CHINESE GREEN TEA AMELIORATES LUNG INJURY IN CIGARETTE SMOKE-EXPOSED RATS

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Short title: Green tea on smoke-induced lung injury

Summary

Background: Epigallocatechin-3-gallate (EGCG), which has been shown to have potent antioxidant effect, comprises 80% of catechins in Chinese green tea. This study was to investigate whether cigarette smoke (CS) exposure would induce lung morphological changes and oxidative stress in the CS-exposed rat model, and whether Chinese green tea (Lung Chen tea with EGCG as its main active ingredient) consumption would alter oxidative stress in sera and lung leading to protection of CS-induced lung damage.

Methods: Sprague-Dawley rats were randomly divided into four groups, i.e. sham air (SA), 4% CS, 2% Lung Chen tea plus SA or 4% CS. Exposure to SA or 4% CS was performed for 1h/day for 56 days in ventilated smoking chambers. Sera and lung tissues were collected 24h after last CS exposure for histology and all biochemical assays.

Results: Airspace enlargement and goblet cell hyperplasia were observed after 56-day CS exposure alone, which were abolished in the presence of green tea consumption. Serum 8-isoprostane level was significantly elevated ($p<0.01$) as well as lung superoxide dismutase (SOD) and catalase activities in CS-exposed rats compared to SA-exposed rats ($p<0.05$), which returned to the levels of SA-exposed rats after Chinese green tea consumption.

Conclusion: These results indicate that increased levels of systemic oxidative stress after CS exposure play an important role in the induction of lung damage. Chinese green tea may have the ability to suppress CS-induced oxidative stress that leads to protection of lung injury.

Keywords: Catalase, Chinese green tea (Lung Chen), cigarette smoke, airspace enlargement, goblet cell, superoxide dismutase

Introduction

Chronic Obstructive Pulmonary Disease (COPD), the fourth leading cause of death worldwide (1), is characterized by airflow obstruction that is usually progressive, and not fully reversible. The chronic airflow limitation is caused by a mixture of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema) (2). According to the classification of the Global Initiative for Chronic Obstructive Lung Disease (GOLD), there are four stages reflecting different severity of the disease (2). The pathophysiological mechanism for COPD is not completely understood but there are two generally accepted mechanisms: oxidant/anti-oxidant and protease/anti-protease imbalances (3). Smoking is the major cause of COPD as cigarette smoke contains highly concentrated free radicals and other oxidants, which is estimated to have around 10^{16} and 10^{17} oxidant molecules in one puff (4). These oxidants will trigger infiltration of various cell types, for instance, neutrophils and macrophages, which release more oxidants and inflammatory cytokines, leading to an oxidant-inflammation vicious circle (5,6). Oxidative stress has also been implicated in direct damage to epithelial cells, inactivation of anti-proteases, production of pro-inflammatory cytokines and lipid peroxidation (7,8). 8-Isoprostane is a product of free radical catalyzed lipid peroxidation and is used as an oxidative stress marker in cigarette smokers and

COPD patients (9).

The major antioxidant defense system in the body mainly includes enzymatic superoxide dismutase (SOD) and catalase (10). SOD converts superoxide anion ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2), which is then converted to H_2O by catalase. Catalase, the main enzyme scavenging H_2O_2, decomposes H_2O_2 to water and acts in concert with SOD.

Chronic cigarette smoke exposure leads to emphysema in various animal models (11,12). Recent studies have demonstrated that oxidative stress and inflammation can cause emphysema (13,14). Goblet cells are the mucin-producing cells in the respiratory system and normally present in epithelium of cartilaginous bronchus in small number. Goblet cell hyperplasia is induced by chronic smoking and is the major reason for mucus hypersecretion (15,16).

Tea is one of the most popular beverages consumed in the world and green tea accounts for 20% of the total amount. In the past decade, researchers have focused on the health-promoting and beneficial effects of green tea on various diseases. For instance, epidemiologic studies have shown that green tea consumption could protect against coronary heart disease, atherosclerosis and cancers (17-19). Chinese green tea has also been proven to be a powerful antioxidant *in vitro* (20). Epigallocatechin gallate (EGCG), one type of catechins, accounts for more than

80% of all active ingredients in green tea and has been shown to have the greatest anti-oxidant activity among several compounds (21). Consequently, we aim to study the effect of Chinese green tea (Lung Chen) on lung morphological changes, marker of systemic oxidative stress, and various antioxidant enzyme activities in lung.

Methods

Green tea preparation

10% Lung Chen tea (龍井茶) was freshly prepared everyday by brewing 60g dried Lung Chen tealeaves in 600ml hot water (not boiling) for 30 minutes. After cooling, either tea solution (5ml) or tap water (5ml) was given by oral gavage twice daily (a total volume of 10ml) one hour before and after sham air (SA) or cigarette smoke (CS) exposure. Based upon HPLC analysis, the content of EGCG in 10 ml of 10% Lung Chen tea was 36mg, which was equivalent to the amount of drinking freely 2% Lung Chen tea daily in Sprague-Dawley rats (unpublished data).

