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## Journal of Ginseng Research

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## Research Article

# Saponin attenuates diesel exhaust particle (DEP)-induced MUC5AC expression and pro-inflammatory cytokine upregulation via TLR4/TRIF/NF- $\kappa$ B signaling pathway in airway epithelium and ovalbumin (OVA)-sensitized mice

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## ARTICLE INFO

## Article history:

Received 17 November 2021

Received in revised form

20 March 2022

Accepted 31 March 2022

Available online 4 April 2022

## Keywords:

Diesel exhaust particle

IL-6/8

Korean Red Ginseng

MUC5AC

Saponin

## ABSTRACT

**Background:** Diesel exhaust particle (DEP) is a harmful kind of particulate matter known to exacerbate pre-existing respiratory diseases. Although their adverse effects on airway pathologies have been widely studied, the mechanistic analysis of signaling pathways and potential targets in reducing DEP-induced mucin secretion and pro-inflammatory cytokine production remain elusive. We, for the first time, investigated the effects of Korean Red Ginseng (KRG) extracts on mucin overproduction and airway inflammation induced by DEP.

**Methods:** The effects of KRG and saponin on DEP-induced expression of MUC5AC and interleukin (IL)-6/8 were examined by real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) in human airway epithelial NCI-H292 cells. We conducted Western blotting analysis to analyze the associated signaling pathways. To evaluate the effects of saponin treatment on DEP-induced MUC5AC expression and inflammatory cell infiltrations in ovalbumin (OVA)-sensitized mice, immunohistochemical (IHC) staining and real-time PCR were implemented.

**Results:** The KRG extracts markedly attenuated DEP-induced MUC5AC expression in vitro by inhibiting the TLR4/TRIF/NF- $\kappa$ B pathway. Furthermore, KRG and saponin inhibited DEP-induced pro-inflammatory cytokine IL-6/8 production. The in vivo study revealed that saponin blocked DEP-induced inflammation, mucin production and MUC5AC expression.

**Conclusion:** Our study revealed that KRG extracts have inhibitory effects on DEP-induced expression of MUC5AC and the production of pro-inflammatory cytokines. This finding provides novel insights into the mechanism by which saponin alleviates diesel-susceptible airway inflammation, elucidating its potential as a phytotherapeutic agent for inflammatory pathologies of airway.

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## 1. Introduction

Exposure to inhalable particulate matter (PM) from urban vehicles is highly associated with morbid respiratory diseases [1]. In particular, diesel exhaust particles (DEP), a major source of ambient air PM<sub>2.5</sub>, is a mixture of organic chemical compounds like *n*-

hexane, benzene, chloroform, ethyl acetate and methanol [2]. In the previous reports, it has been postulated to trigger inflammatory responses in respiratory system, act as an immunological adjuvant to exacerbate pulmonary diseases and potentiate allergic immune responses as asthma [3–5]. Metabolism of organic carbons from DEP including *n*-hexane and dichloromethane forms reactive oxygen species with potential to provoke inflammation [6,7]. Another main components in DEP, a benzene ring-containing polycyclic aromatic hydrocarbons (PAHs) and its derivatives are suggested to elicit pro-inflammatory cellular effects by specifically binding to

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aryl hydrocarbon receptors in human bronchial epithelial cells [8,9].

One of the therapeutic approaches for respiratory diseases is use of phytopharmaceuticals or herbal medicines by their secretolytic and mucolytic properties [10]. Korean Red Ginseng (KRG), a complementary therapeutic agent in Korean traditional medicine, has been an adjuvant candidate for treating airway inflammation. It comprises ginsenosides, saponins, and non-saponins such as polysaccharides, polyacetylenes, phenolic compounds, alkaloids, vitamins, and oligopeptides [11]. Several studies have reported the immunomodulatory functions of ginseng [12,13]. Herein, we used KRG to ameliorate mucus overproduction and reduce pro-inflammatory cytokine levels.

Since previous studies have shown a strong association between DEP and pathologic airway conditions from a molecular biological point of view [14,15], we investigated the underlying signaling pathways relevant to mucin hypersecretion and airway inflammation. Among 20 types of human mucin genes, we focused on the MUC5AC, a predominant secretory mucins associated with airway inflammation. In addition, we explored the effects of KRG components on pro-inflammatory cytokine IL-6/8 production following DEP exposure to investigate the therapeutic values for alleviating airway inflammation.

## 2. Materials and methods

### 2.1. Materials

Human airway epithelial NCI–H292 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Invitrogen (Carlsbad, CA, USA) provided Roswell Park Memorial Institute (RPMI)-1640 medium, while GIBCO (Grand Island, NY, USA) supplied 10% fetal bovine serum (FBS). DEP was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). KRG and saponin samples were acquired from the Korean Ginseng Corp. (KGC) (Daejeon, Korea).

