

Anti-inflammatory Effect of *Gyulpidaehwangbakcho-tang* (*Jupidahuangpoxiao-tang*) in the Collagen-induced Arthritis Mouse Model

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Objectives: To investigate anti-inflammatory and anti-arthritic effects of *Gyulpidaehwangbakcho-tang* (GDBT) extract in a murine model of rheumatoid arthritis.

Methods: The mice received 100 μ g of bovine type II collagen in Freund's complete adjuvant by intradermal injection at the base of the tail on day 0 and a booster injection on day 21. The mice were orally administered with GDBT (200 or 50mg/kg dissolved in distilled water) daily from day 1 to day 21 after arthritis incidence, and monitored for disease incidence and the severity of arthritis up to day 21. In order to evaluate the effect of GDBT on disease progression, we examined pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α , COX-2 and NOS-II.

Results: GDBT produced a significant and dose dependent inhibition of arthritis and inflammation during the entire duration of the study. This action was characterized by the decreased production of IL-1 β , IL-6, TNF- α , COX-2, and NOS-II in vivo.

Conclusion: We believe that the anti-arthritic activity of GDBT is due to its modulatory effect on the expression of pro-inflammatory cytokine in the synovium. Our results contribute towards validation of the traditional use of GDBT in the treatment of RA and other inflammatory joint disorders.

Key Words : Collagen induced arthritis, *Gyulpidaehwangbakcho-tang*, Citri pericarpium, Rhei radix et rhizome, Natrii sulfas, pro-inflammatory cytokines.

Introduction

Arthritis and related disorders, including rheumatoid arthritis (RA), are common diseases affecting millions¹⁾. RA is characterized by the inflammation of synovial joints infiltrated by CD4+ T cells, macrophages, and plasma cells that play major roles in the pathogenesis of the disease^{2,3)}. Conventional medicine, including treatment with steroids, non steroidal anti-inflammatory drugs (NSAIDs) and such biological agents as tumor necrosis factor alpha (TNF- α) and interleukin-1 beta

(IL-1 β) antagonists⁴⁾, has shown only limited success against all forms of arthritis⁵⁾. The adverse reactions and toxicity associated with the use of these drugs have expeditiously promoted the use of natural plant products or procedures belonging to the diverse traditional systems of medicine by patients with RA and other chronic inflammatory disorders⁶⁾.

The classical herbal formula *Gyulpidaehwangbakcho-tang* (GDBT) is first documented in the book Synopsis of Prescriptions of the Golden Chamber⁷⁾. According to the book, GDBT can be prescribed for a patient

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to treat dyspepsia due to eating raw fish. It consists of three component herbs, *Citri pericarpium*, *Rhei radix et rhizome*, and *Natrii sulfas*. Its main action is to induce diarrhea.

Inducing diarrhea is the one of eight methods of oriental medical treatment. Traditionally this method has been used to treat constipation. But much research has made inducing diarrhea a possible method to treat the various diseases of internal medicine. Inducing diarrhea is available on emergency medicine because of instant relief, and it is also considered to treat chronic diseases caused by retention of undigested food, heat pathogen, fluid accumulation, blood retention, stagnation of phlegm, etc.⁸⁾ In oriental medicine, these pathological conditions can be factors, which directly or indirectly worsen the symptoms of arthritis.

Many herbal formulas have been reported previously to have anti-arthritic effects. However, no study on the anti-arthritic and anti-inflammatory activity of GDBT has been reported. In order to verify its anti-arthritic effects, we investigated the immunomodulatory and anti-inflammatory activities of GDBT. The aim of this study was to evaluate the control activity of GDBT extract on IL-1 β , IL-6, TNF- α , COX-2, and NOS-II production. Therefore, we decided to investigate anti-inflammatory and anti-arthritic effects of GDBT extract in a murine model of rheumatoid arthritis. The effects of GDBT extract on total cell number in draining lymph nodes, paw joints, T cells, regulatory T cells by flow cytometric analysis and cytokine production in paw joints, and spleen by ELISA were determined. Moreover, to determine whether GDBT prevented articular destruction, we analyzed the paw joints of mice.

In this study, the various immunomodulatory effects of GDBT were investigated in order to determine the potential bioactivity of GDBT on RA.

