Large bowl
Qing Dynasty (1644-1911 A.D.)

EXPERIMENTAL STUDY

Pyunkang-hwan (Pyunkang-Tang) ameliorates air pollutant-induced inflammatory hypersecretion of airway mucus and bleomycin-induced pulmonary fibrosis in rats

Hyo-Seok Seo, Hyun Jae Lee, Choong Jae Lee

Hyo-Seok Seo, Department of Pharmacology, School of Medicine, Chungnam National University, Daejeon 35015, Korea
Hyun Jae Lee, Department of Health Management, Sahmyook University, Seoul 01795, Korea
Choong Jae Lee, Department of Pharmacology, School of Medicine, Chungnam National University, Daejeon 35015, Korea
Correspondence to: Dr. Choong Jae Lee, Department of Pharmacology, School of Medicine, Chungnam National University, Daejeon 35105, Korea. LCJ123@cnu.ac.kr
Telephone: +82-42-580-8255
Accepted: June 17, 2015

Abstract

OBJECTIVE: To investigate the effect of Pyunkang-hwan (Pyunkang-tang) extract (PGT) on secretion of airway mucin in an experimental animal model involving hyperplasia of goblet cells and mucus hypersecretion, and to test its effects on bleomycin (BLM)-induced pulmonary fibrosis *in vivo*.

METHODS: The protective activity of orally administered PGT was assessed in two rat pulmonary disease models. Effects on hypersecretion of pulmonary mucin in sulfur dioxide (SO_2)-induced bronchitis in rats were assessed by quantifying the amount of mucus secreted and examining histopathology in the tracheal epithelium. In a rat model for BLM-induced pulmonary fibrosis, toxicity to the pulmonary system was examined by measuring levels of malondialdehyde and hydroxyproline, indicators of lipid peroxides and collagen, respectively, in lung tissue 28 days post-BLM treatment. Serial sections of lung tissue were stained with Masson trichrome to visualize collagen deposition. Effects of PGT on collagen synthesis were also assessed *in vitro*, in a cell culture model.

RESULTS: PGT inhibited mucin secretion and normalized SO_2-induced increased muco- substances in goblet cells. In the BLM- induced model, PGT decreased the characteristic histopathological features of lung fibrosis and inhibited fibrotic lesions, as indicated by decreased hydroxyproline content. PGT also inhibited the BLM-induced increase in malondialdehyde levels, demonstrating its protective effect against lipid peroxidation in cell membranes of the lung. In MLg 2908 mouse lung fibroblast cells, PGT decreased transforming growth factor (TGF)-β-stimulated type I collagen synthesis.

CONCLUSION: PGT can inhibit both hypersecretion of airway mucins and pulmonary fibrosis.

INTRODUCTION

Respiratory mucus is very important for defense against invading pathogenic microbes, noxious chemicals and particles. The protective function of airway mucus is attributed to the viscoelasticity of mucin. Mucins are multimillion-dalton glycoproteins,

present in airway mucus, that are produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. Hypersecretion of airway mucus is one of the major symptoms of severe pulmonary diseases, including asthma, chronic bronchitis, cystic fibrosis and bronchiectasis.[1] Therefore, identifying traditional herbal medicines that control inflammatory hypersecretion of mucins would be valuable. In addition, fibroblast proliferation and extracellular matrix remodeling are characteristic of pulmonary fibrosis, the endpoint of a heterogeneous group of disorders designated as interstitial lung diseases. Half of all cases of pulmonary fibrosis are idiopathic pulmonary fibrosis (IPF). IPF is an intractable disease, with a 5-year survival rate of less than 50%. The current conventional therapy for IPF is treatment with corticosteroids, immunosuppressive drugs and antifibrotic agents.[2-5] However, despite intensive investigation, clinical trials of IPF treatments have been unsuccessful. Therefore, it is important to investigate whether traditional herbal medicines used for diverse inflammatory pulmonary diseases can control pulmonary fibrosis. Bleomycin (BLM), an anti-cancer agent used to treat some carcinomas, is reported to provoke pulmonary fibrosis in patients. Therefore, BLM is used in certain experimental disease models, producing lung injury and fibrotic lesions in the lung interstitium in various animal species.[6] Intratracheal instillation of BLM into the lungs of rodents causes an inflammatory response, alveolar cell damage, fibroblast proliferation and collagen deposition. In the early stages of lung damage, BLM produces lesions with physiological and histopathological similarities to human fibrotic lung disease.[7-15] Interventions designed to regulate the consequences of the inflammatory response, such as corticosteroids, anti-tumor necrosis factor (TNF)-α antibody and anti-transforming growth factor (TGF)-β antibody, and to protect the lung from oxidative damage, such as N-acetylcysteine,[16] metalloporphyrin[17] and Ginkgo biloba extract,[18] can inhibit BLM-induced pulmonary fibrosis. These findings suggest that inhibition of lung inflammation and lipid peroxidation may represent therapeutic strategies for patients with pulmonary fibrosis. In Korea, Pyunkanghwan (Pyunkang-tang, PGT), a traditional herbal preparation consisting of a water extract from six herbs (Table 1), has been used for controlling hypersecretion of airway mucus in bronchitis, tonsillitis, pneumonitis and pulmonary fibrosis.[19] Such information indicates that PGT may have a therapeutic effect on inflammatory hypersecretion of airway mucus and on pulmonary fibrosis.

