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An *Uncaria tomentosa* (cat's claw) extract protects mice against ozone-induced lung inflammation

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Abstract

Ozone (O_3) inhalation has been associated with respiratory tract inflammation and lung functional alterations. To characterize the O_3-induced lung inflammation in mice, the effective dose and exposure time were determined. Total protein levels of bronchoalveolar lavage fluid (BALF), cytological smears, and lung histopathology and morphometry were used to assess and measure the degree of pulmonary inflammation in the mouse model. Ozone inhalation caused acute pneumonitis that was characterized by a high number of infiltrating neutrophils (PMNs) immediately after exposure and increased levels of protein in BALF in mice killed 8 h after O_3 exposure. The anti-inflammatory properties of *Uncaria tomentosa* (UT) have been documented previously. To evaluate the anti-inflammatory effects of UT, male mice were given an UT extract for 8 days, exposed to O_3, and killed 0 or 8 h after O_3 exposure. When compared to untreated controls, UT-treated mice had significantly ($p < 0.05$) lower levels of protein in BALF, lower degree of epithelial necrosis, higher number of intact epithelial cell nuclei in bronchial wall, and decreased number of PMNs in the bronchiolar lumen. Therefore, UT extract appeared to prevent O_3-induced respiratory inflammation in male mice.

Keywords: *Uncaria tomentosa*; Ozone; Anti-inflammatory; Bronchoalveolar Lavage (BAL); Histomorphometry

1. Introduction

Uncaria tomentosa (UT) (Willd) D.C. (Rubiaceae), commonly known as cat's claw or "uña de gato" in Spanish, is a woody, long vine that grows in the highlands of the Amazonian rain forest (Duke and Vasquez, 1994). Since ancient times, the indigenous people of Peru and other South American countries have used its inner bark and root to prepare a decoction to treat many diseases including asthma, arthritis and other inflammatory diseases (Duke and Vasquez, 1994; Sandoval-Chacon et al., 1998). In recent years, studies have provided evidence supporting the anti-inflammatory and antioxidant properties of UT (Aguilar et al., 2002; Aquino et al., 1991; Duke and Vasquez, 1994; Sandoval et al., 2002; Sandoval-Chacon et al., 1998). UT, even when given in large amounts, appears to have very low toxicity levels (Piscoya et al., 2001; Santa Maria et al., 1997).

Although the main active ingredient is not know, the anti-inflammatory activity of UT may be due to multiple secondary metabolites working in synergy (Reinhard, 1999). For example, quinovic acid glycosides found in the bark and roots of UT have been documented to be the most potent anti-inflammatory constituents (Aquino et al., 1991). Additionally, the steroidal fraction of UT has shown the presence of beta-sitosterol (60%), stigmasterol, and campesterol all which have moderate anti-inflammatory activities (Senatore et al., 1989). Other metabolites present in the bark and root, such as indole and tetra- and pentacyclin oxindole alkaloids (Aquino et al., 1989; Laus et al., 1997; Wagner et al., 1985) appear do not influence the antioxidant and anti-inflammatory properties of UT (Sandoval et al., 2002).

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The effectiveness of UT therapy against inflammatory diseases such as asthma and arthritis has been recently documented (Piscoya et al., 2001; Sandoval et al., 2002). UT has the ability to inhibit the production of inflammatory components such as TNF-α and to a lesser extent PGE2 (Piscoya et al., 2001; Sandoval et al., 2002). Lastly, it has been suggested that UT may protect cells against oxidative stress by negating the activation of NF-kappa B (Sandoval-Chacon et al., 1998).

Ozone (O_3), a pollutant associated with large urban areas, remains one of the three most important air pollutants worldwide (Steinberg et al., 1990). O_3 is the main component of air pollution (smog). In the last two decades, evidence suggests that allergic respiratory diseases including bronchial asthma have become more common worldwide, an outdoor pollution has been shown to be a major contributing factor (D'Amato et al., 2001). Environmental levels of O_3 frequently exceed air quality standards in many urban areas (Pino et al., 1992). O_3 is a photochemical oxidant able to damage the function and structure of respiratory epithelium resulting in diffuse inflammation of the respiratory tract (Keller, 1992; Van der Vliet et al., 1995). The O_3-induced oxidative tissue damage is characterized by neutrophilic inflammation, accumulation of protein in air space lumen, and edema (Kleeberger and Hudak, 1992; Mustafa, 1990). Although most of the acute effects of O_3 on the respiratory tract are potentially reversible, there is evidence of chronic health damage by repeated O_3 exposure in populations living in highly polluted areas. Therefore, acute and repeated exposure to O_3 (at moderate concentrations) can induce an acute asthmatic reaction in healthy human airways followed by a long lasting bronchial hyper-responsiveness (Keller, 1992).

The mouse (*Mus musculus*) has been reported to be the most susceptible common experimental species to O_3 (Chitano et al., 1995). Therefore this species was selected for these studies. A preliminary study was conducted to induce, characterize, and establish the measurable end points of lung inflammation caused by O_3 exposure. Then, to determine the preventive anti-inflammatory effects of UT, male mice were administered UT extract for 8 days, exposed to O_3 and killed 0 or 8 h after. To characterize inflammation and measure treatment effects in lung tissue, total protein concentrations and the number and type of epithelial and inflammatory cells were measured in bronchoalveolar lavage fluid (BALF). Histopathological examination of fixed lung tissue allowed quantification of inflammatory and epithelial cells present within a bronchiole's lumen as well as measurement of viable epithelium and mural inflammation in the wall of the same bronchiole. The results obtained by this study provide relevant information about the potential anti-inflammatory properties of UT in preventing or modulating O_3-induced lung injury. In addition, the findings may be useful in documenting a potential alternative treatment for pulmonary inflammatory diseases in human and animals.

