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Nutrition 24 (2008) 375–381

NUTRITION

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Basic nutritional investigation

Mate tea reduced acute lung inflammation in mice exposed to cigarette smoke

Manuella Lanzetti, R.D.[a], Frank Silva Bezerra, M.Sc.[a], Bruna Romana-Souza, M.Sc.[a], Aline Cristina Brando-Lima, Sc.D.[b], Vera Lúcia Gonçalves Koatz, Ph.D.[b,#], Luís Cristóvão Porto, Ph.D.[a,*], and Samuel Santos Valenca, Ph.D.[a]

[a] *Tissue Repair Laboratory, Histology and Embryology Department, Roberto Alcântara Gomes Institute of Biology, Rio de Janeiro State University, Rio de Janeiro, Brazil*

[b] *Medical Institute of Biochemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*

Manuscript received August 1, 2007; accepted January 3, 2008.

Abstract

Objective: Short-term cigarette smoke exposure has been associated with acute lung inflammation (ALI) and oxidative damage. We studied mate tea (*Ilex paraguariensis* infusion) as a possible nutritional resource for ALI.

Methods: C57BL/6 mice ($n = 30$) were administered with mate tea orally (150 mg/kg, CSMO), mate tea intraperitonially (150 mg/kg, CSMIP), or the vehicle (CS) and then exposed to cigarette smoke for 5 d (six cigarettes per day). The control group was sham-smoked ($n = 30$). One day after the final exposure, mice were sacrificed. Bronchoalveolar lavages were performed and lungs removed for biochemical (lung homogenates) and histologic analyses.

Results: Mate tea reduced the increase of alveolar macrophages and neutrophils in bronchoalveolar lavages (cells $\times 10^3$/mL) of the CSMO (214.3 ± 21.4 and 12.2 ± 4.9) and CSMIP (248.3 ± 11.1 and 12.1 ± 2.3) groups compared with the CS group (425.9 ± 28.1 and 140.5 ± 20.1). Mate tea reduced lipid peroxidation (the control group was considered 100%) and tumor necrosis factor-α (picograms per milliliter) in the CSMO group (61.3 ± 11.3 and 185.3 ± 21.8) compared with the CS group (150.0 ± 18.1 and 242.3 ± 13.2). Matrix metalloprotease-9 activity was higher in the CS group and lower in the CSMO group. Oxidative and inflammatory markers in the CSMO group were not different from those in the control group.

Conclusion: These data imply a potential antioxidant role for mate tea on ALI. Further studies are needed to determine such mechanisms and to explore its potential as an anti-inflammatory and nutritional resource in lung damaged by cigarette smoke exposure.

Keywords: Acute lung inflammation; Antioxidant; Cigarette smoke; *Ilex paraguariensis*; Inflammatory cells

Introduction

Chronic obstructive pulmonary disease (COPD) is a serious and increasing public health problem that affects a large proportion of the world population [1]. COPD is characterized by progressive and irreversible deterioration of pulmonary function and can be caused by several risk factors such as air pollution, poor diet, occupational exposure, and mainly cigarette smoke (CS) [2].

The presence of CS in the lung tissue is responsible for acute lung inflammation (ALI) [3]. CS causes an inflammatory response in the lower respiratory tract characterized by the accumulation of alveolar macrophages and neutrophils [4]. These activated inflammatory cells release a variety of mediators, including proteases, oxidants, and toxic peptides, which can damage lung structures and are believed to be a major cause of tissue destruction [5].

Cigarette smoke contains a large number of oxidants, and it has been hypothesized that many of the adverse effects of smoking may appear as a result of oxidative damage to

This study was supported by FAPERJ, FUJB, CNPq, and UERJ.

[#] In memoriam.

* Corresponding author: Tel.: +55-21-2587-6509; fax: +55-21-2587-6511.

E-mail address: lcporto@uerj.br (L. C. Porto).

0899-9007/08/$ – see front matter
doi:10.1016/j.nut.2008.01.002

critical biological substances [6]. CS exposure increases the amount of alveolar oxidants, not only because the smoke itself contains an expressive number of free radicals but also because it increases the number of inflammatory cells in alveoli, which spontaneously release oxidants [7]. Reactive oxygen species from CS or from inflammatory cells (particularly macrophages and neutrophils) lead to decreased antiprotease defenses [8] and antioxidants [9]. Nevertheless, antioxidants have been reported as protector factors to the lung in CS-exposure models [7].

