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Extracts of Magnoliae Cortex and *Zea mays L.* modulate TLR-2 ligand-induced inflammatory reactions

후박 추출물과 옥수수 불검화 추출물이 TLR-2 ligand로 유도된 염증반응에 미치는 영향

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Abstract

Extracts of Magnoliae Cortex and *Zea mays L.* modulate TLR-2 ligand-induced inflammatory reactions

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Periodontal disease results from undesired immune reactions in response to periodontal pathogens. The bark of the stems and roots of *Magnolia officinalis* (Magnoliae Cortex) and *Zea mays L.* (maize) has been found to exhibit pharmacological effects as anti-

inflammatory activity. The aim of this study was to evaluate the capacity of single and combined applications of Magnoliae Cortex and maize to modulate inflammation in RAW 264.7 cells stimulated with TLR-2 ligand. Magnoliae Cortex and/or maize were added to RAW 264.7 cells, and the cells were stimulated with TLR-2 ligand. Cytotoxicity and the capacity to modulate inflammation were determined with a methylthiazol tetrazolium (MTT) assay, nitrite production, enzyme-linked immunosorbent assay (ELISA), and western blotting. The statistical analyses were conducted with the Kruskal-Wallis test, followed by the Mann-Whitney test. Treatment with Magnoliae Cortex and/or maize inhibited nuclear factor kappa B (NF-κB) pathway activation and phospho-p44/42 mitogen-activated protein kinase (MAPK) and inducible nitric oxide synthase (iNOS) protein expression in TLR-2 ligand-stimulated RAW 264.7 cells. Moreover, the treatments suppressed cytokines (prostaglandin E_2 [PGE_2], interleukin [IL]-1β, and IL-6) and nitrite production. Both Magnoliae Cortex and maize exerted an anti-inflammatory effect on TLR-2 ligand-stimulated RAW 264.7 cells, and this effect was more pronounced in inhibition of NF-κB pathway activation and phospho-p44/42 MAPK when the extracts were combined. These results suggest that combination of Magnoliae Cortex and *Zea mays L.* has the potential to inhibit

inflammatory response in periodontal disease.

Keywords: inflammation; *Magnolia*; nuclear factor kappa B; RAW 264.7 cell; TLR-2 ligand; *Zea mays*

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Introduction

Periodontal disease results from undesired immune reactions in response to periodontal pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans* [1]. An exogenous infection cause local release of inflammatory mediators such as cytokines, prostaglandin E_2 (PGE_2) and metalloproteinases (MMP) [2]. Host-derived inflammatory mediators cause the pathologic degradation of collagens and bone resorption in periodontal lesion [2,3]. As the periodontal lesion deepens, the subgingival biofilm becomes more anaerobic and the host response becomes more destructive and chronic [4]. Removing periodontal pathogens by mechanical or chemical means is a key component of controlling periodontal disease. The use of host inflammatory modulation agent is an adjunctive way to treatment of periodontal disease [2]. For instance, subantimicrobial-dose doxycycline (SDD) suppresses host-derived MMP and modulates the host immune reaction [3]. Moreover, omega-3 fatty acids (ω-3) and low-dose aspirin (81 mg/day) in addition to scaling and root planning are more effective slowing periodontal disease progression than scaling and root planning alone, in patients with moderate to severe periodontitis [5].

Patients with aggressive periodontitis have genetic susceptibility of hyper-inflammatory status regardless of the amount of the microbial deposit [6]. The modulation of inflammatory mediators may additionally help to control periodontal disease, along with the mechanical removal of periodontal pathogens [2].

Macrophage activation is crucial for the progression of multiple inflammatory diseases through the release of inflammatory mediators [7]. Inflammatory cytokines and other soluble mediators are expressed in stimulated macrophages through the activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) [8]. The interaction of Toll-like receptors (TLRs) on macrophages with bacterial components such as lipopolysaccharide (LPS), lipoproteins, and endotoxin results in the activation of NF-κB [7,8]. Moreover, *P. gingivalis* LPS shows TLR-2 agonist activity rather than TLR-4, therefore TLR-2 ligand could be used to mimic infection of periodontal pathogens and to activate macrophage [9]. NF-κB is a key transcription factor for pro-inflammatory mediators, including cytokines and chemokines such as interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), and PGE_2, as well as nitric oxide (NO) [7,10]. In addition, TLR engagement initiates signal transduction cascades such as extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun N-

terminal kinase 1/2 (JNK 1/2), and p38 MAP kinase pathways [11].

Magnoliae Cortex, the bark of the stems and roots of *Magnolia officinalis*, is used to treat acute diarrhea, cramping abdominal pain, regurgitation, vomiting, and dyspepsia [12]. Honokiol and magnolol are phenolic compounds that have been isolated from Magnoliae Cortex and are known to suppress the production of LPS-mediated cellular responses such as TNF-α, PGE_2, and NO expression [13]. Honokiol inhibits TNF-α and IL-6 in a dose-dependent manner [14]. Additionally, honokiol has anti-inflammatory effects on activated macrophages by inhibiting TNF-α and NO expression through inhibition of the MAPK, protein kinase C-α (PKC-α), and NF-κB pathways [15]. Magnolol inhibits the IL-6-induced Janus kinase (JAK)/signal transduction and activator of transcription (STAT) 3 signaling pathway by reducing STAT3 binding activity in endothelial cells [16].

The corn silk and corn kernels of *Zea mays L.* (maize) contain zeatin, flavonoids, alkaloids, allantoin, saponins, volatile oils, vitamins, starch, fats, cellulose, and β-sitosterol [17]. Corn silk and corn kernels have been found to exhibit pharmacological effects, including anti-hepatoma and anti-fatigue properties [18,19]. Corn bran inhibits NO production and inducible nitric oxide synthase (iNOS) expression in a dose-dependent manner [20]. Moreover, a

maize husk extract shows anti-inflammatory properties which inhibit acute and chronic inflammatory mediators in vivo [21]. In addition, an unsaponifiable fraction of maize reduces gingival inflammation and tooth mobility in patients with periodontal disease [22]. An unsaponifiable fraction of maize also reduces probing depth in patients with oral hygiene instruction [23], however there is no significant difference with control in patients with periodontal surgery [23, 24]. In previous studies, maize and Magnoliae Cortex promote bone tissue regeneration in rats [25], and facilitate clinical improvement in a dog model of experimental periodontitis [26]. However, the underlying mechanisms have not yet been established.

It is possible to combine more than one substance to modulate multi-targeted inflammation processes and functions. In such cases, the combination can produce stable and synergistic effects. The aim of this study was to evaluate the capacity of Magnoliae Cortex and maize to modulate inflammation in RAW 264.7 cells stimulated with TLR-2 ligand. Magnoliae Cortex and maize were separately or simultaneously applied to cells, and the induced inflammatory reactions were measured as the amount of NO, PGE_2, IL-1β, IL-6, phospho-p44/42 MAPK, iNOS, and NF-κB produced.

