIIB). Thus, it can be seen that the result obtained with the use of DFX were not different from the results attained with the use of NAC alone, except with the oxidation of proteins. It suggests that this result is due to the effective action of CAT and of other antioxidant components like GSH.

The catalytic activity of SOD results in the formation of hydrogen peroxide, that, in the presence of transition metals such as iron, through the Fenton Chemistry, form radical hydroxyl that reacts quickly with biomolecules provoking significant damage to the cell membrane through lipid peroxidation. The reaction of hydroxyl with biomolecules can result in the formation of other less reactive radicals and attack other specific biomolecules (Vallyathan et al., 1998). The CAT can clean the excess H_2O_2, reducing the oxidative effect,

though imbalance in the activity of both enzymes, CAT and SOD, can lead to oxidative stress.

According to Heunks and Dekhuijzen (2000), two possible mechanisms have been proposed to account for the antioxidant properties of NAC. Firstly, the NAC may act as a free radical “scavenger”. ROS can react with the NAC, resulting in the formation of disulphide NAC. The importance of this in living beings is questionable due to the low bioavailability of the NAC present in the organism. However, this effect may be accentuated with intravenous administration or inhalation. It is supposed the latter mechanism is related to the results presented in our study. Second, The NAC can play an important antioxidant effect as it facilitates the biosynthesis of glutathione (GSH).

It is probable that the NAC may attenuate the oxidative effects produced by exposure to coal and consequently favour the balance between the activities of CAT and SOD and may be related to precursor property of the intracellular cysteine, indispensable for GSH synthesis (Sprong et al., 1998; Paterson et al., 2003; Ritter et al., 2004). GSH, in its reduced form, plays an important role in pulmonary defence mechanism against attacks from free radicals (Smith et al., 1994; Zhang et al., 1999) diminishing the H_2O_2 content, altering the balance of the pulmonary oxidant-antioxidant capacity (Repine at al., 1997). Depending on the level in the cell, it may represent an important resource against the inflammatory responses caused by industrial particles (Zhang et al., 1999).

The intracellular level of GSH is regulated by the balance between its utilisation and synthesis. Although GSH is essential for the normal functioning of the cell, many organs do not re-synthesise it and, therefore, need to import extracellular sources (Deneke and Fanburg, 1989).

Rhoden et al. (2004) studied the effects of pre-administration of NAC on the inflammatory response in the lungs and oxidative stress after exposure to concentrated environmental particles. The results show that the production of ROS is dependent on the type and quantity of inhaled particles and that these particles may cause an increase in oxidised lipids and proteins.

In summary, the results suggest that the intratracheal instillation of coal in the lung of rats leads to an inflammatory response and induces significant oxidative damage. However, our hypothesis that the use of NAC might have limitations in inflammatory response and present pro-oxidant effects due to its easy interaction with iron was not confirmed. The administration of NAC alone or in association with DFX reduced to a similar degree the inflammatory response and the oxidative stress in rats exposed to coal dust.

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Table

Table I

Groups	Cell Number Total ($x10^5$)	Protein Concentration (μg/ml)
Saline (n=8)	4.83 ± 0.51	405.13 ± 37.46
NAC (n=8)	3.94 ± 0.31*	295.13 ± 17.30*
NAC+DFX (n=8)	2.9 ± 0.33*	226.35 ± 24.17*

Figures

Figure I

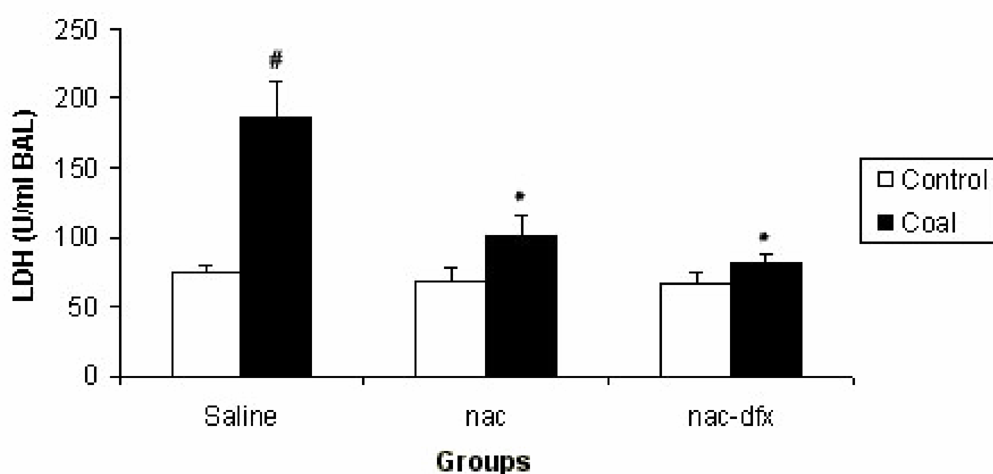


Figure IIA

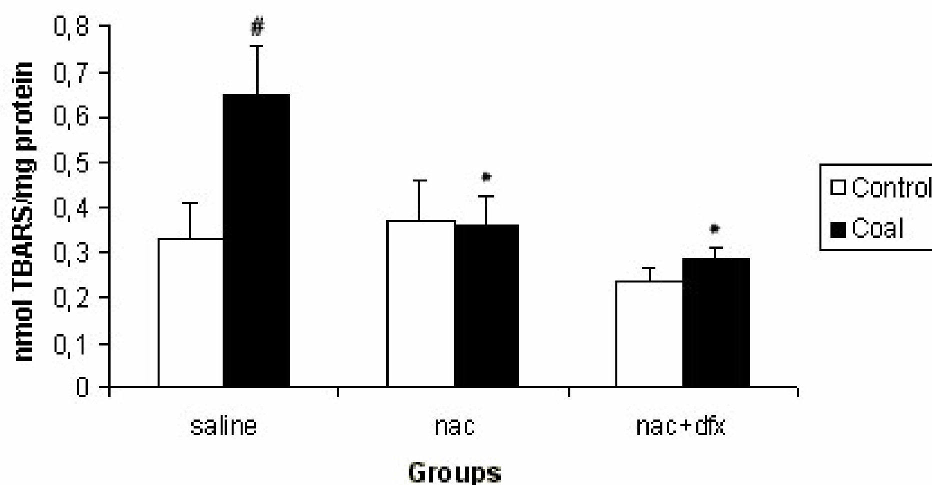


Figure IIB

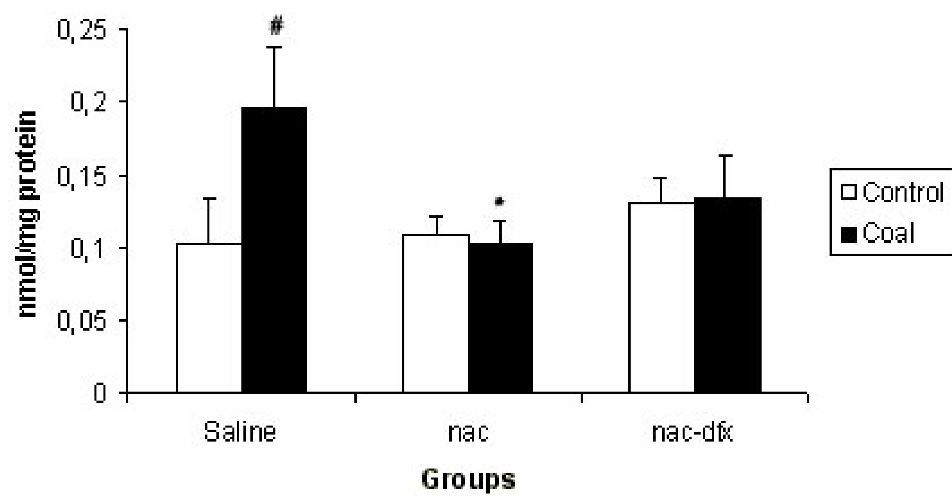


Figure IIIA

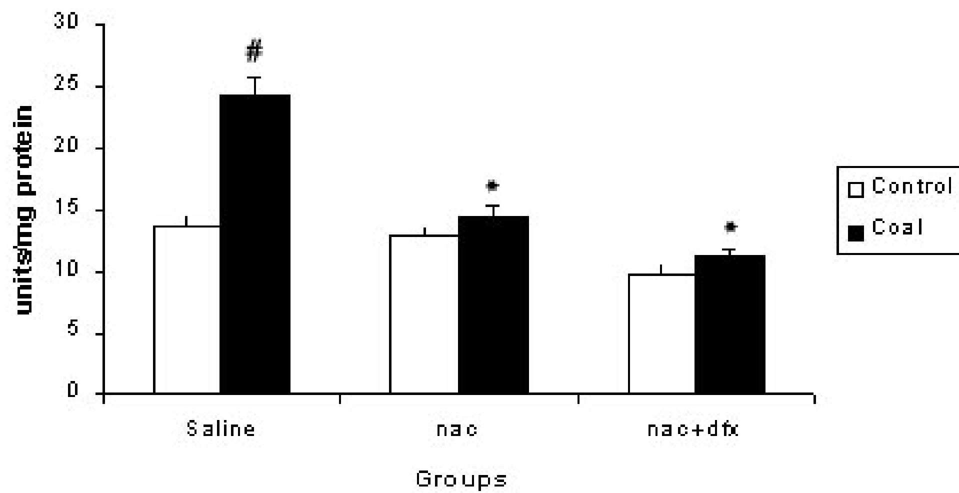
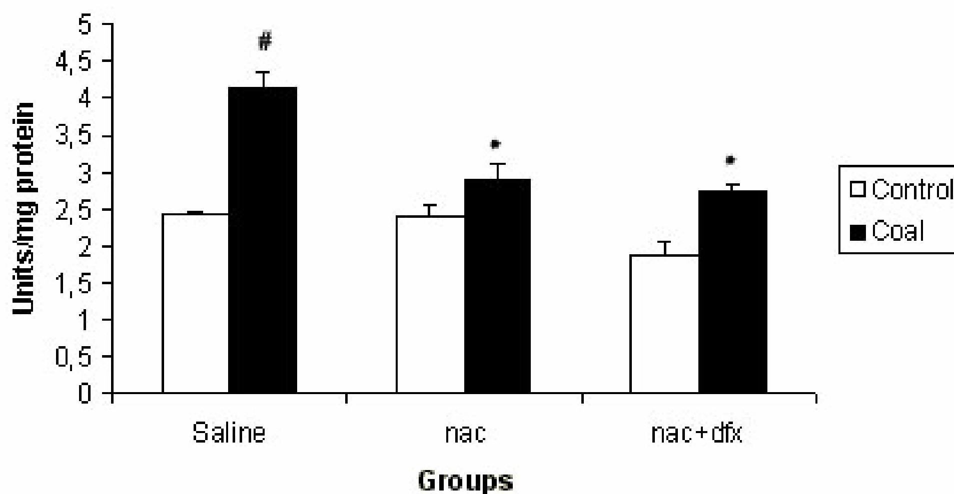


Figure IIIB



Legends

Table I: Total cell count and total protein concentration in BAL of rats after coal dust exposure and supplementation of NAC and NAC+DFX. The methodological proceedings are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between groups (*$p<0.05$).