Ilex paraguariensis (IP) is a potential antioxidant, and IP extract contains a high polyphenol concentration and exhibits high free radical scavenging properties [10]. The mate tea, produced from the IP aqueous extracts, is a beverage whose consumption has increased worldwide [11]. Several works have shown different benefits of mate tea consumption linked to its high polyphenol levels [10,12]. Until now there has been no association relating mate tea effects in mouse lung. This study aimed to verify if IP has protective effects against mobilization of inflammatory cells to alveoli, in lipid peroxidation, matrix metalloprotease-9 (MMP-9), and tumor necrosis factor-α (TNF-α) activation after ALI caused by CS.

Materials and methods

Chemicals and animals

Acrylamide, *n*-butanol, $CaCl_2(H_2O)_2$, Coomassie Blue, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide, Dulbecco's Modified Eagle's Medium, gelatin, thiobarbituric acid, sodium dodecylsulfate, Tris-HCl, Triton X-100, thiobarbituric acid, and $ZnCl_2$ were purchased from Sigma Chemical (St. Louis, MO, USA). A specific enzyme-linked immunosorbent assay for TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Diff-Quik was purchased from Baxter Dade AG (Dudingen, Switzerland). Bradford was purchased from Bio-Rad (Hercules, CA, USA). Ethanol and formalin were purchased from Vetec (Duque de Caxias, RJ, Brazil). C57BL/6 male mice were purchased from Instituto de Veterinária, Universidade Federal Fluminense (Niterói, RJ, Brazil).

Preparation of IP aqueous extract

A sample of commercial "Yerba Mate toasted" was purchased from Leão Junior SA (Curitiba, PR, Brazil). Aqueous extract was prepared under similar conditions as those used traditionally, with some modifications. The plant material was ground to a fine powder and 10 g of each sample was boiled with 200 mL of water for 20 min and cooled at room temperature to 40–45°C [11,12]. After filtration, the extract was lyophilized, yielding 1.30 g of aqueous crude extract. For the nutritional experimental procedure, the freeze-dried sample was dissolved in an amount of distilled water necessary to obtain the required concentration.

CS exposure

To study the effects of mate tea in CS-exposed mice, 30 8-wk-old C57Bl/6 mice (male) were exposed to six commercial filtered cigarettes, distributed three times per day, for 5 consecutive days by using a smoking chamber described previously [3]. Mice exposed only to ambient air and supplemented with distilled water by oral gavages were used as controls ($n = 10$). CS mice were divided in three groups of 10 animals: 1) exposed mice supplemented with distilled water by oral gavages (CS), 2) exposed mice supplemented with mate tea by oral gavages (CSMO, 150 mg/kg), and 3) exposed mice supplemented with mate tea by intraperitoneal injection (CSMIP, 150 mg/kg). Mate tea was prepared daily with distilled water and was administered 1 h before the first inhalation. The smoke of one cigarette produced 300 mg/m^3 of total particulate matter in the chamber. Mice were kept at 22 ± 2°C in an illumination-controlled room (photoperiod 14-h light and 10-h darkness), fed Purina Chow, and allowed unrestricted access to water. All procedures were carried out in accordance with conventional guidelines for experimentation with animals and the local committee approved the experimental protocol. All experiments were performed twice.

Tissue processing

Twenty-four hours after the final CS exposure the mice were sacrificed by cervical displacement and the right ventricle was perfused with saline to remove blood from the lungs. The right lung was ligated and the left lung was inflated by instilling 4% phosphate buffered formalin (pH 7.2) at 25 cmH_2O pressure for 2 min and then ligated, removed, and weighed. Inflated lungs were fixed for 48 h before embedding in paraffin. Serial 5-μm sagittal sections stained with hematoxylin and eosin were obtained from the left lung for histologic analyses.

