

Ginsenosides analysis in the crude saponin fraction extracted from Korean red ginseng, and its efficacious analysis against acute pulmonary inflammation in mice

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Abstract: In this study, we isolated ginseng crude saponin (GCS) from Korean red ginseng (KRG) and determined the ginsenoside content in it to investigate the physiological and pathological effects of GCS on acute pulmonary inflammation induced by intratracheal instillation of cigarette smoke condensates (CSC) and lipopolysaccharide (LPS) solution in BALB/c mice. GCS was orally administered at doses of 10 mg/kg and 25 mg/kg for 3 weeks. The recovery rate of GCS from KRG was 6.5 % and total ginsenosides from GCS was 1.13 %, and the content of Rb1 was the highest among them. Total inflammatory cells in the lung homogenates and bronchoalveolar lavage fluid (BALF) increased following intratracheal administration of CSC and LPS. However, GCS administration impaired this increase. Furthermore, it inhibited the increase in leukocytes in the blood, considerably decreased neutrophils in BALF, and declined infiltration of inflammatory cells and deposition of collagen in the tracheal and alveolar tissue. In this study, GCS was found to have a protective effect against acute pulmonary inflammation and it may be beneficial in preventing various respiratory diseases.

Key words: ginsenosides analysis, crude ginseng saponin, pulmonary inflammation, pathophysiological analysis

1. Introduction

In many ancient oriental books, the medical efficacy of Korean ginseng has been recorded. Long-term administration demonstrated a recovery in vigor and strength, a protective effect on the internal organs, an improvement in mental health, brightness of ageing eyes, the modulation of body weight, and prolonged life. Scientific research on Korean ginseng was not conducted until the 1950s.¹ In a recent study, Korean

ginseng demonstrated various important pharmacological effects such as antimicrobial, antioxidant, anti-inflammatory, and antitumor activity in vitro and in vivo.²⁻⁴ In particular, ginsenosides Rb1, Rb2, Rc, Rd, Rg1, and Re were identified as compounds responsible for biological activation of Korean ginseng. Ginsenoside Rb1 showed a protective effect in liver⁵ and ginsenoside Rd demonstrated anti-inflammatory properties by decreasing the activation of NF- κ B to reduce the iNOS and COX-2 activities.⁶

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In addition, ginsenoside Rh2 effectively suppressed the growth of human cancer cell.⁷

Chronic obstructive pulmonary disease (COPD) characterized by pulmonary inflammation is a respiratory disease with high prevalence and mortality rate. According to a World Health Organization (WHO) report, 210 million people are estimated to be suffering from COPD.⁸ In Korea, COPD, being the seventh leading cause of mortality, is responsible for 15.7 % of deaths per 100,000 people.⁹ In addition, 630,000 patients with COPD are treated in Korea annually at an estimated cost of 490 billion, which is increasing the social and economic burden.¹⁰ The factors contributing to the development of COPD include smoking, exposure to occupational particles, air pollution, genetics, age, gender, and lung growth.¹¹ Smoking is considered the primary cause of COPD.¹² Chemotaxis of neutrophils that allows migration toward sites of infection or inflammation is stronger in patients with COPD who smoke than in healthy individuals.¹³ In addition, interleukin-8 (IL-8), a chemoattractant cytokine, is upregulated in smokers, which results in an increase in neutrophils in the bronchoalveolar lavage fluid (BALF).¹⁴ During acute inflammation, IL-6 levels in the blood increases and promotes the production of C-reactive protein (CRP) in smokers.¹⁵ An increase in CD8+ T cells and B cells is observed in patients with COPD,¹⁶ along with reduced forced expiratory volume in 1 second (FEV1).¹¹ COPD symptoms, including pulmonary inflammation, can be alleviated by creating an inner environment of fresh air and leading a nature-friendly life, along with drug interventions.¹⁷ Prescription drugs, such as beta 2-agonist and corticosteroids, have been used to improve lung function and stabilize disease symptoms;¹⁸ however, side effects have been reported.¹⁹ Therefore, recent studies have actively explored natural substances that can reduce side effects and increase the effectiveness of chronic respiratory diseases prevention and treatment.²⁰⁻²⁹

Although Korean red ginseng (KRG) has been reported to extend potent therapeutic effects against various diseases, its potential protective effect against pulmonary inflammation has been hardly studied.

