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Original Article
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Improvement Effect of Soyeom Pharmacopuncture on Gout via NLRP3 Inflammasome Regulation

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Objectives: Gout is an inflammatory arthritis of the joints and soft tissues occurring due to deposition of monosodium urate (MSU) crystals, which are caused by persistent hyperuricemia. Soyeom pharmacopuncture is one treatment method that has been traditionally used for pain management in Oriental medicine. However, studies on its effect in reducing gout pain have been insufficient. Therefore, we selected Soyeom pharmacopuncture among natural products used in Korea as the new target of our study.
Methods: The effects of Soyeom pharmacopuncture were examined in mouse models of acute gout induced by injection of MSU crystals into footpads. IL-1β, IL-6, and TNF-α production were examined by immunoblotting and enzyme-linked immunosorbent assay as hallmarks of NLRP3 inflammasome and cytokine activation.
Results: Soyeom pharmacopuncture reduced foot edema in gout-induced mice, as well as IL-1β, nitrite, IL-6, and TNF-α production. Moreover, Soyeom pharmacopuncture also reduced MSU-induced gout inflammatory gene expressions, specifically those in the NF-kB pathway.
Conclusion: Pharmacopuncture may serve as a new solution for other inflammatory diseases as well. Through active follow-up studies, we could thoroughly understand the clinical value of Soyeom pharmacopuncture.

Keywords: gout, monosodium urate, nlrp3, inflammasome, soyeom, pharmacopuncture

INTRODUCTION

Gout is the most common inflammatory arthritis in the joints and soft tissues due to deposition of monosodium urate (MSU) crystals, which are caused by elevated serum uric acid levels [1]. Hyperuricemia is defined as uric acid concentrations exceeding 7.0 mg/dL, either by underexcretion or overproduction of uric acid [2]. Gout can be divided into three stages: (1) asymptomatic hyperuricemia, characterized by absence of symptoms but with persistently increased uric acid levels in the blood; (2) acute gout, characterized by inflammation from MSU precipitation; and (3) chronic gout, which is caused by deposition of tophi from persistent hyperuricemia. Additionally, the patients remains asymptomatic between gout flares. Acute gout is accompanied by painful seizures, which are self-limited after 7-14 days without treatment, whereas chronic gout with tophi can cause permanent joint deformation [2].

Gout has been continuously studied in the fields of epidemiology and medicine. The number of gout patients has been increasing, due to the increase of meat and protein consumption since the economic growth in the 20th century [3]. Since uric acid, the main contributor of gout, is the product of purine metabolism, its prevalence is deeply related to the degree of environmental exposure to purine-containing food (e.g. meat and fish) [4]. Additionally, the increase in gout prevalence is related to lifestyle, particularly obesity, metabolic syndrome, hyperten-

sion, and the use of diuretics [4, 5]. It also occurs more commonly in men and adults usually in their 30s and 50s [4].

Despite advances in medical knowledge, further progress is needed in various aspects of gout to provide a wider range of treatments, with knowledge on their side effects and cost. As current clinical practices have steadily required new treatment methods [2, 6-8], we selected Soyeom pharmacopuncture among natural products used in Korea as the new target of our study.

Pharmacopuncture combines the therapeutic action of meridians and acupuncture points with the pharmacological action of drugs. It is practiced by extracting the active ingredient of the drug and injecting it into acupuncture points or common areas where it is most effective [9]. Soyeom pharmacopuncture is used when Cheongyeol action is required for Oriental medicine-patterned diagnosis. Soyeom pharmacopuncture is classified as a type of distillate acupuncture, and its physiochemical and pharmacological effects have been studied.

We hypothesized that Soyeom pharmacopuncture can be used for acute gout, of which its main mechanism is NLRP3 inflammasome activation [10]. Therefore, this study aims to identify the inhibitory effects of Soyeom pharmacopuncture on acute gout.

