

Anti-inflammatory effect of *Chrysanthemum zawadskii*, peppermint, *Glycyrrhiza glabra* herbal mixture in lipopolysaccharide-stimulated RAW264.7 macrophages

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Abstract. The normal inflammatory reaction protects the body from harmful external factors, whereas abnormal chronic inflammation can cause various diseases, including cancer. The purpose of the present study was to investigate the anti-inflammatory activity of a mixture of *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra* (CPG) by analyzing the expression levels of inflammatory mediators, cytokines and transcription factors in lipopolysaccharide (LPS)-stimulated Raw264.7 cells. A nitric oxide assay, ELISA, western blotting and immunofluorescence staining were performed to investigate the anti-inflammatory activity of the CPG mixture. Pretreatment of Raw264.7 cells with CPG inhibited the increase of inflammatory mediators (inducible nitric oxide synthase, cyclooxygenase-2 and IFN- β) induced by LPS. Additionally, it inhibited the production of pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β). CPG suppressed LPS-induced phosphorylation of STAT1, AKT, I κ b and NF- κ B. Furthermore, CPG inhibited the translocation of NF- κ B into the nucleus. In summary, CPG could inhibit LPS-induced inflammation, which occurs primarily through the AKT/I κ b/NF- κ B signaling pathway in RAW264.7 cells.

Introduction

Inflammation is a protective response to external stimuli such as pathogens, physical damage, and chemicals. It can inactivate or destroy invading organisms to maintain normal tissues (1). The inflammatory reaction begins with the release of arachidonic acid from cell membrane phospholipids. Through lipoxygenase or cyclooxygenase (COX) action, various mediators of inflammatory reactions such as leukotriene, thromboxane, and prostaglandin are produced (2). These mediators also include nitric oxide (NO), prostaglandin E₂ (PGE₂), and inflammatory cytokines (3). Moderate amounts of NO can kill bacteria or tumors. However, excessive NO production by iNOS can aggravate inflammatory responses, causing tissue damage, genetic mutation, and nerve damage (4). PGE₂, one of major mediators of inflammatory responses, contributes to tumor formation by inhibiting apoptosis and inducing angiogenesis (5). Cyclooxygenase (COX) is an enzyme that can promote the transformation of arachidonic acid into prostaglandin. In particular, COX-2 is induced by various stimulations of inflammation, growth factors, and cytokines caused by cancer cells (6). Tumor necrosis factor (TNF- α), interleukin (IL-6), and IL-1 β are typical pro-inflammatory cytokines whose expression levels are increased during inflammatory reactions. Macrophages secrete them to mediate various inflammatory reactions and induce NO and PGE₂ production (7). Normal inflammatory reaction protects the body. However, when inflammation continues and progresses to chronic inflammation, cancer can develop due to excessive secretion of inflammatory mediators. Insulin resistance can also increase, thereby acting as a mediator of diabetes and various diseases (8,9). Therefore, effective methods for controlling inflammation are needed. Nuclear factor kappa-B (NF- κ B) and mitogen-activated protein kinase (MAPK) are important signaling molecules in the Toll-like receptor (TLR) pathway. Studies are actively underway to control inflammation by inhibiting these molecules (10).

Chrysanthemum zawadskii is a perennial herb of the Asteraceae family. It is cultivated in China, Russia, Mongolia, and Japan. It has been reported that *C. zawadskii* possess

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antioxidant, antifungal, and anti-inflammatory effects (11). Peppermint (*Mentha piperita* L.), one of the most popular herbal tea, has been used as a folk remedy and alternative medicine to relieve gastritis, enteritis, biliary disorders, indigestion, and flatulence (12). Licorice (*Glycyrrhiza glabra*), one of the most commonly used herbal medicine, has been found to possess anti-inflammatory, antiviral, and immunomodulatory activities (13). The purpose of this study was to investigate the anti-inflammatory effect of a herbal mix of *C. zawadskii*, peppermint, and *G. glabra* (CPG) and its mechanism of action in mouse macrophages.