Bronchoalveolar lavage fluid

Airspaces were washed with buffered saline solution (500 μL) three consecutive times in the right lungs (final volume 1.2–1.5 mL). The fluid was withdrawn and stored on ice. Total mononuclear and polymorphonuclear cell numbers were determined in a Zi Coulter counter (Beckman Coulter, Carlsbad, CA, USA). Differential cell counts were performed in cytospin preparations (Shandon, Waltham, MA, USA) stained with Diff-Quik. At least 200 cells per bronchoalveolar lavage (BAL) fluid sample were counted using standard morphologic criteria [13]. After mice were sacrificed, right lungs were immediately homogenized 10% (w/v) in phosphate buffered saline (pH 7.3) and then cen-

trifuged at 3000 × *g* for 5 min. Supernatants were stocked in a freezer for posterior biochemical analysis.

Cell metabolic activity

The method for cell metabolic activity was done according to Putnam et al. [14]. Briefly, MTT (5 mg/mL) in phosphate buffered saline, pH 7.4, was prepared. BAL cells per well (2×10^5), from each mouse, were settled into the wells from a plate (1 h at 37°C). Twenty microliters of MTT solution was added to 200 μL of Dulbecco's Modified Eagle's Medium in each well and the plate was incubated at 37°C for 3 h. Each well was drained and then 200 μL of dimethyl sulfoxide was added. The absorbance was measured at 540 nm. MTT levels are presented as percentages of control mice values.

Determination of oxidative damage (thiobarbituric acid-reactive substances)

The oxidative damage was measured by thiobarbituric acid-reactive substances (TBARS) concentration using a spectrophotometric method. Samples of right lung extracts (250 μL) were deproteinized with 500 μL of 10% trichloroacetic acid and centrifuged at 900 × *g* for 10 min. The supernatant was mixed with 750 μL of 0.67% TBA. The mixture was heated for 15 min in boiling water and then cooled. The organic phase containing the pink chromogen was extracted with 750 μL of *n*-butanol and absorbance at 535 nm was measured using a Beckman Spectrophotometer (model DU 640; Beckman Instruments, Fullerton, CA, USA). TBARS levels (malondialdehyde equivalents per milligram) are presented as percentages of control mice values [15].

Enzyme-linked immunosorbent assay

Tumor necrosis factor-α in lung homogenates was quantified with a specific enzyme-linked immunosorbent assay using a rat anti-mouse monoclonal antibody, with a detection limit of 10 pg/mL, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Mouse recombinant TNF-α standard was used in this assay.

Gelatin zymography

Aliquots of BAL (30 μg of protein) were subjected to electrophoresis on a 4% acrylamide stacking gel/8% acrylamide separating gel containing 1 mg/mL of gelatin in the presence of sodium dodecylsulfate under no reducing conditions. After electrophoresis, 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 $CaCl_2 \cdot 2(H_2O)$, and 2 μM $ZnCl_2$, pH 8.4. The gels were stained with 0.25% Coomassie Blue and destained in a solution of 50% ethanol, 10% acetic acid, and 40% distilled water. Gelatinase activities appeared as clear bands against a blue background. The molecular weight of the gelatinolytic band was estimated using a placental sample (30 μg of protein). Enzyme amount was quantified by measuring the intensity of the negative bands using a densitometric analyzer (Scion Image Software, Scion Co., Frederick, MD, USA). Results are expressed as variation of positive control (placenta) [16].

Statistical analysis

Data are expressed as means ± SEMs. Differences among BAL cells and TNF-α were compared among control, CS, CSMO, and CSMIP groups were tested with one-way analysis of variance followed by the Student-Newman-Keuls post test ($P < 0.05$). Differences from cell metabolic activity, TBARS, and gelatin zymography among groups were tested with the Kruskal-Wallis test followed by the Dunn post test ($P < 0.05$). InStat 3.00 for Windows was used to perform the statistical analyses (GraphPad Software, San Diego, CA, USA).

Results

Histology

Pulmonary tissue analysis showed that the control group exposed to ambient air presented normal-size air spaces and normal alveolar septa (Fig. 1a). In the CS group the alveolar spaces were similar to those in the control group, but leukocytes were more frequently observed into the alveoli (Fig. 1b). The CSMO group presented the same histology as the control group (Fig. 1c).