2. Material and methods

2.1. Animals

CD-1 pathogen-free male mice, approximately 33 days old, were obtained from Harlan Sprague Dawley (Indianapolis, IN). An adaptation period of two weeks in the laboratory was allowed prior to the experimental period. Mice were housed four per cage in polycarbonate cages with corn cob bedding in animal rooms at 24 °C with a 12 h artificial light cycle (6 a.m. to 6 p.m.). The mice were maintained on commercial laboratory rodent diet 5015 (LabDiet, Richmond, IN), and UT extract or water ad libitum, respectively. The study was conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

2.2. Ozone (O_3) generation

O_3 was generated by XL-15 Air Purification System (Sensidyne, Clearwater, FL). A sealed glove box was used as an experimental exposure chamber. O_3 levels were monitored during exposure using the Gastec Standard Detector Tube System (Nextteq LLC, Tampa FL).

2.3. Preparation of *Uncaria tomentosa* extract

Bark of UT was purchased in Ecuador (Plantas Medicinales de Nuestra Amazonia, Certif. Ministerio de Salud: 047-OSB-C96 R.U.C. 2/114610) and directly shipped to our laboratory. The origin of the plant material used was confirmed to be UT by the company. The night before the extract was to be used; an aqueous decoction was prepared by boiling dry UT bark (20 g/L) in deionized water for three hours. After cooling down overnight at room temperature, it was filtered using paper. Approximately 280 ml of extract were obtained from each 20 g of dry bark, yielding about 14 ml of aqueous extract per gram of dry bark.

2.4. Experimental design

Two experimental phases were included:

2.4.1. Phase I (characterization of O_3-induced acute pneumonitis)

To achieve a measurable characterization of O_3-induced inflammation in an animal model, 40 mice were randomly assigned to four groups (10 mice each). One group (control) was exposed to room air only. The other three remaining groups were exposed to 3.00 ppm O_3 for 4 h and killed 0, 4, and 8 h post-exposure, respectively.

2.4.2. Phase II (prevention of O_3-induced acute pneumonitis by UT)

To determine the anti-inflammatory effects of UT in mice exposed to O_3, 96 mice were randomly assigned to three groups (32 mice each). One group (untreated control) received distilled water ad libitum. The other two groups received 50 and 100% UT decoction diluted with distillated water ad libitum instead of distilled water, for an 8-day period. Immediately after the treatment period, the three groups were exposed to 3.00 ppm O_3 for 4 h, and killed 0 or 8 h after O_3 exposure.

2.5. Sedation and sacrifice

At baseline and one day prior to O_3 exposure, animals were weighed and identified (randomly assigned to necropsy time groups). The percent body weight gain or loss during the treatment period was calculated and recorded for each animal using the following formula $((BW_1 - BW_0)/BW_0)100$, where BW_1 is body weight after O_3 exposure and BW_0 is body weight before O_3 exposure. After O_3 exposure, a premixed combination of ketamine hydrochloride (200 mg/kg) and xylazine (10 mg/kg) was injected intraperitoneally to sedate and anesthetize the animals in order to perform a tracheotomy and conduct the bronchoalveolar lavage. An overdose of the same sedative combination was used to deeply sedate and kill the animals for necropsy and tissue collection.

In Phase I, approximately half of each group ($n \approx 5$) was used to determine the BALF total protein content and to characterize the cytology of BALF at 0, 4, and 8 h post-exposure. The other half of each group was used to collect lung tissue for histopathologic assessment. In Phase II, 16 of the 32 mice per group were killed and necropsied immediately after O_3 exposure (time 0). Approximately half of the 16 mice per group were subjected to BALF analysis, while the other half was the object of bronchiolar histomorphometry. The procedure was repeated for the remaining 16 mice in each group, but 8 h after O_3 exposure.

2.6. Tracheotomy

Anesthetized mice were placed in a supine position on a necropsy table. A ventral midline incision of the skin was made extending from the umbilicus to the mandibular angle. Careful dissection of muscle tissue with smaller-size scissors was performed over the trachea for proper exposure. The trachea was then secured by inserting suture material under it and lifting slightly over the adjoining tissue. For tracheal cannulation, the epiglottis was exposed and a 19 gauge hypodermic needle was inserted through the trachea. The cannula and trachea were then tied with the suture material.

2.7. Bronchoalveolar lavage

Lungs of anesthetized mice were subject to lavage in situ with 0.1 M physiologic-buffered-saline (PBS) to recover and quantify luminal free cells for cytology and lung fluid for protein content determinations. Immediately prior to the lung lavage, the abdomen was dissected and the diaphragm incised to release pressure and permit the collapse of the lung lobes for proper perfusion. The abdominal aorta was cut to eliminate the continuous blood supply to the lung. The lavage was then performed by infusing 1 ml of 0.1 M PBS at 37 °C into the trachea and then withdrawing the liquid. This was repeated three times and the fluid collected. The volume recovered was recorded and transferred to a plastic tube. Samples were stored at 4 °C to minimize sample degradation. The BALF was placed in 15 ml centrifuge tubes and centrifuged at 1800 × *g* for 10 min (Fisher Centrific Centrifuge). Total protein in the supernatant was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as a standard. The remaining cell pellet was used for smear preparations. Cytology smears were Wright-stained for cell type determination and quantification under light microscope.

2.8. Tissue collection

The second set of mice from each Phase was killed and necropsied, and the lung and trachea removed immediately from the thoracic cavity. The total wet lung weight was recorded. The lung weight to body weight ratio (%) was calculated and recorded for each animal using the following formula (wet lung weight/BW_1)100. The left lung was immersion-fixed in 4% paraformaldehyde for 24 h, and then changed into 70% ethanol. After fixation, the left lung, which is not lobulated or divided, was longitudinally sectioned at approximately 3 mm from the acinar region, placed in a cassette, processed, and embedded in paraffin. Five-micron sections were mounted onto microscopic slides and stained with hematoxylin and eosin (H&E) for histopathologic and histomorphometric analyses.

