APÊNDICE A – Declaração da Comissão de Ética para cuidado e uso de animais experimentais

UNIVERSIDADE DO ESTADO DO RIO DE JANEIRO
INSTITUTO DE BIOLOGIA ROBERTO ALCANTARA GOMES

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS

CERTIFICADO

Certificamos que o Protocolo nº **CEA/023/2009** sobre **"Efeitos do extrato de própolis sobre a resposta inflamatória pulmonar induzida por fumaça de cigarro em camundongos"**, sob a responsabilidade de **Luís Cristóvão de M. S. Porto,** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética Para o Cuidado e Uso de Animais Experimentais do Instituto de Biologia Roberto Alcântara Gomes da UERJ (CEA), em **08/04/2009.** Este certificado expira em **08/04/2013.**

Rio de Janeiro, 08 de Abril de 2009.

Prof. Israel Felzenszwalb
CEA/IBRAG/UERJ

Israel Felzenszwalb
Diretor
IBRAG - UERJ
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Profa. Patricia Cristina Lisboa
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Profª Adj. do Depto. Ciências Fisiológicas / IBRAG / UERJ
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COMFERE COM O ORIGINAL
Lei nº 5069/2007.
Ass: _____/Mat.: _____

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APÊNDICE B – Carta de submissão do artigo do mestrado para a revista Evidence-based Complementary and Alternative Medicine

Evidence-Based Complementary and Alternative Medicine **15 de outubro de 2011**
<lamiaa.younes@hindawi.com> **17:30**
Para: lcporto@uerj.br
Cc: lamiaa.younes@hindawi.com, alan.alawave@gmail.com, thinhodabio@gmail.com, manuellalanzetti@yahoo.com.br, karlampires@gmail.com, retiscoski@hotmail.com, ari@correio.nppn.ufrj.br, ajorge@correio.nppn.ufrj.br, samuelv@ufrj.br

Dear Prof. Porto,

The Research Article titled "ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIONS OF PROPOLIS ON MICE EXPOSED TO SHORT-TERM CIGARETTE SMOKE," by Alan Aguiar Lopes, Thiago dos Santos Ferreira, Manuella Lanzetti, Karla Maria Pires, Renata Tiscoski Nesi, Ari Miranda Silva, Antonio Jorge Ribeiro da Silva, Samuel Santos Valença and Luís Cristóvão Porto has been received and assigned the number 280303.

The special issue for which the paper is being processed is
"Evidence-Based CAM for Respiratory Health"

An editor will be assigned to handle the review process of your manuscript, and he/she will inform you as soon as a decision is reached.

All authors will receive a copy of all the correspondences regarding this manuscript. However, only the submitting author will be able to upload any revisions to the journal's Manuscript Tracking System.

Thank you for submitting your work to Evidence-Based Complementary and Alternative Medicine.

Best regards,

Lamiaa Younes
Editorial Office
Hindawi Publishing Corporation
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APÊNDICE C – Carta de aceitação de publicação de artigo para a revista Food International Research

> From: assantana@usp.br
> To: samuelv@ufrj.br
> Date: Thu, 21 Jun 2012 21:14:37 +0100
> Subject: Your Submission
>
> Ms. Ref. No.: FOODRES-D-11-01836R2
> Title: Ready-to-drink matte® tea shows anti-inflammatory and antioxidant properties on a cigarette smoke exposure model
> Food Research International
>
> Dear samuelv,
>
> I am pleased to confirm that your paper "Ready-to-drink matte® tea shows anti-inflammatory and antioxidant properties on a cigarette smoke exposure model" has been accepted for publication in Food Research International.
>
> Thank you for submitting your work to this journal.
>
> With kind regards,
>
> A.G. Marangoni, PhD
> Editor-in-Chief
> Food Research International
>

APÊNDICE D – Artigo aceito para publicação durante ao período de mestrado

Int. J. Exp. Path. (2012)

ORIGINAL ARTICLE

Time course of inflammation, oxidative stress and tissue damage induced by hyperoxia in mouse lungs

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Summary

In this study, our aim was to investigate the time courses of inflammation, oxidative stress and tissue damage after hyperoxia in the mouse lung. Groups of BALB/c mice were exposed to 100% oxygen in a chamber for 12, 24 or 48 h. The controls were subjected to normoxia. The results showed that IL-6 increased progressively after 12 ($P < 0.001$) and 24 h ($P < 0.001$) of hyperoxia with a reduction at 48 h ($P < 0.01$), whereas TNF-α increased after 24 ($P < 0.001$) and 48 h ($P < 0.001$). The number of macrophages increased after 24 h ($P < 0.001$), whereas the number of neutrophils increased after 24 h ($P < 0.01$) and 48 h ($P < 0.001$). Superoxide dismutase activity decreased in all groups exposed to hyperoxia ($P < 0.01$). Catalase activity increased only at 48 h ($P < 0.001$). The reduced glutathione/oxidized glutathione ratio decreased after 12 h ($P < 0.01$) and 24 h ($P < 0.05$). Histological evidence of lung injury was observed at 24 and 48 h. This study shows that hyperoxia initially causes an inflammatory response at 12 h, resulting in inflammation associated with the oxidative response at 24 h and culminating in histological damage at 48 h. Knowledge of the time course of inflammation and oxidative stress prior to histological evidence of acute lung injury can improve the safety of oxygen therapy in patients.

Keywords
hyperoxia, inflammation, lung, mouse, oxidative stress

Acute lung injury (ALI) affects a large number of patients worldwide, with reported mortality rates of 35–40% (Rubenfeld & Herridge 2007). Many patients with ALI require oxygen supplementation to maintain adequate tissue oxygenation, leading to hyperoxia (Fisher & Beers 2008). However, exposure to hyperoxia can have pathological effects, such as lung inflammation and oedema accompanied by epithelial and endothelial cell death, suggesting that oxygen supplementation, although necessary, may potentially perpetuate or exacerbate ALI (Bhandari *et al.* 2006; Bhandari 2008). Paradoxically, hyperoxia may cause ALI and damage to components of the extracellular matrix (Murray *et al.* 2008). Moreover, hyperoxia has been linked to the production of reactive oxygen species (ROS) and subsequent oxidative stress (Huang *et al.* 2009). Reactive oxygen species are important mediators in ALI, attacking biological molecules and causing lipid peroxidation, protein oxidation and DNA breakage (Papaiahgari *et al.* 2006).

Under physiological conditions, living organisms maintain a balance between the formation and removal of ROS (Owuor & Kong 2002). The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase and non-enzymatic antioxidants, such as α-tocopherol, vitamin-C, carotenoids and the glutathione system, all prevent the formation of toxic levels of ROS. Oxidative stress occurs when the generation of ROS in a system exceeds the system's capacity to neutralize and eliminate the ROS (Sies 1997). This imbalance can result from a deficiency of the

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antioxidant system owing to decreased synthesis or increased consumption linked to an over-abundance of ROS from an environmental or behavioural stressor, such as hyperoxia (Ghezzi *et al.* 2005). In addition, hyperoxia incites the release of a large number of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Ogawa *et al.* 2007). The exact mechanisms of hyperoxia-induced toxicity in the lung are complex, but evidence suggests that inflammation and oxidative stress are important co-mediators of ALI (Reddy *et al.* 2009).

We have previously demonstrated the effects of short-duration hyperoxic exposure and different doses of hyperoxia on Wistar rat lungs. In the first study, after 90 min of exposure, hyperoxia induced alterations in rat lung parenchymas, although no structural damage was evident; inflammatory cell influxes, extravasation of red blood cells and oedema were the most important alterations identified (Valenca Sdos *et al.* 2007). In the second study, Wistar rats exposed to 50% or 75% oxygen for 90 min did not exhibit lung alterations, whereas 100% oxygen for the same duration induced interstitial oedema and large numbers of red blood cells in the alveoli (Nagato *et al.* 2009). In this study, our aim was to investigate the time courses of inflammation, oxidative stress and tissue damage after hyperoxia in BALB/c mouse lungs. To achieve this goal, we used different periods of hyperoxia exposure: 12, 24 and 48 h. BALB/c mice were used in this study because this strain is sensitive to hyperoxia and presents an appropriate response to lung inflammation and damage (Ho 2002; Whitehead *et al.* 2006).

Materials and methods

Animals

Male BALB/c mice (8 weeks old; 20–24 g) were purchased from the Instituto de Veterinária – Universidade Federal Fluminense (Niterói, Brazil). The mice were housed in an environment-controlled room with constant 24-h light/dark cycle conditions (12-h light/12-h dark, lights on at 6 pm). The ambient temperature was maintained at 25 ± 2 °C, and the relative humidity was approximately 80%. The animals were provided with water and food (Purine Chow) *ad libitum*. The study was conducted according to the Brazilian Federal Guidelines for Laboratory Animal Use and Care (Brazilian Law 11,794 from 12/08/2008). The experimental design was approved by the Ethics Committee of the Instituto de Biologia Roberto Alcântara Gomes, with protocol number CEA/010/2009.

Hyperoxia protocol

The animals were exposed to hyperoxic conditions in an inhalation chamber (length = 30 cm, width = 20 cm and height = 15 cm) as previously described by our group (Valenca Sdos *et al.* 2007; Nagato *et al.* 2009). Oxygen was acquired from White Martins® (White Martins Praxair Inc., São Paulo, Brazil). The oxygen tank was coupled to the inhalation chamber using a silicone conduit. The mice were randomly divided into four experimental groups of ten animals each: the control group was exposed to normoxia; the 12-h group was exposed to 100% oxygen for 12 h; the 24-h group was exposed to 100% oxygen for 24 h; and the 48-h group was exposed to 100% oxygen for 48 h. The oxygen concentration in the chamber was monitored continuously using an oxygen cell (C3, Middlesbrough, UK). The animals were euthanized by cervical dislocation immediately after being exposed to hyperoxia. The blood was flushed from the pulmonary vasculature, and the lungs were then harvested. The right lung was set aside for histology, and the left lung was used to perform bronchoalveolar lavage (BAL) and to prepare tissue homogenates. This experimental design was repeated twice.

Bronchoalveolar lavage (BAL)

The left lung airspaces were washed with a buffered saline solution (500 µl) three times (final volume 1.2–1.5 ml). The BAL fluid was withdrawn and stored on ice. The total amount of mononuclear and polymorphonuclear cells was determined using a Zi Coulter counter (Beckman Coulter, Carlsbad, CA, USA). Differential cell counts were performed using cytospin preparations (Shandon, Waltham, MA, USA) stained with Diff-Quik. At least 200 cells per BAL sample were counted using the standard morphologic criteria.

Biochemical analyses

All chemicals were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA) unless otherwise specified. All measurements described below were taken on lung homogenates, unless otherwise specified, using a spectrophotometer (Beckman model DU 640; Fullerton, CA, USA) or a microplate reader (Bio-Rad model 550, Hercules, CA, USA). Superoxide dismutase activity was assayed by measuring the inhibition of adrenaline auto-oxidation as the absorbance at 480 nm (Bannister & Calabrese 1987). Catalase (CAT) activity was determined from the rate of decrease in the concentration of hydrogen peroxide, which was determined from the absorbance at 240 nm (Aebi 1984). Myeloperoxidase (MPO) activity was determined in BALs with hydrogen peroxide, hexadecyltrimethylammonium bromide and 3,3′,5,5′-tetramethylbenzidine by measuring the absorbance in a plate reader at 630 nm (Kuebler *et al.* 1996). The ratio of reduced glutathione (GSH) to disulphide glutathione (GSSG) was based on the reaction of GSH or GSSG with 5,5′-dithiobis-(2-nitrobenzoic acid), which produces the 2-nitro-5-thiobenzoate chromophore that absorbs at 412 nm (Rahman *et al.* 2006). To determine the GSSG levels, samples were treated with 2-vinylpyridine, which covalently reacts with GSH but not with GSSG. The level of malondialdehyde (MDA) was measured during an acid-heating reaction with thiobarbituric acid and was determined from the absorbance at 532 nm (Draper & Hadley 1990).

The presence of nitrite was determined in BALs using a method based on the Griess reaction (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthalenediamine dihydrochloride in water) followed by measuring the absorbance in a plate reader at 550 nm (Green *et al.* 1982). The presence of hydroxyproline (OH-proline) was determined using a colorimetric method based on the reaction of chloramine-T, perchloric acid and p-dimethylbenzaldehyde. The colour profile that developed was measured at 557 nm (Woessner 1961). The cytotoxicity was evaluated using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in BAL cells cultured with Dulbecco's modified Eagle's medium. After incubation, the absorbance was measured at 540 nm (Putnam *et al.* 2002). The total protein contents in the BALs and lung tissue homogenates were determined according to Bradford's method (Bradford 1976).

Cytokine analysis

The levels of pro-inflammatory mediators including tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) were measured in the lung homogenates using ELISA with the respective duo-antibody kits (PeproTech Inc., Rocky Hill, NJ, USA).

Zymography

Aliquots of lung homogenates (30 μg protein) were subjected to electrophoresis in 8% acrylamide stacking gel–7% acrylamide separating gel containing 1 mg/ml gelatin in the presence of sodium dodecylsulphate (SDS) under non-reducing conditions. After electrophoresis, the gels were washed twice with 2.5% Triton X-100, rinsed with water and incubated at 37 °C overnight in 50 mM Tris–HCl, 5 mM $CaCl_2$ and 2 nM $ZnCl_2$ (pH 8). The gels were stained with Coomassie blue and destained in a solution of 25% ethanol and 10% acetic acid. The matrix metalloproteinase (MMP)-2 and/or MMP-9 activities appeared as clear bands against the blue background. The molecular weights of the gelatinolytic bands were estimated using 30 μg of protein from a placental sample (Niu *et al.* 2000).

Western blotting

Aliquots of lung homogenates (30 μg protein) were subjected to electrophoresis through 8% acrylamide stacking gel–7% acrylamide separating gel in the presence of SDS under non-reducing conditions. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA). Rainbow markers (Amersham Pharmacia Biotech) were run in parallel to estimate the molecular weights. The membranes were blocked with Tween–TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.5% Tween-20) containing 2% bovine serum albumin and probed with a specific goat anti-mouse IL-6 primary antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After extensive washing in Tween-TBS, the PVDF membranes were incubated with biotin-conjugated donkey anti-goat IgG (1:1000) for 1 h and then incubated with horseradish peroxidase-conjugated streptavidin (1:1000) (Santa Cruz Biotechnology). The immunoreactive proteins were visualized using 3,3′-diaminobenzidine staining. The membranes were stained with Ponceau to estimate the total protein. No differences were identified among the groups.

Histology, morphometry and stereology

The left lung was ligated, and the right lung of each mouse was inflated by instilling 4% formalin buffer at 25 cm H_2O pressure for 2 min to avoid formalin leaks; the lungs were then ligated and removed. The inflated lungs were fixed for 48 h before being embedded in paraffin. Serial sagittal sections were obtained for histological, stereological and morphometric analyses. Five-micron-thick tissue sections were stained with Giemsa and haematoxylin and eosin (H&E). For the immunohistochemistry, the primary antibody used was goat anti-mouse TNF-α (Santa Cruz Biotechnology). For detection, a biotinylated secondary antibody and an avidin–biotin peroxidase with 3,3′-diaminobenzidine were used according to the manufacturer's instructions (Santa Cruz Biotechnology). After staining for TNF-α, the lung sections were counterstained with haematoxylin. Lung sections stained with an isotype control-matched primary antibody were used to confirm non-specific expression.

The total numbers of alveolar macrophages (AMs) and neutrophils (PMNs) were estimated by counting ten different fields in the Giemsa-stained sections (five random fields of two sections), which were analysed using a 40× objective lens on a video microscope (Carl Zeiss model Axiolab, Oberkochen, Germany) linked to a colour video camera (JVC model TK-C380, Tokyo, Japan) and a Sony Trinitron colour video monitor (model PVM-14N2U, London, UK). A total area of 0.77 mm^2 was analysed to determine the numbers of AMs and PMNs in each lung section, as previously described (Valenca *et al.* 2004; Menegali *et al.* 2009). The number of macrophages that were positive for TNF-α was estimated by counting ten different fields, as described above. The AMs positive for TNF-α were counted and divided by the total number of AMs (negative and positive) to obtain an immunohistochemical index (IHI) (Valenca & Porto 2008).

The lung parenchymas were quantified stereologically using a test system composed of 42 points in a known test area delineated by the forbidden line to avoid overestimating the number of structures. The test system was attached to a monitor connected to a microscope. The points (PP) that intercepted the alveolar septa [Vv lung parenchyma ($Vv_{[par]}$)] were counted and compared with the total number of points of a test system (PT). Therefore, the volume density (Vv) is equal to the PP divided by the PT (Davies 1991). Ten fields (five non-overlapping fields in two different sections) were randomly analysed using a 20× objective lens on

a video microscope, as described above. A total area of 1.54 mm^2 was analysed to determine the $Vv_{[par]}$ values in the H&E-stained sections. Two investigators took all of the measurements by counting blinded sections.

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). For continuous data, we used a one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test (for SOD, CAT, MDA, nitrite, OH-proline, protein, IL-6, TNF-α, BAL and morphometry results). For non-continuous data, we used the Kruskal–Wallis test followed by the Dunn's *post hoc* test (for GSH/GSSG, MTT and stereology results). In all instances, the significance level was set at 5% ($P < 0.05$).

Results

The time course of hyperoxia in relation to biochemical analysis

The biochemical markers of redox balance (SOD and CAT), lung injury (MPO), oxidative stress (GSH/GSSG), oxidative damage (MDA), nitric oxide action (nitrite), collagen breakdown (OH-proline) and fluid extravasation (protein) were measured in BALs or lung tissue homogenates, and the results are shown in Table 1. SOD activity decreased by approximately 30% ($P < 0.01$) after 12 h of hyperoxia and by 22% ($P < 0.01$) after 24 and 48 h compared with the control group. CAT activity increased by approximately 50% after 48 h of hyperoxia when compared with the control group ($P < 0.001$). MPO activity increased by approximately 52% ($P < 0.001$) after 24 h and by 32% ($P < 0.05$) after 48 h of hyperoxia compared with the control group. The GSH/GSSG ratio decreased by approximately 23% after 12 h ($P < 0.01$) and by 21% ($P < 0.05$) after 24 h of hyperoxia compared with the control group. Malondialdehyde increased by approximately 92% after 12 h ($P < 0.001$) and by 50% ($P < 0.05$) after 24 h of hyperoxia compared with the control group. Nitrite increased by approximately 41% after 12 h and by 48% after 24 h of hyperoxia ($P < 0.001$) compared with the control group. Hydroxyproline increased by approximately 48% after 12 h and 24 h of hyperoxia ($P < 0.001$) compared with the control group. Finally, the level of total proteins in the BALs decreased by 30% ($P < 0.05$) after 12 h and by 20% ($P < 0.05$) after 24 h of hyperoxia but increased by approximately 20% ($P < 0.05$) after 48 h compared with the control group. The level of total proteins in lung homogenates increased by 18% ($P < 0.05$) after 12 h and by 22% ($P < 0.01$) after 24 h of hyperoxia compared with the control group.

A biochemical marker of cytotoxicity (MTT level) was assayed using inflammatory cells recovered from BAL fluid. MTT decreased by approximately 50% ($P < 0.05$) after 24 and 48 h of hyperoxia compared with the control group (Figure 1).

The time course of hyperoxia in relation to MMP-2 and MMP-9 activities

MMP-2 and MMP-9 activities are shown in Figure 2. The lung homogenates from mice exposed to hyperoxia for 48 h exhibited increases in MMP-2 and MMP-9 activities compared with the control group.

The time course of hyperoxia in relation to IL-6 and TNF-α

IL-6 expression by western blotting is shown in Figure 3. The lung homogenates from mice exposed to hyperoxia

Table 1 Biochemical analysis of oxidative stress and inflammatory markers in BAL fluid or lung tissue homogenates from mice exposed to 100% oxygen for 12, 24 and 48 h

Group	Control	12 h	24 h	48 h
SOD activity (U/mg protein)	64.43 ± 2.70	45.40 ± 2.14**	50.20 ± 2.75**	50.30 ± 0.60**
CAT activity (U/mg protein)	0.68 ± 0.04	0.57 ± 0.03	0.64 ± 0.04	1.02 ± 0.06***
MPO† activity (mU/mg protein)	3.25 ± 0.05	3.43 ± 0.17	4.94 ± 0.27***	4.30 ± 0.24*
GSH/GSSG ratio	1.25 ± 0.06	0.96 ± 0.04**	0.98 ± 0.02*	1.11 ± 0.03
MDA (nM/mg protein)	0.14 ± 0.01	0.27 ± 0.02***	0.21 ± 0.02*	0.13 ± 0.01
Nitrite† (mM/mg protein)	86.48 ± 2.66	146.60 ± 5.39***	167.30 ± 11.83***	100.90 ± 2.96
OH-proline (ng/mg protein)	172.70 ± 11.15	255.10 ± 6.47***	257.4 ± 14.27***	160.70 ± 8.21
Protein† (μg/μl)	0.10 ± 0.00	0.07 ± 0.01*	0.08 ± 0.01*	0.12 ± 0.01*
Protein‡ (μg/μl)	6.85 ± 0.28	8.07 ± 0.15*	8.35 ± 0.20**	6.96 ± 0.30
IL-6 (ng/ml)	0.99 ± 0.10	3.10 ± 0.21***	3.58 ± 0.17***	1.89 ± 0.19**
TNF-α (ng/ml)	1.06 ± 0.06	1.29 ± 0.08	2.05 ± 0.09***	2.80 ± 0.13***

SOD, superoxide dismutase; CAT, catalase; MPO, myeloperoxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; OH-proline, hydroxyproline; IL-6, interleukin 6; TNF-α, tumour necrosis factor alpha; BAL, bronchoalveolar lavage.
†Measurements taken in BAL fluid.
‡Measurements taken in tissue homogenates.
Values are the means ± standard error of the mean. For statistical evaluation of SOD, CAT, MPO, MDA, nitrite, OH-proline, protein levels, IL-6 and TNF-α, we used a one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. For the GSH/GSSG ratio, we used the Kruskal–Wallis test followed by the Dunn's *post hoc* test. In all instances, the significance level was set at 5%. *$P < 0.05$ compared to the control group; **$P < 0.01$ compared to the control group; ***$P < 0.001$ compared to the control group. $N = 10$ per group.

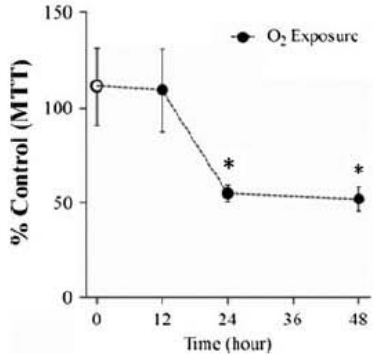

Figure 1 The cytotoxicity was evaluated using the MTT assay with the bronchoalveolar lavage cells cultured in DMEM. After incubation, the absorbance was measured at 540 nm. The cytotoxicity is represented by a black circle after 12, 24 and 48 h of hyperoxia or with a white circle after normoxia. The values are expressed as percentages relative to the control group by the mean ± standard error of the mean (SEM). The groups were tested for significance using the Kruskal–Wallis test followed by the Dunn *post hoc* test; the significance level was set as 5%. $^*P < 0.05$ compared with the control group ($N = 5$ per group). This test was performed in the first experiment only.

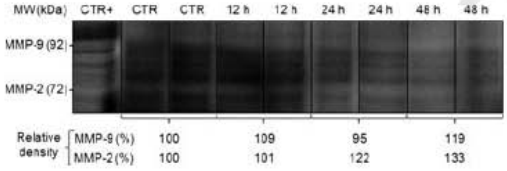

Figure 2 MMP-2 and MMP-9 activities in the lung tissue homogenates from mice exposed to normoxia (control group) or hyperoxia (12, 24 and 48 h). The samples (30 μg protein) were subjected to electrophoresis in a separating gel containing gelatin. After electrophoresis, the gel was stained with Coomassie blue, and the MMP-2 and/or MMP-9 activities appeared as clear bands against the blue background. The positive control was human placenta. The negative bands were assessed using densitometry ($N = 2$ per group). This test was performed in the first experiment only.

showed a progressive increase in IL-6 expression from 12 to 24 h, with a reduction at 48 h compared with the control group. IL-6 and TNF-α levels were determined using ELISA and are shown in Table 1. The lung homogenates from mice exposed to hyperoxia showed a progressive increase in IL-6 levels from 12 h ($P < 0.001$) to 24 h ($P < 0.001$), with a reduction at 48 h ($P < 0.01$) compared with the control group. We also observed a progressive increase in TNF-α levels from 24 h ($P < 0.001$) to 48 h ($P < 0.001$) compared with the control group.

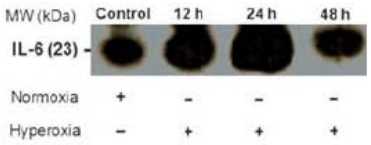

Figure 3 Western blotting for IL-6 expression in the lung tissue homogenates from mice exposed to normoxia (control group) or hyperoxia (12, 24 and 48 h). The samples were subjected to electrophoresis through a separating gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes, and the specific primary antibody for IL-6 was incubated with the membrane overnight. After incubation with the secondary antibody, DAB was used to detect the immunoreactive bands. This procedure was repeated two times. The membrane was stained with Ponceau red as the control ($N = 1$ per group). This test was performed in the first experiment only.