Therefore, in this study, we investigated effects of PGT in two *in vivo* rat models. Its effects on secretion of airway mucins were examined in a model involving hyperplasia of goblet cells and mucus hypersecretion. In this model, hypersecretion of pulmonary mucins, resulting from SO_2-induced bronchitis, is assessed by quantifying the amount of mucus secreted and examining histopathology of the tracheal epithelium. In addition, we examined effects of PGT on BLM-induced pulmonary fibrosis. Besides investigating whether PGT affected the pathologic and biochemical features of pulmonary fibrosis in the rat model, we also examined effects of PGT *in vitro*, on collagen production by the MLg 2908 murine lung fibroblast cell line.

MATERIALS AND METHODS

Materials
All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Preparation of PGT
PGT is a preparation consisting of a water extract of six herbs (Table 1). The six herbs were purchased from Dae-won-dang Oriental Drug Store (Seoul, Korea) and identified by Emeritus Professor of Herbology, Chang Soo Yook (College of Pharmacy, Kyung Hee University, Seoul, Korea). The following voucher specimens were deposited at the Herbarium of the College of Pharmacy, Kyung Hee University, Seoul, Korea: Lonicerae Flos (Lonicera japonica Thunberg, Caprifoliaceae), KHUOPS 2013-75; Liriopis Tuber (Liriope platyphylla Wang et Tang, Liliaceae), KHU-OPS 2013-76; Adenophorae Radix (Adenophora tryphilla var. japonica Hara, Campanulaceae), KHU-OPS 2013-77; Xanthii Fructus (Xanthium strumarium Linne, Compositae), KHUOPS 2013-78; Selaginellae Herba (Selaginella tamariscina Spring, Selaginellaceae), KHUOPS 2013-79; and Rehmanniae Radix Preparata (Rehmannia glutinosa Liboschitz var. purpurea Making, Scrophulariaceae), KHUOPS 2013-80. The six herbs were soaked in 500-mL double-distilled deionized water and decocted for 150 min at 100 °C. The resulting extract was filtered through sterile gauze, concentrated in a rotary vacuum evaporator and lyophilized. PGT (1 g) was prepared from 65 g of a mixture of the six herbs (yield: 1.5%) and stored at − 70 °C until assayed for its biological actions.

Animals
Pathogen-free male Sprague-Dawley (SD) rats (Daehan Biolink, Seoul, Korea), 5 weeks old and weighing 200-220 g, were used. The animals were housed five per cage and were provided distilled water and food ad libitum. They were kept under a 12 h light/dark cycle (light 08:00-20:00) at constant temperature (22.5 °C) and humidity (55%). Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals regulated by Chungnam National University, Daejeon, Korea, throughout all of the experimental procedures.

Experimental design

To test PGT in the BLM-induced fibrosis model, 30 rats were randomly divided into the following six groups: control; BLM-only instillation; BLM instillation with PGT at 157 mg/kg; BLM instillation with PGT at 314 mg/kg; BLM instillation with PGT at 785 mg/kg; and BLM instillation with dexa- methasone at 0.5 mg/kg. BLM was administered to the rats intratracheally (5 mg/kg in 2 mL) and PGT (2 mL of a dilution providing the indicated dose) was administered per os. The positive control, dexa- methasone, was administered by intraperitoneal injection. The control group was exposed to fresh air in a similar environment without BLM administration. The day of intratracheal instillation of BLM was defined as day 0. Rats were euthanized 28 days after BLM administration. The lung vasculature was perfused to remove blood and the left lungs were removed from the trachea and hilar nodes and weighed. Half of the left lung was fixed in 4% phosphate-buffered para-formaldehyde for histo-pathology, while the other half was frozen in liquid nitrogen for measurement of collagen and malondialdehyde (MDA). All parameters were detectable for all groups. To test PGT for effects on mucus secretion in the rat bronchitis model, another 30 rats were randomly divided into the following six groups: control; SO_2-only exposure; SO_2 exposure with PGT at 157 mg/kg; SO_2 exposure with PGT at 314 mg/kg; SO_2 exposure with PGT at 785 mg/kg; and SO_2 exposure with dexamethasone at 0.5 mg/kg. The rats were exposed to SO_2 by inhalation and PGT was administered per os. A positive control, dexamethasone, was administered *via* intraperitoneal injection. A 10% solution of sodium metabisulfite was aerosolized into a Plexiglas exposure chamber, using an ultrasonic humidifier (Samsung Electronics Inc., Seoul, Korea). The concentration of SO_2 gas generated by this apparatus was measured at 150 ppm. Rats were exposed to SO_2 for 3 h per day, 5 days per week, for 3 weeks, and PGT was administered during the last 2 weeks. The control group was exposed to fresh air in a similar environment but without SO_2 exposure.

