© Med Sci Monit, 2011; 17(8): BR187-195
**PMID:** 21804454

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Basic Research

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**Received:** 2010.08.10
**Accepted:** 2010.11.15
**Published:** 2011.08.01

# Grape skin extract reduced pulmonary oxidative response in mice exposed to cigarette smoke

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**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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**Source of support:** This work was supported by grants from FAPERJ, CNPq and ACHÉ Pharmacological Laboratory S.A.

## Summary

**Background:** Oxidative stress has been implicated in the pathogenesis and progression of chronic obstructive pulmonary disease (COPD), and cigarette smoke (CS) is known to be one of the major sources of oxidants in the lungs. We postulated that acute administration of GSE (grape skin extract) would either reduce or protect the ALI (acute lung inflammation) produced by CS via NO release.

**Material/Methods:** We adopted a nutritional approach by investigating the inflammatory cells, metalloproteinase 9 (MMP-9) activity, and oxidative stress markers (superoxide dismutase – SOD; catalase – CAT; glutathione peroxidase (GPx) activities and malondialdehyde – MDA – levels) that play a role in the development of acute lung inflammation (ALI). Therefore, we tested an orally active antioxidant produced from grape skin manipulation (grape skin extract – GSE), in mice exposed to CS from 6 cigarettes a day for 5 days. In addition, we used a separate group treated with NG-nitro-L-arginine methyl ester (an NO inhibitor) to confirm nitric oxide (NO) involvement in GSE effects.

**Results:** We showed for the first time that administration of GSE inhibited ALI and oxidative damage induced by CS. This is associated with decreased MMP-9 activity, decreased number of inflammatory cells in the bronchoalveolar lavage fluid, and reduced levels of lipid peroxidation. Our results indicate that beneficial effects of GSE are NO-dependent.

**Conclusions:** The study indicates that alteration of the oxidant-antioxidant balance is important in the pathogenesis of CS-induced ALI and suggests lung protective effects of GSE treatment in the mouse.

**key words:** **acute lung injury • cigarette smoke • grape skin extract • oxidative stress**

**Abbreviations:** **ALI** – Acute lung inflammation; **AM** – Alveolar macrophage; **CAT** – Catalase; **COPD** – Chronic obstructive lung disease; **CS** – Cigarette smoke; **GPx** – Glutathione peroxidase; **GSE** – Grape skin extract; **MDA** – malondialdehyde; **MMPs** – Metalloproteinases; **NO** – Nitric Oxide; **PMN** – Polymorphonuclear; **SOD** – Superoxide dismutase; **TBARS** – Thiobarbituric acid reactive substances

**Full-text PDF:** http://www.medscimonit.com/fulltxt.php?ICID=881895
**Word count:** 3645
**Tables:** –
**Figures:** 8
**References:** 57

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## BACKGROUD

Worldwide, chronic obstructive pulmonary disease (COPD) is predicted to become one of the top third causes of death and disability by 2020 [1,2]. Cigarette smoking is the greatest risk factor for this disease [3]. It is therefore important to understand the development of this disease in order to develop strategies for its prevention, treatment and cure. Cigarette smoke (CS) is a major source of oxidants such as free radicals, including semiquinone and hydroxyl radicals, nitric oxide and hydrogen peroxide, in the lungs [4,5]. Oxidative stress has been implicated in the pathogenesis and progression of COPD [6,7]. Both reactive oxidant species (ROS) from inhaled CS and those endogenously formed by inflammatory cells constitute an increased intrapulmonary oxidant burden [8]. Oxidative stress is closely linked to lung inflammation [9]. The inflammatory process in COPD is dominated by macrophages and neutrophils [3]. Increased production of mediators such as interleukin-1β (IL-1β) and tumor necrosis factor-α, which both attract inflammatory cells and increase oxidant production by these cells, has been found [10]. Attenuation of oxidative stress would be expected to result in reduced pulmonary damage, contributing to attenuation of the progression of acute lung inflammation (ALI) caused by CS [11].

Moderate consumption of red wine is associated with a decrease in the incidence of cardiovascular events; the cardioprotective effects have been partially attributed to polyphenolic compounds present in purple grapes [12]. The bioactivity of polyphenols includes antioxidant and free radical-scavenging properties that lead to a decrease in low density lipoprotein oxidation, an increase in high density lipoprotein level, prevention of platelet aggregation, and leukocyte adhesion [13,14]. Grape skin extract (GSE) contain a large number of polyphenols with powerful free radical scavengers [15,16]. GSE has been reported to possess a broad spectrum of pharmacological and therapeutic effects including anti-inflammatory activity and reduced apoptotic cell death [17]. Red wine polyphenols have been shown to increase endothelial nitric oxide synthase (eNOS) expression and subsequent endothelial NO release [18,19].

