Effects of Gyejijakyakjimo-tang extract on inhibition of PGE$_2$ synthesis and NO production in murine raw 264.7 macrophage cells

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SUMMARY

Gyejijakyakjimo-tang is a multi-herbal formula that is composed of nine medicinal herbs. Gyejijakyakjimo-tang has been reported to have antipyretic and analgesic effects. Gyejijakyakjimo-tang has traditionally been used for goat and rheumatoid arthritis. However, analgesic and anti-inflammatory effects of Gyejijakyakjimo-tang has not been clarified yet. In this study, we investigated the analgesic and anti-inflammatory effect of the aqueous extract of Gyejijakyakjimo-tang. We evaluated the aqueous extract of Gyejijakyakjimo-tang on Lipopolysaccharide (LPS)-induced inflammation in murine raw 264.7 macrophage cells. For this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), prostaglandin E$_2$ (PGE$_2$) immunoassay, and nitric oxide (NO) detection were performed. Gyejijakyakjimo-tang suppressed PGE$_2$ synthesis and NO production by inhibiting the LPS-induced expressions of COX-2 and iNOS mRNA in murine raw 264.7 macrophage cells. These results show that Gyejijakyakjimo-tang has the analgesic and anti-inflammatory effect by mostly suppressing COX-2 and iNOS expressions, and resulting in the inhibition of PGE$_2$ synthesis and NO production.

Key words: Gyejijakyakjimo-tang; Lipopolysaccharide; Cyclooxygenase-2; Inducible nitric oxide synthase; Prostaglandin E$_2$

INTRODUCTION

Inflammation is a complex process which commences with a primary reaction in tissues. Cardinal signs of inflammation are heat, redness, swelling, and pain. Of these, pain is important clues of immunological activity (McGeer and McGeer, 1995). Lipopolysaccharide (LPS) initiates a number of major cellular responses that play vital roles in the pathogenesis of inflammatory responses. LPS expresses the production of inflammatory mediators, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukins, prostaglandin E$_2$ (PGE$_2$), and leukotrienes (Lee et al., 1992; Kubes and McCafferty, 2000).

PGE$_2$ mediates the important features at inflammatory sites where it contributes to local blood flow increases, edema, and pain sensitization (Park et al., 2007). PGE$_2$ is converted from arachidonic acid by cyclooxygenase (COX). There are two isoforms of COX: COX-1 and COX-2. While COX-1 is a constitutively expressed form in normal physiologic function, COX-2 is expressed by pro-inflammatory mediators and mitogenic stimuli including cytokines, endotoxins, and growth factors

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As a neuromodulator, NO is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues (Bredt and Snyder, 1994). NO is produced by nitric oxide synthase (NOS) enzyme as a by conversion of L-arginine to L-citrulline. Three types of NOS have been identified: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) (Moalem and Tracey, 2006). Of these, iNOS is an important enzyme involved in the regulation of inflammation (Yui et al., 1991).

Gyejijakyakjimo-tang is composed of nine medicinal plants, including Atractylodis Rhizoma(白朮), Aconiti Tuber(附子), Glycyrrhizae Radix(甘草), Paeoniae Radix(芍藥), Cinnamomi cassiae Ramulus(桂枝), Ephedrae Herba(麻黃), Zingiberis Rhizoma(生姜), Ledebouriellae Radix(防風), and Anemarrhenae Rhizoma(知母). Following traditional medical literatures, Gyejijakyakjimo-tang has been reported to have antipyretic and analgesic effect. That’s why it has been mainly used to treat gout, rheumatoid arthritis (Kim et al., 1995). However, the effect of Gyejijakyakjimo-tang on analgesic and anti-inflammatory has not been clarified yet.

In this study, we investigated the analgesic and anti-inflammatory effect of the aqueous extract of Gyejijakyakjimo-tang. We evaluated the effect of the aqueous extract of Gyejijakyakjimo-tang on LPS-induced inflammation in murine raw 264.7 macrophage cells. For this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE2 immunoassay, and NO detection were performed.