Bronchoalveolar lavage fluid (BALF) collection and quantitation of in vivo mucins in BALF

Rats were euthanized on the last day of the experiment and the trachea was cannulated using a sterile polyethylene tube. Bronchoalveolar lavage (BALF) was performed four times with 5.0 mL ice-cold phosphate-buffered saline (PBS, pH 7.4) with an 80% recovery rate. Floating cells and cell debris were removed by centrifugation of the BALF at 12 000 × *g* for 5 min. The BALF supernatants were stored at − 70 ℃ until assayed for mucin content. The amount of mucin in each BALF sample was measured by enzyme-linked immunosorbent assay (ELISA). The BALF samples were diluted at 1 : 10 with PBS and 100 mL of each sample was incubated at 42 ℃ in a 96-well plate until

dry. The plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) (fraction V) in PBS for 1 h at room temperature. The plates were again washed three times with PBS and then incubated with 100 mL per well 45M1 (Neo-Markers, Fremont, CA, USA), a mouse monoclonal MUC5AC antibody diluted at 1 : 200 in PBS containing 0.05% Tween 20. After 1 h, the wells were washed three times with PBS, and 100 mL horseradish peroxidase-goat anti-mouse IgG conjugate (1 : 3000 in PBS) was dispensed into each well. After 1 h, the plates were washed three times with PBS. The color reaction was developed with 3, 3′, 5, 5′-tetramethylbenzidine (TMB) peroxide solution and stopped with 1 N H_2SO_4. The absorbance was read at 450 nm.

Histopathologic analysis of tracheal and lung tissues

Sections were stained with hematoxylin and eosin and also with Alcian blue for detection of acidic mucins (stained blue) in tracheal tissues. Formaldehyde-fixed, paraffin-embedded tracheal tissues were cut into 5 mm sections. The sections were stained with hematoxylin and eosin followed by the standard Alcian blue (pH 2.5) method. Alcian blue at pH 2.5 stains all acidic mucins blue.[19] Rat lung tissue samples were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) and processed for routine paraffin embedding. Serial sections (5 mm) were cut and stained with Masson trichrome stain and then systematically scanned with a light microscope to assess the degree of fibrosis. Using a 40 × objective lens, randomly selected fields of each slide were assessed for gray to greenish blue-stained collagen.

Preparation and analysis of lung tissue homogenates

Lung tissue samples were homogenized in cold Tris-HCl-buffered saline (pH 7.4, 10 mmol Tris-HCl, 0.1 mmol EDTA-2Na, 10 mmol saccharose and 0.8% sodium chloride solution) at 4 ℃ with a Polytron homogenizer. The tissue homogenates were prepared at 10 % (w/v). Samples were centrifuged at 3000 × *g* for 10 min at 4 ℃ and MDA levels and hydroxyproline contents in the supernatants were then determined. MDA level, an indicator of lipid peroxidation, was determined using a commercial ELISA kit according to the manufacturer's instructions (OxiSelect ™ MDA Adduct ELISA Kit, Cell Biolabs, San Diego, CA, USA). To estimate the collagen content of the lung, hydroxyproline was measured using a commercial kit according to the manufacturer's instructions (Hydroxyproline Assay Kit, Sigma-Aldrich).

Murine lung fibroblast culture

MLg 2908, a murine lung fibroblast cell line, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum in the presence of penicillin (100 units/mL), streptomycin (100 μg/mL), and HEPES (25 mM) at

37 ℃ in a humidified water-jacketed incubator equillibrated with 5% CO_2 and 95% air. Cells were passaged by trypsinization, split 1:4 once weekly, and used when nearly confluent (70%). Cells were trypsinized and seeded onto a 24-well plate at a density of 5×10^4 cells per well. At confluence, the medium in the 24-well plates was replaced with serum-free RPMI 1640. After 24-h incubation, the cells were cultured in the presence of recombinant human transforming growth factor-β (TGF-β) (10 ng/mL) and serial concentrations of PGT for 24 h. The supernatants were then collected for quantification of type Ⅰ collagen.

Quantification of type I collagen

Type Ⅰ collagen was measured by ELISA. Supernatants were diluted with PBS at a 1 : 10 ratio and 100 mL of each diluted sample was then dispensed into a 96-well plate. Plates were incubated for 2 h at room temperature, washed three times with PBS, and then blocked with 2% BSA (fraction V) in PBS for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 mL type Ⅰ collagen monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was diluted at 1 : 500 in PBS containing 0.05% Tween 20. After 1 h, wells were washed three times with PBS and then 100 mL horseradish peroxidase-goat anti-mouse IgG conjugate (1 : 3000 in PBS) was dispensed into each well. After 1 h, plates were washed three times with PBS. The color reaction was developed with TMB peroxide solution and stopped with 1 N H_2SO_4. Absorbance was read at 450 nm.