Materials and methods

Materials. *Chrysanthemum zawadskii*, Peppermint (*Mentha piperita*) and *Glycyrrhiza glabra* were purchased from Kyeondong market. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin antibiotics were purchased from Gibco; Thermo Fisher Scientific, Inc. Griess reagent and LPS (L2630) were procured from Sigma-Aldrich; Merck KGaA. Quanti-Max™ WST-8 Cell Viability Assay Kit has gotten from Biomax. Goat anti-mouse IgG (H+L) Alexa Fluor plus 488 conjugated secondary antibodies were purchased from Invitrogen; Thermo Fisher Scientific, Inc. Enzyme-linked immunosorbent assay (ELISA) kit PGE₂, TNF- α , IL-6, and IL-1 β were procured from R&D System. Mouse IFN Beta ELISA Kit (TCM, Serum) was supplied by BL Assay Science. HO-1 activity kit was purchased from Cusabio. Radio-immunoprecipitation assay buffer (RIPA buffer) came from Thermo Fisher Scientific, Inc. Bradford's assay reagent was purchased from Bio-Rad Laboratories, Inc. 5X SDS-PAGE loading buffer was purchased from Biosesang. Antibodies against iNOS, COX-2, p-NF- κ B, p-I κ B, p-Akt, p-STAT1, STAT1, and β -actin were purchased from Santa Cruz Biotechnology, Inc. HO-1 antibody was procured from Abcam. Horseradish peroxidase (HRP)-IgG secondary antibodies and diamidino-2-phenylindole (DAPI) were purchased from Cell Signaling Technology, Inc.

Preparation of CPG (FHH-CZ). *Chrysanthemum zawadskii* was extracted with distilled water at 85°C. Peppermint was extracted with 50% ethanol at 50°C. *Glycyrrhiza glabra* was extracted with distilled water at 50°C. All plant materials were extracted for 6 h. These extracts were filtered twice, concentrated, and freeze-dried. *Glycyrrhiza glabra* extract was mixed with 40% dextrin before freeze-drying. Freeze-dried samples were stored at 4°C before experiments. The CPG mixing ratio was *Chrysanthemum zawadskii* 15: Peppermint 75: *Glycyrrhiza glabra* 10. The mixture was named FHH-CZ.

Cell culture. RAW264.7 cells (ATCC, SC-6003) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics at 37°C with 5% CO₂ in an incubator. These cells were not serum-starved for all experiments.

Cell viability. RAW264.7 cells (2x10⁵ cells/ml) were cultured in 96-well plates for 24 h. CPG was used to treat cells at various concentrations (0 μ g/ml ~ 500 μ g/ml). After incubating for 20 h, 10 μ l of Quanti-Max™ was added. After 4 h of further incubation, the absorbance of each well was measured using a

spectrophotometer (Tecan) at 450 nm. The absorbance of each well conformed to RAW264.7 cell viability.

Nitric oxide assay. RAW264.7 cells (2x10⁵ cells/ml) were seeded into a 96-well plate and incubated for 24 h. These cells were then treated with CPG (0, 50, 100, 200 μ g/ml) for 1 h and then stimulated with LPS (1 μ g/ml) for 16 h. After stimulation, 100 μ l of Griess reagent was mixed with 100 μ l of supernatant and incubated at room temperature (RT) for 15 min. The absorbance at 540 nm was measured using a spectrophotometer (Tecan). NaNO₂ standards were diluted and used to determine NO concentration.

Assay of cytokine production. RAW264.7 cells (2x10⁵ cells/ml) were cultured in 60 mm cell culture dishes for 24 h, treated with CPG (0, 50, 100, 200 μ g/ml), and then incubated for 1 h in an incubator. After that, cells were stimulated with LPS (1 μ g/ml) for 16 h. Cell culture supernatant was then obtained to measure levels of PGE₂ (R&D KGE004B), TNF- α (R&D MTA00B), IL-6 (M6000B) and IL-1 β (MLB00C). Using ELISA kits following the manufacturer's protocol without modification.

Protein extraction and western blot. Raw264.7 cells (2x10⁵ cells/ml) were cultured in 60 mm cell culture dishes for 24 h, treated with CPG (0, 50, 100, 200 μ g/ml), and incubated for 1 h in an incubator. After that, cells were stimulated with LPS (1 μ g/ml) for 16 h. Whole proteins were then extracted from each sample using protease and phosphatase inhibitors treated-RIPA buffer. After quantification using Bradford protein analysis, proteins (30 μ g) present in each sample were separated for 1 h at 100 V on 12% polyacrylamide gels. After separation, proteins were transferred onto PVDF membranes for 1 h at 100 V. After blocking with 5% bovine serum albumin (BSA) for 1 h, membranes were washed three times with TBST solution (10 min each wash) and then incubated with each antibody (iNOS, COX-2, p-NF- κ B, p-I κ B, p-Akt, p-STAT1, STAT1, HO-1, β -actin) at 4°C overnight. Membranes were washed three times with TBST solution for 10 min and then incubated with mouse or rabbit HRP-conjugated secondary antibodies containing BSA for 2 h at RT. Subsequently, membranes were washed three times with TBST solution for 10 min and visualized with an imaging system (Alliance version 15.11; UVITEC) using EZ-western Lumi Pico Alpha chemiluminescent reagent. Band densities were analyzed using ImageJ (developed by the National Institutes of Health) and converted into a graph.