Table 2 Inflammatory scores of mouse lungs exposed to 100% oxygen for 12, 24 and 48 h

Groups	Alveolar congestion	Haemorrhage	Infiltration of leucocytes	Alveolar wall thickness
Control	–	–	–	–
12 h	–	–	2	–
24 h	2	–	2	–
48 h	2	3	3	2

Acute lung injury was scored in each sample (median of $n = 5$ for each group) according to the following four items: alveolar congestion, haemorrhage, infiltration of leucocytes in lung tissue and thickness of the alveolar wall/hyaline membrane formation. Each item was graded according to a 5-point scale: 0, minimal (little) damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, maximal damage.

The time course of hyperoxia in relation to lung histology, BAL, morphometry and stereology

Table 2 presents a score for the evaluation of ALI in accordance with what we have observed in lung histology. The alveolar septa and pulmonary capillaries were preserved both in the control group and in the mice exposed to hyperoxia for 12 h, with no evidence of ALI (Figure 4a,b). However, at 12 h, we observed an increase in the interstitial cellularity, mainly in terms of leucocytes. After 24 h of hyperoxia, low levels of septal oedema were observed (Figure 4c), and infrequently after 48 h, red blood cells in the alveoli could be seen together with alveolar macrophages and neutrophils (Figure 4d). The TNF-α staining was negligible in lung parenchymas after 12 h (Figure 5b) and 24 h (Figure 5c) of hyperoxia, but the decrease was much more evident after 48 h (Figure 5d) compared with the control group (Figure 5a). In contrast, TNF-α was more evident in the bronchial epithelia after 12 h of hyperoxia (Figure 6b) than after 24 h (Figure 6c) and 48 h (Figure 6d) compared with the control group (Figure 6a). The macrophage influx

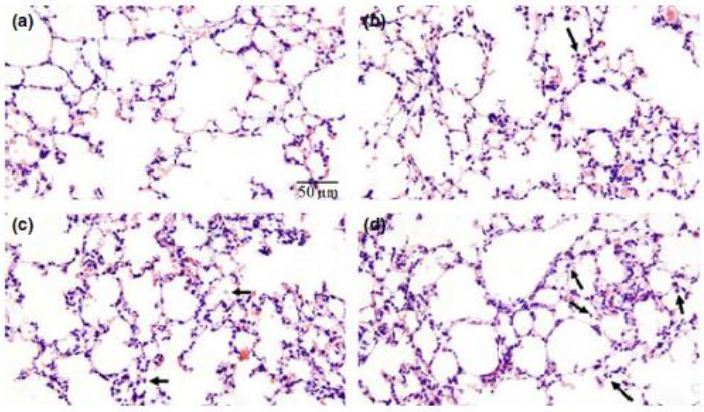

Figure 4 Lung histology in sections stained with haematoxylin and eosin. (a) Control mouse exposed to normoxia with alveolar septa and pulmonary capillaries preserved. (b) Mouse exposed to hyperoxia for 12 h with alveolar septa and pulmonary capillaries preserved, but with interstitial cellularity increased. (c) Mouse exposed to hyperoxia for 24 h with few alveolar macrophages and neutrophils. (d) Mouse exposed to hyperoxia for 48 h with alveolar macrophages and neutrophils. These images are representative of all mice analysed; $N = 10$ for all experiments. The arrows indicate leucocytes (alveolar macrophages or neutrophils). This test was performed in the first experiment only.

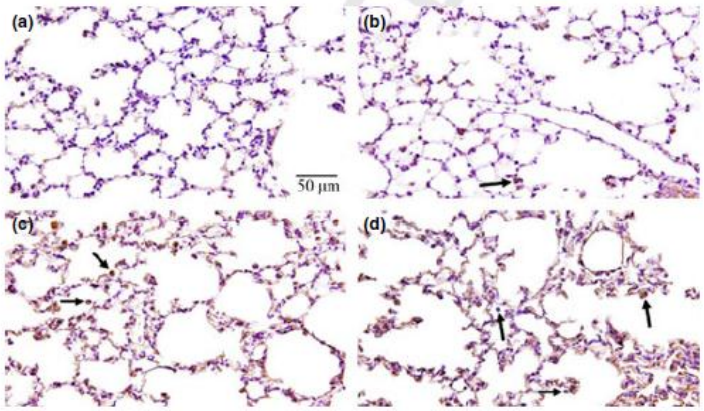

Figure 5 Lung histology in sections immunostained for TNF-α. (a) Lung parenchyma from a control mouse exposed to normoxia with reduced TNF-α expression. (b) Lung parenchyma from a mouse exposed to hyperoxia for 12 h with reduced TNF-α expression. (c) Lung parenchyma from a mouse exposed to hyperoxia for 24 h with a high TNF-α expression level, mainly in alveolar cells. (d) Lung parenchyma from a mouse exposed to hyperoxia for 48 h, with cells expressing TNF-α. These images are representative of all mice analysed; $N = 10$ for all experiments. The arrows indicate leucocytes (alveolar macrophages or neutrophils) expressing TNF-α. This test was performed in the first experiment only.

into the BAL was increased by 90% after 24 h ($P < 0.001$) and by 36% after 48 h ($P < 0.05$) of hyperoxia compared with the control group (Table 3). Although there was a trend towards the reduction in macrophages after 48 h of hyperoxia, the influx of PMNs into the BAL increased progressively by eightfold in 24 h ($P < 0.001$) and by 11-fold in 48 h ($P < 0.001$) compared with the control group (Table 3). The number of AMs was approximately 37% ($P < 0.05$) lower after 12 h of hyperoxia compared with the control ($P < 0.05$); after 24 h of hyperoxia, the AM numbers had increased to approximately 87% ($P < 0.001$) of the control values (Table 3). The number of PMNs was approximately 78% ($P < 0.001$) lower after 12 h of hyperoxia compared with the control, whereas the number of PMNs increased by approximately 77% ($P < 0.01$) after 24 h and by 238% ($P < 0.001$) after 48 h of hyperoxia compared with the

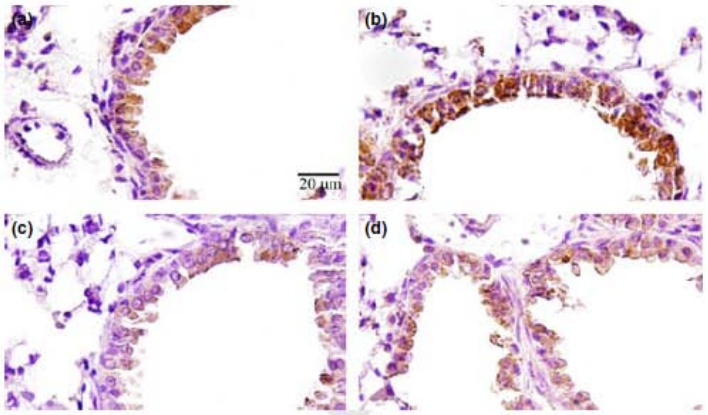

Figure 6 Lung histology in sections immunostained for TNF-α. (a) Bronchi from a control mouse exposed to normoxia with bronchial cells expressing TNF-α. (b) Bronchi from a mouse exposed to hyperoxia for 12 h with bronchial cells expressing TNF-α. (c) Bronchi from a mouse exposed to hyperoxia for 24 h with reduced TNF-α expression. (d) Bronchi from a mouse exposed to hyperoxia for 48 h with reduced TNF-α expression. These images are representative of all mice analysed; $N = 10$ for all experiments. This test was performed in the first experiment only.

Table 3 BAL, morphometry and stereology of mouse lungs exposed to 100% oxygen for 12, 24 and 48 h

Group	Control	12 h	24 h	48 h
IHI (%)	35.3 ± 2.9	47.7 ± 3.8	60.3 ± 2.3*	59.3 ± 7.2*
Macrophages ($\times 10^3$/ml)†	183.2 ± 7.6	205.8 ± 13.1	348.2 ± 14.4***	249.2 ± 14.8*
Neutrophils ($\times 10^3$/ml)†	30.1 ± 4.4	57.2 ± 4.6	257.0 ± 5.1***	348.4 ± 12.2***
Macrophages/mm²‡	197.5 ± 9.2	122.5 ± 11.9*	370.0 ± 20.2***	180.0 ± 21.2
Neutrophils/mm²‡	62.5 ± 3.2	13.7 ± 1.2***	111.0 ± 11.7**	211.0 ± 23.6***
Vv lung parenchyma (%)	50.5 ± 2.9	49.0 ± 3.3	57.2 ± 1.9	64.7 ± 3.2*

IHI, immunohistochemical index (AMs positive for TNF-α/total AMs) × 100; Vv, volume density; BAL, bronchoalveolar lavage.
†Cell counts performed in BAL by using a Zi Coulter counter; differential cell counts were performed on cytospin preparations.
‡Cell counts were performed in lung tissue by using a 40× objective lens on a video microscope linked to a colour video camera and a colour video monitor. Vv was performed using a 20× objective lens as described above.
For more details, see the Materials and Methods section. Values are the means ± standard error of the mean. For macrophages and neutrophils, we used a one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. For IHI and Vv, we used the Kruskal–Wallis test followed by the Dunn's *post hoc* test. In all instances, significance levels were set at 5%. $^*P < 0.05$ compared to the control group; $^{**}P < 0.01$ compared to the control group; $^{***}P < 0.001$ compared to the control group. $N = 10$ per group.

control group (Table 3). The IHI increased by 71% after 24 h and by 68% after 48 h of hyperoxia ($P < 0.05$) compared with the control group (Table 3). The volume density of the parenchyma ($Vv_{[par]}$) increased by 28% ($P < 0.05$) after 48 h of hyperoxia compared with the control group (Table 3).

Discussion

Here, we have demonstrated that the time course of hyperoxia-induced ALI is initiated coincident with inflammation and oxidative stress. We observed significant changes in the antioxidant system and markedly increased lipid peroxidation at 12 and 24 h of hyperoxia; however, at 48 h, all of these outcomes were normalized. This finding is unexpected because the majority of published data indicate that at high levels of hyperoxia, rodents exhibit high mortality by 72-96 h. The burden of inflammation and oxidative stress was progressive between 12 and 24 h of hyperoxic exposure, although a reduction in some parameters (e.g. SOD, MPO, GSH/GSSG ratio, MDA, nitrite, OH-proline, protein in the lung tissue, IL-6 and macrophage counts) was evident after 48 h. Interestingly, after 48 h of hyperoxia, we observed histological evidence of lung damage. Therefore, we suggest that lung tissue damage induced by hyperoxia, observed mainly at 48 h, is preceded by an inflammatory and oxidative response (observed moderately at 12 h and strongly at 24 h). The novel findings in this study are the antioxidant enzyme activities and the concomitant inflammatory response together with the oxidative response. We cannot say from the experiments conducted here whether the inflammatory or the oxidative response occurs first; however, owing to the inflammatory cell influxes in parallel with TNF-α and IL-6 detection after 24 h compared with early redox imbalance (SOD, CAT, MPO and MDA) and reduced cells (AMs and PMNs counted in the lung tissue) observed at 12 h, we suggest that the oxidative stress is present before any inflammatory cellular events.

Our study established that oxidative stress is already detectable after 12 h of hyperoxia. In these experiments, it was

likely that ROS acted as inflammatory mediators and contributed to the lung damage via both lipid peroxidation and protein and DNA oxidation (Lee & Choi 2003). Reactive oxygen species accumulation was progressive within the endothelial cells of the lung capillaries during the exposure of the mice to hyperoxia (Brueckl *et al.* 2006). In this report, SOD and CAT activities were evaluated to understand their contributions to the redox imbalance during the time course of hyperoxia. A reduction in SOD activity results in lower levels of O_2^- dismutation; consequently, there is a greater amount of free O_2^- contributing to oxidative stress (Levy *et al.* 2001; Oury *et al.* 2002). Interestingly, there were no differences in CAT activities after 12 and 24 h in the hyperoxia group compared with the control group. A later increase in CAT activity, observed after 48 h, may be related to the influxes of PMNs because these cells express large amounts of CAT (Rister & Baehner 1977). Additionally, the increased MPO activity in the BAL fluid occurred concurrently with the high interstitial cellularity observed in the lung histology. However, the leucocytes in the alveolar interstitium were not discriminated here. Additionally, we observed a few AMs and PMNs in the BAL fluid at 12 h of hyperoxia, but this result is relevant only in the alveolar space and may represent an apoptosis mechanism of these cells during the first 12 h of hyperoxia exposure but not after. Finally, IL-6 expression and protein levels were increased after 12 and 24 h of hyperoxia, in parallel with the interstitial cellularity. The reduction in IL-6 expression and protein levels observed after 48 h of hyperoxia, with a concomitant reduction in several parameters of inflammatory and oxidative responses, could be related to lung damage processes (Powell *et al.* 1992). The MTT assay performed with bronchoalveolar cells highlighted a toxic effect of hyperoxia at 24 and 48 h; this result is also compatible with the lung damage observed mainly at 48 h. We did not discard cell deaths at 24 and 48 h as a direct effect of hyperoxia, but, in contrast, we counted BAL cells. MTT appears to indicate cytotoxicity rather than viability in this case.

We observed a thickening of the alveolar septa, which was already distinct at 24 and 48 h of hyperoxia. Nevertheless, after 48 h, we observed red blood cells in the alveoli; although we did not investigate the alveolar-capillary barrier, the level of total proteins in the BAL fluid increased over the same time period. We observed that the level of total proteins in the lung tissue increased at 12 and 24 h, whereas the level of total proteins in the BAL fluid decreased over the same time period of hyperoxia. However, at 48 h of hyperoxia, we observed a decrease in the level of total proteins in the lung tissue. These results confirm other reports of increased lung epithelial permeability (Bhandari 2008). Endothelial damage, alveolar oedema and haemorrhage are other hallmarks of ALI that have been reported in previous studies (Valenca Sdos *et al.* 2007; Bhandari 2008; Fisher & Beers 2008; Nagato *et al.* 2009); some of these pathologies were identified in this study in mice exposed to hyperoxia for 24 and 48 h. Interestingly, we observed a reduction in the GSH/GSSG ratio after 12 and 24 h of hyperoxia, when no histological evidence of lung damage was noted, although a high interstitial cellularity was already present. Reduced glutathione is the major cellular thiol antioxidant and redox recycler and is more concentrated in the epithelial lining fluid than in the plasma (Rahman *et al.* 2005). GSH has an important protective role in the alveoli and intracellularly in epithelial cells (Rahman 1999). Several studies have suggested that GSH homoeostasis may play a central role in the maintenance of the integrity of the lung airspace–epithelial barrier, an observation supported by the report that decreased levels of GSH in epithelial cells lead to the loss of the barrier function and an increased permeability (Rahman *et al.* 2001). In this sense, the protein content of the BAL fluid increased after 48 h of hyperoxia, in parallel with the histological evidence of ALI. Although the GSH/GSSG ratios in the hyperoxic and control groups were similar after 48 h, the mechanism of the cellular events culminating in lung tissue damage at 48 h was already detected at 12 h. Finally, the cytotoxicity caused by hyperoxia was noted at 24 and 48 h, with the BAL fluid cells from the hyperoxia-exposed mice exhibiting a 50% survival rate compared with the control cells.

We also evaluated the time course of hyperoxia relative to the oxidative damage parameters. The redox imbalance (SOD and CAT) associated with the subsequent PMN influxes occurred concomitantly with the increased levels of extracellular matrix MMP-2 and MMP-9 activities. We also observed lung immunostaining and the levels of TNF-α during the time course of hyperoxia. Surprisingly, TNF-α labelling in the bronchial epithelia was observed at a different time than TNF-α labelling in the lung parenchymas and AMs was observed. The discrepancy in the TNF-α labelling between the bronchi and lung parenchymas could be explained by the increase in the number of leucocytes in the alveolar space and the interstitium. Thus, TNF-α, acting as a pro-inflammatory cytokine, could be produced and released in the lung structures that are directly affected by the hyperoxia exposure. Moreover, we did not observe bronchial damage (Figure 6). The MDA content was already increased at 12 and 24 h after hyperoxia exposure. We suggest that inflammation, which is represented by TNF-α immunolabelling and protein levels, and oxidative stress, which is quantified using the GSH/GSSG ratio, contributed to the oxidative damage. Nevertheless, these findings are in accordance with the observation in rats that MDA levels increased gradually with the duration of exposure to 100% oxygen for 10, 30 and 90 min, concomitant with the levels of inflammatory cells (Valenca Sdos *et al.* 2007). Additionally, we demonstrated previously that oxidative damage is related to the oxygen concentration (50, 75 and 100%) at 90 min. Finally, the nitrite content (a product of the nitric oxide radical) was increased in the BAL fluid after 12 and 24 h of hyperoxia, as reported by another study that used nitrite as a marker of inflammation in ALI (Robbins *et al.* 1995).

Our study has some limitations. (i) Mice are not people. Many results regarding inflammation and oxidative stress can be found in the literature, but it is unclear which are feasible to be realized in clinical practice in humans. Some

analyses described here could be performed using the plasma (Smith *et al.* 1993; Garcia-de-la-Asuncion *et al.* 2011), in biopsies (Stellingwerff *et al.* 2005), using sputum (Takasaki & Ogawa 1999) and using samples from bronchoscopy (Mousavi *et al.* 2011). Future clinical studies that measure some of these parameters under the ALI + hyperoxia conditions analysed here can confirm (or refute) the findings of our manuscript and those of others, thus allowing a more effective treatment guidance. In contrast, other analyses related to inflammation and oxidative stress in experimental models of hyperoxia were not performed here such as a haeme oxygenase-1 (Siner *et al.* 2007), nitric oxide synthase 2 (iNOS) (Bhandari *et al.* 2011), NADPH oxidase (Carnesecchi *et al.* 2009), tumour growth factor beta (TGF-beta) (Alejandre-Alcazar *et al.* 2007) and NRF2 (Reddy *et al.* 2009). (ii) The decreases observed in most of the parameters measured in Table 1 are likely due to the changes in endothelial cell permeability and protein leakage into the lung tissues and alveolar spaces (as verified by the BAL protein contents). The fluid moving into the lung is 'plasma-like' in nature and affects the total protein contents of the lung tissues and the BALs. This fluid movement results in larger denominators for most of the parameters and thus smaller values at 48 h; however, this movement cannot be prevented. (iii) Western blotting and zymography were performed with only a few samples, and loading controls were not adequately provided (for western blots, we used Ponceau red staining). (iv) Although it is not exceptionally novel or mechanistic, the study of the time course–dependent endpoints in a single report increases our understanding of the complexity of hyperoxic injury.

In conclusion, oxygen is necessary and important for the treatment for illnesses associated with hypoxia. However, oxygen therapy can still be improved in terms of identifying safer exposure limits to avoid perpetuating and exacerbating ALI or becoming the cause of ALI. Although our study was performed in mice, the literature from human studies confirms our findings of the time course of hyperoxia-induced lung injury. Alterations in lung histology, such as alveolar size and thickening of the alveolar septa at 24 h, which subsequently increased by 48 h, appear to be temporary; however, these alterations are important evidence of damage after ALI. Because there are wide-ranging clinical uses for oxygen in the treatment of individuals worldwide, investigations of the time course of inflammation and oxidative stress prior to the histological evidence of ALI can help us to improve the safety of oxygen delivery to patients.

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APÊNDICE E – Artigo publicado durante ao período de mestrado - I

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RESEARCH ARTICLE

Low dose of fine particulate matter (PM2.5) can induce acute oxidative stress, inflammation and pulmonary impairment in healthy mice

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Abstract
Air pollution is associated with morbidity and mortality induced by respiratory diseases. However, the mechanisms therein involved are not yet fully clarified. Thus, we tested the hypothesis that a single acute exposure to low doses of fine particulate matter (PM2.5) may induce functional and histological lung changes and unchain inflammatory and oxidative stress processes. PM2.5 was collected from the urban area of São Paulo city during 24 h and underwent analysis for elements and polycyclic aromatic hydrocarbon contents. Forty-six male BALB/c mice received intranasal instillation of 30 µL of saline (CTRL) or PM2.5 at 5 or 15 µg in 30 µL of saline (P5 and P15, respectively). Twenty-four hours later, lung mechanics were determined. Lungs were then prepared for histological and biochemical analysis. P15 group showed significantly increased lung impedance and alveolar collapse, as well as lung tissue inflammation, oxidative stress and damage. P5 presented values between CTRL and P15: higher mechanical impedance and inflammation than CTRL, but lower inflammation and oxidative stress than P15. In conclusion, acute exposure to low doses of fine PM induced lung inflammation, oxidative stress and worsened lung impedance and histology in a dose-dependent pattern in mice.

Keywords: Particulate matter; PM2.5; lung injury; oxidative stress; respiratory mechanics

Introduction

Particulate matter (PM) is a heterogeneous mixture of gases, liquid and solid particles of different origins and sizes in suspension in the air, keeping close physical and chemical interaction. PM is classified as coarse (2.5–10 µm aerodynamic diameter, PM10), fine (0.1–2.5 µm aerodynamic diameter, PM2.5), and ultrafine (≤0.1 µm aerodynamic diameter) (Donaldson et al., 2001).

Fine particles remain suspended in the atmosphere for a long time (days and weeks), disperse uniformly, and deposit in the extrathoracic airways or, depending on air flow and diffusion, penetrate deeper into the smaller airways and alveoli, where they can remain for weeks or months. Particles can be eliminated by the mucociliary system in the conductive airways and/or undergo phagocytosis by macrophages, when deposited in the alveolar regions (Donaldson & Stone, 2003). Moreover, components leached from PM2.5 can enter the circulation and modify circulatory homeostasis, thus increasing the risk factors for adverse cardiovascular events, such as arrhythmias, sudden death, increased plasma viscosity, change in blood parameters such as fibrinogen levels or red blood cells counts (Seaton et al., 1999; Stone & Godleski, 1999; Gardner et al., 2000; Schwartz, 2001; Kodavanti et al., 2002).

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Saldiva et al. (1992a) observed that chronic exposure to the air of São Paulo significantly compromises the properties of the mucociliary defense and predisposes experimental animals to respiratory infection. Acutely, increases in PM2.5 concentration are associated with exacerbation of asthmatic bouts and respiratory symptoms, as well as with higher hospital admissions by respiratory causes, such as rhinitis, sinusitis, bronchitis, asthma and pneumonia.

The mechanisms involved in the relationship between PM and adverse health effects are not well understood. Nevertheless, it is believed that an association between inflammatory process and oxidative stress exists (Donaldson & Tran, 2002). PM inhalation or instillation promotes inflammatory responses in animals and humans (Clarke et al., 1999; Ghio & Devlin, 2001) characterized by cytokine release, increased oxidative stress and vascular permeability with concomitant neutrophil recruitment (Li et al., 1996), as well as increased expression of genes related to NF-κB activation, including TNF-α, TGF-β and IL-6 (Shukla et al., 2000).

Considering that exposure to high or even daily allowed doses of PM2.5 can induce pulmonary dysfunction as well as systemic oxidative damage, we tested the hypothesis that acute instillation of low doses of PM2.5 may trigger pulmonary alterations in mice, mainly at the lung periphery. To that end, central airways and lung periphery mechanics and histology as well as inflammatory and biochemical markers were analyzed after exposure to PM2.5.

Materials and methods

PM sampling

PM samples were collected from downtown São Paulo, Brazil, by an impactor sampler located at the intersection of two very busy avenues between August 2005 and April 2006. In this place, 89% of fine particles are generated by traffic (CETESB, 2006). PM was collected during 24 h on polycarbonate filters (cat. no. ATTP03700, Millipore Corporate, Billerica, MA) using a Harvard impactor (Air Diagnostics, Harrison, ME) with a flow of 10 $L \cdot min^{-1}$. The filters were dried (24 h at 50°C) and weighed, before and after PM collection. The weight difference corresponded to the total collected mass. Particles were extracted in distilled water by ultrasonic sonication during 8 h, to a final particle:volume ratio of 1:2 (1 μg:2 μL), based on the total collected mass of PM2.5.

Animals and experimental groups

BALB/c mice were provided by the animal facilities of the Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "*Guide for the Care and Use of Laboratory Animals*" prepared by the National Academy of Sciences, USA. The work was carried out in accordance with EC Directive 86/609/EEC. The experiments were approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (Protocol IBCCF 046).

Forty-six male BALB/c mice (25–30 g BW) were randomly assigned to three main groups. Control group (CTRL $n=9$) received an intranasal (i.n.) instillation of 30 μL (3 × 10 μL) of saline (0.9% NaCl). Groups P5 and P15 ($n=16$ each) received PM as extracted by sonication. The solution concentration was 1 μg:2 μL. Thus, P5 group was instilled with 10 μL of the extract plus two 10-μL doses of saline solution. P15 received three 10-μL doses of the extract. Five animals (FILTER group) were instilled (i.n.) with 30 μL of a solution obtained after the extraction procedure from naive polycarbonate filters, as described earlier. For i.n. instillation, mice were anesthetized with sevoflurane, and saline or suspended particles were gently instilled into their snouts with the aid of a precision pipette. The animals recovered rapidly after instillation.

Particle composition

Elements content (Na, Al, Si, P, S, K, Ca, Ti, V, Fe, Ni, Cu, Zn and Pb) of PM2.5 was analyzed by spectrometry (EDX 700HS, Shimadzu Co, Kyoto, Japan) (Mauad et al., 2008). Polycyclic aromatic hydrocarbons (PAHs) were determined by liquid chromatography (LC-10AS, Shimadzu Co., Kyoto, Japan) with a fluorescence detector (RF-10 AxL, Shimadzu Co., Kyoto, Japan), as previously described (Mazzoli-Rocha et al., 2008). Briefly, PAHs were extracted consecutively with an acetone/n-hexane mixture in an ultrasonic bath (90°C for 20 min). Isooctane (1 mL) was added prior to each PAH extraction step. Then, extracts were filtered, combined, and concentrated (1 mL) by vacuum rotatory evaporation. Reagents were supplied by Tedia Co. (Fairfield, OH).