We analyzed ginsenosides in GCS extracted from KRG for the quality control of experimental materials used and investigating the inhibitory effect of GCS on acute pulmonary inflammation induced by cigarette smoke condensates (CSC) and lipopolysaccharides (LPS) in BALB/c mice.

2. Materials and Methods

2.1. Preparation of GCS fraction

Korean ginseng (*Panax ginseng* C.A. Meyer) used in this study was red ginseng produced at the Buyeo ginseng factory of Korea Ginseng Company. The GCS fraction was prepared by butanol extraction method.²² Red ginseng powder (50 g) was added to 70 % methanol solution, extracted at 80 °C, and concentrated by vacuum evaporation. This procedure was repeated thrice. The concentrated extract was dissolved in 100 mL of distilled water and mixed with 100 mL of butanol in a separating funnel. After shaking, the butanol layer and water layer were separated. The water layer was recovered and added to 100 mL of butanol. This procedure was repeated twice. The separated butanol layer was evaporated under vacuum. The GCS content in the butanol extract was measured.

2.2. Analysis of ginsenosides from GCS

Crude saponin powder (0.1 g) extracted from Korean red ginseng was weighed and added into a 10 mL volumetric flask and mixed with 10 mL of methanol. Dissolution was performed in an ultrasonic cleaner (60 Hz, Wiseclean, Seoul, Korea) for 30 min. Then, the solution was filtered (0.2 µm, Acrodisc, Port Washington, NY, USA) and injected into the ultra-performance liquid chromatography [UPLC; Waters Acquity (Waters, USA)] system.

The UPLC system was equipped with a photo diode array detector (Waters, USA), sample manager module (autosampler), and column compartment/heater (both enabling temperature control). Data were collected and processed using Empower chromatographic software (Waters, USA). An acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 µm particles)

was used. The column temperature was 40 °C, the flow rate was 0.6 mL/min, and the injection volume was 2 µL. The mobile phase consisted of deionized water (A) and acetonitrile (B). UPLC gradient conditions were as follows: 0.5–14.5 min (15–30 % B), 14.5–15.5 min (30–32 % B), 15.5–16.5 (32–40 % B), 16.5–17.0 min (40–55 % B), 17.0–21.0 min (55–90 % B), 21–25 min (90–15 % B), 25–27 min (15 % B). The detection wavelength was set at 203 nm. The total ginsenoside content in GCS was measured by UPLC using ginsenosides as the standard.

2.3. Animals

BALB/c mice (18–20 g and 8 weeks of age) were purchased from Orient Bio Inc. (Sungnam, Korea). All the animals were fed solid food (crude protein \geq 22.1 %, crude fat \leq 8.0 %, crude fiber \leq 5.0 %, crude ash \leq 9.9 %, calcium \geq 0.6 %, and phosphorus \geq 0.4 %) in the form of a standard laboratory diet (Samyang feed, Korea). The room was maintained at a temperature of 22 °C \pm 2 °C and relative humidity of 55 % \pm 15 % in a 12-hour/12-hour light/dark cycle. All animal breeding and experiments were performed according to the guidelines of the institutional animal care and use committee (IACUC). Animals were divided into 5 treatment groups (n = 6 per group) as follows: (1) normal group receiving only water, (2) control group treated with LPS (Sigma, USA)+CSC, (3) LPS+CSC+dexamethasone (3 mg/kg; manufactured by Joongwae Pharma Co., Seoul, Korea) group, (4) LPS+CSC+GCS (10 mg/kg) group, and (5) LPS+CSC+GCS (25 mg/kg) group. All the animals were anesthetized with 7 % chloral hydrate (C8383, Sigma, USA) before sacrifice. To induce acute pulmonary inflammation, 50 µL of a solution containing LPS (100 mg/mL) and CSC (4 mg/mL) was administered through intratracheal injection once a week for 3 weeks.

