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Original Article

보골탕이 Monosodium Iodoacetate 유도 골관절염과 Interleukin-1β 유도 연골세포에 미치는 보호 효과

성진욱* · 이해웅† · 강경화‡ · 김경민§ · 조성우*
동의대학교 한의과대학 한방재활의학과교실*, 예방의학교실†, 생리학교실‡, 한방내과학교실§,

Protective Effects of Bogol-tang on Monosodium Iodoacetate-induced Osteoarthritis and Interleukin-1β-treated Primary Chondrocytes

Jin Wook Sung, K.M.D.*, Hai Woong Lee, K.M.D.†, Kyung Hwa Kang, K.M.D.‡, Kyoung Min Kim, K.M.D.§, Sung Woo Cho, K.M.D.*
Departments of Korean Rehabilitation Medicine*, Preventive Medicine†, Physiology‡, and Oriental Internal Medicine§, College of Korean Medicine, Dong-eui University

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CORRESPONDING TO
Sung Woo Cho, Department of Korean Rehabilitation Medicine, College of Korean Medicine, Dong-eui University, 52-57 Yangjeong-ro, Busanjin-gu, Busan 47227, Korea

TEL (051) 850-8671
FAX (051) 867-5162
E-mail luxy@daum.net

CO-CORRESPONDING TO
Kyung-Hwa Kang, Department of Physiology, College of Korean Medicine, Dong-eui University, 52-57 Yangjeong-ro, Busanjin-gu, Busan 47227, Korea
TEL (051) 850-7423
FAX (051) 853-4036
E-mail ghkang@deu.ac.kr

Objectives Bogol-tang has clinically been used to protect joint cartilage and to treat osteoarthritis. Our objective was to study the protective effect of Bogol-tang extract (BGT) in functional impairment, behavioral disorders, cartilage loss and pathological changes in a monoiodoacetate (MIA)-induced murine osteoarthritis (OA) model and interleukin (IL)-1 β -treated primary rat chondrocytes.
Methods Mouse knee joints were injected with MIA, a chemical that inhibits glycolysis and causes joint inflammation and matrix loss. MIA-OA induced mice orally administered BGT or acetaminophen (AAP) for 18 days by daily. Primary rat chondrocytes were pretreated with BGT or dexamethasone (DEX) and followed by co-incubation with IL-1 β (10 ng/mL).
Results In MIA-OA mice model, BGT led to delayed response on hot plate analysis, and suppressed the cartilage loss and damages in joint tissues. BGT suppressed the elevated levels of inflammatory mediators, nitrite and PGE_2, the gene expression of matrix degrading enzymes, and extracellular-signal-regulated kinases 1/2 and c-JunN-terminal kinase phosphorylation in IL-1 β -treated primary rat chondrocytes.
Conclusions Our results suggest that BGT improve the knee joint function and delay the cartilage damages by anti-nociceptive, anti-inflammatory and ant-catabolic effects, which indicate BGT could be a potential candidate for osteoarthritis treatment. **(J Korean Med Rehabil 2019;29(2):101-113)**

Key words Bogol-tang, Osteoarthritis, Monosodium iodoacetate, Nociception

Introduction

Osteoarthritis (OA) is the most common form of joint disorders in aging populations over 65 years of age[1]. The development of OA is promoted by the risk factors such as previous knee injury, female gender, aging, obesity[2]. Due to the progressive destruction of joint cartilage and inflammatory response, patients with OA

experience pain, stiffness, and functional impairment in their damaged joints[3,4]. It results in a substantial economic burden and impaired quality of life[5].

Although analgesics and non-steroid anti-inflammatory drugs (NSAIDs) are commonly used for symptomatic treatment for OA[6], no drug has been approved by other agencies around the world, Food and Drug Administration (FDA) or OA disease modification.

Korean traditional therapies including medications, acupuncture, moxibustion and pharmacopuncture have been applied for degenerative osteoarthritis[7-12]. Recently, scientific efficacy studies have been active on the treatment of Korean medicine for osteoarthritis[13].

Animal models and primary rat chondrocytes that mimic degenerative arthritis pathology were used in the experiment. Intra-articular injection of monosodium iodoacetate (MIA) into joints inhibits glyceraldehye-3-phosphate dehydrogenase activity in chondrocytes, causing the disruption of glycolysis and eventual cell death. The loss of chondrocytes results in pathological symptoms similar to arthritis in humans[14].

Bogol-tang (BGT) is a prescription made by the author based on Palmul-tang and has been operated for 15 years in clinical practice for degenerative osteoarthritis. This prescription consists of drugs improve the function system of liver and kidney and strengthen the muscles and bones, and medicines that help the digestive function in Korean medicines. Medicines strengthen the muscles and bones are Cibotii Rhizoma, Astragali Radix, Coicis Semen, Drynariae Rhizoma, Curcumae Radix, Dioscoreae Rhizoma, Acanthopanacis Cortex, Psoraleae Semen, Eucommiae Cortex Carbonisatum, Lycii Fructus, Rehmanniae Radix Preparata, Carthami Fructus, Clematidis Radix, Asiasari Radix et Rhizoma and Cervi Cornus Colla. And medicines helping digestive function are Atractylodis Rhizoma Alba, Cyperi Rhizoma, Citri Unshius Pericarpium, Poria Sclerotium, Amomi Fructus, Glycyrrhizae Radix et Rhizoma.

This study is to evaluate the efficacy of the BGT on the MIA-induced models of OA in the C57BL/6N mouse knee joint and IL-1β-treated primary rat chondrocytes.

Therefore, we measured the body weights, the weight of organs, pain sensitivity, gait alteration, and cartilage changes in MIA-induced OA mice. Also, we investigated inflammatory mediator, matrix degrading enzyme associated genes, phosphorylation of microtubule-associated protein kinase (MAPKs) activation in IL-1β-treated rat primary cultured chondrocytes.