Statistical analysis

The means for each individual group were converted to percent control and expressed as mean ± standard error of the mean (SEM). Differences among groups were assessed using one-way analysis of variance and the Holm-Šidák test as a post-hoc test using SigmaStat ver4.0 (Systat Software, San Jose, CA, USA). $P < 0.05$ was considered significantly different.

RESULTS

Effect of PGT on histopathologic changes in tracheal tissue and secretion of in vivo airway mucins from airway goblet cells of rats exposed to SO_2

Rats subjected to SO_2 exposure for 3 weeks had a significant increase in mucosubstances (acidic mucins, stained blue, Figure 1) and mucin secretion in tracheal tissues (Figure 2) compared with the control group. However, orally administered PGT inhibited both the increase of mucosubstances (acidic mucins, stained blue) in goblet cells of the tracheal tissues and mucin secretion detected in the BALF. The amounts of mucin in the BALF samples were 100% ± 19% , 294% ± 27%, 217% ± 11%, 155% ± 13%, 129% ± 9% and 147% ± 23% for the control, SO_2-only, SO_2 plus PGT
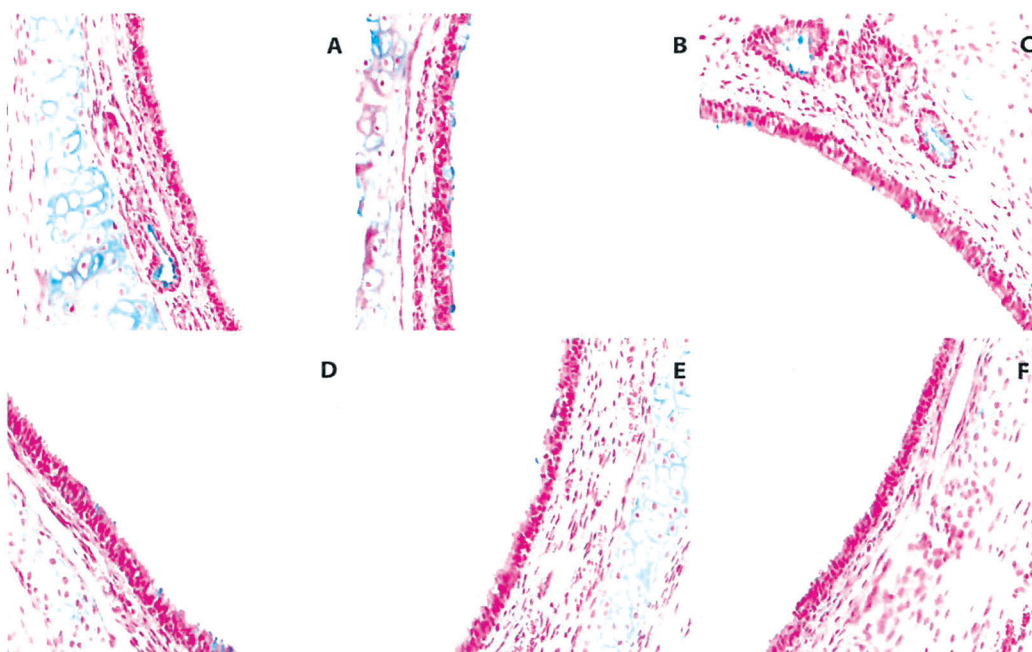

Figure 1 Effect of PGT on epithelial mucosubstances in the trachea of rats exposed to sulfur dioxide (hematoxylin and eosin staining, × 200)

Rats were inhalationally exposed to sulfur dioxide and the effect of orally administered PGT on epithelial mucosubstances (acidic mucins) in the trachea was investigated, as described in Materials and Methods. The blue staining indicates mucins. A: normal control group, rats were treated by no specific agent and exposed to fresh and clean air; B: sulfur dioxide group, rats were treated by no specific agent and exposed to sulfur dioxide; C: section of trachea from rat treated by dexamethasone, a positive control, intraperitoneally, and exposed to sulfur dioxide; D: section of trachea from rat treated by PGT 157 mg/kg and exposed to sulfur dioxide; E: section of trachea from rat treated by PGT 314 mg/kg and exposed to sulfur dioxide; F: section of trachea from rat treated by PGT 785 mg/kg and exposed to sulfur dioxide. PGT: Pyunkang-hwan (Pyunkang-tang) extract; SO2: sulfur dioxide.
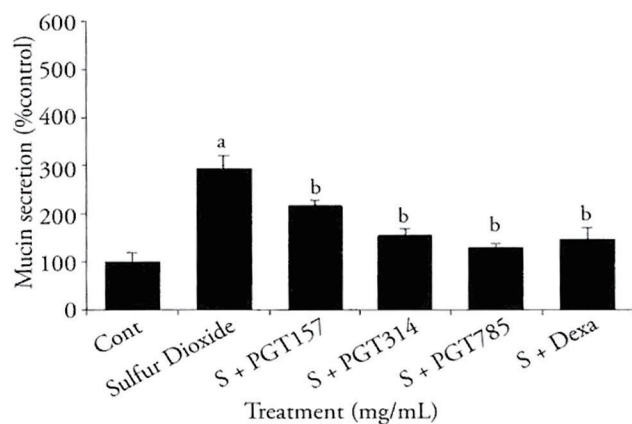
Figure 2 Effect of PGT on secretion of *in vivo* airway mucins from rats exposed to sulfur dioxide