Material and Methods

Sample preparation

The soft 75% ethanol Magnoliae Cortex extract was provided by Dongbang FTL (Seoul, Korea). The extract was produced by Dongbang FTL as follows. Magenoliae Cortex was ground into fine particles, and reflux was performed with 75% ethanol at 65° C for 30 minutes. The sample was filtered, and the residue was subjected to reflux with 75% ethanol at 65° C for 30 minutes; the sample was filtered. The two filtrates were mixed and subsequently evaporated under vacuum to obtain the soft extract. The titrated, unsaponifiable maize extract fraction was provided by Dongkook Pharmaceutical Co., Ltd. (Seoul, Korea). Briefly, corn oil was mixed with ethanol and sodium hydroxide, and reflux was performed at 80 ° C for 3 hours. The samples were then cooled to room temperature (RT) and filtered under vacuum. The residue was extracted with 75% ethanol at 80° C and filtered. The two filtrates were mixed and concentrated to 1/3 of the volume at 80° C and then cooled to RT. The samples were subsequently extracted with ethyl acetate, followed by addition of potassium hydroxide and washing with purified water. The samples were concentrated to 1/3 of the volume at 80 ° C, and then cooled to RT. Next, ethanol was added, followed

by mixing and evaporation under vacuum to obtain the titrated unsaponifiable maize extract fraction. Ibuprofen (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. Dimethyl sulfoxide (DMSO) was used as a solvent. The final soft 75% ethanol Magnoliae Cortex extract concentration was 60 μg/mL in 1% DMSO; this was denoted as M. The final concentration of the titrated unsaponifiable maize extract fraction was 300 μg/mL in 1% DMSO; this was denoted as Z. The combined treatment of M and Z was denoted as MZ. The final ibuprofen concentration was 10 mM in 1% DMSO; this was denoted as IBU.

Cell culture

RAW 264.7 cells (murine) were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) and cultured in a 5% CO_2 atmosphere at 37° C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Invitrogen) (complete DMEM). The cells were inoculated in 10-cm dishes at a density of 1.9×10^4/cm^2, 24-well plates at a density of 5×10^4/cm^2, and 96-well plates at a density of 3.3×10^4/cm^2 and

cultured for 24 hours. The cells were pre-treated with M, Z or MZ, with or without Pam3CSK4 (InvivoGen, San Diego, CA, USA), a synthetic TLR-2 ligand. IBU was used as a positive control.

Cell viability measurements using the MTT assay

RAW 264.7 cells were inoculated into a 96-well plate and incubated for 24 hours in complete DMEM. The culture medium was subsequently discarded and replenished as follows: with medium alone; with the soft 75% ethanol Magnoliae Cortex extract (0.6, 6 or 60 μg/mL); with the titrated, unsaponifiable maize extract fraction (3, 30 or 300 μg/mL); or with a mixture of one of the extracts and IBU (10 mM) for 24 hours. Filtered methylthiazol tetrazolium (MTT) solution in DMEM was added to each well (5 mg MTT/mL), and the cells were incubated at 37° C for 4 hours. The unreacted dye was then removed. The insoluble MTT formazan crystals were allowed to dissolve in DMSO at room temperature for 15 minutes, and absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Nitrite production measurements

RAW 264.7 cells were inoculated into 24-well plates and incubated for 24 hours in complete DMEM as follows: with medium alone; with medium and Pam3CSK4 (10 μg/mL); with the soft 75% ethanol Magnoliae Cortex extract (0.6, 6, or 60 μg/mL) and Pam3CSK4 (10 μg/mL); with the titrated, unsaponifiable maize extract fraction (3, 30, or 300 μg/mL) and Pam3CSK4 (10 μg/mL); with a mixture of one of the extracts and Pam3CSK4 (10 μg/mL); or with IBU (10 mM) and Pam3CSK4 (10 μg/mL). To each well, 80 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride in water; Sigma-Aldrich) was added, and the mixture was incubated for 5 minutes in the dark. The total nitrite level was measured and calculated on the basis of absorbance at 540 nm using a microplate reader (Molecular Devices).

ELISA for PGE_2, IL-1β, and IL-6 release measurements

Cytokines were measured by enzyme-linked immunosorbent assay (ELISA). RAW 264.7 cells were plated in a 24-well plate and

cultured in DMEM containing 10% FBS for 24 hours. These cells were then serum-starved overnight in 0.5% FBS-containing medium. For PGE_2 release measurement, the cells were pretreated for 30 minutes before treatment with Pam3CSK4 (10 μg/mL) for 24 hours in a 5% CO_2 incubator at 37° C with medium alone; with the soft 75% ethanol Magnoliae Cortex extract (6 or 60 μg/mL); with the titrated, unsaponifiable maize extract fraction (30 or 300 μg/mL); or with a mixture of one of the extracts and IBU (10 mM). The collected media were stored at −70° C for cytokine analysis. The supernatant was assessed using PGE_2-specific ELISA kits (R&D system, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was read in a microplate reader (Molecular Devices). For IL-1β and IL-6 release measurements, the cells were pretreated with M (60 μg/mL), Z (300 μg/mL), MZ (60 μg/mL M and 300 μg/mL Z), or IBU (10 μg/mL) for 30 minutes prior to treatment with Pam3CSK4 (10 μg/mL) for 24 hours in a 5% CO_2 incubator at 37° C. The collected media were stored at −70° C for cytokine analysis. The supernatant was evaluated using a bead-based multiplex cytokine assay for IL-1β and IL-6 (Milliplex, Millipore, Billerica, MA, USA) and analyzed with a Luminex 200 System (Luminex, Austin, TX, USA).

iNOS, COX2, and phospho-p44/42 MAPK measurements by western blotting

RAW 264.7 cells were plated in 10-cm dishes and cultured in DMEM containing 10% FBS for 24 hours and then serum-starved overnight in 0.5% FBS medium. The cells were pretreated with M (60 μg/mL), Z (300 μg/mL), MZ (60 μg/mL M and 300 μg/mL Z), or IBU (10 μg/mL) for 30 minutes prior to treatment with Pam3CSK4 (10 μg/mL) for 24 hours (iNOS and COX-2) or 30 minutes (phospho-p44/42 MAPK) in a 5% CO_2 incubator at 37° C. The cells were washed with ice-cold phosphate-buffered saline and harvested with protein extraction solution (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% NP-40, and 1 mM PMSF) (Elpisbio, Daejeon, Korea) according to the manufacturer's protocol. Total cell lysates (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour, and primary antibodies against iNOS (Cell Signaling Technology, Danvers, MA, USA), cyclooxygenase-2 (COX-2; Cell Signaling

Technology), phospho-ERK1/2 (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), and β-actin (Sigma-Aldrich) were added to the Tris-buffered saline (TBS)-T solution containing 5% BSA and incubated overnight at 4° C. After 3 washes with TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) in TBS-T-containing 5% BSA for 1 hour. The membranes were subsequently washed 3 times with TBS-T buffer and analyzed with enhanced chemiluminescence detection reagents (Dogen, Innotech, Daejeon, Korea).

NF-κB activation measurements

RAW 264.7 cells were plated in 10-cm dishes and cultured in DMEM containing 10% FBS for 24 hours and then serum-starved overnight in 0.5% FBS medium. The cells were pretreated with M (60 μg/mL), Z (300 μg/mL), MZ (60 μg/mL M and 300 μg/mL Z), or IBU (10 μg/mL) for 30 minutes prior to treatment with Pam3CSK4 (10 μg/mL) for 30 minutes in a 5% CO_2 incubator at 37° C. NF-κB activation was measured using an NF-κB p65 ActiveELISA kit (Imgenex Corp, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, the cells were washed with

ice-cold phosphate-buffered saline and lysed with hypotonic buffer and 10% detergent solution (Imgenex Corp) at 4° C. The supernatant was collected for cytosolic NF-κB p65. The nuclear pellets were subsequently lysed with nuclear lysis buffer (Imgenex Corp), and the supernatant were stored at −80° C for nuclear NF-κB p65. The supernatants of cytosolic NF-κB p65 were combined with the supernatants of nuclear NF-κB p65 for total NF-κB p65 analysis. Absorbance at 405 nm was determined using a microplate reader (Molecular Devices).