Smoking rat model

Forty Sprague-Dawley (S-D) rats (150-200g) were purchased from the Laboratory Animal Unit (LAU) of the University of Hong Kong. The S-D rats were divided into four groups, with 10 rats in each group. CS and Tea/CS groups were exposed 4% CS for one hour daily with commercially available cigarette (Camel; filter, R.J. Reynolds, Winston-Salem, NC, USA) for 56 days using the modified ventilated smoking exposure chambers as previously described by Chow *et al* (22). Briefly, a ventilated 20-litres chamber containing 5 rats was filled with fixed concentration of smoke, which was kept constant at 4% (vol/vol, smoker/air) by

using a peristaltic pump (Masterflex; Cole-Parmer Instrument Co, Niles, IL, USA) to deliver fresh cigarette smoke from burning cigarettes at a constant rate (40 ml/min), while another pump was used to deliver fresh air from outside simultaneously at a constant rate (960 ml/min) to mix. SA and Tea/SA groups were subjected to the same procedures in another ventilated chamber but exposed only fresh air (0%, vol/vol, smoke/air) simultaneously. Rats were exposed to 12 cigarettes in an hour daily. This CS model was proven to neither disturb the normal physiological functions of the animals, such as acid/base balance and O_2/CO_2 in the blood, heart rate and blood pressure, nor to impose any stress on the animals (22). Since cotinine is the major metabolite of nicotine (23), serum cotinine was measured as a marker of cigarette smoking at various time intervals. Rat body weight was measured biweekly until the day of sacrifice. After 56-day exposure, the rats were sacrificed by overdose of pentobarbitone and blood taken by cardiac puncture. Lungs were excised from the animals for biochemical measurements including superoxide dismutase (SOD) and catalase activities in rat lung homogenates and serum was separated from whole blood for measurement of cotinine and 8-isoprostane. This protocol was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) at the University of Hong Kong.

Histology and morphometric analysis

The excised largest lobe of left lung was inflated with 1 ml formalin under constant pressure before fixing with formalin, then dehydrated with ethanol, and embedded in paraffin. The lung lobe was cut at 5 μm. Sections were stained with hematoxylin and eosin for histologic examination. Airspace enlargement was measured by mean linear intercept (Lm) using a modified method (24). Images of 10 fields for each lung section were captured randomly at x40 magnification. Each image was then analyzed using AxioVision (Ziess, Germany). The number of alveolar intercepts was counted to obtain Lm. Furthermore, Alcian blue (AB)-periodic acid-Schiff (PAS) staining was used to examine goblet cell hyperplasia. Images of 3 fields for epithelium in cartilaginous bronchus were captured randomly at x100 magnification and the AB-PAS-positive areas and the total epithelial areas were measured using the Leica QWIN Imager Analyzer (Cambridge, UK). Results were expressed as the ratio of AB-PAS-positive areas to total epithelial areas.

Protein Extraction

Frozen lung tissues were grinded up with a mortar and pestle in liquid nitrogen. The tissue samples were then homogenized with T-PER$^{@}$ tissue protein

extraction reagent (PIERCE, IL, USA) in the presence of protease inhibitors (Calbiochem, NJ, USA) following the manufacturer's instruction. Protein concentration was assayed by Bradford method using bovine serum albumin as standard.

Quantitations of cotinine, and 8-isoprostane

Serum levels of cotinine (OraSure Technologies Inc, PA, USA), and 8-isoprostane (Oxis Research, Texas, USA) were measured using commercially available kits following the manufacturer's instructions. All measurements were performed in duplicate and in adjacent wells to minimize variability.

Superoxide dismutase activity assay

SOD activity in lung homogenates was determined from the rate of reduction of cytochrome C (25), as one unit (U) of SOD activity was defined as the amount of SOD required to inhibit the rate of cytochrome c reduction by 50%. Xanthine oxidase (Sigma, St. Louis, MO) was added at a sufficient concentration to induce a 0.020 change in absorbance per minute at 550 nm. The SOD activity was expressed as U per mg protein.

Catalase activity assay

The measurement of catalase activity in lung homogenates was based on the reaction with hydrogen peroxide as previously described (26). Briefly, the initial rate of disappearance of H_2O_2 (0 to 60s) was recorded spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was defined as the rate constant of the first-order reaction. The catalase activity is expressed as units (U) per mg protein.

Statistical analysis

Results are expressed as means ± SEM. Between-group comparisons were performed by using student's *t* tests or analysis of variance with Bonferroni's method as post hoc analysis where appropriate; within-group comparisons were performed by using repeated-measures analysis of variance with Bonferroni's method as post hoc analysis. All statistical analyses were performed using computer software (Prism 3.0, GraphPad, SanDiego, CA). A value of $p<0.05$ was regarded as statistically significant.

Results

Rat body weight

In this study, one animal from each group except SA group died accidentally during the experimental period. At baseline, the body weight of all rats was similar between different groups. Within-group comparisons, the body weight showed significant increases over times ($p < 0.001$). Between-group comparisons at week 2, green tea consumption alone (Tea/SA group) or in combination with CS (Tea/CS group) showed significant lower body-weight gain than SA group at all time intervals ($p < 0.01$). At week 8, CS group showed a lower weight gain than SA group, but not reaching statistical significance (478 ± 19 g vs. 539 ± 18 g for CS and SA group respectively, $p > 0.05$) (Figure 1).