The combined extracts were passed through a chromatography column filled with 7 g of Al2O3/Na2SO3 deactivated with 11% of water, and n-hexane (20 μL) was used to elute PAHs. Cleaned extracts were concentrated and eluted with 35 mL of n-hexane/ethyl ether (3:1, v/v). The eluents were concentrated and then diluted with 0.5 mL of acetonitrile.

A 20-μL aliquot of the acetonitrile extract was analyzed by liquid chromatography (LC-10AS, Shimadzu Co., Kyoto, Japan) with a fluorescence detector (RF-10 AxL, Shimadzu Co., Kyoto, Japan). The separation was performed on a Shimadzu CLC-ODS II column (180.0 × 4.1 mm i.d.), isocratic run was completed with a mobile phase composed of water:acetonitrile mixture (20:80, v/v), and detection was determined at 255/325, 253/350, 333/390, 287/462, 280/430, 294/404, 300/500, and 300/421 Ex/Em wavelength steps. The following PAHs were analyzed: naphthalene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene (B[a]P), and benzo[ghi]perylene. The software Borwin 1.2 (Jmbs Developments, Le Fontanil, France) was used for the quantification.

Pulmonary mechanics

Twenty-four hours after instillation the animals were anesthetized (diazepam 1 mg and pentobarbital sodium 20 mg·kg·BW^{-1} i.p.), paralyzed (pancuronium bromide 0.1 mg·kg^{-1} i.p.), and ventilated (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with constant tidal volume (0.2 mL) and flow (1 mL·s^{-1}). A positive end-expiratory pressure (2 cmH_2O) was applied to the expiratory limb of the ventilator, and the anterior chest wall was surgically removed. A pneumotachograph was connected to the tracheal cannula for airflow measurements. Lung volume was determined by digital integration of the flow signal. The pressure gradient across the pneumotachograph was determined by a differential pressure transducer (Validyne MP45-2, Engineering Corp., Northridge, CA). Transpulmonary pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp. Northridge, CA). Signals were amplified (Beckman type R Dynograph, Schiller Park, IL), low-pass filtered (8-pole Bessel filters, 902LPF, Frequency Devices, Haverhill, MA) at 100 Hz, sampled at 200 Hz and analog-to-digital converted (DT2801A, Data Translation, Marlboro, MA). All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung mechanics static elastance (E_{st}), and elastic component of viscoelasticity (ΔE), as well as resistive (ΔP_1), viscoelastic (ΔP_2), and total (ΔP_{tot}) pressures was determined by the end-inflation occlusion method (Bates et al., 1985a, 1988).

Pulmonary histology and immunohistochemistry

Heparin (1000 IU) was intravenously injected immediately after the determination of pulmonary mechanics. The trachea was clamped at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly killed the animals. Thereafter, the lungs were removed *en bloc*.

The left lung was fixed by immersion in modified Millonig's buffered formaldehyde solution for 24 h and routinely processed for histological analyses. Four-µm-thick lung slices were stained with hematoxylin and eosin. The analyses were blindly done by two independent researchers. Morphometric analysis was performed with an integrating eyepiece with a coherent system made of a 100-point and 50-line (known length) grid coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The fractional areas of collapsed and normal alveoli were determined by the point-counting technique at a magnification of ×400 across 10 random non-coincident microscopic fields per animal. Points falling on normal or collapsed alveoli were expressed as percentage of the total number of points in the grid (Weibel, 1990).

For the determination of macrophages, neutrophils, IL-6 and TNF-α, the lung sections were deparaffinized and hydrated. After blocking the endogenous peroxidase, antigen retrieval was performed with high temperature citrate buffer (pH = 6.0). The following primary antibodies were used in the study: rat monoclonal antibody anti-mouse F4/80 and anti-mouse neutrophil (1:100, AbD Serotec, Raleigh, NC); goat polyclonal anti-mouse TNF-α and anti-mouse IL-6 (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA). ABC staining system (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as secondary antibody; 3′3 diaminobenzidine (DAB, Sigma, St. Louis, MO) was used as chromogen. The sections were counterstained with hematoxylin. For negative controls, the first antibody was omitted from the procedure and BSA was used instead. Analysis was performed at ×400 magnification on an Olympus microscope (model BH2, Tokyo, Japan). Fifty to sixty fields of 26,000 µm^2 (10 fields/animal in 5–6 animals from each group) were counted by two investigators at different occasions. The investigators counted non-identified sections. Total number of macrophages and neutrophils in air spaces were expressed as total cells per µm^2. The intensity of the parenchymal staining for TNF-α and IL-6 was semi-quantitatively analyzed by means of a visual analog scale (0 = absent; 1 = very weak; 2 = weak; 3 = moderate; 4 = strong) (Lopes et al., 2009).

For the determination of 8-isoprostane, 4-µm-thick lung slices, mounted on silanized slides, were deparaffinized and hydrated. After blocking the endogenous peroxidase, antigen retrieval was performed with trypsin. Polyclonal goat anti-8-epi-PGF2α (1:1200, Oxford Biomedical Research, Oxford, England) was used as primary antibody. The Vectastin ABCKit (Vector Laboratories, Burlingame, CA) was used as secondary antibody; DAB (Sigma, St. Louis, MO) was used as chromogen. The sections were counterstained with hematoxylin. For negative controls, the first antibody was omitted from the procedure and BSA was used instead. The expression of 8-isoprostane was assessed at ×400 magnification. Two different observers performed independent measurements. The intensity of the parenchymal staining for 8-isoprostane was semi-quantitatively analyzed by means of a visual analog scale (0 = absent; 1 = very weak; 2 = weak; 3 = moderate; 4 = strong) (Lopes et al., 2009).

Lung biochemical analysis

The right lung was removed, homogenized (model NT 136, Novatécnica, São Paulo, Brazil) in a buffer solution (potassium phosphate 100 mM + EDTA 5 mM, final solution volume: 1 mL), centrifuged at 7000*g* (model 243M, FANEM, São Paulo, Brazil) for 10 min, and the supernatants were collected for biochemical analysis. Protein concentration was estimated by the Bradford's method (Bradford, 1976).

Myeloperoxidase

Inflammatory changes were examined by myeloperoxidase (MPO) activity in the supernatant of lung homogenates, by using hydrogen peroxide (H_2O_2), hexadecyltrimethylammonium bromide, and 3,3′,5,5′-

tetramethylbenzidine. Absorbances were determined at 655 nm using a plate reader (Model 550, Bio-Rad, Hercules, CA) (Suzuki et al., 1983). MPO activity was expressed in mU/mg protein.

Malondialdehyde assay

As an index of lipid peroxidation, we used the thiobarbituric acid reactive substances (TBARS) method for analyzing malondialdehyde products during an acid-heating reaction (Draper & Hadley, 1990). Briefly, samples from lung homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid. The samples were then heated in a boiling water bath for 30 min. TBARS were determined by absorbance at 532 nm and expressed as malondialdehyde equivalents (nm/mg protein).

Catalase

Catalase (CAT) activity was measured by the rate of decrease in hydrogen peroxide content at 240 nm (Aebi, 1984). Final activity values were corrected by the protein content in each sample and expressed as U/mg protein.

Reduced glutathione/oxidized glutathione

The assay was based on the reaction of glutathione (GSH) or oxidized glutathione (GSSG) with 5,5-dithiobis-(2-nitrobenzoic acid) that produces the 2-nitro-5-thiobenzoate (TNB) chromophore (Rahman et al., 2006). To determine GSSG, samples of lung homogenate were treated with 2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine was neutralized with triethanolamine. The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH or GSSG in the sample. The concentration of an unknown sample was calculated from the linear equation or the regression curve generated from several standards of GSH or GSSG. The final result was presented as GSH/GSSG ratio.

Statistical analysis

SigmaStat 9.0 statistical software package (SYSTAT, Point Richmond, CA) was used. The normal distribution of the data (Kolmogorov–Smirnov test with Lilliefors' correction) and the homogeneity of variances (Levene median test) were tested. In all instances, both conditions were satisfied, and one-way analysis of variance was used followed by Tukey test for multiple comparisons when needed. The morphometric parameters underwent an arcsine transformation so that a normal distribution resulted. The significance level was always set at 5% ($p < 0.05$).

Results

Analysis of the PM

Table 1 shows element and PAH composition of PM2.5. It can be seen that iron and zinc were the prevalent metals, whereas phenanthrene represents the most abundant PAH.

Pulmonary mechanics

Figure 1 depicts ΔP_1, ΔP_2, ΔP_{tot}, E_{st} and ΔE measured in all groups. ΔP_2, ΔP_{tot}, E_{st} and ΔE were significantly higher in P15 than in CTRL and FILTER. P5 showed significantly larger ΔP_2, ΔP_{tot} and ΔE than CTRL and FILTER. No significant difference was detected between P5 and P15 in all instances, although P15 always tended to show higher values than P5. No functional difference was observed between FILTER and CTRL groups, supporting the absence of any bias due to the extraction process. Therefore, the other parameters were determined only in the three main groups, i.e. CTRL, P5, and P15.

Histopathological analysis

Figure 2 depicts photomicrographs of pulmonary parenchyma in CTRL, P5 and P15 mice. Normal areas in CTRL, P5 and P15 amounted to 91.0±2.7%, 90.4±2.7%, and 86.8±1.8% (SEM) of tissue area, respectively, whereas alveolar collapse was detected in 9.0±2.7%, 9.4±2.5%, and 13.1±1.8% (SEM) of tissue area, respectively. P15 displayed a significantly higher amount of collapsed areas than P5 and CTRL. No difference was observed between CTRL and P5.

Inflammatory signaling

P15 presented significantly higher macrophage and neutrophil content in lung parenchyma than CTRL. Neutrophil influx was higher in P15 than in P5, whereas no difference was observed between CTRL and P5 for both cell types (Figure 3A). The activity of MPO, a marker of neutrophil activation, was higher in P5 and P15 than in CTRL (Figure 3B). P15 also showed significantly

Table 1. Polycyclic aromatic hydrocarbons and elements in PM2.5.

PAH	ng/g	Element	PPM
Naphthalene	798.0	Na	3615±3802
Acenaphthylene	483.9	Al	157±789
Fluorene	233.6	Si	1300±2697
Phenanthrene	3309.9	P	0.23±0.22
Fluoranthene	1157.4	K	1884±4313
Pyrene	127.9	Ca	861±1894
B[a]anthracene	5.0	Ti	242±359
B[b]fluoranthene	9.4	V	99±65
B[k]fluoranthene	1.4	Fe	3595±4342
B[a]pyrene	5.8	Ni	67±69
B[ghi]perylene	0.4	Cu	322±253
		Zn	1620±2164
		Pb	172±225

Concentrations of polycyclic aromatic hydrocarbons (PAHs) quantified using the software Borwin 1.2 by integration of the chromatograms and calculation the concentration of composites. For the identification of composites, the time of retention of each one was compared with the chromatogram of a standard solution. 102 samples were used in the calculation of the concentration of elements (mean ± SD).

higher staining scores for IL-6 and TNF-α in lung tissue than CTRL and P5, whereas no difference was observed between the latter groups (Figure 3C and 3D).

Oxidative damage and stress

P15 showed significantly higher staining scores for 8-isoprostane and TBARS expression than CTRL (Figure 4A and 4B). Both parameters were slightly increased in P5 group, but without significant differences in relation to CTRL or P15. CAT activity increased progressively after PM2.5 exposure, being significantly higher in P15 than in CTRL and P5. GSH/GSSG decreased only in P15 group compared with CTRL and P5 (Figure 4C and 4D).

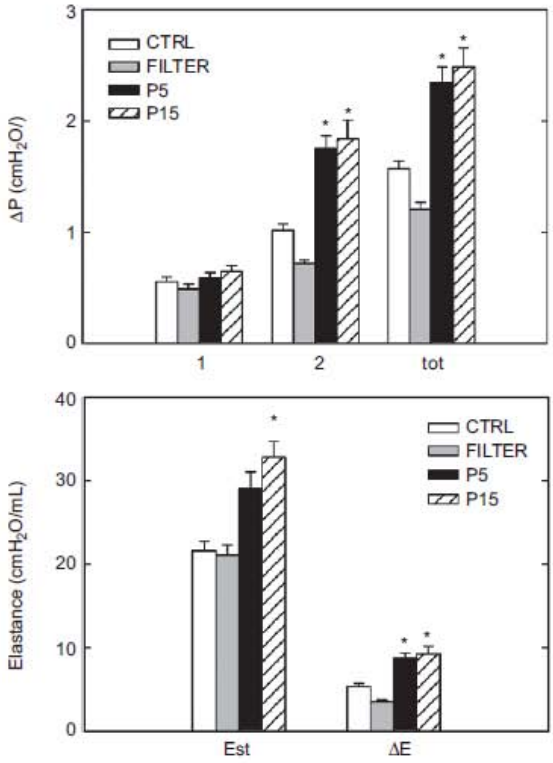

Figure 1. Pressures dissipated to overcome pulmonary viscous (ΔP_1), viscoelastic/inhomogeneous (ΔP_2), the sum of both pressures (ΔP_{tot}), static elastance (*E*st), and the elastic component of viscoelasticity (ΔE) in groups that did not receive PM2.5 (CTRL, $n=9$, and FILTER, $n=5$) and in those that received 5 or 15 μg of PM2.5 (P5 and P15, respectively, $n=16$ in each). Values are mean + SEM. *Significantly different from CTRL and FILTER groups ($p<0.05$).

Discussion

Our results showed that acute exposure to low doses of urban PM2.5 (5 and 15 μg of PM2.5) impaired lung function, characterized by increased elastic and viscoelastic components of lung mechanics. Low dose exposure to PM2.5 also induced lung inflammation and oxidative damage. Lung inflammation was evidenced by increased MPO activity and neutrophil influx into lung parenchyma, as well as increased expression of proinflammatory cytokines such as TNF-α and IL-6. Oxidative damage was expressed by increased reactive substances to thiobarbituric acid and 8-isoprostane, whereas oxidative stress was characterized by increased expression of CAT and reduction in GSH/GSSG.

The used doses were based on the mean concentration of PM2.5 in São Paulo (23 μg/m³), and considering that on a peak pollution day, this amount increases considerably reaching a four-fold value (CETESB, 2008). Furthermore, it should be noted that only a fraction of the nasally instilled PM2.5 reaches the alveolar region (Sabaitis et al., 1999). Indeed, our doses were lower than those usually found in experimental models of air pollution (Gavett et al., 2003; Rivero et al., 2005; Kooter et al., 2006; Mantecca et al., 2010), hence allowing the observation of pulmonary effects induced by ambientally relevant levels of PM2.5. It is noteworthy that even a single low dose exposure to PM2.5 yielded both functional and histological/biochemical pulmonary changes (Figures 1–4).

We focused our measurements on the responses present at 24 h after exposure, because epidemiological studies indicate a 24-h lag between exposure and respiratory symptoms (Braga et al., 2002). Indeed, previous experimental studies from our group using total suspended particles (Mazzoli-Rocha et al., 2008) and diesel PM (Laks

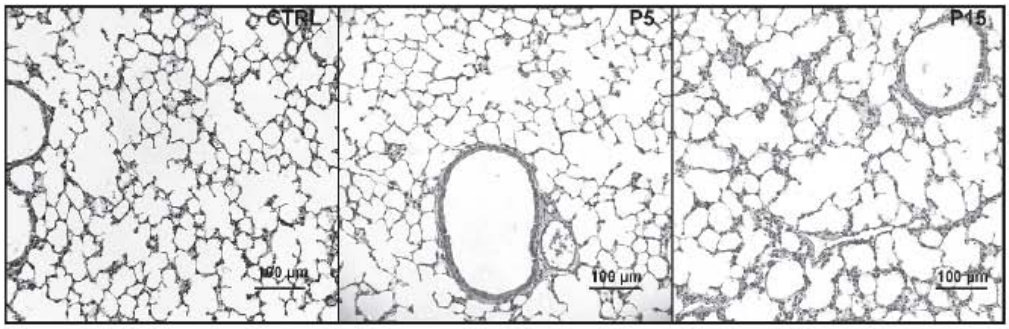

Figure 2. Photomicrographs of pulmonary parenchyma. Representative images from a mouse that did not receive PM2.5 (CTRL) and from those that were instilled with 5 or 15 μg of PM2.5 (P5 and P15, respectively). Original magnification: ×200.

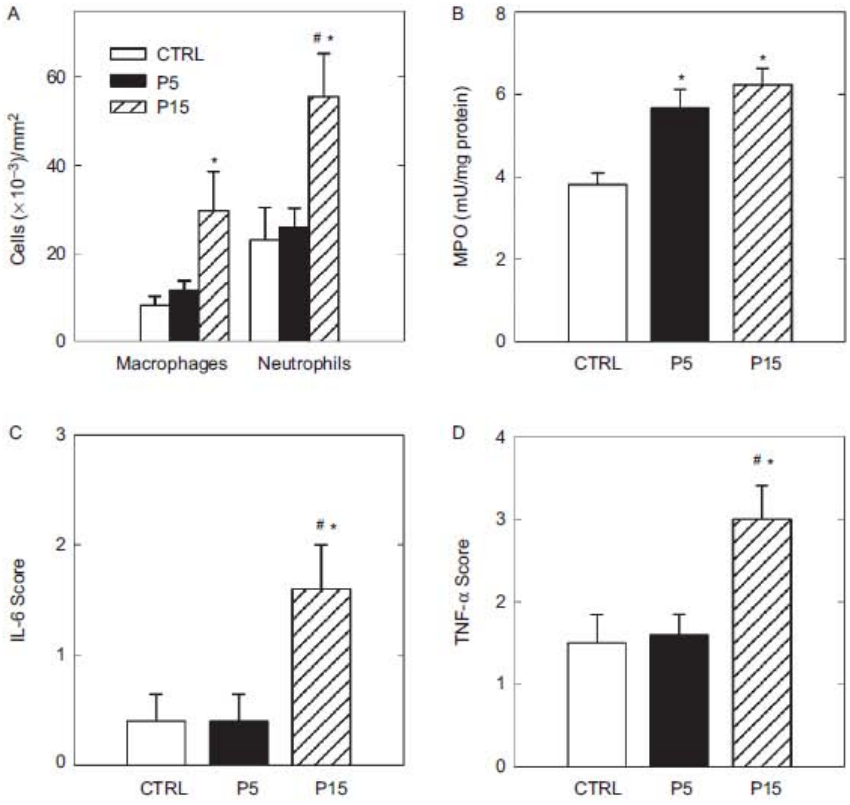

Figure 3. Inflammatory signaling in mice that did not receive PM2.5 (CTRL) and in those that received 5 or 15 μg of PM2.5 (P5 and P15, respectively). In panel A, the amounts of macrophages (mononuclear cells) and neutrophils (polymorphonuclear cells) per area of lung parenchyma in CTRL ($n=6$), P5 ($n=5$) and P15 ($n=5$) groups are shown. Values are mean ± SEM of 5–10 fields per slide. Panel B depicts the activity of myeloperoxidase (MPO) in the samples from lung homogenates of CTRL ($n=5$), P5 ($n=5$) and P15 ($n=5$) groups. Panels 2C and 2D show that tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were higher in the lung tissue of P15 mice ($n=5$), than in P5 ($n=5$) and CTRL ($n=6$) that did differ between them. Values are mean ± SEM of 5–10 fields per slide. *, # indicate values significantly different from CTRL and P5, respectively ($p<0.05$).

et al., 2008) have already demonstrated adverse respiratory effects 24 h after exposure to PM, thus supporting the epidemiological findings.

The suspended PM (extract of sonicated PM2.5) was administered to mice by i.n. instillation. Nasal breathing is characterized by the retention of large particles in the extrathoracic regions of the respiratory system. This model of administration affects the distribution of particles according to their aerodynamic size and, thus, our results may not necessarily reflect the interactions of particles with the respiratory system as if they were inhaled by aerosol. Nevertheless, it is very useful for comparative studies of particle toxicity (Terashima et al., 1997; Laks et al., 2008; Mazzoli-Rocha et al., 2008). It should be stressed that we used PM2.5 suspended in distilled water and, thus, the sonication and dilution processes may have somewhat modified the particle characteristics.

Previous studies disclosed that PMs from different sources are able to induce inflammatory processes triggered by their components (Sørensen et al., 2003; Park et al., 2006). It is also well determined that PM composition varies according to different factors, such as source of air pollution, humidity, and temperature. PM2.5 from the urban area of São Paulo city is mainly produced by traffic and showed high concentration of elements and PAHs with inflammatory characteristics (Table 1). Our results support the concept that ambient particles induce acute respiratory inflammation and oxidative damage (Figures 3 and 4).

The variety of pollutants found in ambient air and their capacity to interact synergistically render difficult the establishment of a cause-effect relationship between a specific compound and the observed toxicity of a given air sample. Previous studies show that the metal composition of the PM can generate pulmonary injury (Prahalad et al., 2001). The most commonly found elements in airborne PM are Cr, Co, Ni, Mn, Zn, Cu, and, mainly, Fe (Billet et al., 2007). The latter appears in higher concentrations in particles generated by fossil fuel burning than in PMs from other origins (Park et al., 2006). In this context, sodium and iron were the most abundant elements present in the PM2.5 used in this study (Table

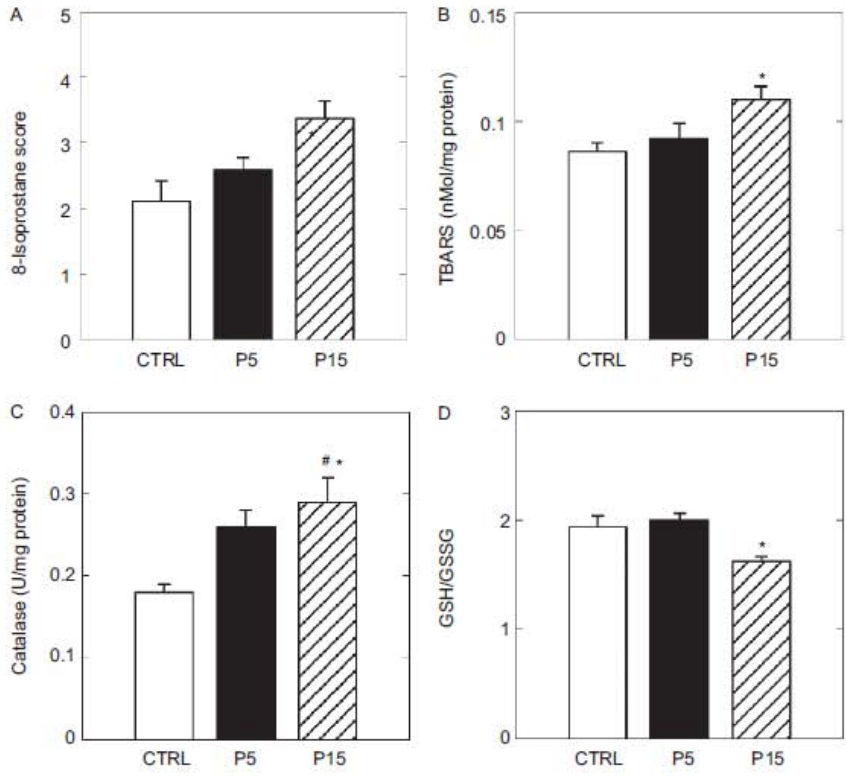

Figure 4. Markers of oxidative damage and stress in mice that did not receive PM2.5 (CTRL) and in those that received 5 or 15 μg of PM2.5 (P5 and P15, respectively). Panel A: semi-quantitative score of staining intensity for 8-isoprostane in CTRL ($n=6$), P5 ($n=5$) and P15 ($n=5$). Values are mean + SEM of 5–10 fields per slide. Panel B shows the values of thiobarbituric acid reactive substances (TBARS) in CTRL, P5, and P15 animals ($n=7$, 10, and 11, respectively). Panel C shows catalase activity in the lungs of CTRL ($n=7$), P5 ($n=10$), and P15 ($n=10$) groups. Panel D shows the relationship between the activities of reduced (GSH) and oxidized (GSSG) glutathione in the lungs of mice in CTRL ($n=8$), P5 ($n=13$) and P15 ($n=11$) groups. In panels B–D values are mean + SEM of samples from lung homogenates. *, # indicate values significantly different from CTRL and P5, respectively ($p<0.05$).

1). Fe is described as deeply linked to the production of oxidative stress (Park et al., 2006) by generating reactive oxygen species (ROS) and facilitating superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) conversion to hydroxyl ions (OH^-) (Stohs & Bagchi, 1995; Park et al., 2006). The resultant pulmonary effects would include surfactant dysfunction (Chauhan & Misra, 1991), epithelial damage, increased vascular permeability and inflammatory response, followed by impaired pulmonary function (Soukup et al., 2000; Dye et al., 2001). Other elements, such as V, Mn, Zn, Cu, Ni, Co, and Cr, also present in our sample, are known to generate ROS (to a lesser extent than Fe) that lead to inflammation and oxidative stress (Stohs & Bagchi, 1995; Sørensen et al., 2005). The initial phase of the pulmonary response to PM exposure seems to be influenced by single elements, while a persistent response would reflect complex interactions among different components present in a composite (Dreher et al., 1997; Antonini et al., 2004).