Rats were inhalationally exposed to sulfur dioxide and the effect of orally administered PGT on secretion of in vivo airway mucins was investigated, as described in Materials and Methods. Normal control group, rats were treated by no specific agent and exposed to fresh and clean air; Sulfur dioxide group, rats were treated by no specific agent and exposed to sulfur dioxide; Section of trachea from rat treated by dexamethasone, a positive control, intra-peritoneally, and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 157 mg/kg and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 314 mg/kg and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 785 mg/kg and exposed to sulfur dioxide. Cont: control; Dexa: dexamethasone; PGT: Pyunkang-hwan (Pyunkang-tang) extract; S: sulfur dioxide. Compared with normal control group, $^aP < 0.05$; compared with sulfur dioxide group, $^bP < 0.05$.

157 mg/kg, SO_2 plus PGT 314 mg/kg, SO_2 plus PGT 785 mg/kg and SO_2 plus dexamethasone 0.5 mg/kg groups, respectively. Dexamethasone, the positive control, significantly inhibited mucin secretion and the increase of mucosubstances (acidic mucins) in tracheal tissues, because of its strong anti-inflammatory activity.

Effect of PGT on histopathologic changes in lung tissue with BLM-induced pulmonary fibrosis

To elucidate histopathological changes associated with BLM-induced pulmonary fibrosis and the efficacy of PGT, left lungs from the different treatment groups were collected at day 28 post-BLM administration. Sections were stained with Masson trichrome to identify collagen. Sections from the control group displayed normal structures and no pathologic changes under a light microscope. In the BLM-only group, marked histopathologic changes, such as large fibrous areas (deposited collagen, stained gray to greenish blue) and collapsed alveolar spaces, were evident. In the BLM-PGT and BLM-dexamethasone groups, the extent of fibrosis was markedly less severe compared with in the BLM-only group, as shown in Figure 3.

Effect of PGT on lipid peroxidation in lung tissue with BLM-induced pulmonary fibrosis

The extent of lipid peroxidation was determined by measuring lung MDA levels. MDA levels in the lung of the BLM-only group were significantly increased compared with the control group. MDA levels in the BLM-PGT groups and the BLM-dexamethasone group were lower than in the BLM-only group, as shown in Figure 4.

Effect of PGT on hydroxyproline content in lung tissue with BLM-induced pulmonary fibrosis

Deposition of collagen in lung tissue is an established biomarker of pulmonary fibrosis and hydroxyproline level is an index of collagen content. The concentration of hydroxyproline on day 28 after BLM instillation was significantly higher in the BLM-only group than in the normal control group. In the groups treated with PGT, the hydroxyproline content was significantly lower than that in the BLM-only group (Figure 5).

Effect of PGT on murine fibroblast type I collagen synthesis in vitro

TGF-β significantly increased production of soluble collagen by MLg 2908 cells. The increase in soluble collagen levels induced by TGF-β was significantly inhibited by treatment of the cells with PGT (Figure 6).

DISCUSSION

Hypersecretion of airway mucus is one of the major symptoms associated with severe respiratory inflammatory diseases. There are two methods of removing excess mucus from the airways: (a) eliminating the mucus by physical methods such as aspiration after dilution of mucus, and (b) pharmacological suppression of mucus secretion. However, in patients, the physical method irritates the airway luminal wall and stimulates hypersecretion of mucus through a reflex mechanism. Therefore, pharmacological regulation of mucin secretion is important to control airway mucus hypersecretion. Though glucocorticoids inhibit hypersecretion of airway mucins, they have various limitations to their use for this application.[1] An alternative approach for controlling airway mucus hypersecretion would be to regulate excessive mucin secretion using traditional herbal medicines that are already used for management of respiratory inflammatory diseases. Modulation of inflammatory reactions would be one of the most appropriate courses of action for treating diverse pulmonary diseases, including asthma, cystic fibrosis and chronic obstructive pulmonary diseases. It is quite difficult to achieve such modulation with conventional pharmacological agents, while traditional herbal medicines are potentially an alternative solution, as indicated by their multiple actions to alleviate the plural symptoms observed in diverse respiratory inflammatory diseases.