MATERIALS AND METHODS

1. Animals and cell culture

C57BL/6 mice (Daehan Bio, Korea) were acclimatized to the absence of specific pathogens for at least 1 week before the experiment. The mice were placed in a room maintained at a temperature of 23 ± 3℃ and a relative humidity of 40%-60%. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee at Woosuk University approved this study (approval ID: WS2020-05). Bone marrow samples from the mice were isolated, and the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). Afterwards, 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 20% L929 were added, and samples were cultured for 7 days. Adherent cells were used as macrophages. Lipopolysaccharides (LPSs) were purchased from List Biological Laboratories Inc. (Campbell, CA, USA) and dissolved in endotoxin-free water from Sigma-Aldrich (St. Louis, MO, USA). Urate crystals were purchased from Invivogen (San Diego, CA, USA). Once the mice were aged 7-8 weeks, they were assigned to each Soyeom pharmacopuncture group.

2. Reagents

DMEM, L-glutamine, FBS, and penicillin-streptomycin solutions were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Various solvents for buffering were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against IL-1β, nitrite, IL-6 and TNF-α were purchased from R&D Systems

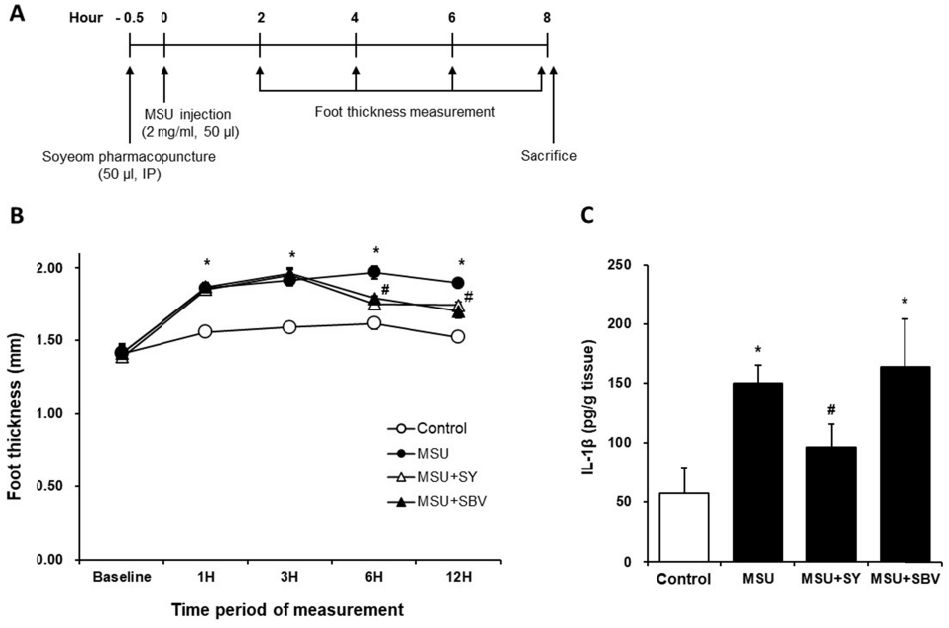

Figure 1. Measurement of foot thickness and IL-1β level. Soyeom pharmacopuncture reduces foot thickness and IL-1β level in MSU-induced gout mouse. Data are presented as mean ± SD (n = 6 mice per group). *p < 0.05 vs. Control group, #p < 0.05 vs. MSU group. MSU, monosodium urate; SY, Soyeom; SBV, sweet bee venom.

(Minneapolis, MN, USA). Soyeom and sweet bee venom (SBV) pharmacopuncture were purchased from AJ Pharmacopuncture (Seoul, Korea).

3. Experimental design

Twenty-four mice were equally divided into four groups. To induce gouty arthritis, MSU crystals was injected at 8 weeks of age, as previously described [11, 12]. MSU (2 mg/mL in 50 μL of sterile, endotoxin-free phosphate buffered saline [PBS]) was subcutaneously injected into both footpads of the mice, and 50 μL of Soyeom and sweet bee venom (SBV) pharmacopuncture was injected intraperitoneally (IP). The foot thickness of each mouse was measured using a digimatic micrometer (Mitutoyo, Japan) at 1, 3, 6, and 8 hours after the start of the experiment. Afterwards, the soles of the paws were cut and ground for protein analysis. Paw tissues were homogenized in RIPA buffer (500 mM Tris-HCl, pH7.4, 10% NP-40, 10% sodium deoxycholate, 1.5 M NaCl, 100 mM EGTA, 100 mM PMSF, 100 mM Na3VO4, 10 μg/mL of aprotinin and 10 μg/mL of leupeptin). The supernatant was collected for enzyme-linked immunosorbent assay (ELISA) and stored at –80℃ until use (Fig. 1A).