Materials and Methods»»»»

1. Animals

Male C57BL/6N mice of 11 week-old were purchased from SAMTACO. Co. Ltd. (Osan, Korea). Mice were housed in cages, 4 animals per cage, were fed standard laboratory chow and water ad libitum, and exposed to a daily 12 h light/dark cycle. Mice were acclimatized for 1 week prior to starting the experiment.

Mice were divided into four groups as follows: Normal saline administration (Control [CON] group, n=8); OA with normal saline administration (MIA group, n=8); OA with 0.46 g/kg/day BGT administration (BGT group, n=8); OA with 0.066 g/kg/day acetaminophen (AAP) treatment (AAP group, n=8). The animals in this study were cared for according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., WA, USA). The protocol was approved by the Committee on Animal Research and Ethics of Dong-eui University (Busan, Korea) (The committee's reference number: R2017-028).

2. Induction of MIA-induced OA

For induction of MIA-induced OA, the mice were intramuscularly anesthetized with a mixture of ketamine hydrochloride (20 mg/kg, Ketalar, Yuhan Co., Seoul, Korea) and xylazine (20 mg/kg, Rumpun, Bayer Korea Ltd., Seoul, Korea), which was diluted 1:1 with normal

saline. Once the mice were under anesthesia, mice were given a single intra-articular injection of 1 mg MIA (Sigma Chemical Co., St. Louis, MO, USA) through the infrapatellar ligament of the right knee[15] and killed at 18 days post injection. MIA was dissolved in sterile saline and administered in a volume of 10 μL using a 30 gauge needle. The left contralateral control knee was injected with 10 μL of sterile saline (0.9%) as a control.

3. Preparation of BGT

BGT are the extracts purified from the twenty-three medicinal herbs, including *Cibotium barometz* J. Smith and *Astragalus membranaceus* Bunge et al., as shown in Table I. The herbs were obtained from HANION HERB Co. (Busan, Korea) and authenticated based on their microscopic and macroscopic characteristics by the classification and identification committee of the Institute of Korean Medicine of Dong-eui University. The total mixture (600 g) of herbs was washed thoroughly with distilled water, cut into pieces, and were submerged into distilled water (1:10, v/v), was boiled at 100°C for 2 h and the extract was filtered, lyophilized and subsequently stored at -20°C. The yield of BGT aqueous extract was 13.73% (w/w).

Table I. The Ingredients of Bogol-tang (BGT)

Herb name	Amount (g)
Cibotium barometz J. Smith	12
Astragalus membranaceus Bunge	12
Coix lacryma-jobi	8
Drynaria fortunei J. Smith	8
Curcuma wenyujin Y. H. Chen et C. Ling.	8
Dioscorea batatas Decaisne	8
Acanthopanax sessiliflorum Seeman	8
Psoralea corylifolia Linné	8
Eucommia ulmoides Oliver	8
Poria cocos Wolf	8
Lycium chinense Miller	6
Atractylodes japonica Koidzumi	6
Cyperus rotundus Linné	6
Citrus unshiu Markovich	6
Rehmannia glutinosa Liboschitz ex Steudel	6
Angelica gigas Nakai	4
Paeonia lactiflora Pallas	4
Carthamus tinctorius Linné	4
Amomum villosum Loureiro	4
Clematis manshurica Ruprecht	4
Asiasarum heterotropoides F. Maekawa	4
Glycyrrhiza uralensis Fischer	4
Cervus nippon Temminck	4
Total	150

4. Treatment

The effect of BGT was studied in four groups of 8 mice after induction of OA. The BGT group was administered orally by gavage at a daily 0.46 g/kg dose of BGT, the AAP group was administered orally by gavage at a daily 0.066 g/kg dose of AAP and the CON and MIA group received water. Histopathological changes in knee joints were studied after 18 days.

5. The weights of body and organs

The mice were recorded the changes of body weights at intervals of 3–4 days for 18 days after intra-articular injection of MIA and oral administration. When the mice were sacrificed, the organs (liver, kidney, and spleen) were harvested and their wet masses were determined.

6. Timeline of experiments

We performed a pilot experiment on a small batch of mice to determine which analyses to use. In the final experiment from which we collected data, the behavioral and functional analyses were performed in the order of least stress to most stress for the mice. To account for habituation formation, all mice were tested 2 times after MIA-induction OA at specific time points. The scheme of the study is illustrated in Figure 1.

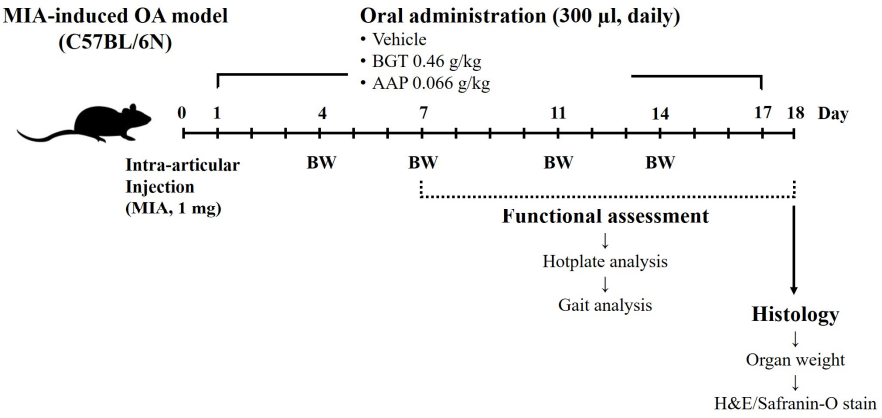

Fig. 1. Scheme of this experimental process. Osteoarthritis (OA) was induced by monoiodoacetate (MIA) injection into the joint of mice. Body weight (BW) was measured at regular intervals after intra-articular injection of MIA. Mice were orally administrated with Bogol-tang extract (BGT) or acetaminophen (AAP) once a day for 18 days. We performed two kinds of functional assessments and postmortem assessment after the final functional analysis. H&E: hematoxylin-eosin.