Based on this information, we investigated the effects of PGT on secretion of airway mucins in an experimental animal model involving hyperplasia of
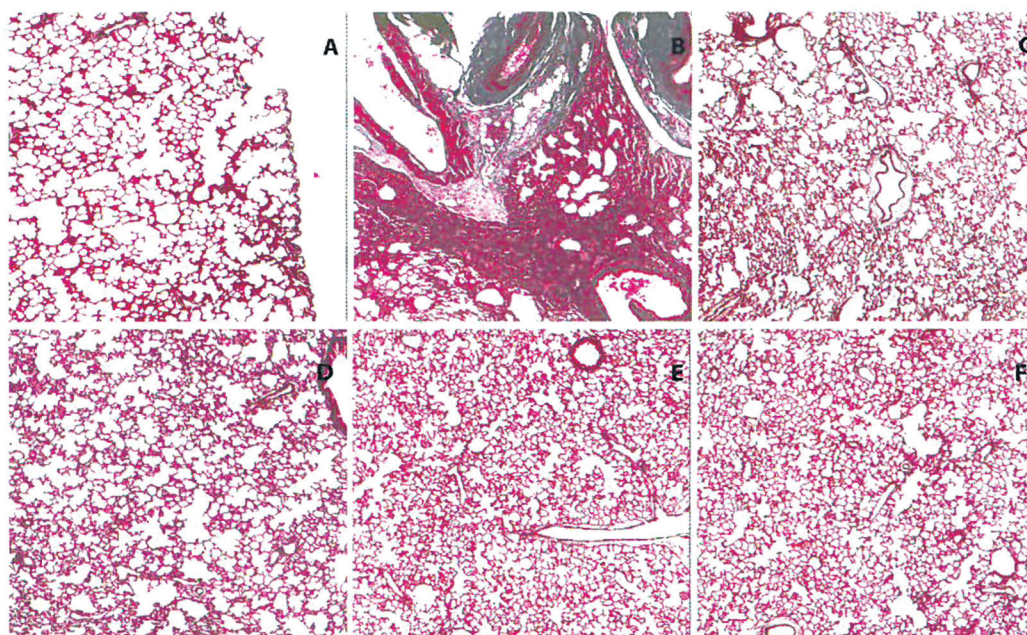
Figure 3 Effects of PGT on histopathologic changes of the lung induced by bleomycin

Rats were exposed to BLM intratracheally to induce fibrosis and treated as described in Materials and Methods. Twenty-eight days post-BLM, samples were stained with Masson trichrome and all panels are shown at the same magnification, original magnification × 40. A: normal control group, rats were treated by no specific agent and exposed to fresh and clean air; B: bleomycin group, rats were treated by no specific agent and exposed to bleomycin; C: section of trachea from rat treated by dexamethasone, a positive control, intraperitoneally, and exposed to bleomycin; D: section of trachea from rat treated by PGT 157 mg/kg and exposed to bleomycin; E: section of trachea from rat treated by PGT 314 mg/kg and exposed to bleomycin; F: section of trachea from rat treated by PGT 785 mg/kg and exposed to bleomycin. BLM: bleomycin; PGT: Pyunkang-hwan (Pyunkang-tang) extract.
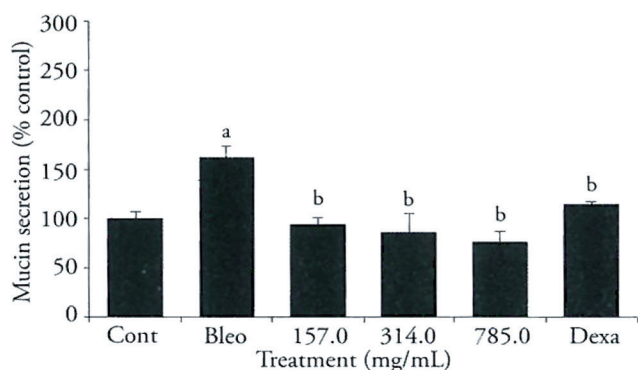

Figure 4 Effect of PGT on MDA levels in lung tissue from rats with BLM-induced pulmonary fibrosis

The experiment was conducted as described for Figure 3, with lung tissue collected twenty-eight days after BLM. Except for the control, all groups received BLM. Normal control group, rats were treated by no specific agent and exposed to fresh and clean air; Sulfur dioxide group, rats were treated by no specific agent and exposed to sulfur dioxide; Section of trachea from rat treated by dexam-n ethasone, a positive control, intraperitoneally, and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 157 mg/kg and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 314 mg/kg and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 785 mg/kg and exposed to sulfur dioxide. Cont: control; Bleo/BLM: bleomycin; Dexa: dexamethasone; MDA: malondialdehyde; PGT: Pyunkang-hwan (Pyunkang-tang) extract. Compared with normal control group, $^aP<0.05$; compared with sulfur dioxide group, $^bP < 0.05$.