Figure I: Lactate dehidrogenase activity in BAL of rats after coal dust exposure and NAC or NAC plus DFX supplementation. Values are presented as MEAN±SEM and the results were expressed in unit per litre of BAL. Significant different as compared between control (n=6)[#] and supplemental (n=8)* groups (*$p<0.05$).

Figure IIA: Lipoperoxidation in lung of rats after coal dust exposure and NAC or NAC plus DFX supplementation. Values are presented as MEAN±SEM and the results were expressed in nmol TBARS per milligram of protein. Significant different as compared between control (n=6)[#] and supplemental (n=8)* groups ($p<0.05$).

Figure IIB: Protein carbonilation in lung of rats after coal dust exposure and NAC or NAC plus DFX supplementation. Values are presented as MEAN±SEM and the results were expressed in nmol of carbonyl per milligram of protein. Significant different as compared between control (n=6)[#] and supplemental (n=8)* groups ($p<0.05$).

Figure IIIA: Superoxide dismutase activity in lung of rats after coal dust exposure NAC or NAC plus DFX supplementation. Values are presented as MEAN±SEM and the results were expressed in unit per milligram of protein. Significant different as compared between control (n=6)# and supplemental (n=8)* groups ($p<0.05$).

Figure IIIB: Catalase activity in lung of rats after coal dust exposure and NAC or NAC plus DFX supplementation. Values are presented as MEAN±SEM and the results were expressed in unit per milligram of protein. Significant different as compared between control (n=6)# and supplemental (n=8)* groups ($p<0.05$).

CAPÍTULO IV

IMPAIRMENT IN CAT/SOD ACTIVITIES IN RATS SUBMITTED TO TREADMILL TRAINING EXERCISE

Ricardo A. Pinho[1e2*] MSc, Michael E. Andrades[1] MSc, Marcos R. Oliveira[1] BSc, Aline C. Pirola[2] BSc, Morgana S. Zago[2] BSc, Paulo C. L. Silveira[2] BSc, Felipe Dal-Pizzol[1e3] PhD, José C. F. Moreira[1] PhD

[1]Centro de Estudos em Estresse Oxidativo/Departamento de Bioquímica/UFRGS
[2]Laboratório de Fisiologia e Bioquímica do Exercício//UNESC
[3]Laboratório de Fisiopatologia Experimental/UNESC

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ABSTRACT

The association between physical exercise and oxidative damage in skeletal musculature has been the focus of many studies in literature, but the balance of superoxide dismutase and catalase activity and oxidative damage parameters is not well established. Thus, the aim of the present study was to investigate the association between regular treadmill physical exercise, oxidative damage and antioxidant defenses in rat's skeletal muscle. Fifteen male Wistar rats (8-12 months) were randomly separated into two groups (trained n=9 and untrained n= 6). Trained rats were treadmill-trained for 12 week in progressive exercise (velocity, time, and inclination). Training program consisted in a progressive exercise (10m/min without inclination for $10min/day^{-1}$). After one week period the speed, time and inclination were gradually increased until $17m/min^{-1}$ at 10% during $50min/day^{-1}$. After training period, animals were killed and gastrocnemius and quadriceps were surgically removed to the determination of biochemical parameters. Lipid peroxidation, protein oxidative damage, catalase, superoxide dismutase, citrate synthase activities and muscular glycogen content were measured in the isolated muscles. We observed that there was a different modulation in CAT and SOD activities in trained rats skeletal muscle when compared with untrained rats (CAT activities decreased and SOD activities increased) and TBARS levels were significantly reduced, in contrast, with a significant increase in protein carbonilation. The results suggest a differential adaptation of skeletal muscle against excercise-induced oxidative stress.

Key Words: oxidative stress, physical exercise, free radical, skeletal muscle

INTRODUCTION

The association between physical exercise and oxidative damage has been the focus of many studies in literature. Physical exercise, which is in association with a remarkably enhanced rate of oxygen utilization increases accumulation of free radicals as a response to the oxygen need[1]. Elevated metabolic rates as a result of exercise may dramatically increase oxygen consumption (VO2max) to 20 fold over steady state. This increase in metabolism is followed by a concomitant increase in free radicals generation[2]. In spite of exercise-induced free radicals, elaborated oxidative defense systems result from a regular physical exercise program[3].

Evidences indicate that physical exercise, especially aerobic, generated reactive oxygen species such as superoxide anion and hydrogen peroxide, able to cause muscular damage and inflammation[4]. Oxidative damage can be reversed by the stimulation of antioxidant defenses, keeping the balance between ROS-induced damage and antioxidant-repair systems[5,4].

Physical exercise associated to oxidative damage depends on the type and intensity of exercise. However, studies have demonstrated that endurance training improves the antioxidant defense[5,4] as well as oxidative capacity in skeletal muscle[6,5,7,8].

Thus, the aim of the present study was to investigate the association between regular treadmill physical exercise, oxidative damage and antioxidant defenses in skeletal muscle of rats.

MATERIAL AND METHODS

Animal Preparation: Fifteen male Wistar rats weighing 300 to 350 g at 8-12 months old, were housed in a temperature-controlled room (24°C) with a 12:12-h reverse light-dark cycle (07:00-19:0 h dark: 19:00-07:00h light) and had free access to food and water. The animals were looked after in accordance with the "Guiding Principles in the Care and uses of Animals"[9] and were approved by the local ethic committee.

Animals were randomly distributed into two groups: trained rats (TR, n=9) and untrained rats (UT, n= 6). All rats were accustomed to treadmill running. The trained group was undergone to a 12-week training program with a progressive physical exercise (10 m/min^{-1} without inclination for 10 min/day^{-1}.) After 1 week period, velocity, time and inclination were gradually increased until 17/m/min^{-1} at 10% inclination for 50 min/day^{-1}. Forty eight hours after the last training session the animals were killed by decapitation and gastrocnemius (mixed fibers) and quadriceps (red fibers) were surgically removed and immediately stored at – 80°C for posterior analyses. Glycogen content and citrate synthase (CS) activity was immediately determined after decapitation.

Muscular Glycogen content: Muscular glycogen was quantified by the reaction with $KI+I_2$ [10]. 300 mg of tissue was boiled for 20 min in 30% of KOH and after that the muscle glycogen was precipitated by the addition of 60% of ethanol. The solution was heated during 10 min at 70°C; followed by an incubation in ice by 15 min and centrifugation by 9 min at 600g. Then the supernatant was redissolved in Milli Q water and 100μl aliquot was mixed in a

solution of KI-I_2 in saturated solution of CaCl. Muscle glycogen was determined by the absorbance at 460nm.

Citrate Synthase Activity: Citrate synthase methods measure the SH groups by using 5.5 dithiobis-(2-nitrobenzoic acid – DTNB)[11]. 100mg muscle (gastrocnemius and quadriceps red) was homogenized in PBS and centrifuged during 10 min at 1000g, 4°C. The medium assay contained 100μl of 1mM-5.5’dithiobis-(2-nitrobenzoic acid), dissolved in 10ml of 1M-Tris/HCl (pH 8.1), 30μl of 10mM-Acetyl-CoA, dissolved in Milli Q water, 20μl of homogenate, 800μl solution (1mM-EDTA/Triton X100 0.05%, pH 7.4 at 25°C). The absorption at 412nm is followed for 3 min and incubated by 7 min. The process is repeated and citrate synthase reaction is then started by the addition of 50μl of oxaloacetate, dissolved in 0.1M-Tris/HCl. It was used a molar absorption coefficient of 13.600M^{-1}.

Catalase (CAT) and Superoxide Dismutase (SOD) Activities: In order to determine CAT activity, organ systems were sonicated in 50mM phosphate buffer and the resulting suspension was centrifuged at 3000g during 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240nm[12]. SOD activity was assayed by measuring the inhibition of adrenaline self-oxidation absorbance at 48nm[13].

Thiobarbituric Acid Reactive Species (TBARS): As an index of lipid peroxidation these researchers used the formation of TBARS during an acid-heating reaction[14]. Briefly, the samples were mixed with 1ml of trichloroacetic acid 10% and 1ml of thiobarbituric acid 0.67%, then they were heated in a

boiling water bath for 30 minutes. TBARS were determined by the absorbance at 535nm.

Protein Carbonyls: The oxidative damage to protein was achieved by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH)[15]. Proteins were soon precipitated by the addition of 20% trichloroacetic acid and reacted with DNPH. After, that the samples were redissolved in 6M-guanidine hydrochloride and carbonyl contents were determined from the absorbency at 370nm using a molar absorption coefficient of 22.0000M^{-1}

Protein Determination: The amount of protein in the assays of CS, catalase, SOD and TBARS was assayed using the Lowry technique[16] and protein carbonyl by Broadford assay[17].

Statistical Analysis: Students' tests were used to compare between sedentary rats and exercise-trained ones. The level of significance was set at 95% ($p<0.05$). The software used for analysis of the data was "Statistical Package for the Social Sciences (SPSS) version 10.0 for Windows."

