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ORIGINAL ARTICLE

Yukgunja-tang, a Traditional Herbal Formula, Attenuates Cigarette Smoke-induced Lung Inflammation in a Mouse Model

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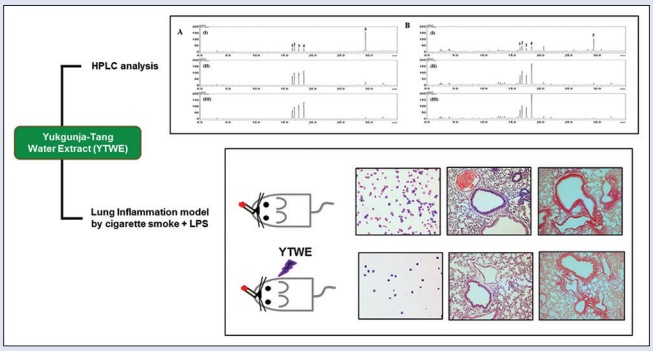
ABSTRACT

Background: Chronic obstructive pulmonary disease is a progressive lung disease that involves airway inflammation, chronic bronchitis, and emphysema. Yukgunja-tang, one of the traditional Asian herbal medicines, has been used widely in treating patients with gastrointestinal diseases in Korea. **Objective:** Here, we investigated its efficacy on the inflammatory response using a mouse model of cigarette smoke (CS) exposure together with lipopolysaccharide (LPS) treatment. **Materials and Methods:** Over 4 weeks, mice were exposed to CS on 5 days/week and instilled intranasally with LPS on days 8 and 23. Yukgunja-tang water extract (YTWE) was administered to mice on the same 5 days. **Results:** YTWE administration significantly reduced the numbers of inflammatory cells and levels of pro-inflammatory cytokines in bronchoalveolar lavage fluid compared with CS plus LPS-exposed mice. Moreover, YTWE inhibited the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and IκBα proteins induced by CS plus LPS treatment. Histologically, YTWE attenuated the infiltration of inflammatory cells into peribronchial lesions, thickening of alveolar walls and accumulation of collagen in the lung tissues. **Conclusion:** Our findings suggest that YTWE prevents CS plus LPS-induced lung inflammation by inhibiting p38 MAPK and IκBα signaling. Therefore, YTWE might be a potential drug for the treatment of lung inflammation induced by CS exposure.

Key words: Chronic obstructive pulmonary disease, cigarette smoke, inflammatory cells, lung inflammation, Yukgunja-tang

SUMMARY

- Main components, a liquiritin apioside, liquiritin, narirutin, hesperidin, and glycyrrhizin, in Yukgunja-tang water extract (YTWE) were quantitatively analyzed by high-performance liquid chromatography
- YTWE administration in cigarette smoke plus lipopolysaccharide (LPS)-exposed mice suppressed the numbers of inflammatory cells and the levels of pro-inflammatory cytokines in BALF with the reduced phosphorylation of p38MAPK and IκBα proteins in lung tissues
- YTWE administration in cigarette smoke plus LPS-exposed mice inhibits histologically the recruitment of inflammatory cells and fibrosis in lung tissues.



Abbreviations used: BALF: Bronchoalveolar lavage fluid; COPD: Chronic obstructive pulmonary disease; CS: Cigarette smoke; HPLC: High-performance liquid chromatography; IL: Interleukin; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein Kinase; NF-κB: Nuclear factor kappa-B; ROF: Roflumilast; TNF-α: Tumour necrosis factor-alpha; YTWE: Yukgunja-tang water extract.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD), a progressive lung disease characterized by persistent airflow limitation that is poorly reversible, is most common in people older than 60 years of age and is considered a significant global health problem.[1,2] COPD involves an abnormal inflammatory response including the release of various inflammatory mediators as well as inflammatory cell infiltration in lungs and airways, and leads to narrowing of peripheral airways, the destruction of lung parenchyma and mucus overproduction, together with fibrosis.[3] The development of COPD is linked to exposure to various factors such as cigarette smoke (CS), chemical fumes, mineral dusts, and industrial pollution. Among them, CS is known as the major risk factor for the development of COPD.[4] CS, which contains high levels of oxidants, leads to lung inflammation by recruiting inflammatory cells and their activating mediators. In patients with COPD induced by CS, increased numbers of inflammatory cells such as macrophages, neutrophils, and lymphocytes have been found in bronchoalveolar lavage fluid (BALF), and in lung parenchyma and its airways.[5] In addition, it has been reported that patients with COPD induced by CS show elevated levels of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 and IL-8.[6] Activation of intracellular signaling molecules such as mitogen-activated

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protein kinases (MAPKs) and nuclear factor kappa-B (NF-κB), which are stimulated by several factors including pro-inflammatory cytokines and oxidative stress, has also been associated with the development of COPD induced by CS.[7,8]