MATERIALS AND METHODS

Cell culture
Murine raw 264.7 macrophage cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) at 37°C in 5% CO2 in a humidified cell incubator. The cells were plated onto culture dishes at a density of 2 × 10⁴ cells/cm² 24 h prior to drug treatments.

Preparation of the aqueous extract of Gyejijakyakjimo-tang
Gyejijakyakjimo-tang was composed of nine medicines: 30 g Atractylodis Rhizoma, 6 g Aconiti Tuber, 12 g Glycyrrhizae Radix, 18 g Paeoniae Radix, 24 g Cinnamomi cassiae Ramulus, 12 g Ephedrae Herba, 30 g Zingiberis Rhizoma, 24 g Ledebouriellae Radix, and 24 g Anemarrhenae Rhizoma. Total 180 g of dried ingredients was added to distilled water, and extraction was performed by heating at 90°C for 2 h, concentrating with rotary evaporator (Eyela, Tokyo, Japan) and lyophilizing by a drying machine (Eyela, Tokyo, Japan) for 24 h. The resulting powder, weighting 32.5 g (yield of 18.06%) was diluted to the concentrations needed with autoclaved distilled water and filtered through a 0.22 μm syringe filter before use.

MTT cytotoxicity assay
Murine raw 264.7 macrophage cells were grown in a final volume of 100 μl culture medium per well in 96-well plates. In order to determine the cytotoxicity of Gyejijakyakjimo-tang, the cells were treated with Gyejijakyakjimo-tang at concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml, and 10,000 μg/ml for 24 h. Cultures of the cells of the control group were left untreated. After adding 10 μl of the MTT labeling reagent containing 5 mg/ml MTT in phosphate-buffered saline to each well, the plates were incubated for 2 h. Solubilization solution 100 μl containing 5% sodium dodecyl sulfate in 0.01 M hydrochloric acid was added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (BioTek, Winooski, VT, USA) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density (O.D.)
was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

RNA isolation and RT-PCR
To identify the expression of COX-2 and inducible iNOS mRNA, RT-PCR was performed. The total RNA was isolated from murine raw 264.7 macrophage cells using RNAzol™B (TEL-TEST, Friendswood, TX, USA). One μg of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 15 min. 1 μl of AMV reverse transcriptase (Promega), 5 μl of 2.5 mM dNTP (Promega), 0.5 μl of RNasin (Promega), and 8 μl of 5 × AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 40 μl volume with diethylpyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42°C for 2 h.

PCR amplification was performed in a reaction volume of 40 μl containing 2 μl of the appropriate cDNA, 0.5 μl of each set of primers at a concentration of 10 pm, 2 μl of 10 × RT buffer, 1 μl of 2.5 mM dNTP, and 0.2 units of Taq DNA polymerase (Takara, Shiga, Japan). For mouse COX-2, the primer sequences were 5′-CCAGATGCTATCTTTGGGGAGAC-3′ (a 23-mer sense oligonucleotide) and 5′-CTTGCATTGATGGTGGCTG-3′ (a 19-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5′-CAAGAGTTTGACCAGAG-GACC-3′ (a 21-mer sense oligonucleotide) and 5′-TGGAACCACTCGTTACTGGGA-3′ (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5′-ACCCCACCGTGTCTTCGAC-3′ (a 20-mer sense oligonucleotide) and 5′-TGGGCACTGATCGATTG-3′ (a 20-mer anti-sense oligonucleotide). Gene-specific PCR primers were selected with the following NCBI database Accession Nos: COX-2 (NM_011198), iNOS (NM_010927), and cyclophilin (NM_001008741).

For COX-2, the PCR procedures was carried out using a PTC-0150 MiniCycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with an additional extension step at the end of the procedure at 72°C for 10 min. For iNOS, the PCR procedures was under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, with an additional extension step at the end of the procedure at 72°C for 10 min. For cyclophilin, under the following conditions: initial denaturation at 94°C for 5 min, followed by 25 amplification cycles, each consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, with an additional extension step at the end of the procedure at 72°C for 10 min. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Imaging-Pro® Plus (Media Cyberbetics Inc., Silver Spring, MD, USA).