Mate tea modulated cell influx

Mate tea, in an oral supplementation or intraperitoneally administered, reduced the influx of alveolar macrophages and neutrophils in BALs from the CS group compared with the control group (Fig. 2). The alveolar macrophage numbers in BAL fluid (cells × 10^3/mL) was elevated ($P < 0.001$) in the CS group (425.90 ± 28.10) compared with the control (202.30 ± 15.30), CSMO (214.39 ± 21.43), and CSMIP (248.31 ± 11.12) groups. The same occurred with the neutrophil number in BAL fluid (cells × 10^3/mL), an increase ($P < 0.001$) in the CS group (140.50 ± 20.10) compared with the control (9.90 ± 1.70), CSMO (12.20 ± 4.90), and CSMIP (12.10 ± 2.30) groups. No statistical differences were found between the control group and the CSMO or CSMIP group for alveolar macrophage and neutrophil numbers.

Mate tea reduced cell metabolic activity

Metabolic activity was measured by the MTT method (Fig. 3). Mate tea reduced ($P < 0.01$) MTT (percentage)

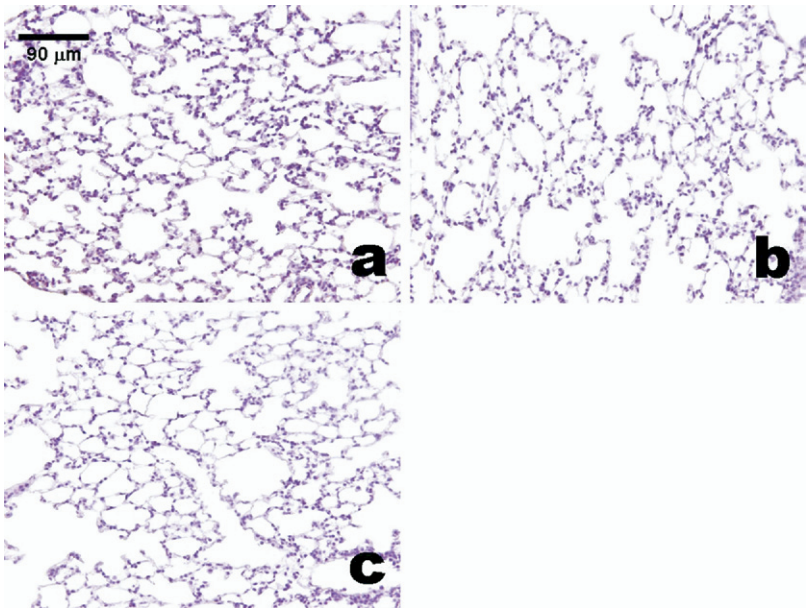

Fig. 1. Photomicrographs of lung sections stained with hematoxylin and eosin. (a) In the control group (mice exposed to ambient air), rare leukocytes are observed, with a normal-size airspace and thin alveolar septa. (b) Mice exposed to six cigarettes per day for 5 d show normal-size airspace and thin alveolar septa with an increase of leukocytes. (c) Mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavages show a histologic pattern similar to the control group, with occasional leukocytes.

from BAL cells in the CSMO group compared with the CS group. Nevertheless, MTT in the CSMO group was similar to that in the control group. MTT (percentage) levels were 100 ± 9 in the control group, 223.6 ± 19 in the CS group, and 89.6 ± 20 in the CSMO group.

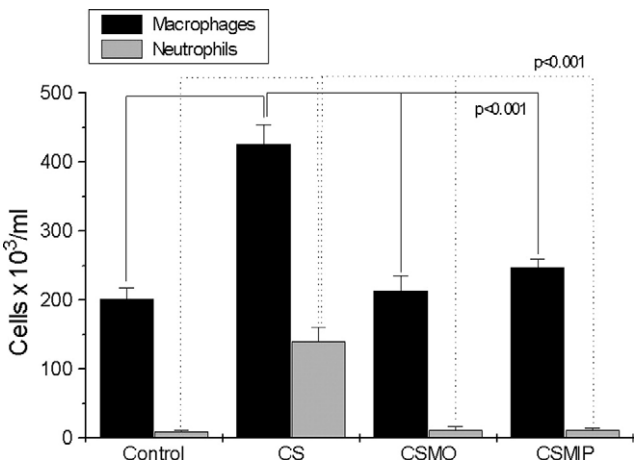

Fig. 2. Effects of mate tea supplementation on cellular influx in bronchoalveolar lavage fluid from the control, CS, CSMO, and CSMIP groups. Alveolar macrophages and neutrophils were statistically increased ($P < 0.001$) only in the CS group. At least 200 cells per bronchoalveolar lavage sample were counted using standard morphologic criteria (mean ± SEM). Control group, mice exposed to ambient air; CS group, mice exposed to six cigarettes per day for 5 d; CSMIP group, mice exposed to six cigarettes per day for 5 d plus daily administration of 150 mg/kg of mate tea by intraperitoneal injection; CSMO group, mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavage.