Materials and Methods

1. Preparation of GDBT extract

Herbs used in the GDBT were purchased from Daejeon University Oriental Medical Hospital (Daejeon, Korea) after confirming the morphology under microscopy. GDBT consisted of 2g *Citri pericarpium* (*Citrus unshiu* Markovich, Rutaceae), 4g *Rhei radix et rhizome* (*Rheum officinale* Baillon, Polygonaceae), 4g *Natrii sulfas* ($\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$). In total, 10g of mixed dried herbs was boiled with 300mL of distilled water for 2 h at 100°C based on the way of decocting GDBT in the clinical use. The suspension was filtered, lyophilized, yielding 20.17% of powder, and kept at -20°C. An aqueous mixture of GDBT was extracted by using a rotary vacuum evaporator (Büchi B-480, Switzerland) and programmable freeze dryer (EYELA FDU-540, Japan). Prepared water extracts of GDBT were ground using a commercial electronic pulverizer and stored in a desiccator, then protected from light and moisture until used. The formulated GDBT was subjected to high performance liquid chromatography (HPLC) finger printing analysis in which major peaks were identified as the marker compounds to their originating individual herbs (unpublished data). The HPLC trace of the initial extract is showed in Fig. 1.

2. Animal preparation

Six-week-old female DBA/1 OlaHsd mice were obtained at Harlan Laboratories, Inc. (Indianapolis, USA). All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

3. Preparations of murine collagen-induced arthritis (CIA) model

All of the animal procedures were approved by

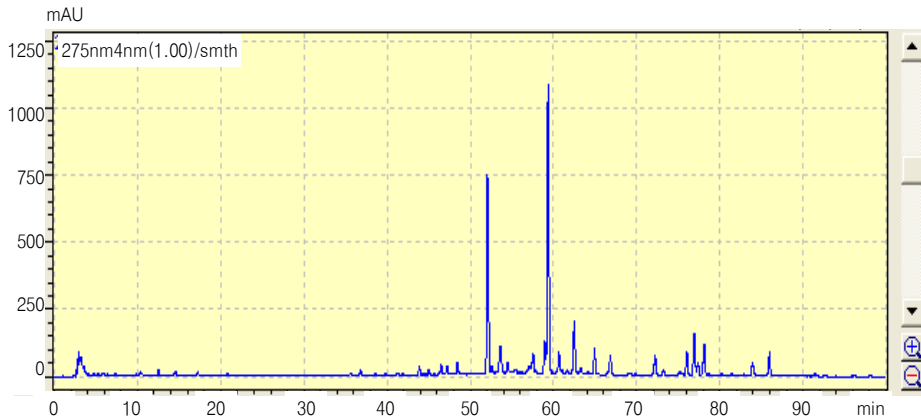


Fig. 1. HPLC analysis of GDBT. The major representative peaks of herbs in the formula are shown.

the Experimental Animal Commission of the Institute of Traditional Medicine and Bioscience at Daejeon University. Murine CIA model mice were induced in a modification of the previously established method^{9,10}.

In brief, female DBA/1 OlaHsd mice (eight-week-old; Harlan Laboratories, Inc., Indianapolis, USA) were kept under controlled environmental conditions (22°C±2°C relative humidity 55%±15%, 7am to 7pm alternate light-dark cycles, food and water ad libitum). The mice received 100µg of bovine type II collagen (Sigma, USA) in Freund's complete adjuvant (Sigma, USA) by intradermal injection at the base of the tail on day 0 and a booster injection on day 21 (n=6 mice per group). Mice were monitored daily for signs of arthritis, and each paw was scored individually as follows: 0=normal, 1=slight erythema and edema, 2=increased edema with loss of landmarks, 3=marked edema, and 4=marked edema with ankylosis on flexion. Each mouse was assigned an arthritis score (articular index) that equaled the sum of the scores for each paw, so that the possible maximum score per mouse was 16. In the prophylactic dosing model, mice were orally administered with GDBT (200 or 50mg/kg dissolved in distilled water) daily from day 1 to day 21 after arthritis incidence, and monitored for disease incidence and the severity of arthritis up to day 21. CIA control mice received an intraperitoneal injection

of PBS alone. The control group of mice received the vehicle (water) by gavage on the corresponding days. A third group of arthritic mice was fed methotrexate (MTX) (Sigma, USA) (2mg/kg), an established antiarthritic compound, as a positive control. All these mice were observed regularly for signs of arthritis.

On the final day of the experiment, all of the mice were anesthetized with ethyl ether and then blood was collected from each by cardiac puncture; the mice were then killed by cervical dislocation. The mice spleen, thymus, and lymph nodes were taken out and used for ELISA analysis and total cell counts.