7. Animal behavioral tests

1) Hot plate analysis

The analgesic response was the latency observed from the time the mouse was placed on the heated surface until the first overt behavioral sign of nociception. Mice were transferred to the room for analysis at least 30 min before the experiment. Then, the mouse was placed on the hot plate at 55°C one at a time (Columbus Instruments, Columbus, OH, USA). The latency period for hind limb response (e.g., shaking, jumping, or licking) was recorded as response time. Each trial has a maximum time of 45 s. The mouse was removed from the hot plate immediately after a response was observed.

2) Gait analysis

The paws of the mice were brushed with ink by an examiner blinded to the MIA-induced OA procedures. Right and left hind paws were colored with ink of different color. Immediately after ink was applied, the mice were allowed to run on a 60 cm long, 6 cm wide Plexiglas track with white paper on the bottom. A dark chamber was present in the end of the track to entice the mice. Upon completion of the test, paper was scanned at 300 dpi. The distance of the same paw between two steps were defined as stride. The areas of third to the fifth set of footsteps were quantified by image J software (U.S. National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/, 1997-2018) N=8 in each group.

8. Histopathology

Mice were sacrificed at 18 days following BGT administration. The knee joint of each mouse was decalcified in 14% ethylenediaminetetraacetic acid for 5 days at room temperature. After dehydration and embedding in paraffin, the sagittal section (5 μm) of the knee joint were stained with hematoxylin-eosin (H&E) and Safranin-O Fast green method. The stained sections were photographed under a light microscope using a Leica microscope (Leica Microsystems, Wetzlar, Germany).

9. Cell examination

1) Culture of primary rat chondrocytes

The tibial and femoral cartilages of 4–5-week old Sprague Dawley rats were dissected, added to 10 mL of 0.2% trypsin at 37°C for 45 min, and then 0.2% (w/v) collagenase Type II and digested for 45 min at 37°C,

two times. The cartilage pieces were retrieved and placed in 10 mL of 0.2% collagenase Type II solution at 0.5 mg/mL overnight at 37°C. Primary rat chondrocytes were cultured at 37°C in a 5% CO_2 incubator in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

2) Cell viability assay

Cell proliferation were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For cell proliferation assay, primary rat chondrocytes were seeded at 1.5×10^5 cells/mL, incubated for 5 days, and treated with varying concentrations of BGT for 24 h. The crystallized formazan was dissolved in dimethyl sulphoxide, and the absorbance was measured at 570 nm on a SpectraMax M2 Microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3) Nitric oxide assay

Primary rat chondrocytes were seeded at 1.5×10^5 cells/mL in 24-well plates. Primary rat chondrocytes were pretreated with varying concentrations (0.25, 0.5, and 1 mg/mL) of BGT for 24 h and subsequently cultured with IL-1β (10 ng/mL) for 24 h. Nitrite (NO_2) accumulation in the culture medium was measured as an indicator of NO production, based on the Griess reaction. In brief, 100 μL of each supernatant from BGT-treated samples was mixed with an equal volume of Griess reagent (1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid and 0.1% [w/v] naphthylethylenediamine) in a dark room for 10 min. Absorbance was then measured at 540 nm on a SpectraMax M2 Microplate reader (Molecular Devices, Sunnyvale, CA, USA). The nitrite concentration was determined by comparison with a standard curve of sodium nitrite.

4) Prostaglandin E_2 assay

To measure prostaglandin E_2 (PGE_2), primary rat chondrocytes were plated at 1.5×10^5 cells/mL in a 24-well plate. Primary rat chondrocytes were pretreated with varying concentrations (0.25, 0.5, and 1 mg/mL) of BGT for 24 h and subsequently cultured with IL-1β (10 ng/mL) for 24 h. The concentration of PGE_2 was measured using the PGE_2 Parameter Assay Kit (R&D system Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

Table II. The Primer Sequences Used in This Study

Gene	Primer sequences	Size (bp)
Aggrecan	Forward: 5′-TGAGTGTGAGCATCCCTCAACCAT-3′ Reverse: 5′-ATGCTGTTCACTCGAACCTGTCCT-3′	211
Collagen type II	Forward: 5′-ATGTCAGCCTTTGCTGGCTTAGGA-3′ Reverse: 5′-AGTCATCTGGACGTTAGCGGTGTT-3′	471
MMP-1	Forward: 5′-GGTGAAGACGTCCAAGCTAAA-3′ Reverse: 5′-CTCTGTAGAAGGCGAACACAATA-3′	361
MMP-3	Forward: 5′-GTGGTACCCACCAAATCTAACT-3′ Reverse: 5′-ATCGATCTTCTGGACGGTTTC-3′	348
MMP-13	Forward: 5′-AAGATGTGGAGTGCCTGATG-3′ Reverse: 5′-CCAGTGTAGGTATAGATGGGAAC-3′	462
ADAMTS-5	Forward: 5′-ATGGTGTGTCTGACCAAGAAG-3′ Reverse: 5′-ACACTGCAGGAACGGTATATG-3′	275
GAPDH	Forward: 5′-TGACTCTACCCACGGCAAGTTCAA-3′ Reverse: 5′-TCTCGTGGTTCAC ACCCATCACAA-3′	269

MMP: matrix metalloproteinase, ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

5) Isolation of total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After isolation of the total RNA from primary rat chondrocytes, RT-PCR was performed with 1 μg of total RNA using TaKaRa One Step RNA PCR Kit (TaKaRa, Tokyo, Japan) using a PCR machine (GeneAmp, PCR system 9700, Applied Biosystems, Forster City, CA, USA). The PCR product was subjected to agarose gel electrophoresis to determine the gene induction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. The primer sequences used in the present study are summarized in Table II.