Mate tea reduced oxidative damage

Oxidative damage was analyzed by the TBARS protocol. Figure 4 shows the effects of supplementation with mate tea by oral gavages on TBARS detection. The reduced ($P < 0.01$) TBARS (percentage) levels in the CSMO group compared with the CS group were not different from the control group ($P < 0.05$). TBARS values were 100 ± 3.46% in the

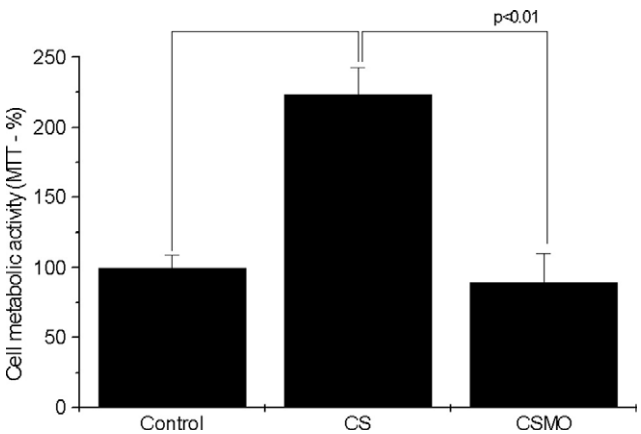

Fig. 3. Cellular metabolic activity of the control, CS, and CSMO groups as measured by the MTT assay. A significant increase ($P < 0.01$) in the percentage of MTT from the CS group was observed when compared with the control group. We found no differences between the CSMO and control groups. Control group, mice exposed to ambient air; CS group, mice exposed to six cigarettes per day for 5 d; CSMO group, mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavage; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide.

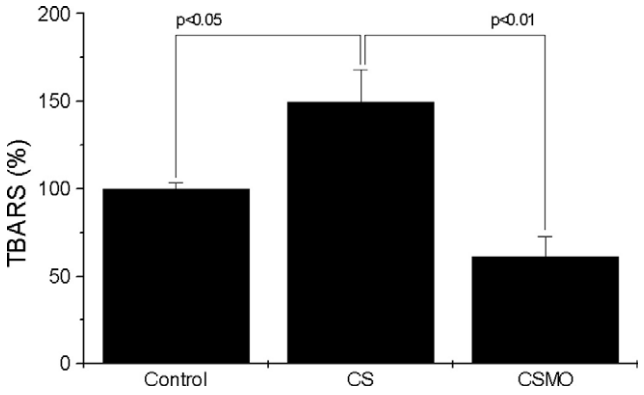

Fig. 4. Effects of mate tea supplementation on TBARS (percentage) in the control, CS, and CSMO groups. A statistical increase from oxidative damage in the CS group was observed in comparison with the control group. We found no differences between the CSMO and control groups. Control group, mice exposed to ambient air; CS group, mice exposed to six cigarettes per day for 5 d; CSMO group, mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavage; TBARS, thiobarbituric acid-reactive substances.

control group, 150 ± 18.18% in the CS group, and 61.33 ± 11.32% in the CSMO group.