4. Antibodies and flow cytometric analysis

Anti-CD3-PE, anti-CD4-FITC, anti-B220-PE, anti-MHC class-II-PE, anti-Gr-1-PE, anti-CD8-FITC, anti-B220-FITC, anti-CD11c-FITC, BD Cytotfix/Cytoperm plus kit, anti-CD3 mAb, anti-foxp3-PE, anti-CD28 mAb for flow cytometric analysis were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA). Cells from lymph nodes and spleen were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN₃) for 10min on ice, and analyzed by two color flow cytometry on a FACScan using CellQuest software (BD Biosciences, Mountain View, CA). Absolute cell numbers were counted manually in a hemocytometer chamber

(Fisher). The 2×10^3 cells were spun onto glass slides (Cytospin centrifuge, Cellspin, Hanil, Korea). Differential count was made according to standard morphologic criteria. Absolute numbers of various immune cells were quantified by multiplied number. Total number multiplied by gating percent (CD area in facs data) in lymph nodes and spleen made absolute number.

5. H&E (hematoxylin and eosin) and M-T (Masson trichrome) staining of paw joints in CIA induced murine RA model

For histologic analysis of the knee joints, the hind limbs of the mice were removed postmortem and fixed in 10% neutral buffered formalin, decalcified in 5% formic acid, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or Masson's trichrome.

6. Statistical analysis

Results were expressed as the mean \pm standard deviation (SD). Statistical analysis of the data was

carried out by Student's t-test. A difference from the respective control data at the levels of $p < 0.05$ was regarded as statistically significant.

Results

1. Effects of GDBT on the clinical characteristics of GDBT

To determine whether GDBT suppresses the immune-mediated pathologic process in arthritic mice, we investigated the effect of GDBT on the arthritis incidence and articular index of CIA in DBA/1 OlaHsd mice. GDBT had no significant effect on the mean body weight of the mice nor did it elicit any behavioral changes in the mice in either treatment group, which suggests that GDBT is not toxic *in vivo* at the concentrations that were tested (results not shown). In our results, 5 weeks of treatment with GDBT (200, 50mg/kg) beginning 1 day after the booster injection of collagen, apparently inhibited arthritis progression (Fig. 2).

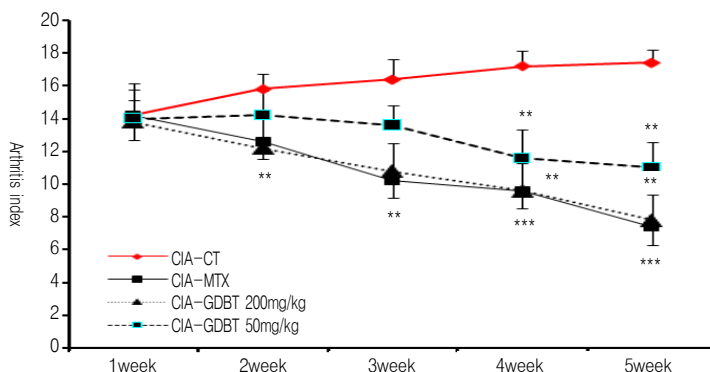


Fig. 2. Suppressive effects of GDBT on arthritis index of paw joint in CIA mice.

Arthritis index were scored according to Mann-Whitney U test at every six-day interval. Following boosterization of collagen, mice were divided into four groups and each group was treated with normal saline 0.1 ml(CIA-CT), MTX 2mg/kg(CIA-MTX), GDBT 200, 50mg/kg(CIA-GDBT) on a daily basis.

CIA-CT: Control group with collagen-induction arthritis

CIA-MTX: Group treated with methotrexate (2mg/kg, p.o)

CIA-GDBT: Group treated with GDBT (200, 50mg/kg, p.o)

** : $p < 0.01$ (Compared with CIA-CT), *** : $p < 0.001$ (Compared with CIA-CT).

2. Inhibitory effects of GDBT on total cell accumulation in draining lymph node and paw joint

To evaluate the effect of GDBT on CIA model mice, we investigated the recruitment of total cells to draining lymph nodes and paw joints. GDBT-treated group cell numbers were lower compared with CIA model control group (Fig. 3).

3. Determination of FACS analysis

1) Inhibitory effect of GDBT on CD4+ T cells, CD8+ T cells, CD4+CD25+ regulatory T cells population in GDBT model mice

The cell surface expression of CD4+ T helper was analyzed by flow cytometry. To detect CD4+ expression in draining lymph nodes and paw joints, two-color immunofluorescence staining was performed using FITC-conjugated anti-CD8 with PE-conjugated anti-CD4 mAb as described in Section 2.

