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Lilium lancifolium Thunb. extract attenuates pulmonary inflammation and air space enlargement in a cigarette smoke-exposed mouse model

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ABSTRACT

Ethnopharmacological relevance: *Lilium lancifolium* Thunb. (Liliaceae) has long been used as a traditional medicine in Korea and China to treat bronchitis, pneumonia, and other pulmonary ailments.
Aim of the study: Cigarette smoke (CS) is a major risk factor for the development of pulmonary inflammatory response; it also triggers pulmonary alveoli enlargement. In the present study, we investigate the effects of *Lilium lancifolium* Thunb. root extract on pulmonary inflammatory responses in a CS-exposed mouse model.
Materials and methods: Water extract of *Lilium lancifolium* Thunb. root was fed to C57BL/6 mice prior CS exposure every day for 3 weeks. The numbers of macrophages and neutrophils in bronchoalveolar lavage fluid (BALF) were counted. The relative inflammatory factors, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), monocyte chemotactic protein-1 (MCP-1), and matrix metalloproteinase-12 (MMP-12) were measured by real-time PCR, ELISA, or Western blot analysis. The average alveoli size was determined by lung histology.
Results: *Lilium lancifolium* Thunb. root extract was found to significantly inhibit the numbers of macrophages and neutrophils in BALF due to CS exposure. *Lilium lancifolium* Thunb. root extract also reduced the protein secretion levels of TNF-α, IL-6, IL-1β, and MCP-1 in BALF and the RNA expression levels of TNF-α, IL-6, IL-1β, MCP-1, and MMP-12 in lung tissue compared with mice only exposed to CS. Moreover, MMP-12 in serum was down regulated in *Lilium lancifolium* Thunb. root extract treated mice compared with CS-exposed mice. Finally, a morphometric analysis of the lungs of *Lilium lancifolium* Thunb. root extract treated mice demonstrated a significant reduction in airspace size compared to mice only exposed to CS.
Conclusion: Our results show that *Lilium lancifolium* Thunb. root extract reduces lung inflammation and airspace enlargement in a CS-exposed mouse model. These data indicate that *Lilium lancifolium* Thunb. root extract is a therapeutic candidate for pulmonary inflammation and emphysema caused by CS.
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1. Introduction

Cigarette smoke (CS) is a toxic mixture of 4000 chemicals, making inhaled CS hazardous to the human body. It reaches deep into the lungs, causing several problems (Canales et al., 2012). One such problem is pulmonary inflammation, and this inflammation evokes various lung diseases, including severe chronic bronchitis, emphysema, and chronic obstructive pulmonary disease (COPD) (Churg et al., 2004). These diseases are characterized by enlarged airspaces, narrowing of small airways, and destruction of the lung extracellular matrix. Furthermore, an increase in activated macrophages and neutrophils is seen in the sputum of patients with these diseases (Barnes et al., 2003).

Generally, various cytokines and chemokine are assumed to regulate the activation and recruitment of inflammatory cells into the airways during inflammatory responses (Bracke et al., 2006). Increased cytokine and chemokine profiles of TNF-α, IL-6, IL-1β, and MCP-1 have been observed after CS exposure (Kubo et al., 2005).

The normal responses to CS include an increase in inflammatory cells such as macrophages and neutrophils (Ofulue et al., 1998; Rinaldi et al., 2012). In particular, macrophages directly release inflammatory mediators, which promote both inflammation and tissue-destructive emphysema (Maeno et al., 2007). These cells can secrete proteases such as matrix metalloproteinase (MMP) (Shapiro and Senior, 1999; Shapiro et al., 2003). MMP-12, which is released by macrophages, is also a major chemotactic factor for macrophages in CS-induced lung disease (Houghton et al., 2006), and mediates the release of TNF-α (Churg et al., 2002, 2003). TNF-α, a central cytokine in the immune response to CS, regulates inflammation through endothelial activation and boosts

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neutrophil recruitment. Accumulated neutrophils in bronchoalveolar lavage fluid (BALF) also secrete injurious products such as tissue-destructive proteases (MacGregor et al., 1997; Churg et al., 2003).

Herbal extracts have long been used as part of traditional Korean medicine, and have been used effectively to treat a variety of inflammatory disorders (Lim et al., 2009; Oh et al., 2009; Ra et al., 2010; Xue et al., 2011). For example, according to the DonguiBogam, a book edited by the royal physician Heo Jun and published in 1613 during the Joseon Dynasty of Korea, the root of *Lilium lancifolium* Thunb.(Liliaceae) (LL) can be used to treat pulmonary disease, cough, bronchitis, pneumonia, asthma, and other conditions. One recent report characterized the anti-inflammatory activity of LL on lipopolysaccharide-stimulated Raw 264.7 cells (Kwon et al., 2010), while another report demonstrated the antibacterial activities of various LL extracts (Tsai et al., 2001). However, there have yet to be any studies characterizing LL activity in reducing pulmonary inflammation in a CS-exposed mouse model. In the present study, we investigate the effect of LL extract therapy on various inflammatory responses in CS-exposed C57BL/6 mice.

