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ORIGINAL ARTICLE

GCSB-5 regulates inflammatory arthritis and pain by modulating the mitogen-activated protein kinase signaling pathway in a murine model of rheumatoid arthritis

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ABSTRACT

Objectives: This study aimed to determine whether GCSB-5 has anti-inflammatory and antinociceptive effects in mice with collagen-induced arthritis (CIA), an animal model of rheumatoid arthritis (RA), and investigate the influence of GCSB-5 on the mitogen-activated protein kinase (MAPK) pathway.

Materials and methods: The experimental animal study was designed to include five groups: CIA mice treated with GCSB-5 (300 mg/kg), GCSB-5 (600 mg/kg), celecoxib (60 mg/kg), or saline for four weeks, and nontreated control mice. The clinical severity of arthritis was scored. Nociceptive thresholds were measured by using a von Frey dynamic plantar analgesimeter. The MAPK pathway was evaluated in mouse synovium. The expression of channels associated with pain signaling was assessed by western blot and immunohistochemical staining.

Results: GCSB-5 treatment diminished the severity of clinical arthritis and increased the nociceptive threshold in mice with CIA. Celecoxib, a positive control drug, also showed comparable changes. Clinical arthritis scores were inversely related to mechanical thresholds. GCSB-5 administration decreased the levels of anti-type II collagen antibody and inflammatory cytokines in the sera of mice with CIA. Furthermore, ERK, p38 MAPK, and JNK phosphorylation were downregulated and TRPV1 and ASIC3 expression were decreased in the synovium of GCSB-5-treated mice compared to saline-treated mice. Interleukin-6-induced TRPV1 and ASIC3 upregulation were also inhibited by GCSB-5 in human RA fibroblast-like synoviocytes *in vitro*.

Conclusion: GCSB-5 decreased inflammatory arthritis and pain in a murine model of RA. The results present evidence that GCSB-5 may be beneficial for relieving pain as well as decreasing inflammation in autoimmune arthritis, such as RA.

Keywords: Anti-inflammatory agents, ASIC3, plant extracts, rheumatoid arthritis, TRPV1.

Rheumatoid arthritis (RA) is an inflammatory disease with a chronic course characterized by synovitis, synovial proliferation, and joint destruction.[1] Proinflammatory cytokines, which are critically involved in RA pathogenesis, can be mediators of pain by directly influencing the nociceptive system.[2] Thus, the inflammatory process in RA can cause pain, and this inflammatory pain is closely correlated with poor quality of life in patients with RA.[3]

The transient receptor potential vanilloid type 1 (TRPV1) and the acid-sensing ion channel 3 (ASIC3) are reportedly associated with inflammatory pain. TRPV1 is a family of nonselective cation channels stimulated by heat, capsaicin, endogenous lipids, such as endovanilloids, and other endogenous compounds, such as ammonia, adenosine triphosphate, and polyamines.[4,5] TRPV1 plays a critical role in the pathogenesis of RA,

with many studies supporting that it can be activated by inflammatory cytokines and also cause the release of inflammatory molecules.[6,7] Accordingly, the expression of inflammatory cytokines increased in synovial fibroblasts derived from RA patients when stimulated with a TRPV1 agonist.[8] Furthermore, a study revealing that the inhibition of TPRV1 reduces joint edema supports the possible targeting of this channel receptor for the amelioration of joint swelling in RA.[9] Along with TRPV1, ASIC3 is also involved in RA development. ASIC3 is highly sensitive to pH changes. ASIC3 channels contribute to hyperalgesia by sensing acidic pain produced during inflammation.[10] ASIC3 is associated with maintaining chronic RA pain.[11] ASIC3 upregulation has also been observed in neurons innervating knee joints in a model of carrageenan-induced arthritis.[12]

GCSB-5 is an extract purified from the mixture of six herbs and has been traditionally accepted as an effective treatment for inflammatory and bone disorders in Eastern Asia.[13] Previous studies have shown that GCSB-5 decreases interleukin (IL)-1 and tumor necrosis factor-alpha (α) serum levels in an experimental arthritis rat model and suppresses cyclooxygenase-2 (COX-2) enzyme levels in macrophages.[14,15] GCSB-5 has also inhibited ear swelling caused by 12-O-tetradecanoylphorbol-13-acetate and paw swelling induced by carrageenan.[16] GCSB-5 exhibits protective effects against peripheral nerve injury in rats via reduction of oxidative stress.[17] Most of the previous studies on GCSB-5 have been conducted in osteoarthritis.(OA) The activities of matrix metalloproteinase-2 and-9 have been attenuated by GCSB-5 in a murine model of OA.[18] Additionally, GCSB-5 reduced bone loss and cartilage degradation in OA-induced rats.[18,19] A few clinical trials have investigated the safety and efficacy of GCSB-5 for the treatment of OA.[20,21] However, little is known about the effect of GCSB-5 on inflammatory arthritis and pain in RA.

Herein, we evaluated the effects of GCSB-5 on the severity of inflammatory arthritis and pain perception in a murine model of RA. The impact of GCSB-5 on pain-related receptors was also investigated, and its underlying mechanism was explored. To the best of our knowledge, this is the first study to reveal the effects of GCSB-5 in RA.

MATERIALS AND METHODS

GCSB-5 ingredients

GCSB-5 is a dried extract of a mixture of six herbs (Saposhnikovia divaricate Schischk, Achyranthes japonica Nakai, Acanthopanax sessiliflorus Seem, Glycine max Merrill, Cibotium barometz J. Smith, and Eucommia ulmoides Oliver) at a fixed ratio (4.444:4.444:4.444:2.778:2.778:1.389 ratio, respectively). GCSB-5 was prepared using the following method. The six herbs were made into a powder at a fixed ratio and heated for 3 h with 1 L of distilled water. Constituents with molecular weight over 10,000 g/mol were discarded through ultrafiltration of the resulting extract. The filtrate was freeze-dried and kept at 4°C. The regulations of the Korea Food and Drug Administration were applied to the standardization of GCSB-5 for quality control. GCSB-5 was kindly presented by the Green Cross Corporation (Yongin, Republic of Korea).

