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Supplementation with vitamins C and E improves mouse lung repair

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Abstract

Cigarette smoke (CS) induces emphysema by tissue destruction through the production of oxidants and metalloproteinases [matrix metalloproteinases (MMPs)]. The possibility of lung repair after emphysema remains unclear. Our aim was to study the effects of vitamins C and E on mouse lung repair evaluated by catalase (CAT), superoxide dismutase (SOD) and MMP-9 activities; by the amount of tumor necrosis factor (TNF)-α in lung homogenates; by cell quantification in bronchoalveolar lavage (BAL) fluid; and by lung histology. Male C57BL/6 mice (n=25) were exposed to nine cigarettes per day, 7 days/week, for 60 days in a whole-body exposure chamber. The control group was sham smoked (n=10). After 60 days of CS exposure, a group of animals was sacrificed (n=5) and the others were divided into two groups: (a) CSv (n=10) supplemented with saline and olive oil (vehicles) for 60 days and (b) CSr (n=10) supplemented with vitamins C and E (50 mg/kg/day) for 60 days. These mice were then sacrificed; BAL was performed and the lungs were removed for biochemical and histological analysis. The results demonstrated that CAT activity was decreased in the CSv and CSr groups compared to the control group. SOD activity was higher in the CSv group than in the control and CSr groups. The CSv group showed a higher neutrophil count in BAL fluid, associated with more TNF-α in lung homogenates, than the control or CSr groups. Finally, emphysema in the CSv group was associated with fewer collagen and elastic fibers than in the control and CSr groups. These results indicate a possible role of vitamins C and E in lung repair after emphysema induced by long-term CS exposure in mice.

Keywords: Vitamin C; Vitamin E; Emphysema; Oxidative stress; Stereology; Lung repair

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major and increasing global health problem, which is predicted to become the third commonest cause of death and the fifth commonest cause of disability in the world by 2020 [1,2]. Cigarette smoke (CS)-induced emphysema is one of the major causes of COPD [3,4], but there are several other risk factors, including air pollution (particularly indoor air pollution from burning fuels) [5], poor diet [6] and occupational exposure [7]. Emphysema is characterized by enlargement of alveolar spaces together with destruction of alveolar walls in the absence of obvious fibrosis [8]. CS causes an inflammatory response in the lower respiratory tract characterized by accumulation of alveolar macrophages associated with a recruitment of fewer neutrophils [9,10]. 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 the tissue destruction found in emphysema [11].

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteinases that regulate the destruction of extracellular matrix components in lung [12]. There is increasing evidence for a role for MMPs in COPD [4]. The concentration, expression and activity of lung MMP-9 (gelatinase B) are increased in emphysema patients [13,14]. The interest in MMPs has also been heightened by the demonstration that emphysema induced by chronic CS exposure is prevented in MMP-12$^{-/-}$ mice [15].

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Oxidative stress plays a key role in the pathophysiology of COPD and amplifies the inflammatory and destructive process [16,17]. Reactive oxygen species (ROS) from CS or from inflammatory cells (particularly macrophages and neutrophils) result in several damaging effects in COPD [18], including decreased antiprotease defenses [19] and antioxidants. Many of the adverse effects of smoking may result from oxidative damage to critical biological molecules [9]. Epidemiological evidence indicates that reduced dietary intake of antioxidants may be a determinant of COPD [20]; moreover, population surveys have linked a low dietary intake of the antioxidant vitamins C and E to declining lung function [21]. Supplementary treatment with vitamin E (100 mg/kg/day) reduced bacterial colonization in the lower respiratory tract of smoke-treated rats [22]. Moreover, pretreatment (100 mg/kg/day) of either ascorbic acid or α-tocopherol acetate before smoke inhalation completely prevented single-strand DNA breaks in the lung, stomach and liver [23]. Also, α-tocopherol (50 mg/kg/day) and ascorbic acid (100 mg/kg/day) protected sperm by inhibiting the induction of ROS generation by Aroclor 1254 [24]. Bezerra et al. [9] showed that inflammatory alveolar cells and levels of tumor necrosis factor (TNF)-α, NF-κB and MMP-12 in lungs of mice exposed to CS for a short period (5 days) were reduced to control levels by ascorbic acid, α-tocopherol or, more efficiently, supplementation with both vitamins. However, it remains unclear whether vitamins C and E may play a role in mouse lung repair after emphysema induced by long-term CS exposure.