Serum Cotinine level

In Table 1, the serum cotinine levels were undetectable in SA and Tea/SA groups at all time points. However, serum cotinine levels were elevated in CS and Tea/CS groups at various time points. There were no significant differences in serum cotinine levels within CS and Tea/CS groups over time. At day 56, Tea/CS group had lower serum cotinine level than CS group ($p < 0.01$).

Histological examination and Lm measurement

In Figure 2 (panels A-D), it was found that cigarette smoke exposure after 56 days obviously produced alveolar space enlargement and green tea abolished such damage. In Figure 3E, the Lm of CS group was significantly higher than that of SA group (31.28 ± 1.75 μm vs. 19.79 ± 0.81 μm for CS and SA groups respectively, $p<0.001$). Tea/CS group showed a reduced Lm (19.01 ± 0.56 μm, $p<0.001$) compared to CS group. Green tea itself had no such effect.

Quantification of Goblet cell hyperplasia

In Figure 3 (panels A-D), there was a significant increase in the number of goblet cells in the surface epithelium of the cartilaginous bronchus in CS group compared to SA group (percentage +ve area = 0.69 ± 0.24 vs. 0.12 ± 0.03 for CS and SA groups respectively, $p<0.01$; Figure 4E). The CS-induced increase was abolished by green tea administration (percentage +ve area = 0.21 ± 0.08, $p<0.05$).

Serum 8-isoprostane level

There was a gradual increase in the elevation of serum 8-isoprostane levels of CS-exposed rats over time. At day 7, serum 8-isoprostane levels did not reach statistical significance but it rose to more than 2 folds at day 28 in comparison to

SA-exposed rats (2.13 ± 0.37 vs. 1.00 ± 0.15 for CS and SA groups respectively, $p < 0.01$) (Figure 4A). At day 56, serum 8-isoprostane levels of CS-exposed rats elevated more than 3 folds compared to SA-exposed rats (3.91 ± 0.99 vs. 1.00 ± 0.21 for CS and SA groups respectively, $p < 0.01$). After green tea consumption in combination with CS exposure, the serum levels returned to the levels of SA-exposed rats ($p < 0.01$). Green tea consumption alone had no effect on serum 8-isoprostane levels (Figure 4B).

SOD activity

In CS-exposed rats, there were significant elevations in lung SOD activities compared to SA-exposed rats (19.0 ± 2.8 U/mg protein vs. 37.5 ± 8.1 U/mg protein for SA and CS groups respectively, $p < 0.05$). After green tea administration in combination with CS exposure, the SOD activities returned to the levels of SA-exposed rats ($p<0.05$). Consumption of green tea alone had no effect on SOD activity (Figure 5A).

Catalase activity

In Figure 5B, lung catalase activities markedly elevated after CS exposure in comparison to SA exposure (261.5 ± 49.3 U/mg protein vs. 124.5 ± 24.9 U/mg

protein for CS and SA groups respectively, $p<0.05$). Green tea consumption reduced CS-induced catalase activities significantly (145.5 ± 18.6 U/mg protein, $p<0.05$), but without effect on the catalase activities of SA-exposed rats.

Discussion

This study was undertaken to evaluate the contribution of CS-induced oxidative stress in producing lung injury. The results demonstrated that cigarette smoking caused airspace enlargement shown by an increase in quantitative Lm, goblet cell hyperplasia by an increase in percentage of AB-PAS +ve staining areas, systemic and local oxidative stress by the elevations in serum 8-isoprostane levels and activities of lung antioxidant enzymes (SOD and catalase). Administration of Chinese green tea followed by CS exposure ameliorated lung injury, which was associated with the decreased serum 8-isoprostane and lung antioxidant enzyme activities, suggesting that oxidative stress might be responsible in part for producing organ injury after CS exposure. As a major metabolite of nicotine (23), cotinine had been used as a marker of cigarette smoke exposure. There were no significant differences in serum cotinine levels within CS and Tea/CS groups over time. At day 56, the serum cotinine level of Tea/CS group was significantly lower than CS group despite identical smoking exposure protocol, suggesting that green tea might have a direct effect on nicotine metabolism after long-term administration.

The lower weight gain in CS group compared to SA group was observed that might be resulted from increased metabolism and loss of appetite after cigarette smoke inhalation (27). Green tea itself also showed body weight-suppressive effect,

possibly due to increased energy consumption, fat oxidation and reduced food intake (28,29). Nonetheless, green tea consumption and cigarette smoking did not show any synergistic effect on weight gain over time in this study. Though a 20% less of weight gain after green tea consumption was quite large in our model, the actual body weight-suppressive effect in human was rather small (30,31), which might reflect on the different dosages of green tea consumption.