RESULTS

Citrate Synthase (CS) Activity and Muscular Glycogen Content (MG): CS activity and MG content were used as an indicator of the effect of exercise training. The results indicate that the treadmill-training program used was sufficient to increase the oxidative metabolism in skeletal muscle. CS activity in red-quadriceps muscle in TR (figure IA) was significantly higher than in UT (TR= 0.58 ± 0.01 U/mg protein, UT= 0.42 ± 0.04 U/mg protein, $p<0.05$). MG content

in TR (figure IB) was significantly increased in red-quadriceps (TR= 1.02 ± 0.037 μg/mg muscle, UT= 0.5 ± 0.033 μg/mg muscle, $p<0.05$) and gastrocnemius (TR= 0.9 ± 0.095 μg/mg muscle, UT= 0.55 ± 0.11 μg/mg muscle, $p< 0.05$) than in UT rats.

Catalase and Superoxide Dismutase activities. Catalase and SOD activities were showed in figure IIA, IIB. Catalase in trained rats (figure IIA) was significantly decreased in all studied muscles (red-quadriceps TR= 0.482 ± 0.064 U/mg protein, UT= 1.733 ± 0.024 U/mg protein; mixed-gastrocnemius TR= 0,449 ± 0.007 U/mg protein, UT= 1.316 ± 0.276 U/mg protein, $p<0.05$). SOD activity in trained rats (figure IB) was significantly increased in red-quadriceps (TR= 0.19 ± 0.04 U/mg protein, UT= 0.014 ± 0.01 U/mg protein, $p<0.05$) and mixed-gastrocnemius muscles (TR= 0.30 ± 0.04 U/mg protein, UT= 0.19 ± 0.01 U/mg protein, $p< 0.05$).

Lipid Peroxidation and Protein Carbonyls: Trained rats showed lower TBARS levels in red-quadriceps when compared with UT rats (TR=0.006 ± 0.0006 nmol/mg protein, UT= 0.028 ± 0.008 nmol/mg protein, $p< 0.05$) and mixed-gastrocnemius muscle (TR= 0.005 ± 0.0008 nmol/mg protein, UT= 0.003 ± nmol/mg protein, $p>0.05$) (figure IIIA). Protein carbonyls content were significantly higher in red-quadriceps muscle (TR= 0.2076 ± 0.009 nmol/mg protein, UT= 0.142 ± 0.024 nmol/mg protein, $p<0.05$) and mixed-gastrocnemius muscle (TR= 0.2315 ± 0.031 nmol/mg protein, UT= 0.158 ± 0.027 nmol/mg protein, $p<0.05$) of trained rats compared to untrained rats (figure IIIB).

DISCUSSION

Citrate Synthase activity and Muscular Glycogen content: CS activity and MG content were used to stress the effect of exercise training. CS activity is one of the key regulatory enzymes in the energy-generating metabolic pathway that catalyses the condensation of oxaloacetate and acetyl coenzyme A to create a citrate in tricarboxylic acid cycle[18]. Glycogen muscular content represents one of the main fuels mobilized to support muscle energy demands during several types of resistance exercise[19].

Several studies have been carried out to determine the influence of acute exercise training in the mitochondrial enzyme adaptation in skeletal muscle of rats[20,21,18]. Few studies however have demonstrated the response of CS in endurance treadmill training.

As it is shown in figure IA, we did not observe differences in citrate syntase activity in gastrocnemius muscle, whereas this enzyme activity was increased in the red quadriceps of trained rats. This could be related to mixed white and red fibers during the gastrocnemius preparation process. In another study, after a swimming period of training, rats did not show any important changes in CS activity in gastrocnemius muscle[6]. Yet Radák and colleagues showed an important increase in CS activity and gastrocnemius muscle after 9 weeks of swimming training with young and middle-aged rats[7]. Another study, after endurance treadmill training with young rats, has shown gastrocnemius increase in CS activity[2]. The difference in the results might be related to differences in the training intensity, duration of the session, training period, and the type of the exercise.

Studies accomplished with other muscles have also been presenting controversial results[22]. In a recent study it was reported that the endurance treadmill training in rats increased the CS activity in skeletal muscle not in the cardiac ventricle. According to the authors[22], it is possible that such differences are related to different molecular adaptations (translational and/or posttranslational modifications) of CS adaptation to exercise training.

The maintenance of an appropriate level of glycogen is assuring better efficiency in the physical effort. The increasing of stock in the skeletal muscle is a great challenge. The speed of depletion of the muscular glycogen is directly related to the intensity of work that the muscle is undergone[23] and the complete repletion of the glycogen stocks after a resistance exercise depends on the presence of precursors (endogenous carbon sources) and of the carbohydrates ingestion during the recovery periods[19]. Studies have shown that trained athletes can increase glycogen concentrations to a greater extent and at a faster rate compared to this to untrained individuals.

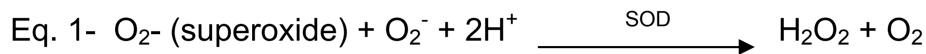
We observed that muscular glycogen content in trained rats (figure IB) has significantly increased more in red-quadriceps and gastrocnemius than in untrained rats. It was recently believed that the increasing or the glycogen stock was due to the increasing of the activity of glycogen synthase as a result of training. More recent studies do not confirm this hypothesis and suggest that an enhanced ability to transport glucose, by GLUT-4 is the primary mechanism that accounts for the faster increase of the replenishment in trained muscle[24].

Although this possibility is accepted, the mechanism that elevates the glycogen stocks in muscular fibers after training programs is not yet well known.

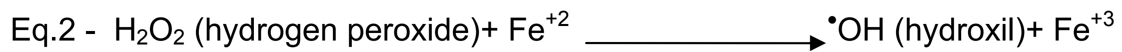
Catalase and Superoxide Dismutase activities: Studies published in the literature demonstrated the increase in the skeletal muscle antioxidant enzymes activities in response to the physical training is associated mainly to vigorous exercises[25]. In the present study, catalase activity in red-quadriceps and mixed-gastrocnemius muscles of trained rats were significantly lower when compared to untrained rats (figure IIA). These results are in agreement with others[26,27]. In contrast, some studies demonstrated an increase in the catalase activity in skeletal muscle and other organ systems after training[28,29,30]. The exhaustive exercise increased catalase activity in liver, heart, kidney, and lung tissues of male and female rats[8]. These controversial results are also observed with SOD activity[31,28,5,32]. We observed that SOD activity of trained rats was significantly higher in red-quadriceps and mixed-gastrocnemius muscles when compared to untrained rats.

We observed that there is a different modulation of SOD and CAT in skeletal muscle in trained rats when compared to untrained rats. After training, SOD activity seems to increase in skeletal muscle (figure IIB), probably as a response to oxidative stress induced by training. The most striking finding was the remarkable increase of SOD activity observed in trained rats without a proportional increase of CAT activity (figure IIA). Cells that over express SOD or transgenic mice for human SOD showed some abnormalities related to oxidative stress. It seems that any concentration of SOD other than an optimal one leads to increase lipid peroxidation and therefore to decrease cell viability[33]. SOD activity results in the production of hydrogen peroxide (eq.1), which can mediate membrane damage by lipid peroxidation or react with iron to generate hydroxil radicals via Fenton chemistry, which is thought to be the most toxic

oxygen reaction species *in vivo* (eq.2). CAT could clean an excess of peroxide, diminishing the oxidative effects of hydrogen peroxide (eq.3).



Eq. 1- O_2- (superoxide) + $O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$



Eq.2 - H_2O_2 (hydrogen peroxide)+ $Fe^{+2} \longrightarrow {}^{\bullet}OH$ (hydroxil)+ Fe^{+3}

Eq. 3 - $2H_2O_2 \xrightarrow{CAT} O_2 + H_2O$

Thus, an imbalance between SOD and CAT activity can take to oxidative stress and could participate in the genesis of several diseases[34]. We couldn't find any study that discuss the imbalance of CAT and SOD during exercise, but role that this proposed imbalance in exercise induced oxidative stress must be confirmed in future studies.

Lipid Peroxidation and protein carbonyls content: Changes at lipoperoxidation is the most frequent evidence cited to support the approach oxidative stress in skeletal muscle[3]. Different forms of exercise result in different levels of oxidative stress, and the influence of exercise on oxidative stress is still controversial. Some studies reported no alteration in lipid peroxidation and protein carbonyls after training exercise[32,3,30]. On the other hand, several other studies demonstrated oxidative stress after anaerobic, isometric, submaximal exercise[35,36,32,7]. In the present study, TBARS levels in red-quadriceps and mixed-gastrocnemius muscles of trained rats were significantly reduced when compared to untrained rats (figure IIIA), suggesting that regular exercise-induced beneficial effects in skeletal muscle.

Although those results are significant, Oh-Ishi and colleagues suggested that the main effect of exercise training on lipid peroxidation might be not lower the basement level of lipid peroxidation but to improve the resistance of tissues to exercise-induced lipid peroxidation[5]. Other studies had not shown difference in skeletal muscle after exercise training. Radák and colleagues showed the effect of the training of the swimming on oxidative damages in skeletal muscles of middle age rats. The results showed that the rate of lipid peroxidation did not change significantly, suggesting that there is a balance between ROS-induced damage to lipids and antioxidant-repair systems[7]. These differences could be related to different exercise protocols, different muscles analyzed or animal ages.

In contrast to lipoperoxidation results, a significant increase in protein carbonilation level may be observed in red-quadriceps and mixed-gastrocnemius muscles of trained rats compared to untrained rats (figure IIIB). Other studies have also shown that endurance training induce to a significant increase in protein oxidation of the skeletal muscles and lungs[35,37]. It is possible that those differences could be secondary to a reduction of protein turnover rate. It is well known that oxidative modified proteins are less degraded by proteassome. This is reinforced by the increase of the proteossome activity as an adaptative process secondary to protein oxidation after exercise[38,7]. Further studies will determinate if this oxidative alteration is restricted to contractile apparatus or is widespread to all muscle protein.

CONCLUSION

The present study suggests a differential adaptation of skeletal muscle against exercise-induced oxidative stress; i.e. an increase in protein carbonyls and a decrease in lipid peroxidation. This is accompanied by a differential modulation of antioxidant defenses that could result in hydrogen peroxide accumulation and potentialization of oxidative damage.