4. ELISA

Anti-mouse IL-1β, nitrite, IL-6 and TNF-α capture monoclonal antibodies were coated on a 96-well plate at 1 μg/mL and left at 4℃ for 12 hours. After coating, a blocking buffer composed of phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) was added to prevent non-specific binding sites and was left at 37℃ for 2 hours. After four times of washing with PBS solutions containing 0.05% Tween 20, 100 μL of recombinant mouse IL-1β, nitrite, IL-6, and TNF-α, culture supernatants of each sample were added to each well and left at 37℃ for 2 hours. After four more times of washing with PBS containing 0.05% Tween 20, biotinylated anti-IL-1β, nitrite, IL-6, and TNF-α, solutions were diluted to 0.05 μg/mL using PBS containing 1% BSA and left for 2 hours at 37℃. After seven times of washing with buffer, each well was treated with 2.5 μg/mL of avidin-conjugated enzyme, left at 37℃ for 30 minutes, and rewashed for seven times. Then, 100 μL of ABTS substrate solution was added to each well to induce color development for 10 minutes, and IL-1β, nitrite, IL-6, and TNF-α levels were measured at a wavelength of 450 nm using an ELISA reader.

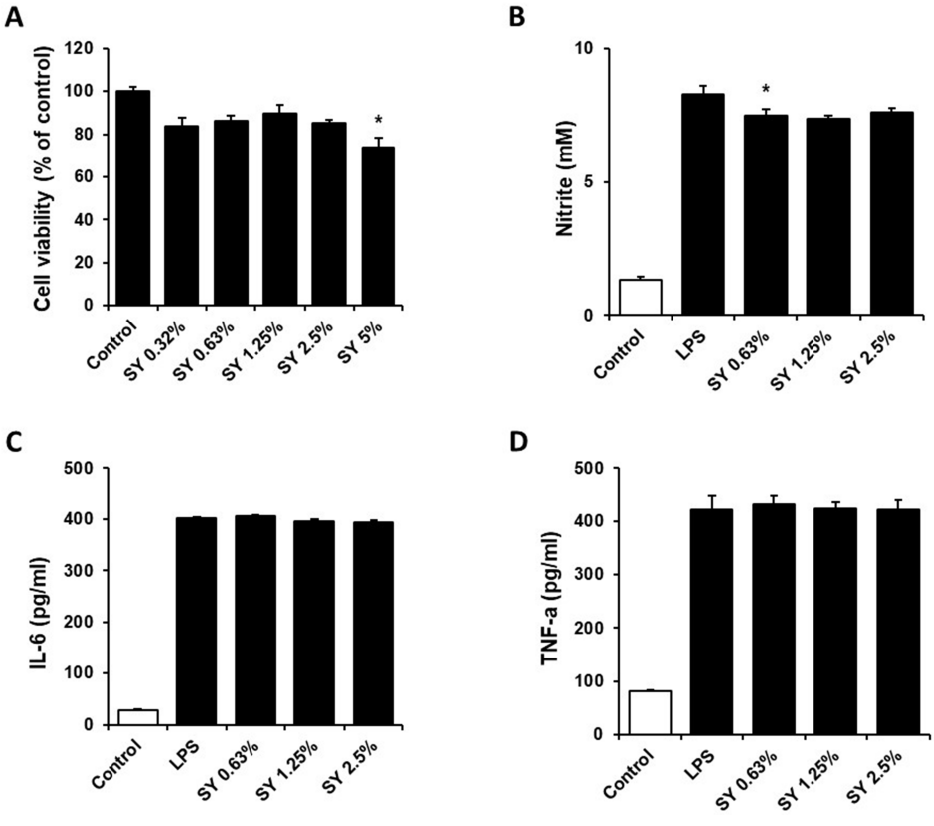

Figure 2. Measurement of cytotoxicity and inflammatory cytokines level on BMDMs. BMDMs were treated with different concentration of Soyeom pharmacopuncture (0.32, 0.63, 1.25, 2.5 and 5%). Cytotoxicity of Soyeom pharmacopuncture showed in 5%. LPS primed inflammatory cytokines on BMDMs were treated with different concentration of Soyeom pharmacopuncture (0.63, 1.25, and 2.5%). Data are presented as mean ± SD (3 wells of cell per group). *p < 0.05 vs. Control group. LPS, lipopolysaccharide; SY, Soyeom; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha.