Roflumilast (ROF), one of the medications commonly used in treating patients with COPD, improves lung function through apoptosis and inhibition of inflammatory cell infiltration and cytokine production *in vitro*.[9] Despite these anti-inflammatory effects, randomized clinical trials have shown that ROF causes adverse side effects such as diarrea, weight loss, nasopharyngitis, and headaches.[10] However, it has been reported that several traditional herbal medicines, with fewer side effects than synthetic drugs, have anti-inflammatory activities in models of COPD. Extracts of the tuber of *Alisma orientale* in a mouse model have shown a repressive effect on the pathogenic features of COPD, which involve lung inflammation, emphysema, and autophagy.[11] Moreover, an extract of *Callicarpa japonica* Thunb. attenuated CS-induced neutrophil inflammation and mucus secretion *in vivo* and *in vitro*.[12] Therefore, it seems desirable to develop efficacious alternative medicines for treating COPD.

Yukgunja-tang, also known as Liu-Jun-Zi-Tang in Chinese and Rikkunshi-to in Japanese, is a traditional herbal medicine containing the extracts of six herbs: *Pinellia ternata* Breitenbach, *Atractylodes macrocephala* Koidzumi, *Citrus unshiu* Markovich, *Poria cocos* Wolf, *Panax ginseng* C. A. Meyer, and *Glycyrrhiza uralensis* Fischer. Yukgunja-tang is widely used in Korea for treating patients with gastrointestinal diseases including dyspepsia,[13] gastrointestinal and gastroesophageal reflux disease,[14] and symptoms arising after gastrectomy.[15] Recently, some studies have been reported the efficacy of Yukgunja-tang against various disease. In acute lung injury rodent model, Yukgunja-tang administration exerts the protective effect on injury of alveolar epithelial cells with reduction of lung inflammation and lung fibrosis.[16] Yukgunja-tang in postoperative ileus rodent model also ameliorates the symptoms of postoperative ileus through anti-inflammatory activity.[17] Yukgunja-tang treatment in small intestinal cells has the cytoprotective ability on mucosal damage.[18] Moreover, a clinical case study reported that Yukgunja-tang appears to improve aspiration pneumonia.[19] Considering the efficacy of this medicine in inflammatory diseases including acute lung injury and postoperative ileus, it might also be effective against CS-induced lung inflammation. However, the therapeutic effects of Yukgunja-tang in this regard have not yet been elucidated. Therefore, in this study, we investigated whether administration of Yukgunja-tang would have anti-inflammatory efficacy using a mouse model of CS exposure coupled with lipopolysaccharide (LPS) treatment as a model of pulmonary inflammation.

MATERIALS AND METHODS

Chemicals and reagents

The chemical standards, liquiritin (purity ≥99.0%) and glycyrrhizin (purity ≥99.0%), were purchased from Wako Pure Chemicals, Inc., (Osaka, Japan). Narirutin (purity ≥99.0%) and hesperidin (purity ≥98.0%) were purchased from Biopurify Phytochemicals (Chengdu, P. R. China). Liquiritin apioside (purity ≥98.0%) was purchased from Shanghai Sunny Biotech (Shanghai, China). High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and water were obtained from J. T. Baker (Phillipsburg, NJ, USA). Formic acid was obtained from Sigma-Aldrich Corp., (St. Louis, MO, USA).

Plant materials

The six medicinal herbs comprising Yukgunja-tang were purchased from Kwangmyungdag Medicinal Herbs (Ulsan, Korea) in February 2016 and were identified by Dr. Goya Choi from the K-herb Research Center, Korea Institute of Oriental Medicine (KIOM). A voucher specimen (2016–EBM111-1 to EBE111-6) has been deposited at the K-herb Research Center, KIOM.

Preparation of Yukgunja-tang water extract

To obtain the lyophilized powder of Yukgunja-tang water extract (YTWE), the six raw herbs, *Pinellia ternata* Breitenbach (231 g), *Atractylodes macrocephala* Koidzumi (231 g), *Citrus unshiu* Markovich (154 g), *Poria cocos* Wolf (154 g), *Panax ginseng* C. A. Meyer (154 g), and *Glycyrrhiza uralensis* Fischer (77 g), were mixed and extracted in a 10-fold volume of water (10 L) at 100°C for 2 h using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The solution was filtered using a standard sieve (No. 270, 53 μm; Chung Gye Sang Gong Sa, Seoul, Korea) and then the filtered solution was processed to give a powder using a PVTFD10RS freeze dryer (IlShinBioBase, Yangju, Korea). The amount of YTWE thus prepared was 305.7 g (yield 30.6%).