Because of the enormous burden of COPD and escalating health care costs, there is now renewed interest in the underlying cellular and molecular mechanisms and a search for new treatment strategies. Vitamins C and E are antioxidants and can therefore be expected to change the oxidant–antioxidant imbalance induced in lungs by CS. Our aim was to study the effects of vitamins C and E on mouse lung repair as evaluated by catalase (CAT) and superoxide dismutase (SOD) activities, by quantification of TNF-α and MMP-9 zymography in lung homogenates, by cell quantification in bronchoalveolar lavage (BAL) fluid and by morphometric and stereological parameters.

2. Materials and methods

2.1. Reagents and animals

Thiobarbituric acid, adrenaline, acrylamide, gelatin, sodium dodecyl sulfate (SDS), Triton X-100, Tris–HCl, $CaCl_2$, $ZnCl_2$, Coomassie Blue, hematoxylin–eosin, orcein and Sirius red were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Specific enzyme-linked immunosorbent assay (ELISA) for TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Diff-Quik was purchased from Baxter Dade AG (Dudingen, Switzerland). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Formalin, 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).

2.2. CS exposure

To study the effects of vitamins C and E on mouse lung repair after emphysema induced by long-term CS exposure, we exposed 25 eight-week-old C57Bl/6 mice (male) to nine commercial filtered cigarettes per day for 60 consecutive days using a smoking chamber as described previously [9,25]. Each cigarette smoked produced 300 mg/m^3 total particulate matter in the chamber. After 60 days of CS exposure, five animals were sacrificed, and histological analysis of the lungs showed evidence of emphysema (first part of protocol). The remaining animals were divided into two groups: (a) CSv (n=10) supplemented with saline (0.2 ml) and olive oil (0.2 ml) for 60 days (vehicles) and (b) CSr (n=10) supplemented with vitamins C (50 mg/kg diluted in 0.2 ml of saline/day) and E (50 mg/kg diluted in 0.2 ml of olive oil/day) for 60 days (second part of protocol). Vehicles or vitamin C and E supplements were administered by oral gavages. Control mice were exposed to ambient air during the first part of the protocol and received only vehicles during the second part. All procedures were carried out in accordance with conventional guidelines for animal experimentation, and the local committee approved the experimental protocol.

2.3. Tissue processing

Sixty-one days after the supplementation with vehicle or vitamins C and E commenced, each mouse was sacrificed by cervical displacement and the right ventricle was perfused with saline to remove blood from the lungs. The left lung was clamped to make BAL fluid from the open right lung (see Section 2.4). Afterward, we clamped the right lung, where BAL was performed, 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 [26,27]. Then, another clamp was made in the left extrapulmonary bronchus and the lungs were removed, weighed and fixed en bloc, to avoid formalin loss, for 48 h before embedding in paraffin. Serial sagittal 5-μm sections were obtained for morphometric and histological analyses. Sections stained with hematoxylin–eosin, orcein and Sirius red were analyzed.

2.4. BAL fluid

The lung air spaces were washed with buffered saline solution (500 μl) three 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 using 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 BAL fluid sample were counted using standard morphological criteria.

After BAL, the right lungs were immediately homogenized at a concentration of 10% (w/v) in PBS (pH 7.3) and then centrifuged at 3000×*g* for 5 min. Supernatants were stored in the freezer for later biochemical analysis.

2.5. CAT and SOD activities

To determine CAT and SOD activities, we used lung homogenates. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm [28]. SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation as absorbance at 480 nm [29]. The total protein content in the right lung samples was determined by the method of Bradford [30].