CSC was extracted using CORESTA Monitoring cigarette No. & (CM5) from Heiner Borgwaldt (Hamburg, Germany). CM7 was smoked with an automatic smoking machine RM20 (Hamburg, Germany) according to ISO 3308. CSC was trapped in a 92-mm Cambridge filter pad, extracted by 2-

propanol for 1 day at room temperature and then concentrated by vacuum evaporator (Eyela, Tokyo, Japan). All other reagents and solvents used were special grade.

2.4. Collection of BALF and lung cells

To collect BALF from the trachea and lungs, a syringe containing 1 mL of 10 % fetal bovine serum (FBS) in Dulbecco's modified Eagle medium (DMEM) was inserted into the trachea of anesthetized mice and airways and pulled out the washing fluids off. This procedure was repeated thrice. For the separation of lung cells, lung tissue was minced in FBS-free DMEM and placed on a shaking incubator (KMC480S, VISION Sci., Korea) for 30 min. This procedure was also repeated thrice.

2.5. Measurement of inflammatory cells

After collection of BALF, the samples were incubated with ammonium chloride for 5 min at 37 °C to lyse the erythrocyte. The samples were washed with FBS/DMEM and stained with 0.04 % trypan blue, and the number of leukocytes was counted. Separated lung cells were washed with D-phosphate buffered saline (D-PBS) and passed through a cell strainer (352350, Falcon, USA) to remove impurities.

2.6. Histological analysis

Lungs were finely minced and fixed in a 10 % formaldehyde solution for 24 hours, followed by washing under running tap water for 8 hours. After embedding the samples in epoxy resin, we prepared thin sections on a microtome and stained them with hematoxylin & eosin (H&E) and Masson's trichrome stain (M-T). To examine goblet cells, the samples were stained with periodic acid-Schiff (PAS). The sliced tissue was examined under a 400 \times optical microscope (333246, Nikon, Japan).

2.7. Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis of differences between groups was performed by Student's t-test. Significance was assumed at $p < 0.05$ or $p < 0.01$.

3. Results and Discussion

3.1. Analysis of ginsenosides from crude saponin of Korean red ginseng

Since the separation of ginsenosides from ginseng using TLC was introduced in 1966,²³ many ginsenoside separation methods have been developed for rapid, accurate, and economical analysis.^{22,24-25} Fig. 1 shows the UPLC profile of ginsenosides separated from the GCS fraction, and Table 1 presents the content of each ginsenoside present in the GCS fraction. The crude saponin content in Korean red ginseng powder was 6.5 % (w/w), and the total content of all ginsenosides in the GCS fraction was 1.13 % (w/w). These results were similar to the results published by Kim.²²

These results were similar to the results published by Kim.²² However, A limitation observed when GCS was extracted using 1-butanol was that impurities present in Korean ginseng co-eluted with ginsenosides under most HPLC conditions and it took a long time

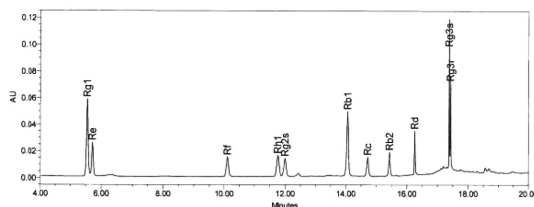


Fig. 1. UPLC ginsenosides profile of GCS fraction.

Table 1. The each ginsenosides content in GCS fraction

	Peak Name	RT (min)	Area	Amount (µg/g)
1	Rg1	5.554	226746	193.150
2	Re	5.720	98539	101.130
3	Rf	10.117	78680	60.890
4	Rh1	11.771	88540	59.410
5	Rg2s	12.002	74254	89.200
6	Rb1	14.070	206004	250.710
7	Rc	14.724	58016	70.320
8	Rb2	15.434	60379	71.030
9	Rd	16.263	68072	66.120
10	Rg3s	17.381	123103	104.120
11	Rg3r	17.426	84528	60.180
Total				1,126.3

with poor resolution. In our study, clear ginsenoside separation with narrow band and increased sensitivity was observed without any impurities peaks on the UPLC profile. And total time for ginsenosides separation was reduced within 40 min at about 120 min with an increased resolution capability. Actually, our method can identify ginsenosides from Korean ginseng easily and accurately.