High-performance liquid chromatography analysis of Yukgunja-tang water extract

The HPLC system for quantitative analysis used the Prominence LC-20A series equipment and LCsolution software (v. 1.24) for data processing (Shimadzu Co., Kyoto, Japan). The analytical column used for separation of the major components was a Phenomenex Gemini C18 column (250 × 4.6 mm, 5 μm, Torrance, CA, USA) with a column oven temperature of 40°C. The mobile phases consisted of distilled water (A) and acetonitrile (B), containing both 0.1% (v/v) trifluoroacetic acid. Gradient elution of the mobile phase system was done as follows: 5%–60% B for 0%–30 min, 60%–100% B for 30–40 min, 100% B for 40–50 min, 100%–5% B for 50–60 min, and 5% B for 60–70 min. The flow rate was 1.0 mL/min and the injection volume was 10 μL. For quantitative analysis of YTWE using an HPLC, 200 mg of lyophilized YTWE was dissolved in 20 mL of distilled water and then extracted by sonication for 30 min. The solution was filtered through a 0.2 μm syringe filter (PALL Life Sciences, Ann Arbor, MI, USA) before HPLC injection.

Animals

Specific pathogen-free 6-week-old male C57BL/6N mice weighing 20–25 g was purchased from Orient Bio Inc.(Seoul, Korea) and used after quarantine and acclimatization for 1 week. All mice were provided with standard chow and water *ad libitum*. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the KIOM Institutional Animal Care and Use Committee. Animals were cared for in accordance with the dictates of the National Animal Welfare Law of Korea.

Experimental procedures

To generate the experimental model, we followed an experimental procedure as described previously.[20] Briefly, mice were divided randomly into four groups (n = 6/group): normal control (NC group), CS exposure with intranasal LPS instillation (CS/LPS group), 10 mg/kg of ROF, *per os* (p. o.) + CS/LPS (ROF group), and 200 mg/kg of YTWE, p. o. + CS/LPS (YTWE group). The smoke was generated from 3R4F research cigarettes (Kentucky reference cigarettes, University of Kentucky, KY, USA). On 5 days/week, mice were exposed to the smoke from eight cigarettes in a smoke-exposure chamber (Dae Han Bio Link, Incheon, Korea) for 4 weeks. On days 8 and 23, LPS (10 μg dissolved in 50 μL of phosphate-buffered saline, PBS) was instilled intranasally with the animal under anesthesia 1 h after the final CS exposure. ROF and YTWE were administered to mice by oral gavage 2 h before CS exposure.

Measurement of inflammatory cells in bronchoalveolar lavage fluid

Samples of BALF from euthanized mice were obtained and processed as previously described.[21] Briefly, these were collected by infusing ice-cold PBS (0.5 mL) into the lung and withdrawing it, and this procedure was repeated three times up to a total volume of 1.5 mL. For differential cell counting, 100 μL aliquots of BALF were centrifuged onto slides at 200 g for 10 min at 4°C using a Cytospin centrifuge (Hanil Science Industrial, Seoul, Korea). After drying the slides, the cells were fixed in 4% paraformaldehyde and stained with Diff-Quik® staining reagent (B4132-1A; IMEB Inc., Deerfield, IL, USA) according to the manufacturer's instructions. The BALF supernatants were stored at −70°C for biochemical analyses.

Histology

After BALF samples had been collected, portions of lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin wax. Sections (5 μm) were deparaffinized, rehydrated, and stained with Mayer's hematoxylin (MHS-16, Sigma-Aldrich) and eosin (HT110-1-32, Sigma-Aldrich) (H and E) solutions using standard procedures. The sections were mounted with mounting medium (Invitrogen, Carlsbad, CA, USA) and observed under light microscopy with bright-field illumination (Olympus, Tokyo, Japan).

Measurement of pro-inflammatory cytokines

The levels of TNF-α and IL-6 in BALF were measured using an enzyme-linked immunosorbent assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Western blot analysis

Lung tissue was homogenized in CelLytic™ MT Cell Lysis Reagent (1/10 w/v, Sigma-Aldrich) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Aliquots of 30 μg of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was incubated with blocking solution (5% skim milk) in tris-buffered saline with Tween 20 buffer for 1 h at room temperature and probed with primary antibodies against p38 MAPK, phospho-p38 MAPK, IκBα, and phospho-IκBα (Cell Signaling, Denver, MA, USA) overnight at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibodies, signals were visualized using SuperSignal West Femto Maximum Substrate System (Thermo Scientific, Rockford, IL, USA) and then detected using a ChemiDoc™ XRS imaging system (Bio-Rad Laboratories).

Histological detection of collagen

The histological visualization of collagen in paraffin wax-embedded lung tissue sections was assessed using Picro Sirius Red Staining Kits (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, tissue sections were deparaffinized and stained with Picro Sirius Red Solution for 1 h. Slides were washed with 0.5% acetic acid solution, dehydrated in absolute alcohol, and then mounted with mounting medium (as above). Images were acquired using light microscopy with bright-field illumination (Olympus).