2.6. TNF-α quantification

TNF-α in the lung homogenates was quantified by ELISA using a rat antimouse monoclonal antibody with a detection limit of 10 pg/ml according to the manufacturer's instructions (R&D Systems, UK). A mouse recombinant TNF-α standard was used in this assay.

2.7. MMP-9 gelatin zymography

Aliquots of lung homogenates (30 μg protein) were subjected to electrophoresis on an 4% acrylamide stacking gel/8% acrylamide separating gel containing 1 mg/ml gelatin in the presence of SDS under nonreducing 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, 5 mM $CaCl_2$ and 2 nM $ZnCl_2$ (pH 8.4). The gels were stained with Coomassie Blue and destained in a solution of 25% ethanol and 10% acetic acid. Gelatinase activity appeared as clear bands against the blue background. The molecular weights of gelatinolytic bands were estimated using a placental sample (30 μg protein). The enzyme was quantified by measuring the intensity of the negative bands densitometrically using Scion Image Software (Scion Co., Frederick, MD, USA). The results are expressed as deviations from the positive control (placenta) [31].

2.8. Morphometry

To assess the extent of lung damage (emphysema), we performed morphometric analysis on hematoxylin–eosin-stained sections. The pulmonary mean linear intercept (MLI), an indicator of air-space size, was calculated for each sample on the basis of 16 random fields observed at a

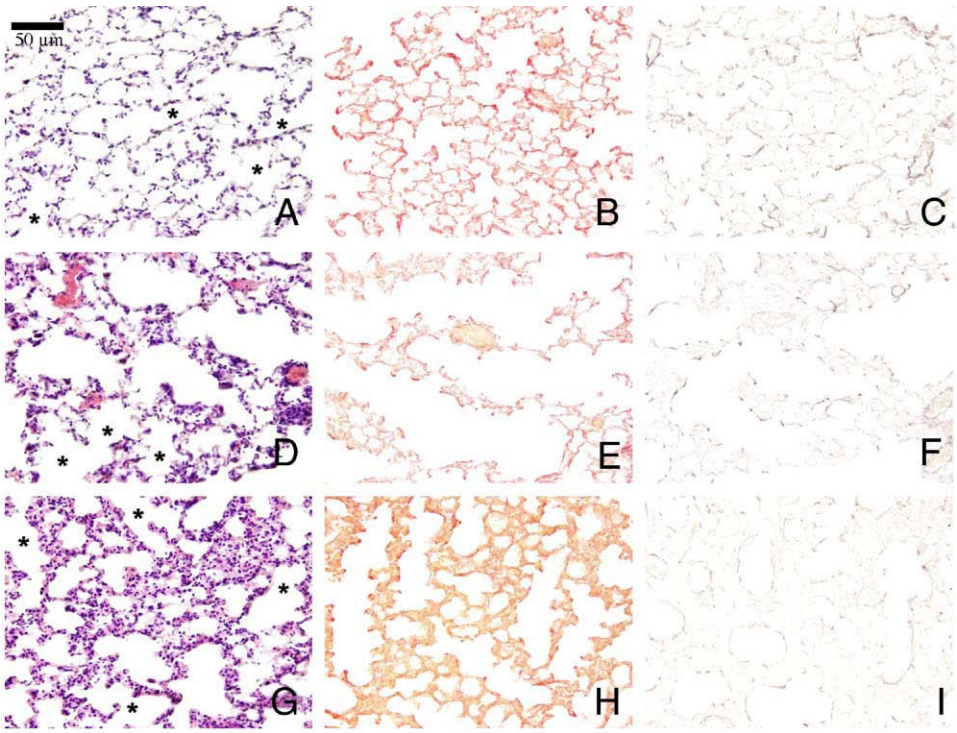

Fig. 1. Lung photomicrographs. (A) The control group lungs were histologically normal, with parenchyma consisting of alveoli connected to alveolar ducts (*), separated from each other only by thin alveolar septa. (B and C) Collagen and elastic fibers in the alveolar septa were characterized by delicate branching fibers. (D) The lungs of all CSv mice showed emphysematous lesions, areas of alveolar septa disruption and enlarged air spaces among alveolar ducts (*). (E and F) Collagen and elastic fibers were fragmented and irregular. (G) The lungs of the CSr mice were histologically similar to the control group, but it was possible to observe some enlarged alveoli, ducts (*) and disrupted and thickened alveolar septa. (H and I) No apparent changes in collagen and elastic fibers were observed, despite some difference in arrangement from the control group.

