Effects of GHX02 on Chronic Obstructive Pulmonary Disease Mouse Model

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Objectives: Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation and irreversible airflow. This study aimed to evaluate the effects of GHX02 in a COPD-induced mouse model.

Methods: The COPD mouse model was established by exposure to cigarette smoke extract and lipopolysaccharide which were administered by intratracheal injection three times with a 7 day interval. GHX02 (100, 200, 400 mg/kg) and all other drugs were orally administrated for 14 days from Day 7 to Day 21.

Results: GHX02 significantly decreased the neutrophil counts in bronchoalveolar lavage fluid (BALF) and the number of CD4+, CD8+, CD69+, and CD11b+/GR1+ cells in BALF and lung cells. GHX02 also suppressed the secretion of tumor necrosis factor-alpha (TNF-α), interleukin-17A, macrophage inflammatory protein 2 (MIP2), and chemokine (C–X–C motif) ligand 1 (CXCL-1) in BALF and ameliorated the lung pathological changes.

Conclusions: Thus, GHX02 effectively inhibited airway inflammation by inhibiting migration of inflammatory cells and expression of pro-inflammatory cytokines. Therefore, GHX02 may be a promising therapeutic agent for COPD.

Key Words: chronic obstructive pulmonary disease, cigarette smoke, airway inflammation, herbal medicine, GHX02

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of mortality worldwide and is predicted to be the 3rd leading cause of death in 2020, due to high smoking rates, air pollution, and an increase in the elderly population1). It is characterized by chronic inflammation and airflow limitation that is usually progressive and irreversible caused by environmental exposure to cigarette smoke, air pollution and various particles or gases2). Cigarette smoke contains reactive oxygen species (ROS) and many other chemical components, which triggers the release of chemoattractants, promoting the recruitment of neutrophils and other inflammatory cells3,4). This non-specific immune system mediates the processes of inflammation, fibrosis and proteolysis5). In particular, an increase in neutrophils is associated with development of COPD and has been observed in bronchoalveolar lavage fluid (BALF) and sputum...
samples obtained from COPD patients. Other inflammatory cells indicate the severity of the disease\(^6\). Current conventional treatment for COPD is based on bronchodilators according to present guidelines\(^7\), although it has limitations in reducing inflammation of the lung and promoting lung functions\(^8\). Corticosteroids are also widely used for COPD patients despite its resistance\(^9\) and high risk of side effects such as skin bruising, reduction of bone density, etc\(^10\). Thus, the need for alternative treatments such as herbal medicines has been increased\(^11\) and several herbal medicines including socheongryong-tang\(^12\), maekmoondong-tang\(^13\), chungsangboha-tang\(^14\), sagan-tang\(^15\) and gwaruhaengryeon-hwan\(^16\) have been studied for COPD patients.

In this study, we developed herbal medicine, GHX02, which contains four herbs originated from gwaruhaengryeon-hwan (GRHRH) in Donguibogam (Principles and Practice of Eastern Medicine)\(^17\) and planned to evaluate anti-inflammatory effects of GHX02 on COPD-induced mice. GHX02 was formed by adding Scutellariae Radix to GRHRH. GRHRH already demonstrated its anti-inflammatory effects on COPD and particulate matter induced lung injury on a mouse model\(^16\) and previous studies reported that Scutellariae Radix inhibits the production of several inflammatory cytokines\(^18\) and has antioxidant effects\(^19\). We first established COPD mouse model by exposure to the cigarettes smoke extract (CSE) and lipopolysaccharide (LPS). Then, flow cytometric analysis, Enzyme-linked immunosorbent assays (ELISA) and lung histopathological study was done to assess the immunotherapeutic potential of GHX02.

### Materials and Methods

**Preparation of GHX02**

GHX02 originated from gwaruhaengryeon-hwan (GRHRH) consists four herbs including Trichosanthis Semen (TS), Armeniaca Semen (AS), Coptidis Rhizoma (CR) and Scutellariae Radix (SR), which were purchased from Human Herbs Co. Ltd. (Kyeongsan, Korea) (Table 1). GHX02 was suspended in distilled water (Choongwae Pharma, Korea) and formulated by boiling 2 times in distilled water at 100 °C for 2 hours. The volume of distilled water was 10 times of the total herb weight. The mixture was then filtered through a Whatman No. 2 filter (Maidstone, UK), concentrated under vacuum, and freeze-dried. The water extract was evaporated at 45°C and subsequently lyophilized. GHX02 showed a yield of the dried ratio of 14.97% and TS, AS, CR and SR showed 5.34, 14.23, 14.81 and 41.01% yield of the dried ratio respectively.