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5. RNA sequencing assay and data analysis

Stimulation was induced by priming with 100 ng/mL of LPS in bone marrow-derived macrophages (BMDM) for 4 hours, and treating with 5% of medicinal needles and 500 μg/mL of MSU for 1 and 4 hours, respectively. Total RNA was isolated using Trizol reagent according to the manufacturer's protocol. RNA quantification and sequencing were performed by E-biogen Inc. (Korea). RNA-sequencing data mining and graphic visualization were performed with ExDEGA (Excel-based Differentially Expressed Gene Analysis, E-biogen Inc., Korea).

6. Statistical analysis

Data were expressed as means ± standard errors of measurement (SEMs). One-way ANOVA was performed using GraphPad Prism7 (GraphPad Software, San Diego, CA, USA). Tukey's test was performed for post-hoc analysis. p-values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ denoted statistical significance.

RESULTS

1. Soyeom pharmacopuncture decreased foot thickness and IL-1β levels in gout-induced mice models

Foot thickness was measured at 1, 3, 6, and 12 hours after injection. The foot thickness of the Soyeom pharmacopuncture group was significantly decreased compared to the MSU-injected group. Additionally, additional SBV pharmacopuncture significantly decreased the foot thickness after 3 hours (Fig. 1A). MSU injection for NLRP3 inflammasome-induced gout increased IL-1β levels, whereas Soyeom pharmacopuncture sig-