In the present study, PM concentrations of PAH (Table 1), particularly benzo[a]anthracene, benzo[b] fluoranthene, benzo[k]fluoranthene, and benzo[a] pyrene, are considered of potential carcinogenic risk, in accordance with IARC (Castaño-Vinyals et al., 2004). Mazzoli-Rocha et al. (2008) detected benzo[a]pyrene and benzo[a]anthracene in samples of total suspended PM from São Paulo, confirming the high levels of PAH in this city. The presence of PAH in PM has been associated with the triggering of inflammation, generation of ROS, and lipid peroxidation (Sørensen et al., 2003), especially in alveolar macrophages and epithelial cells (Li et al., 2002). In this line, our study detected markers of inflammation (Figures 2 and 3) and production of ROS (Figure 4) in P15 mice. According to Caricchia et al. (1999), the amount of PAH can decrease in a sample of PM when the analysis takes place 24 h after collection. This was exactly the case in the present study, thus implying that the ambient levels of PAH (and their effects) may have been underestimated.

Although the simple chemical analysis of particles, as in the present work, does not warrant the establishment of a direct correlation between composition and effect, the presence of metals and PAHs in our PM2.5 could have induced a pulmonary impairment similar to that reported in a study with diesel particles (Laks et al., 2008) and also in the face of total suspended particles and

biomass burning-derived particles (Mazzoli-Rocha et al., 2008). Studies on pulmonary retention of PAH in solution, after intratracheal instillation in rats, indicate that PAHs are quickly absorbed by the respiratory tract (Wolff et al., 1989). Noteworthy, inhaled PAH is predominantly adsorbed to ultrafine particles, penetrating in the cells of the bronchial epithelium, where it is metabolized, activated and biochanged (Sørensen et al., 2003; Castaño-Vinyals et al., 2004). Finally, it is not possible to exclude the contribution of other unmeasured compounds to our results.

To discard any eventual bias in our data secondary to the extraction process itself, which could potentially remove and suspend undesired filter components, an extra control group was analyzed (FILTER group). In this group, animals were instilled with a solution obtained from naïve filters submitted to the same extraction protocol used for PM filters. FILTER and CTRL groups presented similar mechanical results, thus excluding the possibility of a biased conclusion due to the extraction process. It should be stressed that ΔP_2, ΔP_{tot} and ΔE pertaining to FILTER mice seem smaller than those in CTRL group, although no statistically significant difference was disclosed. The smaller number of mice in FILTER group could have induced the apparent differences.

To our knowledge, there are few studies describing the independent contributions of central and peripheral airways/tissue to pulmonary resistance, as previously characterized (Bates et al., 1988; Saldiva et al., 1992b), after exposure to pollutants. Our results, as well as those obtained from total suspended particles (Mazzoli-Rocha et al., 2008), revealed that central airway resistance was not modified after particle exposure, as indicated by ΔP_1. In contrast, ΔP_1 was increased after diesel exposure in mice (Laks et al., 2008). TSP samples used by Mazzoli-Rocha et al. (2008) were represented mainly by coarse particles, but originated from the same place as our PM2.5 samples. On the other hand, diesel particles, usually characterized mainly by fine particles, came from a different source showing different metal and PAH contents. E_{st} and ΔE increased in a dose-dependent manner (Figure 1). Taken together, these findings may suggest that the apparent discrepancies among ΔP_1 behavior could result from particle composition rather than particle size. The resistive component of tissue viscoelasticity (ΔP_2) was increased in both PM5 and PM15 groups (Figure 1), in accordance with previous reports after TSP and diesel exposure (Mazzoli-Rocha et al., 2008; Laks et al., 2008). These mechanical alterations could be attributed to the PMs capacity to penetrate to the alveolar region, triggering an inflammatory reaction (Figure 3), and generating oxidative stress and damage in lung tissue (Figure 4) (see below). These functional changes induced by i.n. instillation of low doses of PM2.5 are herein reported for the first time in the literature.

The aforementioned functional findings were accompanied by morphologic alterations in pulmonary parenchyma (Figure 2). The alveolar collapse and septal distortion observed in P15 (Figure 2) may have caused mechanical parenchyma heterogeneity, potentially contributing to the increase in ΔP_2, E_{st} and ΔE (Bates et al., 1985a, 1985b). Additionally, inflammation and eventual surfactant dysfunction may have contributed to morphological and, thus, functional changes (Liu et al., 1996). In this line, airborne metal pollutants (as Fe, Cd, Ni and Co) interact with proteins and phospholipids of the surfactant, modifying its composition and physicochemical properties, besides jeopardizing its secretion by type II pneumocytes. As a result, alveolar surface tension may increase yielding alveolar collapse (Srivastava & Misra, 1986; Chauhan & Misra, 1991), as also present in our study.

P15 group presented a significant inflammatory response, as indicated by MPO activity (Figure 3B), influx of defense cells, as well as IL-6 and TNF-α expression (Figure 3A). Although inflammation could be observed in P5 group too, a milder response was found: only MPO was higher in P5 than in CTRL. It should be noted that even though the amount of neutrophils in the lung did not differ between CTRL and P5 groups (Figure 3A), these cells were probably activated in the latter mice, since MPO was expressed by P5 animals but not expressed by CTRL (Figure 3B). Other experimental studies report recruitment of neutrophils and alveolar macrophages 24 h after intratracheal instillation of 500 μg (Oberdörster et al., 1992) or 380 μg/m³ (André et al., 2006) of ultrafine particles, as well as i.n. instillation of 100 and 500 μg of PM2.5 from São Paulo in healthy rats (Rivero et al., 2005). In this context, the exposure to PM increases the expression of genes related to NF-κB activation and TNF-α, TGF-β and IL-6 (Shukla et al., 2000; Ding et al., 2010), as depicted in Figure 3C and 3D. Finally, inflammation per se not only modifies the structure of the alveolar septa, but also jeopardizes surfactant action, thus yielding an increased percentage of collapsed alveoli, as aforementioned.

Free radicals and oxidative stress are extensively implicated in the inflammatory response associated with exposure to PM (Donaldson & Stone, 2003; Pereira et al., 2007). Oxidative stress mediated by PM can originate from different sources, involving: (I) direct generation of ROS in the presence of free and oxidant radicals on the particle surface, (II) soluble fractions as organic components and transition metals, (III) alteration in mitochondrial function or NADPH-oxidase, and (IV) activation of inflammatory cells able to generate ROS and reactive nitrogen species (Risom et al, 2005). Many inflammatory mediators, induced by PM exposure, are regulated by transcription factors susceptible to oxi-reduction, as NF-κB, AP-1 and C/EBP, suggesting an increased production of ROS after PM exposure (Li et al., 2002; González-Flecha, 2004; Ohyama et al., 2007). The capacity to generate ROS would be related with particle size and surface area, independently of its composition (Brown et al., 2000). However, evidence suggests that metals and PAH can act synergistically, producing

ROS (Park et al., 2006; Ohyama et al., 2007). To assess the possible involvement of PM2.5 in the genesis of oxidative stress and damage to the pulmonary tissue, we measured some compounds involved in its initial (CAT and GSH/GSSG ratio) and late stages (TBARs and 8-isoprostane). In all instances, P15 presented higher values than CTRL and P5 displayed a nonsignificant, not so intense response (Figure 4). Supporting our findings that P15 mice presented pulmonary oxidative damage (Figure 4A and 4B), it has been shown that soluble and insoluble substances present in PM are able to increase TBARS (Ghio et al., 1996; Bae et al., 2010). Moreover, recent studies (Ding et al., 2010; Martin et al., 2010) showed that low doses of PM were able to increase oxidative stress and induce an inflammatory process in the lung.

Oxidative stress was addressed by the determination of reduced and oxidized glutathione (GSH and GSSG, respectively), since GSH formation falls while GSSG production rises, leading to a reduction in intracellular GSH/GSSG ratio in that condition (Rahman et al., 1999). We found a reduced GSH/GSSG in P15 group (Figure 4C) in accordance with Li et al. (2003), who studied pulmonary alveolar macrophages and bronchial epithelial cells in culture and found similar results, and Ding et al. (2010), who reported a diminished GSH/GSSG in the face of intratracheally instilled low doses of PM2.5 in neonates rats. Additionally, Li et al. (2000) showed that PAH present in diesel particles is related to the reduction in GSH/GSSG. Interestingly, Powell et al. (1994) described reduced GSH levels in adults and asthmatic children, stressing its relation with the inflammatory process. Finally, there may be three possible mechanisms accounting for the decline in GSH in response to PM exposure: (i) via GSH peroxidase (GPx) reaction, (ii) via the GSH transferase reaction, and (iii) via GSH efflux (Deneke & Fanburg, 1989).

The present work disclosed an increased CAT activity in P15 group (Figure 4D). In this context, many authors report consumption of antioxidant enzymes and increased lipid peroxidation in the liver and kidney in diverse species (Weng et al., 2007) in the face of oxidative stress (Sicinska et al., 2006), supporting our results. Under physiological conditions, GSH and CAT play a key antioxidant role, which protects against oxidative damage especially mediated by free radicals and lipid peroxidation. Their decrease induced by fine PM may reduce the protection against oxidative stress (Ding et al., 2010).

In conclusion, this study evaluated for the first time early alterations in the elastic and viscoelastic pulmonary mechanical components of mice exposed to low doses of PM2.5 gathered in a densely populated urban center. These mechanical modifications could probably be explained by histological, inflammatory, and oxidative derangements induced by these fine particles. Our results provide experimental support to epidemiological findings of an association between respiratory impairment and acute exposure to PM2.5.

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Declaration of interest

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APÊNDICE F – Artigo publicado durante ao período de mestrado - II

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Mate tea ameliorates emphysema in cigarette smoke-exposed mice

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ABSTRACT

Exposure to cigarette smoke (CS) is associated with lung inflammation, oxidative stress, and emphysema. The aim of this work was to study Mate tea as a possible natural antioxidant resource against emphysema development. C57BL/6 mice were distributed into 4 groups: exposed to ambient air (control), exposed to the smoke of 12 cigarettes (CS), exposed to ambient air and treated with Mate (500 mg/kg/day diluted in 100 μL) (Mate), and exposed to CS and treated with Mate (CS+Mate). All mice were treated for 60 days. On day 61 the mice were killed. Right and left lungs were removed for histology and biochemical analysis, respectively. Emphysematous lesions and inflammatory cell influxes in the CS group were evident by histological analysis. Cells showed higher 4-hydroxynonenal labeling in the CS group than in the CS+Mate group. Myeloperoxidase activity was reduced in the CS+Mate group compared to the CS group. Superoxide dismutase and catalase activities were significantly higher in the CS+Mate group compared to the CS group. The ratio of reduced to oxidized glutathione was greater in the CS+Mate group than in the CS group. CS-induced emphysema in C57BL/6 mice was prevented by Mate in association with a reduction in inflammatory and oxidative stress parameters.

KEYWORDS cigarette smoke, emphysema, mate tea, oxidative stress

The imbalance between oxidants and antioxidants is believed to be an important trigger in emphysema pathogenesis [1]. In this context, cigarette smoke (CS) is the major risk factor for emphysema, characterized by destruction of lung extracellular matrix and enlarged airspaces [2]. The mechanisms involved in the pathogenesis of this disease are not yet fully elucidated. However, it is well known that CS is rich in oxidant compounds, containing an estimated 10^{14} free radicals per puff. Moreover, many of these radicals, such as tar semiquinone, can generate other reactive oxygen species (ROS) such as hydrogen peroxide by the Fenton reaction [3]. Furthermore, the presence of CS in airways induces migration and activation of inflammatory cells, especially macrophages and neutrophils, which add to the oxidant burden by generating more ROS via the reduced nicotinamide adenine dinucleotide phosphate oxidase system [4].

Oxidative stress induces damage to proteins, DNA, and lipids, which may cause direct lung injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species [5]. Chronic exposure to CS is associated with a decline in antioxidant defenses, both enzymatic and nonenzymatic, through their continuous depletion in response to the oxidative burst. Administration of antioxidant donors could be an efficacious strategy to improve the redox status, thus ameliorating the effects of CS.

Teas are an excellent source of compounds with antioxidant properties, such as polyphenols and catechins, and can be easily introduced into the daily diet. Mate tea, produced from the roasted *Ilex paraguariensis* herb, is a typical beverage of South America, including southern Brazil, northern Argentina, Paraguay, and Uruguay [6], and its consumption has increased worldwide [7]. Mate tea is rich in

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polyphenolic compounds such as chlorogenic acid and caffeic acid derivatives [8], with antioxidant properties such as inhibition of xanthine oxidase [9]. These polyphenolic compounds may also act indirectly as antioxidants by inhibiting the activity of prooxidant enzymes such as myeloperoxidase (MPO) [10] and by increasing the production of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [11]. Indeed, we showed that Mate tea was effective in reducing inflammatory markers in a model of acute lung inflammation induced by CS exposure over 5 days [12], prompting our suggestion of a potential beneficial effect of Mate tea use in a mouse model of CS-induced emphysema [13–15]. Here we tested that hypothesis, and report that CS-induced emphysema in mice was prevented by Mate tea in association with a reduction in inflammatory and oxidative stress parameters.

MATERIALS AND METHODS

Reagents and Animals

3,3′,5,5′-Tetramethylbenzidine, 3,3′-diaminobenzi dine, 5,5′-dithiobis-(2-nitrobenzoic acid), catalase, chloramine-T, adrenaline, hexadecyltrimethylammonium bromide, *p*-dimethylbenzaldehyde, perchloric acid, triethanolamine, and 2-vinylpyridine were purchased from Sigma Chemical (St. Louis, MO). 4-Hydroxynonenal and secondary antibody were purchased from Santa Cruz Biotechnology (San Francisco, CA). Bradford reagent was purchased from Bio-Rad (Hercules, CA). Formalin and hydrogen peroxide were purchased from Vetec (Duque de Caxias, Brazil). Commercial samples of Mate tea were purchased from Leão Jr. S.A. C57BL/6 8-week-old male mice (18 to 22 g) were purchased from Instituto de Veterinária, Universidade Federal Fluminense (Niterói, Brazil). All procedures were carried out in accordance with conventional guidelines for experimentation with animals, and the local committee approved the experimental protocol. The mice were fed Purina chow and allowed unrestricted access to water in a controlled environment kept at 18°C to 22°C and 50% to 70% humidity.

CS Exposure and Procedures

An infusion of roasted Mate tea was prepared daily as for human consumption by adding 6.25 g of sample to 50 mL of boiled water (500 mg/kg). Before treatment the infusion was filtered and cooled to room temperature. The total polyphenol content, in the fresh tea (at the dose of 500 mg/kg), was estimated in 98.6 mg/mL by the Folin-Ciocalteu method, according to Lakenbrink et al. [16]. Briefly, a tea sample was put to react with the Folin-Ciocalteu reagent at room temperature for 2 hours. Gallic acid was used as standard, and the product was measured spectrophotometrically at 700 nm (Beckman Spectrophotometer model DU 640; Fullerton, CA).

Mice ($n = 40$) were divided into 4 groups: 2 groups exposed to ambient air (AA) for 60 days and 2 groups exposed to CS for 60 days. The 20 mice exposed to AA were treated with vehicle ($n = 10$, mineral water, 100 μL: "control") or with Mate tea ($n = 10$, 500 mg/kg/day diluted in 100 μL: "Mate"). The other 20 mice were exposed to 12 commercial filtered Virginia cigarettes per day (4 cigarettes in the morning, 4 cigarettes at noon, and 4 cigarettes in the afternoon) by using a smoking chamber, as described previously [13–15, 17], and treated either with vehicle ($n = 10$, mineral water, 100 μL: "CS") or with Mate tea ($n = 10$, 500 mg/kg/day diluted in 100 μL: "CS+Mate"). Vehicle or Mate tea was administered by oral gavages daily 1 hour after the last exposure to AA or CS. Briefly, animals were placed in the inhalation chamber (40 cm long, 30 cm wide, and 25 cm high), inside an exhaustion chapel. A cigarette was coupled to a plastic 60-mL syringe so that puffs could be drawn in and subsequently expelled into the exposure chamber. One liter of smoke from 1 cigarette was aspirated with this syringe, and then each puff was immediately injected into the chamber. The animals were maintained in this smoke-air condition (± 3%) for 6 minutes. Then the cover was removed from the inhalation chamber, and, by turning on the exhaust fan of the chapel, the smoke was evacuated within 1 minute. Carboxyhemoglobin (COHb) levels were measured by using the method described by Beutler and West [18], and the treatment was not toxic, as described by us previously [17]. Total polyphenols in the plasma from mice were assayed by the method of Pueyo and Calvo [19]. The total polyphenol contents (mg/mL) were 10.08 ± 1.89 in the control, 16.95 ± 0.98 in the Mate group, and 18.81 ± 1.46 in the CS+Mate group. Each cigarette produced 300 mg/m^3 of total particulate matter in the chamber. In a previous experiment in which we gave Mate tea at 150 mg/kg/day concomitant with CS exposure over 60 days, we did not observe a protective effect on the lungs (data not shown).

Bronchoalveolar Lavage Fluid (BALF) Analysis

One day after the last AA or CS exposure, the mice were killed by cervical displacement. Bronchoalveolar lavage was performed in the left lung of all animals from each group by using a canula inserted into

the trachea after the right lung was clamped. The airspaces were washed with buffered saline solution (500 μL) 3 times (final volume 1.2 to 1.5 mL). The fluid was collected and stored on ice for subsequent biochemical analysis (see below).

Tissue Processing and Morphometry

The right ventricle was perfused with saline. The right lung of all animals from each group was then inflated by instilling 4% phosphate buffered formalin (pH 7.2) at 25 cm H_2O pressure for 2 minutes and then ligated, removed, and weighed. Inflated lungs were fixed for 48 hours before embedding in paraffin. Serial sagittal 4-μm sections obtained from the right lungs were stained with hematoxylin and eosin (H&E) for histological analyses and Weigert's elastic stain.

For the oxidative damage assay, we used 4-hydroxynonenal (4-HNE), detected immunohistochemically, as a marker of lipid peroxidation. The primary antibody was rabbit anti-mouse 4-HNE. The biotinylated secondary antibody and avidin–biotin peroxidase with 3,3′-diaminobenzidine were used according to manufacturer's instructions. After staining for 4-HNE, the lung sections were counterstained with hematoxylin.

The total number of alveolar macrophages and neutrophils was estimated by counting 10 different fields (5 random fields of 2 different blinded sections) in lung sections. Numbers of 4-HNE–positive macrophages and type II pneumocytes were estimated by counting 10 different fields (5 random fields of 2 different sections) in lung sections. Pulmonary emphysema, quantified by morphometry, was carried out by using a test system composed of 21 points and in a known test area delineated by the forbidden line, in order to avoid overestimation of the number of structures. The test system was attached to a monitor connected to a microscope. The points (PP) that intercepted the airspaces (Vvair) and elastic fibers (Vvef) were counted and compared with the total number of points of a test system (PT). Therefore, the volume density (Vv) is equal to the PP divided by the PT [20]. Ten fields (5 nonoverlapping fields in 2 different blinded sections) were randomly analyzed by using a video microscope (20× objective lens, Carl Zeiss model Axiolab [Oberkochen, Germany], linked to a color video camera, JVC model TK-C380 [Osaka, Japan]) and a Sony Trinitron color video monitor (model PVM-14N2U; London, United Kingdom). A total area of 1.54 mm^2 was analyzed to determine the volume density of total airspaces (Vvair) in sections stained with H&E and the volume density of elastic fibers (Vvef) in sections stained with Weigert's. In addition, the alveolar airspace enlargement was determined by mean linear intercept (Lm) as described previously [17], using a video microscope (20× objective lens, Zeiss-Axioplan; Carl Zeiss), and a test system superimposed on the monitor screen. The Lm was measured by placing a 100 × 100-μm grid over each field. The total length of each line of the grid divided by the number of alveolar intercepts provides the average distance between alveolated surfaces, or the Lm. Two investigators performed all the measurements by counting coded sections.

Biochemical Analyses

The 10 left lungs of each group were removed and immediately homogenized (Homogenizer NovaTécnica model NT 136; Piracicaba, Brazil) in 1.0 mL potassium phosphate buffer (pH 7.5), centrifuged at 7000 × *g* (Centrifuge FANEM model 243 M; São Paulo, Brazil) for 10 minutes, and the supernatants were collected for biochemical analysis. Protein concentration was estimated by Bradford's protocol, using bovine serum albumin as a standard [21].

MPO is a pro-oxidant enzyme released from neutrophils and an important marker of lung injury [22]. MPO activity was measured by using hydrogen peroxide (H_2O_2), hexadecyltrimethylammonium bromide (HTAB) and 3,3′,5,5′-Tetramethylbenzidine (TMB). Initially, 100 μL of lung homogenate samples were centrifuged with 900 μL of HTAB at 14,000 × *g* for 15 minutes. Seven-five microliters of supernatant was incubated with 5 μL of TMB for 5 minutes at 37°C. The mixture was then incubated with 50 μL of H_2O_2 for 10 minutes at 37°C, after which 125 μL of sodium acetate buffer was added. The reaction was read in a microplate reader (Bio-Rad model 550; Hercules, CA) at 630 nm. SOD activity in lung homogenates was determined according to the method of Bannister and Calabrese [23]. Estimation of SOD activity is made by measuring the level of inhibition of adrenaline auto-oxidation read at 480 nm (Beckman Spectrophotometer model DU 640; Fullerton, CA). Enzyme activity was expressed as U/mg protein. CAT activity in lung homogenates was determined according to the method of Aebi [24]. Estimation of CAT activity is made by measuring the level of H_2O_2 depletion, and monitored at 240 nm. Enzyme activity was expressed as U/mg protein. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is considered a marker of oxidative stress. This assay is based on the reaction of GSH or GSSG with 5,5′-dithiobis-(2-nitrobenzoic acid) that produces the 2-nitro-5-thiobenzoate (TNB) chromophore [25]. To determine GSSG, lung homogenates were treated with

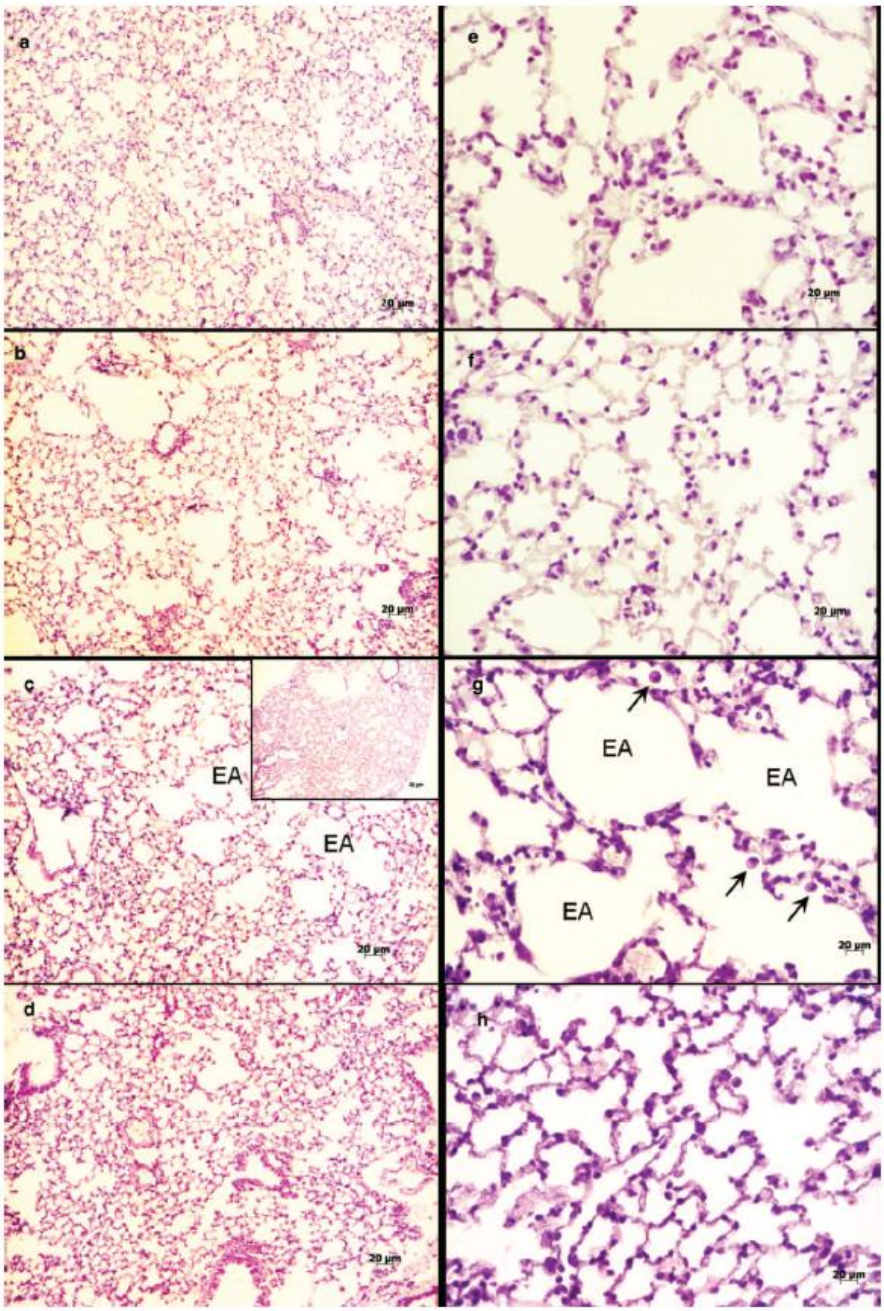

FIGURE 1 Lung photomicrographs stained with H&E of mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). (**a** and **e**) Control group exposed to ambient air, presenting normal alveoli and thin alveolar septa. (**b** and **f**) Mate group exposed to ambient air and treated with Mate tea presenting normal alveoli and thin alveolar septa. (**c** and **g**) Mice exposed to CS and treated with vehicle (CS group) presenting alveolar leukocytes (arrows) and enlarged alveoli (EA). Inset shows image obtained from CS group at a lower magnification (5×). (**d** and **h**) Mice exposed to CS and treated with Mate tea (CS + Mate) presenting few leukocytes and normal alveoli. Scale bar: 20 μm. Original magnification, 10× (**a**–**d**) and 40× (**e**–**h**).