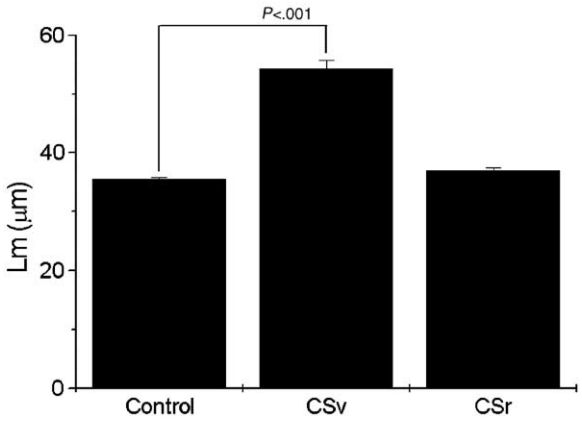

Fig. 2. Air-space sizes in the CSv, CSr and control groups after emphysema induced by long-term CS exposure. There is a 52% increase in Lm for the CSv group compared to the CSr or control groups. Supplementation with vitamins C and E improved lung repair in the CSr group with reduced air-space sizes equivalent to the control group. Data are expressed as means±S.E.M.

total magnification of ×200 using a cross line. The total length of the cross line divided by the number of alveolar walls intersecting the test lines gave the MLI [32].

2.9. Stereology

The volume densities of collagen (Vvcol) or elastic fibers (Vvel) were determined as previously described [25,33]. Briefly, to obtain uniform and proportionate lung samples, we randomly analyzed 18 fields (6 nonoverlapping fields in each of 3 different sections) using a video microscope (Zeiss-Axioplan — 20 objective lens and JVC color video camera linked to a Sony Trinitron color video monitor; Carl Zeiss, Oberkochen, Germany) and a cycloid test system superimposed on the monitor screen. The reference volume was estimated by point counting using the test point systems (PT). The points hitting the collagen or elastic fibers (PP) were counted to estimate the volume densities (Vv) of these structures (Vv=PP/PT). A total area of 1.94 mm^2 was analyzed to determine the Vvcol and Vvel in sections stained with Sirius red and orcein, respectively. Two investigators performed the counting on nonidentified sections.

2.10. Statistical analysis

Data are expressed as means±S.E.M. One-way ANOVA was performed followed by the Student–Newman–Keuls posttest ($P<.05$) to test for differences in BAL cells, CAT, SOD, TNF-α and morphometry among the control, CSv and CSr groups of mice. The normality of the data was verified by the Kolmogorov–Smirnov test and Bartlett's test for all groups prior to ANOVA. Kruskal–Wallis was performed followed by Dunn's posttest ($P<.05$) to examine densitometric differences among the three groups in zymography and stereology. We used the GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA) to perform the statistical analyses.

3. Results

3.1. Vitamins C and E improved lung repair after emphysema

Histological changes are illustrated in Fig. 1. The control group lungs were histologically normal, with parenchyma consisting of alveoli connected to alveolar ducts, separated from each other only by thin alveolar septa (Fig. 1A). Collagen and elastic fibers in the alveolar septa were characterized by delicate branching fibers (Fig. 1B and C).

The lungs of all CSv mice showed emphysematous lesions, areas of alveolar septa disruption and enlarged air spaces (Fig. 1D). Collagen and elastic fibers were fragmented and irregular (Fig. 1E and F). The lungs of the CSr mice had a similar histological pattern to the control group, but some alveoli were enlarged and disrupted alveolar septa with many noninflammatory cells were observed (Fig. 1G). No apparent changes in collagen and elastic fibers were observed, despite some degree of disarrangement in comparison to the control group (Fig. 1H and I).