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Figures

Figure IA

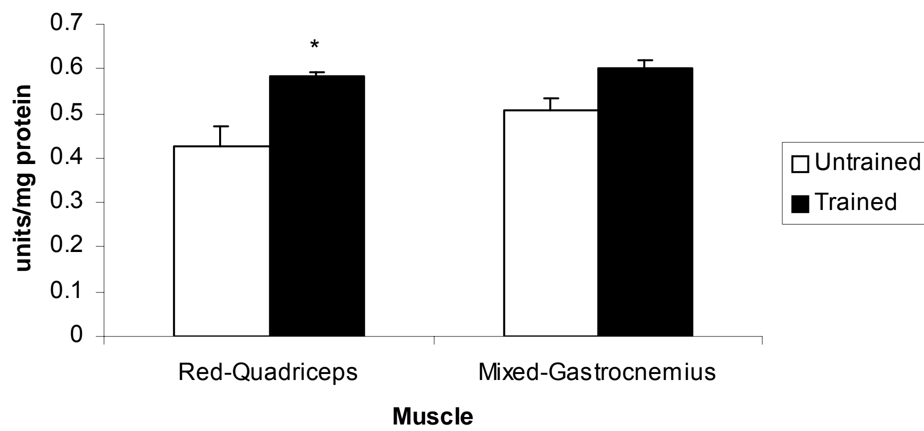


Figure IB

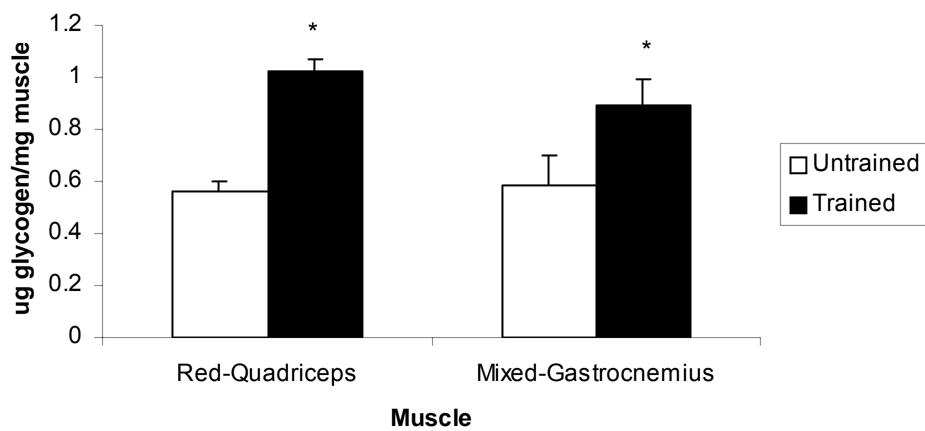


Figure IIA

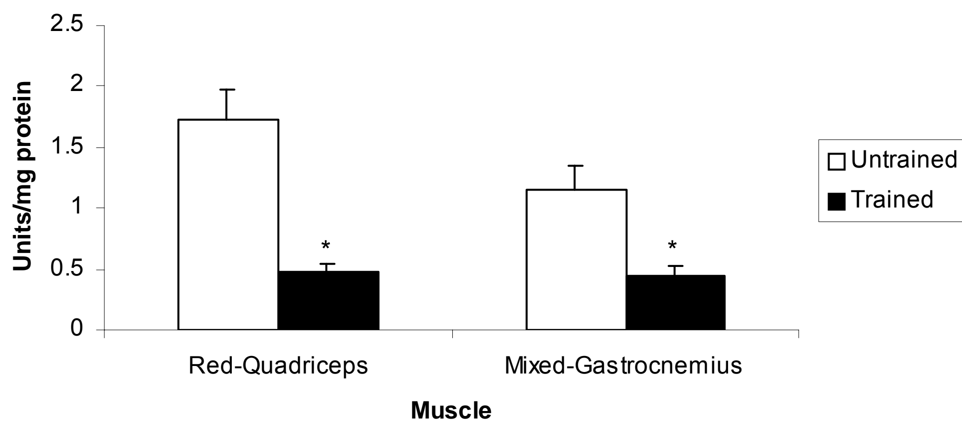


Figure IIB

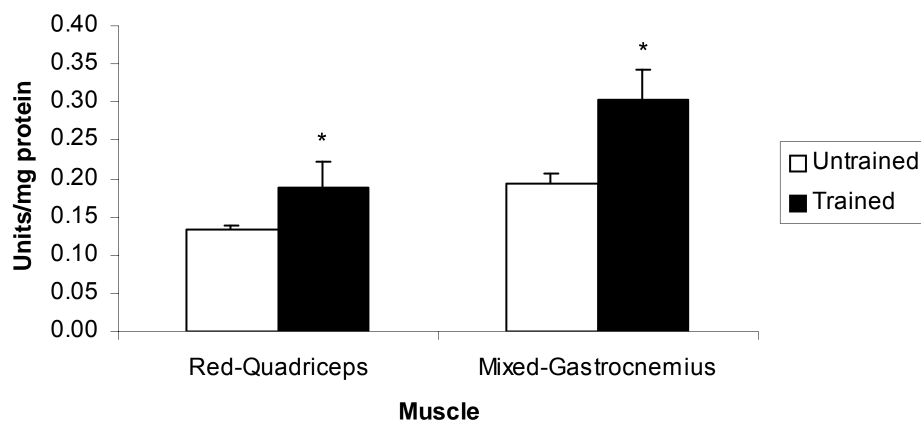


Figure IIIA

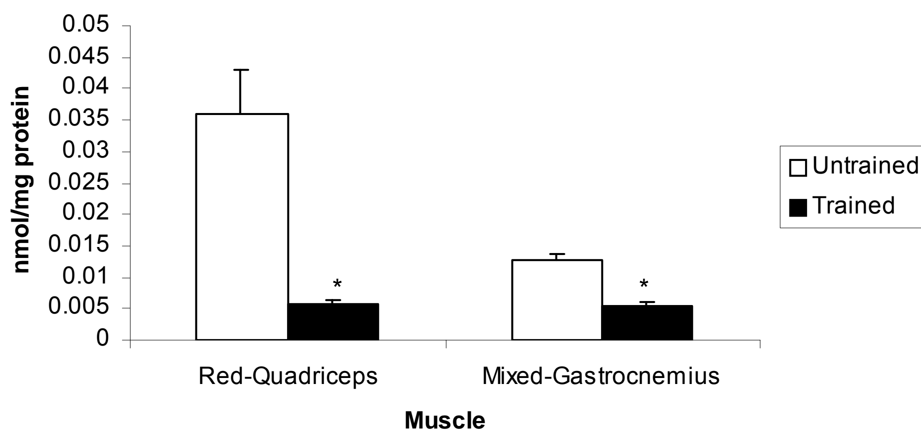
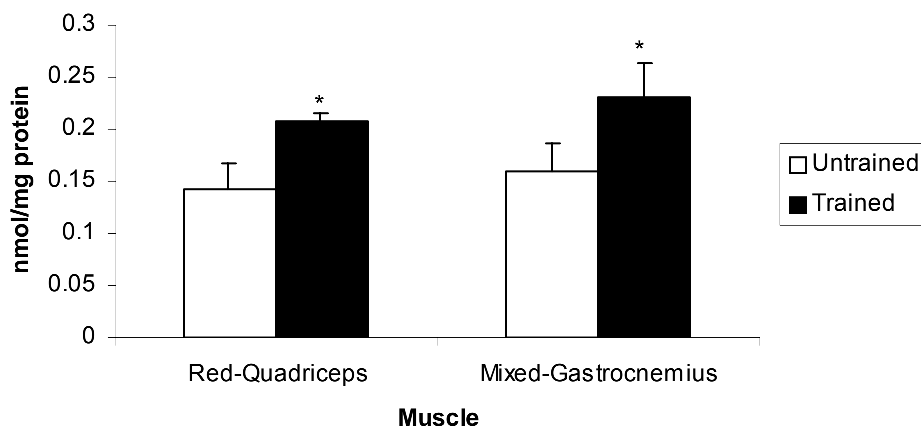


Figure IIIB



Legends

Figure IA: Citrate Synthase activity in skeletal muscle (red-quadriceps, mixed-gastrocnemius and diaphragm) of trained (n=9) and untrained (n=6) rats. Treadmill-training and enzyme assays are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between the trained and untrained groups ($^{*}p<0.05$).

Figure IB: Glycogen content in skeletal muscle (red-quadriceps, mixed-gastrocnemius and diaphragm) of trained (n=9) and untrained (n=6) rats. Treadmill-training and enzyme assays are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between the trained and untrained groups ($^{*}p<0.05$).

Figure IIA: Catalase activity in skeletal muscle (red-quadriceps, mixed-gastrocnemius and diaphragm) of trained (n=9) and untrained (n=6) rats. Treadmill-training and enzyme assays are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between the trained and untrained groups ($^{*}p<0.05$).

Figure IIB: Superoxide dismutase activity in skeletal muscle (red-quadriceps, mixed-gastrocnemius and diaphragm) of trained (n=9) and untrained (n=6) rats. Treadmill-training and enzyme assays are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between the trained and untrained groups ($^{*}p<0.05$).

Figure IIIA: Lipid peroxidation in skeletal muscle (red-quadriceps and mixed-gastrocnemius) of trained (n=9) and untrained (n=6) rats. Treadmill training and assays are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between the trained and untrained groups ($^{*}p<0.05$).

Figure IIIB: protein carbonyl in skeletal muscle (red-quadriceps and mixed-gastrocnemius) of trained (n=9) and untrained (n=6) rats. Treadmill training and assays are described in the Material and Methods section. The values are presented as MEAN±SEM. Significant different as compared between the trained and untrained groups ($^{*}p<0.05$).