Daeil Lab (Seoul, Korea) supplied the EZ-Cytox Cell Viability Assay Kit. The primer sequences of MUC5AC were purchased from Qiagen (QT01329615; Hilden, Germany). Primary antibodies against MUC5AC (rabbit anti-MUC5AC, #sc-20118), horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit immunoglobulin G-HRP, #sc-2004) and TLR4 antibody (#sc-293072) were procured from Santa Cruz (CA, USA). Phosphorylated form of NF- $\kappa$ B p65 rabbit mAb (#3033) and NF- $\kappa$ B p65 rabbit mAb (#4764) were acquired from Cell Signaling Technology (Danvers, MA, USA). For phosphorylation study, NOVUS donkey anti-rabbit (1:4000) secondary antibody was used. Sigma-Aldrich (St. Louis, MO, USA) supplied ovalbumin (OVA) and aluminum hydroxide.

### 2.2. Cell culture and treatment

We cultured human airway epithelial NCI–H292 cells in the RPMI-1640 medium, supplemented with 10% FBS, 2 mM glutamine, and 1% antibiotics (100  $\mu$ g/ml streptomycin and 100 U/ml penicillin G) at 37 °C in 5% CO<sub>2</sub> and fully humidified air. When confluency reached 80–90%, 24 h-incubation of the cultured cells in a serum-starved medium and rinsing with phosphate-buffered saline (PBS) were executed. Afterward, the cells were incubated with the indicated concentrations of DEP. In addition, the culture was pretreated with KRG (0.25 mg/ml) or saponin (25  $\mu$ g/ml) for 24 h before incubating with 20  $\mu$ g/ml DEP for 4 h.

### 2.3. Cell viability assay

The EZ-Cytox Cell Viability Assay Kit was used to analyze the proliferation and cytotoxicity of NCI–H292 cells by assessing the morphological changes under a microscope. The cells were cultured in 96-well plates at a density of  $2 \times 10^4$  cells per well for 24 h. We washed the cells with a fresh medium and subsequently treated the stimulus. After incubation for 2 days, 10  $\mu$ L of water-soluble tetrazolium salt-1 (WST-1) was added as a marker of cell viability, followed by 4 h-incubation. The quantity of formazan salt formed was measured by its absorbance at 450 nm (reference; 600–650 nm) using microplate reader (Tecan Austria GmbH, Austria).

### 2.4. Real-time polymerase chain reaction

Using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in accordance with the recommendations of manufacturer, real-time polymerase chain reaction (PCR) was performed to detect and quantify mRNA expression. In brief, reverse-transcription of 1 ng/ml total RNA was progressed to generate cDNA, maximally 2 ml of each cDNA sample was used per PCR mixture (20 ml). Using the CFX96 real-time PCR system C1000 thermal cycler (Bio-Rad, Hercules, CA, USA), we conducted 50 cycles of denaturation at 95 °C for 15 s and primer-specific annealing at 60 °C for 45 s. To assess the specificity of amplified PCR products, we performed a melt-curve analysis instructed by Roche Applied Science (Penzberg, Germany). 5'-TCA ACG GAG ACT GCG AGT ACA C-3' and 5'-CTT GAT GGC CTT GGA GCA-3', respectively, were forward and reverse primer sequences utilized for quantitative real-time PCR of MUC5AC. Data were normalized versus a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), of which the primer sequences are 5'-CCT CCA AGG AGT AAG ACC CC-3' (forward) and 5'-AGG GGT CTA CAT GGC AAC TG-3' (reverse).

### 2.5. Enzyme-linked immunosorbent assay

After incubation for 24 h, DEP was treated to the NCI–H292 cells as previously described. The protein levels of MUC5AC were evaluated by ELISA. Supernatants of NCI–H292 cells were collected. The samples diluted with PBS were transferred to an F96 certified MaxiSorp Nunc-Immuno Plate (Fisher Scientific, Lenexa, KS, USA) and incubated overnight at 4 °C. Subsequently blocked with 2% bovine serum albumin (BSA) for 1 h and incubated with 1:200 dilution of rabbit anti-MUC5AC antibody (#sc-20118; Santa Cruz Biotechnology, USA), the samples were incubated with an HRP-conjugated secondary antibody. After 1 h, we incubated each well with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution, terminating the reaction with 2 N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>). Using EL800 ELISA reader by BIO-TEK Instruments (Winooski, VT, USA), we measured optical density at 450 nm and indicated the data as a fold increase to the control.

### 2.6. Western blotting analysis

The cells harvested in a radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, IL, USA; 200  $\mu$ L) were incubated for 20 min at 4 °C with a phosphatase inhibitor cocktail from Roche (Mannheim, Germany). After the centrifugation at 2500 $\times$ g at 4 °C for 10 min, the supernatants (whole-cell lysate) were obtained. Proteins (20  $\mu$ g) were separated by SDS-PAGE (10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto a membrane of polyvinylidene difluoride. It was blocked with 5% BSA in Tris-buffered saline (TBS) with 0.1% Tween 20 and incubated overnight with the primary antibodies at 4 °C. Subsequently

washing with TBS and 0.1% Tween 20, the blots were incubated with a secondary antibody for 1 h. We developed the signals equivalent to the protein of interests using an enhanced West Pico Chemiluminescent substrate (Thermo Fisher, Boston, MA, USA) and detected the bands by the Chemiluminescence image system (FUSION-FX7-820wL, Eberhardzell, Germany).