Statistical analysis

The data are presented as the mean ± standard error of the mean. Statistical significance was calculated by one-way analysis of variance followed by a multiple comparison test with Bonferroni adjustment. The data were considered statistically significant at $P < 0.05$.

RESULTS

Quantitative analysis of the five marker compounds in Yukgunja-tang water extract

Established HPLC methods were applied successfully for simultaneous analysis of four flavonoids (liquiritin apioside, liquiritin, narirutin, and hesperidin) and one triterpenoid (glycyrrhizin) in YTWE. These were separated within 30 min. The retention times of liquiritin apioside, liquiritin, narirutin, hesperidin, and glycyrrhizin were 16.45, 16.78, 17.57, 18.42, and 29.28 min, respectively, and typical HPLC chromatograms of standard solutions and YTWE are shown in Figure 1. The regression equations of liquiritin apioside, liquiritin, narirutin, hesperidin, and glycyrrhizin were y = 14,150.06x–2,900.76 (coefficient of determination, $r^2 = 0.9996$), y = 25,781.85x–13,691.60 ($r^2 = 0.9996$), y = 15,610.66x–8,009.40 ($r^2 = 0.9996$), y = 16,801.04x– 15,456.21 ($r^2 = 0.9996$), and y = 7,315.91x–9,811.09 ($r^2 = 0.9996$), respectively. Detection of the five marker compounds was carried out at ultraviolet wavelengths of 254 nm (glycyrrhizin), 275 nm (liquiritin apioside and liquiritin), and 280 nm (narirutin and hesperidin). The amounts of the liquiritin apioside, liquiritin, narirutin, hesperidin, and glycyrrhizin were detected 4.19 ± 0.02, 2.70 ± 0.03, 3.25 ± 0.04, 6.86 ± 0.01, and 9.98 ± 0.03 mg/g lyophilized powder of YSTE, respectively.

Yukgunja-tang water extract reduced the infiltration of inflammatory cells into bronchoalveolar lavage fluid of cigarette smoke/lipopolysaccharide-exposed mice

A mouse model of CS/LPS-induced lung inflammation was generated, and the animals were treated simultaneously with YTWE for 28 days as illustrated in Figure 2a. BALF of the CS/LPS group showed an increased influx of inflammatory cells including macrophages, neutrophils, and lymphocytes [Figure 2b and c]. However, the ROF group had fewer inflammatory cells in BALF compared with the CS/LPS group. Like the ROF group, the YTWE group also exhibited a reduced influx of inflammatory cells in BALF compared with the CS/LPS group [Figure 2b and c].

Yukgunja-tang water extract suppressed the levels of pro-inflammatory cytokines in bronchoalveolar lavage fluid of cigarette smoke/lipopolysaccharide-exposed mice

The production of pro-inflammatory cytokines such as TNF-α and IL-6 is associated with the progression of lung inflammation. Therefore, the levels of these cytokines in BALF samples were examined to address whether the inhibitory effect of YTWE on inflammatory cell influx was linked with the regulation of pro-inflammatory cytokines. As shown in Figure 3a, the BALF of the CS/LPS group exhibited increased levels of TNF-α compared with the NC group, whereas BALF of both YTWE and ROF groups showed markedly decreased levels of TNF-α compared with the CS/LPS group. In addition, changes in the IL-6 levels in BALF were like those of TNF-α. The CS/LPS group had increased levels of IL-6 in BALF compared with the NC group, but both the YTWE and ROF groups showed reduced levels of IL-6 compared with the CS/LPS group [Figure 3b].