In our study, airspace enlargement with a 58% increase in Lm was observed after 56-day CS exposure, in agreement with Lee *et al* (32) who also found that exposure of Sprague-Dawley rats to cigarette smoke caused a 74% increase in Lm after longer exposure. In contrast, Steveson and colleagues (16) did not show airspace enlargement until 8 months after CS exposure. The difference is likely due to the age of rat for the time of CS exposure. Our rats exposed to cigarette smoke were weighed about 150-200 g (~ 6 to 7-week old), similar to Lee *et al* (32). Stevenson *et al* (16) exposed their rats weighing 350-400 g (~ 10 to 11-week old). It appears that age at exposure is an important stipulation for consideration of long-term effects of smoking. It is well known in human studies that young children are much more susceptible to the effects of exposure to environmental tobacco smoke (ETS) compared to adults. Although the effects of ETS exposure in adults are hard to demonstrate, maternal smoking during pregnancy has been consistently

associated with poor lung function in both infants (33) and older children (34). Environmental tobacco smoke-induced early lung damage has also been found in healthy male adolescents (35). Our finding on lung damage from cigarette smoke exposure in younger rats corroborates with observations in humans. Smoking effects may diminish progressively with age in rats. Although the factors that affect lung maturity are not well understood, lung morphogenesis is a highly regulated process that could be impaired by both genetic and environmental factors. Our findings are important as our young rat model might provide a means of studying the mechanisms that control normal lung development and strategies for prevention of chronic obstructive pulmonary disease (COPD) in adult life. The other difference is likely to be the protocol for cigarette smoke exposure, which determines the amount of cigarettes taken, i.e. 12 cigarettes per day in this study. We also observed globlet cell hyperplasia in the epithelium of cartilaginous bronchi in CS-exposed rats, which might account for mucus hypersecretion in COPD patients (36).

8-Isoprostane is produced during free radical-induced lipid peroxidation and viewed as an important systemic oxidative stress marker *in vivo*. From our data, the serum level of 8-isoprostane from CS group showed a gradual increase with cigarette smoke exposure time. The greatest fold increase occurred at day 56 (3-fold of the SA group), which is similar to previous human findings (9,37). Exposure to

CS also increased both SOD and catalase activities in lung homogenates, which might reflect an antioxidant defense mechanism due to the surge of inhaled oxidants from cigarette smoke and oxidants released by epithelial cells and/or inflammatory cells such as alveolar macrophages. The activities of these enzymes were being boosted up to clear up excess amount of oxidants locally but this was insufficient to protect the lungs from injury due to oxidative stress. In agreement, MuCusker and co-workers (38) found that SOD and catalase activities were increased in human alveolar macrophages in chronic smokers. Others also demonstrated SOD and catalase were significantly upregulated both *in vivo* rat model and in smokers (1,39-41). In this model, Chinese green tea (Lung Chen) probably arrests multiple harmful mechanisms of lung injury through protection of cells and tissues from oxidative damage by scavenging oxygen-free radicals generated from cigarette smoke exposure, in agreement with previous reports (42,43). Consequently, we observed a protection of lung morphology after 56-day CS exposure.

The precise mechanisms of the protective role of green tea against CS-induced lung injury are currently unclear. Lung Chen tea contains the largest amount of EGCG when compared with other Chinese teas (31) and EGCG has the highest antioxidant capacity among different catechins and dietary compounds such as vitamin C, E and black tea (21). We found no changes in lung SOD and catalase

activities after green tea administration alone, in line with previously published report (44) that found no effects on hepatic SOD and catalase activities in rats given 2.5% aqueous green tea extract. In contrast, EGCG has been found to elevate hepatic SOD and catalase activities in mice (45).

There are potential limitations in our study. Firstly, due to high cost of pure EGCG powder, brewed green tea was used instead, which may contain other chemicals with unknown effects. Secondly, we did not measure the plasma EGCG levels in our rats. However, Nagaya and co-workers showed that the intake of green tea could increase plasma EGCG concentration 2 hours after a single consumption (46). Thirdly, the green tea regimen in our study was initiated on the same day before the CS exposure, so that its effects might largely be preventive. It is difficult to discern in an *in vivo* study the cause and effect relationship between the beneficial effects of green tea on lung structural damage.

In conclusion, our data demonstrate that increased levels of systemic oxidative stress after CS exposure may play an important role in the induction of lung damage. Chinese green tea (Lung Chen) has a protective effect on CS-induced airspace enlargement, goblet cell hyperplasia as well as a suppressive effect on systemic and local oxidative stress. The detailed mechanisms by which the individual or the combination of the purified major green tea polyphenols acts to

prevent CS-induced lung injury still remain to be determined. Therefore, Chinese Green tea may have the ability to slow down the CS-induced disease progression through prevention of airspace enlargement and goblet cell hyperplasia after suppression of oxidative stress.

Competing interests

None of the authors have a conflict of interest to declare in relation to the contents of this paper.

Acknowledgements

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Figure Legends

Figure 1. The body weight curves of different groups during the experimental period. ◆ represents water/sham air (SA group); ● represents water/cigarette smoke (CS group); ▲ represents tea/sham air (Tea/SA group) and ■ represents tea/cigarette smoke (Tea/CS group). $^{*}p < 0.01$ represents the statistical differences between Tea/SA and SA group at week 2, 4, 6 and 8. $^{\#}p < 0.01$ represents the statistical differences between Tea/CS and SA group at week 2, 4, 6 and 8. Means ± SEM are shown.