6) Western blotting

The cell lysate was homogenized with ice-cold lysis buffer, which consisted of 20 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 2 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l NaF, 1% Igepal CA-630, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l Na_3VO_4, and protease inhibitor cocktail. Following 10 min incubation on ice, the homogenized suspension was centrifuged at 2,000×g for 15 min at 4°C, and the supernatant was used to determine protein concentrations. The protein concentration in the tissue lysates was determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins (20 μg from each sample) were then separated by l0% sodium dodecyl sulfate- polyacrylamide gel electrophoresis and were transferred to nitrocellulose transfer membranes (Whatman; GE Healthcare Europe GmbH, Freiburg, Germany). The membranes were blocked with 5% skim milk in TBS- Tween (TBST) buffer (10 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl and 0.05% Tween 20) for 1 h, and were then incubated with total antibodies to extracellular- signal-regulated kinases (ERK), c-JunN-terminal kinase (JNK), p38, and β-actin and phospho-specific antibodies to ERK, JNK, and p38. The membranes were incubated with the primary antibodies (diluted in 5% skim milk in TBST) overnight at 4°C and were then washed. The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG anti - bodies (diluted in 5% skim milk in TBST). Immunoreactive bands were developed using enhanced chemiluminescence (ECL) regents (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's protocol.

10. Statistical analyses

Statistical analysis was performed using GraphPad Prism ver. 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as mean±standard deviation (SD). Organ weights, NO, and PGE_2 levels were compared using repeated measures one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Body weight and behavioral data were compared using two-way ANOVA at each time point with post-hoc analysis by Bonferroni corrections. Significant differences were defined as $p<0.05$.

Results»»»»

1. Animal

1) The changes of body weights and organ weights

The body weight of mice was examined at 0, 4-, 7-, 11-, and 14-day post intra-articular MIA injection and oral administration of saline, BGT, and AAP. There was no statistical difference in body weight between other experimental groups except AAP group at each time. AAP group showed significant difference in weight loss compared to Con group since 7-day post intra-articular MIA injection (Fig. 2A).

The weight the organs (liver, kidney, and spleen) were

estimated within the experimental group. There were no different among the experimental groups in weight of liver, kidney, and spleen (Fig. 2B).

2) The effect of BGT on hot plate analysis

The hot plate analysis is mainly performed for an assessment of pain sensitivity to thermal stimulus. In this study, we evaluated the pain sensitivity of BGT using hot plate (Fig. 3A).

MIA group showed no significant differnce compared to CON group at 7 and 17 days post intra-articular MIA injection in the latency of the withdrawal response to heat stimulation, BGT and AAP group showed the significant decrease compared to CON group at 7 days ($p<0.05$), however there was no significant differnce between the groups at 17 days in the latency of the withdrawal response to heat stimulation on hot plate. While BGT and AAP group showed the significant increase ($p<0.05$, $p<0.01$ respectively) between 7 and 17 days post intra-articular MIA injection in the latency of the withdrawal response to heat stimulation on hot plate (Fig. 3B).

3) The effect of BGT on gait alteration

After intra-articular MIA injection, mice tended to stand on their toes instead of standing on their feet. To test whether MIA-induced OA caused a gait alteration, we assessed hind paw foot print area and stride by gait analysis (Fig. 4A). No significant differences in hind foot print area were detected between the groups. After intra-articular MIA injection, hind paw foot print area of the groups showed a declining tendency, how-

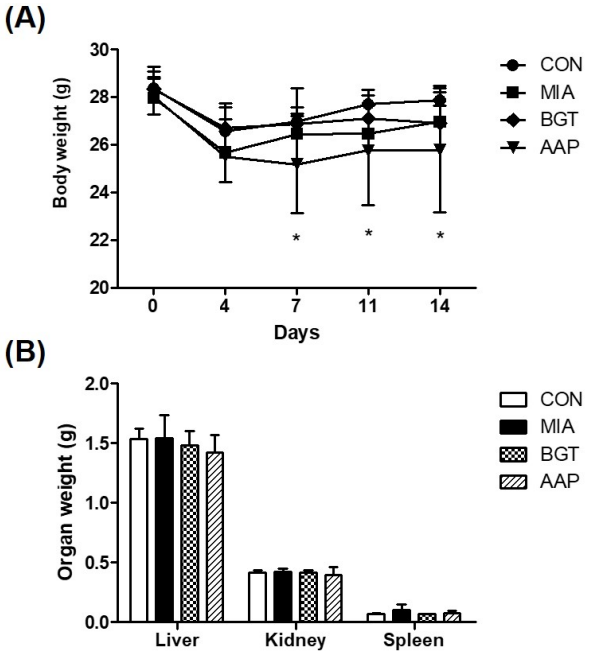

Fig. 2. The changes of body weight and organ weights. (A) Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc comparisons at each time point. Values are presented as mean±standard deviation (SD). (B) Data were analyzed by one-way ANOVA followed by Dunnett's post hoc comparisons. Values are presented as mean±SD. CON: control, MIA: monoiodoacetate, BGT: Bogol-tang extract, AAP: acetaminophen. $^{*}p<0.05$ vs. CON.