No currently available treatments have been shown to slow the progression of COPD; however, a better understanding of the cellular and molecular mechanisms involved in COPD is likely to lead to new treatments focusing its inflammatory mechanisms. In the present study, we adopted a nutritional approach by investigating the inflammatory cells, metalloproteinase 9 (MMP-9) activity and oxidative stress markers that play a role in the development of ALI by testing GSE, an orally active antioxidant produced from grape skin manipulation, in mice exposed to CS. To confirm NO involvement in GSE effects, we used a separate group treated with NG-nitro-L-arginine methyl ester (an NO inhibitor). We postulated that acute administration of GSE would either reduce or protect the ALI produced by CS via NO release.

## MATERIAL AND METHODS

### Preparation of GSE

The dried and powdered skin fruits of *Vitis vinifera* L. (Vitaceae) were extracted by an aqueous solution at 100°C with occasional shaking for about 120 minutes. The solution was then introduced in a column with the ion-exchange resin (cationic). The resin was washed sequentially by ethanol, ethanol: $H_2O$ (1:1) and $H_2O$. The $H_2O$ fraction was discarded. The ethanolic and hydroalcoholic fractions were placed together and evaporated under vacuum at 60°C, and then the concentrated solution was dried by spray-dryer (inlet temperature 190°C and outlet temperature of 85°C). The extract obtained in the process is a fine powder soluble in $H_2O$, which has about 30% of total polyphenols according to the Folin-Ciocalteau [20] and 3% Malvidin-3-O-glucoside [21]. This statement was the subject of a patent application (PI0605693 A2-8).

### LC/UV-DAD analysis of the grape-skin ACH09 extract

LC/UV analysis of the dried hydro-alcoholic grape skin extract was performed on a Hewlett-Packard (Waldbronn, Germany) Series 1100 photodiode array detector (DAD) liquid chromatography system. HPLC/UV/DAD with a Symmetry RP-18 column (4 µm; 250×3.9 mm i.d.; Waters) using a Methanol-Water with 0.5% of formic acid, gradient (20:80→100:0) in 25 min. The detection was performed at 210 nm, 254 nm and 540 nm.

### LC/UV/APCI-MS$^n$ analysis

LC/MS$^n$ was performed directly after UV-DAD measurements. A Finnigan LCQ ion trap (Finnigan MAT, San Jose, CA, USA) with APCI interface was used with the following conditions: capillary temp. 150°C; vaporizer temp. 370°C; positive mode; sheath gas flow: 60 psi (1psi =6894.76 Pa), corona needle current 5 µA. The MS$^n$ experiment was performed by programming dependent scan events. The first event was a full MS scan Mr (150.0–1500.0) (MS$^1$); during the second event the main ion recorded was isolated and selectively fragmented in the ion trap (MS$^2$). The collision energy was set to 15 eV.

### Identification of the main compounds

Compound (**1**). UV ($\lambda_{max}$) 522 nm; LC/APCI-MS (positive mode): *m/z* 463.1.13 $[M]^+$, 301 $[M-162]^+$. Compound (**2**). UV ($\lambda_{max}$) 515 nm; LC/APCI-MS (positive mode): *m/z* 479.1 $[M]^+$, 317 $[M-162]^+$. Compound (**3**). UV ($\lambda_{max}$) 524 nm; LC/APCI-MS (positive mode): *m/z* 493.13 $[M]^+$, 331 $[M-162]^+$. Compound (**4**). UV ($\lambda_{max}$) 317, 532 nm; LC/APCI-MS (positive mode): *m/z* 801.22 $[M]^+$, 639 $[M-162]^+$, 493 $[M-162-147]^+$, 331 $[M-162-162-147]^+$.

### LC-UV and LC-MS analysis sample preparation

HPLC analysis of the dried hydro-alcoholic grape skin extract: 10 mg of the extract was dissolved in 1 mL Methanol/$H_2O$ (1:1) with 0.5% of formic acid (HPLC quality). The mixture was filtered using a Millex®-LCR syringe-driven filter (0.45 µm); 20 µl was analyzed by HPLC.

### Reagents and animals

Thiobarbituric acid, Adrenaline, Acrylamide, Gelatin, sodium duodecyl sulphate (SDS), Triton X-100, Tris-HCl, $CaCl_2$, $ZnCl_2$, NG-nitro-L-arginine methyl ester (L-NAME), NADPH, Coomassie blue, Hematoxylin-eosin, were purchased from

Sigma Chemical (St. Louis, MO, USA). Diff-Quik was purchased from Baxter Dade AG (Dudingen, Switzerland). Bradford was purchased from Bio-Rad (Hercules, CA, USA). Formalin, Methanol, Ethanol, Acetic acid and hydrogen peroxide were purchased from Vetec (Duque de Caxias, Brazil). C57BL/6 male mice were purchased from Instituto de Veterinária – Universidade Federal Fluminense (Niterói, Brazil).