2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine is neutralized with triethanolamine. The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH or GSSG in the sample. The concentration of an unknown sample is determined by calculating from the linear equation or the regression curve generated from several standards of GSH or GSSG. The hydroxyproline (OHpro) content in BALF samples was determined by a colorimetric method as previously described [26]. Initially, 8 μL of the each BALF sample were incubated with 40 μL of 0.05 M chloramine-T for 20 minutes at room temperature in test tubes. The mixture was then incubated with 40 μL of 3.17 M perchloric acid for 5 minutes at room temperature. Finally, the mixture was incubated with 40 μL of 20% *p*-dimethylbenzaldehyde for 20 minutes at 60°C. The samples were read in a microplate reader at 550 nm.

Statistical Analyses

Values for all measurements are expressed as means ± standard error of the mean. Analysis of parametric data (MPO, SOD, CAT, and OHpro) was carried out by using 1-way analysis of variance (ANOVA) followed with Tukey post hoc test ($P < .05$). Analysis of nonparametric data (stereology, morphometry, and GSH/GSSG ratio) was carried out by using the Kruskal-Wallis test followed by Dunn's post hoc test ($P < .05$). GraphPad Prism software was used to perform the statistical analyses (GraphPad Prism version 5.0; San Diego, CA).

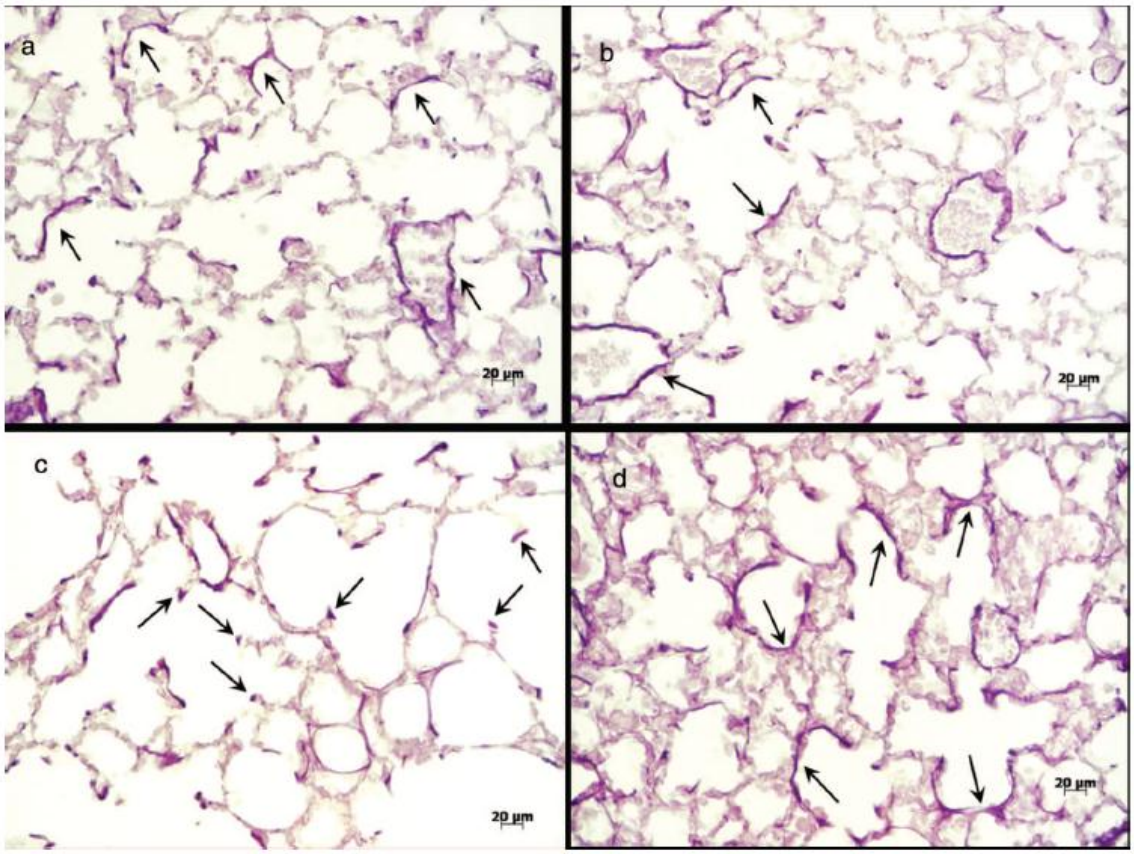

FIGURE 2 Lung photomicrographs stained with Weigert's resorcin-fuchsin of mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). (**a**) Control group exposed to ambient air presenting normal elastic fibers in the alveolar septa (*arrows*). (**b**) Mate group exposed to ambient air and treated with Mate tea presenting normal elastic fibers in the alveolar septa (*arrows*). (**c**) Mice exposed to CS and treated with vehicle presenting breakdown of elastic fibers in the alveolar septa (*arrows*). (**d**) Mice exposed to CS and treated with Mate tea presenting septal elastic fibers more elongated and preserved than in CS group (*arrows*). Scale bar: 20 μm. Original magnification, 40×.

RESULTS

Mate Tea Protects Pulmonary Tissue

To investigate the protective potential of Mate tea against lung damage caused by CS, 2 groups of mice were exposed to CS (from 12 cigarettes per day) and treated with Mate tea ("CS+Mate" group; $n = 10$) or vehicle ("CS" group; $n = 10$) daily for 60 days. Two further groups of mice were exposed to ambient air and treated with Mate tea ("Mate" group; $n = 10$) or vehicle ("control" group; $n = 10$). After 60 days, there were no significant differences in body weight between any of the groups. However, examination of H&E sections revealed the pulmonary histology of the CS group to be altered compared to both the control and Mate groups. The morphological changes observed are shown in Figure 1, where enlargement of alveoli can be seen in the CS group (Figure 1c) compared to the control group (Figure 1a). Strikingly, Mate treatment was effective in preserving the lung histoarchitecture of mice exposed to CS (Figure 1d), resembling that of the control lung, with alveolar spaces preserved.

Figure 2 shows examples of injured parenchyma in which breakdown of elastic fibers can be seen in the CS group (Figure 2c). In the CS+Mate group, by contrast, the elastic fiber network is better preserved (Figure 2d); the fibers are more elongated, similar to those of the control and Mate groups (Figure 2a and b).

Inflammatory cells (alveolar macrophages and neutrophils) were increased in the CS group compared with the control group ($P < .01$ and $P < .001$) (Table 1 and Figure 1e and g). Mice exposed to CS and treated with Mate (CS+Mate group) presented a significant reduction in neutrophil numbers compared with the CS group ($P < .001$; Figure 1g and h); there was no difference in neutrophil counts between the CS+Mate and control groups (Figure 1e and h). In addition, alveolar macrophage numbers were elevated in the CS+Mate group, although the increase was not significant compared to the control group.

Macrophages positive for 4-HNE (a marker of lipid peroxidation) were increased in the CS group ($P < .05$) when compared to the control group (Figure 3a and c). Mice exposed to CS and treated with Mate (CS+Mate group) presented a no significant reduction in positive macrophages for 4-HNE when compared to the CS group (Table 1, Figure 3c and d). The number of pneumocyte type II cells positive for 4-HNE was increased in the CS group when compared to the control group ($P < .001$). Mice exposed to CS and treated with Mate (CS+Mate group) presented a significant ($P < .001$) reduction in pneumocyte type II cells positive for 4-HNE when compared to the CS group (Table 1, Figure 3a to d).

Volume densities of airspaces and elastic fibers (Table 1) in lung parenchyma were quantified morphometrically. The CS group presented an increase in Vvair when compared with the control ($P < .05$) or Mate groups ($P < .001$). The Vvair of the CS+Mate group was not different from that of the control group. The volume density of elastic fibers in the CS group was reduced compared with that of the control group ($P < .05$) or Mate group ($P < .01$). The volume density of elastic fibers was not significantly different between the CS+Mate and control groups. The CS group also presented an increased Lm compared to the control ($P < .001$), whereas CS+Mate was identical to the control and reduced in comparison to the CS group ($P < .001$) Table 1.

Once the Mate group presented morphologic parameters similar to the control, the analysis related to the oxidative stress were performed only in the control, CS, and CS+Mate groups.

TABLE 1 Morphometrical Analysis of Mouse Lung Exposed to Ambient Air or to Cigarette Smoke (CS) and Treated With Vehicle or Mate Tea (500 mg/kg/day)

Groups	Control	Mate	CS	CS+Mate
MΦ/mm^2	53 ± 17	52 ± 5	200 ± 26**	150 ± 25
PMNs/mm^2	8.6 ± 5.7	2.9 ± 2.9	71 ± 8.0***	27 ± 4.1###
4HNE$^+$ AL/mm^2	10 ± 2.1	6.4 ± 2.7	21 ± 5.7*	10 ± 4.3
4HNE$^+$ EC/mm^2	61 ± 6.8	30 ± 4.8	108 ± 11***	27 ± 4.8###
Vvair (%)	67 ± 2.0	61 ± 3.3	78 ± 1.9**	71 ± 1.3#
Vvef (%)	9.5 ± 0.5	9.8 ± 0.4	5.2 ± 1.3*	8.1 ± 0.8
Lm (μm)	63.9 ± 1.4	60.4 ± 1.7	81.3 ± 3.0***	65.6 ± 1.8###

Note. Values are mean ± SEM. Kruskal-Wallis test followed by Dunn's post hoc test. $N = 5$ for all analysis.
MΦ = macrophages; PMNs = neutrophils; 4-HNE$^+$ AL = positive alveolar leukocytes for 4-hydroxynonenal; 4-HNE$^+$ EC = positive epithelial cells for 4-hydroxynonenal; Vvair = volume density of total air spaces; Vvef = volume density of elastic fibers; Lm = mean linear intercept.
*$P < .05$ when compared to control; **$P < .01$ when compared to control; ***$P < .001$ when compared to control; #$P < .05$ when compared to CS; ###$P < .001$ when compared to CS.

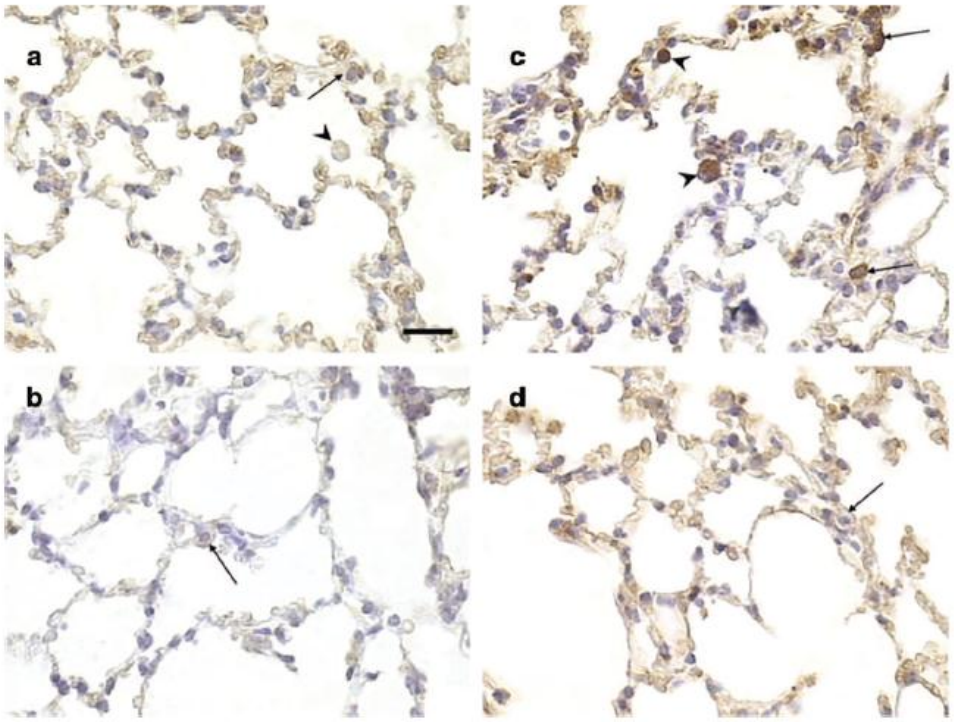

FIGURE 3 Lung photomicrographs immunostained with 4-hydroxynonenal of mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). (**a**) Control group exposed to ambient air presenting weak expression on alveolar leukocyte (*arrowhead*) and epithelial cell (*arrow*). (**b**) Mate group exposed to ambient air and treated with Mate tea presenting nonexpression on epithelial cell (*arrow*). (**c**) Mice exposed to CS and treated with vehicle presenting strong expression on alveolar leukocytes (*arrowheads*) and epithelial cells (*arrows*). (**d**) Mice exposed to CS and treated with Mate tea presenting nonexpression on epithelial cell (*arrow*). Scale bar: 50 μm.

Mate Tea Reduces Oxidative Stress

At the end of 60 days of treatment, BALF samples and lung homogenates were prepared for biochemical analysis. MPO activity (mU/mg protein), a marker of lung injury, was increased ($P < .05$) in the CS group compared to the control group. MPO activity in the CS+Mate group was reduced compared to that of the CS group ($P < .05$) and was similar to that of the control group (Figure 4). SOD activity (U/mg protein) was decreased in the CS group compared to the control group ($P < .001$). Although the CS+Mate group also presented a reduction in SOD activity ($P < .001$) when compared with the control group, this reduction was higher in the CS group (Figure 5). The CS group also presented a significant reduction in CAT activity ($P < .05$) in comparison to the control; the CAT activity of the CS+Mate group was not different from that of the control and was increased when compared to that of the CS group ($P < .001$) (Figure 6). The GSH/GSSG ratio was reduced significantly ($P < .05$) in the CS group compared to the control group (Figure 7). The GSH/GSSG ratio of the CS+Mate group was significantly higher than that of the CS group ($P < .05$). The hydroxyproline level (μg/mg protein), a marker of collagen breakdown, was increased ($P < .05$) in the CS group when compared with the control group (Figure 8). The CS+Mate group presented a reduction ($P < .05$) in hydroxyproline content when compared with the CS group.

DISCUSSION

Oxidative stress is a critical aspect of lung inflammation contributing to the development of emphysema. Pulmonary emphysema is caused mainly by CS and characterized by enlargement of alveolar spaces

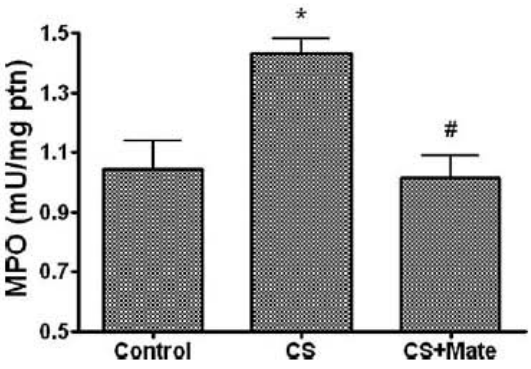

FIGURE 4 Myeloperoxidase (MPO) activity of lung homogenates in mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). MPO activity was increased in the CS group when compared to the control group. MPO activity in the CS+Mate group was reduced compared with that of the CS group. Data are presented as mean ± SEM and were analyzed by 1-way ANOVA followed by Tukey post hoc test ($P < .05$). $^{*}P < .05$ when compared to the control group and $^{\#}P < .05$ when compared to the CS group. n = 8 for all groups.

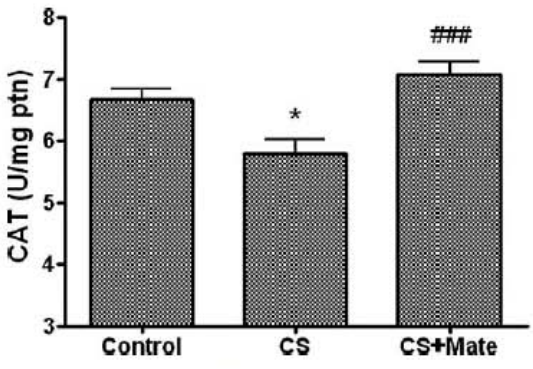

FIGURE 6 Catalase (CAT) activity of lung homogenates in mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). CAT activity was reduced in the CS group when compared to the control group. CAT activity in the CS+Mate group was increased when compared to the CS group, but was not different from the control. Data are presented as mean ± SEM and were analyzed by 1-way ANOVA followed with Tukey post hoc test ($P < .05$). $^{*}P < .05$ when compared to the control group and $^{***}P < .001$ when compared to the CS group. n = 8 for all groups.

with destruction of alveolar septa and migration of inflammatory cells [27]. We previously reported that CS-induced emphysema could be ameliorated with antioxidant vitamin supplementation [13]. Here we used another type of antioxidant source, Mate tea, which could be beneficial for protecting mouse lung from damage caused by CS exposure. In a first study, we showed that Mate tea was capable of lowering inflammatory and oxidative damage markers in a model of acute lung inflammation [12], using a dose 150 mg/kg/day. However in a pilot study using a chronic model, this dose was not able to protect mouse lung

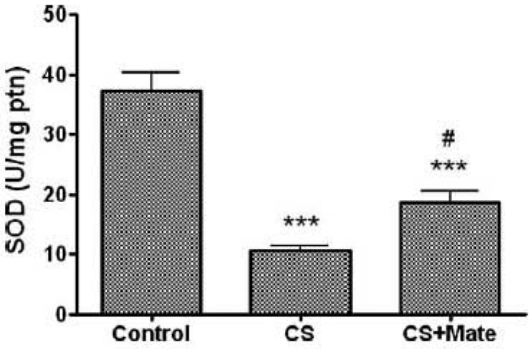

FIGURE 5 Superoxide dismutase (SOD) activity of lung homogenates in mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). SOD activity in the CS and CS+Mate groups was reduced when compared to the control group. SOD activity in the CS+Mate group was increased compared to that of the CS group. Data are presented as mean ± SEM and were analyzed by 1-way ANOVA followed with Tukey post hoc test ($P < .05$). $^{***}P < .001$ when compared to the control group and $^{\#}P < .05$ when compared to the CS group. n = 8 for all groups.

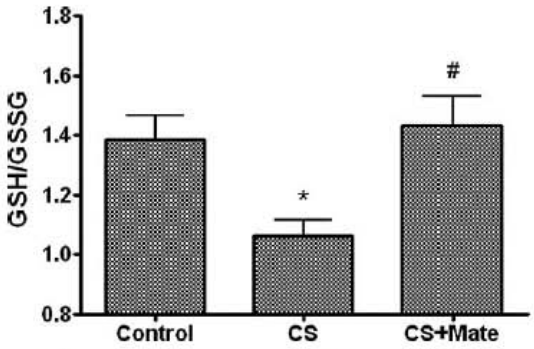

FIGURE 7 Reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio of lung homogenates in mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). GSH/GSSG ratio in the CS group was reduced when compared to the control group. GSH/GSSG ratio in the CS+Mate group was increased when compared with the CS group. Data are presented as mean ± SEM and were analyzed by Kruskal-Wallis followed by Dunn's post hoc test ($P < .05$). $^{*}P < .05$ when compared to the control group and $^{\#}P < .05$ when compared to the CS group. n = 7 for all groups.

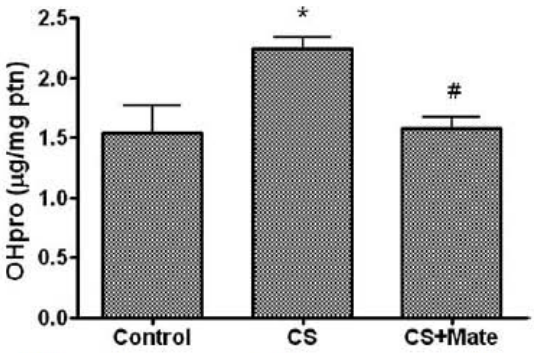

FIGURE 8 Hydroxyproline (OHpro) of bronchoalveolar lavage fluid in mice exposed to ambient air or to cigarette smoke (CS) and treated with vehicle or Mate tea (500 mg/kg/day). OHpro content in the CS group was increased when compared to the control group. OHpro content in the CS+Mate group was significantly reduced compared to that of the CS group. Data are presented as mean ± SEM and were analyzed by 1-way ANOVA followed with Tukey post hoc test ($P < .05$). $^{*}P < .05$ when compared to the control group and $^{\#}P < .05$ when compared to the CS group. n = 8 for all groups.

against CS effects; therefore, in the present study we used a higher dose (500 mg/kg/day) to ensure lung benefits without compromising mouse health [28]. We did not test isolated components, but the literature supports the presence of important concentrations of polyphenols such as chlorogenic acid [29], caffeic acid derivatives [30], and xanthines [31] in Mate tea.

In the present study, Mate tea modulated the effects of CS exposure in a chronic model of lung inflammation in the mouse. One explanation is that the antioxidant properties of Mate tea were responsible for preserving the alveolar spaces in the CS+Mate group of mice, a region that became enlarged in the CS group due to destruction of parenchyma. Furthermore, we also observed preservation of elastic fibers in the CS+Mate group, indicating that Mate tea at least partially inhibited elastolysis stimulated by CS. According to Barnes [32], oxidative stress may impair the function of antiproteases and thereby accelerate the breakdown of elastin in lung parenchyma [32]. Moreover, Mate tea also acted by reducing the influxes of alveolar macrophages and neutrophils into the alveolar spaces. Because these cells are responsible for the inflammatory response, their reduced numbers could contribute to fewer cytokines, metalloproteinases, and ROS being produced and released [33–35]. Furthermore, Evans and Pryor [36] suggested that oxidative stress caused by inflammatory cells could also contribute to the breakdown of extracellular matrix that leads to emphysema. We suggest that Mate tea treatment may help to protect against all of these injurious effects in the mouse lung. Indeed, according to Havsteen [37], the flavonoids, a polyphenol subgroup, can act as tissue inhibitors of matrix metalloproteinases. Moreover, Suzuki et al. [38] suggested that the treatment with curcumin, an antioxidant, was capable to attenuate porcine pancreatic elastase (PPE)- and CS-induced pulmonary inflammation and emphysema in mice, inhibiting macrophage and neutrophil recruitment and air space enlargement.

Another parameter that we used to assess lung damage was hydroxyproline level. According to Churg et al. [39], hydroxyproline in BALF is derived from degradation of lung extracellular matrix and is a good marker of connective tissue breakdown. This fact explains the elevated hydroxyproline level observed in the CS group, suggesting severe degradation of lung extracellular matrix and connective tissue damage. In contrast, the hydroxyproline level in the CS+Mate group was significantly reduced, demonstrating that Mate tea attenuated the CS-induced breakdown of connective tissue. However, in the CS group the collagen fibers may be degraded by proteases released from inflammatory cells and epithelial cells [40]. In the CS+Mate group, we suggest that Mate partially protected the collagen fibers from proteases.

We also analyzed lung injury caused by CS by using a biochemical assay for MPO, an enzyme released from neutrophils. MPO activity was found to be lower in the CS+Mate group than in the CS group, consistent with the observed reduction in neutrophil influx in the former compared to the latter group. MPO is a pro-oxidant enzyme, which yields superoxide radical and hydrogen peroxide for generating more oxidants [41]. MPO activity was elevated only in the CS group, thus corroborating the importance of oxidative stress from CS in emphysema. Tsoumakidou et al. [42] have recently demonstrated increased MPO and interleukin-8 levels during severe exacerbations of chronic obstructive pulmonary disease (COPD) compared with the stable state. MPO is produced mainly by neutrophils [41, 43], and in our experimental design, emphysematous mice exhibited increased neutrophil infiltration into the alveolar space that was mitigated by Mate. We suggest an indirect role for Mate in lowering neutrophil levels in the lungs, although we cannot rule out an action of Mate in regulating the expression of MPO.

SOD provides the main defense against oxidative damage caused by superoxide anion [44]. The reduction in SOD activity observed in the CS group suggests that its depletion was a function of high oxidant concentrations. Gongora et al. [45] recently

reported that even a modest reduction of this enzyme leads to lung injury and death. The CS+Mate group also presented a reduction in SOD activity; however, the magnitude of the reduction was smaller than that observed in the CS group. Probably because of the "protection" given by Mate tea, the cellular necessity for SOD (i.e., level) was reduced, and thus lower SOD activity was noted. The demand for SOD activity could be proportional to the amount of SOD substrate [23]. This hypothesis, although not confirmed here, is reinforced by the observed change in the levels of CAT activity, whereas in the presence of Mate the CAT activity was maintained even with CS exposure. CAT is a major mediator of H_2O_2 catabolism, and the ability of Mate to maintain CAT activity represents an important protective mechanism against the oxidative stress generated by CS. According to Flick et al. [46], exogenous CAT protects airway epithelium in vivo against H_2O_2 generated by neutrophils sequestered in the pulmonary microvasculature. Additional data that contribute to this hypothesis is the GSH/GSSG ratio, which is an important parameter of oxidative stress. Only the CS group presented a significant reduction in GSH/GSSG ratio, indicating substantial oxidation of thiol molecules. GSH can interact directly with oxidants, neutralizing them [5]. Thus, Mate tea helped protect intracellular GSH against oxidation. However, according to Galloway et al. [47] and Tu and Anders [48], phenolic antioxidants are able to increase the levels of glutamate cysteine ligase subunits in human endothelial cells. This enzyme controls the rate of GSH synthesis [49]. Thus, phenolic antioxidants can protect cells from oxidants by either scavenging these molecules or by regulating intracellular GSH levels [50]. Furthermore, tea polyphenols can act as antioxidants in vitro by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions. They may also function indirectly as antioxidants through (i) inhibition of the redox-sensitive transcription factors, nuclear factor-kappaB, and activator protein-1; (ii) inhibition of "pro-oxidant" enzymes such as inducible nitric oxide synthase, lipoxygenases, cyclooxygenases, and xanthine oxidase; and (iii) induction of phase II and antioxidant enzymes such as glutathione *S*-transferases and SODs [9].