Statistical analysis

Data were analyzed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). The results are expressed as means±standard deviations. The statistical analyses were conducted with the Kruskal-Wallis test, followed by the Mann-Whitney test. Differences were considered to be significant when the P values were <0.05.

Results

Preliminary experiments to determine concentrations and the mixture ratio of M and Z

In preliminary experiments, various concentrations of the Magnoliae Cortex extract, the maize extract and ibuprofen were examined with MTT assay and NO production measurement. The optimal concentrations with minimal cytotoxic effects and maximum inhibition of NO production were 60 μg/mL for M, 300 μg/mL for Z and 10 mM for IBU (data not shown). Nuclear NF-κB p65 were measured with various mixture ratios of the Magnoliae Cortex extract and the maize extract, concentrations with minimal cytotoxic effects, to determine the mixture ratio. The optimal mixture ratio of Magnoliae Cortex extract and the maize extract was 1: 5 (60 μg/mL of M : 300 μg/mL of Z; data not shown).

Cytotoxicity of M and Z treatments in RAW 264.7 cells

Concentrations of 0.6, 6 and 60 μg/mL of the Magnoliae Cortex extract and/or 3, 30 and 300 μg/mL of the maize extract and 10

mM of ibuprofen exhibited similar cell viability with control group, and there were no significant difference (Figure 1).

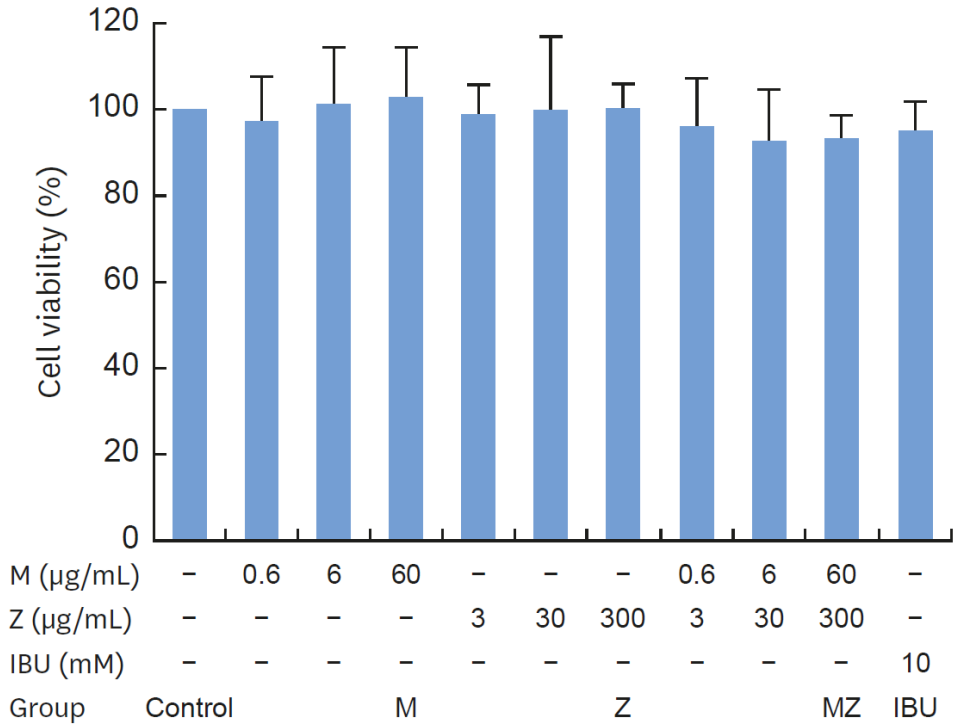

Figure 1. Cytotoxicity of M and Z treatments in RAW 264.7 cells. The data are expressed as mean±standard deviation, as obtained from 6 separate experiments.

M: soft 75% ethanol Magnoliae Cortex extract (60 μg/mL in 1% DMSO), Z: titrated unsaponifiable maize extract fraction (300 μg/mL in 1% DMSO), IBU: ibuprofen (10 mM in 1% DMSO), DMSO: dimethyl sulfoxide, MZ: the combination treatment of M and Z.

Effects of M and Z on Pam3CSK4-induced NO production by RAW 264.7 cells

To evaluate the potential anti-inflammatory effects of M and Z on Pam3CSK4-stimulated RAW 264.7 cells, NO production was measured. Pam3CSK4 treatment alone dramatically induced NO production in comparison with the control group, whereas the M and Z treatments significantly suppressed NO production in Pam3CSK4-induced RAW 264.7 cells (Figure 2). Concentrations of 0.6 μg/mL of the Magnoliae Cortex extract and 3 μg/mL of the maize extract and combination of 0.6 μg/mL of the Magnoliae Cortex extract and 3 μg/mL of the maize extract displayed similar NO production with Pam3CSK4 group, and there were no significant difference. However, combination of 6 μg/mL of the Magnoliae Cortex extract and 30 μg/mL of the maize extract significantly suppressed NO production compare with Pam3CSK4 group, meanwhile single treatment of 6 μg/mL of the Magnoliae Cortex extract and 30 μg/mL of the maize extract showed no significant difference with Pam3CSK4 group. Z significantly inhibited NO production compare with Pam3CSK4 group, also significantly difference with IBU. M and MZ significantly inhibited NO production compare with Pam3CSK4 group, exhibited no significant difference

with IBU (Figure 2).

