# Supplementation of French Maritime Pine Bark Extract (Pycnogenol®) Prevents Lung Injury and Lipid Peroxidation in Nude Mice Exposed to Side-Stream Cigarette Smoke (SSCS)

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## Abstract

Side-stream cigarette smoke (SSCS) is a major component of environmental tobacco smoke. The purpose of this study was to investigate the development of lung injury and lipid peroxidation in the lung and liver of immunodeficient (Nude) mice exposed to acute SSCS (a total 5 hours of exposure). The effects of French maritime bark extract (Pycnogenol®) supplementation of the mice were also determined. SSCS increased pulmonary resistance and lipid peroxidation in these mice. Pycnogenol® supplementation increased vitamin E levels in lung and liver. In addition, Pycnogenol® attenuated SSCS-mediated lung injury and lipid peroxidation. It appears that the enhanced resistance against SSCS-induced lung injury and lipid peroxidation may be primarily due to the antioxidant property of Pycnogenol® in supplemented mice.

Key words: French maritime bark extract (Pycnogenol®), side-stream cigarette smoke (SSCS), lung function, nude mice

## INTRODUCTION

During the last decades, tobacco smoke has become regarded as a significant contributing factor in the etiology of respiratory and cardiovascular disorders. While the risks associated with smoking have been fairly well documented, the risks and effects of constant exposure to side-stream cigarette smoke (SSCS), which has recently come to the forefront of the social debate pitting smokers against nonsmokers, are not well known or accepted. Ironically, SSCS without the proper filtration system may be more toxic than main stream smoke at lower combustion temperature due to differences in chemical composition (1). It has been represented that exposure to short-term SSCS resulted in an increase in oxidative stress that was indicated by an alteration of antioxidant enzymes in the heart, liver, and lung tissues. It has been documented that more than 200 semivolatile phenols and chemical compounds with SSCS are capable of generating reactive oxygen species (ROS) during metabolism. The oxidative stress due to ROS is believed to play a role in carcinogenesis by oxidative damage to DNA and depletion of antioxidants in the tissues by inducing

lipid peroxidation. Numerous studies have shown that susceptibility to lipid peroxidation is greatly influenced by tissue concentrations of antioxidants (2). Depletion of vitamin E and other antioxidants by increased free radical production due to smoke exposure may facilitate lipid peroxidation in the lung.

Pycnogenol®, a standardized extract from the French maritime pine bark (Pinus pinaster Ait.), consists of a concentrate of polyphenols whose main constituents are procyanidins, pharmacologically active biopolymers composed of catechin and epicatechin units. In addition, Pycnogenol® contains the bioflavonoids of catechin and taxifolin and a number of phenolic acids (3). Pycnogenol® is know to exhibit efficient antioxidant activity (4,5) and it may act as a modulator of metabolic and antioxidant enzymes (4) and other cellular functions. Several studies have demonstrated that Pycnogenol® inhibits LDL oxidation, lipid peroxidation in phospholipid liposomes, lipid peroxidation caused by *t*-butylhydroperoxide, and inflammation in capillary tissues (5,6). SSCS contains powerful oxidants and toxins that may stimulate macrophages to produce oxidants by responding to foreign antigens in SSCS; T cell-activated macrophages may also secrete

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large quantities of oxidants and free radicals that deteriorate the function of lung tissue. Although it is true that most studies have focused on the antihypertensive effect of Pycnogenol$^{®}$, there is also the possibility that oxidative damage of SSCS in lung tissue might be prevented by removing ROS. Since the nude mice lack a thymus, and therefore functional T cells, they produce less oxidants and consequently have less oxidative damage than conventional mice with fully functional immune systems. We therefore have begun to develop them as a model to study the direct SSCS damage without accentuation by T lymphocytes, using lipid peroxides as indicators of oxidative damage.

This study investigated the development of lung injury and lipid peroxidation in the lung and liver of nude mice briefly exposed to SSCS, and the effects of Pycnogenol$^{®}$ supplementation on preventing smoking-induced lung damage and tissue lipid peroxidation in the absence of T cells were examined.

