Ligustrum lucidum Fruits Extract Inhibits Acute Pulmonary Inflammation in Mice

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ABSTRACT: Traditionally, Ligustrum lucidum fruits (LL) is one of the well-known oriental herb used in the treatment of skin and lung inflammation. This study investigated anti-inflammatory effects of LL in the pathogenesis of acute pulmonary inflammation in mice. Acute pulmonary inflammation was induced by intratracheal instillation of cigarette smoke condensate (CSC) and lipopolysaccharide (LPS) 5 times within 12 days in mice. LL extract was administered orally at a dose of 50 or 200 mg/kg. Administration of LPS and CSC significantly elevated airway hyperresponsiveness (AHR) to mice, and increased in the levels of inflammatory cells and mediators in mice. However, the LL extract significantly reduced the elevated AHR, and the increase of neutrophils, CD4+/CD3+ cells and CD8+/CD3+ cells, along with reducing the expression of TNF-α, IL-6, and MIP-2. Moreover, the LL extract alleviated the infiltration of inflammatory cells in expanded airway walls histologically. These results indicate that the LL extract has an inhibitory effects on acute pulmonary inflammation and AHR in murine model, and plays a crucial role as an immunomodulator which possess anti-inflammatory property.

Key Words: Ligustrum lucidum fruits, Acute Pulmonary Inflammation, Cigarette Smoke Condensate, Lipopolysaccharide

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease in the lungs and airways characterized by airflow restriction. These inflammations in the lungs are associated with the recruitment of inflammatory cells and mediators, an imbalance between proteases and anti-proteases and the oxidative stress (MacNee, 2003). In particular, the inflammatory cells such as neutrophils, macrophages and lymphocytes have a pivotal role in the pathogenesis of COPD. In addition, the increase of inflammatory mediators is related to the accumulation of inflammatory cells at the inflammatory site in the lungs. These responses are observed at the parts of injured lungs and airways (Kwak and Lim, 2011; Sarir et al., 2008).

The inflammatory responses in COPD are caused by several reasons including smoking, industrial dusts and chemicals, indoor and outdoor air pollution, genetic factors. Cigarette smoke is the best risk factor among the reasons for COPD (MacNee, 2003). The exposure to cigarette smoke is implicated in abnormal immune responses as well as the acute damage of lungs (Taylor, 2010). Hence, cigarette smoke has been used to induce of the pulmonary diseases as the main source (Brusselle et al., 2006; Chan et al., 2009; Zheng et al., 2009). Lipopolysaccharide (LPS) is a compound of cigarette smoke which has been used to induce acute pulmonary diseases in murine models (Brusselle et al., 2006). Some studies have induced acute
respiratory diseases using cigarette smoke and LPS (Doz et al., 2008; Le Quement et al., 2008).

In oriental medicine, the fruits of *Ligustrum lucidum* Ait. (LL, Oleaceae) have been used for protecting kidney and liver, and this plant has been reported to have beneficial effects such as anti-inflammatory, anti-tumor, anti-aging and immunomodulatory activities (Lin et al., 2007). LL contains various compounds such as triterpenes, flavonoids and glucoside (He et al., 2001). In particular, oleanolic acid and ursolic acid, the main bioactive compounds of LL, have been reported to have inhibitory effects on respiratory inflammation (Santos et al., 2011; Kim et al., 2013). In spite of these results, no studies have reported an inhibitory effect of LL on acute pulmonary inflammation as medicinal herb. Therefore, the present study investigates the protection effect of LL extracts on acute pulmonary inflammation induced by CSC and LPS in mice.

**MATERIALS AND METHODS**

1. **Materials and regents**

*Ligustrum lucidum* fruits (LL) was purchased from the Kyoungdong Herb Co. (Seoul, Korea) and its quality was assessed by the Korean pharmaceutical traders association (Seoul, Korea). LL (100 g) was extracted in boiling water (distilled water, 2 L) for 3 hours. The yield of water extracts from LL was 18.23%. After filtration, the solvent was evaporated under vacuum and the extract was then lyophilized by freeze-drying. The freeze-dried crude extract was stored in the deep-freezer (−84°C) until use.