To evaluate the efficacy of GDBT treatment on CD4+ T cells and CD8+ T cell population, we compared the effects of GDBT on intracellular CD4+

T cells and CD8+ T cell expression in CIA model mice by using flow cytometry. The absolute number of CD4+ cells in the GDBT-treated group decreased

compared with the control group in draining lymph nodes and paw joints. The absolute number of CD8+ cells in the GDBT-treated group decreased when compared with the control group in draining lymph nodes. There were marked changes in numbers of CD4+CD25+ regulatory T cells in the CIA model compared to the control group (Fig. 4).

2) Inhibitory effect of GDBT on the level of mRNA of IL-1 β , IL-6, TNF- α , COX-2, NOS-II in paw joints of CIA model mice.

IL-1 β , IL-6, TNF- α , COX-2 and NOS-II have central roles in the maintenance of chronic inflammation and tissue damage during the progression of RA. Especially, TNF- α is known to play a critical role in the pathogenic mechanisms of a number chronic inflammatory diseases, including RA.

To study whether GDBT was related to inflammatory cytokine production, the mRNA of IL-1 β , IL-6, TNF- α , COX-2, and NOS-II in paw joints were analyzed by ELISA, respectively. As shown in Fig. 5, the mRNA generations of IL-1 β , IL-6, TNF- α , COX-2 and NOS-II were suppressed by GDBT. These results support the conclusion that GDBT suppressed the generation of

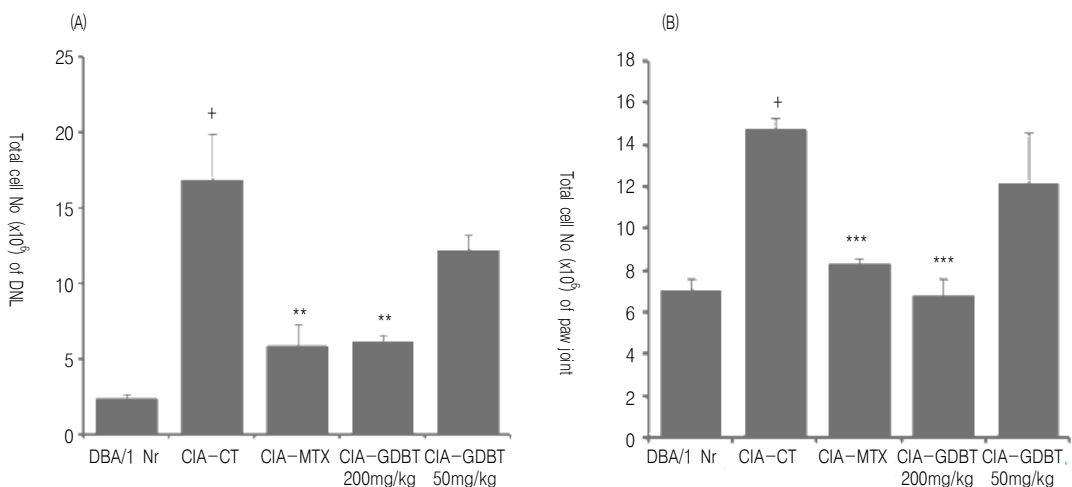


Fig. 3. The effect of GDBT on the total cell number of DLN (A) and paw joint (B) in CIA mice.