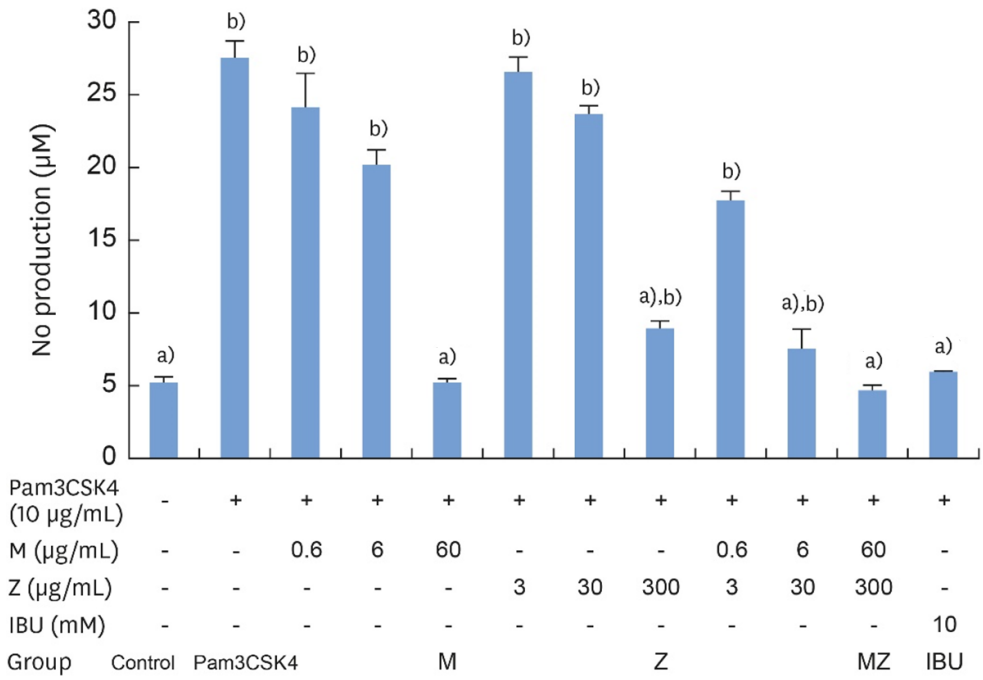

Figure 2. Effects of M and Z on Pam3CSK4-induced NO production in RAW 264.7 cells. Cells (5×10^4/cm^2) were treated with M, Z, MZ, or IBU and 10 μg/mL Pam3CSK4 for 24 hours at 37° C. In experiments without Pam3CSK4, the medium alone was used as a negative control, and IBU was used as a positive control. The data are expressed as mean±standard deviation, as obtained from 6 separate experiments.

Pam3CSK4: synthetic Toll-like receptor-2 ligand, NO: nitric oxide

[a)] $P<0.05$ in comparison with Pam3CSK4; [b)] $P<0.05$ in comparison with the IBU

Effects of M and Z on PGE_2, IL-1β, and IL-6 production in Pam3CSK4-induced RAW 264.7 cells

To determine the effects of M and Z on PGE_2 formation, cells were treated with M, Z, MZ, or IBU for 30 minutes and then stimulated with 10 μg/mL of Pam3CSK4 for 24 hours. PGE_2 was significantly induced in Pam3CSK4-stimulated cells. However, the addition of M and Z significantly suppressed PGE_2 production in Pam3CSK4-induced RAW 264.7 cells. Moreover, M and Z inhibited PGE_2 production in a manner similar to that of IBU treatment, and there were no significant difference. Combined application of 6 μg/mL of the Magnoliae Cortex extract and 30 μg/mL of the maize extract and single application of 6 μg/mL of the Magnoliae Cortex extract significantly suppressed PGE_2 production compare with Pam3CSK4 group, meanwhile single treatment of 30 μg/mL of the maize extract showed no significant difference with Pam3CSK4 group (Figure 3).

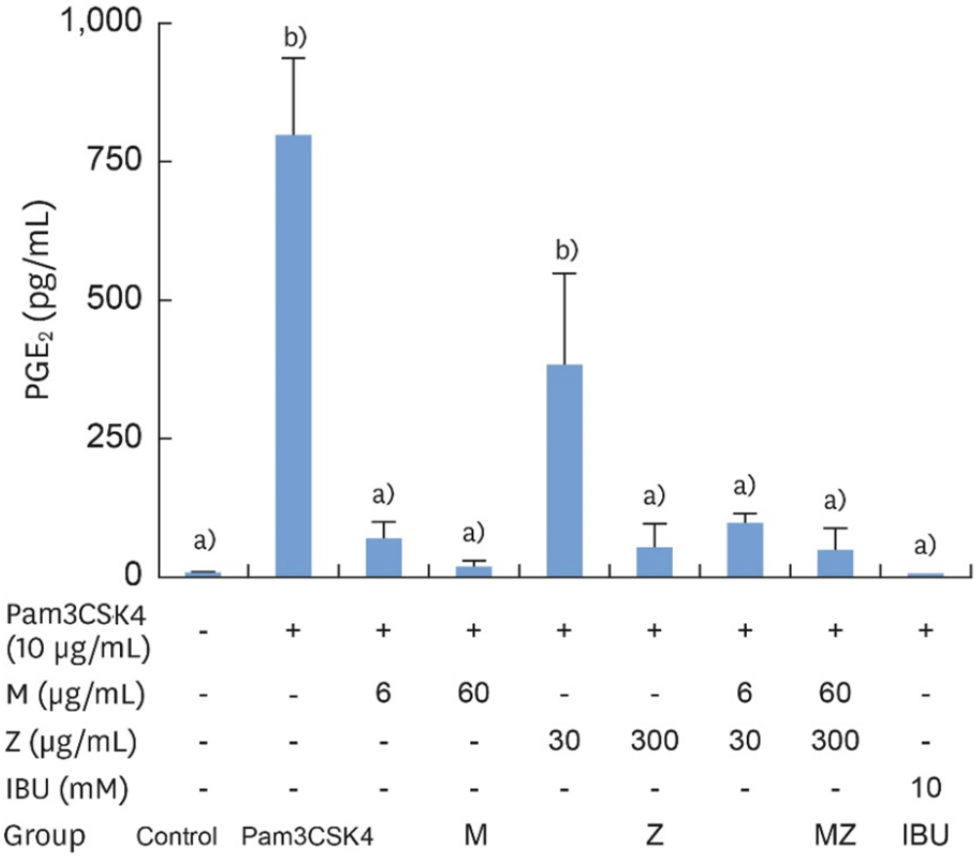

Figure 3. Effects of M and Z on Pam3CSK4-induced PGE_2 formation in RAW 264.7 cells. Cells ($5\times10^4/cm^2$) were treated with M, Z, MZ, or IBU for 30 minutes before treatment with 10 μg/mL Pam3CSK4 for 24 hours at 37° C. The data are expressed as mean±standard deviation, as obtained from 6 separate experiments. PGE_2: prostaglandin E_2

[a] $P<0.05$ in comparison with Pam3CSK4; [b] $P<0.05$ in comparison with the IBU

According to our results, Pam3CSK4 significantly elevated IL-1β and IL-6 levels in RAW 264.7 cells (Figures 4 and 5), but IL-1β and IL-6 production was strongly reduced when the cells were treated with M and Z. Treating the cells with M and MZ significantly suppressed IL-1β production compare with Pam3CSK4, and there were significant difference with Z and IBU (Figure 4). This result shows that with regard to IL-1β, M treatment inhibited inflammation more effectively than Z. However, Z and MZ treatments more significantly inhibited IL-6 levels than M and IBU (Figure 5), indicating that with regard to IL-6, Z was more efficient at suppressing inflammation, even when considering the possibility of a synergistic effect of MZ on IL-6 suppression. Interestingly, IBU treatment promoted IL-6 production to a significantly greater degree than Pam3CSK4 treatment alone (Figure 5).