Mate tea reduced TNF-α

Tumor necrosis factor-α was measured in lung samples as an inflammation marker (Fig. 5). Mate tea supplementation reduced TNF-α content in the CSMO group ($P < 0.05$) compared with the CS group. The CSMO group was not different from the control group. TNF-α contents (picograms per milliliter) were 180.6 ± 14.4 in the control group,

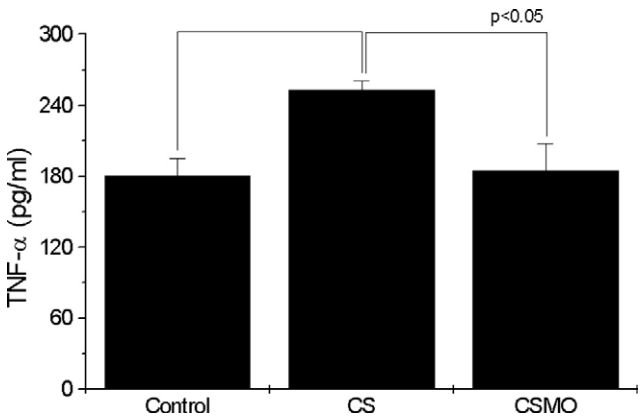

Fig. 5. Effects of mate tea supplementation on TNF-α content in the control, CS, and CSMO groups. There was increase of TNF-α content in the CS group compared with the control group. Mate tea supplementation reduced TNF-α content in the CSMO group compared with the CS group and was similar to the control group. Control group, mice exposed to ambient air; CS group, mice exposed to six cigarettes per day for 5 d; CSMO group, mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavage; TNF-α, tumor necrosis factor-α.

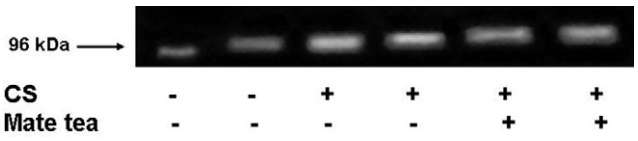

Fig. 6. Effects of mate tea supplementation on gelatin zymography in the control, CS, and CSMO groups. A negative signal indicates ambient air exposure and/or no mate tea supplementation. A positive signal indicates CS exposure and/or mate tea supplementation. Negative bands were reduced in the CSMO compared with the CS group. Negative bands in the CSMO group were more evident than in the control group. The control group presented lower negative bands in comparison with the CS and CSMO groups. Densitometry of negative bands is shown in Figure 7. Control group, mice exposed to ambient air; CS group, mice exposed to six cigarettes per day for 5 d; CSMO group, mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavage.

253.6 ± 7.2 in the CS group, and 185.3 ± 21.8 in the CSMO group.

Mate tea reduced MMP-9 activity

The MMP-2 and MMP-9 activities were analyzed by gelatin zymography. MMP-2 activity was not detected in mice samples. However, MMP-9 activity (Fig. 6 and as demonstrated by densitometry in Fig. 7) was decreased by mate tea supplementation ($P < 0.001$) in the CSMO compared with the CS group. Mate tea reduced MMP-9 activity in the CSMO group, but the band was more evident ($P < 0.01$) than in the control group. Densitometry of the bands indicated values of 107.47 ± 5.91 in the control group, 177.62 ± 4.07 in the CS group, and 130.91 ± 3.42 in the CSMO group.

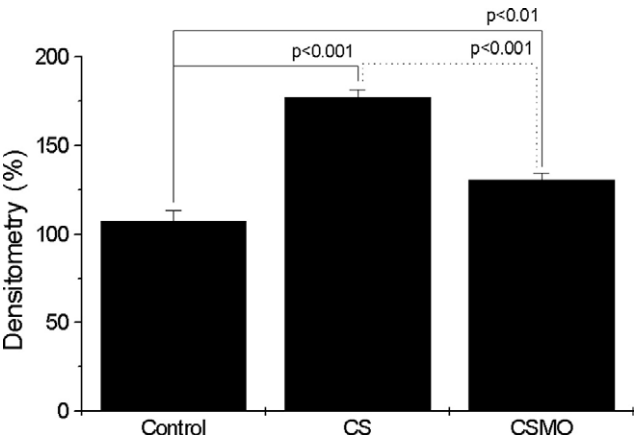

Fig. 7. Densitometry of negative bands from Figure 6. Cigarette smoke induced an increase in matrix metalloproteinase-9 activity compared with the control and CSMO groups ($P < 0.001$); however, the CSMO group was different from the control group to some degree ($P < 0.01$). Control group, mice exposed to ambient air; CS group, mice exposed to six cigarettes per day for 5 d; CSMO group, mice exposed to six cigarettes per day for 5 d plus daily supplementation of 150 mg/kg of mate tea by oral gavage.