2.9. Lung tissue analyses

In Phase I, a subjective histolopathological evaluation of lung sections was conducted. The subjective evaluation focused on the tissue damage severity and number of neutrophils or polymorphonucleated cells (PMNs) present in the bronchial lumen and bronchial wall, and the number quantified in BALF smears.

In Phase II, histomorphometry of lung tissue was conducted. Proper section orientation during embedding allowed for the same main terminal bronchiole to be identified in all samples. The orientation of each lung was determined by its shape, with the anterior most cranial extremity having a blunted end in comparison to the more tapered posterior end. To identify the same bronchiole in each lung section, the pulmonary vein and artery in the centro-acinar area were visualized. The third most anterior terminal bronchiole was chosen in each lung sample for histomorphometric analyses.

Histomorphometry was conducted using a microscope with a camera lucida, and a digitizing tablet connected to a computer with Bioquant IV software (R&M Biometrics, Inc., Nashville, TN). For each terminal bronchiole, several direct and derived parameters were measured and reported here. Direct measurements included: bronchiolar lumen area (LA, μm^2); epithelial mucosal area not including smooth muscle (EMA, μm^2); smooth muscle area (SMA, μm^2); number of desquamated bronchiolar epithelial cells within the LA (EC in LA, #); number of PMNs within the LA (PMN in LA, #); bronchiolar epithelial cell height (ECH, distance from basement membrane to luminal surface, μm); bronchiolar wall epithelial perimeter (EP, mm); number of bronchiolar epithelial cell nuclei present on the EP (EC on EP, #); and the number of mural intra-epithelial PMNs (PMN on EP, #). Derived or reference-based measurements from the direct parameters included: bronchiolar epithelial cell nuclei per unit area of bronchiolar lumen area (EC/LA, $\#/\mu m^2$); number of PMNs per unit area of bronchiolar lumen area (PMN/LA, $\#/\mu m^2$); epithelial cell nuclei per unit distance or length of epithelial perimeter (EC/EP, #/mm); and number of PMNs per unit length of epithelial perimeter (PMN/EP, #/mm). On the average, the percent of total calculated EP with respect to total lumen perimeter (EP/LP, %).

Intra-observer variability or precision was calculated by measuring the same histological section six times, with repositioning. The coefficient of variation (CV%) was <2%. Within section variability was also measured. One paraffin block was sectioned to make 10 serial slides. These ten slides were measured to provide for within-sample variability. For most direct measurements, CV% was >5% due to an expected and gradual narrowing of the bronchiole. However, reference-based values (such as EC/LA) remained consistent and with a coefficient of variation of <4%.

3. Statistical analysis

Basic statistics including means, standard deviations of the mean (S.D.), and standard error of the means (SEM) were calculated, per measurement, for each group using the basic statistical functions of Microsoft Excel™. For all other statistics, Statistica™ for Windows (StatSoft, Inc., Tulsa, OK) was used. Analysis of variance (ANOVA) and *t*-test statistics were used to detect differences among and between groups. However, Levene's test detected non-homogeneous variances for several of the histomorphometric parameters (perhaps due to small sample size and/or tissue variability). For these, the non-parametric Kruskal–Wallis ANOVA by ranks test was used to detect differences among groups, and the Mann–Whitney *U* rank test was conducted to detect differences between groups. Statistical significance was set at $\alpha = 0.05$ for all statistic tests. Trends were set at an $\alpha > 0.05$ but <0.07 for all statistic tests.

4. Results

Phase I characterized the inflammation caused by O_3 exposure by measuring the presence of PMNs and total protein in BALF and examining the histology of lung tissue sections. Total protein in BALF increased with increased time after O_3 exposure (Fig. 1). The highest levels were recorded in mice killed 4 and 8 h after O_3 exposure. At 8 h post-exposure, total protein in BALF was significantly higher ($p < 0.05$) than that of the control group, and of mice killed immediately after exposure.

Differential cytology smears of BALF of animals killed immediately after O_3 exposure was characterized by a high number and a larger percentage (%) of the cells present in the fluid being PMNs, as shown in Fig. 2. The highest PMN% was observed in animals killed immediately after O_3 exposure, and was significantly higher ($p < 0.05$) than in controls and in mice killed 8 h post-exposure. H&E stained lung sections showed a marked increase in the number of PMNs within the bronchiole's lumen and wall (intra-mural) in animals killed immediately after exposure, a moderate number at 4 h, and very low numbers 8 h after exposure. In con-

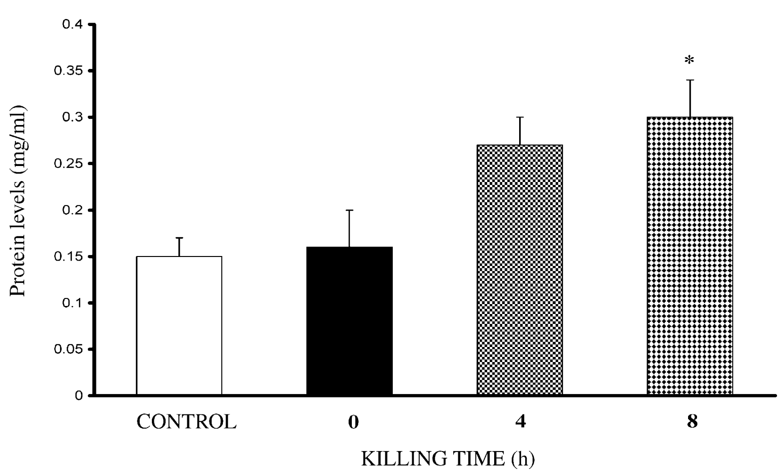

Fig. 1. Total protein in balf of O_3-exposed mice. From left to right: First bar represents control group not exposed to O_3 ($n = 10$). Second, mice killed immediately after 4 h of O_3 exposure ($n = 10$). Third, mice killed 4 h after O_3 exposure ($n = 10$). And fourth, mice killed 8 h after O_3 exposure ($n = 10$), (*) statistically different from control ($p < 0.05$). Bar represents standard error.