CORESTA Monitoring Cigarette No. 6 (CM6) was purchased from ITG Reemtsma Tobacco Co. (Berlin, Germany). Anti-mouse-CD3-PE (phycoerythrin), anti-mouse-CD4-FITC (fluorescein isothiocyanate), anti-mouse-CD8-FITC, anti-mouse-neutrophil mAb, and anti-mouse-IgG-FITC were purchased from Pharmingen Co. (San Diego, California, USA). IL-6 and TNF-α ELISA kits were purchased from BioSource Co. (Camarillo, California, USA). MIP-2 assay kits were purchased from IBL-America Co. (Aramachi, Japan). Cyclosporin A (CSA) was purchased from Choongwae Pharma Co. (Seoul, Korea). All other chemicals used in the study were of special reagent grade.

2. **Cigarette smoke condensate collection**

CM 6 reference cigarettes were conditioned by ISO conditioning at a temperature of 22 ± 2°C, and a relative humidity of 60% ± 5% for 48 h or more. The reference cigarettes were smoked under ISO conditions (puff volume, 35 mL; duration, 60 s; interval, 2 s), by using an automatic smoking machine (Borgwaldt RM20, Germany). Cigarette smoke condensate (CSC) was trapped in a 92 mm Cambridge filter pad, and then extracted with methanol for 3 days at room temperature. After extraction, the solvent was evaporated under vacuum.

3. **Animals and acute pulmonary inflammation model**

Specific pathogen-free (SPF) female BALB/c mice (8-weeks; weight 18-20 g) were purchased from Orient Bio. Inc. (Sungnam, Korea). The protocols for experiments on animals were approved by the Institutional Animal Care and Use Committee of Daejeon University. The mice were housed in a room maintained at 22 ± 2°C, with relative humidity of 55% ± 5% and a 12-h/12-h light/dark cycle. They were given a standard laboratory diet of solid feed (Samyang feed, Korea), and tap water was provided ad libitum. The animals were divided into 5 groups as follows: [1] Normal group (n = 6) was given water; [2] Control group (n = 6), given LPS and CSC; [3] LPS + CSC + cyclosporin A (CSA) group (n = 6), treated with LPS, CSC, and CSA (10 mg/kg); [4] LPS + CSC LL (50 mg/kg) group (n = 6) treated with LPS, CSC, and LL extracts (50 mg/kg); and [5] LPS + CSC + LL (200 mg/kg) group (n = 6) treated with LPS, CSC, and LL extracts (200 mg/kg).

Acute pulmonary inflammation was induced by intratracheal injection of 50 µL of LPS (50 g/mL in PBS) and 50 µL of CSC (10 mg/mL in PBS) for 5 days, after intraperitoneal administration of anesthetics according to the method of Kwak and Lim (2011). On the 5th day, blood was collected from the retro-orbital plexus, and the neutrophil count was measured by flow cytometry to select the mice in which acute pulmonary inflammation had been successfully induced. A crude extract of LL (50 or 200 mg/kg) or 10 mg/kg CSA (used as a positive control) was orally administered to the mice for 12 days (Kwak and Lim, 2011). The following day, the mice were sacrificed.

4. **Measurement of airway hyperresponsiveness (AHR)**

Enhanced pause (Penh), a unitless measure of AHR, was estimated using a plethysmograph (BUXCO BioSystem
XA, USA). Mice were placed in a whole-body chamber, and the baseline airflow was measured for 30 min. Methacholine dissolved in PBS at concentrations of 12.5, 25, and 50 mg/mL was delivered via aerosol for 3 min. After nebulization, Penh was recorded by a flow transducer for 10 min, and calculated using the following calculation: Penh = (Te-Tr)/Tr-PEP/PIP (where Te is the expiratory time, Tr is the relaxation time, PEP is the peak expiratory pressure, and PIP is the peak inspiratory pressure). AHR was measured by a methacholine challenge test on the 11th day.

5. Collection of BALF and enumeration of cells
Following 11 days of LPS and CSC administration, the trachea and airways of the anaesthetized mice were lavaged 3 times using a syringe (containing 1 mL of 10 % FBS/DMEM medium) to obtain bronchoalveolar lavage fluid (BALF)-containing cells from the trachea and the lungs. The BALF was treated with ammonium chloride (ACK) solution for 5 min at 37°C. BALF cells were washed with D-BPS, stained with 0.04% trypan blue, and then, counted. Following bronchoalveolar lavage, the mice lungs were dissected and weighed.