## MATERIALS AND METHODS

### Animals and treatment

Young female C57BL/6 nude mice (nu/nu), weighing about 30 g, were exposed to SSCS with or without Pycnogenol$^{®}$ supplementation for 10 days. They were housed in transparent plastic cages with stainless steel wire lids (4 mice per cage) as required by the University of Arizona Animal Care and Use Committee. The housing facility was maintained at 20°C and 60~80% relative humidity, with a 12 h light:dark cycle. Diet and water were freely available. Body weight, food, and water were measured every 3 days. After one week of housing and consuming the control diet, the mice were randomly assigned to one of the 4 following treatments with eight mice per a group: nonsmoking nude mice given a non-supplemented control diet; nonsmoking nude mice given a Pycnogenol$^{®}$-supplemented diet (120 mg per kg); SSCS-exposed nude mice given control diet; SSCS-exposed nude mice given Pycnogenol$^{®}$-supplemented diet. The nutritional treatment period was 10 days for all groups.

### Side-stream cigarette smoke (SSCS) exposure

The mice were exposed to SSCS for 30 min/day, 5 days/week for 10 days utilizing an IN-TOX (Albuquerque, NM) vacuum-drawn (15 L/min) exposure system modified for cigarette smoke exposure. The total mass concentration of SSCS particulate matter delivered to the mice was 2 mg/exposure, as measured by a seven-stage multi-jet cascade impactor (IN-TOX Products, Inc.). The SSCS-air mixture was drawn from the top of the funnel by a plastic hose connected to an inlet port of the IN-TOX

exposure chamber system. Total particulates over 10 day SSCS exposure were $2 \pm 0.014$ mg (mean $\pm$ SEM). The SSCS particles collected by the cascade impactor were found to have a mass median aerodynamic diameter (MMAD) of 0.34 μm with a geometric standard deviation of 0.046. This SSCS particle size will gain access to the alveolar septal area of the lungs.

The SSCS-exposed mice were placed in the IN-TOX exposure system for a 30-min exposure period. SSCS was generated in the following manner: the first cigarette was lit by a modified syringe device and one puff of cigarette smoke was drawn from the lit cigarette. The lit cigarette was placed upright in a clamp 2.5 cm below the bottom edge of an inverted 220 cm$^3$ funnel and allowed to burn for 7.5 minutes. A second cigarette was lit at the 7 min time point of the 30 min exposure period and replaced the first cigarette in the clamp at the 7.5 min time point. After burning for another 7.5 min, the second was replaced by a third one at the 15 min time point. Then the third was followed by a fourth one at 22.5 time point, which completed the 30 min SSCS exposure period. The IN-TOX exposure system was then thoroughly cleaned before the next exposure trial to prevent the accumulation of cigarette tars and other materials in the exposure ports. Control mice were treated in a similar manner, except that the cigarettes were not lit before being placed in the clamp.

### Pulmonary function assays

On the day of the experiment, the mice were anesthetized intramuscularly with a 1.5 mL/kg mixture of ketamine HCL (50 mg/kg), xylazine (8 mg/kg), and acepromazine maleate (1 mg/kg). The mice were then paralyzed with 6 mL/kg of intraperitoneal gallamine triethiodide and ventilated with a Kent Scientific Co. (Litchfield, CT) pressure-controlled ventilator. Airway pressure was measured with a polyethylene catheter placed at the proximal end of the endotracheal tube. The endotracheal tube for the mice was a specially modified 20-gauge catheter. The airway and esophageal pressure transducers were connected to opposite sides of a differential pressure transducer for measurement of transpulmonary pressure (Ptp). Air flow (V) was calculated with a calibrated heated pneumotachograph (Fleish, Instrumentation Associates, New York, NY) coupled to a Validyne pressure transducer. The Ptp and V signals were input to a PEDS-LAB$^{®}$ computerized pulmonary function system (Medical Associated Services, Hatfield, PA) adapted for mouse pulmonary functions. This system can measure 24 different respiratory variables including pulmonary resistance as determined by the method of Rodarte (7) on a continual basis. After baseline pulmonary functions had been re-

corded, the mice were administered a 0.1 mL bolus of 100 microCuries of technetium-labeled diethylenetriamine pentaacetate ($^{99m}$TC-DTPA, MW=492 amu, physical half-life = 6.02 h) through the endotracheal tube with five tidal volume (0.5 mL) air flushes to disperse the radioactive tracer evenly throughout the lungs. The mice were then again placed on the mechanical ventilator. Pulmonary epithelial clearance of $^{99m}$TC-DTPA is a measure of lung permeability and was determined over 10 min with a Ludlum (Model 44-62, Sweetwater, TX) gamma counter probe placed centrally over the lungs. The gamma probe was connected to a Ludlum (Model 2200) scintillation center. Then, the mice were killed by withdrawing blood from the vena cava, and the lungs and livers of these mice were taken and stored at -70°C until analysis.