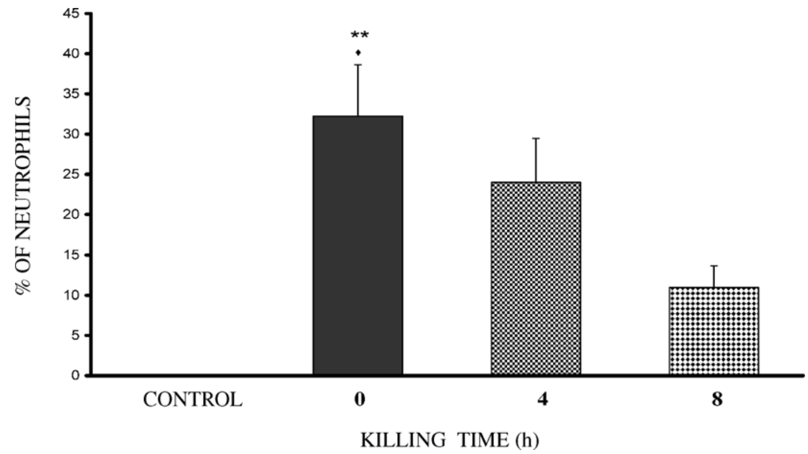

Fig. 2. Percentage of neutrophils in cytology smears in balf of O_3-exposed mice. From left to right: First bar represents control group not exposed to O_3. Second, mice killed immediately after 4 h of O_3 exposure ($n = 10$). Third, mice killed 4 h after O_3 exposure ($n = 10$). And fourth, mice killed 8 h after O_3 exposure ($n = 10$), (★★) statistically different from control ($p < 0.01$). (♦) Statistically different from mice killed 8 h after O_3 exposure ($p < 0.05$). Bar represents standard error.

trast, no PMNs were found in control animals not exposed to O_3.

In Phase II, as shown in Fig. 3, there were no differences in the levels of protein present in BALF when mice were exposed to O_3 for 4 h and killed immediately after. In contrast, an apparent decrease in the protein content of BAFL was seen in both of the UT extract-treated groups sacrificed 8 h after O_3 exposure, with the 100% extract group having significantly lower ($p < 0.05$) protein levels in the BAFL than controls. A subjective analysis of the BAL pellet revealed a lower number of PMNs in animals administered 100% UT extract and killed immediately after exposure (data not shown), when compared to control mice.

Histologically, the bronchioles of control mice not exposed O_3, showed prominent mucosal folds with the epithelium composed of columnar non-ciliated (Clara cells) and ciliated epithelial cells. The relative cell population was not identified, but goblet cells and glands were commonly absent. The smooth muscle formed a layer of variable thickness in close proximity to the epithelial base. In contrast, and as seen in Fig. 4, O_3 exposure caused bronchiolar changes similar to and consistent with those reported previously by Plopper et al. (1973). The changes seen in the bronchiolar epithelium exposed to O_3 consisted of both necrosis and sloughing of bronchiolar epithelial cells (ciliated and non-ciliated), with minimal alveolar and bronchiolar edema. The normally present

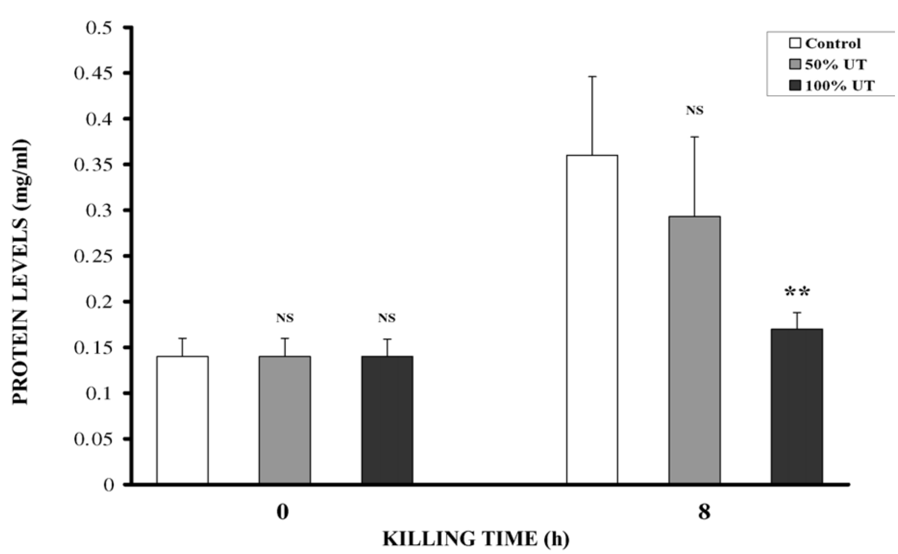

Fig. 3. Total protein in balf of O_3-exposed mice after treatment with *Uncaria tomentosa* extract. From left to right. First bar represents control group killed immediately after 4 h of O_3 exposure ($n = 9$). Second, mice administered 50% UT extract and killed immediately after O_3 exposure ($n = 8$). Third, mice administered 100% UT extract and killed immediately after exposure ($n = 8$). Fourth, control group killed 8 h after O_3 exposure ($n = 7$). Fifth, mice administered 50% UT extract and killed 8 h after O_3 exposure ($n = 8$). Sixth, mice administered 50% UT extract and killed 8 h after O_3 exposure ($n = 7$). (★★) Statistically different from control group killed immediately after O_3 exposure ($p < 0.05$). NS: not statistically different from control killed at 0 or 8 h after O_3 exposure, respectively. Bar represents error.