Determination of nitric oxide production
In order to determine effect of the aqueous extract of Gyejjijakkyjimotang on NO production, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (iNtRON, INC., Seoul, Korea). After collection of 100 μl of supernatant, 50 μl of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at a wavelength of 540 nm. The nitrite concentration was calculated from a nitrite standard curve.

Measurement of prostaglandin E2 synthesis
Assessment of PGE₂ synthesis was performed
using a commercially available PGE₂ competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Supernatant 100 μl from the culture medium and the standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE₂ antibody and peroxidase-conjugated PGE₂ were added to each well, and the plate was incubated at room temperature with shaking for 2 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was then added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H₂SO₄. The absorbance of the content of each well was then measured at a wavelength of 450 nm.

**Statistical analysis**

The results are presented as the mean ± standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan’s post-hoc test using SPSS. The differences were considered statistically significant at \( P < 0.05 \).

**RESULTS**

**Effect of the aqueous extract of Gyejijakyakjimo-tang on viability of murine raw 264.7 macrophage cells**

In order to assess the cytotoxic effect of the aqueous extract of Gyejijakyakjimo-tang on the murine raw 264.7 macrophage cells, the cells were cultured with the aqueous extract of Gyejijakyakjimo-tang at final concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml, 10,000 μg/ml for 24 h, and MTT assays was then carried out. The cells cultured in Gyejijakyakjimo-tang-free media were used as the control. The viability of cells incubated with Gyejijakyakjimo-tang at concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml, 10,000 μg/ml for 24 h was 98.56 ± 1.91%, 94.90 ± 2.38%, 90.06 ± 1.32%, 77.05 ± 1.02%, 23.09 ± 1.34% of the control value, respectively (Fig. 1).

The present results showed that the aqueous extract of Gyejijakyakjimo-tang exerted no cytotoxicity until it was at a concentration of 100 μg/ml. However, at high concentrations (1,000 and 10,000 μg/ml) of Gyejijakyakjimo-tang reduced cell viability. Then, we used the aqueous extract of Gyejijakyakjimo-tang at concentration of 10 and 100 μg/ml for the next experiments.

**Effect of the aqueous extract of Gyejijakyakjimo-tang on NO production**

From the NO detection assay, the amount of nitrite was increased from 1.67 ± 0.15 μM to 25.64 ± 0.66 μM after 24 h of exposure to LPS. NO production was decreased to 22.57 ± 0.55 μM, 20.90 ± 0.93 μM, and 18.52 ± 0.30 μM by pre-treatment for 1 h with the aqueous extract of Gyejijakyakjimo-tang at 10 μg/ml,

![Fig. 1. Effect of Gyejijakyakjimo-tang on murine raw 264.7 macrophage cell viability. The cells were pre-treated for 1 h with Gyejijakyakjimo-tang at concentrations of 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml, then 1 μg/ml LPS was treated for 24 h. The cells were incubated for 24 h. Cells were stained MTT and analyzed by enzyme-linked immunosorbent assay (ELISA). The experiments were repeated four times. (A) Control group, (B) 0.001 mg/ml Gyejijakyakjimo-tang-treated group, (C) 0.01 mg/ml Gyejijakyakjimo-tang-treated group, (D) 0.1 mg/ml Gyejijakyakjimo-tang-treated group, (E) 1 mg/ml Gyejijakyakjimo-tang-treated group, (F) 10 mg/ml Gyejijakyakjimo-tang-treated group. Results are presented as mean ± S.E.M. *P < 0.05 compared to the control.](image-url)
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The present results showed that LPS enhanced NO production in murine raw 264.7 macrophage cells and the aqueous extract of Gyejjakjakjimo-tang suppressed LPS-induced NO production.