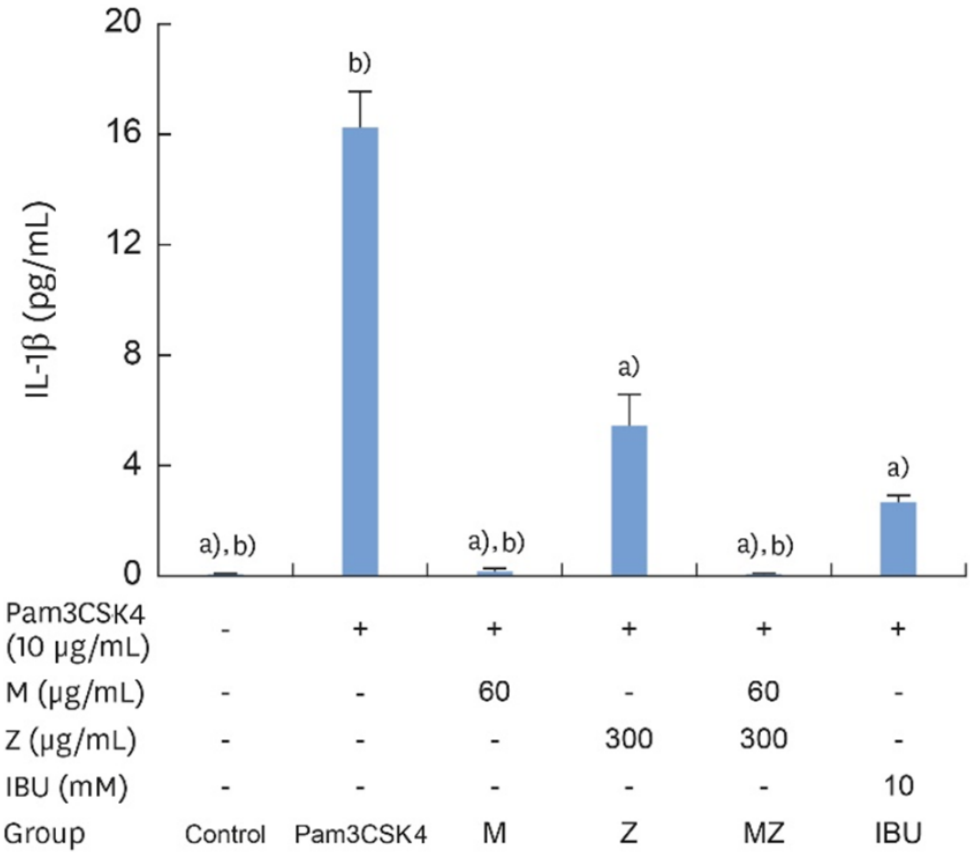

Figure 4. Effects of M and Z on Pam3CSK4-induced IL-1β formation in RAW 264.7 cells. Cells (5×10^4/cm^2) were treated with M, Z, MZ, or IBU for 30 minutes before treatment with 10 μg/mL Pam3CSK4 for 24 hours at 37° C. The data are expressed as mean±standard deviation, as obtained from 6 separate experiments. IL-1β: interleukin-1β

[a] $P<0.05$ in comparison with Pam3CSK4; [b] $P<0.05$ in comparison with IBU.

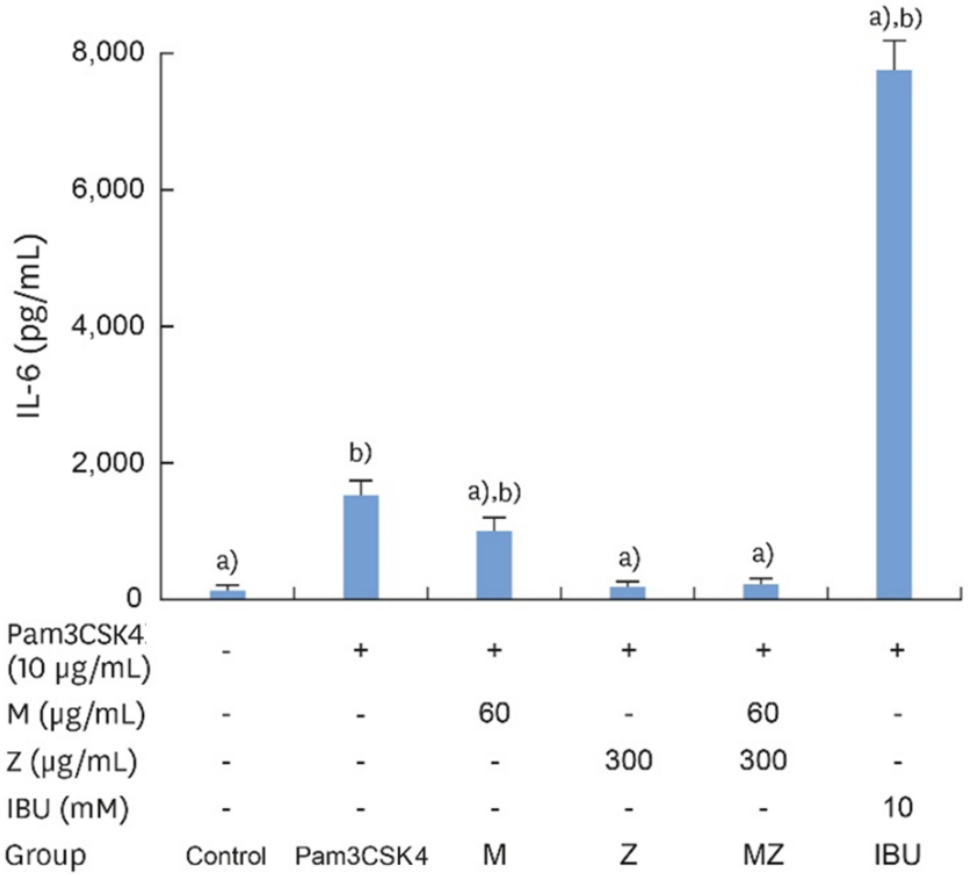

Figure 5. Effects of M and Z on Pam3CSK4-induced IL-6 formation in RAW 264.7 cells. Cells (5×10^4/cm^2) were treated with M, Z, MZ, or IBU for 30 minutes before treatment with 10 μg/mL Pam3CSK4 for 24 hours at 37° C. The data are expressed as mean±standard deviation, as obtained from 6 separate experiments. IL-6: interleukin-6

[a] $P<0.05$ in comparison with Pam3CSK4; [b] $P<0.05$ in comparison with the control

Effects of M and Z on p44/42 MAPK activation in Pam3CSK4-induced RAW 264.7 cells

To assess the effects of M and Z on p44/42 MAPK activation, cells were treated with M, Z, MZ, or IBU for 30 minutes and then stimulated with 10 μg/mL of Pam3CSK4 for 30 minutes. Pam3CSK4 treatment led to significant increases in the levels of phospho-p44/42 MAPK; however, MZ inhibited p44/42 MAPK activation in Pam3CSK4-induced RAW 264.7 cells (Figure 6A and B). M, Z and IBU group had similar levels of phospho-p44/42 MAPK with Pam3CSK4 group, and there were no significant difference (Figure 6A and B).