## 2.7. Animals and husbandry

We acclimatized the mice (Wild-type BALB/c; male, 5–6 weeks old, 15–18 g) purchased from Koatech (Pyeongtaek, Gyeonggi-do, Korea) for 14 days. The humidity (40–45%), temperature (20–25 °C), and 12 h light/dark cycle of polycarbonate cages were controlled for housing environment. They were provided water ad libitum and fed a standard chow diet consistently. Yeungnam University Medical Center Institutional Animal Care and Use Committee (YUMC-IACUC) approved all the experimental protocols for in vivo study (YUMC-AEC2018-043) and we abided by the 'Guidelines for the care and use of laboratory animals' issued by Yeungnam University.

## 2.8. OVA-induced asthma model and DEP exposure

The experimental protocol for asthma model is as follows. On days 0, 7, and 14, OVA (20 µg) and aluminum potassium sulfate (2 mg) in 0.2 ml of sterile PBS were injected intraperitoneally to mice. After the initial sensitization, mice were challenged with 1% OVA in PBS on days 21–25. The OVA challenge was performed using a nebulizer (Buxco, Sharon, CT, USA) for 30 min. For the control group, non-sensitized mice were exposed to a nebulized PBS alone. After the final inhalation challenge with 1% OVA, airway response assessment through whole-body plethysmography was conducted to confirm asthma. On days 26–30, intravenous administration of saponin (KGC, Daejeon, Korea) was completed to the OVA-sensitized asthmatic mice. In sequence, intranasal instillation of DEP (20 µg/ml) was performed to saponin-treated and non-treated OVA-sensitized mice.

## 2.9. Mice inhalation and organ preparation

Anesthesia of mice with isoflurane (Forane; JW Pharmaceutical, South Korea) was done in an oxygen-delivered small rodent gas anesthesia machine (V-1, VetEquip, Pleasanton, CA, USA). It was administered through a mask placed over the mouse's head in an anesthetization chamber. We used ketamine (100 mg/kg) and xylazine (10 mg/kg) to anesthetize the mice and fixed the collected lung tissues in 4% paraformaldehyde at 4 °C overnight. After the fixation, tissues were rinsed twice for 0.5 h in PBS and dehydrated in graded ethanol baths (70%, 80%, 95% ethanol for an hour respectively) followed by 100% ethanol baths twice for 2 h each. After treating xylene twice for 2 h and then incubating in paraffin at 58 °C overnight, paraffin tissue blocks were prepared.

The processing, embedding, and sectioning of paraffin blocks were each performed using an automatic tissue processor (Leica, TP1020 Heidelberg, Germany), tissue embedding system (Leica, EG1150) and rotary microtome (Leica, RM2235), following the manufacturer's protocol. Lung tissues with trachea were mechanically disrupted using a magnetic bead with a homogenizer (Fast-Prep-24, MP Biomedicals, France), to determine the gene expression.

## 2.10. Hematoxylin and eosin, periodic acid-Schiff staining and immunohistochemistry

Paraffin-embedded lung tissue blocks of mice were sectioned into 4-µm thickness and then deparaffinized with xylene. Subsequently, hematoxylin and eosin staining (Sigma-Aldrich, St. Louis, MO, USA) was performed. We analyzed the H&E stained sections under a light microscope (Ti-S 733551, Nikon, Tokyo, Japan) of 200× magnification. The infiltration of inflammatory cells was graded on a subjective scale and compared between the control and OVA-challenged mice.

Using the periodic acid-schiff (PAS) staining kit (Merck Millipore, Darmstadt, Germany), PAS staining was conducted after deparaffinization. Briefly, the samples were incubated in 0.5% periodic acid for 5 min. After washing the sections in running tap water for a minute and immersed in Schiff's reagent for 15 min, we washed the samples in tap water for 3 min and counterstained with hematoxylin solution-modified according to Gill III (Merck Millipore) for 2 min. Next, the samples were washed in tap water for 3 min and dehydrated with 96% alcohol twice. Finally, they were cleared with xylene and mounted with Toluene Permount Mounting Medium of Fisher Scientific (Hampton, NH, USA).

Immunohistochemical study was performed on paraffin-embedded 4-µm sectioned lung tissues. MUC5AC was detected using an unconjugated anti-MUC5AC antibody of 1:100 dilutions (#ab-198294; Abcam, Cambridge, UK) and a goat anti-rabbit HRP-conjugated secondary antibody. Non-immune serum IgG served as a negative control. Counterstaining of the slides with hematoxylin was performed and mounted. Two independent blinded observers performed IHC analyses on three different sections in five tissues per group by a semi-quantitative method. The intensity of staining was scored as 0, negative; 1+, mild; 2+, moderate; and 3+, marked and the extent was graded from 0 (no signal in any cell) to 4+; demonstrating the percentage of positively stained cells. The immunoreactivity score was calculated as multiplication of intensity (0–3) and extent score (0–4), ranged from 0 to 12.