DBA/1 Nr: Normal DBA/1 OlaHsd mice group

** : p < 0.01 (Compared with CIA-CT), *** : p < 0.001 (Compared with CIA-CT), + : p < 0.001 (Compared with DBA/1 Nr)

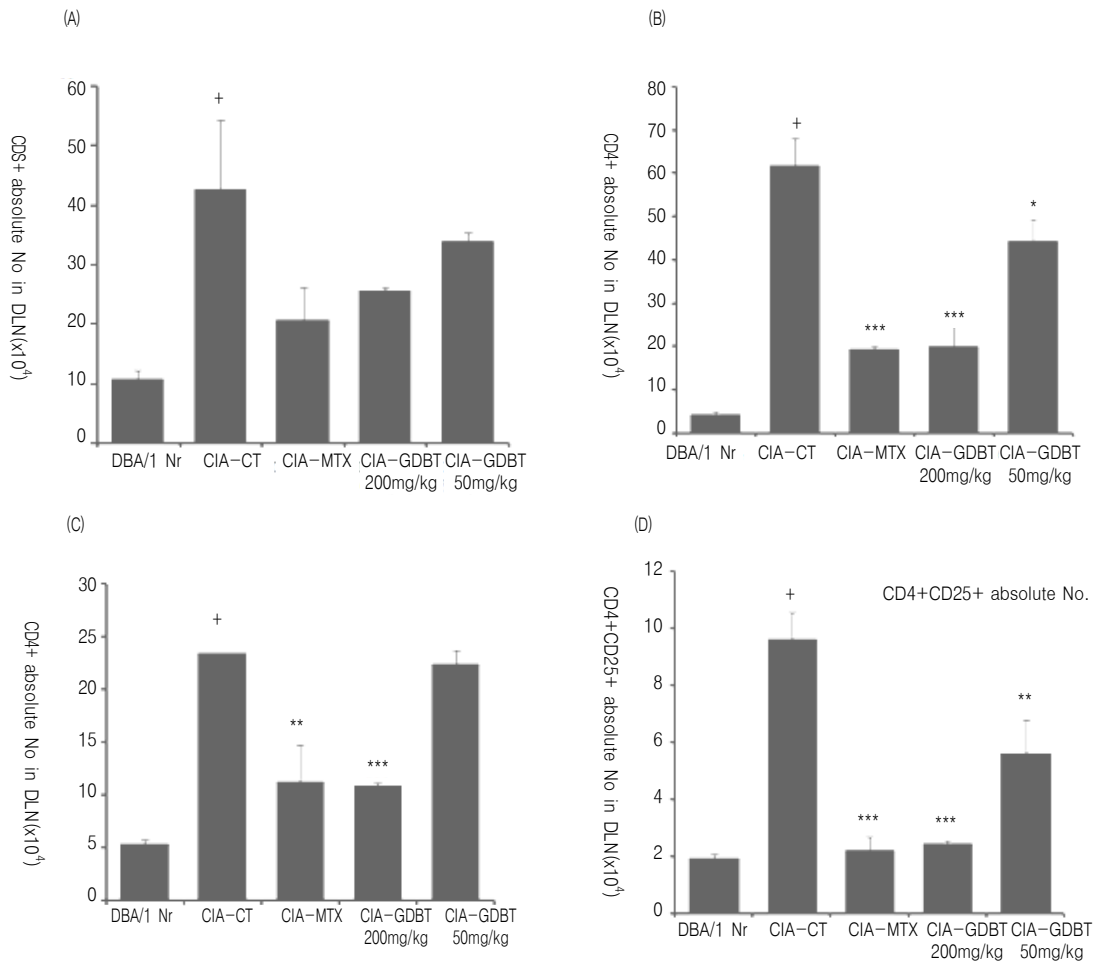


Fig. 4. Effect of GDBT on the changes of total absolute numbers of CD4+ in DLN (A) and in joints (B) of CIA mice. Total absolute numbers of CD8+ (C) and CD4+CD25+ regulatory T cells in DLN (D). After CII booster 5 weeks, CIA mice DLN (5x10⁵ cells/ml) and joints (5x10⁵ cells/ml) were isolated, and the DLN and the joints were washed twice and analyzed by flow cytometer.

pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α , COX-2 and NOS-II (Fig. 5).

DBA/1 OlaHsd mice were immunized with 100 μ g of bovine type II collagen (CII) on CFA on days 0 and 21. Group of 5 mice were treated with MTX 2mg/kg, GDBT 200, 50mg/kg, for 5 weeks beginning on the day after the first collagen immunization, respectively. To analyze the changes of NOS-II mRNA gene expression, real-time-PCR was performed with cDNA of joints

tissues and the other methods for assay were performed as described in Materials and Methods.

Relative quantitation (RQ) studies determine gene expression changes in a target sample, relative to a calibration CIA-CT. When using the comparative or $\Delta\Delta$ CT method, an endogenous control (CIA-CT) normalizes the amount of cDNA that is added to the reaction.