goblet cells and mucus hypersecretion. Orally administered PGT inhibited both the increase of acidic mucins in goblet cells of tracheal tissues and mucin
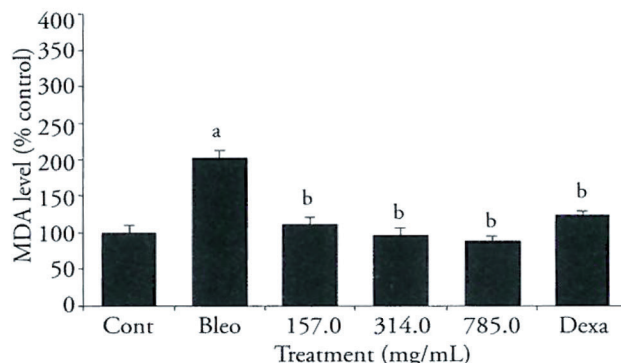
Figure 5 Effect of PGT on hydroxyproline content in rats with BLM-induced pulmonary fibrosis

Normal control group, rats were treated by no specific agent and exposed to fresh and clean air; Sulfur dioxide group, rats were treated by no specific agent and exposed to sulfur dioxide; Section of trachea from rat treated by dexamethasone, a positive control, intraperitoneally, and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 157 mg/kg and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 314 mg/kg and exposed to sulfur dioxide; Section of trachea from rat treated by PGT 785 mg/kg and exposed to sulfur dioxide. Cont: control; Bleo/BLM: bleomycin; Dexa: dexamethasone; PGT: Pyunkang-hwan (Pyunkang-tang) extract. Compared with normal control group, $^aP<0.05$; compared with sulfur dioxide group, $^bP < 0.05$.

secretion in BALF (Figures 1 and 2). To our knowledge, this is the first report on the inhibitory activity of a complex traditional herbal medicine on mucin secretion and the amount of mucosubstances (acidic mucins) in tracheal tissues in animals with pulmonary inflammation.
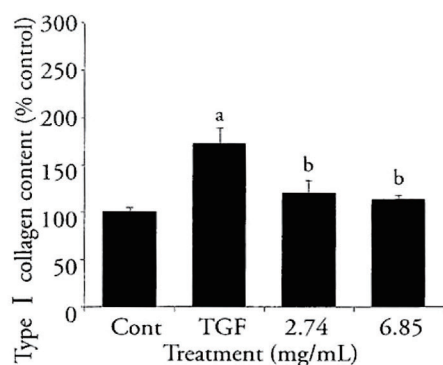
Figure 6 Effect of PGT on TGF-β-stimulated increase in type I collagen synthesis in MLg 2908 cells *in vitro*

Cells were cultured and treated as described in Materials and Methods. Except for the control, all samples were treated with TGF-β (10 ng/mL). Some cells also received PGT at the indicated concentrations of 2.74 or 6.85. Cont: control; PGT: Pyunkang-hwan (Pyunkang-tang) extract; TGF: transforming growth factor-β. Concentration units are mg/mL. Compared with normal control group, $^aP < 0.05$; compared with TGF-β, $^bP < 0.05$.

In addition, to investigate the potential therapeutic effects of PGT on IPF, PGT was administered orally to rats with BLM-induced pulmonary fibrosis and the toxicity to the pulmonary system was assessed through histology and through measurement of MDA and hydroxyproline in lung tissue 28 days after BLM treatment. We found that PGT decreased the characteristic histopathological features of pulmonary fibrosis. BLM binds to iron (Fe^{2+}), undergoes redox cycling, and catalyzes formation of reactive oxygen species (ROS).[20,21] Free radicals target macromolecules, such as protein, lipids, and DNA, leading to progression of lipid peroxidation and resulting in lung damage. Therefore, overproduction of free-radicals plays an important role in BLM-induced pulmonary fibrosis. In this pulmonary fibrosis model, BLM provoked a significant increase in lipid peroxidation, as reflected by MDA levels in the BLM-only group, and treatment with PGT significantly decreased BLM-stimulated lipid peroxidation. Therefore, it is possible that the protective effects of PGT on pulmonary fibrosis may, at least in part, be attributed to its ability to scavenge ROS generated in the pulmonary system. Increased collagen in the lung is associated with increased numbers of interstitial fibroblasts and increased collagen synthesis by these cells. One strategy to regulate pulmonary fibrosis would be to inhibit overproduction of collagen and fibroblast proliferation. We found that PGT decreased the hydroxyproline content, an indicator of collagen deposition, in the lung tissue, compared with that in the BLM-only group, *in vivo*. In addition, PGT inhibited the synthesis of type I collagen stimulated by TGF-β *in vitro*. These findings were consistent with our histopathological observations that PGT inhibited BLM-induced lung fibrosis.

Taken together, these results might explain, at least in

part, the effectiveness of PGT as an herbal remedy for treating several pulmonary inflammatory diseases that are accompanied by hypersecretion of sticky mucus. Additionally, our results suggested that PGT can be used as a remedy for various respiratory diseases provoked by air pollution, since PGT ameliorated mucus hypersecretion induced by exposure to SO_2, a representative air pollutant produced by the combustion of fossil fuels, including coal. Furthermore, our results showed that oral administration of PGT ameliorated BLM-induced pulmonary fibrosis, with the beneficial effects of PGT included inhibiting lipid peroxidation induced by BLM *in vivo* and decreasing collagen synthesis both *in vivo* and *in vitro*. The underlying mechanism of action of PGT on pulmonary fibrosis is not clear at present, though we are investigating whether PGT acts as a potential regulator of the NF-kB signaling pathway in murine lung fibroblast cells. It is important to find traditional herbal medicines and related natural products with specific inhibitory effects on pulmonary fibrosis, to advance both basic and clinical science. Though further studies are required, the results from this study showed that PGT is a potential therapeutic agent for pulmonary fibrosis.