Figure 2. Representative photomicrographs of lung sections stained with haematoxylin and eosin. Panel A, Water/sham air (SA group); Panel B, Water/cigarette smoke (CS group); Panel C, Tea/sham air (Tea/SA group); Panel D, Tea/cigarette smoke (Tea/CS group). Airspace enlargement was obvious after CS exposure (panel B), which was prevented in the presence of green tea administration (panel D). Panel E shows mean linear intercept (μm) of different groups. $^{***}p < 0.001$ represents the statistical difference between SA and CS group. $^{\#\#\#}p < 0.001$ represents the statistical difference between CS and Tea/ CS group. Means ± SEM are shown. Scale bar = 50 μm.

Figure 3. Representative photomicrographs of lung sections stained with alcian blue/periodic acid-Schiff (AB/PAS) showing the cartilaginous bronchus. Panel A, Water/sham air (SA group); Panel B, Water/cigarette smoke (CS group); Panel C, Tea/sham air (Tea/SA group); Panel D, Tea/cigarette smoke (Tea/CS group). Goblet cells appear as purple staining (arrows) over epithelium. Panel E shows quantification of positive AB/PAS staining for goblet cells of different groups. $^{**}p <$ 0.01 represents the statistical difference between SA and CS group. $^{\#}p < 0.05$ represents the statistical difference between CS and Tea/CS group. Means ± SEM are shown. Scale bar = 50μm.

Figure 4. Rat serum 8-isoprostane levels (fold increase relative to sham air group) at different time points (panel A) and in different treatment groups at day 56 (panel B). A: $^{**}p < 0.01$ represents the statistical differences between water/cigarette smoke (CS) and water/sham-air (SA) group at day 28 and 56. B: $^{**}p < 0.01$ represents the statistical difference between CS and SA group at day 56. $^{\#\#}p < 0.01$ represents the statistical difference between CS and Tea/CS group at day 56. Means ± SEM are shown.

Figure 5. Lung superoxide dismutase (SOD) activity (U/mg protein; panel A) and

catalase activity (U/mg protein; panel B) of different groups. *p< 0.05 represents the statistical difference between water/sham-air (SA) group and water/cigarette smoke (CS) group. $^{\#}p$ < 0.05 represents the statistical difference between CS and Tea/CS group. Means ± SEM are shown.

Table 1. Rat serum cotinine levels (ng/ml) at different time points

Group	Day 7	Day 28	Day 56
CS	8.75 (8.23-9.30)	6.83 (4.99-9.16)	7.19 (6.04-8.76)
Tea/CS	9.78 (8.86-10.90)	7.97 (6.34-10.14)	4.97 (3.76-6.53)**

Data are expressed as geometric mean (95% CI).

** $p < 0.01$ represents the statistical difference between Tea/CS and CS group at day 56.

Figure 1

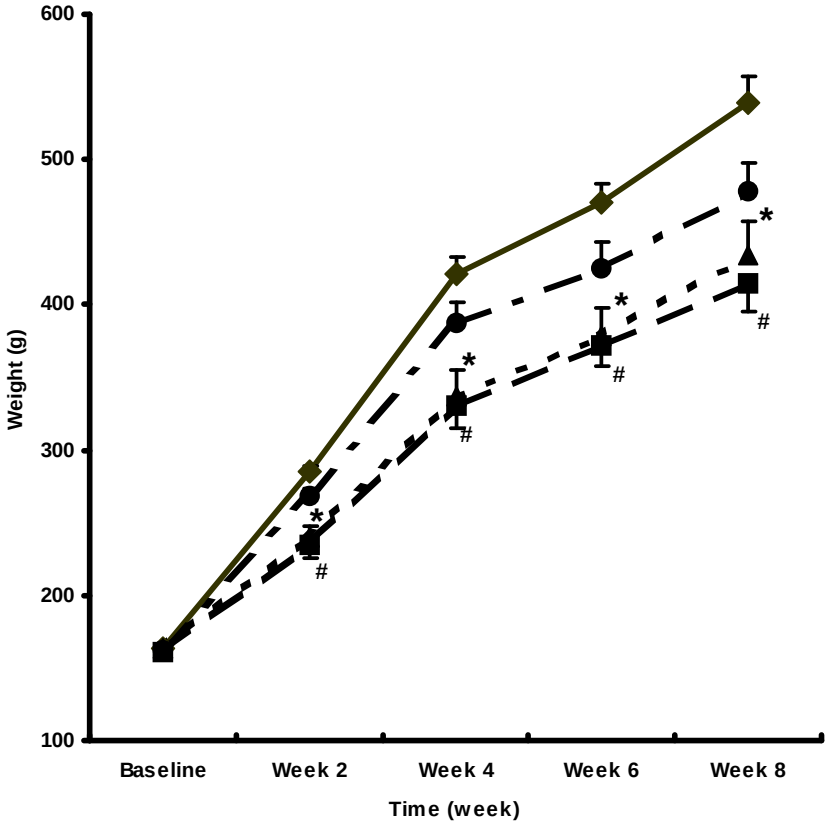
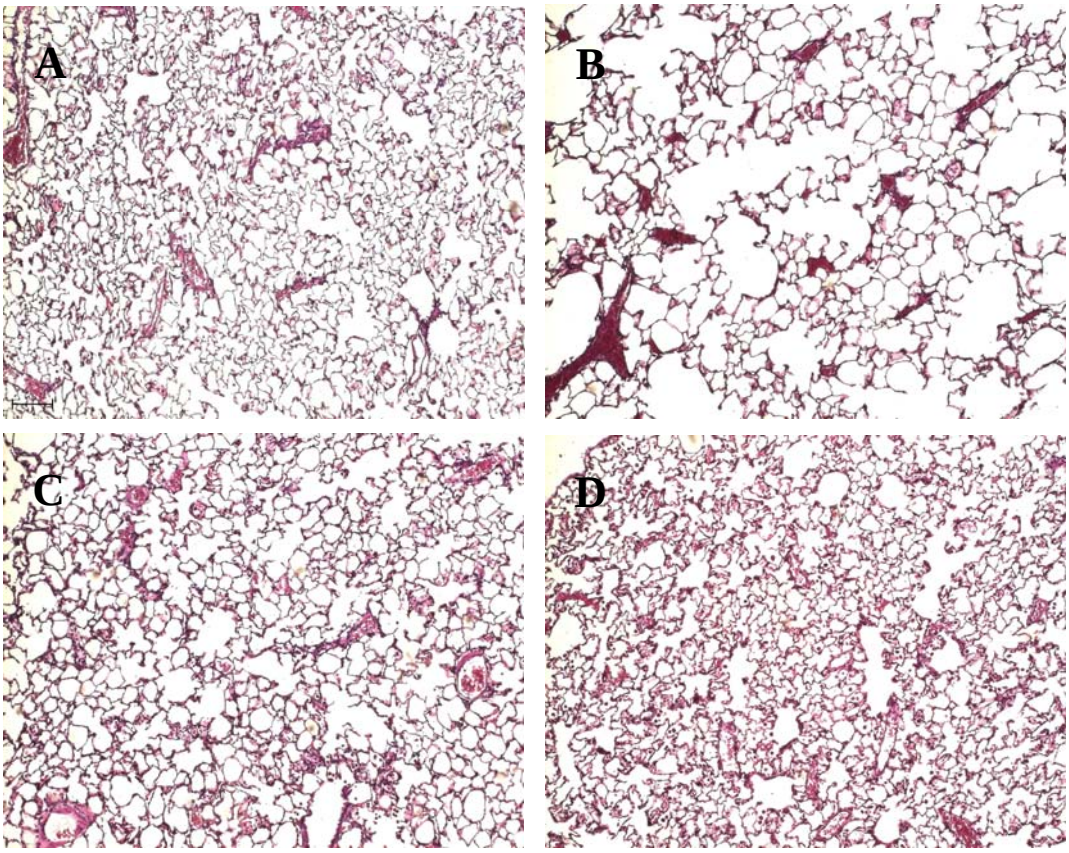