CAPÍTULO V

O EXERCÍCIO FÍSICO REGULAR DIMINUI O ESTRESSE OXIDATIVO PULMONAR EM RATOS APOS EXPOSIÇÃO AGUDA AO CARVÃO MINERAL

Ricardo A. Pinho[1e2], Paulo C. L. Silveira[2], Mariana Piazza[2], Talita Tuon[2], Gabriela A. Slva[2], Felipe Dal-Pizzol[1e3], José C. F. Moreira[1]
[1]Centro de Estudos em Estresse Oxidativo/Departamento de Bioquímica/UFRGS
[2]Laboratório de Fisiologia e Bioquímica do Exercício//UNESC
[3]Laboratório de Fisiopatologia Experimental/UNESC

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RESUMO

Estudos têm apontado o exercício físico regular de baixa a moderada intensidade como um importante agente no combate ao estresse oxidativo. O objetivo desse estudo foi investigar o efeito do exercício físico regular na resposta oxidativa pulmonar após a inalação de pó de carvão mineral. Vinte e quatro ratos Wistar machos (200-250g) foram divididos randomicamente em 2 grupos com respectivos controles (Treinado, n=6 e Não-treinado, n=6). Os animais receberam, por instilação traqueal, pó carvão mineral (3mg/0,5ml salina, 3dias/semana, durante 3 semanas) ou 0,5ml de solução salina 0,9%. Quarenta e oito horas após a última instilação, o grupo treinado foi submetido a um programa de exercício progressivo em esteira durante 12 semanas (até 17m.min^{-1}, 50min.dia^{-1}, 10% de inclinação). Quarenta e oito horas após a última sessão de treinamento, todos os animais foram mortos por decapitação e os pulmões e sóleo foram cirurgicamente removidos para posterior análise bioquímica. A atividade da citrato sintase foi determinada no músculo sóleo e os danos em lipídios e proteínas foram avaliados nos pulmões pelos níveis de TBARS e pela determinação de grupos carbonil, respectivamente. Os resultados mostram que a prática regular de exercício físico reduz significativamente os níveis presentes de TBARS em ratos treinados e diminui os níveis de oxidação em proteínas em ambos os grupos quando comparados aos respectivos controles. Os resultados sugerem que o exercício físico regular em esteira é um agente capaz de amenizar os danos oxidativos pulmonar induzidos pela inalação de partículas de carvão mineral.

Palavras-chave: exercício físico, dano oxidativo, pulmão, radicais livres

ABSTRACT

Studies have shown that regular physical exercise of moderate intensity is an important agent in the oxidative stress control. The aim of this study is to investigate the effect of physical exercise upon pulmonary oxidative responses after inhalation of mineral coal dust. Twenty-four Wistar male rats (200-250 g) were randomly separated into two groups with respective controls (trained n=6 and untrained n= 6). The animals received mineral coal dust by intratracheal instillation (3 mg/0,5 ml saline, 3 days/week, during 3 weeks) or only 0.5 ml of saline 0.9%. Forty-eight hours after the instillation period, the animals were treadmill-trained for 12 weeks in progressive exercise (until 17 m/min^{-1} at 10% during 50 min/day^{-1}velocity, time, and inclination). Forty-eight hours after the training period, animals were killed and their lungs and soleo were surgically removed for posterior biochemical analysis. Citrate synthase activity was determinate in soleo muscles and lipids and proteins damage were evaluated in lungs by TBARS level and by carbonyl groups determination respectively. The results show that the treadmill-training program used was sufficient to increase the oxidative metabolism in skeletal muscle and that physical exercise decrease TBARS and protein oxidation levels in both groups. The results suggest that regular physical exercise in treadmill attenuate pulmonary oxidative damages induced by inhalation mineral coal dust.

Keywords: physical exercise, oxidative damage, lung, free radicals.

INTRODUÇÃO

A inalação de poluentes ocupacionais e atmosféricos, como o carvão mineral, tem contribuído significativamente para o surgimento e progressão de inúmeras desordens respiratórias, como infecções, inflamações, pneumoconiose, bronquite crônica entre outras.

Estudos recentes[1,2,3,4] têm mostrado que a alteração pulmonar, após a inalação de partículas industriais, é mediada, principalmente, pela formação de espécies reativas de oxigênio (ERO). Essa resposta bioquímica, segundo Tao e colaboradores[5], leva à ativação de macrófagos e recrutamento de células polimorfonucleares, as quais induzem ao aumento de mediadores inflamatórios.

As ERO são produzidas normalmente durante o metabolismo celular, principalmente, em nível mitocondrial, sendo fundamentais para diversos processos celulares. Entretanto, sua produção em excesso pode acarretar danos nos constituintes celulares, estando envolvidas em diversos processos fisiológicos e patológicos[6,7]. Essas substâncias podem elevar os efeitos deletérios da doença. Mas, é bem possível que o exercício físico, em longo prazo, possa aumentar o sistema de defesa contra a ação das ERO[8,9].

As intervenções terapêuticas de rotina, especialmente as ações farmacológicas, têm contribuído significativamente para a diminuição da gravidade do problema, mas apresentam limitações. Acredita-se que o exercício físico regular exerça um efeito positivo sobre a capacidade cardiorrespiratória e sobre a resposta bioquímica pulmonar.

Diversos estudos têm descrito as vias metabólicas da produção das ERO durante o exercício[10,11,9,12], porém pouco se conhece sobre a influência do exercício físico regular sobre a liberação de ERO após inalação de partículas minerais. As informações na literatura são apenas pressupostos que necessitam de investigação científica mais apurada.

Assim, o objetivo desse estudo foi investigar o efeito do exercício físico regular na resposta oxidativa pulmonar, após a inalação de pó de carvão mineral. Acredita-se que desvendar esse aspecto é de extrema relevância para uma melhor compreensão do fenômeno bioquímico dos danos oxidativos pulmonares e de sua relação com o exercício físico.

MATERIAIS E MÉTODOS

Todos os procedimentos foram realizados de acordo com "Guiding Principles in the Care and uses of Animals"[13] e aprovados pelo Comitê de Ética da Universidade do Extremo Sul Catarinense.

Preparação do Carvão: 1Kg de carvão mineral bruto foi coletado na Carbonífera Cooperminas, localizada no município de Criciúma, Santa Catarina, Brasil. Trezentos gramas da amostra foram trituradas em um moinho de esfera por 3 horas, numa freqüência de 25hz. O carvão foi analisado no Laboratório de Análises de Solo e Fertilizantes da Universidade do Extremo Sul Catarinense, Criciúma, Santa Catarina, Brasil, apresentando as seguintes características mineralógicas: cobre (0,003%), ferro (2,5%), zinco (0,003%) e sílica (27,3%). As partículas de carvão utilizadas nesse experimento apresentaram um diâmetro até 15 μm.

Instilação do Carvão: Vinte e quatro ratos Wistar machos (200-250g) foram usados no protocolo experimental. Os animais foram agrupados em gaiolas específicas, temperatura ambiente controlada em 22°C, ciclo claro-escuro 12:12h e com livre acesso à água e à comida. Para a instilação de carvão, os animais foram anestesiados com ketamina (80mg/kg de massa corporal, i.p) e xilasina (20mg/kg de massa corporal, i.p) e receberam a administração direta por instilação traqueal de 3mg/0,5ml de salina ou 0,5ml de solução salina 0,9%, uma vez a cada 3 dias durante 9 dias, procedimento adaptado do modelo descrito por Pinho e colaboradores[4]. Os animais foram divididos randomicamente em dois grupos com respectivos controles: Treinado (TR, n=6) e Não-treinado (NTR, n=6).

Protocolo de Treinamento: Quarenta e oito horas após a última instilação de carvão, todos os animais foram ambientados em esteira ergométrica adaptada (10 $m.min^{-1}$ sem inclinação, 10 $min.dia^{-1}$). Os grupos treinados (carvão e salina) foram submetidos a um programa de treinamento progressivo durante 12 semanas com velocidade até 17 $m.min^{-1}$, tempo até 50 $min.dia^{-1}$ e inclinação até 10%, aumentados gradualmente. Quarenta e oito horas após a última sessão de treinamento, todos os animais foram mortos por decapitação e o pulmão e sóleo foram cirurgicamente removidos e imediatamente estocados em – 80°C para posterior análise.

Citrato Sintase (CS): A atividade da CS foi determinada a partir do grupamento SH usando 5. 5'ditiobis - (ácido 2-nitrobenzoico – DTNB)[14]. 100mg de músculo (sóleo) foram homogeneizados em PBS e centrifugados durante 10min, 1000Xg, 4°C. Para a reação foram utilizados 100µl of 1mM-DTNB, dissolvido em 10ml de 1M-Tris/HCl (pH 8,1), 30µl de 10mM-Acetil-CoA,

dissolvido em água de Milli Q, 20μl da amostra, 800μl da solução (1mM-EDTA/Triton X100 0.05%, pH 7,4, 25°C). A atividade enzimática foi medida numa absorbância de 412nm por 3min com incubação por 7min. O processo é repetido e a reação da atividade da CS iniciou quando foram adicionados 50μl de oxaloacetato, dissolvidos em 0,1M-Tris/HCl. Foi utilizado um coeficiente de absorção de 13.600M^{-1}.

Espécies Reativas ao Ácido Tiobarbitúrico (TBARS): como índice de peroxidação de lipídeos, foi verificada a formação de substâncias reativas ao aquecimento do ácido tiobarbitúrico medido espectrofotometricamente (532nm) e expressos como equivalentes de malondialdeído (MDA) (nmol/mg proteína)[15].

Carbonilação de Proteínas: os danos oxidativos em proteínas foram mensurados pela determinação de grupamento carbonil baseados na reação com dinitrofenilhidrazina[16]. O conteúdo de carbonil foi determinado espectrofotometricamente em 370nm usando um coeficiente 22.000 $Molar^{-1}$.

Determinação de Proteínas: a quantidade de total de proteína foi determinada a partir da técnica de Lowry[17].

Análise Estatística: os dados foram expressos em média e erro padrão médio, analisados estatisticamente pelo teste t de student para a análise da atividade da cintrato sintase (CS) e pela análise de variância (ANOVA) one-way, seguido pelo teste post hoc Tukey para os ensaios de danos oxidativos. O nível de significância estabelecido para os testes estatísticos foi de $p<0,05$. Foi utilizado o SPSS (Statistical Package for the Social Sciences) versão 10.0 como pacote estatístico.