Lipid peroxidation, an oxidative damage marker, was measured here by 4-HNE staining. Macrophage and pneumocyte type II cells were reduced in the Mate group, but not significantly. In contrast, the CS group presented a significant increase in 4-HNE–positive cells, and the treatment with Mate concomitant to CS was effective in reducing 4-HNE staining in these cells. In a model of acute lung inflammation, we previously observed an increase in malondialdehyde (MDA) level following CS exposure [12], which is another method to assay lipid peroxidation. We did not measure MDA here because the chemical conditions usually used for the MDA–thiobarbituric acid reaction give an indication of sample oxidizability rather than of sample oxidation [51]. In this context, 4-HNE is more sensitive and reliable [52].

This study examined Mate tea in response to CS exposure in the mouse. The protection observed, by both histological and biochemical analyses, leads us to suggest that Mate tea provides beneficial effects against lung damage caused by CS exposure in the mouse. In the CS+Mate group, few alterations to the alveolar spaces were observed, elastic fibers were preserved, and there were fewer macrophages and neutrophils recruited to alveoli compared to the CS group. Furthermore, Mate tea was capable to improve the redox balance, increasing SOD and CAT activities and GSH level. These results suggest that the mechanism of action of the Mate tea consists in augmentation of the antioxidant defenses, both enzymatic and nonenzymatic that were diminished by CS.

In summary, CS-induced emphysema in C57BL/6 mice was prevented by treatment with Mate tea and was associated with a reduction in inflammatory and oxidative stress parameters. Our results point to Mate tea as a nutritional antioxidant against lung injury in mice exposed chronically to CS and support efforts to investigate the beneficial effects of Mate tea on CS-related lung injury in other animal models and humans.

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APÊNDICE G – Artigo publicado durante ao período de mestrado - III

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Addition of açaí (*Euterpe oleracea*) to cigarettes has a protective effect against emphysema in mice

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ABSTRACT

Chronic inhalation of cigarette smoke (CS) induces emphysema by the damage contributed by oxidative stress during inhalation of CS. Ingestion of açai fruits (*Euterpe oleracea*) in animals has both antioxidant and anti-inflammatory effects. This study compared lung damage in mice induced by chronic (60-day) inhalation of regular CS and smoke from cigarettes containing 100 mg of hydroalcoholic extract of açai berry stone (CS + A). Sham smoke-exposed mice served as the control group. Mice were sacrificed on day 60, bronchoalveolar lavage was performed, and the lungs were removed for histological and biochemical analyses. Histopathological investigation showed enlargement of alveolar space in CS mice compared to CS + A and control mice. The increase in leukocytes in the CS group was higher than the increase observed in the CS + A group. Oxidative stress, as evaluated by antioxidant enzyme activities, mieloperoxidase, glutathione, and 4-hydroxynonenal, was reduced in mice exposed to CS + A versus CS. Macrophage and neutrophil elastase levels were reduced in mice exposed to CS + A versus CS. Thus, the presence of açai extract in cigarettes had a protective effect against emphysema in mice, probably by reducing oxidative and inflammatory reactions. These results raise the possibility that addition of açaí extract to normal cigarettes could reduce their harmful effects.

1. Introduction

Cigarette smoke (CS) is usually associated with development of chronic obstructive pulmonary disease (COPD), a pathological condition characterized by a slow, progressive, and largely irreversible airflow limitation due to chronic bronchitis and/or emphysema (O'Donnell and Parker, 2006). COPD is a major and increasing global health problem: It is currently the 4th leading cause of death worldwide, and is projected to become the 3th leading cause of death and the 5th most common cause of disability by the year 2020 (Lopez and Murray, 1998). Airway oxidative stress, inflammation, and proteolysis are intimately related to the pathophysiology of CS-induced COPD. The increase in oxidative stress in the lungs is due to an imbalance in the production of oxidants and reactive oxygen species (ROS) as well as to a reduction of antioxidant defense mechanisms (Church and Pryor, 1985; Pryor et al., 1983; Schumacher et al., 1977). ROS are constituents of both the tar and gas phases of smoke, which include reactive aldehydes, quinones, and benzo(a)pyrene. These agents, plus reduced pulmonary antioxidant defense mechanisms, result in an oxidative burden that along with chronic inflammation leads to cellular damage and lung remodeling. To reduce the lung damage induced by smoke, the cigarette industry has produced low tar and low nicotine cigarettes; however, it is not clear that reduction of tar and nicotine have decreased the incidence of COPD among smokers (Dobson, 2008; Dunsby and Bero, 2004). Experimental data in animals show that systemically administered antioxidants may

Abbreviations: CS, cigarette smoke; COPD, chronic obstructive pulmonary disease; ROS, reactive oxygen species; ASE, açaí stone extract; COHb, carboxyhemoglobin; H&E, hematoxylin and eosin; BAL, bronchoalveolar lavage; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide; MPO, myeloperoxidase; WB, Western blotting; 4-HNE, 4-hydroxynonenal; MMP-12, metalloelastase.

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reduce lung damage. Specifically, oral administration of antioxidants such as vitamin C (Banerjee et al., 2008; Panda et al., 2000; Silva Bezerra et al., 2006), vitamin E (Graziano et al., 1985; Uneri et al., 2006), black tea (Banerjee et al., 2007), mate tea (Lanzetti et al., 2008), and curcumin (Shishodia et al., 2003; Vanisree and Sudha, 2006) reduce lung damage induced by CS in experimental animal models.

Euterpe oleracea Mart., popularly known as the açaí berry, is widely cultivated in the Amazon region of Brazil. Chemical studies have shown that the açaí berry has a diverse composition that includes hydroxybenzoic acids, antioxidant polyphenolics, flavan-3-ols, and anthocyanins, predominantly cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucuronide (Del Pozo-Insfran et al., 2004; Kang et al., 2010; Lichtenthaler et al., 2005; Rodrigues et al., 2006; Schauss et al., 2006b). Other studies have shown that consumption of the açai berry has other beneficial effects due to its high superoxide anion scavenging properties, its anti-inflammatory effect via inhibition of cyclooxygenases 1 and 2 (Schauss et al., 2006a), its vasodilator effect (Rocha et al., 2007), and its inhibition of nitric oxide production and iNOS activity and expression (Matheus et al., 2006). The antioxidant effect of a fruit and berry juice blended containing açai as the predominant ingredient was demonstrated in an *In vivo* study performed in health subjects (Jensen et al., 2008). The present study was undertaken to determine whether adding a hydroalcoholic extract of açai berry stone to cigarettes reduces the harmful effects of CS in mice.

2. Methods

2.1. Reagents

The following reagents were purchased from Sigma Chemical (St. Louis, MO, USA): acrylamide, adrenaline, bovine serum albumin, bromophenol blue, calcium chloride dehydrate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dipotassium hydrogen phosphate, eosine, glutathione, glutathione disulfide, glycerol, hematoxylin, hexadecyltrimethylammonium bromide (HTAB), 2-mercaptoethanol, monopotassium phosphate, myeloperoxidase, nicotinamide adenine dinucleotide phosphate (NADPH), 2-nitro-5-thiobenzoate (TNB), sodium acetate buffer, sodium chloride, sodium dodecylsulfate, 3,3′,5,5′-tetramethylbenzidine (TMB), thiobarbituric acid, triethanolamine, Tris–HCl, TritonX-100, Tween-20, 2-vinylpyridine, and zinc chloride. Panótico was purchased from Laborclin (Pinhais, Paraná, Brazil). Bradford assay reagents were purchased from Bio-Rad (Hercules, CA, USA). Acetic acid, ethanol, formalin, and hydrogen peroxide were purchased from Vetec (Duque de Caxias, Rio de Janeiro, Brazil).

2.2. Animals

C57BL/6 male mice (8 weeks old; 20–24 g) were purchased from the Veterinary Institute at Fluminense Federal University (Niterói, Rio de Janeiro, Brazil). Mice were housed 5 per cage in a room with a controlled environment, a 12-h light/12-h dark cycle (lights off at 6 pm), and an ambient temperature of 25 ± 2 °C (humidity around 40–60%). The animals had free access to filtered tap water and food (™Nuvilab for rodents, Nuvital Nutrients S.A., Colombo, Paraná, Brazil). Acclimatization was performed for two weeks before the experimental period began. All procedures were carried out in accordance with The Ethics Committee for Experimental Animals Use and Care (CEA) of Instituto de Biologia Roberto Alcântara Gomes/Universidade do Estado do Rio de Janeiro. The CEA follow guidelines from Intramural Animal Care and Use (ACU) program of the National Institutes of Health (NIH). The cigarette smoke protocol was adapted from OECD guidelines for inhalation studies (Draft Updated Test Guideline 413, 2009).

2.3. Preparation and characterization of açaí stone extract (ASE)

E. Oleracea Mart. fruits were obtained from the Amazon Bay (Belém do Pará, Pará, Brazil) excicata number 29052 Museu Goeldi–Belem do Para. Many readers may not be aware that the seed of the acai fruit constitutes as much as 80% of the fruit mass. On that matter, some characteristics of this berry are described next. The açai fruits ($n = 10$) are black, weight 1.612 ± 0.072 g and have an oval shape (1.456 ± 0.032 × 1.215 ± 0.036 cm). The açai stones obtained by removal of the skin of açai fruit are brown ochre, weigh 1.308 ± 0.051 g and have an oval shape (1.310 ± 0.031 × 1.090 ± 0.034 cm). Hydroalcoholic extracts were obtained from a decoction of the berry stone. Approximately 200 g of açaí stone were boiled in 400 mL of water for 5 min, mixed for 2 min, and then boiled again for 5 min. The decoction was cooled to room temperature and then extracted by addition of 400 mL of ethanol with shaking for 2 h. The extract was stored in dark bottles inside a refrigerator (4 °C) for 10 days. After this maceration period, the hydroalcoholic extracts of açaí were filtered through #1 Whatman filter paper, and the ethanol was evaporated using a rotary evaporator (Fisatom Equipamentos Científicos Ltda São Paulo, São Paulo, Brazil) under low pressure at 55 °C. The extract was then lyophilized (LIOTOP model 202, Fisatom Equipamentos Científicos Ltda São Paulo, São Paulo, Brazil) with temperature from −30 to −40 °C and vacum of 200 mm Hg and frozen at −20 °C until use. The concentration of polyphenols in the ASE, as measured by analysis of total phenolic content using the Folin–Ciocalteau procedure (George et al., 2005), was 255.7 ± 15.3 mg/g ($n = 4$, expressed in gallic acid equivalent) of extract. Typically 100 g of stone yielded approximately 5 g of lyophilized extract.

We intended analyzing the composition of ASE by mass spectroscopy, but the results obtained from this equipment in our institution were not reliable. Thus, we decided to use HPLC instead. Therefore ASE was analyzed on a RP-18 column (250 × 4 mm, 5 μm particles) according to a procedure reported by Peng et al. (2001). Elution was made with solvents A [0.2% (v/v) phosphoric acid] and B [82% (v/v) acetonitrile, 0.04% (v/v) phosphoric acid]. Flow rate was 1 ml/min. DAD UV–Vis absorption spectra were recorded on-line during HPLC analysis. The HPLC elution profile observed is strongly indicative of the presence of proanthocyanidins (Peng et al., 2001). The peak eluting at 37.2 min corresponds to catechin as confirmed by co injection of a standard and by comparison of the ultraviolet absorption spectrum. The late elution (at 54.7 min) and UV spectrum of the main peak are consistent with the presence of polymeric proanthocyanidins (Fig. 1).

2.4. CS exposure

The mice were divided into three groups of 20 animals: a control group, a CS group, and a CS + A group. The control group was sham-smoked, i.e. exposed to ambient air using a smoking chamber. The CS group was exposed to smoke from 12 commercially-obtained full-flavor (Marlboro – Philips Morris, Santa Cruz, Rio Grande do Sul, Brazil) filtered Virginia cigarettes (10 mg of tar, 0.9 mg of nicotine, and 10 mg of carbon monoxide) each day for 60 days using a smoking chamber as described previously (Menegali et al., 2009; Valenca et al., 2008a, 2004; Valenca and Porto, 2008). Briefly, mice were placed in the inhalation chamber (40 cm long, 30 cm wide, and 25 cm high) inside a laboratory fume hood. A cigarette was coupled to a plastic 60 mL syringe so that puffs could be drawn in and subsequently expelled into the exposure chamber. One Liter of the puff was expelled into the chamber.

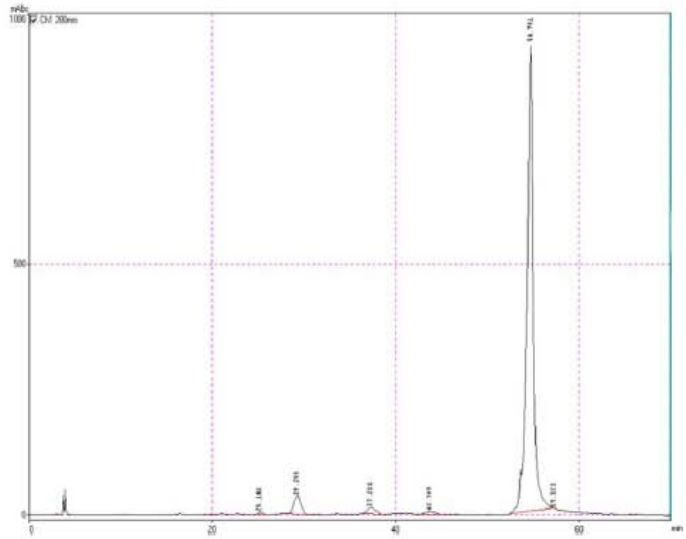

Fig. 1. HPLC analysis of ASE. We used a RP-18 column (250 × 4 mm, 5 μm particles). Elution was made with solvents A [0.2% (v/v) phosphoric acid] and B [82% (v/v) acetonitrile, 0.04% (v/v) phosphoric acid]. Flow rate was 1 ml/min. The peak eluting at 37.2 min corresponds to catechin as confirmed by co injection of a standard and by comparison of the ultraviolet absorption spectrum. The late elution (at 54.7 min) and UV spectrum of the main peak are consistent with the presence of polymeric proanthocyanidins.

The animals were maintained in this smoke-air condition for 6 min (~3%), and the inhalation chamber was opened, by removing its cover, and the smoke evacuated for 1 min. There was thus exposure to four cigarettes i.e. 4 × 6-min exposures that were separated by 1-min intervals in which the chamber was cleared of smoke. This procedure was repeated three times per day (morning, noon, and afternoon) for a total of 72 min/day of exposure to CS from twelve cigarettes. Each cigarette produced 300 mg/m^3 of total particulate matter in the chamber as measured by weighing the material collected on Pallflex filters. Immediately after CS exposure, carboxyhemoglobin (COHb) levels were measured using the method described by Beutler and West (1984). Briefly, 1.2 mL hemolyzing solution (phosphate buffer, pH 6.85, in water, 1:10) is added to 10 mL blood, homogenized and left to stand for 10 min; 100 mL of this solution are pipetted into a test tube containing 2.3 mL reducing solution (25 mg of sodium dithionite in 20 mL of phosphate buffer, pH 6.85, prepared immediately before use), homogenized and left to stand for 10 min. Absorbance at 420 and 432 nm is read using the reducing solution as a reference. Carboxyhemoglobin concentration was obtained by an equation that considered the AR—the ratio of the absorbance values of the solution at 420 and 432 nm considering the factors (reference values) from blood bubbled with 100% carbon monoxide or 100% oxygen. CS-exposed mice had a mean COHb of 8.9 (± 0.96 [SEM]) versus air-exposed mice with a mean COHb of 1.9 (± 0.31 [SEM]).

The CS + A mice were exposed to smoke from 12 commercially-obtained full-flavor filtered Virginia cigarettes plus açaí each day for 60 days using the same smoking chamber described above. ASE (100 mg) was mixed with 1 mL saline and injected into each cigarette (100 mg/cigarette). The cigarettes with açaí were dried at 37 °C for 24 h. Exposure to smoke from the cigarettes with açaí was performed as for CS without açaí. When 'smoked,' each cigarette with açaí produced 370 mg/m^3 of total particulate matter in the chamber as measured by weighing material collected on Pallflex filters. The COHb concentration from the CS-A cigarettes was similar to that from regular CS. The choice of this dose (100 mg/cigarette) was made based on previous data from our group in which 100 mg of açaí (orally) reduced both the influx of inflammatory cells into the mouse lungs and the TNF-α expression after acute cigarette smoke exposure (short-term 5 days, data not published yet). The experimental protocol used in the present paper was very similar to that one, except by the time of exposure (long-term 60 days and short-term 5 days). We performed this protocol with ASE into the cigarettes since we believe that the present result is a novelty on this subject.

2.5. Histology and morphometry

Twenty-four hours after the final exposure to CS, the mice were sacrificed by cervical displacement and the right ventricle was perfused with saline to remove blood from the lungs. The lungs from 10 mice from each group were fixed with 10% buffered formalin pumped through a tube inserted into the trachea using continuously maintained pressure of 25 cm H2O (AVS Projetos, Campinas, São Paulo, Brazil). After addition of formalin, the lungs were clamped and removed en bloc, after which they were immersed in fixing solution for 48 h, processed in increasing alcohol concentrations, cleared in xylene, and embedded in paraffin to obtain sections from the apex, middle third, and base of the lungs. Sections from the right lung (5 μm thick) were stained with hematoxylin and eosin (H&E).

Pulmonary emphysema was quantified based on the degree of alveolar destruction as determined by measuring the mean linear intercept in micrometers (L_m; mean alveolar diameter) as described previously (Valenca and Porto, 2008). In brief, this involves determining the number of times the gas exchange structures in the parenchyma intersect a series of grid lines. When emphysema is present, there are fewer intercepts of the alveolar structures with the grid, indicating alveolar destruction. The L_m is obtained using the equation $L_m = L_{tot}/L_i$, where L_{tot} is the total length of the lines in the microscope field and L_i is the number of intercepts of the alveolar structures with the grid lines. To obtain the L_m, 16 fields

in each section were counted and observed at 200× magnification through a grid attached to the monitor. We used three sections per mouse and five mice per group.

2.6. Bronchoalveolar lavage

The lung air spaces were washed three times with buffered saline solution (500 μL) for a final bronchoalveolar lavage (BAL) fluid volume of 1.2–1.5 mL). The collected BAL fluid was stored on ice. The total number of cells in the BAL fluid was determined using a Neubauer chamber. Differential cell counts were performed on cytospin cell preparations (Cientec CT 2000, Piracicaba, São Paulo, Brazil) stained with Panótico. At least 200 cells per BAL sample were counted using standard morphologic criteria. After BAL, the lungs were removed immediately, homogenized on ice with 10% (w/v) 0.1 M potassium phosphate buffer (pH 7.4) using a tissue homogenizer (NT136; Campinas, São Paulo, Brazil), and centrifuged at 800g for 5 min. The supernatants were stored at −20 °C for biochemical analysis. The protein concentration in the lung homogenate samples was determined by the Bradford method (Bradford, 1976).

2.7. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activity determination

SOD, CAT, and GP*x* activities were determined in the lung homogenates as follows. SOD activity was assayed by measuring inhibition of adrenaline auto-oxidation as absorbance at 480 nm (Bannister and Calabrese, 1987) using a Beckman spectrophotometer (model DU 640; Beckman Instruments, Fullerton, CA, USA). CAT activity was measured by the rate of decrease of hydrogen peroxide concentration at 240 nm (Aebi, 1984). GP*x* activity was measured by monitoring the oxidation of NADPH at 340 nm in the presence of hydrogen peroxide (Flohe and Gunzler, 1984).

2.8. Reduced glutathione (GSH) and glutathione disulfide (GSSG) assay

This assay was based on the reaction of GSH or GSSG with DTNB, which produces the TNB chromophore (Rahman et al., 2006). To determine GSSG, lung homogenate samples were treated with 2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine is neutralized with triethanolamine. The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH or GSSG in the sample. The concentration of an unknown sample is determined using the linear equation or the regression curve generated by measuring several known concentrations of GSH or GSSG. The final determination is presented as the GSH/GSSG ratio.

2.9. Myeloperoxidase (MPO) assay

MPO activity was measured using hydrogen peroxide, HTAB, and TMB. Initially 100 μL of a lung homogenate sample was centrifuged with 900 μL of HTAB at 14,000g for 15 min. The supernatant (75 μL) was incubated with 5 μL of TMB for 5 min at 37 °C. The mixture was then incubated with 50 μL of hydrogen peroxide for 10 min at 37 °C, after which 125 μL of sodium acetate buffer was added. The reaction was read using a microplate reader (Bio-Rad model 550, Hercules, CA, USA) at 630 nm (Suzuki et al., 1983). The concentration of MPO in the samples was determined using a standard curve established using purified MPO.

2.10. Western blotting (WB)

Proteins from lung homogenate samples were denatured by addition of sample buffer (50 mM Tris–HCl, pH 6.8, 1% sodium dodecylsulfate, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heating in boiling water for 3 min. Proteins (50 μg) were resolved by 15% sodium dodecylsulfate polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). Rainbow markers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were run in parallel for estimation of molecular weights. The membranes were blocked with Tween-TBS (20 mM Tris–HCl, pH 7.5, 500 mM sodium chloride, 0.5% Tween-20) containing 2% bovine serum albumin and probed with specific primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA): goat anti-mouse 4-hydroxynonenal (1:2000), goat anti-mouse metalloelastase (1:2000), and goat anti-mouse neutrophil elastase (1:3000). After extensive washing in TBS-Tween, the membranes were incubated with biotin-conjugated donkey anti-goat immunoglobulin G (1:10000) for 1 h (SantaCruz Biotechnology, Santa Cruz, CA, USA) and then developed with the ECL Western Detection Reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. The bands were assessed by densitometry using Scion Image Software (Scion Co., Frederick, MD, USA).

2.11. Statistical analysis

The data are expressed as mean ± SEM. For comparison of L_m, BAL cells, antioxidant enzyme activities, MPO, and densitometry (WB) in the control, CS, and CS + A groups, the data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p < 0.05$). To compare the GSH/GSSG ratio in the control, CS, and CS + A groups, the data were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test ($p < 0.05$). Statistical analyses were performed using GraphPad Prism software (GraphPad Prism version 5; GraphPad Software, SanDiego, CA, USA).

3. Results

3.1. Histopathological investigation

Pulmonary tissue analysis showed that the control group mice, who were exposed to ambient air, had normal-sized air spaces and normal alveolar septa (Fig. 2a). The alveolar spaces were enlarged in mice in the CS group, and leukocytes were numerous in the alveoli (Fig. 2b). The lung parenchyma of the CS + A group had some enlarged areas and some leukocytes in the alveoli (Fig. 2c). The lung histology in the CS + A group mice was not to the same as in the control group mice, but appeared less affected by emphysema than did the lungs of mice in the CS group.

Alterations in the lung parenchyma were quantified based on the L_m (Fig. 3). The L_m was 38% greater in the CS group compared to the control group ($p < 0.001$). The L_m was 25% lower in the CS + A group compared to the CS group ($p < 0.01$) but was not significantly different from the L_m in the control group.

3.2. CS+A modulates cell influx

BAL fluid from mice exposed to CS showed a 400% increase in leukocytes compared with the control group ($p < 0.001$) (Fig. 4). Mice exposed to CS + A showed 65% fewer leukocytes in the BAL fluid compared to the CS group ($p < 0.001$). There were 140% more alveolar macrophages (Fig. 5) in the BAL fluid of CS mice compared with the control group ($p < 0.001$), and 100x more neutrophils (Fig. 6) ($p < 0.001$). Mice exposed to CS + A showed 44% fewer macrophages in the BAL fluid compared with the CS group ($p < 0.001$), while there were 83% fewer neutrophils ($p < 0.001$). However,

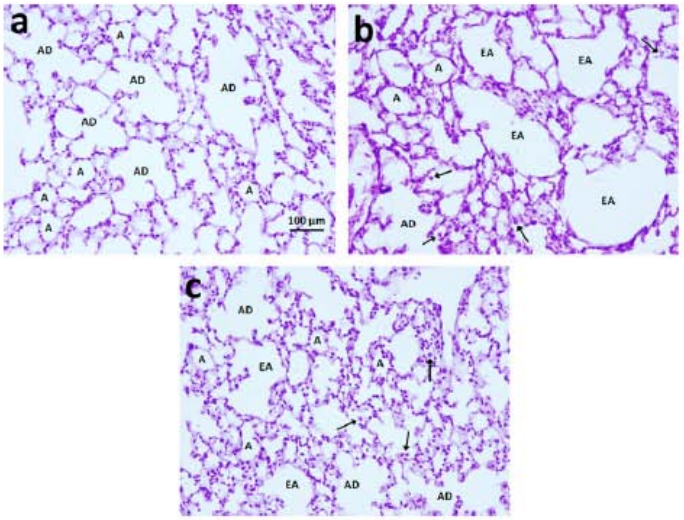

Fig. 2. Photomicrographs of lung sections stained with hematoxylin and eosin (400× magnification). (a) Control group mice were exposed to ambient air. (b) CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. (c) CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and injected into each cigarette. A = alveoli; AD = alveolar duct; EA = enlarged areas; arrows = leukocytes.