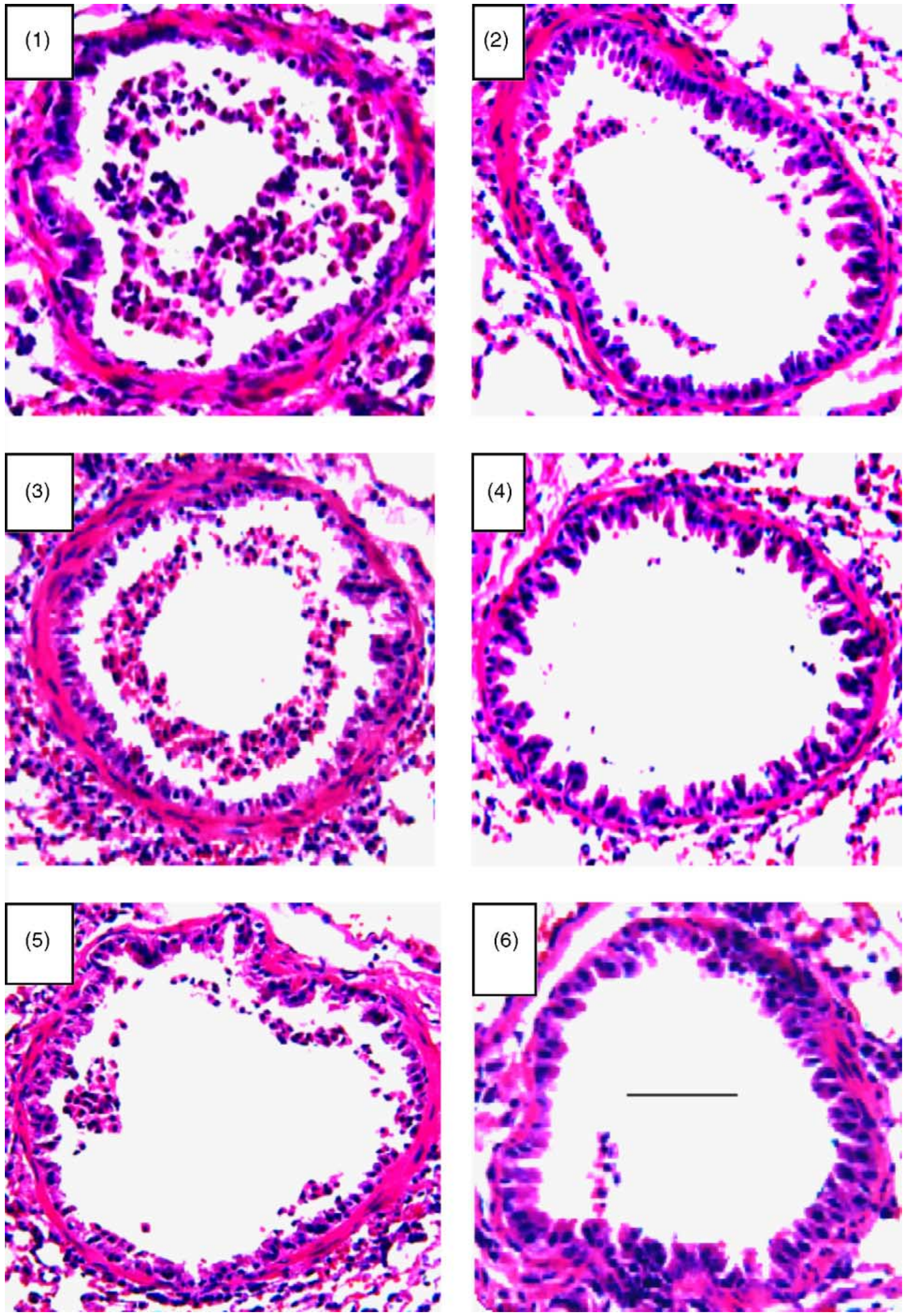

Fig. 4. Bronchioles of mice treated with *Uncaria tomentosa* extract and exposed to O_3. Bronchiolar sections of mice killed immediately after O_3 exposure are presented on the left (1, 3 and 5) while those of mice killed 8 h after exposure are presented on the right (2, 4 and 6). Control mice (1 and 2), 50% *Uncaria tomentosa* extract (3 and 4), and 100% *Uncaria tomentosa* extract (5 and 6). Bar corresponds to 100 microns.

mucosal folds appeared to be decreased both in number and in height (slightly flattened) due to the loss of epithelial cells. The sloughed bronchiolar epithelium often remained in the bronchiolar lumen partially occluding it and forming aggregates of variable size and cell numbers. In addition, numerous PMNs were commonly seen infiltrating the bronchiolar wall and localizing among the remaining and attached bronchiolar epithelium. Also, PMNs were found admixed with the sloughed epithelial cells and fluid present in the bronchiolar lumen. The smooth muscle layer surrounding the bronchioles appeared slightly thickened and more prominent at 0 h after O_3 exposure. All the histologic changes, including the bronchiolar epithelial necrosis and inflammation, appeared to be more severe at time 0 than at time 8 h. As shown by Fig. 4, subjective and qualitative evaluation of the bronchiolar tissues suggested that treatment with UT provided protection against the ozone-induced bronchiolar injury with consumption of 100% UT extract providing the best overall protection.