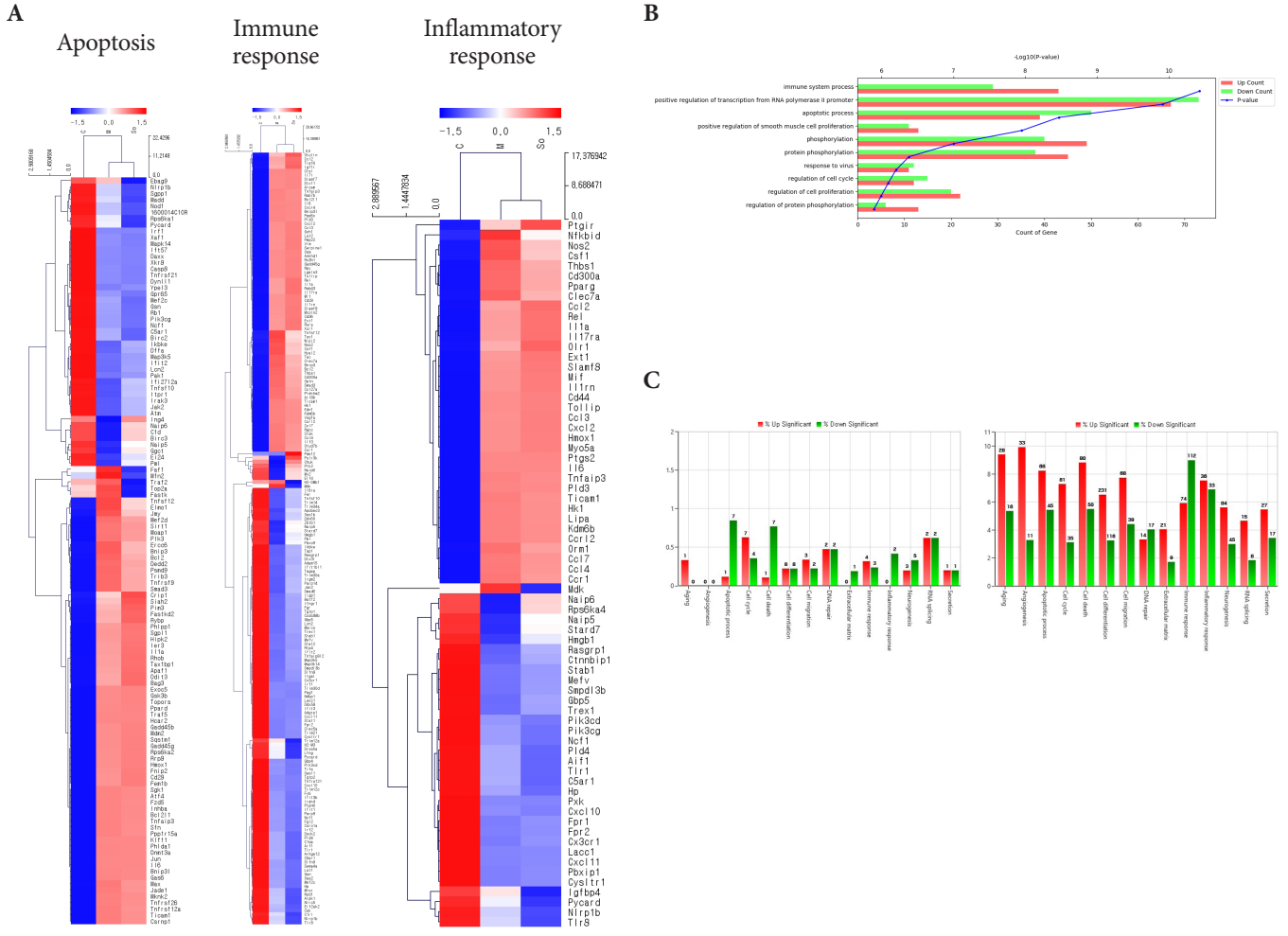

Figure 3. RNA sequencing analysis. Soyeom pharmacopuncture down regulated genes associated with inflammatory response in MSU-induced gout mouse model.

nificantly decreased the IL-1β levels. The SBV pharmacopuncture group showed opposite results (Figs. 1B, C). Therefore, Soyeom pharmacopuncture reduced gouty edema by suppressing NLRP3 inflammasome-induced IL-1β production.

2. Analysis of cytotoxicity and inflammatory cytokines

To investigate the cytotoxicity of Soyeom pharmacopuncture in BMDMs, we prepared various concentrations (0.63, 1.25 and 2.50%) of Soyeom pharmacopuncture. Concentrations < 5% were found to be non-toxic (Fig. 2A). From 8.28 ± 0.54 mM in the LPS solution, the the nitrite level significantly decreased to 7.39 ± 0.16 and 7.6 ± 0.28 mM in the 1.25% and 2.50% pharmacopuncture solutions, respectively (Fig. 2B). From 402.42 ± 3.93 pg/mL in the LPS solution, the IL-6 level decreased to 392.71 ± 5.07, 394.89 ± 8.22, and 393.63 ± 8.64 pg/mL in the 0.63%, 1.25%, and 2.50% pharmacopuncture solutions, respectively (Fig. 2C). From 422.04 ± 43.74 pg/mL in the LPS solution, the TNF-α level decreased to 425.54 ± 36.47, 421.32 ± 18.36, and 422.19 ± 27.68 pg/mL in the 0.63%, 1.25%, and 2.50% pharmacopuncture solutions, respectively (Fig. 2D). However, there were no significant differences in the IL-6 and TNF-α levels among the groups.