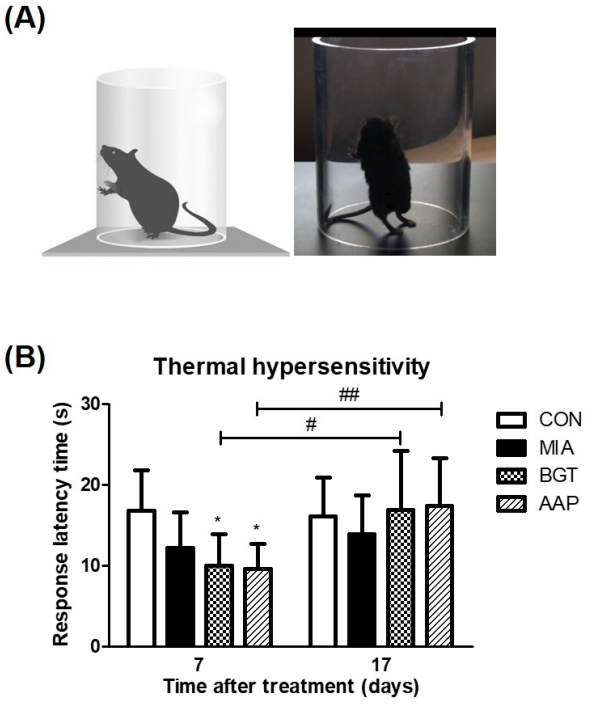

Fig. 3. The response latency time of paws to heat stimulation by hot plate analysis. (A) The experiment device of hot plate analysis. (B) The response latency time of the paws was measured at 7 and 17 days after treatment in each group. N=8. Data were analyzed by one-way analysis of variance (ANOVA) and two-way ANOVA followed by Bonferroni post hoc comparisons at each time point. Values are presented as mean±standard deviation. CON: control, MIA: monoiodoacetate, BGT: Bogol-tang extract, AAP: acetaminophen. $^{*}p<0.05$ vs. MIA. $^{\#}p<0.05$. $^{\#\#}p<0.01$ vs. time.

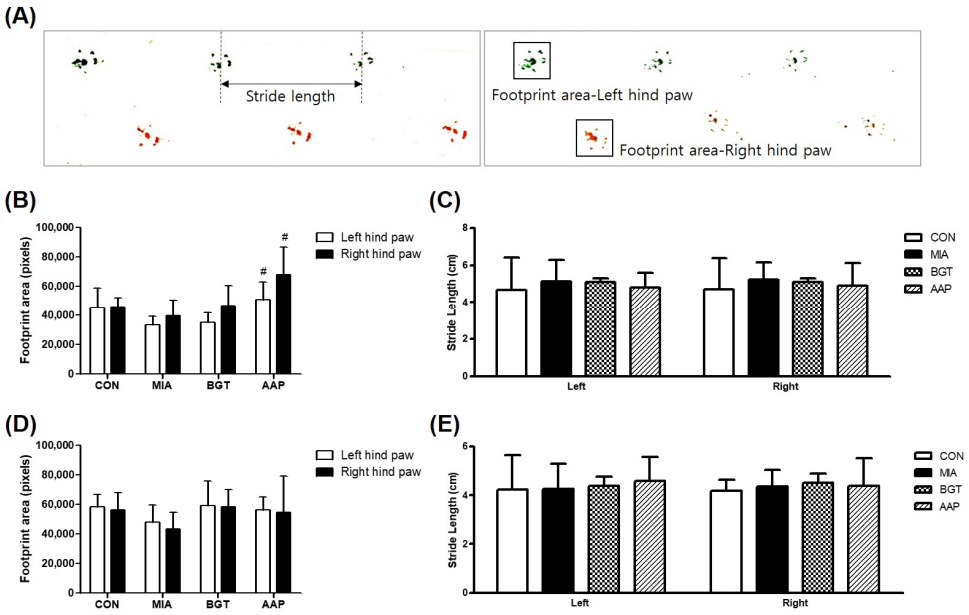

Fig. 4. The gait change of paws by gait analysis. (A) Representative hind paw foot prints for gait analysis. (B, C) Quantification of hind limb footprint area and stride of the mice on 8 days. N=8. (D, E) Quantification of hind paw footprint area and stride of the mice on 18 days. N=8. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc comparisons at each time point. Values were presented as mean±standard deviation. CON: control, MIA: monoiodoacetate, BGT: Bogol-tang extract, AAP: acetaminophen. $^{\#}p<0.05$ vs. MIA.