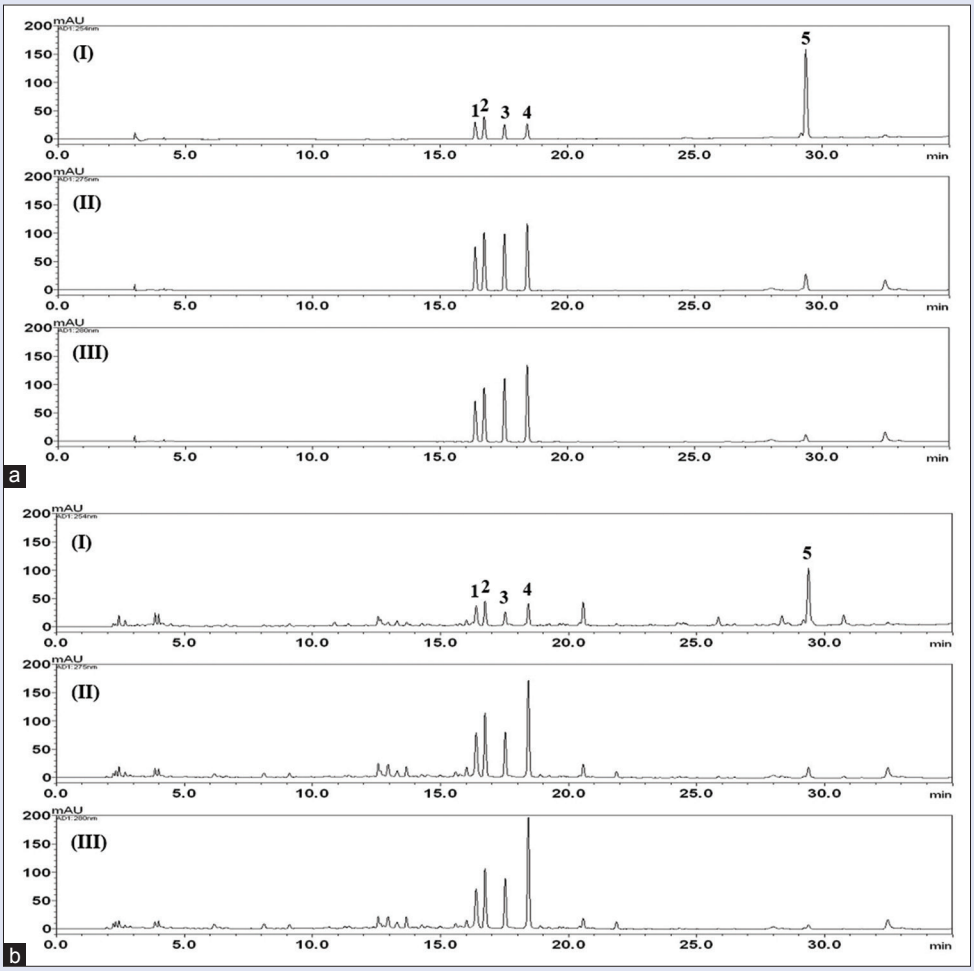

Figure 1: High-performance liquid chromatography analysis of Yukgunja-tang water extract. High-performance liquid chromatography chromatogram of the standard solution (a) and Yukgunja-tang decoction sample (b) at ultraviolet wavelength 254 nm (I), 275 nm (II), and 280 nm (III), showing the results for liquiritin apioside (1), liquiritin (2), narirutin (3), hesperidin (4), and glycyrrhizin (5)

Yukgunja-tang water extract inhibited cigarette smoke/lipopolysaccharide-induced activation of p38 MAPK and IκBα protein

MAPKs and NF-κB signaling pathways are activated by pro-inflammatory cytokines including TNF-α and IL-6. Therefore, the activation of MAPKs and NF-κB signaling pathways on protein levels was examined to elucidate their involvement in regulating the inflammatory response by YTWE. CS/LPS exposure activated the phosphorylation of p38 MAPK, one of the MAPK family, in lung tissue, whereas YTWE administration decreased the levels of phosphorylated p38 MAPK protein compared with CS/LPS exposure [Figure 4]. Furthermore, phosphorylation of the IκBα protein, a moderator of NF-κB signaling, induced by CS/LPS exposure was also reduced by YTWE administration [Figure 4].

Yukgunja-tang water extract attenuated the inflammatory responses in lung tissues of cigarette smoke/lipopolysaccharide-exposed mice

Lung tissues were stained with H and E to analyze the effect of YTWE on the histopathological changes induced by CS/LPS exposure. The CS/LPS group showed extensive recruitment of inflammatory cells into perivascular and peribronchial regions of the lung compared with the NC group [Figure 5a]. However, lung tissues of the YTWE and ROF groups showed reductions in perivascular and peribronchial inflammation compared with the CS/LPS group. Moreover, lesions such as thickening of alveolar wall and infiltration of inflammatory cells were more prominent in the CS/LPS group than in the NC group [Figure 5b]. However, both the YTWE and ROF groups showed reduced alveolar lesions with thinner alveolar walls and fewer infiltrations of inflammatory cells compared with the CS/LPS group.

Yukgunja-tang water extract reduced lung fibrosis in cigarette smoke/lipopolysaccharide-exposed mice

Given the restoration of damaged lung structures by YTWE treatment [Figure 5], we next examined the accumulation of collagen to determine whether YTWE also affected lung fibrosis. Figure 6 shows collagen fiber hyperplasia in the peribronchial vascular space in the CS/LPS group, but not in the NC group. However, lung tissue in the YTWE group had less accumulation of collagen than in the CS/LPS group.