Animals and experimental arthritis

Male DBA/1J mice aged six to eight weeks (Japan SLC Inc., Hamamatsu-shi, Shizuoka, Japan) were used. The mice were housed, provided with water and chow *ad libitum*, and kept under a 12-h light and 12-h dark cycle at 22±1°C with a humidity of 55±10%. As a primary immunization for induction of CIA in mice, an emulsion of bovine type II collagen and complete Freund's adjuvant (Chondrex, Redmond, WA, USA) at a 1:1 ratio was injected into the base of the tail intradermally. Three weeks later, bovine type II collagen emulsified in incomplete Freund's adjuvant was also injected intradermally as a booster immunization. To investigate the influence of GCSB-5 on the severity of arthritis, intragastric infusion of GCSB-5, celecoxib, or saline was administered to collagen-induced arthritis (CIA) mice five times per week after booster immunization. Prior to administration, GCSB-5 and celecoxib were dissolved in saline. The study was designed to include five groups: *(i)* CIA mice treated with GCSB-5 (300 mg/kg), *(ii)* CIA mice treated with GCSB-5 (600 mg/kg), *(iii)* CIA mice treated with celecoxib (60 mg/kg), *(iv)* saline-treated CIA mice, and *(v)* nontreated control mice. Three observers independently scored the arthritis severity. The experimental timeline

is shown in Figure 1a. The arthritis scores were based on the severity and extent of paw swelling and erythematous change, in which 0 represents no swelling, 1 represents mild swelling confined to the ankle or midfoot, 2 represents moderate swelling extending from the ankle to the midfoot, 3 represents severe swelling and erythema from the ankle to the metatarsal joints, and 4 represents ankylosis of the ankle. The arthritis score of each mouse was determined as the sum of the scores of the four extremities.

Radiological assessment

A mammographic imager (Siemens Mammomat Novation DR Digital Mammography; Siemens Healthineers, Erlangen, Germany) was used for plain radiography of the hind paws. Formalin fixation was performed with dissected hind paws for radiography. All radiographic assessments were performed by a radiologist. The radiographic features were based on tissue swelling and bone changes (0, no bone damage; 1, tissue edema; 2, joint erosion; 3, bone erosion and osteophyte formation).[22]

Mechanical withdrawal threshold measurement

An electronic von Frey device (Dynamic Plantar Aesthesiometer; Ugo Basile, Gemonio, Italy) was used for measuring mechanical withdrawal threshold. After an adjustment period of more than 2 h, each von Frey filament was touched to the sole of the hind paw of each mouse. The pressure force was increased at a rate of 0.5 g/sec. The mechanical withdrawal threshold was determined as the pressure force (g) when the mouse took its paw off the device. Five repeated measurements were performed at 10-min intervals.

Histological evaluation

Joint tissues were fixed in 4% paraformaldehyde and decalcified with hydrochloric acid for one week. The decalcified joint tissue was embedded in paraffin. The paraffin-embedded tissue sections cut to a thickness of 4 μm were stained with hematoxylin and eosin for histological evaluation, and the infiltration of inflammatory cells was observed. Safranin O staining was performed on the tissue sections embedded in paraffin to assess cartilage damage. Histological evaluation was performed by a pathologist with scoring based on the degree of synovial inflammation, pannus formation, cartilage damage, and bone resorption.[23] For the histological expression analysis of TRPV1 and ASIC3, the paraffin-embedded joint tissue sections were incubated for 30 min at room temperature with a 20% goat serum for nonspecific protein blocking and subsequently incubated with anti-TRPV1 and anti-ASIC3 antibodies (Alomone Labs, Jerusalem, Israel) overnight at 4°C. Immunohistochemistry was processed using 3,3'-diaminobenzidine as a chromogen according to a standard protocol of the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

Western blot

Synovial tissues were obtained from hind paws and kept at -70°C until use. Proteins were extracted from frozen tissues, homogenized, and lysed with ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitors. An 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate the extracted protein, which was then transferred to a nitrocellulose transfer membrane (Millipore, Billerica, MA, USA). After nonspecific protein blocking, the membrane was washed to remove the blocking solution and incubated with antibodies against TRPV1, ASIC3 (Alomone Labs Ltd, Jerusalem, Israel), p-ERK (extracellular signal-regulated kinase), ERK, p-p38 MAPK, p38 MAPK, p-JNK (c-Jun N-terminal kinase), JNK (Cell Signaling Technologies, Danvers, MA, USA), and β-actin (Sigma-Aldrich, St. Louis, MO, USA). After washing the membrane, horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were added. Protein expression was detected with enhanced chemiluminescent detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden), and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for quantification of protein intensity normalized to the density of β-actin.

Enzyme-linked immunosorbent assay

Anti-type II collagen IgG (Chondrex) was measured by enzyme-linked immunosorbent

assay (ELISA). The levels of IL-17 or IL-6 (R&D Systems, Minneapolis, MN, USA) were measured in mice sera using ELISA kits (R&D Systems) for mouse IL-17 and IL-6 according to the manufacturer's instructions. The absorbance was read at 450 nm by a Victor3 multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

Isolation of rheumatoid arthritis fibroblast-like synoviocytes

The patients fulfilled the American College of Rheumatology criteria.[24] The synovium of RA patients was obtained from the Keimyung Human Bio-Resource Bank, which belongs to National Biobank in Korea. Fibroblast-