## 2.11. Statistical analysis

We conducted statistical analyses by the SPSS version 12.0 (SPSS, Chicago, IL, USA). Data are indicated as mean ± standard deviation (SD) from each obtained quantitative value. After the test of normality, Kruskal–Wallis test was used for comparison as non-parametric statistics. To determine the effects of KRG or saponin on DEP-induced mucin expression, we analyzed by Mann-Whitney *U* test post-hoc. For all the tests conducted, *p* values < 0.05 were considered statistically significant.

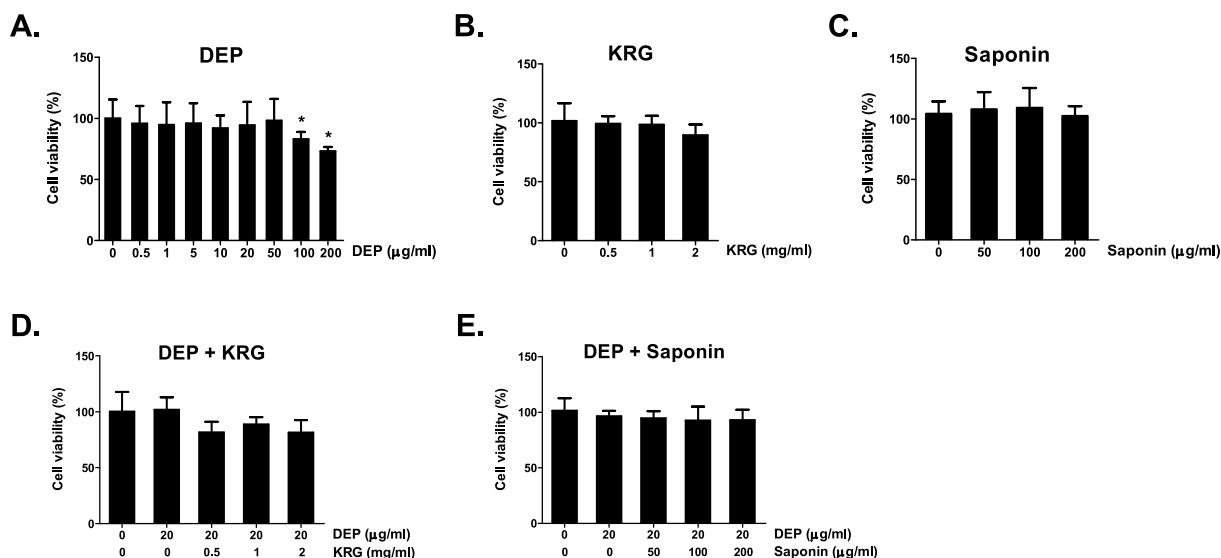
## 3. Results

### 3.1. Cell viability measurement

DEP up to 50 µg/ml did not affect the viability of NCI–H292 cells. However, DEP above 100 µg/ml exerted significant cytotoxic effects (Fig. 1A). KRG up to 2 mg/ml and saponin up to 200 µg/ml had no significant effect on the cell viability (Fig. 1B and C). No significant decrease in cell viability was observed after co-exposure to 20 µg/ml of DEP and the indicated concentrations of KRG extracts or saponin (Fig. 1D and E).

### 3.2. KRG extracts inhibited DEP-induced MUC5AC mRNA expression and protein synthesis

To investigate the effects of KRG extracts on MUC5AC mRNA expression and protein production after DEP exposure, we



**Fig. 1.** Effects of DEP and ginseng extracts on the viability of human NCI–H292 cells. (A) The viability of human airway epithelial NCI–H292 cells treated with 100 µg/ml and 200 µg/ml DEP significantly decreased, whereas cells treated with DEP up to 50 µg/ml remained viable. (B, C) KRG (up to 2 mg/ml) and saponin (up to 200 µg/ml) did not affect the cell survival. (D, E) Exposure to DEP (20 µg/ml) in combination with KRG up to 2 mg/ml or saponin up to 200 µg/ml, respectively, did not affect the cell viability. Bars indicate means ± standard deviation (SD) of three independent experiments conducted in triplicate. \**p* < 0.05 versus the zero value. DEP, diesel exhaust particles; KRG, Korean Red Ginseng.

incubated NCI–H292 cells with KRG (0.25 mg/ml) or saponin (25 µg/ml) with pretreatment of DEP (20 µg/ml). Real-time PCR and ELISA analyses revealed that KRG and saponin significantly decreased DEP-induced overexpression of MUC5AC mRNA (Fig. 2A) and protein overproduction (Fig. 2B).