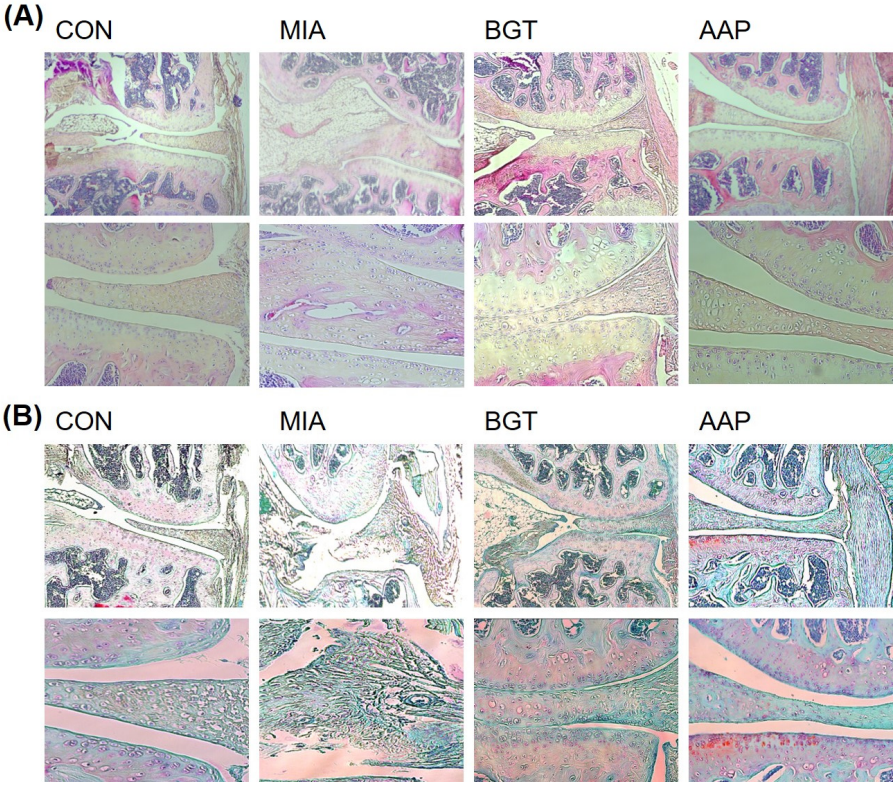

Fig. 5. Histopathologic analyses of knee joint. Histopathological changes in the knee joint after Bogol-tang extract (BGT) administration in monoiodoacetate (MIA)-induced osteoarthritis (OA) mice. (A) In hematoxylin-eosin (H&E) stain, synoviocytes, cartilage lacunae, and chondrocytes were well preserved in the BGT and acetaminophen (AAP) groups (5×, 20× objective). (B) Safranin O-fast staining showed a clear reaction of proteoglycans in the BGT and AAP groups (5×, 20× objective). CON: control.

ever it showed no significant differences compared to CON group. AAP group only showed a bigger hind foot print area compared to CON group at 7 days (Fig. 4B). There are no significant differences in stride length between the groups (Fig. 4C-E).

2. Cell

1) Histological evaluation

Histologic observation for cartilage damage were performed. In H&E stain, synovial membrane was seen to be destroyed in MIA group, while BGT and AAP groups clearly exhibited synovial membrane cells of cartilage and synovial cavity compared to MIA group. In addition, BGT and AAP groups exhibited synovial membrane cells, synovial cavity, and cartilage cells similar to the CON group (Fig. 5A). Safranin O-fast staining showed that the positive reaction of proteoglycans in articular cartilage in MIA group was lower than that of CON group. In addition, in BGT and AAP groups, the deep layer of cartilage showed a higher positive reaction for proteoglycans as compared to MIA group (Fig. 5B).

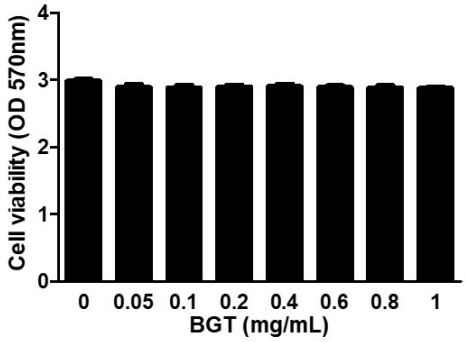

Fig. 6. Effects of Bogol-tang extract (BGT) on the cell viability of rat primary cultured chondrocytes. To evaluate the cytotoxic effect of BGT, rat primary chondrocytes were isolated from the cartilage in knee joints of rat by enzymatic digestion and seeded into 24-well culture plates. Chondrocytes were treated with BGT using MTT assay. Cell viability of chondrocytes was not significantly altered by BGT at various concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL) for 24 h. Results were expressed as percent of the control. Each data point represents the means±standard deviation of three independent experiments. OD: optical density, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

2) Primary rat chondrocytes

(1) The effect of BGT on cell viability

BGT showed no toxicity at all treated concentrations. Therefore, we used 0.25, 0.5, and 1 mg/mL of BGT for subsequent experiments (Fig. 6).

(2) The effect of BGT on IL-1β-treated nitrite and PGE_2 production

The nitrite production (421.4±16.71 μM) in primary rat chondrocytes treated with 10 ng/mL IL-1β was significantly increased compared to CON (26.30±0.85 μM). However, treatment with 0.25, 0.5, and 1 mg/mL BGT (176.1±20.09, 216.2±37.66, and 218.9±22.19 μM) and

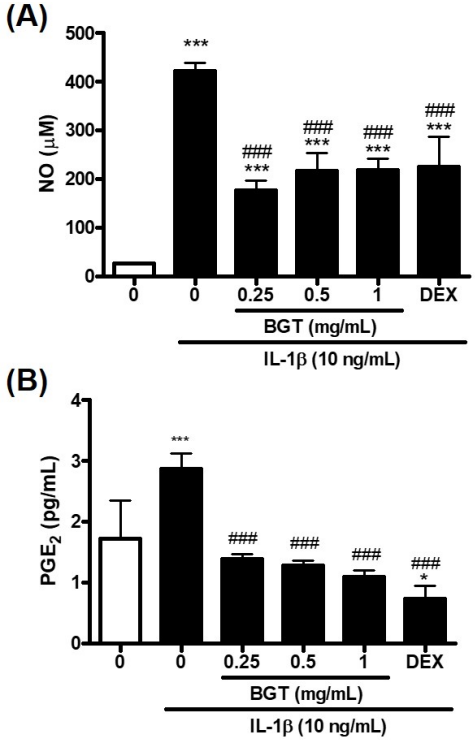

Fig. 7. The effect of Bogol-tang extract (BGT) on interleukin (IL)-1β-treated nitrite and PGE_2 production in rat primary cultured chondrocytes. Cells were pre-treated with 0.25, 0.5, 1 mg/mL of BGT for 24 h, followed by co-incubation with IL-1β (10 ng/mL) for 24 h. (A) Nitrite production in the cell culture supernatant was determined by Griess reagent. (B) PGE_2 production in the cell culture supernatant was determined by ELISA kit(R&D system Inc., Minneapolis, MN, USA). Results were expressed as the means±standard deviation of three independent experiments. $^{*}p<0.05$. $^{***}p<0.001$ vs. untreatment. $^{###}p<0.05$ vs. IL-1β only treatment.