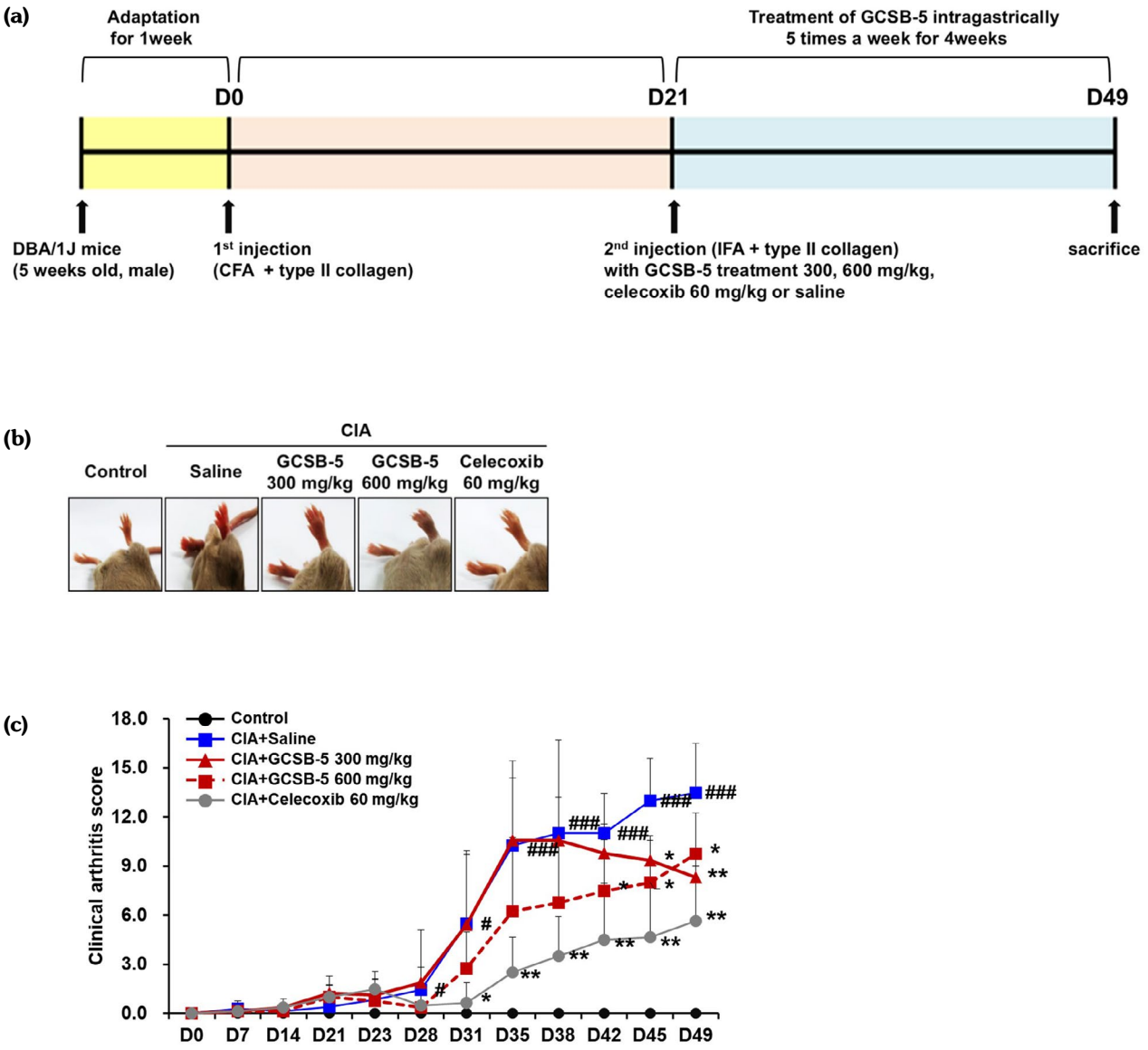

Figure 1. The effects of GCSB-5 on the severity of autoimmune arthritis. **(a)** Experimental timeline. **(b)** Representative photos of hind paws of mice from each group. **(c)** Clinical arthritis scores of each group. Celecoxib is used as a positive control. Values are expressed as mean ± standard deviation.

CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant; CIA: Type II collagen-induced arthritis; # $p<0.05$; ## $p<0.01$; ### $p<0.001$ compared to the control group; * $p<0.05$ and ** $p<0.01$ compared to the CIA + saline group.

like synoviocytes (FLS) of RA patients were isolated from the synovium through tissue explant cultivation, as previously described.[25] Under sterile conditions, synovial tissues were placed in culture dishes with Hanks' Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO; USA), and the connective tissue and fat from synovial tissues were removed. The synovial tissues were divided into small pieces (approximately 1-2 mm^3), treated with 0.5% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA), and incubated in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. The culture medium was replaced with fresh culture medium every two to three days. Adherent cells were routinely trypsinized, split at a 1:2 ratio, and passaged in DMEM containing 10% FBS. FLS at passage numbers 3 to 5 were used in the experiments.

Statistical analyses

Data were analyzed using the IBM SPSS version 26.0 software (IBM Corp., Armonk, NY, USA). Results are presented as mean±standard deviation. Statistical significance was determined by the Kruskal-Wallis H test. A *p*-value <0.05 was considered statistically significant.

RESULTS

Effects of GCSB-5 on the development of arthritis in CIA mice

We first assessed whether GCSB-5 affected the clinical severity of experimental autoimmune arthritis. Representative photos of hind paws in each group are shown in Figure 1b. The GCSB-5-treated group revealed reduced joint swelling compared to the saline-treated CIA group. The mean arthritis score was also lower in the GCSB-5-treated group than in the saline-treated CIA group (Figure 1c).

Effects of GCSB-5 on serum proinflammatory cytokine levels in CIA mice

To investigate whether GCSB-5 affected antibody production against type II collagen and proinflammatory cytokine levels in CIA mice, serum levels of anti-type II collagen IgG, IL-17, and IL-6 were measured by ELISA. Administration of GCSB-5 (300 mg/kg) decreased the serum concentration of the anti-type II collagen IgG (Figure 2a). The serum concentrations of IL-17 in GCSB-5-treated groups (both 300 mg/kg and 600 mg/kg) were also lower than those in the saline-treated CIA group (Figure 2b). Both GCSB-5-treated groups showed comparable serum IL-17 levels to the celecoxib-treated group. Serum concentrations of IL-6 were lower in the GCSB-5 (300 mg/kg)-treated group than those in the saline-treated CIA group; however, GCSB-5 (600 mg/kg) treatment did not significantly reduce serum IL-6 levels (Figure 2c). Additionally, IL-6 serum levels were lower in the GCSB-5