600
500
400
300
200
100
Weight (g)
Baseline
Week 2
Week 4
Week 6
Week 8
Time (week)
*
*
*
*
#
#
#
#

Figure 2



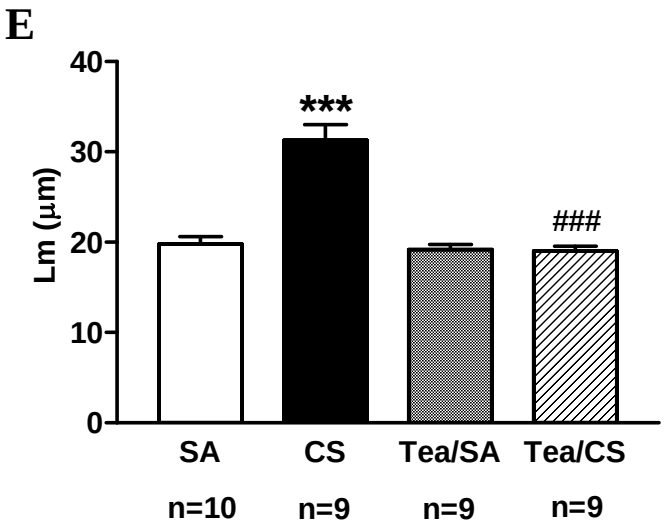
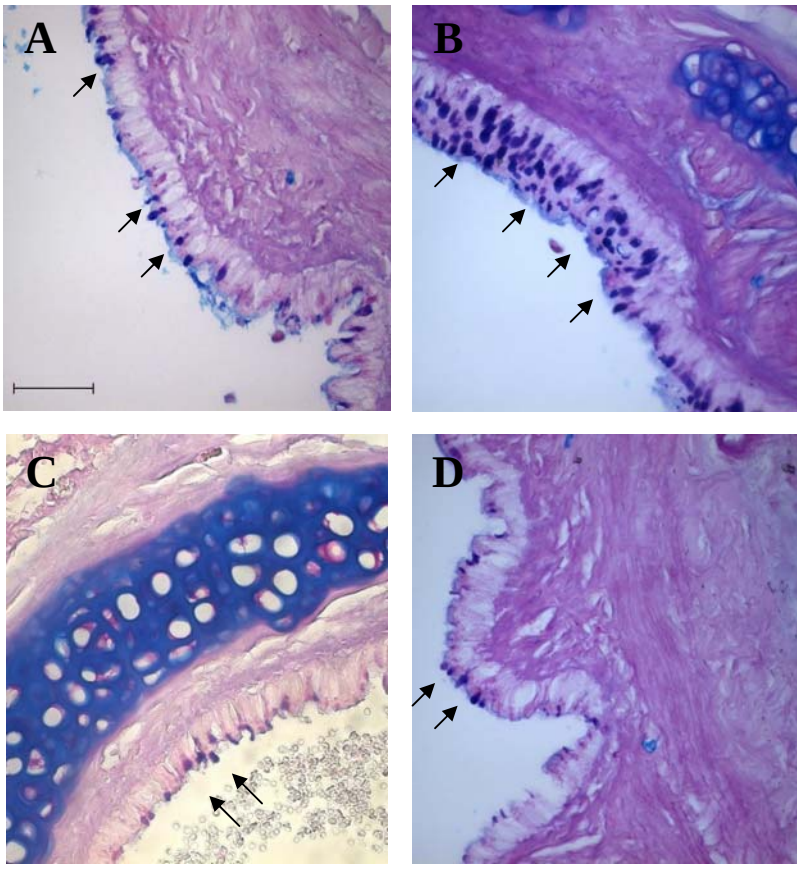