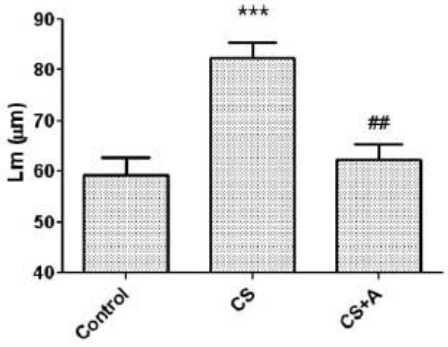

Fig. 3. Morphometry of lung parenchyma. Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and then injected into each cigarette. L_m, the mean linear intercept, indicates the degree of alveolar destruction. Data are expressed as means ± SEM (n = 10 for each group) and were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test ($p < 0.05$). ***$p < 0.001$ compared with the control group. ##$p < 0.01$ compared with the CS group.

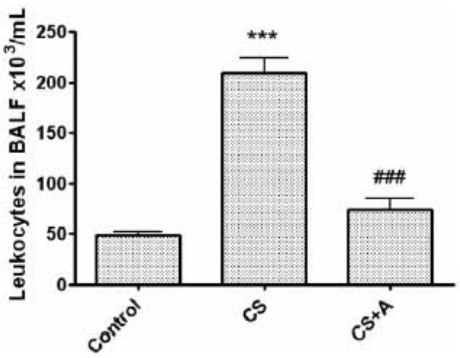

Fig. 4. Leukocytes in bronchoalveolar lavage fluid (BALF). Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and injected into each cigarette. Data are expressed as means ± SEM (n = 10 for each group) and were analyzed by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). ***$p < 0.001$ compared with the control group. ###$p < 0.001$ compared with the CS group.

there were more neutrophils in BAL fluid from CS + A mice than from control mice ($p < 0.01$).

3.3. CS+A reduces oxidative stress

Results of biochemical analysis of oxidative stress parameters are shown in Table 1. SOD activity decreased 34% ($p < 0.001$) in the CS group and 50% ($p < 0.001$) in the CS + A group compared with the control group. CAT activity decreased 16% ($p < 0.001$) in the CS group and 28% in the CS + A group compared with the control group. GPx activity decreased 74% ($p < 0.001$) in the CS group compared with the control group. There was a 5× increase in GPx activity in the CS + A group ($p < 0.001$) than in the CS group. The GSH/GSSG ratio decreased 48% ($p < 0.01$) in the CS group compared with the control group and increased 78% ($p < 0.01$) in the CS + A group compared with the CS group. Finally, MPO increased 123% ($p < 0.001$) in the CS group compared with the control group. MPO was reduced 43% in the CS + A group compared with the control group ($p < 0.001$). Western blotting for 4-hydroxynonenal (4-HNE) expression (Fig. 7) was performed to confirm the biochemical analysis of oxidative stress markers. Expression of 4-HNE was increased in the CS group compared with the control group ($p < 0.05$), but not significantly different in the CS + A group compared to the control group.

3.4. CS+A reduces elastase expression

Metalloelastase (MMP-12 or macrophage elastase) and neutrophil elastase levels were examined using Western blotting; these

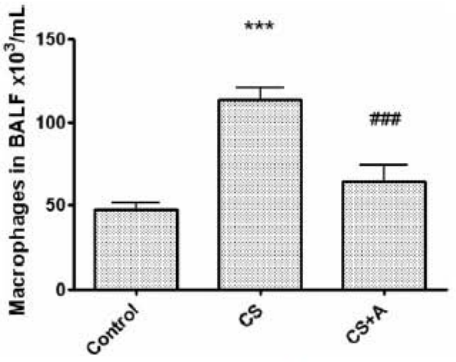

Fig. 5. Macrophages in bronchoalveolar lavage fluid (BALF). Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and then injected into each cigarette. Data are expressed as means ± SEM ($n = 10$ for each group) and were analyzed by one-way ANOVA followed with Tukey's post hoc test ($p < 0.05$). $^{***}p < 0.001$ compared with the control group. $^{###}p < 0.001$ compared with the CS group.

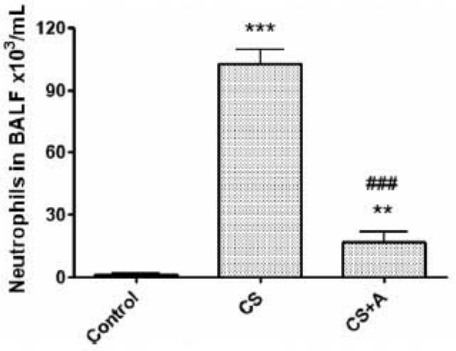

Fig. 6. Neutrophils in bronchoalveolar lavage fluid (BALF). Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and then injected into each cigarette. Data are expressed as means ± SEM ($n = 10$ for each group) and were analyzed by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with the control group. $^{###}p < 0.001$ compared with the CS group.

are two important enzymes that break down extracellular matrix in pulmonary emphysema. MMP-12 expression increased in the CS group ($p < 0.001$) and in the CS + A group ($p < 0.01$) compared with the control group (Fig. 8). However, MMP-12 expression was lower in the CS + A group compared with the CS group ($p < 0.05$). Neutrophil elastase expression was higher in the CS group compared with the control group ($p < 0.05$) (Fig. 9).

4. Discussion

This study demonstrated for the first time that adding a hydroalcoholic extract of açaí stone to cigarettes significantly reduced pulmonary inflammation, oxidative stress, and CS-induced emphysema in mice. We decided to study the effect of açai stone extract, instead of he açaí pulp extract since we found that the extract of stone was more active than the extract of pulp, concerning vasodilation *in vitro* (Rocha et al., 2007). Because lung damage induced by CS is mainly due to inflammation and oxidative stress, it seems likely that açaí's anti-inflammatory and antioxidant properties underlie these protective effects (Lichtenthaler et al., 2005; Rodrigues et al., 2006; Schauss et al., 2006a). Previous studies have shown that antioxidants such as black tea, mate tea, vitamin C and vitamin E can mitigate the deleterious effects of CS in the lungs (Banerjee et al., 2007; Lanzetti et al., 2008; Panda et al., 2000; Silva Bezerra et al., 2006). However, these studies used oral administration of antioxidants, rather than addition to cigarettes, as was done here. The present study demonstrated that açaí extract in cigarettes has a preventive action; that is, the harmful effects of CS can be significantly reduced when the smoke also contains antioxidant compounds. The present study also demonstrated that ASE contain proanthocyanidins, that are polyphenols and occur as a mixture of oligomers and polymers composed of flavan-3-ol units linked mainly through C4 → C8 and/or C4 → C6 bonds (B type). Proanthocyanidins are common constituenst of many plant foods, such as gooseberry, red currant, blackcurrant, barley, grape, wine, sorghum and apple. Several plants of the palm family Arecaceae are reported to contain proanthocyanidins (Esquenazi et al., 2002; Hong et al., 2006). Finally, there are several reports on the health benefits produced by ingestion of food containing proanthocyanidins (Prior and Gu, 2005). Notably, the active compounds in the ASE occurring in the cigarette are heat resistant, since the peak temperatures at the periphery of the coal can exceed 950 °C (Baker et al., 2004).

The oxidant effect of cigarettes is due not only to the high quantity of ROS in the smoke, approximately 10^{14} free radicals per puff, but also to endogenous ROS that are produced in the lungs mainly by inflammatory cells in alveoli that spontaneously release ROS (Babior, 2000; MacNee, 2005). It is likely that CS induces an increase in oxidative stress in alveoli, activating inflammatory reactions. In turn, this leads to recruitment of inflammatory cells with a further increase in the oxidative burden. Ultimately, the result is alveolar lesions and development of emphysema (Tuder et al., 2006). Our results confirmed previous findings that CS induces substantial lung damage in mice (Blake et al., 2010; Iizuka et al., 2005; Sato et al., 2008; Yao et al., 2008).

The present results demonstrated that the CS-induced increase in oxidant stress, as showed by an increase in 4-HNE expression (a marker of lipid peroxidation) in the lungs of mice exposed to CS, is significantly lower in the lungs of mice exposed to CS + A versus CS. The antioxidant activity of ASE probably does not depend on activation of antioxidants enzymes such as SOD and catalase, since levels of these enzymes were not increased in mouse lungs exposed to CS + A compared to those exposed to CS. Instead, this antioxidant activity may be due to the polyphenol content of ASE (Mertens-Talcott et al., 2008; Pacheco-Palencia et al., 2008a,b). The major player in redox balance in the lung is the glutathione system. As assessed by GPx and the GSH/GSSG ratio, the glutathione system was preserved in mouse lungs exposed to CS + A. Catechin is a powerful antioxidant agent for scavenging ROS, such as superoxide anions and hydroxyl radicals, 1,1-diphenyl-3-picrylhydrazyl radical superoxide, hydrogen peroxide, and hydroxyl radicals (Nanjo et al., 1999); interestingly, ASE has a high catechin content (data not shown).

Acute or chronic CS exposure induces substantial inflammation in the lungs of experimental rodents (Castro et al., 2004, 2009; da Hora et al., 2005; Lanzetti et al., 2008; Le Quement et al., 2008; Silva Bezerra et al., 2006; Valenca et al., 2008a,b, 2006, 2004, 2009; Valenca and Porto, 2008). The present study showed a CS-induced increase in leukocyte recruitment in the BAL fluid of mice. However, this increase in macrophages and neutrophils was significantly lower in mice exposed to CS + A, suggesting that the açaí had anti-inflammatory activity. Activation of inflammatory cells by CS induces the release of a variety of mediators, including both

Table 1
Biochemical analyses of antioxidant enzyme activities (SOD, CAT and GPx), GSH/GSSG ratio and MPO.

Groups	Control	CS	CS + A
SOD (U/mg protein)	18.41 ± 2.11	12.14 ± 0.42***	9.53 ± 0.66***
CAT (U/mg protein)	9.10 ± 0.14	7.69 ± 0.25***	6.60 ± 0.27***
GPx (mM/min/mg protein)	0.031 ± 0.001	0.008 ± 0.001***	0.043 ± 0.003###
GSH/GSSG	1.11 ± 0.07	0.65 ± 0.05**	1.13 ± 0.10##
MPO (mU/mg protein)	0.248 ± 0.008	0.555 ± 0.079***	0.316 ± 0.022###

SOD – superoxide dismutase.
CAT – catalase.
GPx – glutathione peroxidase.
GSH – reduced glutathione.
GSSG – glutathione disulfide.
MPO – myeloperoxidase.
Values are means ± SEM of ten mice in each group.
** $p < 0.01$ Compared with the control group.
*** $p < 0.001$ Compared with the control group.
$p < 0.01$ Compared with the CS group.
$p < 0.001$ Compared with the CS group.

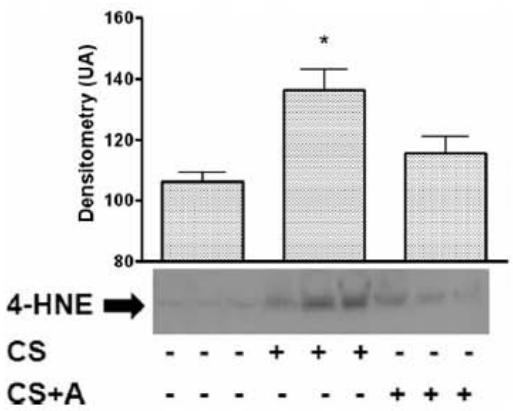

Fig. 7. Detection of 4-hydroxynonenal in lung homogenate samples by Western blotting. Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and then injected into each cigarette. Quantitative densitometry was performed on protein bands after ponceau staining of the membrane. Data are expressed as means ± SEM ($n = 3$ for each group) and were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test ($p < 0.05$). *$p < 0.05$ compared with the control group.

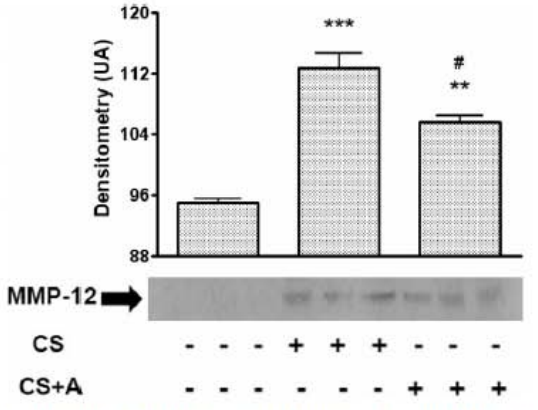

Fig. 8. Detection of metalloelastase (metalloproteinase 12 or MMP-12) in lung homogenate samples by Western blotting. Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and then injected into each cigarette. Quantitative densitometry was performed on protein bands after ponceau staining of the membrane. Data are expressed as means ± SEM ($n = 3$ for each group) and were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test ($p < 0.05$). ***$p < 0.001$ and **$p < 0.01$ compared with the control group. #$p < 0.05$ compared with the CS group.

oxidants and proteases that can damage lung structure and lead to emphysema (Churg et al., 2007; Ma et al., 2005; Selman et al., 2003). Metalloproteinases are a family of zinc- and calcium-dependent endopeptidases that play key roles in lung extracellular matrix degradation (Ohbayashi, 2002). We found that the increase in metalloelastase (MMP-12 or macrophage elastase), an important enzyme that breaks down lung extracellular matrix during emphysema development, was significantly reduced in mice exposed to CS + A. It is likely that açaí has an antiproteinase activity and protected mouse lungs by reacting directly with MMP-12. Neutrophil elastase, another potent proteolytic enzyme that breaks down extracellular matrix, was reduced in mouse lungs exposed to CS + A. The MPO enzyme, a marker of neutrophil influx and lung injury, was reduced in parallel with neutrophil elastase in mouse lungs exposed to CS + A.

We showed recently that nitric oxide, an important modulator of inflammation, plays an important role in CS-induced acute lung inflammation (Valenca et al., 2009). We also demonstrated that a hydroalcoholic extract of açaí induces activation of eNOS (Rocha et al., 2007). Thus, protection from emphysema in mice exposed to CS + A may be partly due to an increase in nitric oxide formation. In conclusion, this study suggests that addition of a hydroalcoholic extract of açai berry stone to cigarettes may reduce pulmonary harm induced by CS.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Roberto Soares de Moura is the inventor of a patent (PCT/BR2009/000274) related to the insertion of the açaí stone extract inside the cigarette. Karla P Pires is a PhD student and this study is part of her Thesis. Due to the patent process and commercial issue related to Roberto Soraes de Moura, she is cited as second author in this study. Karla P Pires and Thiago S Ferreira were

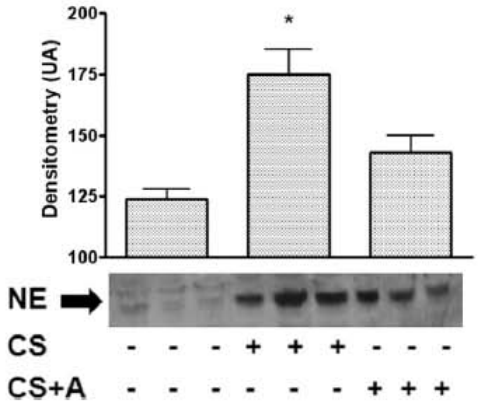

Fig. 9. Detection of neutrophil elastase in lung homogenate samples by Western blotting. Control group mice were exposed to ambient air. CS group mice were exposed to smoke from twelve cigarettes per day for 60 days. CS + A group mice were exposed to smoke from twelve açaí cigarettes per day for 60 days. Açaí extract (100 mg) was mixed with 1 mL of saline and then injected into each cigarette. Quantitative densitometry was performed on protein bands after ponceau staining of the membrane. Data are expressed as mean ± SEM ($n = 3$ for each group) and were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test ($p < 0.05$). *$p < 0.05$ compared with the control group.

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APÊNDICE H – Carta de aceitação de apresentação de resumo em congresso: 10th International Congress on Cell Biology

Luís Cristóvão Pôrto <luis.cristovaoporto@gmail.com> 22 de junho de 2012 06:52
Para: Alan de Aguiar Lopes <alan.alawave@gmail.com>, Samuel dos Santos Valença <samuelv@hotmail.com>

Ola Alan

O trabalho foi selecionado para apresentçao sob a forma de poster e oral
Abstract title: ANTIOXIDANT AND CHEMICAL PROFILE OF BRAZILIAN PROPOLIS: AN IN VITRO STUDY
Abstract title:
Theme: Cell biology and inflammation
Type of presentation: Oral and Poster (first author must be a student or posdoc)
Text:
Background: Cigarette smoke is resource of several chemical noxious substances which cause inflammation and parenchyma destruction. Propolis is resinous substance produced by bee which has anti-inflammatory and antioxidant properties. Aim: In this study, our objective is to investigate propolis action over murine macrophages exposed to cigarette smoke. Methods: Chemical profile of propolis was made in gas chromatograph-mass spectrometry (GC-MS). RAW 264.7 cells (5x105 cells per well, 5-6th passage, 80-90% confluence) were treated with different concentrations of propolis and cigarette smoke extract (CSE), during 24 hours. Analysis of total polyphenols, cell viability (MTT), total reactive oxygen species (ROS), antioxidant potential (DPPH) and malondialdeyde levels (MDA). Results: Four main compounds were found in propolis: hydroxycinnamic acid, coumaric acid, drupanin and artepillin C. Total polyphenol levels was positively related to increasing concentration of Propolis. MTT levels increased in concentration-dependent manner in propolis, on the other way CSE had inverse effect. Propolis (15.6 and 31.2 mg/mL) decreased ROS levels ($p<0.05$, both); 10% CSE group showed the highest ROS level ($p<0.05$). DPPH decreased in the most groups of Propolis ($p<0.05$ and $p<0.001$); 1.25 and 5% CSE groups increased DPPH ($p<0.001$ and $p<0.01$, respectively). Propolis resulted in a decrease in malondialdeylde levels (31.2, 62.5 and 125 mg/mL - $p<0.01$ and $p<0.001$) and 1.25 and 5% CSE groups increased them ($p<0.001$, both). Conclusion: Propolis and its constituents have antioxidant action, especially in lower concentrations and that can be used to study its pharmacological actions against CSE effects on murine macrophages in vitro.

Status: Aprovado

APÊNDICE I – Certificado de participação em congresso internacional – I

Certificate

STATINS EFFECTS IN ACUTE LUNG INFLAMMATORY RESPONSE BY CIGARETTE SMOKE IN MICE

authored by

FERREIRA T. S., LOPES A. A., PIRES K. M. P., ALVES J., LANZETTI M., NESI R. T., SILVA M. A., VALENÇA S. S.

was presented at the Poster Session during the XI Brazilian Symposium on Extracellular Matrix and VI International Symposium on Extracellular Matrix held in Armação dos Búzios, RJ, Brazil, on August 21-24, 2011.

Ruy G. Jaeger — Cecilia Hedin Pereira

Organizing Committee
SIMEC 2011

APÊNDICE J – Certificado de participação em congresso internacional – II

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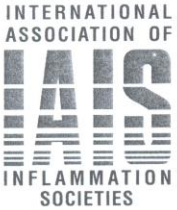
Inflammation Research

The 10th World Congress on Inflammation

25–29 June 2011
Palais des Congrès
(Paris, France)

The Abstracts



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like trypsin to intestinal epithelial cell monolayers reduces permeability as shown by increased transepithelial resistance (R_{TE}). This effect of trypsin is independent of activation of protease-activated receptors, but the underlying mechanism is unknown. Given the central role of epidermal growth factor receptor (EGFR) in epithelial cell biology and homeostasis, we hypothesized that trypsin increases R_{TE} by activating EGFR.
Methods: R_{TE} of confluent SCBN monolayers and mouse colonic tissue was measured in Ussing chambers. To determine a role for EGFR-associated signaling, cells were pretreated with inhibitors of EGFR (PD153035), ErBb2 (AG879), ERK-1/2 (PD98059), PI3 K (LY294002) and MMPs (marimastat, MMT) andthen stimulated apically by trypsin or EGF. Flux of FITC-dextran across the monolayer was measured following treatment with PD153035 or AG879 and trypsin.
Results: Apical trypsin increased R_{TE} by 300% ($p < 0.05$) within 60 min. PD153035 or AG879 caused a significant dose-dependent decrease in trypsin-induced ΔR_{TE}, with IC_{50}s of 0.38 and 2.73 μM, respectively. PD98059, LY294002 and MMT also reduced trypsin-induced ΔR_{TE} by 66, 63 and 25% respectively. Pretreatment of cells with PD153035 or AG879 prevented trypsin-induced reduction of dextran flux. Apical EGF increased R_{TE} by 80%. PD153035 and AG879 reduced this increase by 99 and 84% respectively. Also, soybean trypsin inhibitor decreased R_{TE} in mouse colon in vitro, suggesting that luminal serine proteases maintain barrier function.
Conclusions: Our data suggest that trypsin increases R_{TE} via activation of EGFR and ErBb2 and partially via activation of ERK-1/2, PI3K and MMPs. Serine proteases may strengthen epithelial barrier to enhance the efficiency of electrolyte and nutrient transport, and may represent a method to counter barrier defects caused by intestinal inflammation.
Disclosure of interest: None declared.

P-137
ANTI-INFLAMMATORY EFFECT OF BLACK TEA. EFFECT ON THE ACTION OF NEUTRAL SERINE PROTEASES IN GRANULOCYTES

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Tea has various beneficial effect on health. Green tea contains catechins, theanine; and vitamins such as folic acid and ascorbic acid which exert healthy effect. In contrast to green tea, black tea contains polymers of catechins such as theaflavin or thearubigin other than catechins. In this study we examined the effect of black tea extract, theaflavin, and thearubigin on the activity of neutral serine proteases in granulocytes. Neutral serine proteases in granulocytes such as medullasin and cathepsin G were shown to play important roles in biophylaxis. Both proteases stimulate lymphocyte function and enhance NK cell activity. Medullasin causes inflammation leading to the development of atherosclerosis. Mice fed with diet containing black tea components such as theaflavin or thearubigin revealed decreased medullasin activity dose-dependently, but cathpsin G activity decreased only slightly. In contrast to black tea components, addition of green tea components such as epicatechin, epigallocatechin, and epigallocatechin gallate to the diet of mice caused an increment of both medullasin and cathepsin G activity. From these results described above drinking of black tea is considered to be useful for the prevention of atherosclerosis by decreasing medullasin activity in granulocytes.
Disclosure of interest: None declared.

P-138
ELASTASE–PROTEOLYTIC STIMULUS LEADS TO REDOX IMBALANCE IN THE EARLY STAGE OF PULMONARY INFLAMMATORY RESPONSE

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Pulmonary emphysema has become epidemic worldwide. The mechanisms by which extracellular matrix degradation occurs are not yet fully understood. However, literature described both inflammatory and oxidative processes as critical to parenchyma destruction. Thus, this study focused to investigate the partipation of oxidative stress during the emphysema establishment process induced by elastase in mice. C57BL/6 mice were submitted at a protocol with pancreatic porcine elastase (PPE) instillation (0.05 U, 0.5 U (i.t.) or 3 U (i.n.) of PPE per animal), to induce pulmonary emphysema in vivo ($n = 6$). Lung tissue was collected on days 1, 7, 14 and 21 after PPE instilation. Vehicle treated mice were used as controls. Emphysema typical morphological alterations were seen just at 21 days after PPE 0.5 U. TNF-α was elevated at 7 and 14 days after PPE 0.5 U, and was accompanied by a reduction in IL-10 levels at the same time-points. MPO was elevated at the three time-points with PPE 0.5 U. The levels of Nitrite, TBARS and the SOD activity were also increased at 7 days after PPE 0.5 U, while CAT activity was decreased. When the dose was enhanced to 3 U of PPE and a group with just 1 day after stimulus was performed, CAT activity presented reduced at 1 day, while SOD activity was increased at the same time-point. These results are corroborated with mRNA expression, with an increase in Nrf2, Sirt-1, CAT and GPx. However, there was no difference in mRNA SOD expression between any groups (control, 1, 7, 14 and 21 days). ELISA for TGF-β1 showed a reduction just at 21 days after 3U of PPE. These results indicate that the proteolytic pathway has a link with the oxidative pathway. These data suggest that redox imbalance generated by proteolytic stimulus may be crucial in the early stage of the injury, and has no effect in later stages, when in fact, emphysema is installed. But the stimulus for the antioxidant response remains enhanced, as seen by the RT-PCR.
Disclosure of interest: None declared.

P-139
HEPARANASE AS A SIGNALING MOLECULE IN CANCER AND INFLAMMATION

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Heparanase is a β-D-endoglucuronidase which degrades heparan sulfate, a key component of the extracellular matrix and basement membrane. The degradation of the ECM is essential for both

APÊNDICE K – Certificado de participação em congresso internacional – III

ACTAS

5° Congreso Sudamericano de la Yerba Mate

5 y 6 de Mayo de 2011
Posadas, Misiones - Argentina

ESTUDO DA AÇÃO DO MATE COMERCIAL CONTRA A INFLAMAÇÃO PULMONAR CAUSADA POR FUMAÇA DE CIGARRO EM CAMUNDONGOS

Barroso MV[1], Lanzetti M[2], Cordeiro NC[1], Nesi RT[1], Lopes AA[2], Borges PA[1], Alves JN[2], Brogliato AR[1], Porto LC[2], Benjamim CF[1], Valenca SS[1]

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A fumaça de cigarro (FC) é o principal responsável pelos casos de doença pulmonar obstrutiva crônica. Nós testamos aqui os efeitos do chá mate (CM) antes, durante e após a exposição aguda de camundongos à FC. CM impediu e preveniu o influxo de leucócitos para o pulmão dos camundongos expostos à FC quando comparados ao grupo controle ($p<0.05$ e $p<0.01$). CM impediu o aumento de citocinas pró-inflamatórias (TNF-α, IL-10 e IL13) nos camundongos expostos à FC quando comparados ao grupo controle ($p<0.05$). CM impediu, preveniu e reverteu a produção de ROS nos camundongos expostos à FC quando comparados ao grupo controle ($p<0.05$). CM impediu, preveniu e reverteu o dano oxidativo (MDA e carbonil) nos camundongos expostos à FC quando comparados ao grupo controle ($p<0.05$). Concluímos que o CM é um antioxidante eficaz contra a lesão oxidativa causada pela FC em camundongos.