**3.3. TLR4-mediated TRIF signaling cascade is implicated in attenuation mechanism of KRG extracts against DEP exposure**

To examine whether TLR4/TRIF signaling is involved in attenuation effects of KRG extracts, the mRNA expression of downstream adaptor proteins was measured by Western blotting and real-time PCR analyses. DEP-induced TLR4 mRNA overexpression and protein production declined when cells were treated with KRG (0.25 mg/ml) or saponin (25 µg/ml) (Fig. 3A and B). In addition, dexamethasone, a positive control for KRG extracts, attenuated DEP-induced MUC5AC expressions. As indicated in Fig. 3C and D, KRG and

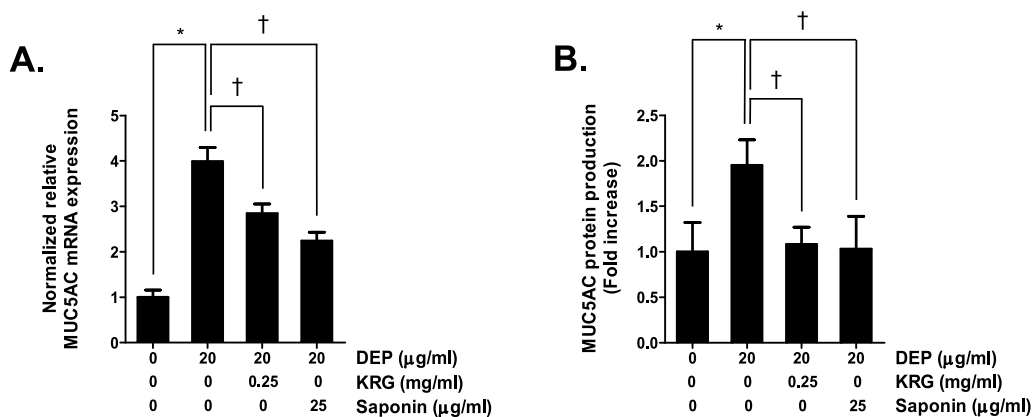
saponin downregulated the mRNA expression of TRIF, but not of MyD88.

**3.4. KRG and saponin attenuated the DEP-induced phosphorylation of NF-κB**

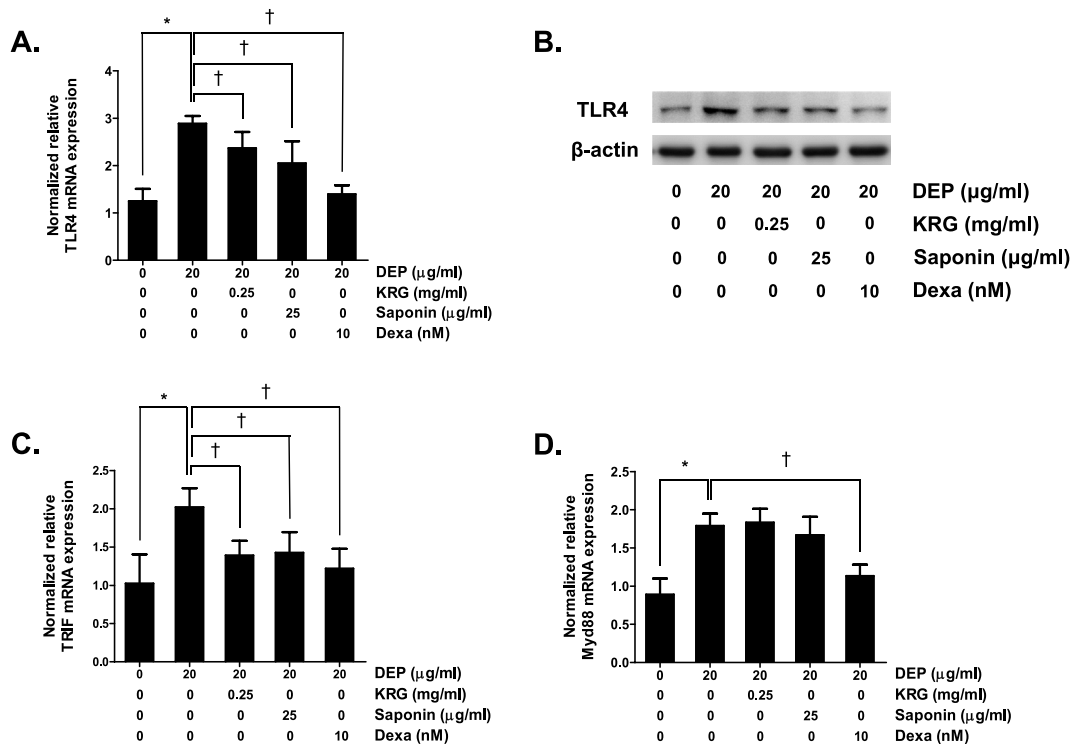
We discovered that DEP activated the phosphorylation of NF-κB (Fig. 4A). In addition, KRG, saponin and dexamethasone significantly attenuated the DEP-induced NF-κB phosphorylation (Fig. 4B). These results confirmed that NF-κB signaling is involved in the DEP-induced MUC5AC expression.