**Effect of the aqueous extract of Gyejjakjakjimo-tang on PGE\(_2\) synthesis**

From PGE\(_2\) immunoassay, the amount of PGE\(_2\) from the culture medium was increased from 21.68 ± 0.01 pg/ml to 53.86 ± 0.88 pg/ml after 24 h of exposure to LPS. PGE\(_2\) synthesis was decreased to 54.96 ± 0.54 pg/ml, 43.97 ± 1.32 pg/ml, and 38.36 ± 1.23 pg/ml by the pre-treatment for 1 h with the aqueous extract of Gyejjakjakjimo-tang at 10 μg/ml, 100 μg/ml, and 400 μM ASA, following 1 μg/ml LPS treatment for 24 h (Fig. 3).

The present results showed that LPS enhanced PGE\(_2\) synthesis in murine raw 264.7 macrophage cells and the aqueous extract of Gyejjakjakjimo-tang suppressed LPS-induced PGE\(_2\) synthesis.

**Effect of the aqueous extract of Gyejjakjakjimo-tang on mRNA expressions of COX-2 and iNOS**

RT-PCR analysis of the mRNA levels of COX-2 and iNOS was performed to estimate the relative level of expressions of these genes. In the present study, the mRNA levels of COX-2 and iNOS in the control cells were set as 1.00.

The level of COX-2 mRNA was markedly increased to 6.93 ± 0.29 following a treatment with 1 μg/ml LPS for 24 h. The level of COX-2 mRNA was decreased to 5.31 ± 0.90, 3.99 ± 0.80, and 3.05 ± 0.50 in the cells pre-treated for 1 h with the aqueous extract of Gyejjakjakjimo-tang at 10 μg/ml, 100 μg/ml, and 400 μM ASA, following LPS treatment for 24 h.

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with 1 μg/ml LPS for 24 h was markedly increased to 4.21 ± 0.58. The level of iNOS mRNA was decreased to 2.78 ± 0.37, 2.00 ± 0.38, and 1.69 ± 0.36 in the cells pre-treated for 1 h with the aqueous extract of Gyejijakyakjimo-tang at 10 μg/ml, 100 μg/ml, and 400 μM ASA, following LPS treatment for 24 h (Fig. 4).

The present results showed that LPS enhanced COX-2 and iNOS mRNA expressions in murine raw 264.7 macrophage cells and the aqueous extract of Gyejijakyakjimo-tang suppressed LPS-induced COX-2 and iNOS mRNA expressions.

DISCUSSION

Gyejijakyakjimo-tang was originally discussed in the quotation “諸肢節疼痛 身體羸弱 腿腫如脫 頭眩短氣 溫溫欲吐 桂枝芍藥知母湯主之” Jinguiyaolue (金要略). Since early times, Gyejijakyakjimo-tang has been used for arthralgia syndrome (bi-zheng, 體肢節疼痛), li-jie-feng (歷節風) or gout (tong-feng, 痛風) (Li, 1989).

In domestic research of the past use of Gyejijakyakjimo-tang, it was reported effects of pain reduction, relaxation, removal of fever, anti-edema, blood vessel permeable suppression and white corpuscle wandering suppression in the male rats and decrease in Ig G anti-collagen antibody (Lee and Shin, 1990). It was reported the effect of pain reduction and anti-edema and it increases the uric acid emission, and has an effect on decrease of blood uric acid although it is not involved in uric acid metabolism (Kim et al., 1995).

In overseas studies, reported a decrease in ESR and CRP after treatment for clinical Rheumatoid arthritis patients (Guo et al., 2006). Of C57BL/6 mice, Zhang reported that in Type II collagen induced arthritis, it suppresses the increase of RA T-Lymphocyte, RA IL-2 and CRP (Zhang, 2005; Zhang, 2006), and it suppresses the increase of TNF-α, IL-1β (Yue et
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Injury and infection stimulate immune system to produce an inflammatory response (Dickerson et al., 1998). With inflammatory diseases, the macrophages produce excess amounts of mediators such as NO, prostanoids and pro-inflammatory cytokines (Ahn et al., 2005).