**CS exposure**

To study GSE effects in CS-exposed mice, 30 8-week-old C57BL/6 male mice were exposed to smoke from 6 commercial filtered cigarettes per day for 5 days (CS, CS+GSE and CS+GSE+L-NAME groups) by using a smoking chamber described previously [11,22–24]. Each cigarette produced 300 mg/m$^3$ of total particulate matter in the chamber. The CS group was treated with saline (0.2 ml/day); the CS+GSE group was treated with grape skin extract (200 mg/kg/day) [25]; and the CS+GSE+L-NAME group was treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day) [26]. All groups were treated concomitantly by oral gavages. Mice exposed to ambient air were used as controls (n=10).

A separate group of C57BL/6 male mice (n=5 for each group) were exposed to ambient air during 5 days by using the same protocol described above and simultaneously treated with vehicle (control group), 200 mg/kg/day of GSE (GSE group), and 50 mg/kg/day of L-NAME (L-NAME group).

All procedures were carried out in accordance with conventional guidelines for experimentation with animals and the local committee approved the experimental protocol. This experimental protocol was performed twice.

**Tissue processing**

Twenty-four h after the last CS exposure, each mouse was 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 cm $H_2O$ pressure for 2 min and then ligated, removed, and weighed. Inflated lungs were fixed for 48 h before embedding in paraffin. Serial sagittal 5-µm sections were obtained for histological analyses. Hematoxylin-eosin, Orcein and Sirius Red stained sections were analyzed. After mice were sacrificed, right lungs were immediately homogenized 10% (w/v) in PBS (pH 7.3) and then centrifuged at 3000 g for 5 min. Supernatants were frozen for posterior biochemical analysis.

**Bronchoalveolar lavage fluid**

Airspaces were washed with buffered saline solution (500 µL) 3 consecutive times (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 on 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.

**Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities**

To determine SOD, CAT and GPx activities, lung homogenates were used. SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation as absorbance at 480 nm [27] (Beckman Spectrophotometer mod DU 640; Fullerton, CA, USA). CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm [28]. GPx activity was measured by monitoring the oxidation of NADPH at 340 nm in the presence of $H_2O_2$ [29]. The total protein content in the samples from right lung tissue was determined by the method of Bradford [30].

**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 as previously described by Draper et al. [31]. 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 levels were determined by absorbance at 532 nm and expressed as malondialdehyde equivalents (nm/mg protein).

**Gelatin zymography (MMP-9 activity)**

Aliquots of lung homogenates (40 µg protein) were subjected to electrophoresis on an 8% acrylamide stacking gel/7% acrylamide separating gel containing 1 mg/mL gelatin in the presence of SDS under nonreducing 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, 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. Gelatinase activities appeared as clear bands against a blue background. Molecular weight of gelatinolytic band was estimated using placental sample (20 µg 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).

**Statistical analysis**

Data are expressed as means ±SEM. For comparison between control and CS groups in respect of differences among BAL cells, SOD, CAT, GPx and MDA One-way ANOVA was performed followed by the Tukey post-test ($p<0.05$). InStat Graphpad software was used to perform the statistical analyses (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, CA, USA).

## RESULTS

**Isolation and Identification of the major constituents of the ACH09 GSE extract**

In order to rapidly identify the active principles in grape skin, the extract was analysed by LC/UV/MS, with an atmospheric pressure chemical ionisation (APCI) interface [32]. All compounds showed the same UV spectra in the

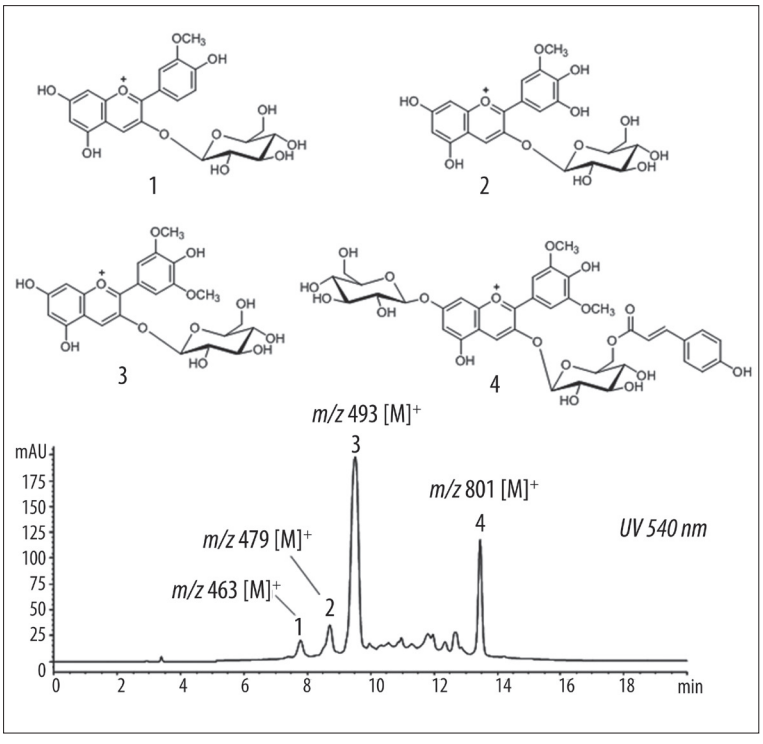


**Figure 1.** LC/UV/MS analysis of GSE . A symmetry RP-18 column using a methanol-water with 0.5% of formic acid by 25 min was used. The detection was performed at 210, 254 and 540 nm. Flow rate was 1 mL/min. The peaks and molecular representations correspond to peonidin-3-O-glucoside (1), petunidin-3-O-glucoside (2), malvidin-3-O-glucoside (3), and malvidin-3-(6-O-*trans-p*-coumaryl)-5-O-diglicoside (4).