Immunofluorescence. RAW264.7 cells (2x10⁵ cells/ml) were cultured in 4-well cell culture slides for 24 h. These cells were then treated with CPG (0, 50, 100, 200 μ g/ml), incubated for 1 h in an incubator, and then stimulated with LPS (1 μ g/ml) for 16 h. Next, cells were fixed in ice cold methanol for 10 min at RT. Slides were washed three times with PBS. After that, slides were incubated with PBS containing 1% BSA for 1 h to prevent non-specific antibody binding. After that, slides were incubated at 4°C overnight with antibodies (NF- κ B, p65) in 1% BSA. Incubated slides were washed three times with PBS. After that, slides were incubated with Alexa Fluor 488-conjugated goat

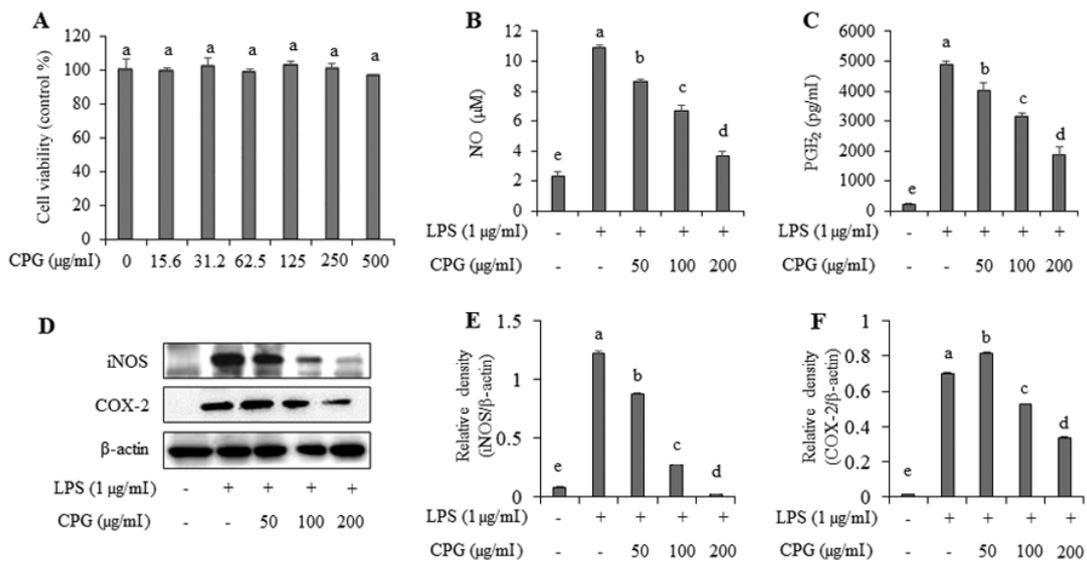


Figure 1. Effects of CPG on cell viability, NO levels, PGE₂ levels, iNOS and COX-2 expression in LPS-stimulated RAW264.7 macrophages. (A) RAW264.7 cells were treated with CPG at the indicated concentrations for 24 h and relative cell viability was assessed using a WST-1 assay. RAW264.7 cells were pretreated with CPG at the indicated concentrations and stimulated with LPS for 16 h. Levels of (B) NO and (C) PGE₂ in the culture supernatants were analyzed using a Griess assay and ELISA, respectively. (D) iNOS and COX-2 expression was determined by western blot analysis. (E) Relative density of iNOS was calculated using ImageJ. (F) Relative density of COX-2 was calculated using ImageJ. Data are presented as the mean ± SD. All groups labelled with the same lower case letter (a-e) were not significantly different from each other (P>0.05), whereas groups labelled with different lower case letters were significantly different (P<0.05). CPG, mixture of *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra*; LPS, lipopolysaccharide; NO, nitric oxide; PGE₂, prostaglandin E₂; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

anti-mouse or rabbit IgG antibody in 1% BSA at RT for 1 h. Subsequently, slides were washed with PBS, mounted with DAPI, and photographed under a ZEISS Fluorescence Microscope.

