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생쥐에서 급성 폐렴에 대한 여정자 열매 추출물의 억제효과

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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 ㎎/㎏. 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 a 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). Lipopolysaccaride (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

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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 ℃) 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℃, 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 ㎜ 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℃, 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 ㎎/㎏); [4] LPS + CSC LL (50 ㎎/㎏) group (n = 6) treated with LPS, CSC, and LL extracts (50 ㎎/㎏); and [5] LPS + CSC + LL (200 ㎎/㎏) group (n = 6) treated with LPS, CSC, and LL extracts (200 ㎎/㎏).

Acute pulmonary inflammation was induced by intratracheal injection of 50 μL of LPS (50 g/mL in PBS) and 50 μL of CSC (10 ㎎/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 ㎎/㎏) or 10 ㎎/㎏ 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 ㎎/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 numeration 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℃. 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℃. 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 ㎛ 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 ㎎/ml ($p < 0.01$). However, the increased Penh level was significantly reduced by the treatment with LL (200 ㎎/㎏) 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 ㎎/㎏) and CSA (10 ㎎/㎏) 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 ㎎/㎏). CSA (10 ㎎/㎏) 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

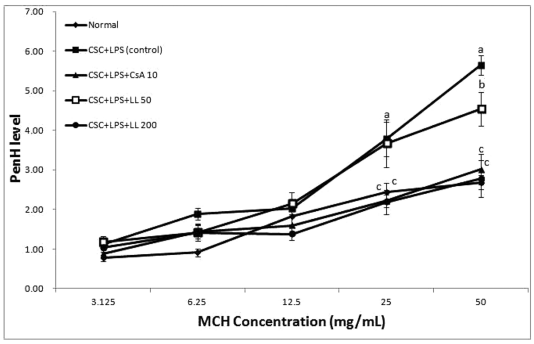

Fig. 1. Airway hyperresponsiveness by MCH in mice. Mice were treated with LPS (50 ㎍/mL) and CSC (10 ㎎/mL) by intratracheal injection 5 times within 12 days. Values are expressed as a mean ± SE (n = 5). Statistical analysis of the data was performed using Student's T-test. a;$p < 0.01$ compared with normal group. b;$p < 0.05$, c;$p < 0.01$ compared with control group. Normal ; Balb/c mice, LPS ; Lipopolysaccharide (50 ㎍/mL), CSC ; Cigarette smoke condensate (10 ㎎/mL), CSA ; Cyclosporine A (10 ㎎/㎏), LL ; Ligustrum lucidum fruits extract, MCH ; Metacholine.

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 ㎎/㎏) ($p < 0.05$) and CSA (10 ㎎/㎏) ($p < 0.01$).

4. Histological analysis

Infiltration of inflammatory cells into the tracheal and alveolar tissues was observed by H&E staining, while collagen deposition was assessed by M-T staining. In this study, an increased damage to the tracheal and alveolar tissues was observed, with infiltration of inflammatory cells and collagen deposition, in the LPS + CSC group compared with the normal group (Fig. 2A, B). In contrast, the LL (50 and 200 ㎎/㎏) and CSA (10 ㎎/㎏) treated groups showed reduced damage to the tracheal and alveolar tissues, as well as reduced infiltration of inflammatory cells and collagen deposition in the lung tissue compared with the LPS + CSC group (Fig. 2C, D, E).

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

Table 1. The number of inflammatory cells in BALF.

Group	Neutrophils (× 10^3)	$CD4^+/CD3^+$cells (× 10^3)	$CD8^+/CD3^+$cells (× 10^3)
Normal	0.216 ± 0.061	0.251 ± 0.059	0.013 ± 0.003*
CSC + LPS (Control)	2.113 ± 0.278^a	2.429 ± 0.036^a	0.460 ± 0.128^{a**}
CSC + LPS + CSA (10 ㎎/㎏)	0.199 ± 0.002^c	0.843 ± 0.116^c	0.156 ± 0.011^{b***}
CSC + LPS + LL (200 ㎎/㎏)	0.696 ± 0.072^c	0.785 ± 0.114^c	0.191 ± 0.042^b
CSC + LPS + LL (50 ㎎/㎏)	1.385 ± 0.070^c	1.559 ± 0.093^c	0.252 ± 0.065

*Values are expressed as the mean ± SE (n = 6).
**$^a p < 0.01$ compared with normal group.
***$^b p < 0.05$, $^c p < 0.01$ compared with control group.

Table 2. Level of inflammatory mediators in BALF.

Group	TNF-α (pg/mL)	IL-6 (pg/mL)	MIP-2 (pg/mL)
Normal	0.764 ± 0.182	2.314 ± 1.200	5.249 ± 2.189*
CSC + LPS (Control)	22.159 ± 1.895^a	56.528 ± 10.766^a	64.306 ± 6.326^{a**}
CSC + LPS + CSA (10 ㎎/㎏)	2.588 ± 0.731^c	11.640 ± 0.097^c	4.110 ± 0.791^{c***}
CSC + LPS + LL (200 ㎎/㎏)	4.390 ± 1.202^c	3.660 ± 0.874^c	14.840 ± 2.349^c
CSC + LPS + LL (50 ㎎/㎏)	14.949 ± 2.613^b	25.516 ± 5.578^c	49.947 ± 4.965

*Values are expressed as the mean ± SE (n = 6).
**$^a p < 0.01$compared with normal group.
***$^b p < 0.05$,$^c p < 0.01$compared with control group.

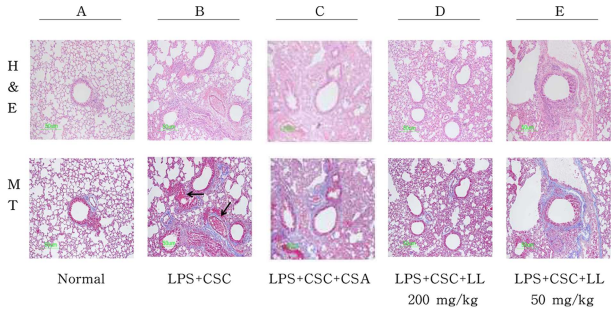

Fig. 2. Histological analysis of lung tissue. ← ; Infiltration of inflammatory cells in airway walls, H & E; hematoxylin-eosin staining, M-T; Masson-Trichrome staining, Normal; Balb/c mice, LPS; Lipopolysaccharide, CSC; Cigarette smoke condensate, CSA; Cyclosporine A (10 ㎎/㎏), LL; Ligustrum lucidum fruits extract.

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 nuetrophil 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.

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