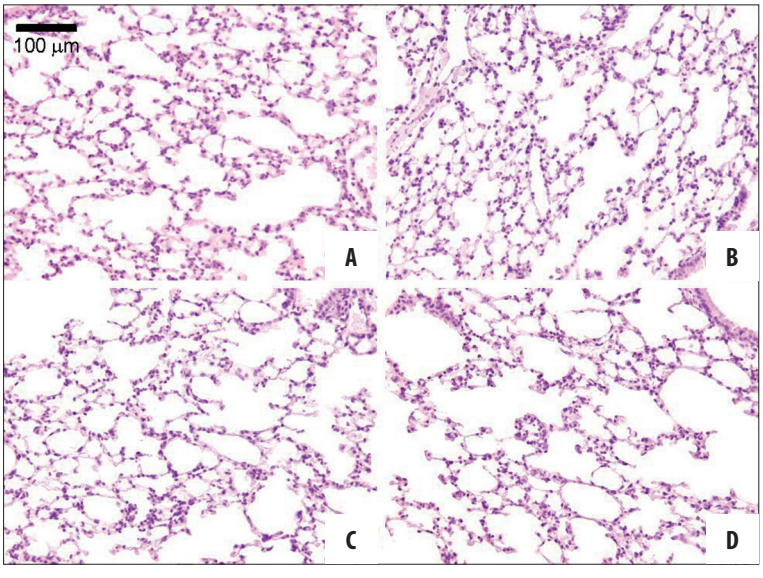


**Figure 2.** Representative photomicrographs of mouse lung sections stained with hematoxylin and eosin (400×). (**A**) Control group: animals exposed to ambient air; (**B**) CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; (**C**) CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day); (**D**) CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day).

LC/UV/DAD analysis characteristic of anthocyanins [33]. Four distinct compounds (named **1, 2, 3** and **4**) presented the molecular ions at *m/z* 463 $[M]^+$, 479 $[M]^+$, 493 $[M]^+$, and 801 $[M]^+$, and all showed a similar fragmentation pattern (Figure 1). Compounds **1**, **2** and **3** present 2 signals corresponding to the molecular ion $[M^+]$ and the fragment resulting from the loss of a glucose molecule $[M\text{-}162]^+$, corresponding to the aglicon. In the case of compound **4**, the MS spectra shows the loss of 1 glucose and a fragment corresponding to the loss of an *p*-coumarylglucoside moiety $[M\text{-}308]^+$ [34]. According to these data, the compounds were identified as peonidin-3-O-glucoside (**1**), petunidin-3-O-glucoside (**2**) malvidin-3-O-glucoside (**3**) and malvidin-3-(6-O-*trans-p*-coumaryl)-5-O-diglicoside (**4**), previously described in different species of *Vitis* spp. [34,35]. This hypothesis was confirmed by the comparison of retention time, UV and MS data in the LC/UV/MS analysis using commercial available standards. Compounds **1-3** are always present in grape extracts, generally in high concentrations, with the exception of **4,** which can be found in low concentration [34].

### GSE effects in lung histology after acute lung inflammation

Histological changes are illustrated in Figure 2. The lungs of control group mice showed parenchyma features consisting of alveoli connected to alveolar ducts, separated from each other only by thin alveolar septa (Figure 1A). The lungs of