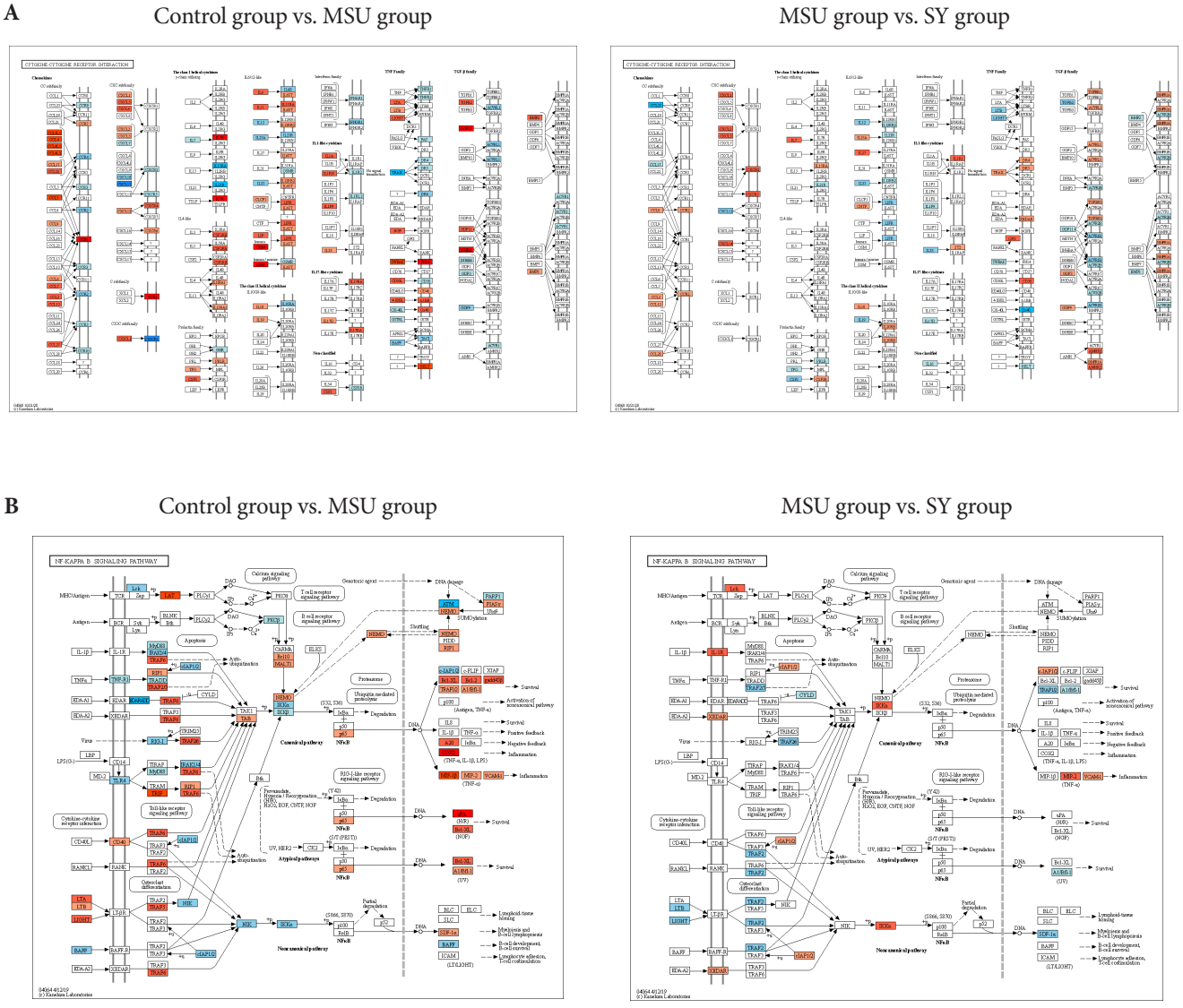

Figure 4. Analysis of gene expression correlated with inflammatory signal pathway. Soyeom pharmacopuncture showed that down regulated genes associated with IL-1β production and NF-κB pathway signal.

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3. RNA sequencing analysis

We induced NLRP3 inflammasome activation in BMDM with MSU, and treated Soyeom pharmacopuncture with RNA-sequencing. Afterwards, the categories to which the genes regulated by Soyeom pharmacopuncture belong were first analyzed (Fig. 3A), and the pathways involved in regulating those genes were analyzed (Fig. 3B). The number of genes increased or decreased by MSU was regulated by Soyeom pharmacopuncture. It was further confirmed that the levels of 1,076 genes were regulated (Fig. 3C) Furthermore, the genes increased by MSU were significantly decreased by Soyeom pharmacopuncture.