NF-κB DNA-binding activities. NF-κB DNA-binding activities were measured in the nuclear protein extracts using the NF-κB transcription factor assay kits. Nuclear extracts (10 µg) and complete binding buffer (0.03 ml) were added into wells pre-fixed with NF-κB p65 DNA target and incubated for 1 h at room temperature with mild agitation. Following the incubation, the wells were washed three times with wash buffers, and 0.1 ml of diluted NF-κB antibodies, which recognize only p65 epitopes that are bound to DNA in the wells, was added to each well and incubated for 1 h at room temperature. Then, the wells were washed as above, and HRP-conjugated antibodies (0.1 ml) were added to each well and incubated for 1 h at room temperature. Following incubation with the HRP-conjugated antibodies, the wells were washed. 0.1 ml of developing solution was added into the wells for 5 min and then, the reaction was stopped by adding 0.1 ml of stopping solution. The absorbance, which corresponded with the DNA binding activities, was read at 450 nm using a spectrophotometer (Tecan).

HO-1 activity. Raw264.7 cells (2x10⁵ cells/ml) were cultured in 60 mm cell culture dish for 24 h. the cells were treated with CPG (50, 100, 200 µg/ml) or without CPG and incubated for 1 h in the incubator. After that, the cells were stimulated by LPS (1 µg/ml) for 16 h. After that, whole proteins were extracted from each sample using protease and phosphatase inhibitors treated-RIPA buffer. And enzyme activity was measured using mouse HO-1 ELISA kit.

Statistical analysis. Data are presented as mean ± SD. Statistically significant differences among groups were determined by one-way analysis of variance (ANOVA) analysis followed by Tukey's post hoc test. P<0.05 was considered statistically significant.

Results

Effect of CPG on NO level and DPPH radical scavenging activity. *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra* were mixed in various ratios to determine the optimal mixing ratio (Table SI). NO assay and DPPH radical scavenging activity were measured for each mixing ratio. As a result, the No. 5 mixture (*Chrysanthemum zawadskii* 15: Peppermint 75: *Glycyrrhiza glabra* 10) showed the highest NO generation inhibitory effect and DPPH radical scavenging activity (Figs. S1 and S2). Therefore, subsequent experiments were conducted using No. 5 Mixture.

CPG inhibits NO and PGE₂ production in LPS-stimulated RAW264.7 macrophages. To investigate anti-inflammatory effects of CPG in LPS-stimulated RAW264.7 cells, we determined cytotoxic effects of CPG on RAW264.7 cells. After cells were treated with various concentrations (0, 15.6, 31.2, 62.5, 125, 250, and 500 µg/ml) of CPG for 24 h, cell cytotoxicity was investigated. CPG did not cause cytotoxicity to RAW264.7 cells at concentration up to 500 µg/ml. Based on such result, we selected concentrations of 50, 100, and 200 µg/ml to study the anti-inflammatory effect of CPG on LPS-stimulated RAW264.7 cells (Fig. 1A). Next, we evaluated effects of CPG treatment on NO and PGE₂ production in LPS-stimulated RAW264.7 cells. We found that LPS treatment significantly

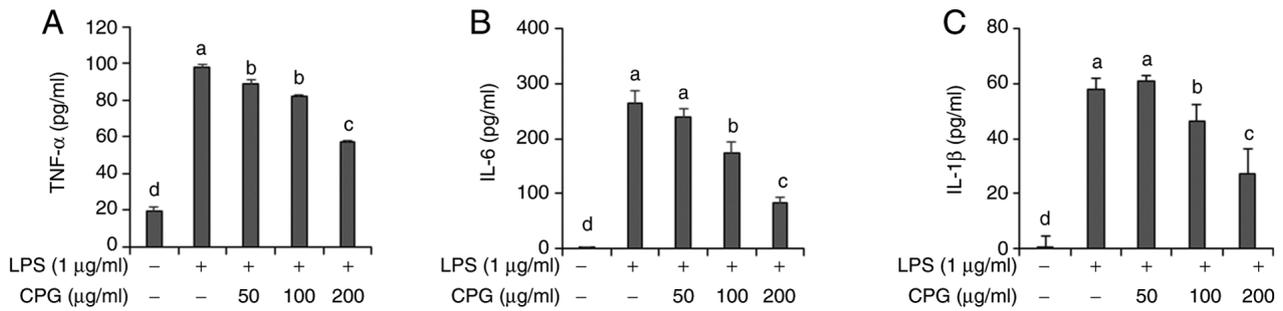


Figure 2. Effect of CPG on TNF- α , IL-6 and IL-1 β production in LPS-stimulated RAW264.7 macrophages. RAW264.7 cells were pretreated with CPG at the indicated concentrations and stimulated with LPS for 16 h. Levels of (A) TNF- α , (B) IL-6 and (C) IL-1 β in the culture supernatants were analyzed using an ELISA. Data are presented as the mean \pm SD. All groups labelled with the same lower case letter (a-d) were not significantly different from each other ($P>0.05$), whereas groups labelled with different lower case letters were significantly different ($P<0.05$). CPG, mixture of *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra*; LPS, lipopolysaccharide.