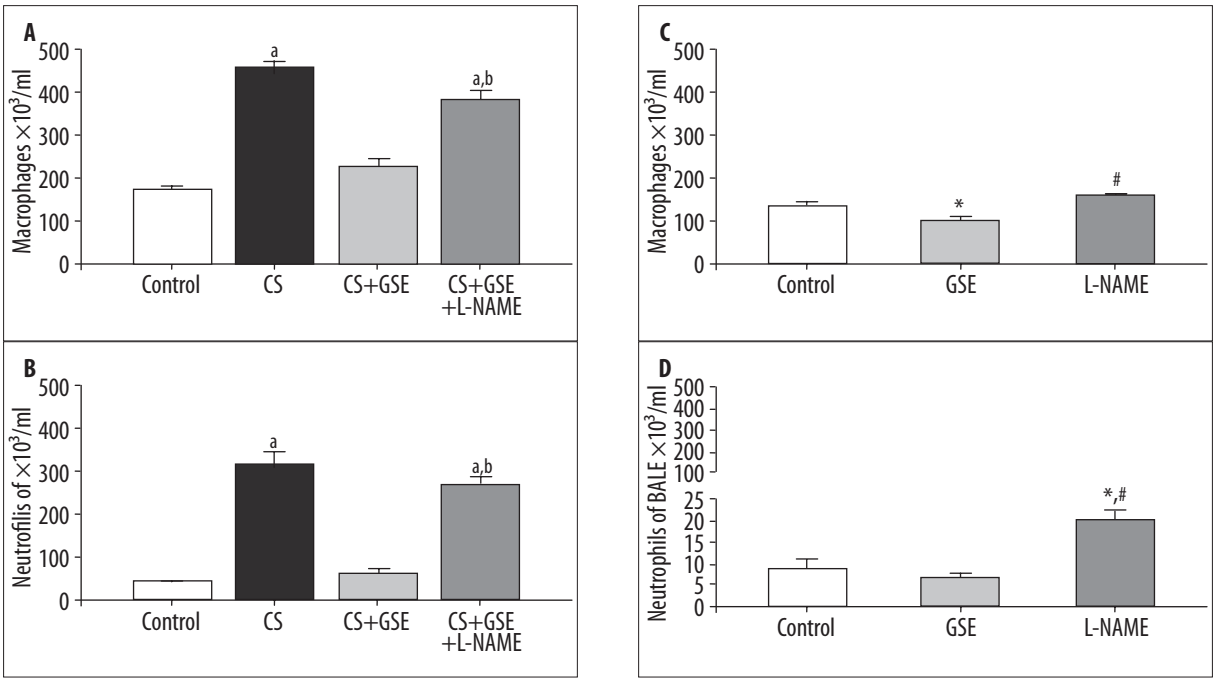


**Figure 3.** Bronchoalveolar lavage (BAL) fluid cellularity at the end of the experiment. Macrophages (**A, C**) and neutrophils (**B, D**) were collected from BAL fluid. Panels on the left show data from cigarette smoke-exposed animals plus treatment; Panels on the right show data from ambient air-exposed animals plus treatment. Control group: animals exposed to ambient air; CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day); CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day); GSE group: animals exposed to ambient air and treated with grape skin extract (200 mg/kg/day); L-NAME group: animals exposed to ambient air and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day); Data are mean values (n 4–6) with their standard errors represented by vertical bars. One-way ANOVA was performed followed by the Tukey post-test was used for statistical analysis. (a) Mean value was significantly different from that of the Control group. (b) Mean value was significantly different from that of the CS group. (c) Mean value was significantly different from that CS+GSE group. (*) Mean value was significantly different from that of the Control group; (#) Mean value was significantly different from that of the GSE group.

all CS mice showed small modifications including increased alveolar macrophages (AMs) and neutrophils (PMNs) in alveoli; however, no changes were observed in lung histoarchitecture (Figure 1B). The lungs of CS+GSE mice were similar to the control group, with few AMs and no or very rare PMNs (Figure 1C). Finally, the lungs of CS+GSE+L-NAME mice were similar to the CS group, with increased in AMs and PMNs. No changes were observed in elastic and collagen fibers of any group exposed to CS or ambient air.

**GSE modulated BAL cells after acute lung inflammation**

The AMs and PMNs numbers in BAL fluid are shown in Figure 3. The 160% increase in AMs number in the CS group when compared to the control group ($p<0.001$) was 55% reduced by GSE treatment ($p<0.001$). However, when L-NAME was given together with GSE, AMs numbers were similar to CS group. In addition, the pattern of PMNs influx was similar to the AMs (Figure 3A). There was a 25% increase in AMs number in C57BL/6 mice that were exposed to ambient air for 5 consecutive days and treated with L-NAME ($p<0.05$; Figure 3C). PMNs increased 950% in CS ($p<0.001$) and 770% CS+GSE+L-NAME ($p<0.001$) when compared to the control group. PMN numbers of the CS+GSE group showed no differences when compared with PMN numbers of the control group (Figure 3B). There was a 95% increase in PNMs number in C57BL/6 mice that were exposed to ambient air for 5 consecutive days and treated with L-NAME ($p<0.001$; Figure 3D).

**GSE reduced oxidative stress after acute lung inflammation**

We analyzed 3 specific enzymes involved in the antioxidant endogenous defense mechanisms. SOD activity is shown in Figure 4. CS exposure increased SOD activity 66% in lung homogenates when compared to the control group ($p<0.001$). GSE administration restored SOD activity compared to the control group. The CS+L-NAME+GSE group showed SOD activity similar to the CS group. Catalase activity increased 45% in lung homogenates after CS exposure ($p<0.05$) (Figure 5). CAT values in the CS+GSE group were similar to the CS group. Glutathione Peroxidase activity is shown in Figure 6. GSE treatment reduced the 250% increase in GPx activity observed in the CS group ($p<0.001$), but it was still high when compared with the control group (105%, $p<0.001$). In contrast, concomitant GSE and L-NAME administrations in animals exposed to CS resulted in a 67% increase of GPx activity when compared to the CS+GSE group ($p<0.001$).