In recent studies, we found that the aqueous extract of Gyejijakyakjimo-tang significantly suppressed LPS-induced COX-2 expression and PGE$_2$ synthesis in murine raw 264.7 macrophage cells. One such enzyme that is particularly important in regards to inflammation is COX. Elevated level of COX-2 expression has been detected in various tumor types, and may account for the excessive production of inflammatory prostaglandins. Specific COX-2 inhibitors are known to attenuate the symptoms of inflammation (Shao et al., 2000). Myers et al. reported that mice deficient in COX-2 displayed significant reduction in synovial inflammation and joint deterioration in a collagen-induced arthritis model (Myers et al., 2000). Kim et al. showed that the COX-2 inhibitor has a beneficial effect in the treatment of mast cell-mediated inflammatory allergy disease (Kim et al., 2007).

In recent results, we find that the aqueous extract of Gyejijakyakjimo-tang significantly inhibited LPS-induced iNOS expression and NO production in murine raw 264.7 macrophage cells. NO is a short-lived free radical that mediates many biological functions, including neurotransmission, vascular homeostasis, and host defense (Moncada et al., 1991; Jaffrey and Snyder, 1995). Overproduction of NO by iNOS has been linked to circulatory shock (Szabo, 1995), inflammation (MacMicking et al., 1997), and carcinogenesis (Ohshima and Bartsch, 1994). Thus, inhibition of NO production by blocking iNOS expression is an important benefit in the treatment of inflammatory diseases. Recently, Cuzzocrea et al. reported that selective inhibitors of iNOS activity have potent effect on collagen-induced arthritis in rodent model (Cuzzocrea et al., 2002).

Many herbal plants are used in the treatment of inflammation in traditional medicine (Talhouk et al., 2007). Herbal medicine has several different pharmacological properties, because synergistic effects between herbs can occur (Lee et al., 1995; Nishizawa et al., 1995; Jang et al., 2003). The herbal constituents are selected to accentuate the therapeutic actions, to enhance the activity of component compounds in clinical application, and reduce the toxicity or other side effects of the compounds (Bansky and Barolet, 1990; Oh et al., 2005). Gyejijakyakjimo-tang is a formula prescription that has been used to treat goat and rheumatoid arthritis. Gyejijakyakjimo-tang is composed of nine medicinal plants. Of these, Ledebouriellae Radix has anti-nociceptive and anti-inflammatory effects on Freund’s adjuvant-induced arthritis in rats (Kim et al., 2002). Aconiti Tuber exerts the anti-nociceptive effect by stimulating kappa-opioid receptors (Omiya et al., 1999). Bioactive glucoside from Paeoniae Radix suppressed production of interleukin-1 (IL-1), PGE$_2$, IL-6, vascular epidermal growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF) in synoviocyte of the rats with adjuvant arthritis (Zheng et al., 2007). Zingiberis Rhizoma has an analgesic effect related to the inhibition of prostaglandin and leukotriene biosynthesis (Srivastava and Mustafa, 1992). Of significance, it was reported that with Atractylenolid I and Atractylenolid III, the components of Atractylodis Rhizoma, inhibited LPS-induced TNF-α and NO production in macrophages (Li et al., 2007).

In this study, we have shown that the aqueous extract of Gyejijakyakjimo-tang exerts analgesic and anti-inflammatory effects by mostly suppressing COX-2 and iNOS expressions, and resulting in the inhibition of PGE$_2$ and NO production.

CONCLUSION

In this study, we investigated the analgesic and anti-inflammatory effect of the aqueous extract of Gyejijakyakjimo-tang by using in vitro and vivo
In vitro study, we evaluated the aqueous extract of Gyejijakyakjimo-tang on LPS-induced inflammation in murine raw 264.7 macrophage cells. Gyejijakyakjimo-tang suppressed PGE$_2$ synthesis and NO production by inhibiting the LPS-induced expressions of COX-2 and inducible iNOS mRNA in murine raw 264.7 macrophage cells. These results show that Gyejijakyakjimo-tang has the analgesic and anti-inflammatory effect by mostly suppressing COX-2 and iNOS expressions, and resulting in the inhibition of PGE$_2$ synthesis and NO production.

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