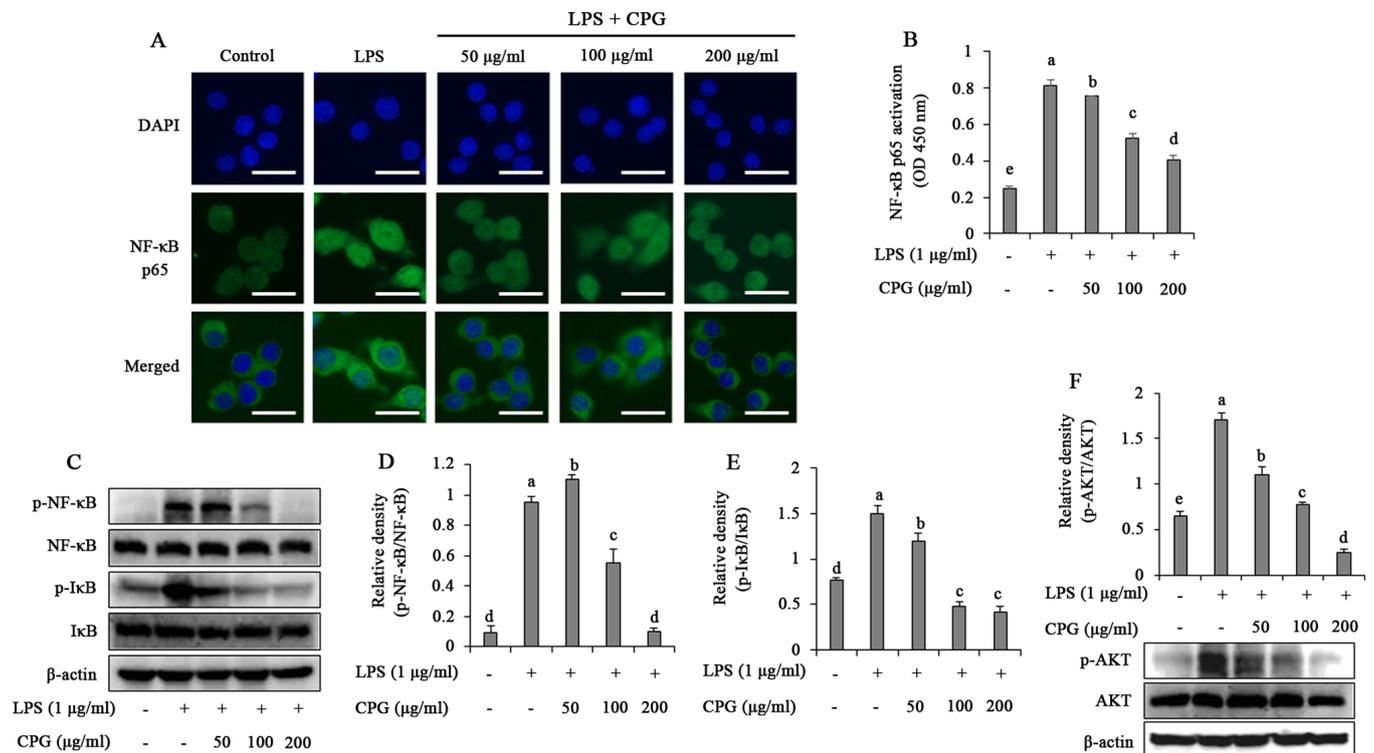


Figure 3. Effects of CPG on LPS-induced NF- κ B activation and Akt phosphorylation in RAW264.7 macrophages. RAW264.7 cells were pretreated with CPG at the indicated concentrations and stimulated with LPS for 30 min. (A) Translocation of NF- κ B p65 were determined using immunofluorescence. NF- κ B (green) was detected and nuclei were counterstained with DAPI (blue). Scale bar, 10 μ m. (B) DNA binding activity of NF- κ B p65 were determined using ELISA. Phosphorylation levels of (C) NF- κ B p65, I κ B and (F) Akt were measured by western blot analysis. (D) Relative density of p-NF- κ B p65 was calculated using ImageJ software. (E) Relative density of p-I κ B was calculated using ImageJ software. (F) Relative density of p-AKT was calculated using ImageJ software. Data are presented as the mean \pm SD. All groups labelled with the same lower case letter (a-e) were not significantly different from each other ($P>0.05$), whereas groups labelled with different lower case letters were significantly different ($P<0.05$). CPG, mixture of *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra*; LPS, lipopolysaccharide; p-, phosphorylated; OD, optical density.

increased NO production in RAW264.7 cells. However, cells pre-treated with CPG before administration of LPS showed significant decreases of NO and PGE₂ production in a dose-dependent manner compared to cells treated with LPS only (Fig. 1B and C).