To check whether genes upregulated or downregulated by MSU led to changes in inflammation or immune response, we identified gene changes corresponding to the signal pathway through Kyoto Encyclopedia of Genes and Genomes (KEGG). Expression of interacted pathways among cytokines and corresponding receptors, chemokines, and MSU-related cytokines were decreased by Soyeom pharmacopunture (Fig. 4A). In the NF-kB pathway, a representative inflammatory response signaling pathway, MSU-induced gene expression was decreased by Soyeom pharmacopuncture (Fig. 4B). Thus, we demonstrated that Soyeom pharmacopuncture regulates inflammation and immunity-related genes whose expression is increased by MSU.

DISCUSSION

Acute gout flares are accompanied by pain, fever, erythema and swelling, and are usually found in the lower extremities, especially in the first metatarsal joint [2]. Gout flares, of which its pain is reportedly equivalent to that of childbirth or visceral colonic pain, are temporary, self-limited phenomena. However, in case of recurrence, prompt treatment is important [2, 13]. Gout is also commonly associated with other diseases, including hypertension, obesity, cardiovascular disease and diabetes [4, 5]. Therefore, gout patients have more contraindications to drugs than patients with other diseases, suggesting a very narrow range of drug choices [14]. Owing to the steady demand for new treatments, understanding the exact mechanism of gout is essential to develop effective treatments.

Treatment of acute gout focuses on powerful anti-inflammatory drugs that can quickly relieve gout flare-associated pain [15]. Nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, and corticosteroids are currently used in the clinical setting to treat acute gout. Such medications are used alone or in combination, depending on each patient's individual comorbidities and accompanying drugs [6].

Soyeom pharmacopuncture, which was used for analgesic and anti-inflammatory purposes in this study, is a new pharmacopuncture treatment in traditional Korean medicine that combines pharmacopuncture and herbal medicine [16]. Injecting herbal extracts directly into acupoints and areas of tenderness makes it applicable for easy dosage control and immediate effect [17]. Currently, in Korea and China, pharmacopuncture is used to treat many diseases, such as musculoskeletal disorders, obesity, and asthma [17]. Thus, we hypothesized that pharmacopuncture used in Korean medicine would effectively treat acute gout. We chose Soyeom pharmacopuncture due to its inflammatory effect [18]. Overall, our results indicate that Soyeom pharmacopuncture can be used as a new acute gout analgesic.

Our study results suggested a significant anti-inflammatory effect of Soyeom pharmacopuncture. Similarly to colchicine, which is one primary drug for acute gout treatment in clinical practice, pharmacopuncture reduced feet thickness and inhibited caspase-1 and IL-1B production. Additionally, the production of nitrite, IL-6, and TNF-a was inhibited in the RAW264.7 cell experiment, indicating that Soyeom pharmacopuncture exhibited significant anti-inflammatory effects with respect to NLRP3 inflammasomes. Edema and inflammation were directly suppressed by Soyeom pharmacopuncture. This makes Soyeom pharmacopuncture a possible new and effective treatment method for acute gout. Additionally, our study results provide a basis for clinical application of acupuncture by demonstrating the mechanism of pharmacopuncture, which had not been clarified in previous studies. Though our study confirms the anti-inflammatory effect of Soyeom pharmacopuncture in relation to NLRP3, an experiment on the effect of pharmacopuncture on NLRP3 inflammasome-mediated inflammation was not conducted. It is necessary to conduct further research on its exact effect on NLRP3, such as its relationship with signal 1, which is related to NF-kB, or signal 2, in which the inflammasome is assembled [19-21]. We also did not identify the ingredient responsible for the anti-inflammatory effect in Soyeom. Therefore, follow-up studies on Soyeom pharmacopuncture, particularly on its ingredients, side effects, and optimal route of administration, should be conducted.

Our study confirmed the anti-inflammatory effect of Soyeom pharmacopuncture on acute gout, suggested a promising possibility of using Soyeom pharmacopuncture as the first-

choice treatment during acute gout flares. NLRP3 inflammasome components are expressed not only in macrophages but also in various cell types of different organs [22, 23].

CONCLUSION

The efficacy of Soyeom pharmacopuncture in acute gout flares, we believe that it may treat other inflammatory diseases as well. Through active follow-up studies, we hope to thoroughly understand the clinical values of Soyeom pharmacopuncture.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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