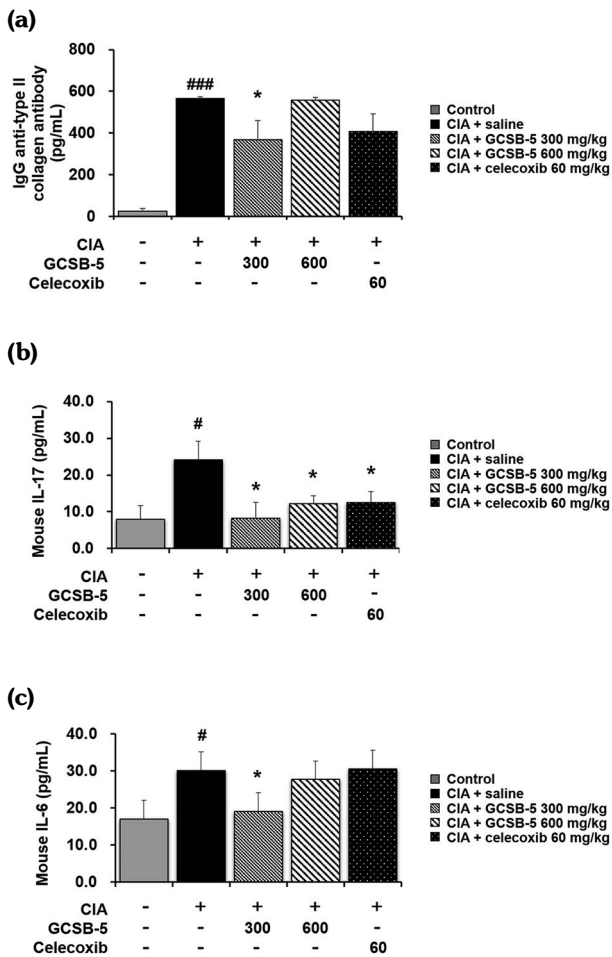

Figure 2. The effects of GCSB-5 on proinflammatory cytokine serum levels. **(a)** Pro-inflammatory cytokines such as IL-17, **(b)** IL-6 and **(c)** are measured by ELISA. Values are expressed as mean ± standard deviation.

CIA: Type II collagen-induced arthritis; IL: Interleukin; ELISA: Enzyme-linked immunosorbent assay; # p<0.05 and ### p<0.001 compared to the control group; * p<0.05 compared to the CIA + saline group.

(300 mg/kg)-treated group ($p<0.001$) and in the GCSB-5 (600 mg/kg)-treated group ($p<0.01$) compared to those in the celecoxib-treated group.

Effects of GCSB-5 on radiographic and histologic changes in CIA mice

On plain radiographs, multiple erosive changes were found in the joints of hind paws from the saline-treated CIA group, whereas only minimal erosions were observed in the GCSB-5-treated group (Figure 3a). Moreover, the GCSB-5 (300 mg/kg)-treated and celecoxib-treated groups had significantly decreased radiographic arthritis scores compared to the CIA group (Figure 3b). Furthermore, radiographic arthritis scores were comparable between the GCSB-5-treated groups and the celecoxib-treated group.

Histologic sections of the mouse hind paw joints are shown in Figure 3c. Marked cartilage and bone damage were observed in

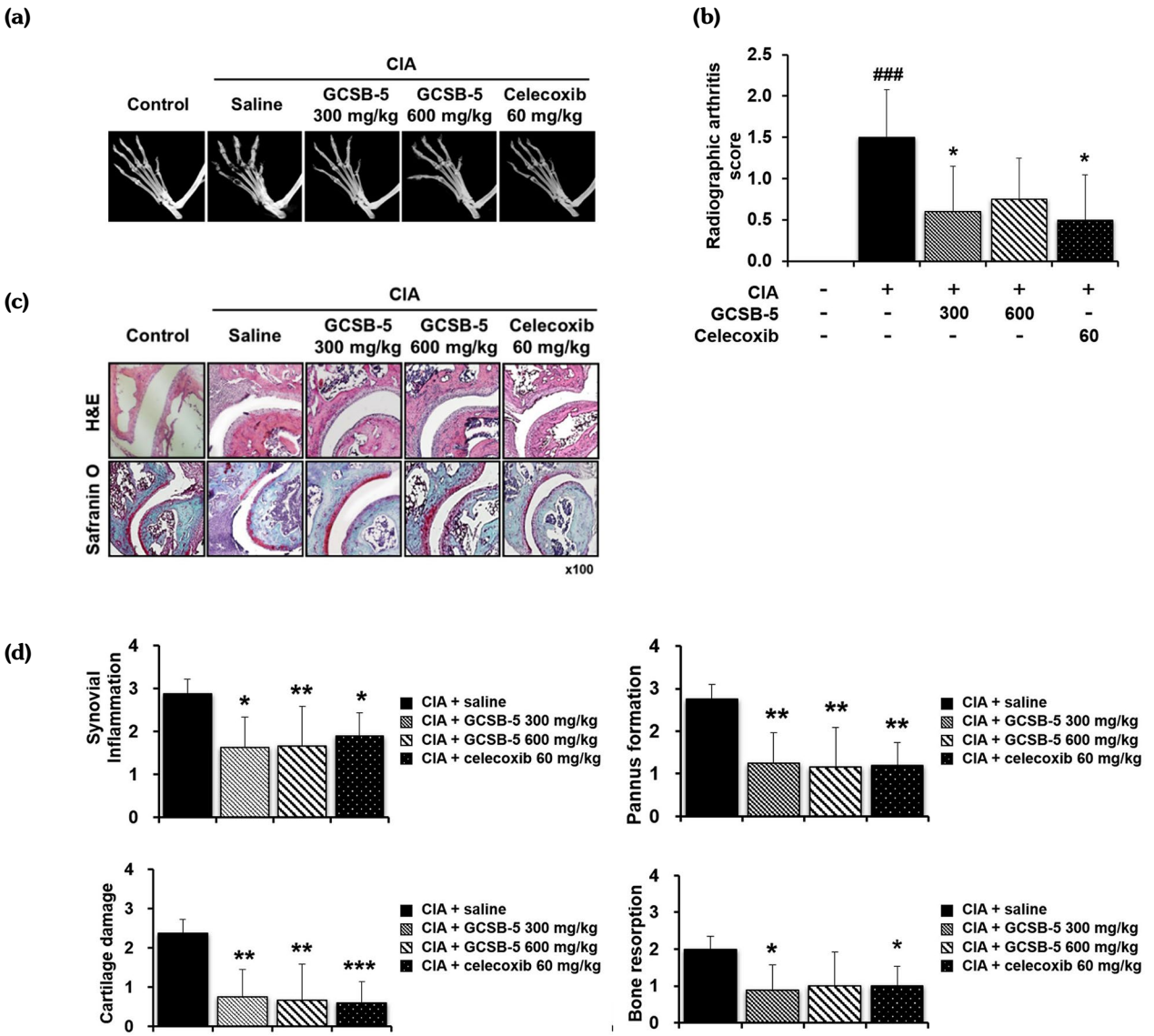

Figure 3. The effects of GCSB-5 on radiographic and histologic changes. **(a)** Representative plain radiographs of each group. **(b)** Radiographic scores of each group. **(c)** H&E and safranin O staining of each group. **(d)** Histologic scores of each group. Values are expressed as mean ± standard deviation.