CPG inhibits iNOS and COX-2 expression levels in LPS-stimulated RAW264.7 macrophages. To investigate inhibitory effects of CPG on NO and PGE₂ production, we examined iNOS (enzyme generating nitric oxide) and COX-2

(enzyme producing PGE₂) expression in LPS-stimulated RAW264.7 cells. Results showed that LPS-only treated cells had elevated expression levels of iNOS and COX-2 while cells pre-treated with CPG before administration of LPS showed significant decreases of iNOS and COX-2 expression compared to cells treated with LPS only (Fig. 1D-F).

CPG inhibits TNF- α , IL-6 and IL-1 β production in LPS-stimulated RAW264.7 macrophages. Next, we evaluated effects of CPG on the production of pro-inflammatory mediators such as IL-6,

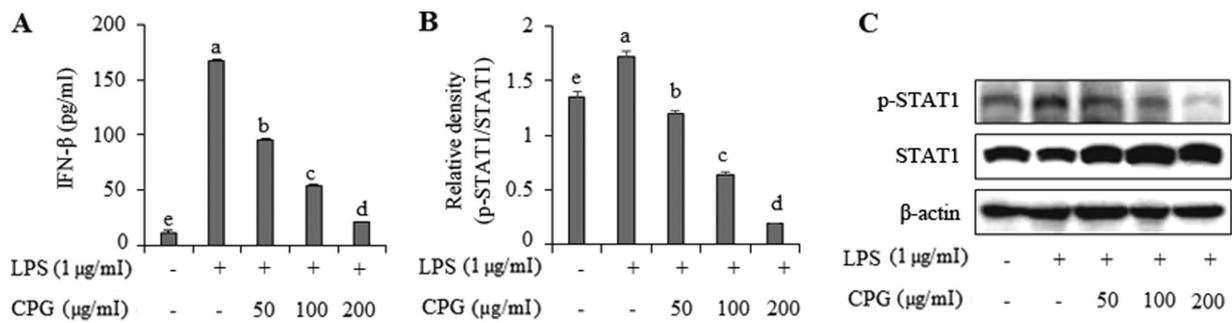


Figure 4. Effects of CPG on IFN- β expression and STAT1 activation in LPS-stimulated RAW264.7 macrophages. RAW264.7 cells were pretreated with CPG at the indicated concentrations and stimulated with LPS for 16 h. (A) IFN- β expression in culture supernatant was determined using ELISA. RAW264.7 cells were pretreated with CPG at the indicated concentrations and stimulated with LPS for 3 h. (C) STAT1 phosphorylation was determined by western blot analysis. (B) Relative density was calculated using ImageJ software. Data are presented as the mean \pm SD. All groups labelled with the same lower case letter (a-e) were not significantly different from each other ($P>0.05$), whereas groups labelled with different lower case letters were significantly different ($P<0.05$). CPG, mixture of *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra*; LPS, lipopolysaccharide; p-, phosphorylated.

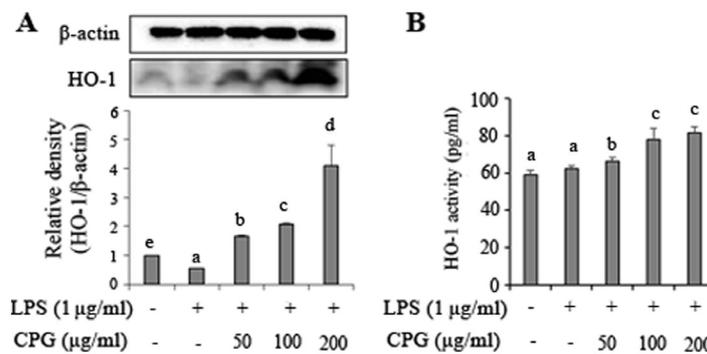


Figure 5. Effects of CPG on HO-1 expression in LPS-stimulated RAW264.7 macrophages. RAW264.7 cells were pretreated with CPG at the indicated concentrations and stimulated with LPS for 16 h. (A) Expression levels of HO-1 were determined by western blot analysis. (B) HO-1 activity was evaluated using an ELISA. Data are presented as the mean \pm SD. All groups labelled with the same lower case letter (a-e) were not significantly different from each other ($P>0.05$), whereas groups labelled with different lower case letters were significantly different ($P<0.05$). CPG, mixture of *Chrysanthemum zawadskii*, peppermint and *Glycyrrhiza glabra*; LPS, lipopolysaccharide; HO-1, heme oxygenase-1.