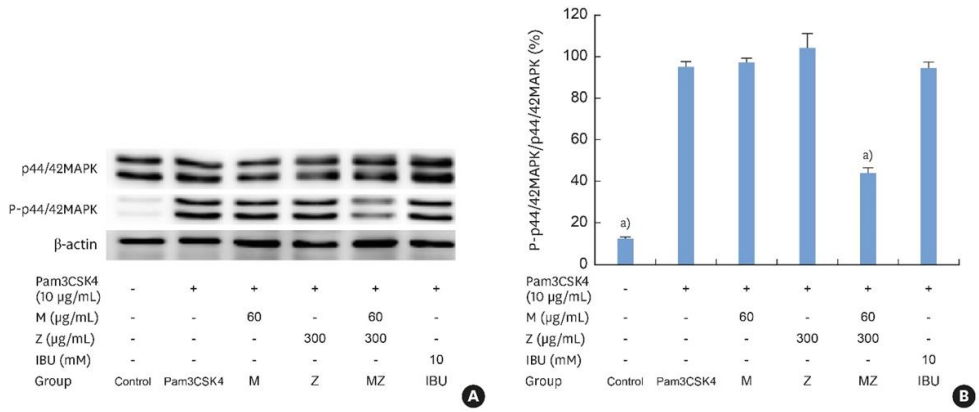

Figure 6. Effects of M and Z on phospho-p44/42 MAPK expression levels in Pam3CSK4-induced RAW 264.7 cells. The levels of phospho-p44/42 MAPK and β-actin expression were detected by western blotting using specific antibodies. Cells ($1.9\times10^4/cm^2$) were treated with M, Z, MZ, or IBU for 30 minutes before being treated with 10 μg/mL Pam3CSK4. To detect phospho-p44/42 MAPK expression, Pam3CSK4 was added for 30 minutes at 37° C. The blots shown here are representative of 3 independent experiments. Each sample contained 10 μg of total protein. (A) The levels of p44/42 MAPK, phospho-p44/42 MAPK and β-actin expression; (B) ratio of phospho-p44/42 MAPK to p44/42 MAPK expressed as a percentage.

MAPK: mitogen-activated protein kinase

[a)] $P<0.05$ in comparison with Pam3CSK4

Effects of M and Z on iNOS, and COX-2 expression in Pam3CSK4-induced RAW 264.7 cells

To evaluate the effects of M and Z on iNOS and COX-2 expression, cells were treated with M, Z, MZ, or IBU for 30 minutes and then stimulated with 10 μg/mL of Pam3CSK4 for 24 hours. iNOS expression increased after Pam3CSK4 stimulation in RAW 264.7 cells, however M, Z, MZ, and IBU significantly inhibited iNOS expression (Figure 7A and B). There was no change in COX-2 expression following treatment with Pam3CSK4, M, Z, MZ and IBU (Figure 7A).

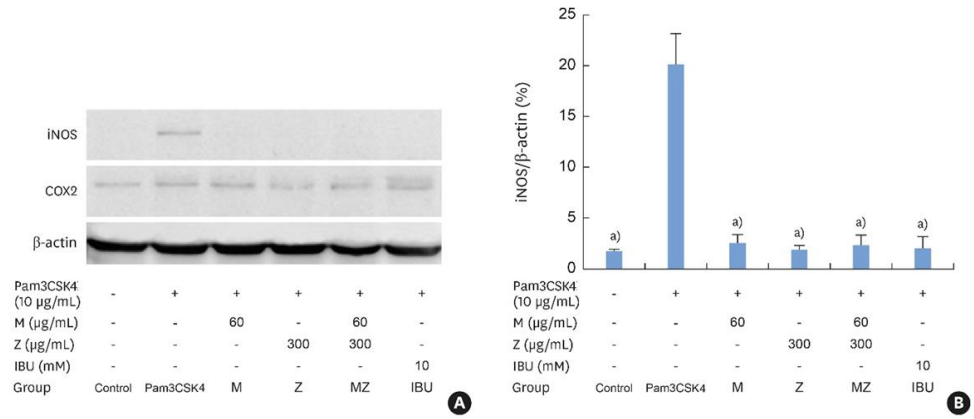

Figure 7. Effects of M and Z on nuclear iNOS and COX-2 expression levels in Pam3CSK4-induced RAW 264.7 cells. The levels of iNOS, COX-2, and β-actin expression were detected by western blotting using specific antibodies. Cells (1.9×10^4/cm^2) were treated with M, Z, MZ, or IBU for 30 minutes before being treated with 10 μg/mL Pam3CSK4. To detect iNOS and COX-2 expression, Pam3CSK4 was added for 24 hours at 37° C. The blots shown here are representative of 3 independent experiments. Each sample contained 10 μg of total protein. (A) The levels of iNOS, COX-2, and β-actin expression; (B) ratio of iNOS to β-actin expressed as a percentage.

iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase-2

a) $P<0.05$ in comparison with Pam3CSK4

Effects of M and Z on Pam3CSK4-induced NF-κB transactivation in RAW 264.7 cells

To determine whether M and Z could affect the level of p65 expression in RAW 264.7 cells, cells were treated with M, Z, MZ, or IBU for 30 minutes and then stimulated with 10 μg/mL of Pam3CSK4 for 2 hours. M significantly reduced the level of p65 compare with Pam3CSK4 group (Figure 8). Moreover, MZ significantly inhibited NF-κB transactivation to the control level, and there were significant difference with IBU (Figure 8). However, Z and IBU had similar level of p65 with Pam3CSK4 group, and there were no significant difference (Figure 8). These results show a synergistic effect of M and Z on Pam3CSK4-induced RAW 264.7 cells with respect to NF-κB inhibition.

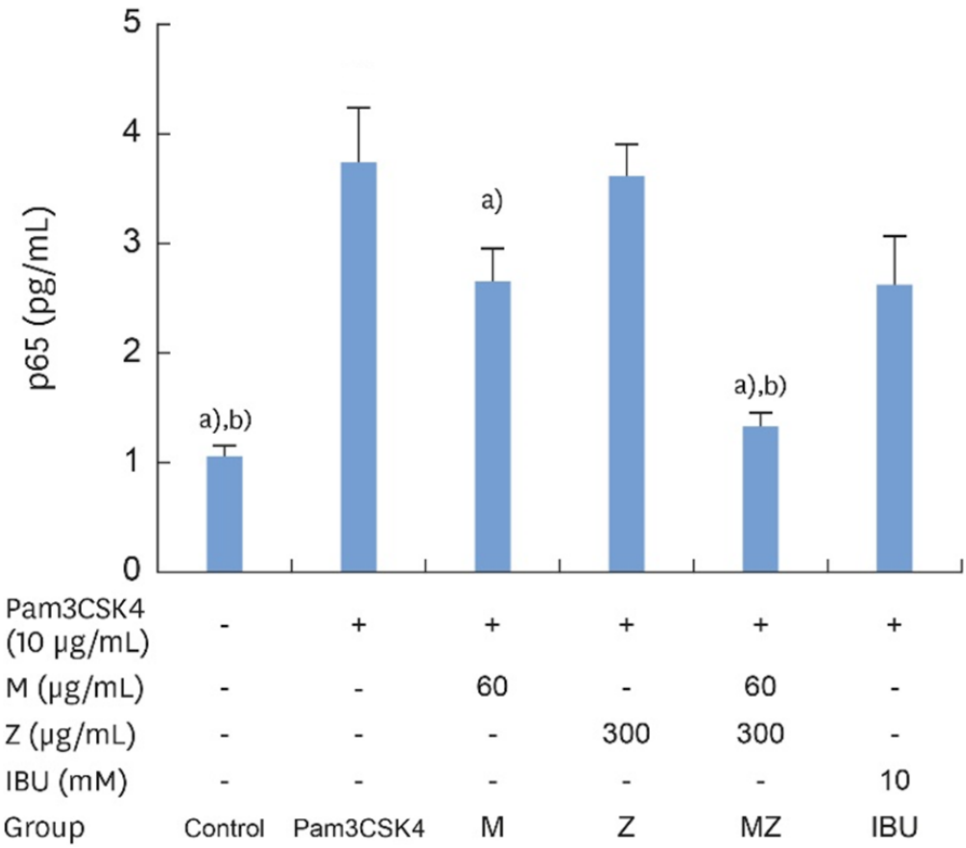

Figure 8. Effects of M and Z on Pam3CSK4-induced NF-κB transactivation in RAW 264.7 cells. Cells (5×10^4/cm^2) were treated with M, Z, MZ, or IBU for 30 minutes prior to treatment with 10 μg/mL Pam3CSK4 for 2 hours at 37° C. The data are expressed as mean±standard deviation, as obtained from 6 separate experiments. NF-κB: nuclear factor kappa B, p65: transcription factor p65.