## Measurement of vitamin E

Vitamin E was measured by HPLC as described previously (8). Briefly, about 0.2 g of lung or liver tissue was homogenized in 1.0 mL of water. Butylated hydroxytoluene was added to prevent oxidation of $\alpha$-tocopherol. Pentane, ethanol and sodium dodecyl sulfate were used to extract $\alpha$-tocopherol from the homogenate. Extracts were evaporated under a steady flow of nitrogen gas at 20°C and then redissolved in 0.5 mL of methanol and injected onto a C18 column (3.9×150 mm NovaPak, Millipore, Bedford, MA). A mobile phase composed of methannol:1 mol/L sodium acetate in the ratio of 98:2 (by volume) at a flow rate of 1.5 mL/min was used. $\alpha$-tocopherol, eluting at 6.5 min, was monitored by a fluorescence detector (Millipore) at 290 nm excitation and 320 nm emission wavelength. A set of $\alpha$-tocopherol solutions with different concentrations were analyzed to make a standard curve and to verify calibration.

## Determination of conjugated dienes and lipid fluorescence

Approximately 0.2 g of lung or liver tissue was homogenized in 5.0 mL of Folch solution (2:1 v/v chloroform: methanol). After protein separation, a 0.1 mL fraction was dried in a steady flow of nitrogen gas at 55°C and used to determine conjugated dienes as previously described (9). Conjugated diene fatty acids were determined by obtaining absorbency of the solution at 237 nm in a Shimadzu UV 160 UV recording spectrophotometer (Tokyo, Japan) using an appropriate blank. Lipid fluorescence was measured in an Aminco Bowman fluorescence spectrophotometer (Rochester, NY). Maximum fluorescence at 470 nm was measured. The activation wavelength was at 395 nm.

## Determination of phospholipids

The phospholipid contents of lung and liver were determined by the method of Raheja et al. (10). This method does not require predigestion of phospholipids. Briefly, 0.5 mL chloroform was added followed by 0.2 mL of a coloring reagent and 3.0 mL of carbon tetrachloride. Phospholipid concentration was determined by obtaining absorbency of the chloroform solution at 710 nm in a Shimadzu 160 UV recording spectrophotometer (Tokyo, Japan). Dipalmitoyl phosphatidylcholine was used as a standard.

## Determination of total cholesterol and triglycerides

The total cholesterol levels of lung and liver were determined by the method of Zak (11). Briefly 0.3 mL of Folch extract was dried under air at 70°C. Then 3.0 mL Zak's reagent was added followed by 2.0 mL of sulfuric acid. Total cholesterol was determined by obtaining the absorbency of the solution at 570 nm in a Shimadzu 160 UV recording spectrophotometer (Yokyo, Japan) comparing to cholesterol standards (Sigma, St. Louis, MO). Triglycerides were determined colorimetrically (12).

## Statistics

Mean (SEM) data were calculated for each group of mice. The statistical tests for comparison among groups were done by using analysis of variance (ANOVA) for the lung function data. Vitamin E and lipid peroxidation data were analyzed by NCSS software (Kaysville, UT) using Friedman's Block/Treatment test, followed by Duncan's Multiple Range Test between any two groups. Differences between groups were considered significant at $p < 0.05$.

# RESULTS AND DISCUSSION

## Diet intake and body weight

The average diet intake was 4.45 g/mouse/day. No significant differences were observed in food consumption between groups (data not shown). Pycnogenol® intake in supplemented mice was 641.8 µg/mouse/day, while in unsupplemented 28.3 µg/mL/day. SSCS exposure did not affect the amount of Pycnogenol® consumption in the diet. Body weight was not affected by SSCS exposure or Pycnogenol® supplementation.

## $^{99m}$TC-DTPA lung clearance and resistance after an acute exposure to SSCS and Pycnogenol® supplementation

Lung clearance of particulates is an indicator of pulmonary injury. SSCS causes an increase in alveolar epithelial permeability to small solutes as indicated by the changes that we observed in $^{99m}$TC-DTPA lung clearance. Our experiment demonstrated that $^{99m}$TC-DTPA lung clearance tended to be increased by SSCS exposure and reduced by Pycnogenol® supplementation, although the differences were not statistically significant (Table 1).