MDA levels were significantly increased (130%, $p<0.01$) in the CS group when compared to the control group

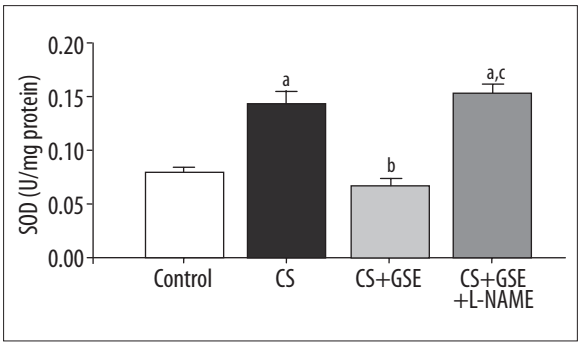


**Figure 4.** Measurements of superoxide dismutase activity in lung homogenates from all experimental groups. Control group: animals exposed to ambient air; CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day); CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day). Data are mean values (n 4–6) with their standard errors represented by vertical bars. One-way ANOVA was performed followed by the Tukey post-test was used for statistical analysis. (a) Mean value was significantly different from that of the Control group. (b) Mean value was significantly different from that of the CS group. (c) Mean value was significantly different from that CS+GSE group.

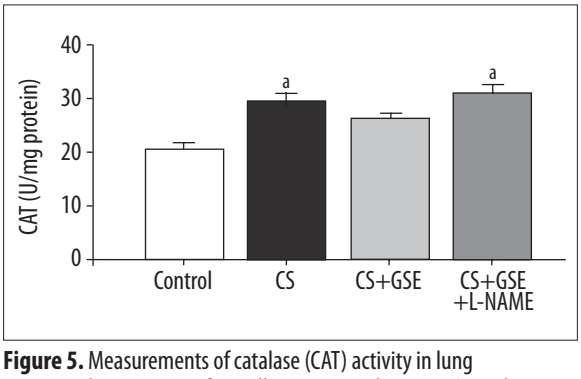


**Figure 5.** Measurements of catalase (CAT) activity in lung homogenates from all experimental groups. Control group: animals exposed to ambient air; CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day); CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day). Data are mean values (n 4–6) with their standard errors represented by vertical bars. One-way ANOVA was performed followed by the Tukey post-test was used for statistical analysis. (a) Mean value was significantly different from that of the Control group. (b) Mean value was significantly different from that of the CS group. (c) Mean value was significantly different from that CS+GSE group.

(Figure 7A). GSE administration resulted in MDA levels comparable to the control group; however, the CS+GSE+L-NAME

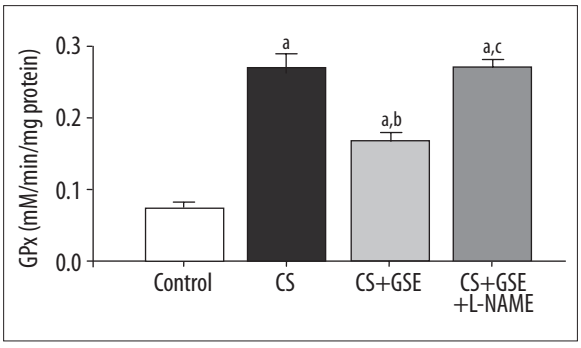


**Figure 6.** Measurements of gluthatione peroxidase (GPx) activity in lung homogenates from all experimental groups. Control group: animals exposed to ambient air; CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (150 mg/kg/day); CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day). Data are mean values (n 4–6) with their standard errors represented by vertical bars. One-way ANOVA was performed followed by the Tukey post-test was used for statistical analysis. (a) Mean value was significantly different from that of the Control group. (b) Mean value was significantly different from that of the CS group. (c) Mean value was significantly different from that CS+GSE group.

group showed a significant increase in MDA levels (110%, $p<0.01$) when compared to the CS+GSE group. A separate group of C57BL/6 mice was exposed to ambient air for 5 consecutive days and treated with vehicle, GSE, or L-NAME There was no alteration in MDA in mice treated either with GSE or L-NAME and exposed to ambient air (Figure 7B).

### GSE reduced MMP-9 activity after acute lung inflammation

MMP-9 activity increased after CS exposure (Figure 8A). Densitometric analyses of MMP-9 bands showed increases activity of this enzyme after CS exposure (Figure 8B). The CS+GSE group showed a decrease in MMP-9 activity in lung homogenates; however, concomitant GSE and L-NAME administration during CS resulted in MMP-9 activity densitometry similar to the CS group.