[a] $P<0.05$ in comparison with Pam3CSK4; [b] $P<0.05$ in comparison with IBU

Discussion

The primary etiology of periodontal disease is infection by periodontal pathogens such as *P. gingivalis*, *P. intermedia,* and *A. actinomycetemcomitans* [1-3,27]. Conversely, periodontal disease progresses in response to the host immune inflammatory reaction to periodontal pathogens and their products [2,3,27]. TLR-2 ligands from periodontal pathogens stimulate immune cells, such as neutrophils, monocytes, and macrophages, and immune cells produce cytokines by multiple processes [2,28]. Degradation action on collagen fibers is performed by MMPs released by host cell that responds to the inflammatory cytokines [6,29]. Therefore, the inflammatory mediators, such as IL-1β, IL-6, and PGE_2, cause local tissue destruction and alveolar bone loss [2,3,27].

Aggressive periodontitis generally involves rapid attachment loss and bone destruction regardless of the amount of the microbial deposit [6,27,30]. Single nucleotide polymorphisms (SNP) on genes encoding cytokines, receptors, metabolic modulators and proteins result in increased disease susceptibility or overexpression of the host innate inflammatory reaction [27]. The local inflammatory reaction by the microbial deposit, overexpressed by hyper-responsiveness of inflammatory mediators, resulted in local tissue

destruction in the host with these SNPs [27].

Modulators of inflammatory mediators, such as SDD, suppress host-derived MMPs in the periodontal lesion and inhibit local tissue destruction [2,3]. Waite et al. reported that the patients taking non-steroidal anti-inflammatory drugs had significantly shallower depths of periodontal pockets [31]. El-Sharkawy et al. published that ω-3 and low dose of aspirin adjunctive with scaling and root planning significantly reduced probing depths compared with scaling and root planning in patients with moderate to severe periodontitis [5]. In the model of experimental periodontitis in non-human primates, IL-1 and TNF antagonist inhibited loss of tissue attachment [32]. In the model of experimental periodontitis in beagle dogs, subcutaneous injections of IL-11, known to downregulate inflammatory mediators, reduced attachment loss and bone resorption [33]. The modulators of inflammatory mediators may be beneficial for slowing the progression of periodontal disease, especially aggressive periodontitis, along with the mechanical removal of oral pathogens [2-4].

Magnolol and honokiol, the main components of Magnoliae Cortex, have been reported to suppress inflammatory reactions both *in vitro* and *in vivo* [15,34]. One of the main components of maize, β-sitosterol, has been shown to exhibit anti-inflammatory effects that

suppress monocyte activation by IL-6 and TNF-α and might be useful as a treatment for rheumatoid arthritis [35]. Although anti-inflammatory effects of Magnoliae Cortex and maize have been presented in previous studies, details regarding the mechanism through which these effects occur have not been reported [20-22,34]. Herein, we demonstrated that Magnoliae Cortex and/or maize can modulate the inflammatory response by suppressing TLR-2 ligand-induced signaling and pro-inflammatory molecule production.

NO, an inflammatory mediator, is a reactive molecule that has a variety of effects depending on its relative concentration [36]. Small amounts of NO are produced by constitutive nitric oxide synthase (NOS) enzymes (i.e., endothelial NOS and neural NOS), and are essential for physiological homeostasis [36]. In contrast, the large amounts of NO produced by inducible NOS have been closely linked to the pathophysiology of numerous inflammatory diseases [36,37]. In the model of experimental periodontitis in rat, ligation caused a significant increase in the iNOS activity [38]. However, the treatment of a selective inhibitor of iNOS resulted in a significant inhibition of alveolar bone loss [38]. In the present study, the high level of NO production observed in Pam3CSK4-stimulated macrophages was strikingly suppressed by M and Z, with an

effectiveness that was almost the same as that of IBU. NO inhibition by iNOS downregulation in macrophages modulates host immune inflammation induced by TLR-2 ligand, and the results presented herein correspond well with those of earlier studies. For example, previous studies demonstrated that honokiol and magnolol, the major active components of Magnoliae Cortex, inhibited NO release and iNOS expression in a dose-dependent manner [13,15,34]. In addition, a study of corn bran showed that 80% of an ethanolic corn bran extract inhibited NO production and iNOS expression in macrophages stimulated with bacterial products [20].

The NF-κB pathway, which is considered to be a prototypical pro-inflammatory signaling cascade, is activated by TLRs that recognize microbial molecular patterns [39]. NF-κB binds to the iNOS gene promoter, thereby promoting iNOS expression and NO production [36]. However, some drugs, such as salicylates, only inhibit iNOS expression without affecting NF-κB-mediated iNOS mRNA expression [40]. In the present study, M and MZ inhibited Pam3CSK4-induced NF-κB activation, whereas Z had similar level of NF-κB activation with Pam3CSK4 group. These findings are different from those of Kim et al., who reported that corn phenolic amides contributed to the inhibition of NO production by downregulating the level of NF-κB-mediated iNOS gene

expression [20]. Moreover, Rho et al. also reported that Zea mays husk extract inhibited NO production by suppressing the level of NF-κB-mediated iNOS gene expression [41]. These results indicate that the various components of corn interact with inflammatory processes via different pathways. In the present study, MZ significantly suppressed NF-κB activation, and this activity was more pronounced than that of M, suggesting that M and Z exerted a synergistic effect with respect to NF-κB inhibition.

In addition to the NF-κB signaling pathway, JAK-STAT signaling regulates inflammatory processes [36,42]. Cytokines such as interleukins and interferons activate the Janus family of tyrosine kinases (JAK1, JAK2, JAK3, and tyrosine kinase 2 [Tyk2]), and activated JAK kinases phosphorylate members of the STAT family [42]. In addition to interleukins and interferons, other cytokines, such as IL-6, also activate JAK1 and STAT3 [16]. Suppression of IL-6-mediated JAK-STAT signaling is important for controlling inflammation [16]. In the present study, M and Z significantly suppressed Pam3CSK4-induced IL-6 production in RAW 264.7 cells, and Z was more effective at inhibiting IL-6 secretion than was M. These results are assumed that Z does not suppress the NF-κB pathway, but instead suppresses the JAK-STAT pathway, and that M suppresses both pathways to exert its anti-

inflammatory effects. These results are consistent with those of an earlier study reporting that magnolol extracted from Magnoliae Cortex suppressed IL-6-induced STAT3 phosphorylation and downstream target gene expression in endothelial cells [16]. Interestingly, the results of present study show that IBU significantly increased Pam3CSK4-induced IL-6 production, but not IL-6 production without Pam3CSK4 stimulation (data not shown). These findings correspond well with those of earlier studies, in which ibuprofen significantly increased Pam3CSK4-induced IL-6 production [43] but had no inhibitory effects on IL-6 bioactivity [44]. Although IBU suppressed inflammation, further studies are needed to reveal the precise mode of action with regard to inflammatory suppression.