**Animal experiments**

C57BL/6 mice (6–8 weeks old) were obtained from the Orient Bio Inc. (Seongnam, Korea) and used after 1 week of acclimatization. The mice were housed under pathogen-free conditions with a standard laboratory diet and maintained at a temperature of 22-24 °C, a humidity of 50 ± 10% and with controlled day and night cycles\(^20\). The animal protocol was approved by the committee

### Table 1. Components of GHX02

<table>
<thead>
<tr>
<th>Herb</th>
<th>Latin name</th>
<th>Family name</th>
<th>Lot No.</th>
<th>Amount (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gwaruin</td>
<td><em>Trichosanthis Semen</em></td>
<td>Cucurbitaceae</td>
<td>K2714011</td>
<td>4,212</td>
</tr>
<tr>
<td>Haengin</td>
<td><em>Armeniaca Semen</em></td>
<td>Rosaceae</td>
<td>H0114041</td>
<td>2,106</td>
</tr>
<tr>
<td>Hwangryeon</td>
<td><em>Coptidis Rhizoma</em></td>
<td>Ranunculaceae</td>
<td>H0313121</td>
<td>2,106</td>
</tr>
<tr>
<td>Hwangkeum</td>
<td><em>Scutellariae Radix</em></td>
<td>Labiatae/Lamiaceae</td>
<td>H1114031</td>
<td>4,212</td>
</tr>
</tbody>
</table>

http://dx.doi.org/10.13048/jkm.18040 127
for animal welfare at Daejeon University (DJUARB2016-009). All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and guidelines of the Institutional Animal Care and Use Committee of the South Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

**Preparation of cigarette smoke extract**

CM6 (CORESTA approved Monitor No. 6) reference cigarettes were conditioned under ISO conditions (one puff/min., 35 mL puff volume over 2 s, every 60 s) of temperature (22 ± 2 °C) and relative humidity (60 ± 5 %) for 48 h or more. The cigarettes were smoked using an automatic smoking machine (Borgwaldt RM20, Germany) by ISO conditions (puff volume: 35 mL; duration: 60 s; interval: 2 s). Cigarette smoke was trapped on a Cambridge filter pad (0.22 μm, Ø4 mm) and extracted with isopropanol as a solvent. Total particulate matter (TPM) was prepared for the whole 2-year period and stored at -80°C. TPM was tested immediately after preparation (T0), after 1 month (T1) and 3 months (T3).

**Establishment of COPD mouse model and GHX02 administration**

A COPD mouse model was established by exposure to the CS extract (CSE) and LPS (Sigma–Aldrich, St. Louis, MO, USA) according to previous literature (Fig. 1). After intraperitoneal injection of anesthetics, CSE (1 mg/mL) and LPS (100 μg/mL) were administered by intratracheal injection three times with a 7 day interval. Dexamethasone and all other drugs were orally administrated for 14 days from Day 7 to Day 21. The mice were divided into the following ten groups as follows: (i) C57BL/6_Nr (no treatment), (ii) control (CT) group (CSE/LPS), (iii) dexamethasone group (positive control, CSE/LPS + Dexa 3 mg/kg), (iv) TS group (CSE/LPS + TS 200 mg/kg), (v) AS group (CSE/LPS + AS 200 mg/kg), (vi) CR group (CSE/LPS + CR 200 mg/kg), (vii) SR group (CSE/LPS + SR 200 mg/kg), (viii) GHX02-100 group (CSE/LPS + GHX02 100 mg/kg), (ix) GHX02-200 group (CSE/LPS + GHX02 200 mg/kg), and (x) GHX02-400 group (CSE/LPS + GHX02 400 mg/kg). All mice were observed daily for their general condition, including the behavior, physical appearance, respiratory murmur, hair condition, sensitivity and liveliness.