3.2. Effects of GCS on inflammatory cells in lung homogenates and BALF

In general, if inflammation exacerbates, the total number of inflammatory cells increases. The signs of acute pulmonary inflammation are usually evident in the pulmonary alveoli and airways and are known to be closely associated with the type and number of inflammatory cells in the bronchi. In a study by Lee *et al.*, the number of total cells and neutrophils in BALF of patients with COPD who were smokers increased compared with that in patients with COPD who did not smoke.²⁶ Therefore, the measurement of total number of cells in the lung homogenates and BALF is important to identify acute inflammation pathogenesis.

Table 2 shows the number of total inflammatory cells in lung homogenates and BALF. The number

Table 2. The number of total inflammatory cells in Lung and BALF

Group	Numbers of total cells	
	Lung($\times 10^7$)	BALF($\times 10^5$)
Normal	0.15 \pm 0.02	1.90 \pm 0.60
CSC + LPS	0.58 \pm 0.07 ^{*a)}	13.5 \pm 3.17 ^{*a)}
CSC + LPS + Dexamethasone (3 mg/kg)	0.21 \pm 0.05 ^{*b)}	3.60 \pm 1.15 ^{*c)}
CSC + LPS + KRGCS (10 mg/kg)	0.27 \pm 0.04 ^{*b)}	8.50 \pm 4.60
CSC + LPS + KRGCS (25 mg/kg)	0.25 \pm 0.02 ^{*b)}	6.00 \pm 0.75 ^{*c)}

Values of the number of total cells in bronchoalveolar lavage fluid (BALF) and lung are expressed as mean \pm S.E (N=6). Statistical analysis of data was analyzed using Student's T-test. ^{*a)}p < 0.01 compared with normal group. ^{*b)}p < 0.05 and ^{*c)}p < 0.01 compared with control group.; Normal groups : Balb/c mice, CSC : Cigarette smoke condensate, LPS : Lipopolysaccharide, KRGCS : Korean red ginseng crude saponin.

of total cells was $(0.15 \pm 0.02) \times 10^7$ and $(1.90 \pm 0.60) \times 10^6$ in the lung homogenates and BALF of the normal group, respectively. These values indicated good physical condition without any abnormalities such as inflammation in mouse breeding.²⁷ However, the total cells in the lung homogenates and BALF of the CSC+LPS group significantly increased compared with the normal group ($p < 0.01$). On the other hand, the total cells in the lung homogenates and BALF of the CSC+LPS+dexamethasone (3 mg/kg) group significantly decreased compared with the CSC+LPS group ($p < 0.05$). In addition, the total cells in the lung homogenates and BALF of the CSC+LPS+GCS (10 mg/kg) group and CSC+LPS+GCS (25 mg/kg) group significantly decreased compared with the CSC+LPS group ($p < 0.05$), similar to the CSC+LPS+dexamethasone (3 mg/kg) group. A significant difference in the number of total inflammatory cells in BALF, but not in the lung homogenates, was observed between the CSC+LPS+GCS (10 mg/kg) and CSC+LPS+GCS (25 mg/kg) groups. Therefore, we concluded that the intratracheal administration of CSC and LPS in the mice increased the number of total inflammatory cells in the lung homogenates and BALF, which is a sign of acute pulmonary inflammation. However, this increase can be inhibited by treatment with GCS.

3.3. Effects of GCS on leukocytes in blood

Leukocytes cause inflammation at any site in the body in response to an immune reaction. The inflammation is resolved through chemical signaling by neutrophils, monocytes, eosinophils, and mast cells.²⁸ Furthermore, the incidence of COPD along with severity correlate with the increase in the number of polymorphonuclear leukocytes, macrophages, eosinophils, and lymphocytes related to inflammation.¹⁶ In general, markers, such as C-reactive protein (CRP), lipopolysaccharide binding protein (LBP), the soluble TNF receptor p55 (sTNF-R55), have been used to identify the induction of inflammation. CRP and sTNF-R55 are upregulated in patients with COPD, along with the number of leukocytes.²⁹⁻³⁰ We deduced that the investigation of the number of leukocytes in the blood can be an