Reagentes: Ácido tiobarbitúrico, DTNB, acetil-CoA, oxaloactato, dinitrofenilhidrazina, adrenalina e peróxido de hidrogênio foram adquiridos da Sigma Chemical (St. Louis, MO).

RESULTADOS

Citrato Sintase (CS): A atividade da CS foi usada como indicador do efeito do exercício físico. Os resultados (tabela I) indicam que o programa de treinamento realizado em esteira foi suficiente para aumentar a capacidade oxidativa do músculo esquelético (TR= 0,58 ± 0,01 U/mg proteína, NTR= 0,32 ± 0,04 U/mg proteína, p<0,05).

Lipoperoxidação: Como índice de dano oxidativo em lipídeos de membrana, avaliamos a formação de TBARS, após exposição ao carvão mineral. Conforme figura I, os resultados indicam um aumento significativo na lipoperoxidação em ratos expostos ao carvão (Carvão NTR= 0,123 ± 0,025 nmol/mg proteínas; Salina NTR=0,082 ± 0,014 nmol/mg proteínas, p<0,05). Os resultados ainda mostram que a prática regular de exercício físico reduz significativamente a formação de TBARS em ratos treinados comparados com não treinados (TR= 0,533 ± 0,009 nmol/mg proteína e NTR= 0,669 ± 0,004 nmol/mg proteína, p<0,05).

Carbonilação de Proteínas: para verificar os danos oxidativos em proteínas, avaliamos os grupos carbonil baseados na reação com dinitrofenilhidrazina. Conforme figura II, os resultados não mostram uma diferença significativa no dano em proteína, em ratos, após exposição ao carvão mineral, quando

comparados ao grupo salina, entretanto, verificou-se que o exercício físico regular diminui a resposta oxidativa em ambos os grupos comparados aos respectivos controles (Carvão TR= 0,136 ± 0,01 nmol/mg proteína e Carvão NTR= 0,407 ± 0,074 nmol/mg proteína; Salina TR= 0,118 ± 0,018 nmol/mg proteína e Salina NTR= 0,249 ± 0,027 nmol/mg proteína, $p<0,05$).

DISCUSSÃO

A citrato sintase é uma enzima regulatória na produção de energia aeróbia que catalisa a condensação do oxaloacetato e acetil CoA para citrato no ciclo do ácido tricarboxilíco[18]. Vários estudos têm utilizado a atividade de enzimas mitocondriais para confirmar ou não a influência do exercício físico na adaptação oxidativa do músculo esquelético de ratos[10,1918]. Porém, poucos estudos têm demonstrado a resposta da CS após treinamento de endurance em esteira.

Na tabela I, observamos um aumento significativo na atividade da CS no músculo sóleo de ratos, após o programa de treinamento. Da mesma forma, Alessio e Goldfarb[20] mostram que o treinamento em esteira em ratos jovens aumenta a atividade da CS no gastrocnemius duas vezes a mais do que o grupo controle. Em outro estudo, Rádak e colaboradores[12] mostraram um importante aumento na atividade da CS em diferentes músculos esqueléticos de ratos jovens e de meia idade, após nove semanas de treinamento em água. Embora nosso estudo corrobora com esses resultados, outros estudos citados por Pereira e colaboradores[21] apresentam respostas contrárias. Provavelmente, as diferenças entre os estudos estão relacionadas com os diferentes tipos de fibras musculares utilizadas, ou diferentes protocolos de

treinamento, ou diferentes métodos para determinação da atividade enzimática, ou ainda, com as adaptações moleculares diferenciadas (modificações translacional e/ou pós-translacional) na adaptação da CS durante e após o treinamento físico[22].

Recentes estudos[23,24,5,4] mostram que a resposta pulmonar por poluentes ocupacionais e atmosféricos, como carvão, provoca uma resposta inflamatória em cadeia mediada por ativação de macrófagos e recrutamento de células polimorfonucleares, citocinas, quimocinas e espécies reativas de oxigênio (ERO). Após a inalação desses poluentes, a produção de ERO pode ser aumentada significativamente ocasionando danos nos constituintes celulares[2].

A resposta oxidativa ao exercício físico é determinada pelo tipo, freqüência, duração e intensidade, que, por um lado, podem gerar a formação de ERO capazes de causar danos celulares e inflamação[25] e, por outro lado, o exercício físico regular de endurance pode tornar mais eficiente o sistema de defesa antioxidante, estabelecendo um equilíbrio entre os danos induzidos pelas ERO e os sistemas de reparos antioxidantes[26]. Portanto, em função desses dois extremos observados na resposta oxidativa ao exercício físico, os resultados obtidos com o treinamento físico ainda não são conclusivos.

Em nosso estudo, hipotetizamos que a prática regular e contínua de exercícios físicos aeróbios possa ser um agente capaz de amenizar os danos em proteínas e lipídios de membrana causados pela exposição ao carvão mineral.

Os danos em lipídios, a partir de sua oxidação, resultam da reação de radicais livres com ácidos graxos polinsaturados presentes em lipoproteínas de

membranas. Mudanças nos marcadores de lipoperoxidação são evidências mais freqüentes observadas no tecido pulmonar, após exposição a poluentes atmosféricos[4] e na resposta ao exercício físico[26].

Os resultados obtidos em nosso estudo mostram que o exercício físico regular diminui significativamente os níveis de lipoperoxidação pulmonar em ratos expostos ao carvão mineral após o treinamento físico (figura I). É possível que esses resultados sejam decorrentes do aumento nos níveis de enzimas antioxidantes, entretanto, embora esses resultados sejam importantes, Oh-Ishi e colaboradores[11] sugerem que o efeito do treinamento físico sobre a redução nos níveis de lipoperoxidação não é tão significativo quanto a melhora na capacidade do tecido em resistir aos efeitos da lipoperoxidação induzida pelo próprio exercício físico.

As ERO podem modificar aminoácidos por reações em cadeias através de agregados de proteínas suscetíveis a degradações proteolíticas. Durante esse processo, alguns aminoácidos são convertidos em derivados de carbonil[7]. A exposição ao carvão mineral também estimula a produção de grupos carbonil[4], o que pode, nesse caso específico, ser amenizado pela prática regular de exercício físico, conforme os resultados mostrados nesse estudo.

Conforme figura II, observamos uma redução significativa na carbonilação de proteínas, após o programa de exercício. Embora sem utilizar poluentes atmosféricos para induzir danos pulmonares, Radák e colaboradores[12] mostraram resultados contrários. O treinamento de endurance induz a um significante aumento na oxidação de proteínas em pulmão de ratos. É possível que essas diferenças em estudos possam estar relacionadas a

quatro aspectos: primeiro, o uso ou não de agentes estimuladores de danos pode apresentar repostas diferenciadas induzidas pelo exercício físico; segundo, a metodologia utilizada para determinar os níveis de oxidação protéica; terceiro, o tipo, duração, freqüência e intensidade do exercício utilizado; quarto, é possível que essas diferenças estejam relacionadas à redução na taxa do turnover de proteínas. Está bem estabelecido na literatura que as proteínas modificadas ou oxidadas são menos degradadas por proteossomas. Essa hipótese é reforçada pelo aumento na atividade de proteossomas como um processo adaptativo secundário à oxidação de proteínas após o exercício[27]. Embora ainda seja uma hipótese, pode ser a principal justificativa para as diferenças encontradas em diversos estudos.

Em resumo, os resultados apresentados neste estudo mostram evidências de que o exercício físico regular em esteira possa ser um agente capaz de amenizar os danos oxidativos pulmonares induzidos pela inalação de partículas de carvão mineral.

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Tabela I

Grupo	Média	EPM#
Treinado (TR)	0,589 U/mg proteína*	0,010
Não-treinado (NTR)	0,327 U/mg proteína	0,042

#EPM = Erro Padrão Médio

Figuras

Figura I

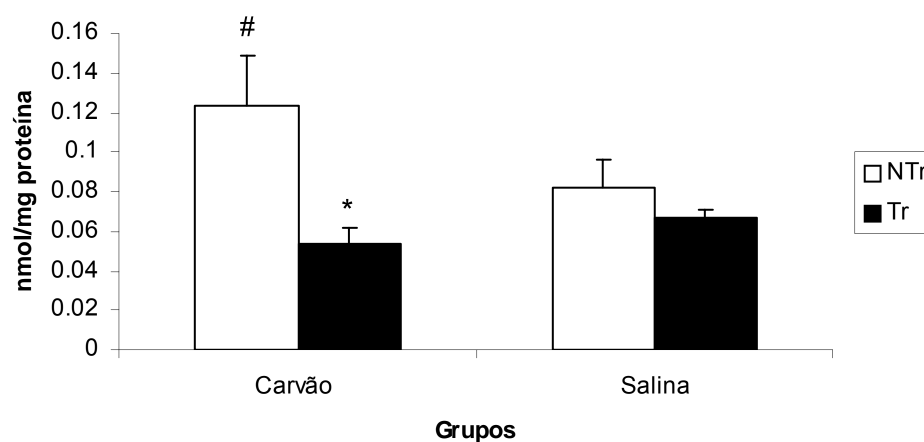
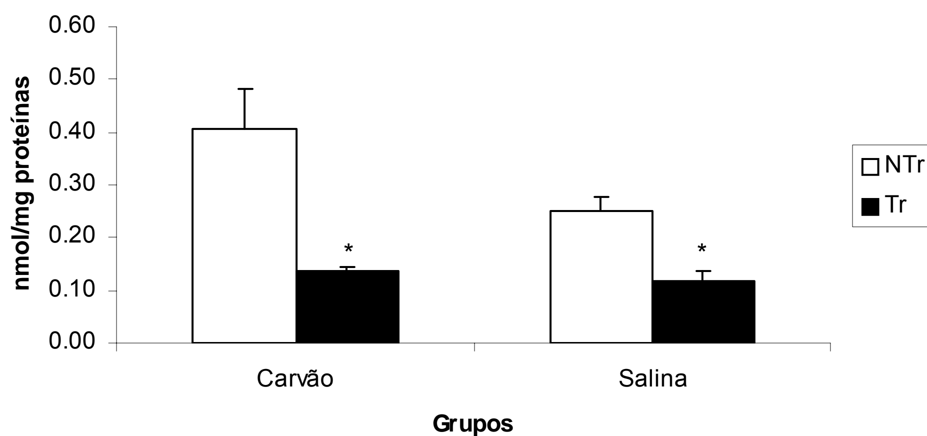


Figura II



Legendas

Tabela I: Atividade da Citrato Sintase em músculo esquelético (sóleo) de ratos treinados e não-treinados em esteira (TR=6, NTR=6). O treinamento em esteira e o método enzimático estão descritos no material e métodos. Os valores são apresentados em Média±EPM. A diferença significativa entre os grupos foi de *p<0.05.