10 μM dexamethasone (DEX) (224.8±61.20 μM) significantly reduced the nitrite production in the presence of IL-1β (Fig. 7A).

The PGE_2 production in primary rat chondrocytes treated with 10 ng/mL IL-1β was significantly increased (2.86±0.62 pg/mL) compared to CON (1.72±0.62 pg/mL). Treatment with 0.25, 0.5, and 1 mg/mL BGT and DEX decreased the PGE_2 production (1.38±0.86, 1.28±0.08, 1.09±0.11, and 0.73 ± 0.22 pg/mL) in the presence of IL-1β (Fig. 7B).

(3) The anti-catabolic effects of BGT on the expression of IL-1β-treated matrix degrading enzymes

BGT increased the mRNA level of collagen type II and aggrecan, it more effectively counteracted the IL-1β-treated catabolic effects at low concentrations (Fig. 8A). As shown in Fig. 8B, mRNA expression and activation of matrix degrading enzymes were significantly upregulated in the chondrocytes treated with IL-1β. In contrast, the IL-1β-treated upregulation of matrix degrading enzymes was antagonized by BGT and DEX. These results show that the protective effect of BGT against the IL-1β-treated catabolic effects was mediated by the suppression of matrix degrading enzymes.

(4) The effect of BGT on MAPKs in IL-1β-treated rat primary cultured chondrocytes

As shown in Figure 9, IL-1β-treated chondrocytes alone significantly increased the phosphorylation of ERK1/2 and JNK, when compared with the control levels. However, pre-treatment with BGT at all the concentration and DEX suppressed the phosphorylation of ERK1/2 and JNK, respectively. In addition, the total protein levels of the MAPKs did not differ among the compared groups. However, activation of p38 was unaffected by BGT and DEX (Fig. 9). These results suggested that the BGT ef-

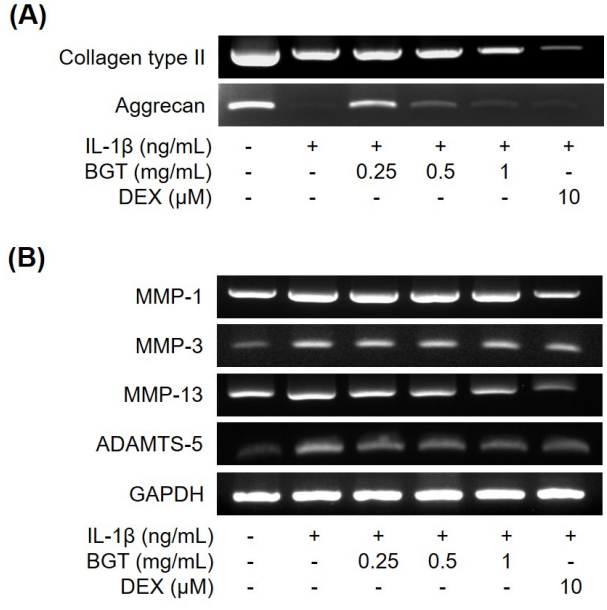

Fig. 8. The effect of Bogol-tang extract (BGT) on the expression of interleukin (IL)-1β-treated matrix degrading enzymes. (A) BGT slightly increased the anabolic effects through up-regulation of collagen type II and aggrecan antagonized the IL-1β-treated catabolic effects. (B) BGT suppressed the induction of IL-1β-treated matrix degrading enzyme associated genes. MMP: matrix metalloproteinase, DEX: dexamethasone, ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs, +: treatment, -: untreatment.

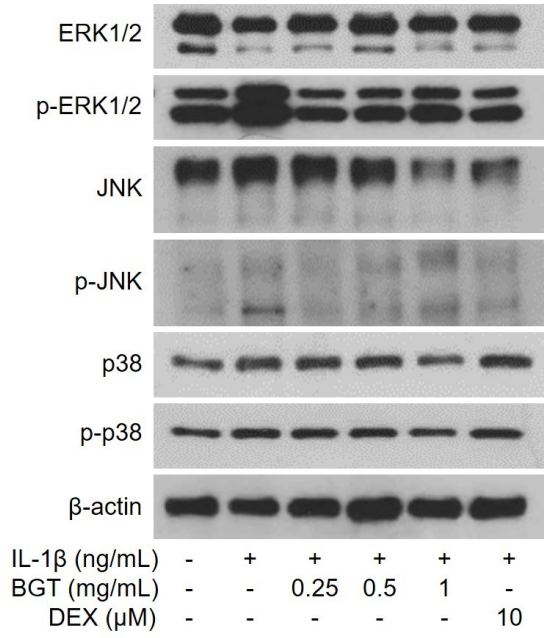

Fig. 9. The effect of Bogol-tang extract (BGT) on phosphorylation of microtubule-associated protein kinase (MAPKs) activation in interleukin (IL)-1β-treated rat primary cultured chondrocytes. Cells were pre-treated with 0.25, 0.5, 1 mg/mL of BGT for 24 h, followed by co-incubation with IL-1β (10 ng/mL) for 1 h. Phosphorylation of MAPKs was determined by western blotting. DEX: dexamethasone, +: treatment, -: untreatment.

fectively suppressed ERK1/2 and JNK phosphorylation during the IL-1β—treated inflammatory response in rat primary chondrocytes.

Discussion»»»»

In the study, we used the intra-articular MIA injected OA mice, because this model has become a standard for modelling joint disruption in OA[15].

Intra-articular injection of MIA disrupts chondrocyte glycolysis by inhibiting glyceraldehyde-3-phosphatase dehydrogenase and results in chondrocyte death, cartilage destruction, subchondral bone necrosis and collapse, as well as inflammation[15]. The morphological changes of the articular cartilage and bone disruption are reflective of some aspects of patient pathology. Along with joint damage, MIA injection induces referred pain sensitivity in the hind paw and gait alteration that are measurable and quantifiable. These behavioral changes are similar to the symptoms reported by the patient with OA.