Data from morphometry and stereology confirmed the histological changes. The MLI (µm) was 35.6±0.2 in the control group, 54.3±1.4 in the CSv group and 36.9±0.6 in the CSr group (Fig. 2). The volume densities (%) of collagen fibers (Vvcol) were 22.8±0.8 in the control group, 11.8±0.4 in the CSv group and 18.8±1.7 in the CSr group (Fig. 3). The volume density (%) of elastic fibers (Vvel) was 15.2±1.3 in the control group, 6.7±0.4 in the CSv group and 8.5±0.5 in the CSr group (Fig. 3). The decrease of Vvcol paralleled the

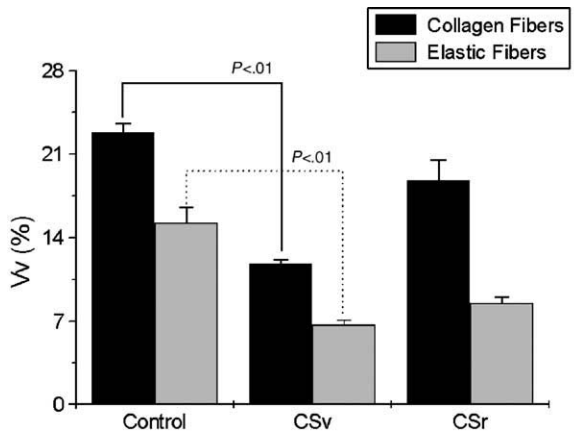

Fig. 3. Volume density of collagen (Vvcol) and elastic fibers (Vvel) in the CSv, CSr and control groups after emphysema induced by long-term CS exposure. There is a 51% decrease in Vvcol and a 44% decrease in Vvel in the CSv group compared to the CSr or control groups. Vitamin supplementation stimulated regeneration of collagen and elastic fibers in the CSr group with values equivalent to the control group. Data are expressed as means±S.E.M.

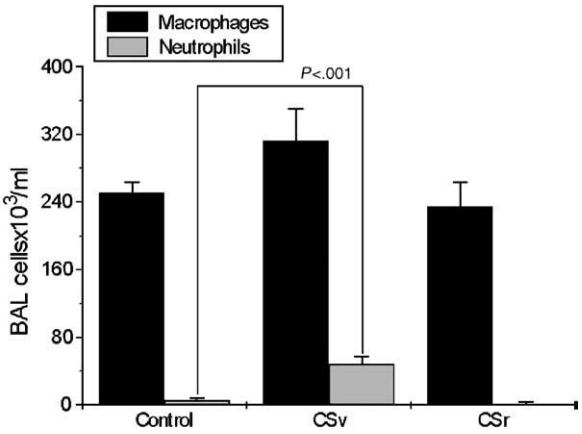

Fig. 4. BAL fluid from the CSv, CSr and control groups after emphysema induced by long-term CS exposure. There were almost 10× increases of neutrophils in BAL fluid from the CSv group compared to the CSr group or the control group. No statistically significant differences were observed among the alveolar macrophage numbers in BAL fluid from the three groups. Supplementation with vitamins C and E reduced neutrophil influx into the CSr mouse lungs. Data are expressed as means±S.E.M.

decrease in Vvel and corresponded inversely to the increase of MLI in the CSv group.

3.2. Vitamins C and E modulated cell influx after emphysema

The alveolar macrophage numbers in BAL fluid (cells×10^3/ml) were 250.9±12.8 in the control group, 312.5±38.7 in the CSv group and 234.4±28.8 in the CSr group. The numbers in the CSv group were higher but not statistically different from those in the CSr and control groups. However, neutrophil numbers were significantly higher in the CSv group (P<.001) than in the CSr and control groups (Fig. 4). The neutrophil numbers in BAL fluid (cells×10^3/ml) were 4.9±1.9 in the control group, 47.8±9.1 in the CSv group and 1.7±1.1 in the CSr group.