Figura I: Lipoperoxidação em pulmões de ratos treinados (TR) e não-treinados (NtR) após exposição ao carvão mineral. Os procedimentos de exposição ao carvão e método de dosagem do dano oxidativo estão descritos no Material e Métodos. Os valores são apresentados em Média±EPM e os resultados foram expressos em nmol de TBARS/mg de proteínas. A diferença significativa entre os grupos controle* e grupo não-treinado# foi de p<0.05.

Figura II: Carbonilação de proteínas em pulmões de ratos treinados (TR) e não-treinados (NtR) após exposição ao carvão mineral. Os procedimentos de exposição ao carvão e método de dosagem do dano oxidativo estão descritos no Material e Métodos. Os valores são apresentados em Média±EPM e os resultados foram expressos em nmol de carbonis/mg de proteínas. A diferença significativa entre os grupos controle* e grupo não-treinado# foram de p<0.05.

CAPÍTULO VI

DISCUSSÃO GERAL

Os objetivos dessa tese foram pautados na possível relação entre os efeitos do exercício físico e da suplementação de antioxidantes e as alterações nos parâmetros oxidativos pulmonares decorrentes da exposição ao pó de carvão mineral. Para tanto, partimos das seguintes evidências científicas previamente descritas na literatura:

a) A inalação e o depósito de partículas industriais sobre o tecido pulmonar provocam alterações sobre parâmetros bioquímicos oxidativos (Castranova et al., 2002). Mossman (2003) sugere que essas partículas, quando inaladas, ativam células fagocitárias alveolares, como macrófagos, e aumentam, concomitantemente, a produção de anion superóxido. Vários autores têm demonstrado que as Espécies Reativas de Oxigênio (ERO), após inalação de partículas industriais, são importantes mediadores de propriedades inflamatórias e da toxidade pulmonar (Mosmann e Churg, 1998; Borges et al. 1999), que incluem danos em células endoteliais, formação de fatores quimostáticos, recrutamento de neutrófilos, peroxidação lipídica e danos em DNA (Zhang et al., 1999), produção de TNF-α, IL-1β e formação de peroxidonitrito (Blackford et al., 1997; Dorger et al., 2002).

b) A suplementação de antioxidantes melhora a resposta de defesa oxidativa pulmonar (Repine et al., 1997). Os antioxidantes agem contra os efeitos deletérios e a propagação dos radicais livres. Além dos de origem endógena, os antioxidantes também podem ser acrescidos no organismo via alimentação, suplementação ou, ainda, como recurso terapêutico. Um dos principais antioxidantes mais utilizados na prática clínica e em estudos experimentais é a N-acetilcisteinea (NAC). Além da propriedade mucolítica, a NAC é um tiol que atua como um antioxidante por ser precursor da cisteína intracelular,

aumentando a produção de glutationa (Sprong et al., 1998). Vários estudos apresentados por Repine et al. (1997) sugerem que as propriedades antioxidantes da NAC estão associadas à diminuição do peróxido de hidrogênio, à diminuição da inativação de alfa-1-antitripsina mediada por HOCl e à diminuição de anormalidades em células polimorfonucleares, macrófagos alveolares, fibroblastos e células epiteliais. O tratamento com a NAC também atenua, em ratos, a hiperplasia celular induzida pelo cigarro (Heunks e Dekhuijzen, 2000).

c) O exercício físico de endurance melhora a resposta ao estresse oxidativo pulmonar. A associação entre exercício físico e estresse oxidativo tem sido objeto de muitos estudos, devido ao fato de o exercício físico aumentar a taxa de utilização de oxigênio e, concomitantemente, o aumento de radicais livres (Carmeli et al., 2000). Essa situação pode causar danos celulares e inflamação (Liu et al., 2000). Entretanto, os efeitos do aumento na produção de radicais livres estão diretamente relacionados ao tipo, duração e intensidade do exercício. Terblanche et al. (2000) sugerem que o exercício físico intenso leva ao estresse oxidativo, porém, Oh-Ishi et al. (1997) mostram que o treinamento físico de endurance melhora as defesas antioxidantes assim como a capacidade oxidativa muscular. Sobre a função pulmonar, estudos têm apontado uma redução significativa dos marcadores de estresse oxidativo após prática regular de exercício físico aeróbio (Heunks e Dekhuijzen, 1999; Polidori et al, 2000). Embora esses resultados tenham sido evidenciados em pacientes com DPOC, acredita-se que esses efeitos possam ser alcançados em outras gravidades pulmonares.

Com base nessas evidências, hipotetizamos que o carvão mineral e seus constituintes apresentam efeitos degenerativos similares a outras partículas industriais como asbestos e sílica sobre o tecido pulmonar e que a NAC e o exercício físico, como agentes farmacológicos e terapêuticos respectivamente, melhoram as respostas de defesas oxidativas pulmonares em animais previamente expostos ao carvão mineral.

Dessa forma, com o objetivo de avaliar o papel do carvão mineral como indutor de dano tecidual e oxidativo, estabeleceu-se um modelo animal de dano pulmonar por instilação traqueal. Os resultados desse estudo, apresentados no capítulo II, confirmam a hipótese de que o carvão mineral provoca alterações significativas sobre a estrutura pulmonar, as quais são mediadas por radicais livres. Embora o próprio carvão não seja um agente fibrogênico, acredita-se que outros elementos agregados em sua composição durante a extração, como a sílica e o ferro, por exemplo, depois de inalados, são responsáveis em alterar tanto a estrutura física quanto a fisiologia e bioquímica pulmonar.

Nós demonstramos que a exposição ao carvão mineral provoca uma resposta inflamatória pulmonar em duas fases específicas: uma fase aguda marcada por uma infiltração linfocitária e recrutamento de neutrófilos e uma fase crônica com presença predominante de macrófagos alveolares. A presença marcante de linfócitos e macrófagos não nos permite garantir a formação de processos fibrogênicos sobre o parênquima pulmonar, entretanto, embora não tenhamos especificado o tipo de linfócito presente, Gross e Hunninghake (2001) sugerem que elevada presença de linfócito Th2 demonstra uma possível resposta fibrogênica. Assim, é possível supor que a resposta linfocitária observada em nosso estudo possa induzir a uma fase crônica

subseqüente e levar a um processo inicial de resposta fibrótica na presença de carvão mineral.

Sobre a resposta bioquímica, observamos que a presença de partículas de carvão mineral altera substancialmente os marcadores de estresse oxidativo. Essa resposta pode estar diretamente associada com a resposta inflamatória. O aumento de fagócitos observado sobre o pulmão causa uma produção local de $O_2^{\bullet-}$ e formação de H_2O_2. Na presença de ferro, o H_2O_2 é convertido em radical hidroxil pela reação de Fenton (Vallyathan et al., 1998). De acordo com Kim et al. (2000), a presença de ferro em partículas minerais é o principal mediador da toxidade pulmonar. Adicionalmente, o $O_2^{\bullet-}$ é um inibidor da alfa-1-antripsina, uma glicoproteína que inibe a ação de várias proteases, o que pode causar destruição tecidual e enfisema pulmonar (Olszewer, 1999). A presença de macrófagos sobre o tecido pulmonar, observada nesse estudo, também, contribui para aumento na formação de radicais livres. Segundo Zhang et al. (1999), os macrófagos são fontes importantes de radicais livres e podem contribuir para o processo de danos celulares, principalmente de lipoperoxidação, após a exposição ao carvão mineral.

Com base nesses achados e nas evidências apontadas por outros estudos, constatamos que a lesão pulmonar induzida pelo carvão, no modelo proposto, é mediada pela ação de radicais livres.

A partir dessas constatações, elaboramos outro estudo em que o objetivo foi verificar se a administração de antioxidante (NAC) de forma isolada ou associada com deferoxamina (DFX) reduziria significativamente os

indicadores inflamatórios e danos oxidativos em pulmão de ratos expostos ao pó de carvão mineral.

Hipotetizamos que o acúmulo de peróxido de hidrogênio na presença de ferro, durante o processo inflamatório induzido pela exposição aguda ao carvão, poderia levar à formação da radical hidroxil. Adicionalmente, o uso isolado de NAC pode ter limitações e apresentar efeitos pro-oxidantes, provavelmente, pela sua fácil interação com ferro (Ritter et al. 2004). Dessa forma, o uso de deferoxamina (DFX), um quelante de ferro, poderia melhorar a resposta ao uso de NAC.

A NAC é considerada um importante agente terapêutico, utilizada comumente na prática clínica por apresentar propriedades capazes de manter a capacidade antioxidante pulmonar. NAC é um doador de tiois, que atua como precursor da cisteína intracelular, aumentando a produção de glutationa (GSH). Sugere-se que o potencial efeito da NAC é diminuir os níveis de H_2O_2, alterando o equilíbrio da capacidade oxidante-antioxidante pulmonar (Repine et al. 1997).

Os resultados desse estudo, apresentados no capitulo III, não confirmaram nossa hipótese de que o uso isolado de NAC poderia ter limitações na reposta inflamatória, por apresentar possíveis efeitos pro-oxidantes pela sua fácil interação com ferro. Os resultados ainda mostraram que a administração isolada da NAC ou associada com a DFX reduz similarmente a resposta inflamatória e o estresse oxidativo em ratos expostos à poeira de carvão mineral.

A resposta positiva ao uso da NAC pode estar associada a dois mecanismos propostos por Heunks e Dekhuijzen (2000): 1 - a suplementação

da NAC pode atuar com um scanveger de radicais livres resultando na formação de NAC dissulfida; 2 - a NAC pode exercer um importante efeito antioxidante por facilitar a biossíntese da glutationa (GSH).