REFERENCES

1   **Evans CM**, Koo JS. Airway mucus: the good, the bad, the sticky. Pharmacol Ther 2009; 121(3): 332-348.

2   **Hunninghake GW**, Kalica AR. Approaches to the treatment of pulmonary fibrosis. Am J Respir Crit Care Med 1995; 151(3 Pt 1): 915-918.

3   **Mapel DW**, Samet JM, Coultas DB. Corticosteroid and treatment of idiopathic pulmonary fibrosis. Past, present and future. Chest 1996; 110(4): 1058-1067.

4   **Lasky JA**, Ortiz LA. Antifibrotic therapy for the treatment of pulmonary fibrosis. Am J Med Sci 2001; 322 (4): 213-221.

5   **Mason RJ**, Schwarz MI, Hunninghake GW, Musson RA. NHLBI workshop summary pharmacological therapy for idiopathic pulmonary fibrosis. Past, present and future. Am J Respir Crit Care Med 1999; 160(5 Pt 1): 1771-1777.

6   **Hay J**, Shahzeidi S, Laurent G. Mechanisms of bleomycin-induced lung damage. Arch Toxicol 1991; 65 (2): 81-94.

7   **Zia S**, Hyde DM, Giri SN. Development of a bleomycin hamster model of subchronic lung fibrosis. Pathology 1992; 24(3): 155-163.

8   **Lazenby AJ**, Crouch EC, McDonald JA, Kuhn C, 3rd ed. Remodeling of the lung in bleomycin-induced pulmonary fibrosis in the rat. An immunohistochemical study of laminin, type IV collagen, and fibronectin. Am Rev Respir Dis 1990; 142(1): 206-214.

9   **Thrall RS**, Swendsen CL, Shannon TH, et al. Correlation of changes in pulmonary surfactant phospholipids with compliance in bleomycin-induced pulmonary fibrosis in the rat. Am Rev Respir Dis 1987; 136(1): 113-118.

10 Crouch E. Pathology of pulmonary fibrosis. Am J Physiol 1990; 259(4 Pt 1): L159-L184.

11 Aso Y, Yoneda K, Kikkawa Y. Morphologic and biochemical study of pulmonary changes induced by bleomycin in mice. Lab Invest 1976; 35(6): 558-568.

12 Jules-Elysee K, White DA. Bleomycin-induced pulmonary toxicity. Clin Chest Med 1990; 11(1): 1-20.

13 Lazo JS, Hoyt DG, Sebti SM, Pitt BR. Bleomycin: a pharmacologic tool in the study of the pathogenesis of interstitial pulmonary fibrosis. Pharmacol Ther 1990; 47 (3): 347-358.

14 Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. Int J Exp Pathol 2002; 83(3): 111-119.

15 Osanai K, Takahashi K, Sato S, et al. Changes of lung surfactant and pressure-volume curve in bleomycin-induced pulmonary fibrosis. J Appl Physiol 1991; 70(3): 1300-1308.

16 Hagiwara SI, Ishii Y, Kitamura S. Aerosolized administration of N-acetylcysteine attenuates lung fibrosis induced by bleomycin in mice. Am J Respir Crit Care Med 2000; 162(1): 225-231.

17 Oury TD, Thakker K, Menache M, Chang LY, Crapo JD, Day BJ. Attenuation of bleomycin-induced pulmonary fibrosis by a catalytic antioxidant metalloporphyrin. Am J Respir Cell Mol Biol 2001; 25(2): 164-169.

18 Daba MH, Abdel-Aziz AA, Moustafa AM, Al-Majed AA, Al-Shabanah OA, El-Kashef HA. Effects of L-carnitine and ginkgo biloba extract (EG b 761) in experimental bleomycin-induced lung fibrosis. Pharmacol Res 2002; 45 (6): 461-467.

19 Jang IM. Treatise on Asian herbal medicines. Seoul: Haksul-pyunsu-kwan in Research institute of natural products of Seoul National University, 2003: 1438.

20 Gutteridge JM, Xiao Change F. Protection of iron-catalysed the radical damage to DNA and lipids by copper (II) bleomycin. Biochem Biophys Res Commun 1981; 99(4): 1354-1360.

21 Martin WJ 2nd, Kachel DL. Bleomycin-induced pulmonary endothelial cell injury: evidence for the role of iron-catalyzed toxic oxygen-derived species. J Lab Clin Med 1987; 110(2): 153-158.