**3.5. KRG inhibited DEP-induced IL-6/8 mRNA expression and protein synthesis**

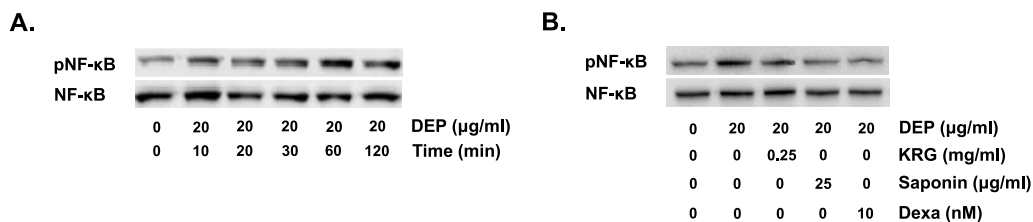
To determine the effects of KRG extracts on pro-inflammatory cytokine IL-6/8 production induced by DEP, real-time PCR and ELISA analyses were undertaken. DEP significantly increased the



**Fig. 2.** Inhibitory effects of KRG extracts on DEP-induced MUC5AC mRNA expression and protein production. To confirm the effects of ginseng extracts on MUC5AC mRNA expression and protein synthesis induced by DEP, we performed real-time PCR and ELISA analyses at different pretreatment combinations. (A, B) KRG (0.25 mg/ml) and saponin (25 µg/ml), respectively, inhibited DEP-induced MUC5AC mRNA expression and protein production. Bars indicate the means ± SD of three independent experiments conducted in triplicate. \**p* < 0.05 versus the zero value, †*p* < 0.05 versus DEP (20 µg/ml). DEP, diesel exhaust particles; KRG, Korean Red Ginseng; SD, standard deviation.



**Fig. 3.** Attenuation effects of KRG and saponin on TLR4-mediated MUC5AC expression against DEP exposure. (A, B) KRG, saponin, and dexamethasone significantly inhibited DEP-induced mRNA expression and protein levels of TLR4. (C) KRG and saponin significantly inhibited DEP-induced expression of TRIF mRNA, as dexamethasone. (D) Unlike dexamethasone, KRG and saponin did not attenuate the Myd88 mRNA expression induced by DEP. Bars indicate the means ± SD of three independent, triplicate experiments. \**p* < 0.05 versus the zero value, †*p* < 0.05 versus DEP (20 μg/ml). DEP, diesel exhaust particles; Dexa, dexamethasone; KRG, Korean Red Ginseng; SD, standard deviation.



**Fig. 4.** Effects of KRG and saponin on DEP-induced NF-κB phosphorylation. (A) Western blotting data indicated that DEP treatment activated the phosphorylation of NF-κB. The phosphorylated NF-κB peaked at 60 min after DEP exposure. (B) KRG (0.25 mg/ml) and saponin (25 μg/ml) significantly attenuated the DEP-induced expression of phosphorylated NF-κB, approximate as dexamethasone. DEP, diesel exhaust particles; Dexa, dexamethasone; KRG, Korean Red Ginseng.

levels of IL-6/8, whereas KRG, saponin and dexamethasone substantially attenuated the DEP-induced pro-inflammatory cytokine production (Fig. 5A and B).

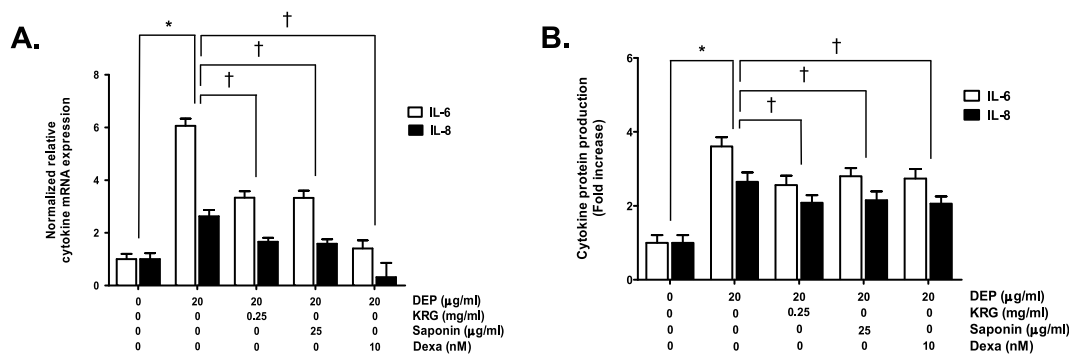
### 3.6. Saponin alleviated DEP-exacerbated airway inflammation and MUC5AC mRNA expression in OVA-challenged asthma mice

To investigate whether the in vivo study showed consistent findings with the previous results, we conducted staining and semi-quantitative histological analyses in a mouse model sensitized with OVA. The H&E stained bronchiole sections of OVA-challenged mice represented an increase of inflammatory cell infiltration compared to the control (Fig. 6A). In addition, PAS staining and immunohistochemistry for MUC5AC in OVA-challenged mice indicated increased mucus secretion and MUC5AC production, respectively (Fig. 6B and C). In comparison with the OVA-sensitized mice without DEP exposure, the inflammatory cell infiltration, mucus secretion, and MUC5AC production were prominent in DEP-treated OVA-sensitized mice. Saponin