Figure 3


A
B
C
D

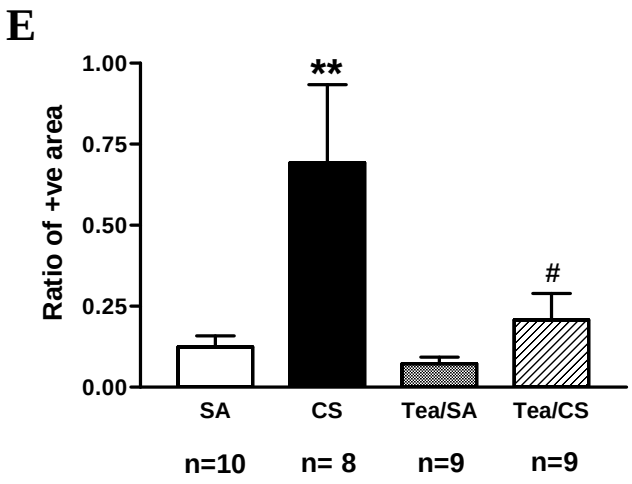

E
Ratio of +ve area
1.00
0.75
0.50
0.25
0.00
**
#
SA
CS
Tea/SA
Tea/CS
n=10
n= 8
n=9
n=9

Figure 4

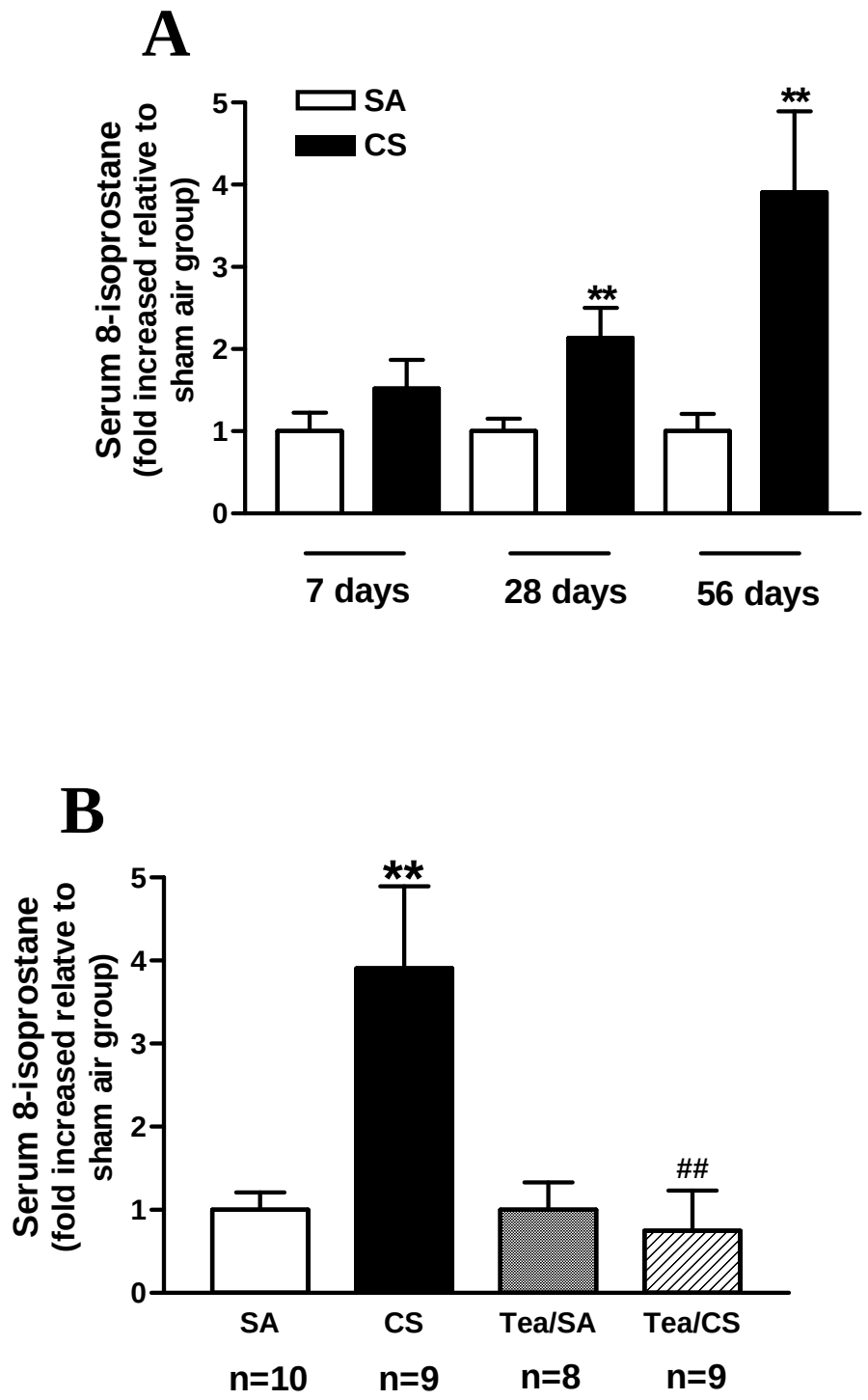
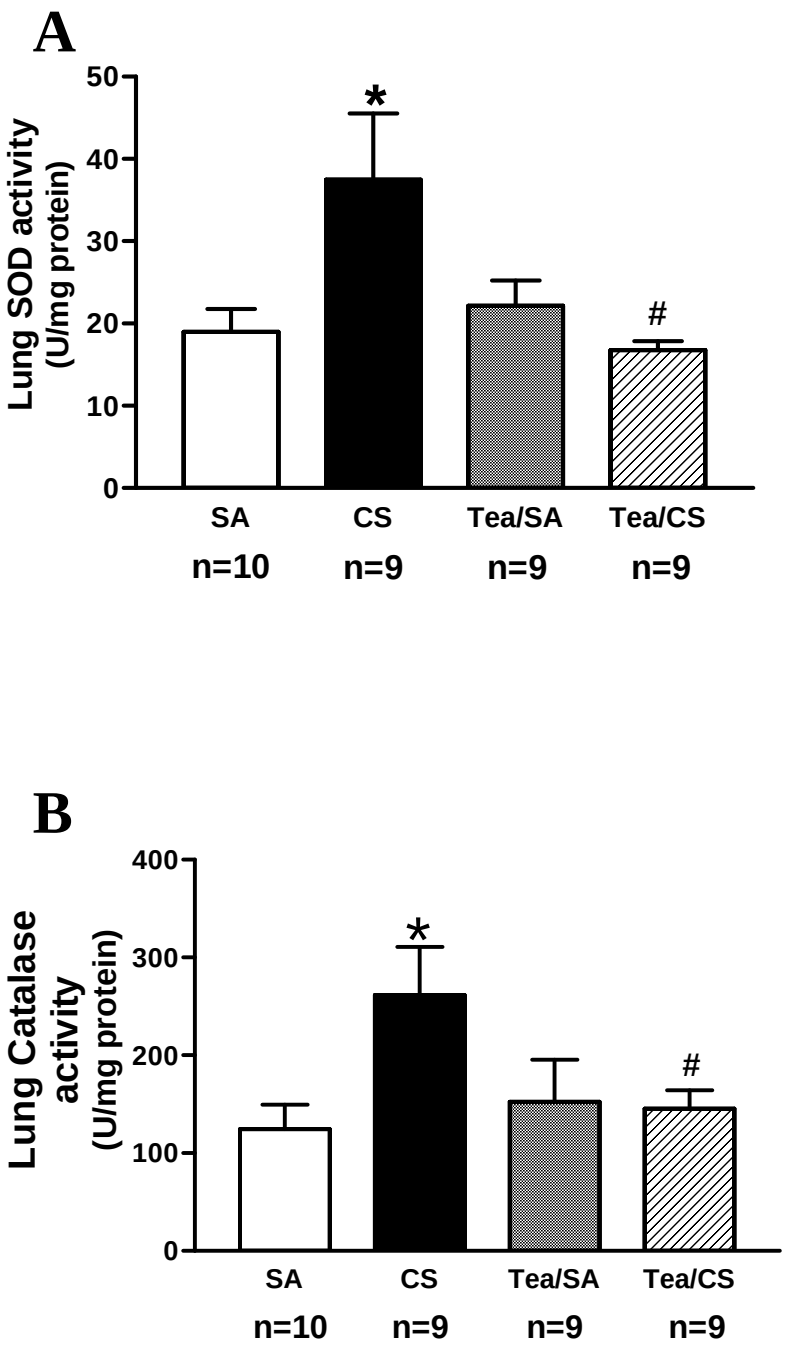

A
SA
CS
Serum 8-isoprostane
(fold increased relative to
sham air group)
0
1
2
3
4
5
**
**
7 days
28 days
56 days
B
Serum 8-isoprostane
(fold increased relatve to
sham air group)
0
1
2
3
4
5
**
##
SA
CS
Tea/SA
Tea/CS
n=10
n=9
n=8
n=9

Figure 5


A
Lung SOD activity (U/mg protein)
50
40
30
20
10
0
*
#
SA
CS
Tea/SA
Tea/CS
n=10
n=9
n=9
n=9
B
Lung Catalase activity (U/mg protein)
400
300
200
100
0
*
#
SA
CS
Tea/SA
Tea/CS
n=10
n=9
n=9
n=9