DISCUSSION

COPD is a progressive lung disease characterized by chronic airflow limitation and an abnormal inflammatory response. Here, we investigated

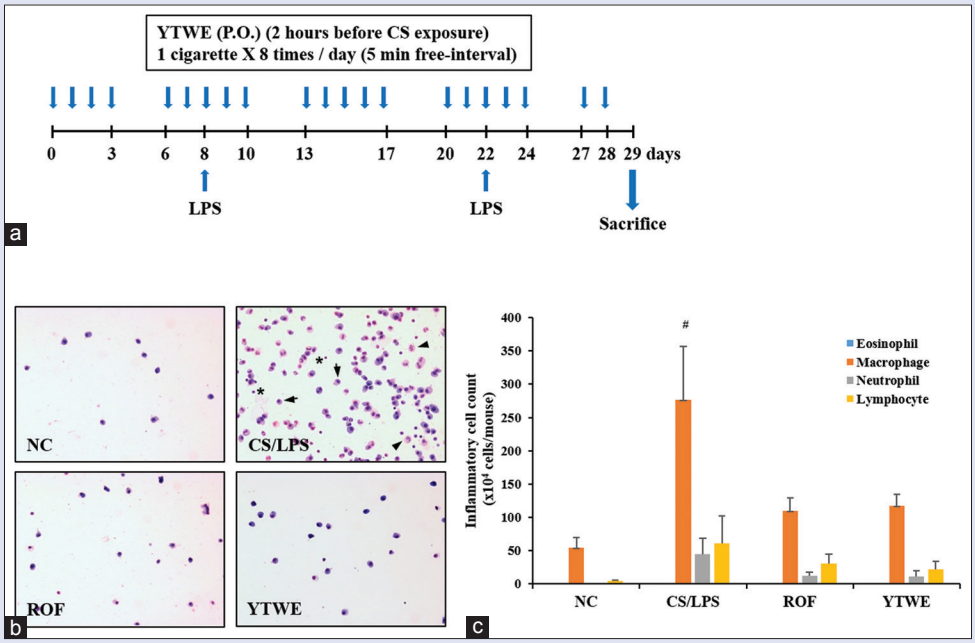

Figure 2: Yukgunja-tang water extract reduced the numbers of inflammatory cells in BALF samples of cigarette smoke/lipopolysaccharide-exposed mice. (a) Schematic diagram of the experimental protocol. On 5 days/week, mice were administered with vehicle, roflumilast, or Yukgunja-tang water extract for 2 h and then exposed to cigarette smoke of eight cigarettes. Mice were treated with lipopolysaccharide twice on days 8 and 23. (b) The image of macrophages (arrow), neutrophils (arrowhead), and eosinophils (asterisk; magnification, ×400). (c) Differential cell counts in the NC, cigarette smoke/lipopolysaccharide, roflumilast, and Yukgunja-tang water extract groups. Values are presented as the mean ± standard error of the mean ($n = 6$). $^{\#}P < 0.05$, compared with the NC group

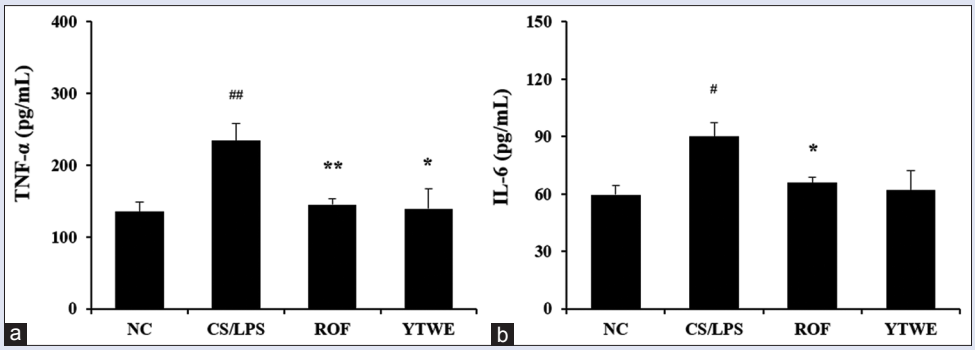

Figure 3: Yukgunja-tang water extract treatment reduced the levels of pro-inflammatory cytokines in cigarette smoke/lipopolysaccharide-exposed mice. Tumor necrosis factor-alpha (a) and interleukin-6 (b) concentrations in bronchoalveolar lavage fluid samples were determined using enzyme-linked immunosorbent assay kits in the NC, cigarette smoke/lipopolysaccharide, roflumilast, and Yukgunja-tang water extract groups. Values are presented as the mean ± standard error of the mean ($n = 5$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the NC group; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the cigarette smoke/lipopolysaccharide group

the anti-inflammatory effects of YTWE on airway and lung inflammation using a CS/LPS-exposed mouse model. YTWE significantly inhibited the recruitment of inflammatory cells and the production of cytokines such as TNF-α and IL-6 in CS/LPS-exposed BALF samples. YTWE treatment attenuated the infiltration of inflammatory cells and fibrosis in lung tissues induced by CS/LPS exposure. In addition, YTWE decreased the phosphorylated levels of p38 MAPK and IκBα proteins induced by CS/LPS exposure.