We investigated the body weight, organs, pain sensitivity, gait alteration, and histological analysis of knee joint to assess if the BGT leads to altered OA progression in MIA-induced OA mice.

First, we found that BGT had no effects body weight by in MIA-induced OA mice. However, AAP group showed significant difference in weight loss compared to CON group since 7-day post intra-articular MIA injection. There were no different among the experimental groups in weight of liver, kidney, and spleen. These results suggest that BGT has no toxic effect in experimental animals.

We observed whether BGT causes behavioral changes by hot plate and gait analysis in MIA-induced OA mice.

The hot plate analysis is mainly performed for an assessment of pain sensitivity to thermal stimulus. Many studies have reported changes in pain sensitivity and sensitization in patients with OA[16,17].

BGT and AAP group showed the significant increase ($p<0.05$, $p<0.01$ respectively) between 7 and 17 days post intra-articular MIA injection in the latency of the withdrawal response to heat stimulation on hot plate. To test whether MIA-induced OA caused a gait alteration, we assessed hind paw foot print area and stride by gait analysis. No significant differences in foot print area and stride length of hind paw were detected between the groups. These results suggest that BGT and AAP has diminishing effect on pain sensitivity but no effect on gait alteration.

Histologic findings for cartilage damage were observed in the MIA-OA knee joint. MIA group showed that the destruction of synovial membrane and chondrocytes, erosion and fibrillation of cartilage surface, subchondral bone exposure and loss of proteoglycan in cartilage, while BGT and AAP groups clearly exhibited synovial cavity, and a higher positive reaction for proteoglycans in the deep layer of cartilage as compared to MIA group.

We also estimated the production of inflammatory mediators, the expression of matrix degrading enzyme associated genes, the phosphorylation and activation of MAPKs to assess if the BGT leads to regulation of inflammation and enzymes in IL-1β-treated rat primary cultured chondrocytes.

The homeostasis of cartilage is maintained by the balance between catabolic and anabolic enzymes in chondrocytes[4] and this homeostasis is disrupted in progressive OA. OA patients are usually observed increased expression of catabolic genes such as, matrix metalloproteinases (MMPs) and decreased expression of anabolic genes including collagen type II and aggrecan in the chondrocytes of articular cartilage[4]. Generally, increase of catabolic enzymes results in softening of the articular cartilage. Additionally, IL-1β-treated inflammatory responses further promote the expression of catabolic enzymes[18]. Thus, joint cartilage destruction and inflammation may be two aspects of interaction in the progressing of OA.

In cell viability assay, BGT showed no toxicity at all

treated concentrations in primary rat chondrocytes. Therefore, we used 0.25, 0.5, and 1 mg/mL of BGT for subsequent experiments.

We assessed whether BGT suppresses nitrite and PGE_2 production treated by IL-1β in primary rat chondrocytes. We found that BGT has inhibitory effect on IL-1β-treated nitrite and PGE_2 production in primary rat chondrocytes. These results suggested that BGT could have anti-inflammatory effects by the suppression of IL-1β-treated inflammatory mediators.

To investigate the effects of BGT against the IL-1β-treated catabolic effects, primary rat chondrocytes were pretreated with 0.25, 0.5, and 1 mg/mL BGT and 10 μM DEX and followed by co-incubation with IL-1β (10 ng/mL) for 24 h. The cultured chondrocytes were then harvested to isolate total RNA, which was converted to cDNA to verify the alteration of anabolic and catabolic genes. We found that BGT increased the mRNA level of collagen type II and aggrecan, and antagonized the IL-1β-treated upregulation of matrix degrading enzymes. These results show that the protective effect of BGT against the IL-1β-treated catabolic effects was mediated by the suppression of matrix degrading enzymes.

The MAPKs are fundamental regulators in the inflammatory response. We estimated the effect of BGT to stimulate IL-1β-treated phosphorylation of ERK1/2, JNK, and p38 in IL-1β-treated rat primary chondrocytes. We found that IL-1β-treated chondrocytes alone significantly increased the phosphorylation of ERK1/2 and JNK. However, pre-treatment with BGT at all the concentration and DEX suppressed the phosphorylation of ERK1/2 and JNK, respectively. In addition, the total protein levels of the MAPKs did not differ among the compared groups. However, activation of p38 was unaffected by BGT and DEX.

These results suggested that the BGT effectively suppressed ERK1/2 and JNK phosphorylation during the IL-1β -treated inflammatory response in rat primary chondrocytes.

From these results, it was considered that BGT has the potential effect to improve the knee joint function and delay the cartilage damages similar to AAP. BGT reduces the pain sensitivity and the structural damage of knee joint after MIA injection in mice. In addition, BGT suppresses the production of inflammatory mediators, the expression of matrix degrading enzymes and the phosphorylation of ERK1/2 and JNK associated with OA pathology in chondrocytes.

Therefore, it is suggested that the BGT might be a good candidate for delaying OA progression.

Conclusion»»»»

In this study, we obtained the following results for BGT: (1) Intra-articular MIA injection cause the higher pain sensitivity, functional impairment, and cartilage transformation; (2) BGT shows no differnces in body weight and organ weight; (3) BGT improves the pain sensitivity; (4) BGT showed the inhibition of damages of synovial membrane and cartilage in knee joint tissue; (5) BGT suppresses the production of IL-1β-treated inflammatory mediators; (6) BGT protectes the IL-1β-treated catabolic effects by the suppression of matrix degrading enzymes; (7) BGT suppresses ERK1/2 and JNK phosphorylation during the IL-1β -treated inflammatory response in rat primary chondrocytes.

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