**Table 1.** $^{99m}$TC-DTPA lung clearance and pulmonary resistance

| Treatment | | $^{99m}$TC-DTPA lung | Pulmonary |
|---|---|---|---|
| SSCS | Pycnogenol$^{®}$ | clearance (%/min) | resistance |
| - | - | 0.96±0.48[1] | 16341±1013 |
| - | + | 0.81±0.54 | 12082± 872* |
| + | - | 2.61±0.53 | 13939±1143 |
| + | + | 1.69±0.45 | 15743± 752[#] |

[1] Values are presented as mean±SEM (n=8).
*Significantly different (p<0.05) from untreated mice that were not exposed to SSCS or Pycnogenol$^{®}$.
[#] Significantly different (p<0.05) from Pycnogenol$^{®}$ supplemented mice but not exposed to SSCS.

In addition, SSCS exposure significantly (p<0.05) increased pulmonary resistance, known as a criterion of lung function, in Pycnogenol$^{®}$-supplemented mice, but did not cause a significant change in the unsupplemented group (Table 1). Pycnogenol$^{®}$ supplementation decreased pulmonary resistance in non-smoking mice with statistical significance (p<0.05), but not in smoking mice. Theses observation may be related to the generation of oxidative stresses that induce damage and increased resistance in the lung tissue. Cigarette smoke exposure recruits neutrophils and macrophages to the lungs (13). These activated cells release increased amounts of myeloperoxidase and superoxide anion (14), causing lung cell damage. Similarly, main stream and side-stream cigarette smoke increases the activities of cytochrome P450 A1 and 2B1 in the lungs of adult rats as well as oxidative stress (15). In addition, cigarette smoke contains large amounts of oxidants and free radicals that directly initiate and promote oxidative damage in the lungs. Oxidative-induced lung injury also results indirectly from reactive oxygen species generated by the increased number and activity of pulmonary alveolar macrophages and neutrophils. This was likely very limited in nude mice as they lack T cells to assist in activation of macrophages. We found that SSCS significantly increased oxidation damage in the lung tissue even after a short exposure. Oxidation damage is a powerful agent, which may suppress and damage the immune system locally in lung and also systemically, which increases cancer risk and other smoking-related disorders.

## Lipid peroxidation levels in lung and liver tissues after Pycnogenol$^{®}$ supplementation and acute exposure to SSCS

Pycnogenol$^{®}$ functions as a scavenger of the different free radicals by working as an antioxidant (16). Since the oxidative damage is a major factor involved in the toxicity of cigarette smoke, the level of tissue vitamin E plays a role in mediating the development of smoking-related disorders. There is increased utilization of vitamin

E that may be associated with cigarette smoke, as we showed here for SSCS. Thus Pycnogenol$^{®}$ supplementation may substitute for the role of vitamin E and help restore it to normal levels in tissues. The tissue and serum vitamin E concentrations have been shown to be significantly lower in male, and female smokers as compared to nonsmokers (17). This trend is also associated with higher levels of lipid peroxidation products. Pacht et al. (18), also showed that vitamin E levels were significantly lower in alveolar fluid of smokers than in that of nonsmokers. They also suggested that smokers had a faster rate of vitamin E utilization and smoking might predispose it to enhanced oxidant attack on their lung parenchymal cells. Our experiments demonstrated that SSCS exposure significantly decreased vitamin E levels of lung and liver only in Pycnogenol$^{®}$-supplemented mice but not in unsupplemented mice (Fig. 1). This observation may mean
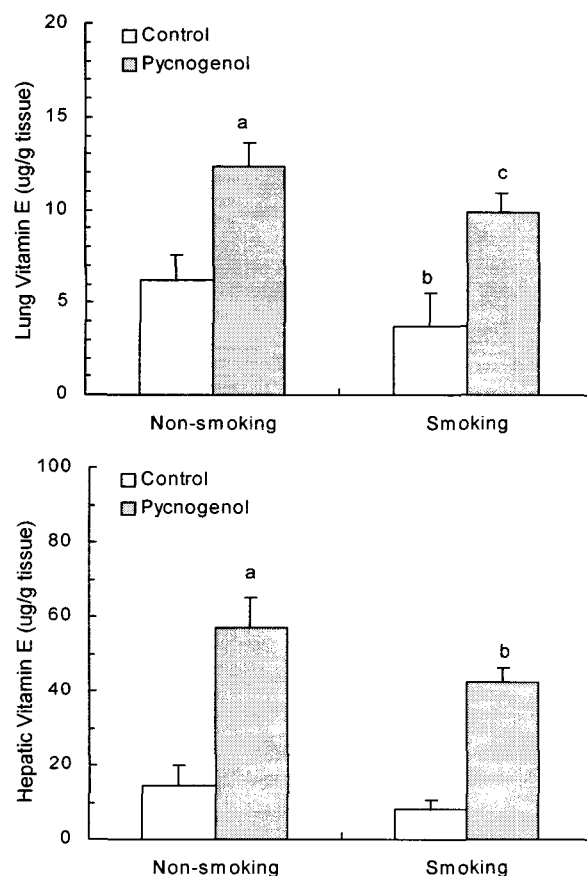