Table 1
Effect of *Uncaria tomentosa* on body and lung weight in mice exposed to ozone

Group	N	Basal body weight (g)	Body weight at necropsy (g)	%Body weight change	Wet lung weight (g)	Lung/body weight ratio (%)
Time 0, killed immediately after exposure						
Control	6	26.5 ± 1.4	29.2 ± 1.3	10.4 ± 4.1	0.2 ± 0.0	0.6 ± 0.1
50%	7	26.3 ± 1.1	29.5 ± 0.9	12.2 ± 4.6	0.2 ± 0.1	0.8 ± 0.1
100%	7	26.2 ± 1.1	28.1 ± 1.3	7.6 ± 4.7	0.2 ± 0.1	0.7 ± 0.1
Time 8, killed 8 h after exposure						
Control	8	26.5 ± 1.3	28.8 ± 1.2	8.7 ± 4.7	0.2 ± 0.0	0.6 ± 0.1
50%	8	26.6 ± 1.4	28.3 ± 1.5	6.8 ± 7.0	0.2 ± 0.0	0.7 ± 0.1
100%		26.4 ± 0.8	27.7 ± 1.7	4.6 ± 4.0	0.2 ± 0.0	0.6 ± 0.0

N: number of samples included. Mean ± S.D.

Table 1 presents the body and absolute wet lung weight (means ± standard deviation (S.D.)) for mice treated with UT extract prior to O_3 exposure. No treatment effects were evident in either of these parameters. Regardless of treatment, the mice gained equivalent weight during the treatment period. Also, the relative lung weights (%, adjusted to body weight) were equivalent among groups.

Table 2 includes histomorphometric data for lumen area, epithelial mucosal area, and smooth muscle area. No treatment effects were observed in the first two parameters (LA and EMA) among or between groups. The greatest smooth muscle area was recorded in mice receiving 100% UT extract and immediately after exposure. Although this value was not statistically different from controls, it was significantly different ($p < 0.05$) from that of mice administered 50% UT extract. In contrast, mice administered 50% UT extract and killed 8 h after O_3 exposure had the greatest smooth muscle area recorded, and was significantly higher ($p < 0.05$) than their respective controls, but no different from that of mice administered 100% UT extract. An explanation for the observed effects in smooth muscle area cannot be provided, but smooth muscle activity is required for proper airway and fluid movement and if these are increased the muscle should be able to hypertrophy. This hypertrophy could lead to increased total measurable area.

Table 3 shows the effects of UT treatment on the cell population present in bronchioles of mice treated with UT prior to O_3 exposure. Mean counts of exfoliated epithelial cells present in bronchiolar lumina decreased with time after O_3 exposure and with treatment. A greater number of exfoliated epithelial cells were seen in bronchioles of mice killed immediately after O_3 exposure. A dose-related effect was observed for mice treated with UT. When compared to controls, significantly lower ($p < 0.05$) values were observed in mice administered 100% UT extract and killed at 8 h and the effect remained after adjusting for bronchiolar lumen area. Similarly to the data on epithelial cell exfoliation, the number of PMNs present in bronchiolar lumina decreased with time after exposure and with treatment. PMNs per area of bronchiole lumen measured histomorphometrically are presented in Fig. 5. Immediately after exposure, when compared to control, significantly lower numbers of PMNs ($p < 0.05$) were observed in mice administered 50% UT extract. However, only trends ($p < 0.07$) with lower numbers of PMNs approaching significance were observed in mice administered 100% UT extract. At 8 h after exposure, no treatment effect was detected.

Table 4 presents the effects of UT on bronchiolar mural cell population in control animals and mice treated with UT extract prior to O_3 exposure. No treatment effect on epithelial cell height was observed in mice killed immediately after

Table 2
Effect of *Uncaria tomentosa* on bronchiole histomorphometric parameters in mice exposed to ozone

Group	N	LA (μm^2)	EMA (μm^2)	SMA (μm^2)
Time 0, killed immediately after exposure				
Control	6	32.3 ± 10.1	9.8 ± 3.3	8.5 ± 2.1
50%	7	31.3 ± 18.6	9.1 ± 1.9	7.4 ± 1.4
100%	7	27.7 ± 10.4	12.9 ± 5.2	12.1 ± 5.8[b]
Time 8, killed 8 h after exposure				
Control	8	40.2 ± 19.0	11.4 ± 3.9	7.7 ± 1.2
50%	8	36.4 ± 21.5	12.5 ± 3.7	9.4 ± 1.7[a]
100%	9	38.7 ± 13.9	15.0 ± 5.9	9.0 ± 3.3

N: number of samples included; LA: bronchiolar lumen area; EMA: epithelial mucosal area; SMA: smooth muscle area, mean ± S.D.
[a] $p < 0.05$ vs. Control.
[b] $p < 0.05$ vs. 50%.

Table 3
Effect of *Uncaria tomentosa* on cell population present in bronchiole mice exposed to ozone

Group	N	EC (#)	PMNs (#)	EC/LA (#/μm^2)	PMNs/LA (#/μm^2)
Time 0, killed immediately after exposure					
Control	6	62.8 ± 24.6	10.3 ± 7.0	2.2 ± 1.0	0.3 ± 0.2
50%	7	54.7 ± 35.5	3.4 ± 1.7[a]	1.9 ± 1.5	0.1 ± 0.1[a]
100%	7	42.4 ± 34.8	3.1 ± 2.7[a]	1.7 ± 1.7	0.1 ± 0.1[b]
Time 8, killed 8 h after exposure					
Control	8	30.3 ± 16.2	8.5 ± 7.5	0.9 ± 0.5	0.2 ± 0.2
50%	8	24.5 ± 22.8	3.3 ± 2.1	0.8 ± 0.7	0.1 ± 0.1
100%	9	14.4 ± 7.2[b]	6.2 ± 4.6	0.4 ± 0.3[a]	0.2 ± 0.2

N: number of samples included; LA: bronchiolar lumen area; EC: total number of desquamated bronchiolar epithelial cells within the LA; PMNs: total number of neutrophils within the LA; EC/LA: bronchiolar epithelial cells per unit area of bronchiolar lumen area; PMNs/LA: number of PMNs per unit of bronchiolar lumen area; mean ± S.D.
[a] $p < 0.05$ vs. Control.
[b] $p < 0.07$ vs. Control.