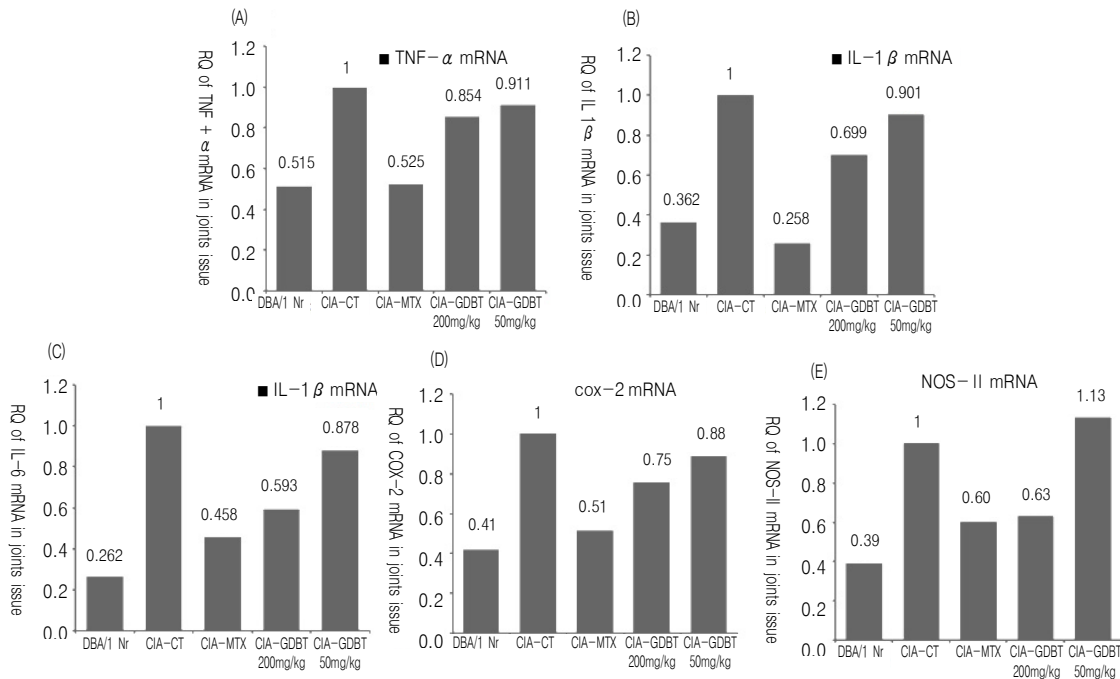


Fig. 5. The effect of GDBT on the level of TNF- α mRNA (A), IL-1 β mRNA (B), IL-6 mRNA (C), COX-2 mRNA (D), and NOS-2 mRNA (E) expression in paw joints in CIA mice.

4. Histological analysis of paw joints from CIA model mice

To determine whether GDBT prevented articular destruction, we analyzed the paw joints of mice histologically. Cartilage erosion and synovial cell infiltration were severe in mice with CIA, but GDBT administered at a concentration of 200mg/kg prevented these signs of disease severity considerably. A small increase in synovial cell infiltration was detected in the joints of animals receiving 50mg/kg of GDBT, but no discernable cartilage erosion was observed in the knee joints of these animals. These histopathologic results suggest that GDBT suppresses the immune-mediated pathologic process in CIA model mice (Fig. 6).

DBA/1 mice were sacrificed, their hind limbs were removed, and the paws were processed for histology and stained with hematoxylin-eosin and Masson's

trichrome staining. Normal wild-type DBA/1 OlaHsd mouse (A, C), CIA-control (B, D), MTX 2mg/kg (E, H), GDBT 200mg/kg (F, I), and GDBT 50mg/kg (G, J) were analyzed with histopathology of joints of murine CIA. Intra-articular exudate, marginal erosion, necrotic chondrocytes, and relative loss proteoglycans in the articular cartilage present panel (B). JC; synovial joint cavity, SM; synovial membrane, ST; synovial tissue, B; bone, CPJ; cartilage pannus junction, P; pannus, S; synovium, NB; new bone, IP; invasion pannus, JS; joint space and resulting in severe cartilage and bone degradation (arrow). Original magnifications: X 20.

Discussion

The results of this study, based on CIA model mice, have revealed that GDBT, a traditional oriental medicine-based herbal formula, can suppress ongoing