Palavras-chave: chá mate, fumaça de cigarro, antioxidantes, inflamação pulmonar, estresse oxidativo.

CHÁ MATE PREVINE ENFISEMA PULMONAR EM CAMUNDONGOS EXPOSTOS À FUMAÇA DE CIGARRO

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Exposição à fumaça de cigarro (FC) é associada à inflamação pulmonar, estresse oxidativo e enfisema. Nosso objetivo foi estudar o chá mate (CM) como uma fonte de antioxidante contra o enfisema em camundongos. Após 60 dias de exposição à FC (grupo CS, 12 cigarros por dia, durante 60 dias) a histologia do grupo tratado com CM (500 mg/kg) apresentou uma redução das características enfisematosas em comparação ao grupo CS. Nós observamos também no grupo CS lipoperoxidação, aumento da atividade de mieloperoxidase ($p<0.05$), redução da razão entre glutationa reduzida e glutationa oxidada ($p<0.05$), e redução de SOD ($p<0.05$) e CAT ($p<0.001$) quando comparamos os mesmos parâmetros nos grupos controle e tratado com CM. Nós concluímos que o enfisema observado em camundongos expostos à FC foi prevenido pelo tratamento com CM, associado a uma redução dos parâmetros de inflamação e estresse oxidativo.

Palavras-chave: chá mate, estresse oxidativo, enfisema, fumaça de cigarro

EFEITOS DO CHÁ MATE (NATURAL E DIET) SOBRE O PERFIL REDOX E INFLAMATÓRIO EM PULMÕES SAUDÁVEIS DE CAMUNDONGOS IDOSOS

Cordeiro NC[1], Lanzetti M[2], Barroso MV[1], Lopes AA[2], Alves JN[2], Nesi RT[1], Santos JC[3], Pires KMP[2], Porto LC[2], Benjamim CF[1], Ribeiro ML[3], Valenca SS[1]

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O chá mate (CM) é uma bebida amplamente consumida em diversos países. Entretanto suas propriedades antioxidantes e antiinflamatórias não são claras. Nosso objetivo foi observar os efeitos do CM (natural [N] e diet [D]) no pulmão de camundongos durante o processo de envelhecimento. Para tanto, camundongos receberam água filtrada, CMN ou CMD durante cinco meses. Nós observamos uma redução da expressão das enzimas antioxidantes SOD ($p<0.01$), CAT ($p<0.01$) e GPx ($p<0.05$) nos grupos CMN e CMD quando comparados ao grupo controle, em contraste a um aumento dos reguladores transcricionais antioxidantes SIRT-1 e NRF2 ($p<0.05$). Observamos também redução na expressão de citocinas próinflamatórias (TNF-α, IL-6 e iNOS) nos grupos CMN e CMD quando comparados ao grupo controle ($p<0.05$). Entretanto, redução de ROS foi observada somente no grupo CMD quando comparado ao grupo controle ($p<0.05$). Sugerimos que o CM pode retardar o processo de envelhecimento e proteger o pulmão de camundongos agindo como antioxidante e antiinflamatório.

Palavras-chave: estresse oxidativo, mate comercial, envelhecimento, inflamação pulmonar.

ESTUDO DO EFEITO PROTETOR DE EXTRATO AQUOSO DE *ILEX PARAGUARIENSIS* SOBRE MACRÓFAGOS ALVEOLARES EXPOSTOS À FUMAÇA DE CIGARRO

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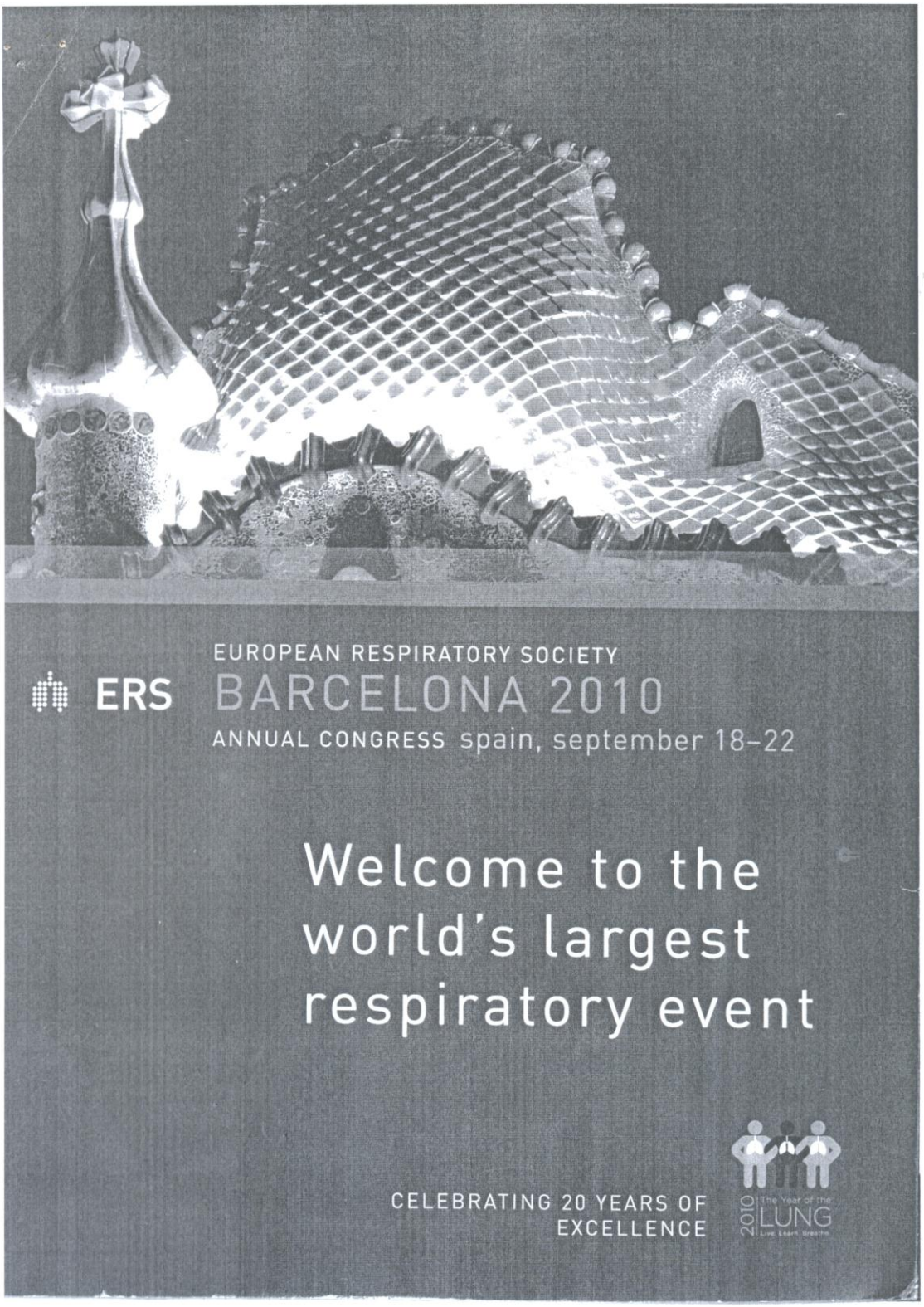
O estresse oxidativo causado pela fumaça de cigarro tem importante papel no desenvolvimento de doenças pulmonares e a *Ilex paraguariensis* tem potentes propriedades antioxidantes. Nosso objetivo foi estudar diferentes doses de extrato aquoso da *Ilex paraguariensis* (EIP) em macrófagos alveolares (AMs) expostos ao extrato aquoso da fumaça de cigarro (EFC). AMs foram tratados com EFC5% (v/v) juntamente com diferentes concentrações de EIP (1%, 10% e 50%-v/v) por 1h. A atividade da Catalase diminuiu nos grupos EFC e EFC+EIP10% e aumentou nos grupos EFC+EIP1% EFC+EIP50%. Em contrapartida, os níveis de malondialdeído aumentaram nos grupos EFC e EFC+EIP50% e reduziram nos grupos EFC+EIP1% e EFC+EIP10%. Citotoxicidade, mensurada pelo ensaio de MTT, foi aumentada no grupo EFC sem alterações em EFC+EIP1% e EFC+EIP10%. O aumento do MTT correlacionou-se negativamente com a Catalase e positivamente com o malondialdeído. Concluímos que a menor dose de EIP protegeu AMs contra o dano oxidativo causado pela EFC.

Palavras-chave: *Ilex paraguariensis*, estresse oxidativo, fumaça de cigarro, macrófago alveolar

APÊNDICE L – Certificado de participação em congresso internacional – IV



BUDAPEST (HALL 3) SESSION 347 08:30 - 10:30

E-Communication Session: New insights into reactive oxygen species

Chairs: S. Uhlig (Aachen, Germany), A. Millar (Bristol, United Kingdom)

E3718 Late Breaking Abstract: Paraoxonase 1 activity and protein thiol oxidation in sera of COPD patients
T. Zanic Grubisic, L. Rumora, M. Grdic Rajkovic, G. Puclin, I. Cepelak (Zagreb, Croatia)

E3719 Late Breaking Abstract: Cerebrovascular responses to hypercapnia and oxidative stress in women with COPD
S. Hartmann, V. Pialoux, R. Leigh, N. Eves, A. Beaudin, M. Pun, M. Poulin (Calgary, Canada; Lyon, France)

E3720 Protective effects of peroxiredoxin 6 on cecal ligation and puncture (CLP) induce lung and distal organ injury
X. Wang, X. J. An, D. Yang, J. Bi, C. X. Bai (Shanghai, China)

E3721 TNF-α protects lung cells against iron and oxidation
L. Persson (Sweden)

E3722 Protective effects of peroxiredoxin 6 on ventilation-induced lung injury
X. Wang, X. J. An, D. Yang, L. Tong, C. X. Bai (Shanghai, China)

E3723 Multipotent stromal cell-derived stanniocalcin-1 reduces reactive oxygen species and promotes survival of injured A549 lung cancer cells through upregulation of uncoupling-protein 2 and induction of the Warburg effect
S. Okochi, G. Block, K. Ahmed, P. Darwin, T. Nukiwa (Sendai, Japan; Seattle, Temple, United States of America)

E3724 Acai and propolis reduced acute lung inflammation caused by cigarette smoke in mouse lungs
S. Valença, A. Lopes, T. Ferreira, M. Lanzetti, R. Nesi, A. Resende, R. Soares-de-Moura, P. Souza, L. C. Porto (Belém, Brazil)

E3725 The effect of thymoquinone on lung pathology and cytokines of ovalbumin sensitized guinea-pigs
R. Keyhanmanesh, M. H. Boskabady, M. A. Ebrahimi Saadatlou, S. Khamneh, F. Mirzaei Bavil (Islamic Republic of Iran)

E3726 Reduced airway cell antioxidant defences in COPD reflect normal age-related declines and are not related to disease severity or smoking status
R. Dove, N. Larsson, A. F. Behndig, E. Roos-Engstrand, A. Blomberg, I. S. Mudway (United Kingdom; Umeå, Sweden)

E3727 Ethyl pyruvate prevents lung injury through inhibition of the NF-kappa B pathway, but does not prevent bleomycin-induced lung injury
A. Mizutani, K. Sugahara, H. Yamamoto (Nishihara, Japan)

E3728 NADPH oxidase 4 deficiency protects from bleomycin-induced epithelial cell death and pulmonary fibrosis
S. Carnesecchi, C. Deffert, B. Hinz, Y. Donati, B. Moore, B. Banfi, J. C. Pache, C. Barazzone-Argiroffo, K. Karl-Heinz (Switzerland; , Canada; Ann Arbord, Iowa City, United States of America)

E3729 Bleomicin-induced pulmonary fibrosis results in strain dependent oxidative stress
K. M. Pires, M. A. Silva, E. Trajano, R. Nesi, L. C. Porto, S. Valenca (Brazil)

E3730 Effect of pulse therapy with glucocorticoid and cyclophosphamide on lung fibrosis due to paraquat poisoning in rat
S. Seifirad, N. Hasanzadeh Ghavifekr, A. Ghaffari, E. Mostafidi, S. Talghini (Islamic Republic of Iran)

E3731 The effect of intravenous immunoglobulin on active oxygen forms production in patients with community acquired pneumonia
U. Farkhutdinov, A. Mirchaidarov, S. Farkhutdinov (Ufa, Russian Federation)

E3732 Short-term mechanical ventilation induces oxidative stress and inflammation in healthy mouse lungs
K. M. Pires, E. Trajano, M. Lanzetti, M. Machado, N. Casquilho, A. Schmidt, W. Zin, L. C. Porto, S. Valença (Brazil)

E3733 Antioxidant properties of ambroxol in chronic obstructive pulmonary disease
U. Farkhutdinov, V. Petryakov, S. Farkhutdinov (Ufa, Russian Federation)

E3734 Cigarette smoke induces polymers of Z α_1-antitrypsin
S. Alam, Z. Li, S. Janciauskiene, R. Mahadeva (Cambridge, United Kingdom; Malmoe, Sweden)

E3735 Induction of antioxidant defense mechanisms by amifostine in lung tissue attenuates ventilator induced lung injury
P. Fu, A. Birukova, D. Grdina, K. Birukov (Chicago, United States of America)

IZMIR (HALL 3) SESSION 348 08:30 - 10:30

E-Communication Session: Tuberculosis in children

Chairs: B. Kampmann (London, United Kingdom), M. Altet Gomez (Barcelona, Spain)

E3736 The characteristics of tuberculosis among adolescents in Vojvodina (Serbia)
M. Ilic, V. Kuruc, S. Pavlovic, I. Kopitovic, M. Kojicic, S. Kasikovic-Lecic (Sremska Kamenica, Republic of Serbia)

E3737 Epidemiology of childhood tuberculosis in Poland
M. Korzeniewska-Kosela, I. Szczuka, K. Lewandowska, J. Kus (Warsaw, Poland)

E3738 Changes in the clinical pattern of tuberculosis in children hospitalized in the department of Children's Lung Diseases and Allergy of Warsaw Medical University in the years 1973-2002
Z. Jerzy, J. Chadzynska, K. Marta, T. Bielecka (Warsaw, Poland)

P4339 Influence of smoking on airway and systemic inflammation in healthy subjects and COPD patients
J. J. P. Goldring, R. I. Craggs, G. R. Arthur, M. J. Saunders, J. A. Wedzicha (Loughborough, United Kingdom)
P4340 Expansion of helper T cells with a non-regulatory function in smoking and COPD
E. Roos-Engstrand, J. Pourazar, A. F Behndig, A. Bucht, A. Blomberg (Umeå, Sweden)
P4341 Increased production of reactive oxygen species of systemic PMN from COPD patients in comparison to healthy controls
A. Strassburg, I. Muenckel, K. Dalhoff (Luebeck, Germany)
P4342 Mast cells in different phenotypes of smokers emphysema
A. Ballarin, E. Bazzan, D. Zanovello, R. Hernandez, S. Baraldo, G. Turato, M. Saetta, M. G. Cosio (Padova, Italy; Montreal, Canada)
P4343 Protein carbonyls in the lung parenchyma of patients with COPD
T. Hackett, J. Warner (Southampton, United Kingdom)

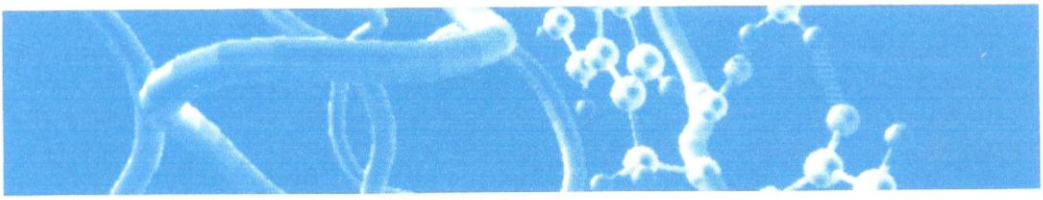
HALL 3-20 SESSION 390 12:50 - 14:40

Thematic Poster Session: Macrophages: cell biology and disease-specific characteristics

Chairs: L. Donnelly (London, United Kingdom), C. Taggart (Belfast, United Kingdom)

P4344 Late Breaking Abstract: Suppressed rhinovirus (RV) induced type III interferon production in alternatively activated macrophages
A. Nikonova, S. Traub, M. Khaitov, L. Stanciu, S. Johnston (London, United Kingdom; Moscow, Russian Federation)
P4345 Late Breaking Abstract: The role of galectin-3 on macrophages in CNT-induced mouse lung inflammation
F. Tian, G. Estrada, W. Kreyling, T. Stoeger (Munich, Germany)
P4346 Phenotype polarization of alveolar macrophages in COPD patients
F. Polverino, S. Baraldo, M. Cosio, M. Saetta, A. Agustí, J. Sauleda, B. Cosio (Cava dei Tirreni, Padova, Italy; Palma de Mallorca, Barcelona, Spain)
P4347 Reduced phagocytosis of bacterial pathogens by macrophages from COPD patients
C. Thomas, M. Saunders, J. Wedzicha, P. J. Barnes, L. E. Donnelly (London, Charnwood, United Kingdom)
P4348 Are complexemia and expression of FcγR and CR receptors on peripheral blood monocytes involved in the etiopathogenesis of sarcoidosis?
M. Smigielska, A. Dubaniewicz, P. Trzonkowski (Poland)
P4349 Influence of Vitamin D on the innate immune response in cigarette smoke exposed macrophages
C. Herr, M. Branscheid, C. Vogelmeier, R. Bals (Germany)
P4350 Expression of dopamine receptors in human alveolar macrophages
A. Ricci, L. Pisani, P. Bruno, L. Tabbì, V. Pietrangeli, M. Del Forno, D. Scozzi, F. Fioretti, S. Mariotta (Roma, Italy)
P4351 Inhibition of LPS-induced cytokine/chemokine production from human lung macrophages by roflumilast is enabled by autocrine PGE_2
A. Buenestado, S. Grassin-Delyle, E. Naline, A. Chapelier, H. Tenor, P. Devillier (Suresnes, France; Konstanz, Germany)
P4352 Phenotyping human lung macrophages
K. Chana, T. Woolley, P. Barnes, L. Donnelly (Loughborough, United Kingdom)
P4353 Differential effects of diesel exhaust particles and endotoxin on phagocytosis and cytokine release by monocyte-derived macrophages
G. Sehra, P. Fenwick, D. Rogers, L. Donnelly (United Kingdom)
P4354 Defect of sirtuin, an anti-aging molecule, reduces anti-oxidative stress capacity in THP-1 cell line
L. Nunez-Naveira, N. Mercado, K. Ito (London, United Kingdom)
P4355 Nitric oxide and N-acetylcysteine reduced oxidative stress in alveolar macrophages exposed to cigarette smoke extract
M. Lanzetti, C. Rueff-Barroso, R. Fonseca, A. Lopes, T. Ferreira, F. Albuquerque, L. C. Porto, S. Valença (Brazil)
P4356 Cigarette smoke extract induces the generation of microparticles by human monocytes/macrophages
C. Cordazzo, T. Neri, Y. Carmazzi, S. Petrini, P. Paggiaro, A. Celi (Pisa, Italy)
P4357 Production of alpha-1 antitrypsin by pro- and anti-inflammatory macrophages
E. F. A. van 't Wout, A. van Schadewijk, N. D. L. Savage, J. Stolk, P. S. Hiemstra (The Netherlands)
P4358 Increased expression of microRNA-146a negatively regulates both the LPS and Poly (I:C) induced inflammatory response in monocytes
M. Perry, E. Tsitsiou, M. Lindsay (United Kingdom)
P4359 Role of PPAR-γ in airway inflammation induced by monocyte-derived microparticles
T. Neri, C. Cordazzo, Y. Carmazzi, S. Petrini, P. Paggiaro, A. Celi (Pisa, Italy)
P4360 Expression of toll-like receptor 3 in alveolar macrophages from patients with COPD
A. Koarai, S. Yanagisawa, H. Sugiura, T. Ichikawa, M. Kanda, K. Furukawa, K. Akamatsu, T. Hirano, K. Matsunaga, Y. Minakata, M. Ichinose (Wakayama, Japan)
P4361 Genetic and pharmacological evidence for critical role of macrophage elastase in emphysema formation in βENaC-overexpressing mice
S. Diemer, J. B. Trojanek, A. Cobos-Correa, S. C. Schubert, J. Schatterny, S. Hirtz, M. A. Mall, C. Schultz (Heidelberg, Germany)

APÊNDICE M – Certificado de participação em congresso nacional - I



FeSBE 2011

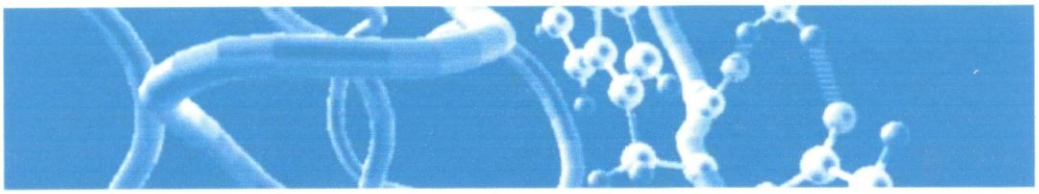
24 a 27 de agosto de 2011
Rio de Janeiro – RJ

Certificamos que o resumo 08.020 - **"Temporal analysis of oxidative effects on pulmonary inflammatory response in mice exposed to cigarette smoke"**, autoria de GONÇALVES, E. G. ; DOURADO, V. A. ; FERREIRA, B. S. B. ; LOPES, A. D. A. ; LANZETTI, M. ; SILVA, M. E. ; VALENÇA, S. S. ; LIMA, W. G. ; COSTA, D. C. ; BEZERRA, F. S., foi apresentado na XXVI Reunião Anual da Federação de Sociedades de Biologia Experimental (FeSBE), realizado de 24 a 27 de agosto de 2011 no Rio de Janeiro, RJ.

Comissão Organizadora

CAPES CNPq FAPESP FAPERJ Organização Pan-Americana da Saúde

APÊNDICE N – Certificado de participação em congresso nacional – II



FeSBE 2011

24 a 27 de agosto de 2011
Rio de Janeiro – RJ

CERTIFICADO

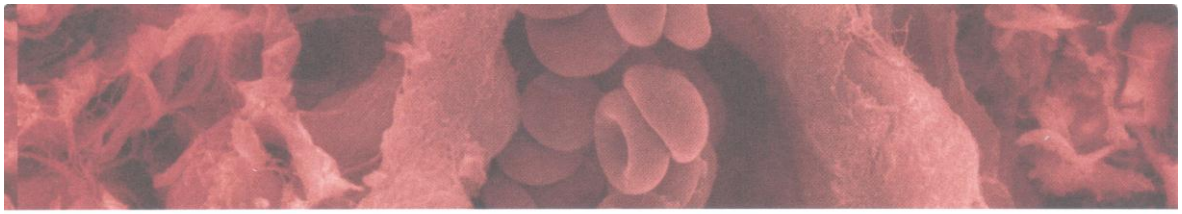
Certificamos que o resumo 08.071 – COMMERCIAL MATE TEA BENEFICIAL ACTION IN ACUTE PULMONARY INFLAMMATION MODEL BY CIGARETTE SMOKE – EFFECTS AS AN ANTIOXIDANT, autoria de BARROSO, M. V.; LANZETTI, M.; CORDEIRO, N. C.; NESI, R. T.; LOPES, A. A.; BORGES, P. A.; ALVES, J. N.; BROGLIATO, A. R.; PORTO, L. C.; BENJAMIN, C. F.; VALENÇA, S. S., foi apresentado na XXVI Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, realizado de 24 a 27 de agosto de 2011 no Rio de Janeiro, RJ.

Comissão Organizadora

CAPES CNPq FAPESP FAPERJ Organização Pan-Americana da Saúde

Para verificar a autenticidade deste certificado, acesse www.fesbe.org.br/certificados

APÊNDICE O – Certificado de participação em congresso nacional – III



FeSBE 2010

25 a 28 de agosto de 2010
Águas de Lindóia – São Paulo

CERTIFICADO

Certificamos que

o resumo 25.071 intitulado EFEITOS DO L-NAME E DA L-ARGININA SOBRE O EQUILÍBRIO REDOX EM CÉREBROS DE CAMUNDONGOS EXPOSTOS À FUMAÇA DE CIGARRO de autoria Lopes, A. A., Ferreira, T. S., Alves, J. N., Pires, K. M. P., Porto, L. C., Valença, S. S. - Laboratório de Reparo Tecidual, UERJ Laboratório de Inflamação, Estresse Oxidativo e Câncer, UFRJ, foi apresentado sob a forma de painel na

XXV Reunião Anual da Federação de Sociedades de Biologia Experimental – FeSBE, realizada na cidade de Águas de Lindóia – SP, de 25 a 28 de agosto de 2010.

Comissão Organizadora

CAPES CNPq FAPERJ FAPESP Organização Pan-Americana da Saúde