IL-1 β , and TNF- α in LPS-stimulated RAW264.7 cells. Results demonstrated that cells treated with LPS only had significantly increased production of IL-6, IL-1 β , and TNF- α cytokines in culture media of cells compared to untreated cells (Fig. 2). However, production levels of TNF- α , IL-6 and IL-1 β were found to be decreased in cells pre-treated with 100 or 200 μ g/ml of CPG before the administration of LPS (Fig. 2).

CPG inhibits NF- κ B and Akt activation in LPS-stimulated RAW264.7 macrophages. To determine molecular mechanisms underlying the anti-inflammatory effects of CPG, we evaluated NF- κ B pathways in LPS-stimulated RAW264.7 macrophages. As shown in Fig. 3A, NF- κ B p65 subunit was translocated into the nucleus in LPS-stimulated RAW264.7 cells. However, such translocation induced by LPS was markedly suppressed in CPG pretreated RAW264.7 cells. DNA binding activity of NF- κ B was also significantly increased in LPS-stimulated RAW264.7 cells. However, when cells were pre-treated with CPG before administration of LPS, the DNA binding activity of NF- κ B was significantly reduced in a dose-dependent manner (Fig. 3B). We further investigated whether CPG affected the phosphorylation of NF- κ B and I κ B in LPS-stimulated RAW264.7 cells. Results revealed that cells treated with LPS alone had significantly increased phosphorylation of NF- κ B and I κ B.

However, phosphorylated NF- κ B and I κ B were reversed when cells were pre-treated with 50 to 200 μ g/ml of CPG before administration of LPS (Fig. 3C-E). We also determined effects of CPG on Akt activation in LPS-stimulated RAW264.7 cells. Akt phosphorylation was significantly increased in cells treated with LPS alone. However, when cells were pre-treated with 50 to 200 μ g/ml of CPG before administration of LPS, the phosphorylation of Akt was significantly reduced in a dose-dependent manner (Fig. 3F).

CPG inhibits IFN- β production and STAT1 activation in LPS-stimulated RAW264.7 macrophages. Next, we analyzed the effect of CPG on IFN- β production in LPS-stimulated RAW264.7 cells. As shown in Fig. 4A, the production of IFN- β was significantly increased in cells treated with LPS only. However, cells pre-treated with 50 to 200 μ g/ml of CPG before administration of LPS showed a significant and dose-dependent decrease in IFN- β production compared to cells treated with LPS only. To further analyze the mechanism of action involved in the effect of CPG on IFN- β production, we determined STAT1 activation in LPS-stimulated RAW264.7 cells. As shown in Fig. 4B and C, when cells were treated with LPS alone, the phosphorylation of STAT1 was significantly increased in RAW264.7 cells. However, the phosphorylation of

STAT1 was significantly decreased when cells were pre-treated with 50 to 200 $\mu\text{g/ml}$ of CPG before administration of LPS.

CPG induces HO-1 expression in LPS-stimulated RAW264.7 macrophages. To further determine anti-inflammatory effects of CPG, we investigated HO-1 expression in LPS-stimulated RAW264.7 cells. As shown in Fig. 5A, cells treated with LPS alone showed slightly reduced HO-1 expression than non-treated cells. However, HO-1 expression was significantly increased when cells were pretreated with 50 to 200 $\mu\text{g/ml}$ of CPG before the administration of LPS. We also investigated HO-1 activity in LPS-stimulated RAW264.7 cells (Fig. 5B). Results showed that HO-1 activity was increased in cells pre-treated with 50 to 200 $\mu\text{g/ml}$ of CPG before the administration of LPS.

Discussion

As a result of measuring antioxidant and anti-inflammatory effects of *C. zawadskii*, peppermint, and *Glycyrrhiza glabra* in our preliminary study, it was confirmed that there was a synergistic effect when these herb were combined than a single substance. Therefore, in the present study, we investigated anti-inflammatory effects of CPG in LPS-treated RAW264.7 cells. LPS can cause inflammation in the human body via excessive production of pro-inflammatory mediators such as NO, PGE₂, iNOS, COX-2, TNF- α , IL-6, and IL-1 β (14,15). In the present study, we found that CPG significantly decreased LPS-elevated NO, PGE₂, iNOS, COX-2 production in RAW264.7 cells. The production of TNF- α , IL-6 and IL-1 β in stimulated cells was also suppressed by CPG. Several studies have found that herbal mixture can suppress NO, PGE₂, iNOS, COX-2, TNF- α , IL-6, and IL-1 β in macrophages (16,17). Thus, CPG might be useful for suppressing inflammatory mediators.