## Discussion

We present here for the first time that antioxidant and anti-inflammatory properties of GSE were able to inhibit ALI in mice. CS increased macrophages and neutrophils counts in BAL, as well as increased MMP-9, SOD, CAT, GPx and MDA. Conversely, GSE treatment reduced all these parameters, except CAT. In addition, we showed that lung protective effects of GSE were regulated by NO release, since both inflammatory and oxidative markers in the CS+GSE+L-NAME group were comparable to the CS group.

Reports in the literature have described a variety of lipid peroxidation biomarkers, including lipid hydroperoxides

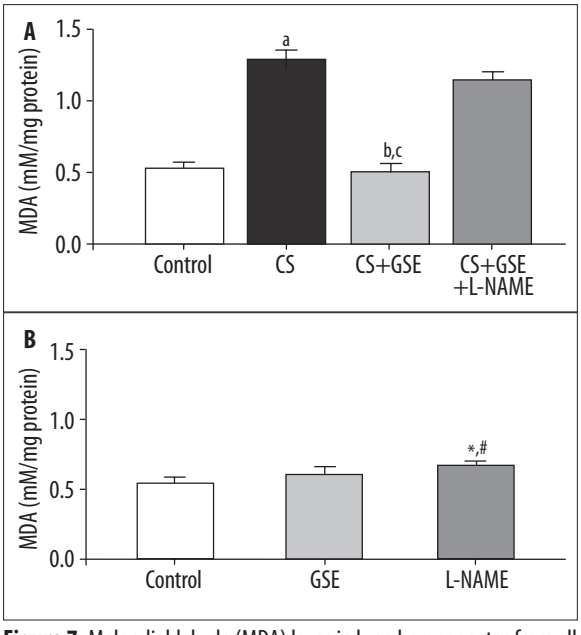


**Figure 7.** Malondialdehyde (MDA) leves in lung homogenates from all experimental groups. (**A**) shows data from cigarette smoke-exposed animals plus treatment; (**B**) shows ambient air-exposed animals plus treatment. Control group: animals exposed to ambient air; CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day); CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (150 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day); GSE group: animals exposed to ambient air and treated with grape skin extract (200 mg/kg/day); L-NAME group: animals exposed to ambient air and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day); Data are mean values (n 4–6) with their standard errors represented by vertical bars. One-way ANOVA was performed followed by the Tukey post-test was used for statistical analysis. (a) Mean value was significantly different from that of the Control group. (b) Mean value was significantly different from that of the CS group. (c) Mean value was significantly different from that CS+GSE group. (*) Mean value was significantly different from that of the Control group; (#) Mean value was significantly different from that of the GSE group.

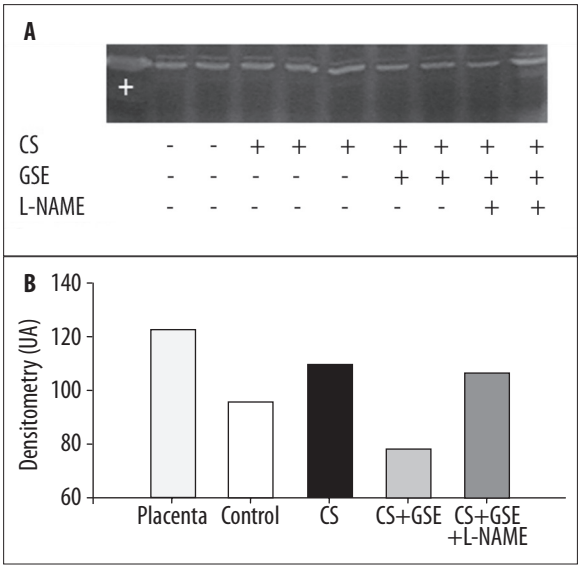


**Figure 8.** Gelatin zymography of MMP-9 activity in lung homogenates from all experimental groups (**A**). Representative bands of MMP-9 activity are shown for each group (**B**). CS group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days; CS+GSE group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day); CS+GSE+L-NAME group: animals exposed to 6 commercial filtered cigarettes per day for 5 consecutive days and treated with grape skin extract (200 mg/kg/day) plus NG-nitro-L-arginine methyl ester (50 mg/kg/day).

[36], isoprostanes [37], aldehydes and oxidized phospholipids[38]. In this regard, we have used MDA when it has become widely accepted as a useful method of analyzing lipid peroxidation in biological materials [23,24,39,40]. MDA levels were observed to be increased in CS mice, but were shown to be reduced following GSE treatment. However, MDA levels were increase in lung homogenates of animals from the CS+GSE+L-NAME group and, in a lower scale, MDA levels were also increased following L-NAME administration in ambient air-exposed mice. These data suggest a clear action of GSE in protecting lung cells from oxidative damage via NO release. Nevertheless, high SOD, CAT and GPx activities were also important in maintaining oxidative status in CS mice exposed for 5 days. We observed a reduction in both SOD and GPx activities in CS+GSE in comparison with CS. The reduction in redox status during GSE treatment is indicative of a reduction of oxidative stress and restoration of balance among oxidants and antioxidants.