6. Flow cytometry
After enumeration, the BALF cells were stained using immunofluorescence, by incubation with anti-mouse-CD3, anti-mouse-CD4-FITC, anti-mouse-CD8-FITC, anti-mouse-neutrophil mAb, and anti-mouse-IgG-FITC for 30 min at 4°C. Then, all the cells were washed 3 times with PBS. Cluster of differentiation antigen CD4+/CD3+, CD8+/CD3+, and neutrophils were analyzed with a flow cytometer using Cell Quest software, and the absolute numbers in each tissue were calculated on the basis of the total cell count.

7. Measurement of inflammatory mediator levels
The levels of macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor-a (TNF-α), and interleukin-6 (IL-6) in the serum and BALF were determined using ELISA kits, according to the manufacturer's instructions (BioSource for TNF-α and IL-6 and IBL-America for MIP-2).

8. Histological analysis
The dissected lung tissue was fixed in a 10% formaldehyde solution for 24 h, rinsed in water for 8 h, dehydrated, and then, embedded in paraffin by standard methods. Tissue sections, 5 µm thick, were stained with hematoxylin-eosin (H&E) and Masson-Trichrome (M-T) to aid examination by light microscopy.

9. Statistical analysis
Data are expressed as a mean± standard error of the mean (SEM). Statistical analysis of comparisons between groups was performed using Student's T test. Significance was assigned using a p-value of 0.05 or 0.01.

RESULTS

1. Inhibitory effects of LL on AHR
AHR to methacholine was determined in the Penh level. Dose-response curves for the Penh level are shown in Figure 1. Penh value changes are indicative of the severity of airflow obstruction and are associated with the respiratory disease such as COPD and asthma. Additionally, enhanced AHR was observed in COPD patients. (Hamelmann et al., 1997; Daheshia, 2005). Compared with the normal group, the LPS + CSC group showed a significant increase in Penh level in response to methacholine doses of 25 and 50 mg/ml (p < 0.01). However, the increased Penh level was significantly reduced by the treatment with LL (200 mg/kg) group, compared with the LPS and CSC challenged group.

2. Effects of LL on inflammatory cells in BALF
To identify the inhibitory effects of LL on acute pulmonary inflammation, the inflammatory cells in the mouse BALF were counted using flow cytometry (Table 1). The number of neutrophils was significantly increased by the treatment with LPS + CSC (p < 0.01). The treatment with LL (50 and 200 mg/kg) and CSA (10 mg/kg) inhibited the increase of neutrophils by the LPS + CSC (p < 0.01). The number of CD4+/CD3+ and CD8+/CD3+ cells significantly increased in the LPS + CSC treated inflammatory group (p < 0.05). The increased CD4+/CD3+ and CD8+/CD3+ cells were inhibited by the treatment with LL (50 and 200 mg/kg), CSA (10 mg/kg) also inhibited the increase of CD4+/CD3+ and CD8+/CD3+ cells (p < 0.01).

3. Effects of LL on the inflammatory mediators in BALF
The concentrations of inflammatory mediators in the BALF are presented in Table 2. Levels of TNF-α, IL-6
and MIP-2, inflammatory mediators, in BALF were significantly elevated by the treatment with LPS + CSC ($p < 0.01$). However, the concentrations of the elevated inflammatory mediators was significantly reduced by the treatment of LL (50 and 200 mg/㎏) ($p < 0.05$) and CSA (10 mg/㎏) ($p < 0.01$).

### DISCUSSIONS

COPD include various respiratory diseases such as chronic obstructive lung disease and emphysema and is involved in the acute pulmonary inflammation. Moreover, these inflammatory responses play a pivotal role in the pathogenesis of the respiratory disease (MacNee, 2003). LPS and CSC have been associated with the development of COPD in animal models (Brusselle et al., 2006; Chan et al., 2009; Zheng et al., 2009). In addition, some studies have proven the acute inflammation in the lungs.
and airways induced by LPS and CSC (Doz et al., 2008; Le Quement et al., 2008). Many medicinal plants have been reported to have the medicinal effect for COPD. These medicinal natural products affect the pulmonary inflammation through traditional medication and experimental studies (Kim et al., 2011; Ram et al., 2011; Lim and Kim, 2012). LL has also been reported to have the anti-inflammatory effects (Xia et al., 2012). Therefore, we investigated the inhibitory effects of the LL extract on acute pulmonary inflammation induced by LPS and CSC.