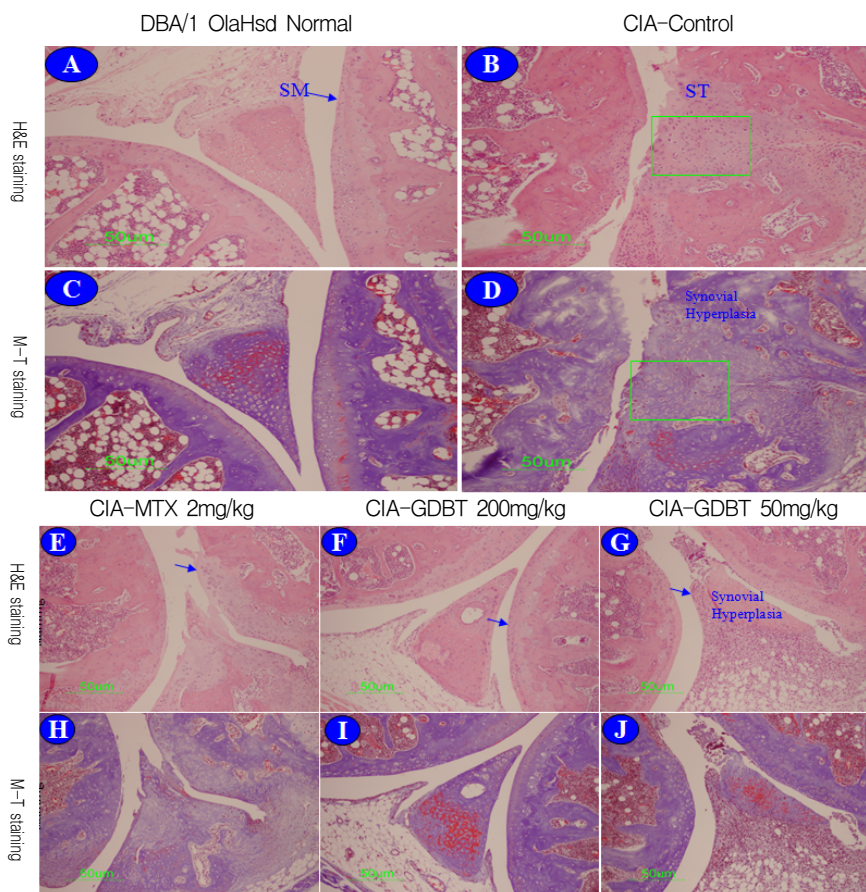


Fig. 6. Histological section of knee joints from CIA mice.

arthritis. The antiarthritic activity of this complementary and alternative medicine modality was associated with significant changes in the T cell proliferative and cytokine profile in the draining lymph nodes and paw joints of CIA model mice.

CIA is a well-established *in vivo* model that has been used in numerous studies to investigate the pathogenesis of RA and for identification of potential therapeutic targets¹¹. Collagen-induced arthritis and human RA have been shown to exhibit common immunological and pathological features^{12,13}, including the involvement of inflammatory mediators in the arthritic etiology.

In oriental medicine practice, the early acute stage

of arthritis is usually considered an excess-heat syndrome (Hot Bi), and heat-clearing herbs are most suitable for this stage of treatment^{14,15}.

GDBT consists of three herbs, namely Citri pericarpium, Rhei radix et rhizome, and Natrii sulfas. The active ingredients in Citri pericarpium are hesperidin and nobiletin, both of which exhibit anti-inflammatory effects^{16,17}. Rhei Rhizoma extracts reportedly have beneficial biological effects such as lowering serum cholesterol and improving diabetic nephropathy as well as protecting pro-oxidation^{18,19}. Natrii sulfas is a medicinal material used to clean away heat, relax the bowels and treat periappendicular abscesses. Natrii sulfas is not absorbed in the intestine but dissolves

in the intestine to create a highly concentrated salt solution that inhibits moisture absorption and stimulates the intestinal membrane to increase the vermiculation of the intestine. This medication may be the most typical Oriental herbal medication that has a cathartic action to cause diarrhea²⁰⁾. The cathartic action causes the excretion of toxic substances within the internal bowels, improves blood circulation and stimulates the metabolism²¹⁾.

This might explain our observation that the formula showed no effect when used in the chronic stage of symptoms in this CIA mice model but showed significant anti-arthritis effects in the earlier stage. The findings of our study seem to point up the importance of syndrome differentiation stressed in oriental medical theory and suggest that this formula may be useful in treating the acute stage of inflammatory arthritis²²⁾.

T cells play a fundamental role in the initiation and perpetuation of RA immunopathology, leading to downstream inflammation and, ultimately, soft tissue and joint destruction. CD4+ T-helper cells and macrophages infiltrate the synovial membrane (SM) in chronic, destructive rheumatoid arthritis (RA) and probably play a central role in promoting and maintaining the disease process²³⁾.