2. Materials and methods

2.1. Preparation of LL

LL granules (The root of *Lilium lancifolium* was extracted with 100 °C water and the lyophilized extract was granulated with excipient containing starch.) were purchased from Sun Ten Pharmaceutical (Taipei, Taiwan). It was weighed to precisely 50 mg and then dissolved in 10 ml of distilled water (DW) by stirring overnight at room temperature. Next, the solution was centrifuged for 10 min at 3000 rpm (Eppendorf, Hamburg, Germany), after which the supernatant was sterilized by passing the solution through a 0.22 μm syringe filter. The stock was stored at 4 °C until use. And also, for the Ultra performance liquid chromatography (UPLC) analysis, 20 mg of LL granules were extracted with 100 ml of methanol by sonication for 2 h at 50 °C to remove excipient. The extract was filtered with filter paper (Hyundai Micro Co., Ltd., Korea). The residual was dissolved in 930 μl of 5% acetonitrile and 70 μl of DMSO to yield a final concentration of 46 mg/ml.

2.2. UPLC–ESI–MS analysis

UPLC (Ultra performance liquid chromatography) instrument ACQUITY UPLC H-Class system running Empower software (Milford, USA). The PDA detector was recorded between 210 and 400 nm. The Brownlee SPP C18 column (2.1 × 75 mm, 2.7 μm) was selected for the UPLC study (PerkinElmer, USA). The monitoring wavelength was set to 280 nm. The mobile phase was comprised of acidified acetonitrile with formic acid (0.1%, solvent A) and acidified water with formic acid (0.1%, solvent B). All solvents were filtered through a 0.2 μm filter. The gradient program was 0–2 min, 2% of solvent A; 4 min, 7% of solvent A; 5.5 min, 15% of solvent A; 10.5 min, 20% of solvent A; 13.5 min, 24% solvent A; 18.5 min, 100% solvent A, at a flow rate of 0.2 ml/min using a commercial splitter. The injection volume was 2 μl.

AccuTOF® single-reflectron time-of-flight mass spectrometer was equipped with an ESI source (Electrospray ionization, JEOL, USA) and was operated with Mass Center system version 1.3.7b (JEOL, USA). In the positive ion mode, the atmospheric pressure interface potentials were typically set to the following values: orifice 1 = 80 V and ring lens and orifice 2 = 10, 5 V, respectively. The ion guide potential and detector voltage were set to 2000 V and 2300 V, respectively. ESI parameters were set as follows: needle electrode = 2000 V, nitrogen gas was used as a nebulizer, desolating and their flow rate were 1 and 3 l/min, desolating chamber temperature = 250 °C, orifice 1 temperature = 80 °C. Mass scale calibration was accomplished with YOKUDELNA calibration kit (JEOL, Japan) for accurate mass measurements and calculations of the elemental composition. MS acquisition was set with a scan range of *m*/*z* 100–2000.

2.3. Animals

Specific pathogen-free female C57BL/6 mice aged 6–7 weeks and weighing 16–17 g were purchased from Orient Bio Inc. (Seoul, South Korea). The animals were housed at 20 °C on a 12:12-h day–night cycle in sterile micro-isolators and fed a standard sterile rodent diet (Purina Mills, St. Louis, MO, USA) with water given ad libitum. Individual body weights were measured every other day on a balance with an accuracy of 0.1 g (Sartorius, Gottigen, Germany) and averaging functions to correct for animal movement. All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of Kyung Hee University.

2.4. Cigarette smoke exposure and drug administration

Female C57BL/6 mice were divided into four groups: a control group (CON) exposed to air and treated with DW. The other groups were exposed to CS for 3 weeks. The CS group was exposed to CS and DW only, the DEX group was exposed to CS with 1 mg/kg of dexamethasone, and the LL group was exposed to CS with 50 mg/kg of LL extract. In a preliminary dose-response study, three LL extract amounts (50 mg/kg, 100 mg/kg, and 150 mg/kg) were assessed anti-inflammatory effects. The results of the three concentrations were similar (data shown in the graphical abstract); therefore, among these three concentrations, 50 mg/kg was chosen as the experimental study concentration of LL extract. Mice were exposed to air in a whole-body chamber or to CS from reference cigarettes 3R4F (University of Kentucky, Lexington, Kentucky) using a smoking apparatus. Exposures were conducted for 2 h/day (3 cigarettes, 1 h fresh air break after each 30 min period of exposure), 5 day/week for 3 weeks. DEX and LL were administered by oral gavages daily before the first CS exposure. Mice were sacrificed after 3 weeks.

2.5. Collections of serum and BALF

Prior to the mice sacrifice, blood was collected and sera were obtained by centrifugation and stored at −80 for western blot analysis. And after the sacrifice, PBS (phosphate-buffered saline, pH 7.2; kept at room temperature) was slowly infused into the lungs and withdrawn via a cannula inserted through the trachea. Retrieved PBS was kept on ice. After three lavages, the BALF was centrifuged for 10 min at 1300 rpm. Cell concentrations were then determined using a hemocytometer, and differential cell counts were measured on slides prepared by cytocentrifugation and Diff-Quick staining. After approximately 500 cells were counted, the BALF were centrifuged and the cell-free supernatant was stored at −80 °C until subsequent measurement of TNF-α, IL-6, IL-1β, and MCP-1.

2.6. RNA extraction and real-time PCR

Real-time PCR was performed to measure the expressions of TNF-α, IL-6, IL-1β, MCP-1 and MMP-12 using a Thermal Cycler Dice Real Time PCR System (Takara, Japan). Total RNA was extracted from the whole lung. The tissue was lysed in 500 μl of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), after which total RNA was isolated according to the manufacturer's protocols. RNA (2 μg) was

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