**Fig. 1.** Effect of SSCS exposure and Pycnogenol$^{®}$ supplementation on lung and hepatic vitamin E concentrations in nude mice. Every sample from each mouse was measured in triplicate. Values are mean±standard error; n=8. Letters indicate significant differences at p<0.05: a, statistical analysis within non-smoking mice groups with or without Pycnogenol$^{®}$ supplementation; b, statistical analysis between non-smoking and smoking mice groups without Pycnogenol$^{®}$ supplementation; c, statistical analysis within smoking groups with or without Pycnogenol$^{®}$ supplementation.

that Pycnogenol®-supplemented mice may be more sensitive and susceptible to SSCS damage, which may explain why SSCS exposure could significantly increase pulmonary resistance in Pycnogenol®-supplemented mice, but not in the unsupplemented group. Pycnogenol® caused about a two-fold increase, with statistical significance ($p <$ 0.05), in lung vitamin E levels of both non-smoking and smoking mice, respectively (Fig. 1). Hepatic vitamin E content was increased about 3 to 5-fold in both non-smoking ($p < 0.05$) and smoking mice after Pycnogenol® supplementation for 10 days. A possible explanation is that Pycnogenol® supplementation removed the oxidative stress caused by SSCS to restore the level of vitamin E in both lung and liver. In addition, these results suggest that the antioxidant capacities of the lung and liver of Pycnogenol®-supplemented mice is higher than those of the controls. However, the supplementation dose in our experiment may have been insufficient to compensate for

the damaged vitamin E content by free radicals in smoking-exposed lung during the 5 hours of SSCS treatment.

Conjugated dienes are major products of lipid peroxidation. Our data revealed that SSCS exposure does not significantly increase the level of conjugated dienes in lung tissue (Fig. 2). However, SSCS did cause a significant ($p < 0.05$) increase in hepatic conjugated dienes, which might be caused by the differences in the amount of lipids between the two organs. Pycnogenol® supplementation significantly ($p < 0.05$) decreased the level of conjugated dienes in the lung tissue in smoking and hepatic conjugated dienes in both non-smoking and smoking mice. Lipid fluorescence is another indicator of lipid peroxidation. SSCS exposure significantly ($p < 0.05$) increased lipid fluorescence in both lung and liver tissues (Fig. 3). Pycnogenol® supplementation significantly ($p < 0.05$) prevented the increased lipid fluorescence levels of lung and liver in smoking mice, but not in non-smoking mice.
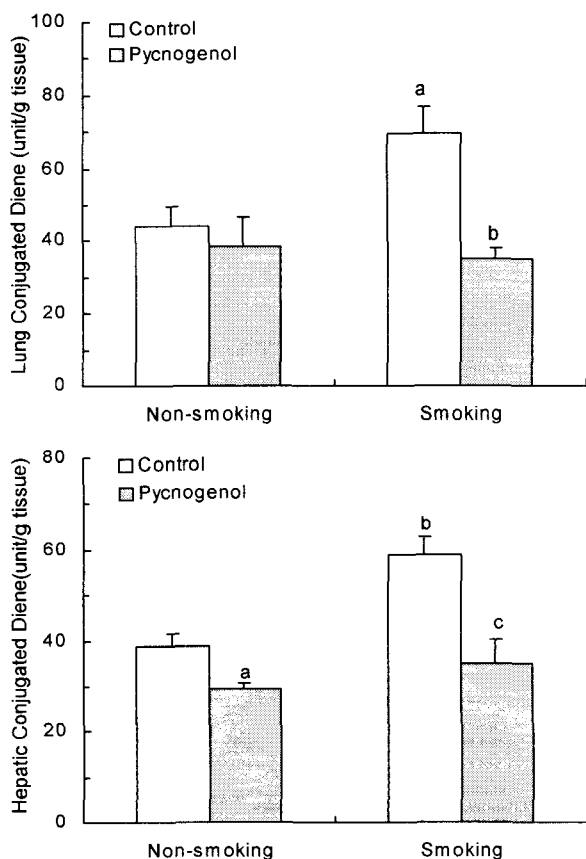