CIA: Type II collagen-induced arthritis; H&E Hematoxylin and eosin; ### $p<0.001$ compared to the control group; * $p<0.05$ and ** $p<0.01$ compared to the CIA + saline group.

the CIA group compared to the GCSB-5-treated or celecoxib-treated group. In contrast, the joints of GCSB-5-treated or celecoxib-treated mice exhibited decreased levels of synovial inflammation, pannus formation, cartilage damage, and bone resorption (Figure 3d). There were no differences between the GCSB-5-treated groups and the celecoxib-treated group in histologic changes, such as synovial inflammation, pannus formation, cartilage damage, and bone resorption.

Effects of GCSB-5 on pain hypersensitivity in CIA mice

The effect of GCSB-5 on mechanical hyperalgesia in CIA mice was evaluated using von Frey filaments. The CIA group showed a marked decrease in the paw withdrawal threshold compared to the control group (Figure 4a). After CIA induction, the paw withdrawal threshold in the CIA group was 5.04±0.82 g, whereas it was 9.95±0.14 g in the control group. The paw withdrawal threshold of mice in the GCSB-5 (300 mg/kg)-treated group was higher than that of mice in the CIA group (Figure 4a). The baseline paw withdrawal latency after CIA induction was 1.29±0.19 sec, whereas that of the control group was 2.52±0.59 sec (Figure 4b). Figure 4b also shows that intragastric administration of GCSB-5 (300 mg/kg) increased the paw withdrawal latency of mice in this group compared to that of mice in the CIA group. The celecoxib group exhibited increased paw withdrawal threshold and latency compared to the CIA group (Figures 4a and b). Withdrawal threshold and withdrawal latency were also both inversely correlated with clinical arthritis scores (Supplemental Figure 1).

Effects of GCSB-5 on TRPV1, ASIC3, and MAPK signaling pathways in CIA mice

Immunohistochemical staining of the hind paw joints showed that TRPV1 and ASIC3 expression was markedly increased in CIA mice and that GCSB-5 treatment reduced the expression of these pain-related proteins (Figure 5a). TRPV1 and ASIC3 expression levels in GCSB-5-treated CIA mice were also comparable to those in celecoxib-treated CIA mice (Figure 5a). We used western blotting to measure TRPV1 and ASIC3 expression in the mouse hind paws. GCSB-5 administration suppressed the elevated TRPV1 and ASIC3 expression in CIA mice (Figure 5b). Administration of GCSB-5 (both 300 mg/kg and 600 mg/kg) reduced the expression of TRPV1, and administration of GCSB-5 (300 mg/kg) decreased ASIC3 expression in mice synovia within these groups compared to those in the saline-treated CIA group.

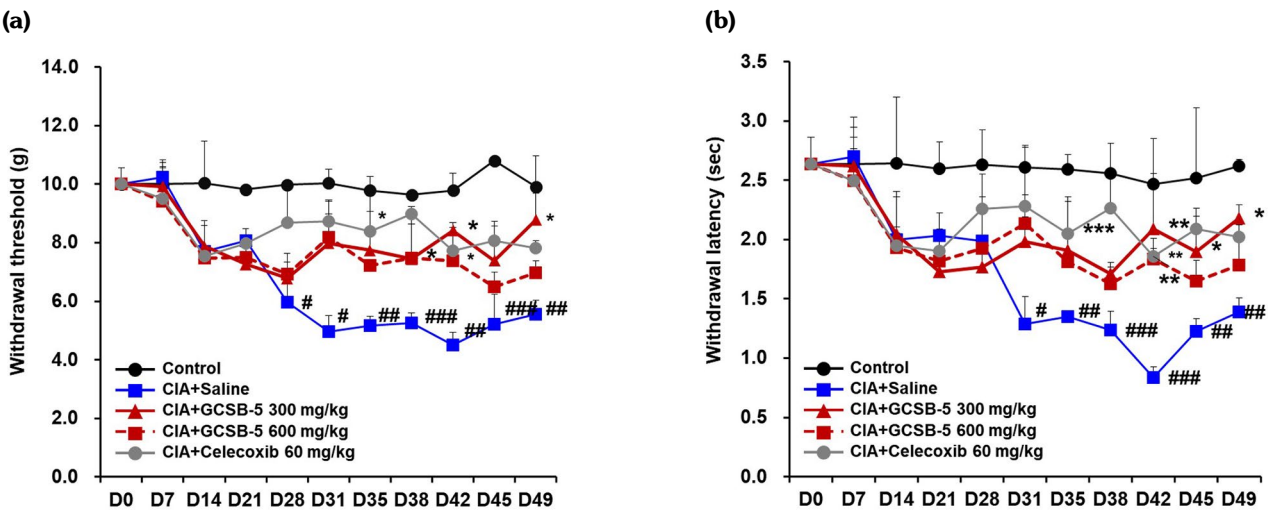

Figure 4. The effects of GCSB-5 on mechanical nociception evaluated using a von Frey dynamic plantar analgesimeter. **(a)** Withdrawal threshold and **(b)** withdrawal latency of each group. Values are expressed as mean ± standard deviation.

CIA: Type II collagen-induced arthritis; # $p<0.05$ and ## $p<0.01$; compared to the control group; * $p<0.05$ and *** $p<0.01$ compared to the CIA + saline group.

The influence of GCSB-5 on the MAPK signaling pathway was also evaluated using synovial tissues from the ankle joints of mice in each group by western blot. ERK, p38 MAPK, and JNK phosphorylation were enhanced in the saline-treated CIA group compared to the control group (Figure 6). The GCSB-5 (300 mg/kg)-treated group exhibited suppressed ERK, p38 MAPK, and JNK phosphorylation compared to the saline-treated CIA group (Figure 6). Similarly, ERK phosphorylation was inhibited in the GCSB-5 (600 mg/kg)-treated group compared to the saline-treated CIA group (Figure 6).