**Collection of bronchoalveolar lavage fluid**

On the last day of drug administration, the trachea and airways of the anaesthetized mice were lavaged 3 times using a syringe (containing 1ml of FBS free DMEM medium) to obtain BALF containing cells from the trachea and lungs. The BALF was centrifuged at 4 °C, 2,000 rpm for 5 minutes and the supernatant of BALF was stored at -25 °C for the determination of cytokines. The cells isolated from BALF were treated with ammonium chloride (ACK) solution for 3 min and washed with 1% FBS free DMEM medium for cell counting using a hemocytometer.
Flow cytometric analysis

Antibodies against CD4 (RM4-5, rat IgG2a), CD8 (53-6.7, rat IgG2a), CD69 (H1.2F3, rat IgG), and granulocytic marker GR1 (RB6-8C5, rat IgG2b), for flow cytometric analysis, were purchased from Becton Dickinson Pharmingen (San Diego, CA, USA). Cells from the lung and BALF were incubated with fluorescein isothiocyanate- and phycoerythrin-labeled monoclonal antibodies (30 min), washed with phosphate-buffered saline (PBS), fixed with 0.5% paraformaldehyde (20 min), washed again with PBS, and then stored at 4 °C until analysis by two-color flow cytometry on a FACS Calibur™ instrument (BD Biosciences, Mountain View, CA, USA).

Enzyme-linked immunosorbent assays for inflammatory mediators

The levels of macrophage inflammatory protein 2 (MIP2), tumor necrosis factor-α (TNF-α), interleukin-17A (IL-17A), and chemokine (C–X–C motif) ligand 1 (CXCL-1) in BALF were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The absorbance was measured at 450 nm using an ELISA reader.

Lung histopathological study

Mice were perfused with saline, and the whole lung was inflated with fixatives. Lung tissue was fixed in a 10% formaldehyde solution for 24 h. The tissues were embedded in paraffin, sectioned at 5μm thickness, and stained with hematoxylin–eosin (H&E) and Masson's trichrome (Sigma-Aldrich Korea). Three separate H&E-stained sections were evaluated for each mouse under light microscopy at 100× magnification. To determine the severity of inflammatory cell infiltration, mucus production, peribronchial cells, and goblet cell hyperplasia were quantified in the airway epithelium in a blind manner using the five-point (0–4) grading system as follows: 0, no cells; 1, a few cells; 2, ring of cells two cell layers deep; 3, ring of cells two–four cell layers deep; 4, ring of cells four cell layers deep.22

Statistical analysis

Data are expressed as the mean ± standard error and analyzed using a one-way analysis of variance. Statistical significance was calculated by a nonparametric Mann–Whitney test, followed by Dunnett’s multiple comparison test (SPSS version 19.0; IBM, USA). P < .05 was considered statistically significant.

Results

Inhibitory effect of GHX02 on airway neutrophil accumulation in BALF of COPD mouse model

To evaluate the effects of GHX02 on the recruitment of cells to the airway, we investigated neutrophils in BALF as neutrophil mediated inflammation plays an important role in the development and pathogenesis of COPD.23 The number of neutrophils was significantly higher in the BALF cytospin of the COPD-induced mice (CSE+LPS) than in that of untreated group (C57BL/6_Nr) (Fig. 2). Then, a significant decrease was observed in the dexamethasone and TS, AS, SR, CR, GHX02 (100, 200, 400 mg/kg) groups, compared with the control group. Herbal formula, GHX02 showed its dose-dependent inhibitory effect better than individual herbs of TS, AS, SR and CR respectively and was not inferior to dexamethasone.
Inhibitory effects of GHX02 on absolute numbers of immune cell subtypes in BALF and lungs of a COPD mouse model

Flow cytometric analysis was used to evaluate the effects of GHX02 on immune cell subtypes. The numbers of CD4⁺, CD8⁺, CD69⁺, and CD11b⁺/GR1⁺ cells in the BALF and lungs of the COPD-induced mice were significantly higher than those in the untreated group and were significantly lower in the GHX02-treated mice than in the control mice (Table 2). Thus, GHX02 showed profound inhibitory effects on airway inflammation in the mouse model of COPD, and these effects were caused by suppression of Th2-type cytokines and neutrophil infiltration.