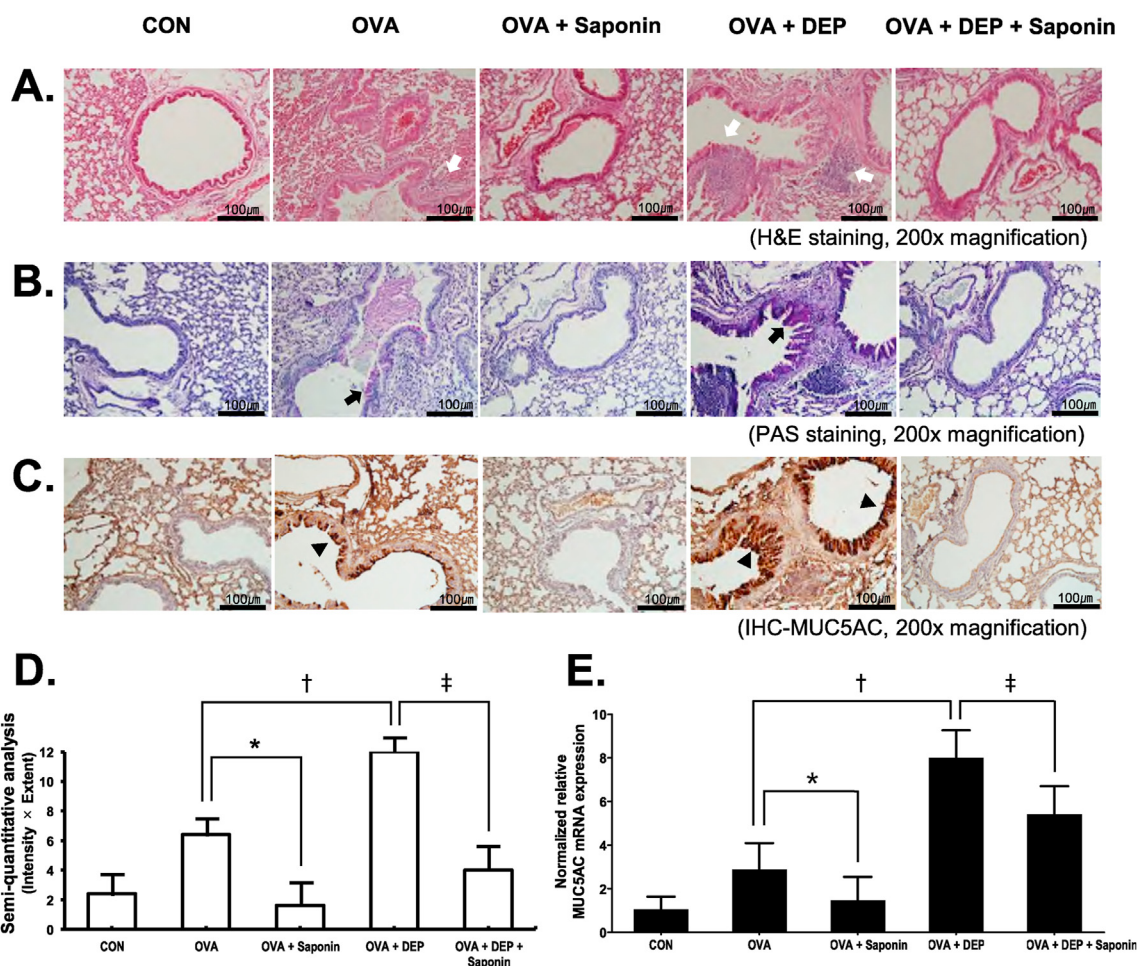
substantially alleviated these changes both in DEP-treated and non-treated OVA-sensitized mice, suppressing the infiltration of inflammatory cells, mucin hypersecretion and MUC5AC over-production (Fig. 6A–C). In a semi-quantitative analysis of immunoreactivity and real-time PCR, saponin significantly reduced mRNA expression levels of MUC5AC in both OVA-challenged mice with and without DEP (Fig. 6D and E).

## 4. Discussion

Exposure to DEP is known to be responsible for mucin over-expression in the human respiratory epithelium. Previously, it was reported to elevate the expression of MUC5AC via TLR4-mediated activation of the MAPK signaling pathway [16]. Because over-produced mucin may precipitate mucus accumulation, DEP was issued as a precipitating factor to exacerbate chronic airway inflammation. In the same manner that KRG and ginsenoside Rg3 suppressed mucin production induced by Asian sand dust through NF-κB inhibition in bronchial epithelial cells [17], we adopted DEP



**Fig. 5.** Effects of KRG and saponin on mRNA and protein levels of pro-inflammatory cytokines (IL-6/8) against DEP exposure. (A, B) KRG, saponin, and dexamethasone significantly inhibited DEP-induced mRNA expression and protein production of IL-6/8. Bars represent the means ± SD of three separate experiments conducted in triplicate. \**p* < 0.05 compared to the control, †*p* < 0.05 compared to DEP (20 μg/ml). DEP, diesel exhaust particles; Dexa, dexamethasone; KRG, Korean Red Ginseng; SD, standard deviation.