Effects of GCSB-5 on TRPV1 and ASIC3 in FLS derived from patients with RA

To investigate whether GCSB-5 affects TRPV1 and ASIC3 regulation in human samples, we analyzed TRPV1 and ASIC3 expression

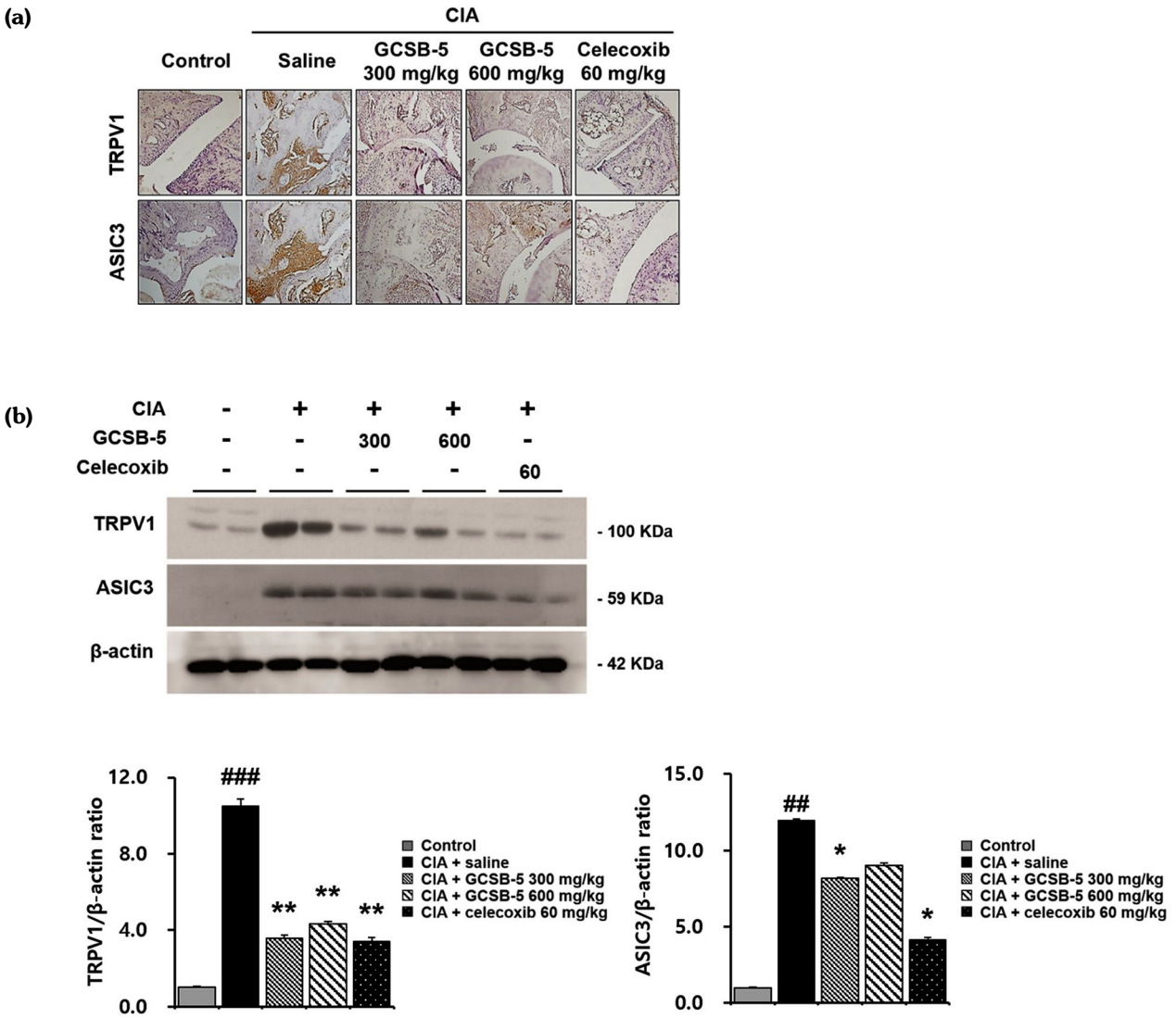

Figure 5. Regulatory effects of GCSB-5 on the expression of pain-related ion channels. **(a)** representative immunohistochemical staining for TRPV1 and ASIC3 in the joints of mice from each group. **(b)** Western blot analysis using TRPV1 and ASCI3 antibodies in protein extracts from synovial tissues. Values are expressed as mean ± standard deviation.

TRPV1: Transient receptor potential vanilloid channel type 1; ASIC3: Acid-sensing ion channel 3; CIA: Type II collagen-induced arthritis; ## p<0.01; ### p<0.001 compared to the control group; * p<0.05 and ** p<0.01 compared to the CIA + saline group.

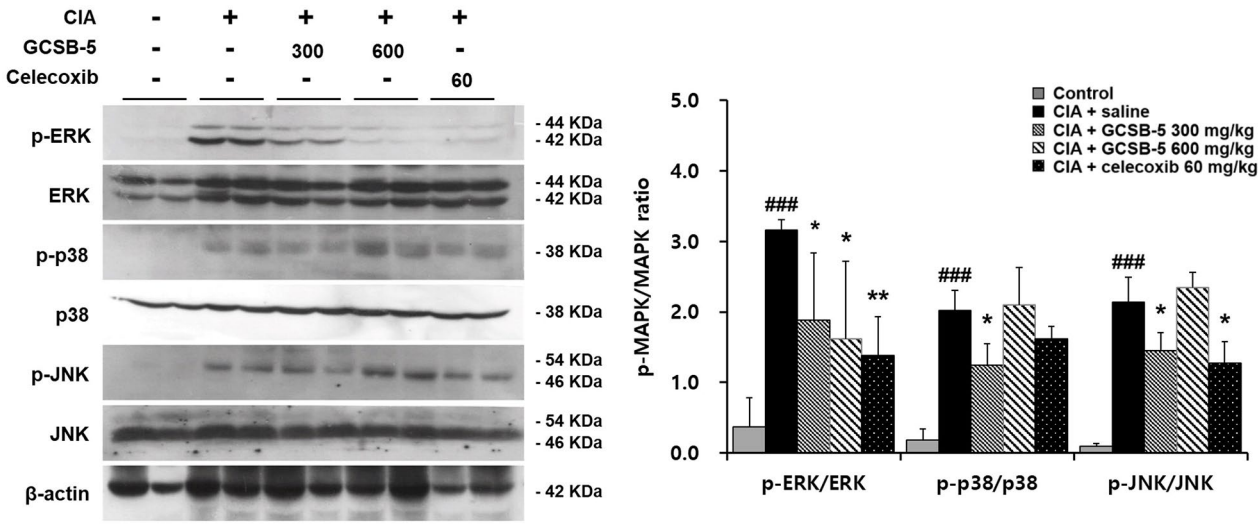

Figure 6. The effects of GCSB-5 on MAPK signaling pathway. The expression and phosphorylation of ERK, p38 MAPK, and JNK are analyzed in protein extracts from synovial tissues using western blot. Representative images of western blot (left panel) and densitometric quantification phosphorylation/total of ERK, p38 MAPK, and JNK expression (right panel). Values are expressed as mean ± standard deviation.