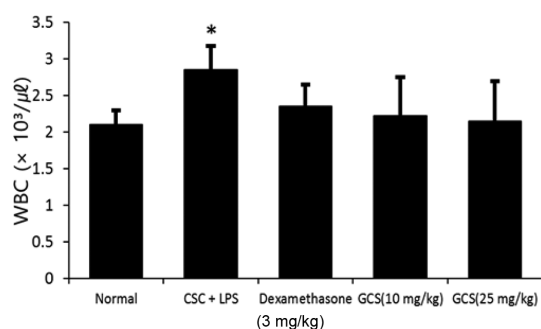


Fig. 2. The number of leukocytes in blood of mice. The number of leukocytes in blood was counted. Values are expressed as mean \pm S.E (N=5). Statistical analysis of data was analyzed using Student's T-test. * $p < 0.05$ compared with normal group.; Normal groups : Balb/c mice, Dexamethasone. : CSC+LPS+dexamethasone (3 mg/kg) group. KRGCS : Korean red ginseng crude saponin, WBC : white blood cell (leukocyte).

important basis for the evaluation of the degree of inflammation progress.

Fig. 2 shows the number of leukocytes in the blood of each group. The number of leukocytes significantly increased in the CSC+LPS group compared with the normal group ($p < 0.05$). However, the number of leukocytes in the CSC+LPS+dexamethasone (3 mg/kg) group did not significantly increase compared with the CSC+LPS group. Added dexamethasone (3 mg/kg) treatment resulted in a 15 % reduction in the leukocyte number induced by CSC and LPS without significance. In case of GCS treatment, the leukocyte production induced by CSC and LPS declined by approximately 19 % with the addition of 10 mg/kg of GCS and by 22 % with the addition of 25 mg/kg of GCS. There was no significant difference between the added GCS group and without GCS group. In general, many studies have reported difficulty in observing a significant change in inflammatory cells in the whole blood, but not in observing a significant change in markers or at any specific site.²⁸ Therefore, we concluded that additional GCS treatment only inhibited the increase in leukocyte production in the blood of BALB/c mice, induced by the intratracheal administration of CSC and LPS.

3.4. Effects of GCS on neutrophils in BALF

Neutrophils recognize as an indicator of all kinds

of inflammation at any site in various diseases. Many research have been shown that acute inflammation such as COPD and pneumonia increased the number of neutrophils in BALF and sputum.²⁹ Generally, the role of neutrophils is to remove the damaged tissues by initiating secretion of serin protease, neutrophils elastase, cathepsin G and protease 3.³⁰ In addition, movement of neurophils from the alveoli to the airways results in increased levels of cytokines such

as TNF- α and IL-1.³¹

Table 2 shows the number of neutrophils in the BALF of each group. The number of neutrophils in the BALF from normal group was $(1.60 \pm 0.43) \times 400$. The one from the CSC+LPS group was significantly increased as compared with the normal group ($p < 0.01$). However, the one from CSC+LPS+dexamethasone group (3 mg/kg) was significantly declined as compared with the CSC+LPS group. In addition, the ones from the CSC+LPS+GCS (10 mg/kg) and CSC+LPS+GCS (25 mg/kg) were reduced by 70 % and 77 %, as compared with the CSC+LPS group, respectively. Therefore, we concluded in this experiment that GCS treatment to the mouse dramatically decreased the number of neutrophils in the BALF which were increased by the intratracheal administration of CSC and LPS and acted positively in acute pulmonary inflammation.

Table 3. The number of neutrophils in BALF

Group	BALF
	Neutrophils ($\times 400$)
Normal	1.60 ± 0.43
CSC + LPS	$231.90 \pm 23.11^{*a)}$
CSC + LPS + Dexamethasone (3 mg/kg)	$35.50 \pm 6.15^{*b)}$
CSC + LPS + KRGCS (10 mg/kg)	$71.20 \pm 10.10^{*b)}$
CSC + LPS + KRGCS (25 mg/kg)	$53.80 \pm 18.72^{*b)}$

The number of neutrophils in BALF was counted using the Diff-Qick staining. Values are expressed as mean \pm S.E (N=6). Statistical analysis of data was analyzed using Student's T-test. ^{*a)} $p < 0.01$ compared with normal group. ^{*b)} $p < 0.01$ compared with control group.