Fig. 2. Effect of SSCS exposure and Pycnogenol® supplementation on lung and hepatic conjugated diene content of nude mice. Every sample from each mouse was measured in triplicate. Values are mean±standard error; n=8. Letters indicate significant differences at $p < 0.05$: a, statistical analysis within non-smoking mice groups with or without Pycnogenol® supplementation; b, statistical analysis between non-smoking and smoking mice groups without Pycnogenol® supplementation; c, statistical analysis within smoking groups with or without Pycnogenol® supplementation.
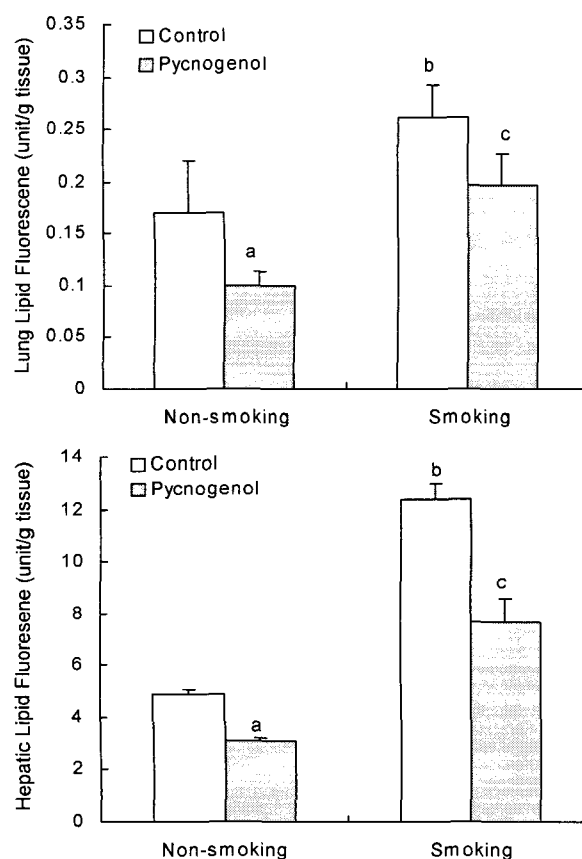
Fig. 3. Effect of SSCS exposure and Pycnogenol® supplementation on lung and hepatic lipid fluorescene content of nude mice. Every sample from each mouse was measured in triplicate. Values are mean±standard error; n=8. Letters indicate significant differences at $p < 0.05$: a, statistical analysis within non-smoking mice groups with or without Pycnogenol® supplementation; b, statistical analysis between non-smoking and smoking mice groups without Pycnogenol® supplementation; c, statistical analysis within smoking groups with or without Pycnogenol® supplementation.

However, there was no significant difference in phospholipid, total cholesterol or triglyceride levels of lung or liver caused by SSCS exposure or Pycnogenol® supplementation (data not shown).

Conjugated dienes and $\alpha$-tocopheryl quinone were significantly higher in the lung tissues of rats exposed to cigarette smoke (19). These rats had an increased sensitivity to ischemia-reperfusion injury due to the increased levels of free radicals generated by cigarette smoke exposure (20). Supplementation with the antioxidant, Pycnogenol®, has been shown to result in significantly less mitochondrial oxidative damage in both rats and mice (21). The protective effect of Pycnogenol® supplementation on mitochondrial activity is a function of free radical scavenging ability. However, the exact mechanism of how Pycnogenol® acts on improving cigarette smoke-induced lung injury is largely unexplored. The increased antioxidant capacity of the mouse tissues in our experiment seems to be the major effect of supplementation with Pycnogenol®. The enhanced resistance of Pycnogenol®-supplemented nude mice against SSCS-mediated lung injury and lipid peroxidation may be due to this effect.

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