CIA: Type II collagen-induced arthritis; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated protein kinase; JNK: c-Jun N-terminal kinase; ### p<0.001 compared to the control group; * p<0.05 and ** p<0.01 compared to the CIA + saline group.

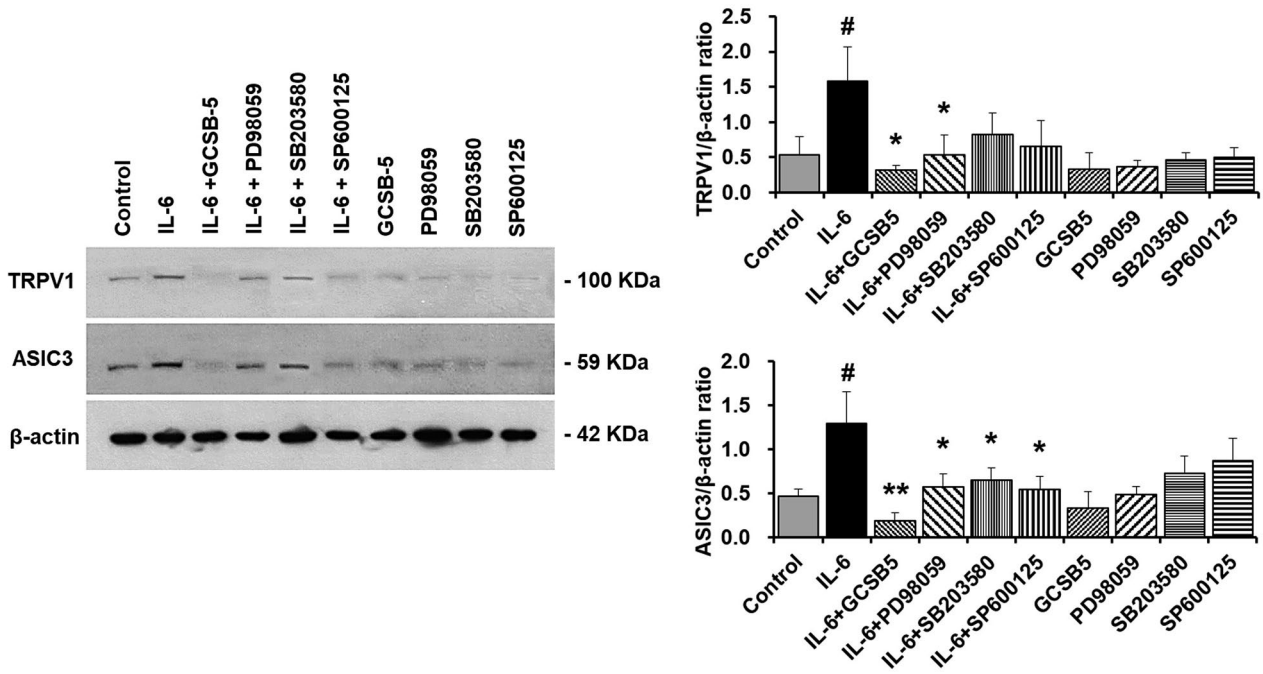

Figure 7. The effects of GCSB-5 on IL-6-induced TRPV1 and ASIC3 expression in RA-FLS. Western blot analysis of TRPV1 and ASIC3 in human RA-FLS (left panel). Optimal density ratios of TRPV1/β-actin and ASIC3/β-actin (right panel). Values are expressed as mean ± standard deviation.

TRPV1: Transient receptor potential vanilloid channel type 1; ASIC3: Acid-sensing ion channel 3; RA-FLS: Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis; PD98059: Extracellular signal-regulated protein kinase (ERK) inhibitor; SB203580: p38 mitogen-activated protein kinase (MAPK) inhibitor; SP600125X c-Jun N-terminal kinase (JNK) inhibitor; IL-6: Interleukin-6; # p<0.05 compared to the control group (IL-6 unstimulated group); * p<0.05 and ** p<0.01 compared to the IL-6 stimulated group.

in FLS derived from RA patients (RA-FLS) (Figure 7). A 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide assay was applied to evaluate the cytotoxic potential of GCSB-5 in RA-FLS with no effect observed in cell viability from 0 to 3,000 μg/mL (Supplemental Figure 2). IL-6, one of the key inflammatory cytokines in RA pathogenesis, enhanced TRPV1 and ASIC3 expression in RA-FLS. In contrast, GCSB-5 treatment (800 μg/mL) reduced IL-6-induced (0.2 ng/mL) TRPV1 expression. PD98059, an ERK inhibitor, also suppressed IL-6-induced TRPV1 expression. Similarly, IL-6-induced ASIC3 expression was inhibited by GCSB-5 (800 μg/mL) in RA-FLS. Furthermore, PD98059 (50 μM), SB203580 (p38 MAPK inhibitor, 25 μM), and SP600125 (JNK inhibitor, 25 μM) reduced IL-6-induced ASIC3 expression. The results demonstrated that TRPV1 and ASIC3 could be induced by IL-6 in human RA-FLS. Moreover, the results imply that the MAPK signaling pathway might be involved in IL-6-induced TRPV1 and ASIC3 expression in RA-FLS.

DISCUSSION

Rheumatoid arthritis is a chronic autoimmune disease that causes abnormal proliferation of synovium of the joints, with accompanying troublesome pain and limitation of joint mobility.[26,27] The final goal of treating RA is improving the quality of life of patients by controlling inflammation, reducing joint damage, maintaining joint function, and regulating pain.[28] Celecoxib, a selective COX-2 inhibitor, which we chose as a positive control drug in this study, is commonly used for symptomatic control in RA with an excellent anti-inflammatory and analgesic effect; nevertheless, it is associated with gastrointestinal toxicity and cardiotoxicity.[29,30] Previous studies have shown that GCSB-5 exhibits medicinal effects comparable to that of celecoxib in OA, with the advantage of being safe in terms of gastrointestinal toxicity.[20,31] Several studies have also shown that GCSB-5 improves symptoms in patients with OA and that it is as safe as celecoxib for the long-term treatment of knee OA.[20,21,31] However, evidence regarding whether GCSB-5 acts against inflammation and alleviates symptoms associated with inflammatory reactions in RA remains scarce. We believe that the present study is the first to investigate the anti-inflammatory effect and analgesic mechanisms of GCSB-5 in a murine model of RA. Although our results are based on an *in vivo* animal model and *in vitro* human RA-FLS, this study supports the beneficial effects of GCSB-5 in reducing inflammation and relieving pain in RA.