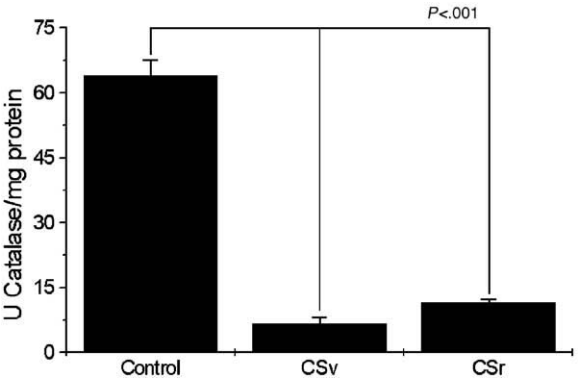

Fig. 5. CAT activity in lung homogenates from the CSv, CSr and control groups after emphysema induced by long-term CS exposure. There is an almost 10× reduction of CAT activity in the CSv and CSr groups compared to the control group. Supplementation with vitamins C and E has no effect on CAT activity. Data are expressed as means±S.E.M.

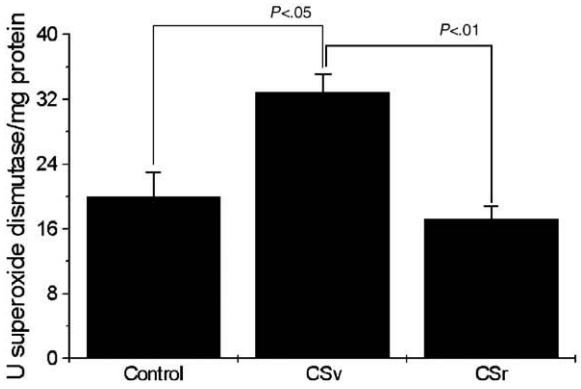

Fig. 6. SOD activity in lung homogenates from the CSv, CSr and control groups after emphysema induced by long-term CS exposure. There is a 64% increase of SOD activity in the CSv group compared to the control group. Supplementation with vitamins C and E reduced SOD activity in the CSr group by 90% compared to the CSv group. No differences were found between the CSr and control groups. Data are expressed as means±S.E.M.

3.3. Vitamins C and E reduced oxidative stress after emphysema

Oxidative stress was analyzed by CAT and SOD activities (Figs. 5 and 6, respectively). CAT activity was lower in the CSv and CSr groups (P<.001) than in the control group: 64.1±3.4 U/mg protein in the control group, 6.7±1.3 U/mg protein in the CSv group and 11.5±0.8 U/mg protein in the CSr group. SOD activity was increased in the CSv group (32.9±2.3 U/mg protein) compared to both the control group (20.0±2.9, P<.05) and the CSr group (17.3±1.5, P<.01).

3.4. Vitamins C and E reduced TNF-α after emphysema

TNF-α was measured in lung samples as an inflammatory marker. The TNF-α content was higher in the CSv group (Fig. 7) than in the CSr group (P<.05) or the control group

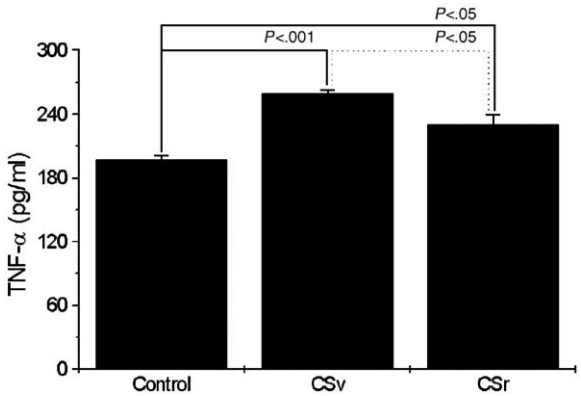

Fig. 7. TNF-α content in lung homogenates from the CSv, CSr and control groups after emphysema induced by long-term CS exposure. There is a 31% increase of TNF-α content in the CSv group compared to the control group. Supplementation with vitamins C and E reduced the TNF-α content in the CSr group by 12% compared to the CSv group. The TNF-α content was increased by 16% in the CSr group compared to the control group. Data are expressed as means±S.E.M.