Intracellular inflammatory signaling pathways such as MAPK and NF-κB are associated with the pathogenesis of COPD induced by CS, LPS, or inflammatory oxidants. The MAPK pathway, composed of three subtypes of kinases, p38 MAPK, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinases (JNKs), is activated by a variety of extracellular stimuli such as pro-inflammatory cytokines, growth factors and oxidative stress, and regulates cell proliferation, apoptosis, and differentiation as well as inflammation.[22] Among these subtypes, p38 MAPK is the most specific signal transducer in regulating pro-inflammatory responses and an increase in its active form, the phosphorylated p38 MAPK, has been found in the lungs of patients with COPD.[23,24] The increased phosphorylation of p38 MAPK in epithelial cells and macrophages of lung tissue from such patients activates pro-inflammatory cytokine production. In addition, p38 MAPK inhibitors suppress the inflammatory processes in lung cells of such patients.[24,25] Consistent with previous reports, our study showed that CS/LPS-exposed mouse model increased the phosphorylated levels of p38 MAPK but reduced them by YTWE

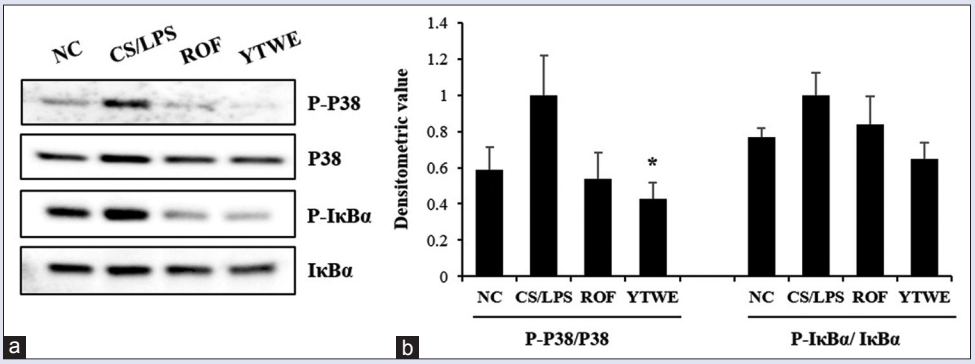

Figure 4: Yukgunja-tang water extract inhibited the phosphorylation of p38 MAPK and IκBα proteins induced by cigarette smoke/lipopolysaccharide exposure. (a) Lung homogenates were processed for Western blot analysis with anti-phospho-p38, anti-p38 MAPK, anti-phospho-IκBα, and anti-IκBα antibodies. (b) Densitometry of protein bands on Western blots is shown for the NC, cigarette smoke/lipopolysaccharide, roflumilast, and Yukgunja-tang water extract groups. Values are presented as the mean ± standard error of the mean ($n = 5$)

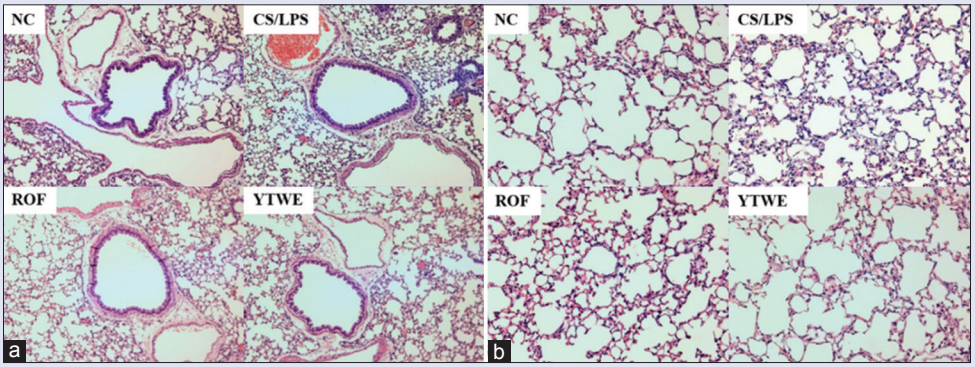

Figure 5: Yukgunja-tang water extract treatment repressed airway inflammation in lung tissues of cigarette smoke/lipopolysaccharide-exposed mice. Representative H and E histology of peribronchial lesions (magnification, × 100) (a) and alveolar lesions (magnification, × 200) (b) in lung tissues for the NC, cigarette smoke/lipopolysaccharide, roflumilast, and Yukgunja-tang water extract groups