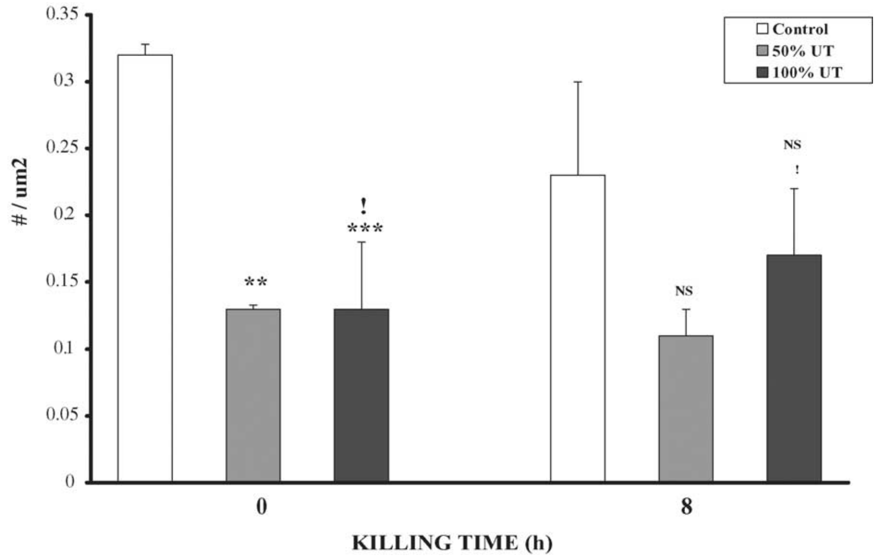

Fig. 5. Mean numbers of pmn cells per bronchiole lumen area in mice treated with *Uncaria tomentosa* extract and exposed to O_3. From left to right. First bar represents control group killed immediately after 4 h of O_3 exposure ($n = 6$). Second, mice administered 50% UT extract and killed immediately after O_3 exposure ($n = 7$). Third, mice administered 100% UT extract and killed immediately after exposure ($n = 7$). Fourth, control group killed 8 h after O_3 exposure ($n = 8$). Fifth, mice administered 50% UT extract and killed 8 h after O_3 exposure ($n = 8$). Sixth, mice administered 100% UT extract and killed 8 h after O_3 exposure ($n = 9$). #/μm^2: number of PMN cells per μm^2 of lumen. (✲✲✲) Statistically different from control group killed immediately after O_3 exposure ($p < 0.05$). (!) Statistically different from control group killed immediately after O3 exposure ($p < 0.07$). (!) Not statistically different from mice administered 50% UT extract. NS = not statistically different from controls killed 8 h after O_3 exposure. Bar represents standard error.

exposure. However, after 8 h, significantly greater ($p < 0.05$) epithelial height measurements were seen in mice treated with both 50% and 100% UT extract when compared to controls. Also, the numbers of epithelial cells per bronchiole epithelium length increased with time of exposure and with treatment. Mice administered 100% UT extract, regardless of whether they were killed immediately after or 8 h after O_3 exposure, showed consistently and significantly higher ($p < 0.05$) numbers of epithelial cells compared to controls. Compared to control mice, mice administered 50% UT extract had lower numbers of PMNs per length of bronchial epithelium, with significant differences ($p < 0.05$) achieved only at 0 h.

Table 4
Effect of *Uncaria tomentosa* on bronchiolar mural cell population in mice exposed to ozone

Group	N	ECH (μm)	EC/EP (#/mm)	PMNs/EP (#/mm)
Time 0, killed immediately after exposure				
Control	6	15.0 ± 2.9	131.50 ± 47.8	6.80 ± 4.2
50%	7	13.3 ± 2.4	156.60 ± 51.4	2.70 ± 2.6[b]
100%	7	16.5 ± 3.4[c]	195.0 ± 25.3[a]	3.50 ± 3.9
Time 8, killed 8 h after exposure				
Control	8	13.0 ± 2.8	146.7 ± 30.3	3.30 ± 4.6
50%	8	17.9 ± 5.5[a]	167.6 ± 54.9	1.70 ± 3.1
100%	9	16.6 ± 3.7[a]	193.4 ± 36.5[a]	3.60 ± 3.2

N: number of samples included; ECH: bronchiolar epithelial cell height; bronchiolar wall epithelial perimeter (EP, mm); EC/EP: number of bronchiolar epithelial cell nuclei per EP; and PMNs/EP: number of mural intraepithelial PMNs per EP, (PMNs/EP), mean ± S.D.

[a] $p < 0.05$ vs. Control.

[b] $p < 0.07$ vs. Control.

[c] $p < 0.07$ vs. 50%.

5. Discussion

As previously reported, administration of UT extract at the levels provided in this study appeared to be relatively safe and non-toxic (Mur et al., 2002; Piscoya et al., 2001; Riva et al., 2001; Rizzi et al., 1993; Sandoval et al., 2000; Santa Maria et al., 1997; Sheng et al., 2000a; Sheng et al., 2000b). No clinical events, physiological alterations or sporadic deaths were observed during the experimental period. At necropsy, no differences in body weight, body weight gain, lung weight or lung/body weight ratios were seen among the experimental groups. Lastly, no gross or microscopic alterations were seen in animals ingesting UT for a period of 8 days.