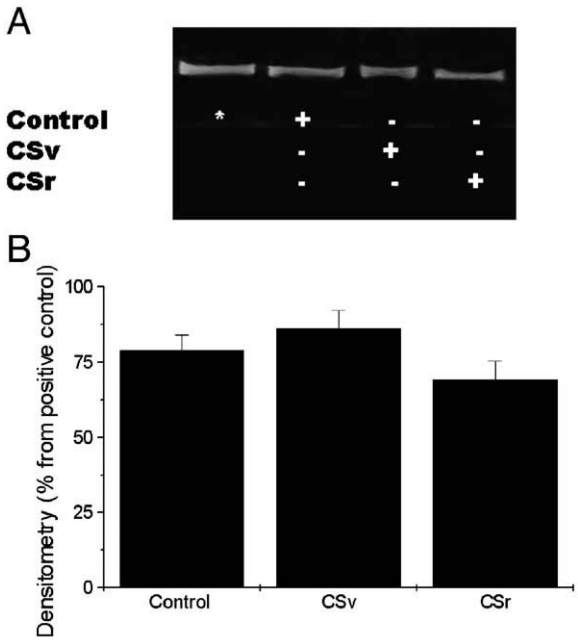

Fig. 8. (A) Gelatin zymography in lung homogenates from the CSv, CSr and control groups after emphysema induced by long-term CS exposure. The first band represented by * is the positive control (placenta). Representative bands of MMP-9 activity are shown. No differences in MMP-9 activity were found among the three groups. (B) Densitometry of gelatin zymography in lung homogenates (complement to Panel A). No MMP-9 activity was evident after emphysema induced by long-term CS exposure. MMP-9 activity in the CSr group was not affected by supplementation with vitamins C and E.

($P<.001$). The TNF-α content was also higher in the CSr group ($P<.05$) than in the control group. Supplementation with vitamins C and E reduced the TNF-α content of the CSr group but not to control level. The TNF-α content (pg/ml) was 197.6±3.1 in the control group, 259.7±3.2 in the CSv group and 230.6±9.3 in the CSr group.

3.5. Gelatin zymography after emphysema

MMP-9 activity was more intense in the CSv group than in the CSr and control groups (nonsignificant difference — Fig. 8A). Densitometry was performed on the negative bands (Fig. 8B).

4. Discussion

The present study reports lung repair after emphysema induced by long-term CS exposure in mice supplemented with vitamins C and E. A marked neutrophilia associated with high TNF-α content and emphysema was observed in mice previously exposed to smoke for 60 days (CSv group), but the inflammatory status was reduced by vitamins C and E with improved lung histology.

In COPD, MMPs play an important role in the destruction of lung tissue [3,11]. MMPs attack and degrade extracellular matrix components such as collagens and elastic fibers [4,25]. MMP-9 is elevated in BAL fluid from subjects with emphysema, suggesting that it may be important in the pathogenesis of the disease [14]. These observations have been supported and extended by Russell et al. [13], who showed that more MMP-9 was secreted by macrophages from COPD patients than from healthy volunteers when stimulated with IL-1β, endotoxin or CS-conditioned medium. We expected to find high MMP-9 activity in the CSv group, but surprisingly, it was not different from the CSr or control groups and it was not associated with the lung inflammatory status indicated by neutrophilia and high TNF-α levels in the CSv group. Alveolar macrophages secrete proteolytic enzymes, including MMP-2 and MMP-9, which contribute to the destruction of lung parenchyma [34]. Macrophages are the likely source of the progressively increased levels of MMP-9 in mouse emphysema [35,36], and MMP-9 is more abundant than MMP-2 in COPD subjects [37]. Thus, the accumulation of macrophages in mouse lung may explain, in part, the very marked increase in MMP-9. Our result, therefore, may be explained by the restoration of mouse alveolar macrophages to control levels when smoking was stopped after 60 days. Further confirmatory studies should be performed using extended observation times and different intervals after cessation of smoking to determine when the lung MMP-9 activity returns to control level.