3.5. Pathological analysis

Pathological characteristics of acute pulmonary inflammation such as COPD and pneumonia include

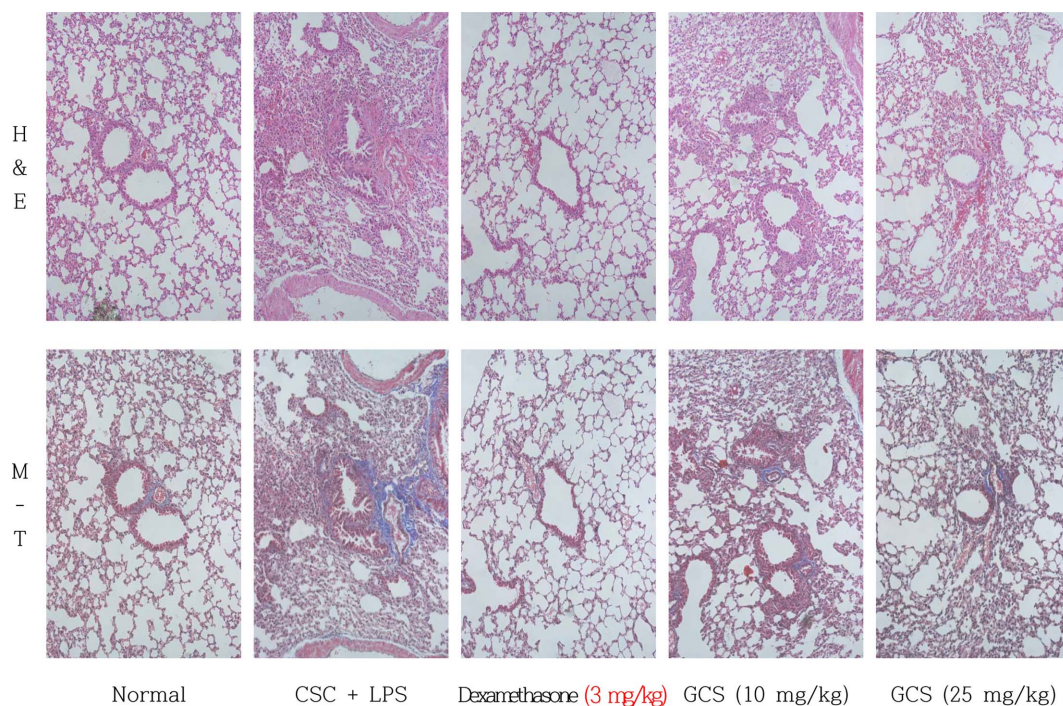


Fig. 3. The histological analysis of lung tissue.

a reduced rate of respiration that results in upregulation of collagen, with increased severity observed in lung arteries.³²⁻³³

Fig. 3 shows pathohistological analysis of lung tissue using H & E and M-T staining. The CSC+LPS group showed an increased infiltration of inflammatory cells and an elevated collagen deposition in the tracheal and alveolar tissue as compared with the normal group. In contrast, the decrease in inflammatory cell infiltration and collagen deposition was observed in two LPS+CSC+ GCS groups as compared with the LPS+CSC group. Therefore, we concluded with pathological analysis that GCS treatment to the mouse modulate the inflammatory cells infiltration and collagen deposition which enhanced by intratracheal administration of CSC+LPS in the tracheal and alveolar tissue.

4. Conclusions

The quality control of used experiment material before test is very important to research the efficiency of natural products. This study introduces a new method being able to determine ginsenosides from CGS for the quality control, and separate each of ginsenoside without any impurities easily and accurately with good resolution in our UPLC condition. The total ginsenoside content of the GCS we used to investigate the ginseng efficacy against acute pulmonary inflammation was 1.1%. Acute pulmonary inflammation in mice increased the number of total inflammatory cells in the lung homogenates and the BALF, the leukocyte in the blood, and neutrophils in the BALF, however, added GCS modulated these increases, and also declined pathologically the inflammatory cells infiltration and collagen deposition in the tracheal and alveolar tissue. We concluded that GCS has a protective potentials against acute pulmonary inflammation and may be beneficial for the prevention of various respiratory diseases.

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