É provável que a NAC possa estar diminuindo os efeitos oxidativos gerados pela exposição ao carvão, por ser precursora da cisteína intracelular, indispensável para a síntese de GSH (Sprong et al., 1998; Paterson et al., 2003; Ritter et al., 2004). A GSH, em sua forma reduzida, tem um importante papel no mecanismo de defesa do pulmão contra ataques de radicais livres (Smith et al., 1994; Zhang et al., 1999), por diminuir o conteúdo de H_2O_2 e alterar o equilíbrio da capacidade oxidante-antioxidante pulmonar (Repine et al., 1997). Dependendo do nível na célula, pode ser uma fonte importante contra respostas inflamatórias causadas por partículas industriais (Zhang et al., 1999).

No capítulo IV, objetivou-se estabelecer um protocolo de exercício físico, em esteira, para ratos e avaliar a associação entre exercício físico, danos oxidativos e defesas enzimáticas antioxidantes. Os resultados desse estudo mostraram que a atividade enzimática da citrato sintase aumentou significativamente em resposta ao programa de treinamento, apontando a influência do exercício físico na adaptação oxidativa do músculo esquelético. Os resultados ainda sugerem uma adaptação diferenciada nos músculos esqueléticos contra os danos oxidativos induzidos pelo exercício físico. Embora esses resultados sejam relevantes, outros estudos apontam resultados contrários. Segundo Leek et al. (2001), as diferenças entre estudos podem estar relacionadas com alguns parâmetros: a) tipos de fibras musculares utilizadas nos ensaios; b) protocolos de treinamento diferentes (intensidade,

duração, freqüência e tipo do exercício); c) diferentes métodos para determinação da atividade enzimática e d) adaptações moleculares diferenciadas na regulação da CS durante e após o treinamento físico.

No capítulo V, objetivou-se avaliar os efeitos do exercício físico regular sobre os parâmetros de danos oxidativos pulmonares em lipídios de membrana e proteínas, após inalação de carvão mineral. Pressupõe-se que o exercício físico possa ser, também, um importante agente capaz de atenuar os efeitos deletérios da lesão pulmonar induzida pelo carvão mineral. Isso se deve a diversos estudos que têm apontado que a prática regular de exercício físico aeróbio de baixa a moderada intensidade contribui significativamente para tornar mais eficiente o sistema de defesa antioxidante, estabelecendo um equilíbrio entre os danos induzidos pelas ERO e os sistemas de reparos antioxidantes (Leaf et al., 1999; Power et al., 1999, Carmeli et al., 2000). Além disso, outros estudos têm demonstrado aumento na atividade de enzimas antioxidantes decorrentes do treinamento físico aeróbio (JI, 1999; Oh-Ishi et al.,1997; Rádak et al., 1999).

Embora a literatura não apresente nenhum estudo que estabeleça a relação entre a prática de exercício físico e os danos pulmonares causados por carvão mineral, supõe-se que, em se tratando de lipoperoxidação, a redução observada nesse estudo, após o programa de treinamento, decorre do aumento nos níveis de enzimas antioxidantes, embora Oh-Ishi e colaboradores (1997) sugiram que esse efeito esteja mais relacionado com a capacidade do tecido em resistir aos efeitos da lipoperoxidação induzida pelo próprio exercício físico. Sobre a carbonilação de proteínas após treinamento físico, a literatura apresenta resultados conflitantes com nossos achados. Isso decorre,

possivelmente, de quatro aspectos: primeiro, o uso ou não de agentes estimuladores de danos, como o carvão, o que pode representar repostas diferenciadas induzidas pelo exercício físico; segundo, a metodologia utilizada para determinar os níveis de oxidação protéica; terceiro, o tipo, duração, freqüência e intensidade do exercício utilizado; quarto, é possível que essas diferenças estejam relacionadas à redução na taxa do turnover de proteínas.

A reposta oxidativa ao exercício físico é determinada por vários fatores que, por um lado, podem gerar a formação de ERO capazes de causar danos celulares e inflamação (Liu et al., 2000) e, por outro lado, podem tornar mais eficiente o sistema de defesa antioxidante, estabelecendo um equilíbrio entre os danos induzidos pelas ERO e os sistemas de reparos antioxidantes (Carmeli et al., 2000).

Os resultados apresentados neste estudo apontam evidências de que o exercício físico regular melhora a resposta ao dano oxidativo induzido pela inalação de partículas de carvão mineral. Porém, convém ressaltar, na comparação com outros estudos, que devem ser observadas as características do treinamento físico realizado, quais sejam: tipo, freqüência, intensidade e duração do exercício. Essas variáveis são determinantes para a análise dos resultados alcançados.

CAPÍTULO VII

CONCLUSÕES

Atentos aos objetivos iniciais desta tese, chegamos às seguintes conclusões:

1) O carvão mineral provoca alterações significativas na estrutura pulmonar, as quais são mediadas por radicais livres.
2) A administração de N-acetilcisteína (NAC) de forma isolada ou associada com deferoxamina (DFX) reduz significativamente os indicadores inflamatórios e os danos oxidativos em pulmão de ratos expostos ao pó de carvão mineral.
3) O protocolo de exercício utilizado foi suficiente para alterar marcadores de aptidão aeróbia, e ainda, provocar uma adaptação oxidativa muscular diferenciada acompanhada por uma modulação, também diferenciada, das defesas antioxidantes enzimáticas.
4) O exercício físico regular de baixa a moderada intensidade reduz os marcadores de danos oxidativos pulmonares em lipídios de membrana e proteínas, após inalação de carvão mineral.

PERSPECTIVAS

Os resultados apresentados nessa tese mostraram que o uso de N-acetilcisteína e a prática regular de exercício físico reduzem significativamente os indicadores de danos oxidativos sobre o tecido pulmonar de ratos após a inalação de carvão mineral. Embora a consistência dos resultados permita sugerir o uso desses agentes terapêuticos no tratamento de danos pulmonares induzidos pelo carvão em animais, pretende-se:

1) Dosar outros antioxidantes como GSH e GPX;

2) Determinar a expressão gênica das enzimas antioxidantes associadas ao exercício por rt-pcr;

3) desenvolver estudos similares com seres humanos;

4) fazer a associação da suplementação de NAC com o treinamento físico.

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ANEXOS

Protocolo de Avaliação Anatomopatológica

1. Brônquios:
1.1 Alterações estruturais
( ) normais ( ) dilatados ( ) avaliação prejudicada ( ) estenosados
1.2- Alterações Inflamatórias
( ) presente ( ) neutrocitária ( ) mononucleares ( ) plasmocitária ( ) linfocitária
1.3 Folículos linfóides
( ) ausentes ( ) presentes
1.4 Alterações vasculares
( ) ausentes ( ) presentes
1.5 Fibrose
( ) ausente ( ) presente
2. Septos alveolares
2.1 Alterações estruturais
( ) normais ( ) alterados
2.2 Alterações inflamatórias
( ) neutrocitária ( ) linfocitária ( ) plasmocitária ( ) histiocitária
( ) leve ( ) moderada ( ) acentuada
2.3 Fibrose intersticial
( ) ausente ( ) presente
( ) colagênica ( ) não-colagênica
( ) leve ( ) moderada ( ) acentuada
2.4 granulomas
( ) ausentes ( ) imunológicos sarcóides ( ) imunológicos tuberculóides ( ) de tipo corpo estranho
2.5 Vasculite
( ) ausente ( ) presente
2.6 Outras alterações:

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3. Interstício
3.1 Alterações estruturais
( ) normais ( ) alterados
3.2- Alterações inflamatórias
( ) neutrocitária ( ) linfocitária ( ) plasmocitária ( ) histiocitária
( ) leve ( ) moderada ( ) acentuada
3.3 Fibrose intersticial
( ) ausente ( ) presente
( ) colagênica ( ) não-colagênica
( ) leve ( ) moderada ( ) acentuada
3. 4 granulomas
( ) ausentes ( ) imunológicos sarcóides ( ) imunológicos tuberculóides ( ) de tipo corpo estranho
3.5 Vasculite
( ) ausente ( ) presente
3.6 Outras alterações

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4. Luz alveolar
( ) sem alterações

( ) descamação de macrófagos
( ) neutrófilos
( ) proliferação fibroblástica
( ) células gigantes
( ) ____________________

5. Pleura
( ) sem alterações
5.1 hiperplasia mesotelial
( ) focal ( ) difusa
5.2 Fibrose
( ) focal ( ) difusa
( ) colagênica ( ) não-colagênica
( ) leve ( ) moderada ( ) acentuada
5.3 Granulomas
( ) ausentes ( ) imunológicos sarcóides ( ) imunológicos tuberculóides ( ) de tipo corpo estranho
6. Em presença de granulomas
6.1 pesquisa de bacilos alcool ácido resistentes
( ) negativa ( ) positiva
6.2 pesquisa de fungos
( ) negativa ( ) positiva

PROTOCOLO DE TREINAMENTO PARA RATOS

Semana	Dia	Velocidade(km/h)	Grau (%)	Duração (min)
1(adaptação)	1-5	0,6	0	5-10
1	1-2	0,6	0	15
	3-5	0,6	0	20
2	1-2	0,6	0	25
	3-5	0,6	0	30
3	1-5	0,6	0	40
4	1-2	0,8	0	25
	3-5	0,8	0	30
5	1-5	0,8	0	40
6	1-5	0,8	0	40
7	1-5	0,8	5	30
8	1-5	0,8	5	40
9	1-5	0,8	5	50
10	1-5	1	10	30
11	1-5	1	10	40
12	1-5	1	10	50

Protocolo adaptado de Brooks: para ratos idosos ou com problemas cardiorrespiratório.

Importante: Fazer um aquecimento (5 min) com uma velocidade inferior.
Fazer um teste de esforço com carga progressiva pré e pós-treinamento e coletar sangue antes e após cada teste.

Teste de Esforço: Iniciar o teste numa velocidade de 0,6 km/h e incrementar a carga com 0,2 km/h a cada 2 minutos. Interromper o teste na exaustão do animal ou quando atingir uma velocidade de 1,6 km/h (15 minutos). (adaptado de Gava et al.1995).