The data presented here indicate that neutrophils may be related to inflammatory status in ex-smokers. The increased neutrophil number in the CSv group was combined with high TNF-α content. However, supplementation with vitamins C and E reduced alveolar neutrophil numbers, although the TNF-α level remained elevated compared to the control group. These findings raise the possibility that TNF-α is associated with emphysema, as shown by Shapiro et al. [38] and Churg et al. [32]. In addition, TNF-α transgenic mice have increased lung volumes, loss of elastic recoil, destruction of alveolar walls and pulmonary hypertension [39]. Since these changes progress with CS exposure, chronic inflammation induced by TNF-α plays an important role in developing emphysema [32,39]. Our results indicate that a self-perpetuating inflammatory status is present in ex-smoker mice and that vitamin supplementation is more efficient in reducing cell influx to alveoli, probably by reducing the generation of ROS, although the TNF-α level in mouse lung homogenates remains high. Emphysema was confirmed by morphometry and stereology. The MLI, an indicator of air-space destruction, was increased in the CSv group in association with decreased collagen and elastin volume densities. Treatment with vitamins C and E improved the de novo formation of collagen and elastic fibers to some degree. Although elastin fiber destruction and repair are well described in elastase-induced emphysema, collagen and elastin fiber remodeling in chronic CS exposure is poorly characterized. Cells in the alveolar septa of the CSr group may be fibroblasts, myofibroblasts or leukocytes. Our BAL data indicate that the leukocytes in the alveolar septa

did not enter the alveoli. We can speculate that tissue remodeling activity was more intense, as reflected by Vvcol and Vvel. The mechanisms of alveolar septa repair and de novo formation of collagen and elastic fibers were not studied here, but we believe that antioxidant supplementation by vitamins C and E may stimulate fibroblast activation and proliferation [40]. Vitamin C plays an essential role in collagen formation because it acts as a cofactor for prolyl hydroxylase, which catalyzes hydroxyproline synthesis. Hydroxyproline is required for effective production of type I and type III collagens by fibroblasts and endothelial cells; these collagens represent approximately 90% of all the collagen in the lung [41]. Vitamin E may participate in collagen production by regulating genes responsible for tumor growth factor-β and some extracellular matrix proteins [42].

Oxidative stress has been implicated in the pathogenesis of emphysema [16]. Free radicals from inhaled CS and oxidants endogenously formed by inflammatory cells expose the lung to an increased oxidant burden [17]. Despite these observations, studies assessing the benefits of antioxidants for COPD have yielded mixed results. Antioxidants such as vitamins C and E have been shown to decrease smoke-induced oxidative damage in animal [9] and human studies [20]. In addition, numerous epidemiological studies have shown a positive correlation between dietary intake of vitamins C and E and lung function [20]. However, clinical trials evaluating the efficacy of these antioxidants have been less impressive. The formation of H_2O_2 and superoxide is the first event in the development of lung oxidative injury [18]. The major enzymatic antioxidants in the airways are CAT and SOD [17]. SOD is the primary enzyme in defending the lung against the damaging effects of superoxide. It does this by converting superoxide into hydrogen peroxide, which can then be broken down into water by antioxidants such as CAT and glutathione [16]. SOD is a key mediator in the pathophysiological responses that lead to the development of emphysema. We found CAT activity in both ex-smoker groups when compared to the control group. In this context, improvement was independent of vitamin supplementation, in contrast to the SOD activity, which was restored only in the CSr group. The number of neutrophils into the alveoli was only comparable to the control levels in the treated mice. This result confirms that the reduction of oxidative stress in the lung should be considered to decrease the influx of inflammatory cells into the alveoli [22].

Despite the importance and rising prevalence of COPD, little progress has been made toward developing effective drug therapies. One treatment strategy is to suppress the inflammatory and destructive processes that underlie this disease. In this context, we observed that supplementation with vitamins C and E can ameliorate the lung structure in ex-smoker mice. These data suggest that interactions among these individual antioxidants, with beneficial mutual protection and regeneration, may represent a complementary treatment for human emphysema.

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