Neutrophils play as indicator cells in the pathogenesis of respiratory disease. The migration of neutrophils is believed to contribute to structural changes in the airways and cause lung damage by creating an imbalance between proteases and anti-proteases (Quint and Wedzicha, 2007). The recruitment of inflammatory cells such as neutrophils and lymphocytes at the site of injured lung tissue is implicated in the development of acute pulmonary inflammation. In particular, numerous activated T lymphocytes are associated with lung damage and cytokine expression (Sarir et al., 2008). CD3+ as a protein is expressed on the surface of T cells. It develops a T-cell receptor (TCR) complex by association with the TCR and is involved in TCR signal transduction (Yamaguchi et al., 2008). CD4+ and CD8+, glycoproteins, are expressed on the surfaces of helper T and cytotoxic T cells, and they affects a subset of Class I and II MHC molecules to form antigen-presenting cells (Zamoyska, 1998). CD4+ cells play an important role in cell-mediated immunity, macrophage activation, B cell maturation, and allergic inflammation, whereas CD8+ cells have been reported to be related with cell-mediated immunity and antigen-specific cytotoxicity (Larosa and Orange, 2008). The number of T lymphocytes is indicative of the severity of COPD, and increased T lymphocyte counts is observed in the lungs and airways of COPD patients (Daheshia, 2005). In this study, we found that the enhanced accumulation of neutrophils and the elevated number of CD4+/CD3+ and CD8+/CD3+ cells by LPS and CSC decreased upon treatment with the LL extract in mice. These data indicate that the LL extract inhibits the increase of T lymphocytes in acute pulmonary inflammation induced by LPS and CSC.

Neutrophil accumulation is correlated with MIP-2 expression. Increased MIP-2 levels have been observed in damaged lung tissue and BALF (Tsujimoto et al., 2002). In addition, MIP-2 is also expressed by macrophages and epithelial cells, and it is thought to play a role in the inflammatory response and oxidative stress in the injured lung (Driscoll, 2000). Our results demonstrated that MIP-2 expression is linked with increased neutrophil counts and that LL extract may suppress the recruitment of neutrophils through reduced MIP-2 expression in the acute pulmonary inflammation induced by LPS and CSC. In addition, results of this study demonstrate that the LL extract has an inhibitory effect on both neutrophil and MMP-2 levels in acute pulmonary inflammation.

Various types of inflammatory mediators, including cytokines and chemokines, are linked with inflammatory reactions (Sarir et al., 2008). TNF-α is thought to play a key role in acute pulmonary inflammation, and it is expressed by neutrophils and T lymphocytes (Ram et al., 2011). In addition, TNF-α expression is associated with the infiltration of inflammatory cells (Ram et al., 2011). IL-6, an inflammatory and fibrogenic cytokine, also plays a critical role in the development of pulmonary inflammation and in the fibrotic process in lung tissue (Song et al., 2001). Furthermore, elevated levels of TNF-α and IL-6 are observed in the BALF of COPD patients (Song et al., 2001; Sarir et al., 2008). In this study, we found that the LL extract significantly decreased the elevated TNF-α and IL-6 levels by LPS and CSC in the BALF of acute pulmonary inflammation.

Infiltration of inflammatory cells in expanded airway walls is a histological change observed in the pathogenesis of COPD (MacNee, 2003). In addition, chronic inflammation in the bronchi causes a structural change in
the airway accompanied with collagen deposition (Zheng et al., 2009). Our histological results indicate that administration of the LL extract markedly reduced the infiltration of inflammatory cells and collagen deposition in the lung tissue. These results also support the protection effect of LL on acute pulmonary inflammation.

In conclusion, the results of this study indicate that LPS and CSC exacerbates AHR and induces the accumulation of inflammatory cells and mediators in the BALF, leading to histological changes. However, these inflammatory responses are inhibited by treatment with LL extract. Therefore, LL may be used as a natural, plant-based therapy for acute pulmonary inflammation related with COPD.

**LITERATURE CITED**


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