To the best of our knowledge, this is the first study to report that GCSB-5 relieves arthralgia and the related mechanisms through a CIA model, a representative murine model of RA. Synovial TRPV1 and AISC3 expression were demonstrably reduced by GCSB-5. Both TRPV1 and ASIC3 are pain-related receptors, which can be induced during inflammatory responses. TRPV1 and ASIC3 expression are regulated by the MAPK signaling pathway, which is known to play a significant role in the pathogenesis of RA.[32-34] This finding led us to investigate the MAPK signaling pathway in this study.

Nevertheless, this study had some limitations. First, we did not investigate the roles of each component of this herbal combination drug. Therefore, the pharmacokinetic characteristics of this compound should be investigated further to provide supporting evidence on how the 300 mg/kg GCSB-5 treatment induced better responses than 600 mg/kg GCSB-5 in several of our experiments. Second, this study observed the severity of clinical arthritis for only 49 days, which is a widely adopted period of observation for a murine model of RA. During the observation period, although the analgesic effect of GCSB-5 through regulation of TRPV1 and ASCI3 was comparable to that of celecoxib, the antiarthritic effect of GCSB-5 seemed to be milder and delayed compared to that of celecoxib. The GCSB-5-treated group exhibited a delayed treatment effect and gradual improvement of clinical arthritis compared to the celecoxib-treated group. A longer observation period might show better clinical outcomes comparable to that of celecoxib. Further studies are needed to confirm delayed inhibitory effect of GCSB-5 on murine autoimmune arthritis. Third, as celecoxib is not classified as a disease-modifying antirheumatic drug and is used as an adjuvant in the control of RA, GCSB-5 also has limitations as an adjunctive drug in RA for reducing

inflammation and pain, although GCSB-5 was effective for inflammation and pain in our experiments. Further studies are needed to determine whether GCSB-5 alters the course of RA. Fourth, as we obtained synovial samples already retained in the Bio-Resource Bank, there are limitations in obtaining sufficient clinical data, such as disease activities or therapeutic modalities of RA patients who donate synovial samples used in our *in vitro* study. An analysis of the association between clinical information and *in vitro* study results could have provided more accurate information for the experiment.

In conclusion, GCSB-5 reduced the severity of inflammatory arthritis by regulating the MAPK signaling pathway in a murine model of RA in this study. GCSB-5 has beneficial effects in relieving pain. Suppressed expression of pain-associated receptors, such as TRPV1 and ASIC3, by GCSB-5 treatment was confirmed both *in vivo* and *in vitro*. These results suggest the possibility that GCSB-5 could be an effective anti-inflammatory and pain-relieving agent for autoimmune inflammatory arthritis, including RA.

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Ethics Committee Approval: All animal experiments were conducted in accordance with the principles for laboratory animal use and care provided by US guidelines (NIH publication #85-23, revised in 1985). The study was approved by Keimyung University Institutional Animal Care and Use Committee for animal experiments (ethics approval number: KM-2015-21R1).

Patient Consent for Publication: A written informed consent was obtained from each patient.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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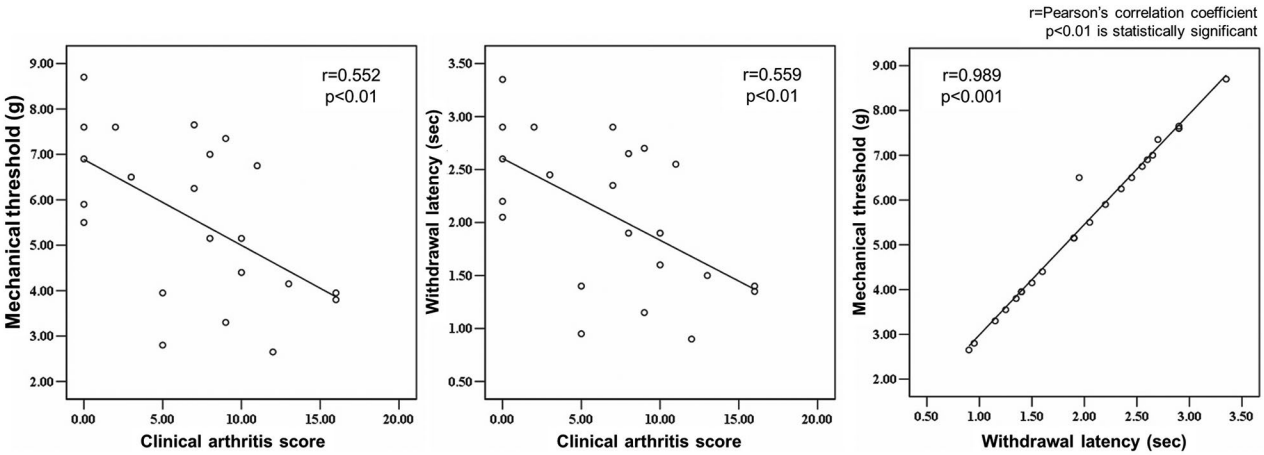
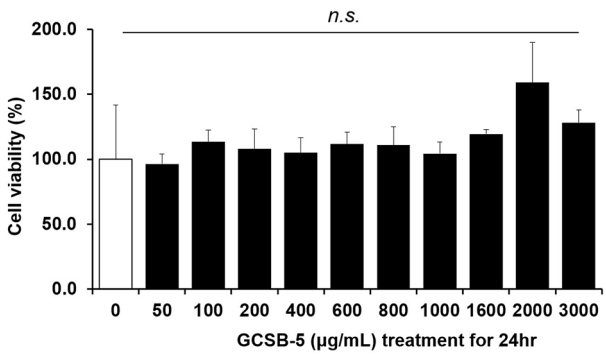


Supplemental Figure 1. Correlations between mechanical hyperalgesia and clinical arthritis severity.



Supplemental Figure 2. Assessment of cell viability in RA-FLS.

RA-FLS: Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis; n.s: No significant difference.