In COPD, oxidants and metalloproteinases (MMPs) complement each other in the potential to destroy lung tissue [41,42]. Oxidative stress can be defined as an imbalance between oxidants and antioxidants in a cell, tissue or extracellular fluid [9]. Oxidative stress induces inflammatory response in the lungs by activating cells through signal transduction mechanisms which involve the redox-sensitive transcription factors NF-κB and AP-1 [6,43]. Evidence suggests that oxidative stress enhancement is associated with lipid peroxidation [44] and by an increase in the amount MMP-9 activity [45,46]. MMPs are enzymes able to attack and degrade extracellular matrix components such as collagens and elastic fibers [47]. Regarding emphysema features, if antiproteolytic protective screen of the lung is insufficient, unregulated proteolytic enzymes such as neutrophil elastase also are able to digest collagens and elastic fibers [48]. 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 [49]. MMP-9 is elevated in bronchoalveolar lavage fluid of subjects with emphysema, suggesting that MMP-9 may be important in the pathogenesis of the disease [41]. This observation has been supported and extended by Russell et al. [42], who have shown that macrophages from COPD patients secreted more MMP-9

than macrophages from healthy volunteers when stimulated with IL-1β, endotoxin or CS-conditioned medium. We studied MMP-9 activity of all groups. We observed not only a high MMP-9 activity in CS, but also increased lipid peroxidation indicated by high MDA levels. MMP-9 activity increase may be due to the increase in AMs and PMNs numbers in the CS and CS+GSE+L-NAME groups when compared to the control group and the CS+GSE group. GSE exerted an antiproteolytic defense due the decreased MMP-9 activity in the CS+GSE group.

We studied inflammatory cells that are a hallmark of the inflammatory process. In the present study and as previously shown by Valenca et al. [23], L-NAME administration was able to increase, in lower scale, the inflammatory cell numbers in the BAL of mice exposed to ambient air. These data help confirm the hypothesis that NO modulates cell recruitment, as it has been shown to stimulate endothelial cell signaling towards neutrophils adhesion [50] and increases neutrophil migration into the airspaces in sepsis [51] and ischemia-reperfusion injury [52]. However, little is known regarding the effect of CS on NO production and cellular influx to the lung. The present study suggests that NO may also influence cell influx into BAL in mice.

GSE treatment was effective in impairing AMs and PMNs influx into the BAL, with values comparable to the control group. However, inhibition of endogenous NO production affected GSE protective action by resulting in increase in AMs and PMNs numbers in the BAL. The mechanisms of NO release by GSE treatment with anti-inflammatory properties are not elucidated here, but we believe that NO has a potential role in modulating not only inflammatory cells influx, but also the oxidative stress process once some pivotal oxidative markers were altered due to L-NAME administration. We also cannot exclude other beneficial effects of GSE treatment, such as the release and action of polyphenols.

CS contains a large number of oxidants, and it has been hypothesized that many of the adverse effects of CS may appear as a result of oxidative damage to critical biological substances [8]. CS exposure increases the amount of alveolar oxidants, not only because CS itself contains an impressive number of free radicals, but also because it increases the number of inflammatory cells in alveoli [9], which spontaneously results in the release of oxidants [16]. Leukocytes from smokers release more oxidants, such as the superoxide anion and hydrogen peroxide, than leukocytes from nonsmokers [6,43]. The role of oxidative stress in lung inflammation is highlighted by the fact that antioxidant treatment prevents transcription factor activation [43,53]. Also, oxidative stress modulates antioxidant concentration in the lungs and antioxidants protect lungs and cells against oxidative stress [54]. The inflammatory status in lung mediated by CS via oxidative stress can be ameliorated by antioxidant treatment [55].

Nowadays, only a few therapies are available for COPD, and have limited efficacy [43,56,57]. Therefore, new anti-inflammatory strategies that also affect oxidative pathways are needed to improve the control of symptoms and to prevent the progression of COPD. A better understanding of the cellular and molecular mechanisms involved in COPD is likely to lead to new therapies that target the underlying inflammatory mechanisms. There is clearly a need for a broad-spectrum treatment, since corticosteroids, which are so effective in suppressing the inflammation in asthma, are ineffective in COPD [9,54].

## Conclusions

In the mouse model of ALI shown in the present study, we showed for the first time that administration of GSE inhibited ALI and oxidative damage induced by CS. This is associated with evidence of decreased MMP-9 activity, decreased number of inflammatory cells in the bronchoalveolar lavage fluid, and reduced levels of lipid peroxidation. The study indicates that alteration of the oxidant-antioxidant balance is important in the pathogenesis of CS-induced ALI, and suggests lung protective effects of GSE treatment in the mouse.

**Acknowledgments**

KMPP was PhD student of BHEx-UERJ. SSV during the experimental design was Visiting Professor from UERJ. EFQ and DDCM were supported by Aché Laboratórios Farmacêuticos, S.A.

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