In this study, we report the effect of GDBT on CD4+ cells in lymph nodes and paw joints with collagen-induced arthritis model mice. CD4+ cells in periphery take an important role in the induction and development of CIA. CD4+ T cells are required for the induction of CIA, and CD8+ T cells might have a suppressive role in the etiology of CIA²⁴⁾. CD4+ and CD8+ T cells are involved in resistance to arthritis, though the relative importance of each subset changes during the course of the process, leading to the development of resistance to CIA²⁵⁾. In our study, GDBT could lower the absolute number of CD4+ T cells in draining lymph nodes and paw joints, which suggests that GDBT might induce immunosuppressive response by lowering the CD4+ T cells and CD8+ T cells.

Studies in multiple mouse models of inflammatory arthritis have indicated that CD4+CD25+ regulatory T cells are capable of modifying disease, and the role of regulatory T cells has been most extensively studied in the collagen-induced arthritis models. As seen in human arthritis, CD4+CD25+ regulatory T cells can be found in the synovial fluid, joints, and draining lymph nodes of arthritic mice^{26,27)}. CD4+CD25+ T cells isolated from arthritic animals are capable of exerting suppressor function in *in vitro* assays^{28,29)}. We observed that GDBT treatment down regulated the CD4+CD25+ T cells. This suggests that GDBT treatment may drive reduction of the CD4+CD25+ T cells population directly, although we can not assume that regulatory T cells decreased just because of the decrease of CD4+/CD25+ cells. It is necessary to observe that there are increases of CD62L, CD152 and Foxp3 genes which will become regulatory T cells, and there is a need to differentiate between effector T cells and regulatory T cells. Thus, to conclude that GDBT treats arthritis by controlling the regulatory T cells, additional research as above will be needed.

GDBT inhibited various aspects of the inflammatory process: the secretion of pro-inflammatory cytokines. At present, which compound is responsible for each of these anti-inflammatory effects is not clear. However, the anti-inflammatory effect of GDBT has been known. In our observation for the inhibition of IL-1 β mRNA, IL-6 mRNA, TNF- α mRNA, COX-2 mRNA, NOS-II mRNA, it was not clear if the various anti-inflammatory effects observed were mediated by CD4+CD25+ regulatory T cells. However, it is noteworthy that GDBT extract decreased the concentration of the pro-inflammatory cytokines TNF- α mRNA and IL-1 β mRNA at the local inflammation site in the CIA model compared with CIA model control group. IL-1 β and TNF- α originate from activated macrophages, and TNF- α is also produced by antigen-primed helper T cells³⁰⁾. These cytokines have been documented as critically important in RA in humans^{4,31)}. They contribute

to many features of arthritic inflammation, including synovial tissue inflammation, synovial proliferation, and cartilage and bone damage³⁰. GDBT also decreased the level of IL-6 mRNA expression in paw joints in CIA mice. Studies have shown that IL-6 overproduction (i.e., detection in synovial fluid) correlates to increased RA disease activity^{32,33}. Murine models indicate that IL-6 deficiency delays the onset and reduced the severity of collagen-induced arthritis³⁴; blocking the IL-6 receptor leads to diminished joint disease and a decrease in anti-type II collagen antibodies in similar murine models³⁵.

Other inflammatory mediators are known to participate in RA: inducible nitric oxide synthases (iNOS) levels increase significantly³⁶ and cyclooxygenase (COX)-2-deficient mice are less susceptible to CIA³⁷. At the site of inflammation, the production of oxygen free radical scavengers such as NO, hydrogen peroxide, superoxide and hydroxyl radicals contribute to tissue damage. As inhibitors of NOS activity reduce the development of arthritis, NO appears to be implicated in the pathophysiology of CIA³⁸. COX-2 is highly expressed by endothelial cells³⁹. Furthermore, COX-2 is markedly upregulated at sites of inflammation⁴⁰.

This study provides evidence of less COX-2 and iNOS expression in the paw joint tissue of CIA mice orally administered with GDBT.

In summary, GDBT has profound effects on the collagen-induced arthritis model through suppression of IL-1 β , IL-6, TNF- α , COX-2 and NOS-II. The therapeutic activity of GDBT on RA in oriental medicine may be partly related to CD4+ T cells, CD4+CD25+ regulatory T cells, and pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α .

Hence, the results indicated that GDBT could act as an immunomodulator which possess anti-inflammatory and anti-arthritic property also by downregulating IL-1 β , IL-6, TNF- α , COX-2 and NOS-II.

In addition, we suggest that GDBT be considered for further evaluation of its efficacy as a Complementary and Alternative Medicine modality for the treatment

of RA patients.

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