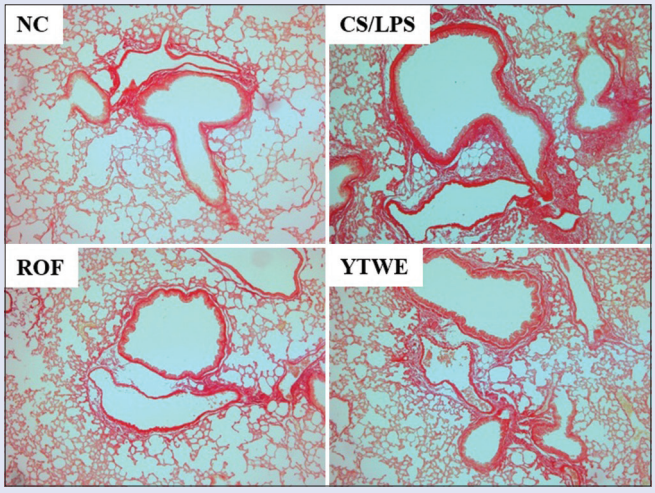

Figure 6: Yukgunja-tang water extract treatment repressed collagen accumulation in lung tissues of cigarette smoke/lipopolysaccharide-exposed mice. Collagen fibers in lung tissues were stained with Sirius Red for the NC, cigarette smoke/lipopolysaccharide, roflumilast, and Yukgunja-tang water extract groups (×100)

administration, indicating that YTWE inhibited CS/LPS-induced lung inflammation.

Concerning the NF-κB signaling pathway, this cytokine forms a heterodimer and acts as a transcription factor to control DNA transcription, cytokine production, cell survival, and metabolism.[26,27] In its canonical pathway, activation of NF-κB is regulated by interaction with its inhibitory IκBα protein. In unstimulated cells, NF-κB is located in the cytoplasm, complexed with IκBα. Extracellular signals induce the activation of IκB kinases, which then phosphorylate IκBα. This in turn dissociates from NF-κB and is subjected to proteasomal degradation. Activated NF-κB is then translocated into the nucleus and eventually acts as a transcription factor. IκBα is important in the link between NF-κB activation and inflammation. IκBα-deficient mice showed phenotypes such as dermatitis and histological alternations in the liver and spleen with an increase of inflammatory cells.[28] Decreased levels of IκBα protein were found in the lung tissues of smokers and patients with COPD.[29] Here, we found that YTWE attenuated the increased levels of phosphorylation of IκBα as well as p38 MAPK induced by CS/LPS treatment. Furthermore, in testicular Sertoli cells, MAP phosphatase-1, a negative regulator of the inflammatory response, attenuated LPS-induced inflammation through the inhibition of p38 MAPK and IκBα signaling.[30] Taken together, YTWE appears to promote anti-inflammatory activity through the inhibition of p38 MAPK and NF-κB signaling.

The inflammatory response in patients with COPD induced by CS leads to changes in lung structure that eventually result in airflow limitation.[31] Mucous secretions and bronchiolar fibrosis in the proximal airways and remodeling of small airways and alveolar walls are involved in structural

changes in such lungs. Here, YTWE treatment attenuated collagen accumulation in bronchioles and reduced the thickening of alveolar walls [Figures 5 and 6], showing that YTWE treatment was effective in limiting lung inflammation induced by CS/LPS treatment. Nevertheless, further studies are necessary to elucidate the mechanisms that are associated with this inhibition of lung remodeling.

A number of studies have demonstrated that flavonoids, natural compounds synthesized by plants, have pharmacological activities against various diseases.[32,33] Among many, four flavonoids, liquiritin apioside, liquiritin, narirutin, and hesperidin, are present in YTWE. The protective effects of these four flavonoids on inflammation-related diseases are well known. In a CS-exposed mouse model, liquiritin apioside treatment promoted anti-inflammatory effects through the reduction of cytokine production and inflammatory cell infiltration.[34,35] In a mouse model of myocardial fibrosis with diabetes, liquiritin inhibited the inflammatory response associated with MAPK and NF-κB signaling.[36] Narirutin repressed airway inflammation in a mouse model of asthma[37] and attenuated LPS-induced inflammation through inhibition of MAPKs and NF-κB signaling.[38] Hesperidin, abundantly found in citrus fruits, was also shown to play a protective role against many disorders through its antioxidant and anti-inflammatory properties.[39,40] Consistent with these findings, here, we confirmed that YTWE promoted anti-inflammatory activity in lung inflammation induced by CS/LPS exposure.

CONCLUSION

We demonstrate that YTWE attenuated airway and lung inflammation induced by CS/LPS treatment by reducing the infiltrations of inflammatory cells, producing pro-inflammatory cytokines and fibrosis, and modulating p38 MAPK and IκBα signaling. These findings suggest that YTWE could be a potential therapeutic agent in the treatment of airway and lung inflammation induced by CS.

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Conflicts of interest

There are no conflicts of interest.

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