Table 2. Quantification of Immune Cell Subtypes in the Lungs and BALF of Mice by FACS Analysis

<table>
<thead>
<tr>
<th>Source</th>
<th>Cell phenotypes</th>
<th>Mouse groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>CD4⁺/CD3⁺ (1 × 10⁵ cells)</td>
<td>2.120 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>CD8⁺/CD3⁺ (1 × 10⁵ cells)</td>
<td>0.468 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>CD69⁺/CD4⁺ (1 × 10⁵ cells)</td>
<td>0.514 ± 0.044</td>
</tr>
<tr>
<td></td>
<td>GR1⁺/CD11b⁺ (1 × 10⁵ cells)</td>
<td>1.684 ± 0.045</td>
</tr>
<tr>
<td>BALF</td>
<td>CD4⁺/CD3⁺ (1 × 10⁵ cells)</td>
<td>0.484 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>CD8⁺/CD3⁺ (1 × 10⁵ cells)</td>
<td>0.074 ± 0.0020</td>
</tr>
<tr>
<td></td>
<td>CD69⁺/CD4⁺ (1 × 10⁵ cells)</td>
<td>0.517 ± 0.023</td>
</tr>
</tbody>
</table>

Results are expressed as total absolute numbers (mean ± SEM; n = 8 mice per group). ††† P < .001 versus the vehicle group; *** P < .001 versus the CTL group. BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; CTL, control; Dexa, dexamethasone; FACS, fluorescence-activated cell sorting; LPS, lipopolysaccharide.

Inhibitory effects of GHX02 on inflammatory cytokines in BALF of COPD mouse model

We measured the levels of COPD-accompanying cytokines in BALF to determine the mechanisms underlying the GHX02-mediated inhibition of airway inflammation. In mice exposed to CSE and LPS, the levels of TNF-α, IL-17A, MIP2, and CXCL-1 in BALF were significantly higher than those in the untreated group. However, administration of GHX02 and individual herbs of TS, AS, SR, CR significantly suppressed the increases in TNF-α, IL-17A, MIP2, and CXCL-1 in BALF compared with those in the COPD-induced untreated mice (Fig. 3). The inhibitory
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Fig 3. Effect of GHX02 and individual herbs on TNF-α, IL-17A, MIP2 and CXCL-1 production of BALF in COPD mice. Mice were challenged by aspiration of LPS+CSE (Control), and then treated with Dexa (dexamethasone 3 mg/kg), TS (Trichosanthis Semen 200 mg/kg), AS (Armeniacae Semen 200 mg/kg), CR (Coptidis Rhizoma 200 mg/kg), SR (Scutellariae Radix 200 mg/kg) and GHX02 (400, 200, 100 ㎎/㎏) for 21 days (n=8). *: Significantly different from the Control group (* p<0.05, ** p<0.01, *** p<0.001).

Fig 4. Effects of GHX02 and individual herbs on (A) histopathological changes and (B) histology scores in the lung of a CSE/LPS-induced murine model of chronic obstructive pulmonary disease. Untreated mice received the vehicle, whereas model mice were induced by intratracheal injection of CSE/LPS and then treated with the vehicle (CTL), Dexa (dexamethasone 3 mg/kg), TS (Trichosanthis Semen 200 mg/kg), AS (Armeniacae Semen 200 mg/kg), CR (Coptidis Rhizoma 200 mg/kg), SR (Scutellariae Radix 200 mg/kg) and GHX02 (400, 200, 100 mg/kg) for 21 days. Lungs were fixed, sectioned, and stained with hematoxylin and eosin (H&E) and Masson’s trichrome (M–T) stains. (A) Representative sections from each treatment group are shown. (B) Quantitative analysis of the degree of lung tissue damage in the sections. Data are from individual mice, with the arithmetic means shown in the histogram. All values are mean±SEM. *: Significantly different from the Control group (* p<0.05, ** p<0.01, *** p<0.001).
Effect of GHX02 was shown to be greater than that of individual herbs, TS, AS, SR, and CR.