**Fig. 6.** Saponin alleviated DEP-exacerbated airway inflammation and MUC5AC expression in OVA-sensitized mice. (A–C) Light microscope images of bronchiole tissues from control, saponin-treated and non-treated OVA-sensitized mice with or without DEP exposure (200-x magnification). In comparison to the mice sensitized with OVA independently, DEP exacerbated the inflammation, mucus overproduction, and MUC5AC expression in OVA-challenged asthmatic mice. (A) H&E staining: Saponin alleviated inflammatory cell infiltrations (white arrow) in OVA-sensitized mice with and without DEP. (B) PAS staining: Saponin inhibited mucus production (black arrow) in both DEP-treated and non-treated OVA-sensitized mice. (C) Immunohistochemistry for MUC5AC: Saponin reduced MUC5AC-positive cells (black arrowhead) in OVA-challenged mice with and without DEP. (D) Semi-quantitative analysis of immunoreactivity score for MUC5AC: Saponin significantly lowered the MUC5AC-IHC scores in both OVA-sensitized mice with and without DEP. (E) DEP-exposed OVA-sensitized mice indicated a significant increase in MUC5AC mRNA expression, compared with OVA-sensitized mice without DEP. Saponin significantly blocked MUC5AC expression in both OVA-challenged groups with and without DEP treatment. Scale bars, 100 μm \*and †*p* < 0.05 in comparison to OVA, ‡*p* < 0.05 in comparison to OVA + DEP. DEP, diesel exhaust particles; OVA, ovalbumin; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; IHC, immunohistochemistry.

as a ligand of TLR4 and revealed the inhibitory properties of KRG and its ginsenosides on MUC5AC expression [18]. Downregulation of DEP-induced MUC5AC mRNA and protein expression in human NCI–H292 cells by KRG or saponin indicated their potentials for treating mucus-producing respiratory diseases.

Korean Red Ginseng has been known for anti-inflammatory and antioxidant properties recently, suggesting rationale for its phytotherapeutic use. For instance, it has been reported to alleviate respiratory tract infections by reducing pro-inflammatory cytokine expression and enhancing cell-mediated immunity [19]. In this study, KRG extracts significantly inhibited DEP-induced expression of the pro-inflammatory cytokines. Since DEP exposure implicated morbid chronic airway pathologies relevant to pro-inflammatory cytokine activation [20], we evaluated the efficacy of KRG as an anti-inflammatory metabolite to alleviate DEP-aggravated inflammation. However, whether ginseng saponin alleviates disease-susceptible inflammatory responses needed more evidence through further studies.

Based on the concept that ameliorating the DEP-mediated signaling pathway would be instrumental in understanding the pathophysiology of bronchial asthma and pulmonary diseases sharing etiology [21], we demonstrated, for the first time, the attenuation effects of KRG extracts on mucin expression and airway inflammation against DEP. Moreover, our findings implied the crucial involvement of TLR4-mediated TRIF and NF- $\kappa$ B signal transduction in the process. In an attempt to determine the TLR4/TRIF signaling activity, further investigation to elucidate its precise regulatory mechanism and downstream signaling molecules in both gene and protein levels is needed.

The airway inflammation significantly increased in DEP-treated mice with OVA sensitization, whereas the concurrent treatment with saponin attenuated this increase to a certain degree. Consistent with a previous research suggesting that KRG reduced nasal allergic inflammatory reaction [22], the inflammatory cell infiltration and MUC5AC-positive cells in mice bronchioles were significantly lower in saponin treatment groups. Although Th2 cytokine reduction counted as possible mechanism, we focused on airway inflammation mediated by Th1-type cytokines herein, especially IL-6 and IL-8, as previously discussed in asthma murine model [23,24]. It suggested the value of ginseng saponin as complementary and alternative medicine to prevent mucus secretion and inflammation in lower respiratory tracts resulting from DEP.

However, to adopt saponin as a therapeutic adjuvant against airway inflammatory diseases, its associated intermediates and detailed subprocesses further need to be investigated. Understanding the underlying molecular mechanism could pave the way for developing pharmaceuticals implicated in the downstream signaling pathways. Further studies on specific metabolites of ginseng saponin, such as protopanaxadiol including Rb1/2, Rc, and Rd [25,26] may reveal the components of KRG responsible for its anti-inflammatory property and the mechanism by which it inhibits pro-inflammatory reaction induced by DEP.

According to our data, the attenuation effects of saponin on DEP-induced mucin expression and pro-inflammatory cytokine synthesis were similar to those of dexamethasone, in accordance with the glucocorticoid-like effects of ginsenosides in allergic murine model [22]. Its clinical application as an alternative to dexamethasone, however, would depend on patient selection, particularly in cases of relative contraindication to the steroid treatment due to patient comorbidity. Clinical trials to investigate a long-term efficacy of KRG and its ginsenosides are prerequisites for proving its therapeutic potential to lessen the progression and disease burden of chronic airway inflammation.

## 5. Conclusion

The KRG and saponin inhibited DEP-induced mucin overproduction by inactivating TRIF and NF- $\kappa$ B, the downstream signaling mediators of TLR4. This study demonstrated that TLR4/TRIF/NF- $\kappa$ B signaling cascade plays a pivotal role in MUC5AC overexpression, following the exposure of NCI–H292 cells to DEP. Immunomodulatory effects of saponin on OVA-induced asthma mice inferred that saponin components derived from KRG prevented DEP-aggravated mucus hypersecretion and its associated airway inflammation.

## Declaration of competing interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

This research was supported by the 2017 Korean Society of Ginseng.

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