Functional pulmonary alterations have been observed in animals exposed to O_3 at concentrations as low as 0.24–0.34 ppm, with the mouse LC_{50} reported to be 21 ppm (Chitano et al., 1995). Measurable lung inflammatory effects have been described at levels of 0.3 ppm for several hours (Kodavanti et al., 1995; Plopper et al., 1973, 1979). Similarly to those reports, our experiments showed that exposure to O_3 at 3.00 ppm for 4 h induced a measurable and reproducible inflammatory reaction.

O_3 exposure causes pulmonary tissue damage characterized by neutrophilic inflammation (Kleeberger and Hudak, 1992; Last et al., 1983; Mustafa, 1990; Suzuki et al., 1992). In Phase I of our study, O_3 exposure appeared to cause an immediate increase in the number of neutrophils present in the bronchiolar lumen and wall. The number of inflammatory cells and severity of tissue damage appeared to decrease over time suggesting a spontaneous recovery or physiological healing process after cessation of O_3 exposure. A similar inflammatory process has been previously described (Keller, 1992). The high number of PMNs observed in smears of BALF pellets and H&E-stained tissue sections of mice killed immediately after O_3 exposure suggested a rapid and strong chemotactic response to O_3-induced lung injury. In Phase II of this study, the O_3-induced increase in the influx and number of PMNs within the bronchiolar lumen was prevented or at least ameliorated by the administration of 50% or 100% UT extract for 8 days prior exposure. Furthermore, lower numbers of PMNs per unit length of bronchiolar epithelium were observed in mice administered 50% UT extract, and killed either immediately or 8 h after exposure. These results suggested that inhibition of PMN extravasation and tissue movement with release of enzymatic granules could be one of the mechanisms by which UT anti-inflammatory effects are elicited.

Accumulation of protein-rich fluid in the alveolar and bronchiolar lumen can result from oxidative damage to alveolar and/or bronchiolar epithelium, with extensive capillary leakage (Kleeberger and Hudak, 1992; Mustafa, 1990). In this study all the exposed groups presented higher levels of protein in BALF compared to non-exposed animals, and potentially the O_3 exposure caused damage to both alveolar (not presented here) and bronchiolar epithelium resulting in leakage of protein-rich serum into the airways. The lower levels of protein present in BALF from mice administered UT extract suggest some capillary protective effect which translated to a lower level of edema and protein loss into the airways of the treated mice. The effect appeared to be dose-dependent, with the protective effect being more pronounced in mice administered 100% extract.

The lung is the primary target of O_3, with the central acinar region being the most affected area (Pino et al., 1992; Plopper et al., 1973). This area was analyzed in Phase II to determine the anti-inflammatory effects of UT. Epithelial cell necrosis was induced by O_3 exposure, as previously reported (Last et al., 1983; Pino et al., 1992; Suzuki et al., 1992), and was observed in this study. The number of exfoliated epithelial cells in bronchial lumen was higher in mice killed at 0 than 8 h after exposure. These results suggested that damage of bronchiolar epithelium and cell desquamation was almost immediate and most severe immediately after O_3 exposure. However, the 8 h time period seems to represent a post-healing time point at which some tissue repair and physiological response has taken place. While no major treatment effects were observed immediately after exposure, at 8 h, significant improvement was detected for the bronchiolar epithelial cells, especially in mice treated with 100% UT extract. This observation suggests that UT given in drinking water ad libitum for 8 days prior to the O_3 exposure elicited a lung tissue protective effect.

The numbers of epithelial cells lining the bronchiole and numbers of epithelial cells per epithelial length were significantly higher in animals treated with 100% extract and killed 0 and 8 h after exposure. Concomitantly, the bronchial epithelial height was significantly higher in mice administered both 50% or 100% UT extract and killed 8 h after exposure. These results suggest maintenance of epithelial cell morphology and a decrease in the O_3-induced cell damage in UT treated mice. Apparently more epithelial cells of control mice died and/or desquamated due to O_3 exposure. The more severe desquamation seen in controls probably required more extensive repair of bronchial epithelial surfaces. However, lower numbers of epithelial cells were affected by O_3 exposure in UT-treated mice. Therefore, the epithelial cells remaining in UT-treated mice might be involved in the reparative process and protect the submucosal surfaces leading to cells reaching near-normal cell heights quicker and in greater numbers that in control mice. This condition may be interpreted as a decreased susceptibility to O_3 insult in UT-treated mice, and therefore implies some protective effects of UT.

The anti-inflammatory effects of UT have been attributed to the synergistic effects of various secondary metabolites present in the bark (Reinhard, 1999). Results reported in this study suggest a positive relationship between extract concentration and measured inflammatory markers. The more concentrated bark extract (100%) appeared to provide the best results in this study. The mechanism of action of UT lung protection against O_3 exposure cannot be provided by our study, but perhaps involves a protective effect on the exposed cells that might involve the antioxidant capacity reported previously (Sandoval et al., 2002; Sandoval-Chacon et al., 1998). Antioxidants help to maintain a critical redox balance in the cell during oxidative changes. UT administration apparently assisted the cells with its protective effects, and in turn the decreased cell necrosis lead to lower production and release of lipid inflammatory mediators resulting in reduced tissue damage.

6. Conclusion

The reported results provide the first evidence of a direct protective effect of cat's claw on lung bronchiolar epithelium that might be indicative of anti-inflammatory activity preventing or modulating O_3-induced lung injury. Additional in vivo and in vitro studies oriented to evaluate UT as a potential alternative treatment for pulmonary inflammatory diseases in humans and animals should be conducted before recommending this form of therapy to human and animal patients.

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