**Effects of GHX02 on lung inflammation in COPD mouse model**

The lungs of the COPD mice showed histopathological features typical for inflammatory lung tissues, such as distinct infiltration of leukocytes, goblet cell hyperplasia, and erosion in perivascular and peribronchial areas. In contrast, histological sections from the dexamethasone, TS, AS, CR and GHX02-treated mice revealed significantly reduced airway inflammation in lung tissues (Fig. 4A). GHX02 treatment markedly attenuated the damage when compared to that of the COPD-induced mice. The index score, representing the degree of histopathological changes, increased in COPD-induced mice compared with that of the untreated mice. However, the GHX02-treated mice showed significantly decreased index score compared with that in COPD-induced mice. This result indicates that the COPD model was successfully established and GHX02 obviously improved the pathological injury of COPD (Fig. 4B).

**Discussion**

In the circumstances that the worldwide prevalence of COPD has substantially increased and no direct treatment has yet been developed, various herbal medicines are being used in the Oriental countries. In this study, we demonstrated anti-inflammatory and lung injury inhibitory effects of herbal medicine GHX02 on COPD mouse model and suggest GHX02 as a promising therapeutic agent for COPD.

COPD is associated with chronic inflammation of the lungs and airways, caused by environmental exposure to cigarette smoke (CS) and air pollution. CS provokes severe inflammation mediated by NF-κB-dependent production of cytokines, inducing the transcription of various inflammatory mediators including IL-1β, IL-6 and TNF-α. Those pro-inflammatory cytokines and chemokines recruit neutrophils to the lung tissue causing lung injury. Exposure to CSE and LPS induced COPD-like pathological changes such as inflammatory cell accumulation in the lung parenchyma, hyperplasia of goblet cells, enlargement of airway space, and increase of elastin and collagen. This model encompasses major risk factors of COPD, including both smoking and acute exacerbation. In this model, inhibitory effect of GHX02 on inflammation was evaluated.

GHX02 and all of individual herbs of TS, AS, CR and SR significantly reduce the number of neutrophils in BALF and the number of CD4+, CD8+, CD69+, and CD11b+/GR1+ cells in BALF and lung cells in all dosage of GHX02 (100, 200, 400 mg/kg). This result demonstrates that GHX02 and individual herbs suppress Th2-type cytokines and neutrophil infiltration, although herbal formula of GHX02 showed better effects than individual herbs.

GHX02 also suppresses the increase of the pro-inflammatory cytokines, TNF-α and IL-17. TS, AS, CR and SR also showed inhibitory effects on TNF-α and AS, CR showed effects on IL-17. Cigarette smoke can activate macrophages to produce TNF-α and is measured in large quantities in COPD, especially during exacerbations. TNF-α induces the gene expression of many pro-inflammatory cytokines via NF-κB transcription factor activation. Interleukin (IL)-17A correlates with the presence of neutrophils and the severity of loss of lung function. IL-17A plays a critical role for the innate immune response through its ability to indirectly mobilize neutrophils, promoting airway neutrophils through the induction of granulocyte chemokines and growth factors. Therefore, GHX02 is considered to have an inhibitory effect on neutrophil-related...
inflammation by decreasing pro-inflammatory cytokines. In addition, GHX02 effectively inhibits airway inflammation by regulating the expression of inflammatory cytokines through the blockade of MIP2 and CXCL-1 secretion. GHX02 and SR significantly suppressed the increases in CXCL-1 and MIP2 was decreased in treatment with GHX02 and AS, SR, CR. CXCL-1 is known to increase in the sputum of patients with COPD, and CXCL-1 and MIP2, which mainly secreted from macrophages, are reported to accumulate neutrophil and other inflammatory cells into lesions. Overall, GHX02 exhibited anti-inflammatory effects by inhibiting migration of inflammatory cells and expression of pro-inflammatory cytokines in a COPD mouse model. This anti-inflammatory effect could also be demonstrated by the result that GHX02 markedly attenuated the damage of lung.

This study provides evidence that the treatment of GHX02 and individual herbs exert preventive and therapeutic effects against COPD mouse model. Among individual herbs, CR significantly showed inhibitory effect on airway neutrophil accumulation and SR decreased the level of CXCL-1 effectively. Our results are consistent with previous reports indicating anti-inflammatory effects of GRHRH and as well as TS, AS, CR, SR. These data demonstrated that GHX02 may be a promising strategy for treatment of COPD. Further studies to discover the therapeutic mechanisms of GHX02 and clinical trials for COPD patients will be conducted.

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References


26. Gan WQ, FitzGerald JM, Carlsten C, Sadatsafavi


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