Parallel to the NF-κB and JAK-STAT pathways, the ERK 1/2 pathway is also key for regulating inflammation at the transcriptional level. Recognition of bacterial components by TLRs leads to the phosphorylation of interleukin-1 receptor-associated kinase (IRAK), which in turn activates tumor necrosis factor receptor-activated factor 6 (TRAF6) [8]. TRAF6 activates IκB kinase and MAPK, which phosphorylate downstream kinases. Activated IκB kinase and MAPK then activate p38 and ERK 1/2 as well as NF-κB, leading to IL-1β, TNF-α, and NO transcription

and secretion [8]. In the present study, MZ significantly reduced phospho-p44/42 MAPK expression in Pam3CSK4-stimulated macrophages, but neither M nor Z could inhibit the ERK 1/2 pathway alone. These results indicate that M and Z exerted a synergistic effect in inhibiting the ERK 1/2 pathway, suggesting that they may control inflammation more effectively when used together.

The increased release of active IL-1β is a hallmark of auto-inflammatory diseases and chronic inflammatory diseases. IL-1β entering the circulation from a local inflammation site induces IL-1 itself as well as systemic inflammation [45,46]. Both IL-1β bursts and IL-1β polymorphisms are closely related to the slowly progressive inflammatory processes that occur in periodontitis, coronary heart disease, osteoarthritis, and type 2 diabetes [45-47]. As a result, blocking IL-1β has been the focus of auto-inflammatory and chronic inflammatory disease treatments [46]. In the present study, M and Z efficiently blocked IL-1β secretion in Pam3CSK4-stimulated RAW 264.7 cells. Additionally, M significantly and more effectively blocked IL-1β secretion than IBU. These results suggest that M and Z can modulate not only chronic inflammatory reactions but also auto-immune inflammatory reactions.

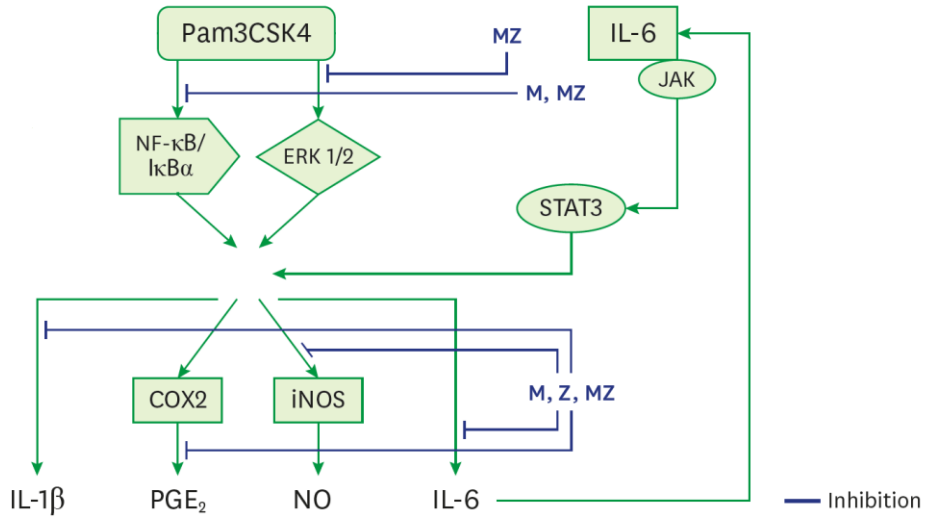

Figure 9. Schematic model for the anti-inflammatory mechanism of M and Z in Pam3CSK4-induced RAW 264.7 cells. ERK 1/2: extracellular signal-regulated kinase 1/2, NF-κB/IκBα: nuclear factor kappa B pathway, JAK/STAT3: Janus kinase/signal transduction and activator of transcription 3 signaling pathway

In conclusion, both the Magnoliae Cortex and maize extracts modulated the downstream targets of multiple inflammatory pathways, and decreased TLR-2 lignad-induced inflammatory reactions by inhibiting NO, PGE_2, phospho-ERK 1/2, iNOS, IL-1β, IL-6, and NF-κB levels. However, a difference in their modes of action was observed, in that Magnoliae Cortex was more effective at decreasing NO, IL-1β, and NF-κB levels, while maize was more effective at reducing IL-6 levels. In all experiments, the combination of Magnoliae Cortex and maize showed either the same or enhanced results relative to the use of Magnoliae Cortex or maize alone. Additionally, greater efficacy was obtained with respect to NF-κB and phospho-ERK 1/2 inhibition when both extracts were added simultaneously (Figure 9). These results suggest that combination of Magnoliae Cortex and maize has the potential to inhibit inflammatory response in periodontitis. Further study is needed to elucidate the precise anti-inflammatory signaling pathways targeted by each of the components of Magnoliae Cortex and maize.

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국문초록

후박 추출물과 옥수수 불검화 추출물이 TLR-2 ligand로 유도된 염증반응에 미치는 영향

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치주질환은 치주원인균의 자극에 의한 염증반응에 의해 시작된다. 후박과 옥수수는 예전부터 염증조절 용도로 많이 사용되어 왔고, 최근에 다양한 연구들이 이를 뒷받침하고 있다. 이 연구의 목적은 TLR-2 ligand에 의해 자극된 RAW 264.7 세포에 후박 추출물 및 옥수수 불검화 추출물을 각각 또는 함께 적용하였을 때 염증반응의 조절능력을 알아보는 것이다. RAW 264.7 세포에 후박추출물 및 옥수수 불검화 추출물

을 각각 또는 함께 처리한 상태에서 TLR-2 ligand로 세포를 자극시켰다. 각 추출물의 세포독성과 염증조절능력을 세포독성실험 [methylthiazol tetrazolium (MTT) assay], 아질산염생성측정법 (nitrite production), 효소면역분석법 (ELISA; enzyme-linked immunosorbent assay) 및 웨스턴 블롯 (western blot) 등으로 분석했다. RAW 264.7 세포에 후박 추출물 및 옥수수 불검화 추출물을 각각 또는 함께 처리 후 TLR-2 ligand로 자극했을 때, 각 추출물은 RAW 264.7 세포의 nuclear factor kappa B (NF-κB) 경로 활성과 인산화된 p44/42 mitogen-activated protein kinase 그리고 inducible nitric oxide synthase (iNOS) 단백질 생성을 억제했다. 또한 각 추출물은 RAW 264.7 세포의 사이토카인들 (prostaglandin E_2 [PGE_2], interleukin [IL]-1β, and IL-6) 과 아질산염 생성을 억제했다. 후박 및 옥수수 불검화 추출물 모두 TLR-2 ligand에 의해 유도된 RAW 264.7 세포의 염증반응을 억제하였고, 두 추출물을 함께 처리하였을 때 NF-κB 경로 활성 억제 및 인산화된 p44/42 MAPK 억제에 더 효과적이었다. 이러한 결과는 후박 및 옥수수 불검화 추출물이 치주질환의 염증반응을 억제할 수 있다는 가능성을 보여준다.

주요어 : 염증; 후박; nuclear factor kappa B; RAW 264.7 세포; TLR-2 ligand; 옥수수

학번: 2011-30651