Discussion

The evidence shows that CS plays a key role for the development of COPD [17]. The presence of more than 4700 chemical compounds, including high concentrations of free radical and other oxidants in CS, increased the importance of the imbalance between oxidants and antioxidants in the pathogenesis of COPD [18].

In our study, the short-term cigarette smoke exposure induced an ALI response in mice, characterized by the influx of inflammatory cells, mainly macrophages and neutrophils in BALs. The effects of mate tea in the lung were observed in the reduction of the influx of inflammatory cells into the alveolar space in both groups treated by an oral or intraperitoneal route. Although there is little knowledge and few reports about the availability of mate by any route of administration, the similar BAL results induced us to focus on IP by an oral route because it is more physiologic and represents the human form of consumption. Therefore, the following assays were realized only with the CSMO group. Cell metabolic activity was analyzed by MTT and we demonstrated a lesser metabolic activity in the CSMO group compared with the CS group.

According to Bixby et al. [11], IP extract is the beverage with the highest polyphenol content [19] when compared with green tea and white and red wines. The aqueous extract was prepared under similar conditions as those used traditionally, with some modifications [11,12], and probably also by other populations in the world, to obtain nutritionally relevant conclusions. Mate tea was used in an attempt to decrease the effects of CS in the lung, especially to diminish the oxidative stress that was indicated by lipid peroxidation, a well-established mechanism of cellular injury [20]. Together with the generation of 10^{14} free radicals per puff in CS, endogenous reactive oxygen species are also produced in the lung in normal cellular processes [21]. Malondialdehyde levels have been used as a convenient index of lipid peroxidation–related oxidative damage from smokers [22].

The TBARS are also markers of lipid peroxidation and were usually associated with other parameters for evaluation of oxidative stress [23]. In this study the CSMO group showed decreased levels of TBARS (similar to the control group) compared with the CS group. Among the reasons stated before, this result could be explained because of an increase in endogenous antioxidant mechanisms such as catalase, glutathione peroxidase, or superoxide dismutase activities [10].

In the exposure models of CS, oxidants and MMPs complement each other in the potential to destroy lung tissue [24]. Nevertheless, MMPs also play a role in regulating inflammation through the generation of cytokines, such as TNF-α, and by blazing trails for cells through tissue barriers [25]. We studied MMP-2 and MMP-9 activities in the CS, CSMO, and control groups. MMP-9, the most elastolytic of the MMPs, is predominantly produced by macrophages but is also found in neutrophil granules, whereas MMP-2 is produced in smaller quantities by macrophages but is also released by fibroblasts [26]. No MMP-2 activity was detected in any group and the highest MMP-9 activity found in the CS group may be due the elevated macrophage and neutrophil influxes and in response to inflammatory cytokines such as TNF-α. Nevertheless, a recent study connected the role of reactive oxygen species in MMP activation [27]. We observed an increase of MMP-9 activity in the CSMO group compared with the control group ($P < 0.01$), although it was smaller than in the CS group, probably due to less cell inflammatory influx into the airspace, and the oxidative stress provoked by CS, which was attenuated by mate tea antioxidant action.

Alveolar macrophages stimulated by TNF-α may release MMP, suggesting that proinflammatory stimuli might regulate MMP activity in CS-exposure models [25]. Therefore, we expected to find in our exposure model high TNF-α levels and MMP activity. TNF-α expression was increased in the CS group compared with the CSMO and control groups, as was MMP activation. These data suggest that mate tea supplementation in an ALI model can induce an anti-inflammatory profile, because decreases in inflammatory cytokine expression, cell influx, and cellular metabolic activity were observed in the groups treated with mate tea. Corbel et al. [28] proved that TNF-α administration induces airway neutrophilia and increases MMP-9 activity.

Conclusion

Our data demonstrate that daily administration of mate tea attenuates the initial lung inflammatory response induced by CS in mice. Mate tea reduced oxidative stress, inflammatory cell influx, cellular metabolic activity, and MMP-9 and TNF-α expression. New data are needed to clarify the nutritional antioxidant properties found in this study by analyzing other inflammatory and oxidative markers, and perhaps to investigate a possible nutritional role for mate tea as a potential antioxidant in a long-term CS-exposure model. These results in mice should drive future studies with mate tea extracts as a potential protective resource against CS inflammation in humans.

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