The production of pro-inflammatory cytokines by LPS is implicated in the NF- κ B signaling pathway. Therefore, NF- κ B has been studied as a key target in the treatment of inflammation (18). NF- κ B activation was investigated in this study to elucidate the precise mechanism involved in the effect of CPG on LPS-induced inflammation in RAW264.7 cells. When LPS is recognized by Toll-like receptor 4 (TLR4), NF- κ B is activated through the activation of MyD88 with phosphorylation and decomposition of I κ B α (19). I κ B α , an inhibitor of NF- κ B, is regulated by I κ B kinase (IKK). When activated, NF- κ B subunit p65 separates from I κ B α and migrates from the cytoplasm to the nucleus, causing transcription of pro-inflammatory cytokine genes (20). Previous studies have reported the anti-inflammatory effect of a herbal mixture is due to inhibition of NF- κ B signaling pathway (21,22). Akt is activated by PI3K. It is involved in the activation of transcription factors including NF- κ B (23). Our results revealed that CPG inhibited the translocation and DNA binding activity of NF- κ B in LPS-stimulated RAW264.7 cells. CPG also suppressed the phosphorylation of Akt, I κ B, and NF- κ B p65 in LPS-stimulated RAW264.7 cells. Results of the present study suggest that the anti-inflammatory effect of CPG might be due to the inactivation of NF- κ B, at least in part.

LPS stimulation can induce IFN- β production in macrophages through the TRIF/TBK1/IRF3 signaling pathway. IFN- β production is related to NF- κ B and MAPK activation (24). STAT1 activation is also required for IFN- β

production (25). Previous studies have reported that a herbal mixture can attenuate inflammatory responses by suppressing the inhibition of NF- κ B and IFN- β /STAT1 activation in macrophages (26). Consistent with previous studies, our results demonstrated that CPG could suppress IFN- β production and STAT1 phosphorylation increased by LPS stimulation in RAW264.7 cells. These findings suggest that CPG could suppress inflammatory responses by regulating IFN- β /STAT1 activation and NF- κ B pathway.

In immune responses, HO-1 plays an important role in the regulation of NO production. HO-1 induction is regulated by Nrf2 transcription factor, a strong antioxidant/anti-inflammatory regulator (27). Our results showed that CPG increased levels of HO-1 expression induced by LPS stimulation in RAW264.7 cells. Many studies have reported that natural products and herbal mixture possess anti-inflammatory activities by regulating Nrf2/HO-1 expression in macrophages (28,29). Therefore, Nrf2 transcription factor needs to be investigated in the future.

Herbal medicine is believed to have less side effects compared to drugs such as chemical counterparts or synthetic anti-inflammatory agents. Its effectiveness has been proven empirically for a long time (30). Anti-inflammatory and antioxidant functions of *C. zawadskii*, peppermint, and licorice have been reported previously (11-13). Linarin, the major bioactive substance of *C. zawadskii*, has been reported to exhibit anti-inflammatory, antioxidant, hepatoprotective, antibacterial, and anticancer activities (31). Limonene, one of major ingredients of peppermint, has been reported to have anti-inflammatory, antioxidant, antibacterial, and anti-cancer effects (32). A previous study has shown that glycyrrhetic acid exhibits anti-inflammatory effects through NO scavenging in LPS-stimulated RAW264.7 cells (33). Our results also found that linarin, limonene, and glycyrrhetic acid could inhibit NO production increased by LPS treatment in RAW264.7 cells (data not shown). Therefore, the anti-inflammatory effect of CPG might be due to bioactive substances contained in these three extracts used in the present study.

In conclusion, CPG herbal mixture could suppress the production of NO, iNOS, COX-2, and other pro-inflammatory mediators including PGE₂, TNF- α , IL-6, IL-1 β , and IFN- β in LPS-stimulated RAW264.7 macrophages. CPG also could suppress LPS-induced inflammation through Akt/NF- κ B and STAT1 signaling pathways in RAW264.7 cells. Moreover, CPG could inhibit LPS-induced inflammation through HO-1 induction in RAW264.7 cells. Results of the present study demonstrates that CPG should be considered as anti-inflammatory natural materials for the treatment of inflammatory diseases. In addition, animal model studies are required to verify whether the effect of CPG, which was shown at the cellular level, appears in animals.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BOC and SIJ conceived the study and developed the methodology. SIJ provided resources, reviewed and edited the manuscript, and supervised the study. JYS, HJK, JHP, SH and FW participated in acquisition, analysis and interpretation of data. BOC and JYS wrote the original draft. BOC, JYS and SIJ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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